



Synthesis and Characterisation of Poly(L-Lysine) for Directed Transfection of siRNA

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ABSTRACT

This work focuses primarily on the synthesis of the cell penetrating peptide poly(L-lysine). This 30mer peptide (K_{30}) has been shown to be extremely useful in delivery of genetic material to the cell for gene therapy. So far it has shown transgene expression of up to twelve weeks, using DNA, which was delivered to the brain.[1] Lung cells have also been successfully transfected by this method.[2, 3]

In this research we aim to use a polymer conjugate of K_{30} for the delivery of small interfering RNA (siRNA), a new exciting alternative to gene therapy, which operates downstream in the transcription pathway, through modulation of mRNA to produce gene silencing. Once optimised, it is hoped that PEGylated K_{30} can be used as a universal delivery agent for any number of siRNA therapeutics in vivo.

K_{30} can be classified as a 'difficult peptide sequence'. This so-called terminology is used in the literature to describe any peptide which cannot be synthesised by classical means, involving routine automated peptide synthesis.[4-7] Generally, automated synthesis is reliable to a length of approximately twenty residues[8], so it is not surprising that K_{30} has been difficult to make by these means.

Various methods were attempted towards the synthesis of K_{30} , including (i) modification of the standard automated procedure with double coupling from a PEG-polystyrene composite resin, (ii) stepwise synthesis of two unprotected fragments to be ligated orthogonal to amide bond chemistry, and (iii) the synthesis of protected fragments to be coupled in solution to form the amide bond.[8]

In the end, monitoring of the sequence assembly was used to overcome the limitations associated with the solid phase synthesis of this peptide and the polylysine sequence was successfully elongated by this approach.

After a certain point, about 18 residues, the peptide begins to aggregate on the solid support, preventing the reagents from reaching and reacting with the N-terminal of the peptide. To overcome this aggregation, a resin with a high hydrophilic content and a low substitution was chosen. The hydrophilicity of this resin repels the growing hydrophobic, protected chain. Furthermore the sparsity of linkers reduces the contact between adjacent chains. This NovaPEG Low Loading resin, combined with double coupling chemistry yielded the peptide K₃₀.

Other work included synthesis of host defence and cell penetrating peptide P17, peptide PEGylation by thiol-maleimide ligation and azo-modified amino acids for use in Click chemistry with alkynyl PEG.

ABBREVIATIONS

Ac	Acetyl
BrAc	Bromoacetyl
BrEA	2-Bromoethylamine Hydrobromide
Bnz	Benzyl
CPP	Cell Penetrating Peptide
DEAE	Diethylaminoethyl cellulose
DIEA	<i>N,N</i> -Diisopropylethylamine
DMF	Dimethylformamide
DVB	Divinylbenzene
EDT	Ethane Dithiol
Et	Ethyl
HBTU	<i>O</i> -Benzotriazole- <i>N,N,N',N'</i> -Tetramethyl-Uronium-Hexafluoro-Phosphate
HOBt	1-Hydroxy-Benzotriazole
<i>iv</i>	Intravenous
K ₃₀	H-(Lys) ₃₀ -NH ₂
MALDI	Matrix-Assisted Laser Desorption/Ionisation
Me	Methyl
miRNA	Micro Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-Sulfonyl
PEG	Poly(Ethylene Glycol)
pH	Potentiometric Hydrogen Ion Concentration
siRNA	Small Interfering Ribonucleic Acid
R ₈	Octaarginine
rDNA	Ribosomal Deoxyribonucleic Acid
RISC	RNA-Induced Silencing Complex
rRNA	Ribosomal Ribonucleic Acid
SPPS	Solid Phase Peptide Synthesis
TA	Thioanisole
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoro Acetic Acid
TFE	Tetrafluoroethylene
TIS	Triisopropyl Silane
TLC	Thin Layer Chromatography
Trt	Trityl

LIST OF COMPOUNDS:

1. H-K₃₀-NH₂
2. HCK₃₀-NH₂
3. mPEG-CK₃₀-NH₂
4. mPEG-mal
5. P17
6. Fmoc-Lys(N₃)-OH
7. Fmoc-Lys(NH₂)-OH
8. H-Phe-OH
9. N₃-Phe-OH
10. H-K₁₅-NH₂
11. H-C β AK₃₀-NH₂
12. H-CK₁₅-NH₂
13. H-K₁₅C-NH₂
14. BrAc-K₁₅-NH₂
15. H-C(Acm)K₁₄-SBnz
16. N ^{α} -ethyl, S-Trt Cysteine
17. Boc-C(Trt)[K(Boc)₁₅]-OH
18. H-K(Boc)₁₅-NH₂
19. H-K₁₅C-S-K₁₅-NH₂
20. Ac-K₁₅-NH₂
21. S-EtNH₂-Cysteine
22. H-C(Acm)K₁₄CK₁₅-NH₂
23. H-C(Acm)K₁₄-SPh
24. N ^{α} -Et-N ^{α} -Fmoc-S-Trt-Cysteine
25. H-C(Acm)K₁₄-N ^{α} -ethyl-Cys-NH₂
26. H-C(Acm)K₁₄-S-N ^{α} -ethyl-Cysteine
27. H-C(Acm)K₁₄K_cK₁₅-NH₂
28. H-K₂₀-NH₂
29. H-K₂₅-NH₂
30. H-DLLGLCEQK-NH₂
31. mPEG-K₃₀-NH₂
32. mPEG-N ^{ϵ} -K₃₁-NH₂
33. N₃-K₃₀-NH₂
34. H-K(N₃)K₃₀-NH₂
35. Imidazole-1-sulfonyl azide 1.HCl
36. N₃-Lys(Boc)-OH

LIST OF COMPOUNDS (continued):

37. H-CK₁₄K_cK₁₅-NH₂
38. N^α-Me, S-Trt Cysteine
39. H-K₁₅-MeCl
40. H-K₁₅-Me-S-CK₁₅-OH
41. Fmoc-Lys(Boc)-OH
42. Fmoc-βAla-OH
43. Fmoc-Cys(Trt)-OH
44. H-C(Acm)[K(Boc)]₁₄-SBnz
45. Fmoc-Cys(Acm)-OH
46. H-Cys(Trt)-OH
47. Boc-Cys(Trt)-OH
48. mPEG-DLLGLCEQK-NH₂
49. Fmoc-Phe-OH
50. Fmoc-Ala-OH
51. Fmoc-Leu-OH
52. Fmoc-His(Trt)-OH
53. Fmoc-Pro-OH
54. Fmoc-Trp(Boc)-OH

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CHAPTER 1 – INTRODUCTION

1.1 Drug Delivery

Directed drug delivery involves the design of complex molecules and composites capable of locating their target, and acting exclusively on them. The potential to direct a drug exclusively to its target has many important advantages, for example reduced side effects and reduced dosage, and therefore costs.

A large proportion of the side effects associated with drugs are due to undesired side reactions in the body at sites other than the target. This is mainly due to the ability of the drug to interact with more than one receptor or target molecule, due to similar structural requirements. It can also be due to reactions with the same molecule but in tissues other than the therapeutic target. These side reactions can be minimised by delivering the drug to its target tissue before release.

Furthermore, many drugs never make it to their target, due to the many diverse control mechanisms and barriers in the body. If ingested, the drug must survive digestion in the stomach and intestines, be absorbed through the gut wall, survive metabolism in the liver, then enter the circulation and reach its specific target without being removed by the kidneys. Similarly, an *iv* injected drug will have spread throughout the body through the blood before reaching its target.

One class of chemotherapeutic agents act preferentially on rapidly dividing cells, the reason being that during division the nuclear membrane breaks down, allowing access of the drug to the nuclear contents. Cisplatin is such a drug. Cancer cells divide rapidly, but so too do hair follicle cells, stomach cells and cells lining other areas of the gut. Hence, chemotherapy can cause hair loss, vomiting, and diarrhoea.[9, 10] Chemotherapy is a notoriously unpleasant experience, and the side effects are potentially fatal. Considering

cancer is so widespread in today's society, the benefits of more specific, directed action cannot be overemphasised, in terms of reduced dose and costs and increased patient compliance.

Exclusive action and delivery of a larger proportion of a therapeutic compound to its site of action is also much more cost effective, as it diminishes the amount of drug wasted on sites where it is not needed. This makes drugs cheaper to use and to buy. Clearly this has very positive implications for many diseases, especially for those medications which are currently expensive to make, such as certain HIV drugs, which are unaffordable for many people who need them.

The aim of this project is the synthesis of drug delivery vectors for siRNA for the treatment of cancer. Small interfering RNA (siRNA) works by silencing genes at the messenger RNA (mRNA) level. Genes which have been linked to cancer can be silenced by siRNA by selecting the appropriate sequence to target their mRNA. However, siRNA needs a delivery vector to successfully enter the cell, where it functions.

Poly(L-lysine) (H-K₃₀-NH₂ **(1)**, or H-CK₃₀-NH₂ **(2)** containing a standard linker) is such a vector. Its positively charged side chains coat the negatively charged siRNA, and it instigates transport across the cell membrane. In addition, a linear poly(ethylene glycol) (PEG) is conjugated to this peptide, to produce (mPEG-CK₃₀-NH₂, **(3)**) which then surrounds the composite particle. This PEG shell improves the pharmacological profile of the vesicle *in vivo* by increasing its bioavailability and reducing its clearance from the body.

Hopefully, in the future these composites will also include targeting molecules, such as the RGD integrin binding motif, for specific delivery to the tumour vasculature.

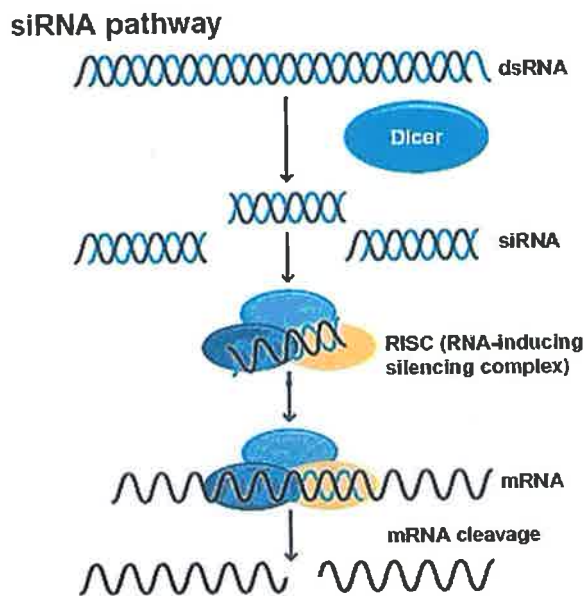


Fig. 1: The Pathway of siRNA in the Cytoplasm [11]

siRNA is a duplex, which associates with Dicer, an RNase III nuclease. Nucleases are a class of proteins in the body which cleave nucleic acids, and RNases are a subclass of nucleases which act specifically on RNA. The siRNA duplex, once associated with RNase III, is then cleaved by an ATP dependent helicase. Helicases are a class of proteins which unwind nucleic acids.

The antisense strand then associates with the RNA-induced silencing complex (RISC) which guides the cleavage of complementary strands of mRNA. By doing so, they prevent transcription of this mRNA into proteins. This complex is also recycled, creating an extended and prolonged action. [12]

siRNA is a synthetic analogue of a natural process of translational control. Micro RNA (miRNA) is an endogenous component of all cells, transcribed in the nucleus, it then undergoes post-transcriptional modifications in the nucleus to become pre-miRNA, and then it enters the cytoplasm. siRNA mimics this pre-miRNA, and both compounds follow the same biochemical pathway after this point, whereby they are both cleaved by Dicer.

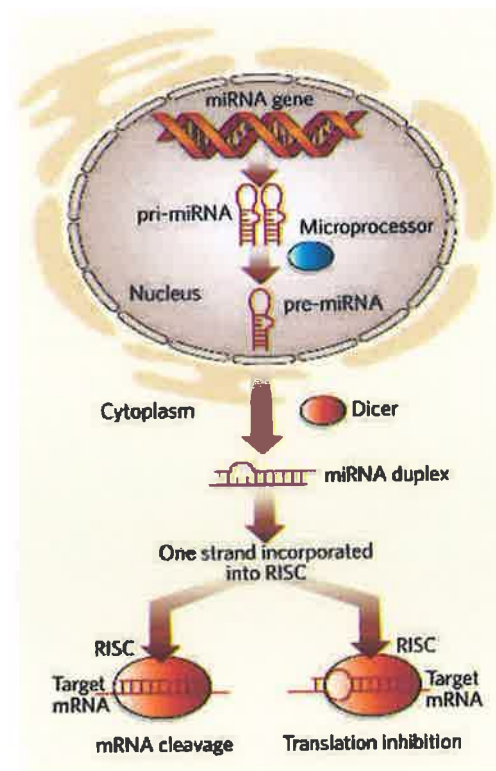


Fig. 2: miRNA Pathway [13]

The potential of siRNA as a therapeutic agent is very important and very broad. Its ability to modulate gene expression, through silencing of protein transcription, could be used to treat many different genetic disorders, such as the genetic mutations of cancer.

siRNA is a highly anionic molecule, due to the repeating negative charge of the phosphate backbone. This high concentration of negative charge makes it impossible for RNA strands to enter the cell. Furthermore, they are the target of many ribonucleases, which destroy foreign nucleic acids in the body, a host defence mechanism likely to have evolved to fight against viral RNA infection.

Hence, siRNA requires some delivery conjugate to enter the cells. There are a wide variety of transfection agents for siRNA, all of which contain some type of cationic moiety. [12] Cell-penetrating peptides, such as those used in this research, are one type of siRNA transfecting agent.

The cell-penetrating peptide K₃₀ (**1**) has been shown to associate with the anionic phosphate groups of the DNA backbone. It does so through non-covalent ionic interactions. The two associate to form neutral nanoparticles, of varying shape, dependent on the peptide salt (ellipsoid for TFA, rod-shaped for acetate). This nanoparticle is then resistant to nucleases.[2] It is reasonable to assume that K₃₀ (**1**) will associate with siRNA in a similar manner.



Fig .: Electron micrograph of CK₃₀PEG10k-compacted DNA (Bar, 100 nm) [2]

1.3 Cell Penetrating Peptides

The cell membrane is a highly organised structure, consisting of two layers of phosphatidyl choline, with many various embedded protein, fat and carbohydrate structures for support and functionality. The phosphatidyl choline has a negatively charged, polar head which faces outwards into surrounding environment and into the cytoplasm. The hydrophobic tail faces toward the other molecule, and hence lines the inside of the membrane.

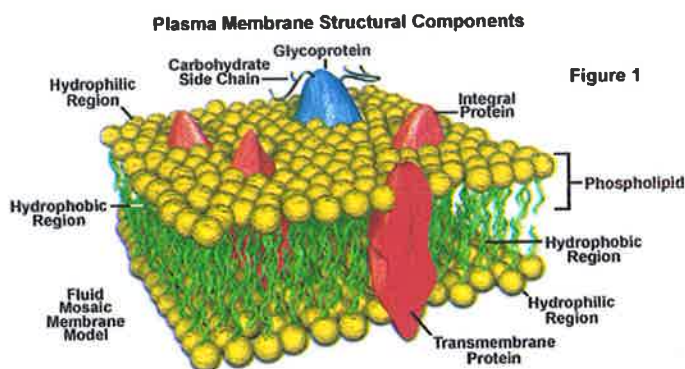


Fig. 4: The Plasma Membrane [14]

Cells, therefore, are a primary consideration in drug and drug delivery vector design. Furthermore, if the target is inside the cell (or on the basal side of a layer of cells) the drug must first be able to cross the cell membrane. This can be quite a difficult task, as entry into the cell and survival inside is tightly controlled. In general, the membrane is highly impermeable, especially to non-hydrophobic molecules.

There are some protein membrane-bound transporters, but they are very selective and usually only carry small molecules. There is an endocytotic mechanism, but a molecule must have the specific physicochemical properties to activate this process. To cross the membrane directly, a drug must have the correct balance of polarity to approach the membrane, and lipophilicity to traverse it. However, if a molecule is active as a drug but fits none of the cell-membrane traversing criteria, attachment to a molecule which can cross the membrane can sometimes bypass this problem.

Cell-penetrating peptides (CPPs) can be used for this purpose. Drugs can be attached to them directly, through a linker. However, CPPs are highly cationic and so association can also occur through non-covalent interactions.

Cell-penetrating peptides are a class of peptides, with a large degree of positive charge, capable of traversing the cell's lipid bilayer. In 1965 a report was published by H. Ryser, which demonstrated that cationic peptides (polylysine, polyornithine, arginine-rich histones, spermine and quaternary amine polymers like diethylaminoethyl cellulose (DEAE)-dextran) can direct the import of macromolecules such as proteins into eukaryotic cells.[15, 16] Since then, there has been a large body of work on CPPs, most of which in the last fifteen years or so. Recently, there has been much work, and much controversy, about the way in which CPPs enter cells.

The way in which CPPs translocate across the lipid membrane has been comprehensively studied. It is now known that these peptides cross the cell membrane by a number of mechanisms, including various types of endocytosis and direct uptake. The process or processes involved are dependent on the specific peptide and also the cargo attached to it. These various mechanisms include macropinocytosis, clathrin-mediated endocytosis, caveolae/lipid-raft mediated endocytosis and endocytosis-independent (direct) uptake. [17]

The process of endocytosis is energy-dependent and involves engulfment by the membrane, creating an internal vesicle containing the peptide. Generally, this endocytotic vesicle then fuses with a lysosome to be processed before direct cytoplasmic entry. Efficient escape from this vesicle, before being broken down by digestive enzymes in the lysosome is then an important consideration.

In many studies chloroquine has been used effectively *in vitro* as part of CPP-siRNA composites to destabilise the vesicle after endocytosis.[18-23] Other methods are still under investigation; for example, a proton sponge can be used

to neutralise the acidic protons of the vesicle.[24] This causes swelling of the vesicle, which eventually ruptures.

In most cases, energy-independent direct translocation to the cytoplasm is more desirable to avoid degradation in an endosome, and studies are currently taking place to examine what conditions will optimise this process. In preliminary studies it has been shown that direct entry occurs at high CPP concentrations through specific nucleation zones on the cell surfaces, which has also been found to be dependent on heparan sulphate proteoglycan-mediated transport.[17] This study examined octaarginine (R_8) among other CPPs, although K_{30} (**1**) was not examined.

In a different study in 2008, it was shown that mPEG-CK₃₀-NH₂ (**3**) condensed nanoparticles are transported by colocalisation with nucleolin at the cell surface, with subsequent trafficking to the nucleus [25]. This mode of transport allows mPEG-CK₃₀-NH₂ (**3**) to avoid endosomal encapsulation and degradation. It also provides intact nanoparticles of condensed DNA capable of traversing the nuclear membrane pore. This mode of transport has not been shown for other CPPs.

Nucleolin is a protein found in the nucleolus of cells, the sub-nuclear structure, which produces ribosomal subunits. Nucleolin has a variety of functions, interacting with different proteins and RNA sequences. Research has shown that it is involved in chromatin structure, ribosomal DNA (rDNA) transcription, ribosomal RNA (rRNA) maturation, ribosome assembly and nucleocytoplasmic transport.[26].

Once in the cell, there are still many different paths the drug can take. It can react with many various cytoplasmic components, still complexed with the polymeric carrier, or after detachment. Alternatively, the vesicle, the composite or detached drug, can try to gain access to the nucleus. To do this, it must pass through another membrane, the nuclear membrane, or the nuclear membrane pore. However, it can only do so if compacted in a chromatin-like state, as is

the case for the K₃₀-nucleic acid nanoparticle, because the nuclear membrane pore is only 26nm in diameter.

This work focussed primarily on the CPP K₃₀ (**1**) which has proven to be a difficult sequence to synthesise. A variety of strategies were adopted, focussing on different aspects of the chemistry involved. Through experimentation it was discovered that aggregation of the growing peptide was occurring, thus preventing further elongation. The strategies adopted included separate synthesis of shorter chains with subsequent ligation, and changing the microenvironment of the peptide by utilising resin supports with different polymer compositions.

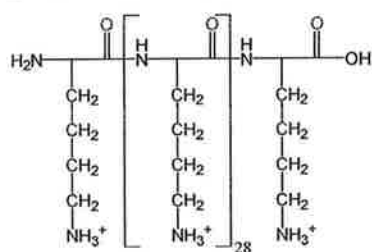


Fig 5: Structure of K₃₀ (**1**)

This peptide has protonated basic functions on the side chains at physiological pH, making it highly cationic. It has been shown that the H-K₃₀-NH₂ (**1**) chain is capable of transfection, and of carrying other conjugated or complexed molecules along with it, as mentioned above.

1.4 Poly(L-Lysine)

Poly(L-lysine) is one of a number of cell penetrating peptides capable of cell transfection, and additionally of transporting other molecules of interest with it. There are a number of examples in the literature of successful cell transfection with K₃₀ (**1**) and composites containing it. [2, 25, 27-30] However there is no detailed published procedure for the synthesis of this peptide. In all documented examples of its use, the peptide was bought from one of a select number of companies.

K₃₀ (**1**) is of particular interest in the field of siRNA delivery. As a delivery agent, it has been shown to be very resilient, effective and capable of prolonged therapeutic action. As a result it has the potential to be a very flexible and universal mode of drug delivery. Its synthesis, however, may prove problematic, due to its long chain length. Generally, solid phase synthesis is reliable to an average peptide length of about 20 residues.[8] Often sequences larger than this are difficult to obtain in high purities allowing efficient recovery of the final product by standard solid phase peptide synthesis (SPPS). Generally, this problem arises due to aggregation of adjacent peptide chains on the resin, peptide folding, or steric hindrance from numerous bulky side chain groups.

The free peptide adopts an alpha helical conformation, as seen in figure 6; however, on the resin the presence of bulky protecting groups would drastically change the steric properties and conformation of the peptide chain.

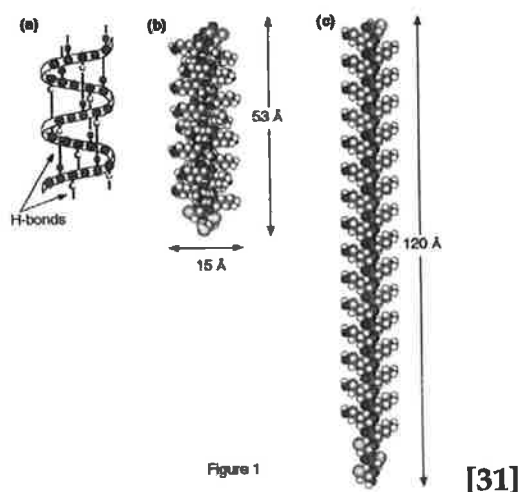


Fig. 6: (a) Ribbon model of basic α -helix structure
(b) Structural Model of $C_3K_{30}C$
(c) The Elongated State

Once synthesised, the next step is polymer attachment to improve bioavailability and stability of the final composite. In our studies PEG was the primary polymer used for this purpose. Availability of a specific linker to attach PEG must also be considered when synthesising the peptide.

1.5 Poly(Ethylene Glycol)

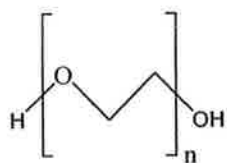


Fig. 7: Poly(Ethylene Glycol)

PEG is a relatively simple polymer, but it has met significant success as a drug delivery agent. Unlike many other polymers, it is biocompatible. This is due to its solubility in water and low charge content. Generally, charged polymers become opsonised by the immune system, which causes rapid clearance, and can also produce a toxic response. PEG, however, improves the solubility of compounds in the blood and decreases their clearance rates.[32] It has been shown to improve the pharmacological profile of many drugs, a number of which are already on the market. These include Pegasys (PEG – Interferon 2 α conjugate) for hepatitis C and Oncaspar (PEG – Asparaginase conjugate) for leukaemia.[33, 34]

This research involved a linear PEG, terminally blocked on one side as a methoxy group and the other modified with a maleimide linker (mPEG-mal, (4)), which can be used for the attachment of a cysteine-modified peptide. The maleimide linker reacts efficiently and relatively selectively with the nucleophilic cysteine thiol by a Michael addition.[35-38], see figure 8, page 22. This reaction is very convenient as it allows the reaction of cysteine thiols either contained in the peptide intermediate or liberated from the peptide homodimer upon reduction of its disulfide bond by tris(2-carboxyethyl)phosphine (TCEP). [39] Eventually all thiols will react with the maleimide irreversibly, driving the reaction to completion.

