Solid phase peptide synthesis: New resin and new protecting group

2018

Shaveer Ramkisson

211514242

Solid phase peptide synthesis: New resin and new protecting group

211514242

Shaveer Ramkisson

2018

This thesis is submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, to satisfy the requirements for the degree of Master of Medical Science in Pharmaceutical Chemistry.

This thesis presents work of two distinct research projects with a common link, Solid Phase Peptide Synthesis. Chapter one consists of an introduction and a brief literature background of the two projects. Chapter two highlights the work conducted for project one and Chapter three will highlight project two. Chapter two and three are independent and will have their own conclusion.

This is to certify that the contents of this thesis are the original research work of Mr. Shaveer Ramkisson, carried out under our supervision at Peptide Sciences Laboratory and the Catalysis and Peptide Research Unit, University of KwaZulu-Natal, Westville Campus, Durban, South Africa.

As the candidate's supervisor, I have approved this thesis for submission.

Supervisor:

Signed: _____ Name: **Prof. Fernando Albericio,** Date: _____

Co-Supervisor:

Signed: _____ Name: **Prof. Beatriz G. de la Torre,** Date: _____

ABSTRACT

Solid phase peptide synthesis is the common approach used today in synthesizing peptides in a research scale and production. Success in this approach are governed by several factors. These are; (1) the solid support on which synthesis is to be carried out, (2) linker/spacer on which the first amino acid is linked to the support to allow stepwise growth of the peptide chain, (3) protecting groups of amino acids to allow a clean synthesis without disruption of the growing peptide chain and (4) coupling reagents for improved amide bond formation yielding peptides in the shortest amount of time and with the highest quality. The following thesis shows work conducted on some of these aspects. Chapter two is based on the application of a novel resin Fmoc-Rink-Amide PEG Octagel surface resin. Chapter three describes the development of a novel protecting group for histidine amino acid.

A new PEG-PS based resin called Octagel has been developed by Aapptec. Fmoc-Rink-Amide Octagel PEG surface resin is a unique highly uniform surface-active resin. To study the resin's performance, two peptides Aib-enkephalin pentapeptide and Aib-ACP decapeptide were synthesized and results were compared to Polystyrene and ChemMatrix resins. Swelling and microscope imaging studies were also conducted on each of the resins to highlight their performance associated with Solid Phase Peptide Synthesis. Results have demonstrated that Octagel resin has the potential to synthesize peptide sequences with high purity and therefore to be a good alternative to those currently in the market.

Histidine is an important amino acid used in SPPS. It contains a reactive imidazole side group that can cause side reactions in SPPS if left unprotected. Fmoc-based SPPS is the most commonly used strategy in synthesizing peptides today. A protecting group was created for Fmoc-Histidine. SPPS was carried out on the protected histidine and results show that the group is stable in acidic conditions.

DECLARATION

Plagiarism declaration

I, **Shaveer Ramkisson** declare that

1. The research work reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other person's data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. In cases where other written sources have been quoted:
    a. Their words have been re-written, but the general information attributed to them has been referenced.
    b. Where their exact words have been used, then their writing has been placed inside quotation marks, and referenced.
5. This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and in the references sections.

Signed

_____

Shaveer Ramkisson

ACKNOWLEDGMENT

I would like to give many thanks to the following:

- Firstly, Almighty God for giving me strength, knowledge and courage to persevere through this journey
- My Mum and Dad for allowing me to pursue my own goals and ambitions. My undying love goes out to you both
- My supervisors, Prof Fernando Albericio and Prof Beatriz G. de la Torre for the continuous support and guidance through it all. You guys are really one of a kind and I am truly grateful for this opportunity you both have given me
- Dr Anamika Sharma and Dr Ashish Kumar, two magnificent scientists who pushed me and taught me so much, not only in the lab but about life. Your wisdom, knowledge and not forgetting the scolding has allowed me to grow so much throughout these years
- The peptiders, from the old to new and the ones who left and who remain, I would like to say that we linked together forms the strongest peptide bond ever, teamwork! Many thanks towards you all, I have learnt so much from each one of you guys, especially Dr Yahya Jad
- The entire team at CPRU, thank you for all the help and support when I needed it.
- The staff of the Chemistry department, Myler and the guys from Chemical stores and Mr Raj Somaru. Thank you for all the help you have given me.
- My girlfriend, Kimel Pillay for her undying love and support throughout this journey. Thank you for always being with me when times were tough

