Developing polymer micelles conjugated with antimicrobial peptides

Hannah Merlan Anor

Thesis submitted for the degree of
Master of Science in Chemistry
60 credits

Institute of Chemistry
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

[August / 2023]

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Abstract

Antimicrobials peptides (AMP) are the new antibiotics. Antibiotics have in the recent years lost a lot of its ability to defeat microbial infections. This is due to overuse leading to resistance bacterial strains flourishing. AMPs, however, cannot avoid the proteolytic enzymes found in the body.

Therefore, in this study method development on how cys-indolicidin could be conjugated to the polymer Brij 100 was investigated, to promote stability and protection. First approach was to modify Brij, by changing the hydroxyl end group to carboxylic acid. The product would then further be functionalized with maleimide, which is used in conjugation reaction such as the thiol-maleimide reaction. The approach was changed when the experiments gave undesired results. Instead of modifying Brij 100, the use of 6-maledimidohexanoic acid was explored instead, by using Steglich-esterification. An esterification which is carried out under mild conditions. Purification of esters produced was proven difficult, and the compound was not able to be used in the next step of making the peptide conjugated product.

To confirm the effectiveness of the thiol-maleimide reaction without using the target polymer. DSPE-PEG-Mal, a PEGylated lipid derivative, was used to conduct the procedure to see if it was functional. The conjugated product with DSPE-PEG-Mal showed promising result.

Preface

The work presented in this thesis was executed at the Department of Chemistry, University of Oslo in the research group of soft matter. Under the supervision of Professor Reidar Lund, alongside his co-supervisors PhD candidate Szymon Szotak and PhD candidate Nora Uggerud. The work was conducted in the period of August 2021 – August 2023 and was the continued work of the bachelor's project that was conducted the spring of 2021.

Thank you to Reidar Lund for giving me the opportunity to complete a master thesis in the group. Thank you to Szymon and Nora for helping me with the experiments even if it seemed like nothing was going to plan. Thank you to the rest of the group for being a great support even though we worked on different things, especially thanks to my fellow master students in the group that I could ask my stupid chemistry questions.

Lastly, I would like to thank all my friends and family for the support even when you did not understand anything of what I was talking about. The support was really needed!

All figures are rendered in Chemdraw, Biorender, Pymol or Microsoft PowerPoint.

Acronyms and abbreviations

WHO World Health Organization

AMR Antimicrobial resistance

AMP Antimicrobial peptide

NP Nanoparticle

CAMPs Cationic antimicrobial peptide

PEG Polyethylene glycol

PEO Polyethylene oxide

NMR Nuclear magnetic resonance

HPLC High-performance liquid chromatography

UV Ultraviolet

SAXS Small angle x-ray scattering

SAS Small angle scattering

TFA Trifluoroacetic Acid

Cys Cysteine

Mal Maleimide

Mhx 6-maleimidohexanoic acid

RT Room temperature

ESRF European Synchrotron radiation facility

PBS Phosphate buffered saline

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1 Introduction

World Health Organization, WHO, declared antimicrobial resistance as one of the 10 global public health treats.[1] Antimicrobial resistance or AMR, are making microbial infections harder to treat, leading to longer hospitals stays, expensive treatments, and in some cases death.

Since the discovery of Penicillin in 1928 by Alexander Flemming (1881 – 1955), coupled with the work of Howard Walter Florey (1898–1968) and Ernest Boris Chain (1906–1979) in the 1940s made treatment with antibiotics accessible. Antibiotics have been effective at treating bacterial infections, however antibiotics are been observed to be gradually losing their ability to treat infections in the past decades.[2, 3] Antibacterial resistance is not a new phenomenon. Since the dawn of time, bacteria have had the need to protect themselves from natural organic molecules that have inhibited their growth and killed them. For survival bacteria have needed to develop resistance genes. Medical exploitation of antibiotics facilitates an environment for resistant bacteria to grow, as the antibiotic kills of the non-resistant, bacteria, the resistant strains are left to flourish.[4] Methicillin-resistant Staphylococcus aureus (MRSA) is an example of overuse of antibiotics and bacterial strains becoming resistant to antibiotics. Therefore, a need for new antibacterial compounds or new sources.

A potential candidate that has been researched in recent years are peptides with antibacterial properties. Antimicrobial peptides, abbreviated to AMPs, are found in the innate immune system of everything living.[5] Bacteria, plants, and animals have shown to have these antimicrobial peptides that not only affect bacteria, but with a broader spectrum where some also affects fungi, virus, and some even cancers.[6, 7] Which is unlike most antibiotics which are selective and only affect one or two microorganisms.[8] The first discovery of an AMP was done by Dubos from *Bacillus* spp. found in soil, making the first AMP discovered the peptide named gramicidin[9,10]. Bacteria are less likely to produce the same resistance to AMPs as with conventional antibiotics such as penicillin. [11]

However, a downfall to using AMPs is that they can easily be degraded by enzymes found in the body, such as proteases found in the digestive system and in serum. Meaning admission of AMPs is limited and may result in lessened effect or the AMP having no effect at all. Not

only can peptides undergo proteolysis, but they have also shown some cytotoxicity towards human cells. To overcome these problems several solutions have been proposed, one being changing the natural l-amino acids to the d-amino acid or other amino acid derivatives to avoid cleavage of proteases. [12,13] Another solution may be changing the chemical properties of the peptide, to become a prodrug that will undergo a chemical transformation to become active once administered into the body.[13, 14]

Another potential solution is to increase the stability of the peptide is by incorporating in nanoparticles (NPs). Which can be done in different ways, one example is encapsulating the peptides or coating NPs with peptide.[15–17]

2 Aim of study

The aim of this study is to create polymer micelles with commercial surfactant, Brij S 100, conjugated with peptides with antimicrobial properties. As a proof-of-concept indolicidin was chosen as the target peptide. The conjugation will be performed as a Michael-addition, by modifying indolicidin with an extra amino acid, cysteine. As the indolicidin used in this study has been modified, it will be referred to as cys-indolicidin to separate from indolicidin that is modified with cysteine.

As mentioned in the introduction, conventional antibiotics seem to slowly lose their ability in fighting of infections. AMP have therefore been studied to be a potential new alternative. However due to AMPs being peptides they are easily degradable and is needed to be protected in a way.

Brij 100 by itself can not directly be attached to cys-indolicidin, there is a need of modification of the polymer as well. The conjugation reaction that was chosen to be explored was the facile thiol-maleimide reaction. Can the brij 100 be functionalized with a maleimide group to be able to conjugate it to cys-indolicidin?

The study involved exploring procedures to conjugate the surfactant Brij S 100 with indolicidin, and for this procedure to be further used to conjugate other AMPs on Brij S 100 that can be used in making mixed liposomes which can be an alternative to today's antibiotics.

3 Background

3.1 Peptides

Peptides are chains built up off 20-50 amino acids linked by peptide bonds similarly to proteins, which are more complex with longer chains of amino acids often consisting of peptides bound together in complex structures. Peptides are produced naturally in bacteria, plants, and animals, but can also enter biological systems through consumption. A peptide that may be more familiar is insulin. Insulin is a hormonal peptide and functions as signal-molecule in the body for many important biological processes, such as regulating uptake of nutrients after a meal.[18] Similar to insulin there are other peptides that are biologically active and contribute to other biological processes for example peptides with antimicrobial properties. These types of peptides have been given the name antimicrobial peptides due to this, and the name are often shortened to AMP.

3.1.1 Therapeutic peptides

Insulin, as mentioned previously is a hormone peptide. Some cannot produce insulin naturally and needs it supplemented as a drug for the metabolic reaction to function properly. This insulin deficiency is most known as Diabetes mellitus. Now due to insulin being able to be used as a drug, people with diabetes are able to live a relatively normal life.[18]

3.1.2 Antimicrobial peptides

The primary role of AMPs is protecting the host against harmful pathogens, and they play important parts in the innate immune system of these organisms. In humans there have been found that innate AMPs are most abundant in areas where organs are most exposed to airborne pathogens, such as in wounds, the skin, and in mucosa membranes – lines cavities and surfaces such as nasal cavity, mouth and eyes. [19, 20] AMPs pathogen targets are not only bacterial as several AMPs such as LL-37 also have shown to have an effect on cancerous pathogens, viral pathogens, and fungal pathogens as well[21]. Several peptides may exhibit action towards more than one pathogen target. However due to the evolution of antibacterial resistance, the primary focus have been in the antibacterial peptides – ABPs[22].

AMPs are generally peptides with an overall net charge of +2 to +9 [6], with an amphipathic structure having separate hydrophobic and hydrophilic domains[23]. It is speculated that the positive charge of the peptide plays a central role in its mode of action in getting rid of the harmful pathogen. Where several modes of action is still being investigated, such as membrane interference and interaction with intercellular mechanisms.[24–27]

Indolicidin is another peptide that has been seen to have antimicrobial properties. Indolicidin, with the amino sequence - ILPWKWPWWPWRR, is a short peptide consisting of only 13 amino acids. It is found in bovine neutrophils, a sort of white blood cells which helps overcome infections. Indolicidin is in the group of cationic AMPs – CAMPs, having a positive net charge. The amino acids lysine, Lys, and arginine, Arg, are responsible for the overall positive net charge of the peptide.

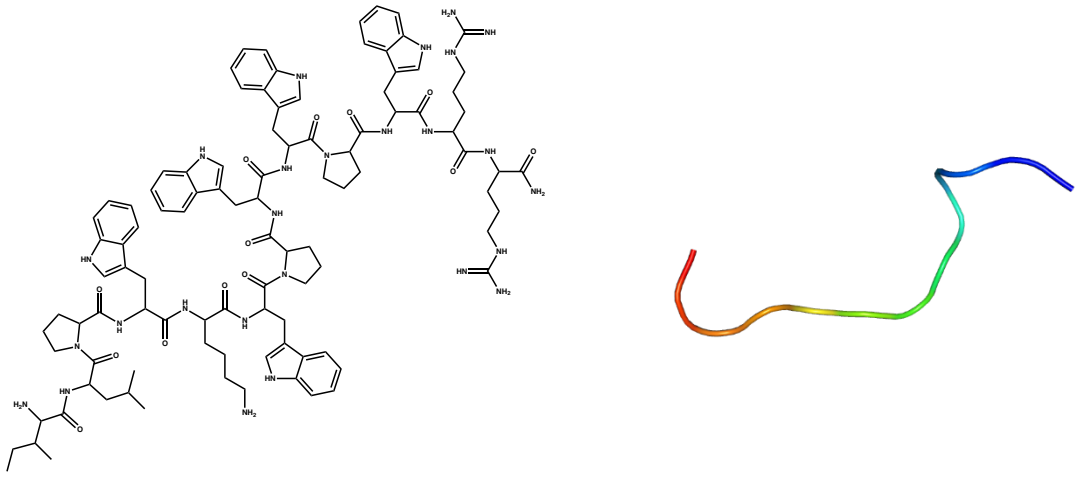

Figure 1: Indolicidin, structures rendered in Chemdraw (left) and Pymol (right)

The structure of indolicidin is linear and can be observed in Figure 1. The secondary structure of indolicidin is categorized as an extended structure, other major structure groups which peptides are categorized in are α-helical, β-sheet and loop.[22]

As peptides are already used as a pharmaceutical, there can be a possibility that antimicrobial peptides can aswell.

3.2 Bioconjugation

As previously stated, AMPs are easily degraded by peptide degrading enzymes, proteases, and have a need to be modified. One of this modification is to conjugate the peptides to other molecules, such as block copolymers, that will form nanoparticles.

When a molecule of biological origin is conjugated with another molecule this is called bioconjugation. In some case the conjugated complex may be entirely synthesized, but it is used towards biological applications. These processes can be modified to be highly selective by choosing different types of reaction, reagents and reaction conditions.[28]

Polymer bioconjugation is generally described as a synthetic macromolecules conjugated to a biological molecule, such as lipids, nucleic acids, proteins, peptides and carbohydrates.[29] This is used to give either macromolecule or the biological molecule other physical and chemical properties.

One of the common macromolecules used are polyethylene glycol (PEG), used as a shielding agent, for active substances. Conjugation with PEG increases its size and hides the protein/peptides structure, preventing the attack of antibodies and antigen and reducing degradation by proteases[30]

3.3 Polymers and surfactants

Along with peptides we also have polymers which also is a large molecule. Both often referred alongside each other as macromolecules. Polymers, similar to peptides, consist of smaller “building blocks” called monomers. These are covalently bound together making these long polymer chains. The monomeric unit that builds up the polymer need to have at least two bonding sites such that it can be linked to other monomers and form the long polymer chains. A polymer can consist of one species of monomer or more. When they only contain one species it is simply referred to as polymer. With two different monomer units in a chain, it is known as copolymer, and with three in one chain is a terpolymer. Copolymers can then be divided into four categories: 1. Statistical copolymer, 2. Alternating copolymer, 3. Block copolymer, and 4. Graft copolymer.[31]

3.3.1 Polyethylene oxide / polyethylene glycol

Polyethylene oxide or also referred to as polyethylene glycol, are more commonly abbreviated to PEO and PEG correspondingly. PEO is a polymer consisting of the repeating monomer of polyethylene oxide, general structure seen in Figure 2

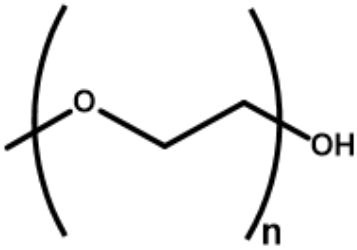

Figure 2: General structure of PEG

3.3.2 Surfactants

The name surfactant comes from the word surface active agent, they lower the surface tension or tension between liquids, solid and liquid or liquid and gas. Surfactants are commonly detergents, emulsifiers, wetting agents, foaming agents

The Brij family of surfactants are a group of non-ionic surfactants with a polyoxyethylene part and a polymethylene chain, giving the molecules a hydrophilic and hydrophobic part respectively and making them amphiphilic structures. Meaning the molecules have a part which is water loving and the other part being water-hating. These molecules can be simplified to a concept structure shown in Figure 3.

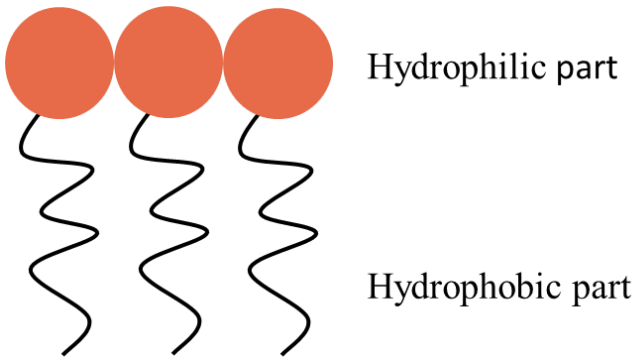

Figure 3: Amphiphilic structure

Brij S100 is a non-ionic surfactant, and have the simplified structure shown in Figure 4, with the chemical name polyoxyethylene (100) stearyl ether. Surfactants such as Brij S 100 and other in the Brij family have long been used in pharmaceutics as solubilizers and emulsifiers to aid poorly soluble drugs.

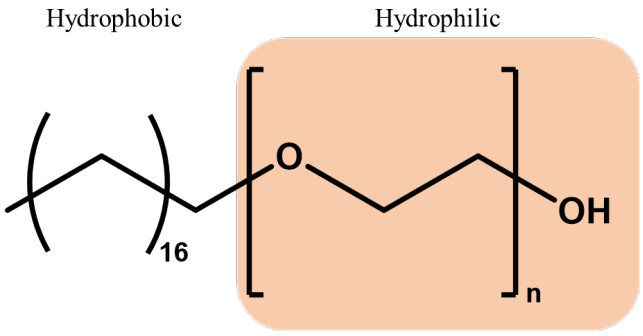

Figure 4: Simplified structure of Brij S 100, a surfactant

Surfactants have shown biocompatibility, and conjugating the peptides which is easily enzymatic degraded there is opportunity to make a bioavailable antimicrobial compound.

The Brij 100 have to become functionalized before the peptide can be conjugated.

3.3.3 PEG lipid derivatives

DSPE-PEG is PEG derivative containing lipid molecules, DSPE – 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol. DSPE-PEG conjugated with other end groups such as amine, biotin, and carboxylic acid to change functionality. In Figure 5 the structure of DSPE-PEG-Maleimide, showcasing the different parts of the molecule.

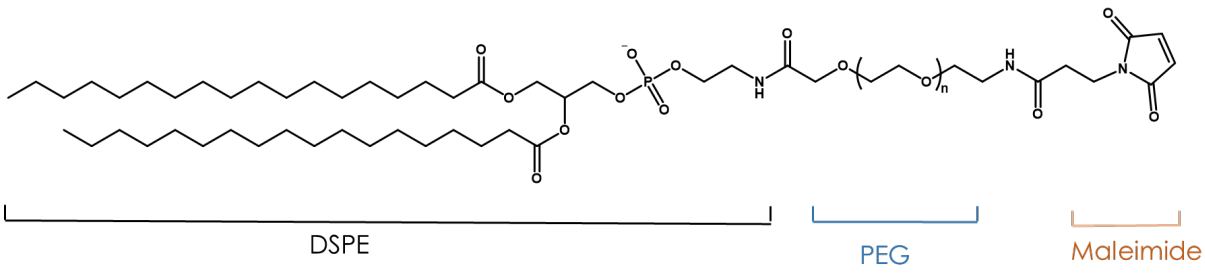

Figure 5: General structure of DSPE-PEG-Mal

DSPE is the hydrophobic element of the molecule and conjugating it with PEG increases the hydrophilicity of the molecule. The hydrophobic properties of the DSPE makes it possible to

encapsulate hydrophobic drugs, while the increase of hydrophilicity of the molecule due to the polyPEG increases the solubility allowing for delivery of the drug.

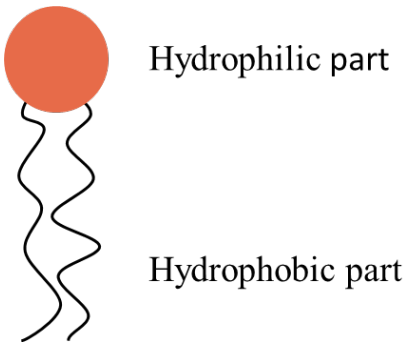

Figure 6: Simplified visualized structure of lipids

DSPE-PEG is an amphiphilic block copolymer used in drug delivery, as it is biocompatible and biodegradable and can be functionalized with various functions as mentioned previously. The polyPEGs in it can self-assemble to liposomes or micelles, and by functionalization with maleimide we give it the functionality of selectively reacting with thiol such as in cysteine. DSPE-PEG-Maleimide is therefore popular to use in bioconjugation of protein, peptides, and antibodies.[32]

3.4 Self-assembly

Surfactants, polymers, and lipids are used in drug delivery as many newly discovered pharmaceuticals may have low solubility in aqueous solutions. Some can also be easily degraded by enzymes or the low pH in the stomach, leading to problems with administration and decreasing bioavailability and effect.

3.4.1 Micelles

Micelles are formed by self-assembly of amphiphilic structures, such as lipids and surfactants. The structures will assemble such that it's more thermodynamically stable, in a micellar structure, where the hydrophobic part of the molecule will orient away from the water forming a hydrophobic core. Hydrophobic drugs can sit in the core away from water making micelles capable as a drug carrier.

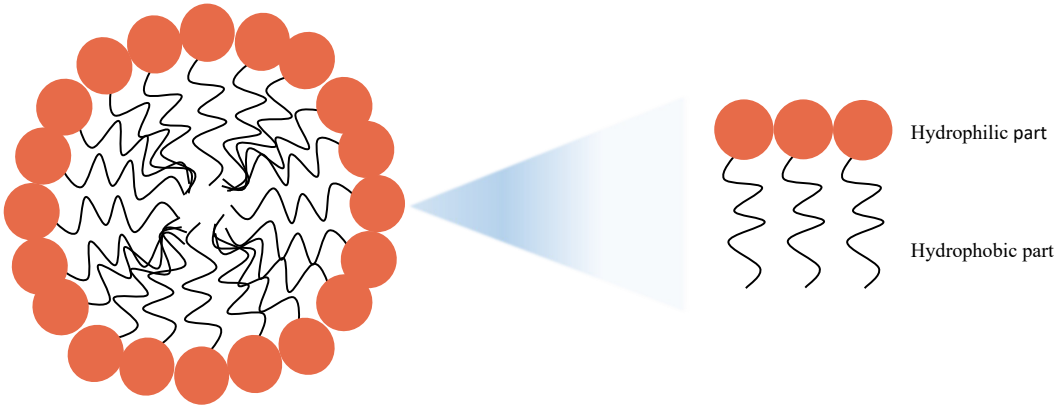

Figure 7: micelle structure

The surfactant Brij S 100 forms micelles in aqueous solution as it is an amphiphilic molecule. The micelle structure can be seen in Figure 7. With its long hydrocarbon tail consisting of 18-carbons, making this the hydrophobic part, and the PEO chain giving the hydrophilic part.

4 Theory

4.1 Oxidation of primary alcohol with Bobbitt's salt

Alcohols are often oxidized by using acidified dichromate solutions or permanganate, and other oxidizing agents like Dess-Martin periodinane and Jones reagent. However, the issue with using these sorts of oxidizing agents is degradation of the polymer chain and structural changes. Making the oxidation selective is therefore essential. A better alternative for an oxidizing agent will be the use of "Bobbitt's salt", 4-(Acetylamino)-2,2,6,6-tetramethyl-1-oxo-piperidinium tetrafluoroborate, shown in Figure 8.

Bobbitt's salt is an oxoammonium salt derived from 4-acetamido- (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl, with the trivial name TEMPO[33], developed by James M. Bobbitt

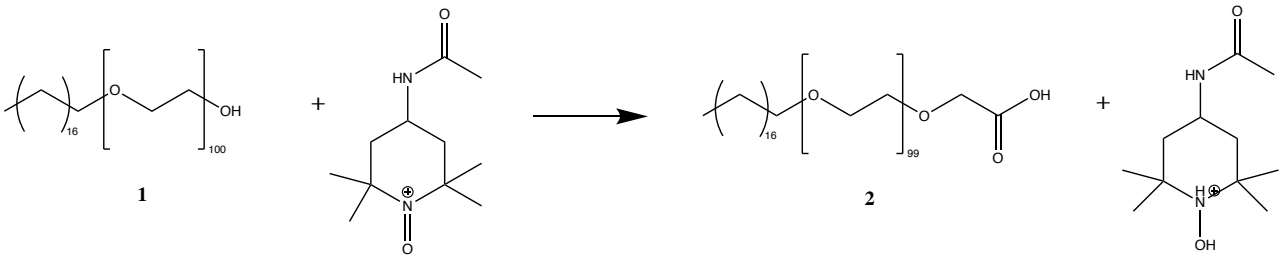

Figure 8: Scheme of oxidation of Brij® S 100 with Bobbitt's' salt

Oxoammonium salts are favored due to them being able to do oxidation under mild conditions, and will not have the same drawbacks as other oxidizing agents such as heavy metals, which can be carcinogenic and can cause pollution to the environment.[34,35] They also have the possibility of being recycled and are very user friendly. One more overlooked property of these salt are they are colorimetric. Meaning that while the reaction is progressing color change may be observed, making it possible to physically view that the reaction is taking place and progressing.

In oxidation with TEMPO, NaClO, sodium hypochlorite, must be present as a second oxidative agent, as well as Br^- as a catalyst for the reaction.[36] The use of "Bobbitt's salt" eliminates the need for other extra agents. Additionally, excess NaClO can cause chain degradation and avoiding this may be preferable. The reaction with Bobbitt's salt and Brij® S 100 is described in, where Brij® S 100 is compound 1 reacting with Bobbitt's salt, giving an oxidized product which is seen as compound 2 and a reduced Bobbitt's salt derivative.

The oxidation will be a two-step reaction, where the alcohol will oxidize to an aldehyde, and with an excess of oxidizing agent, further oxidize into carboxylic acid. The oxidized polymer will still retain its properties, and the molecular weight will be close to that of the original un-carboxylated polymer.

4.2 Diels-Alder reaction

A Diels-alder reaction requires two components, a diene and a dienophile ("diene loving" compound). These two form a cyclohexene product, as seen in Figure 9, making the Diels-Alder reaction a cycloaddition reaction. [37]

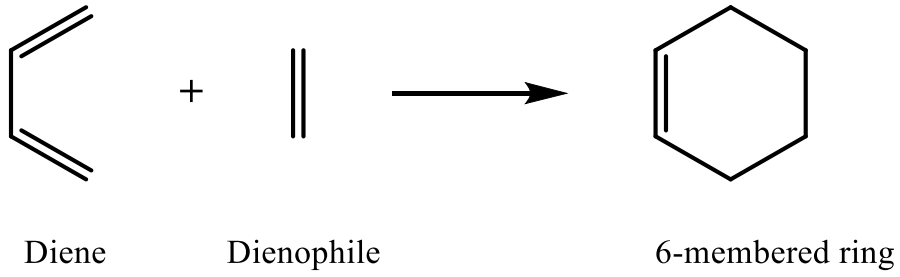

Figure 9: Scheme of Diels-Alder cycloaddition

For the Diels-Alder reaction to occur the diene need to be conjugated, meaning the double bonds have to be adjacent to each other, see Figure 10 below.