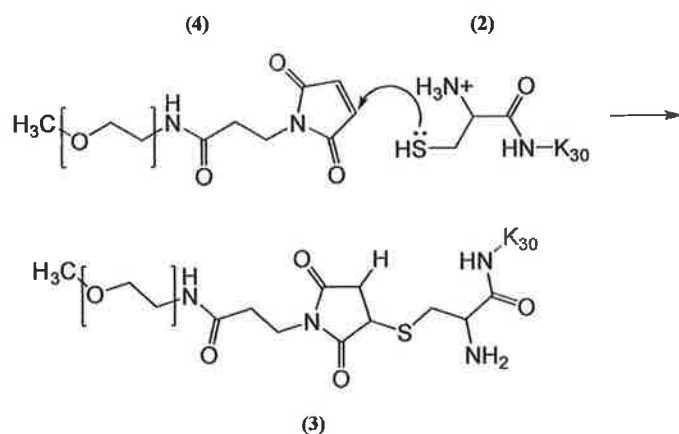
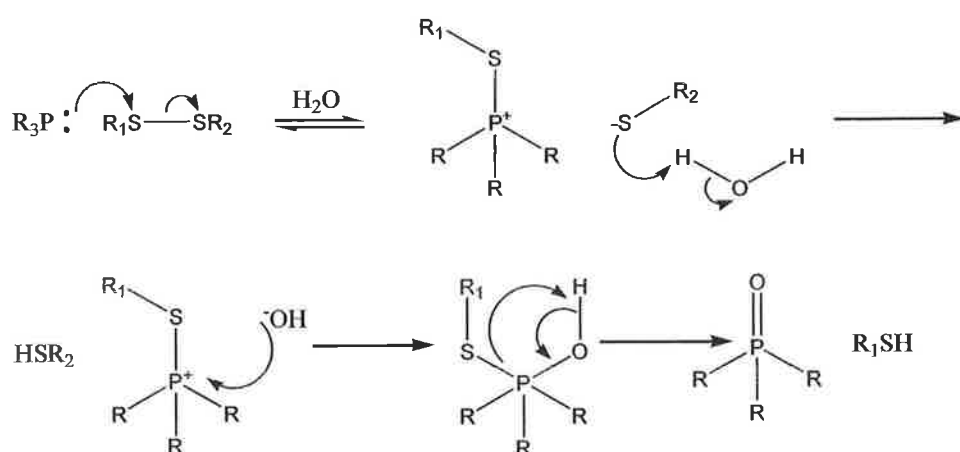


Fig. 8: Conjugation of PEG to Peptide using Cys Thiol and the Maleimide Linker



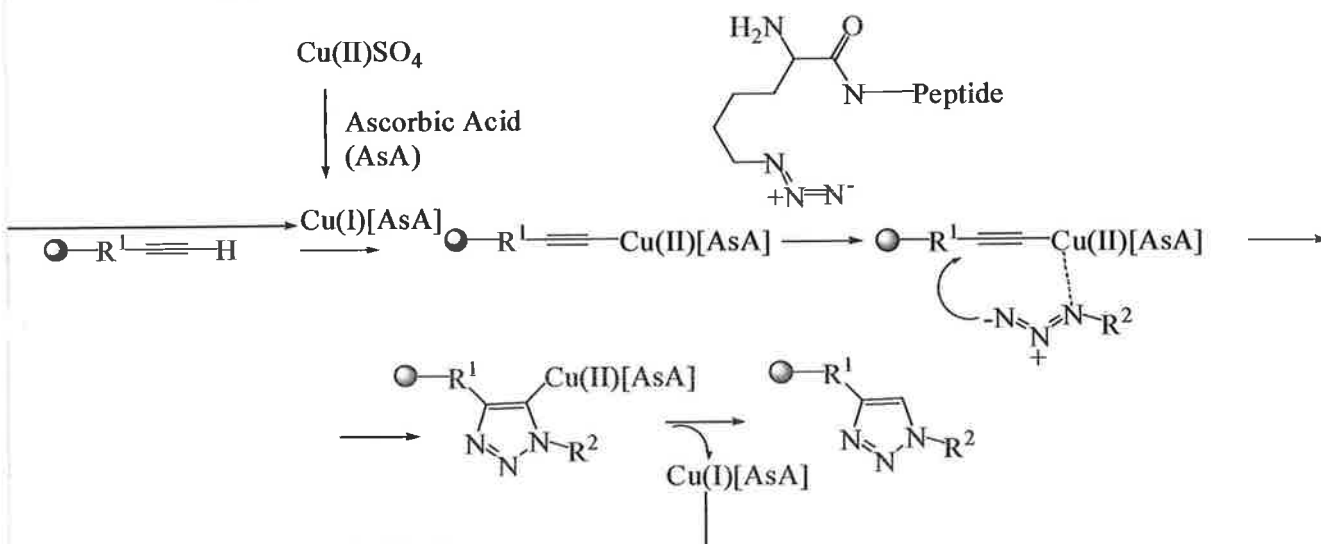
Where R_3P is Tris(2-carboxyethyl)phosphine

Fig. 9: Mechanism of TCEP-mediated Disulfide Reduction

Any number of other linkers can be used; however, for the orthogonal ligation of unprotected peptides, as mentioned previously, we must ensure that any unprotected side groups or peptide termini do not interfere with the chemistry of this ligation. In the case of the maleimide linker, this group can react with nucleophilic functions such as amino groups, of which there are many in our peptide, however this reaction occurs at a much higher pH than the reaction involving cysteine residues, which can be performed at pH6.5, where all amines are protonated.[35, 40]

Another example of orthogonal reaction for selective PEGylation of a peptide is that of an alkynyl-functionalised PEG and an azido-containing

peptide, by a 'Click chemistry' reaction [41-47]. The alkyne is activated by a copper catalyst, which assists the concerted cycloaddition of the azide.



● Refers to the polymer resin support and R^1 is the resin linker and peptide

Fig. 10: Addition of Azido-Peptide to Alkynyl Polymer by Click Chemistry [48-50]

The azido-peptide is prepared by either manually coupling an α -azido-acid (as in our case) to the penultimate N-terminal residue of a peptide chain, or by direct N-terminal modification by a diazotransfer reaction applied on a polymer-bound peptide, where all amino side chains are still protected and only the N-terminal amine is free.

P17: KWKFKKLPKFLHLAKKF

It has been shown to have potent antitumor properties [51], and may represent another valuable drug delivery vector due to its ability to translocate cell membranes. Like K₃₀ (**1**), it has a high net charge (+9). It's mode of cell penetration, however is quite different.

This mode of transport can be a destructive process, destabilising the cell membrane, however, it does promote the direct uptake into cells, which could be useful for our purposes, due to the fact that siRNA works on mRNA in the cytoplasm.

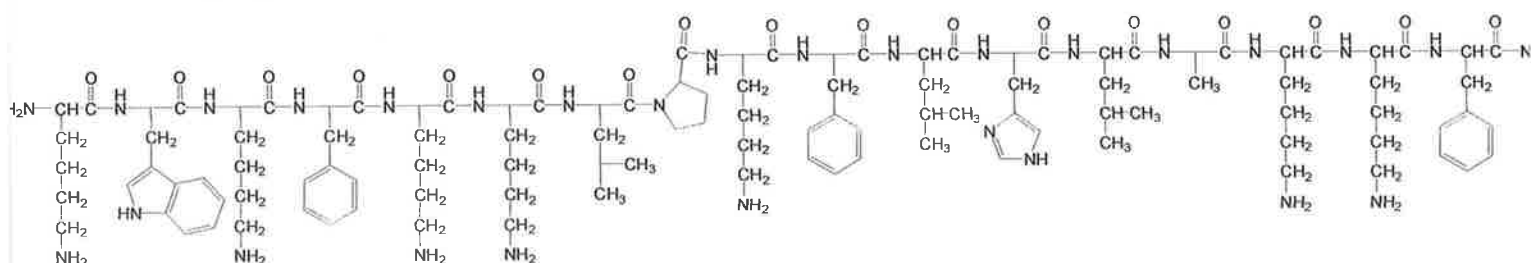


Fig. 11: Chemical Structure of P17 (5)

1.7 Peptide Synthesis

1.71 Stepwise Synthesis

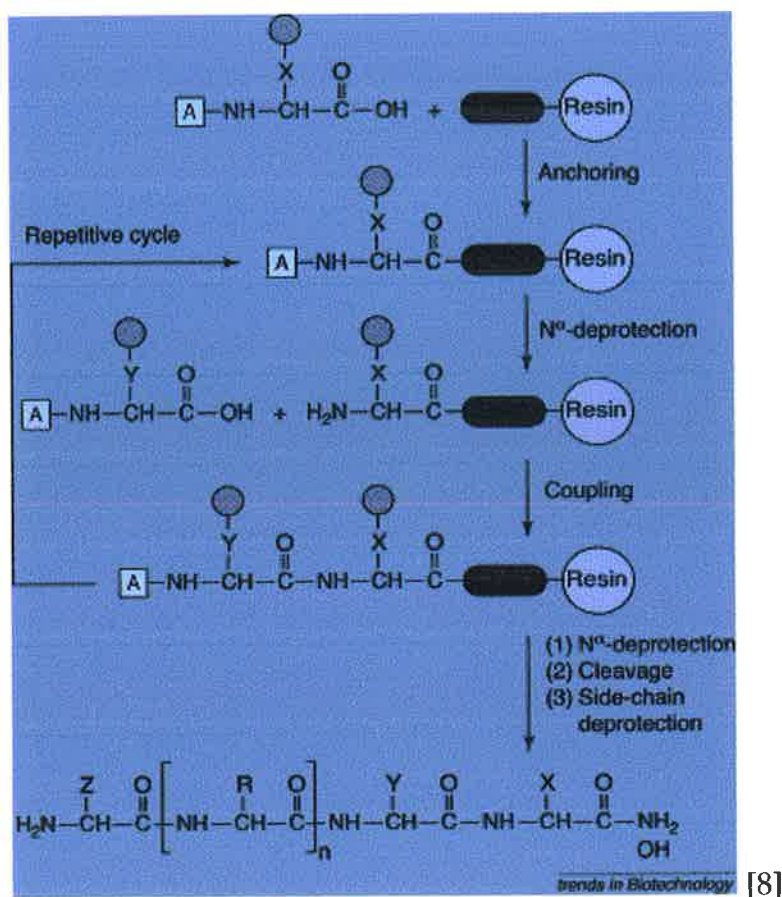


Fig. 12: Stepwise Synthesis

This work employs the Fmoc/*t*-Bu strategy for SPPS. In general, it involves a sequential stepwise procedure of single amino acid activation and coupling, followed by N-terminal deprotection and cycle repetition. Functional groups contained in the side-chains remain protected while bound to the resin support, and can be generally be deprotected by acidic treatment during the cleavage of the peptide from the resin, alternatively side chains can remain blocked depending on the protecting groups and chemistry used.

Coupling is generally achieved by activation of the incoming amino acid carboxyl with *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluorophosphate (HBTU) and 1-hydroxy-benzotriazole (HOBt) in dimethylformamide (DMF). *N,N*-diisopropylethylamine (DIEA) deprotonates

the amino acid prior to activation and is used as a proton scavenger after formation of the amide bond.

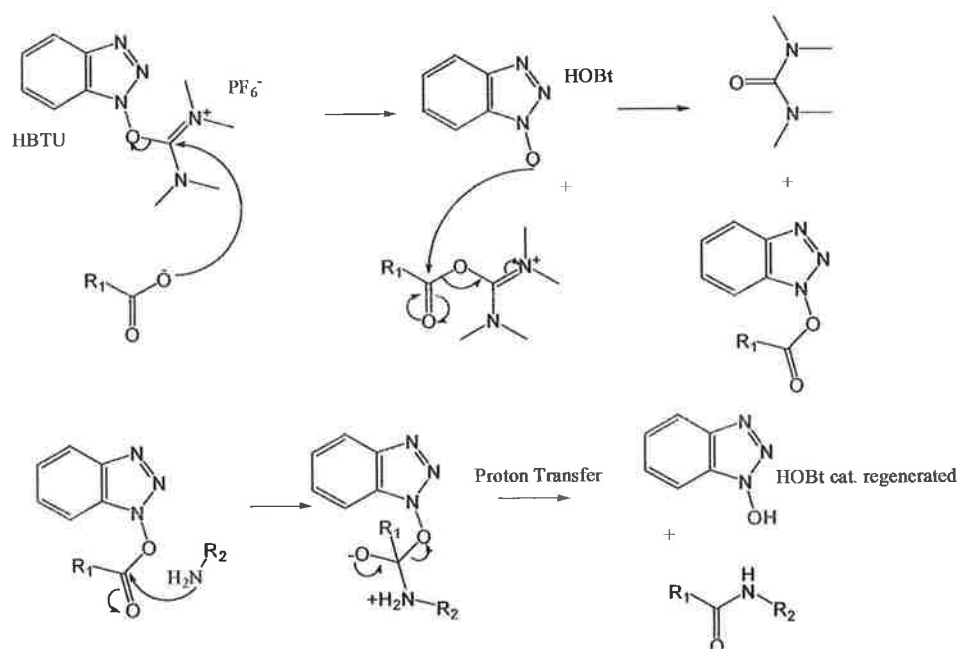


Fig. 13: Amino Acid Coupling using HBTU and HOBT

Removal of the *N*^α Fmoc group is achieved by treatment with a solution of piperidine (typically 20% in DMF). This deprotection is base mediated to avoid deprotection of the side chain protecting groups during sequence assembly.

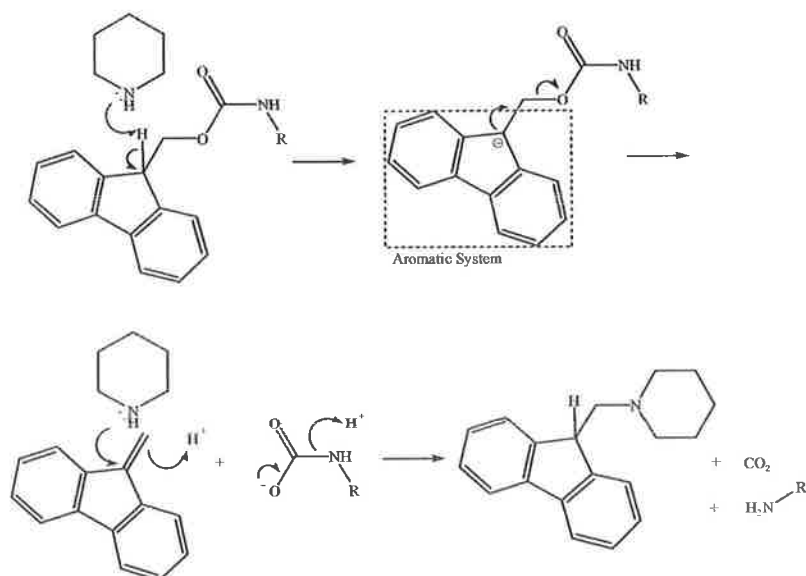


Fig. 14: Fmoc Deprotection by Piperidine

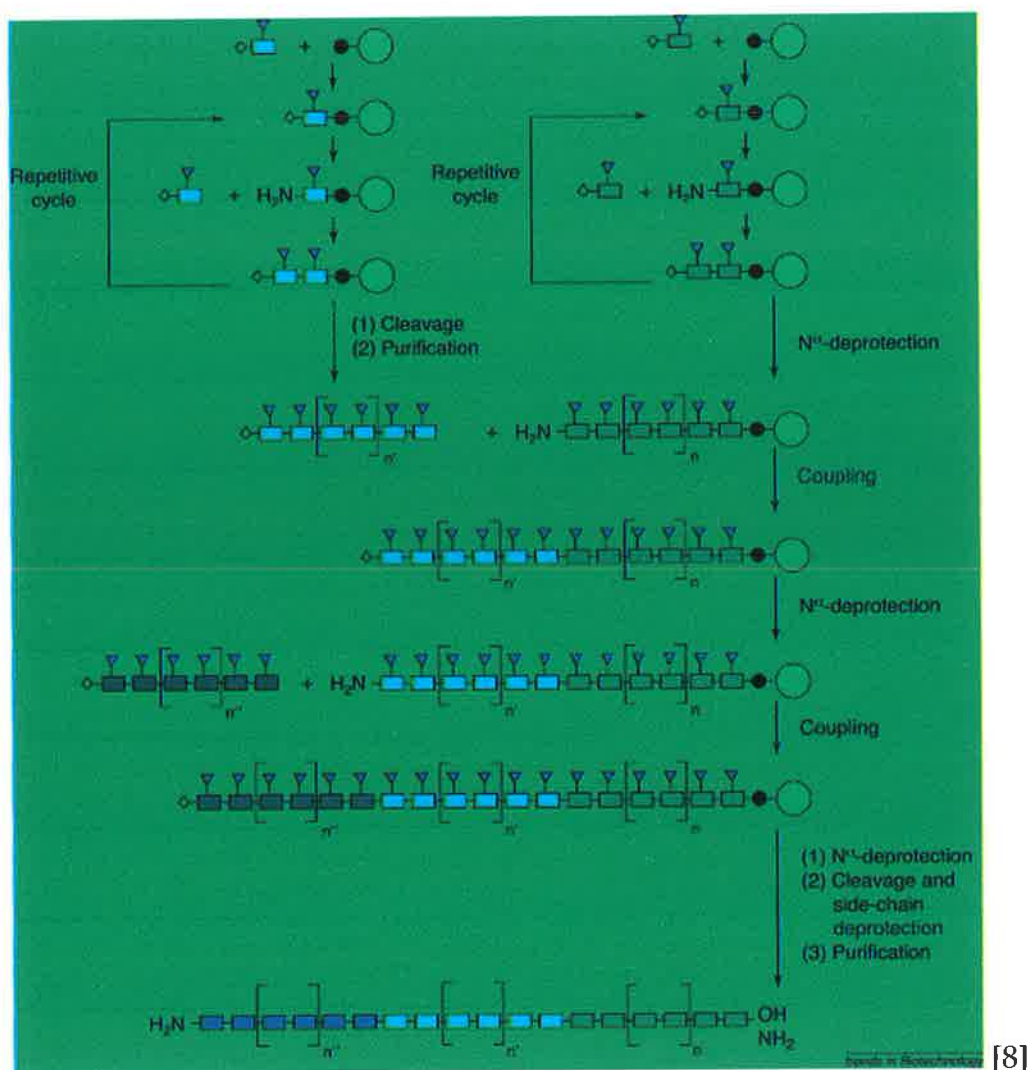


Fig 15: Convergent Synthesis of Protected Fragments

Convergent synthesis of fully protected fragments involves the stepwise synthesis of two peptide fragments, isolated as fully protected sequences. These two protected fragments can then be coupled in solution with subsequent removal of the side chain protecting groups. Usually a resin is chosen which requires different cleavage chemistry than the standard acidic type which affects both resin linkage and side chain protecting groups. On the other hand, amino acid starting materials can be chosen whose protecting groups do not respond to standard cleavage conditions.

Cleavage of protected peptides usually leads to much lower yields, due to poor solvation of the peptide and steric hindrance at the cleavage site,

caused by bulky constituents, which would normally be removed in the cleavage process. Special resins are used to obtain these protected fragments, for example Sieber amide resin for protected peptide C-terminal amides and 2-chlorotrityl chloride resin for protected peptide C-terminal acids.

1.73 Chemoselective Ligation of Unprotected Fragments

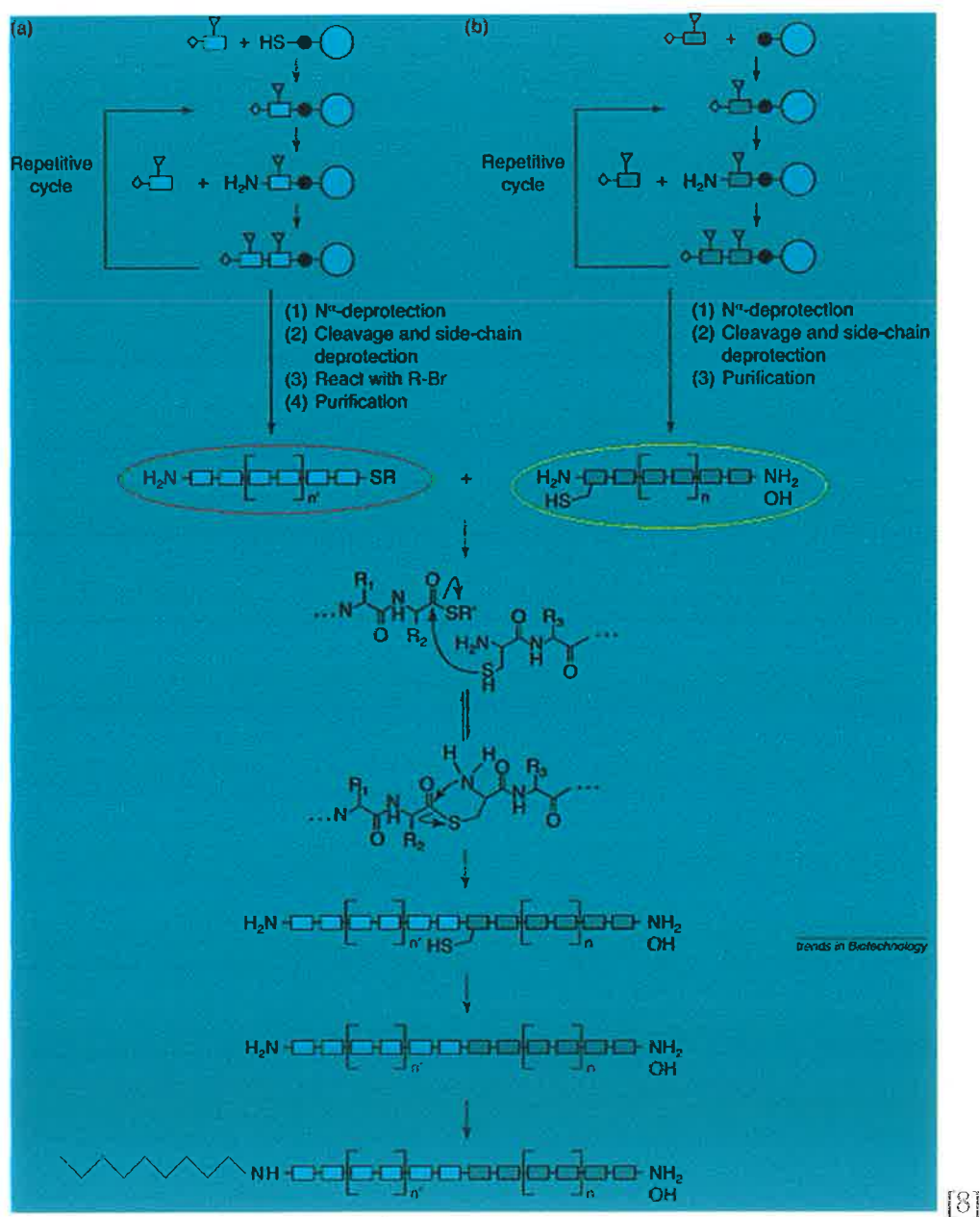


Fig. 16: Orthogonal Ligation of Unprotected Fragments

Chemoselective ligation of unprotected fragments involves the stepwise synthesis of two peptide fragments which are subsequently ligated in their unprotected form using functional groups orthogonal to amide chemistry. The

functional groups can be native groups of the peptide, for example a cysteine thiol, or non-native, added to the peptide for the purpose of ligation. These groups can be added in a variety of ways, including addition while cleaving from the resin, such as by thioester formation upon cleavage from a 4-sulfamylbutyryl NovaSyn TG resin, or by amide coupling at the N-terminal with a group other than an amino acid, for example bromoacetic acid.

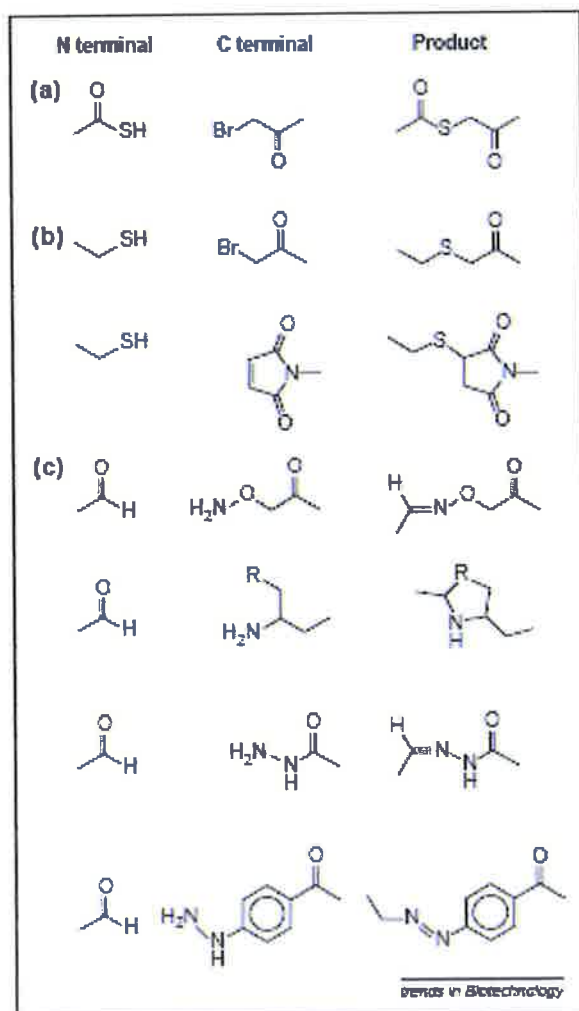


Fig. 17: Ligation Strategies with Functional Groups and End Products[8]
N- and C-terminal groups used for non-native chemoselective ligation. The product structures shown are produced when the groups react under aqueous conditions.

1.74 Resins

As mentioned previously, a variety of resins can be used for SPPS. These resins vary in their polymer composition. This provides a different microenvironment for the growing peptide chain. They can also possess different linkers for

peptide attachment. These linkers then determine the chemistry for peptide attachment, cleavage and the C-terminal functionalisation of the cleaved peptide.

(i) Rink Amide MBHA Resin

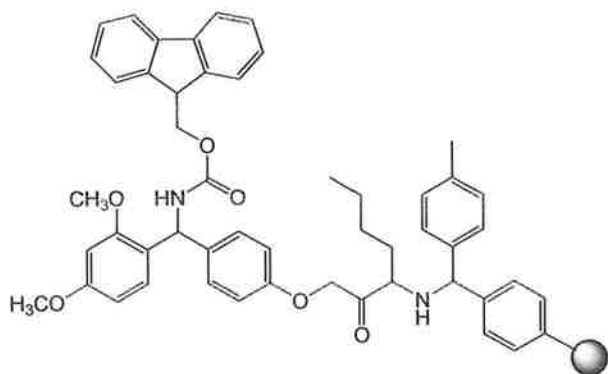


Fig. 18: Rink Amide MBHA Resin

MBHA resin contains a copoly(styrene-1% divinylbenzene (DVB)) polymer support. This is the standard resin used for routine synthesis of peptide sequences by stepwise solid phase synthesis of peptide amides. Peptide amides are useful as the C-terminal is less reactive than the native carboxy functionalisation. This resin displays a reasonably high loading capacity, although low loading versions are also available.

(ii) NovaPEG Rink Amide Low Loading Resin

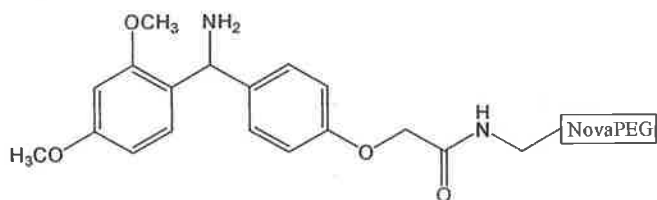


Fig. 19: NovaPEG Rink Amide Low Loading Resin

The NovaPEG resin is more hydrophilic, as it is composed totally of crosslinked PEG. This makes it more suitable for very hydrophobic sequences, as it separates the peptide chains, and helps prevent aggregation. Low loading (LL) refers to a resin which has less linkers per gram resin, creating further separation between growing peptide chains.

(iii) 4-Sulfamylbutyryl NovaSyn TG resin

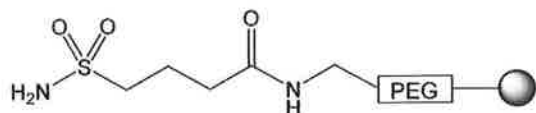


Fig. 20: 4-Sulfamylbutyryl NovaSyn TG Resin

NovaSyn TG resin is a composite of PEG and low cross-linked polystyrene. It has good swelling properties and diffusion rates. The 4-sulfamylbutyryl linker assists in thioester formation on the C-terminal when the peptide is cleaved, by N-activation with iodoacetone nitrile then ligand replacement with benzylmercaptan. This C-terminal peptide thioester can then be used in one of the orthogonal ligation strategies shown in figure 16, page 28, which involves native chemical ligation, discussed more in depth in section 2.4.

(iv) 2-Chlorotrityl Chloride Resin

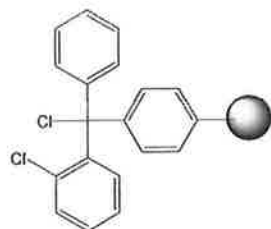


Fig. 21: 2-Chlorotrityl Chloride Resin

This resin is a low loading polystyrene resin, capable of producing a cleaved, side chain protected peptide upon treatment with 1-5% TFA. The resin is based on a super-acid sensitive linker, and therefore requires much milder conditions for cleavage than those needed for side chain deprotection. Upon cleavage, a C-terminal carboxylic acid is produced. This carboxylic acid can then be used for in solution coupling, while all other groups are still protected.

