Final Thesis

Solid-phase bio-organic synthesis to create intelligent surfaces

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LITH-IFM-EX--04/1320—SE
Bioorganisk fastfas syntes för att skapa intelligenta ytor

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Solid-phase synthesis, peptide design, SAM, cyclic peptide
Abstract

This thesis investigates three different surface modifications, and the route to design and synthesize them. The thesis is therefore divided into three sub-projects. (i.) Design and synthesis of a peptide which secondary structure could be controlled by a negatively charged surface. (ii.) Design and synthesis of a cyclic peptide, that would self-organize prior to surface interaction, using the type I anti-freeze protein of a winter flounder as template. (iii.) The use of solid-phase synthesis to make the synthesis of SAM-molecules easier.
Solid-phase bio-organic synthesis to create intelligent surfaces
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>CP8</td>
<td>Cyclic peptide containing 8 amino acids</td>
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<tr>
<td>DCM</td>
<td>Dichloromethane</td>
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<tr>
<td>DIPEA</td>
<td>Diisopropyl ethylamine</td>
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<tr>
<td>DIPCDI</td>
<td>Diisopropylcarbodiimide</td>
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<tr>
<td>DMF</td>
<td>N, N’-Dimethylformamide</td>
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<tr>
<td>DTT</td>
<td>Dithiothreithol</td>
</tr>
<tr>
<td>EDT</td>
<td>Ethandithiol</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>HOBr</td>
<td>1-Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>MPA</td>
<td>3-Mercaptopropionic acid</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NMM</td>
<td>N-Methylmorpholine</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OAII</td>
<td>Allyl</td>
</tr>
<tr>
<td>OtBu</td>
<td>tert-Butoxy</td>
</tr>
<tr>
<td>PbF</td>
<td>2, 2, 4, 6, 7-Pentamethyl-dihydrobenzofurane-5-sulfonyl</td>
</tr>
<tr>
<td>R2A</td>
<td>Peptide containing 8 arginines</td>
</tr>
<tr>
<td>R1A</td>
<td>Peptide containing 4 arginines</td>
</tr>
<tr>
<td>rf</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>TBTU</td>
<td>O-Benzotriazol-1-yl-N,N,N’,N’-tetramethyluronium tetrafluoroborate</td>
</tr>
<tr>
<td>tBu</td>
<td>tert-Butyl</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilan</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>Trt</td>
<td>Trityl</td>
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1. Introduction

The ability to tailor make surfaces with well-defined chemical properties has been, and still is, a major research field. Well-defined surfaces can be used to investigate biological systems (protein chips), to create nano-devices (nano-electronics), to design new materials etc. the applications are almost endless. Examples in the literature include the growth of inorganic crystals on SAMs\(^1\), constructions of nanopens and nanopenciles, nanowires of silver, nanotrails from ATPase\(^2\) can be mentioned.

If one, at the time of writing, makes a search on self-assembled monolayers in the American chemical society’s publications, 4978 documents are presented. Doing a search on the same page but on the word surface generates 13318 documents. This gives a hint on how important the understanding of surfaces and their chemistry is.

This thesis uses solid-phase synthesis to synthesize molecules that will arrange themselves as monolayers. The use of solid-phase synthesis will hopefully increase the yields in every step and also make it easier to synthesize more complex molecules.

There are a vast number of different surfaces that can be used. Mainly pure metal surfaces are studied but also metal oxides, metal nitriles, carbines, carbon oxides, plastic and glass should be mentioned. In this project studies have been made on gold and silicon oxide.

By combining organic synthesis and surface science one gets a very powerful tool, and by further including molecular biology, inorganic chemistry, medicine and/or biology the possibilities become almost endless. There are many methods used to analyze chemically modified surfaces, such as IR-spectroscopy, XPS, SPR, ellipsometry and AFM. They can give information of for example the thickness of the modification, the interactions with surfactants, the physical properties of the molecules, hydrogen bonding between molecules and charged interactions.

One way to create well defined surfaces is to use nature as a source of inspiration for synthesizing. By combining natures fine solutions and laboratory organic chemistry one gets a powerful tool and also the opportunity to increase the understanding of how natural systems works.

The work done in this thesis is mostly concerned with the use of peptides, and one might ask “Why use peptides and not other organic molecules”? To answer that
question one has to consider multiple aspects. First, the synthesis of peptides is fairly easy; it is made automatically with a peptide synthesizer machine using a solid support as a starting point and gives a high yield. Secondly, purification of synthesized peptides also becomes simplified, since there is no purification between each synthetic step. The peptide is purified using HPLC when the synthesis of the target molecule is done.

There are other advantages of using peptides for surface modification besides those described above. The natural occurring amino acids contain a number of chemically modifiable side-chains that gives the possibility to incorporate more functionality. One is of course not limited by the naturally occurring amino acids, but can also use artificial ones and other compounds that can form amide bonds.

Peptides can also be designed to bind with each other to high specificity which can be used to create supramolecular systems that will be organized in solution prior to the surface modification.

This thesis is primarily concerned with development of novel methods for synthesis of complex surface modifying agents using solid-phase synthesis to create complex bio-organic molecules. The molecules can be categorized in three groups; self-assembled mono-layer molecules, pre-organized surface modifications and surface induced organized molecules.

By creating well-defined surfaces on a molecular scale one can further introduce functional groups with known distances. This opportunity gives the starting ground for a vast number of further applications that there is no possibility to include in this report.
2. Peptide design

2.1 Structural theories

When designing a peptide one has to consider multiple interactions and behaviors of the constituting amino acids. Here the interactions of a peptide will be broken down in smaller parts to describe the different aspects that have to be considered.

The first, and probably most important, aspect of a peptide and its behavior is the peptide backbone. The backbone is composed of amides and the α-carbons. The backbone becomes stabilized by the partial double bond of the amides. Also the secondary structure is stabilized by the backbone, through the hydrogen bonds between the carbonyl oxygen and the hydrogen attached to the nitrogen. Depending on the molecular environment around these groups, their hydrogen bonds give rise to the different secondary structures α-helices, β-sheets and turns.

The behavior of amino acids in a chain is also affected by the side-chains of the participating amino acids, their geometry as well as functional groups. The side-chains make the amino acids more suitable for different secondary structures and/or interactions with neighboring molecules.

It has been an important research field to understand the amino acids preference of the different secondary structures. Chou-Fasmans rules summarizes one explanation of these results and is based on the statistical distribution of how the amino acids prefer to behave (Appendix 1). This rule can give a hint of how a protein or peptide will fold just by looking at the amino acid sequence.

Even though these aspects are taken under consideration, while designing a peptide one can not be sure of the outcome of the design. Nature does not follow the rules of man and even if they did there are other parameters to consider. For example salt, like NaCl, can disturb the conformation. Also temperature, molecular environment and denaturants such as urea influence the outcome of the peptide structure.

2.2 Peptide structure

For two of the surface modifications that was going to be examined, a cyclic peptide for attachment on gold surfaces and a peptide that would go from a random coil to a
α-helix when a negative charge is introduced, the structure/sequence of the molecules had to be designed. The thought was to use as many natural amino acids as possible.

Four designs of the random coil to helix peptide were presented in the startup phase of the project. Two of that contained eight arginines and two that contained four. When designing these peptides a helical wheel was used to more easily see which amino acids that would be designed to interact and how they would be positioned in the helix. The designs were labeled R1A for arginine – one row – all, R1V, arginine – one row – varied, and R2A, arginine – two rows – all and R2V, arginine – one row – varied. R2A was the first peptide to be designed and was then used as a template for the other three, although there were major changes made in the different designs.

R1A and R2A were chosen for further investigation. R1V and R2V were discarded as they were predicted to have less chance to be successful.

![Figure 1. Schematic picture of the random coil to helix peptide. (i.) The peptide in solution. (ii.) Introduction of the negatively charge surface. (iii.) Helix formation on the surface.](image)

In the above described project the molecule-surface interaction is strictly electrostatic. This is one way of making surface modifications; another is to use covalent bonding. The project described below uses the covalent approach for surface interaction, but still peptides are used as the target molecules for the modification.