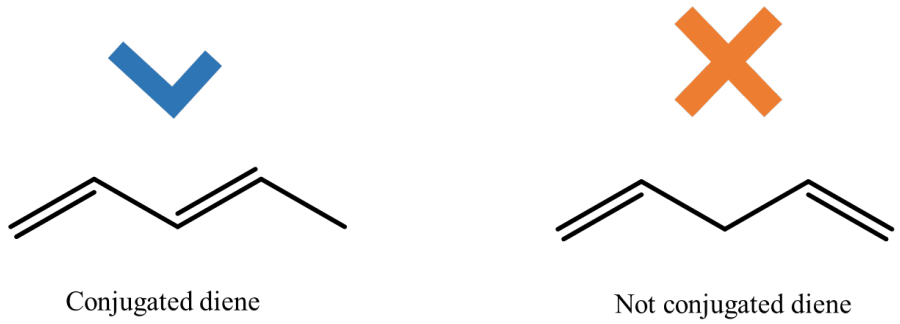

Figure 10: Conjugated diene and not conjugated diene

The diene and dienophile break three C-C π bonds (double bond) and form two new C-C σ bonds (single bond) and one new C-C π bond. The mechanism for which bonds are formed and broken can be viewed in Figure 11. The bond making and breaking happens in one single step, with no observable intermediates.

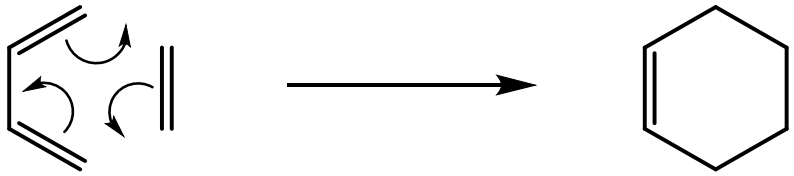

Figure 11: The mechanism of a Diels-Alder reaction

4.3 Steglich esterification

Esterification of carboxylic acids and alcohols are usually performed with a Fischer esterification, which uses a strong acid as a catalyst. An alternative method of esterification was reported by Wolfgang Steglich and Berhard Neisen in 1978 utilizing the amide coupling agent *N,N'*-dicyclohexylcarbodiimide (DCC) and the organocatalyst 4-dimethylaminopyridine (DMAP)[38].

The Steglich-esterification makes it possible for sterically demanding and acid labile substrates to form esters, as the esterification proceeds under mild conditions, involving ambient temperatures and often a neutral pH. The general reaction can be viewed in Figure 12.

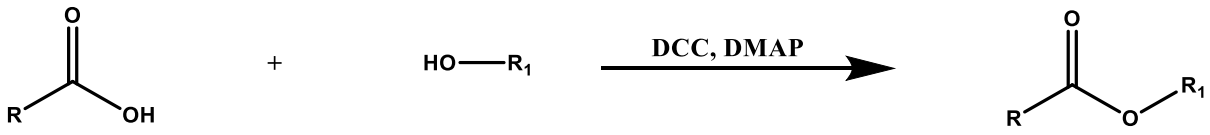

Figure 12: General scheme of a Steglich reaction.

4.4 Thiol-maleimide reaction

The thiol-maleimide reaction also known as Michael- addition or Michael reaction. The Michael reaction is a conjugate addition of a carbon nucleophile to the β-carbon of an unsaturated compound, resulting in a new bond. [37]

In bioconjugation, maleimide or maleic acid anhydride is popular group to utilize due to it reacting easily with thiol. The double bond in the maleimide undergoes an alkylation reaction with the sulfhydryl (-SH) group to form a thioether bond[39]. The usual reaction of thiols and maleimides can be observed in the scheme in Figure 13 below.

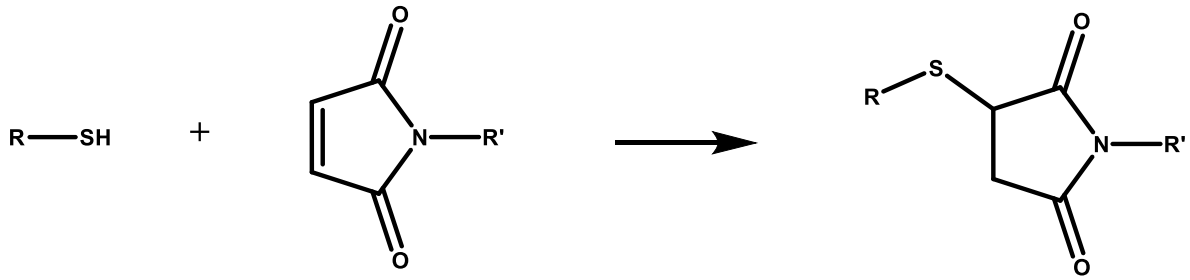

Figure 13: Scheme of thiol-maleimide conjugation

In biological systems this reaction is often performed with the natural amino acid, cysteine which contains a sulfhydryl group. Due to cysteines low abundance in proteins and peptides, and enhanced nucleophilicity it commonly used as modification target.[40,41]

The facile reaction of maleimide and thiols are pH dependent. The maleimide is selective for thiols in the pH range of 6.5 and 7.5. With higher pH there may be cross-reaction with amino groups.[39] Similar to oxidation of Bobbitt's salt, maleimide reaction are easily observable while the reaction is progressing. However, instead of the progress being able to be observed with the naked eye, the reaction may be followed spectrometrically, with for example an UV-detector. UV-detectors are described in more detail in subsection 4.6.1. The absorbance of the double bond at 300nm will disappear as it reacts [42].

4.4.1 Thiols and disulfide bridges

As mentioned above the abundance of cysteine residues in proteins and peptides are low. Even so, an important aspect to cysteine in proteins and peptides are the formation of disulfide bridges. These bridges form from a reaction of the sulfhydryl groups on two cysteine residues either in the same molecule or residues on different molecules, creating intramolecular or intermolecular disulfide bonds. These bonds are a central structural element in folding and stabilization of peptides and proteins.[40,43,44]

However, in reactions with the sulfhydryl having it in these bonds makes it inaccessible. To prevent this, proteins and peptides can be reduced with reduction agents, for instance glutathione and ascorbic acid. Glutathione and ascorbic acid are natural reduction agents found in the cells and their concentration is a factor in if the peptides or proteins form

disulfide bridges. With abundance of oxidized gluthathione (GSSH) formation of cysteine bridges are most likely to occur.[44,45]

In protein chemistry a more commonly used reductant is Dithiothreitol, shortened to DTT, given the name Cleland's reagent after the one that first described the reduction reaction. The reduction reconstitutes the -SH group which can then be reacted further. The reaction proceeds completion as the formation of the 6-membered ring is favored. [46,47] Reaction mechanism can be viewed below in Figure 14.

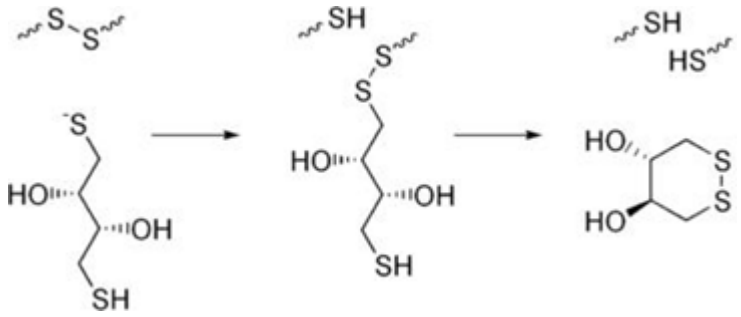

Figure 14: Reaction of DTT and disulfide bonds, picture from blogpost from AG Scientific. [48]

4.5 Nuclear magnetic resonance – NMR

Nuclear magnetic resonance, or more frequently referred to as the abbreviation NMR was used in this project for confirming and to validate the structure of molecules of the experiments performed.

NMR makes it possible to determine molecular structures based on nuclei behavior. This behavior is referred to as a specific orientation or spin orientation. Not all nuclei behave like this. Nuclei with even atomic mass and atomic number such as ^{12}C and ^{16}O will not exhibit NMR and will not be observable. However, both proton ^{1}H and carbon ^{13}C nucleus have an odd number and will then be observable. Signals are observed by applying a magnetic field, the nucleus with spin will orient in relation to the magnetic field and flip, resulting in a signal. However not all ^{1}H nuclei and ^{13}C nuclei absorb at the same magnetic frequency, if this was the case it wouldn't make it possible to see individual atoms in the molecule and therefore not seeing the whole structure. The different nuclei have different absorption frequency in relation to the environment, meaning the surrounding atoms. All the different nuclei in a molecule have electrons surrounding them and setting up tiny magnetic fields. The small magnetic fields affect the neighboring nuclei. The neighboring nuclei is therefore shielded from the

applied magnetic field, giving lower signals. The nucleuses will have a slightly difference in shielding making it possible to observe a distinct signal for each of the distinct ^{1}H or ^{13}C nuclei. However these two nuclei cannot be observed at the same time on a spectrometer, this is due to the amount of energy needed to flip the different nuclei.[49]

In a NMR spectrum the position of where the different nuclei signals are observed is called chemical shift. The difference in chemical shift is as mentioned above, the small difference of the magnetic field due to the surrounding electrons. The nuclei that are more strongly shielded by electrons require a stronger applied magnetic field to be brought to resonance and are absorbed on the right side of the spectrum, while less shielded nuclei are observed on the left of the NMR spectrum. Most proton have a chemical shift between 0-10, and by knowing where the different shifts of protons it is possible to tell immediately which proton a molecule contains. See Figure 15 for the chemical shifts given by the different protons in selected functional groups.[50]

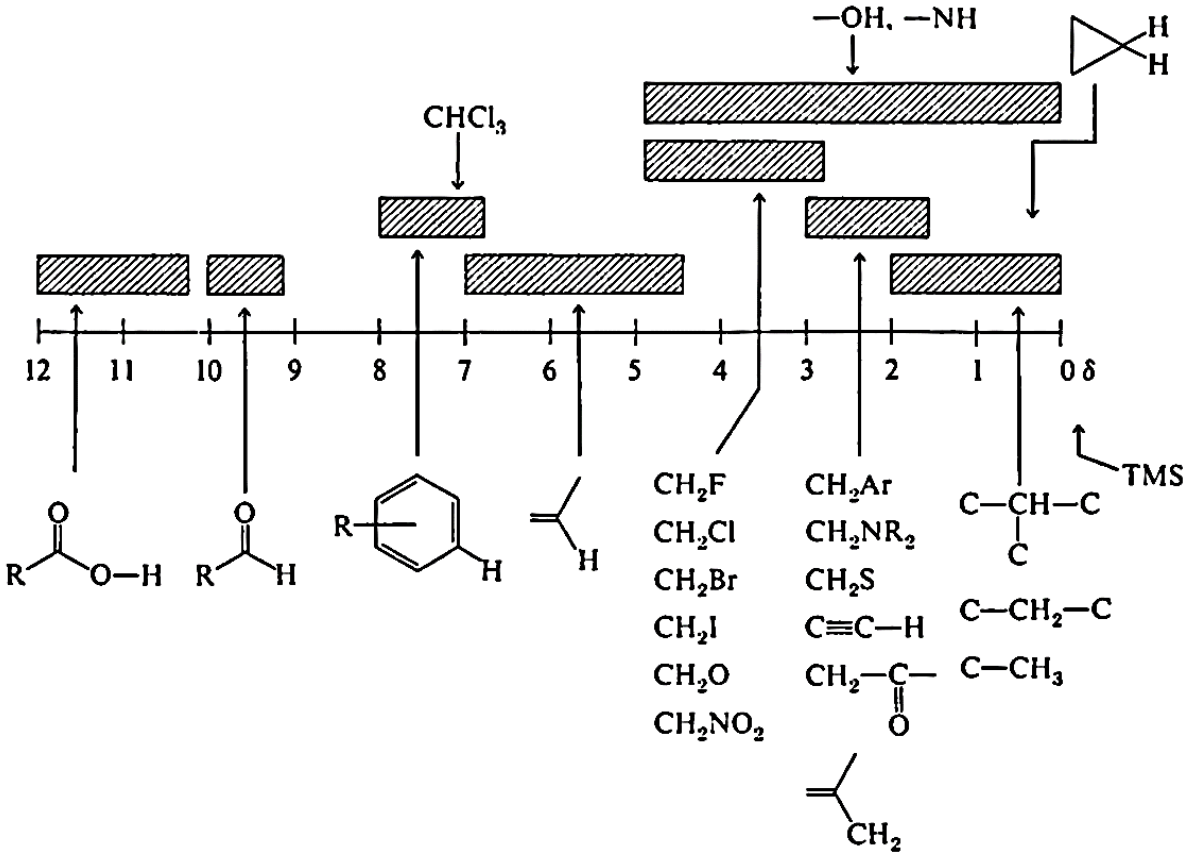

Figure 15: Proton chemical shift ranges for different functional groups, figure from Pavia (2015). [50]

4.6 Chromatography of peptides

Chromatography is a technique in analytic chemistry used to separate and quantify components in a mixture by utilizing two different phases, a stationary and a mobile phase. A mobile phase is used to move the components trough the stationary phase. The components will interact with both the stationary phase and the mobile phase. The chemical and structural differences of the components will make them interact differently with the phases making it possible to separate them.

High-performance liquid chromatography abbreviated to HPLC, formerly known as high-pressure liquid chromatography. HPLC is one liquid chromatography method which utilizes high pressure. The mobile phase is a liquid, and the stationary phase is found in a column. For components to be able to be separated by HPLC they need to be soluble and not volatile. A typical HPLC system, shown as a schematic diagram in Figure 16, contains the following: a pump, an injector, a separation column, a detector – there can also be several for one system, and a data system where the data can be viewed [51, 52].

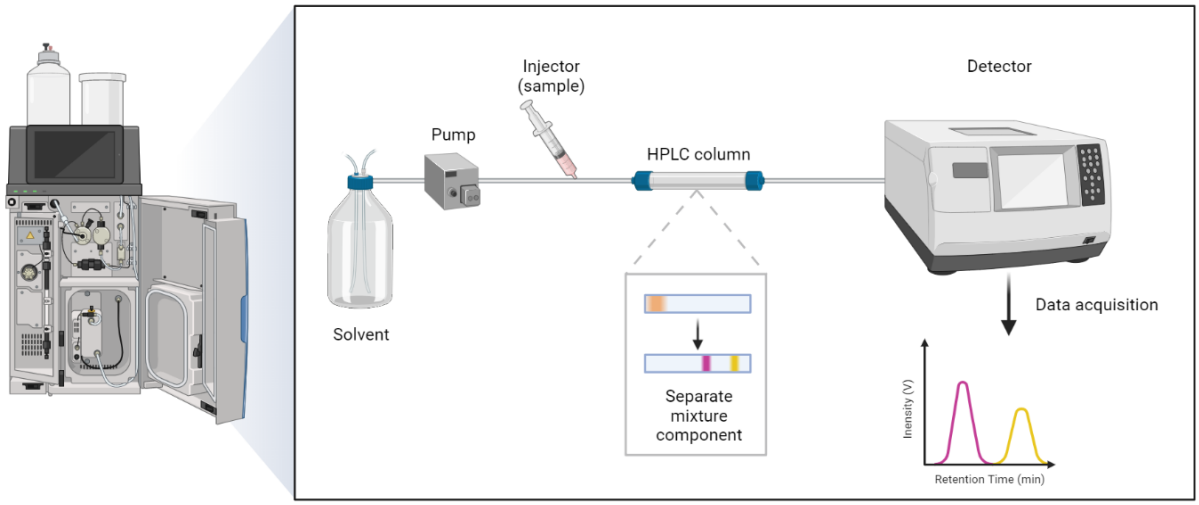

Figure 16: A typical HPLC system. The figure was created using Biorender.

The mobile phase travel through the injector, bringing the sample to the separation column also known as the stationary phase in this system. On the column the different components in the sample are separated. The different components will eluate differently granting different retention times. By varying the mobile phase to have more interaction with the analytes (components of interest) will shorten the retention time. This is due to the analytes having a higher affinity to the mobile phase and will travel faster trough the column. By varying the

stationary phase to granting more interactions with the analytes will result in slower elution time and retention time will be longer.[51]

How does separation of small molecules and peptide differ? Since peptides are larger molecules how they are separated are slightly different. For the peptides to eluate the critical concentration of organic modifier or also known as the less polar organic phase to water, needs to be reached[53]. That is why a gradient of the mobile phase is run when separating peptides – meaning the composition of aqueous solvent and organic is changed over time. Small molecules have a continuous interaction with the column and the mobile phase, while peptides are too large and will get stuck on the column until the right concentration. The peptide will stick to the column with what is called "hydrophobic foot", the peptides will then be separated based in differences in the "foot". These differences come from difference in sequence or conformation[53, 54].

4.6.1 Detector

Detectors are used to measure the analytes in the sample, they are responsible for the quantification and qualification of the analytes. Different types of detectors can be used when analyzing the compounds. The most common detectors coupled to HPLCs for peptide analysis are UV-vis and RI, where UV-vis is the most used.

UV

UV-vis stands for ultraviolet and visible, as it uses ultraviolet light and visible light. UV-vis is one kind of UV detector. The detector measures the amount of UV light and visible light that are absorbed by the compounds. Meaning that for compounds to be detected they need to be chromophoric. Most pharmaceuticals are chromophoric, however there are some that are not like amikacin. Needing to have chromophoric compound is limiting for use of UV and will be a need to rather use universal detectors such as RI. Additionally the mobile phase when using UV needs to be optical transparent [55].

The principle for UV detection is based on Beer-Lambert law. See Equation 1, below.

Equation 1. $A = \varepsilon cb$

Where A is absorbance, ε is the molar absorptivity constant ($M^{-1}cm^{-1}$), c is the concentration of the sample (M), and b is the path length (cm)[51].

4.7 SAXS

SAXS – Small angle x-ray scattering, is a fundamental tool used in different science fields to study the structure of materials on a nanometer scale. When X-rays interact with matter it scattered and redirected in different directions. By analyzing the scattering pattern, it is possible to gain information of shape and size, and arrangements of atoms. [56–59]

5 Instrument and method

The instruments and methods used for this study are all mentioned below

5.1 NMR

All NMR experiments were performed in-house, using mainly Bruker AV 400 NEO NMR spectrometer on automation. Some of the experiments were performed on a Bruker AVI 600 NMR spectrometer and Bruker AVIII HD 400 NMR instrument. The instruments are controlled by TopSpin, and the same software was used to analyze and showcase the spectra. Solvents used in the experiments were mostly DMSO-d6, however in aqueous solutions 10% D_2O was added.

5.2 HPLC

LC experiments were performed using a Synkam gradient pump coupled with a Waters 486 tunable absorbance detector. The column utilized was Avantor Vydac 218TP104 C18 HPLC column, particle size 10 microm 4.6x250 mm.

All compounds containing the peptide cys-indolicidin was run with a 25-75% B in 40 min H_2O(0.1% TFA):Acetonitrile (0.085% TFA). The absorbance was set to 220nm on the Waters detector. All the experiments were performed in RT.

5.3 SAXS

All SAXS data were measured at the European Synchrotron radiation facility (ESRF), on the BioSAXS beamline BM29. The measurements were performed in 25˚C / 298K.

6 Experimental

The water used in experiments is Milli-Q® Type 1 Ultrapure water, in the experiment are only noted as water for simplicity. For dialysis experiments Elix® Type 2 Pure water was used, also noted as water in the respective experiments containing dialysis.

6.1 Oxidation with Bobbitts' salt

Procedure was given from Dr. Willner of Forschnungszentrum Jülich, which was work from a previous student under Dr.Willner's supervision. The polymer that was used at Jülich was C18-PEO_5.

The oxidation experiment was performed on both commercially available polymer Brij® S 100 and in-house made C18-PEO_5.

6.1.1 Oxidation of Brij100

Table 1: Table of reactants and solvents used in oxidation of Brij 100

Chemical	**Supplier**	**Molecular weight**	**Amount**	**Mole**
Brij® S 100	Sigma-Aldrich	Mn ~4670 g/mol	113,3 mg	0,024mmol
4-(Acetylamino)-2,2,6,6-tetramethyl-1-oxo-piperidinium tetrafluoroborate (Bobbitt's salt)	Sigma-Aldrich	300.20 g/mol	60,9 mg	0,202mmol
Sodium hydroxide 20% solution (NaOH)	Sigma-Aldrich	40 g/mol	0.3 mL	
Sodium bisulfite solution	Sigma-Aldrich		0.3 mL	

($NaHSO_3$)				
Hydrochloric acid 37% (HCl)				
Ethyl acetate	Sigma-Aldrich		5mL	
Chloroform	Riedel-de Haën		2.5mL	

All the chemicals and the amount can be viewed in Table 1: Table of reactants and solvents used in oxidation of Brij 100 above.

Brij 100 was dissolved in 1mL water, the Bobbitt's salt was then added, and the flask was flushed with nitrogen and left under nitrogen overnight.

The next day the 20% NaOH solution and $NaHSO_3$ solution was added to the reaction and left for 24h under stirring. Precipitation formed, the solution was colorless while the solids were and slight orange color. The next day the pH was adjusted with 1M HCl until pH 4-5, the precipitation dissolved.

The mixture was then extracted with ~1mL EtAc five times. The aqueous solution was then extracted with ~0.5mL $CHCl_3$ five times. The $CHCl_3$ phase was the dried with $MgSO_4$ and filtered. The solvent was removed under pressure.

48mg of product was extracted.

Solvent change

The procedure was slightly modified based on the procedure performed in the paper from Qiu et al.[34]

Table 2: A tablet of reactants and solvents used in oxidation of Brij100

Chemical	Supplier	Molecular weight	Amount	Mole
Brij® S 100	Sigma-Aldrich	Mn ~4670 g/mol	110 mg	0.024mmol
4-(Acetylamino)-2,2,6,6-tetramethyl-1-oxo-piperidinium tetrafluoroborate (Bobbitt's salt)	Sigma-Aldrich	300.20 g/mol	38.2 mg	0.13mmol
Acetonitrile	Sigma-Aldrich			
Diethyl ether	Sigma-Aldrich		10mL	
Hydrochloric acid 37% (HCl)				
Sodium chloride	Sigma-Aldrich		Dissolved in water to make saturated solution	

The list of chemicals and the amount can be viewed in Table 2 above.Table 2: A tablet of reactants and solvents used in oxidation of Brij100

The oxidation on Brij 100 was also performed with a different solvent than water. Water was replaced with CH_3CN:H_2O (90:10).

Brij was dissolved in 1mL CH_3CN:H_2O, Bobbitt's salt was added and it was left over three days for the solution to turn orange and cloudy. The solution was then left for another week, a total of 10 days, until the solution turned transparent orange/yellow. 1mL of water was then added.

The mixture was then extracted with 2mL diethyl ether five times. The aqueous phase remained yellow. The organic phase was washed with 10% HCl, the aqueous phase remained yellow again. Lastly it was washed with NaCl brine, and dried with $MgSO_4$ and the solvent was removed under pressure.

12mg of product was obtained.

6.1.2 Oxidation of C18-PEO5

The polymer, C_{18}-PEO_5 was made in house, for previous research completed in the group. The same procedure as explained in subsection 6.1.1 was utilized for the C_{18}-PEO_5 polymer.

C_{18}-PEO_5 was dissolved in 1mL water, the Bobbitt's salt was then added. The flask was flushed with nitrogen and left in nitrogen overnight.

The next day 3mL of 20% NaOH solution and 3mL NaHSO solution was added to the flask. Orange precipitation appeared. The pH of the ration mixture was adjusted with 1M HCl until pH 4. The precipitation did not dissolve, the mixture was left under vigorous stirring until everything was dissolved.

The solution was extracted with 10mL EtAc five times, the organic phase gained a slight orange color. The aqueous solution was then shaken with 10mL $CHCl_3$ four times. Lastly the $CHCl_3$ phase was washed with water. The $CHCl_3$ phase was then dried with $MgSO_4$ and filtered. Solvent was removed under pressure.

From the chloroform phase 32mg of product was extracted. The EtAc solution was also evaporated leaving 63mg of additional product. Giving a total of 95mg product.

6.2 Diels-Alder reaction, furan-maleimide adduct

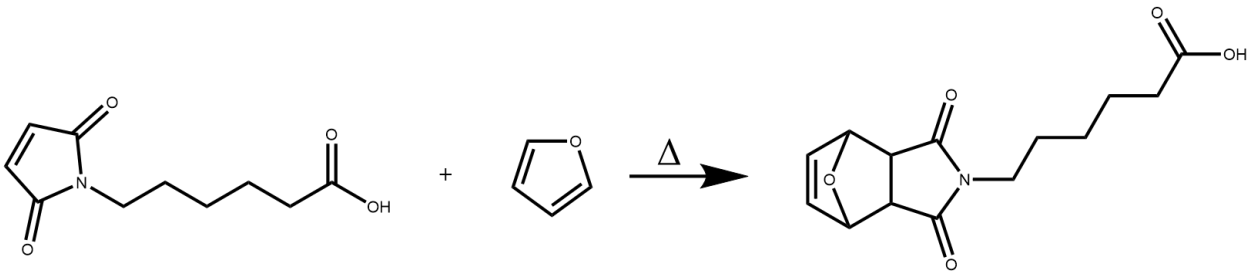

Figure 17: Reaction scheme of the furan-maleimidohexanoic acid adduct

Protecting of maleimidohexanoic acid (Mhx) with furan, performing of a Diels-Alder reaction as described in section 4.2

Table 3: A table of the reactants and solvents used in the Diels-Alder recation

Chemical	**Supplier**	**Molecular weight**	**Weight**	**Mole**
Maleimidohexanoic acid	Sigma-Aldrich	211.21 g/mol	2.12 g	10.0mmol
Furan	Thermo fisher Scientific	68.07 g/mol	1,404 g ~ 1,5 mL	20.6 mmol

To produce the furan-Mhx adduct, seen in Figure 17, furan was first dissolved in 100mL water. The Mhx was then added to the furan-water solution. The solution was heated to 60°C. A cooler was used to prevent solvent loss.

