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Determination of Cysteine Connectivity in Bioactive Peptides

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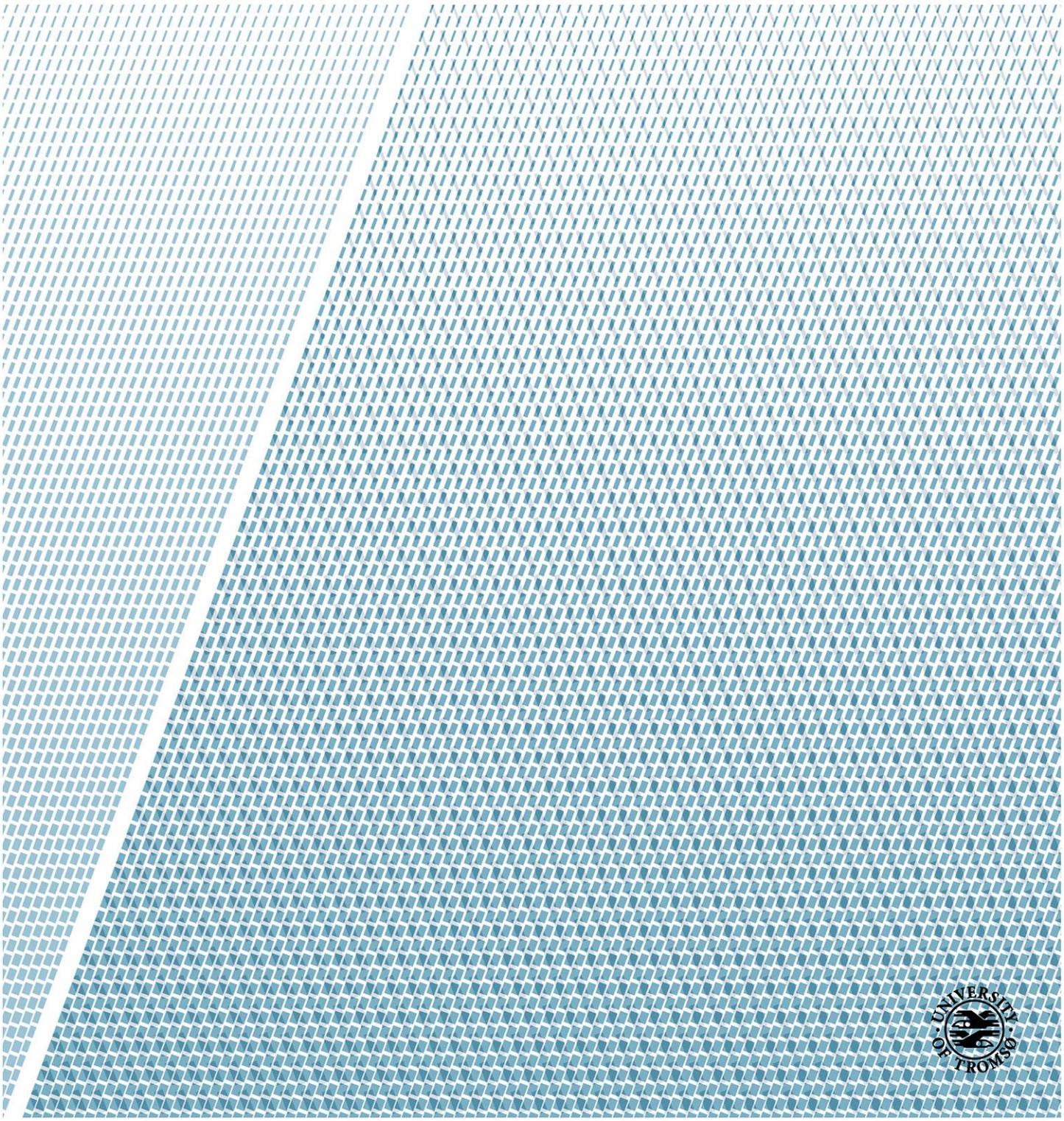
Master thesis in Pharmaceutical science, May 2014



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Abstract

In recent years, the use of biologically- and peptide-based therapeutics as a template for new lead compounds has increased rapidly. A common characteristic of the peptides used in drug development, is the high frequency of disulfide bridges. It is crucial for the biological effect of the peptide that the formation of these bridges is correct. Presently, there is no generic method for deciding the sulfide connectivity in a disulfide-rich peptide. As toxins are neuro-pharmacologically active, it is an interesting group to examine. Conotoxins are diverse and well-studied, and is therefore suited as a model to develop a general method for analyzing disulfide-rich peptides. To investigate the folding of both commercially available and self-synthesized Conotoxins, an UHPLC-MS/MS method was developed where one disulfide-bridge was reduced at the time and alkylated with different maleimides. The results from the UHPLC-MS/MS method were examined in a peptide- and protein-structure determination software.

TCEP was used to reduce the disulfide-bridges. Available literature about reduction of peptides and proteins does not discuss breaking of single disulfide-bridges, but of how to effectively fully reduce all bridges. A small activity study of TCEP was conducted to assess effective concentrations and if the effect was influenced by pH.

The Cysteine-connectivity of Conotoxin α ImI was confirmed by use of an in-vial reduction and alkylation method. The method developed was also used to establish the Cysteine-connectivity in two synthesized and oxidized disulfide-rich peptides. The method was further developed to include peptide reduction on a solid phase extraction column.

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Abbreviations

AA	amino acid
Acm	acetamidomethyl
Can	acetonitrile
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
Boc	t-butyloxycarbonyl
CID	collision induced dissociation
Cys	Cysteine
Cα	Conotoxin alpha ImI
Cα-PhM_2	Conotoxin alpha ImI with one bridge reduced and alkylated with PhM
Cα-PhM_2-NMM_2	Fully reduced Conotoxin alpha ImI alkylated with both PhM and NMM
Cω	Conotoxin ω- GVIA
Cω-PhM_2	Conotoxin ω- GVIA with one bridge reduced and alkylated with PhM
Cω-PhM_2-NEM_2	Conotoxin ω- GVIA with two bridges reduced and alkylated with PhM and NEM
Cω-PhM_2-NEM_2-NMM_2	Fully reduced Conotoxin ω- GVIA alkylated with PhM, NEM and NMM
DCM	Dichloromethane
DIPEA	N, N-diisopropylethylamine
DMF	N, N-dimethylformamide
DRP	Disulfide-rich peptide
DTNB	5, 5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothretiol
FA	formic acid
Fmoc	9-fluorenylmethoxycarbonyl

Gly	Glycine
HCTU	O-(1H-6-Chloro-benzothiazole-1-yl)-1,1,3,3-tetramethyluronium-hexafluoro-phospate
HPLC	High Performance Liquid Chormmatography
pI	Isoelectric point
$[M+H]^+$	Protonated molecule ion
MeOH	Methanol
MS	mass spectrometer
NEM	N-ethylmaleimide
NMM	N-methylmaleimide
OtBu	t-butyl ester
Pbf	2, 2, 4, 6, 7-pentamethyl-dihydrobenzofuran-5-sulfonyl
PDA	photo diode array
PhM	Phenylmaleimide
PP	"Protein Prospector"
Pro	Proline
QTOF	quadropole – time of flight
Ser	Serine
SPE	solid phase extraction
SPPS	solid phase peptide synthesis
tBM	Tert-Butylmaleimide
tBu	t-butyl ether
TCEP	tris(2-carboxyethyl)phosphine
TFA	trifluoroacetic acid
TIS	triisopropylsilane
TMP	2,2,6,6-Tetramethylpiperidine
Trp	Tryptophan
Trt	triphenylmethyl
UHPLC	ultra high performance liquid chromatography

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1. Introduction

Traditionally, most drug discovery programs concentrate on small-molecule natural products as a template for a new lead compound. In recent years, biologically- and peptide-based therapeutics has gained on the small-molecule drugs. Currently the success rate for launching biologics and peptide-based drugs is doubled compared to traditional small-molecule drugs (1). Peptide-based drugs vary in size from monoclonal antibodies with four peptide-chains creating a quaternary structure (2), to small disulfide-rich peptides with a single peptide-chain of less than 100 amino acids and derivatives thereof (1).

1.1 Disulfide-Rich Peptides

Cysteine (Cys) occurs in about 2.3 % of mammalian proteins. In small disulfide-rich peptides however, Cysteine residues accounts for more than 10 % of the sequence. These Cysteine residues are able to form reversible, covalent intra- and intermolecular disulfide-bridges. The bridges form post translational between the side-chain sulfhydryl groups. It is one of the most common post-translational alterations to occur. The oxidation of the two sulfhydryl side-groups into a disulfide-bridge stabilizes the three-dimensional structure of the folded peptide-chain. This stabilization is of high importance for the biological function of the peptide. The peptide-chain in small disulfide-rich peptides (DRPs) normally folds into a limited number of secondary structure elements that are stabilized by two or more disulfide-bridges. It is crucial for the biological effect of the peptide that the forming of these bridges is correct. The natural oxidation process is complex, with bonds forming between two Cys-residues just to break again so a different bond may be formed involving a different Cys-residue. The final, stable product of this process is called the peptides native fold.

The number of possible isomers formed through natural oxidation increases with increasing numbers of Cys-residues in the peptide. The mathematical formula is given in Equation 1. The equation shows that a peptide with four Cys-residues will have three possible isomers, six Cysteines have 15 isomers and eight Cys-residues give the possibility of 105 different isomers.

$$N_{2n}^{j} = \frac{(2n)!}{2^{j}(2n-2j)!j!}$$

Equation 1: Possible isomers formed in a random oxidation processes increases with increasing numbers of Cys-residues according to this formula, where N is the number of isomers formed through random oxidation of n thiol-groups to form *j* sulfide-bridges.

The venom-derived peptides conotoxins from marine cone snail venom, ST enterotoxins and other small disulfide-rich peptides such as plant-derived cyclotides, knottins and highly knotted antimicrobial defensins peptides are highly constrained peptides with a wide range of therapeutic applications. Interestingly, some of these peptides are potential peptide-based templates for drug design in addition to their wide range of pharmaceutical activities (1). As toxins are neuro-pharmacologically active, it is an interesting group to examine. Conotoxins are diverse and well-studied (3), and is therefore suited as a model to develop a general method for analyzing disulfide-rich peptides.

1.2 Conotoxins

While poisons are absorbed by oral intake or through the skin, venoms are actively injected by the venomous animal. The peptide and protein components of venoms are called toxins. Toxins from all four main groups of arthropods target voltage-gated ion channels (4). Conotoxins are toxins from the cone snails. Due to the vast variety of prey, each species of the conus family has developed a large array of toxins. There are conotoxins that interact with the pore-forming subunit of Na^{+}, K^{+} and Ca^{2+} channels of its prey (3), others interact with a diversity of membrane receptors and other transporters (5). So far five different groups of conotoxins are characterized. They are named α-, δ-, κ-, μ- and ω-conotoxins and they each interact with a different target (6).

Conotoxin alpha ImI (Cα) from *conus imperialis* was one of the first conotoxins to be characterized. It is part of the α-family of the conotoxins which interact antagonistic with nicotinic-acetylcholine-receptors (3). The amino acid sequence of Cα is Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys-NH_2, with two disulfide-bridges; one between Cys-

residue 2 and 8, the other between Cys-residue 3 and 12 (7). Figure 1 is graphic display of the bioactive, native isomer of the peptide.

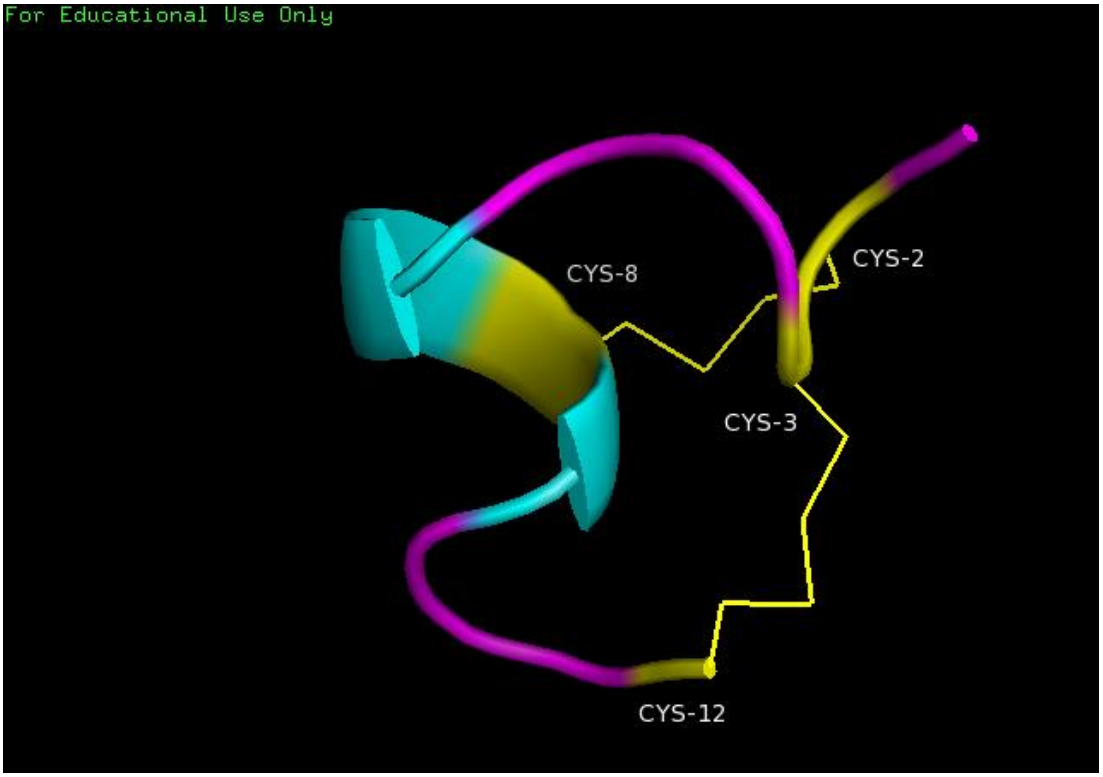


Figure 1: Conotoxin α ImI. Loops colored purple, helix turquoise and Cys-residues yellow. Disulfide-bridges are stabilizing the fold of the peptide, giving it its active isomer.

Conotoxin omega GVIA (Cω) comes from the species *conus geographus*, targets Ca^{2+} channels, has three disulfide-bridges and the sequence Cys-Lys-Ser-Hyp-Gly-Ser-Ser-Cys-Ser-Hyp-Thr-Ser-Tyr-Asn-Cys-Cys-Arg-Ser-Cys-Asn-Hyp-Tyr-Thr-Lys-Arg-Cys-Tyr-NH_2 (3). The three bridges are formed between residue 1 and 16, residue 8 and 19 and between 15 and 26 (8). Figure 2 shows a graphic display of the bioactive, native isomer. Note that Conotoxin ω GVIA is a β-sheet structure while Conotoxin α ImI has an α-helix as secondary structure.

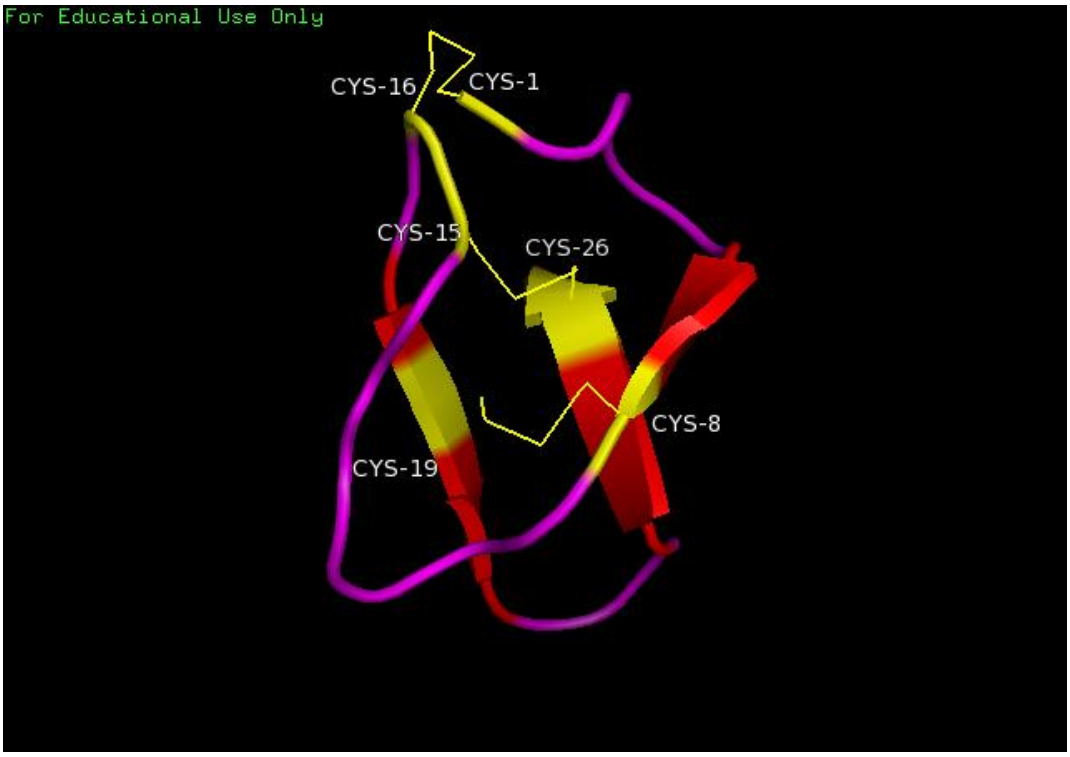


Figure 2: Conotoxin ω GVIA, β-sheets colored red, loops purple and Cys-residues yellow. The disulfide-bridges are drawn as in the active isomer.

2. Theoretical Background

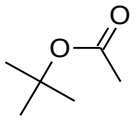
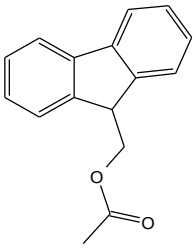
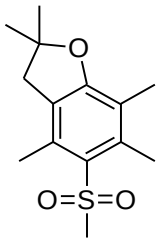
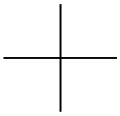
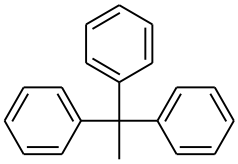
2.1 Peptide Synthesis

Solid phase peptide synthesis involves, as the name implies, a solid support for the peptide to be assembled on to. The C-terminal of the first amino acid in the peptide-chain is attached to an insoluble support via its carboxyl-group. If the side-chain of the amino acid has a functional group i.e. aspartic acid, it needs to be masked by a protecting group that is not affected by the reaction conditions employed during the assembly of the peptide-chain. This is referred to as orthogonal protection, and is necessary to avoid agglomeration, formation of dimers and even branching of the peptide-chain. The N-terminal of the amino acid is also protected, but is removed when the coupling stage is finished. This allows the next amino acid in the sequence to attach and make a peptide bond between the first and the second amino acid. A coupling reagent is added to facilitate the reaction. After the coupling, the peptidyl is washed of excess reagents and the N-terminal is deprotected before the process is repeated until the desired peptide-chain has been assembled. The fully assembled peptide is then chemically cleaved from the resin. Protection-groups on the side-chains are removed during the cleavage step. The protection-groups that are removed from the side-chains are still reactive. It may re-form the attachment to the side-group, or form a new attachment on a different residue. To prevent this, the cleaving mixture has to contain scavengers that react with the de-attached protector and render it unreactive (9).

9-fluorenylmethoxycarbonyl (Fmoc) - protection of the N-terminus is currently favored over t-butyloxycarbonyl (Boc) - protection, since Fmoc uses milder conditions.

Boc is still frequently used as a protector of active side-chains in Fmoc-SPPS. t-butyl ester (OtBu), 2, 2, 4, 6, 7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf), t-butyl ether (tBu) and triphenylmethyl (Trt) are other recommended side-chain protectors in Fmoc-SPPS. Table 1 show the recommended protectors for Fmoc-amino acids and gives the molecular structure of these.

Table 1: Name and molecular structure of Fmoc and the side-chain protecting groups recommended in Fmoc-SPSS, and their removing agent

Name	Molecular structure	Removing agent
Boc		95 % TFA
Fmoc		20% Piperidine in DMF
Pbf		TFA
tBu/OtBu		95 %TFA
Trt		TFA

There are recommendations on which protecting groups that are suitable for which amino acid. The different protectors have different chemical properties and will need different reaction-terms to be removed, i.e. reaction time and reagents, and are listed in Table 2.

Table 2: The amino acids, the functionality of the side-chains, and their recommended protecting groups; NA = not applicable.

Amino acid	Functional side-chain protected	Recommended protecting group
Alanine	NA	NA
Arginine	guanidino N	Pbf
Asparagine	Amide	Trt
Aspartic acid	Carboxylic acid	tBu
Cysteine	Sulfhydryl	Trt
Glutamic acid	Carboxylic acid	Trt
Glutamine	Amide	tBu
Glycine	NA	NA
Histidine	Imidazole	Trt
Isoleucine	NA	NA
Leucine	NA	NA
Lysine	Amino	Boc
Methionine	NA	NA
Phenylalanine	NA	NA
Proline	NA	NA
Serine	Hydroxyl	tBu
Threonine	Hydroxyl	tBu
Tryptophan	Indole	Boc
Tyrosine	Phenol	tBu
Valine	NA	NA

Chemical oxidation is a method used to ensure the correct oxidation of the sulfhydryl side-chains of the Cysteine residues into the native isomer of the peptide. This is done by protecting Cys-residues with protectors with different qualities. The protectors, triphenylmethyl (Trt) and acetamidomethyl (Acm) cleave off the side-chain of Cys at different chemical environments. Trt is removed by TFA in the cleaving-step, while Acm is stable in acidic environment. Acm will therefore still be attached to the Cysteine sulfhydryl-group even after cleaving, and needs to be removed by iodine in a separate step. The molecular structure of Acm is presented in Figure 3. By protecting the two Cys-residues of

one of the native disulfide-bridges with the same protecting-group they are deprotected at the same time and therefore oxidize to form a disulfide-bridge. The second native bridge is then the only remaining option for the next Cysteine pair that is deprotected when Acm is removed.

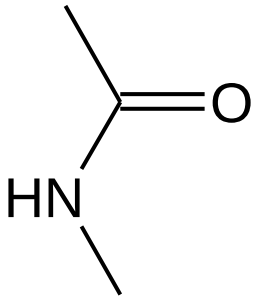


Figure 3: Molecular structure of Acm

2.2 Activity Studies of TCEP

5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) is previously used to study the activity of reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) (10). DTNB is also known as Ellman's reagents with the molecular structure presented in Figure 4. One molecule of DTNB is reduced to two molecules of 2-nitro-5-thiobenzoate (NTB).

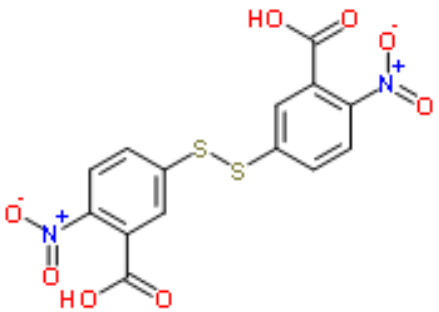


Figure 4: DTNB is color-free and has no absorption at 412 nm unlike the reduced structure with its distinct yellow color and clear absorption at this wavelength.

The proposed mechanism of reaction between DTNB and TCEP is shown in Figure 5 and occurs by breaking the bond between the two sulfur-atoms which can be compared to a disulfide-bond in DRPs. TNB has a molar extinction coefficient of 14.150 $M^{-1}cm^{-1}$ at 412 nm (10). By measuring the absorbance of reduced DTNB after different reaction times and at different pH, this fact can be utilized in activity studies of reduction agents used in proteomics to reduce disulfide-bridges. The stronger the absorbance, the more DTNB has been reduced, thus the peak performance area of the reducing agent can be found.

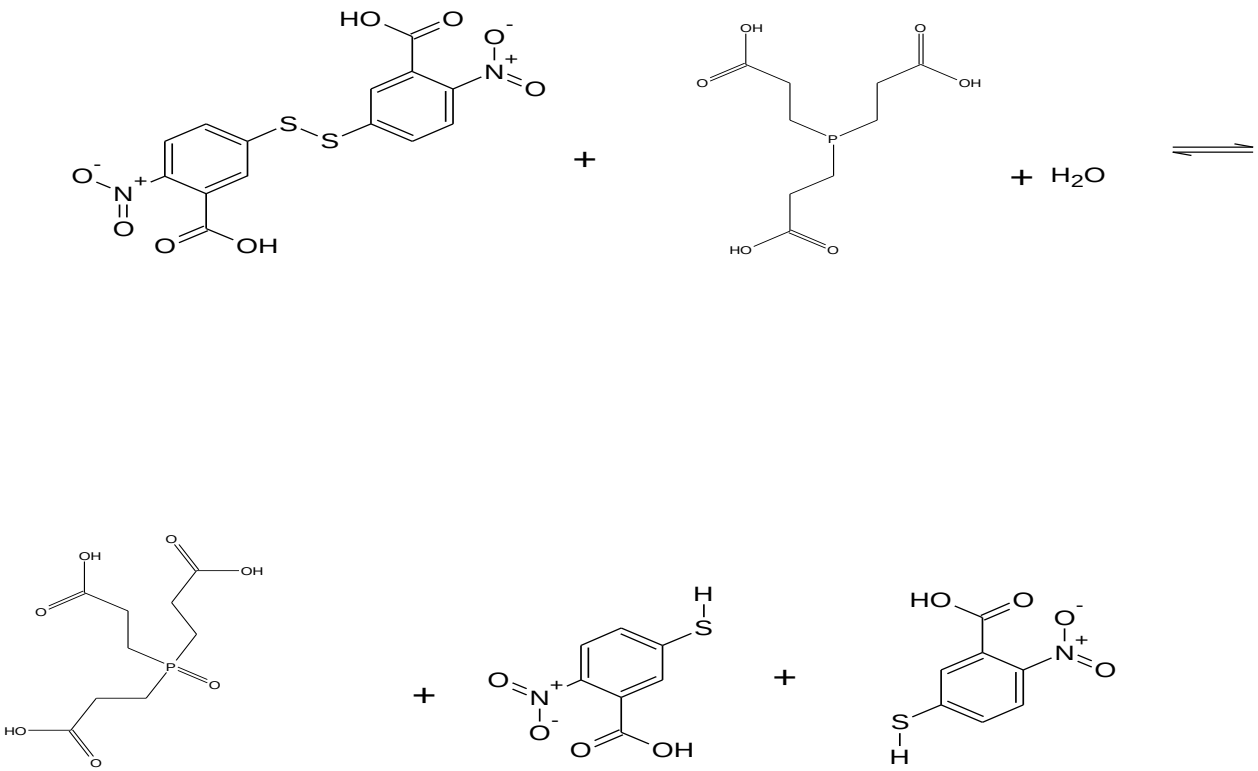


Figure 5: Reaction between DTNB and TCEP. The product of the reaction oxidized TCEP and 2 molecules NTB. The mechanism is the same as when TCEP is used to reduce disulfide bonds in peptides.

2.3 Reduction and Alkylation of Disulfide-Bridges

The oxidation of cysteine residues to form disulfide-bridges is one of the most common post-translational alterations to occur. The natural process of oxidative folding is very complex (1). To reverse the oxidation is not as complicated, but it does not happen spontaneously after the native fold has been made. Thus a reduction agent is needed. Dithiothretiol (DTT) and TCEP are two reagents used in proteomics to reduce disulfide-bridges. The latter is deemed more effective and suitable for disulfide-bonds in proteins. TCEP is claimed to be efficient at acidic pH where DTT is not, and does not react with the peptide-chain's active side-groups where DTT does (11).

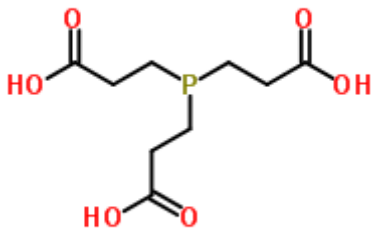


Figure 6: Structural formula of TCEP

TCEP is colorless, odorless and readily soluble in water (10). An aqueous solution will have a pH of 2.5, and a buffer is needed to adjust the pH of the TCEP solution to the desired value.

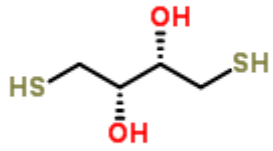


Figure 7: Structural formula of DTT

DTT is soluble in water and a variety of organic solvents. It is colorless as an aqueous solution, but do have an odor due to the sulfhydryl-groups.

The proposed mechanism of reaction involves the phosphine group that forms a radical intermediate involving two electrons followed by the de-protonation of the sulfhydryl group that produces the final oxidation product after direct nucleophilic attack by water (TCEP), or a nucleic attack from the deprotonated sulfhydryl group with the lowest pKa (DTT). In both cases, the oxidation is irreversible and occur in one single step (11). Due to its reaction mechanism involving deprotonation of a sulfhydryl group, the DTT loses its reducing qualities at pH lower than 5, and optimal pH range is between 6.5 and 9 (10).

After the reduction of the disulfide bond, an alkylating agent is used to ensure that the sulfhydryl-group of the Cysteine residues is inactivated. There are several alkylating agents commercially available, amongst these are maleimides.

Maleimides is a group of alkylating reagents with a preserved ring-structure. The difference is the composition of the side-chain of the nitrogen-atom in the ring-structure. In its simplest form the side-chain is a mere proton. In methyl-maleimide (NMM) the side-chain is indeed a methyl-group as the name suggests, see Figure 8.

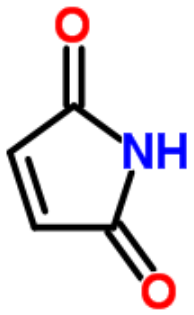


Figure 8: 5-Hydroxy-2H-pyrrol-2-one (maleimide). The reaction happens between the sulfhydryl-group of the peptide and the electrons of the double-bond to form a thioether.

Maleimides block the sulfhydryl-groups of the Cys side-chain by forming a stable thiolether. The reaction is irreversible and occurs in a single step. See Figure 9 for proposed reaction between NMM and the sulfhydryl-group of Cysteine in a DRP.

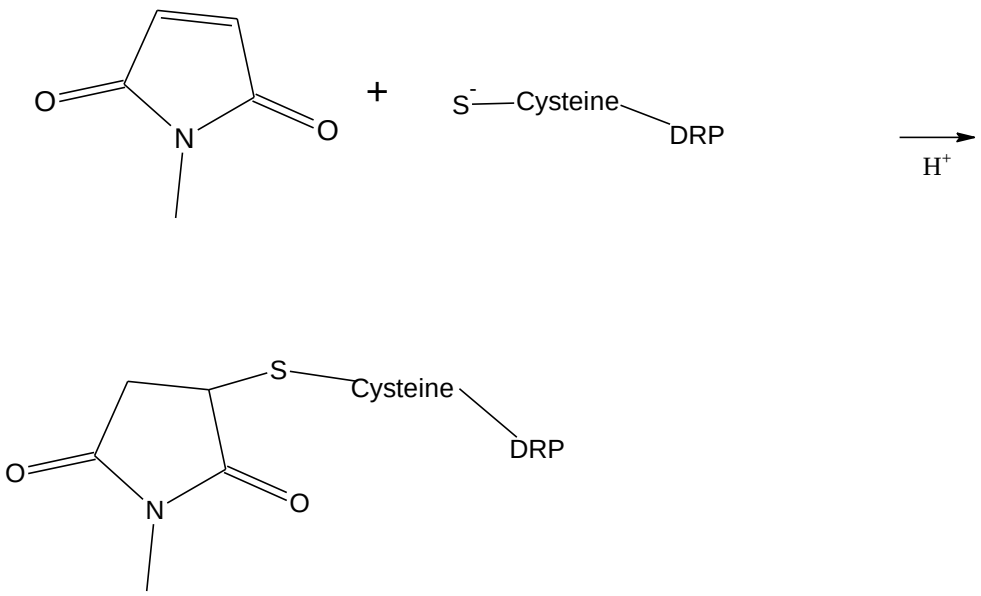


Figure 9: The reaction between NMM and Cysteine's sulfhydryl-group to form a thiolether.

Most maleimides are poorly soluble in water due to its ring-structure and hydrophobic side-chain. The electronegative oxygen-atoms bound to the ring by double bonds give the molecule polarity. When the side-chain is a proton, the molecule is polar enough to dissolve in water (12). Methylmaleimide is also readily soluble in aqueous solutions. An increase in side-chain size and hydrophobicity gives decreasing water-solubility of the maleimide.

The alkylation reaction between the maleimide and the sulfhydryl-group is instantaneous and complete at pH 8. At a lower pH, the reaction is still complete given enough time; it is no longer instantaneous. At higher pH, the base-catalyzed ring opening of the maleimides incapacitates their activity (13).

Figure 10 shows a simplified schematic reaction for the reduction and following alkylation of a DRP with two disulfide-bridges. Only the peptide backbone is drawn, with the disulfide bridges oxidized, reduced and alkylated respectively. The alkylating agents are of different size, weight and hydrophobicity. This can be used to differentiate between the Cysteine-residues using the analytical tools of liquid chromatography and mass spectrometry coupled with proteomic software.