(v) Sieber Amide Resin

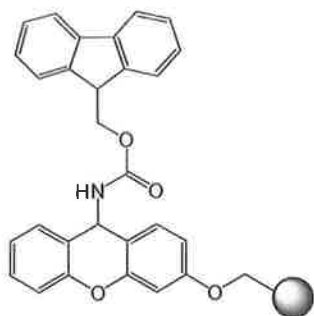


Fig. 22: Sieber Amide Resin

This polystyrene-based resin has a moderate loading capacity. The amino-xanthen-3-yloxy linker is also super acid sensitive, but in this case allows for cleavage of protected amides. In this case the C-terminal is blocked from reaction, along with side chain functional groups. Hence, the N-terminal may be deprotected by Fmoc removal, leaving only this amine to react in solution.

1.75 Standard Cleavage and Deprotection

TFA cleaves the protecting groups of the standard commercially available Fmoc-amino acids from the peptide, such as Boc, and the linker of a number of resins, including Rink amide MBHA resin and Rink amide NovaPEG LL resin. Water, triisopropyl silane (TIS), thioanisole (TA), and ethanedithiol (EDT) are soft nucleophiles, acting as scavengers for the groups which are released during deprotection. Without these scavengers, the released protecting groups would react again with the various side chains to form irreversible products. EDT is used only in the case of deprotected thiol side chains, to provide an alternative reacting point.

1.8 Characterisation Techniques

1.81 MALDI TOF MS

Matrix-Assisted Laser Desorption/Ionisation (MALDI) time of flight (TOF) mass spectrometry (MS) is the primary way in which these peptides are characterised. For large biomolecules, under well defined reaction conditions, molecular weight gives a good indication of whether or not the product is the target compound, as it is generally unlikely to find a side product with an identical mass, and co-eluting during purification by chromatography (HPLC).

This technique involves the ionisation of the compound and flight through a magnetic field, in which ions are separated according to their charge/mass ratio. MALDI TOF MS is a relatively soft ionisation technique, and does not cause the same degree of fragmentation as seen in other forms of MS, such as electrospray MS. For this reason, it is used quite often for biological molecules and macromolecules. For larger compounds the degree of fragmentation increases, thus creating a more complicated spectrum.

The matrix assists in the vaporisation and ionisation of the compound. It also protects the molecules from being destroyed by the laser beam. The matrix used in these experiments is α -cyano-4-hydroxycinnamic acid (CHCA), as shown below.

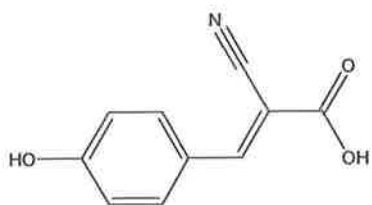


Fig. 23: α -Cyano-4-Hydroxycinnamic Acid (CHCA)

The conjugated pi system allows this molecule to absorb the laser beam irradiation, and the carboxy group provides an acidic proton to assist in ionisation. Other matrices used in MALDI TOF MS include 2,5-dihydroxy benzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (SA), and 4-

hydroxy-3-methoxycinnamic acid (ferulic acid), all of which contain an aromatic group and acidic groups.

1.82 Kaiser Test

This test is very useful in measuring the extent of deprotection of the *N*-terminal Fmoc group between peptide couplings, and is determined by a change in colour. Ninhydrin reacts with free primary amines to produce a chromophore. This reaction cannot occur with secondary or tertiary amines as the reaction proceeds via a Schiff base. [53, 54] Positive reaction with a primary amine produces a highly colourful blue chromophore, with a large conjugated π -system. Negative binding maintains the standard yellow colour of the solution.

1.83 NMR

Nuclear magnetic resonance (NMR) spectroscopy is a method of characterisation where a magnetic field interacts with nuclear spins, usually of the hydrogen and carbon nuclei and their response to the magnetic field analysed. This technique is very accurate, and gives information about the exact structure and conformation of the molecule. However, it is much more useful for small molecules than larger ones, as in the latter signals become more complex and overlapping.

CHAPTER 2 – RESULTS AND DISCUSSION

2.1 Introduction

The main focus of this project was the synthesis of H-K₃₀-NH₂ (**1**) and its PEGylation for use as a transfection agent of siRNA. Many strategies were attempted, as this peptide proved quite difficult to synthesise by standard SPPS. Through each attempt, knowledge was gained about the mechanism of formation of this peptide and the challenges created by the nature of the compounds involved. It was found that the hydrophobicity of the chain was a major barrier to chain elongation; hence this factor was addressed for successful synthesis of the peptide. In contrast, P17 (**5**) was successfully synthesised by standard SPPS, on the first attempt.

PEGylation of K₃₀ derivatives was unsuccessful, although work on this process was only preliminary due to time constraints, and has since been successfully completed by Dr. Stephane Desgranges, another member of the peptide group. This was achieved by applying a 'Click chemistry' approach instead of maleimide-thiol conjugation.

Synthesis of azido acids from their amino acid starting materials was also studied for use in 'Click chemistry' reactions as an alternative to maleimide-thiol ligation. Fmoc-Lys(N₃)-OH (**6**) was successfully synthesised from Fmoc-Lys(NH₂)-OH (**7**), although attempts with another amino acid H-Phe-OH (**8**) to produce N₃-Phe-OH (**9**) were unsuccessful.

2.2 Poly-lysine (30-mer peptide)

Difficult sequences in peptide terminology are those which cannot be synthesised by standard stepwise solid phase methods [4-7, 54-60]; K₃₀ (**1**) is such a sequence. During the course of this work, a number of variations in the stepwise procedure were undertaken, using different methods and resins. The

methods used included stepwise synthesis, orthogonal ligation of unprotected fragments, and protected fragment coupling.[8]

A variety of resins were used, some to provide different micro-environments for the growing peptide chain, for example the hydrophilic Nova-PEG Low Loading resin. Others produced either protected or unprotected fragments with varying termini, for example Sieber Amide resin for protected peptide amides. Also, a variety of orthogonal groups for ligation were explored, which would not interfere with the unprotected groups of the fragments. Cysteine was added at the N-terminus of the poly-lysine sequence for the purpose of attachment to PEG at a later stage. In some experiments β -alanine was also used as a spacer between the active peptide and the cysteine thiol attachment point.

This peptide, H-CK₃₀-NH₂ (**2**) was eventually synthesised by a combination of Rink Amide NovaPEG LL resin and stepwise synthesis with double coupling after the first 20 residues had been added to the chain. Below is a detailed discussion of the varying methods attempted towards the synthesis of K₃₀ (**1**) and its derivatives.

2.3 Stepwise Synthesis I - Rink amide MBHA Resin (Methods 4.3)

The first method attempted was performed according to the Fmoc/*t*-Bu strategy and a single coupling chemistry, from a Rink amide MBHA resin (0.7mmol/g loading). This is the standard procedure for routine preparation of peptides, based on a common resin for peptide amide synthesis. This was initially applied to the synthesis of the 15-mer peptide, H-K₁₅-NH₂ (**10**) whose integrity was verified by MALDI TOF MS (H⁺: 1939.0708 m/z, Na⁺: 1962.1239 m/z, K⁺: 1978.0737 m/z. See Appendix 5.1, page 74). Assembly of the complete 30-mer sequence, H-K₃₀-NH₂ (**1**) was then attempted after this checkpoint. However, this method failed, and a complex mixture of deleted and protected peptides

was obtained, as demonstrated by MS analysis, in which the target sequence could not be identified.

In a second attempt of standard stepwise synthesis, described in method 4.32, page 59, the full peptide sequence was assembled on an automated synthesiser, including the N-terminal cysteine and the β -alanine spacer, in an attempted production of H-C β AK₃₀-NH₂ (**11**). A double coupling procedure was applied from the 21st residue. It was hoped that the use of this procedure and of uninterrupted elongation would overcome the difficulties associated with interchain associations. Unfortunately, a complex mixture of deletion peptides with and without cysteine, β -alanine, and potentially remaining protecting groups was again obtained.

To simplify the number of potential by-products arising from the incomplete coupling and/or deprotection of some residues, stepwise synthesis of H-K₃₀-NH₂ (**1**) was repeated (method 4.33, page 59), this time omitting the N-terminal cysteine and β -alanine. A double coupling protocol was again applied from the 21st residue.

A mixture of deletion peptides was again obtained, but it was possible to identify that a 19-mer poly-lysine sequence was one obtained sequence in this attempt. It was therefore concluded that the limit of chain elongation was somewhere in the region of 15-19 residues, as the 15mer (**10**) is obtainable by standard stepwise procedures.

With the knowledge gained from the stepwise synthesis from a Rink amide MBHA resin, the fragment condensation of two 15-mer poly-lysine peptides appeared to be a viable option, as we had established that a 15-mer (**10**) sequence could be easily assembled from this resin, and with good purities. The addition of a cysteine residue on one fragment was also a consideration, to provide a method for polymer attachment.

In later experiments (method 4.51 (i), page 61) it was found that the H-CK₁₅-NH₂ (**12**) sequence could be successfully assembled by standard coupling

methods from a Rink amide MBHA resin, while the H-K₁₅C-NH₂ (**13**) peptide could not be obtained under these conditions (method 4.41 (i), page 60). The difference here is the presence of the sterically hindered trityl protecting group at the end or start of the assembled chain, respectively. This bulky trityl group may have required multiple coupling cycles for the next amino acid, which were not performed. In addition, this group would have disrupted the conformation of the proceeding groups.

It is noteworthy that in studies dedicated to difficult peptide sequences Lys(Boc)₆ has been used as a pre-sequence at the C-terminus to prevent growing chain aggregation, its structure assists by disruption of β -sheet formation and adoption of a random coil conformation [61]. This result is counter-intuitive, based on results found here, however we can conclude that it is only after as reasonable elongation has occurred that the chain is capable of aggregating with adjacent chains.

These factors may contribute to the difference in synthetic results obtained with H-CK₁₅-NH₂ (**12**) and H-K₁₅C-NH₂ (**13**). Further experiments with double and triple coupling procedures, or manual coupling after the initial cysteine under microwave activation, could be performed to further investigate this approach. In this way we could ascertain the relative importance of conformation versus cysteine trityl steric hindrance.

2.4 Fragment Coupling

Fragment coupling can be broadly categorised into two types, chemoselective ligation of unprotected fragments and amide coupling of protected fragments. Both methods have their own advantages and limitations, as discussed in more depth below.

Fragment Coupling I (methods 4.4) involved the orthogonal ligation of bromoacetyl-K₁₅-NH₂ (**14**) and H-K₁₅C-NH₂ (**13**). Bromoacetylation of H-K₁₅-NH₂ (**10**) was unsuccessful, as was synthesis of H-K₁₅C-NH₂ (**13**).

Fragment Coupling II (methods 4.5) involved the orthogonal ligation of the peptide-benzyl thioester, H-C(Acm)K₁₄-SBnz (**15**) and H-CK₁₅-NH₂ (**12**). This reaction of thioester and cysteine thiol is called native chemical ligation, and spontaneously rearranges to form the 'native' amide bond. H-CK₁₅-NH₂ (**12**) was successfully synthesised by standard SPPS on Rink Amide MBHA resin. Two separate strategies were undertaken towards the peptide thioester fragment. The first involved a resin linker designed to produce a thioester upon cleavage from the resin (method 4.51, 4.52). This approach was unsuccessful. The next involved a modified cysteine, *N*^α-ethyl, *S*-Trt Cysteine (**16**) designed to rearrange upon cleavage to form a thioester in place of the amide bond (method 4.53). Synthesis of this precursor, however, was also unsuccessful.

Fragment Coupling III (methods 4.6) involved the in solution coupling of two protected peptide fragments, Boc-C(Trt)[K(Boc)]₁₅-OH (**17**) and H-[K(Boc)]₁₅-NH₂ (**18**), by using 2-chlorotrityl chloride resin and Sieber Amide resin, respectively. H-[Lys(Boc)]₁₅-NH₂ (**18**) was successfully synthesised (Appendix 5.3, 5.4, page 76-77), however, this method was not continued further due to the discovery of a more efficient and effective method through Stepwise Synthesis II – NovaPEG LL resin for H-CK₃₀-NH₂ (**2**), as discussed later in Section 2.5.

In Appendix 5.3, 5.4 it is interesting to note there is a small extent of deletion peptides present in the unpurified sample. This Sieber amide resin is a polystyrene based resin, unlike MBHA which is copoly(styrene- 1% DVB). This result further supports the assertion that the microenvironment provided by the resin is an important consideration in peptide synthesis.

2.41 Fragment Coupling I (Methods 4.4)

Chemoselective Ligation of Unprotected Fragments - A

Generally, it is difficult to selectively obtain a native amide bond between two unprotected peptides. Unprotected side groups interfere with the normal

coupling reaction, particularly in the case of K₃₀ derivatives, where there are thirty ϵ -amino groups to every one reacting α -amine. Alternate ligation strategies use orthogonal groups, which react without affecting the side chains [8]. However, in most cases a native amide bond is not obtained, thioester [62] and thioether linkages [63, 64], for example, are formed. However, some of these hybrids can undergo rearrangement, under the right conditions to form a native amide bond, such as in the case of native chemical ligation between a peptide thioester and cysteine thiol.

The first chemoselective ligation of unprotected strands attempted was that of an N-terminally bromoacetylated fragment (14) and a C-terminally modified cysteine fragment (13). [63] This route forms a thioether conjugate of the two fragments as the final product, H-K₁₅C-S-K₁₅-NH₂ (19). The schematic for the reaction is as follows:

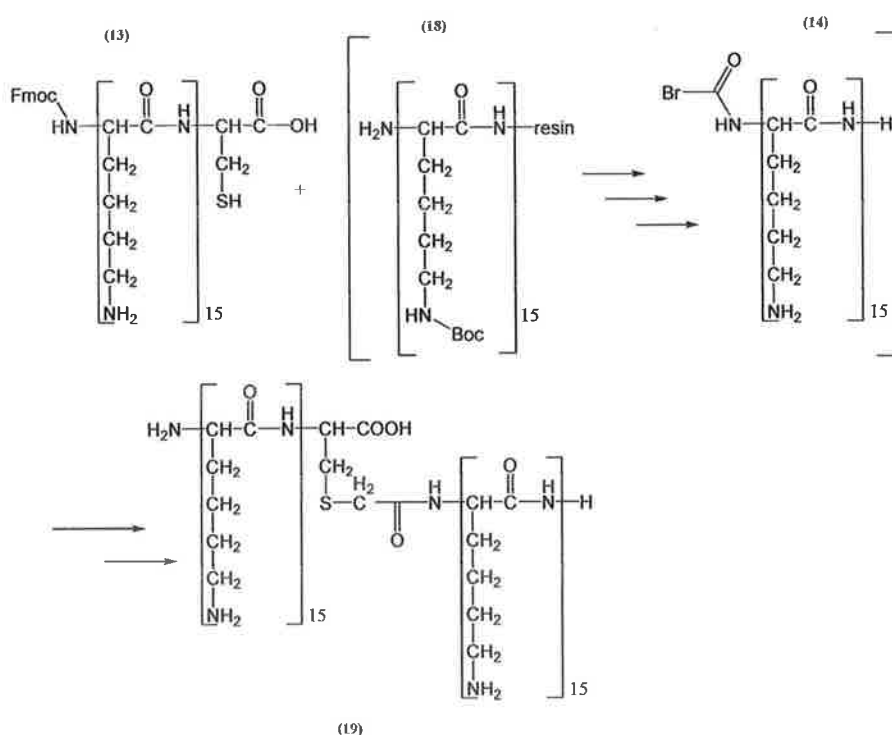


Fig. 24: Bromoligation of Unprotected K₁₅ Fragments

However, preliminary bromoacetylation attempts of the N-terminal H-K₁₅-NH₂ (10) fragment remained unsuccessful under the conditions investigated. Analysis of the product obtained showed that the main reaction

occurring was the nucleophilic substitution of the halogen by the terminal α -amino group to produce an *N*-acetyl- K_{15} -NH₂ (**20**), of structure shown below. Other assays performed with deprotonation of the bromoacetic acid prior to the addition to the resin-bound peptide conjointly with the coupling agent, could possibly overcome this limitation.

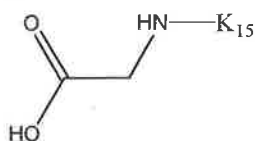


Fig. 25: *N*-Acetyl K_{15} -NH₂ (**20**)

2.42 Fragment Coupling II (Methods 4.5)

Chemoselective Ligation of Unprotected Fragments - B

The next synthetic route to H-CK₃₀-NH₂ (**2**), based on unprotected peptides, investigated involved two modified K_{15} fragments fused by native chemical ligation (NCL), with subsequent transformation of the central cysteine (C) to a pseudo-lysine (*S*-Ethylamino-Cysteine, K_c) (**21**). NCL involves the reaction of a peptide thioester (**15**) and an *N*-terminally cysteine-modified sequence (**12**). Their reaction forms an intermediate thioester which spontaneously rearranges to form the native amide bond, and regenerating the cysteine side chain.

In addition to this central cysteine, a second cysteine was included on the other fragment (**15**), protected by an alternative, acetamidomethyl (Acm) protecting group. The Acm group is used here in place of the usual trityl group, which is resilient towards standard cleavage conditions using concentrated TFA solutions. NCL can be performed to fuse these two peptide fragments to form a new peptide H-C(Acm) K_{14} CK₁₅-NH₂ (**22**). Acm can be removed after peptide modification, with specific oxidative deprotection by treatment with iodine. Then, this free thiol can be used for PEGylation with a maleimide linker.

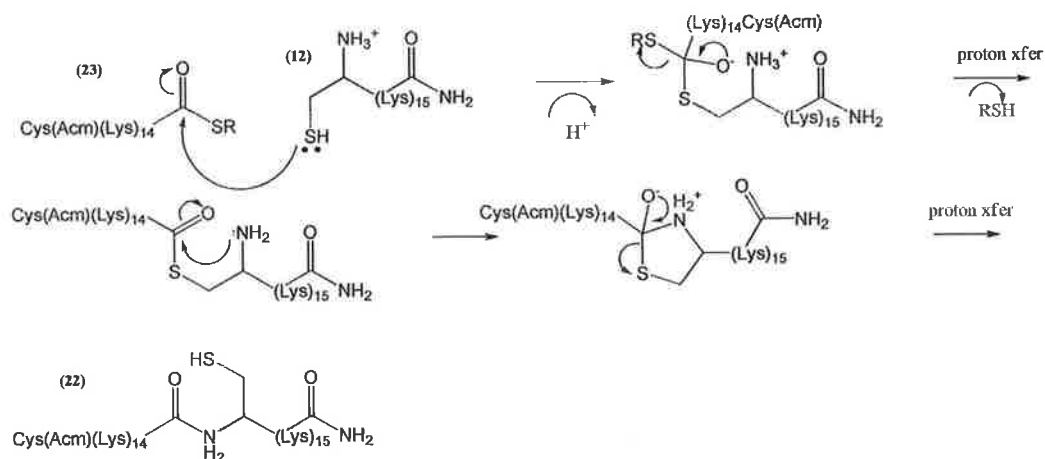


Fig. 26: Synthesis of H-K₁₄KcK₁₅-NH₂ by NCL

(i) Native Chemical Ligation I

This method involved loading the first (lysine) amino acid of the thioester peptide, H-C(Acm)K₁₄-SBnz (**15**), onto a 4-sulfamylbutyryl NovaSyn TG resin. The loading of this first amino acid onto the resin, is performed through an amide bond, formed by PyBOP/DIEA coupling chemistry.

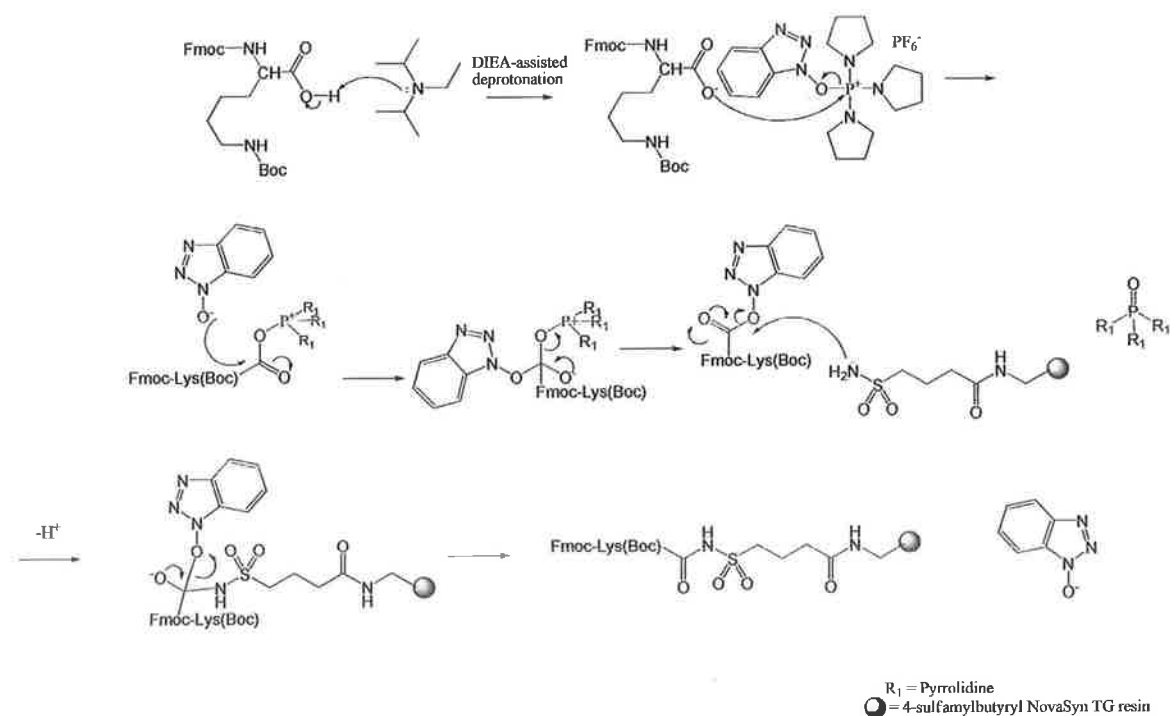


Fig 27: Loading of First Amino Acid onto 4-sulfamylbutyryl NovaSyn TG Resin

The remaining sequence was assembled by automated SPPS according to the Fmoc/*t*-Bu protocol, using HBTU/HOBt/DIEA coupling chemistry. After automated sequence assembly, the linker of the resin-bound peptide was then

activated by iodoacetone nitrile and cleaved by a thiophenol ligand to produce a peptide thioester (H-Cys(Acm)K₁₄-SPh, **(23)**). The thiophenolate ester should be produced in this reaction, being replaced in solution by the more stable thioester of benzylmercaptan **(15)**.

Although the ligation reaction involves unprotected fragments, the peptide is cleaved from this resin in its protected form, and so yields for this experiment were consistently low. As mentioned before, this is attributed to steric hindrance by and poor solvation of the protected sequence.

Activation of Sulfamyl Resin-Bound Peptide for Cleavage

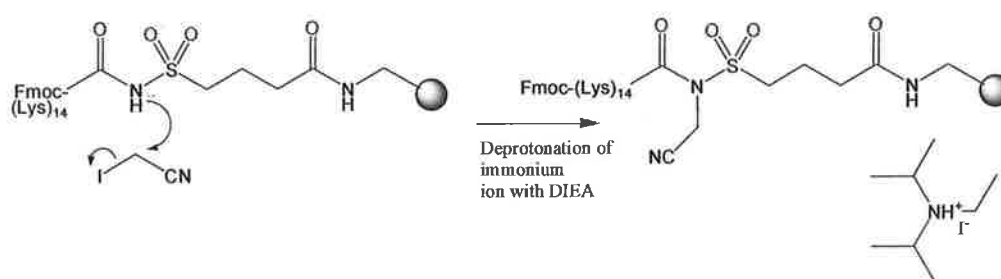


Fig. 28: N-Activation with Iodoacetone nitrile

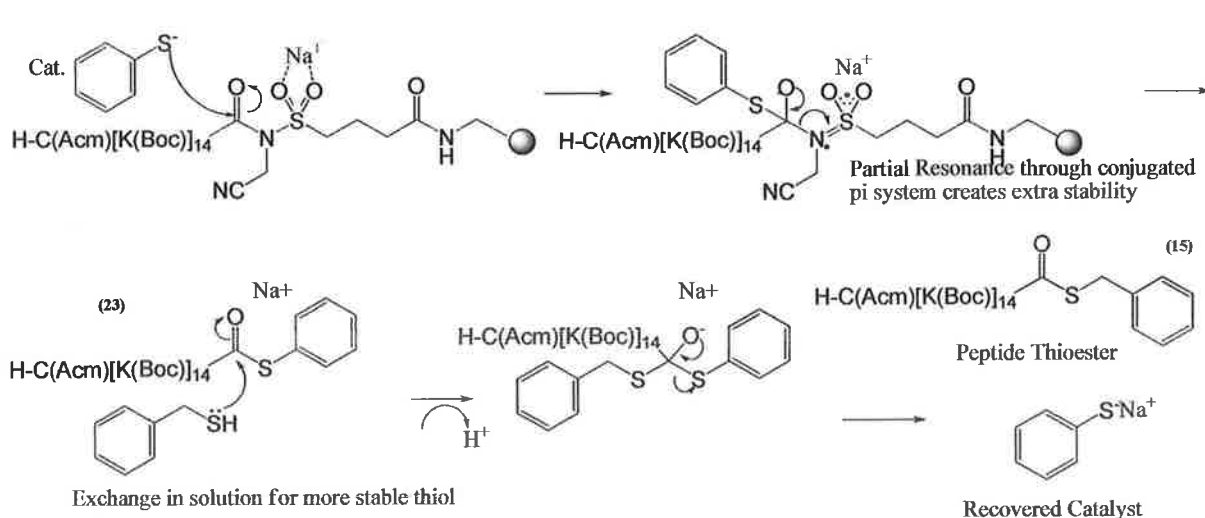


Fig. 29: Thioester Formation with Catalytic NaSPh and Subsequent Thiol Exchange

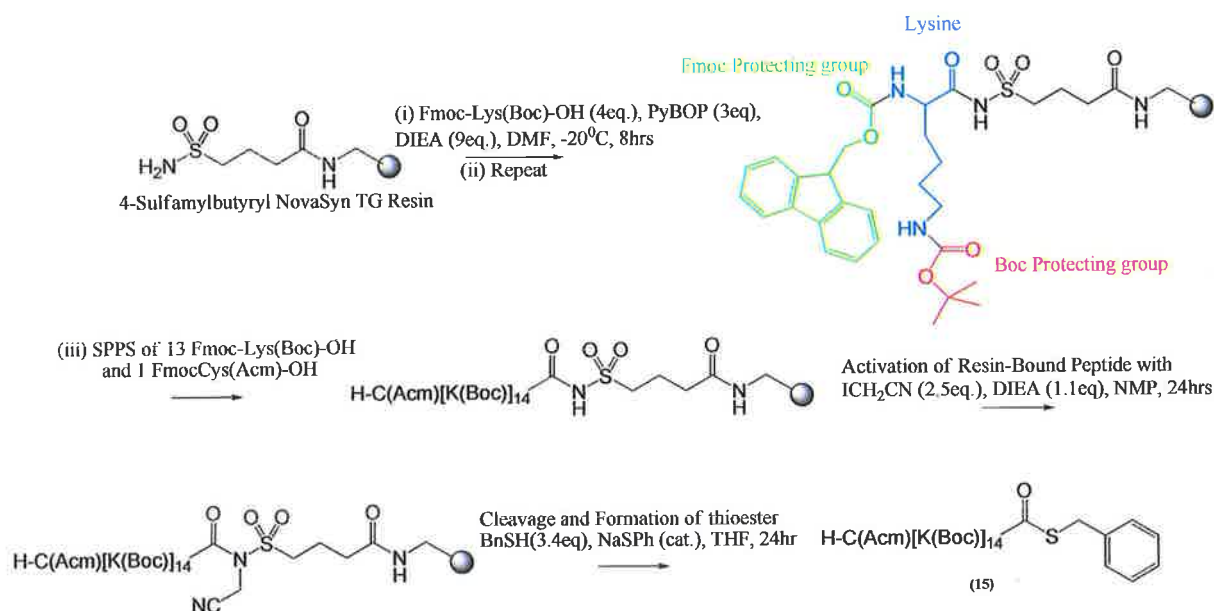


Fig. 30: Synthesis of K₁₄-Thioester

After multiple attempts at the resin cleavage stage, this method proved problematic. With extremely low yields, it also produced a complex mixture of peptides with multiple deletion peptides and possible attachment of iodoacetamide to nitrogens other than the intended sulfonamide nitrogen.