The reaction was conducted under 60°C for 24h. After 24h the reaction was removed from the heat and left to cool down to RT. After cooling solvent was slowly removed under pressure, until it precipitated. About 5-10mL solvent left.

The precipitate was filtered with vacuum and left to dry overnight on the bench. What was left was a fluffy white powder.

Yield: 1.732g, 62%

6.3 Functionalization of Stearic acid

Stearic acid was tried to functionalize with maleimide, the procedure was planned to be executed on Brij® S 100 as well. The procedure is a carbodiimide coupling reaction that are usually used for peptide synthesis and amide bond formation. The general reaction scheme can be observed below in Figure 18.

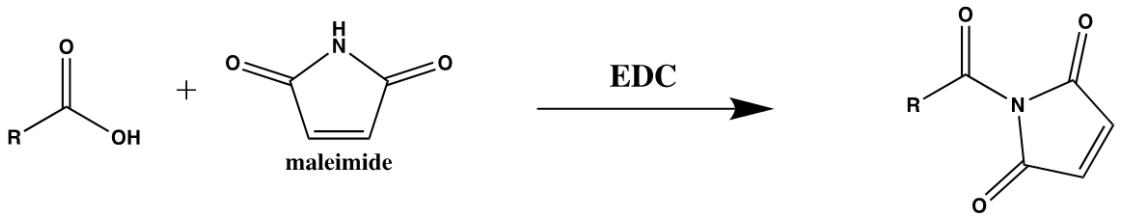

Figure 18: General reaction of acid functionalization with maleimide

Table 4: Table of the reagents in the reaction of functionalizing stearic acid

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**
Stearic acid	Aldrich	284,48 g/mol	1.007 g	0.004 mol
N-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, HCl)	Sigma-Aldrich	155.24 g/mol	0.665g	0.004 mol
Maleimide	Sigma-Aldrich	97.07 g/mol	0.362 g	0.004 mol
N,N-Diisopropylethylamine (Hünig's base)	Sigma-Aldrich	129.24 g/mol (Density - 0.742 g/mL)	1.5 mL	0.01 mol
1-Hydroxybenzotriazole hydrate (Hobt)	Sigma-Aldrich	135.12 g/mol	0.557 g	0.004 mol
Dimethylformamide (DMF)			70 mL	

All the chemicals and the amounts for this reaction can be viewed in Table 4 above.

Stearic acid was mixed with maleimide and Hobt in DMF. The EDC was mixed in to the Hüning's base first, before adding the base mixture to the DMF solution. The reaction was then left under nitrogen for 3 days. The solution started as a milky yellow color, but ended up with a brown color. There was trials in removing solvent and the obtained mass of product was 2.01g which correspond to 146.8%. The product ended up as a sticky mass, since DMF is hard to remove.

To try to get most of the solvent out, the experiment was carried out again, this time only for 24 hours and with half the amount of reagents. Half of the mixture was evaporated with vacuum, this resulted in a wax-like product. The amount from just evaporation was 0.630g

The other half was extracted with ethyl acetate. The solution was first diluted with 25mL water and extracted with 20mL ethyl acetate four times. The DMF:water phase gained a yellow color while the organic phase was cloudy with a pink/red color.

The ethyl acetate was then washed with NaCl brine, and precipitation occurred. The ethyl acetate was collected and removed under pressure. The product obtained was again brown colored and wax-like. The amount from extraction with ethyl acetate was 0.126g.

The total amount obtained from this reaction was then: 0.756g, giving 173.4% yield.

6.4 Steglich esterfication of Brij and Maleimidohexanoic acid

6.4.1 Steglich-estefication with DIC

The same procedure was performed for both protected and unprotected Mhx, meaning with and without furan. The scheme for the esterification with protected Mhx can be spotted in Figure 19, below.Figure 19: Reaction scheme of conjugation of Brij100 to furan-maleimidohexanoic acid adduct

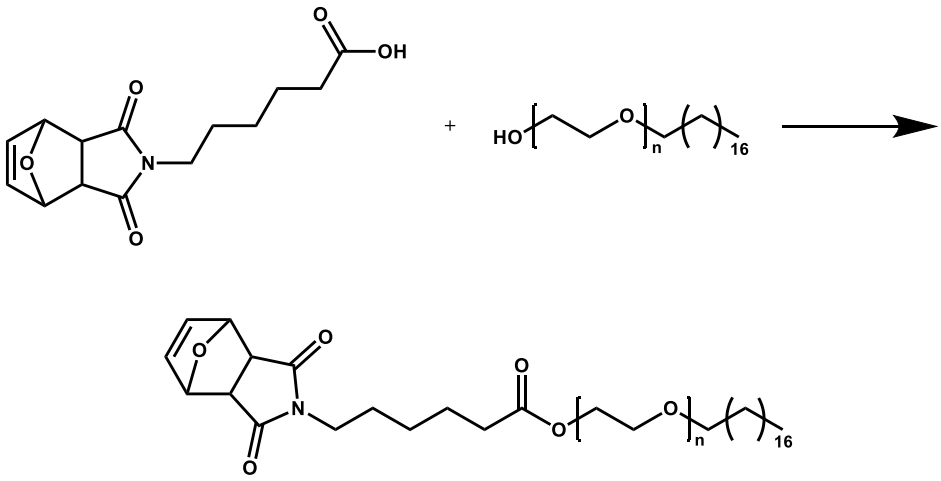

Figure 19: Reaction scheme of conjugation of Brij100 to furan-maleimidohexanoic acid adduct

The procedures for two of the reactions are the same and only differing the acid used.

6.4.1 a)

The experiment was first carried out with unprotected acid. Everything used and their amount is presented in Table 5.Table 5: The chemicals used in esterfication of Brij 100 and maleimidohexanoic acid.

Table 5: The chemicals used in esterfication of Brij 100 and maleimidohexanoic acid.

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**
6-Maleimidohexanoic acid		211.21 g/mol	129 mg	0.61mmol
Brij S 100	Aldrich	Mn ~4670 g/mol	1 g	0.219 mmol
4-dimethylaminopyridine, DMAP	Aldrich	122.17 g/mol	65 mg	0.54 mmol
N,N'-Diisopropylcarbodiimide, DIC	Thermo fisher scientific	126.20 g/mol (Density – 0.806 g/mL)	83 µL	0.53 mmol
Dichloromethane, DCM	Sigma-Aldrich		15 mL	

Diethyl ether	Sigma-Aldrich		45 mL	

Brij 100 and Mhx were dissolved in DCM with DMAP. The mixture was then put under nitrogen and molecular sieve beads (3Å) were added to the solution. DIC was then added in, the reaction mixture was left under nitrogen overnight. The next day the solution had turned dark brow/red. The solvent was removed under vacuum using a rotary evaporator.

The collected crude sample was 0.904g, yield % = 80.5%.

To purify the product the sample was washed with cold diethyl ether. 15mL of cold diethyl ether was used. When adding the diethyl ether, the product remained as precipitation while solution gained a milky-pink color. The product was vacuum filtered and carefully washed over with more of the cold diethyl ether. The washing was repeated two more times, the solution turned less cloudy and white with each washing. The product was left to dry further on a glass plate.

After the washing the collected sample was 0.436g, giving a yield of 38.8%

6.4.1 b)

Table 6: The chemicals for the esterfication reaction of Brij100 and furan-maleimidohexanoic acid adduct using.

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**
Furan-Mhx adduct (from section 6.2)		279.29 g/mol	142 mg	0.51mmol
Brij S 100	Aldrich	Mn ~4670 g/mol	1.024 g	0.219 mmol
4-dimethylaminopyridine, DMAP	Aldrich	122.17 g/mol	65.8 mg	0.54 mmol
N,N'-Diisopropylcarbodiimide, DIC	Thermo fisher scientific	126.20 g/mol	82.6 µL	0.53 mmol

		(Density – 0.806 g/mL)		
Dichloromethane, DCM	Sigma-Aldrich		15 mL	

The furan-Mhx adduct gotten from experiment described in 6.2 was used in this procedure. The amount and the rest of the chemicals used in the procedure is in Table 6, above.

DMAP was dissolved in dry DCM with some molecular sieve beads (3Å). Brij 100 was then added and left to dissolve, then the furan-Mhx was added and the solution was put under nitrogen. After a couple of minutes DIC was added to the mixture and the reaction was left under the nitrogen overnight.

The solution started clear, after 2 hours the solution had turned a slight orange color. After 24 hours the color was orange/brown in color. The solvent was removed under pressure using a rotary evaporator.

For purification it was dissolved in diethyl ether. The product did not dissolve the product. There was no obtained yield as during the purification the product was spilled.

6.4.1 c) Change in molar ratio of DMAP

The procedure was slightly changed based on the paper by Gholizade et al.[60]

Table 7: Table of chemicals for esterficationof Brij100 and furan-Mhx with molar change of DMAP

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**
Furan-Mhx adduct (from section 6.2)		279.29 g/mol	140.7 mg	0.5 mmol
Brij S 100	Aldrich	Mn ~4670 g/mol	2.37 g	0.51 mmol
4-dimethylaminopyridine, DMAP	Aldrich	122.17 g/mol	6.16 mg	0.05 mmol

N,N'-Diisopropylcarbodiimide, DIC	Thermo fisher scientific	126.20 g/mol (Density – 0.806 g/mL)	85.6 µL	0.55 mmol
Dichloromethane, DCM	Sigma-Aldrich		10 mL	

The major changes in reagents were only the amount of polymer and the amount of DMAP. There were also some changes in how the reagents were added to the reaction mixture. The amount and the other reactants are listed in Table 7, above.

The acid (Furan-Mhx adduct) was dissolved in DCM and cooled down to 0°C, and the flask was flushed with nitrogen. When the solution was cooled down Brij was added. When the Brij polymer was dissolved DMAP was added as well.

When everything was dissolved and cold, the DIC was added. After five minutes the ice bath was removed, and reaction was left in room temperature for 48 hours. After the two days the reaction had a slight orange/pink color. The solvent was evaporated with vacuum.

1.817g was collected, some remained in the flask as the solvent bubbled a lot up the sides of the flask during evaporation. Yield: 72.2%

6.4.2 Steglich -esterification with EDC-HCl

The esterification was again performed, but with a change in the carbodiimide reagent. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC-HCl, was utilized instead of the previous DIC, mentiond in 6.4.1. The amount and the rest of the chemical used in the experiment is listed in Table 8, below.

Table 8: Chemicals for the esterfication of Brij100 and furan-Mhx using EDC-HCl

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**

Furan-Mhx adduct (from section 6.2)		279.29 g/mol	101.4 mg	0.36 mmol
Brij S 100	Aldrich	Mn ~4670 g/mol	1.66 g	0.36 mmol
4-dimethylaminopyridine, DMAP	Aldrich	122.17 g/mol	17.66 mg	0.15mmol
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC-HCl		191.7 g/mol	86.02 mg	0.45 mmol
Dichloromethane, DCM	Sigma-Aldrich		4 mL	

Brij 100 and furan-Mhx were dissolved in 3mL dry DCM. The flask was then flushed with nitrogen and DMAP was added to the solution. Meanwhile EDC-HCl was dissolved in 1mL DCM and added to the mixture with a syringe. The mixture was left to stir under nitrogen. After the 22 hours an additional 20mL of DCM were added and the solution was filtered. The solvent was evaporated under pressure using rotary evaporator. 1.7g of product was obtained, giving a yield of 97.1%

6.5 Peptide conjugation

6.5.1 Conjugation of PEG-Maleimide and indolicidin

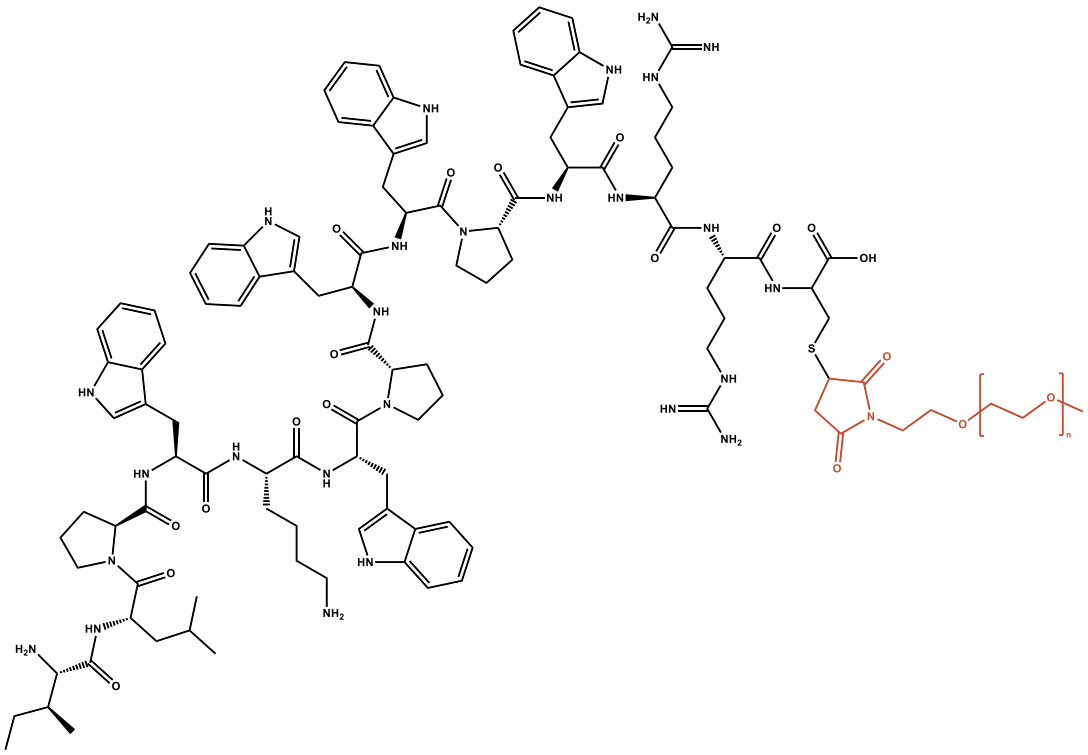

Figure 20: The product of PEG-Mal with indolicidin, PEG-Mal in orange.

Procedure obtained from Broadparm on conjugation with thiol and maleimide.[61]

Table 9: Table of reactants and solvent used in the conjugation of PEG-Maleimide and indolicidin

Chemicals	**Supplier**	**Molecular weight**	**Amount**	**Mole**
Methoxy polyethylene glycol maleimide (PEG-Mal)	Sigma-Aldrich	M_n ~5000 g/mol	251.5 mg	0.05 mmol
Cys-indolicidin	TAG Copenhagen	2010.0 g/mol	10mg	0.005 mmol
Phosphate buffered saline (PBS buffer pH 7.4)	Gibco		12.5mL	

The amount and the reagents are listed in Table 9 above.

First a maleimide solution was made, the PEG-maleimide was dissolved in 2.5mL of buffer. The liquid was clear. Indolicidin was then dissolved in 10mL PBS buffer, the liquid was cloudy, and some precipitation occurred.

The two solutions were mixed and shaken, and the resulting mixture was then refrigerated overnight. Due to not having an available freeze dryer at the moment of when this reaction was performed the mixture was not dried and a yield was not obtained. However, NMR experiment was still performed on the sample.

6.5.2 DSPE-PEG-Mal and cys-indolicidin

Model reaction, to see if maleimide-thiol conjugation would work appropriately. Procedure for conjugation is modified from Ji et. Al [62]

Table 10: Table of reactants and solvents for conjugation of DSPE-PEG-Mal and cys-indolicidin

Chemical	**Supplier**	**Molecular weight, Mw (g/mol)**	**Amount**	**Mole, n**
DSPE-PEG-Mal	Biopharma PEG	2k	**6.5.2 a** 5mg **6.5.2 b** 5mg	0.0025 mmol
Cys-indolicidin	TAG Copenhagen	2010 g/mol	**6.5.2 a** 5,2mg **6.5.2 b** 5,0mg	0.0026mmol 0.0025mmol
HEPES, 0.1M buffer pH 7.0	Sigma Aldrich		5mL	

All the reactants and solvents are listed in Table 10 above. Two reactions were carried out in parallel, and the only significant difference between them is the buffer used in the experiment. Other than that, the procedure is the same. The experiment carried out in HEPES buffer will have everything noted as 6.5.2 a. The experiment which was carried out in water is noted under 6.5.2 b. Buffer in the procedure is then either a = HEPES or b = water.

DSPE-PEG was dissolved in 1mL buffer, while the peptide was dissolved in 4mL. The DSPE-PEG solution was added to the peptide solution. This mixture was then left on a stirring plate for six hours. The solution was then dialyzed for 3 days. After dialysis the sample was freeze dried.

6.5.2 a

The collected sample from the HEPES buffer reaction was 4.9mg, yield % = 48.0%

6.5.2 b

The total collected sample from the water reaction was 7,8mg, yield % = 72.2%

6.5.3 DSPE-PEG-Mal conjugation with reduced dialyzing time

The procedure described in 6.5.2 a was performed again, with a change of the amount of time the sample was dialyzed. The time of dialysis was shortened to one day, instead of three as previously. However, before dialysis the sample was freeze dried. The collected product was 98,8mg. The dried sample was again dissolved in 5mL water and dialyzed for 24 hours.

The dialyzed sample was again freeze dried. The collected sample was then 31.7 mg

The sample was once again rehydrated and dialyzed for a second time for a total of five days. After the second time dialyzing the sample was freeze dried again.

The end yield after dialyzing and freeze drying several times where 9.2mg, 92%

6.5.4 DSPE-PEG-Mal conjugated with reduced cys-indolicidin

The procedure of conjugation was again performed, but with the cys-indolicidin reduced with DTT prior to the conjugation. Experimental of reduction of cys-indolicidin is below in section 6.7.

To the dialyzed sample of reduced cys-indolicidin, experimental details of the reduction can be viewed under 6.7.3 b.

7.1mg of DSPE-PEG-Mal was added, the solution was left on a shaking plate overnight. The solution was dialyzed and freeze dried. The collected sample was 4.2mg, 34.4%

6.6 Quantification of unbound cys-indolicidin

It was necessary to determine the quantity of unbound cys-indolicidin in the DSPE-PEG-peptide conjugate, for an upcoming bacterial testing. To determine the concentration of free indolicidin a series of concentration was analyzed with HPLC coupled with UV-detector.

For the experiments cys-indolicidin was dissolved in water, creating a stock solution of 1mg/mL. From the stock solution a diluted concentration series: 10µg/mL, 7.5µg/mL, 5µg/mL, 2.5µg/mL, and 1µg/mL, was generated by further dilution with water.

6.7 Reduction of cys-indolicidin

To check that the peptides did not form thiol bridges and dimerize, reductant such as ascorbic acid and DTT was used and to see if a change in HPLC eluation.

6.7.1 Ascorbic acid

Table 11: Table of reactants used for reduction of cys-indolicidin with ascorbic acid

Chemical	**Supplier**	**Molecular weight, Mw (g/mol)**	**Amount**	**Mole, n**
(+)-Sodium L-ascorbate	Sigma Aldrich	198.11 g/mol	9.99 mg	0.05mmol
Cys-indolicidin	TAG Copenhagen	2010.0 g/mol	3.91 mg	0.002mmol

The amount of ascorbic acid and peptide is listed in Table 11 above.

The peptide was dissolved in 2mL water. Ascorbic acid was dissolved in 1mL water and shaken to dissolve everything. 88µL of the ascorbic acid solution was added to the 2mL

peptide solution. And additional 1912µL was added to make the final volume 4mL. The mixture was left on a shaking plate overnight.

The solution was then measured on HPLC, 10µg/mL. It was further diluted to make the dilution series: 7.5 µg/mL, 5 µg/mL, 2 µg/mL and 1 µg/mL.

6.7.2 Gluthathione

Table 12: Chemicals for the reduction of cys-indo with GSH

Chemical	**Supplier**	**Molecular weight, Mw (g/mol)**	**Amount**	**Mole, n**
Gluthathione, reduced (GSH)	Thermo fisher scientific	307.33 g/mol	20.2mg	0.066mmol
Cys-indolicidin	TAG Copenhagen	2010.0 g/mol	2mg	0.001mmol
HEPES, 0.1M buffer, pH 7	Sigma Aldrich (Stock solution made in lab)		5mL	

The amount of reductant and peptide is shown in Table 12.

The peptide was dissolved in 2mL buffer, and the glutathione was dissolved in 3mL water. The solutions were mixed and left for 30 minutes on a shaking plate. The sample was dialyzed for a day. Before the sample was dialyzed a small amount was taken out to be measured on the HPLC.

6.7.3 Dithiothreitol

6.7.3 a

The procedure was sourced from the blogpost by AG scientific "*Dithiorthreitol (DTT) Applications You Must Know*" [48]

All the chemicals used is listed in Table 13, below.

Table 13: Table of chemicals used for reduction of cys-indolicidin with DTT.

Chemical	**Supplier**	**Molecular weight, Mw (g/mol)**	**Amount**	**Mole, n**
Dithiothreitol	Thermo fisher scientific	154.25 g/mol	4.1mg	0.027mmol
Cys-indolicidin	TAG Copenhagen	2010.0 g/mol	4,2mg	0.002mmol
HEPES buffer (0.1M, pH 7)	Sigma-Aldrich		5mL	

Cys-indolicidin was dissolved in 2mL buffer, making a 1mM peptide solution. The Dithioreitol was dissolved in 3mL buffer, making a 9mM solution. The two solutions were combined and left on a shaking plate for 30 minutes. After the 30 minutes the sample was dialyzed for 3 days. A small amount was taken out to be measured with HPLC, before the dialyzing process. After dialyzing the sample was freeze dried. Resulted in a sticky substance that could not be removed from the flask.

6.7.3 b

Cys-indolicidin was reduced again by using DTT, but with the procedure from Konigsberg.[46]

The amount of peptide and reductant is listed in Table 14.Table 13: Table of chemicals used for reduction of cys-indolicidin with DTT.

Table 14: Chemicals for the reduction of cys-indolicidin with DTT (Konigsberg).

Chemical	**Supplier**	**Molecular weight, Mw (g/mol)**	**Amount**	**Mole, n**

Dithiothreitol	Thermo fisher scientific	154.25 g/mol	23.05mg	0.15mmol
Cys-indolicidin	TAG Copenhagen	2010.0 g/mol	5.12mg	0.003mmol
Tris buffer (0.1M, pH 8)	Sigma-Aldrich		5mL	

Cys-indolicidin were dissolved in 3mL buffer, and DTT was dissolved in 2mL. The solutions were mixed and left on a shaking plate for 2 hours. After the two hours the solution was dialyzed for 2 days. The dialyzed product was used further in a conjugation experiment, experimental details for this reaction is presented in 6.5.4.

7 Results

7.1 Oxidation with Bobbitts salt

Both the in-house synthesized C_{18}-PEO_5 polymer and the commercially available Brij S 100 showed no oxidation in the NMR spectra.