When the designs of the cyclic peptide were done, two sequences were suggested. One containing eight amino acids (CP8) and the other containing twelve (CP12). When designing the cycles the idea was to duplicate the function of the anti-freeze protein, where every twelfth amino acid is a threonine and there is a high abundance of alanines. The resemblance to the protein was more pronounced in the CP12. Due to the larger flexibility that CP12 would have, and to more easily understand the interactions involved, it was later decided that initially experiments would only be preformed on CP8.
2.3 Design of R2A

The task was to design a peptide that were to be a random coil in solution and would take the shape of a helix when it was exposed to a negative surface. When designing the peptide, Chou-Fasmans rules were kept in mind, as well as how the side chains would interact with the surface. Also the time for the total synthesis were taken in account.

Since the surface would be negatively charged, the amino acids that were going to interact with it ought to be positively charged. Among the twenty naturally occurring amino acids three contain a positive charge; arginine, lysine and histidine. The histidine was removed due to its low pK\textsubscript{a}; it would become uncharged in the basic environment that would be used. In choosing between arginine and lysine the choice fell on arginine. The decision was based on the fact that arginine has a higher pK\textsubscript{a} than lysine and the observation that three lysines close to each other may lower the pK\textsubscript{a} of the lysine in the middle\textsuperscript{3}. If that would be the case the middle lysine would become deprotonated and losess the positive charge and that could interfere with the interaction with the surface. A loss of the positive charge would decrease the possible interactions with the surface and thereby destabilize the helix once it is formed.

The length of the peptide was decided to be 28 amino acids to get a more pronounced $\alpha$-helix when the peptide is introduced to the surface. 28 amino acids will generate four helical turns. To get as much interaction between the peptide and the surface as possible at least one arginine should be present in every helical turn. It was thought that a high concentration of positive charges would favor the attachment on the surface.

To get the charges of the peptide to become even more concentrated the row R3 and R7 were filled with hydrophobic side-chains. This was to protect the positive arginines from the surrounding charges when the helix would form and thus concentrate the charges on the surface. The eight arginines already incorporated in the design are according to Chou-Fasmans rules helix inducers, and since the peptide were supposed to be a random coil in solution, the amino acids in row R3 and R7 were to be a mix of both helical inducers and amino acids with less good helical properties. The amino acids with aromatic side-chains were excluded due to their bulkiness. Also excluded were proline, since it is to good a helical breaker, and
methionine in which the sulfur can undergo unwanted side reactions i.e. oxidation to sulfone. Of the four hydrophobic amino acids left, two have helical preference; alanine and leucine, and

![Helical wheel of R2A with only arginine present.](image)

two have β-sheet preference; isoleucine and valine. A mix of these four was incorporated in the structure; four helical inducers, three leucines and one alanine. In an attempt to control the secondary structure in solution the less helical inducing leucine was taken to a higher extent than alanine. Their helical properties would hopefully be enough to induce the formation of the helix when the peptide is introduced to the surface.

The amino acids that were inserted in row R4 and R6 have two functionalities. One; they were to stabilize a close packing of the helix on the surface and, secondly, to isolate the negative charges that were to be incorporated in row R5. By using polar side-chains the thought was that they would form hydrogen bond, thus arrange themselves close to each other. The polarity would also stabilize the charges in row R5. Still the balance between helical inducers and none-helical inducers had to be controlled. To achieve this, amino acids in these two rows were chosen to be a mix of helical inducing glutamine, and less helical inducing, threonine, asparagines and serine. The last three also works as helix stabilizers if they occur in the first turn of the helix, due to hydrogen bonding to the peptide backbone.
When appointing the amino acids for the final row, R5, the row furthest away from the surface, two properties were taken into account. First, the amino acids should repel the charged surface and thus hopefully twist the peptide backbone to easier undertake the helical conformation. Secondly, the amino acids should contain chemically modifiable side chains, like the ability to attach fluorescent molecules. To get negative charges that would repel the surface only two naturally occurring amino acids are available, glutamic acid and aspartic acid. To further reduce the helical structure aspartic acid were chosen, although kept in mind that aspartic acid next to a glycine can generate unwanted side reactions with the peptide backbone.

![Figure 3. Spontaneous formation of peptide succinimides and product of hydrolysis from Asp residues.](image)

More than one of the amino acids can undergo chemical modification. Aspartic acid and glutamic acid can form both amides and esters, but since aspartic acid is already incorporated in the sequence and is used to repel the surface, a chemical modification on a carboxylic acid could disrupt the helix formation. Therefore another functionality, which would not disturb other interactions, had to be incorporated. Histidine can be modified, but as explained earlier bulky side-chains were to be avoided, and like lysine and arginine, which can also be modified, they contain positive charges and could therefore be attracted by the surface. The side-chains of tyrosine and tryptophan are also subjects for modifications, but are bulky and therefore dismissed. The alcohol group of threonine and serine could in theory also be modified, but one has to use extreme conditions and by using one of these for modification the polarity of rows R4 and R6 could be disrupted. By just considering naturally occurring amino acids the only chemically modifiable amino acid left, which also is a good nucleophile and can be modified using mild conditions, is cysteine. Cysteine, which does not have helical preference, was placed between the two repelling aspartic acids. When the amino acid sequence had been set the helical preference of the peptide where calculated using Chou-Fasmans rules. The number of
amino acids that have a surface helical preference were divided by the total number of amino acids in the sequence, and the quota were multiplied by 100 to get the value in percent. The helical preference of the peptide, R2A, is roughly 50%, which ought to be enough to keep the peptide as a random coil in solution and have high enough helical preference to be able to form a $\alpha$-helix when introduced to the negative surface. Using the same kind of calculations to see how high the $\beta$-sheet preference is gives a rough value of 20%.

![Helical wheel of R2A with all amino acids present. Amino acids in bold have helical preference. The amino acid sequence of R2A is shown under the wheel.](image)

**Figure 4.** Helical wheel of R2A with all amino acids present. Amino acids in bold have helical preference. The amino acid sequence of R2A is shown under the wheel.

### 2.4 Design of R1A

When designing R1A, R2A had already been designed, so R2A were used as a template for the design. The length of the peptide was kept at 28 amino acids. Other similarities were the basic ideas of two rows that would attach to the surface, shielded by two rows of hydrophobic amino acids. One row that would repel the surface, the
two rows beside that one that would stabilize the negative charge and help ordering the peptides on the surface.

Although the above mentioned criteria’s were kept in mind there were major changes in the design of R1A compared to the design of R2A. R1A were designed to contain one row of arginines, R2A has two. In row R2, which in R2A also contains only arginines, the amino acid asparagine was incorporated. It was thought that the electropositive amide hydrogen’s would be attracted to the negative surface and thus help inducing the helical structure.

To parry the decrease in $\alpha$-helix preference due to the change from helical inducing arginines to asparagines, which occurs more in reversed turns, the amino acids in row R3 and R7 were changed as well. In the design of R2A, these rows where filled with a mixture of hydrophobic helical inducers and breakers. The thought was to keep the hydrophobicity and to increase the helical preference of the peptide. This resulted in a change to alanine in all this positions. Alanine, which has the highest $\alpha$-helix preference next to glutamine, should with their slightly hydrophobic nature still concentrate the “binding sites” as the hydrophobic side chains in R2A were supposed to do.

As for row R5 in R2A the row R5 was going to be both surface repelling and be able to modify chemically. To keep the balance between helical inducers and none-helical inducers and to abide the recently given parameters the row was directly copied from the R2A design, hence getting the row sequence $D_7C_{14}D_{21}Q_{28}$. The glutamine in this row was inserted to be the N-terminal amino acid and to work as a built-in end-caper. This is done due to the fact that glutamine as an N-terminal spontaneously cyclize to form a pyrrolidone carboxylic acid.