It was later identified when comparing the results obtained with the NovaPEG LL resin experiments (methods 4.7) that solvation during the automated peptide elongation would need to be increased. It was expected that this increased solvation (method 4.52, page 63) would improve the yield and purity of the peptide, as observed with the NovaPEG LL resin. Unfortunately, increased solvation did not improve yields in the present case, or diminish the occurrence of deletion peptides.

This may have been due to the composition of the resin. Although the NovaSyn TG resin is also a low loading resin, it is a composite of PEG and cross-linked polystyrene, unlike NovaPEG resin which is composed totally of PEG. Hence this resin is less hydrophilic, and therefore less efficient at preventing aggregation of adjacent hydrophobic chains. The 4-sulfamylbutyryl linker is not currently commercially available on the NovaPEG solid support.

An alternative method towards the thioester for NCL was therefore implemented.

(ii) Native Chemical Ligation II

This new method relied not on the 4-sulfamylbutyryl linker to produce the thioester, but on a modified cysteine. It involved, instead, the cleavage of a deprotected peptide from the resin, with subsequent thioester formation. In general, unprotected peptide cleavage is much more convenient and is associated with higher yields. This alternative method employed the synthesis of *N*^α-ethyl-*N*^α-Fmoc-S-Trityl-cysteine (**24**), to be attached to the Rink amide MBHA resin, as the C-terminus of the peptide, method 4.53, page 63.

Upon cleavage of H-C(Acm)K₁₄-*N*^α-ethyl-Cysteine-NH₂ (**25**), it has been shown that this residue undergoes a reversible *N*- to *S*-acyl transfer, to produce the thioester by reaction with the thiol side-chain, resulting in H-C(Acm)K₁₄-*S*-*N*^α-ethyl-Cysteine (**26**) [65-67].

The alkylation of the cysteine amino group gives extra stability and electron-donation to the α-nitrogen, once involved in the amide bond. This allows the rearrangement to occur, by attack of the cysteine thiol and detachment of the amide-bound, ethylated nitrogen. It has been reported that this cysteine-based thioester can be displaced by benzyl mercaptan similar to the 4-sulfamylbutyryl approach, to produce (**15**).[67]

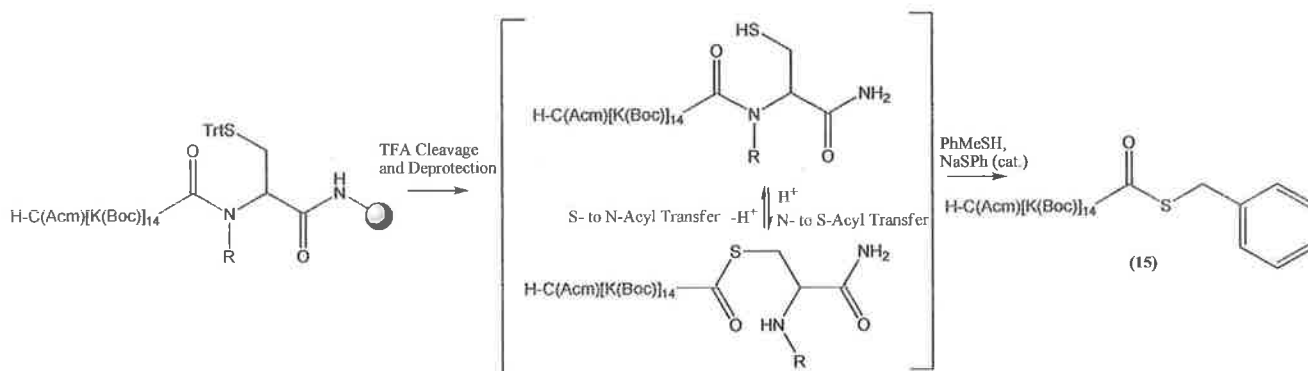


Fig. 31: Post-SPPS *N*- to *S*-Acyl Transfer to Produce a Peptide Thioester

It was anticipated that the synthesis of **(18)** by a standard procedure from the Rink amide MBHA resin could be challenging; as described above, despite one example of a successful synthesis by Hojo *et al* [67]. Loading of the second amino acid, lysine, should be performed manually with repeated couplings and monitoring by the Kaiser test, to overcome the steric hindrance of the alkylated N-terminal, which greatly reduces reactivity.

As regards the synthesis of *N*^α-ethyl, *S*-Trt Cysteine (**(16)**), which in our case was unsuccessful; the expected reaction steps are as shown in the scheme below, with Schiff base formation involving the condensation of the amine with ethanal and subsequent reduction to form the secondary amine. The reaction should be performed in basic conditions (KOH) to maintain the deprotonation of the amine group, and to avoid side reactions at the carboxyl by creation of the potassium salt. An Fmoc group should also be subsequently added to prevent the amino acid from coupling with itself, instead of the resin during peptide synthesis, resulting in *N*^α-ethyl-*N*^α-Fmoc-*S*-Trityl-cysteine (**(23)**).

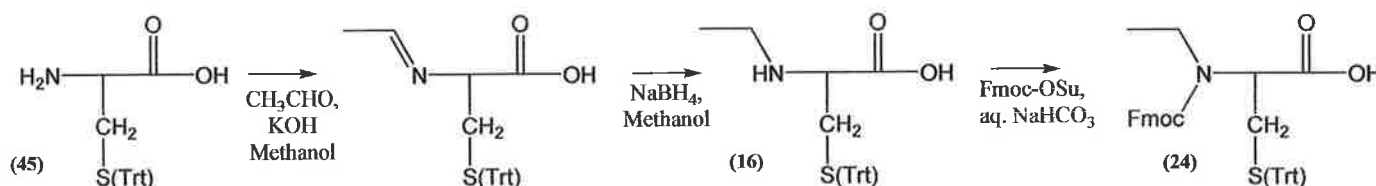


Fig. 32: Reaction Scheme for Synthesis of *N*^α-ethyl, *N*^α-Fmoc, *S*-Trt Cysteine

After multiple attempts under various conditions and with guidance from the authors of the original reference, **(16)** could be produced, however with only partial conversion. The low yields were further exacerbated by the difficult recovery of the desired product from the reaction mixture. Several isolation procedures were attempted, including extraction after acidification, to facilitate the separation of the amino acid from the secondary amine product, method 4.53 (iv), page 65. However, no significant improvement of the yields was obtained, and so this method was discarded.

The proposed NCL-based synthetic route, using this precursor, would be as follows. The two peptide fragments H-C(Acm) K_{14} -SBnz (**(15)**) and H-CK K_{15} -

NH₂ (**12**) would be ligated, unprotected, to produce H-C(Acm)K₁₄CK₁₅-NH₂ (**22**).[62, 68-70] The thiol group of the central cysteine would then be alkylated to produce a pseudo-lysine residue (K_c (**20**)), with a thioether replacing one carbon of the typical Lys side-chain, resulting in H-C(Acm)K₁₄K_cK₁₅-NH₂ (**27**).[71-73]

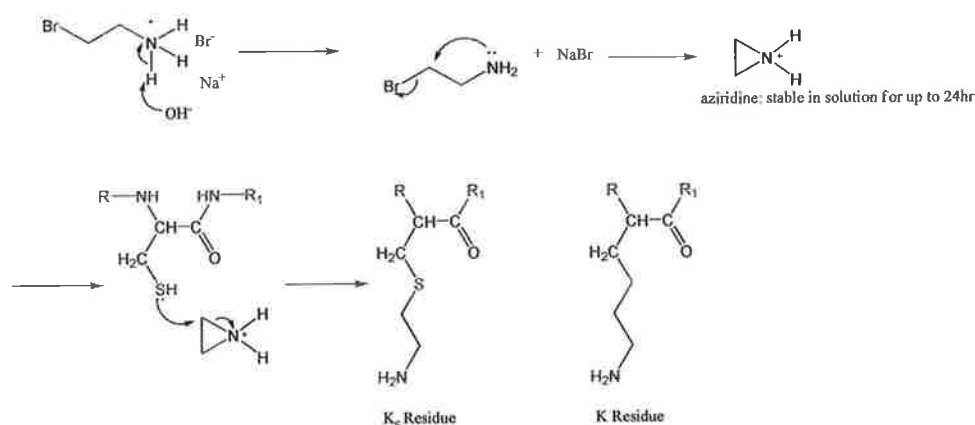


Fig. 33: Ethylation of Cysteine with BrEA

After ligation and central cysteine alkylation, the N-terminal Cysteine would be deprotected with iodine [74] and finally PEGylated.[39]

2.43 Fragment Coupling III (Methods 4.6)

Convergent Synthesis of Protected Peptide Fragments

The next synthetic route devised towards the synthesis of H-CK₃₀-NH₂ (**2**) involved the coupling of two protected chains in solution, each fragment containing 15 lysines and being assembled from super-acid sensitive resins.

H-K(Boc)₁₅-NH₂ (**18**) was produced from a Sieber amide resin. The C-terminal residue is loaded by standard coupling with formation of an amide bond. The synthesis of this peptide is conveniently performed by automated synthesis directly from the Sieber resin. MALDI-TOF MS analysis was performed on the deprotected peptide, which confirmed the presence of H-K₁₅-NH₂ (**10**) (H⁺: 1939.1220 m/z, Na⁺: 1960.9486 m/z), Appendix 5.3, 5.4, page 76-77,

as peptides with protected basic groups usually give poor signals in MALDI-TOF mass spectrometry.

The second peptide Boc-C(Trt)[K(Boc)]₁₅-OH (**17**) was synthesised from a 2-chlorotrityl chloride resin. The N-terminal is protected instead with a Boc group for this peptide so that it may be deprotected later on alongside the side chain groups. This resin requires manual loading of the first amino acid under inert conditions and exclusion of water. The extent of this esterification reaction was determined by the Kaiser test. Deprotection of this peptide for characterisation purposes was not completed, due to success with other methods undertaken in parallel.

Although not performed, after assembly and cleavage of these protected peptide fragments, coupling of the N-terminally Fmoc protected fragment (**17**) displaying a free C-terminal carboxyl, obtained from the Trityl resin, and of the C-terminally amide protected fragment (**18**) with a free N-terminal amine, obtained from the Sieber resin, can be performed selectively. The combination of these peptides provides only one possible outcome, with no side reactions. Fmoc removal followed by side-chain deprotection of the full length peptide would then be performed after the in solution coupling step.

The assembly of these two peptide fragments and their cleavage as protected sequences has been investigated to date. This route was not investigated further, as the stepwise assembly from a PEG-based resin, described below, successfully delivered the target H-CK₃₀-NH₂ (**2**) sequence.

2.5 Stepwise Synthesis II - Rink amide NovaPEG LL resin (Methods 4.7)

Performed in parallel to the approaches described above, was the stepwise synthesis from a resin with a greater hydrophilicity than a polystyrene-based support and with a low loading capacity, to prevent intermolecular association of the growing protected peptide chains. The Rink amide NovaPEG LL resin,

known to facilitate the synthesis of peptides containing a large hydrophobic content, was selected for this purpose.

The increased hydrophilic nature due to the PEG content of this solid support is likely to prevent its interaction with the protected peptide chains. This separation would, in turn, reduce the likelihood of adjacent chain aggregation of peptide chains by β -sheet formation. Furthermore, this resin has a very low loading of linkers for peptide attachment and elongation. Generally, a reasonably high loading is preferred in the interest of economy. However in this case, the greater distance between the linkers should assist in peptide strand separation.

On the first attempt of H-K₁₅-NH₂ (**10**) from NovaPEG LL resin (method 4.71, page 67), the synthesis failed, as this PEG-based support possesses significantly greater swelling properties than a polystyrene-based resin and required much greater solvation than usually performed from 0.1mmol of resin with the 0.1mmol Fast Fmoc method.

Subsequent experiments were performed by maintaining the quantities of resin at 0.1mmol, but using the 0.25mmol synthetic scale for the solvents and reagents. H-K₁₅-NH₂ (**10**) was successfully produced by this method (Appendix 5.4, page 77). The resin was split for further experiments into 0.05mmol batches, which were used for further elongation of the sequence with the 0.25mmol method. This elongation was performed by sequentially adding 5 additional lysines at a time and performing a check point at each stage, *i.e.* by verifying the integrities of the 20-mer (H-K₂₀-NH₂ (**28**), Appendix 5.10, page 83), 25-mer (H-K₂₅-NH₂ (**29**), Appendix 5.6, page 77) and 30-mer (H-K₃₀-NH₂ (**1**), Appendix 5.7, page 80) peptides.

Each elongation step was performed with 0.05 mmol of resin on a 0.25 mmol scale and a double coupling procedure from residue 21, *i.e.* with an excess of reagents to resin of 20 fold up to K₂₀ (**28**) and 40 fold thereafter. The

double coupling was employed for the last 10 residues, to avoid potential aggregation as was seen in the MBHA resin method.

The H-K₃₀-NH₂ (**1**) sequence was then N-terminally modified with cysteine, according to a double coupling procedure to produce H-CK₃₀-NH₂ (**2**) (Appendix 5.8, page 81 5.9, page 82). This peptide was also successfully produced directly from H-K₂₀-NH₂ (**28**) (Appendix 5.11, page 84, Appendix 5.12, page 85) by using 0.05mmol of peptide resin, the 0.25mmol method and a double coupling procedure for the last six residues.

Purification of H-CK₃₀-NH₂ (**2**) by reverse phase HPLC proved difficult. Due to the significant presence of protonated amines in this peptide, it was considerably hydrophilic, and so had very little interaction with the C18 stationary phase, and therefore minimal separation. Separation was attempted on portions of the peptide using varying gradients and flow rates, none of which improved the separation of the peptide from its impurities.

2.6 K₃₀ PEGylation

Due to the unsuccessful purification by HPLC, purification was postponed to the final stage, after PEGylation, to produce mPEG-CK₃₀-NH₂ (**3**). PEG attachment was consequently performed with the crude peptide. As the PEGylated peptide would also be particularly hydrophilic, it was not expected that it could be purified by reverse-phase chromatography either, but this approach, with crude peptide (**2**), was favoured to secure enough material to investigate the PEGylation step. The final purification step, post-PEGylation, was planned by size-exclusion chromatography.

The PEGylation reaction was performed according to a reported procedure successfully implemented previously in our group.[43] MALDI-TOF MS analysis showed that in the case of H-CK₃₀-NH₂ (**2**) this method was unsuccessful as both the separate signals of the free peptide and unmodified

PEG could be identified, but no signal corresponding to the PEGylated peptide could be detected.

In order to ascertain what caused this method to fail, a different cysteine-modified peptide candidate, H-DLLGLCEQK-NH₂ (**30**) was subjected to the same reaction, but no PEGylation occurred either in this experiment.

Due to time constraints, further experiments could not be completed to resolve this problem; however, the pH of these reaction mixtures was recorded, and shown to be 3.86 in the case of H-CK₃₀-NH₂ (**2**) and 3.91 in the case of H-DLLGLCEQK-NH₂ (**30**). In the literature it has been shown that this reaction is only successful at a pH under 8 [36, 75], as hydrolysis of the maleimide linker can occur. Furthermore, all amines must be protonated in solution to avoid their reaction with the linker. No mention was found of effect of low pH, however in many papers the optimum pH is stated as 6-7.

2.7 P17 (Methods 4.8)

P17 (**5**) was successfully synthesised from a Rink amide MBHA resin, using a standard Fmoc/*t*-Bu procedure, with no double coupling except for Leu⁷. Generally double coupling after a proline residue is required, due to the termination of the growing peptide chain by an endocyclic secondary nitrogen. Secondary amines have increased nucleophilicity, but are sterically hindered. P17 (**5**) was cleaved on a small scale to reveal the crude peptide in excellent purity, by MALDI TOF MS (H⁺: 2188.4408 m/z, Na⁺: 2210.4901 m/z), Appendix 5.13, page 86. It is therefore a convenient cell permeable peptide candidate, for use as a possible delivery agent of siRNA.

2.8 Azido-Amino Acids (Methods 4.9)

Azido amino acids were designed as an alternate route for PEG attachment. Indeed azides and alkynes undergo a concerted, ring forming reaction, which allows the quantitative and selective ligation of complex, multifunctional,

molecules [41, 44-47]. This reaction has also been shown to have an excellent economy of atoms, and can be ideally suited for PEGylation. The application of this 'Click chemistry' reaction to the synthesis of mPEG-K₃₀-NH₂ (**31**) or mPEG-N^ε-K₃₁-NH₂ (**32**) was planned by using an azido-peptide, modified at either the α-nitrogen of N₃-K₃₀-NH₂ (**33**) or the ε-nitrogen of H-K(N₃)K₃₀-NH₂ (**33**) and an alkyne-functionalised PEG.

The reagent for diazo-transfer, imidazole-1-sulfonyl azide 1.HCl (**35**), was initially produced. This compound was conveniently synthesised, and its NMR data ¹H NMR [(400MHz, D₂O) δ: 7.453 (dd, 1H, H-4), 7.829 (dd, 1H, H-5), 9.054 (dd, 1H, H-2)], Appendix 5.15, and ¹³C NMR [(100MHz, D₂O) δ: 119.68, 124.84, 137.67], Appendix 5.16, page 88-89 were in agreement with the values reported in the literature [41]. The azido moiety was also clearly visible by IR (2168cm⁻¹), Appendix 5.14, page 87. This reagent is very useful, as it requires mild reaction conditions, is shelf stable, inexpensive to produce and provides a regiospecific azide product.

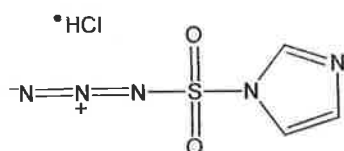


Fig. 34: Imidazole-1-Sulfonyl Azide Hydrochloride 1.HCl

The first amine to azide conversion reaction was performed with phenylalanine. N₃-Phe-OH (**9**) is potentially a very useful reagent for peptide chemistry, as its incorporation into the sequence can be measured quantitatively by UV using the signal of its aromatic ring. The azido acid of phenylalanine could not be obtained however under the experimental conditions investigated and only the H-Phe-OH (**8**) starting material could be identified during monitoring of the reaction by TLC.

The amine to azide conversion was then performed on Fmoc-Lys-OH (**7**), to produce Fmoc-Lys(N₃)-OH (**6**). There are two possible sites on lysine for conversion to the azide, the ε-amino group of the side chain, and the α-amino

function (N₃-Lys(Boc)-OH, **(36)**), in common with all other amino acids with the exception of proline. Here the ϵ -amino group was selected, as this residue acts as an extended linker, rather than forming part of the active K₃₀ peptide derivative. A linker can be useful when PEGylating a peptide, as it reduces the risk of steric hindrance by the polymer, when the active peptide must react with its target site.

Fmoc-Lys(N₃)-OH (**6**) was successfully synthesised, and its MS data (Na⁺ 416.9885 m/z), Appendix 5.18, page 91, and ¹H NMR spectrum [(CDCl₃, 400MHz) δ : 1.48 (m, 2H), 1.61 (m, 2H), 1.75 (m, 1H), 1.98 (m, 1H), 3.20 (t, 2H), 4.13-5.31 (5H), 7.22 (t, 2H), 7.32 (t, 2H), 7.39 (m, 1H), 7.45 (m, 1H), 7.68 (d, 2H)], Appendix 5.17, page 90 was in agreement with literature values [76]. Its application to the synthesis of a PEGylated K₃₀ derivative (mPEG-N ^{ϵ} -K₃₁-NH₂, **(32)**) by 'Click chemistry' was not undertaken, although the stepwise synthesis from a low substituted hydrophilic resin explored in parallel, afforded the synthesis of the required conjugate.

Application of the procedure developed in the work reported here was indeed subsequently successfully applied for the reliable and efficient production of the PEGylated 30-mer polylysine, by another member of the group, Dr. Stephane Desgranges, using the procedure outlined in this work, but removing TCEP before subsequent thiol-maleimide reaction.

CHAPTER 3 – CONCLUSIONS AND FUTURE WORK

H-CK₃₀-NH₂ (**2**) was successfully synthesised from a Rink amide NovaPEG LL resin, on a 0.05mmol scale, according to the Fmoc/*t*-Bu strategy, with a single coupling procedure for the first 24 cycles, using a 20-fold excess of amino acid and coupling reagents, followed by a double coupling procedure for the last six cycles, using a 40-fold excess of amino acid and coupling reagents overall.

Further experiments facilitated by the present work have shown that this synthesis can be performed on a 0.1mmol scale (S. Desgranges, unpublished results). This process can afford sufficient quantities of well characterised peptide which can be PEGylated to produce the target material, mPEG-K₃₀-NH₂ (**30**) in this case by the 'Click chemistry' approach. To the best of our knowledge, this is the first detailed and fully documented procedure for the synthesis of a PEGylated 30-mer poly-lysine reported to date.

Synthesis of H-CK₁₄K_cK₁₅-NH₂ (**37**), by use of an *N*-ethyl cysteine derivative (**24**) for the preparation of a poly-lysine thioester fragment (**15**), with subsequent native chemical ligation and pseudo-lysine (K_c, (**21**)) formation can potentially extend the application of the NCL approach, where the cysteine residue is converted to a lysine at the ligation site. An *N*-methyl cysteine (**38**) derivative could also be investigated as a C-terminal thioester precursor, as it was shown to be only marginally less effective than the *N*-ethyl derivative (**16**) [74]. Furthermore this compound is widely commercially available, avoiding the need for lengthy synthesis optimisation and purification.

Synthesis of H-K₃₀-NH₂ (**1**) in solution by coupling of protected fragments could also provide distinct advantages. Its yield after optimisation can be compared with those provided by the NCL and stepwise strategies. The availability of protected fragments could also be used to combine different fragments of cell penetrating peptide sequences to generate novel candidates

and compare their transfection efficiencies. However, possible racemisation of the C-terminal lysine in the *N*-terminal fragment would need to be studied.

Ligation by thioether formation through alkylation of cysteine by nucleophilic substitution could also be a viable option for the synthesis of PEGylated poly-lysine. As the H-K₁₅C-NH₂ fragment (**13**) could not be synthesised in good purity, but since the preparation of the H-CK₁₅-NH₂ (**12**) peptide was reliable, the strategy could be modified to undertake the conjugation of a chloromethyl ketone poly-lysine peptide (H-K₁₅-MeCl (**39**)) with an *N*-terminally cysteine-modified poly-lysine fragment (**12**). This reaction would produce the thioether H-K₁₅-Me-S-CK₁₅-OH (**40**). In this case the reaction scheme would be as follows:

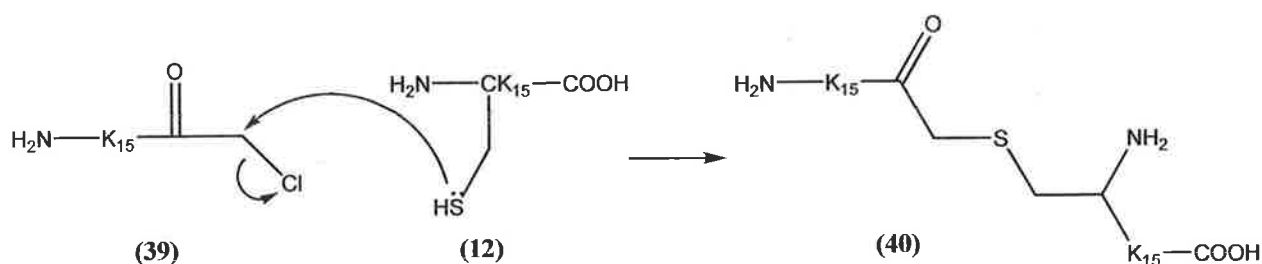


Fig. 35: Reaction of a chloromethyl ketone peptide fragment with a cysteine-modified peptide fragment

Peptide synthesis is an expensive process and H-CK₃₀-NH₂ (**2**) is a relatively long peptide. The decrease in yield is compounded with every additional coupling step. It therefore stands to reason that coupling of two 15mer chains could produce higher yields than one 30mer chain. Although the reduced yields due to protected fragment cleavage would also need to be taken into account. If K₃₀ (**1**) proves to be an efficient delivery agent of siRNA, as has been shown with DNA, it will be important to find the most cost effective method of its production.

An alternative PEGylation approach can also be attempted by 'Click chemistry' of an alkynyl PEG with an azido-peptide. This latter approach provides a novel compound, and PEGylation by 'Click chemistry' may prove to be a useful alternative to maleimide-thiol conjugation. The triazole ring

produced by the cyclo-addition reaction of an azide and a terminal alkyne can furthermore be considered as a peptide bond isostere and constitutes a linker which integrates better in the peptide structure than the thioether linkage based on pyrrolidine-2,5-dione.

Future studies with PEGylated K₃₀ could also investigate the attachment of targeting molecules to the composite to increase the occurrence of the therapeutic agent at its target site. Attachment of other polymers could also be studied, to compare their transfection efficiency and bioavailability. Composites containing P17 (**5**) as the transfecting peptide, could also be studied, and its effectiveness and mode of action compared to K₃₀ (**1**) and other CPPs, such as oligo-arginine.

CHAPTER 4 – MATERIALS AND METHODS

4.1 Solid Phase Synthesis by Fmoc/t-Bu Chemistry

Peptide synthesis was performed by Solid Phase Peptide Synthesis [77], according to the Fmoc/t-Bu protocol [78] with HBTU/HOBt/DIEA coupling chemistry in NMP solvent. Single coupling cycles, using a 10-fold excess of Fmoc-amino acid derivatives to resin-bound peptide were employed. The syntheses were carried out on a 1.0×10^{-4} mol scale (Fast Fmoc 0.1mmol method). Alternatively double coupling cycles, using a 4-fold excess of Fmoc-amino acid derivatives were employed on a 2.5×10^{-4} mol resin scale (Fast Fmoc 0.25mmol method).