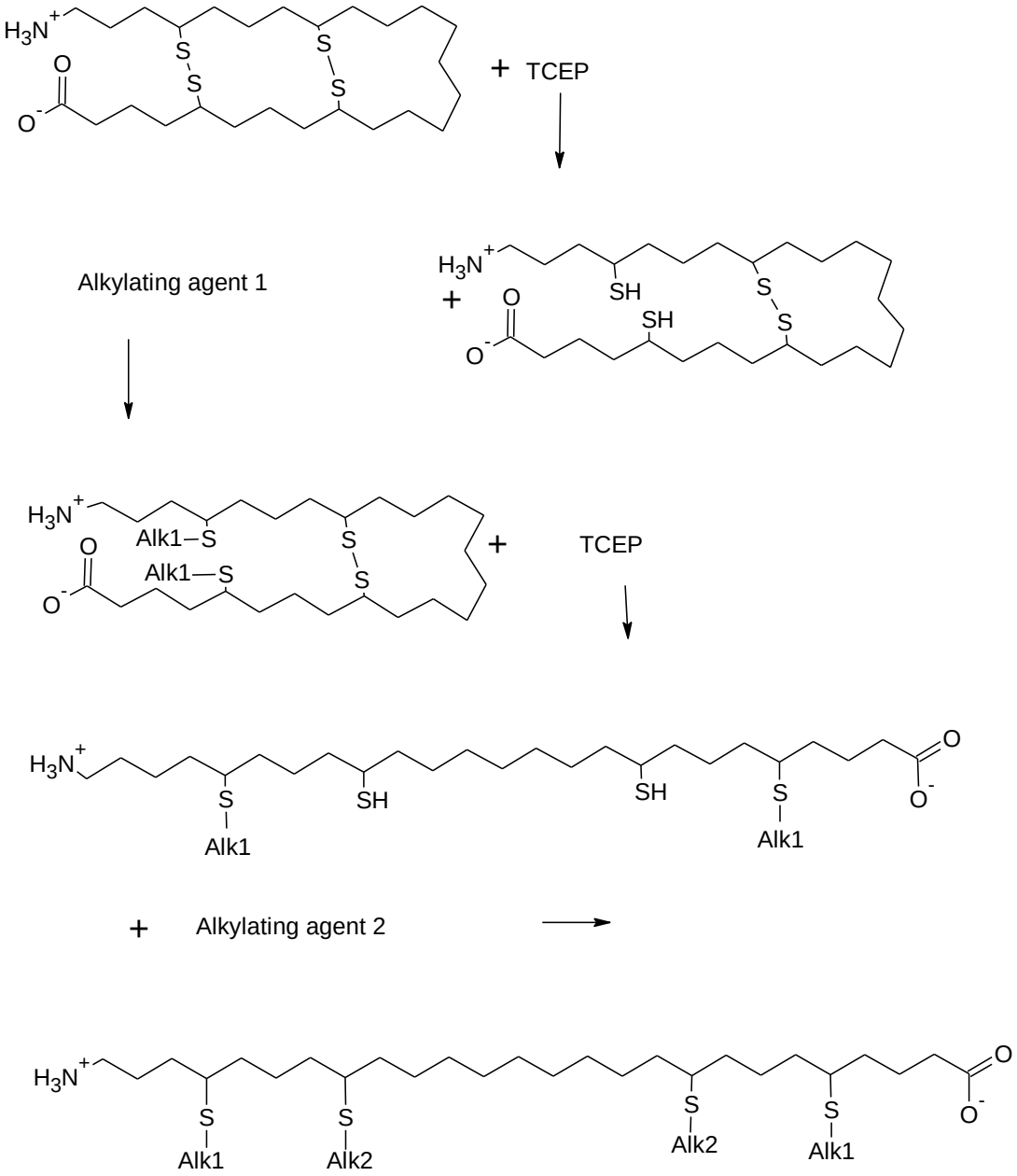


Figure 10: Simplified sketch of the selective reaction between a peptide with two disulfide bridges, TCEP and two alkylating agents.

2.4 Solid Phase Extraction

Solid phase extraction (SPE) is a sample preparation method mainly used on aqueous solutions, but is also used on organic solutions. There are several types of SPE-columns available; reversed phase, normal phase, ion exchange and even mixed columns. Within each category there are also several choices. This makes SPE a versatile preparation method that easily can be optimized to fit a wide range of analytes. One of the before mentioned extraction columns packed with stationary phases of choice is loaded with a sample. Contaminants are washed of the column, and the analyte is there after eluted. See Figure 11 for illustration.

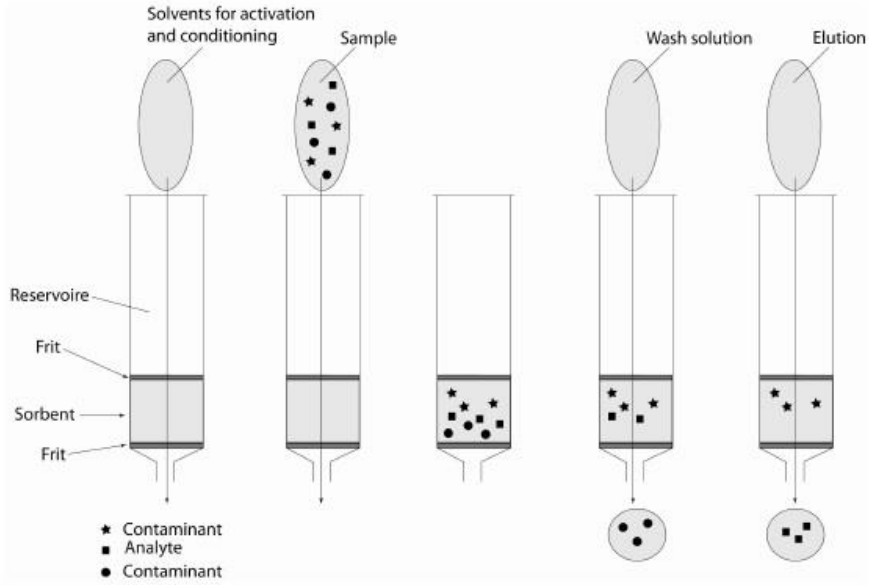


Figure 11: Schematic illustration of a SPE experiment. Used with permission by Terje Vasskog (14).

A vacuum manifold is used to drag the washing solutions with its contaminants and the eluent with the analyte through the column. The technique is based on the difference in affinity to the solid phase between the wanted analyte and the contaminating components of the sample. To extract relatively non-polar analytes from aqueous solutions it is a good choice to use a hydrophobic stationary phase. The stationary phase can be based on silica particles with varying length of hydrophobic group from an ethyl group, trough aromatic and cyclic compounds to an octadecyl group. The octadecyl-silica columns are referred to as C_{18} columns. Retention of an analyte will increase with the strength of its interaction with the adsorbent surface of the solid phase, and a C_{18} column will therefore give strong retention to a non-polar compound. Composition and pH of the washing solution must ensure the retention of the analyte through the washing step of the extraction while more polar contaminations are

washed away. The eluent must be strong enough to elute the analyte, but preferably not strong enough to elute hydrophobic contaminations (15).

2.5 UHPLC-MS/MS

2.5.1 Ultra High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique often used in the analysis of a wide range of compounds, hereunder peptides. The technique is based on the difference in affinity to the mobile- and solid phase between the compounds one wants to separate. Retention of an analyte will increase with the strength of its interaction with the adsorbent surface of the solid phase. Composition and pH of mobile phase both affect the retention of the analyte, as will the composition of the solid phase (16). Ultra high performance liquid chromatography (UHPLC) is a further development of the HPLC technique. In UHPLC mobile phase pumps can withstand a higher backpressure, and the limitations to particle size and column length can be extended. The particles of the solid phase are smaller and more spherical, and the diameter of the column is often smaller than in HPLC. This gives greater resolution per unit time and narrower peaks.

Generally the most widespread used is reversed phase UHPLC where the solid phase is hydrophobic and the mobile phase polar (16). Reversed phase UHPLC gives a wide range of possibilities in composition of both mobile- and solid phase. The mobile phase may vary in pH and polarity as suits the analyte. The pH can either be acidic or alkaline throughout the analysis, or a pH gradient can be made. The aqueous-to-organic ratio in the mobile phase will change according to a preprogrammed gradient. The gradient moves from mainly aqueous to mainly organic during the analysis, ensuring a separation of components of the sample. The solid phase is most commonly made up from molecules of varying length and hydrophobicity linked to a silica core.

2.5.2 Electrospray Ionization

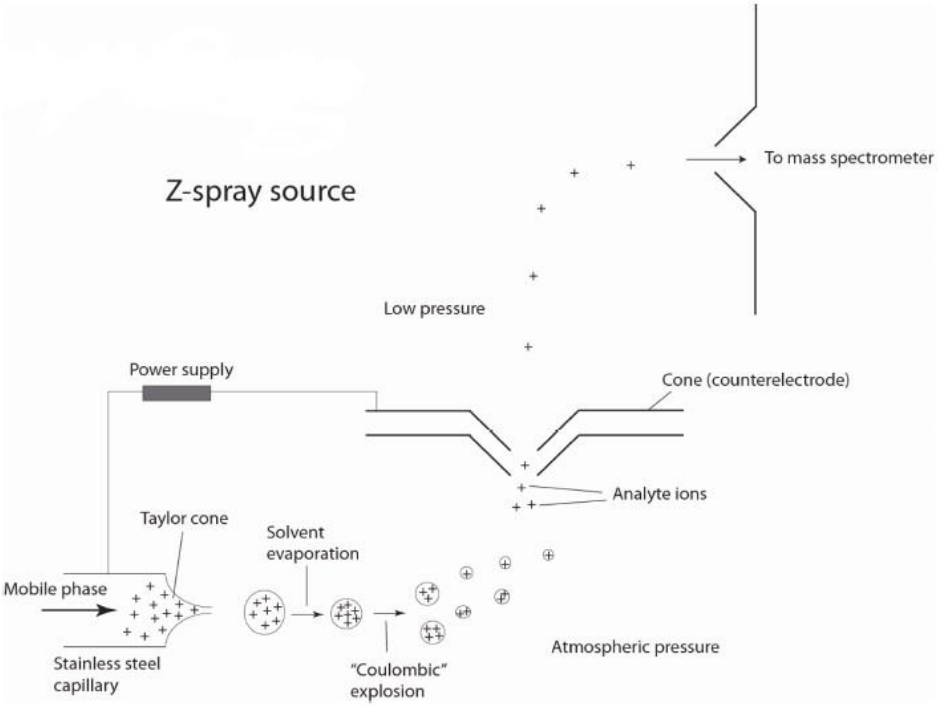


Figure 12: Illustration of an ESI z-spray source. Used with permission by Terje Vasskog (17).

Due to so called soft ionization methods such as matrix-assisted laser desorption ionization and electrospray ionization (ESI), which is able to form molecule ions from biologically derived samples, MS/MS can be used to analyze peptides, proteins and nucleic acids (18). As illustrated in fFigure 12, the mobile phase from the UHPLC flows through a capillary and an aerosol is formed with help from a nebulizing gas that flows along the tip of the capillary. There is a steel capillary in the continuation of the UHPLC-capillary with an electrical potential of up to 5 kV. The electrode, gives an electric potential to the small drops of mobile phase in the aerosol. The volatile mobile phase evaporates, and the evaporation is helped by a drying gas. After mobile phase evaporation the analyte ions are extracted through the sample cone into the vacuum area of the MS (15).

In ESI, the number of charges a molecule will get depends on its molecular weight and number of available potential charge sites. Potential charge sites include sites for protonation, deprotonation, anionization and cationization. Since the MS measures a mass-to-charge ratio, a high charge state will allow big molecules such as proteins to be analyzed on standard MS instruments. Molecules of low mass gives only singly charged ions in ESI. Higher mass molecules will form doubly, triply and multiply charged ions that all will show up in the mass spectrum of the compound (19).

2.5.3 Mass Spectrometry

A mass spectrometer (MS) is an instrument that detects atomic or molecular ions based on their mass to charge ratio (*m/z*). The MS might also form fragment-ions from the original molecular ion, which can be used in structure determination of unknown analytes. An instrument capable of two stages of *m/z* analysis is referred to as a MS/MS. There is a range of available systems to combine two MS, here under the tandem Quadropole, Quadropole-Time-of-Flight (QTOF) and Orbitrap instruments.

The hybrid MS/MS instrument QTOF is commonly used in MS/MS analysis of peptides. In the QTOF the first MS is a quadropole mass filter (MS1), the collision cell is an ion guide of some kind (i.e. T-wave collision cell or collision cell consisting of six or eight metal rods), and the final mass analysis occurs in a Time-of-Flight MS (MS2) (18). The quadropole in MS1 consists of four parallel hyperbolic or round rod electrodes in a square. The opposite rods have the same potential at any given time. A periodic voltage is applied to the rods, and the ions will be attracted and repulsed accordingly as the electric force changes. In the collision cell an inert collision-gas may be present. Argon and Nitrogen are frequently used for this purpose. The collision gas fragments the precursor-ions by increasing the internal energy of these through collisions. The increased energy breaks the weakest bonds in the precursor-ion and form fragments, known as product ions. Finally, the time-of-flight analyzer measures, as indicated by the name, the time any given ion uses to move a given distance. The principle is that the smaller and therefore lighter ions will reach the detector earlier than the heavier ions (19).

A MS/MS measures the compounds monoisotopic mass, not the average mass usually used when calculating molar masses and so on. Peptides and proteins are high mass molecules, and often carry multiple charges due to alkaline side-chains of AA. The charge state of an analyte ion can be determined by calculating the *m/z* difference between its isotopic peaks. The mass difference between the C_{12} and the C_{13} isotopic peak is one amu. At charge state one the *m/z* difference between the isotopic peaks is one. If the charge state is four, the mass difference between the isotopes is still one, but the *m/z* difference measured will be ¼.

2.5.4 CID-Fragmentation of Peptides

Collision induced dissociation (CID) is a fragmentation method widely used in MS/MS of small ions, but has extended to incorporate larger biological compounds (16).

CID of peptide-ions is an important tool in MS/MS analysis of peptides. It is a mechanism by which to fragment molecular ions by colliding them with inert gas molecules. The kinetic energy of the collision results in fragmentation of the precursor-ion (18). A peptide is a chain of amino acids (AA) chained together by the peptide bond. The C-terminus of the first AA bonds to the N-terminus of the second, creating a water-molecule and a covalent bond between the two AAs. When this AA-chain is fragmented, it may break at any bond in the peptide back-bone, dependent on the fragmentation technique used. A classification system has been made to generalize the names of the fragment-ions that occur. Figure 13 shows a peptide chain of n amino acids with the fragment names and numbers according to this system. The names and numbering of the fragments follows the sequence of the peptide.

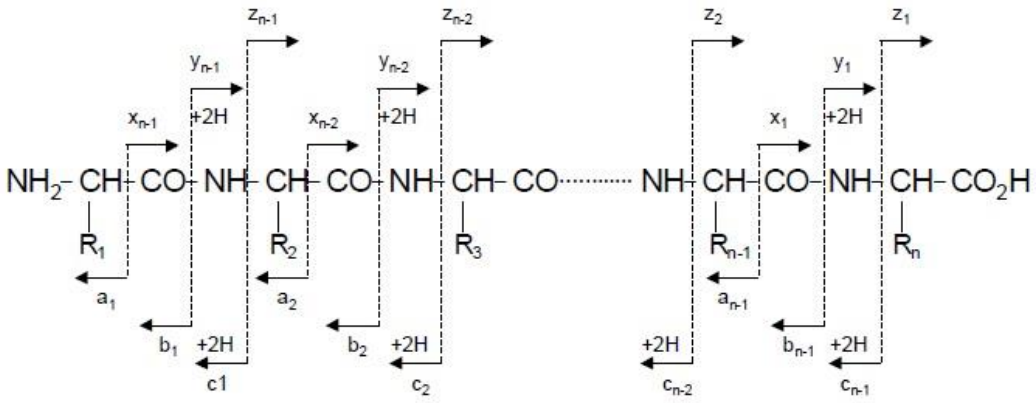


Figure 13: Fragment-ions of peptides. The chain breaks in different positions forming a-x, b-y and c-z fragments. Note the two protons added to c- and y-ions.

In CID-fragmentation, the majority of the fragmentations happen at the amide bond CO-NH, which gives dominant b- and y-ion series. Figure 14 shows two residues in a peptide-chain and the b- and y-ions that would be the result of a CID fragmentation.

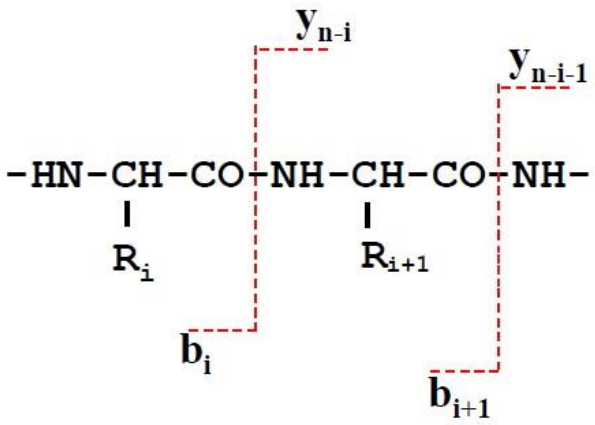


Figure 14: Numbering of b and y fragment-ions follows the AA sequence.

A peptide containing Proline (Pro) will undergo a Proline induced fragmentation. Proline is a special case, since its side-chain folds back on its back-bone amide. A Pro-residue will therefore bend the peptide back-bone differently than the other AAs, and stabilize the fold of the native isomer of the peptide. When the peptide is linearized, this bend will destabilize the structure, and fragmentation will occur more frequently at this residue than other residues in the sequence. The peptide will fragment on the N-terminal side in a Pro-residue. The resulting y-ion will most likely be the base peak in a MS/MS spectrum.

2.6 Software Mediated Structure Determination

To be able to interpret the signals from the MS/MS and determine the disulfide-bridges, the sequence of the linear peptide and the monoisotopic mass of the chosen alkylating agents has to be known. There are several databases that offer help to interpret the MS/MS output and thus structure determine proteins and peptides. Amongst these is “Protein prospector”. The database is programmed to calculate theoretical masses of fragments arising from CID-fragmentation of the peptide. On the front page of the Protein prospector database there are links to underlying pages such as “MS-digest”, “”MS-fit” and “MS-product”. These pages use different software to calculate fragments. “MS-digest” for instance, calculates fragments made from digesting the peptide with digestive enzymes such as trypsin. “MS-product” is used to calculate CID-fragments, and the known peptide sequence is manually submitted to the database using the one-letter abbreviations of the amino acids. Since the program calculates theoretical masses of fragments, it needs information on whether or not the C-

terminal is amidated. Protein prospector allows adjustments in the peptide-chain by exchanging residues in the sequence with the small letters u, v, x and y. These substitute residues needs to be manually defined in the database, by submitting monoisotopic masses and molecular formulas.

In the case of a peptide with four Cys-residues and two bridges, two and two Cysteines will be alkylated with the same alkylating agents. The small letters will correspond to an alkylated Cys-residue, and monoisotopic masses of the alkylated Cys-residues needs to be allocated to the small letter of choice. Following the theoretical fragmentation in Protein prospector, the theoretical fragment-masses can be compared with the masses of the actual fragments that were formed in the collision cell of the MS/MS. Since there are three possible isomers of a peptide with two disulfide-bridges, all three possibilities needs to be submitted to the database and compared with the actual fragments to decide which isomer is the one at hand. If the native AAs are left out of the sequence, the three isomers can be illustrated by the letter combinations vvuu, vuvu and uvvu in the database. Uuvv is the same isomer as vvuu and is not submitted to the database. The same situation is valid for uvuv and vuuv, which are the same as vuvu and uvvu respectively. These letter combinations will be used to illustrate the three isomers throughout this study.

Not all of the ions made from CID-fragmentation will be helpful towards identifying the fold of the DRP. If an ion can assist in identifying the fold, it is called a diagnostic ion. To be a diagnostic ion, the fragmentation of the sequence has to separate the four Cys-residues. A fragment containing one to three Cys-residues is therefore a diagnostic ion.

The MS/MS spectra are compared to the three possible isomers, and diagnostic ions are found. To conclude on an isomer, at least two diagnostic fragment-ions per bridge are needed. A total of six diagnostic ions in the case of a peptide with two disulfide-bridges are sufficient to verify the fold.

3. Aim of the Thesis

The aim of this thesis is to find a method to determine which Cysteine residues are linked by disulfide-bridges in a disulfide-rich bioactive peptide with three or more Cysteine residues. This will be done by reducing commercially available disulfide-rich peptides one disulfide-bridge at the time, followed by alkylation of the different bridges with different maleimides. The reduced and alkylated peptide is then analyzed on an UHPLC-MS/MS and the bridges allocated by alkylation. The final structural determination is accomplished by software analysis of the MS/MS chromatograms. The method will be tested on different commercially available disulfide-rich bioactive peptides with different sequence-length and number of disulfide-bridges. Finally, the method will be used to verify correct bridging in a self-synthesized peptide.

4. Methods and Materials

4.1 Chemicals

For the reduction- and alkylation steps of the different peptides Tris(2-carboxyethyl)phosphine (TCEP), N-methylmaleimide(NMM), N-ethylmaleimide(NEM), Phenylmaleimide (PhM), tert-Butylmaleimide(tBM), 5,5'-dithiobis(2-nito-benzoic acid) (DTNB) from Sigma Aldrich, St Louis, MO, USA were used. Conotoxin α ImI and Conotoxin ω GVIA was purchased from Tocris bioscience, Bristol, UK. TCEP and the peptides were buffered with ammonium formate buffer with with pH 3, made from ammonia solution 28-30 % for analysis and formic acid 98-100 % for analysis both from Merck, Darmstadt, Germany. The acetonitrile (AcN) used was HiPerSolv Chromanorm Acetonitrile lc-ms grade from BDH Prolabo, Leuven, Belgium. Purified water was obtained from a Rios 100 milliQ purification unit from EMD Millipore corp, Billerica, MA, USA, hereby referred to as simply milliQ water.

In the synthesis of Conotoxin α-ImI, the protected amino acids Fmoc-Gly-OH, Fmoc-Cys(trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Asp(otBu)-OH, Fmoc-Pro-OH, Fmoc-Arg(pbf)-OH, Fmoc-Ala-OH, Fmoc-Trp(Boc)-OH and Fmoc-Cys(Acm)-OH were used to build the peptides. The peptide chain was built on a Rink Resin SS, 100-200 mesh, 1 % DVB from Advanced Chemtech, Louisville, KY, USA. Acetic anhydride, Piperidine 99%, triisopropylsilane (TIS) and Dichloromethane (DCM) was purchased from Sigma-Aldrich, St Louis, Missouri, USA. Merck Schuhardt OHG, Darmstadt, Germany produced the 2, 4, 6-trimethylpiridine for synthesis (TMP), the O-(1H-6-Chloro-benzothiazole-1-yl)-1,1,3,3-tetramethyl-uronium-hexafluoro-phospate (HCTU), and the 22rifluoroacetic acid for synthesis (TFA) used. Formic acid 98 – 100 % for analysis (FA) and N, N-Dimethylformamide (DMF) was also obtained from Merck. Finally, N, N-diisopropylethylamine (DIPEA) came from BDH Prolabo, Leuven, Belgium.

4.2 Materials

The main analysis of the peptides was performed on an Acquity UPLC I-class with an Acquity UPLC BEH C18 1.7 µm 2.1*100 mm column for the first 5 months and an Acquity UPLC BEH C18 1.7 µm 2.1*150 mm column for the remaining period. The detector coupled to the UHPLC was a Xevo G2 QTOF MS. All instruments and columns above are from Waters corp., Milford, MA, USA. For the activity studies of TCEP an Agilent tech open-top UV quarts cell 10 mm, 3 mL vol. in a UV spectrometer from Agilent Technologies, Santa Clara, CA, USA, was used. The maleimides were analyzed on an Acquity UPLC BEH C18 1.7 µm 2.1*50 mm column in an Acquity UPLC H-class connected to a UPD pda detector, all from Waters corp. The same instrument was used to separate the peptide with two alkylated sulfhydryl-groups from the unreacted peptide still present in the sample.

For the solid phase extractions (SPE), Solid Phase Extraction Cartridge; Empore C18 Standard density from 3M Company, St. Paul, MN, USA on a vacuum manifold from Thermo Fisher Scientific, Waltham, MA, USA was used.

A Prelude Peptide Synthesizer version 2.1.0.192 from Protein Technologies Inc., Tucson, AZ, USA, performed the peptide synthesis. Solvent was evaporated from the synthesized peptides with a Hei-VAP Advantage rotavapor from Heidolph Instruments GmbH & co, Schwabach, Germany. The peptide crudes were purified on an Xselect csh prep C18 5µm OBD 19*250 mm column in a Waters 2545 Binary Gradient Module with a 998 pda detector from Waters corp., Milford, MA, USA, and the fractions were gathered on a Waters fraction collector model 2767 Sample Manager from the same provider. Freeze-drying was performed on a FreeZone 4.5 plus from Labconco, Kansas City, MO, USA.

The UHPLC-MS/MS system, the HPLC-PDA fraction collector system and the UHPLC-PDA were controlled and operated by the software MassLynx version 4.1 copyrighted by Waters corp., Milford, MA, USA. The operating system for the Prelude Peptide Synthesizer was SUser version 2.1.0.391copyrighted by Protein Technologies Inc. The peptide-models were drawn in the educational version of Pymol Molecular Graphics System version 1.3 copyrighted by Schrodinger LLC. Molecular structures and reaction equations are derived from the database http://www.chemspider.com provided by the Royal Society of Chemistry, or drawn with the freeware ChemSketch version 14.01. Paint version 6.1 has been used to add information to structures and chromatograms. The monoisotopic masses were calculated by

use of the web page http://www.lfd.uci.edu/~gohlke/molmass/, a freely available molecular mass calculator developed by Christoph Gohlke, Laboratory for Fluorescence Dynamics, University of California Irvine. The database http://prospector.ucsf.edu/prospector/mshome.htm was used to determine peptide structure and disulfide-bridging.

4.3 Peptide Synthesis

Fmoc-SPPS was chosen as method to synthesize the conotoxins because it demands less use of TFA than Boc-SPPS, and anhydrous hydrogen fluoride is avoided altogether. The synthesis was set at 100 mM scale using Fmoc-amino acids dissolved in N, N-dimethylformamide (DMF) with a concentration of 200 mM. A rink resin with a loading capacity of 0.7 mmole/g was used as solid phase. It is important that the resin is swelled in DMF prior to the first coupling. The swelled resin has an increased number of binding sites accessible to the first residue in the process, compared to the compact, non-swelled resin. O-(1H-6-Chloro-benzothiazole-1-yl)-1,1,3,3-tetramethyl-uronium-hexafluoro-phospate (HCTU) was used as a activator in the coupling step due to its fast reaction time and ability to produce higher purity peptides than HBTU (20). N, N-diisopropylethylamine (DIPEA) was used as base. Base is used together with the activator to ensure an in situ activation of the carboxylic acid, the formation of an ester and thus a fast and complete coupling reaction. The coupling step was repeated for each Fmoc-amino acid. This is referred to as a double coupling, and increases the yield of the synthesis.

A mixture with a 1:1:3 ratio of acetic anhydride: pyridine: DMF capped the unprotected, thus uncoupled amino-acids, to prevent peptides with residues missing in the sequence being synthesized.

Two protection groups were used on the Fmoc-Cysteine; Trt and Acm. In the case of Cα, Cys-2 and Cys-8 were protected with Trt, and Acm was used to ensure formation of the native bridge between Cys-3 and Cys-12.

Since residue 5 in Cα is Aspartic acid, steps must be made to prevent aspartamide formation. Instead of using 20 % piperidine in DMF as is customary, 20 % piperidine in DMF with 5 % FA was used as a deprotecting agent, since this has been proved to be effective (21).

Actions were taken to prevent racemization of Cysteine. The Fmoc-Cys(Acm)-OH was dissolved in 47 % dichloromethane (DCM) in DMF since racemization occurs more frequently in polar solution (22). This was done by dissolving the Fmoc-Cys(Trt)-OH and Fmoc-Cys(Acm)-OH and the base used under the coupling step in a mixture of nonpolar DCM and polar DMF as described in Table 3. The protected Cys-residues were first dissolved in 1000 µL pure DMF before DCM was added due to their poor the solubility in DCM.

Table 3: The volume of DMF and DCM used in the different solutions during the coupling of Cysteine

	DMF(µL)	DCM(µL)
HCTU	1300	0
TMP	250	750
AA	1000	1500
Total	2550 (53 %)	2250 (47 %)

The base used in the coupling reaction together with HCTU was changed from the usual DIPEA to a weaker base, namely 2, 2, 6, 6-Tetramethylpiperidine **(**TMP), for the coupling step of the Fmoc-Cysteine. This was also a step towards preventing racemization (22).

After each step, the resin with the growing peptide-chain attached was washed with DMF to remove excess reagents.

The assembly of the peptide was automated, and the Prelude was programmed to perform all the wanted steps. Four different coupling programs were used to accommodate for the needs in the different coupling stages. The first coupling is different from the others since it involves the resin swell step at the beginning of the sequence. The standard program used for the standard amino acids starts with removing the Fmoc on the N-terminus of the already coupled amino acid, readying it for the next coupling. This program was repeated until the next amino acid was a Cysteine, a Lysine or simply the last amino acid of the peptide chain. Since there are some considerations to be made during the coupling of Cysteine residues, a special program was made just for this coupling. The difference for the Prelude is the base used during the coupling. It was changed from the strong base DIPEA to the weak base TMP. The final coupling was made by yet a different program. A deprotection step at the end of the sequence is added to remove the final Fmoc; the other steps are identical to the standard coupling program. The different programs may be viewed Appendix 1.

The cleaving was done manually, and the cleaving cocktail consisted of 95% TFA, 2, 5% TIS and 2, 5% milliQ water. The resin was kept in 10 mL cleaving cocktail for 3 hours. When the cleaving was completed, the peptide was isolated by filtration. The linear, unprotected peptide was transferred to a round bottom flask. Ether was added and the peptide was left to precipitate. After precipitation, the ether was decanted away, and the precipitate was air dried. The crude peptide was re-dissolved in milliQ water with 0.1 % v/v TFA after weighing.

The quality of the crude was analyzed on an analytical HPLC instrument, and a standard inlet method with milliQ water as mobile phase A and AcN as mobile phase B both with 0.1 % v/v TFA was used. A linear gradient of 0-50 % of mobile phase B (AcN with 0.1% TFA) for 30 minutes was used for the preliminary analysis. The analysis is performed to verify the quality of the crude and to find the retention time of the crude. The retention time was used to program the preparative HPLC by which the crude peptide was purified. Purification chromatography was performed using a linear gradient of 5-35 % mobile phase B over 30 minutes, followed by a column rinse with 100 % B for 5 minutes and finally a 4 minute re-equilibration down to 5 % B before the next sample was run.

The purified peptide solution was then freeze-dried, weighed and subsequently re-dissolved in the mobile phase A described above. The purification step was repeated to ensure a purity of 95% or higher. If the crude did not meet this standard, a new purification step was initiated. The fractions were analyzed on an UHPLC-PDA instrument and fractions with a purity of 95 % or higher were pooled. The pure linear peptide with two Cys-residues still protected by Acm, was then oxidized.

Chemical oxidation was chosen over air oxidation to ensure the correct fold on the finished product. When a peptide is oxidized by chemical oxidation the method must be divided into separate oxidizing steps, one for each bridge. In the case of Cα with two bridges, two steps must be made. The first step oxidizes the Trt-protected Cys-residues. The Trt-group was removed during cleaving, and the Cys-residues two and eight are left deprotected. The freeze-dried linear peptide was dissolved in mobile phase A (milliQ water with 0.1% TFA). Ammonium acetate buffer pH 7 was stirred by a magnetic stirrer in a conical flask. The unreduced, linear peptide was dripped in to the buffer one drop at the time and the concentration of the linear peptide in the buffer solution was 0.1 mg/mL, both measures taken against dimerization. The linear peptide was left to air-oxidize to monocyclic in the buffer overnight, and the oxidation was confirmed by UHPLC-PDA and MS.

The second bridge was oxidized by adding 10 molar equivalents of iodine to the monocyclic peptide. Iodine has a dual quality, as it both deprotects the Acm-Cys residues and oxidizes the free Cys residues 3 and 12. 50 mM iodine solution was made of I_2 dissolved in Acetic Acid. The iodine-peptide solution was left to react while stirred under N_2 –gas. Samples were taken after 0, 10, 20, 30, 50 minutes and analyzed with UHPLC-PDA. The oxidation was duly

stopped by adding ascorbic acid to the iodine-peptide solution when the peptide was fully cyclic. The oxidized peptide was freeze-dried and kept in an air tight container at -20ºC.

Both the freeze-drying steps and the chemical oxidation were performed by my fellow student Børge Renmælmo, and the methods are merely recited here with his permission.

4.4 Activity Studies of TCEP

2 mM solution of DTNB and five 4mM TCEP solutions were made in milliQ water. The five 4 mM TCEP solutions were pH adjusted to pH 2, 3, 4, 5 and 6. The pH adjustment of the pH 2 TCEP solution was performed with FA, and the solutions with pH 3, 4, 5 and 6 were made with 1 M sodium hydroxide (NaOH). Since the UV quartz-cell has a volume of 3 mL and needs to be filled to at least two thirds of this volume to make an accurate measurement, milliQ water was added to obtain a volume of 3 mL in each sample.

Neither the DTNB nor the water was pH adjusted. The DTNB:TCEP ratio was 1:1. The contents of the test tube were immediately transferred to a quarts cell, and the absorbance of the DTNB-TCEP mixture was measured after 0, 15 and 30 minutes. Three parallel samples of DTNB:TCEP for each pH was analyzed, a total of 15 samples. Each sample was measured three times, and an average of these was calculated. An overview of the concentrations and volumes of the 15 samples is presented in Table 4.

Table 4: Concentration (mM), volume (mL) and pH adjustment of the reagents involved in the activity studies.

	Concentration (mM)	Volume (mL)	pH adjusted
DTNB	2	0.50	No
TCEP	4	0.25	Yes
milliQ water	-	2.25	No

In the second study, different ratios of DTNB:TCEP were studied. The pH of the 4 mM TCEP solution was adjusted to 4 with NaOH, and the absorbance of the DTNB-TCEP mixture was measured after 0 and 15 minutes. As in the previous experiment, TCEP was added to a reagent glass where DTNB and milliQ water in the amounts described in Table 5 were already added, and the absorbance was measured for the first time immediately after the TCEP was

added. Three parallel samples of each ratio were made, and the ratios of DTNB:TCEP analyzed were 1:2, 1:5 and 1:10. The absorbance of each sample was measured three times in a row and an average was calculated.

Table 5: Volume of each reagent (mL) in the different ratios measured

Ratio DTNB:TCEP	1:2	1:5	1:10
2 mM DNTB (mL)	0.25	0.25	0.25
4 mM TCEP (mL)	0.50	1.25	2.5
milliQ water (mL)	2.25	1.50	0.25

4.5 Reduction and Alkylation of Disulfide-Bridges

TCEP was chosen over DTT as a reduction agent since it has been proven to be more effective. It is claimed to be active at pH 1 to 12, while DTT has a more limited pH span. In addition TCEP reacts solely with the disulfide bond, leaving other side chains unreacted (11).

To rationalize the project, a TCEP stock-solution with pH 5.5 and concentration 37 mM in milliQ water was made to fit the concentration of the peptide solution. A 37 µM solution of peptide gave an adequate signal on the MS instrument, but further dilution of the sample had to be avoided. By making a 37 mM TCEP stock-solution that could be diluted with milliQ water to the wanted concentration every morning, a TCEP-to-peptide molar ratio of 1:1 could be made and excessive dilution of the sample avoided. NaOH was added to the TCEP solution to acquire pH 5.5, since the pH of the 37 mM TCEP in milliQ water was 2.5. The stock-solution was divided into 1 mL units in Eppendorf-tubes and kept in the dark at -20ºC. This stock-solution was diluted to fit the TCEP-to-peptide ratios to be examined. After three weeks, the reduction-results were no longer reproducible, and it seemed as the TCEP stock-solution had deteriorated in the freezer where it was kept. The TCEP stock-solution was therefore discarded, and a fresh TCEP solution was made at the beginning of each day.