![Figure 5. Spontaneous formation of a pyrrolidone carboxylic acid from an N-terminal glutamine.](image)
Since the base design was a duplicate of R2A, the rows R4 and R6 were to be filled with amino acids containing polar side-chains. By inserting a row of asparagine and thus lowering the helical preference of the peptide, the polar amino acids had to increase the preference as well as the hydrophobic had done. Therefore one extra glutamine was introduced in the sequence instead of the asparagine that had been in R2A. Another of the major changes in design in R1A in comparison to R2A is the use of bulky side chains. Among the polar side chains in row R4 and R6 the amino acid tyrosine has been incorporated. This was done to get a UV-detectable side-chain that could be used for concentration measurements, but also to investigate if the bulkiness would disturb formation of a helix, or if the presumption was wrong.

As for R2A the helical preference for R1A was calculated using the Chou-Fasmans rules and the same type of calculations. The calculations of the $\alpha$-helix preference for R1A gave the same rough value as it had given for R2A, approximately 50%.

**Figure 6.** Helical wheel of R1A. Amino acids in bold have helical preference. The amino acid sequence of R1A is shown under the wheel.
2.5 Design of a CP8

The basic idea for making a cyclic peptide was to create a homogenous and controlled surface using a molecule that would self-organize both on the surface and in solutions. A second objective was the possibility to introduce of a functionality to this surface. The surface of interest for this project is a pure gold surface.

To make a cyclic peptide an easy way is to let the sequence contain D- and L-amino acids. By attaching them every second D- and every second L-amino acid the peptide becomes almost cyclic as it is synthesized. Although this is convenient, the most important reason for using D- and L-amino acids is to get the stacking of the cycle due to hydrogen bonding between the peptide backbones.

To get a well defined surface, stabilization should derive from both side chain – side chain interaction as well as the hydrogen bonding in the peptide backbone. In comparison to α-helixes and β-sheets, the backbone of the cyclic peptide will produce hydrogen bond interactions between the peptide backbone of one of the cycles and the backbone of another cycle. These interactions will hopefully make the ordering of the peptide on the surface easier as well as help the peptides to arrange in a homogenous way.

![Hydrogen bond through space of the peptide backbone. Most of the peptide side chains have been omitted for clarity.](image)

The idea for the functionality was to lower the freezing point of water. By designing the cycle with a type I anti-freeze protein as a template this would hopefully be achieved. The sequence of one of these proteins contains >60% alanine and every
twelfth amino acid is a threonine\textsuperscript{7}. So when designing the peptide a high abundance of alanines was crucial and also the threonine at the top.

\[ \text{H}_2\text{N-DT}_{2}\text{ASDAAAAAALT}_{13}\text{AANAKAAAELT}_{24}\text{AANAAAAAAAT}_{35}\text{AR-COOH} \]

\textit{Figure 8. Amino acid sequence of the Winter flounder is shown as an example\textsuperscript{7}.}

To reduce the synthesis time and to limit the interactions between peptides and between peptides and the surroundings, the length of the peptide was decided to be eight amino acids. When appointing the amino acids for CP8 the following elements were considered. One of the amino acids should work as an attachment site to the gold surface. One of the amino acids would have to be the threonine described above. For the side chain – side chain interaction an amino acid that could form hydrogen bonds were to be chosen. The rest were to be alanines to replicate the anti-freeze protein.

\[ \text{H}_2\text{N-QATAQACA-COOH} \]

\textit{Figure 9. The glutamine side chain hydrogen bond interaction.}

With the criteria’s already described the assembly of the sequence where made. An \(\text{L}\)-cysteine, for the attachment to the surface, was the first amino acid to be incorporated in the design. For the side chain – side chain interaction \(\text{L}\)-glutamine was chosen, and was placed as the third and seventh amino acid. On the top of the cycle a
threonine was placed as to resemble the anti-freeze protein further. In the even positions D-alanine was placed both for the protein replication purpose and to induce the cyclic structure of the octapeptide.

Although the design of the peptide was made using the cysteine as the first amino acid when synthesizing CP8 the order of the amino acids had to be changed. This did not disturb the design; it only made it easier to perform the synthesis.

**Figure 10. The cyclic D,L – peptide, structure and sequence.**

**Figure 11. The CP8 nanotube interactions.**
3. Methods

3.1 Solid-phase synthesis

In this thesis, both peptides and organic molecules have been created by the use of solid-phase synthesis, to create intelligent surface modifications. Solid-phase synthesis is used to get higher yields of the target molecule and to reduce synthesis time. The solid-phase resins are built up by copoly(styrene-1% divinylbenzene) that contains groups that can be chemically modified. There are a vast number of solid supports available and the numbers of synthetic reactions that can be made are innumerous. The groundbreaking work of solid-phase synthesis was developed by Merrifield et al. and is now used in the synthesis of biopolymers, combinatorial solid-phase organic chemistry, synthesis of natural products, catalyst selection, chemical ligation and material development.

In the solid-phase synthesis a surplus of reagents, in respect to the resin, is used. The excess is used to increase the chance of substituting the resin. Non-reacted reagents are washed away between every reaction step to prevent unwanted reactions further on in the synthesis and to diminish the formation of unwanted by-products. All manmade synthesis in this work was done on the same kind of solid support, the resin Fmoc-PAL-PEG-PS.

![Figure 12. The Fmoc-PAL-PEG-PS resin.](image)

The synthesized molecule is removed from the resin using a mixture of mainly TFA but also scavengers such as water, TIS and EDT. The scavengers react with reactive intermediates, such as protection groups and the resin, to prevent unwanted side reactions. When the synthesized molecule is cleaved from this resin it usually
generates a terminal amid, although in this thesis modifications of the resin, in some cases, have been made to create other terminals.

It was also the intention to show that complex molecules more easily could be synthesized using the solid-phase method than the same synthesis done in solution.

**Schematic picture of peptide synthesis.** $R_1$, $R_2$, and $R_n$ represent the amino acid side-chains. $X$, $Y$, and $Z$ represent eventual protection groups.
The procedure of the peptide synthesis system is illustrated above by the schematic picture.

The activation of the amino acid is usually TBTU and DIPEA. TBTU is a good leaving group when the carbonyl carbon reacts with the resin-bound free amine. The Fmoc-protection group is removed using a 20% piperidine in DMF solution. Cleavage of the molecule is done with TFA, and if needed scavengers.

### 3.2 Self-assembled monolayers

Some of the molecules investigated in this work were meant to interact with the surface to form self-assembled monolayers (SAMs) when in contact with a surface. SAMs has been a subject for investigation since the discovery almost sixty years ago. Molecules used to form SAMs attach to a metal surface (i.e. Au, Ag, Cu, etc.) via a specific strong interaction, mainly a sulfur bond. The SAM molecules arrange themselves on the surface in a homogenous way.

![SAM diagram](image)

**Figure 13.** As an example of a SAM a 16-Mecapto hexadecanoic acid is shown.

The interest of SAMs derives from the vast number of applications that exists. Some examples are; chemical sensors, nanoscale electronic devices and single-molecule wires.

Analysis of SAM-layers can be made using techniques such as SPR (Surface Plasmon Resonance), IR-spectroscopy, ellipsometry, XPS (X-ray Photo-electron
Spectroscopy). Measurements done with these techniques have been done in collaboration with Dr. Uvdal and her group.

### 3.3 CD spectroscopy

A useful tool when investigating secondary structure of peptides, and particularly the helical content of the peptide, is to use circular dichroism (CD). CD makes use of the fact that left and right polarized light exhibits chirality and by doing that interacts differently with chiral molecules. The dominating absorption in a CD spectrum in the range of 190-240 nm derives from the amide bond in the peptide backbone. This adsorption is different for the different secondary structures.

The values of CD spectroscopy are often given in ellipticity. The ellipticity is calculated using this formula:

$$\theta = 3298 \times \Delta \varepsilon$$

where $\Delta \varepsilon$ is the difference in molar extinction coefficients of right, $\varepsilon_R$, and left, $\varepsilon_L$, polarized light ($\Delta \varepsilon = \varepsilon_R - \varepsilon_L$).