List of Abbreviations

2-CTC – 2-chlorotrityl chloride

2-Me THF – 2-methyl tetrahydrofuran

AcOH – Acetic acid

ACP – Acyl Carrier Protein

Act – Activating group

Aib – 2-Aminoisobutyric acid

AM – aminomethyl

Arg – Arginine residue

BHA – benzhydrylamine

Boc – tert-butoxycarbonyl

Bom – Benzyloxymethyl

Bum – Tert-butoxymethyl

Bzl – Benzyl

Cbz – Carbobenzoxy

CLEAR – Cross-linker ethoxylate acrylate resin

CM – ChemMatrix

DCM – Dichloromethane

DIC – Diisopropylcarbodiimide

DIEA – diisopropylethylamine

DMF – N,N – Dimethylformamide

Dnp – 2,4-dinitrophenyl

Doc – 2,4 – dimethylpentyl-3-yloxycarbonyl

DVB – Divinylbenzene

equiv. – equivalent

Et_2O – Diethyl ether

EtOAc – Ethyl Acetate

Fmoc – Fluorenylmethoxycarbonyl

Gly – Glycine residue

HAL – Hyper Acid sensitive Linker

HD – High definition

HF – Hydrofluoric acid

His – Histidine residue

HMPA – 4-hydroxymethyl-3-methyoxyphenoxy) acetic

HPLC – High performance liquid chromatography

LC- MS – Liquid chromatography mass spectrometry

MBHA – p-methylbenzhydrylamine

MeCN – Acetonitrile

mg – milligram

$MgSO_4$ – Magnesium sulphate

mL – millilitre

Mmt – monomethoxytrityl

Mtt – methyltrityl

$NaBH_4$ – sodium borohydrate

NMP – N-methylpyrrolidone

NMR – Nuclear Magnetic Resonance

OG – Octagel

Pac-Br – Phenacyl bromide

PAM – 4-(hydroxymethyl)phenylacetamidomethyl

PEG – Polyethylene Glycol

PEGA – Polyethyleneglycol cross-linked dimethyl acrylamide

PG – Protecting group

Phe – Phenylalanine residue

PS – Polystyrene

PS-DVB – Polystyrene crosslinked divinylbenzene

rt – room temperature

SASRIN – Super Acid Sensitive Resin

SEM – Scanning electron microscopy

$SOCl_2$ – Thionyl Chloride

SPPS – Solid Phase Peptide Synthesis

t-Bu – tert-butyl

TEA – triethylamine

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran

TIS – triisopropylsaline

TLC – Thin Layer Chromatography

Tos – Tosyl

Trt – Trityl

TTEGDA – Tetra (ethyleneglycol) diacrylate crosslinker

Z – benzyloxycarbonyl

μL – microlitre

μm – micrometer

μM – micromolar

List of Tables, Figures etc

TABLE OF CONTENTS

CHAPTER ONE

Introduction

The synthesis of peptides has great importance in the field of medicinal chemistry and pharmaceutical research. A peptide is made by linking amino acids together through peptide bonds. A peptide bond is formed by coupling two amino acids, one is protected at its amino end and the other at the carboxylic end. The first free peptide (glycylglycine) was formed in 1901 by Emil Fischer and was in solution [1]. In solution phase peptide synthesis, after each coupling of an amino acid, the intermediate peptide is isolated, purified and characterized before the next amino acid is attached. This is a long and tedious process and has disadvantages such as low solubility of the intermediate peptides and low yield.

Solid-Phase Peptide Synthesis (SPPS) was developed in 1963 by R. Bruce Merrifield [2] as an efficient method in synthesizing peptides. This development uses a solid support to which the growing peptide is bound. Compared to solution phase synthesis, SPPS is a simple technique that creates peptides rapidly and efficiently with high purity and high yield. The strategies used in solution phase have been adapted to work in solid phase.

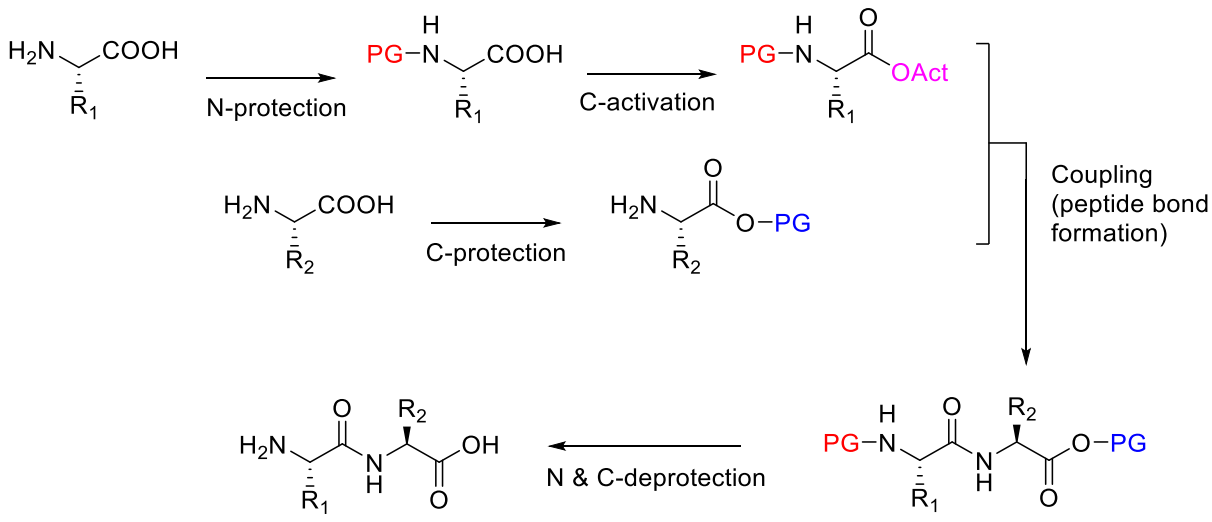
The main component of SPPS is the solid support used to grow the peptide chain. Ever since the invention of SPPS, many research groups have made various types of resins to improve the quality of peptides. In chapter two, a novel support developed by Aaptec Biotechnology will be discussed. Fmoc-Rink-Amide Octagel PEG resin is a surface-active resin that contains polyethylene glycol (PEG) chains on its surface. A comparative study was conducted with Fmoc-Rink-Amide Aminomethyl Polystyrene and H-Rink-Amide ChemMatrix resins. SPPS of ‘difficult’ peptides, Aib-pentapeptide and Aib-ACP decapeptide was carried out on each resin. Also, swelling of each resin under different solvents were tested and SEM and light microscope images of the resins also conducted.

Amino acids are trifunctional molecules that requires protecting groups to block the reactivity of functionalities to ensure a clean formation of the desired peptide. Among the 20 essential amino acids found in nature, few contains reactive side chains that can undergo side reactions during SPPS, e.g. arginine, histidine, tryptophan, aspartic/glutamic acids, lysine. Histidine is an important amino acid used in peptide synthesis. It contains a reactive imidazole side group that can cause side reactions in SPPS if left unprotected. Fmoc-based SPPS is the most

commonly used strategy in synthesizing peptides today as it is a mild approach compared to Boc synthesis. In chapter three the development of a novel protecting group for histidine amino acid and its use in Fmoc – SPPS will be discussed.