7.1.1 Oxidation of Brij S 100

From H NMR taken of the product, can be observed below in Figure 21, it shows no clear sign of oxidation. There would be an observable peak above 10ppm, and a change in the protons neighboring the hydroxyl. The full spectra can be observed in the appendix

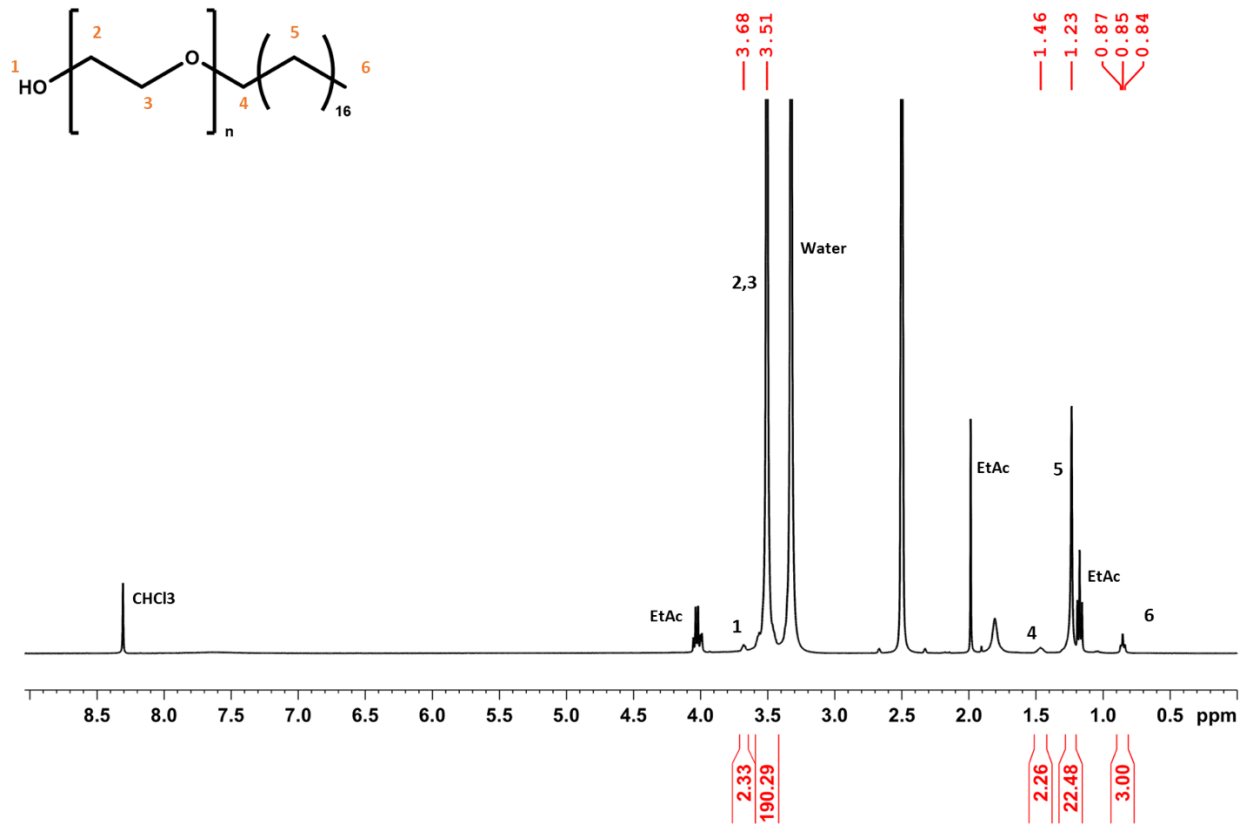

Figure 21: H-NMR of the product from the experiment of oxidation of Brij100

^{1}H NMR (400 MHz, DMSO-d6): δ 3.68 (bs, 1H, **H^1**), 3.51 (s, 4H, **H^{2,3}**), 1.46 (bs, 2H, **H^4**), 1.23 (s, 32H, **H^5**), 0.85 (t, 3H, **H^6**)

The spectra show no difference from Brij 100 straight from the bottle, except the extra peaks which corresponds to the solvent used in the experiment. Meaning no oxidation can be concluded from the spectra.

7.1.2 Oxidation of C_{18}-PEO_5

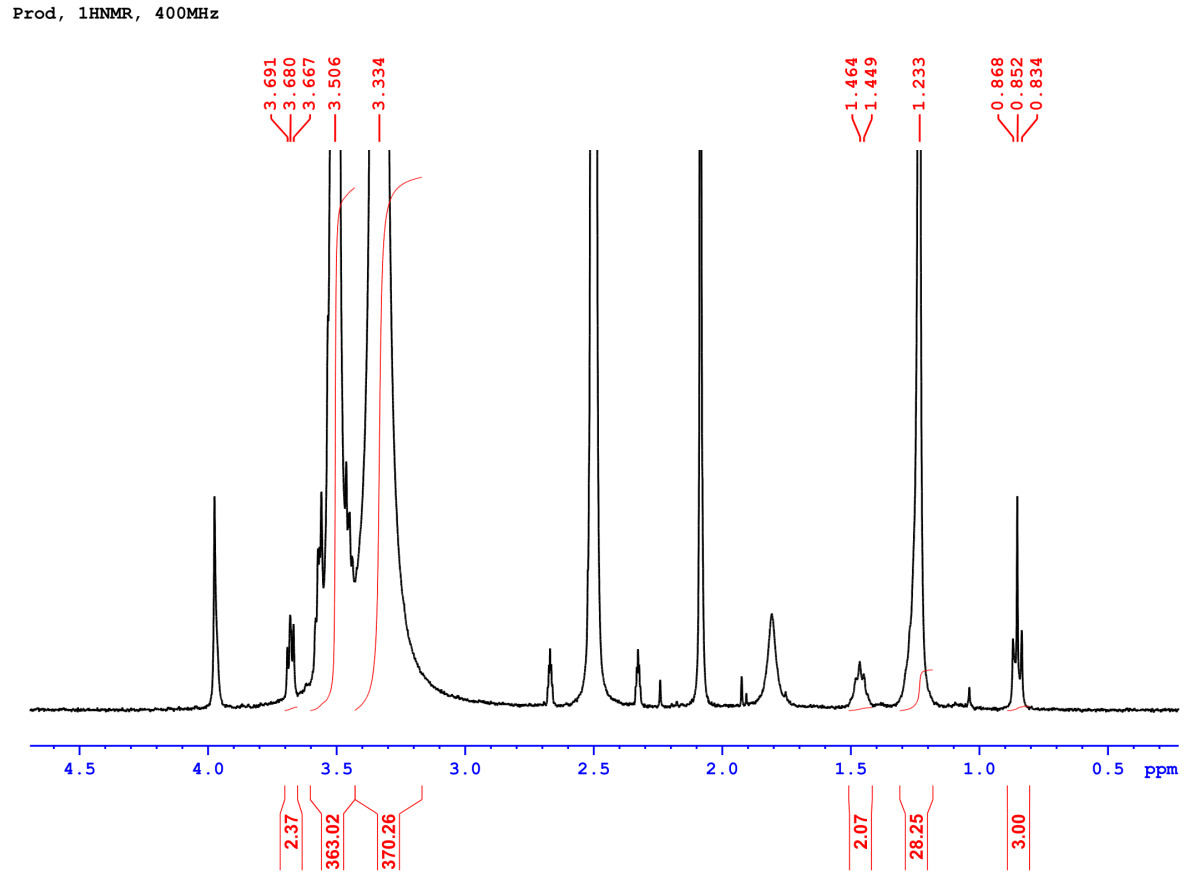

Figure 22: H NMR spectrum of the product from oxidation of the polymer C18-PEO5 with Bobbitt's salt

^{1}H NMR (400 MHz, DMSO-d6): δ 3.68 (t, 1H, **H^1**), 3.51 (s, 4H, **H^{2,3}**), 1.45 (t, 2H, **H^4**), 1.23 (s, 36 **H^5**), 0.85 (t, 3H, **H^6**)

As for the oxidation experiment of C_{18}-PEO_5 the outcome seems to be similar to Brij 100. However, there are some changes from C_{18}-PEO_5 before oxidation. There are two new peaks that can be observed at 3.95 and 2.80ppm.

There is no observable peak at above 10ppm which is again for carboxylic acids, a very apparent broad peak.

7.2 Diels-Alder reaction

The Diels-Alder reaction have resulted in the desired product which is the furan-mhx adduct as seen in Figure 23, below.

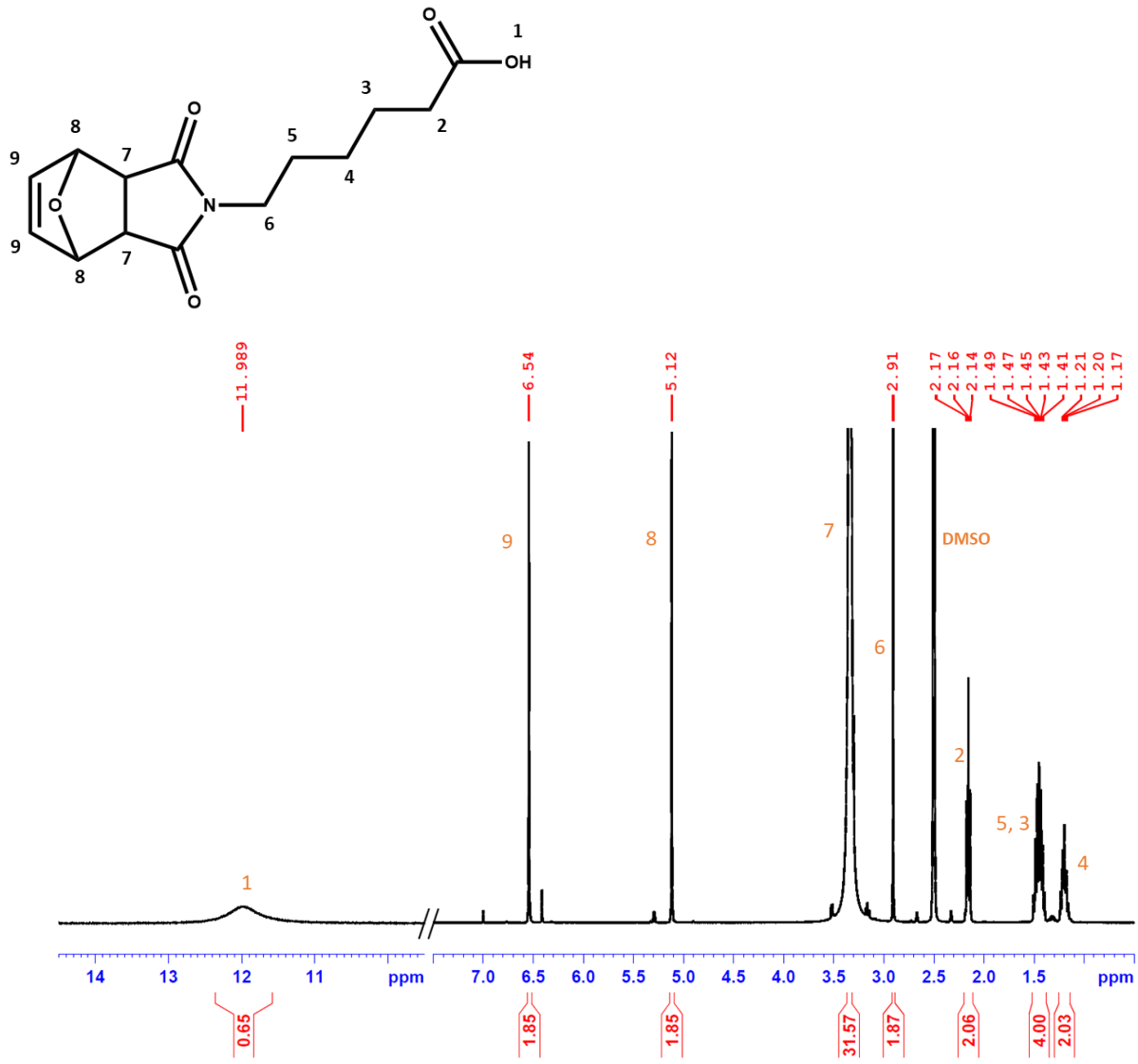

Figure 23: H NMR of product from Diels-Alder reaction. 400MHz in DMSO-d6.

^{1}H NMR (400 MHz, DMSO-d6): δ 11 (bs, 1H, **H^{1}**), 6.54 (s, 2H, **H^{9}**), 5.12 (s, 2H, **H^{8}**), 3.33 (s, 1H, **H^{7}**), 2.91 (s, 2H, **H^{6}**), 2.16 (t, 2H, **H^{2}**), 1.45 (q, 4H, **H^{5,3}**), 1.20 (q, 2H, **H^{4}**),

Peak for H-7, at 3.30ppm is hidden as water appears at 3.33ppm with DMSO-d6 as solvent.

As can be observed in the proton NMR spectrum, we observe two new peaks at 6.54 and 5.15 ppm which corresponds to the hydrogen in the ring, H-9 and H-8. A small residual peak for the acid is also observable in the spectrum at 7ppm. The NMR spectrum for maleimidohexanoic acid can be observed in appendix.

7.3 Maleimide functionalization of Stearic acid

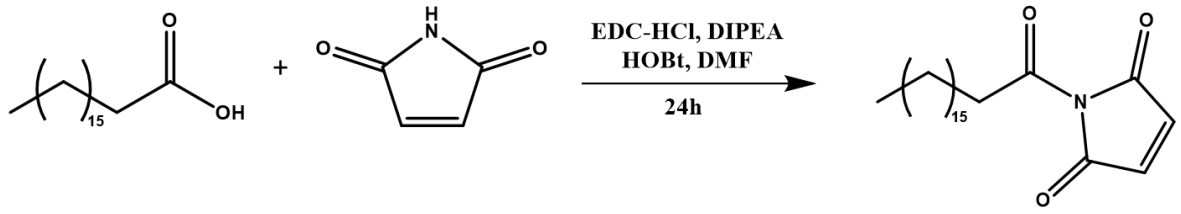

Figure 24: Reaction scheme for functionalization of stearic acid

The reaction scheme for the maleimide functionalization of stearic acid can be viewed above in Figure 24.

As mentioned previously the procedure was meant to be conducted on the Brij polymer, however this experiment was abandoned due to not being able to oxidize the end hydroxyl group to carboxylic acid, and the failure of functionalizing a shorter chain with maleimide. The experiment was changed with another approach of conjugating maleimide with Brij,

As showed in Figure 25 after the reaction and only evaporating the solvent there is still some solvent and reagents left in the product. The other half of the reaction mixture was extracted with ethyl acetate and shows a purer product. DMF is harder to evaporate and is clearly shown in the H NMR as single peaks at 7.95, 2.98 and, 2.73. After extraction with ethyl acetate these peaks have disappeared.

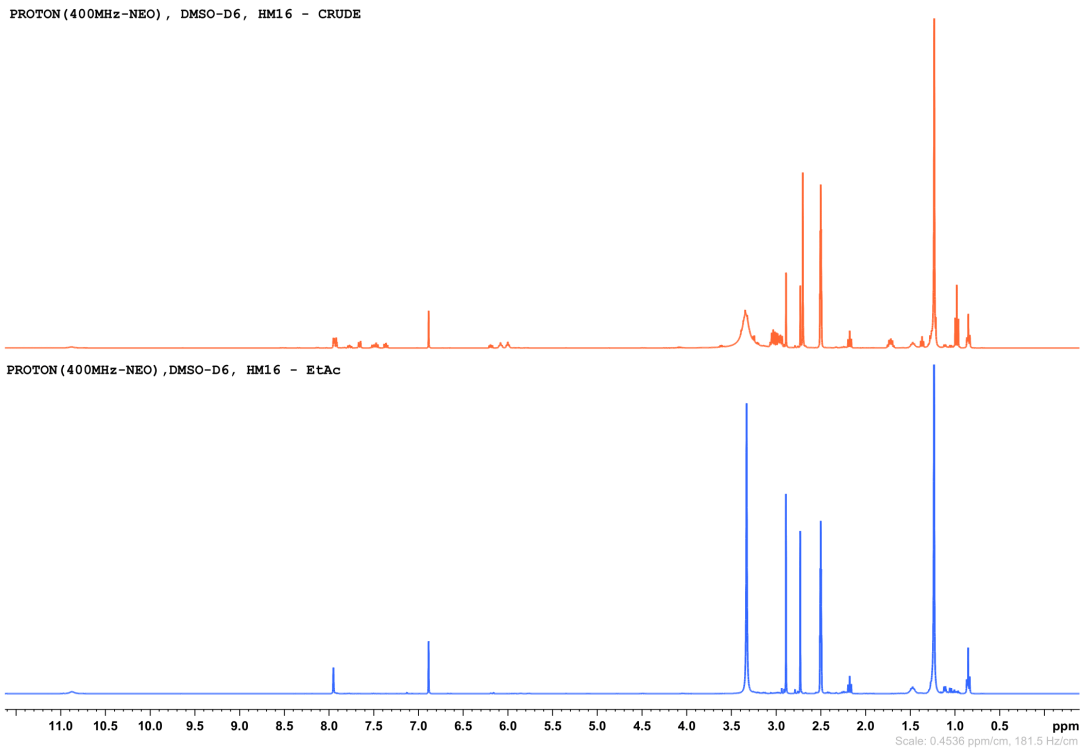

Figure 25: H NMR spectra, comparing crude and purified product

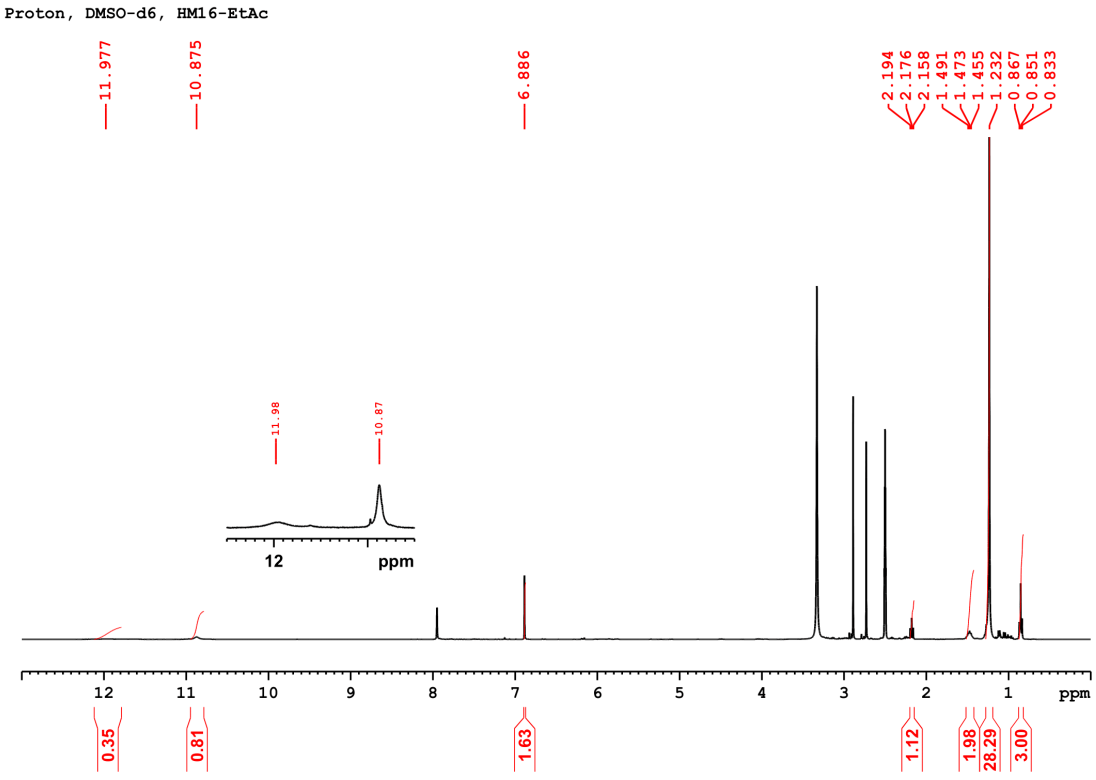

Figure 26: Product of functionalization reaction of stearic acid with maleimide.

^{1}H NMR (400 MHz, DMSO-d6): δ 11.98 (bs, 1H, **H^{x}**), 10.87 (bs, 1H, **H^{x}**), 6.88 (s, 2H, **H^{x}**), 2.17 (t, 1H, ^{x}), 1.47 (t, 2H, **H^{x}**), 1.23 (s, 28H, **H^{x}**), 0.85 (t, 3H, **H^{x}**)..

7.4 Steglich-esterfication of Brij and Maleimidohexanoic acid

The details of alle the esterification experiments are presented in subsection 6.4.

The esterification of Brij100 and maleimidohexanoic acid was performed with three different reactions. The esterification was first performed without the modification of the acid molecule such as seen in Figure 27.

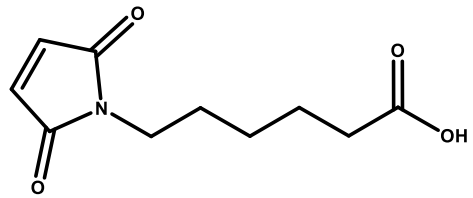

Figure 27: Maleimidohexanoic acid

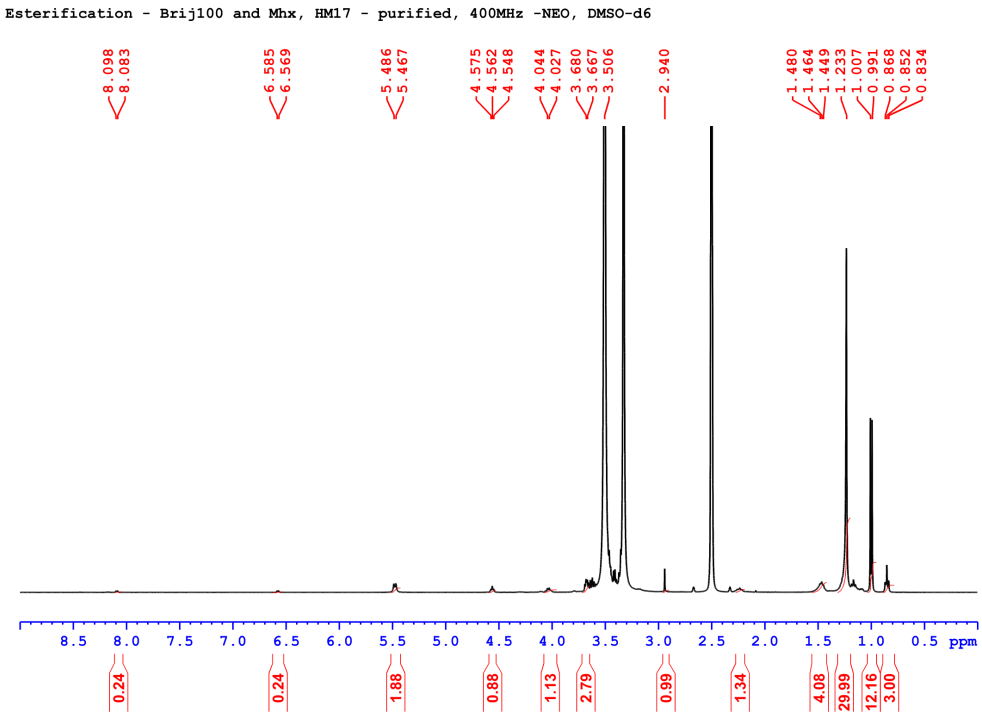

Figure 28: H NMR spectrum from reaction 6.4.1 a

As may be observed from the NMR spectra of reaction 6.4.1 a, the double bond in the maleimide has dissapered hinting to a ring-opening.

The rest of the esterifications were utilizing the furan-maleimidohexanoic acid compound from 7.2.

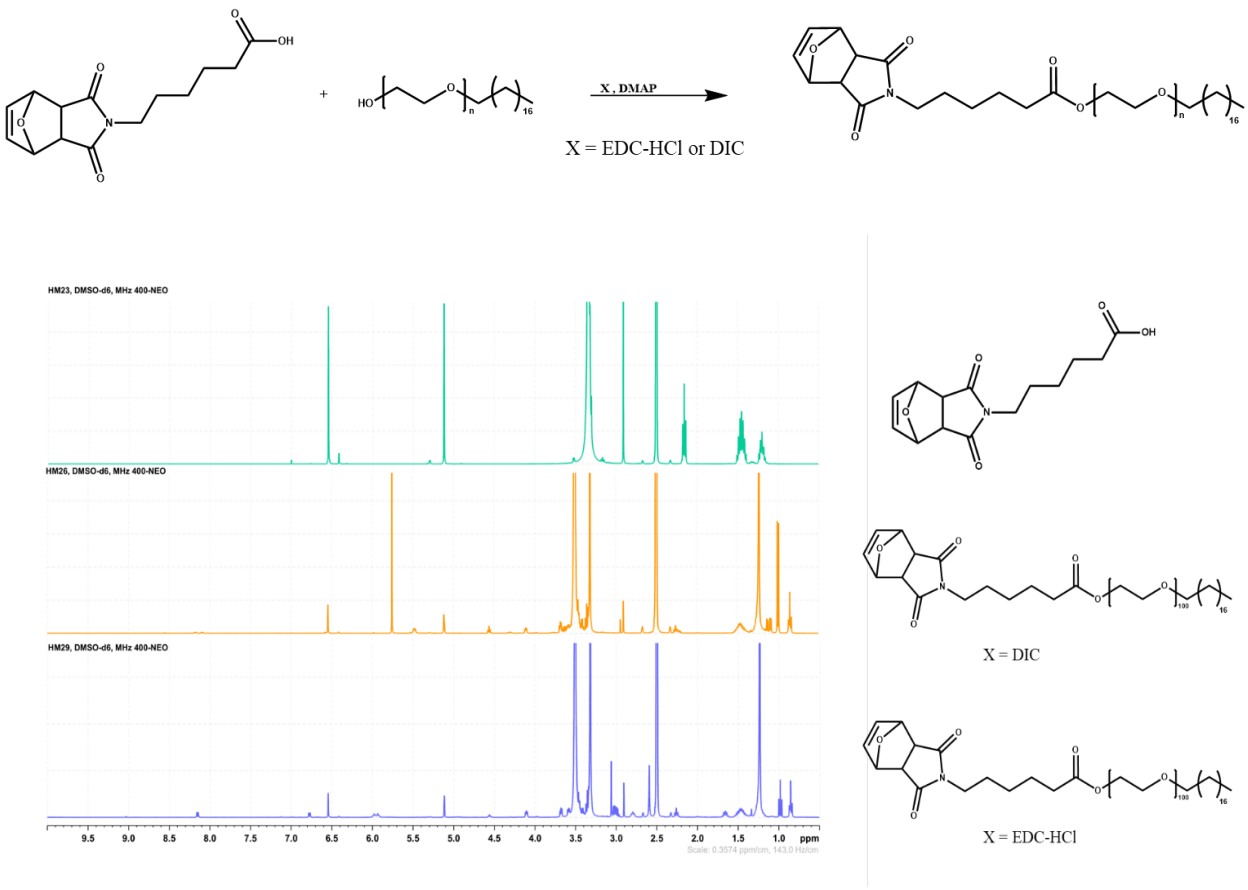

Figure 29: H NMR of the two different Steglich-esterfication reactions and starting molecule. Starting molecule showed in green, reaction 6.4.1 c orange and reaction 6.4.2 in blue.