A right handed $\alpha$-helix has a distinctive double minimum at 208 and 222 nm. The minimum at 222 nm is a good measurement of the helical content of a peptide that contains mostly of helices.

### 3.4 IR spectroscopy

Infrared (IR) spectroscopy is a useful tool for determining the composition of molecules. The information achieved from IR is the constituent of groups, intra- and intermolecular interactions and orientation. Organic molecules vibrate mainly in the mid IR region, 4000-400 cm$^{-1}$. Molecules built up by several different atoms usually have a dipole moment. IR spectroscopy measures the transition dipole moment, $M$

$$M = \partial \mu / \partial Q$$

where $\mu$ is the change in the dipole moment and $Q$ is the vibration coordinate.
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Investigations of thin surface bound substrates uses a method called IRAS (Infrared Adsorption Spectroscopy), this method has been used in this work but will not be investigated further in this text. For more information see references 13 and 14.
4. Results and discussion

4.1 R2A

4.1.1 Synthesis of R2A

The designed peptide R2A was synthesized using a peptide synthesis machine. Each coupling cycle were set to two hours to improve the yield. The N-terminal was acetylated and the C-terminal carboxylic acid was converted into an amide, this was done to cancel out the interactions that the charged terminals would produce. 0.5 grams of the solid-support Fmoc-Gly-PEG-PS, with a substitution level of 0.19 mmol, was used for the synthesis and the theoretical yield would be 0.331 gram. The amino acids were weighted in test tubes and placed in the amino acid dispenser sledge. The synthesis was started and the total synthesis time was estimated to around 62 hours.

When the peptide synthesis was done, the peptide was cleaved from the solid-support with a cleavage cocktail containing scavengers for cleaving peptides containing cysteines. The cocktail recipe was taken from the 2002/3 Catalog Novabiochem. The cleavage cocktail contains mostly TFA with the scavenger’s TIS, water and EDT. The cleavage was preformed for 2 hours. Since the catalog gives the volumes needed in percentage and they give a total of 100.5% (94.5% TFA, 2.5% H$_2$O, 2.5% EDT and 1% TIS) each percent was converted to 100 µl of fluid, giving the total volume of 10.05 ml. The solution was collected in a 50 ml falcon tube and was placed under nitrogen gas until two-thirds of the TFA had been evaporated. Thereafter the peptide was precipitated with cold dimethylether. The precipitate was solved in de-ionized water and lyophilized.

4.1.2 Purification of R2A

The lyophilized peptide was solved in 20% ACN in Milli-Q water giving a total volume of 40 ml. Before purification commenced, 1 µl of the crude peptide was analyzed with MALDI (Figure 14.i) using α-cyano-4-hydroxycinnamic acid as matrix. The purification was done under isocratic conditions. The mobile phase used was 22% ACN in Milli-Q water containing 0.1% TFA. R2A were dissolved in 20% ACN. Note the difference of solvent for R2A and the mobile phase constitution. Tests were done using a mobile phase of 20% ACN but the separation was not efficient enough, also
25 % ACN was tested but then the separation time became way too long. By using 22% ACN the peptide was eluted after 11 to 13 minutes as a free peak. The purified peptide was analyzed on MALDI (Figure 14.ii) to verify the purification. The purified peptide was lyophilized and the weight of the peptide was 0.05773g (17.44%).

![MALDI-spectra](image)

**Figure 14.** (i.) MALDI-spectrum for the crude R2A. (ii.) MALDI-spectrum for the purified R2A with peak at M+1 (3314) and M+2 (1657).

### 4.1.3 Analysis of R2A

#### 4.1.3.1 CD-experiment of R2A

CD spectra were recorded on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, Longjumeau, France), employing constant N\textsubscript{2} flushing. The instrument was calibrated using an aqueous solution of d\textsubscript{10}-(+)-camphorsulfonic acid. The samples were kept at room temperature during the whole experiment. The far-UV region
(~190-260 nm) spectra were recorded by scanning a 0.1 mM sample of R2A in different buffers (20 mM TRIS and 40 mM NaCl with pH 8.4, 20 mM carbonate with pH 9.8 and ~0.35 % NaOH pH ~12) and with and without 1:1 ratio of 6, 9 and 15 nm silica particles. All experiments were conducted with a 0.05 cm quartz cell. The CD data were collected at 0.5 nm intervals with an integration time of 2 s for the region between ~190-240 nm and with 2 nm intervals between 240-260 nm. Each spectrum represents an average of three consecutive scans and before summation the three separate scans were intercompared to detect possible alterations of the sample during the scan period. The peptide spectra were corrected by a spectrum of a reference solution lacking the peptide but otherwise identical. The ellipticity is reported as Delta Epsilon (M⁻¹ cm⁻¹) according to:

![Graphs showing CD-spectra for R2A with different particle sizes](image)

**Figure 15.** (i.) CD-spectrum of R2A in solution (black line) and R2A with 6 nm silica particles (gray line). (ii.) CD-spectrum of R2A in solution (black line) and R2A with 9 nm silica particles (gray line). (iii.) CD-spectrum of R2A in solution (black line) and R2A with 15 nm silica particles (gray line).
Delta epsilon = 32.99*Delta A/(c*l)

where Delta A is the difference between absorbance of left-handed and right-handed circularly light, c is the protein concentration (mol/dm$^3$) and l is the optical path length of the cell (cm).

4.1.3.2 Analytical ultra centrifugation of R2A

The experiments were performed on a Beckman Coulter Optima XL-I Analytical Ultracentrifuge equipped with an An-50 Ti Rotor using 6 sector centerpieces. The sedimentation equilibrium experiments were conducted using rotor speeds between 2500-50000 rpm. Equilibrium at each rotor speed was reached after 20 hours. The sedimentation was monitored by measuring the absorbance at 230, 240 and 280 nm. The samples had a 1:1 ratio between the peptide and 6 and 9 nm particles and the concentration was 0.1 mM. For the R2A samples the reference solution was ~0.35 % NaOH with pH ~12. For samples containing both R2A and particles the reference contained an equal amount of particles as the sample. The temperature was kept at 20 °C for all experiments.

The sedimentation properties were analyzed by using the self-association model in the Beckman software package.
4.1.4 Conclusion

The CD-spectra of peptide R2A indicates a structural change towards a $\alpha$-helix when the negatively charged silica particles are introduced. This can be seen by looking at the minima at $\sim$200 nm which shifts towards 208 nm and also the minima at $\sim$220 nm which decreases in perspective to the silica free peptide solution. Both these minima indicate a helix, but the helical content is not as high as one could have hoped for.

The result from the analytical ultra centrifuge shows that the peptide is “attached” to the silica particle, and that information indicates that the conformational change of the peptide is indeed induced by the particle.

4.2 R1A

4.2.1 Synthesis of R1A

R1A was synthesized on an Fmoc-Gly-PEG-PS solid support, where the first residue glycine is already attached on the resin. Amino acids for peptide synthesis with four mol equivalences surplus was weighted in test tubes and placed in a peptide synthesizing machine. The amino acids used were all natural $\text{L}$- amino acids. Each
synthesis step was set to two hours, giving the total synthesis time of 62 hours, 18 minutes and 56 seconds. When the synthesis was done the resin was transferred from the synthesizing tube to a VacMaster tube. Since the peptide only contains natural amino acids and the sequence is similar to the sequence of R2A the same cleavage cocktail composition could be used (94.5% TFA, 2.5% H₂O, 2.5% EDT and 1% TIS).

To reduce the volume of the solution, two-thirds of the TFA was removed by nitrogen gas. The peptide was then precipitated using cold ether. Precipitation was done three times and then the ether phase was discarded. The precipitate was dissolved in water and lyophilized.