At the beginning of the method development the TCEP solution with pH adjusted to 5.5 was used, as earlier research on the area indicated that this would be optimal (10). The reduction was more or less instant at this pH, and a TCEP-to-peptide ratio was the parameter that could be changed and optimized. Ratios 1:1 through 10:1 were tested. A 1:1 ratio was supposed not

to be enough to reduce the disulfide-bridges sufficiently, and a 10:1 ratio to be an excess and possibly lead to full reduction of all disulfide-bridges. The TCEP, dissolved in milliQ water and with pH adjusted to 5.5, was added to a vial already containing a 37 µM peptide solution. The peptide was dissolved in milliQ water without any pH adjustment. Immediately after adding TCEP, the mixture was analyzed on the UPHLC-MS/MS instrument. As anticipated, 1:1 failed to reduce the peptide when the reaction time was less than one minute, and 10:1 reduced both disulfide-bridges instantaneously. The optimal value was thought to be between 3:1 and 5:1. Ratios 3:1, 3.5:1, 4:1, 4.5:1 and 5:1 were analyzed to find the best ratio to cleave only one disulfide-bridge. However, all ratios examined to this point had three peaks in their chromatograms with an *m/z* corresponding to one cleaved and one intact disulfide-bridge. The non-native isomer, not present in the original Cα sample, was now present. This meant that the native isomer was rearranged into a non-native isomer during the reduction step. To avoid the formation of this non-native isomer, lower ratios and higher reaction times were examined. A sample was made with a 1:1 ratio to be injected into the UHPLC-MS/MS system every 12 minutes. The non-native isomer was already formed after 12 minutes, and a new, shorter UHPLC method was made to be able to follow the reaction more closely. The method had the same linear gradient as the 12 minutes method, but was shortened to four minutes with a reset period for the gradient of one minute. The sample could then be analyzed every five minutes. It became clear that the non-native bridge would appear at any ratio, and reaction time did not alter this. The only parameter left was the pH, which led to disregarding the previous assumption of the optimal pH value being 5.5. When a disulfide-bridge is broken, the sulfhydryl-group of the Cysteine side-chain will be protonated at low pH, and deprotonated at high pH. The pKa value of the sulfhydryl side chain of Cysteine is 8.33, and its isoelectric point (pI) is 5.02 (23). The local environment may induce abnormal pKa-values, and the theoretical pKa value does not give an accurate account for the chemical environment in the peptide chain.

Deprotonated sulfhydryl-groups might react with remaining bridges, breaking these and form new, non-native bridges. This is referred to as disulfide scrambling. This is highly disadvantageous since the aim of the thesis is to prove correct bridging in a synthesized peptide. It was therefore paramount to the study that the regrouping and formation of non-native bridges was avoided. This was ensured by buffering both TCEP and peptide-solutions to pH 3, where the sulfhydryl-groups were in a less reactive state. TCEP was therefore dissolved in an ammonium formate buffer with pH 3, as was the peptides. To make sure the

reaction terms were stable, TCEP was made fresh every morning. The pH of the buffer was regularly measured to ensure a stable pH.

When the pH was adjusted to 3, both the ratio and the reaction time had to be reexamined. Since none of the available literature on TCEP as a reduction agent concerned itself with reduction of one disulfide-bridge but rather high excess of TCEP to reduce all disulfide-bridges in large proteins as a help in sequencing these, ratios in the order 1:1-10:1 were not thoroughly described. One paper concluded that TCEP was active at pH 1-12 (10). Since there was no evidence of the contrary, the experiments at pH 3 continued where the pH 5.5 experiments had ended; with a TCEP-to-peptide ratio between ratio 3:1 and 5:1. This turned out to be far too low because the reactivity of TCEP proved to be much lower at lower pH. Each ratio was examined with the four minutes method and the 12 minutes method. A sample was injected from the same vial several times in a row in order to examine the reaction time. When it became apparent that the ratio had to be higher, the ratios 10:1, 20:1 and 30:1 were examined in the same manner, and also turned out to be too low. Subsequently the ratios 50:1, 100:1, 200:1 and 300:1 were then analyzed. When the optimal ratio was found, the exact reaction time had to be investigated. It seemed as the optimal reaction time would be somewhere between four and eight minutes based on the four minutes UHPLC method. The TCEP was added to the vial already containing the peptide solution and was analyzed with the four minutes method after one minute. Other samples were analyzed after two, three and four minutes reaction time to best pinpoint the optimal reaction time.

4.5.1 In-Vial Reduction and Alkylation

The activity of TCEP depends on pH and concentration (11). Low pH requires higher concentration and longer reaction times. The optimal ratio between TCEP and the peptides with two disulfide-bridges was 100 molar equivalents of TCEP to one mole of peptide. Six minutes reaction time was found to be optimal.

The maleimides were dissolved in AcN to avoid unwanted reactions between maleimides and methanol/water. Since the maleimides react with TCEP (13), the TCEP was left to react with the peptide for six minutes before PhM was added at 450 molar equivalents of the peptide. Even though maleimides instantly and completely react with the available sulfhydryl groups at pH 6-7, this proved not to be the case at pH 3. The maleimides are still able to fully alkylate

the sulfhydryl-groups, but a longer reaction time is needed. 24 minutes was found to be an adequate reaction-time, after analyzing the PhM treated reduced peptide solution with the four minutes UHPLC-MS method. A sample was drawn from the vial every five minutes for 30 minutes. See Table 6 for the optimal values of the parameters in the reduction and following alkylation of the two sulfhydryl-groups involved in the first disulfide-bridge.

Table 6: First step of the reduction and alkylation; reducing one bridge in Cα with TCEP and alkylating it with PhM; Amount of reagent in nmole, concentration of the reagents in mM, volumes in µL and reaction times in minutes; Buffer is ammonium formate pH 3

Reagent	Amount (nmole)	Concentration (mM)	Volume (µL)	Reaction time (min)
Cα dissolved in buffer	2.22	0,037	60	
TCEP dissolved in buffer	222	37	6	6
PhM dissolved in can	1000	100	10	24

For reasons discussed in chapter 5.3, a separation technique was needed to isolate the partially reduced and PhM-alkylated peptide. A manual isolation method was developed on an UHPLC-PDA instrument. The peptide with one of the two disulfide-bridges reduced and alkylated had a retention time of 2.17 on the UHPLC-PDA instrument. Injection volume was 10 µL, and the mobile phase was manually collected in a vial when the signal appeared on the real time chromatogram after 2.17 minutes. The peak width of the correct fraction was 3.6 seconds. To ensure sufficient amount of the PhM-alkylated peptide, the wanted fraction of six injections were gathered in the same vial. Approximately 216 µL were collected (6* 36 µL), based on peak width and mobile phase flow. The collected sample was heated to 50 ºC under a flow of nitrogen to evaporate AcN to further increase the concentration of the peptide solution. The precise amount of peptide present in the sample at this time was not known, but an approximate estimate can be made taking mobile phase flow, injection volume, sample concentration and peak width into consideration. After six injections an estimate of the amount of the peptide with one disulfide-bridge reduced and alkylated with PhM collected was 0,58 nmole, and the concentration of the collected sample after volume reduction was estimated to 4.5 µM .

As there was no need to adjust TCEP concentration to avoid unwanted reduction in the second step, a vast molar excess of TCEP was added to the partially reduced and PhM-alkylated

peptide. A 100 mM solution was made to avoid dilution of the sample; six µL 100 mM TCEP gives a molar excess of more than 1000. The reduction of the second bridge was much slower than was the case with the first bridge, even with a molar excess of more than 1000 of TCEP present. The alkylation step also took considerably longer in the second step than in the first. Because of the reaction between TCEP and the maleimides, the second alkylating agent, NMM, had to be added in molar excess to the TCEP present. Due to the vast amount of TCEP present after the second reduction step, a 0.5 M NMM in AcN solution was made to avoid too much dilution of the sample. This gave over 8 times molar excess of NMM to TCEP in the sample. Table 7 shows the optimal parameters for the second step of reduction and alkylation.

Table 7: Second step of the reduction and alkylation; reducing remaining bridge in Cα-PhM$_2$ with TCEP and alkylating it with NMM; Amount of reagent in mmole, concentration of the reagents in mM, volumes in µL and reaction times in minutes; Buffer is ammonium formate pH 3

Reagent	Amount (µmole)	Concentration (mM)	Volume (µL)	Reaction time (min)
Cα-PhM$_2$	Ca 0.00058	Ca 0.0045	Ca 130	
TCEP dissolved in buffer	0.6	100	6	120
NMM dissolved in AcN	5	500	10	60

4.5.2 Reduction and Alkylation on SPE-Column

A second method involving reduction of one disulfide-bridge on a reversed phase SPE-column was also initiated. The promising results from the first step of investigating whether it would work as a generic method for determination of disulfide bridges in DRPs could never be followed through, due to failure of the UPLC-MS/MS instrument available. Although the most common use of SPE is to extract or purify samples, the main purpose in this thesis was to stabilize the fold of the peptide while reducing the disulfide-bridges and thereby enabling specific reduction of one disulfide-ridge at the time. The SPE-method is represented in tabular form in Table 8.

Table 8: Reduction of the first disulfide-bridge, generic SPE method; Name of compound, volume of compound in µL, and purpose of the chemicals on the SPE-column

Compound	Volume (µL)	Purpose
MeOH	1000	Conditioning
MilliQ water with 0.1% FA	1000	Equilibration
Peptide-solution	60	Analyte
TCEP in ammonium formate buffer pH 3	1000	Reducing agent
TCEP in ammonium formate buffer pH 3	4000	Reducing agent
Ammonium formate buffer pH 3	5000	Wash
80% AcN with 0.1% FA	500	Eluting solvent

A conditioning and equilibration must always be done prior to sample loading to ready the column for sample loading. After the initial preparation of the column, the sample was loaded to the C_{18} column. The samples were small, 60 µL of 37 µM Cα and 33.7 µM Cω, and it was difficult to see if it was loaded onto the solid phase of the column, or was still lingering on top of the filter. If the peptide failed to pass the filter, it would be reduced pre-column when TCEP was added. To minimize possible pre-column reaction between peptide and TCEP, the 100 mM TCEP solution was added in two steps. The flow was approximately 1 mL per 30 seconds for all the following steps. First one mL was loaded and pulled through the column, before four more mL of 100 mM TCEP was washed through. Five mL of ammonium formate buffer with pH 3 was sent through the column to wash away as much of the TCEP as possible. The sample was eluted with an 80% AcN solution with 0.1% FA, and the eluate was collected in an Eppendorf tube containing 50 µL 100 mM PhM. Some of the TCEP still remained on the column after the washing step and was co-eluted with the peptide. The PhM would quench the TCEP and by doing so stop the ongoing reduction of the peptide. To ensure alkylation of the disulfide-bridge of the peptide, a molar excess of PhM to TCEP had to be added. No calculation was made on how much TCEP was still present in the eluate. 5 µmole of PhM was enough to ensure both quenching and alkylation. To concentrate the peptide sample, it was heated to 50ºC under nitrogen gas to evaporate AcN. As PhM is poorly soluble in water, unreacted PhM precipitated when the AcN evaporated and the relative water content of the sample increased. The Eppendorf tube containing the sample, cooled to room temperature after volume reduction, was centrifuged and the supernatant collected for analysis. The PhM pellet was discarded.

4.6 UHPLC-MS/MS

Liquid chromatography was performed using a linear gradient of 5-95 % mobile phase B over 10 minutes with a 2 minute reset period down to 5 % B before the next sample was run. A standard inlet method with milliQ water as mobile phase A and AcN as mobile phase B, both with 0.1 % v/v FA, was used. Midway through the project a column change was made from a C18 1.7 µm 2.1*100 mm to a C18 1.7 µm 2.1*150 mm. The mobile phase flow was set to 0.6 mL/minute on the 100 mm column and 0.5 mL/minute when the 150 mm column was used.

The mass spectrometer was equipped with an ESI ion source and operated in positive mode with lock-spray activated. The lock-spray compound was Leucine enkephalin with a monoisotopic mass of 555.269287 Amu (24), which has a signal of 556.2771 *m/z* in the MS software when operated in positive mode.

The values of several parameters were defined in the MS/MS software. Both low and high mass limits had to be defined. The scan time was set to 0.2 seconds to ensure sufficient amount of scans over the peaks. The lock-spray scan time determined how often the instrument would correct its measurements to the lock-spray compound and adjust the measured *m/z* values accordingly. A capillary voltage of 0.6 kV on the ESI-capillary was optimal to ionize the analyte. The desolvation gas was N_2 at a temperature of 350 ºC and a flow of 800 L/Hour. A cone gas flow at 20 L/Hour, and a cone voltage of 35 V was used. The ion source held a temperature of 130 ºC. In Table 9 the different parameters and their values are listed.

Table 9: Parameters in the MS method; low and high mass (amu), scan time and lock-spray interval (sec), capillary voltage (kV), Cone voltage (V), source and desolvation temperature (ºC), cone gas flow and desolvation gas flow (L/Hr) and polarity

Parameters	Values
Low mass	105
High mass	5000
Scan time (sec)	0.2
Lock-spray interval (sec)	15
Capillary voltage (kV)	0.6
Cone voltage (V)	35
Source temperature (ºC)	130
Desolvation temperature (ºC)	350
Cone gas flow (L/Hr)	20
Desolvation gas flow (L/Hr)	800
Polarity	Positive

The final product was analyzed using both mass filters of the MS. In addition to the parameters described in Table 9, some additional MS/MS parameters had to be defined. The values of these are listed in Table 10. The high and low limits for masses were set lower for the MS/MS method, since the peptides would undergo CID-fragmentation. A narrower mass window led to the ability to apply a shorter scan time. The instrument was set to switch from MS to MS/MS at a total ion count of 2500 per second, and to switch back from MS/MS to MS when the signal dropped to a total ion count of 35, or after 1.5 seconds if the signal continued beyond this time limit. The charge states selected were the ones with the highest intensity signals (total ion count) in the MS analysis of the peptides. Argon was the collision gas of choice, and the MassLynx default settings for the charge state recognition and ramping collision energy method were used. The collision energy parameters and their values may be viewed in Appendix 3.

Table 10: Parameters in the MS/MS method: low and high mass (amu), scan time (sec), Threshold for MS/MS acquisition and stop (TiC or sec), selected charge state

Parameters	Values
Low mass (amu)	80
High mass (amu)	2000
Scan time (sec)	0.036
Threshold for MS/MS acquisition (intensity (total ion count))	2500
Threshold for MS/MS stop (intensity (total ion count),	35
or elapsed time (sec))	or 1.5
Selected charge state	2+, 3+, 4+

Several adjustments may be implemented to the MS/MS method in order to optimize it to the analyte in question. The aim of the thesis was to make a generic method for all DRPs. The interesting task at hand was not to adjust the method to fit Cα perfectly, but rather finding an adequate method to be applied on additional peptides.

The spectra obtained on this MS/MS method, was analyzed with structure determining software according to the method below to affirm that the native fold had been synthesized.

4.7 Structure Determination

Collision induced dissociation is the present method of choice for peptide sequencing when using a MS/MS instrument, and was therefore chosen for this thesis (25). The database "Protein prospector" and its underlying "MS-product" were chosen as a tool to interpret the MS/MS spectra obtained when using the method above.
As described in chapter 2.6, the Cys-residues of the sequence has to be substituted with one of the small letters u, v, x and y. In this thesis, "u" was allocated to Cys-NMM, and "v" to Cys-PhM. The molecular formulas and monoisotopic masses of "u" and "v" were submitted to the database. Cys-NMM and Cys-PhM are illustrated in figure 15.

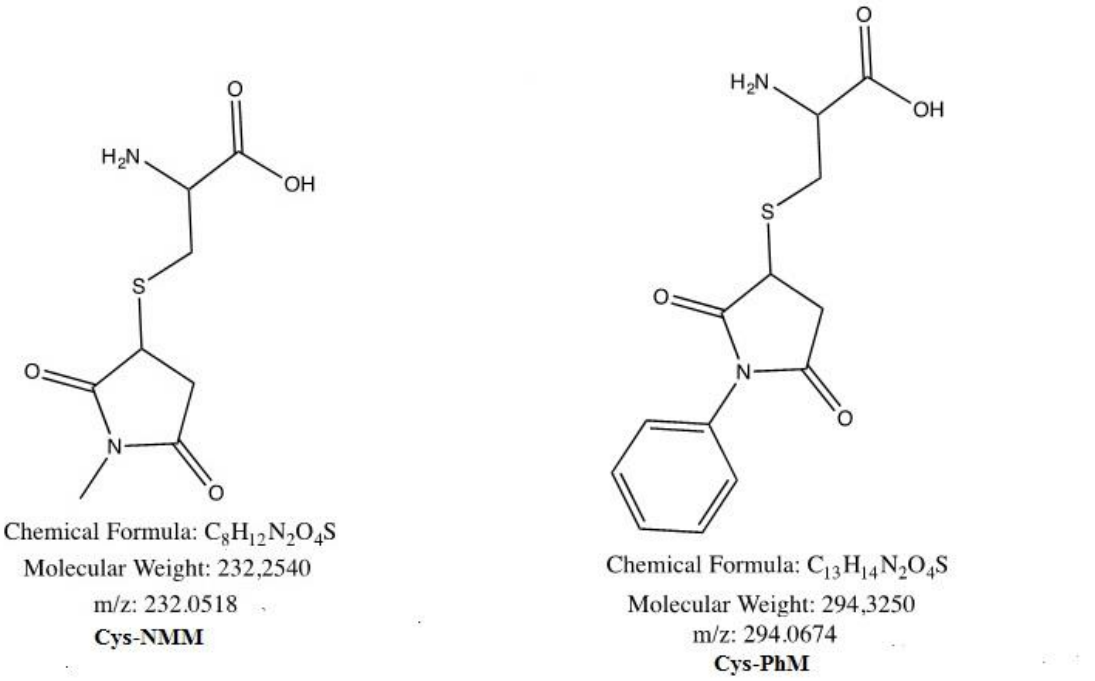


Figure 15: Cysteine alkylated with NMM and PhM. Chemical formula, molecular weight and theoretical *m/z* when z = 1.

In the case of Cα, both commercially bought and self-synthesized, the sequences GvvSDPRuAWRu-NH_2, GvuSDPRvAWRu-NH_2, GvuSDPRuAWRv-NH_2, GuuSDPRvAWRv-NH_2, GuvSDPRuAWRv-NH_2 and GuvSDPRvAWRu-NH_2 were submitted to the software. The sequences and how they are related to the three possible isomers are illustrated in Figure 16. The monoisotopic masses of the two alkylated forms of Cysteine were submitted to the program. Finally, the theoretical CID-fragments were subsequently compared to the actual CID-fragmented reduced and alkylated peptide.

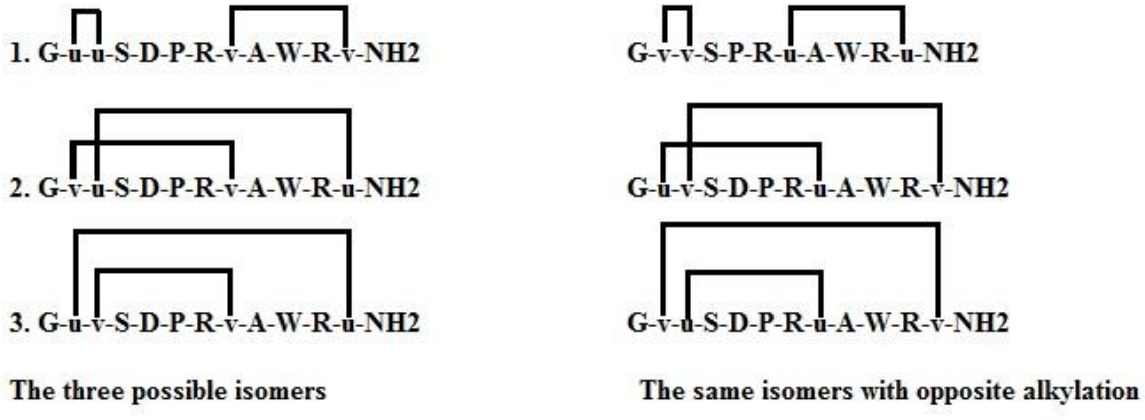


Figure 16: The three possible isomers of Cα with six different sequences depending on alkylation of the Cys-residues.

5. Results and Discussion

5.1 Synthesis of Conotoxin Alpha ImI

The automated synthesis of Cα was performed according to the method described in chapter 4.3. Since the synthesized peptide was linear and the fold of the cyclic peptide could not be established until the final analysis of the end product, the peptide was referred to as DRP1during the process of synthesis, purification and oxidation.

The freeze-dried crude product was weighed to 19 mg, dissolved in 2 mL mobile phase A, and purified on a prep-HPLC instrument. The purified, linear, freeze-dried peptide was weighed to 16 mg, and oxidized by two separate steps.

The initial oxidizing step, from linear to monocyclic state of the peptide, was performed according to the method described in 4.3. An UHPLC-PDA analysis was performed both before and after the air oxidation in ammonium acetate buffer pH 7. A difference in retention time indicated that the free sulfhydryl-groups of the previously Trt-protected Cys-residues had oxidized to form a disulfide-bridge. In Figure 17 the UHPLC chromatogram of samples of the peptide taken before and after oxidation is presented. Linear DRP1 and monocyclic DRP1 had a retention time of 3.06 minutes and 3.11 minutes respectively. At the time of the second sample, there still is some linear DRP1 left.

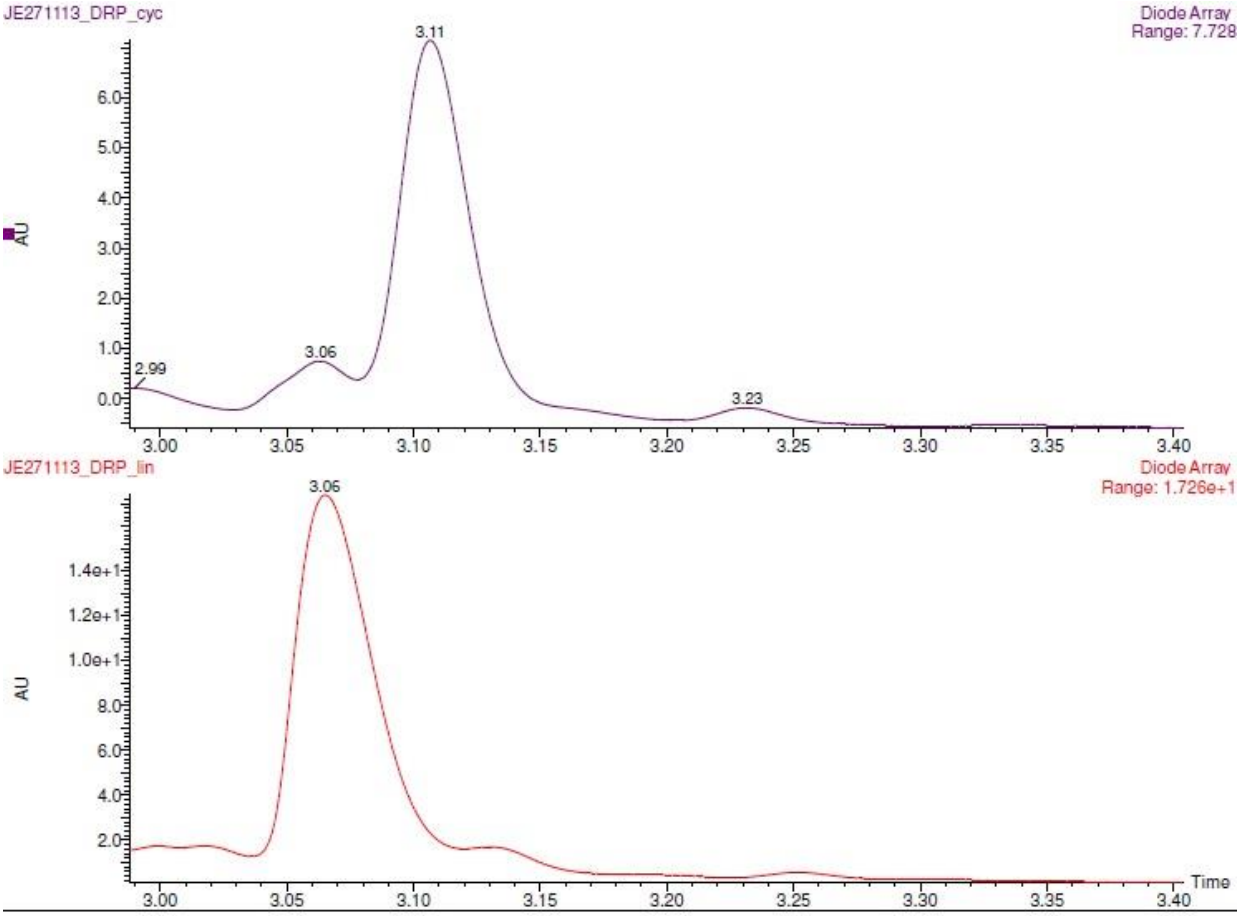


Figure 17: Linear DRP1 with retention time 3.06 minutes, and monocyclic DRP1 with retention time 3.11. The analysis was performed on the same sample, before and after oxidation. Note that there still were some linear DRP1 present when the second sample was taken.

Figure 18 shows the UHPLC chromatograms of samples taken 0, 10, 20, 30 and 50 minutes into the iodine oxidation of the second bridge after quenching the iodine with ascorbic acid. When ascorbic acid was added to the peptide-iodine mixture, the color of the mixture instantly changed from red to blank. The color change was an indicator that the iodine was no longer active, but quenched by the ascorbic acid. Figure 18 shows how the monocyclic species at time = 0 with retention time 3.06, gradually gets smaller and disappears completely after 50 minutes of iodine oxidation. The monocyclic species completely oxidizes into the cyclic species with retention time 3.12 within 50 minutes.

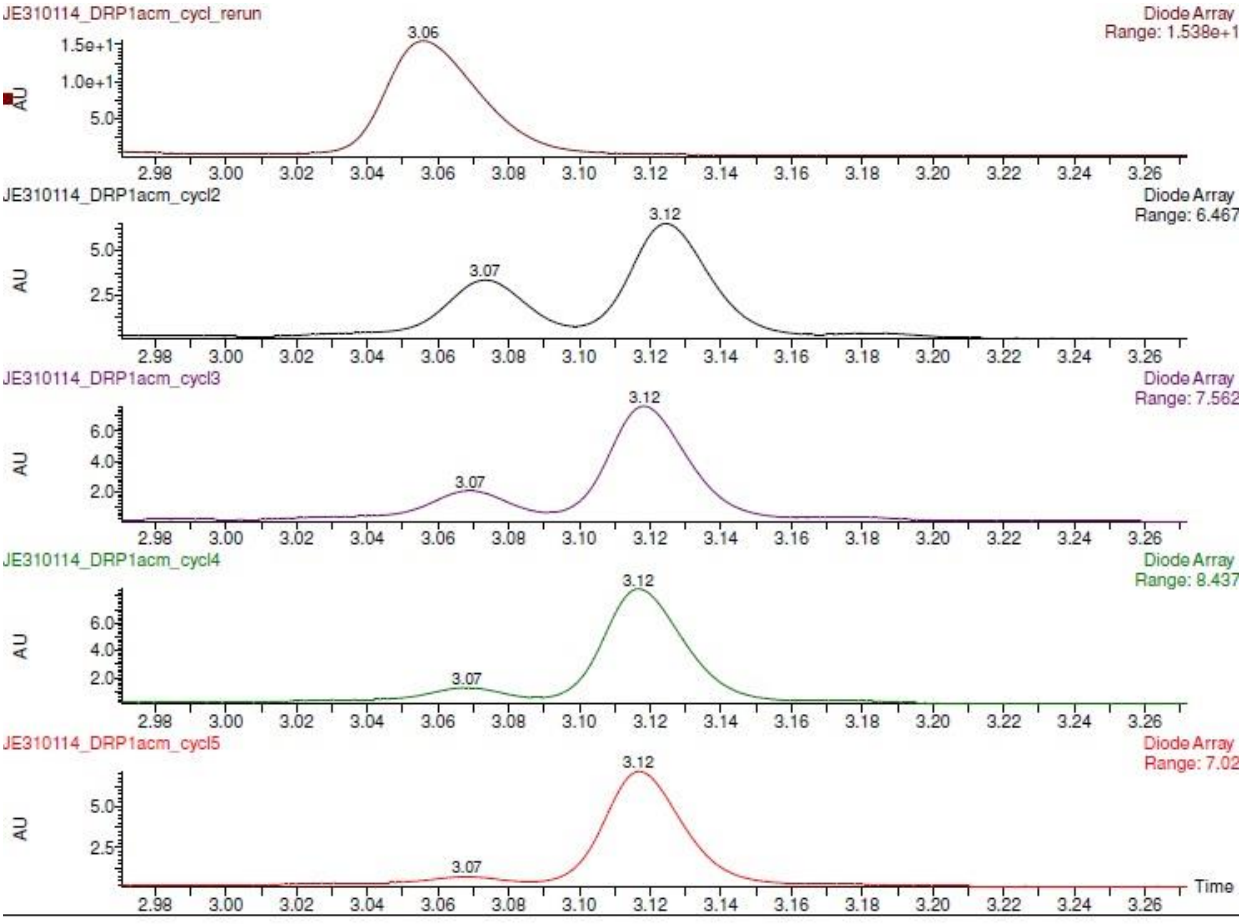


Figure 18: Chemical oxidation of the second bridge of DRP 1. The monocyclic DRP1 has a retention time of 3.06 minutes, and the fully oxidized DRP1 has a retention time of 3.12 minutes. The monocyclic DRP1 is almost fully oxidized within 50 minutes.

5.2 Activity Studies of TCEP

The results of the 1:1 ratio experiment are here presented in Figure 19, and the details of the results can be viewed in Appendix 2.

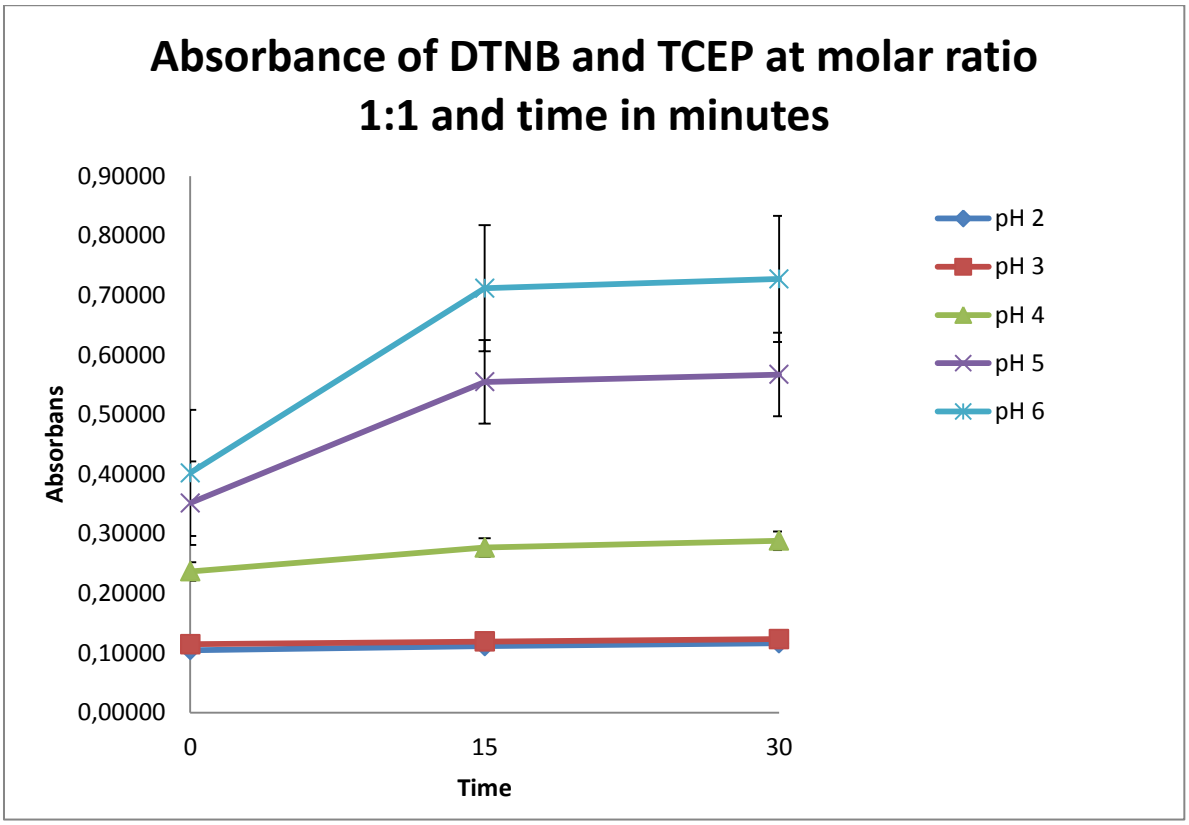


Figure 19: Absorbance of DTNB reduced to NTB by TCEP measured at 412 nm after 0, 15 and 30 minutes.

As seen in Figure 19, the reaction is slower and less of the DNTB is reduced to NTB, the yellow-colored product measured at 412 nm in the UV-spectrometer, at a lower pH. This slower and less potent reaction was later proven to also be the case for the peptide-reduction.

DTNB was also tested with ratios 1:2, 1:5 and 1:10 with TCEP. The samples were measured at 0 and 15 minutes, since it seemed from the previous pH-experiment presented in Figure 19 that the reaction at ratio 1:1 had run its course after 15 minutes. The pH was adjusted to 4 since there seemed to be little reduction taking place at pH 2 and 3 in the previous experiment. The result is presented in Figure 20 and shows a connection between ratio and both speed- and amount of DTNB reduced to NTB. Figure 20 shows that the reaction was significantly faster at ratio 1:10 than both 1:5 and 1:2. Ratio 1:10 gives an instant reduction of

DTNB measured through change in absorbance. The change in absorbance between 0 and 15 minutes at ratio 1:10 and 1:5 was insignificant, whilst at ratio 1:2 the absorbance measured after 15 minutes was higher than the measurement at 0 minutes. Again, this was also seen to be true for the reduction of the peptides.