1.1 Solid Phase Peptide Synthesis

In 1901, the first ever free peptide was synthesized by Emil Fischer and the reaction was done in solution[1]. For a peptide to be synthesized, there are few chemical requirements needed by amino acids. A peptide is synthesized by protecting the carboxylic group of one amino acid and the amino group of the second amino acid. The activation of the free carboxyl group results in the formation of a peptide (amide) bond between the amino acids and the selective removal of the protecting groups results in a dipeptide (scheme 1).

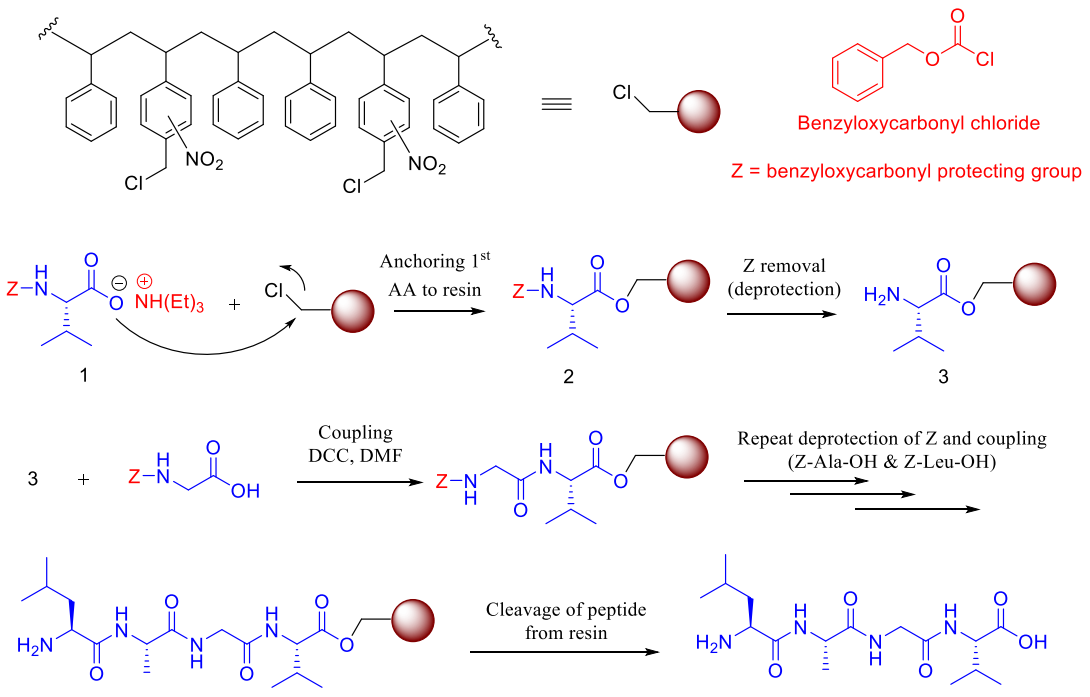


Scheme 1: General chemical reaction of peptide synthesis

Peptide synthesis in solution is limited because each intermediate step of its synthesis must be isolated, purified and characterised before attaching the next amino acid. This time-consuming and laborious approach results in low yield and low solubility of the intermediate peptides with the increase in the peptide chain length. A new approach was needed to synthesize peptides more efficiently as there was a growing need to create larger and more complex peptides with high yield and high purity.

In 1963, R. Bruce Merrifield developed a ground-breaking concept called Solid Phase Peptide Synthesis (SPPS)[2]. In SPPS, the peptide is synthesized in a stepwise manner while the C-terminal end of the peptide was anchored to an inert cross-linked polymer support and the peptide is synthesized from the C-terminal towards the N-terminal (C-N synthesis). Concurrently with Merrifield, Letsinger and Kornet developed a "popcorn polymer support" and synthesized a dipeptide L-leucylglycine[3]. Their strategy was poorly accepted as they anchored the N-terminal of an amino acid to the support. This N-C direction of synthesis does not allow cleavage of the peptide from the support under mild conditions and always had the possibility of racemisation during synthesis. Merrifield's concept was accepted as a new technique of peptide synthesis and is the most used approach in synthesizing peptides today.