### 4.2.2 Purification of R1A

The crude peptide was then solved in 30 ml mobile phase A (10% ACN in 90 % water containing 0.1% TFA) for purification with HPLC. 1 µl of the crude peptide was taken for analysis with MALDI (Figure 17.i) using 2,5-dihydroxybenzoic acid as matrix.
At the start the HPLC was run isocratic using 16.4% ACN in Milli-Q water containing 0.1% TFA but with time the elution changed and a new program had to be done. The new program also used an isocratic mobile phase but this time 34% ACN. All purification was done on a C18 rf column. The HPLC was monitored with a UV-spectrometer at 230 nm. The peptide was eluted after approximately 14 minutes in both programs. The purified peptide was lyophilized and stored in 5ºC.

4.2.3 Analysis of R1A

4.2.3.1 CD experiment of R1A

CD spectra for R1A were recorded using the same CD-spectrochrograph as had been used for the analysis of R2A. The measurements were conducted under constant N₂ flushing. As for R2A the instrument was calibrated with an aqueous solution of d₁₀(+)-camphorsulfonic acid. Samples were handled and kept at room temperature during the whole experiment time. A solution of 0.1 mM of R1A was used to record spectra in the far-UV region (~190-260 nm). The buffers used for this measurements were 2 mM TRIS and 2 mM NaCl with pH 8.4, 5 mM carbonate with pH 9.8 and ~0.35 % NaOH pH ~12 and with and without 1:1 ratio of 6, 9 and 15 nm silica
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particles. As for R2A all experiments were conducted using a 0.05 cm quartz cell. The CD data between ~190-240 nm were collected at 0.5 nm intervals with an integration time of 2 s and with 2 nm intervals between 240-260 nm. Each spectrum represents an average of three consecutive scans and before summation the three separate scans were intercompared to detect possible alterations of the sample during the scan period. The peptide spectra were corrected with a reference solution lacking the peptide but otherwise identical. The ellipticity is reported as Delta Epsilon (M\(^{-1}\)cm\(^{-1}\)) calculated in the same way as for R2A.

Figure 19. (i.) CD-spectrum of R1A in solution (black line) and R1A with 6 nm silica particles (gray line). (ii.) CD-spectrum of R1A in solution (black line) and R1A with 9 nm silica particles (gray line). (iii.) CD-spectrum of R1A in solution (black line) and R1A with 15 nm silica particles (gray line).
4.2.3.2 Analytical ultra centrifugation of R1A

At the time of writing the ultra centrifuge experiments on R1A are still running. The centrifugation is preformed on the same type of ultra centrifuge as for R2A (Beckman Coulter Optima XL-I Analytical Ultracentrifuge equipped with an An-50 Ti Rotor using 6 sector centerpieces). The sedimentation equilibrium experiments are conducted using rotor speeds between 2500-50000 rpm. Equilibrium at each rotor speed will be reached after 20 hours. The sedimentation is monitored by measuring the absorbance at 230, 240 and 280 nm. The samples have a 1:1 ratio between the peptides and 6 and 9 nm particles and the peptides concentration is 0.1 mM. For R1A samples the reference solution is 2 mM TRIS and 2 mM NaCl with pH 8.4, 5 mM carbonate with pH 9.8 and ~0.35 % NaOH pH ~12 measured. The temperature is kept at 20 °C for all experiments.

The sedimentation properties were analyzed by using the self-association model in the Beckman software package.

4.2.4 Conclusion

By comparing the CD-measurements done on R1A with the measurement done on R2A one can see similarities. R1A gives a decrease in delta epsilon at ~220 nm which suggests a helix. There is also a small shift towards 208 nm. Although these results indicate a helical peptide, they are not as pronounced as for R2A.

4.3 CP8

4.3.1 Synthesis of CP8

The route to synthesis a cyclic peptide is the same as for synthesizing a normal peptide with the exception that the first amino acid to be attached to the resin has to have a protection group on the carboxylic acid instead of the usual side-chain protection.

By using this none-side chain protected amino acid as the starting material for the synthesis the side chain will attach to the solid-support and the peptide backbone carboxylic acid can be used for the cyclization reaction. The resin used for this
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synthesis caps the attached carboxylic acid when cleaved, i.e. an amide is formed. This means in this case that the glutamic acid becomes a glutamine after cleavage.

\[
\begin{align*}
&\text{i.} \\
&\text{ii.}
\end{align*}
\]

**Figure 20.** (i) The structure of a glutamic acid, with Fmoc-protection group on the N-terminal and an OtBu-protection group on the side chain, for linear peptide synthesis. (ii.) The structure of a glutamic acid, with Fmoc-protection group on the N-terminal and a O-All-protection group on the side chain, used for the synthesis of branched peptides and in this case for the cyclic peptide (CP8).

The synthesis of CP8 was preformed on a peptide synthesis machine. Starting with the attachment of Fmoc-\(\text{L-}\)Glu (OH)-OAll. Followed by the sequence Fmoc-\(\text{D-}\)Ala-OH, Fmoc-\(\text{L-}\)Cys (Trt)-OH, Fmoc-\(\text{D-}\)Ala-OH, Fmoc-\(\text{L-}\)Gln (Trt)-OH, Fmoc-\(\text{D-}\)Ala-OH, Fmoc-\(\text{L-}\)Thr (tBu)-OH and finally Fmoc-\(\text{D-}\)Ala-OH. Each cycle was two hours, giving a total synthesis time of 18 hours and 39 minutes.

When the synthesis was done, the resin was transferred from the synthesis tube to a VacMaster column, washed with DCM and dried. In a separate beaker the OAll-cleavage mixture was prepared. The mixture was composed of 3 mol equivalence of \((\text{Ph}_3\text{P})_4\text{Pd}\), compared to the peptide, and was dissolved in CHCl\(_3\)/ AcOH/ NMM (37:2:1) with the total volume of 15 ml. The cleavage reaction was preformed in darkness and under \(\text{N}_2\) (g) for two hours, with occasional stirring. The resin was then washed with 0.5% DIPEA in DMF and 0.5% (w/w) Sodium diethyldithiocarbamate (5x10 ml of each) to remove the catalyst and then washed with DMF (3x10 ml). The peptide was treated with DIPCIDI and HOBt, to activate the newly freed carboxylic acid. The free amine of the peptide backbone then attacks the activated carboxylic acid and the formation of the cyclic peptide backbone is made.
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**Figure 21. The cyclization reaction. Most of the peptide has been omitted for clarity.**

(i.) HOBt.

After the cyclization was done yet another Kaiser test (Appendix 2) was made, this time to verify that there were no free amines. When the HOBt-reaction mixture had been removed, the peptide was cleaved from the resin using a mixture of TFA/ EDT/ H₂O/ TIS (94.5:2.5:2.5:1). The total volume was 10.05 ml; each percentage was converted into 100 µl. This cleavage cocktail cleaves the peptide from the resin and removes the protection groups from the side chains.

Two-thirds of the cleavage mixture was evaporated with nitrogen gas and the peptide was precipitated using cold ether. Precipitation was done four times, and the ether phase was then discarded. The precipitate was solved in Milli-Q water and lyophilized.

4.3.2 Purification of CP8

The lyophilized peptide was almost insoluble, only DMF seemed to work. Due to the fact that DMF is an unpleasant solvent for HPLC and impossible to use in MALDI, another purification method than HPLC had to be used. The choice fell on extraction. The peptide was shaken with Milli-Q water 3*40 ml followed by one portion of ACN and the two more times with Milli-Q water giving the pure peptide. The purified peptide was then shaken ones again with Milli-Q water and lyophilized.

From a solubility test using water and ACN, 1 µl of peptide solution were taken for MALDI analysis and yet another µl was taken from the purified peptide solution, both spectra shown in figure 22.
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Figure 22. (i.) MALDI spectra of the crude CP8. (ii.) MALDI spectra of the purified CP8 (the sodium adduct is the biggest peak).

4.3.3 Analysis of CP8

4.3.3.1 Transmission IR

Measurements were made on a Bruker IFS48 Fourier transform infrared spectrometer, continuously purged with N$_2$ gas. Multilayer of CP8 was applied on a CaF$_2$-window by adding droplets of CP8 in DMF on to the surface. The sample was dried with N$_2$-gas. Each spectrum is obtained by averaging 100 interferograms at 2 cm$^{-1}$ resolution using deuterated triglycine sulphate (DTGS) detector.