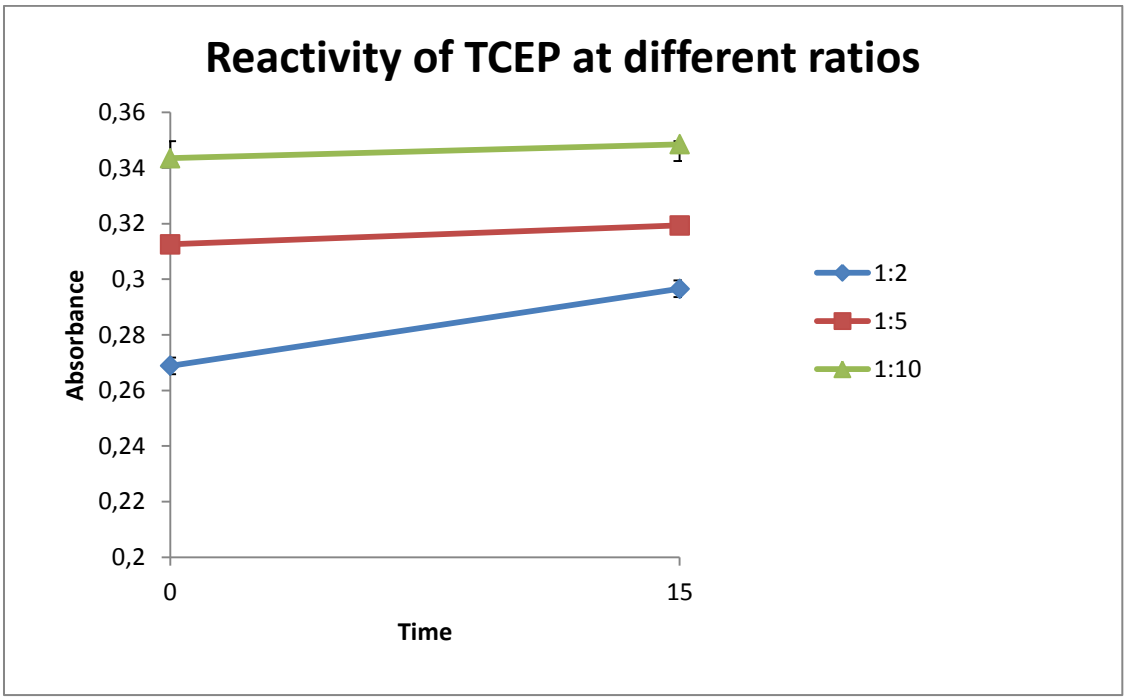


Figure 20: Absorbance of DTNB reduced to NTB by TCEP at ratios 1:2, 1:5 and 1:10 measured at 412 nm after 0 and 15 minutes.

As will be discussed further in chapter 5.3, it was necessary to keep the pH in the peptide experiments at pH 3, which is shown here to be disadvantageous for the TCEP activity. Had the experiment been performed at pH 3, it is likely that there would have been no difference between ratios 1:2, 1:5 and 1:10, and that higher ratios would be needed to get a similar outcome. This based on the ratio necessary to partially reduce the peptide presented in chapter 4.5. According to Santarino IB, Oliveira SCB and Oliveira-Brett AM who did a study on the reduction-qualities of TCEP using DNTB, the reduction of disulfide-bridges at a 1.05:1 ratio between TCEP and disulfide at pH 4.5 was completed in one hour. At higher ratios and pH, others have fully reduced DNTB to NTB within 40 seconds (10).

5.3 Reduction and Alkylation of Disulfide-Bridges

Table 11 shows an overview of monoisotopic masses, *m/z* and charge states of the analytes in this work.

Table 11: Mono-isotopic masses in amu, *m/z* of the precursor ion, *m/z* of base peak in the chromatograms and the charge state of these, and charge states observed in the spectra for the different compounds involved in the reactions.

Compound	Monoisotopic Mass (amu)	*m/z* [M+H$^+$]	*m/z* of base peak, charge state in brackets	Charge states observed
Conotoxin α ImI	1350.484	1351.4918	676.24 (2)	1, 2, 3
Conotoxin ω GVIA	3035.155	3036.1628	1013.06 (3)	2, 3, 4, 5
TCEP	250.0606	251.0684	251.06 (1)	1
Phenylmaleimide	173.0477	174.0555	174.05 (1)	1
N-methylmaleimide	111.0320	112.0398	112.03 (1)	1
N-ethylmaleimide	125.0477	126.0555	125.05 (1)	1

Before any attempt was made to reduce and alkylate the two commercially acquired peptides, they were analyzed on the UHPLC-MS/MS. The retention time of the unreacted peptides could be used to some extent to predict retention time for the reduced species and the alkylated species. It was also interesting to see which charge state was most intense in the spectra, and which would be the *m/z* of the peptide peak in the chromatogram. Figure 21 and Figure 22 show the chromatogram and spectrum of Cα respectively.

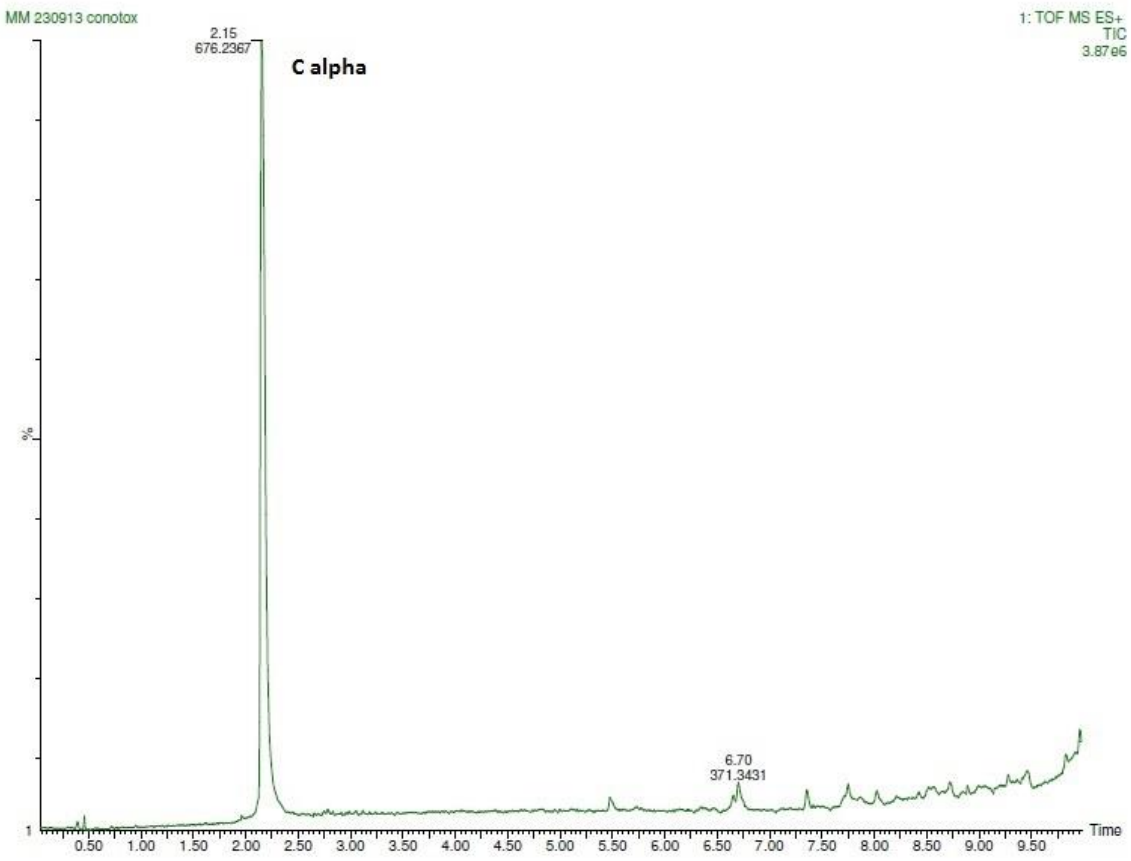


Figure 21: *m/z* 676.2367 is Cα; [M+H$^+$] = 1351.473, z= 2

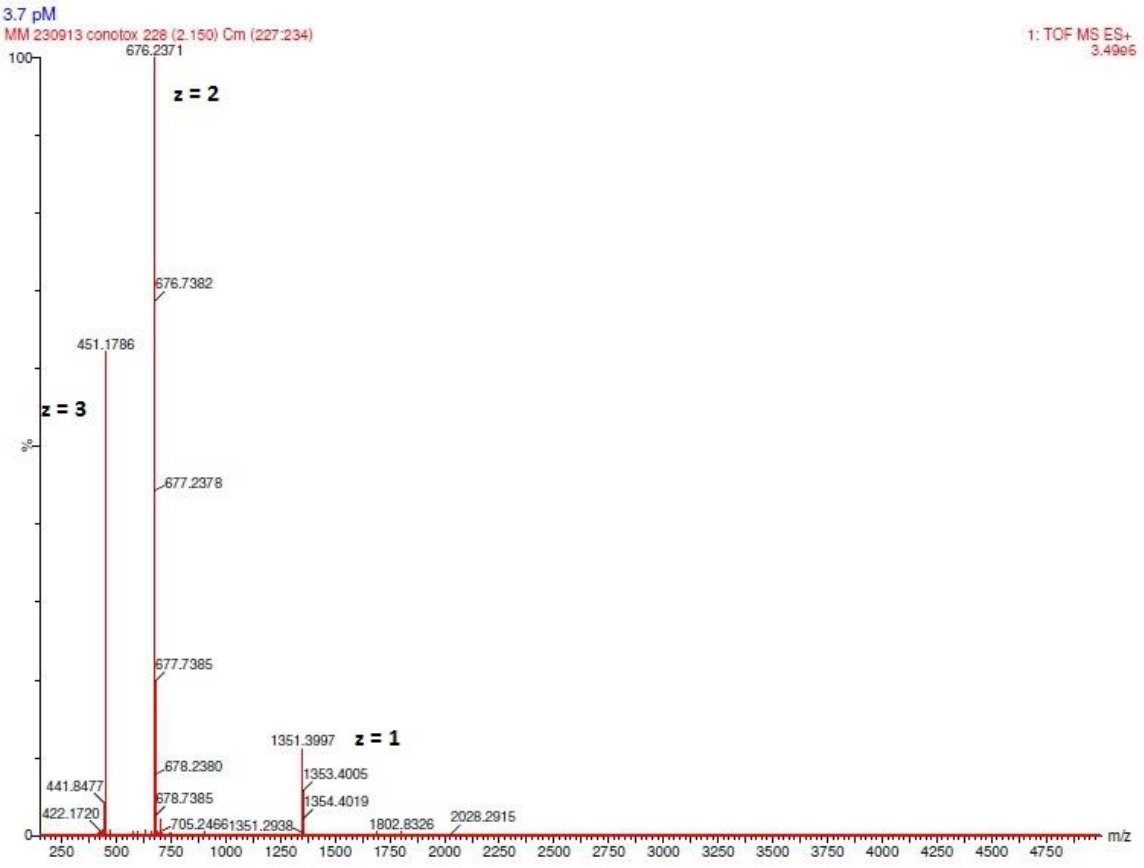


Figure 22: MS spectrum of C*α*. Charge states for C*α*; Theoretical values for *m/z* when [M+H$^+$]= 1351.484: (1350.484/2) + 1 = 676.242, (1350.484/3) + 1= 451.161

Figure 23 and Figure 24 show the chromatogram and spectrum of Cω respectively.

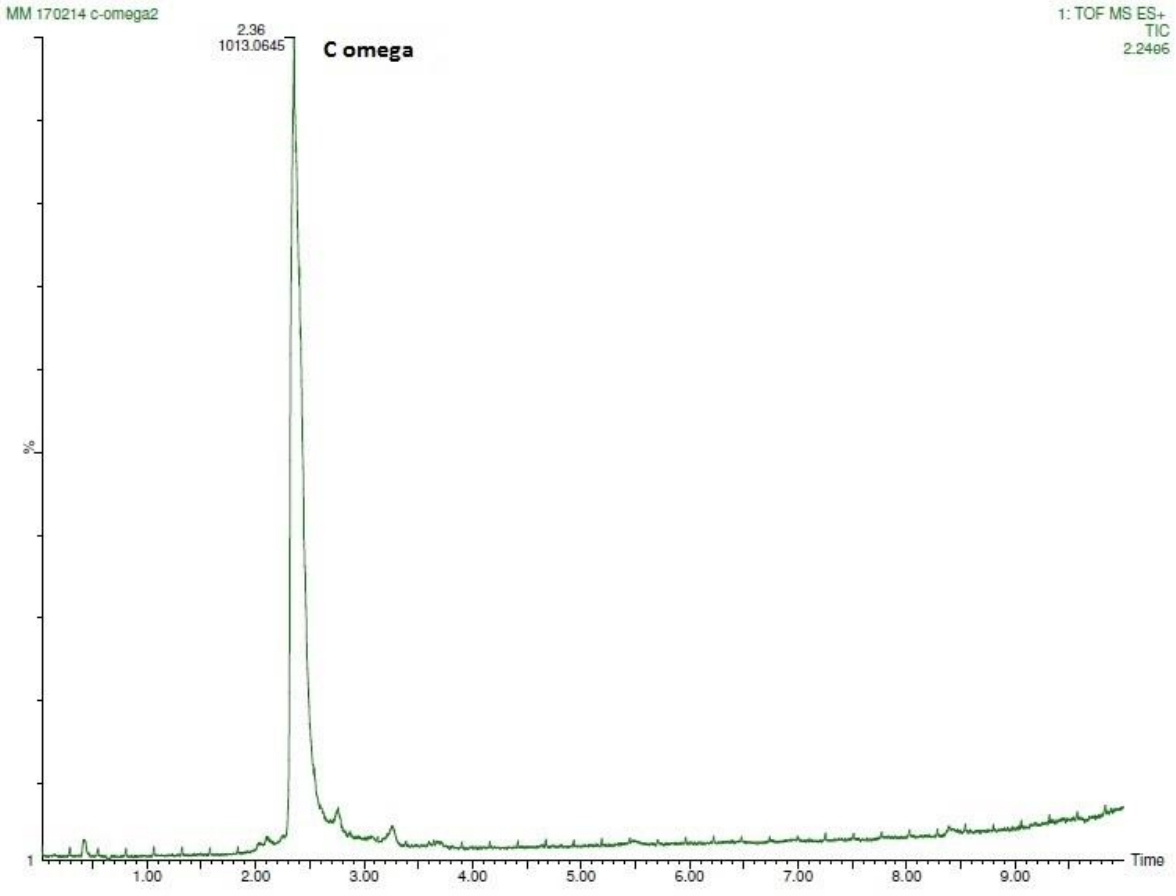


Figure 23: *m/z* 1013.0645 is Cω; [M+H$^+$] = 3036.155, z= 3

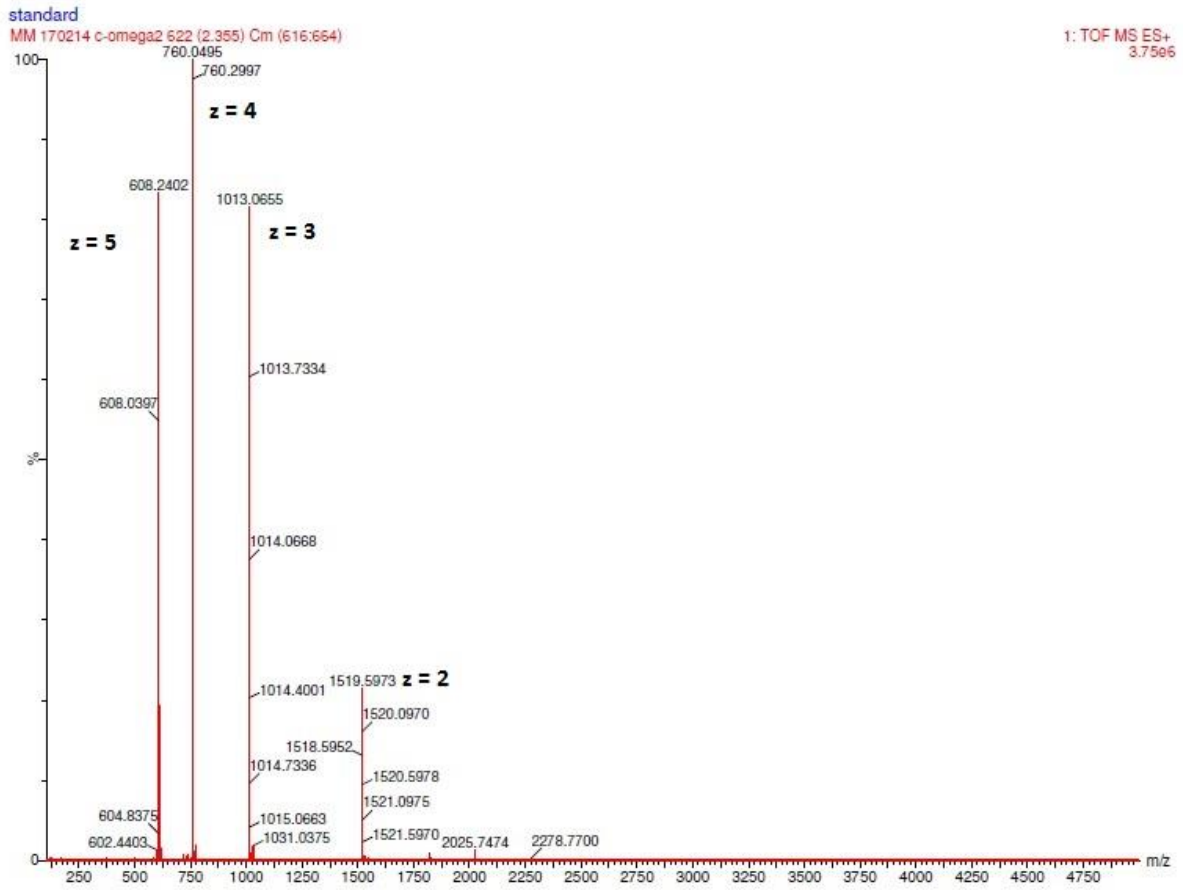


Figure 24: MS spectrum of peak 2.36 in the chromatogram of Cω. [M+H$^+$]=3036.155. Charge states for Cω; Theoretical values for *m/z* when [M+H$^+$]= 3036.155:(3035.155/2) + 1 = 1519.077, (3035.155/3) + 1= 1013.052, (3035.155/4) + 1 = 760.039, (3035.155/5) + 1= 608.031.

5.3.1 In-Vial Reduction and Alkylation

After several trials at different pH, ratios and reaction times, the ones described in the method development chapter was found to be as good as it could get. When the peptide-reduction was performed at pH 5 with four molar equivalents of TCEP, the chromatogram showed five separate peptide signals. See Figure 25 for chromatogram.

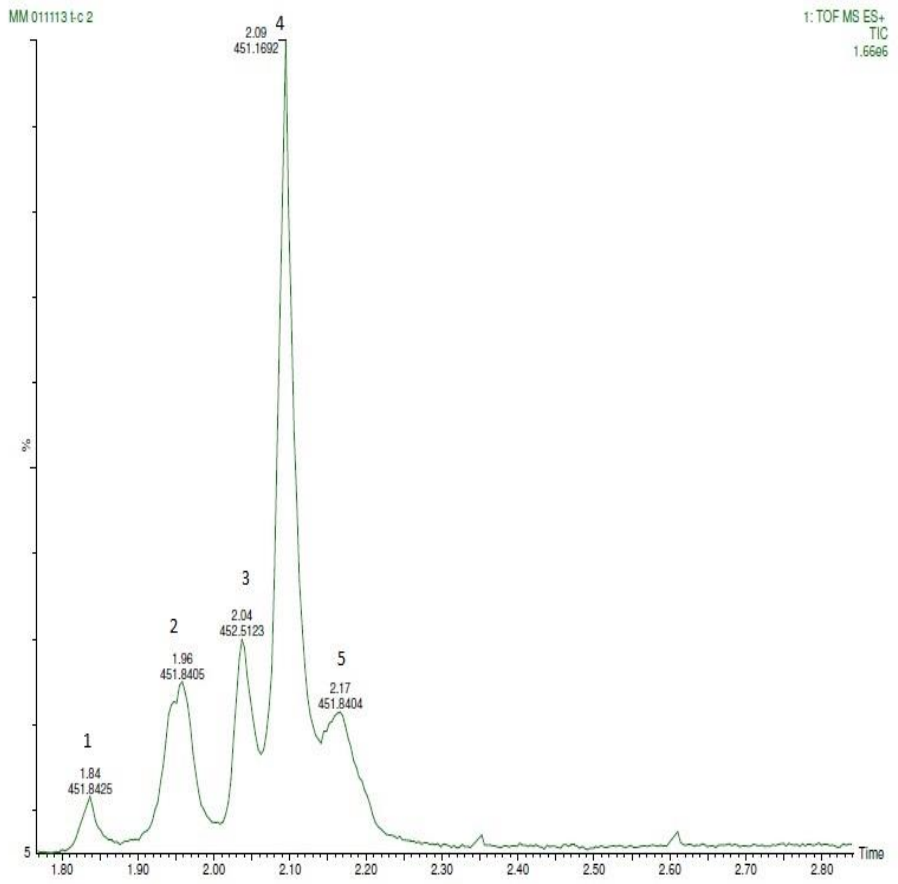


Figure 25: Chromatogram of Cα reduced with TCEP at a ratio 1:4 at pH 5. Peaks are numbered as follows: 1: non-native isomer of Cα formed during reduction 2: Disulfide-bridge 1 in the native isomer of Cα reduced, 3: Fully reduced Cα, 4: Unreacted Cα and 5: Disulfide-bridge 2 in the native isomer of Cα reduced.

The base peak of Figure 25, peak number 4, was from the still unreacted Cα. There was also a strong signal from unreacted TCEP, suggesting that given time, the reaction would go towards full reduction of both the disulfide-bridges. Even though Cα has two disulfide-bridges, there were three peaks with the same *m/z* separated in the UHPLC. These corresponds to three different isomers were one bridge is reduced and the other is intact. This indicates that the third, non-native isomer is formed through rearrangement during the reduction of the first bridge at pH 5, a phenomenon referred to as disulfide scrambling (25). According to the pKa value of the sulfhydryl group in Cysteine (8.33), the sulfhydryl-groups of the Cys-residues should have been 99 % in its protonated, less reactive state, and should not succumb to disulfide scrambling. Since this disulfide scrambling was observed at pH 5.5, it is assumed that the local chemical environment of the peptide changes the pKa value of the sulfhydryl group in Cysteine in Cα. The fully reduced peptide was also present. The chromatographic separation was adequate for the purpose of this project. Figure 26 shows the spectra of the three oxidation stages of Cα: The fully reduced, with only one of two disulfide-

bridges reduced and the native Cα, all with the charge states z = 1, 2 and 3 visible in their spectra.

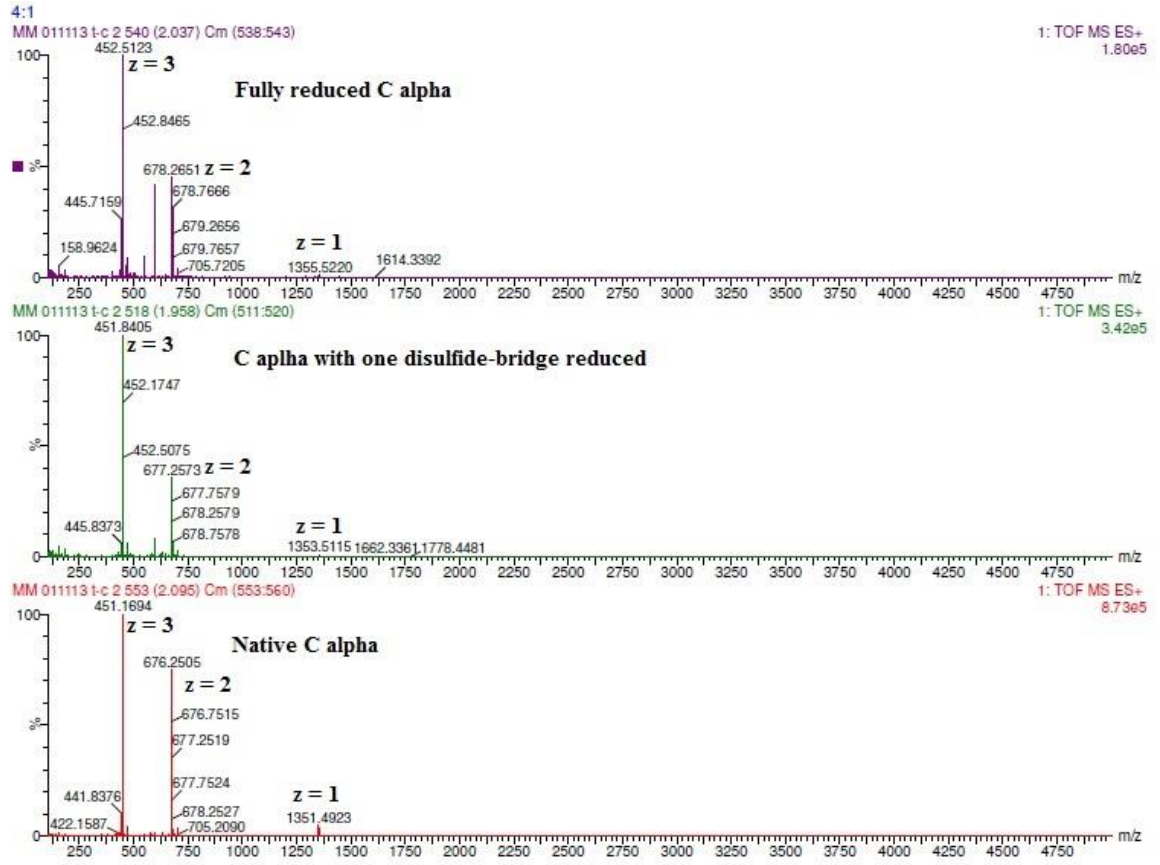


Figure 26: Spectra of the three stages of oxidation for Cα. Fully reduced on top, one reduced disulfide bridge in the middle and unreacted, native Cα at the bottom.

To be able to prove that the peptide has the native fold, the formation of the third, non-native isomer must be prevented. Hence, the re-oxidation of the non-native isomer that happened when a disulfide-bridge was broken at pH 5 had to be avoided. Peak number 1 in Figure 25 is the non-native bridge. At pH 3 there were only two peaks in the chromatogram with an *m/z* corresponding to the peptide with one cleaved and one intact bridge, which indicates that the re-oxidation does not occur at pH 3. Figure 27 shows the chromatogram for the reaction between Cα and TCEP at pH 3.

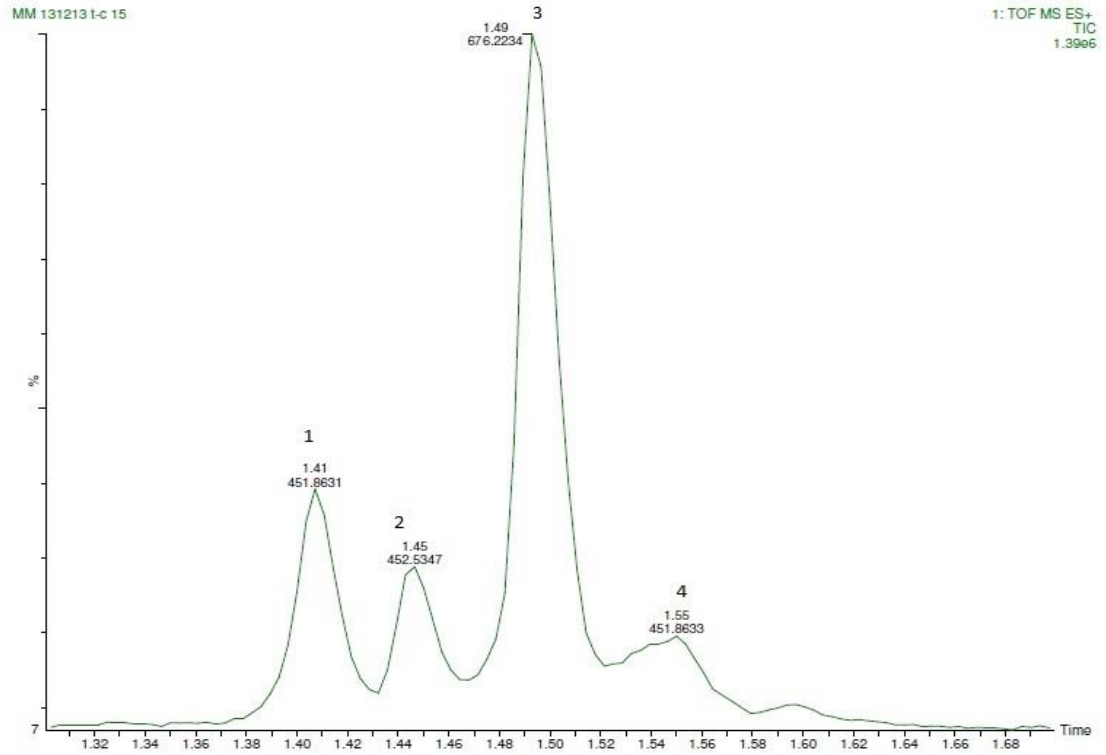


Figure 27: Chromatogram of Cα reduced with TCEP at a ratio 1:100 at pH 3. Peaks are numbered as follows: 1: Disulfide-bridge 1 in the native isomer of Cα reduced, 2: Fully reduced Cα, 3: Unreacted Cα and 4: Disulfide-bridge 2 in the native isomer of Cα reduced.

This is due to the protonation of the sulfhydryl-group. When the sulfhydryl-group is ionic, it is highly reactive and will seek to break other disulfide-bridges. By keeping the pH low, the sulfhydryl-groups are protonated and rendered less active. Figure 25 and Figure 27 are zoomed in images of the peptide peaks in the chromatograms. In addition the TCEP signal was present in both chromatograms. The TCEP signal at pH 3 was even higher than at pH 5, which is to be expected since the ratio between Cα and TCEP is 25 times higher at pH 3 compared to pH 5. The full chromatogram may be viewed in Appendix 3.

At pH 3, the reaction-time and molecular equivalents of TCEP had to be changed. Where at pH 5 the reaction was instantaneous, the reaction time necessary to reduce one bridge in Cα at pH 3 was 6 minutes. 100 equivalents of TCEP had to be added to the Cα at pH 3 to reduce one bridge. This would instantly reduce both bridges at pH 5. When the pH was 5, four times TCEP to one Cα was enough.

Leaving the reaction longer than six minutes at pH 3 did not increase the amount of the wanted isomer, it only increased the amount of fully reduced Cα. As peak four in Figure 27

did not change significantly between the different analyses taken, it seemed the single bridge reduction of the other bridge was stable. This indicates the disulfide-bridge that is reduced first is more accessible to the TCEP than the other. Steric hindrance due to the peptide folding may be an explanation to this observation. When developing the method, a four minute method with a linear gradient from 0-40 % mobile phase B was developed to pinpoint the appropriate reaction time. The development of the reaction could be viewed through the chromatograms. The intact Cα peak, peak number three in Figure 27, would gradually get lower and in the end disappear completely if the reaction was allowed to go far enough. Peak number one in Figure 27 appeared first, before peak number two. Peak number one was the first, more easily accessed bridge. Number two was the second, more shielded and protected bridge that would not be reduced so easily without the first bridge being reduced first. After three analyses of the reduction process lasting four minutes, the first peak was stable and further reduction only gave more of the fully reduced version. The fifth peak in the chromatogram was higher than in the first and second analysis, and if the reaction was allowed to continue, all other peaks would disappear, leaving only the fully reduced version of Cα.

After the first reduction stage, the peptide was alkylated. The alkylating agents of choice, maleimides, are poorly soluble in water and the solubility decreases with the size of the differentiating side-chain. NMM was water soluble, while the other maleimides were all readily soluble in the organic solutions of MeOH and AcN. PhM and tBuM reacted with water and MeOH when dissolved in a 40:60 solution. The reactions were detected upon UHPLC-MS analysis of the maleimides, this data is not shown. AcN was chosen to dissolve all maleimides to avoid such reactions.

Analysis of tBuM on UHPLC-MS/MS indicates that it was not as stable as wanted. Three peaks with *m/z* suggesting tBuM with a missing methyl-group was observed in the chromatogram. It seemed as a loss of each one of the three methyl groups in tBuM gave a de-methylated molecule with different retention-time compared to de-methylation in the two other positions. Why this happened is difficult to explain, since the molecular structure of tBuM suggests that there is free rotation in the molecule, which would give the same isomer regardless of which methyl-group that left the molecule. Regardless of the reason, a maleimide that it self produces four signals in a chromatogram would lead to a messy chromatogram of the alkylated peptide where there was a possibility for as many as four peaks corresponding to the same product, namely one for the unreacted tBuM and one for

each of the de-methylated states of tBuM. This de-methylation was present both in aqueous solution and both organic solutions used in this work.

Alkylation alters the chemical properties of the peptide and therefore also the retention-time. Firstly the alkylation was carried out with NMM. This turned out to be unfavorable, because the retention-time for the first Cα with one reduced and alkylated bridge was the same as for the second non-alkylated isomer. In addition to this co-elution, the separation between the remaining signals was barely acceptable. To shift the signals and thus improve the separation the reduced peptide was alkylated with a more lipophilic maleimide, namely phenyl maleimide. PhM required extended reaction time compared to NMM to ensure full alkylation of the two sulfhydryl-groups in the partially reduced disulfide-bridge. The reaction between partially reduced Cα and NMM was completed within 12 minutes. When PhM was added to the partially reduced peptide and left in room temperature for 12 minutes, a signal with *m/z* corresponding to Cα with only one PhM bound to it was observed. There was also a signal with *m/z* 624 where z equaled 3. This corresponds to a compound weighing 1873 amu, which is Cα with three PhM. This data is not shown. The reaction time was therefore prolonged to 24 minutes to ensure full alkylation of all available sulfhydryl-groups, thus eliminating the signals from Cα with one and three attached PhM. Figure 28 shows the chromatogram of the completed alkylation reaction. The chromatogram shows no signals corresponding to unreacted TCEP, nor any from PhM. There is however a peak with *m/z* 424.1452, corresponding to the end product of a reaction between the two. This reaction is complete and irreversible, and probably fully incapacitates TCEP. It seems that TCEP reaction is more favorable for the maleimides, and therefore happens before the desired reaction between the maleimides and the reduced peptide. A vast molar excess of maleimide must therefore be added to the Cα-TCEP mixture to ensure there is in fact enough free maleimides to react with the sulfhydryl-groups.