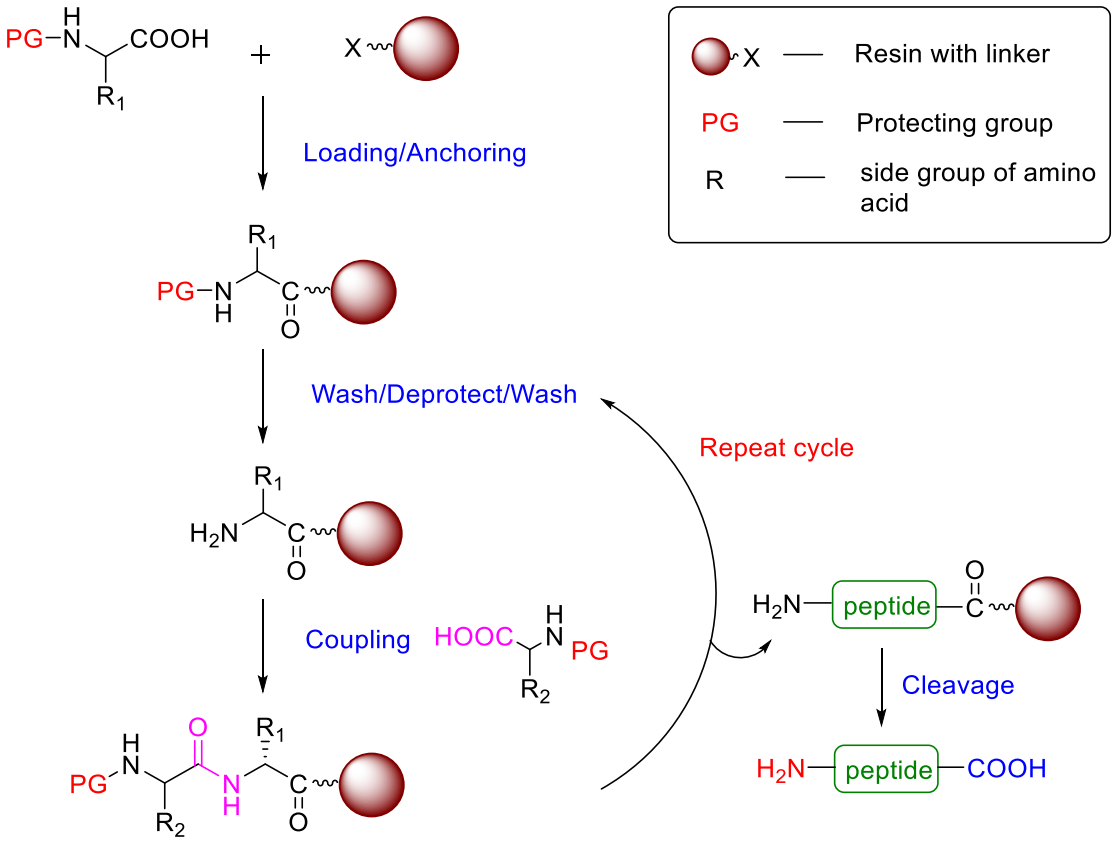
Merrifield used a chloromethylated copolymer of a nitrated polystyrene (PS) and divinylbenzene (DVB) as a support and synthesized a model tetrapeptide (scheme 2). A year later he improved his method by using Boc-protected amino acids which eliminated the need to nitrate the solid support[4]. The chloromethylated copolymer of polystyrene (PS) and divinylbenzene (DVB) resin is now known today as the Merrifield resin.



Scheme 2: Merrifield's first synthesis of a tetrapeptide using solid phase peptide synthesis[5]

1.2. Principles of SPPS

Peptide synthesis using SPPS is carried out by a series of chemical reactions that occurs in a heterogenous reaction mixture using an insoluble polymer support, a soluble protected amino acid and a solvent[6]. A peptide is formed in a stepwise manner by first anchoring or loading an amino acid onto the solid support at its C-terminus (carboxylic end) with its N-terminus end protected (amino end). Thereafter a series of deprotection, washing and coupling reactions on the solid support results in the formation of a peptide (Scheme 3).



Scheme 3: Flow diagram describing SPPS[7]

The synthesis of peptides using this approach has many advantages over peptide synthesis in solution. These are:

i. The peptide is synthesized while being covalently bounded to an insoluble polymer support at its C-terminal. This allows an easy removal of any by-products or excess reagents and solvents from the growing peptide.

ii. The use of excess reagents drives the reaction to completion on a support.
iii. There is no mechanical loss of the peptide as it is retained on the polymeric support in a single reaction vessel during the whole synthetic process.
iv. The final peptide sequence is obtained by a single cleavage step at the end of its synthesis. In this reaction, the side chain protecting groups are also cleaved resulting in a simple work-up and isolation of a peptide.
v. The process of SPPS physically is simple, quick and lends itself towards automation
vi. Some resins used in SPPS can be recycled e.g. 2-CTC resin[8]

Despite the several advantages of SPPS, there are some limitations of SPPS which have been extensively reviewed throughout the years[9]:

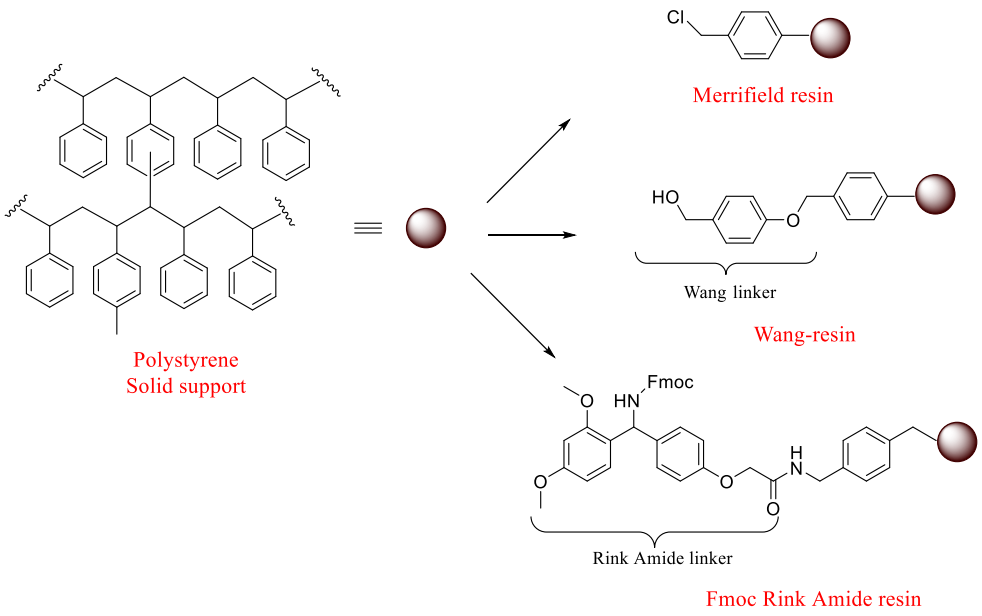
i. Stability of peptide-resin bond through the various reaction conditions.
ii. The formation of truncated and error peptide sequences.
iii. The intra and intermolecular interactions of the growing peptide and the resin beads can change the conformation of the peptide within the resin and therefore difficult the synthesis

These problems are overcome with modifications of the SPPS protocol with several developments like new polymer supports with improved swelling properties to increase solvation of the support and the growing peptide chain and linkers to allow a simple removal of the peptide from the support. Also, the development of protecting groups greatly reduces the formation of truncated and error peptides. In this chapter we look at some of these developments.