The transmission IR spectrum shows how CP8 organizes when the sulfur of the cysteine does not form a covalent bond to the surface. Only the fingerprint region (1900-1300 cm$^{-1}$) is shown. This is where the important vibrations amide I (1660 cm$^{-1}$)
and amide II (1530 cm\(^{-1}\)) is found. The amide I indicate hydrogen bonding between the cycles.

![Image of IR spectrum](image)

**Figure 23.** Transmission IR spectrum of CP8

### 4.3.3.2 IR adsorption spectroscopy

Infrared Reflection Absorption Spectroscopy (IRAS) measurement was performed on a Bruker IFS66 Fourier transform spectrometer equipped with a grazing angle of incidence reflection accessory aligned at 85°. The infrared radiation was polarized parallel to the plane of incidence. Interferograms were apodized with a three term Black-Harris function before Fourier transformation. The instrument was flushed with nitrogen gas for 30 min after mounting a sample. During the measurement the sample chamber was continuously purged with N\(_2\) gas to reduce the water and carbon dioxide signals in the spectra. The spectra were recorded by averaging 2000 interferograms at 4 cm\(^{-1}\) resolution using liquid nitrogen cooled mercury cadmium telluride (MCT) detector.

The gold substrates used were prepared by electron beam evaporation of 2000 Å thick of Au at a rate of 10 Å/s onto a clean single-crystal Si (100) wafer. Before evaporation of gold film, the silicon wafers were first coated with 20-25 Å thick of Ti layer at a rate of 2 Å/s. The gold surfaces were cleaned in a 5:1:1 mixture of Milli-Q
water, 25% hydrogen peroxide, and 30% ammonia for 5 minutes at 80 °C and then thoroughly rinsed in Milli-Q water.

![Figure 24. Maleimide-terminated EG₄ disulfide](image)

The gold surfaces (2) were incubated with a 10 µM maleimide-terminated EG₄ disulfide dissolved in methanol for 24 hours. The surfaces were rinsed in methanol and ultrasonicated for 5 minutes. Before IR-measurements of the maleimide surface the sample plate were dried with N₂ gas. The IR-spectra of the pure maleimide surface is showed in figure 25a.

The maleimide modified gold surface were then rinsed with DMF and immediately incubated in a DMF-based solution containing CP8 (1mM) for 24 hours. The other maleimide modified gold surface were also rinsed with DMF and incubated in a CP8 (1mM) DMF solution but the incubation lasted for five days. After the incubation, the surfaces were rinsed in DMF and then ultrasonicated in DMF for 5 minutes. The surfaces were dried with N₂ gas and immediately analyzed.

The most important peak to consider is the amide I band at 1670 cm⁻¹ and amide II band at 1547 cm⁻¹. The amide I peak position of about 1668 cm⁻¹ indicates hydrogen bonding among the cyclic peptides (Note: Non-hydrogen bonded amide I is around 1685 cm⁻¹).

It is to be noted that the amide I peak assigned is a convolution of peaks coming from the amide part of the maleimide-EG₄ and cyclic peptides. The amide I is observed to be increasing in intensity relative to the peak at 1715 cm⁻¹ (assigned to be the C=O asymmetric stretch from the maleimide molecule). This indicates that the cyclic peptide is adsorbed on the maleimide-modified surface. Increased thickness seen in the ellipsometric measurement also supports the adsorption of cyclic peptide on the maleimide surface. Conformation of the cyclic peptides when adsorbed on a maleimide-modified EG₄ gold surface is hard to determine. The relative intensities of amide I and amide II coming from the cyclic peptides alone is hard to isolate and interpret due to the maleimide-EG₄ amide peaks contribution.
**Figure 25.** IRAS spectrum in the fingerprint region 2000-900 cm\(^{-1}\). (a) Maleimide-modified gold surface\(^{16}\). (b) 24 hours incubation of CP8. (c) Five days incubation of CP8.

### 4.3.4 Conclusion

Both the IR transmission measurement and the IRAS measurement indicate that CP8 self-organize when attached to a surface as well as unattached. The degree of success still needs more investigation, but the results so far are promising.
4.4 16-Mercapto-hexadecanoic acid [2-(3, 4-dihydroxy-phenyl)-ethyl]-amide

4.4.1 Synthesis of 16-Mercapto-hexadecanoic acid [2-(3, 4-dihydroxy-phenyl)-ethyl]-amide

Approximately 0.5 grams of the resin Fmoc-PAL-PEG-PS 1 was placed in a VacMaster tub and let to swell in 10 ml DMF. The DMF was then replaced with 10 ml of 20% piperidine in DMF to remove the Fmoc-protection group to give the free amine 2. In a separate beaker 75 µl of MPA, mixed with DIPCDI and HOBt, was solved in DMF. DIPCDI and HOBt activated the carboxylic acid by forming a pyrrolidinyl ester. The mixture was added to the resin after removal of the piperidine. The free amine then attacks the carbonyl carbon and results in the formation of an amide 3.

The thiol from the newly formed amide was then treated with dithioldipyrridine which forms a disulfide which in turn can be broken by yet another sulfide, the 16-Mercapto hexadecanoic acid to form intermediate 4.

16-Mercapto hexadecanoic acid was solved in 15 ml of DMF and added to the activated resin 5 ml at a time. When the thiopyridine is released from the disulfide bond it gives the solvent a bright yellow color thereby while adding the 16-Mercapto hexadecanoic acid in fractions the reaction could be followed by the decrease of yellow color in the solvent.

Once again a mixture of DIPCDI and HOBt was made and added, to the cleaned (3x5 ml DMF) resin, to activate the carboxylic acid. In a different beaker dopamine and DIPEA was mixed in DMF, and then added to the resin. The DIPEA was added to remove eventual hydrogen on the dopamine amine. The dopamine then forms the amide 5 with the carboxylic acid.

\[ \text{Scheme 1: (i.) Dithioldipyrridine, DMF} \]
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**Scheme 2:**
(i.) piperidine, DMF; (ii.) MPA, DIPCDI, HOBt, DMF; (iii.) (a) dithioldipyrirdine, DMF (b) 16-mercapto hexadecanoic acid, DMF (iv.) (a) DPICDI, HOBt, DMF (b) dopamine, DIPEA, DMF; (v.) DTT, ethyl acetate

The resin was washed with 4x5 ml of DCM to remove as much DMF as possible. Thereafter DTT solved in 10 ml DCM was added to the resin to reduce the disulfide bond and thus free the target molecule (6).

**Scheme 3:** The cleavage of a disulfide by DTT
The solution was collected in a clean beaker and a large portion of the DCM was evaporated. The remaining elute was purified by flash column chromatography using 100% hexane as mobile phase. The solution was evaporated and the clean product was taken for further examination, which are not presented in this work.