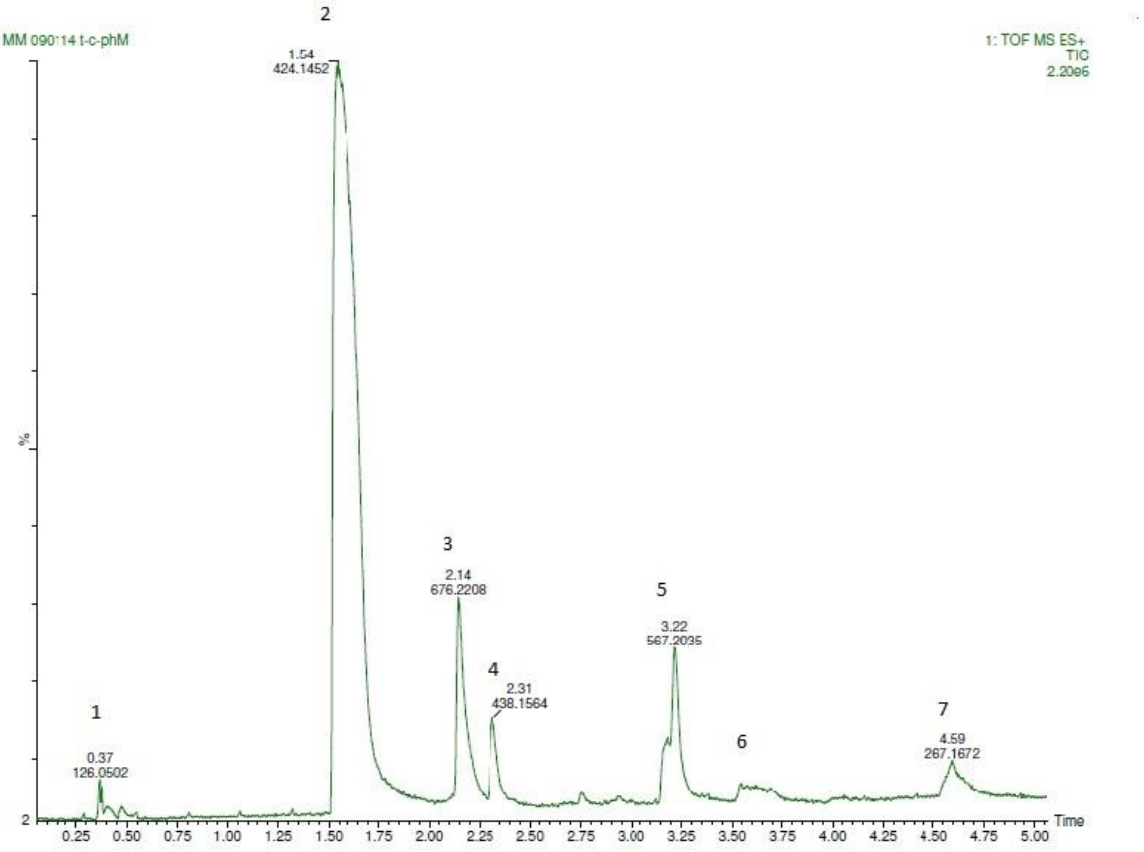


Figure 28: Chromatogram of Cα reduced with TCEP at a ratio 1:100 and alkylated with PhM at pH 3. Peaks are numbered as follows: 1: Injection peak, 2: TCEP and PhM reacted, 3: Unreduced Cα, 4: Contamination, 5: Cα with 2 PhM coupled to the sulfhydryl-group of the Cys-residues formerly attached through a disulfide-bridge, 6: Cα with the other disulfide-bridge reduced and alkylated with PhM, 7: Fully reduced and alkylated Cα.

To reduce and alkylate the remaining bridge with another maleimide, TCEP needs to be reintroduced. TCEP cannot be added directly to the mixture of Cα in different stages of reduction and alkylation presented in Figure 28. The possibility that there was unreacted PhM ready to alkylate the next bridge when reduced could not be ruled out, even if there were no signal corresponding to PhM in the MS. To avoid a reaction between the sulfhydryl-groups of the second bridge and PhM, the Cα with one disulfide-bridge alkylated with PhM needed to be separated from the other compounds. This version of Cα will from now on be referred to as Cα-PhM_2. Peak number 5 with *m/z* 567.2095 in Figure 28 is Cα-PhM_2. An attempt was done to separate the compounds on the available prep-HPLC system. The collection of the wanted peak was analyzed on the MS/MS, but was too diluted to give a signal, and it was clear that this was not the way forward. The separation idea was good, but fruitless since a mobile phase flow of 20 mL/min is needed on the available prep-column. A manual separation method on an analytical column on an UHPLC-PDA was therefore developed.

It was challenging to collect the Cα-PhM_2 from the UHPLC-PDA, since there was a delay between the peak appearing in the chromatogram and the Cα-PhM_2 was actually coming out of the tube. The delay corresponded to one drop of mobile phase. A trial analysis with MS/MS showed that one run was not sufficient; the concentration of Cα-PhM_2 was too low to give a signal strong enough for reliable MS/MS results. 6 injections of 10 µL were gathered in one vial to ensure a sufficient amount of Cα-PhM_2. One injection contained approximately 0.29 nmol peptide, whereof approximately 25% was Cα-PhM_2. The rest of the peptide was either still intact or fully reduced and alkylated.

After gathering the Cα-PhM_2 from the UHPLC-PDA, the AcN from the mobile phase was removed by heating the sample under nitrogen flow. This increased the concentration of Cα-PhM_2. The concentration and amount of Cα-PhM_2 had to be high enough to give adequate signals when analyzed with the MS/MS method.

The actual amount of Cα-PhM_2 that was obtained with this method is highly uncertain. Since the intention was to fully reduce the remaining bridge, there were no concerns regarding the amount of TCEP that was added to Cα-PhM_2. To make sure the reduction was complete, 0.6 µmoles of TCEP was added. To ensure as little dilution of the sample as possible, a 100 mM solution of TCEP was made. It was clear that the reaction-time for the reduction of the second bridge was much longer than the first. A sample that was run after half an hour showed that TCEP failed to fully reduce the Cα-PhM_2 during this time. Only after 2 hours was the second bridge in Cα-PhM_2 fully reduced. It is not clear why this second reaction was slower than the first, but it is likely that the steric hindrances of the second bridge, both from the unfolded peptide chain itself and the added PhM, play a role.

Since a vast excess of TCEP was used to ensure reduction, an even greater excess of NMM had to be added. This was necessary since the available unreacted TCEP instantly would react with available NMM before the NMM could alkylate the peptide. To ensure full alkylation of the second bridge, the Cα-PhM_2-NMM_2 mixture was left for one hour before analyzing it with the MS/MS method. The fully reduced and alkylated Cα will be referred to as Cα-PhM_2-NMM_2. See Figure 29 and Figure 30 for chromatogram and Figure 31 for spectrum of Cα-PhM_2-NMM_2.

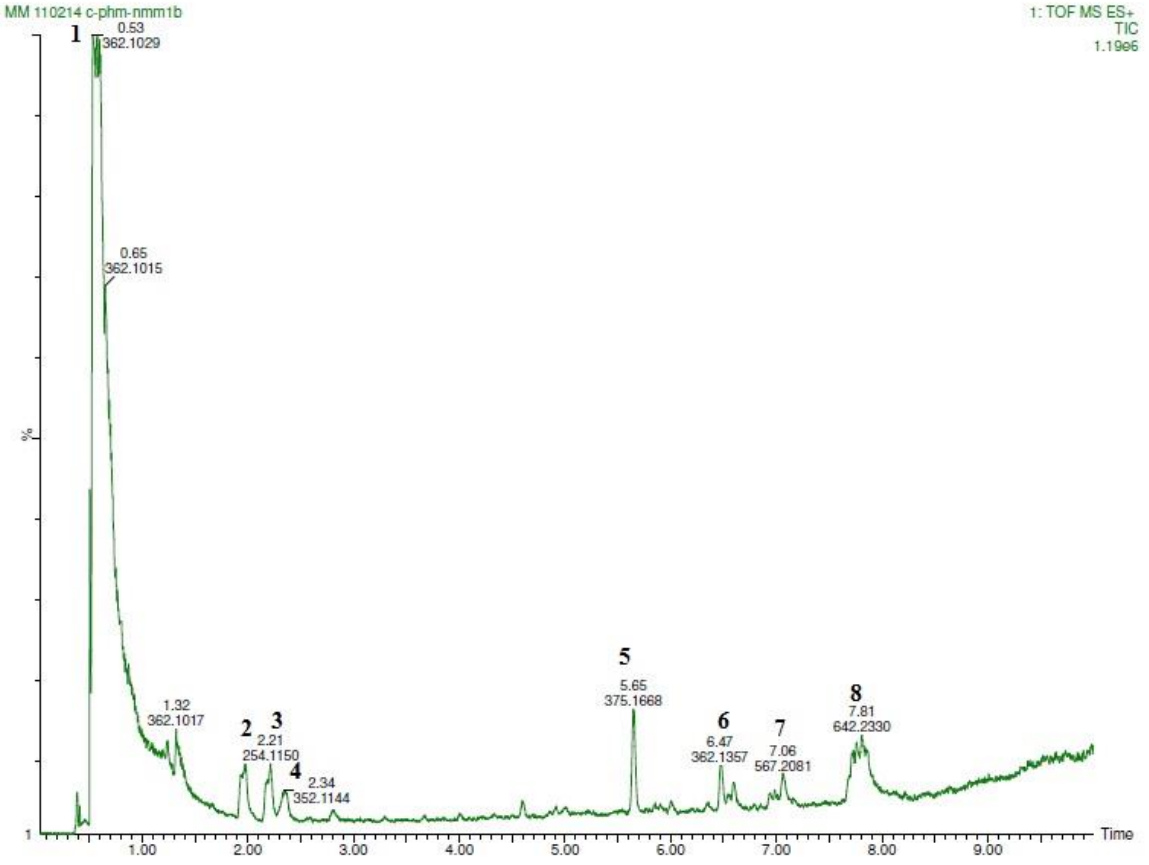


Figure 29: Peak 1: TCEP+NMM, peak 2, 3, 4,5 and 6: Contaminations, peak 7 Cα-PhM$_2$ with the remaining disulfide-bridge reduced, Cα-PhM$_2$-NMM$_2$.

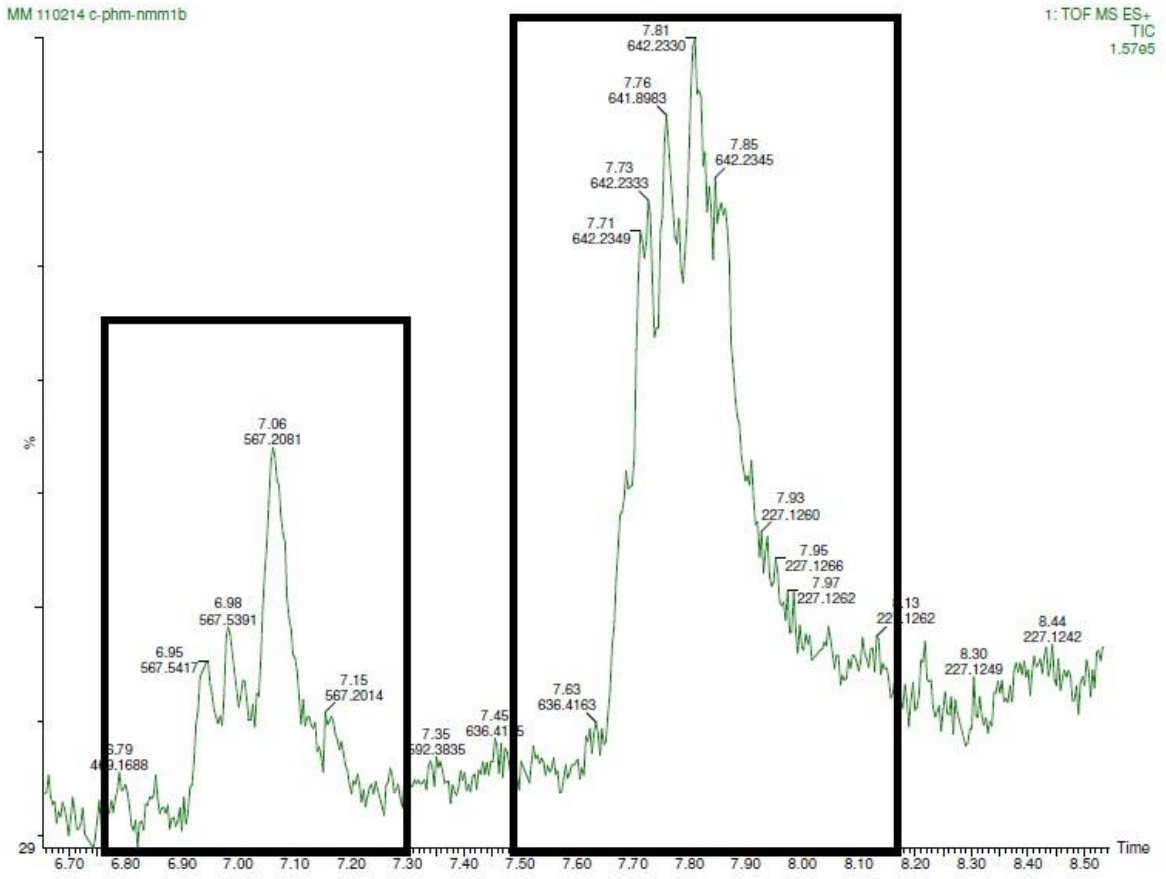


Figure 30: Peak 7 and 8 from the previous figure.

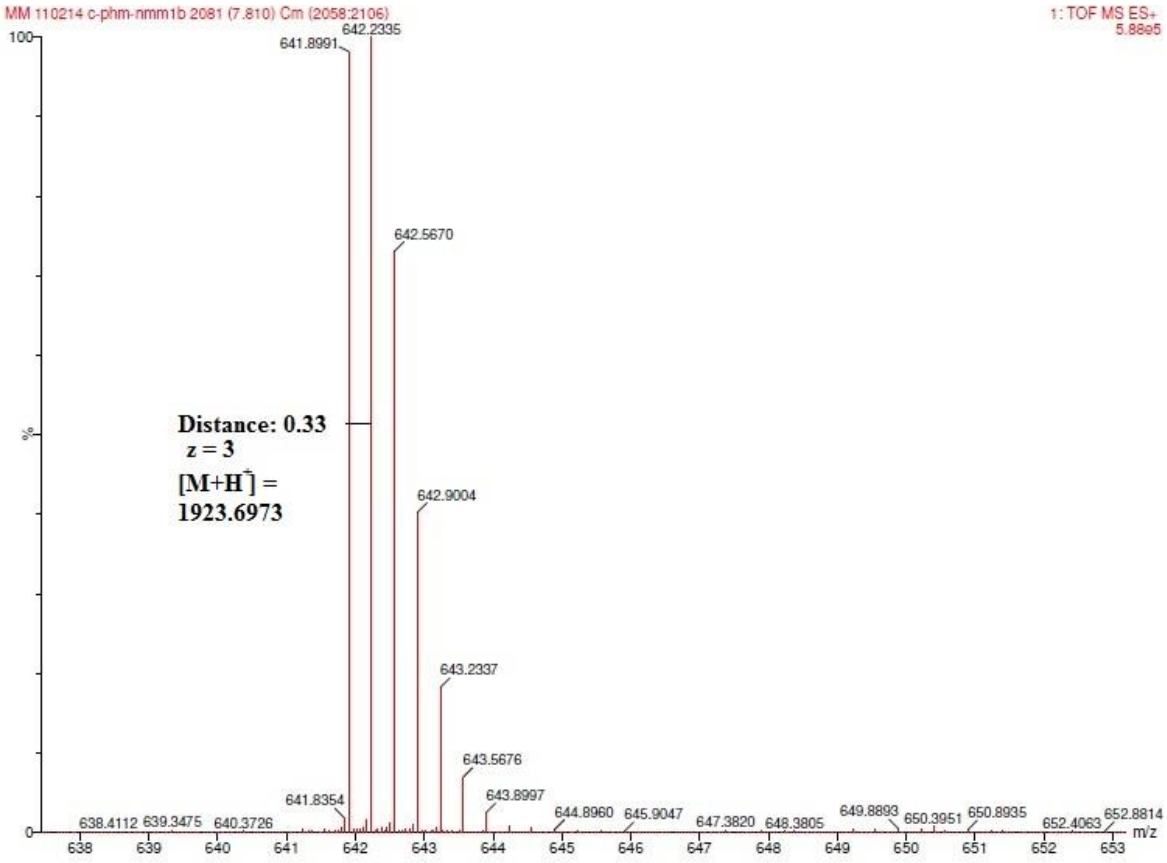


Figure 31: Spectrum of peak with retention time 7.81, verifying that it is Cα-PhM$_2$-NMM$_2$.

Cα was synthesized according to the method described in chapter 4.3. To check if it was oxidized to the native fold, it was analyzed and the bridges were allocated. This peptide was, as before mentioned in chapter 5.1, referred to as DRP1 until it was confirmed that it indeed was the native isomer of Cα.

During a natural oxidation experiment performed by my fellow master student Børge Renmælmo, linear, unprotected DRP1 was left in milliQ water at room temperature for approximately one month. During this time, the peptide oxidized both into a non-native and the native isomer, with a ratio of approximately 1:1. The non-native isomer differed in UHPLC retention-time compared to the native isomer Cα. The chromatograms that show this difference may be viewed in Figure 32.

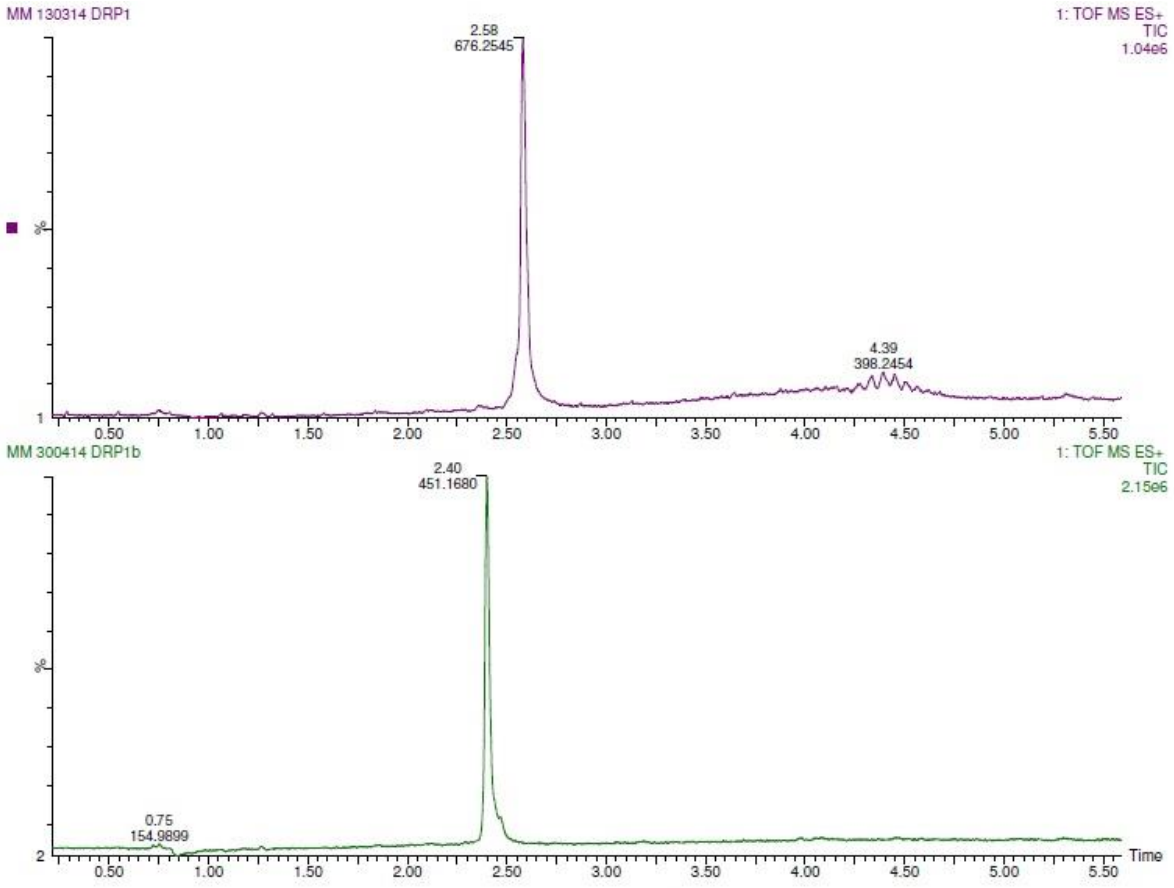


Figure 32: Difference in retention time between DRP1 (2.58 minutes) and DRP1.2 (2.40 minutes)

This non-native isomer will from now on be referred to as DRP1.2. DRP 1.2 was analyzed and the isomer determined. The only difference between the experiments conducted on Cα, DRP1, DRP1.2 and Cω, was the concentration of the peptide solution at hand. The Cα solution had a concentration of 37 µM, DRP1 was 81.5 µM, DRP1.2 was 80 µM and Cω was 65.6 µM. The TCEP/maleimide concentrations were adjusted accordingly to give the appropriate molar ratios as decided in the methods described in chapter 4.5.1.

The method worked well on both DRP1 and DRP1.2, which was expected, since DRP1 is the same peptide as Cα and DRP 1.2 has the same sequence but with different disulfide connectivity. Disappointingly, the bridging of Cω could not be solved by this method. It was not possible to break only one or even two of the three disulfide-bridges of Cω. It was either no reduction or full reduction of the peptide. Since pH needed to be 3, the only variables were the TCEP: Cω ratio and reaction time. The reaction time was first thought to be longer than was the case with Cα, but extending the reaction time only reduced unreduced Cω to fully reduced Cω. A chromatogram of one of the reduction attempts on Cω may be viewed in Appendix 3.

There are several aspects that may lead to this total reduction of Cω. As seen in Figure 2, Cω has a structure built from the secondary structure β-sheets and loops. β-sheets are less stable than α-helices, and a disruption of just one disulfide-bridge may be enough to unravel the whole fold. Less energy is then needed to break the remaining bridges than would be the case in an α-helical structure. When viewing Figure 2, it looks like the position of the disulfide-bridges in the peptide is of a stacking manner, with the topmost bridge of Figure 2 connecting the two end-loops. This stacking may lead to less steric hindrance and therefore easier access for TCEP to the second disulfide-bridge in Cω than is the case for the second bridge of Cα. The properties of the AA in the peptide-sequence, such as hydrophobicity, bulkiness, pKa-value and so on, might be more important factor to whether or not a partial reduction will take place than the number of disulfide-bridges.

Hence, a reduction of just one disulfide-bridge in Cω would need a method that would stabilize the structure while only breaking the most accessible disulfide-bridge. A new method was developed, and since the aim of the thesis was to make a generic method, it was also tested out on Cα.

5.3.2 Reduction and Alkylation on SPE-Column

An attempt was made to develop a SPE method early on in the project. In the early attempt, 2.22 nmole Conotoxin was reduced with a ratio of 50 molar equivalents of TCEP at pH 3 for 6 minutes before it was applied to the conditioned SPE-column. The column was washed with 3 mL milliQ water to rid the sample of excess TCEP. Finally, the peptide was eluted with 0.5 mL 50 mM NMM in AcN. The eluate was examined on UHPLC-MS/MS, and no peptide-signal was detected. These data are not shown. In retrospect, this was probably due to low concentration of peptide in the eluate. Seeing as this attempt failed and the method described in chapter 4.5.1 was a success for Cα, SPE as a tool to specifically break one disulfide-bridge in a DRP was left unresolved until it was clear that the method in chapter 4.5.1 could not be used on Cω.

The SPE method was modified to the one described in chapter 4.5.2. At this stage, DRP1 had been successfully synthesized and the fold was verified by the method described in chapter 4.5.1. For this reason, the amount of available Cα outclassed that of Cω, and Cα was used in

initially in the new SPE trial. Since the aim was to find a generic method, it had to be tested on both Conotoxins available.

The modified SPE method proved a success. A truly specified cleavage of one disulfide-bridge was obtained both with Cα and Cω. Chromatograms of both peptides gave signals corresponding to unreduced and fully reduced and alkylated variants of the peptides present. Figure 33 and Figure 34 are sections of the chromatograms from the sample derived from the SPE-induced reduction and alkylation of Cα and Cω, and show amongst other, the signals from Cα-PhM_2 and Cω-PhM_2 respectively. The strongest signal in both Figure 33 and Figure 34 was the TCEP-PhM complex peak. The co-elution of TCEP and the peptide could not be avoided. It seemed TCEP and the peptide had a similar affinity to the SPE-column, and the PhM had to be introduced to the peptide-TCEP eluate as soon as possible to avoid post-column reduction which would lead towards full reduction of the peptide if not stopped. There were contaminations visible in both chromatograms that had not been observed during the in-vial experiments. A blank eluate, consisting of 80 % AcN with 0.1 % FA, was analyzed on the UHPLC-MS/MS instrument. After examining the chromatogram of the blank eluate, it was clear that the contaminations origins from the SPE-column. Extended washing of the column with solutions of various AcN-to-milliQ water ratios were unable to eliminate the contamination signals. Cα-PhM_2, peak 2 in Figure 33, was the only signal corresponding to a peptide with one disulfide-bridge cleaved and alkylated while the other remained intact. The same was observed for Cω, only one signal where two disulfide-bridges were intact and one cleaved. If no disulfide scrambling occurred, and all three disulfide-bridges were cleaved to leave the peptide with one cleaved and two whole disulfide-bridges, there would be three signals with the same *m/z* in the chromatogram. The fact that there was not, shows that one disulfide-bridge was specifically cleaved. There were signals corresponding to Cω with two disulfide-bridges being cleaved and to the fully reduced Cω. More chromatograms and spectra from the SPE experiments may be viewed in Appendix 3.

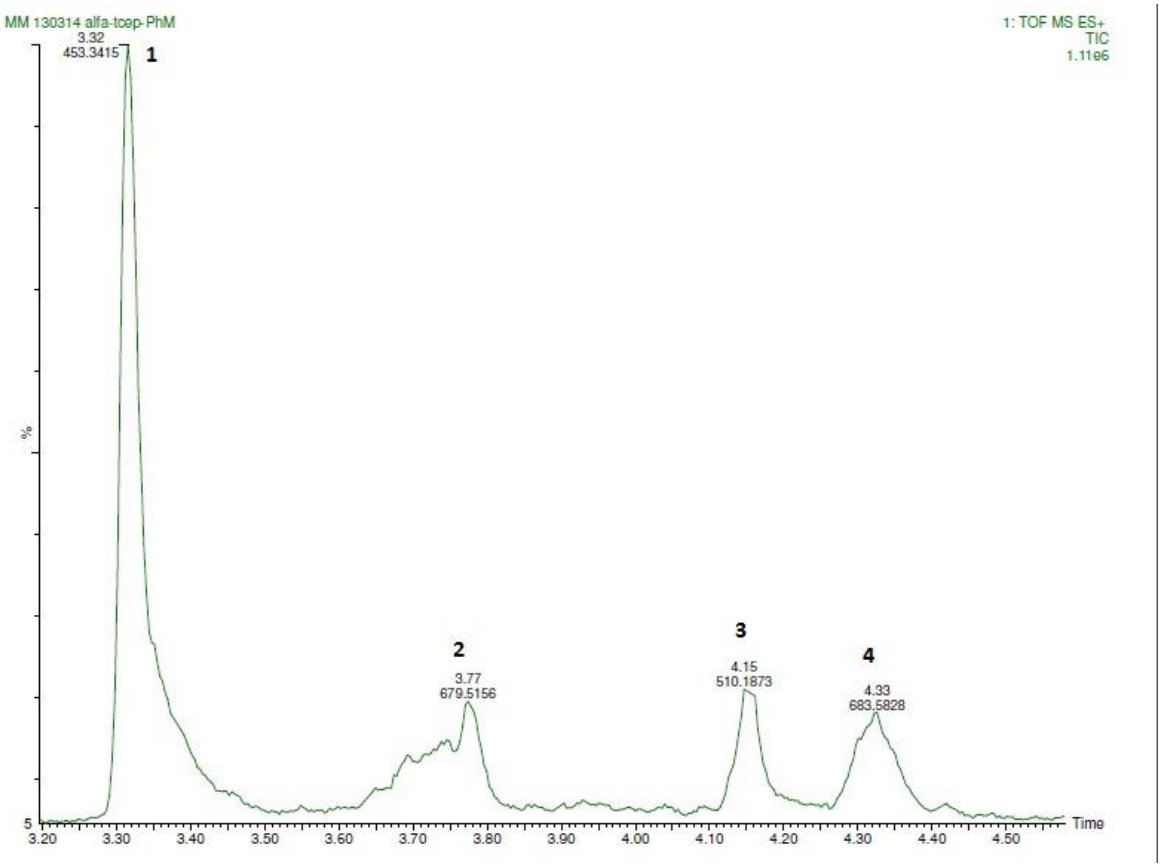


Figure 33: A chromatogram of Cα from the SPE-method, 3.30-4.50 minutes displayed. Signal 1; TCEP-PhM, signal 2; Cα-PhM$_2$ signal 3 and 4 contaminations from the SPE-column.

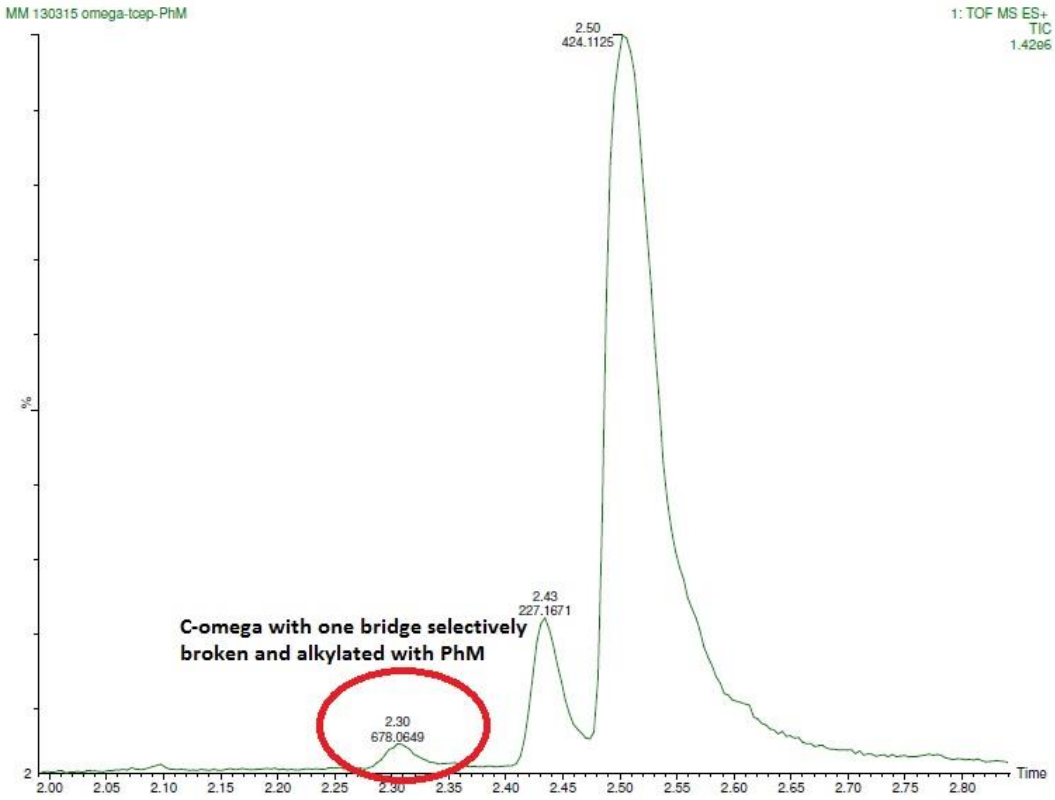


Figure 34: Cω-PhM$_2$ signal with retention time 2.30 dwarfed by the TCEP-PhM signal with retention time 2.50. The peak at 2.43 minutes is a contamination.

After these preliminary tests the aim was to increase the volume of sample added to the SPE column, and adjust the amount of TCEP and PhM accordingly. The Cω-PhM$_2$ would be separated from the rest of the components in the sample through the same manual purification method on the UHPLC-PDA as described in chapter 4.5.1. The prepared Cω-PhM$_2$ would undergo a new reduction step on a SPE column, and eluted into an Eppendorf tube containing NMM. The Cω-PhM$_2$-NMM$_2$ would subsequently be treated equally to the Cω-PhM$_2$ sample. The third and final reduction step would be carried out in the same manner, with the sample eluting in to a NEM solution, creating Cω-PhM$_2$-NMM$_2$-NEM$_2$ ready for software mediated structure determination. Due to unfortunate, long term damage to the UHPLC-MS/MS system, this remains unresolved at the end of the project period. Further development of the method must be continued to see if the method will live up to the current expectations.

5.4 Structure Determination

After a successful analysis, the MS/MS spectra acquired were compared to the theoretical CID- fragments calculated in the software program "MS-product" in the database "Protein prospector". It was possible to identify which Cys-residues were coupled to which maleimide, and thereby establish the Cysteine-connectivity of the alkylated peptide. To simplify the different isomer sequences of Cα or any other two-bridged DRP, these may be illustrated by vvuu, vuvu or uvvu, as previously explained in chapter 4.7 and illustrated in Figure 16. The three sequences were defined in the "Protein Prospector" (PP) software. In Appendix 4, the theoretical values of fragment-ions formed upon CID-fragmentation of Cα-PhM_2-NMM_2 are shown. The fragment-ions actually observed are marked with red squares. The observed fragments are illustrated in Figure 35 with deviation from the theoretical value in ppm.