1.3. Development of Resins

The success of SPPS is dependent on a resin and its performance. In the SPPS context, usually under the word “resin”, there are two parts: the solid support and a spacer/linker. The solid support is considered the heart of SPPS. The main requirement of a solid support is to be chemically stable throughout all conditions of the synthesis. Also, it should allow for an easy interaction of the amino acids towards the growing peptide which is anchored onto the support[10].

A linker/spacer is a chemical entity that is permanently attached onto a polymer support that temporarily links the growing peptide onto the support. Linkers aid in the cleavage of the peptide from the support under specific conditions. A large variety of linkers were developed, all of which are used in polystyrene core resins. Most of these linkers are cleaved in acidic conditions and can result in a modified C-terminal end of a peptide, e.g. peptide acid, amide, hydrazine or sulfonamides (Scheme 4).



Scheme 4: The concept of a solid support with a linker/spacer which together form the resin

Many supports were developed and used for peptide synthesis, most of which are modifications of the common copolymer of polystyrene and divinylbenzene developed by Merrifield whereas others were made of polyamide, polyethylene glycol or a mixture of polystyrene with these two. However, the most commonly used supports are those based on polystyrene or polyethylene glycol or a combination of both.

1.4. Polymer supports

The Merrifield resin is a copolymer PS-DVB which is chloromethylated. PS is hydrophobic in nature and the interactions of amino acids towards the growing peptide can become difficult.

Improvements and modifications were conducted on Merrifield's concept to deal with the rigid hydrophobic environment of the PS-DVB matrix.

Atherton et al developed a polar support, polydimethyl acrylamide resin that has a similar structure to the peptide backbone[11-13]. The similarity of the support and the peptide structure helps in the solvation of the peptidyl resin. This reduces the steric hindrance of the many reactions performed during synthesis (deprotection and coupling). The cross-linked polydimethylacrylamide resin was prepared by persulfate initiated emulsion copolymerisation of dimethylacrylamide and N,N-bisacryloylethylenediamine in water and cellulose acetate butyrate together with Boc-β-alanyl-N-acryloylhexamethylenediamine[14]. Polyacrylamide beads are large and swells well in polar solvents like DMF and AcOH but not so well in non-polar solvents like DCM. The stability of these resins is also less compared to polystyrene supports. A copolymer of PEG with polyamide was also developed for SPPS. Bis-2-acrylamidoprop-1-yl polyethyleneglycol cross-linked dimethyl acrylamide (PEGA) was introduced by Meldal et al as a hydrophilic, biocompatible and flexible support for peptide synthesis[15].

The mixture of PS with PEG polymers are a good class of solid supports. The hydrophobic nature of PS together with the hydrophilic polymers of PEG allows for a more stable and flexible support for SPPS. Li et al conducted a comparison study of PS resins and PS cross-linked with PEG of different percentages and observed faster kinetics as the PEG percentage increased[16]. There is also high swelling of PS-PEG supports in non-polar solvents like DCM and DMF which are suitable solvents used in SPPS.

Bayer et al created Tentagel which was prepared by grafting ethylene oxide onto PS supports[17]. Albericio and Zalipsky developed a support with PEG chains coupled by amide bonds onto a suitable amino functionalized PS support[18]. Champion I and II developed by Adams et al incorporated PEG chains onto PS by attaching a spacer between them to improve stability and loading during SPPS[19]. A copolymer of PS with a tetraethyleneglycol diacrylate crosslinker (TTEGDA) was developed by Pillai et al and resembled a gel rather than a solid support[20]. It had good swelling and good mechanical properties in automated synthesis of larger peptides however, it suffered drawbacks with the crosslinker compared to DVB. A highly crosslinked support by Kempe and Barany called CLEAR (cross-linked ethoxylate acrylate resin) has a trivalent branched linker allowing improved stability compared to PEGA[21].

Recently a fully PEG based support was developed by Côté el al called ChemMatrix resin[22]. This support is mainly made from primary ether bonds and is highly cross-linked. The amphiphilic character of the resin makes it highly stable and biocompatible. In chapter two, work conducted on a novel resin by Aaptec Biotechnology called Fmoc-Rink-Amide Octagel PEG surface resin will be discussed.

1.5. Linkers

The so-called Merrifield resin is made up of a copolymer of a chloromethylated polystyrene crosslinked with 1% divinylbenzene. The chloromethyl entity of the support acts a linker to the peptide and the support. Many linkers were developed to improve the removal of a peptide from the solid support.

Wang developed p-alkoxybenzyl alcohol (1) resin for the synthesis of protected peptides having a free carboxyl group and p-alkoxybenzyloxycarbonyl hydrazide (2) resin for the synthesis of protected peptide hydrazides (Figure 1)[23]. The p-alkoxy group on the benzyl moiety increases the acid lability of the peptide-resin bound where 50 - 90% of trifluoroacetic acid (TFA) can cleave the anchoring bond. These supports can be used for peptide synthesis using Fmoc-amino acids where the Fmoc groups are removed by an organic base.

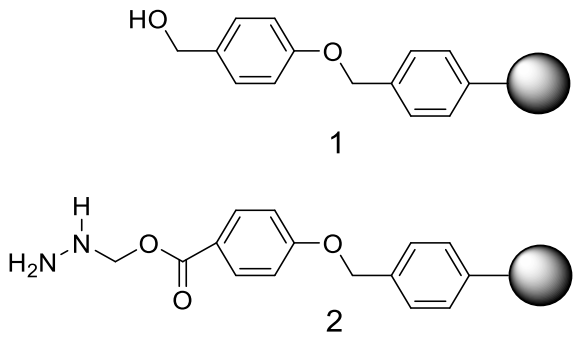


Figure 1: Wang resin derivatives (1) p-alkoxy benzyl (2) p-alkoxy hydrazide

Mitchell et al also developed a linker to combat the loss of the peptide during cleavage through acidolysis[24]. The 4-(hydroxymethyl)phenylacetamidomethyl (PAM) linker is electron

withdrawing which makes the ester bond more stable towards acid compared to the chloromethyl (Merrifield resin) and, of course, the alkoxy benzyl alcohol resin (Figure 2).