4.5 3-Mercapto-propionic amide \((\text{CH}_2\text{CH}_2\text{O})_5\text{CH}_2\text{CH}_2\text{NH} – \text{Nle} – \text{Tyr} – \text{Nle} – \text{Phe} – \text{Leu} – \text{Nle} – \text{Formyl}\)

4.5.1 Synthesis of 3-Mercapto-propionic amide \((\text{CH}_2\text{CH}_2\text{O})_5\text{CH}_2\text{CH}_2\text{NH} – \text{Nle} – \text{Tyr} – \text{Nle} – \text{Phe} – \text{Leu} – \text{Nle} – \text{Formyl}\)
Scheme 4: (i.) 3-Mercapto-propionic acid, DMF (ii.) (a) DIPCDI, HOBT, DMF (b) H₂N(CH₂CH₂O)CH₂CH₂NH₂, DIPEA, DMF; (iii.) Fmoc-L-Nle-OH, DIPCDI, HOBT, DMF; (iv.) Fmoc-L-Tyr (tBu)-OH, Fmoc-L-Nle-OH, Fmoc-L-Phe-OH, Fmoc-L-Leu-OH, Fmoc-L-Nle-OH, TBTU, DIPEA, Piperidine, DMF; (v.) DIPCDI, HOBT, Formic acid, DIPEA, DMF; (vi.) (a) 95% TFA, 5% H₂O (b) DTT, DCM

The synthesis of this biological active molecule was prepared in the same way as the synthesis of 16-Mercapto-hexadecanoic acid [2-(3,4-dihydroxy-penyl)-ethyl]-amide until intermediate 3 was synthesized. The thiol of intermediate 3 was treated with dithioldipyrridine, as was done in the previous synthesis, but to get a better indication of a successful synthesis; the disulfide was then treated with cystamine to generate a free amine, 7, that could be seen using Kaiser. Then 3-Mercapto-propionic acid was added to the resin, breaking the existing disulfide and creating a new, intermediate 8. This step was also monitored with a Kaiser test; the yellow colored result indicated a success. The carboxylic acid was activated using DIPCDI and HOBT, and then a diaminated oligoethylen glycol was added to the slurry forming amid 9. The formation was monitored with a Kaiser test, when a dark blue color was shown the reaction was thought to be complete.

Unfortunately the Kaiser test did not become blue, but instead red. Since Kaiser is not an absolute method the first amino acid, norleucine, pre-activated with DIPCDI and HOBT, was added to the resin. A small amount of the resin was then treated with
20% piperidine in DMF to remove the Fmoc-protection group; the free amine could then be seen with yet another Kaiser test. This indicated that intermediate 10 had been successfully synthesized.

The resin was then placed in a Pioneer™ Peptide Synthesis System. The amino acid sequence Tyr – Nle – Phe – Leu – Nle was synthesized, using two hour cycles. The norleucine already attached (10) worked as an amino acid already on support, and was used as an attachment point for the second amino acid and the following acids were attached to give the hexapeptide 11.

Once the peptide synthesis was done the resin was removed from the peptide synthesizing tube and placed in a VacMaster tube. A pre-prepared mixture, formic acid, DIPCDI and HOBt solved in DMF, were added to the resin. The slurry was stirred occasionally during the hour the reaction was let to go on. The solvent and the reactants were removed leaving the resin-bound intermediate 12.

The resin was washed with DCM several times to remove the DMF. Thereafter DTT solved in DCM was added to reduce the disulfide and free the target molecule 13. The DCM was then evaporated and 13 were solved in Milli-Q water and lyophilized.

The lyophilized product was then solved in ACN: H2O (1:9) + 0.1% TFA. 1 µl of the crude product solution was analyzed by MALDI. This analysis showed that no product at all had formed.

4.5.2 Conclusion

By monitoring the synthesis using Kaiser test the assumption can be made that something goes wrong when the resin is introduced to the conditions of the peptide synthesizer. Why this happens is uncertain.
5. Experimental

5.1 Synthesis and purification of R2A

The synthesis of the peptide R2A was performed on a Pioneer™ Peptide Synthesis System. 0.521 g of the resin Fmoc – Gly – PEG – PS was placed in a synthesis tube. Amino acids were weighted, in test tubes, as follows. Fmoc – L – Ala – OH 0.125 g in tube 22 and 26. Fmoc – L – Cys (Trt) – OH 0.234 g in tube 13. Fmoc – L – Asp (OtBu) – OH 0.165 g in tube 6 and 20. Fmoc – L – Ile – OH 0.141 g in tube 12. Fmoc – L – Leu – OH 0.141 g in tube 5, 7, and 21. Fmoc – L – Asn (Trt) – OH 0.239 g in tube 17. Fmoc – L – Gln (Trt) – OH 0.244 g in tube 9, 16, and 27. Fmoc – L – Arg (Pbf) – OH 0.260 g in tubes 1, 4, 8, 11, 15, 18, and 25. Fmoc – L – Ser (tBu) – OH 0.153 g in tube 2 and 24. Fmoc – L – Thr (tBu) – OH 0.159 g in tube 3, 10, and 23. Fmoc – L – Val – OH 0.136 g in tube 14 and 19. After synthesis the peptide was cleaved for 2 h using 9.45 ml of TFA, 250 µl of Milli-Q water, 250 µl of EDT and 100 µl of TIS. The cleavage solution were reduced to one-third using N₂ (g). The peptide was precipitated using cold dimethyl ether. The precipitated peptide was solved in Milli-Q water and lyophilized. After lyophilizing the peptide was purified using a Dynamax® Solvent Delivery System Model SD-1 with an rf column, using an isocratic mobile phase (22% ACN in water and 0.1% TFA). The detector was a Dynamax® Absorbance Detector Model UV-1. The purification of the peptide was monitored using MALDI (PerSeptive Biosystems Voyager – DE™ STR BioSpectrometry™ Workstation). After purification 0.05773 g of R2A was obtained, corresponding to a yield of 17.44% total exchange.

5.2 Synthesis and purification of R1A

The synthesis of the peptide R1A was performed in the same way as R2A. 0.514 g of the resin Fmoc – Gly – PEG – PS was placed in a synthesis tube. Amino acids were weighted, in test tubes, as follows. Fmoc – L – Ala – OH 0.125 g in tube 5, 7, 12, 14, 19, 21, and 26. Fmoc – L – Cys (Trt) – OH 0.234 g in tube 13. Fmoc – L – Asp (OtBu) – OH 0.165 g in tube 6 and 20. Fmoc – L – Asn (Trt) – OH 0.239 g in tube 1, 8, 15.
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and 22. Fmoc – L – Gln (Trt) – OH 0.244 g in tube 9, 16, 17 and 27. Fmoc – L – Arg (Pbf) – OH 0.260 g in tube 4, 11, 18 and 25. Fmoc – L – Ser (tBu) – OH 0.153 g in tube 2 and 23. Fmoc – L – Thr (tBu) – OH 0.159 g in tube 3. Fmoc – L – Tyr (tBu) – OH 0.183 g in tube 10 and 24. The peptide was cleaved and precipitated using the same conditions as R2A. After lyophilizing the peptide was purified using a Varian ProStar Solvent Delivery Module with an rf column, using an isocratic mobile phase (16.4% ACN in Milli-Q water containing 0.1% TFA). The detector was a Varian ProStar PDA Detector. The purification of the peptide was monitored using MALDI (PerSeptive Biosystems Voyager – DE™ STR BioSpectrometry™ Workstation).

5.3 Synthesis and purification of CP8

0.927 g of the resin Fmoc – PAL – PEG – PS was placed in a small sized peptide synthesis tube. The following amino acids and quantities were weighted in test tubes and placed in Pioneer™ Peptide Synthesis System. Fmoc – d – Ala – OH 0.249 g in tube 2, 4, 6 and 8, Fmoc – L – Cys (Trt) – OH 0.469 g in tube 3, Fmoc – L – Glu (OH) – OAll 0.328 g in tube 1, Fmoc – L – Gln (Trt) – OH 0.489 g in tube 5 and Fmoc – L – Thr (tBu) – OH 0.318 g in tube 7. Each attachment cycle was for 2 h, giving a total synthesis time of 18 hours and 39 min. After synthesis the resin was moved to a VacMaster tube and washed with DCM (4x10 ml). The resin was dried in a vacuum exicator. A Kaiser test was preformed verifying the presence of free amines. To cleave the OAll protection group 0.853 g (Ph₃P)₄Pd solved in CHCl₃/AcOH/NMM (37:2:1) with a total volume of 15 ml was added to the resin. The reaction was let to go for two hours with occasional stirring and under N₂ (g). The reaction mixture was removed and the resin was washed with 0.5% DIPEA in DMF and 0.5% (w/w) Sodium diethylthiocarbamate (5x10 ml of each), followed by 3x10 ml pure DMF. 150 µl DIPCDI was solved in DMF (5 ml) and added to the resin. After 30 min 0.1422 g HOBt solved in DMF (5 ml) was added to the slurry. The reaction was let to go for one hour with occasional stirring. The reaction mixture was removed and the resin was washed with DMF (3x10 ml) and then with DCM (3x10 ml).The resin was dried in preparation for cleavage. The peptide was then cleaved from the solid support using a mixture 9.45 ml of TFA, 250 µl Milli – Q water, 250 µl EDT and 100 µl TIS. Approximately 5 ml of the cleavage solution was evaporated with N₂ (g). The peptide
was precipitated with cold dimethyl ether. The peptide was purified by extraction using 3*40 ml Milli-Q water followed by 40 ml ACN and then 40 ml Milli-Q water.