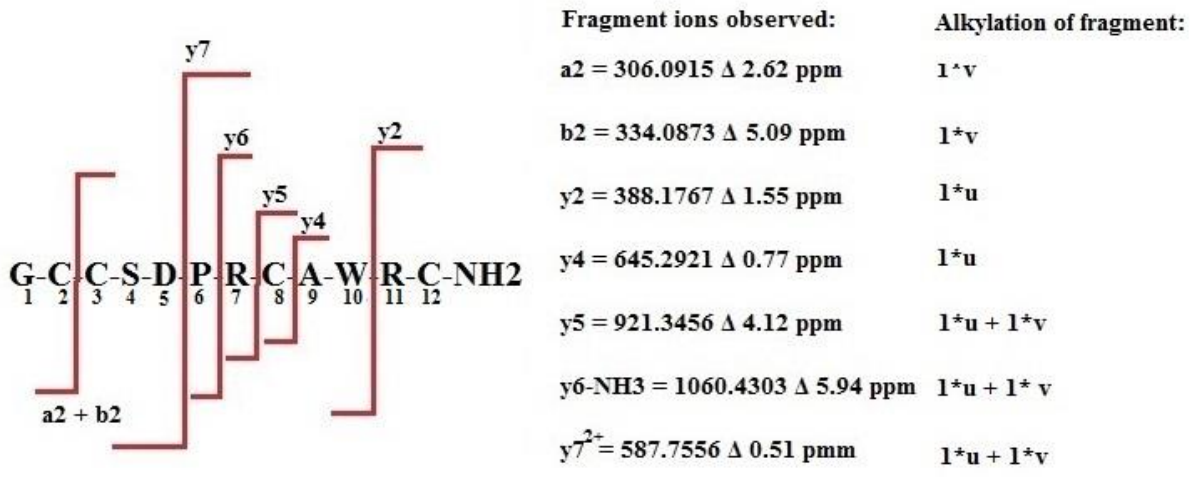


Figure 35: The diagnostic ions observed upon CID-fragmentation of Cα-PhM_2-NMM_2. Theoretical values can be viewed in Appendix 4.

In Figure 36, the fragment with *m/z* 587.7556 is the base peak. This is as expected since Proline (Pro) induces fragmentation, making y_7 the most prominent fragment. The y_7 fragment of Cα was a diagnostic ion. If the isomer vvuu was the native fold with both C8 and C12 alkylated with NMM, the *m/z* of the ion y_7^{2+} would have been 556.7475 according to the PP. As the *m/z* of y_7^{2+} was 587.7556, the sequence at hand must be uvuv, vuvu, vuuv or uvvu. The sequences uvuv and vuvu are the same isomer, only alkylated in the opposite order. The same is true for vuuv and uvvu, as illustrated in Figure 16. Fragmentation at residues four, five, six and seven in the sequence gives different length fragments all containing C8 and C12, and they all give information of the alkylation of these. However, not all of these fragments were observed. In addition to the before mentioned fragment y_7^{2+} the fragments y_5 and y_6-NH_3 were observed, and effectively ruled out the possibility of vvuu being the native sequence. Figure 35 and Figure 36 shows their observed values

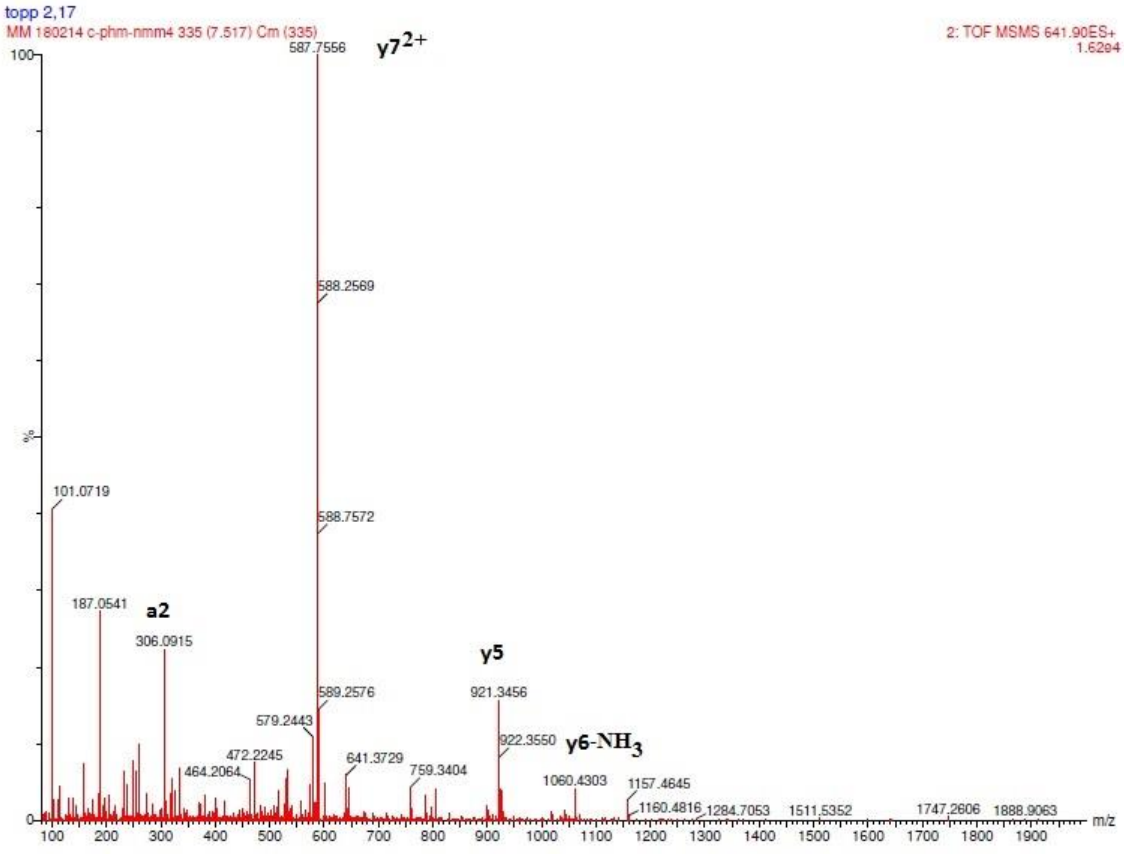


Figure 36: CID-fragments of Cα-PhM_2-NMM_2, diagnostic ions a_2, y_5, y_6 and y_7 shown.

Fragments y_1-y_4 and thus b_8-b_{11} were diagnostic ions and would determine the alkylation of Cys8 and Cys12. Of these possible fragments only y_2 and y_4 were observed, and are shown in

Figure 37 and Figure 38. Fragment y_4 had an *m/z* of 645.2921, which corresponds to the theoretical value of *m/z* 645.2926 if C12 was u (NMM). The y_2-fragment had an *m/z* of 388.1767, where PP's theoretical value was 388.1761 if C12 was u (NMM). The difference between the actual observations and the theoretical fragments were 0.77 and 1.55 ppm respectively. An error of less than 25 ppm was considered acceptable. Hence, C12 was identified as u (NMM). Following this and the previous conclusion that vvuu could not be the isomer, C8 needed to be v (PhM).

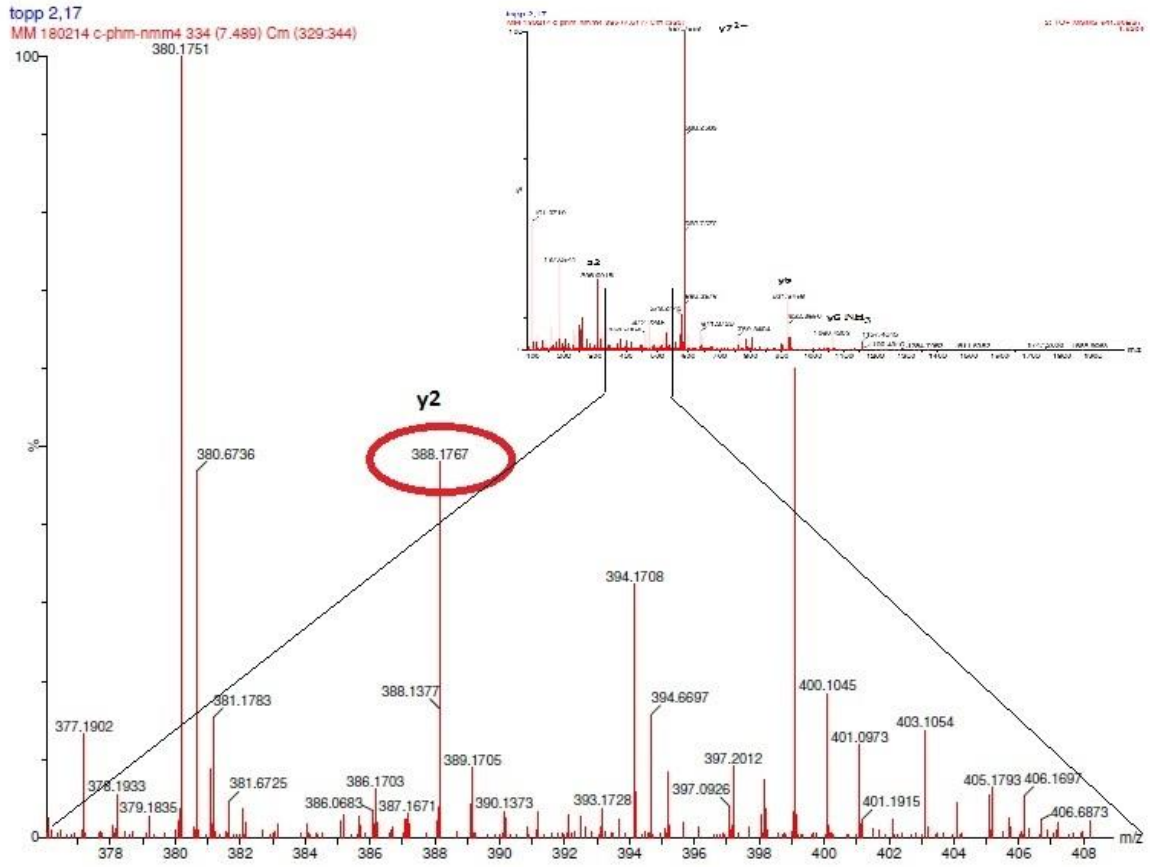


Figure 37: MS/MS spectrum of Cα-PhM$_2$-NMM$_2$ shows the presence of the diagnostic ion y_2 with m/z 388.1767

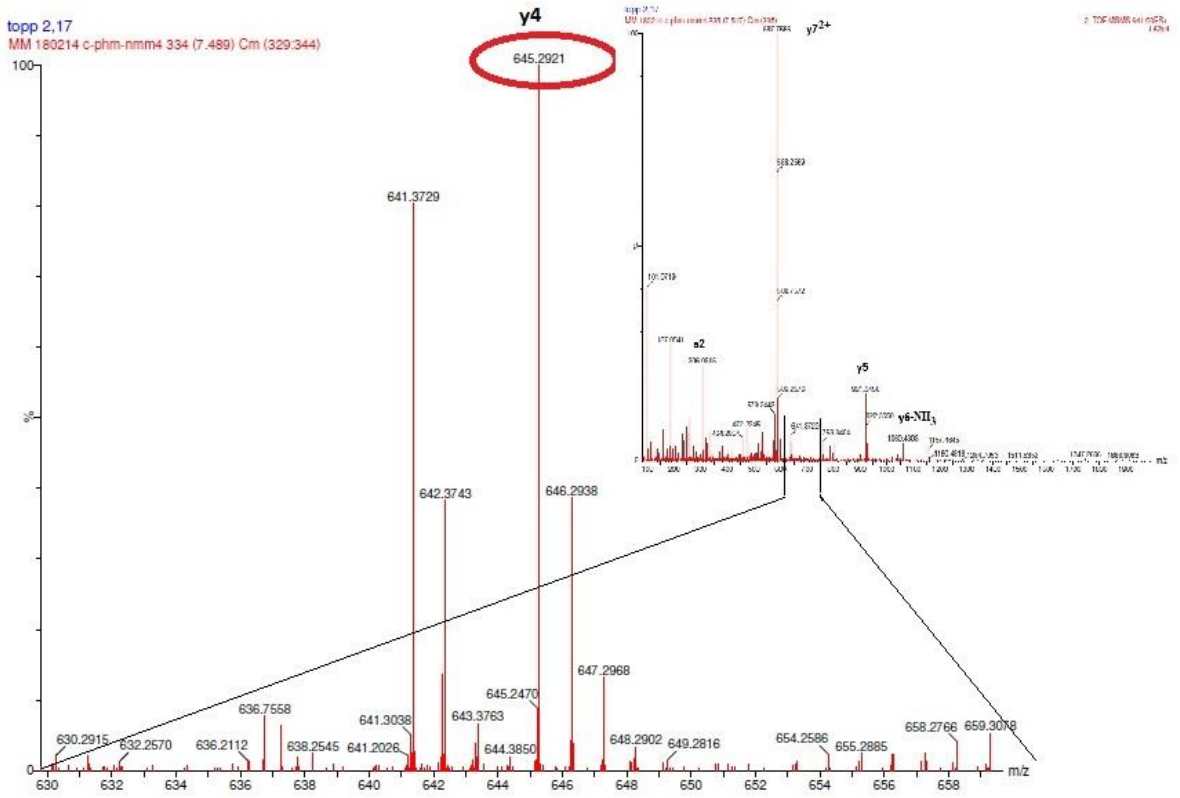


Figure 38: MS/MS spectrum of Cα-PhM$_2$-NMM$_2$ shows the presence of the diagnostic ion y$_4$ with *m/z* 645.2921

In Figure 36, fragment-ion a_2 had an *m/z* of 306.0907, while the theoretical value for this ion whit Cys2 as v (PhM) was 306.0917. The difference between theoretical and empirical value was only 2.62 ppm and the ion therefore diagnostic for Cys2. If Cys2 had been u (NMM), the theoretical value of fragment-ion a_2 was 244.0755. Fragment-ion b_2 also gave support to Cys2 being v (NMM), with empirical value 334.0873 and 334.0856 as theoretical value for a fragment-ion containing v (NMM). Difference between actual and theoretical value was 5.09 ppm.

From these seven diagnostic ions, it was concluded that the sequence was vuvu (PhM-NMM-PhM-NMM), connecting Cys-residues 2 and 8 in a disulfide-bridge and Cys-residues 3 and 12 in a second. This corresponds to previous knowledge of the Cα fold. The Cα fold is illustrated in Figure 39.

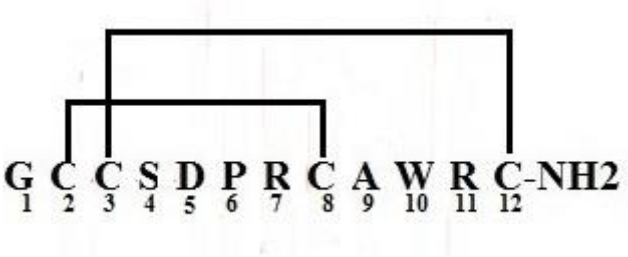


Figure 39: Native fold of Cα ImI verified by UHPLC-MS/MS and PP.

To verify that the synthesized DRP1 was correctly folded into the native fold of Cα, the MS/MS spectra of DRP1-PhM_2-NMM_2 was simply compared to the spectra of the standard Cα-PhM_2-NMM_2. The diagnostic ions found for Cα-PhM_2-NMM_2 were also found for DRP1-PhM_2-NMM_2. The fragments of Cα-PhM_2-NMM_2 and DRP1-PhM_2-NMM_2 are compared in Figure 40. By the reasoning above, DRP1 was thus proven to have the native fold, and could be renamed Cα.

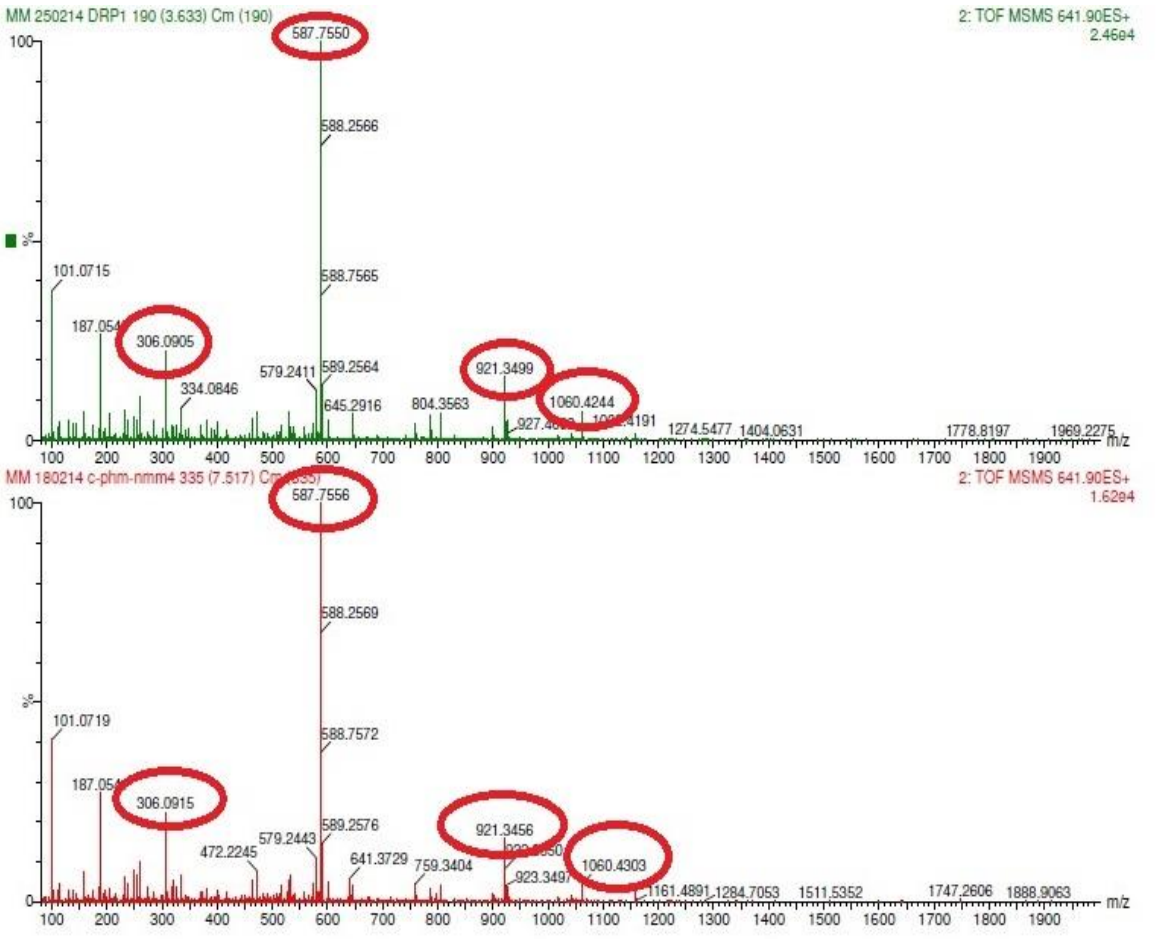
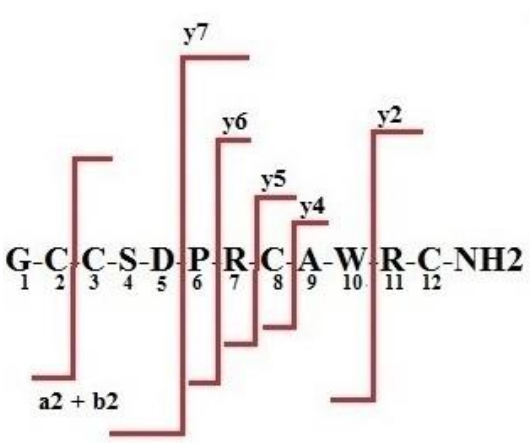


Figure 40: Comparison of the CID-fragment-ions of Cα-PhM_2-NMM_2 and DRP1-PhM_2-NMM_2. The most intense diagnostic ions are marked with red circles. The two spectra are sufficiently similar to conclude that the two peptides are in fact the same.

To further strengthen the conclusion that DRP 1 was the native isomer, MS/MS analysis of both fully reduced Cα and Cα alkylated with NMM in all four positions was performed. The diagnostic ions of Cα-NMM_4 had different *m/z* compared to Cα-PhM_2-NMM_2, since two PhM with monoisotopic mass 173.055 amu was replaced with NMM with monoisotopic mass 111.0399 amu. The spectra may be viewed in Appendix 4. The theoretical values of the fragment-ions that can be formed upon CID-fragmentation of Cα-NMM_4 from PP can be viewed in Appendix 4. The observed values and the deviation from the theoretical values are illustrated in Figure 41.



Fragment ions observed:	Alkylation of fragment:
a2 = 244.0754 Δ 1.64 ppm	1*u
b2 = 272.0705 Δ 1.84 ppm	1*u
y2 = 388.1680 Δ 20.87 ppm	1*u
y4 = 645.2877 Δ 7.59 ppm	1*u
y5 = 859.3369 Δ 3.61 ppm	2*u
y6 - NH3 = 998.4119 Δ 3.51 ppm	2*u
$y7^{2+}$ = 556.7488 Δ 2.33 ppm	2*u

Figure 41: The diagnostic ions observed upon CID-fragmentation of Cα-NMM_4.

The MS/MS spectrum of DRP 1.2-PhM_2-NMM_2, the non-native fold of Cα formed during oxidative folding of the linear synthesized peptide was compared to the theoretical values of the fragment ions in PP. Diagnostic ions y_2, y_4, y_5, y_6 and y_7 all had the same values as in Cα-PhM_2-NMM_2 and DRP1-PhM_2-NMM_2, indicating that there was no difference in the alkylation of C8 and C12. This meant that C8 = v (PhM) and C12 = u (NMM) also in DRP 1.2, based on the knowledge from diagnostic ions $_{y2, y5}$, y_6-NH_3 and y_7^{2+}. Fragment ions a_2 and b_2, had *m/z* 244.0755 and 272.0705 respectively, corresponding to a fragment where C2 was u (NMM) and not v (PhM) as was the case of Cα.

Figure 42 illustrates the observed fragment-ions and the deviation from the theoretical value from PP. The theoretical values from PP may be viewed in Appendix 4. The spectra with the diagnostic ions marked may be viewed in Appendix 4. Figure 42 compares the spectra of

DRP1 and DRP1.2. The figure shows the difference in *m/z* for diagnostic ion a_2 and that *m/z* for the diagnostic ions y_7^{2+} and y_5 are the same in the two peptides.

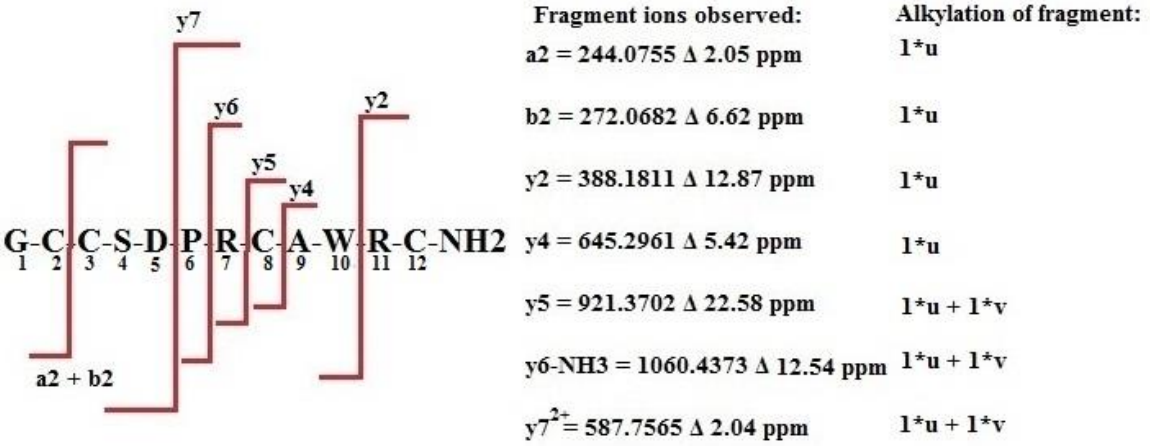


Figure 42: The diagnostic ions observed upon CID-fragmentation of DRP1.2-PhM$_2$-NMM$_2$.

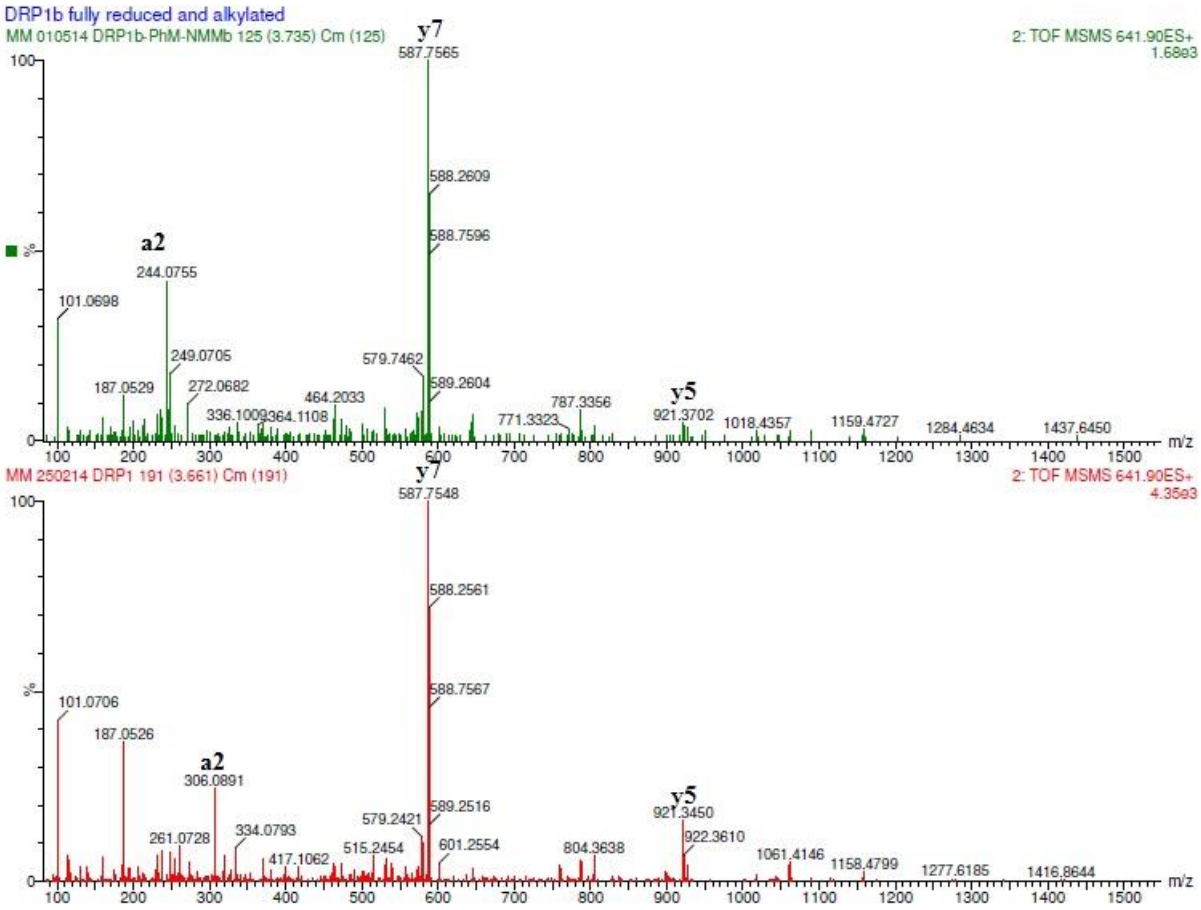


Figure 43: DRP1.2-PhM-NMM and DRP1-PhM-NMM on top of each other. Fragment ion a_2 differs, y_7^{2+} and y_5 are the same.

The seven observed diagnostic ions showed in Figure 42 confirmed the theory that DRP 1.2 was the non-native isomer uvvu shown in Figure 44.

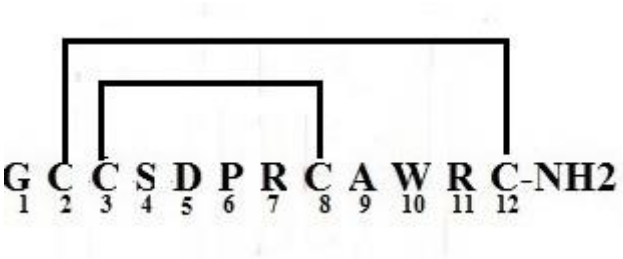


Figure 44: The sequence and Cysteine connectivity of the non-native isomer DRP1.2

6. Future Perspectives

The aim of the thesis was to find a generic method of allocating Cysteine-connectivity for DRPs, and ideally a method that in the course of one work flow could identify samples of minute amount. This was not accomplished through these experiments, and needs further attention.

Since the unfortunate instrument break-down hindered the full exploration of the SPE-method, this needs to be further investigated in the future. Should the method prove to work as anticipated, it needs to be tried on several peptides differing in qualities such as sequence, hydrophobicity, number of Cys-residues and others.

In future experiments the use of digestive enzymes with well-known and –studied active sites which will cut the peptide-chain between foreseen AAs could be used to obtain anticipated fragments. These fragments could be analyzed by other software or databases to determine the final structure and Cysteine-connectivity.

Another path worth further investigation is the use of scavenger columns. The solid phase of these SPE-columns contains thiol-groups that will quench any remaining maleimide from the solution, readying it for further reduction without the time consuming and imprecise fraction-sampling method on the UHPLC.

7. Conclusion

A successful synthesis of Cα was a performed with neither Cysteine racemization nor aspartamide formation detected. The purification of the crude resulted in a 95 % purity of the linear, Acm-protected peptide. The chemical oxidation ensured correct cysteine-connectivity in the peptide.

TCEP solutions were not found to be as effective as proclaimed in previous papers. At pH 3, both the reaction time and ratio needed were higher than at the recommended pH 5.5.

The self-synthesized Cα, the commercially bought Cα, and the naturally oxidized peptide DRP1.2 were all analyzed by the method to determine isomer and Cysteine-connectivity in DRPs with two disulfide-bridges that was found. Both peptide solutions and TCEP-solutions had to be made with ammonium formate buffer with pH 3 to prevent disulfide scrambling. The maleimides had to be dissolved in AcN to prevent unwanted reactions between maleimide and water and/or MeOH. Because of the different reaction stages present in a peptide sample with one bridge reduced and alkylated, the Cα-PhM_2 fraction needed to be isolated.

A generic MS/MS method gave sufficiently adequate results to compare observed fragment-ions with theoretical ions calculated by the chosen software, Protein Prospector. The disulfide-connectivity of the self-synthesized Cα, the commercially bought Cα, and the naturally oxidized peptide DRP1.2 were all verified. This was accomplished by comparing the ions obtained upon CID-fragmentation of the peptides with each bridge alkylated with a different maleimide with the theoretical values of these ions

The generic SPE-method for reducing and alkylating disulfide-bridges of all DRPs regardless of number of bridges shows potential. There is still much work ahead to develop the SPE-method, and other methods such as a method involving scavenger columns or digestive enzymes may be considered should the SPE-method fail to live up to its expectations.

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Appendix

Appendix 1

Peptide Synthesis

Prelude programs;

Appendix table 1: 100 µM First coupling with resin swelling and double coupling; Volume in µL, time in minutes: seconds, concentrations in M. The table is modified from the software of the Prelude peptide synthesizer.

Operation	Volume	Mix time	Repetitions	Reagents/ action
DMF top wash	5000	1:00	3	Resin swell
Deprotection	2500	5:00	1	Piperidine
Deprotection	2500	10:00	1	Piperidine
DMF top wash	2500	0:30	5	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong Base	1000	30:00	1	1.0 M DIPEA
DMF top wash	2500	0:30	1	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong base	1000	30:00	1	1.0 M DIPEA
DMF top wash	2500	0:30	3	DMF

Appendix table 2: Standard 100 µM double coupling and capping; Volume in µL, time in minutes: seconds, concentrations in M. The table is modified from the software of the Prelude peptide synthesizer.

Operation	Volume	Mix time	Repititions	Reagents
Deprotection	2500	5:00	1	Piperidine
Deprotection	2500	10:00	1	Piperidine
DMF top wash	2500	0:30	5	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong Base	1000	30:00	1	DIPEA
DMF top wash	2500	0:30	1	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong Base	1000	30:00	1	DIPEA
DMF top wash	2500	0:30	5	DMF
Capping	2500	15:00	1	20 % acetic anhydride 20 % pyridine in DMF
DMF Top Wash	2500	0:30	3	DMF

Appendix table 3: 100 µM Cysteine double coupling and capping program. Volume in µL, time in minutes: seconds, concentrations in M. The table is modified from the software of the Prelude peptide synthesizer.

Operation	Volume	Mix time	Repititions	Reagents
Deprotection	2500	5:00	1	Piperidine
Deprotection	2500	10:00	1	Piperidine
DMF top wash	2500	0:30	5	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Weak base	1000	30:00	1	1.0 M TMP
DMF Top Wash	2500	0:30	1	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Weak base	1000	30:00	1	1.0 M TMP
DMF top wash	2500	0:30	5	DMF
Capping	2500	15:00	1	20 % acetic anhydride
				20 % pyridine in DMF
DMF top wash	2500	0:30	3	DMF

Appendix table 4: 100 µM final double coupling program; Volume in µL, time in minutes: seconds, concentrations in M. The table is modified from the software of the Prelude peptide synthesizer.