Assembly of the amino acid sequence, starting from a Rink Amide MBHA resin was carried out on an automated peptide synthesizer (Applied Biosystems 433A, Warrington, UK). The resin is swelled and washed between reactions with DCM and NMP, the incoming amino acid is deprotonated with DIEA in NMP then activated and coupled with HOBt and HBTU. After coupling the Fmoc group is removed with piperidine. The synthesiser can be programmed to run a synthesis on either the standard 0.1mmol resin scale (Fast Fmoc 0.1mmol method) or 0.25mmol scale (Fast Fmoc 0.25mmol method).

Side chains were deprotected and the peptide cleaved from the synthesis resin using a mixture of either

- (i) 75% TFA, 10% H₂O, 10% TIS, 5% TA
- (ii) 70% TFA, 10% H₂O, 10% TIS, 5% TA, 5% EDT for side chain deprotection of sulfur-containing peptides (Cys, Met)

at room temperature for 2-3h. Peptides were precipitated and washed three times with 10 ml portions of diethyl ether. They were then dried, dissolved in distilled water and lyophilized.

Chromatographic analysis and purification were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (*PerSeptive Biosystems*) using Gemini columns (Phenomenex, 110Å, C18, 4.6mmd/250mL or 100mmd/250mL, for the analytical or semi-preparative columns, respectively) with single wavelength detection at 214 nm.

Purified peptides were finally characterised by MALDI TOF MS using the α -cyano-4-hydroxy-cinnamic acid matrix.

4.2 Standard Peptide Cleavage and Deprotection

Standard cleavage for all peptides on small aliquot scale (~0.02mmol), unless otherwise stated was: (1mL TFA, 150 μ L H₂O, 150 μ L TIS, 75 μ L TA) for peptides with no sulfur-containing amino acids (Cys, Met), otherwise (1mL TFA, 150 μ L H₂O, 150 μ L TIS, 75 μ L TA, 75 μ L EDT). Cleavage volume was multiplied by five in the same ratio for standard full cleavage.

Once deprotected, peptides were precipitated from the cleavage solution by addition of cold ether. After centrifugation the ether was decanted and the process repeated 2-3 times. The peptide was then dissolved in water and the resulting solution frozen in liquid nitrogen. The solution was then lyophilised to produce the peptide in a powdered, more soluble form.

4.3 Stepwise Synthesis I: K₃₀ from an MBHA resin

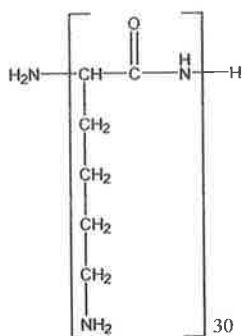


Fig. 36: H-K₃₀-NH₂ (1)

4.31 H-K₃₀-NH₂ (1) - Route 1

(i) H-K₁₅-NH₂ (10)

The Fast Fmoc 0.1mmol coupling method was performed with a Rink amide MBHA resin (0.1mmol, 0.7mmol/g substitution), using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and a single coupling chemistry. The elongation of the sequence was stopped after 15 cycles, a small aliquot cleaved and the isolated product, H-K₁₅-NH₂ (**10**), analysed by MALDI-TOF MS (H⁺: 1939.0708 m/z, Na⁺: 1962.1239 m/z, K⁺: 1978.0737 m/z). See Appendix 5.1, page 74

(ii) H-K₃₀-NH₂

The synthesis of this peptide was attempted from the 15-mer resin-bound poly-lysine peptide by automated synthesis using the Fast Fmoc 0.1mmol method with Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and a double coupling chemistry for all cycles. Standard cleavage of small aliquot was performed for analysis by MALDI-TOF MS. No product retained.

4.32 H-C β AK₃₀-NH₂ (11) - Route 2

The Fast Fmoc 0.1mmol coupling method was performed with a Rink amide resin (0.1mmol, 0.7mmol g⁻¹ substitution). Assembly of the complete sequence H-C β AK₃₀-NH₂ was attempted by automated synthesis, using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle), Fmoc- β Ala-OH (**42**) (1mmol/cycle) and Fmoc-Cys(Trt)-OH (**43**) (1mmol/cycle), with a double coupling chemistry at each cycle after the 20th lysine. Standard cleavage of a small aliquot was performed for analytical purposes (MALDI-TOF MS). No product retained.

4.33 H-K₃₀-NH₂ (1) - Route 3

(i) H-K₃₀-NH₂ (1)

The Fast Fmoc 0.1mmol coupling method was performed with a Rink amide MBHA resin (0.1mmol, 0.7mmol g⁻¹ substitution), using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle), with a double coupling chemistry for cycles 21 to 30. Standard cleavage of a small aliquot was performed for analysis by MALDI-TOF MS. No product retained.

4.4 Fragment Coupling I

Chemoselective Ligation of Unprotected Fragments – A

Orthogonal Binding of H-K₁₅C-NH₂ (**13**) and BrAc-K₁₅-NH₂ (**14**)

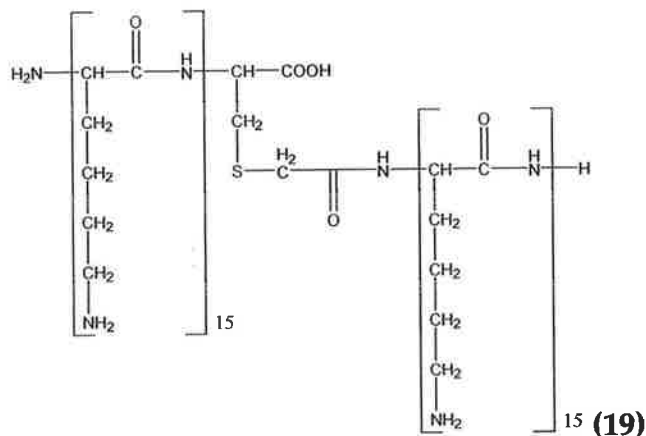


Fig. 37: H-K₁₅-thioether-K₁₅-NH₂ from Nucleophilic Substitution Reaction

(i) H-K₁₅C-NH₂ (**13**)

The Fast Fmoc method was performed on a 0.1mmol synthesis scale from a Rink amide MBHA resin (0.7mmolg⁻¹ substitution), using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and Fmoc-Cys(Trt)-OH (**43**) (1mmol/cycle) with a single coupling chemistry. Standard cleavage of a small aliquot was performed for analysis by MALDI-TOF MS. No product retained.

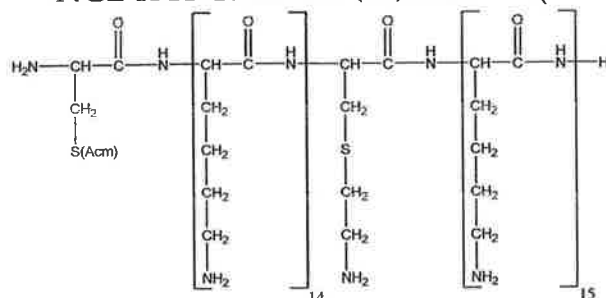
(ii) H-K₁₅-NH₂ (**10**)

This peptide was synthesised as previously described in method 4.31 (i).

(iii) BrAc-K₁₅-NH₂ (**14**)

A solution of HOBt (0.5mmol), HBTU (0.5mmol) and bromoacetic acid (0.5mmol) in DMF (4mL) was added to the resin-bound [K(Boc)]₁₅ peptide. DIEA (0.2mmol) was added to the solution which was then stirred for 3 hours. Standard cleavage of a small aliquot was performed for analytical purposes. No product retained.

Chemo selective Ligation of Unprotected Fragments – B:

NCL of H-CK₁₅-NH₂ (**12**) and H-C(Acm)K₁₄-SBnz (**15**)Fig. 38: H-K₁₄KcK₁₅-NH₂ Peptide from NCL reaction, containing central pseudo lysine residue (27)4.51 H-CK₁₄KcK₁₅-NH₂ - Route 1 (**36**)(i) H-CK₁₅-NH₂ (**12**)

The Fast Fmoc method was performed on a 0.1mmol scale from a Rink amide MBHA resin (0.7mmol/g substitution), using single coupling cycles with Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and Fmoc-Cys(Trt)-OH (**43**) (1mmol/cycle). Standard cleavage of a small aliquot was performed for analysis by MALDI-TOF MS confirmed (**12**) (H⁺: 2042.9569 m/z, Na⁺: 2065.9619 m/z, K⁺: 2081.7832 m/z). See Appendix 5.2, page 75.

(ii) Synthesis of H-C(Acm)[K(Boc)]₁₄-SBnz (**44**)

(a) Loading of first amino acid onto 4-sulfamylbutyryl NovaSyn TG resin:

In a 10ml round-bottomed flask, was introduced the 4-sulfamylbutyryl resin (0.12mmol, 461.5mg, substitution 0.26mmol/g), CHCl₃ (5.48mL, filtered through alumina base plug), DIEA (105μL, 0.6mmol), and Fmoc-Lys(Boc)-OH (**41**) (0.36mmol, 168.678mg). The mixture was stirred for 10mins, and then cooled to -20 °C in an acetone bath. After 20min, PyBOP (0.36mmol, 187.308mg) added. The solution was stirred for 8hr at -20 °C, and then warmed to room temperature overnight. The resin was filtered, washed with CHCl₃ (3 x 5mL, 10min), and resubjected to the same coupling cycle.[79-81]. Complete coupling was measured by Kaiser test (method 4.10).

(b) Sequence assembly from the Fmoc-Lys(Boc)-preloaded resin:

The 4-sulfamylbutyryl NovaSyn TG resin, preloaded with Fmoc-Lys(Boc) (0.1mmol, 0.26mmol⁻¹ substitution) was used for automated peptide synthesis. The Fast Fmoc method was performed on a 0.1mmol scale, using single coupling chemistry with Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and Fmoc-Cys(Acm)-OH (**45**) ((1mmol/cycle).

(iii) Cleavage of H-C(Acm)K₁₄-SBnz – A (**15**)

(a) Activation of the Linker:

NMP (2mL), DIEA (100 μ L, 0.66mmol) were added to the resin-bound peptide (200mg, 0.06mmol). ICH₂CN (108 μ L, 1.5mmol), prefiltered through a plug of basic alumina, was added to the reaction mixture which was protected from light. The resin was agitated for 24hr, filtered, washed with NMP (5 x 3mL, 10min/wash), DCM (3 x 3mL), and THF (3 x 3mL), and then transferred to a 50mL round-bottomed flask.

(b) Cleavage of H-C(Acm)[K(Boc)]₁₄-SBnz (**15**) from resin:

THF (1.8mL), benzylmercaptan (240 μ L, 2.04mmol), and NaSPh (cat.) (0.25mg, 2 μ mol) were added to the flask. The resulting mixture was stirred for 24hr. The resin (0.06mmol) was then separated from the solution by filtration and washed with DCM (3 x 3mL). The filtrates were combined and concentrated [80, 81]

(c) H-C(Acm)K₁₄-SBnz (**15**):

Side chain deprotection was performed by a TFA/water/TIS/TA (2.5ml, 375 μ L, 375 μ L, 187.5 μ L) (100/15/15/7.5) cleavage cocktail, the reaction was performed for 3 hours. The peptide isolated from this cleavage solution was analysed by MALDI-TOF MS. No product retained.

(iv) Cleavage of H-C(Acm)K₁₄-SBnz – B (**15**)

(a) Activation of the linker:

NMP (2mL) and DIEA (100 μ L, 0.33mmol) were added to the resin-bound peptide (200mg, 0.06mmol), followed by ICH₂CN (108 μ L, 1.5mmol), prefiltered through a plug of basic alumina, under the exclusion of light. The resin was

agitated for 24hr, filtered, washed with NMP (5 x 3mL, 10min/wash), DCM (3 x 3mL), and THF (3 x 3mL), and then transferred to a 50mL round-bottom flask.

(b) Cleavage of H-C(Acm)[K(Boc)]₁₄-SBnz from resin: **(44)**

THF (1.8mL), benzylmercaptan (240μL, 2.04mmol), and NaSPh (cat.) (0.25mg, 2μmol) were added to the flask containing the activated 4-sulfamylbutyryl NovaSyn TG resin. The mixture was stirred for 24hr. The resin (0.03mmol) was then separated from the solution by filtration and washed with DCM (3 x 3mL). The filtrates were combined and concentrated [57, 58]

(c) H-C(Acm)K₁₄-SBnz **(15)**

Side chain deprotection was performed by a mixture of TFA/water/TIS/TA (2.5ml, 563μL, 375μL, 187.5μL) (100/15/15/7.5) was used and the reaction was performed for 3hrs. The peptide isolated by precipitation was analysed by MALDI-TOF MS. No product retained.

4.52 H-CK₁₄KcK₁₅-NH₂ - Route 2 (37)

(i) Synthesis of H-C[K(Boc)]₁₄-SBnz (44)

(a) Loading of first amino acid onto 4-sulfamylbutyryl NovaSyn TG resin:

The loading was performed as previously described in method 4.51 (ii) (a).

(b) Sequence assembly from the Fmoc-Lys(Boc)-preloaded resin

The preloaded 4-sulfamylbutyryl NovaSyn TG resin with Fmoc-Lys(Boc)-OH (0.1mmol, 0.26mmolg⁻¹ substitution), was used for automated peptide synthesis, using the Fast Fmoc method on a 0.25mmol synthesis scale and a double coupling procedure.

(ii) Cleavage of H-C(Acm)K₁₄-SBnz (15)

Cleavage was performed as by method 4.51 (iv). 20mg of crude peptide was obtained which was analysed by MALDI TOF MS. No product obtained.

4.53 H-CK₁₄KcK₁₅-NH₂ - Route 3 (37)

(i) Synthesis of N^α-ethyl, S-Trt Cysteine I (16)

(a) 10mL of a solution of 1% KOH in methanol, 10mL, was added under N₂ to H-Cys(Trt)-OH **(44)** (2mmols, 727mg). The solution was stirred with gentle

warming until complete dissolution was obtained. The solution was then cooled to 0-10°C and ethanal (2mmols, 88.1mg, 112.2µL) in methanol (5mL) was added rapidly. The reaction was performed for 5mins at 0°C then the product was precipitated with ether.

- (b) A 5-10% solution of the Schiff base, dissolved in methanol (778.3mg, 2mmol in 10mL) was placed in a three-necked flask fitted with a mechanical stirrer, a dropping funnel and a reflux condenser. A 2-10% solution of sodium borohydride, double the molar amount of Schiff base, was dissolved in methanol (152.3mg, 4mmol in 5ml), and added dropwise to the flask. Once the addition was complete, the solution was refluxed for a further 15min and then cooled (BP 65°C). The product was precipitated with ether and analysed by NMR. [82] No product retained.

(ii) Synthesis of N^{α} -ethyl, S-Trt Cysteine II (16)

A 5mL solution of 1% KOH in anhydrous methanol was added under N_2 to H-Cys(Trt)-OH (**46**) (2mmols, 727mg). The solution was stirred with gentle warming until complete dissolution was obtained. The solution was then cooled to 0-10°C and ethanal (2mmols, 88.1mg, 112.2µL) in anhydrous methanol (5ml) was added rapidly. The reaction was performed for 5mins at 0°C [83] A 2-10% solution of sodium borohydride, double the molar amount of Schiff base, was dissolved in anhydrous methanol (152.3mg, 4mmol in 5mL), and added dropwise to the flask. After complete addition, the solution was refluxed for a further 15min and then cooled. The product was isolated by precipitation with ether and analysed by NMR. No product retained.

(iii) Synthesis of N^{α} -ethyl, S-Trt Cysteine III (16)

To a suspension of H-Cys(Trt)-OH (**46**) (1.0 g, 2.8 mmol) and $NaBH_3CN$ (0.17 g, 2.8 mmol) in methanol (27 mL) was added acetaldehyde (0.23 mL, 4.1 mmol) and the resulting solution was stirred at room temperature for 1h. The solvent was then removed *in vacuo* and the isolated product was dissolved in 1-butanol, washed with water and brine, and the organic phase dried over

MgSO₄. The solvent was removed, again, *in vacuo* and the residual oil was purified by silica gel column chromatography (60g) using CHCl₃-CH₃OH (4:1) as the mobile phase. No product was retained.

(iv) Synthesis of N^α-ethyl, S-Trt Cysteine IV (16)

To a suspension of H-Cys(Trt)-OH (46) (1.0 g, 2.8 mmol) and NaBH₃CN (0.17 g, 2.8 mmol) in methanol (27 mL) was added acetaldehyde (0.23mL, 4.1 mmol) and the resulting solution was stirred at room temperature for 1h. After the solvent was removed *in vacuo*, the product was dissolved in 1-butanol and washed with water and brine, and the organic phase dried over MgSO₄. The solvent was removed, again, *in vacuo* and the residual oil was purified by silica gel column chromatography (60 g) using CHCl₃-CH₃OH (4:1) containing 1% AcOH as the mobile phase. 42mg of solid was recovered; TLC analysis showed that the reaction product still contained a majority of starting material. No product retained.

4.6 Fragment Coupling III

Convergent Synthesis of Fully Protected Fragments

Amide Coupling of Boc-C(Trt)[K(Boc)₁₅]-OH (17) and H-K(Boc)₁₅-NH₂ (18)

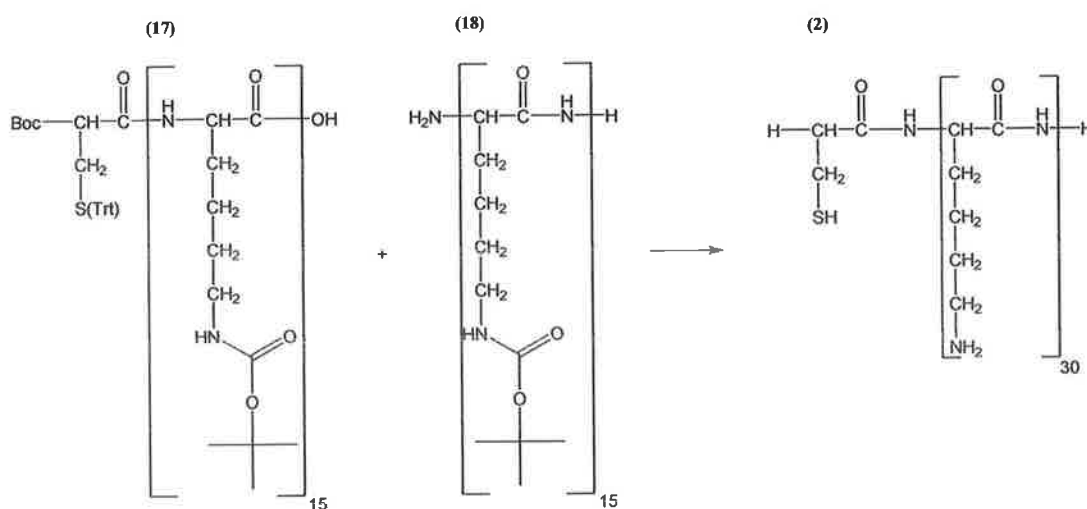


Fig. 39: K₃₀ Synthesis by Coupling of Protected K₁₅ Fragments

(i) Synthesis of H-[Lys(Boc)]₁₅-NH₂ (18)

Peptide assembly was performed from a Sieber amide resin (0.64mmol/g loading) on a 0.1mmol synthesis scale using the Fast Fmoc method and a single coupling chemistry. Cleavage of small aliquot was performed to produce the unprotected peptide, H-K₁₅-NH₂ (10) (for analysis by MALDI-TOF MS (H⁺: 1939.1220 m/z, Na⁺: 1960.9486 m/z). See Appendix 5.3, 5.4, page 76-77.

(ii) Synthesis of Boc-Cys(Trt)[Lys(Boc)]₁₅-OH (17)

(a) Preloading of the first lysine residue onto 2-chlorotrityl chloride resin:

All solvents and glassware were dried. Fmoc-Lys(Boc)-OH (39) (2eq, 0.26mmol, 1.218g) and DIEA (4eq relative to Lys, 1.04mmol, 997μL) were dissolved in dry DCM (10mL/g resin) and the resulting solution was added to the resin (0.13mmol, 77mg) under N₂. The mixture was stirred for 3hr, the resin isolated by filtration and then washed with DCM/MeOH/DIEA (17/2/1) three times, DCM three times, NMP two times, then DCM 2 times. The resin was dried under vacuum and complete loading verified by the Kaiser Test, indicated by colour change of deprotected aliquot.

(b) 0.1mmol of preloaded resin was used for automated solid phase synthesis using Fmoc-Lys(Boc)-OH (41) and Boc-Cys(Trt)-OH (47) and a single coupling procedure.

(c) The peptide was cleaved from resin by treatment with a cleavage cocktail consisting of TFE (1mL), AcOH (1mL) and DCM (3mL) for two hours. The resin was removed by filtration and washed three times with a fresh solution of cleavage mixture. Hexane (300mL) was added to the combined filtrates, to facilitate the evaporation of acetic acid by formation of an azeotrope. 0.1mmol of crude protected peptide was recovered after evaporation. Deprotection for characterisation not performed.

4.7 Stepwise Synthesis II: K₃₀ on NovaPEG LL resin (1)

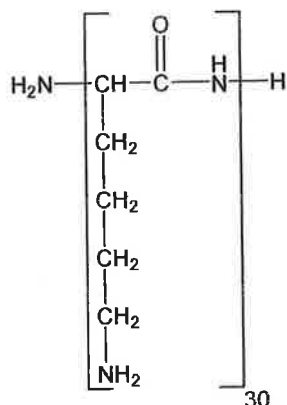


Fig. 40: H-K₃₀-NH₂ (1)

4.71 H-CK₃₀-NH₂ - Route 4 (2)

(i) H-K₁₅-NH₂ (10)

The Fast Fmoc coupling method was performed on a 0.1mmol synthesis scale with a Rink amide NovaPEG LL resin (0.1mmol, 0.17 mmolg⁻¹ substitution), using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and a single coupling chemistry. Cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. No product retained.

4.72 H-CK₃₀-NH₂ Route 5 (2)

(i) H-K₁₅-NH₂ (10)

The Fast Fmoc method was performed on a 0.25mmol synthesis scale with NovaPEG Rink amide resin LL (0.1mmol, substitution 0.17mmol/g), using Fmoc-Lys(Boc)-OH (1mmol/cycle) and a single coupling procedure. Cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. H-K₁₅-NH₂ (**10**) confirmed (H⁺: 1939.0919 m/z, Na⁺: 1961.0453 m/z, K⁺: 1977.0674 m/z). See Appendix 5.5, page 78

(ii) H-K₂₅-NH₂ (29)

The protected poly-lysine (15-mer) supported on the NovaPEG Rink amide resin LL (0.05mmol substitution 0.17mmol/g) obtained from method 4.72 (i) was placed in the 433A synthesiser. The Fast Fmoc method was performed on a 0.25mmol synthesis scale, using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle), and a

single coupling chemistry. Cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. H-K₂₅-NH₂ (**29**) confirmed: (H⁺: 3224.8197 m/z, Na⁺: 3247.0079 m/z). See Appendix 5.6, page 79.

(iii) H-K₃₀-NH₂ (**1**)

The protected poly-lysine (25-mer) supported on the NovaPEG Rink amide resin LL was introduced in the 433A synthesiser. The Fast Fmoc method was performed on a 0.25mmol synthesis scale, using Fmoc-Lys(Boc)-OH (**41**) (1mmol/cycle) and a double coupling procedure for the five incoming residues. Standard cleavage of an aliquot was performed for analysis by MALDI-TOF MS. H-K₃₀-NH₂ (**1**) confirmed (H⁺: 3866.8690, 2H⁺: 1934.0732 m/z). See Appendix 5.7, page 80.

(iv) H-CK₃₀-NH₂ (**2**)

The protected poly-lysine (30-mer) supported on the NovaPEG Rink amide resin LL was placed on the synthesiser. The Fast Fmoc method was performed on a 0.25mmol synthesis scale, using Fmoc-Cys(Trt)-OH (**46**) (1mmol/cycle), with a double coupling protocol. Standard cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS, H-CK₃₀-NH₂ (**2**) confirmed (H⁺: 3971.4185 m/z, 2H⁺: 1985.7368 m/z). See Appendix 5.8, page 81. Full scale cleavage of the peptide from the resin was then performed yielding 23mg of crude peptide (**2**).

The latter was purified by reverse phase HPLC using a Gemini 5micron (250x10.00mm) C18 100A column. Elution was performed with 98% H₂O (0.1% TFA), 2% MeCN (0.1% TFA) for 10mins followed to a gradient to 60% H₂O, 40% MeCN over 10mins, with a flow rate of 2mL/min. 10mg (**2**) was recovered, and analysed by MALDI MS (H⁺: 3967.4228 m/z, Na⁺: 4048.7722 m/z, K⁺: 4131.7363 m/z). Impurities were remaining in sample. See Appendix 5.9, page 82.

(v) mPEG-CK₃₀-NH₂ (3)

H-CK₃₀-NH₂ (2) (10mg) was dissolved in ethanol (125μL), acetonitrile (125μL), PBS buffer (50mM, 50μL, pH6.4); TCEP (1mg), and mPEG-mal (4) (11153g/mol, 57mg) were added and the solution was agitated overnight [43]. The isolated product was purified by reverse phase HPLC using Gemini 5micron (250x10.00mm) C18 100A column. The elution was performed with 98% H₂O (0.1% TFA) 2% MeCN (0.1% TFA) for 15min, followed by a gradient to 60% H₂O (0.1% TFA)/40% MeCN (0.1% TFA) over 30min then to 20 H₂O (0.1% TFA)//80 MeCN (0.1% TFA) over 15min, with a flow rate of 0.5mL/min. Fractions collected were lyophilised and analysed by MALDI-TOF MS. No product retained.

4.73 H-CK₃₀-NH₂ Route 6 (2)

(i) H-K₁₅-NH₂ (10)

The peptide was synthesised as described in method 4.72 (i)

(ii) H-K₂₀-NH₂ (28)

(iii) The protected poly-lysine (15-mer) supported on the NovaPEG Rink amide resin LL (0.05mmol) was introduced in the 433A synthesiser. The Fast Fmoc method was performed on the 0.25mmol synthesis scale, using Fmoc-Lys(Boc)-OH (41) (1mmol/cycle) and a single coupling chemistry. Standard cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. H-K₂₀-NH₂ (28) confirmed. (H⁺: 2581.3417 m/z, Na⁺: 2603.4265 m/z). See Appendix 5.10, page 83.

(iv) H-CK₃₀-NH₂ (2)

The protected poly-lysine (20-mer) supported on the NovaPEG Rink amide resin LL (0.05mmol) was placed on the 433A synthesiser. The Fast Fmoc method was performed on the 0.25mmol synthesis scale, using Fmoc-Lys(Boc)-OH (41) (1mmol/cycle) and Fmoc-Cys(Trt)-OH (46) (1mmol/cycle). The first five (lysine) residues were added by a single coupling protocol, while a double coupling chemistry was used for the next 5 lysine residues and the N-terminal

cysteine. Standard cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. H-CK₃₀-NH₂ (**2**) confirmed. (H⁺: 3970.6793 m/z, 2H⁺: 1985.7167 m/z). See Appendix 5.11, page 84. Full scale cleavage (0.05 mmol) was then performed, yielding 27mg of crude peptide (**2**) which was purified by reverse phase HPLC using Gemini 5micron (250x10.00mm) C18 100A column. A gradient 80% H₂O (0.1% TFA), 20% MeCN (0.1% TFA) to 35 H₂O (0.1% TFA)/65 MeCN (0.1% TFA) was performed over 30min with a flow rate of 4mL/min. Less than 1mg of purified material was recovered and analysed by MALDI-TOF MS (H⁺: 3971.1789 m/z), Appendix 5.12, page 85. Impurities were remaining in sample.