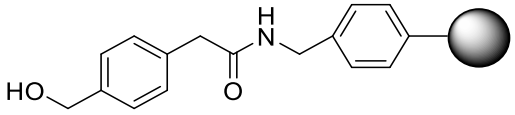


Figure 2: PAM resin

Kaiser and DeGrado developed the Kaiser oxime resin is a p-nitrobenzophenone oxime ester (Figure 3)[25]. It had a major impact in SPPS for peptides using Boc chemistry. The final cleavage from resin is done with nucleophiles to yield hydrazides, N-alkyl amides and peptide esters.

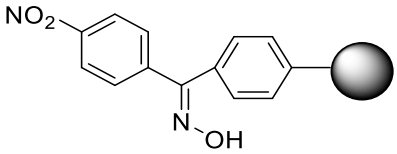


Figure 3: Kaiser oxime resin

The creation of C-terminal peptide amides from a support began with the development of a benzhydrylamine (BHA) linker by Pietta and Marshall[26]. Modifications of this support was done by Matseuda and Stewart forming a p-methylbenzhydrylamine (MBHA) resin to improve the stability of the support[27].

With the use of Wang linkers, N-protected Boc groups that are removed with 30% TFA in DCM cannot be used because the continuous acidic treatment causes a loss of the peptide from the support, and therefore the use of Fmoc for the N-protection was implemented. Fmoc protection can also be used with more acid labile linkers, which were created by Sheppard et al (4-hydroxymethyl-3-methyoxyphenoxy) acetic acid (HMPA)[28] and 2-methoxy-4-benzyoloxybenzyl alcohol-based resins (SASRIN by Mergler et al)[29] and (SASRIN-NH_2 by Albericio and Barany[30]). These linkers are cleaved with 1% TFA in DCM.

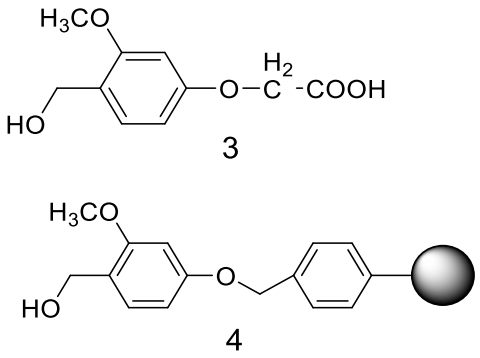


Figure 4: Acid sensitive linkers (3) HMPA type linker and (4) SASRIN

Rink also created a trialkyloxy-diphenyl-methyl ester amide linker to increase the acid lability of supports to create protected peptides[31]. Protected peptides are important for convergent peptide synthesis of larger peptides. Rink acid and amide were created to form peptide acids or peptide amides respectively (Figure 5).

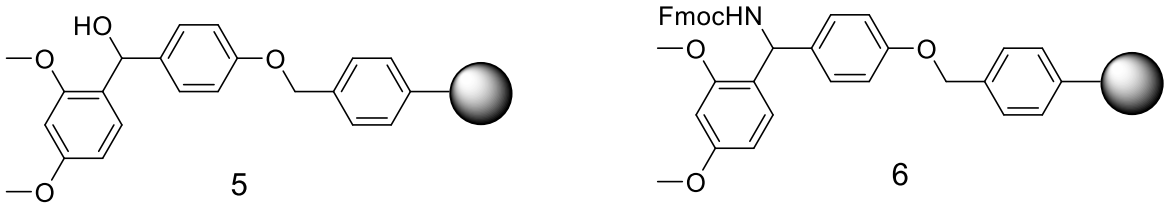


Figure 5: Rink linkers, (5) Rink acid and (6) Rink amide

In 1991, two resins with high acid sensitivity linkers were developed. The 2-chlorotrityl (2-CTC) resin created by Barlos et al is a high acid sensitive resin [32] that cleaves in 1% TFA and links peptides through an electrophilic substitution reaction. Barany and Albericio developed a tris alkoxy benzyl ester linker called HAL (hyper sensitive linker) that is highly labile to acid (Figure 6)[33].

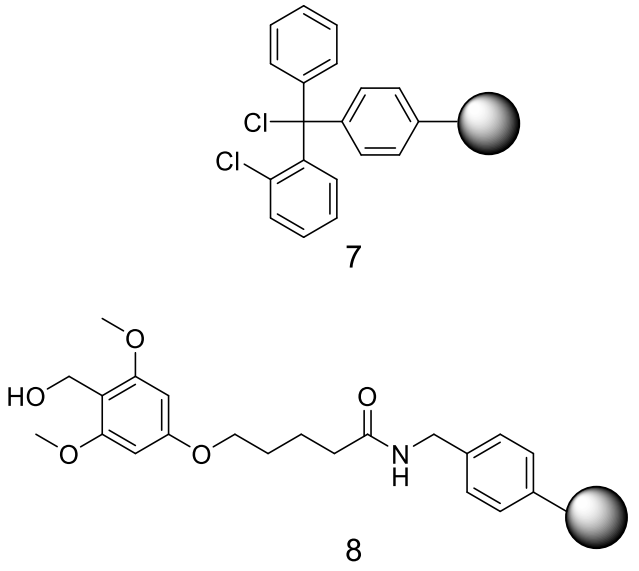


Figure 6: 2-Chloro trityl resin (7) and hyper sensitive linker (HAL) (8)

1.6. Protecting groups

In the synthesis of peptides, from a simple dipeptide to larger complexed ones, the functional groups of amino acids that are not involved in an amide bond formation should be protected or blocked to ensure the correct formation of the desired peptide.