Operation	Volume	Mix time	Repetitions	Reagents
Deprotection	2500	5	1	Piperidine
Deprotection	2500	10	1	Piperidine
DMF top wash	2500	0:30	5	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong Base	1000	30	1	DIPEA
DMF top wash	2500	0:30	1	DMF
AA Building Block	2500	-	1	0.2 M Fmoc solution
Activator	1300	-	1	0.4 M HCTU
Strong Base	1000	30:	1	DIPEA
DMF top wash	2500	0:30	3	DMF
Deprotection	2500	5	1	Piperidine
Deprotection	2500	10	1	Piperidine
DMF top wash	2500	0:30	4	DMF

Appendix 2

Activity Studies of TCEP

Experiment 1; TCEP activity and pH

Appendix table 5: Absorbance of DNTB and TCEP solution measured at pH 2, each parallel was measured three times at three different times; t is measured in minutes.
An average value for each parallel was calculated.

pH 2	t= 0	t= 15	t= 30
Parallel 1, measurement 1	0.10518	0.10726	0.11614
Parallel 1, measurement 2	0.10577	0.10759	0.11622
Parallel 1, measurement 3	0.10448	0.10732	0.1164
Average 1	0.10514	0.10739	0.116253
Parallel 2, measurement 1	0.10577	0.11417	0.11637
Parallel 2, measurement 2	0.10503	0.11424	0.11603
Parallel 2, measurement 3	0.10523	0.11417	0.11625
Average 2	0.10534	0.11419	0.11622
Parallel 3, measurement 1	0.10325	0.11285	0.11699
Parallel 3, measurement 2	0.10445	0.11229	0.11698
Parallel 3, measurement 3	0.10474	0.11298	0.11706
Average 3	0.10415	0.11271	0.11701

Appendix table 6: Absorbance of DNTB and TCEP solution measured at pH 3, each parallel was measured three times at three different times; t is measured in minutes.
An average value for each parallel was calculated.

pH 3	t= 0	t= 15	t= 30
Parallel 1, measurement 1	0,11463	0,12007	0,12419
Parallel 1, measurement 2	0,1149	0,11975	0,12418
Parallel 1, measurement 3	0,11523	0,11984	0,12429
Average 1	0,11492	0,11989	0,12422
Parallel 2, measurement 1	0,1124	0,12089	0,1243
Parallel 2, measurement 2	0,11231	0,12111	0,12466
Parallel 2, measurement 3	0,11257	0,12116	0,12487
Average 2	0,11243	0,12105	0,12461
Parallel 3, measurement 1	0,11711	0,11648	0,12082
Parallel 3, measurement 2	0,11706	0,11728	0,121
Parallel 3, measurement 3	0,11731	0,11714	0,12148
Average 3	0,11716	0,11697	0,1211

Appendix table 7: Absorbance of DNTB and TCEP solution measured at pH 4, each parallel was measured three times at three different times; t is measured in minutes.
An average value for each parallel was calculated.

pH 4	t= 0	t= 15	t= 30
Parallel 1, measurement 1	0.19328	0.2477	0.25418
Parallel 1, measurement 1	0.208	0.24726	0.25412
Parallel 1, measurement 1	0.21877	0.24767	0.25415
Average 1	0.20668	0.24754	0.25415
Parallel 2, measurement 1	0.27793	0.32754	0.33935
Parallel 2, measurement 2	0.28772	0.3262	0.3393
Parallel 2, measurement 3	0.29611	0.32828	0.33945
Average 2	0.28725	0.32734	0.33937
Parallel 3, measurement 1	0.20267	0.25534	0.27149
Parallel 3, measurement 2	0.21932	0.25534	0.27177
Parallel 3, measurement 3	0.22891	0.25655	0.27216
Average 3	0.21697	0.25574	0.27181

Appendix table 8: Absorbance of DNTB and TCEP solution measured at pH 2, each parallel was measured three times at three different times; t is measured in minutes.
An average value for each parallel was calculated.

pH 5	t= 0	t= 15	t= 30
Parallel 1, measurement 1	0.27682	0.53065	0.54416
Parallel 1, measurement 2	0.31957	0.53033	0.54449
Parallel 1, measurement 3	0.36444	0.52986	0.54517
Average 1	0.32028	0.53028	0.54461
Parallel 2, measurement 1	0.32117	0.54466	0.56335
Parallel 2, measurement 2	0.37896	0.54486	0.56258
Parallel 2, measurement 3	0.40832	0.54485	0.5626
Average 2	0.36948	0.54479	0.56284
Parallel 3, measurement 1	0.29957	0.58873	0.59308
Parallel 3, measurement 2	0.37413	0.58977	0.59439
Parallel 3, measurement 3	0.41991	0.58912	0.59537
Average 3	0.36454	0.58921	0.59428

Appendix table 9: Absorbance measured at pH 6, each parallel was measured three times at three different times; t is measured in minutes. An average value for each parallel was calculated.

pH 6	t= 0	t= 15	t= 30
Parallel 1, measurement 1	0.34731	0.68768	0.70256
Parallel 1, measurement 2	0.40101	0.68769	0.70099
Parallel 1, measurement 3	0.44115	0.68707	0.70207
Average 1	0.39649	0.68748	0.70187
Parallel 2, measurement 1	0.35696	0.74112	0.75453
Parallel 2, measurement 2	0.42474	0.74053	0.75439
Parallel 2, measurement 3	0.46488	0.74181	0.75289
Average 2	0.41553	0.74115	0.75394
Parallel 3, measurement 1	0.33268	0.70628	0.72642
Parallel 3, measurement 2	0.40538	0.70637	0.72674
Parallel 3, measurement 3	0.4447	0.7084	0.72686
Average 3	0.39425	0.70702	0.72667

Experiment 2; TCEP activity at different ratios

Appendix table 10: Absorbance of DNTB and TCEP solution measured at ratio 1:2, each parallel was measured three times at two different times; t is measured in minutes. An average value for each parallel was calculated.

Ratio 1:2	t=0	t=15
Parallel 1, measurement 1	0.28075	0.29295
Parallel 1, measurement 2	0.24916	0.29295
Parallel 1, measurement 3	0.27129	0.29342
Average 1	0.26707	0.29311
Parallel 2, measurement 1	0.25882	0.30362
Parallel 2, measurement 2	0.28827	0.30441
Parallel 2, measurement 3	0.27907	0.30457
Average 2	0.27539	0.3042
Parallel 3, measurement 1	0.24529	0.2924
Parallel 3, measurement 2	0.26847	0.29254
Parallel 3, measurement 3	0.27861	0.29217
Average 3	0.26412	0.29237

Appendix table 11: Absorbance of DNTB and TCEP solution measured at ratio 1:5, each parallel was measured three times at two different times; t is measured in minutes. An average value for each parallel was calculated.

Ratio 1:5	t= 0	t= 15
Parallel 1, measurement 1	0.30493	0.3164
Parallel 1, measurement 2	0.31218	0.31624
Parallel 1, measurement 3	0.31187	0.31585
Average 1	0.30966	0.31616
Parallel 2, measurement 1	0.32178	0.32579
Parallel 2, measurement 2	0.32106	0.3267
Parallel 2, measurement 3	0.31694	0.32665
Average 2	0.31993	0.32638
Parallel 3, measurement 1	0.30484	0.31597
Parallel 3, measurement 2	0.30989	0.3155
Parallel 3, measurement 3	0.30972	0.31527
Average 3	0.30815	0.31558

Appendix table 12: Absorbance of DNTB and TCEP solution measured at ratio 1:10, each parallel was measured three times at two different times; t is measured in minutes. An average value for each parallel was calculated.

Ratio 1:10	t= 0	t= 15
Parallel 1, measurement 1	0.33956	0.34259
Parallel 1, measurement 2	0.33951	0.34358
Parallel 1, measurement 3	0.33844	0.34398
Average 1	0.33917	0.34338
Parallel 2, measurement 1	0.34952	0.35012
Parallel 2, measurement 2	0.34951	0.3508
Parallel 2, measurement 3	0.34896	0.35039
Average 2	0.34933	0.35044
Parallel 3, measurement 1	0.34152	0.35167
Parallel 3, measurement 2	0.34267	0.35216
Parallel 3, measurement 3	0.342	0.35178
Average 3	0.34206	0.35187

Appendix 3

MS/MS Method Tables

Appendix table 13: MassLynx settings, charge state +1, the *m/z* value trigger a change in collision energy

Charge state	*m/z*	Collision energy (eV)
+1	300	22
	785	32
	955	55

Appendix table 14: MassLynx settings, charge state +2, the *m/z* value trigger a change in collision energy

Charge state	*m/z*	Collision energy (eV)
+2	400	25
	785	32
	820	37
	1200	57
	1500	72
	2000	90

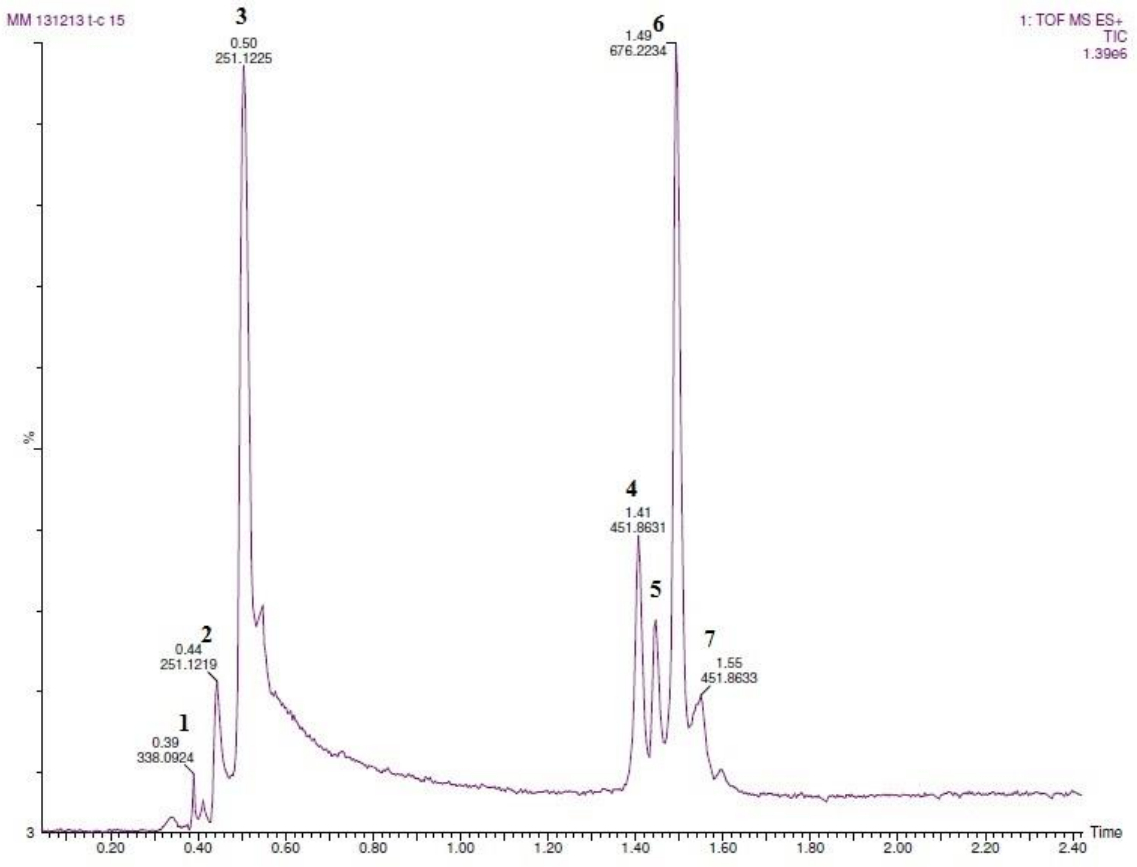
Appendix table 15: MassLynx settings, charge state +3, the *m/z* value trigger a change in collision energy

Charge state	*m/z*	Collision energy (eV)
+3	435	21
	547	28
	605	31
	1000	42
	1500	55
	1800	65

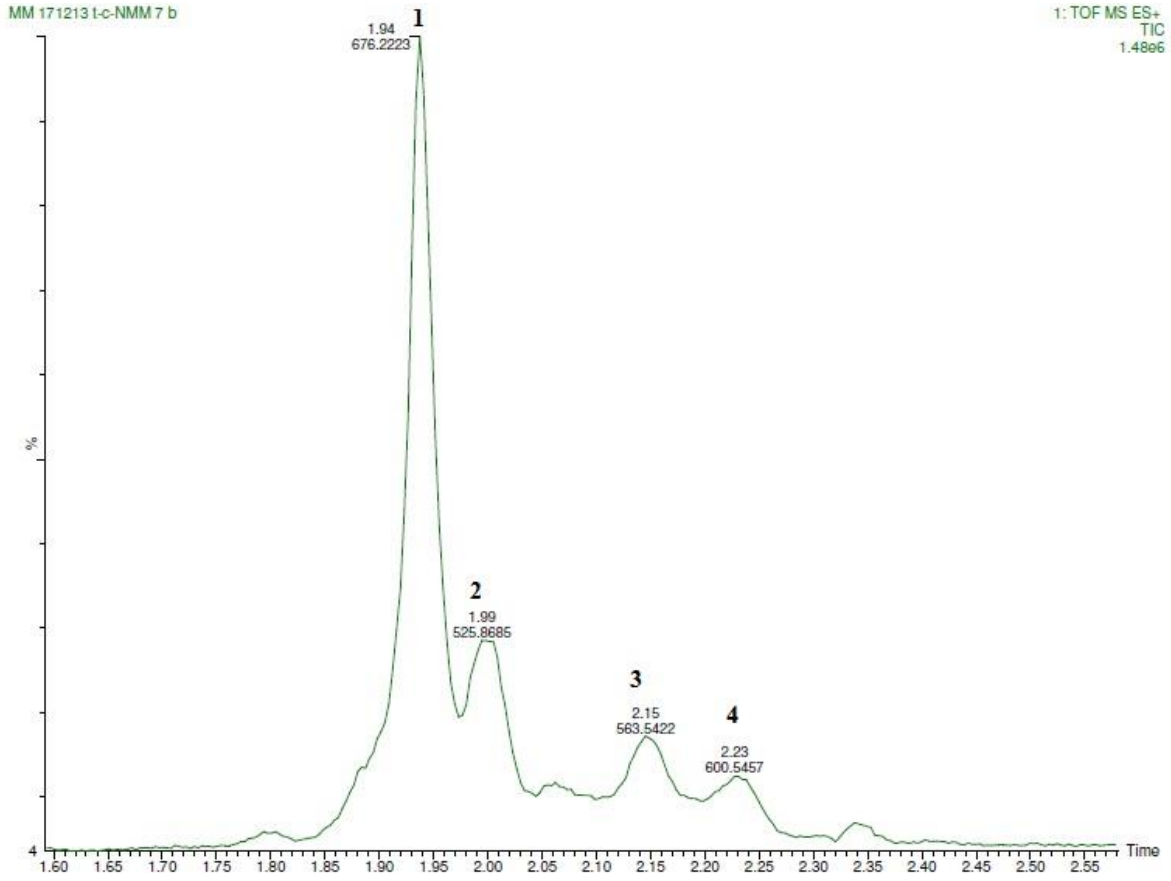
Appendix table 16: MassLynx settings, charge state +4, the *m/z* value trigger a change in collision energy

Charge state	*m/z*	Collision energy (eV)
+4	435	18
	547	25
	605	28
	1000	40

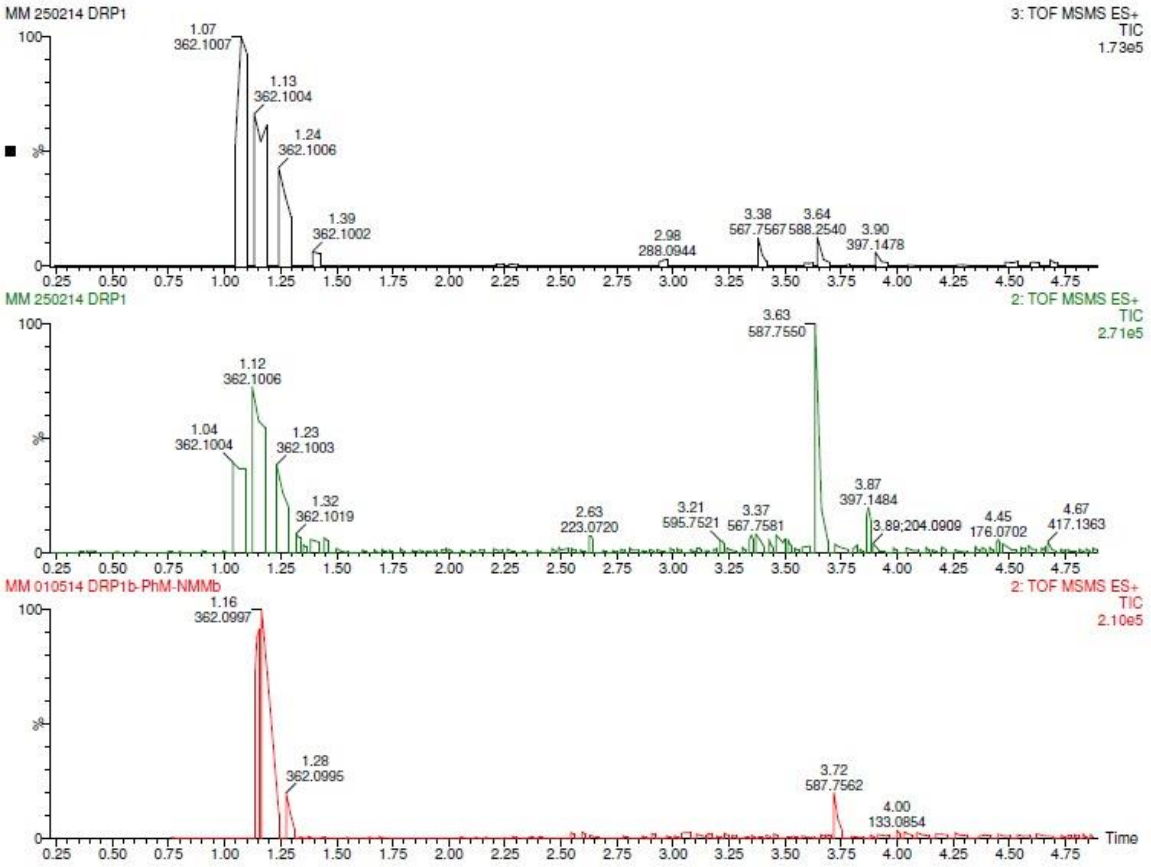
UHPLC Chromatograms and MS Spectra



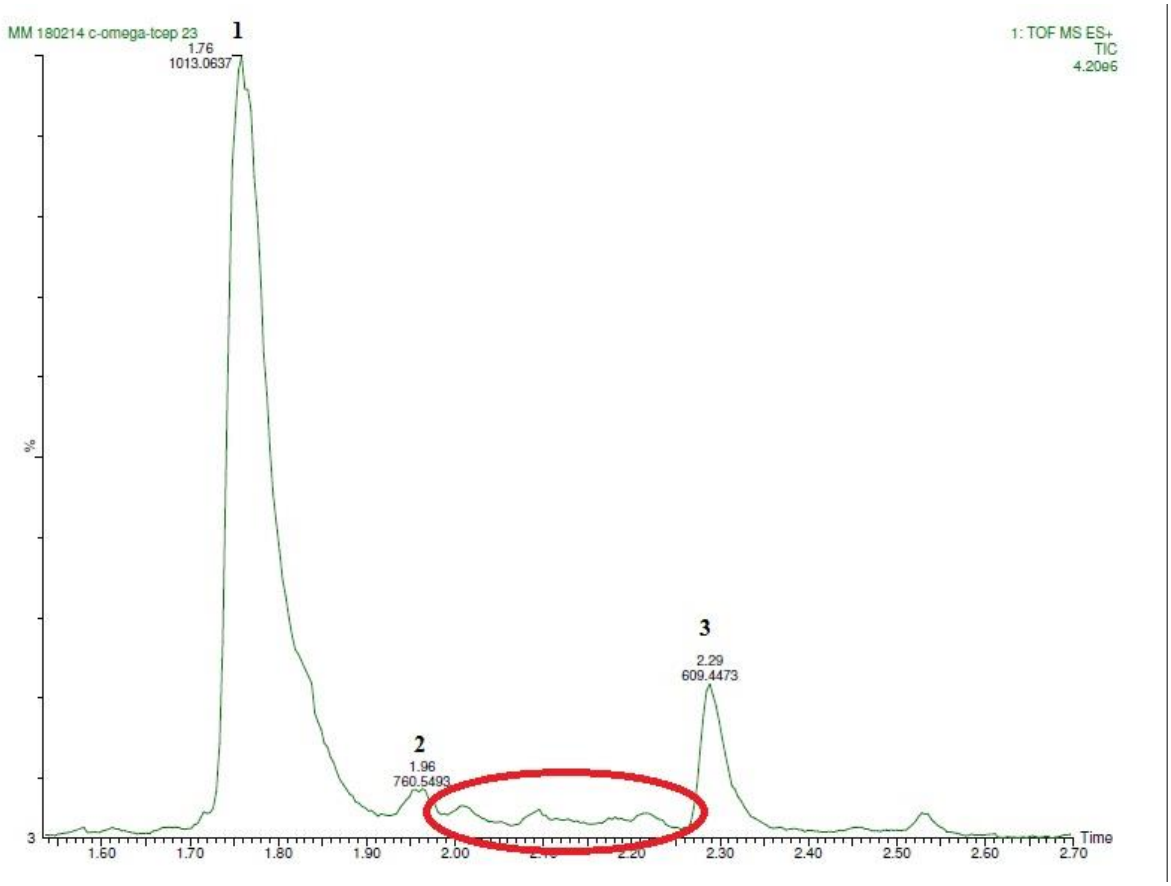
Appendix figure 1: Peak 1: Injection peak, Peak 2: split peak TCEP, peak 3: TCEP, peak 4: One disulfide-bridge reduced, peak: 5 fully reduced Cα, peak 6: unreacted Cα and peak 7 one disulfide-bridge reduced



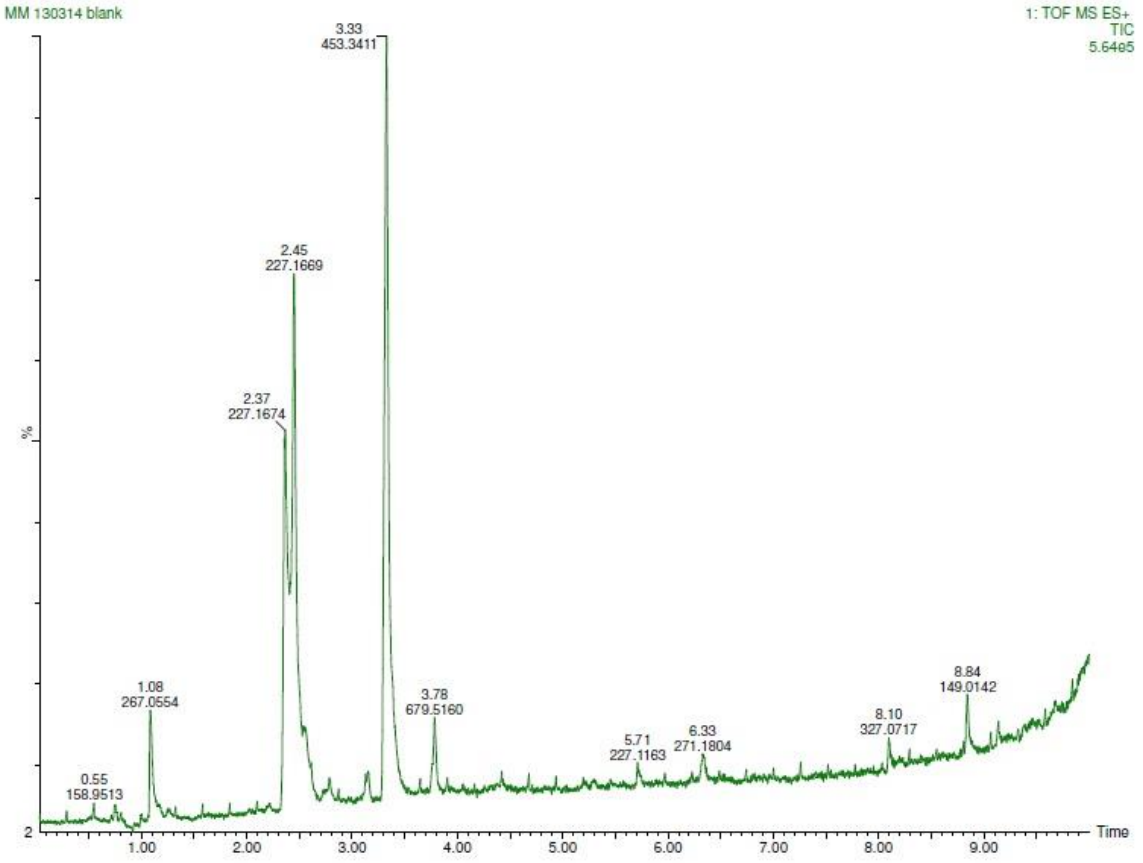
Appendix figure 2: Cα alkylated with NMM. Peak 1: unreacted Cα, peak 2: Cα-NMM_2, peak 3: contamination and peak 4: Cα with 4 NMM



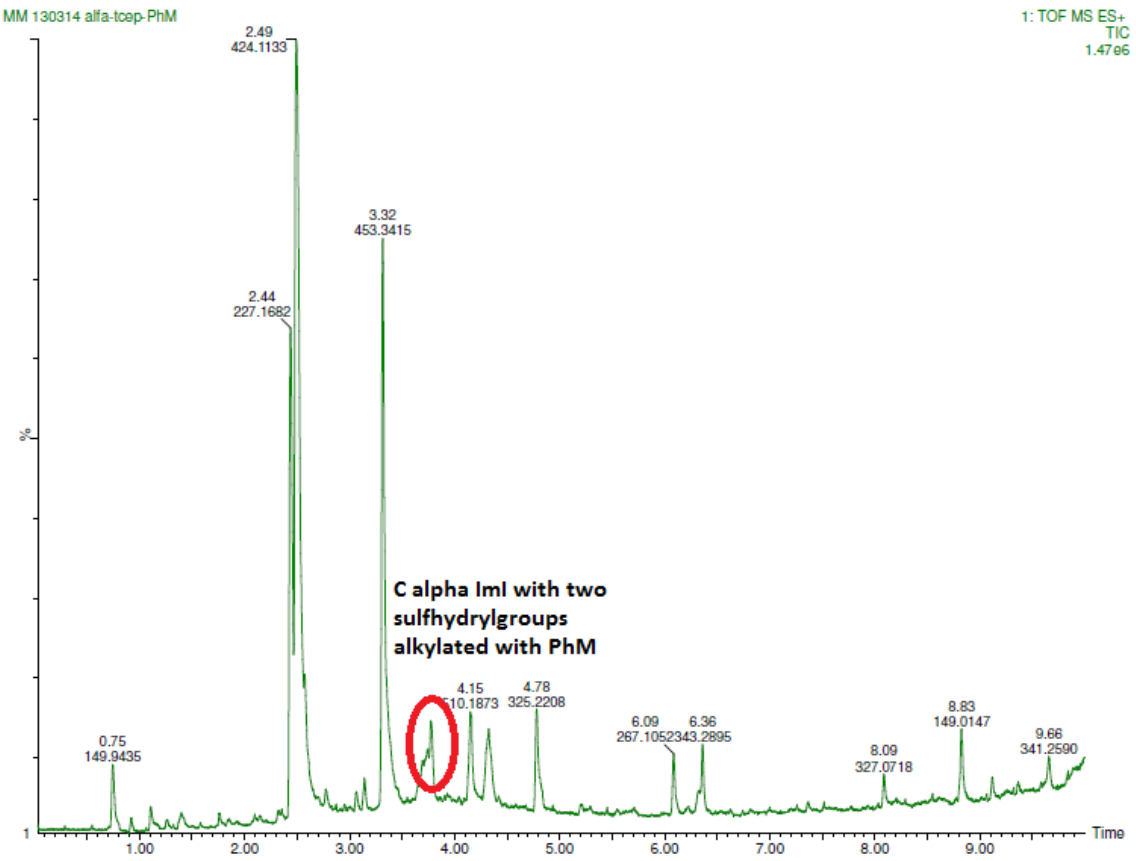
Appendix figure 3: Two MS/MS chromatograms of DRP1 and one from DRP1.2 at the bottom. Retention time and most abundant fragment correlate.



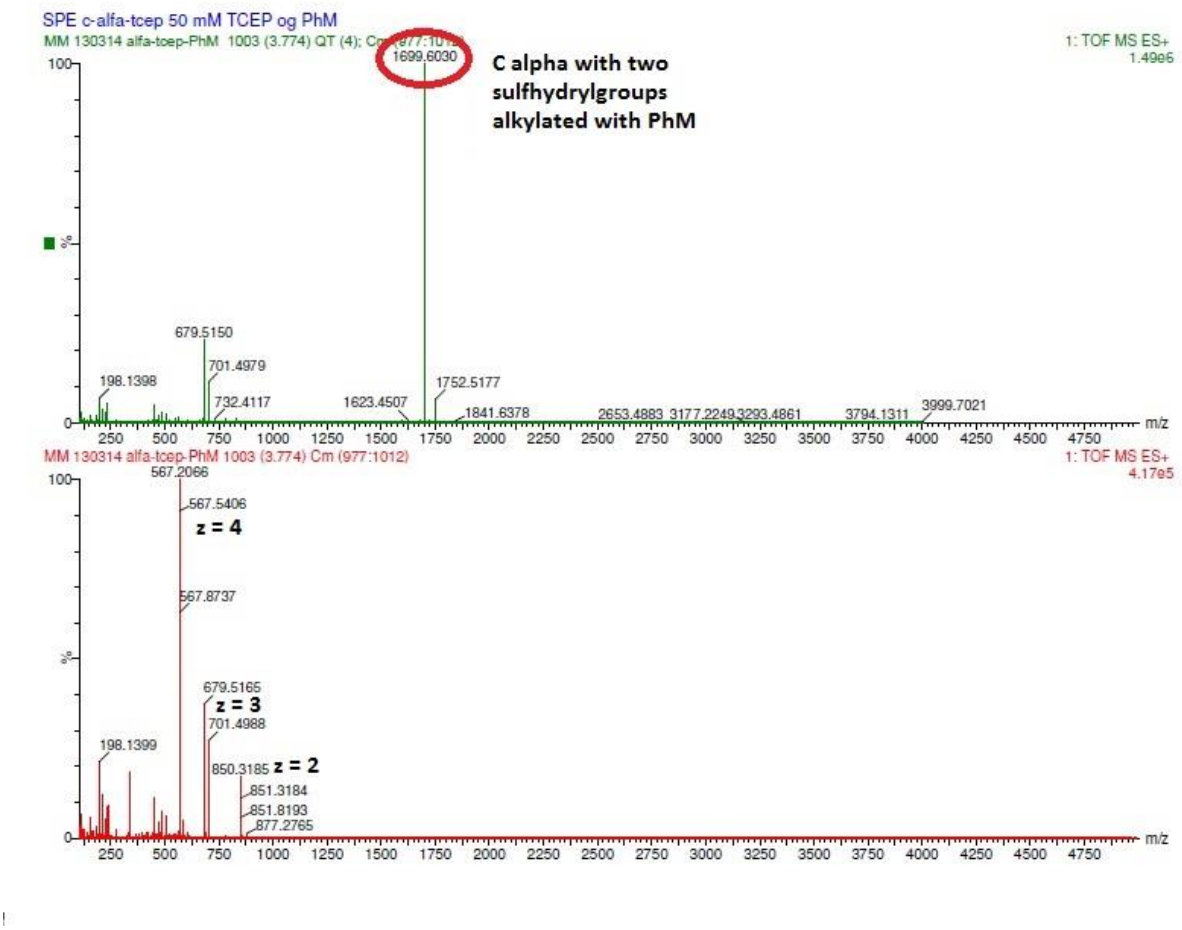
Appendix figure 4: Chromatogram of attempted reduction of one disulfide-bridge in Cω. Peak 1 and 2 and the tailing marked with the red ellipse all come from unreduced Cω. Different folding of the same isomer of the peptide can give several signals. Peak number 3 is fully reduced Cω.



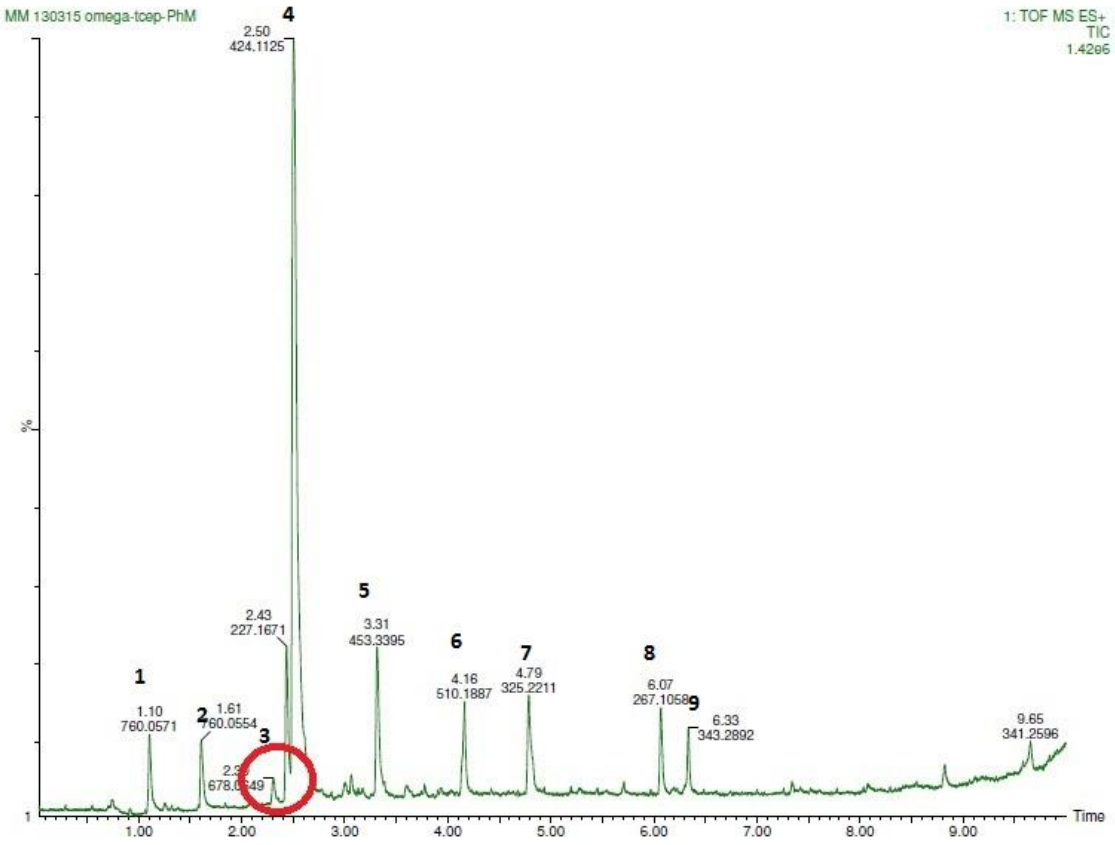
Appendix figure 5: Analysis of blank eluate from the SPE-column. Signals detected are contaminations and not related to the experiment.