To see the difference between the reaction using DIC as the carboxylic activator and EDC-HCl it is observable in Figure 29. The only clear difference in the spectra are peaks from residual carbodiimide that was used, and solvents. Dicloromethane is observable in the sample of 6.4.1 c (orange)

Rest of the spectra and a closer look at the spectra can be seen in appendix.

7.5 Peptide conjugation

For peptide conjugation all the experimental detail are presented in subsection 6.5.

7.5.1 Conjugation of PEG-Mal and cys-indolicidin

The first conjugation reaction performed was cys-indolicidin with PEG-Mal. The spectra of PEG-Mal before the conjugation experiment can be viewed in Figure 30. From the spectra we can still observe the peak at 7ppm that corresponds to the double bond in the maleimide molecule. This indicate that the reaction did not reach completion.

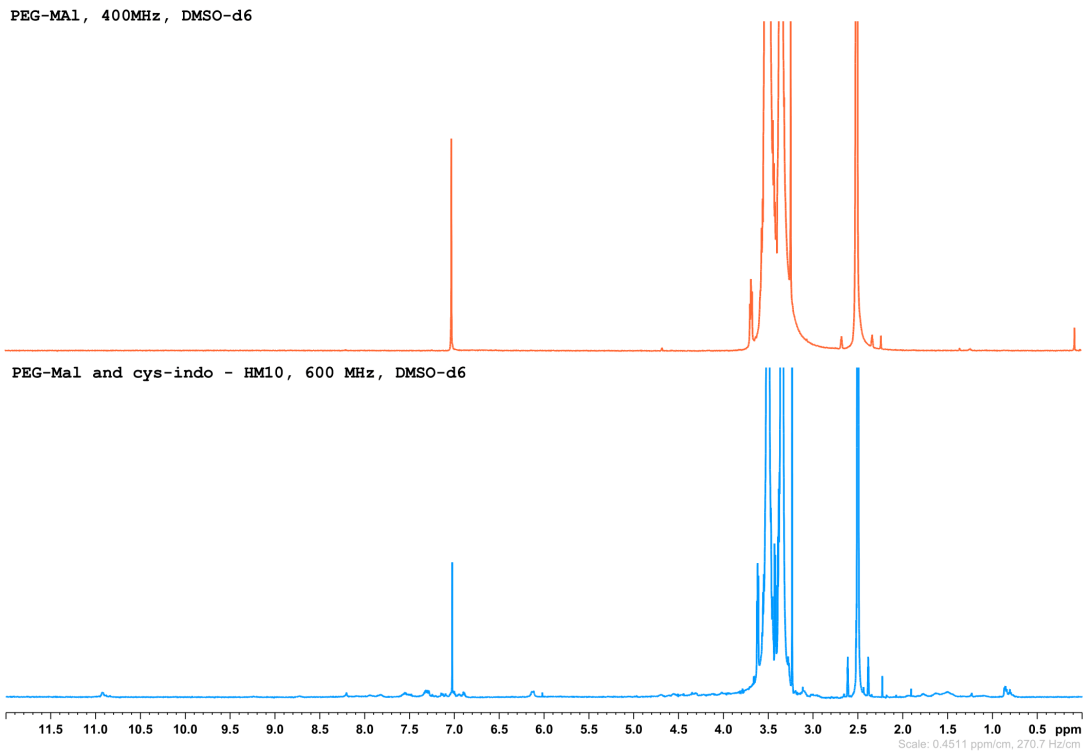

Figure 30: H NMR spectrum of the product from conjugation of PEG-Mal and cys-indolicidin and PEG-Mal before the conjugation. The orange spectrum is PEG-Mal before conjugation, and blue is the product after conjugation.

^{1}H NMR (400 MHz, DMSO-d6): δ 11.98 (bs, 1H, **H^{x}**), 10.87 (bs, 1H, **H^{x}**), 6.88 (s, 2H, **H^{x}**), 2.17 (t, 1H, **H^{4}**), 7.40 (m, 1H, **H^{9}**), 7.29 (ddd, *J* = 6.1, 2.7, 0.9 Hz, 1H, **H^{2}**).

7.5.2 DSPE-PEG-Mal and cys-indolicidin conjugate

The DSPE-PEG-Mal conjugation was carried out several times. The H NMR spectra of the experiments 6.5.2a and 6.5.2b is seen in Figure 31. Comparing the two spectra the product are similar. More importantly the peak at 7ppm given by the double bond found in maleimide is no longer observable and indicating that the reaction reached completion. The spectrum for DSPE-PEG-Mal before the reaction can be seen in the appendix.

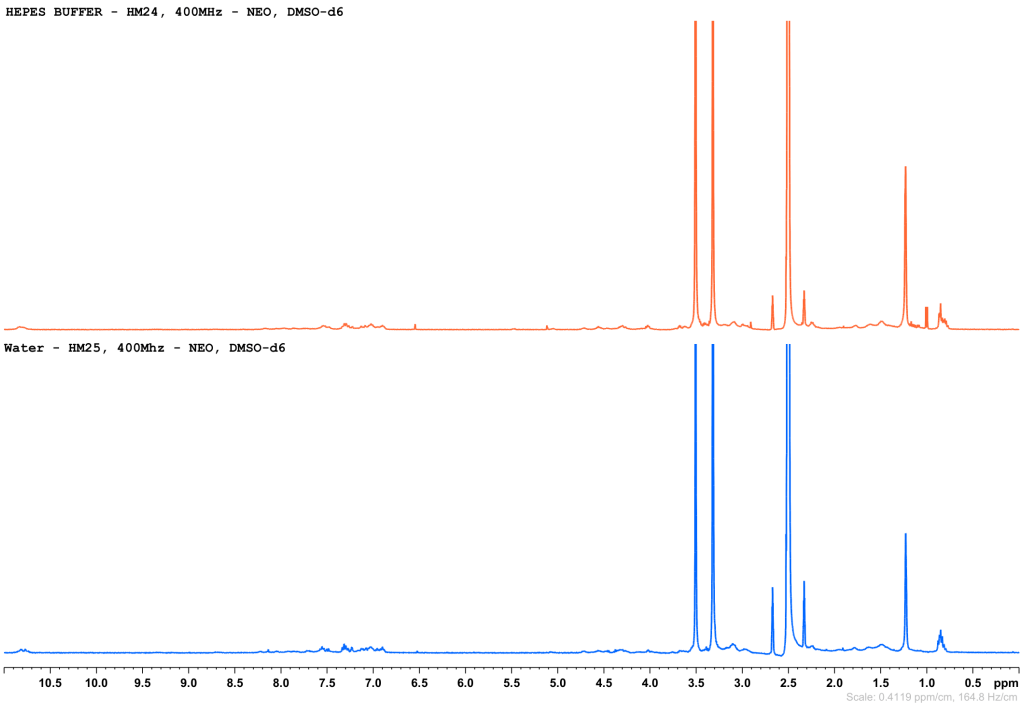

Figure 31: Spectra of the conjugated product from HEPES buffer and the product from water. The orange spectrum is from experiment 6.5.2a and blue is from experiment 6.5.2b.

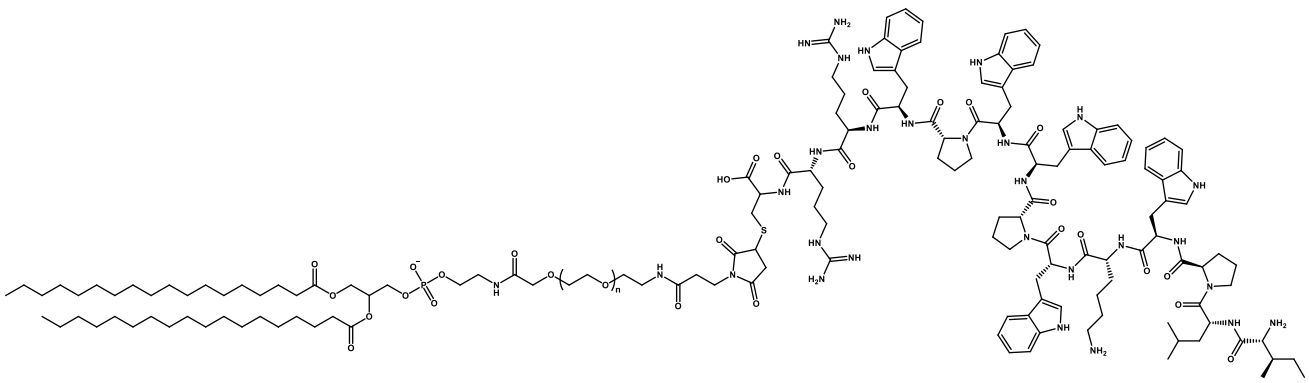

Figure 32: The DSPE-PEG-petide conjugate illustrated, drawn in Chemdraw.

The DSPE-PEG peptide compound, illustrated in Figure 32.

Both the compounds were also measured on the BIOSAXS beamline Grenoble.

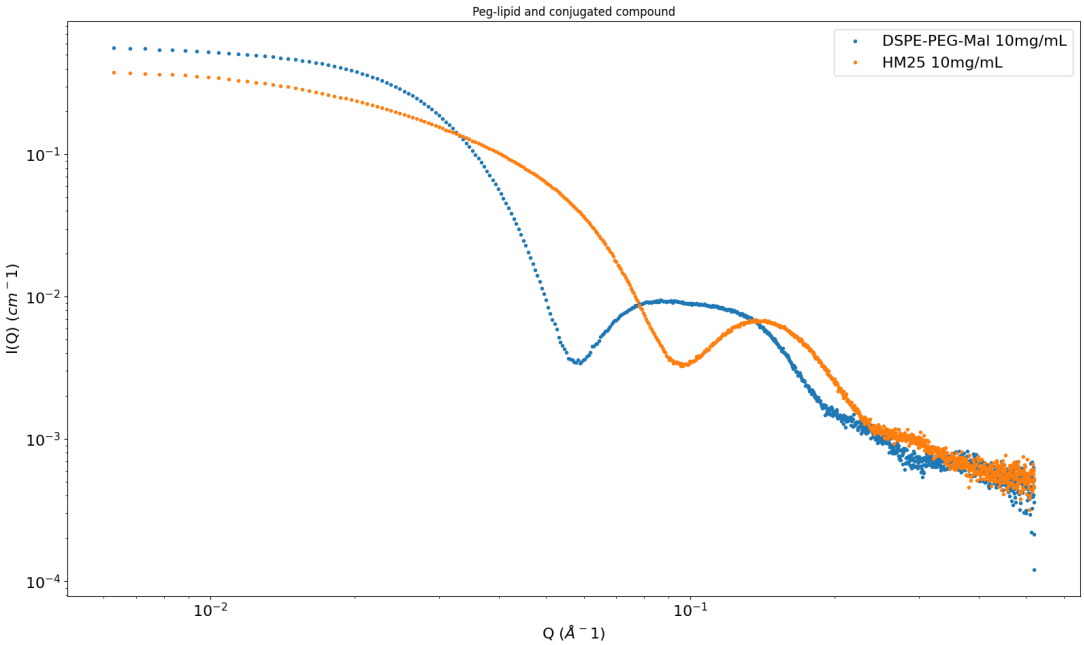

Figure 33: SAXS data of DSPE-PEG-Malimide before and after conjugation. Before conjugation is showed with the color blue and after conjugation is showed as orange.

From the SAXS data above, Figure 33, there is an observable difference in structure of before and after conjugation with cys-indolicidin.

The conjugation reaction was both performed in buffer, in this case HEPES and in water. To compare if there was a difference, NMR and SAXS was executed on both the products. Both the NMR, Figure 31, and SAXS, Figure 34, shows similar products.

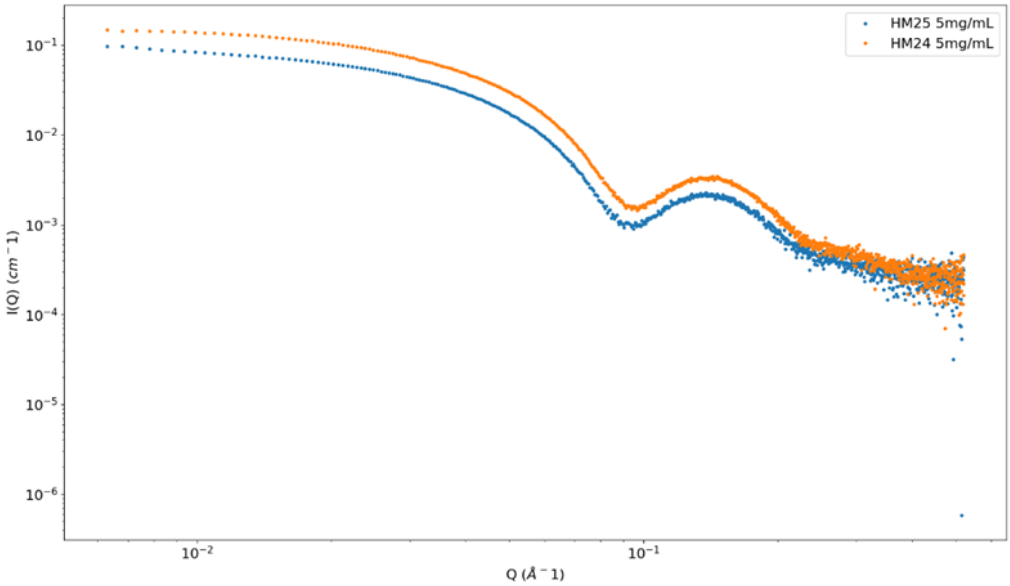

Figure 34: SAXs curves from conjugation experiment of cys-indo with DSPE-PEG-Mal in water and in HEPES buffer. Blue represents the water experiment and orange represent the experiment in HEPES buffer

7.5.3 Conjugation with DSPE-PEG-Mal with reduced cys-indo

The conjugation procedure was performed on reduced cys-indo to observe the possible difference in conjugation with reduced and un-reduced peptide.

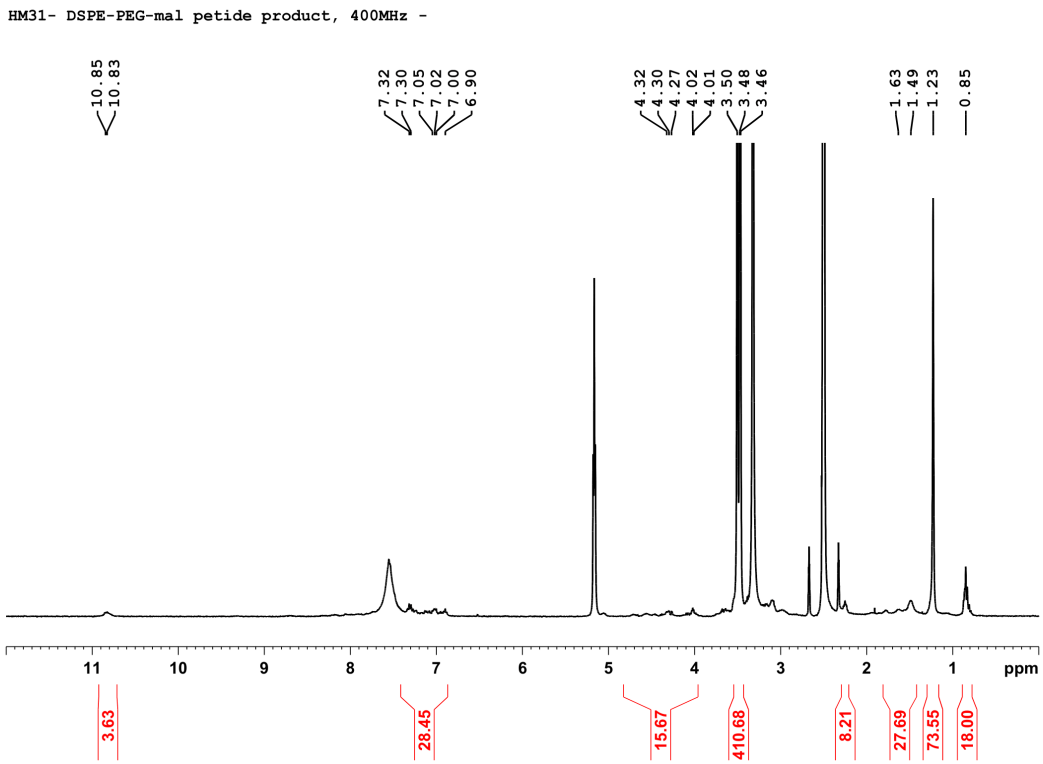

Figure 35: H NMR spectrum of the product from the conjugation recation with reduced cys-indolicidin.

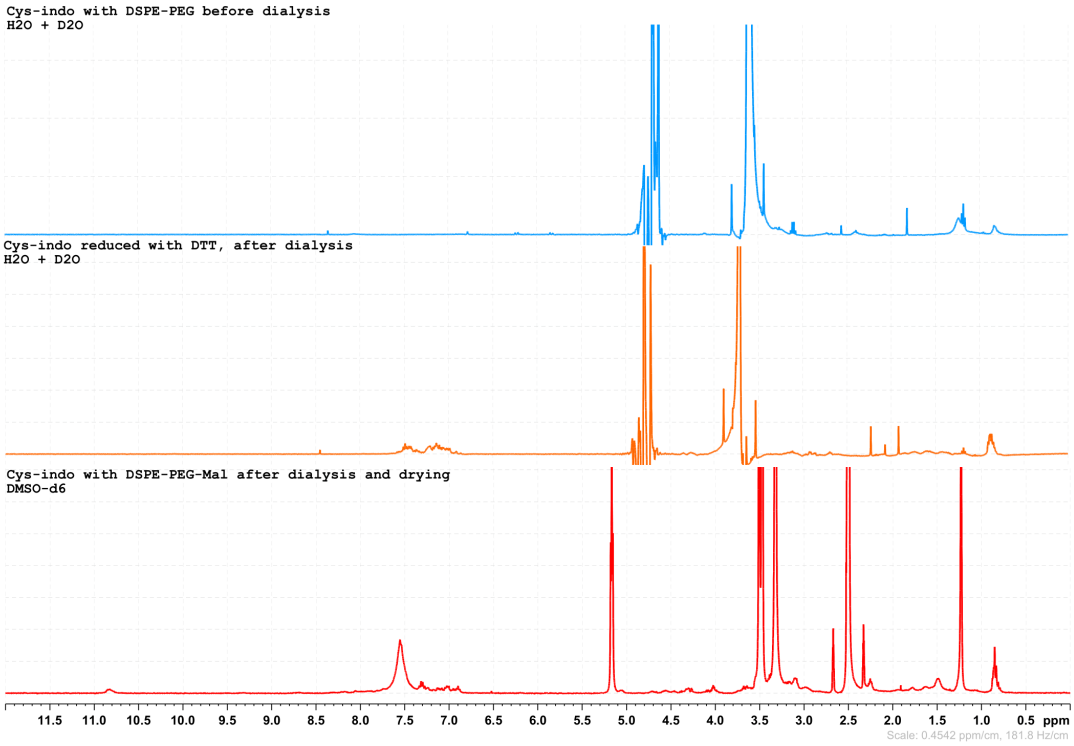

Figure 36: H NMR spectra of the different stages in the conjugation of DSPE-PEG-Mal and DTT reduced cys-indolcidin. The blue spectrum is the cys-indo right after conjgation with DSPE-PEG-Mal, but before dialysis, measured in 90% H2O + 10% D2O. The orange spectrum is cys-indolicidin after reduction with DTT and dialysis, also measured in 90% H2O+10% D2O. The red spectrum is the product after conjugation, and after drying, measured in DMSO-d6.

7.6 Quantification of unbound cys-indolicidin

The concentrations 10µg/mL, 7.5µg/mL, 5µg/mL, 2.5µg/mL, and 1µg/mL was measured to create the curve observed below in Figure 37. The chromatograms of each of the concentrations can be viewed in appendix, along with the table of the measured peak areas for the different concentration used to generate the calibration curve for quantifying unbound cys-indolicidin.

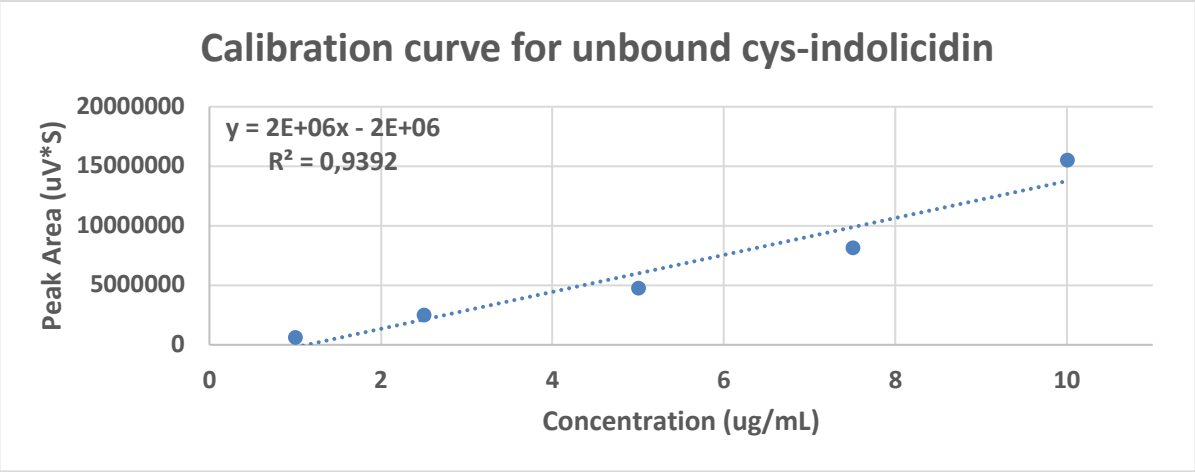

Figure 37: Concentration curve for cys-indolicidin from the HPLC

From the calibration curve the following formula y = 2E+06x-2E+06 was obtained. Where y equals the peak area and x equals the concentration of cys-indolicidin. The measured peak area in the conjugate samples can be seen in Table 15 . Reaction 6.5.2 b had the highest concentration of unbound cys-indolicidin, and reaction 6.5.2 a yielded the lowest concentration of unbound cys-indolicidin. The calculated amount of unbound cys-indolicidin is also presented in Table 15.

Table 15: Measured peak area, the concentrations injected and the calculated amount of unbound cys-indolicidin for the DSPE-PEG-peptide compounds

Compounds	Concentrations injected	Measured peak area	Calculated amount of unbound cys-indolicidin
DSPE-PEG-peptide (6.5.2 a)	20 µg/mL	1737783,3	2,43 µg/mL
DSPE-PEG-peptide (6.5.2 b)	20 µg/mL	4616083,5	4,18 µg/mL
DSPE-PEG-peptide (6.5.3)	10 µg/mL	921609,5	1,94 µg/mL

7.7 Reduction of cys-indolicidin

7.7.1 Ascorbic acid

For chromatogram obtained after reduction of cys-indolicidin with ascorbic acid see Figure 38, for the experiment with 10µg/mL concentration. The experimental details for the

reduction is represented under subsection 6.7.1. Comparing the chromatogram with the chromatogram of unreduced cys-indolicidin the first peak has similar intensity, and the compound eluates at the same time. The difference that is observable is the second peak being significantly smaller than all the LC experiments.

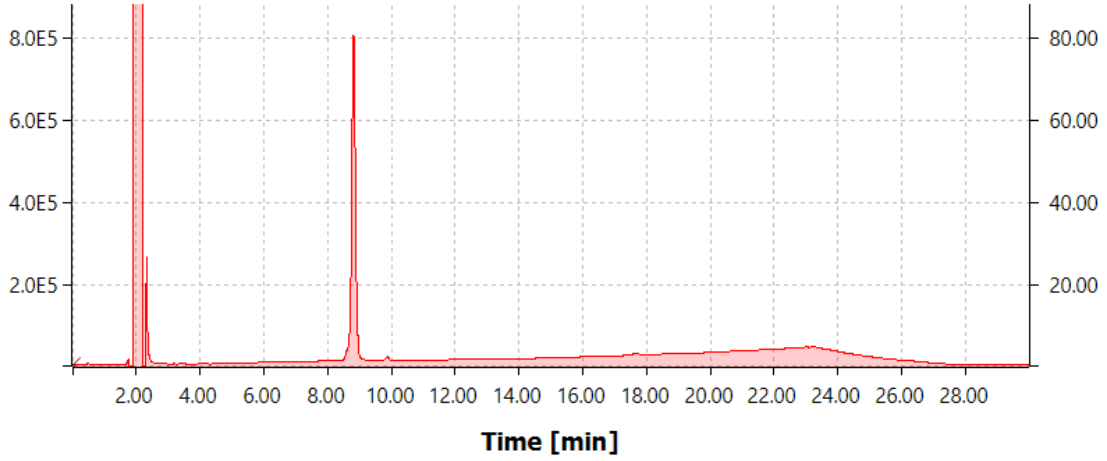

Figure 38: Chromatogram of 10µg/mL cys-indolicidin after reduction with ascorbic acid

The only other experiments that were performed was with 5 µg/mL and 1 µg/mL. These chromatograms can be viewed below in Figure 39 and Figure 40.