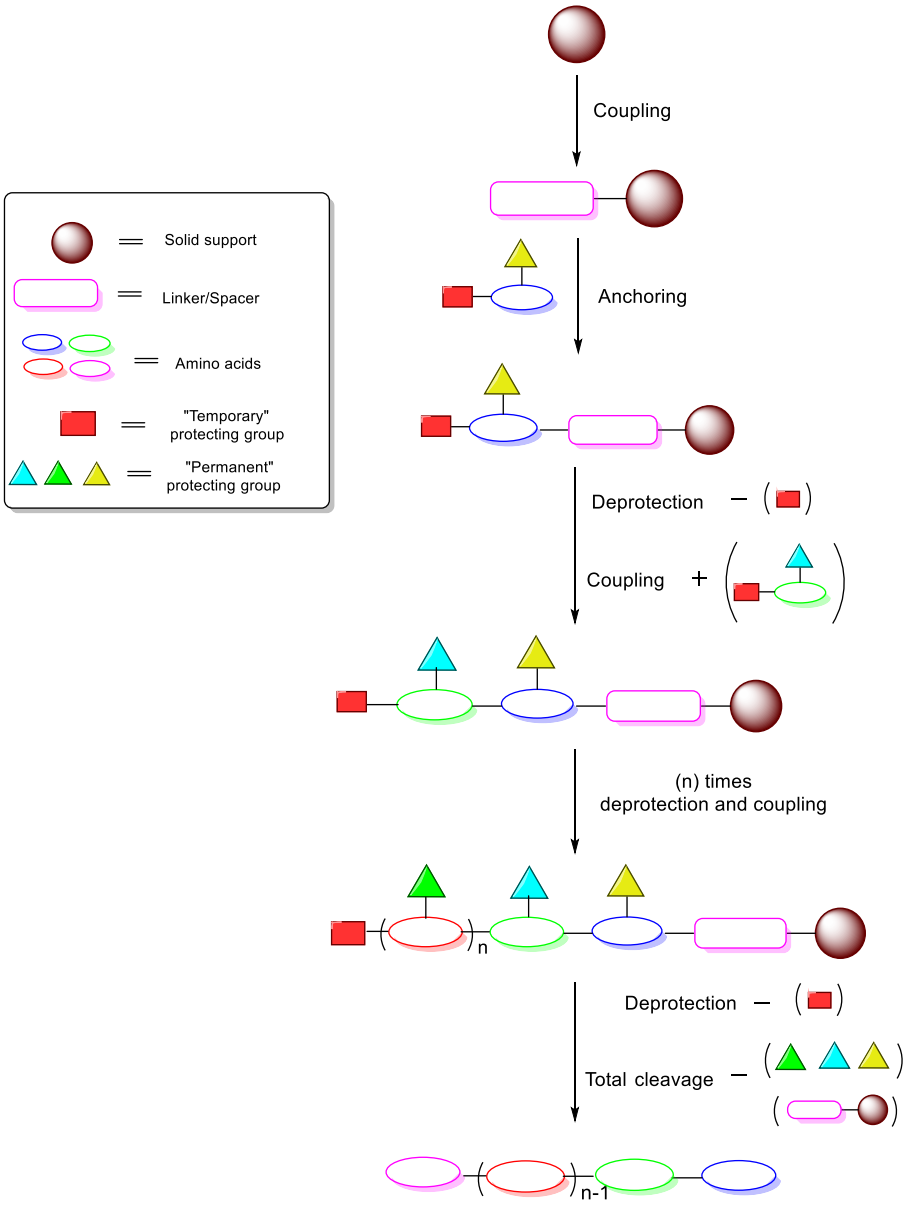
The first protecting group to be developed for peptide synthesis was in 1932 by Bergmann and Zervas called the carbobenzoxy (Cbz or also known as Z)[34]. The carbobenzoxy group was used as a temporary α-amino protecting group. This development allowed for several peptides to be synthesized in solution such as vasopressin[35] and oxytocin by du Vigneaud et al[36], which were the first active peptides synthesized. du Vigneaud and Merrifield, years later, were recognized with the Nobel Prize of Chemistry.

Few years later, Albertson and McKay developed the Boc group as a temporary acid labile protecting group[37]. The Boc group was used in combination with Cbz protecting group to synthesize many peptides thereafter. The use of Boc was employed by Merrifield in the synthesis of Bradykinin[4].

The use of Boc protected amino acids was employed in almost every synthetic strategy of peptide synthesis however its removal in acid during SPPS at that time also encounter loss of the peptide fragments during SPPS, due to the partial instability of the chloromethyl resin with the use of acids.

The development of Fmoc group by Carpino et al as a base-labile amino protecting group mainly for the N-terminal was a great milestone in the synthesis of linear peptides[38]. This mild protecting group that is removed under basic conditions such as 20% piperidine in DMF is common today in amino acids and is most commonly used in synthesizing peptides.

Stepwise SPPS has two main types of protection, being temporary and permanent groups. A schematic representation (scheme 5) explains these different types of protection.



Scheme 5: SPPS strategy depicting the various types of protection. An adaptation of Scheme 3

There are two types of protection strategies associated with SPPS; the Boc/Benzyl and Fmoc/t-Bu strategy. Boc and Fmoc groups act as temporary protecting groups at the N-terminal end of the amino acids and are removed under acidic and basic conditions respectively[39].