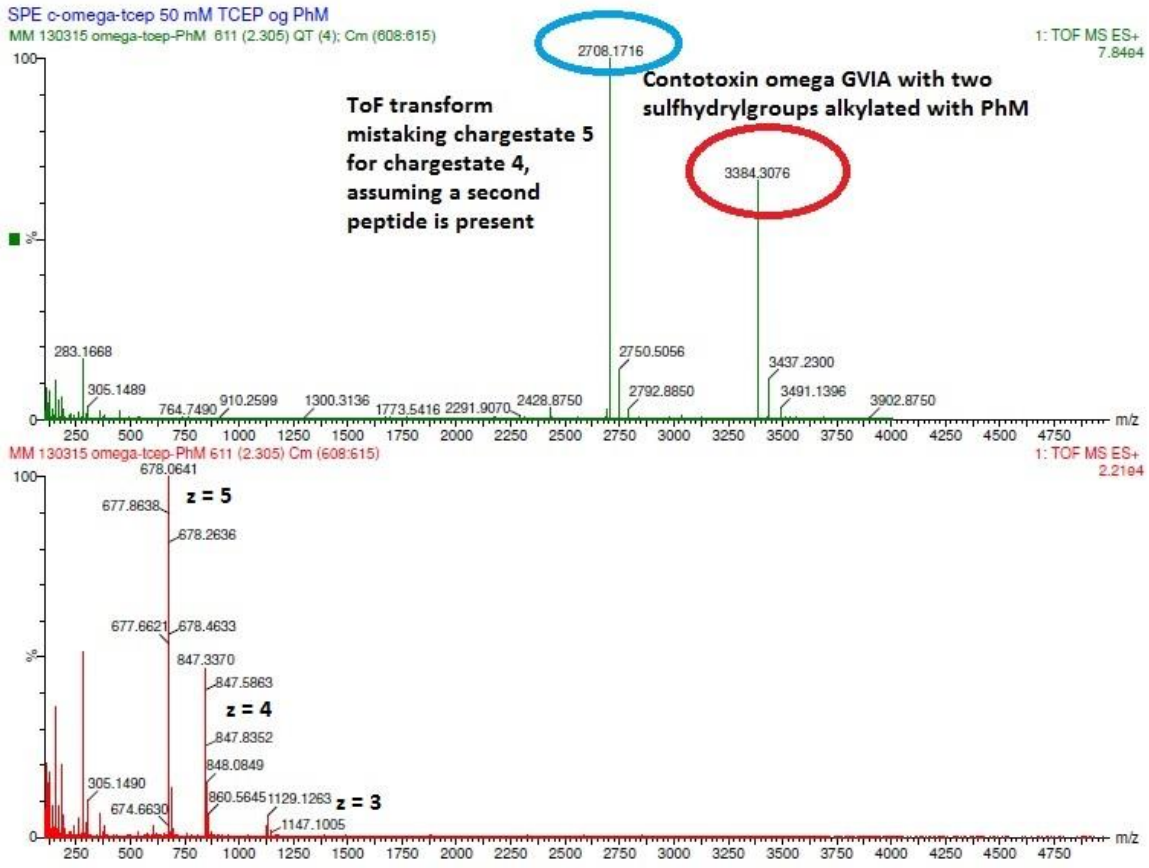
Appendix figure 6: Chromatogram from the SPE-method, Cα-PhM$_2$ marked with a red circle. Compare with the blank eluate to see which peaks are contaminants.



Appendix figure 7: MS-specter of C*α* with one reduced and alkylated disulfide-bridge, result of the SPE-method.



Appendix figure 8: Chromatogram from the SPE-method, Cω-PhM$_2$ marked with a red circle and number 3. Compare with the blank eluate to see which peaks are the contaminants. Signal 1 and 2 are different folds of the native isomer of Cω, *m/z* = 760.0571 and 760.0554 respectively. z in this case is 5. Signal 4 is the TCEP-PhM product. The rest of the signals are contaminants.



Appendix figure 9: MS-specter of Cω with one reduced and alkylated disulfide-bridge, result of the SPE-method.

Appendix 4

Structure Determination

GvuSDPRvAWRu-Amidated

User AA Formula 1: **C8 H10 N2 O3 S1**
User AA Formula 2: **C13 H12 N2 O3 S1**
Elemental Composition: C82 H107 N24 O23 S4

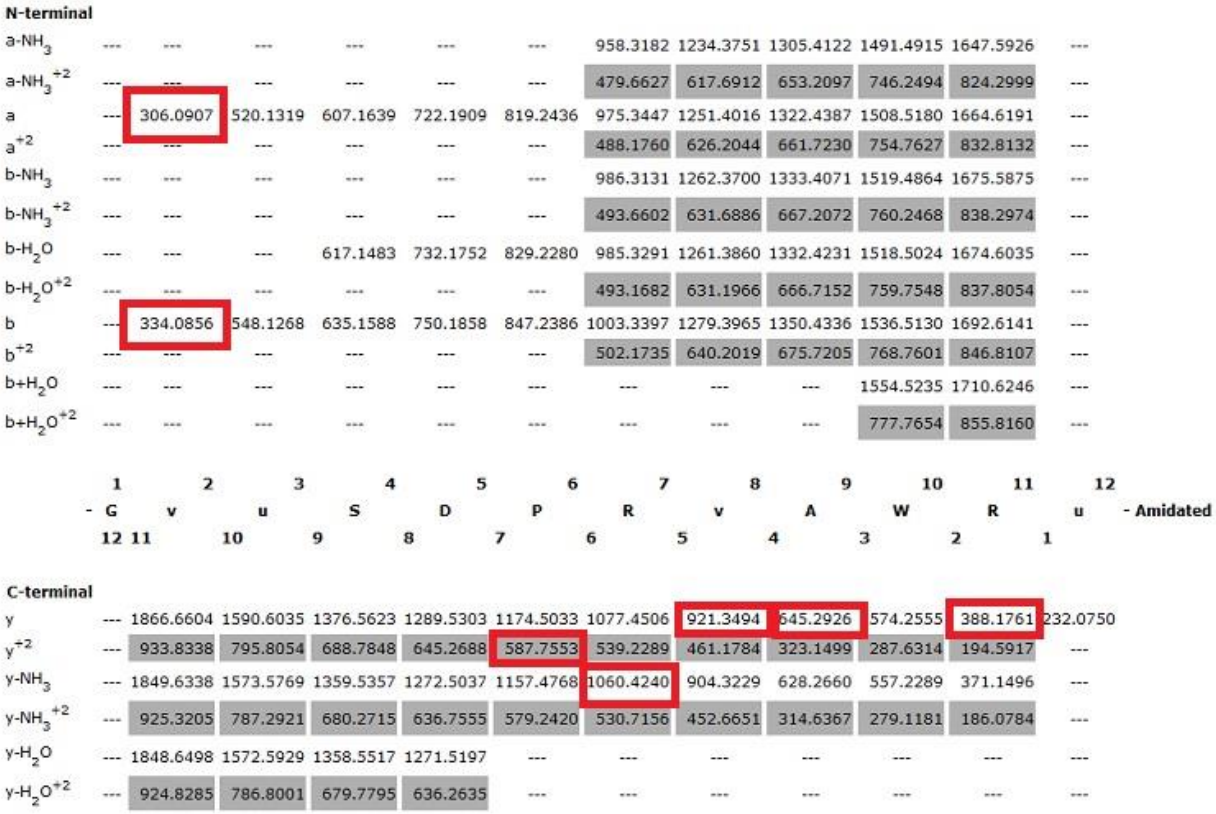
MH^{+1}(av)	MH^{+1}(mono)	MH^{+2}(av)	MH^{+2}(mono)
1925.1682	1923.6818	963.0878	962.3446

[–] Main Sequence Ions

b	b^{+2}				y	y^{+2}
---	---	1	G	12	---	---
334.0856	---	2	v	11	1866.6604	933.8338
548.1268	---	3	u	10	1590.6035	795.8054
635.1588	---	4	S	9	1376.5623	688.7848
750.1858	---	5	D	8	1289.5303	645.2688
847.2386	---	6	P	7	1174.5033	587.7553
1003.3397	502.1735	7	R	6	1077.4506	539.2289
1279.3965	640.2019	8	v	5	921.3494	461.1784
1350.4336	675.7205	9	A	4	645.2926	323.1499
1536.5130	768.7601	10	W	3	574.2555	287.6314
1692.6141	846.8107	11	R	2	388.1761	194.5917
---	---	12	u	1	232.0750	---

Amidated

Appendix figure 10: Theoretical values of b and y ions formed upon CID-fragmentation of Cα-PhM_2-NMM_2, observed ions marked with red squares.



N-terminal

a-NH_3	---	---	---	---	---	---	958.3182	1234.3751	1305.4122	1491.4915	1647.5926	---
a-NH_3^{+2}	---	---	---	---	---	---	479.6627	617.6912	653.2097	746.2494	824.2999	---
a	---	306.0907	520.1319	607.1639	722.1909	819.2436	975.3447	1251.4016	1322.4387	1508.5180	1664.6191	---
a^{+2}	---	---	---	---	---	---	488.1760	626.2044	661.7230	754.7627	832.8132	---
b-NH_3	---	---	---	---	---	---	986.3131	1262.3700	1333.4071	1519.4864	1675.5875	---
b-NH_3^{+2}	---	---	---	---	---	---	493.6602	631.6886	667.2072	760.2468	838.2974	---
b-H_2O	---	---	---	617.1483	732.1752	829.2280	985.3291	1261.3860	1332.4231	1518.5024	1674.6035	---
b-H_2O^{+2}	---	---	---	---	---	---	493.1682	631.1966	666.7152	759.7548	837.8054	---
b	---	334.0856	548.1268	635.1588	750.1858	847.2386	1003.3397	1279.3965	1350.4336	1536.5130	1692.6141	---
b^{+2}	---	---	---	---	---	---	502.1735	640.2019	675.7205	768.7601	846.8107	---
b+H_2O	---	---	---	---	---	---	---	---	---	1554.5235	1710.6246	---
b+H_2O^{+2}	---	---	---	---	---	---	---	---	---	777.7654	855.8160	---

	1	2	3	4	5	6	7	8	9	10	11	12	
-	G	v	u	S	D	P	R	v	A	W	R	u	- Amidated
	12	11	10	9	8	7	6	5	4	3	2	1	

C-terminal

y	---	1866.6604	1590.6035	1376.5623	1289.5303	1174.5033	1077.4506	921.3494	645.2926	574.2555	388.1761	232.0750
y^{+2}	---	933.8338	795.8054	688.7848	645.2688	587.7553	539.2289	461.1784	323.1499	287.6314	194.5917	---
y-NH_3	---	1849.6338	1573.5769	1359.5357	1272.5037	1157.4768	1060.4240	904.3229	628.2660	557.2289	371.1496	---
y-NH_3^{+2}	---	925.3205	787.2921	680.2715	636.7555	579.2420	530.7156	452.6651	314.6367	279.1181	186.0784	---
y-H_2O	---	1848.6498	1572.5929	1358.5517	1271.5197	---	---	---	---	---	---	---
y-H_2O^{+2}	---	924.8285	786.8001	679.7795	636.2635	---	---	---	---	---	---	---

Appendix figure 11: Theoretical values of additional ions formed upon CID-fragmentation of Cα-PhM_2-NMM_2, observed ions marked with red squares.

GuvSDPRvAWRu-Amidated

User AA Formula 1: **C8 H10 N2 O3 S1**
User AA Formula 2: **C13 H12 N2 O3 S1**
Elemental Composition: C82 H107 N24 O23 S4

MH^{+1}(av)	MH^{+1}(mono)	MH^{+2}(av)	MH^{+2}(mono)
1925.1682	1923.6818	963.0878	962.3446

[–] **Main Sequence Ions**

b	b^{+2}				y	y^{+2}
---	---	1	G	12	---	---
272.0700	---	2	u	11	1866.6604	933.8338
548.1268	---	3	v	10	1652.6191	826.8132
635.1588	---	4	S	9	1376.5623	688.7848
750.1858	---	5	D	8	1289.5303	645.2688
847.2386	---	6	P	7	1174.5033	587.7553
1003.3397	502.1735	7	R	6	1077.4506	539.2289
1279.3965	640.2019	8	v	5	921.3494	461.1784
1350.4336	675.7205	9	A	4	645.2926	323.1499
1536.5130	768.7601	10	W	3	574.2555	287.6314
1692.6141	846.8107	11	R	2	388.1761	194.5917
---	---	12	u	1	232.0750	---

Amidated

Appendix figure 12: Theoretical values of b and y ions formed upon CID-fragmentation of DRP1.2-PhM_2-NMM_2, observed ions marked with red squares.

N-terminal

a-NH_3	---	---	---	---	---	---	958.3182	1234.3751	1305.4122	1491.4915	1647.5926	---
a-NH_3^{+2}	---	---	---	---	---	---	479.6627	617.6912	653.2097	746.2494	824.2999	---
a	---	244.0750	520.1319	607.1639	722.1909	819.2436	975.3447	1251.4016	1322.4387	1508.5180	1664.6191	---
a^{+2}	---	---	---	---	---	---	488.1760	626.2044	661.7230	754.7627	832.8132	---
b-NH_3	---	---	---	---	---	---	986.3131	1262.3700	1333.4071	1519.4864	1675.5875	---
b-NH_3^{+2}	---	---	---	---	---	---	493.6602	631.6886	667.2072	760.2468	838.2974	---
b-H_2O	---	---	---	617.1483	732.1752	829.2280	985.3291	1261.3860	1332.4231	1518.5024	1674.6035	---
b-H_2O^{+2}	---	---	---	---	---	---	493.1682	631.1966	666.7152	759.7548	837.8054	---
b	---	272.0700	548.1268	635.1588	750.1858	847.2386	1003.3397	1279.3965	1350.4336	1536.5130	1692.6141	---
b^{+2}	---	---	---	---	---	---	502.1735	640.2019	675.7205	768.7601	846.8107	---
b+H_2O	---	---	---	---	---	---	---	---	---	1554.5235	1710.6246	---
b+H_2O^{+2}	---	---	---	---	---	---	---	---	---	777.7654	855.8160	---

	1	2	3	4	5	6	7	8	9	10	11	12	
-	G	u	v	S	D	P	R	v	A	W	R	u	- Amidated
	12	11	10	9	8	7	6	5	4	3	2	1	

C-terminal

y	---	1866.6604	1652.6191	1376.5623	1289.5303	1174.5033	1077.4506	921.3494	645.2926	574.2555	388.1761	232.0750
y^{+2}	---	933.8338	826.8132	688.7848	645.2688	587.7553	539.2289	461.1784	323.1499	287.6314	194.5917	---
y-NH_3	---	1849.6338	1635.5926	1359.5357	1272.5037	1157.4768	1060.4240	904.3229	628.2660	557.2289	371.1496	---
y-NH_3^{+2}	---	925.3205	818.2999	680.2715	636.7555	579.2420	530.7156	452.6651	314.6367	279.1181	186.0784	---
y-H_2O	---	1848.6498	1634.6086	1358.5517	1271.5197	---	---	---	---	---	---	---

Appendix figure 13: Theoretical values of additional ions formed upon CID-fragmentation of DRP1.2-PhM_2-NMM_2, observed ions marked with red squares.

GuuSDPRuAWRu-Amidated

User AA Formula 1: **C8 H10 N2 O3 S1**
Elemental Composition: C72 H103 N24 O23 S4

MH^{+1}(av)	MH^{+1}(mono)	MH^{+2}(av)	MH^{+2}(mono)
1801.0252	1799.6505	901.0163	900.3289

[–] **Main Sequence Ions**

b	b^{+2}				y	y^{+2}
---	---	1	G	12	---	---
272.0700	---	2	u	11	1742.6291	871.8182
486.1112	---	3	u	10	1528.5878	764.7976
573.1432	---	4	S	9	1314.5466	657.7770
688.1701	---	5	D	8	1227.5146	614.2609
785.2229	---	6	P	7	1112.4877	556.7475
941.3240	471.1656	7	R	6	1015.4349	508.2211
1155.3652	578.1863	8	u	5	859.3338	430.1705
1226.4023	613.7048	9	A	4	645.2926	323.1499
1412.4817	706.7445	10	W	3	574.2555	287.6314
1568.5828	784.7950	11	R	2	388.1761	194.5917
---	---	12	u	1	232.0750	---
			Amidated			

Appendix figure 14: Theoretical values of b and y ions formed upon CID-fragmentation of Cα-NMM_4, observed ions marked with red squares.

N-terminal

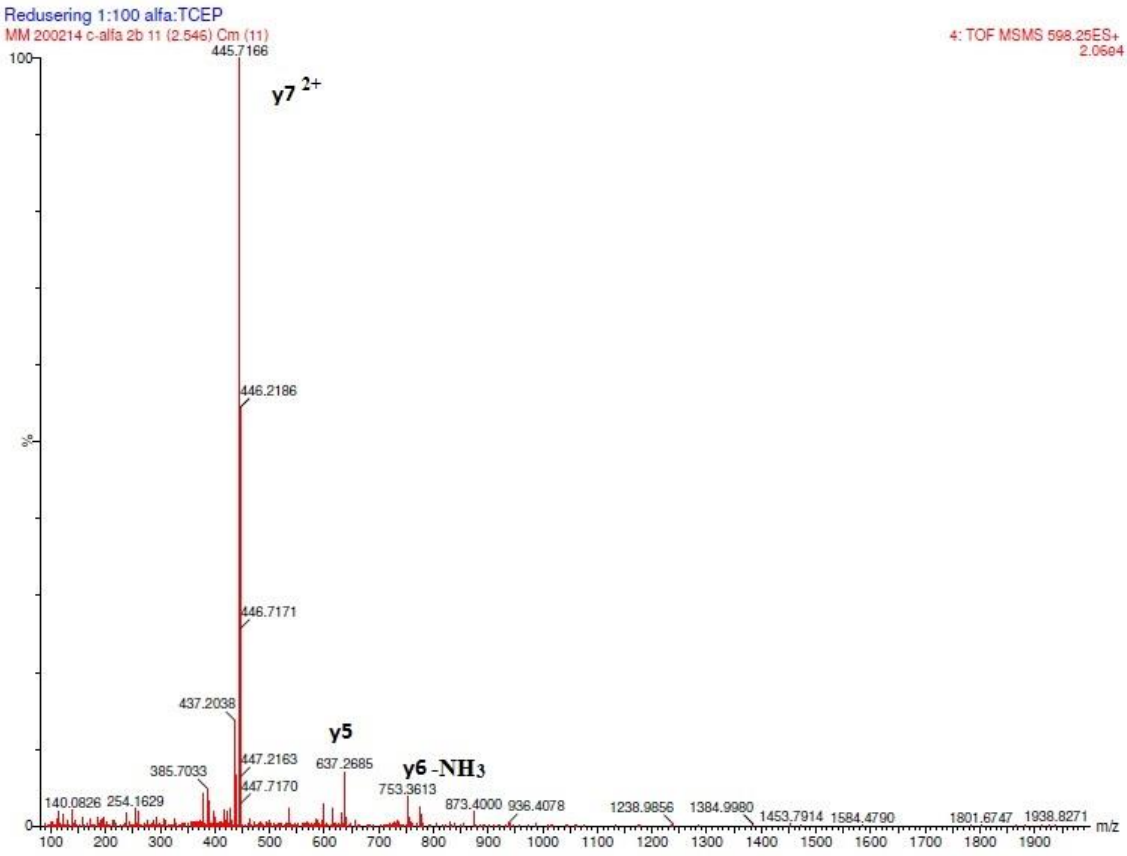
a-NH_3	---	---	---	---	---	---	896.3025	1110.3438	1181.3809	1367.4602	1523.5613	---
a-NH_3^{+2}	---	---	---	---	---	---	448.6549	555.6755	591.1941	684.2337	762.2843	---
a	---	244.0750	458.1163	545.1483	660.1752	757.2280	913.3291	1127.3703	1198.4074	1384.4867	1540.5878	---
a^{+2}	---	---	---	---	---	---	457.1682	564.1888	599.7074	692.7470	770.7976	---
b-NH_3	---	---	---	---	---	---	924.2975	1138.3387	1209.3758	1395.4551	1551.5562	---
b-NH_3^{+2}	---	---	---	---	---	---	462.6524	569.6730	605.1915	698.2312	776.2817	---
b-H_2O	---	---	---	555.1326	670.1596	767.2123	923.3134	1137.3547	1208.3918	1394.4711	1550.5722	---
b-H_2O^{+2}	---	---	---	---	---	---	462.1604	569.1810	604.6995	697.7392	775.7897	---
b	---	272.0700	486.1112	573.1432	688.1701	785.2229	941.3240	1155.3652	1226.4023	1412.4817	1568.5828	---
b^{+2}	---	---	---	---	---	---	471.1656	578.1863	613.7048	706.7445	784.7950	---
b+H_2O	---	---	---	---	---	---	---	---	---	1430.4922	1586.5933	---
b+H_2O^{+2}	---	---	---	---	---	---	---	---	---	715.7497	793.8003	---

	1	2	3	4	5	6	7	8	9	10	11	12	
-	G	u	u	S	D	P	R	u	A	W	R	u	- Amidated
	12	11	10	9	8	7	6	5	4	3	2	1	

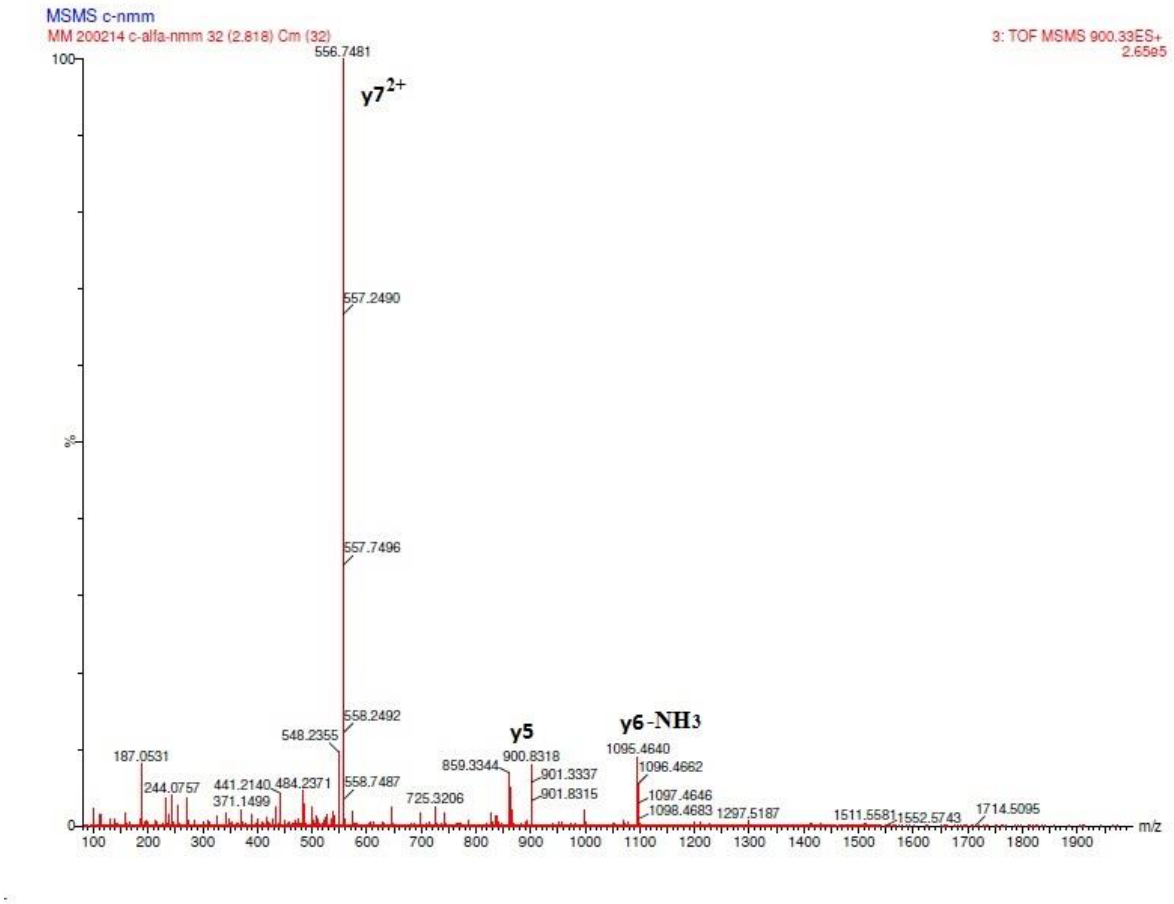
C-terminal

y	---	1742.6291	1528.5878	1314.5466	1227.5146	1112.4877	1015.4349	859.3338	645.2926	574.2555	388.1761	232.0750
y^{+2}	---	871.8182	764.7976	657.7770	614.2609	556.7475	508.2211	430.1705	323.1499	287.6314	194.5917	---
y-NH_3	---	1725.6025	1511.5613	1297.5201	1210.4881	1095.4611	998.4084	842.3072	628.2660	557.2289	371.1496	---
y-NH_3^{+2}	---	863.3049	756.2843	649.2637	605.7477	548.2342	499.7078	421.6573	314.6367	279.1181	186.0784	---
y-H_2O	---	1724.6185	1510.5773	1296.5361	1209.5040	---	---	---	---	---	---	---

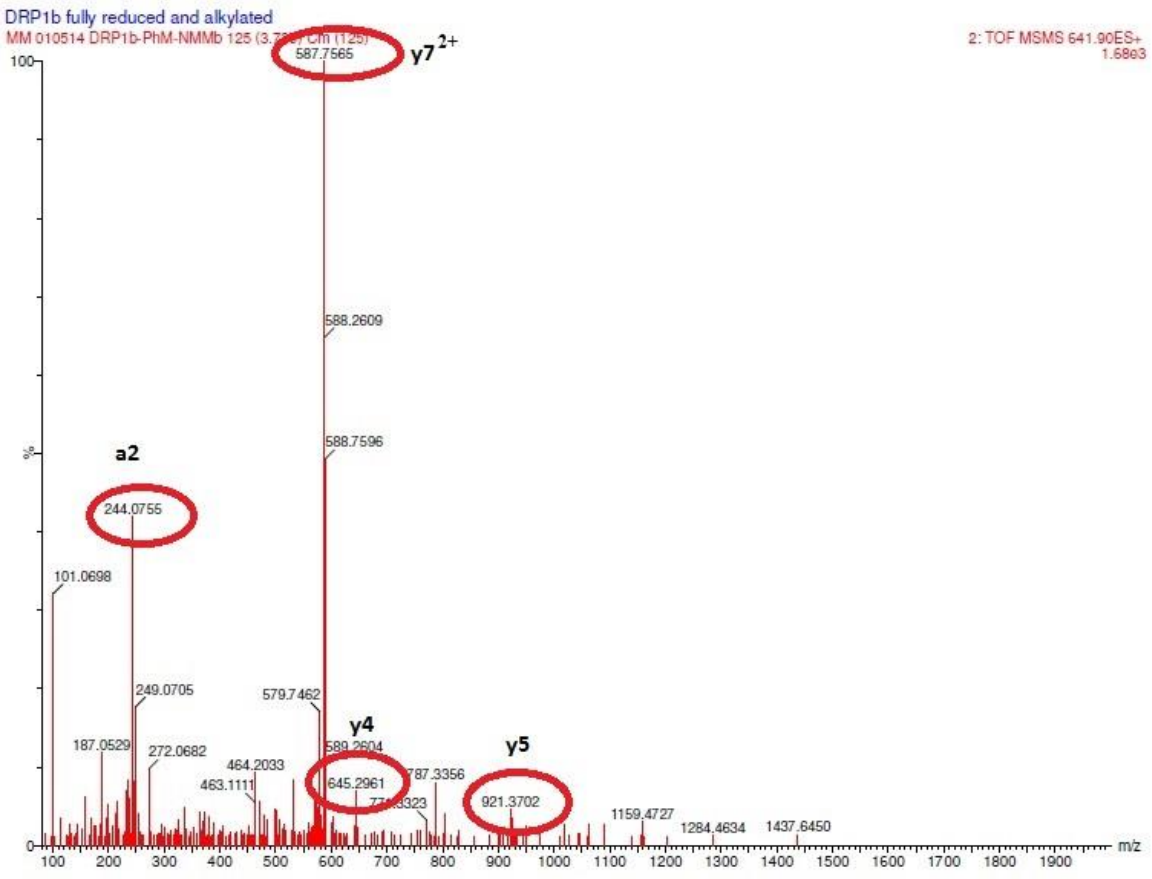
Appendix figure 15: Theoretical values of additional ions formed upon CID-fragmentation of Cα-NMM_4, observed ions marked with red squares.



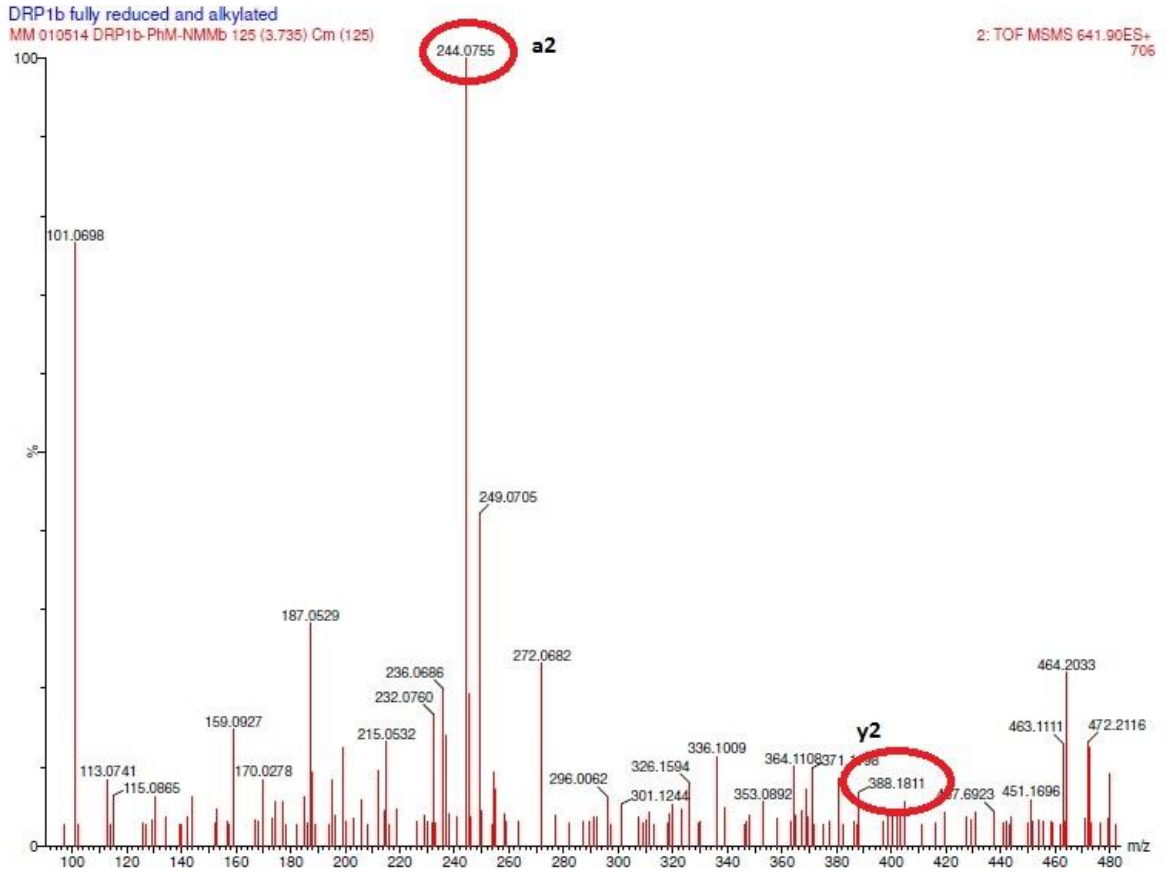
Appendix figure 16: MS/MS of fully reduced Cα, fragment-ions y_5, y_6 and y_7 labeled.



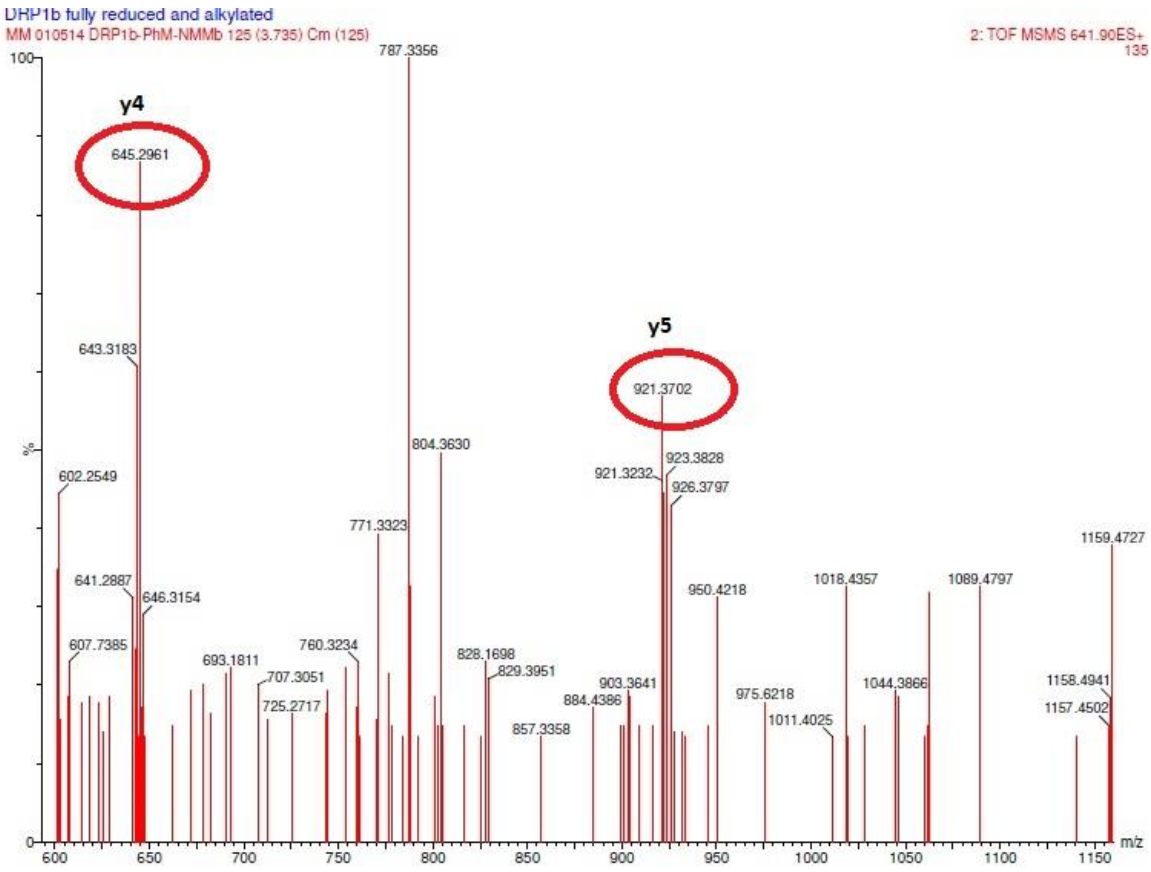
Appendix figure 17: MS/MS of fully reduced Cα with all four sulfhydryl-groups alkylated with NMM. Fragment-ions y_5, y_6 and y_7 labeled.



Appendix figure 18: MS/MS of DRP 1.2, diagnostic ions a_2, y_7^{2+}, y_4 and y_5



Appendix figure 19: MS/MS of DRP 1.2, diagnostic ions a_2 and y_2



Appendix figure 20: MS/MS of DRP 1.2, diagnostic ions y_4 and y_5