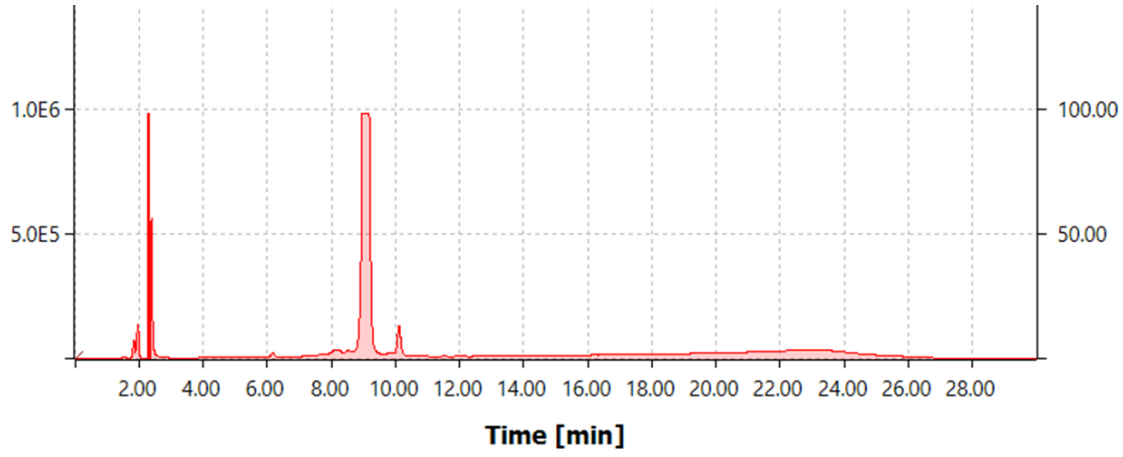

Figure 39: Chromatogram of 5 µg/mL cys-indolicidin reduced with ascorbic acid

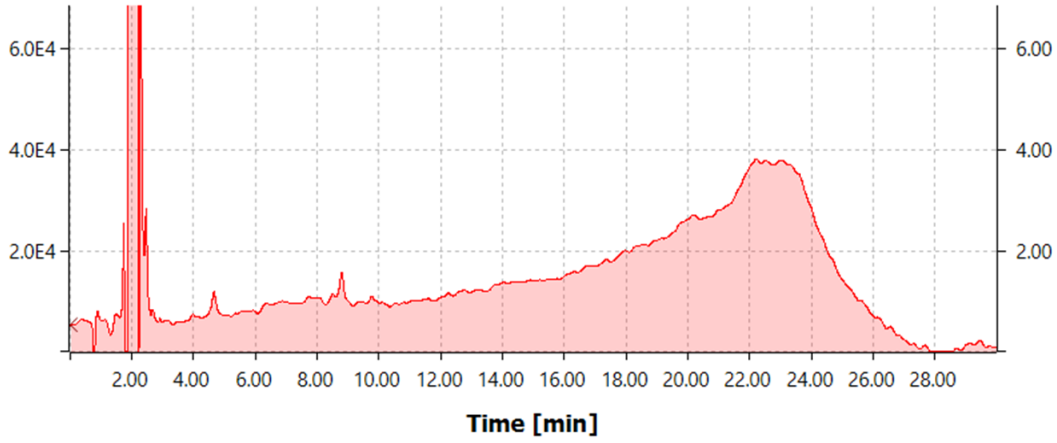

Figure 40: Chromatogram of 1 µg/mL cys-indolicidin with ascorbic acid

Unlike the chromatogram for 10 µg/mL, 5 µg/mL and 1 µg/mL shows an unexpected result. The first peak in the 5 µg/mL shows that the experiment has exceeded the range of the detector, due to its flat top. For 1 µg/mL the peaks are almost non-detectable, the peaks slightly blend in with the background.

7.7.2 Gluthathione

For the reduction with gluthathione or GSH, the only experiments measured were 10 µg/mL, 5 µg/mL, and 1 µg/mL. However the only usable data is from the 5 µg/mL, which can be seen in Figure 41.

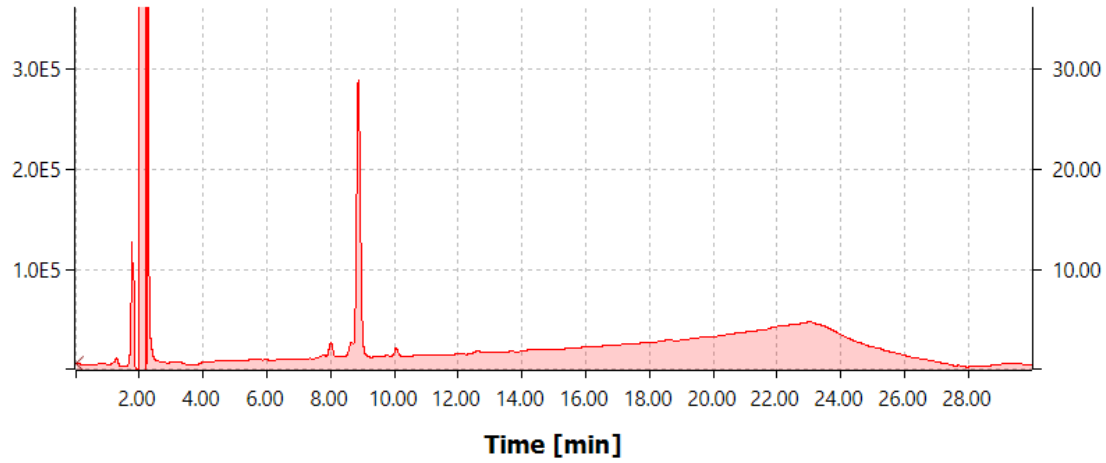

Figure 41: Cromatogram of 5 µg/mL cys-indo after reduction with gluthathione

For the 10 µg/mL experiment the data are invalid as the detector encountered some problems, and the experiment only showed as noise. For the 1 µg/mL, the chromatogram can be viewed in Appendix. The experiment is also invalid as the chromatogram have no observable peaks.

7.7.3 Dithiothreitol

Reduction of dithiothreitol were performed twice, with different approaches. The chromatogram from the LC experiment of 6.7.3 a is below, Figure 42.

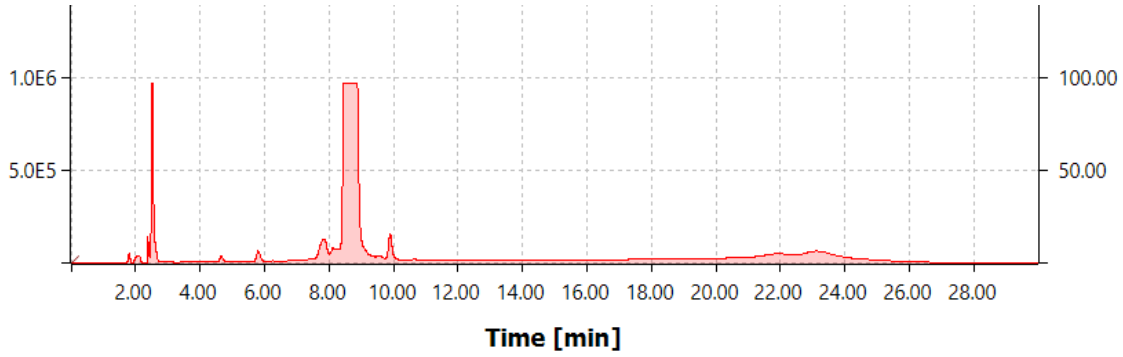

Figure 42: Chromatogram of 10 µg/mL cys-indolicidn after reduction with DTT

The chromatogram shows several peaks that have not been observed previously, and it also show the experiment exceeding the range of detector, as the peak has a flat top. The peak has an increased intensity comparing it with the same concentration of un-reduced peptide.

8 Discussion

8.1 Oxidation with Bobbitts salt

The reaction did not show signs of working properly on Brij 100. To test if this was a problem with the surfactant, a test experiment was performed on the C_{18}-PEO_5 which corresponds to the polymer that were used by the group of Dr.Willner from Forskerzentrum Jülich. When performing this experiment, it seems that the polymer did not oxidize to a carboxylic acid which is what was expected.

For the carboxylic acid there would be an observable peak at 10-12ppm, in neither of the spectra for the C_{18}-PEO_5 and Brij 100, does it show a peak in this area. As these NMR experiments are performed with DMSO-d6 as solvent, we would be able to observe this peak. None of the spectra shows this distinct peak.

However, in the H NMR spectrum from the C_{18}-PEO_5 oxidizing experiment there is two new observable peaks. The peak for hydroxylgroup – OH which is observed at 4.57ppm for the polymer, is no longer apparent. On the other hand, the new peak at 2.5ppm is broader than the other peaks, meaning that this can imply -NH or -OH. This peak does not correspond to any of the solvents or reagents used in the experiment and may only be an impurity. The new peak at 3.95 may indicate a change in the methylene closest to the hydroxyl end group have happened. This have been observed in modification of Brij 35 by Gonzales Garcia et al., by oxidation of the end hydroxyl group to carboxylic acid with TEMPO. [63]

The procedure was also tested by the lab in Jülich on Brij 100, and they gained the same result with no oxidation of the hydroxyl group.

The Bobbitt's oxidation is often colorimetric it is easy to observe the reaction progressing with color change. The color starts as yellow and after completion of oxidation gains an orange hue.

8.2 Diels-Alder reaction

The diels-alder reaction showed to have a good success with the maleimidohexanoic acid and furan. The reaction gave a high yield and from the H NMR showed to be relatively pure, with little impurities from solvent and starting material.

8.3 Maleimide functionalization

Since the oxidation of the polymers did not yield in the carboxylic acid end group, the procedure was tested on stearic acid. Stearic acid was used as it contained a long methylene chain with a carboxylic group at the end.

8.4 Steglich-esterfication of Brij

As the maleimide functionalization stearic acid did not work the approach to add maleimide to Brij 100 was changed. Instead of modifying Brij 100 an esterification between malemidohexanoic acid and Brij was approached instead. The esterification was first perfomed on unaltered malemidohexanoic acid and Brij 100. However as there were suspicion of the maleimide ring opening, the acid was first protected with furan before a new reaction was conducted.

8.5 Peptide conjugation

The conjugation procedure was performed several times, with different reagents and with different buffers.

In all the reaction the H NMR shows the disappearance of the double bond in maleimide indicating that thiol have reacted and formed the thioether bond.

In the SAXS curves we see a change in the structure of the nanoparticle that DSPE-PEG-mal creates before and after conjugation with cys-indolicidin. The working theory is that cys-indolicin, as it coats the surface of the NP forms loops.

8.6 Quantification of unbound cys-indolicidin

Only the first eluated peak in the chromatograms were used to construct the calibration curve used to calculate the concentration of unbound cys-indolicidin in the DSPE-PEG-peptide

conjugate sample. The second peak in the chromatograms of cys-indolicidin showed no trend indicating that this peak is not dependent on the concentration of the sample.

8.7 Reduction of cys-indolicidin

Yield of the conjugation between DSPE-PEG-Mal and cys-indolicidin were consecutively low, there was speculation that the cysteines in the peptides had formed disulfide bridges and dimerized. Therefore, making them unavailable to react with maleimide. The reduction was performed with three different reduction agents, the different procedures is viewable in subsection 6.7.

8.7.1 Ascorbic acid

Considering the chromatograms of the ascorbic acid reduced peptide, it shows no obvious change in structure. With a dimerization there would be expected a change in the eluted compound, or at least if the reduction had not reached full completion there would be an extra peak.

8.7.2 Glutathione

The only usable data from the 6.7.2 experiment was the 5 µg/mL measurement. The two other LC measurements gave unusable chromatograms, as mentioned in 7.7.2. Yet the only valid chromatogram shows similar result to un-reduced peptide. Bearing in mind that the structure will change if the peptide is reduced and the dimer is split, a new or extra peak is expected. This is not observed in the chromatogram.

8.7.3 Dithiothreitol

From the chromatogram of 6.7.3 a it cannot be said if the peptide have been reduced. The main peak that is observed in the chromatogram eluates at the same time as the un-reduced peptide, meaning the majority of the sample is probably the same compound. A theory to why the compounds eluates as the same time before and after elution is that the peptide is not dimerized.

9 Conclusion

The first few approaches to functionalize the polymer of interest, Brij 100, did not show the desired or anticipated outcome. However, the work did not stop there. The approach to the problem was changed. Instead of changing the polymer molecule, there may be other ways the functional group, maleimide, could be incorporated and conjugated to Brij 100. That is when esterification of Brij 100 and 6-maleimidohexanoic acid was investigated, trough the Steglich-esterfication procedure. Here as well there were struggles. The first reactions with maleimidohexanoic acid, showed signs of ring opening making the polymer product unable to react further with cys-indolicidin to make the polymer-peptide compound that was desired. To prevent this, the acid needed to be protected. Other had showed protection of maleimide with furan when conjugation it to other molecules.[64] The protected acid seemed promising, the yield was good and the showed the wanted product form the NMR experiment performed.

The esterification with protected acid also seemed promising on a surface level, as some of the peaks that would be expected are seen however it is hard to conclude if the reaction was successful with the lack of analyzation of the end-product due to troubles with purification and removal of reagents.

The question now was if all the hard work in adding maleimide to Brij 100 worth it. Would the maleimide-thiol reaction even work with cys-indolicidin? Therefore, the use of DSPE-PEG-Mal. If the reaction yielded positive results on DSPE-PEG-Mal which is comparable to Brij 100, the Michael-reaction might prove successful on Brij 100.[65] The DSPE-PEG-Mal peptide conjugate seem to show a desirable product from NMR, and even a change in structure after the reaction with cys-indolicidin. However, a low yield each time was concerning. Was this the fault of the natural disulfide bridge? Reduction was performed with three different reductants. However, no one of them showed a difference in structure after the reduction, meaning that disulfide bond may not have been the reason for low yield.

Even though a lot of the work in this study did not have a desired outcome, some seem to show some promise that can be used in further work.

9.1 Further work

The biggest question I was left with after working on this thesis was, did the modified indolicidin still have the antibacterial properties that indolicidin have shown to have. Will it also be anti-bacterial when conjugated to Brij100.? To investigate this, a bacterial testing to comparing the cys-indolicidin and indolicidin would be needed.

Further work in analyzing the structure of the nanoparticle that was formed by the DSPE-PEG-Mal peptide conjugate would also be of interest.

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Appendix

Additional data and spectra used in the thesis.

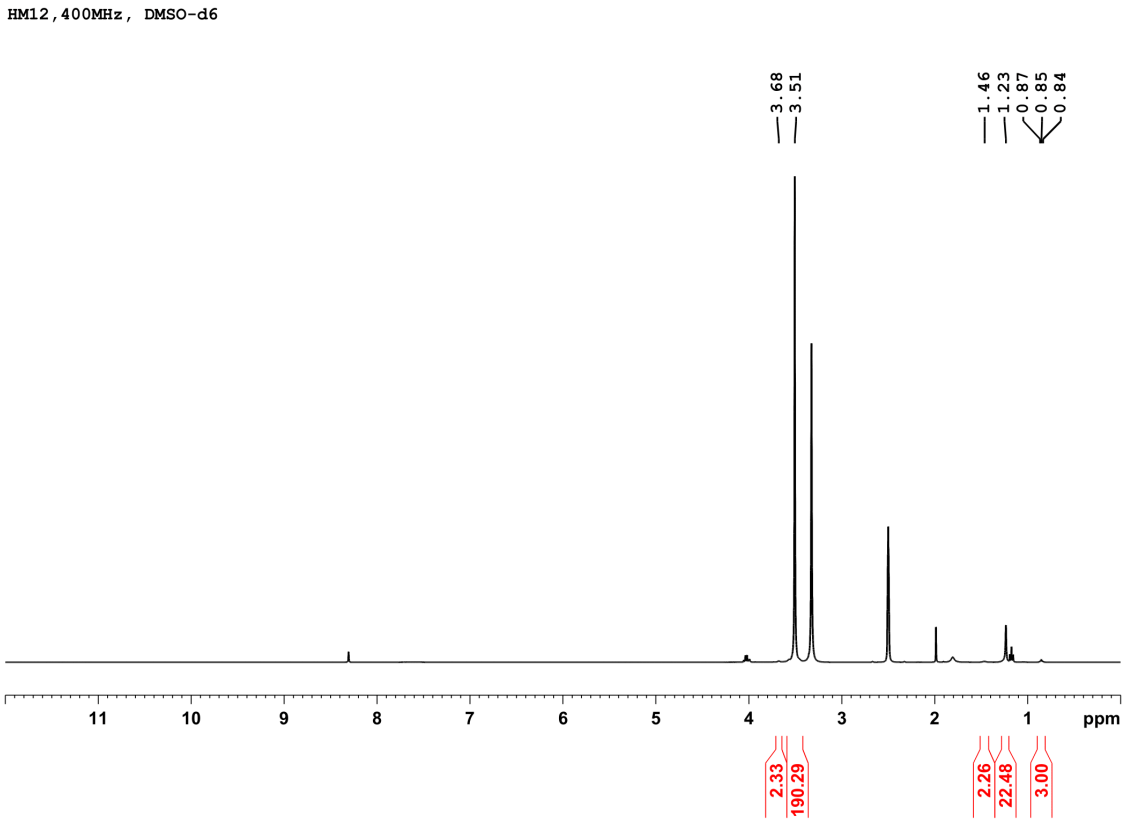
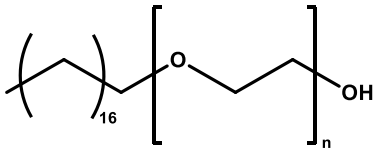


Figure 43: Full spectrum of the oxidation of Brij100



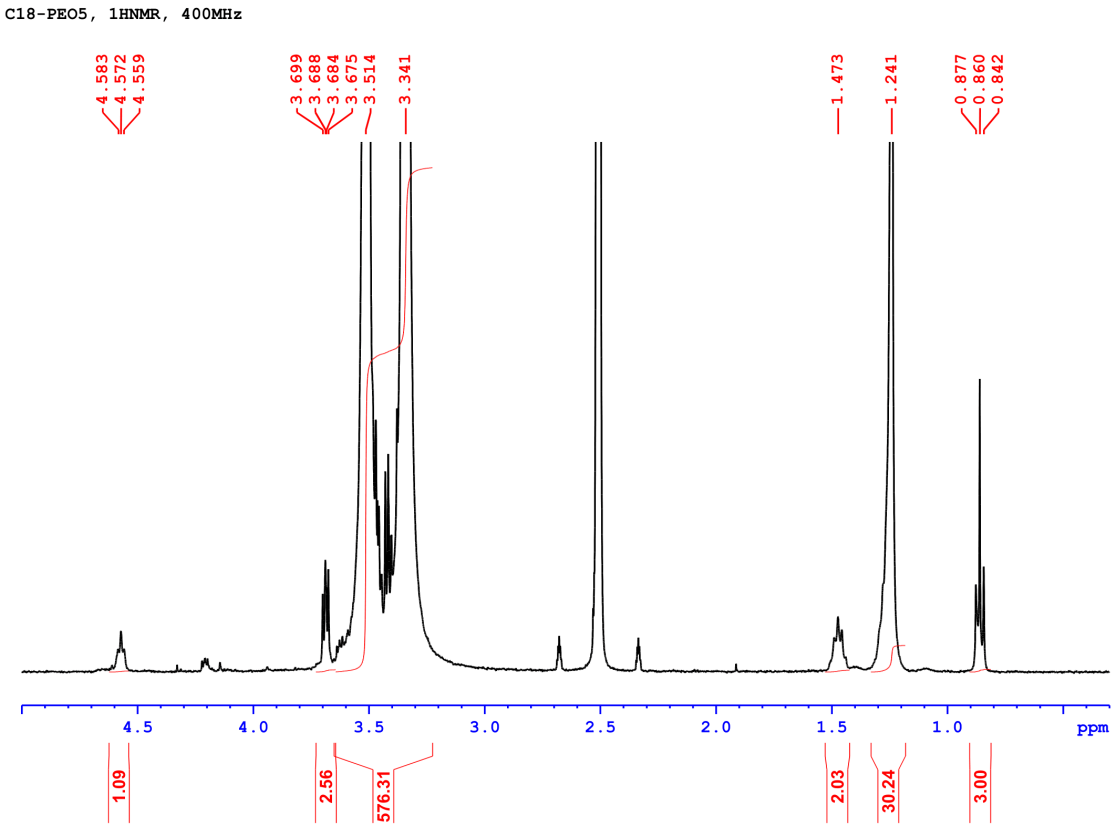


Figure 44: H NMR of C18-PEO before the oxidation with Bobbit's salt

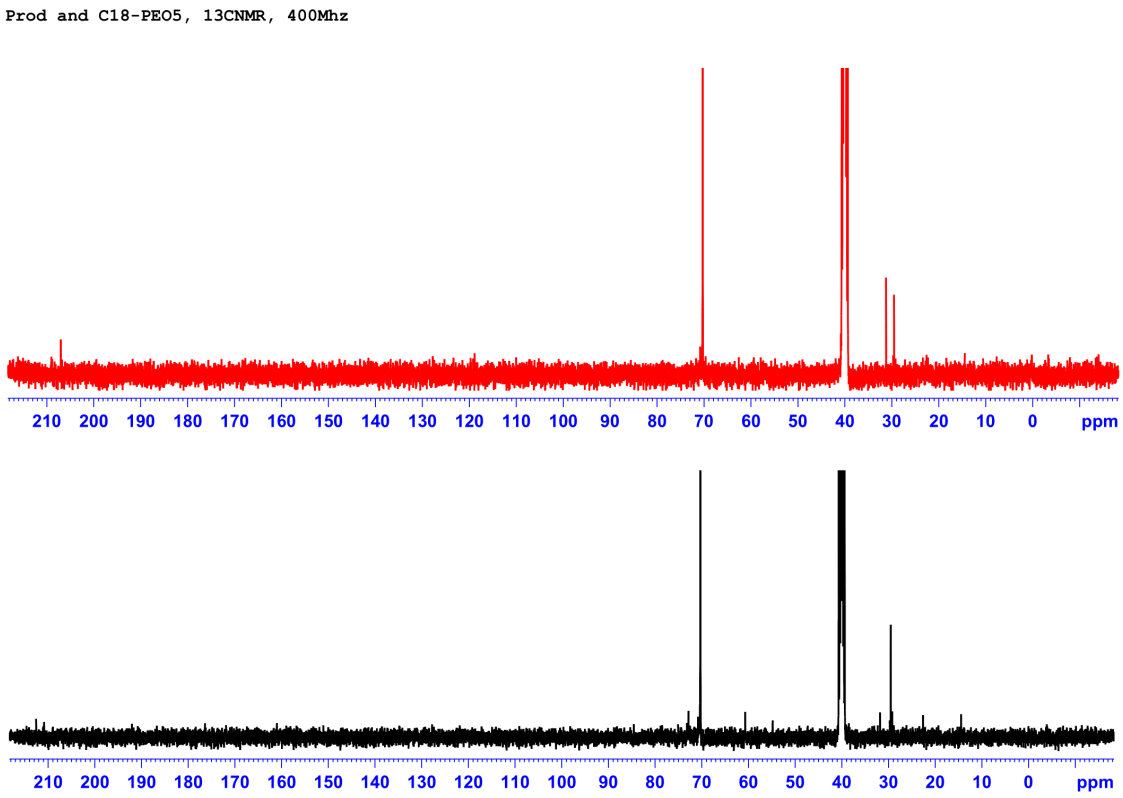
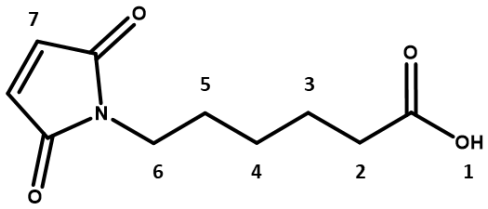


Figure 45: C NMR of the product and C18-PEO5. The red spectrum shows the product after the reaction and the black shows the starting polymer C18-PEO5