(v) mPEG-CK₃₀-NH₂ (**3**)

The PEGylation reaction was repeated on 3mg of material already subjected to this synthetic step (See Appendix 5.11) from method 4.72 (iv), using ethanol (84μL), acetonitrile (84μL), PBS buffer (50mM, 34μL, pH6.4), TCEP (1mg) and mPEG-mal (**4**) (11153g/mol, 17mg) [43]. Monitoring of the reaction was performed by MALDI-TOF MS, by sampling the reaction mixture at 4hours, 12 hours and 3 days. No PEGylation observed.

(vi) mPEG-DLLGLCEQK-NH₂ (**48**)

A sample of purified peptide DLLGLCEQK (**30**) (5mg, MW 1017.25) was used in a control experiment. This test was performed in an eppendorf with ethanol (250μL), acetonitrile (250μL), PBS buffer (50mM, 100μL, pH6.4), TCEP (2mg), mPEG-mal (11153g/mol, 100mg). The solution was agitated overnight, and a sample analysed by MALDI-TOF MS. No PEGylation observed.

4.8 P17 (H-KWKFKKLPKFLHLAKKF-NH₂) (**5**)

The Fast Fmoc method was performed on the 0.1mmol synthesis scale from a Rink amide resin (0.7mmolg⁻¹ substitution), single coupling chemistry was used except for Leu⁷, using 1mmol/cycle of each amino acid (Fmoc-Phe-OH (**49**), Fmoc-Lys(Boc)-OH (**41**), Fmoc-Ala-OH (**50**), Fmoc-Leu-OH (**51**), Fmoc-His(Trt)-

OH (**52**), Fmoc-Pro-OH (**53**), Fmoc-Trp(Boc)-OH (**54**)). Standard cleavage of an aliquot of resin was performed for analysis by MALDI-TOF MS. P17 (**5**) confirmed. (H^+ : 2188.4408, Na^+ : 2210.4901 m/z). See Appendix 5.13, page 86.

4.9 Azido-Functionalised Amino Acids

4.91 **Imidazole-1-sulfonyl azide hydrochloride (35)**

Sulfonyl chloride (16.1mL, 200mmol) was added dropwise to an ice-cold suspension of NaN_3 (13.0g, 200mmol) in MeCN (200mL) and the mixture stirred overnight at RT. Imidazole (25.9g, 380mmol) was added portionwise to the ice-cooled mixture and the resulting slurry stirred for three hours at RT. The mixture was then diluted with EtOAc (400mL), washed with H_2O (2 x 400mL) then saturated aqueous $NaHCO_3$ (2 x 400mL), the organic phase was dried over $MgSO_4$ and filtered. An acidified solution (obtained by dropwise addition of $AcCl$ (21.3mL, 300mmol) to ice-cooled dry ethanol (75mL) was added dropwise to the filtrate while stirring, the mixture was then cooled in an ice bath, filtered and the filtrate washed with EtOAc (3 x 100mL) to give the imidazole-1-sulfonyl azide hydrochloride (**35**) as colourless needles. Product confirmed by KBr IR (azide: $2168cm^{-1}$). Appendix 5.14, page 87, 1H NMR [(400MHz, D_2O) δ : 7.453 (dd, 1H, H-4), 7.829 (dd, 1H, H-5), 9.054 (dd, 1H, H-2)], Appendix 5.15, page 88 and ^{13}C NMR [(100MHz, D_2O) δ : 119.68, 124.84, 137.67], Appendix 5.16, page 89. NMR data were in agreement with literature values.[41]

4.92 **N_3 -Phe-OH (9)**

(i) N_3 -Phe-OH - Route 1 (9)

Imidazole-1-sulfonyl azide 1.HCl (**35**) (0.75g, 3.6mmol) was added to the H-Phe-OH (**8**) (500mg, 3mmol), K_2CO_3 (138mg, 7.1mmol) and $CuSO_4 \cdot 5H_2O$ (7.5mg, 30 μ mol in MeOH (15mL) and the mixture stirred at RT for 12hr. Mixture acidified with $KHSO_4$, then extracted with EtOAc. Fractions combined

and concentrated and purified by flash chromatography for analysis by IR and NMR. No product retained.

(ii) N₃-Phe-OH - Route 2 (9)

Imidazole-1-sulfonyl azide 1.HCl (**35**) (0.75g, 3.6mmol) was added to a solution of H-Phe-OH (**8**) (500mg, 3mmol), K₂CO₃ (138mg, 7.1mmol) and CuSO₄·5H₂O (7.5mg, 30μmol) in MeOH (15mL) and the mixture stirred at RT for 12hr. The mixture was concentrated under vacuum and then diluted with H₂O (45mL), acidified with conc. HCl and extracted with EtOAc (3x30mL). The combined organic layers were dried over MgSO₄, filtered and concentrated. Purification was performed by flash chromatography. No product retained.

4.93 Fmoc-Lys(N₃)-OH (6)

Imidazole-1-sulfonyl azide 1.HCl (**35**) (1g, 4.8mmol) was added to a solution of Fmoc-Lys-OH (**7**) (1.474g, 4mmol), K₂CO₃ (1.2577g, 9.1mmol) and CuSO₄·5H₂O (10mg, 40μmol) in MeOH (20mL) and the mixture stirred at RT overnight. The mixture was acidified, concentrated and then diluted with H₂O (60mL), acidified with conc. HCl and extracted with EtOAc (3x40mL). The combined organic layers were dried over MgSO₄, filtered and concentrated. The product (**6**) was confirmed by ¹H NMR [(CDCl₃, 400MHz) δ: 1.48 (m, 2H), 1.61 (m, 2H), 1.75 (m, 1H), 1.98 (m, 1H), 3.20 (t, 2H), 4.13-5.31 (5H), 7.22 (t, 2H), 7.32 (t, 2H), 7.39 (m, 1H), 7.45 (m, 1H), 7.68 (d, 2H)], see Appendix 5.17, page 90. The NMR data were in agreement with the literature values [76]. Sample also analysed by MALDI TOF MS (H⁺: 416.9885 m/z), see Appendix 5.18, page 91.

4.10 Kaiser Test

The Kaiser test is performed as follows:

Kaiser Solution A: 0.5 g ninhydrin in 10 mL ethanol.

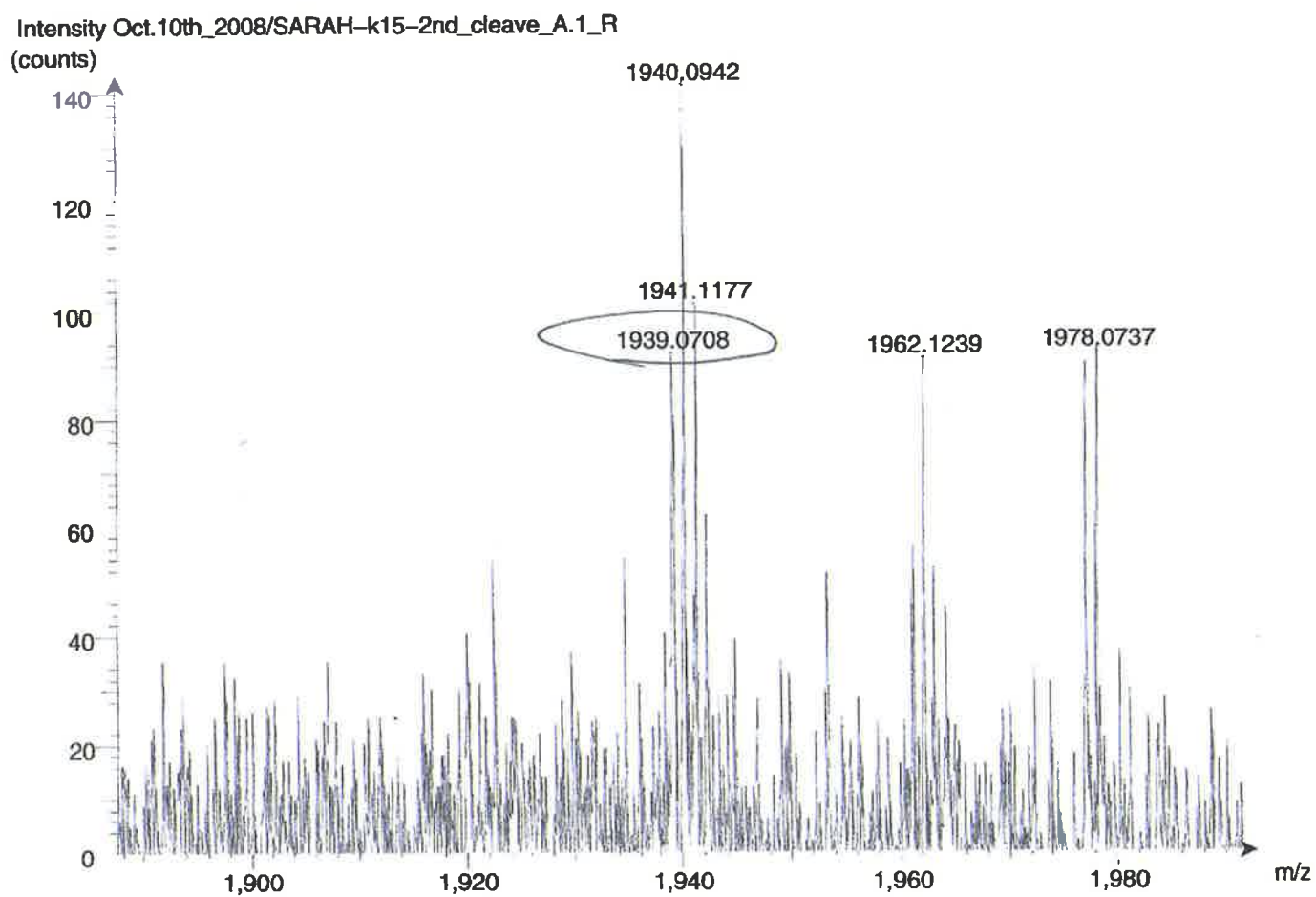
Kaiser Solution B: 0.4 mL of 0.001 M KCN aq. in 20 mL pyridine.

One drop of each solution is placed in a boiling tube with a few resin beads for five minutes at 100°C. [53, 84]

CHAPTER 5 – APPENDIX

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5.1 MALDI-TOF MS of H-K₁₅-NH₂ (10), method 4.31 (i)

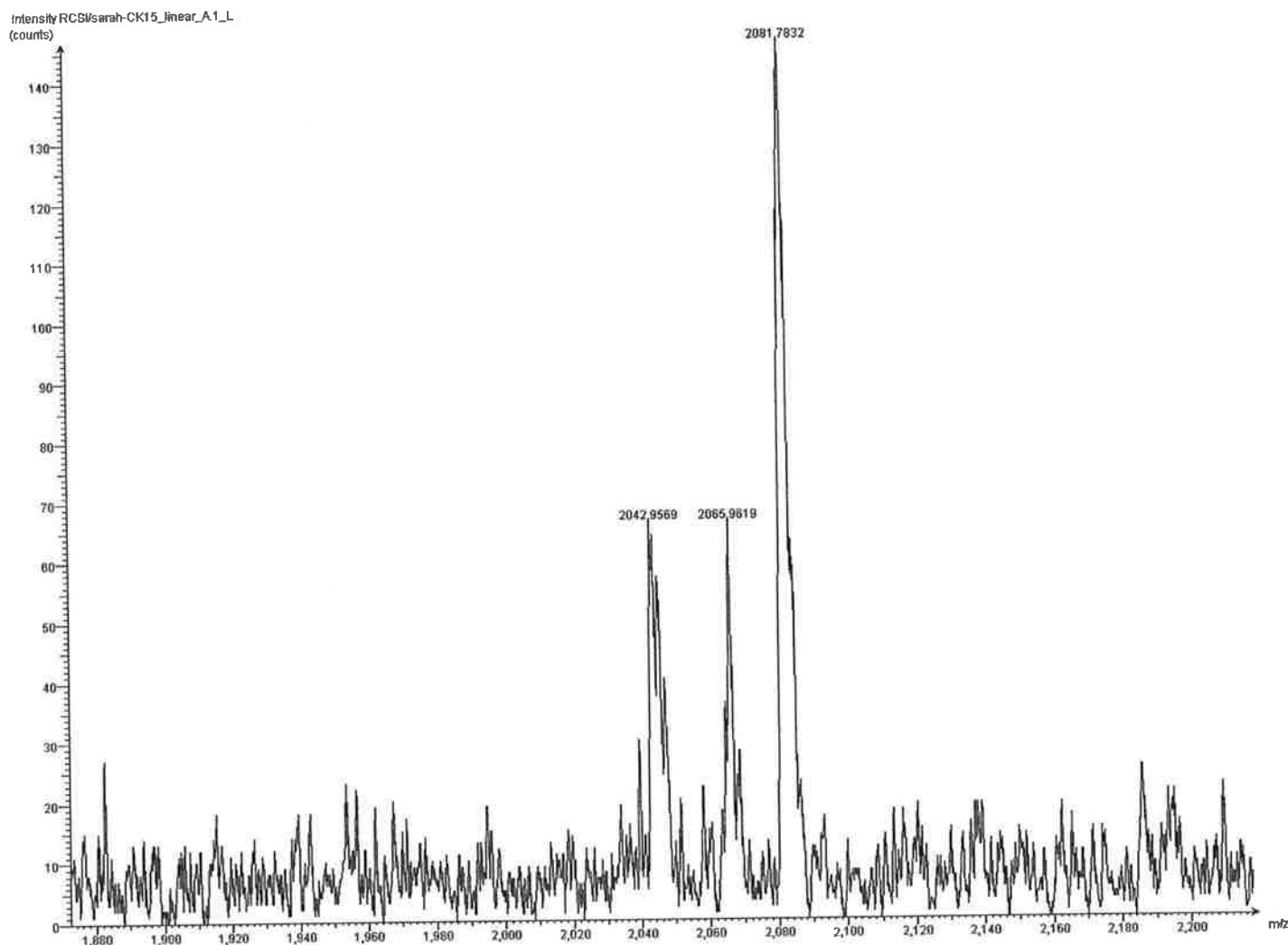


H-K₁₅-NH₂ MW – 1939.61 g/mol

Analysis performed on unpurified peptide.

MS (H⁺: 1940.0942 m/z, Na⁺: 1962.1239 m/z, K⁺: 1978.0737 m/z)

5.2 MALDI-TOF MS of H-CK₁₅-NH₂ (2), method 4.51 (i)

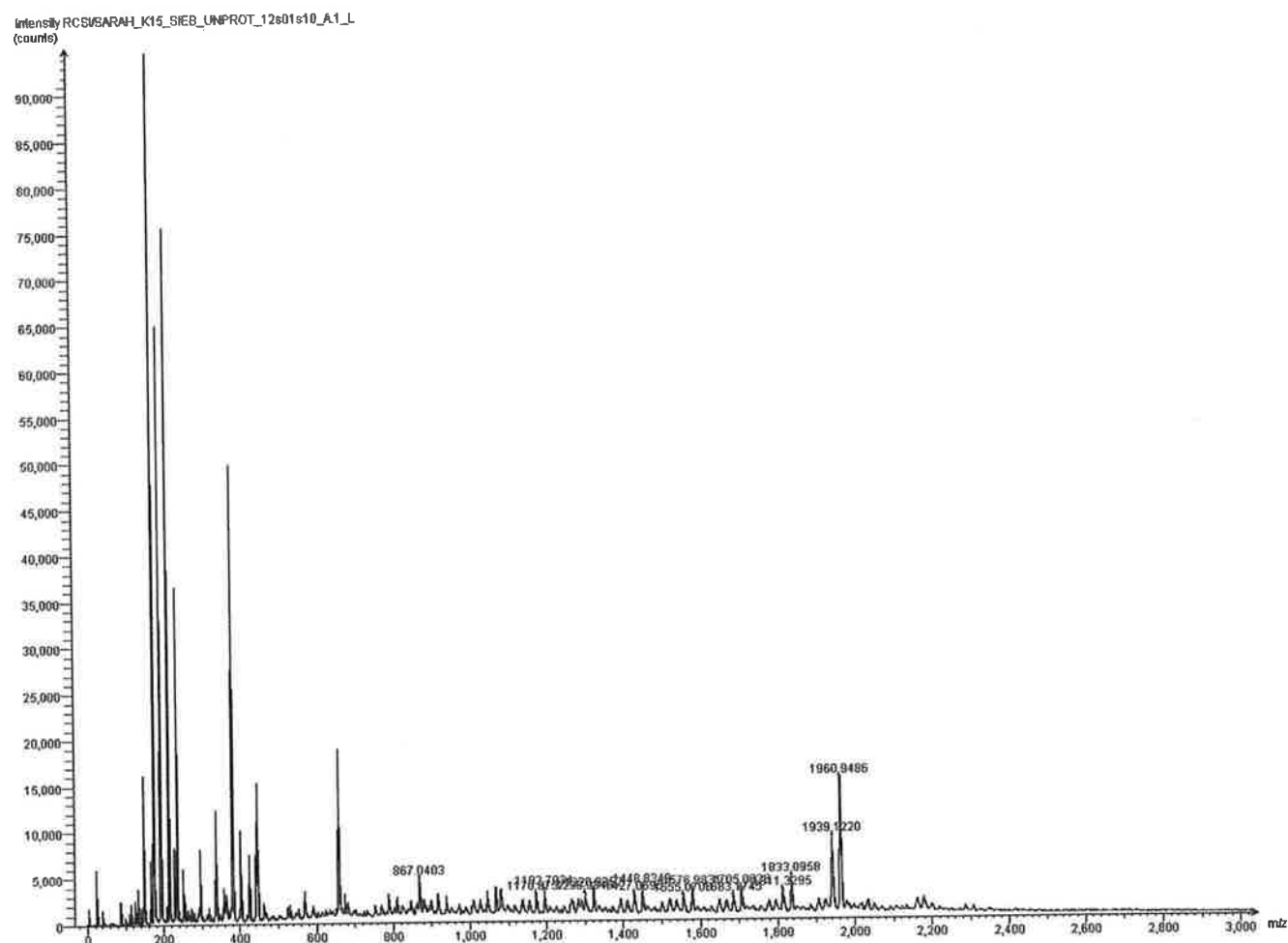


H-CK₁₅-NH₂ MW – 2042.76 g/mol

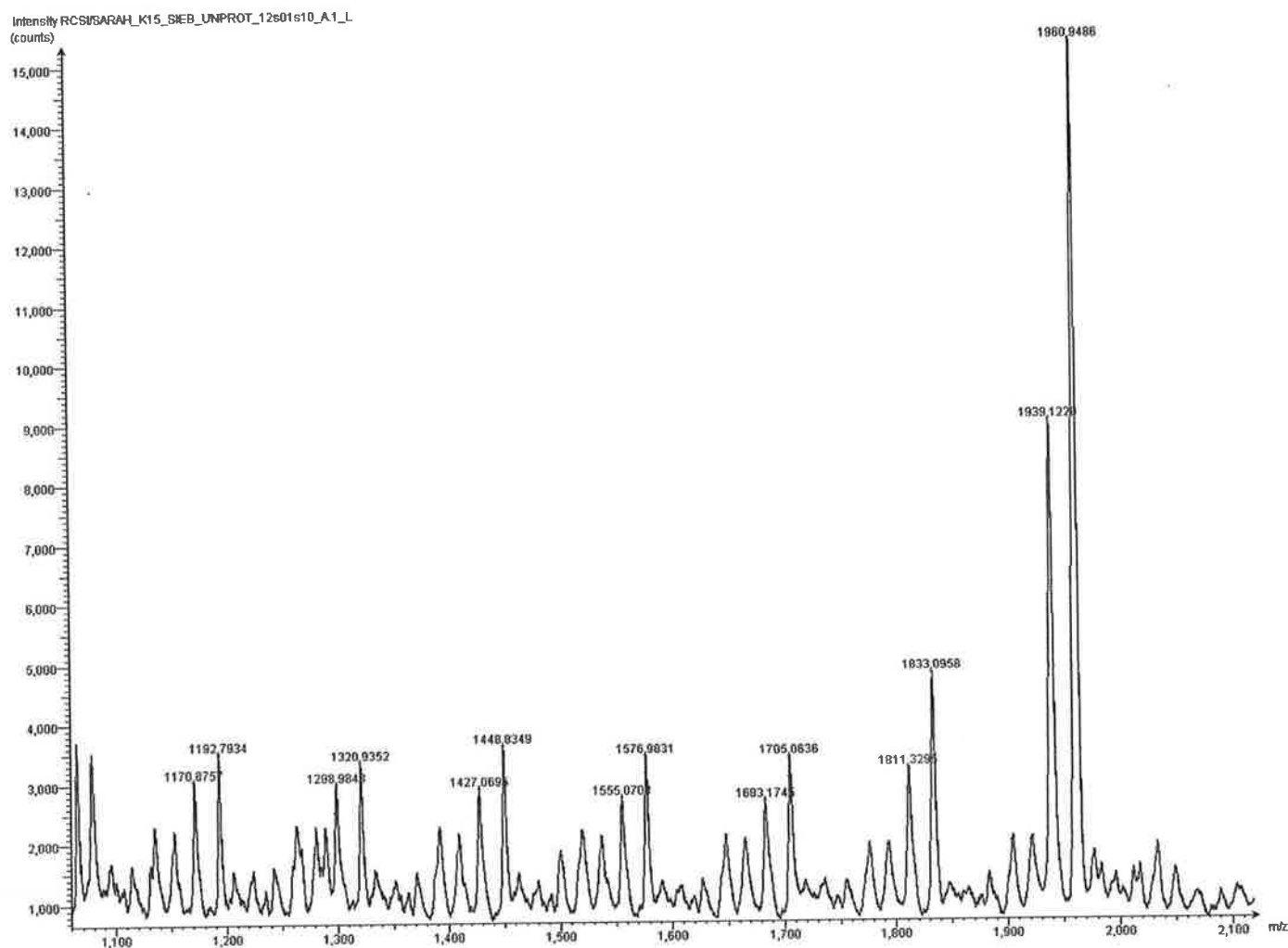
Analysis performed on unpurified peptide.

(H⁺: 2042.9569 m/z, Na⁺: 2065.9619 m/z, K⁺: 2081.7832 m/z)

5.3 MALDI-TOF MS of H-K₁₅-NH₂ (10), method 4.6 (i)



5.4

MALDI TOF MS of H-K₁₅-NH₂ (10), method 4.6 (i)

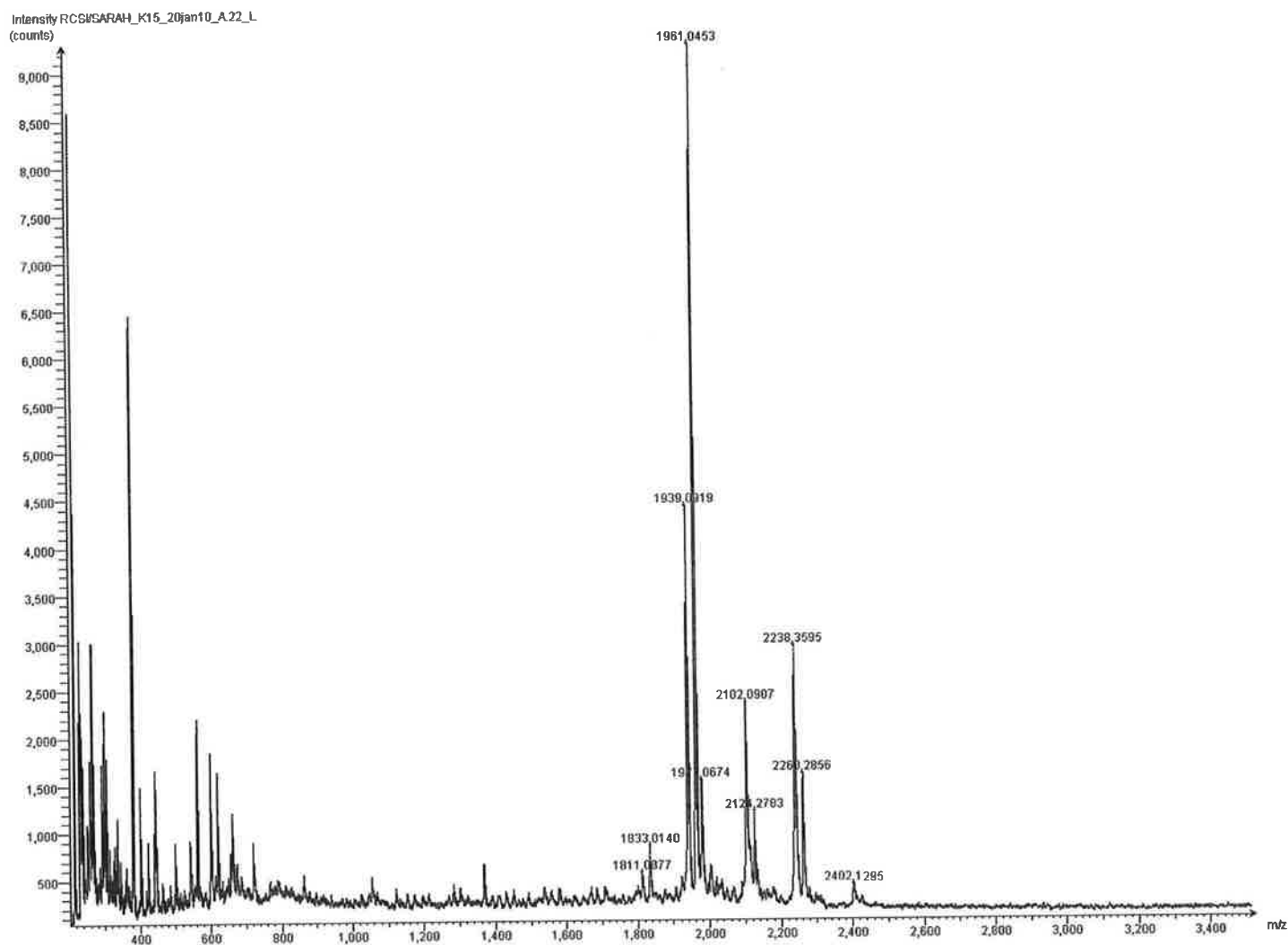
H-K₁₅-NH₂ MW 1939.61g/mol

Analysis performed on unpurified peptide.

(H⁺: 1939.1220 m/z, Na⁺: 1960.9486 m/z).