5.4 Synthesis and purification of 3-Mercapto-hexadecanoic acid [2-(3, 4-dihydroxy-phenyl)-ethyl]-amide

1.04523 g of Fmoc – PAL – PEG – PS (0.24 mmol/g substitution level) were placed in a VacMaster tube and let to swell in 10 ml of DMF for 1 h. Thereafter the resin was treated with 20% piperidine (3x10 ml). The solvent was removed. 0.0602 g (0.47 mmol) DIPCDI and 0.0612 g (0.45 mmol) HOBT was solved in 5 ml DMF and added to the resin. 150 µl MPA solved in 10 ml DMF was added and the mixture was stirred occasionally for one hour. The reaction mixture was removed. A surplus of dithioldipyridine (0.1031 g, 0.47 mmol) was added and the slurry was stirred occasionally for 30 min. The solvent was removed. 0.3842 g (1.33 mmol) of 3-Mercapto-hexadecanoic acid was solved in 15 ml of DMF and was added to the resin 5 ml at a time, the third time no yellow color was detectable by the human eye. The resin was washed with DMF (3x5ml). 0.0583 g (0.46 mmol) DIPCDI and 0.0601 g (0.45 mmol) HOBT was solved in 5 ml DMF was added to the resin and stirred occasionally for 30 min. The solvent was removed. 0.0703 g (0.46 mmol) 3-Hydroxytyramine and 80 µl (0.46 mmol) DIPEA was solved in DMF and added to the resin, and left there for 30 min. The solvent was removed. The resin was washed with DMF (2x5ml) and then DCM (2x5ml). A surplus of DTT was solved in 5 ml DCM and added to the resin. The solution was collected and purified by flash column chromatography with 100% hexane as mobile phase.

5.5 Synthesis and purification of 3-Mercapto-propionic amide (CH$_2$CH$_2$O)$_5$CH$_2$CH$_2$NH – Nle – Tyr – Nle – Phe – Leu – Nle – Formyl

2.17891 g of Fmoc – PAL – PEG – PS (0.21 mmol/g substitution level) were placed in a VacMaster tube and let to swell in 10 ml of DMF for 1 h. The solvent was removed. 0.23672 g (1.7519 mmol) HOBT mixed with 0.265 ml (1.6924 mmol) DIPCDI solved in 5 ml DMF were added, followed by addition of 0.150 ml (1.6916

41
mmol) MPA in 5 ml DMF. The mixture was let to react for 1.5 h with occasional stirring. The reaction mixture was removed. 0.37021 g (1.6804 mmol) 2, 2 – Dithioldipyridine in 10 ml DMF was added and left to react for 30 minutes with occasional stirring. The solvent was removed. 0.5062 g (1.76 mmol) 16-Mercapto hexadecanoic acid solved in 10 ml DMF were added to the resin, and let to react for 1 hour. The solvent was removed. The resin was washed with 2* DMF/MeOH/DCM and placed in vacuum exicator O/N. 1.00543 g of the activated resin was placed in a new VacMaster tube and let to swell for an hour in DMF. The solvent was removed. 0.135 ml (0.86 mmol) DIPCDI and 0.11378 g (84 mmol) HOBr solved in 10 ml DMF were added to the resin and reacted for 1 hour. Reaction mixture was removed. 0.12863 g (1.12 mmol) NHS solved in 10 ml DMF were added, and left to react for 1 hour with occasional stirring. The reaction mixture was removed. The resin was solved in 10 ml of DMF. ~0.23672 g (0.844 mmol) 2-[2-(2-{2-[2-(2-Amino-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethyamine and 295 µl (1.68 mmol) DIPEA solved in 1 ml DMF was added drop-vise, while stirring. The solvent was removed. To the resin a mixture of 0.299 g (0.85 mmol) norleucine, 0.116 g (0.86 mmol) HOBr and 0.120 g (95 mmol) DIPCDI solved in DMF was added. The reaction was let to go for 1 hour. The solvent was removed. The resin was placed in a Pioneer™ Peptide Synthesis System and the amino acid sequence Tyr-Nle-Phe-Leu-Nle was synthesized, using Nle 0.298 g, Leu 0.298 g, Phe 0.327 g, Nle 0.298 g and Tyr 0.386 g. The resin was placed in a VacMaster tube. 0.032 ml (0.85 mmol) formic acid was mixed with 0.135 ml (0.86 mmol) DIPCDI and 0.1141 g (0.84 mmol) HOBr in DMF were poured on the resin, and was left to react for 1 hour. The reaction mixture was removed. 0.13554 g (84 mmol) DTT solved in 10 ml DCM was added and let to react for 90 min. The elute was reacted with 3 mol equiv. dithioldipyridine and then with 3 mol equiv. cystamine. The DCM was evaporated and the product was solved in 15 ml ACN:H2O (1:9). The product was then purified using HPLC.
6. Future approaches

6.1 What to do with the R2A concept

The research on controlling a peptides secondary structure has given positive results but still needs to be investigated further. As mentioned above, the helical content of the peptides while interacting with the silica particles is more a tendency than a clear helix. By re-designing the peptide or modifying the existing designs might improve the helicity. Another approach for further investigations is to make use of the cysteine introduced in the design. Does an attached molecule affect the structural change, or does it still arrange itself towards a helix?

In a different approach; can a peptide be designed to go from a β-sheet to a α-helix, or to a random coil or the reverse, the possible routes are many.

6.2 More research on CP8

The measurements on CP8 have proved that the self-organization of the cycles into tubes works, but the IRAS measurement is disrupted by amides in the surface linker molecule. By synthesizing a linker lacking amides the signals would hopefully improve and the interactions would be more pronounced. If only the amides deriving from CP8 can be seen, the stacking of the molecule would be clear. That is if the molecule is stacking perpendicular or parallel to the surface plane.

Other approaches that can be interesting to investigate are the creation of the slightly mentioned CP12 or keeping the amino acid length of the cycle and make a re-design to be able to compare the results better.

6.3 Solid-phase synthesis of SAM-molecules

In this work both a success and a failure has been presented for solid-phase synthesis. Examining the failed synthesis one sees that something goes wrong when the resin is placed in the peptide synthesizer machine. Can this be circumvented by synthesizing the peptide sequence as a first step using an amino acid as was used in the synthesis of
CP8? Would the synthesis be easier if the one of the amines in the oligo ethylene glycol molecule was protected by for example an Fmoc group? The possibility to improve the synthesis route is many and only time can tell if they will help.
7. Appendix

7.1 Chou-Fasmans rules

<table>
<thead>
<tr>
<th></th>
<th>a. a</th>
<th>α-helix</th>
<th>β-strand</th>
<th>Reversed turn</th>
<th>N-term.</th>
<th>Middle</th>
<th>C-term</th>
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<td>0.67</td>
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7.2 Kaiser test

A small amount of resin is placed in a clean test tube and is washed with three times 2 ml EtOH. The resin is then treated with 2-3 drops of reagents 1, 2 and 3 (see below). The test tube is then placed in a preheated block at 100°C for five minutes. The resin will then be yellow if no free amines are present and blue if there are free amines.

Reagents:
1. Cyanide, 2 ml 0.01 M KCN diluted to 100 ml with pyridine.
2. Ninhydrin, 500 mg ninhydrin in 10 ml BuOH.
3. Phenol, 80 g phenol in 20 ml BuOH.
8. Acknowledgements

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9. References


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