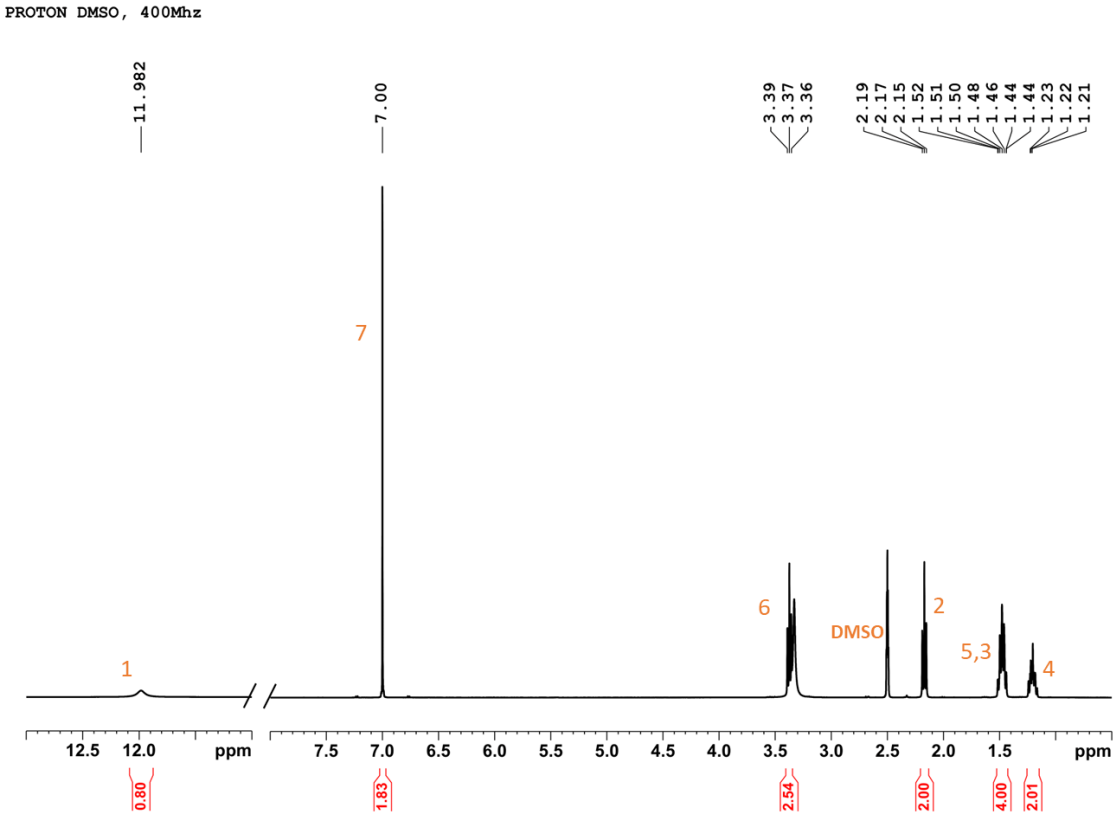


Figure 46: H-NMR spectra of Mhx

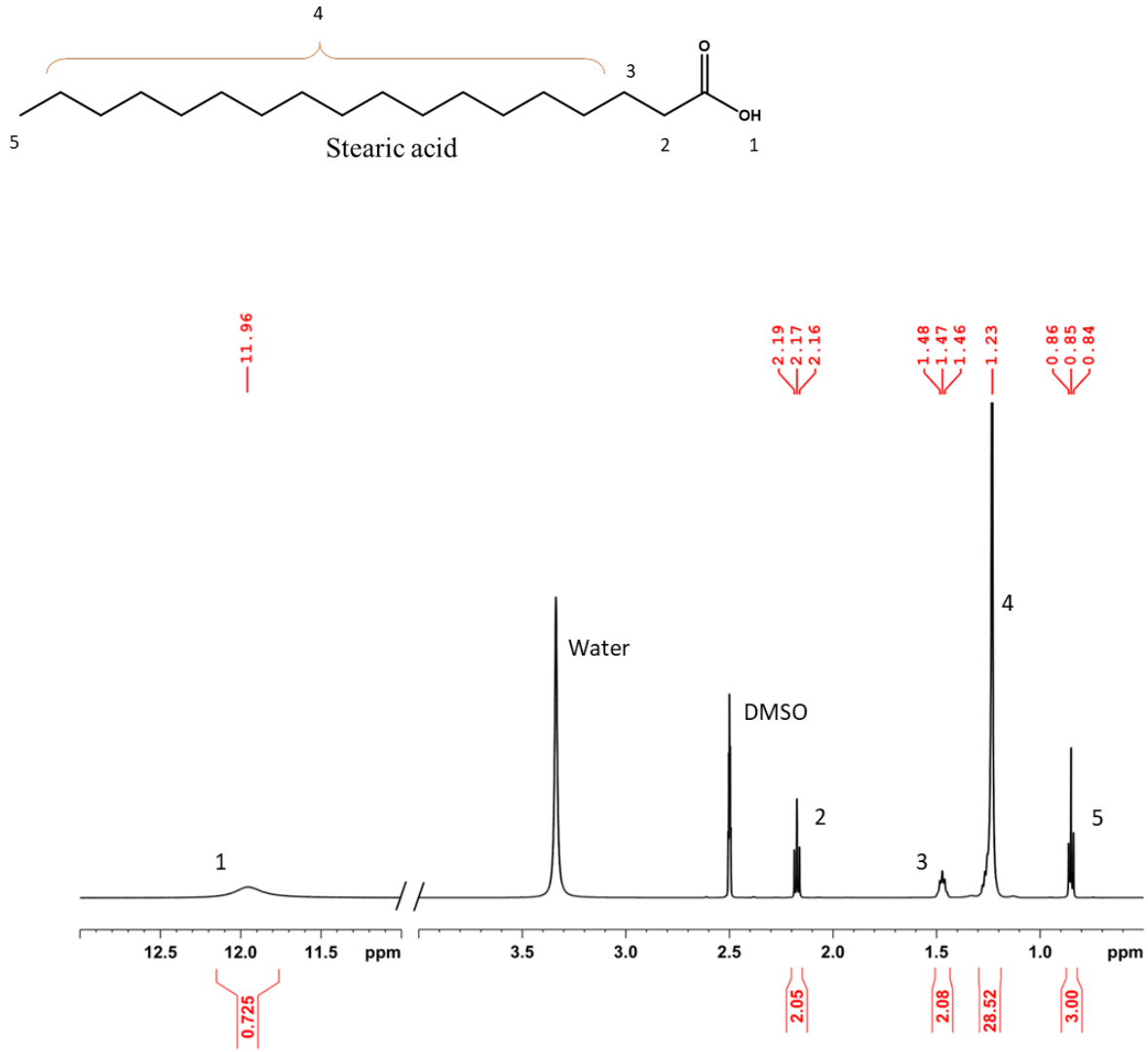


Figure 47: H NMR of stearic acid

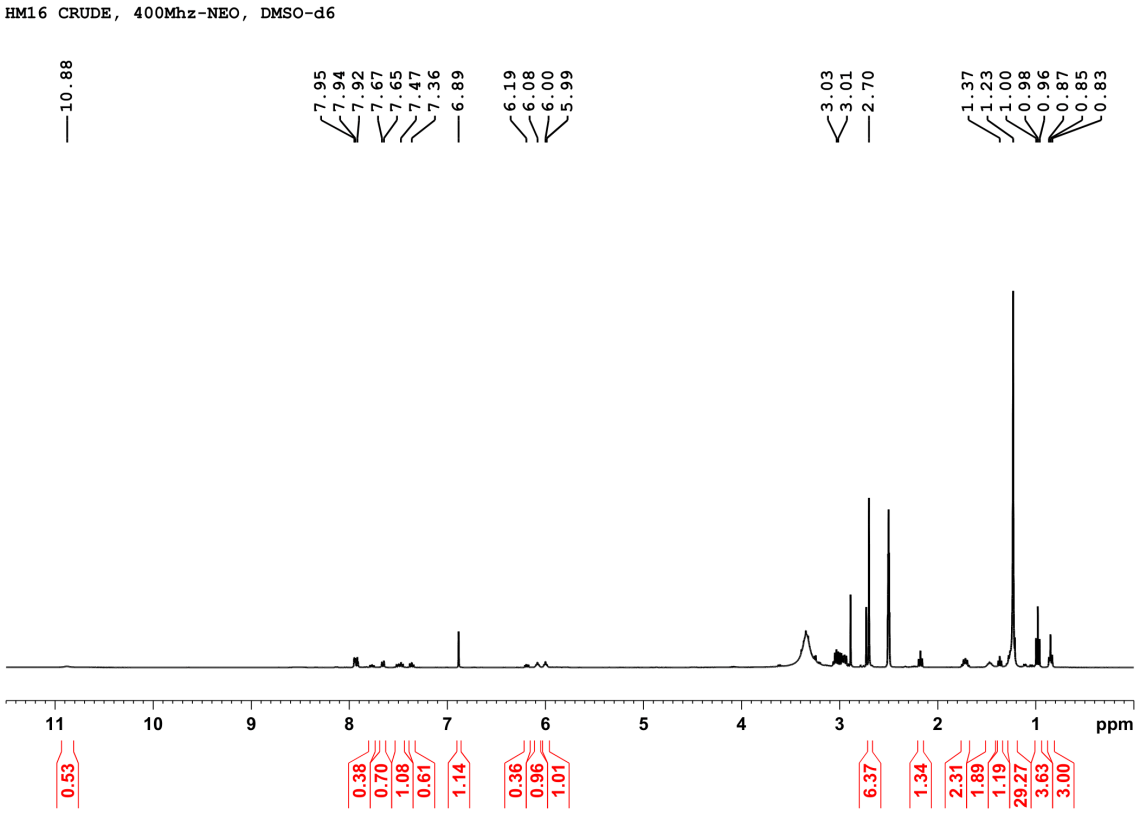


Figure 48: H NMR of crude product from maleimide functionalization of stearic acid

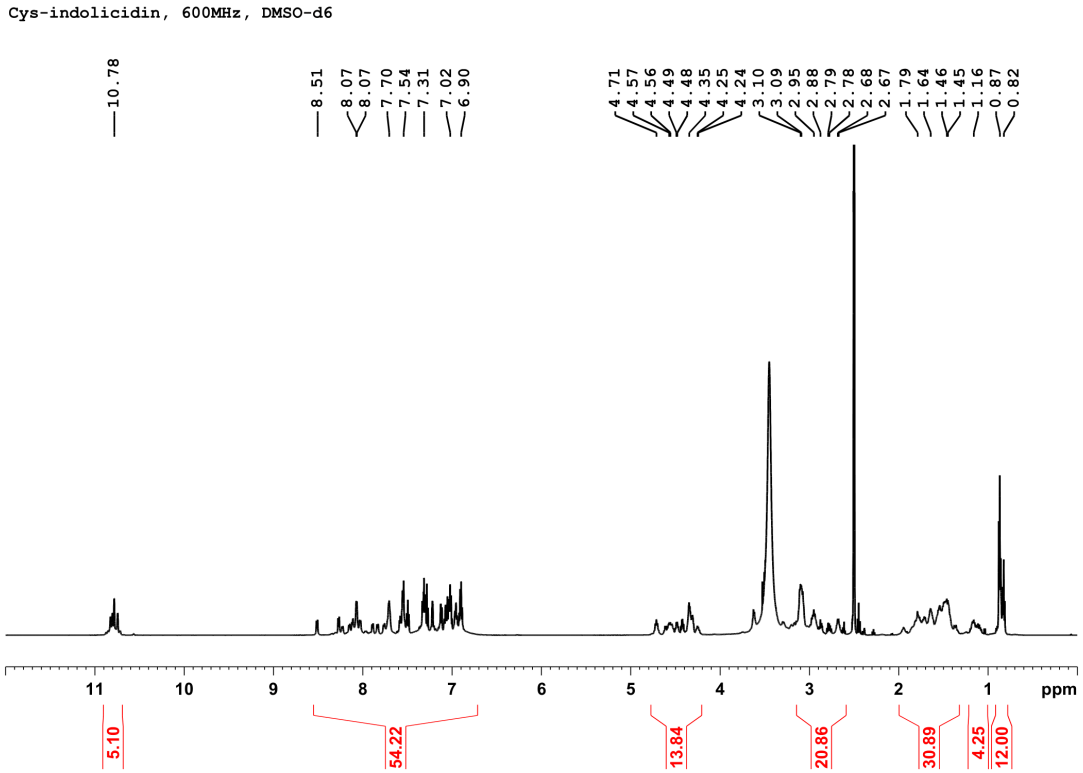


Figure 49: H NMR spectrum of cys-indolicidin

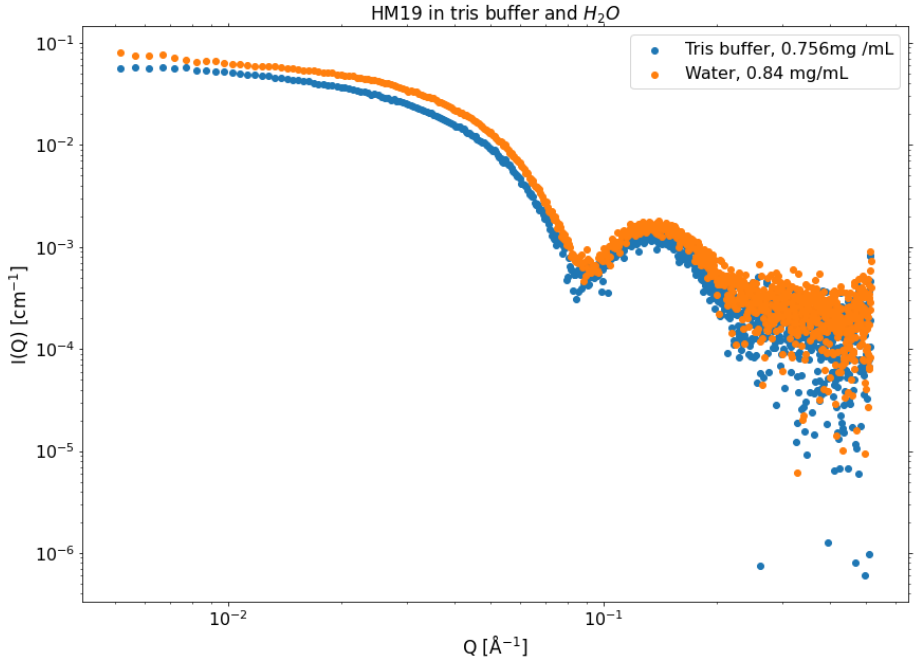


Figure 50: SAXS data of peptide-indolicidin compound in water and in buffer

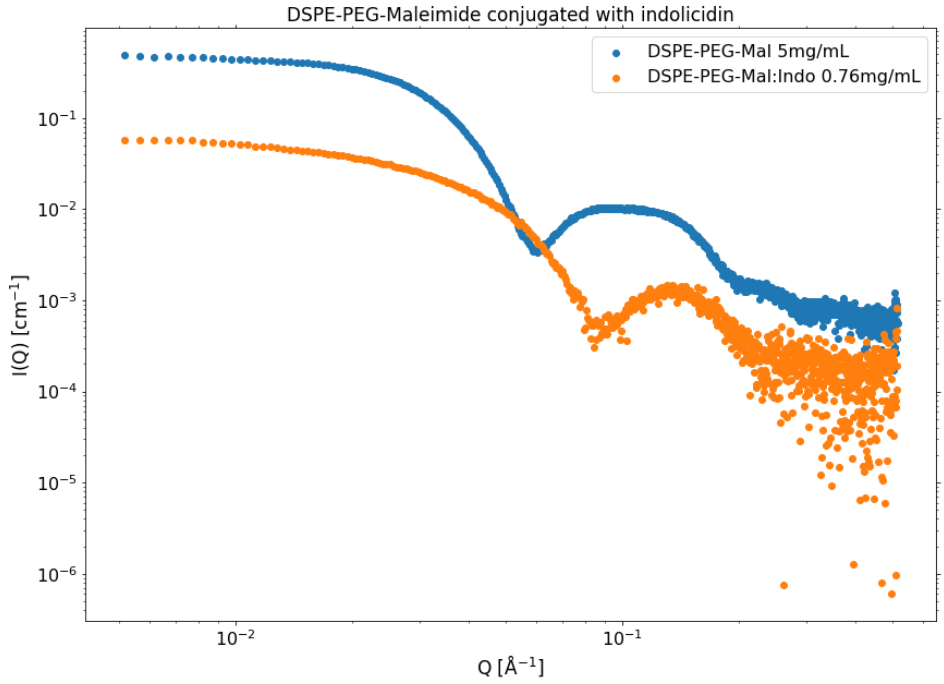


Figure 51: SAXS data of DSPE-PEG-Mal before and after conjugation

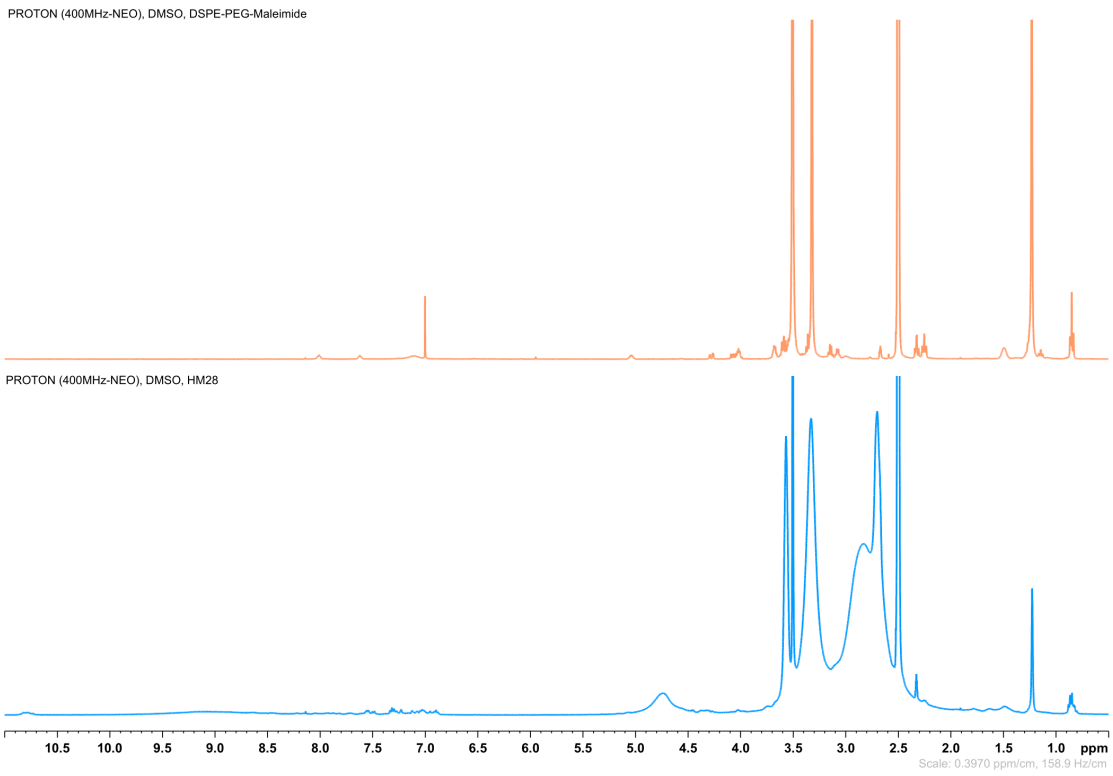


Figure 52: Proton NMR of DSPE-PEG-maleimide before (orange) and after (blue) conjugation with cys-indolicidin

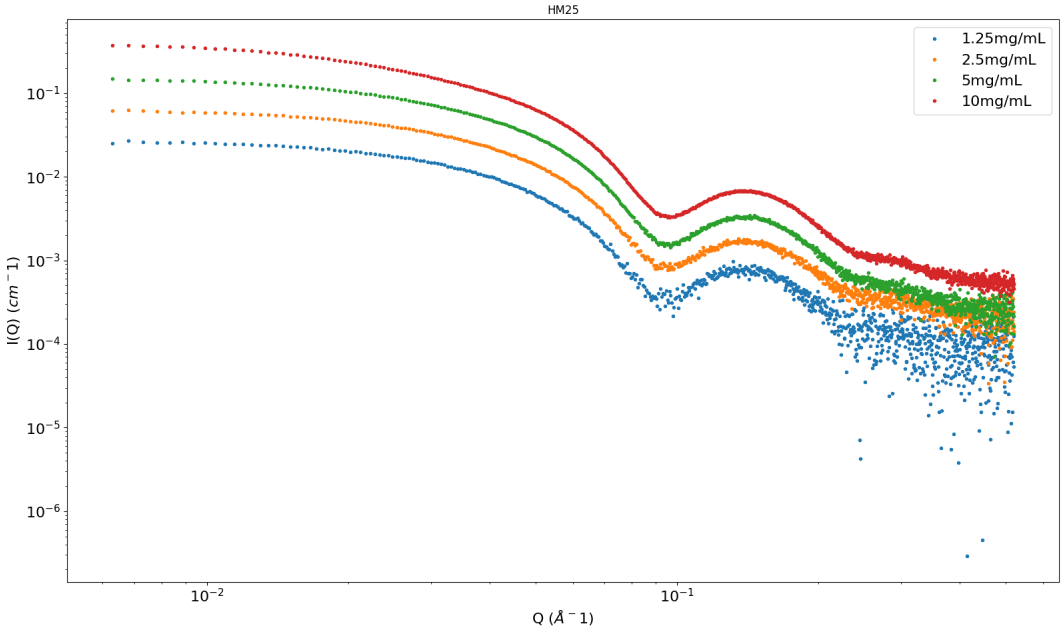


Figure 53: SAXS data of all the concentration measured of compound from experiment 6.5.2 b

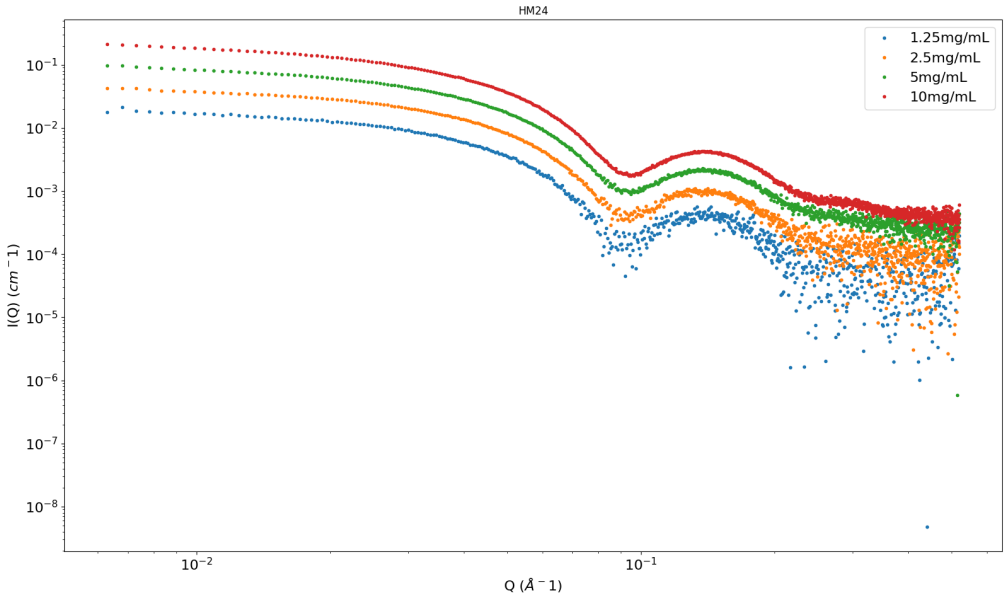


Figure 54: SAXS data of the concentrations measured of compound from experiment 6.5.2a