Note: Small occurrence of extra peaks corresponding to deletion peptides

5.5

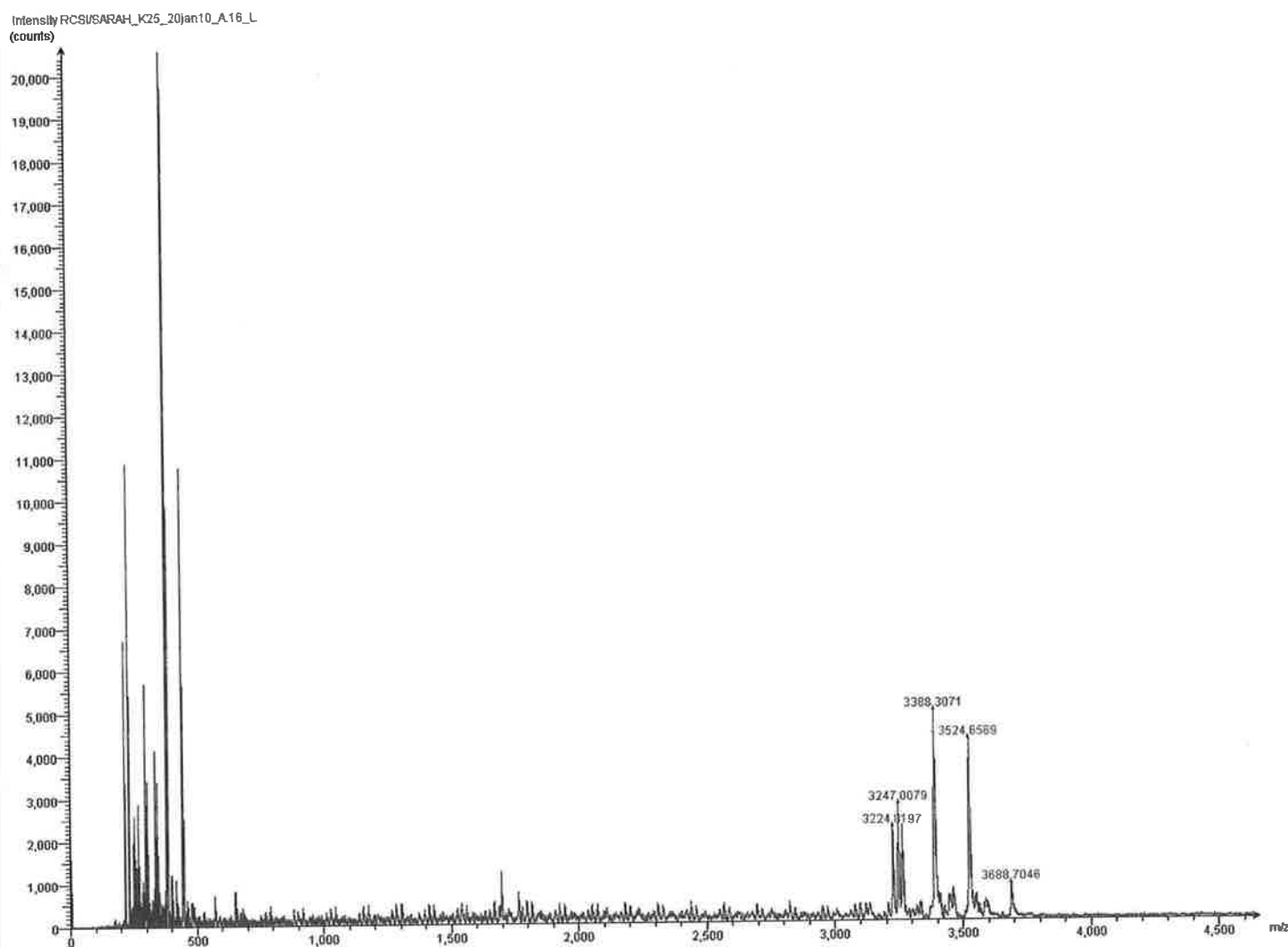
MALDI TOF MS of H-K₁₅-NH₂ (10), method 4.72 (i)

H-K₁₅-NH₂ MW 1939.61 g/mol

Analysis performed on unpurified peptide.

(H⁺: 1939.0919 m/z, Na⁺: 1961.0453 m/z, K⁺: 1977.0674 m/z)

Note: Occurrence of extra peaks above the expected mass of H-K₁₅-NH₂, most likely due to either unprotected side chains, and/or reattachment of reactive protecting groups. These peaks could not be definitively identified.

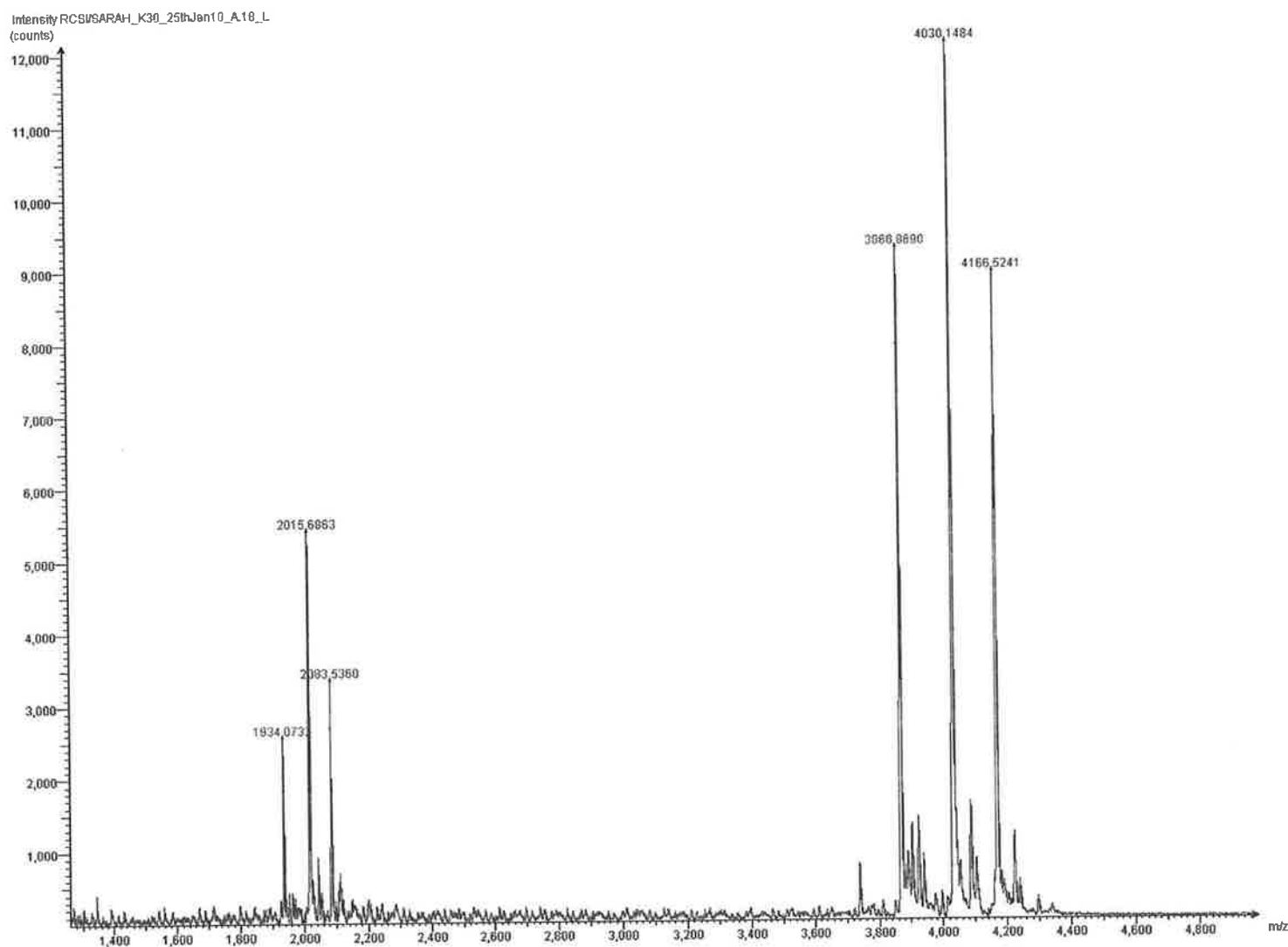


H-K₂₅-NH₂ MW 3221.34 g/mol

(H⁺: 3224.8197 m/z, Na⁺: 3247.0079 m/z).

Note: Small occurrence of deletion peptides, below 3224.8197 m/z and peaks larger than the expected total mass (3388.3071, 3524.6569, 3688.7046 m/z). These are most likely due to the cleavage process, with unprotected side chains and/or reattached reactive protecting groups, which could not be definitively assigned, however mass increase is equivalent to that found for H-K₁₅-NH₂ (10), Appendix 5.5 from method 4.72 (i) (163 and 299 m/z).

5.7

MALDI-TOF MS of H-K₃₀-NH₂ (1), method 4.72 (iii)

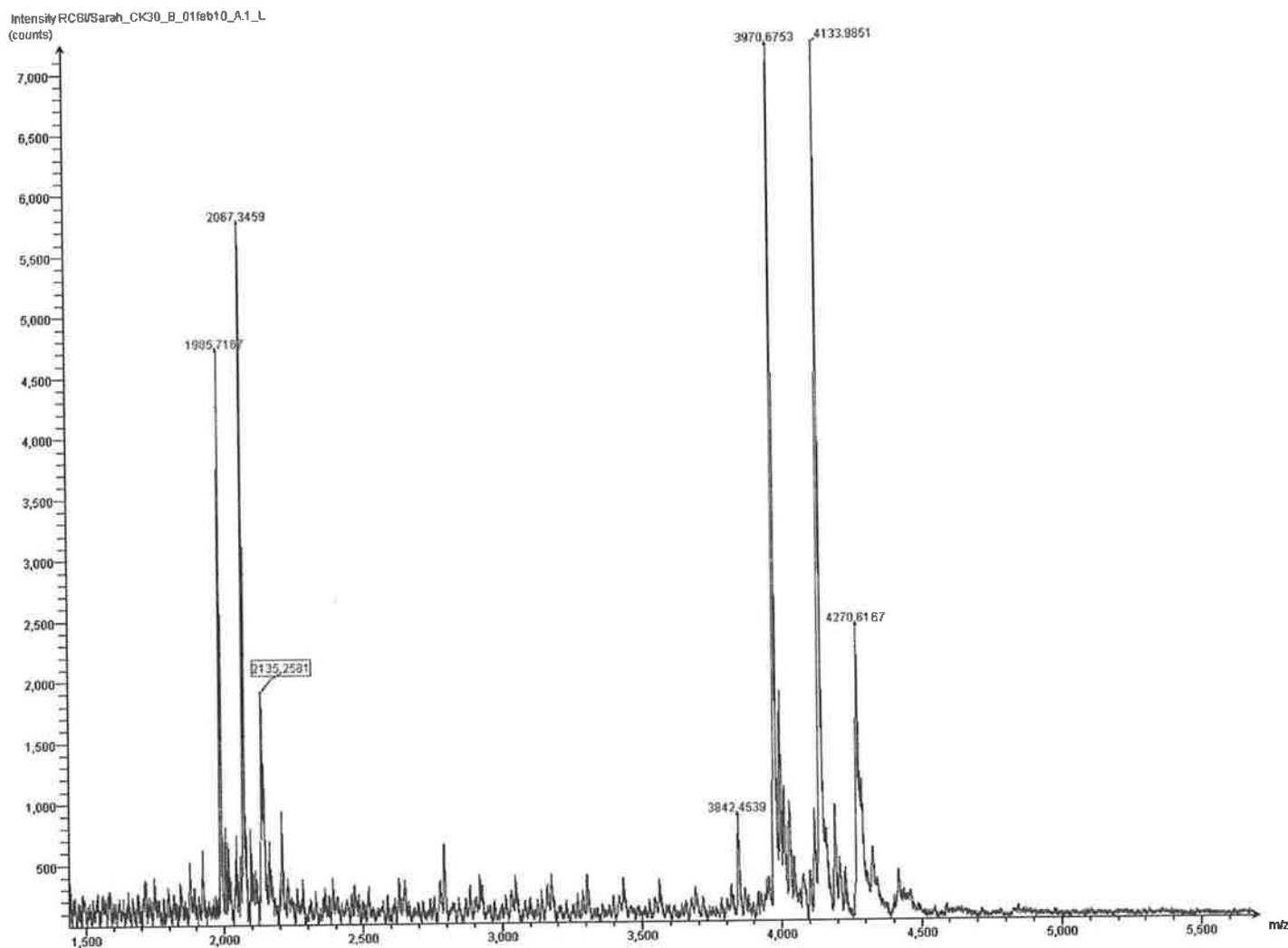
H-K₃₀-NH₂ MW 3862.20 g/mol

Analysis performed on unpurified peptide.

(H⁺:3866.8690 m/z, 2H⁺: 1934.0732, 2Na⁺: 2015.6863, 2K⁺: 2083.5360).

Note: MS displays half masses of the target peptide, through doubly charged moieties. Also, deletion peptides seem to be minimised, but there is still apparent increased-mass impurities, (4030.1484 m/z, 4166.5241 m/z), which also displayed half masses (2015.6863, 2083.5360 m/z). The mass increase is equivalent to those present in the precursors H-K₁₅-NH₂ (**10**) (Appendix 5.5) and H-K₂₅-NH₂ (**29**) (Appendix 5.6).

5.8

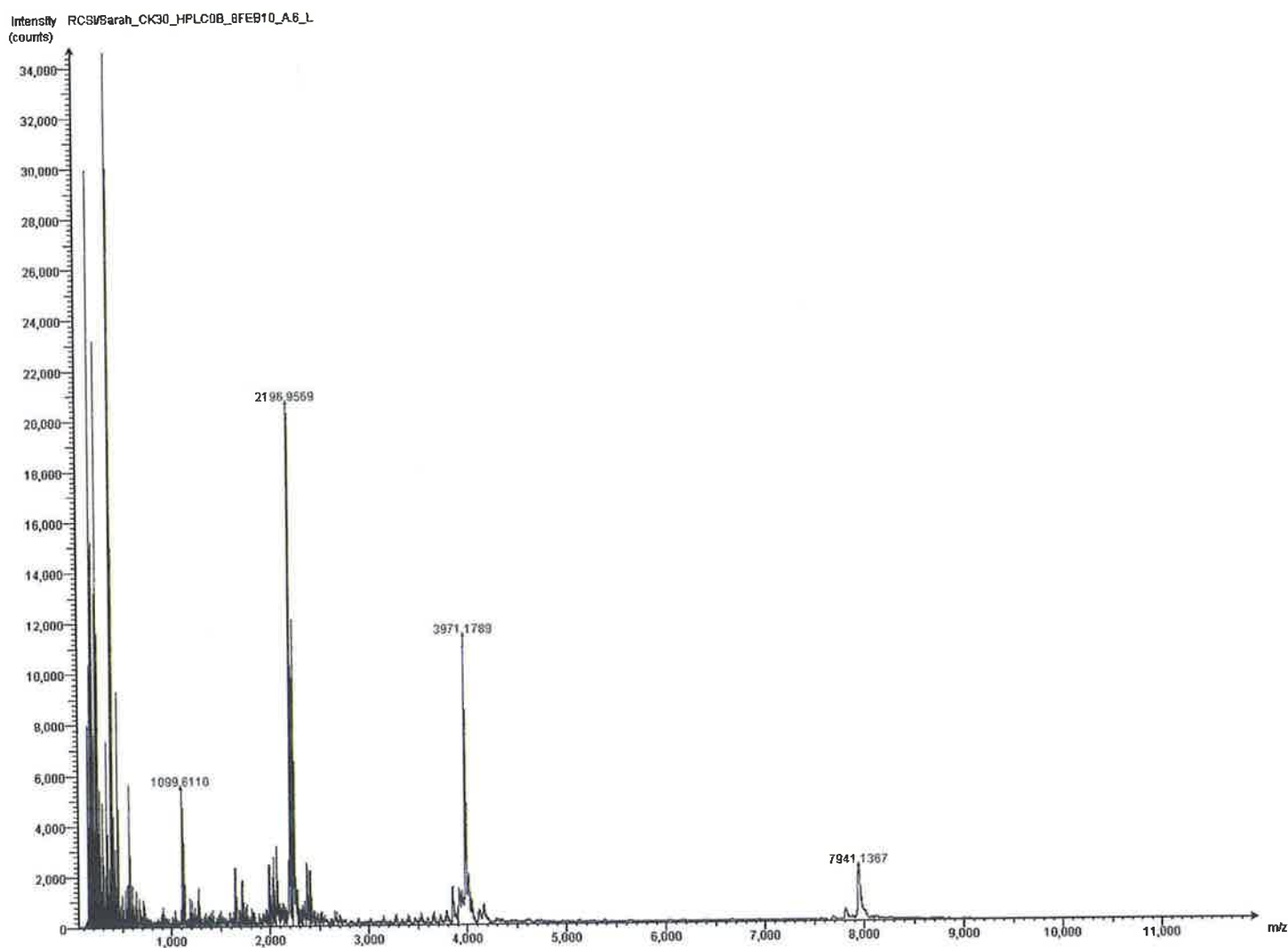
MALDI-TOF MS of H-CK₃₀-NH₂ (2), method 4.72 (iv)

H-CK₃₀-NH₂ MW – 3965.34 g/mol

Analysis performed on unpurified peptide.

(H⁺: 3970.6753 m/z, 2H⁺: 1985.7368 m/z)

Note: Occurrence of half mass by doubly charged peptide, and small occurrence of deletion peptides. However, there is considerable presence in increased-mass impurities (4133.9850, 4270.6167 m/z), as seen in Appendix 4.5-7. Their half masses were visible also (2067.3459, 2135 m/z).



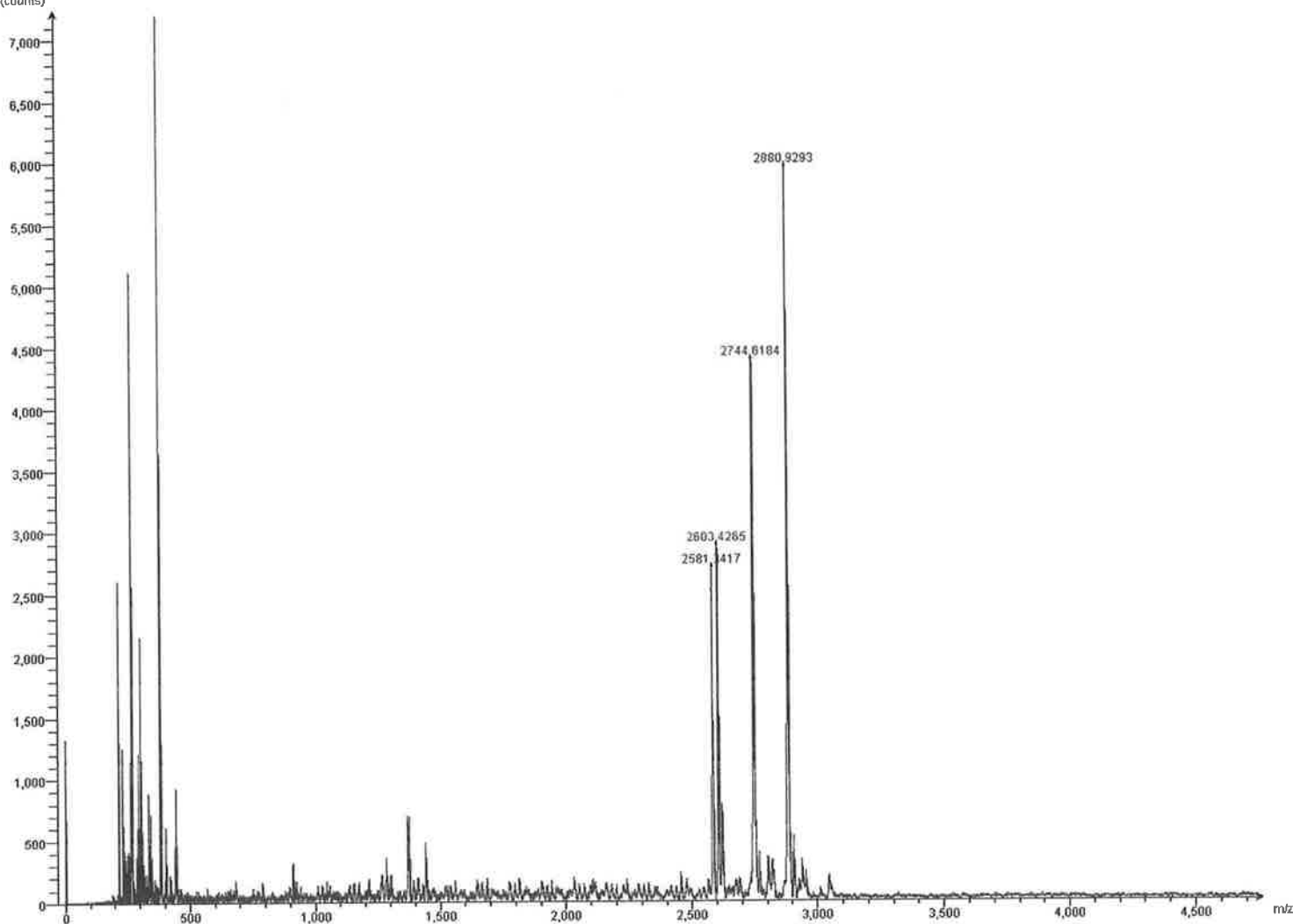
H-CK₃₀-NH₂ MW – 3965.34 g/mol

(H⁺: 3971.1789 m/z)

Note: Occurrence of peptide dimer at 7941.1367 m/z due to disulfide linkage between cysteine thiols. Peaks at 1099.6110 m/z and 2196.9565 m/z could not be identified. Increased mass peak were not seen here.

5.10 MALDI-TOF MS of H-K₂₀-NH₂ (28), method 4.73 (iii)

Intensity RCSVSARAH_K20_20jan10_A.20_L
(counts)



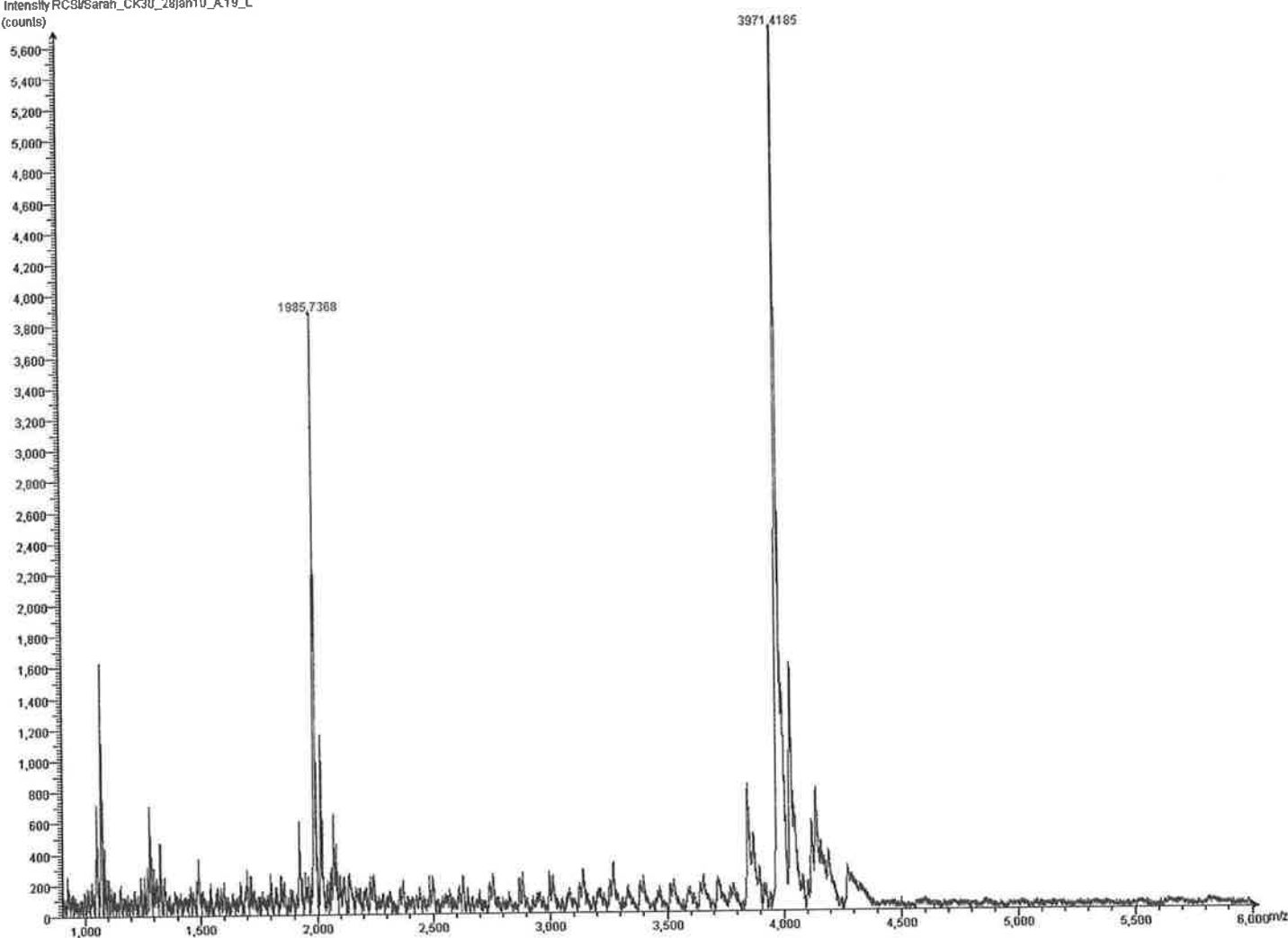
H-K₂₀-NH₂ MW – 2580.48 g/mol

Analysis performed on unpurified peptide.

(H⁺: 2581.3417 m/z, Na⁺: 2603.4265 m/z)

Note: Occurrence of impurities with increased-mass of same quantity as seen in Appendix 4.5-8. These peaks could not be definitively assigned.