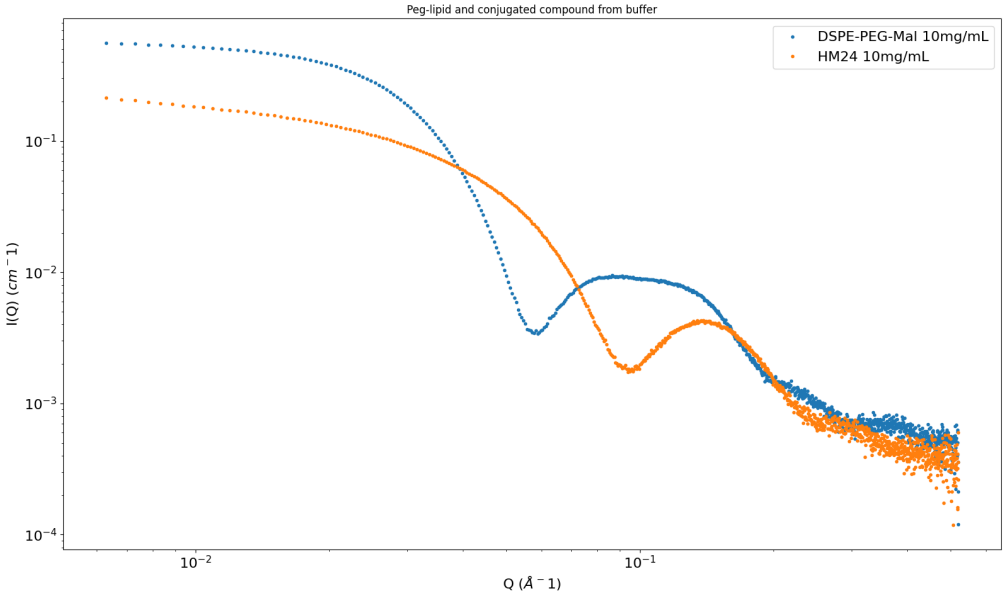


Figure 55: SAXS data of compound from experiment 6.5.2 a and DSPE-PEG-Mal

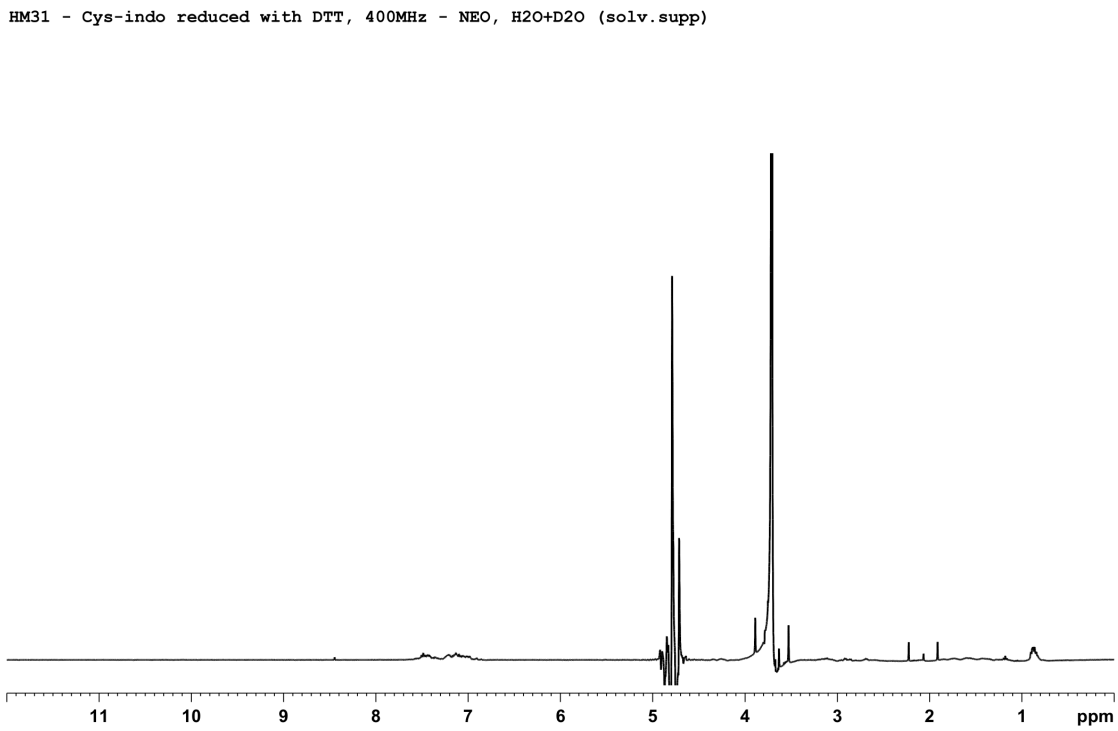


Figure 56: H NMR of cys-indolicidin after reduction with DTT and after dialysing

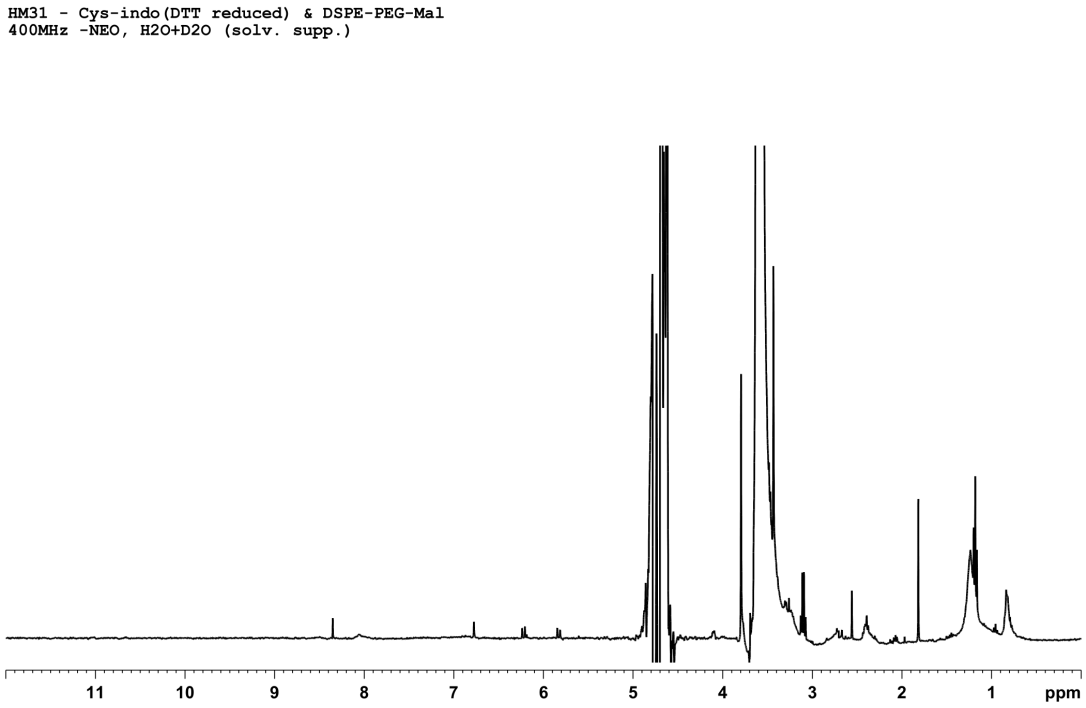


Figure 57: H NMR spectrum of product of conjugation with reduced indolicidin before dialyzing

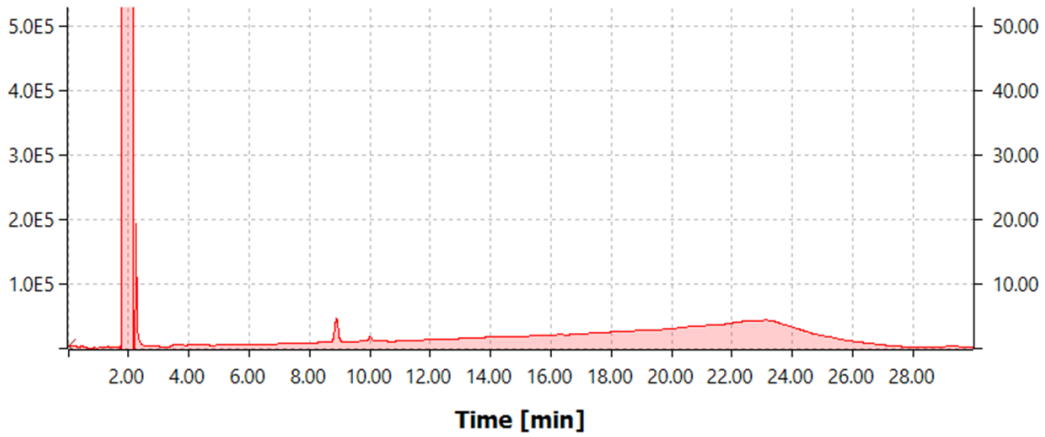


Figure 58: Chromatograph of 1µg/mL cys-indolicidin

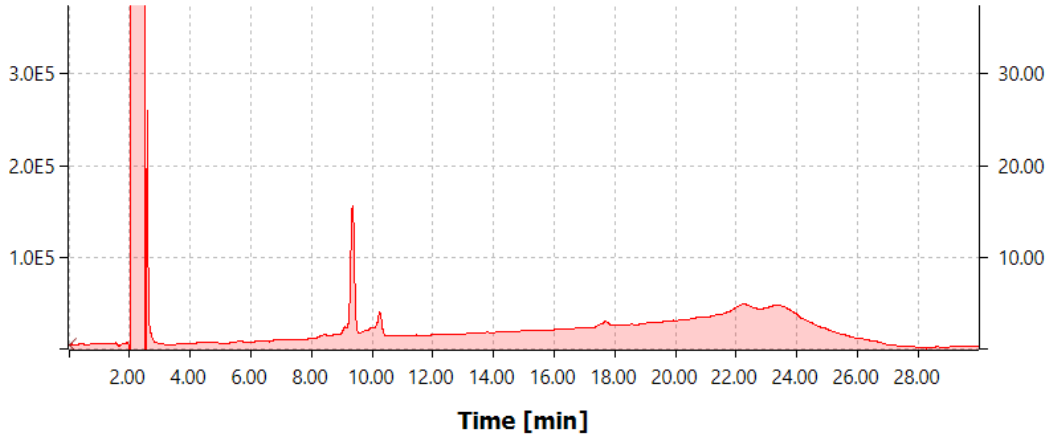


Figure 59: Cromatogram of 2.5 µg/mL cys-indolicidin

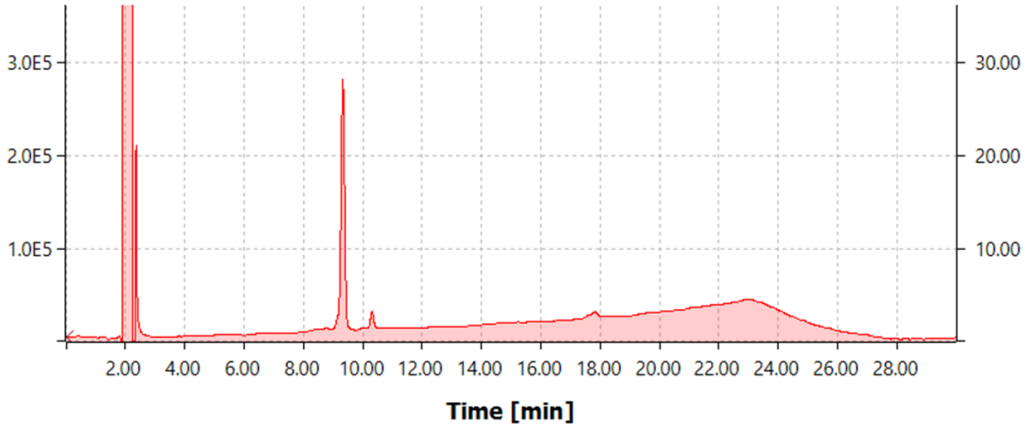


Figure 60: Chromatogram of 5 µg/mL cys-indolicidin

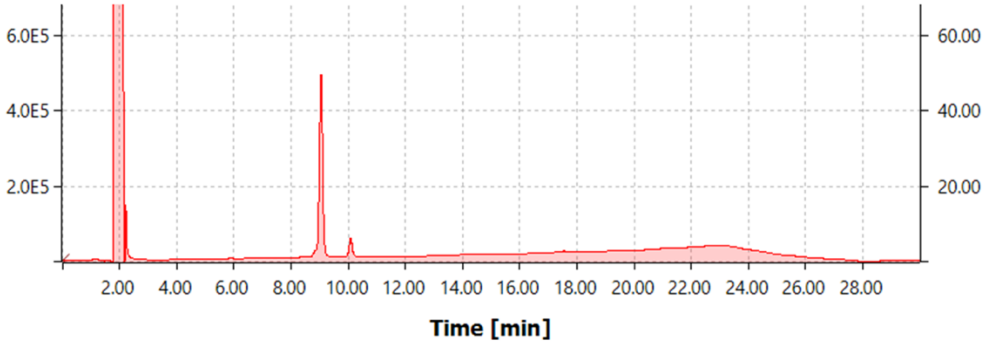


Figure 61: Cromatogram of 7.5 µg/mL cys-indolicidin

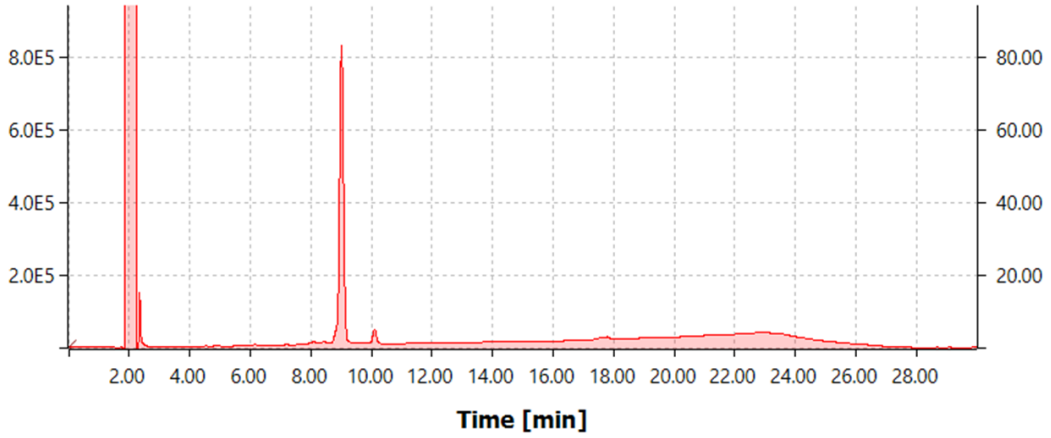


Figure 62: Chromatogram of 10 µg/mL cys-indolicidin

Table 16: Measured peak areas used to create calibration curve

First peak concentration µg/mL	~18 min Area (µV*S)	Second peak concentration µg/mL	~20 min Area (µV*S)
1	623345	1	102014
2,5	2508699,33	2,5	530964,18
5	4769576,4	5	259808,8
7,5	8152362,7	7,5	746499,6
10	15483781,39	10	658156,9

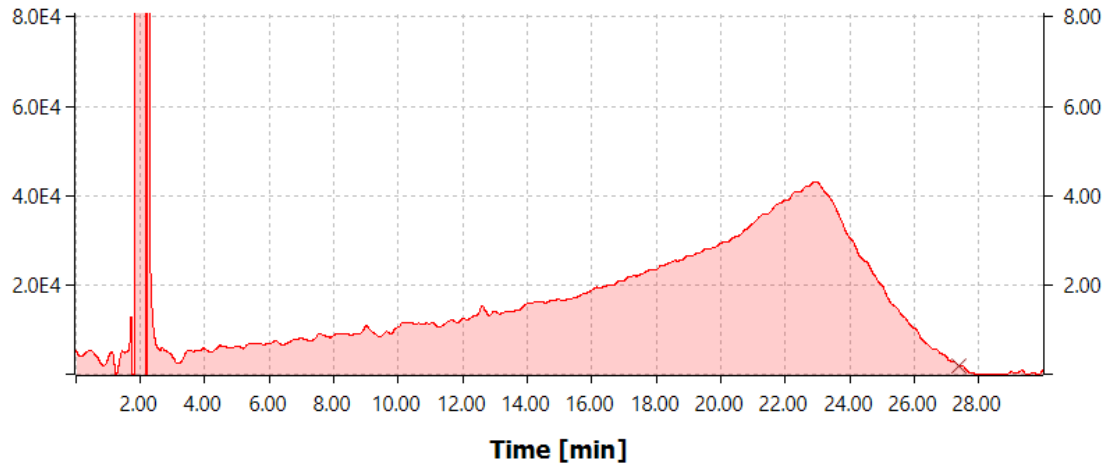


Figure 63: Chromatogram 1 ug/mL of GSH reduced cys-indolicidin

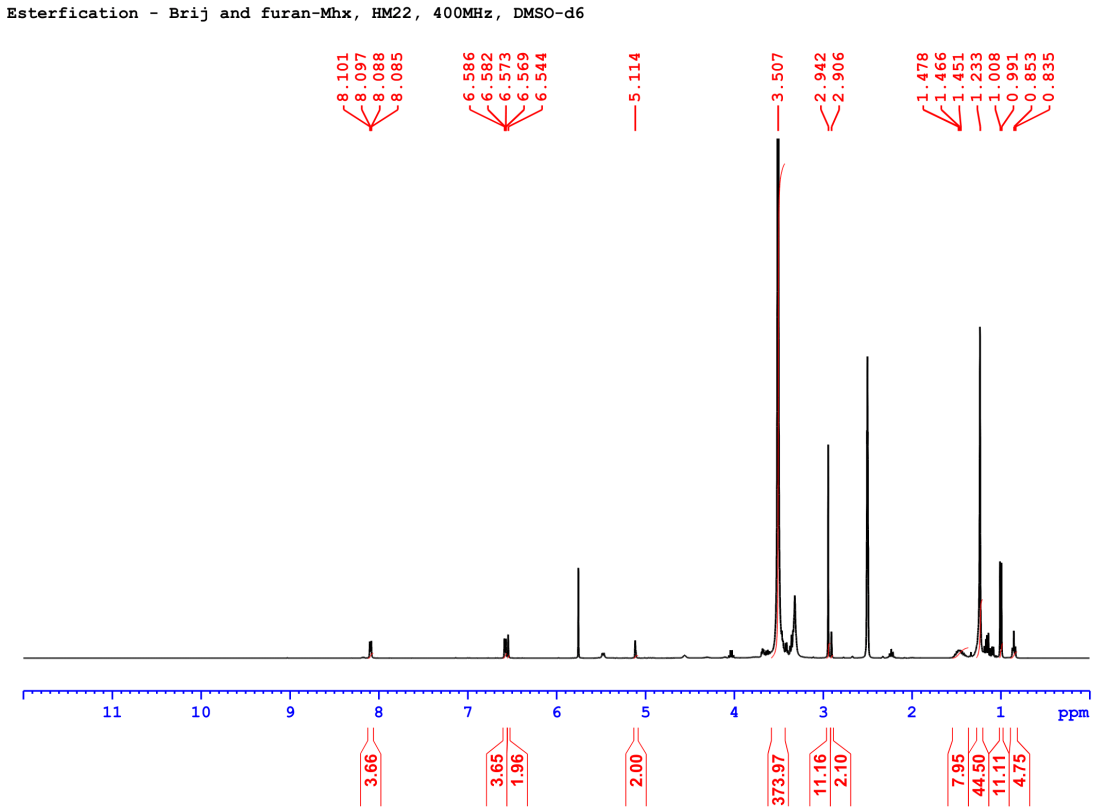


Figure 64: H NMR of experiment 6.4.2 b

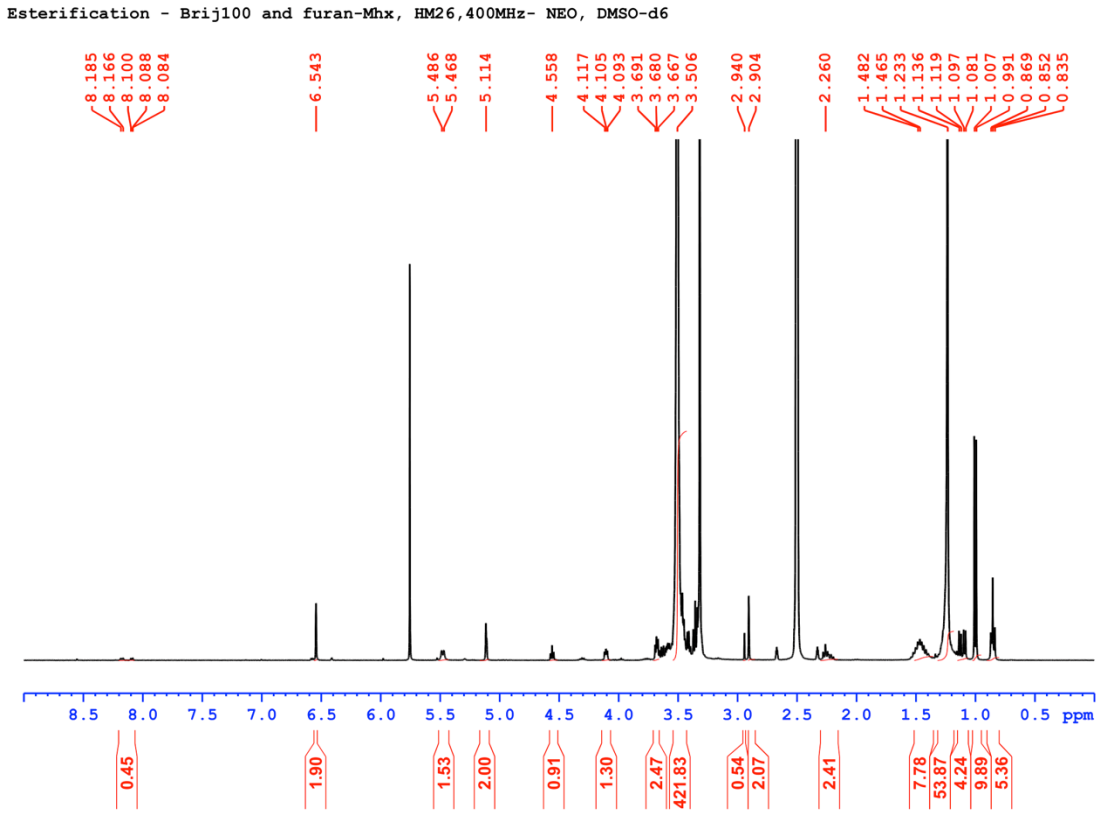


Figure 65: H NMR of experiment 6.4.1 c

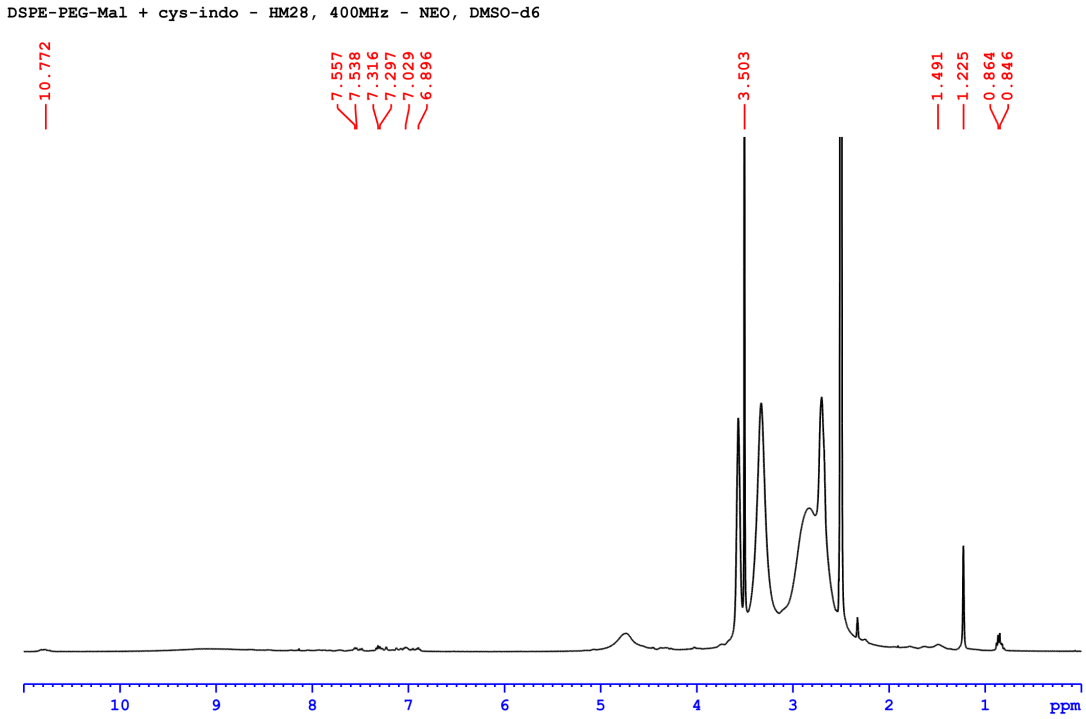


Figure 66: H NMR of experiment 6.5.3

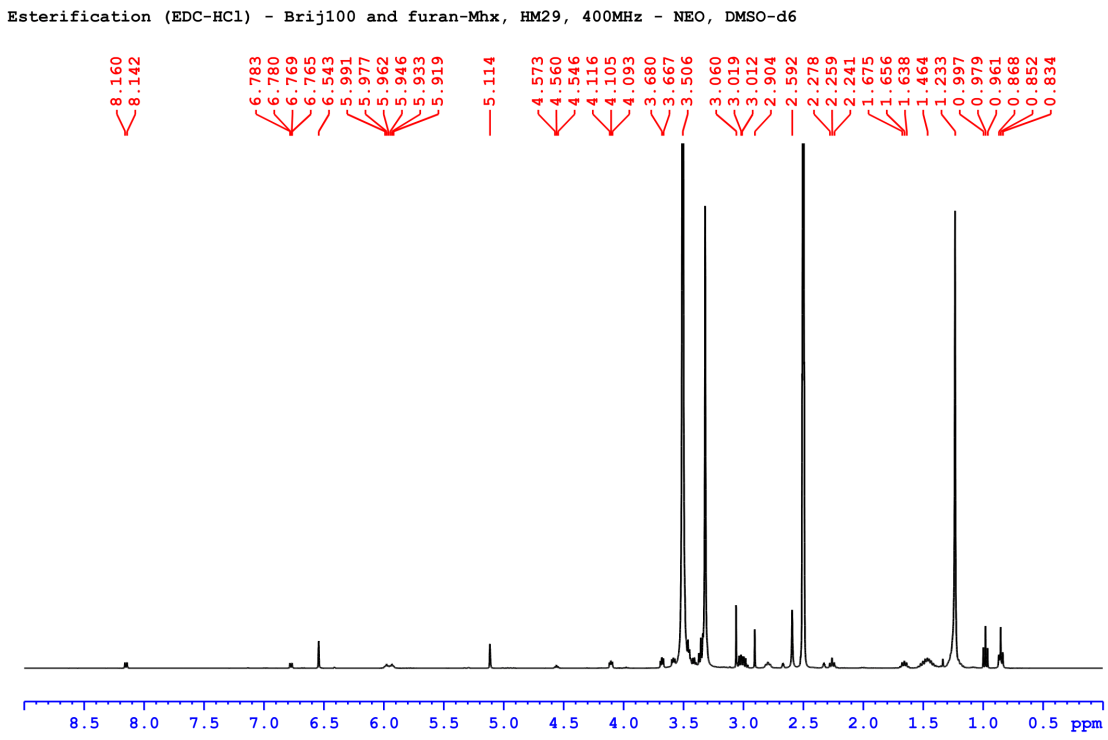


Figure 67: H NMR of experiment 6.4.2