5.11

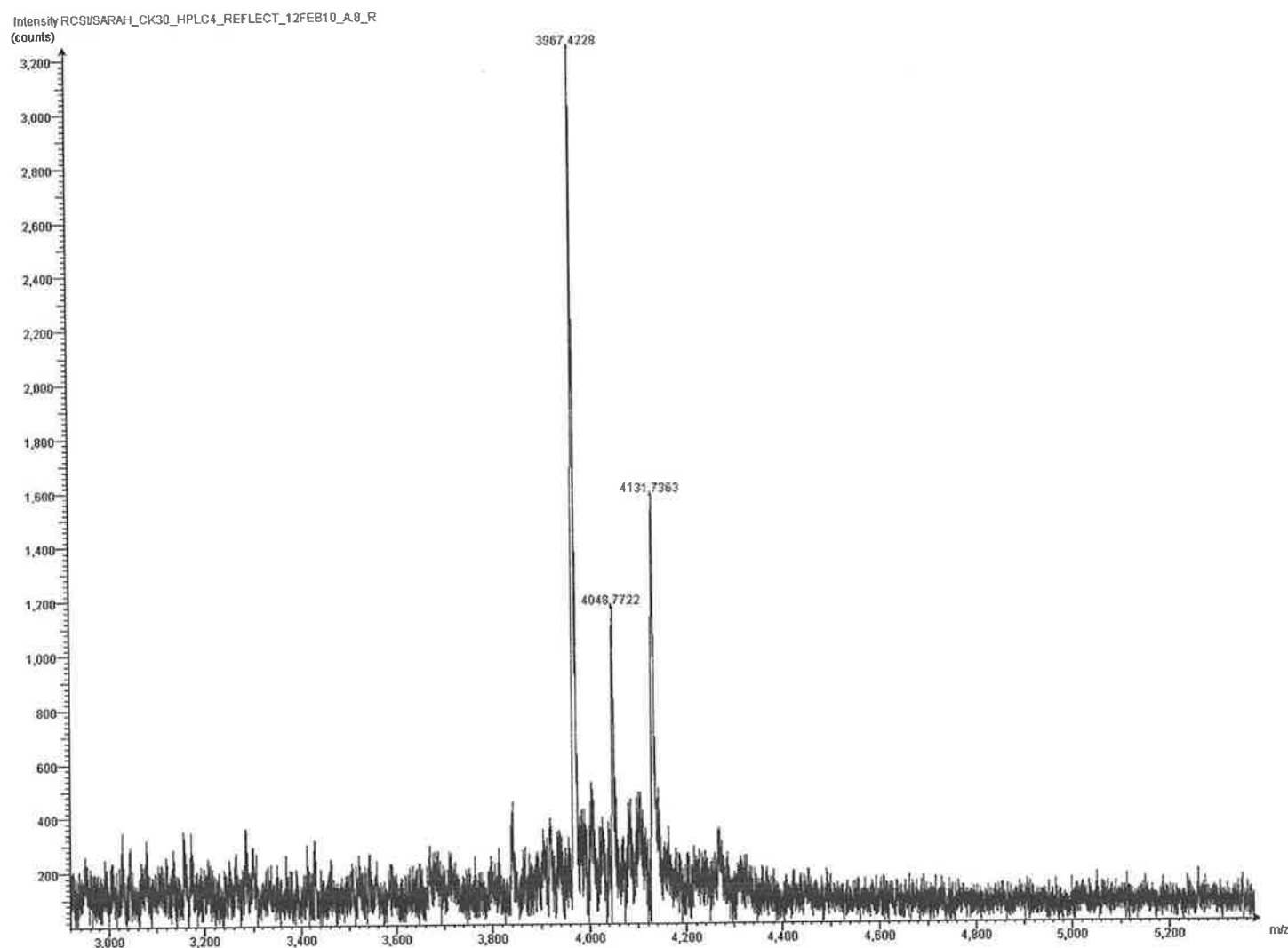
MALDI-TOF MS of H-CK₃₀-NH₂ (2), method 4.73 (iv)Intensity RCSI\Sarah_CK30_28\Jan10_A19_L
(counts)H-CK₃₀-NH₂ MW 3965.34g/mol

Analysis performed on unpurified peptide.

(H⁺: 3970.6793, 2H⁺: 1985.7167 m/z)

Note: Occurrence of a small number of deletion peptides. No occurrence of increased mass impurities.

5.12 MALDI-TOF MS of Purified H-CK₃₀-NH₂ (2), method 4.73 (iv)



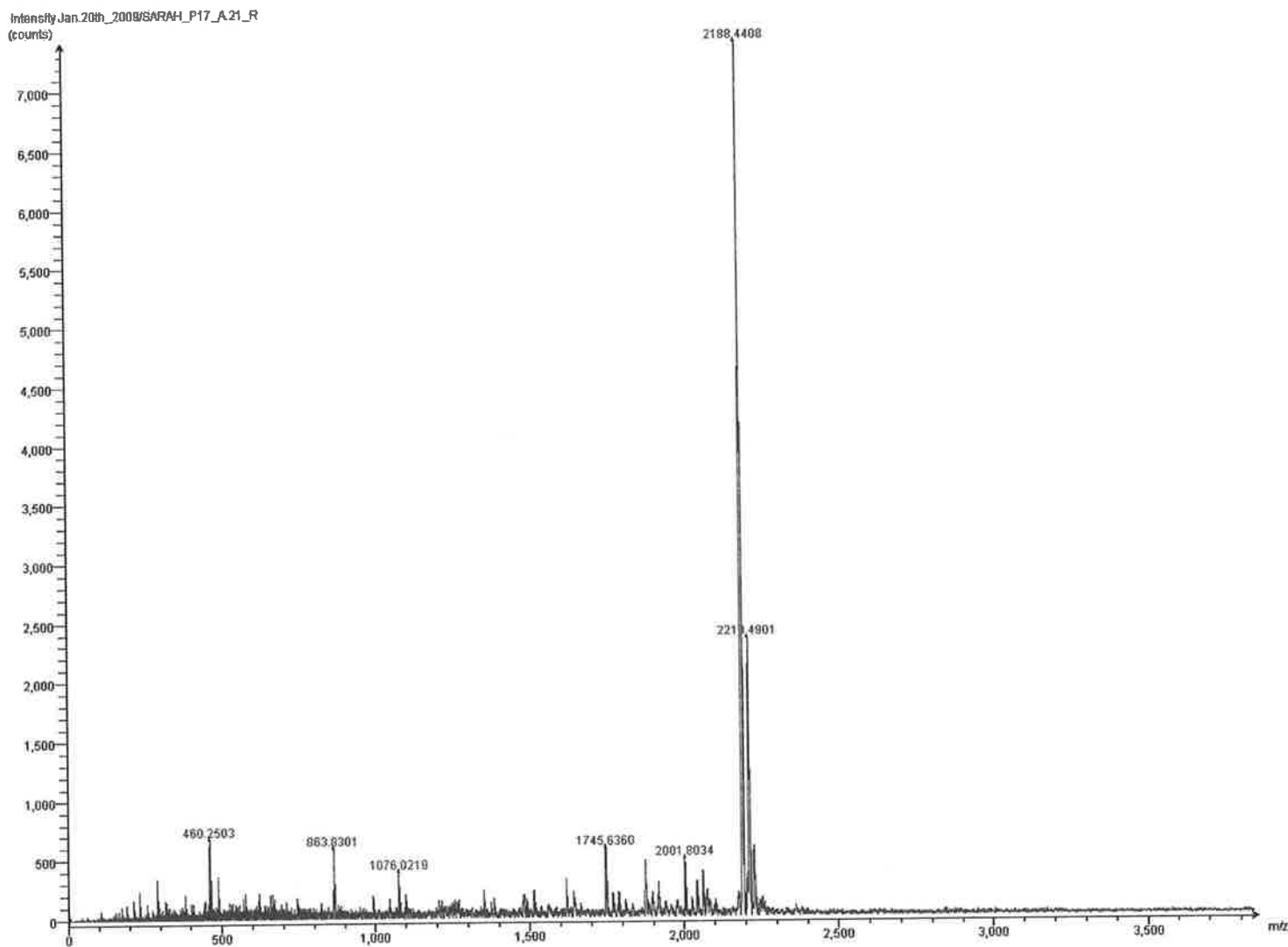
H-CK₃₀-NH₂ MW 3965.34g/mol

Analysis of purified peptide

(H⁺: 3971.1789 m/z)

Note: Occurrence of increased mass impurities (4048.7722, 4131.7363 m/z), with increase of 163 m/z (as before) and 81 m/z. Impurities could not be identified.

5.13. MALDI-TOF MS of P17 (5), method 4.8



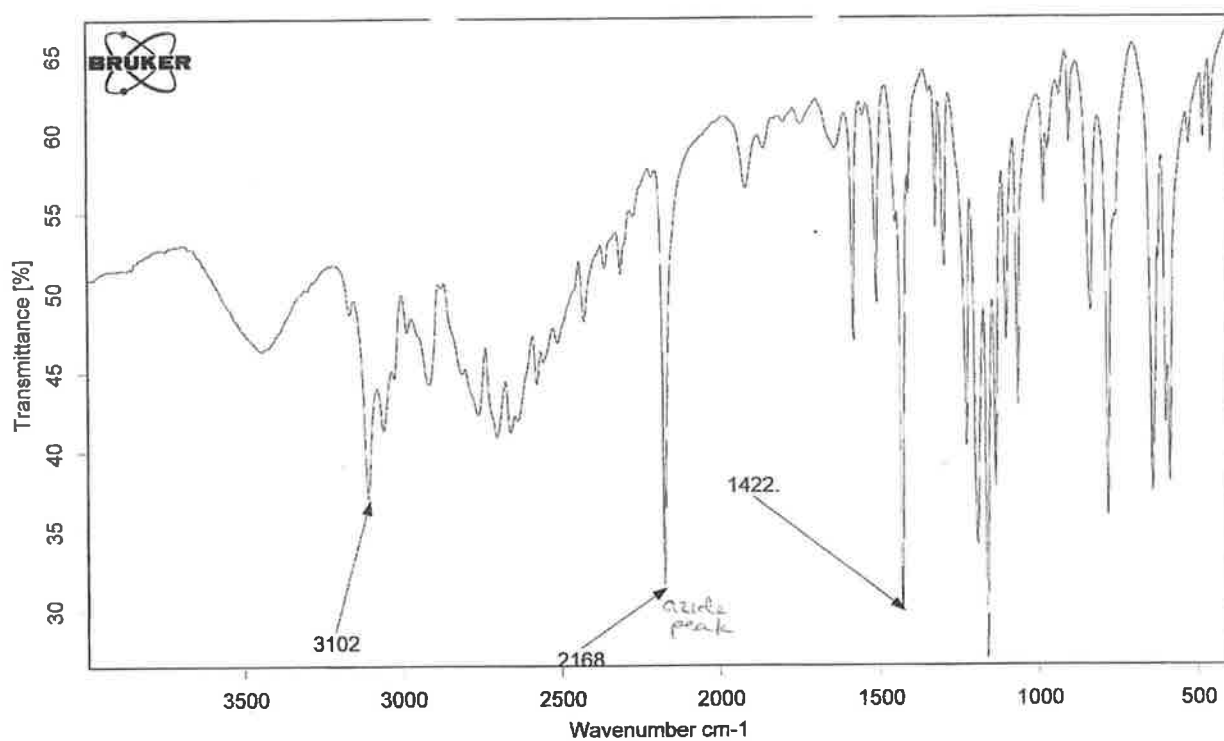
P17 MW 2186.77 g/mol

Analysis performed on unpurified peptide.

(H⁺: 2188.4408 m/z, Na⁺: 2210.4901 m/z)

Note: Small occurrence of deletion peptides.

5.14

IR Spectrum of Imidazole-1-sulfonyl azide 1.HCl (35), method 4.91

Imidazole-1-sulfonyl azide hydrochloride 1.HCl			06/11/2008
C:\Program Files\OPUS\MEAS\WORK.650	Sarah Imidazole-Azide 06/11/08	sample form	

Page 1 of 1

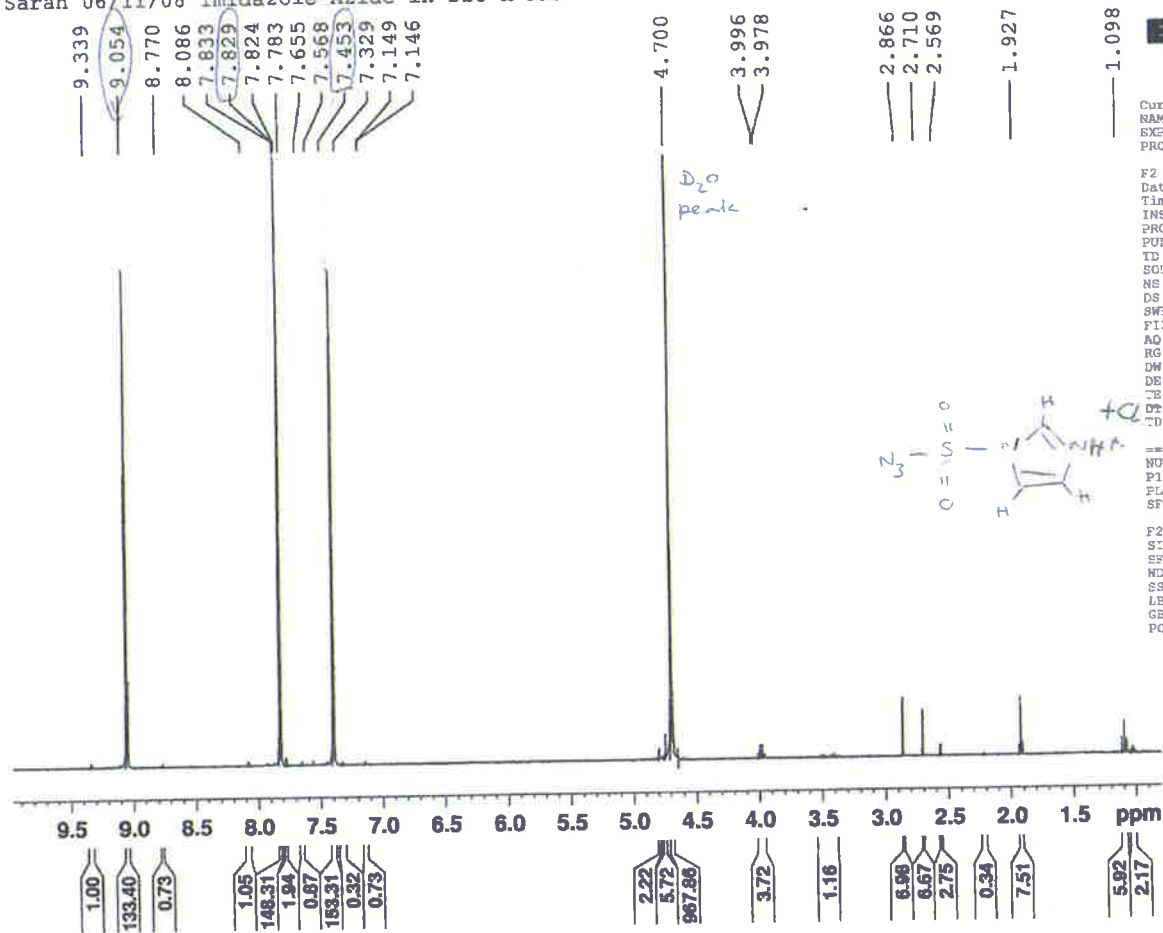
Analysis performed on unpurified compound

IR KBr: 3102 cm^{-1} , 2168 cm^{-1} (azide), 1422 cm^{-1}

5.15 ¹H NMR Spectrum of Imidazole-1-sulfonyl azide 1.HCl (35), method 4.91

Imidazole-1-sulfonyl azide hydrochloride 1.HCl

Sarah 06/11/08 Imidazole-Azide in D2O H NMR



Current Data Parameters
NAME Nov06-2008-nmr
EXPNO 16
PROCNO 1

F2 - Acquisition Parameters
Date_ 20081106
Time 17.37
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 65536
SOLVENT D2O
NS 16
DS 2
SWH 8223.685 Hz
FIDRES 0.125483 Hz
AQ 3.9846387 sec
RG 645
DM 60.800 uS
DE 6.00 uS
TE 292.2 K
DT 1.00000000 sec
TDO 1

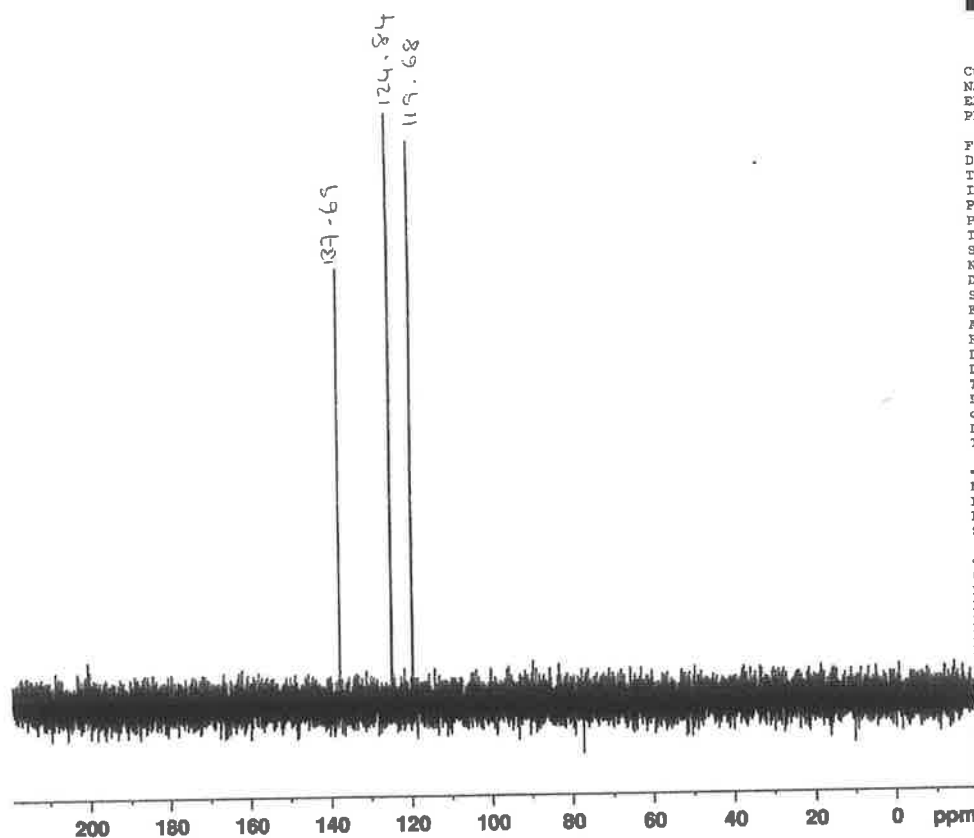
CHANNEL f1
NUC1 1H
P1 13.12 uS
PL1 -3.00 dB
SFO1 400.1324710 MHz

F2 - Processing parameters
SI 32768
SF 400.1300000 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

Analysis performed on unpurified sample

NMR (400MHz, D₂O) δ: 7.453 (dd, 1H, H-4), 7.829 (dd, 1H, H-5), 9.054 (dd, 1H, H-2)

Imidazole-1-sulfonyl azide hydrochloride 1.HCl

Sarah 06/11/08 Imidazole-Azide in D₂O C NMR

Current Data Parameters
 NAME Nov06-2008-nmr
 EXPNO 11
 PROCNO 1

F2 - Acquisition Parameters
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 Time 21.34
 INSTRUM spect
 PROBHD 5 mm PABBO BB-
 PULPROG zgpg30
 ID 65536
 SOLVENT D₂O
 NS 1024
 DS 4
 SWH 24038.461 Hz
 FIDRES 0.366798 Hz
 AQ 1.3631988 sec
 RG 1030
 DW 20.800 usec
 DE 6.00 usec
 TE 293.6 K
 D1 2.00000000 sec
 d11 0.03000000 sec
 DELTA 1.89999999 sec
 TD0 1

CHANNEL f1
 NUC1 13C
 P1 9.50 usec
 PL1 -1.00 dB
 SFO1 100.6228298 MHz

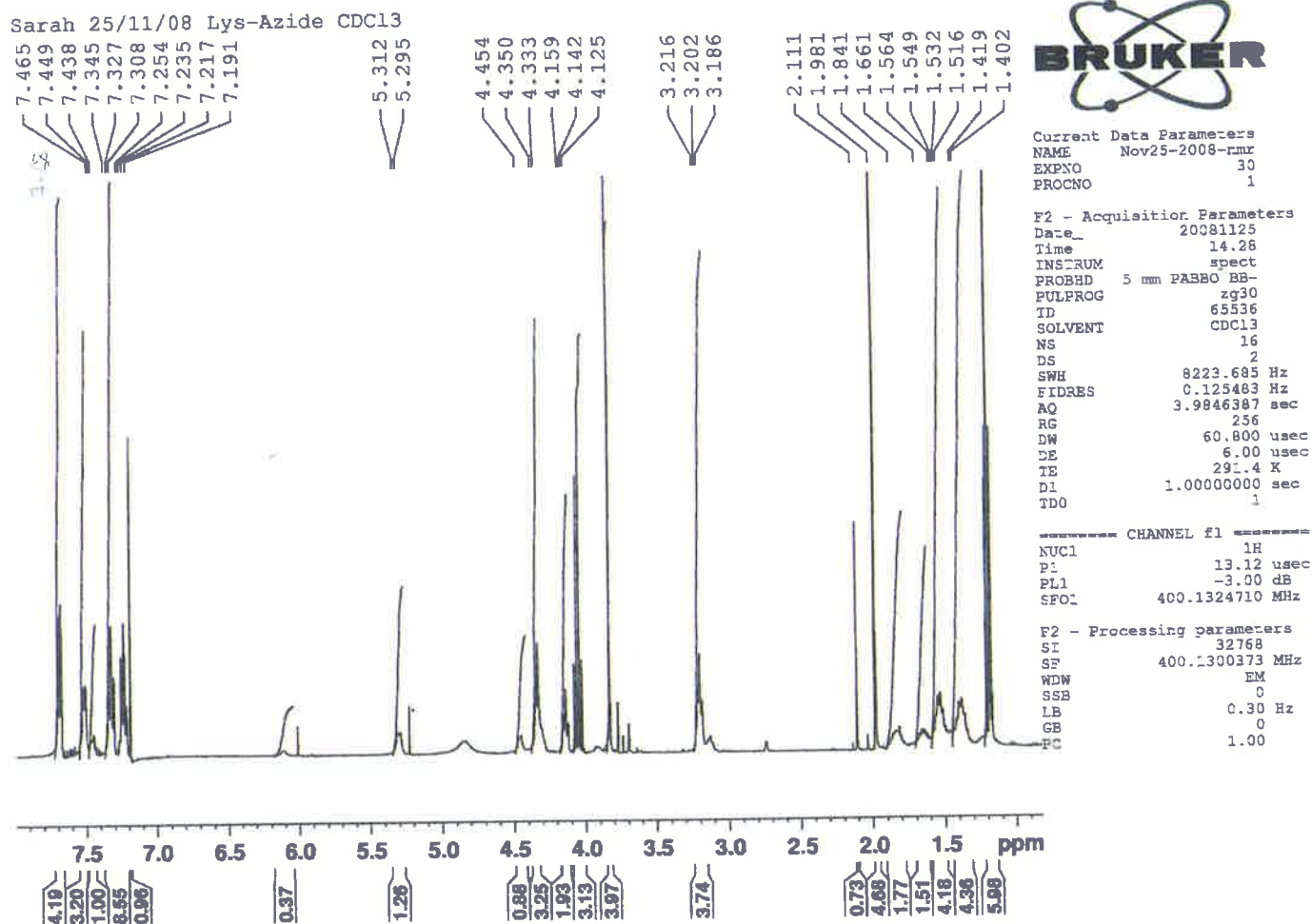
CHANNEL f2
 CPDPRG2 waltz16
 NUC2 1H
 PCD2 80.00 usec
 PL2 -1.00 dB
 PL12 14.00 dB
 PL13 14.00 dB
 SFO2 400.1316005 MHz

F2 - Processing parameters
 SI 32768
 SF 100.6127690 MHz
 WDW EM
 SSB 0
 LB 1.00 Hz
 GB 0
 PC 1.40

Analysis performed on unpurified sample

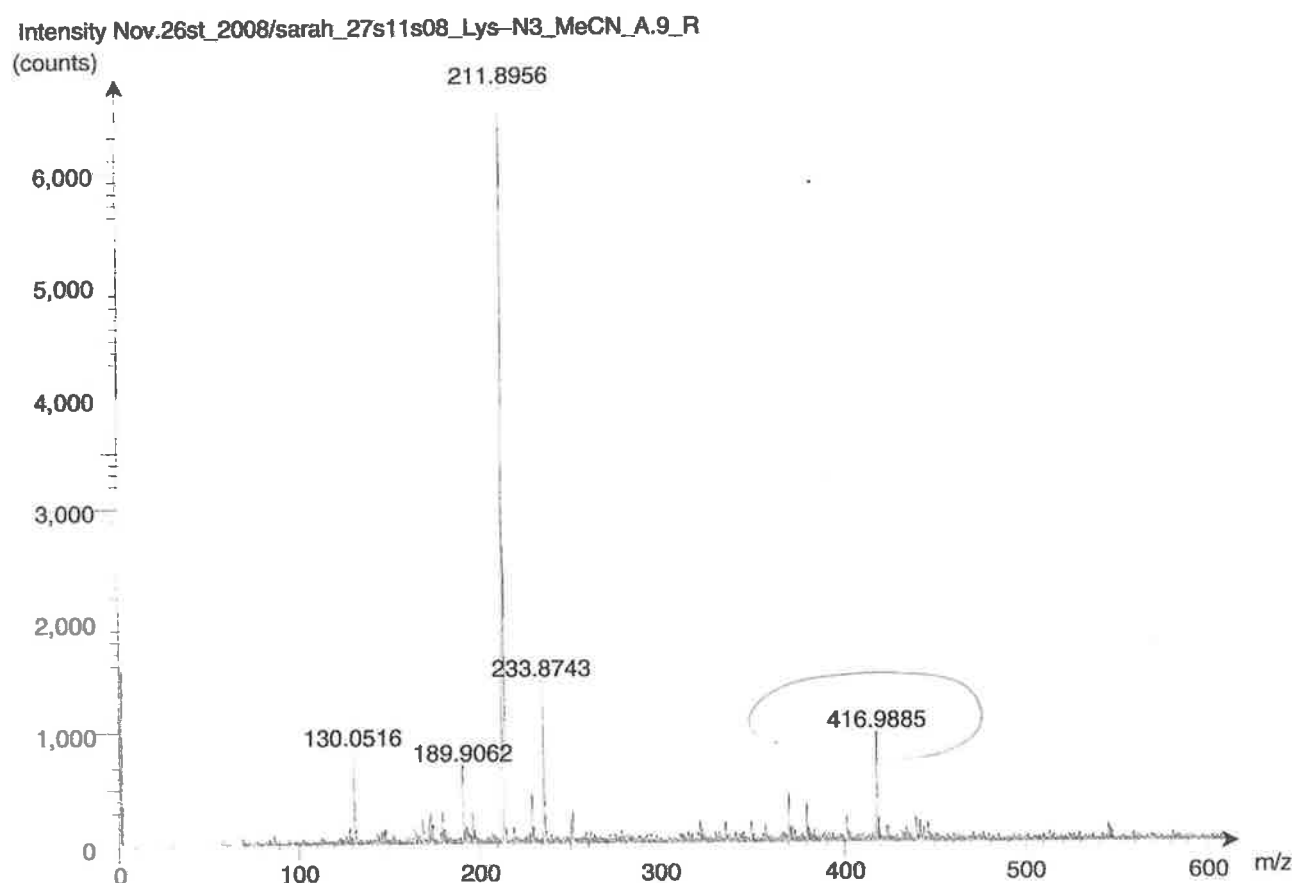
NMR (100MHz, D₂O) δ : 119.68, 124.84, 137.67

5.17 ¹H NMR of Fmoc-Lys(N₃)-OH (6), method 4.93



Analysis performed on unpurified sample

¹H NMR (CDCl₃, 400MHz) δ: 1.48 (m, 2H), 1.61 (m, 2H), 1.75 (m, 1H), 1.98 (m, 1H), 3.20 (t, 2H), 4.13-5.31 (5H), 7.22 (t, 2H), 7.32 (t, 2H), 7.39 (m, 1H), 7.45 (m, 1H), 7.68 (d, 2H)



Fmoc-Lys(N₃)-OH MW – 394.16 g/mol

Analysis performed on unpurified sample

(Na⁺ 416.9885 m/z)

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