The Boc/Bzl strategy depends on acid lability and is usually removed with TFA (25-50% in DCM). The permanent protecting groups, usually Bzl type functionalities, are removed at the end of the synthesis when the peptide is cleaved from the resin under strong acidic conditions using hydrofluoric acid (HF) or trifluoromethanesulfonic acid with scavengers like anisole to capture the formed carbocations. These harsh conditions can lead to damages to the resultant peptide chain and requires special instrumentation to use[39]. The use of the Fmoc/t-Bu strategy is a milder approach that uses an orthogonal protection system. The concept of orthogonality was introduced by Barany et al and explains that two or more protecting groups can be present in a molecule and can be removed in the presence of the other under different conditions[40, 41]. Figure 7 explains the orthogonality of Fmoc/t-Bu protection.

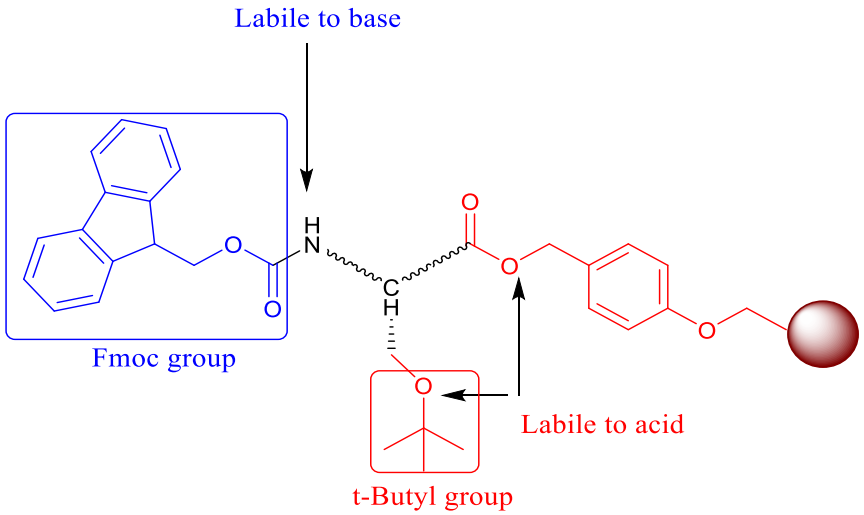
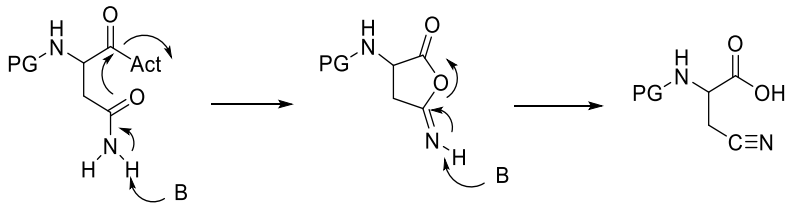
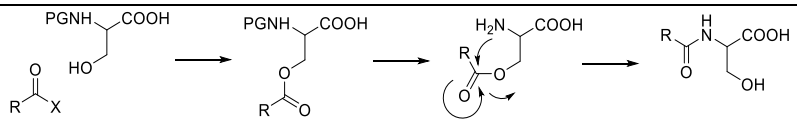
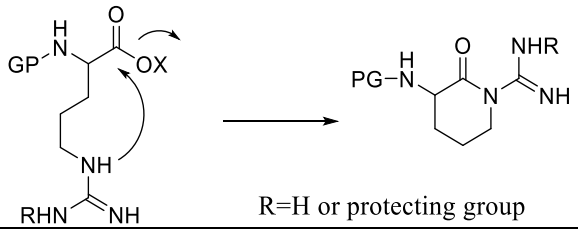


Figure 7: The concept of Orthogonality using Fmoc/t-Bu chemistry

1.7. Histidine amino acid

There are 20 common, naturally occurring amino acids that are used for peptide synthesis. Most of these amino acids contain reactive side chain groups that can be activated during peptide synthesis and requires protecting groups to ensure a clean formation of a peptide e.g. arginine, histidine, serine, aspartic/glutamic acids, lysine etc. Table 1 shows examples of some amino acids that can undergo side reactions without protection of their side chains.

Table 1: Some examples of side chain reactions that can occur in amino acids without the use of protecting groups

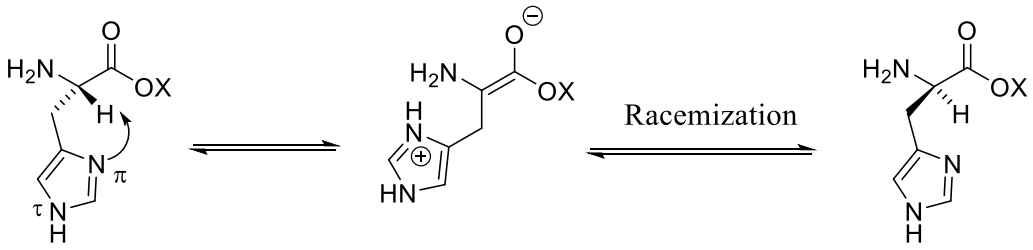
Amino acid	Reaction	Causes
Asparagine		Use of base during coupling[39]
Serine		Acylation[42]
Arginine		Deguanidination due to deprotonation of side chain[39]

Among the many amino acids used in peptide synthesis, histidine has an important presence in nature. Histidine is an essential amino acid and is a precursor to histamine, a compound released via immune response to fight off allergic reactions. Peptide sequences containing histidine have many applications. Histatins, an antimicrobial peptides secreted by the parotid and sub-mandibular salivary glands are rich in histidine amino acids[43]. They are known to possess anti-fungal and anti-bacterial properties. Histidine rich peptides also show application in nucleic acid delivery[44]. Overall, peptides that are rich in histidine have shown great potential in biotechnology and nanomedicine[45].

Histidine is common to cause racemization in peptide synthesis when activated for peptide bond formation[42]. This side reaction is more frequent when histidine is used without protection of its imidazole side-chain. Hence an unprotected imidazole of histidine, in addition to partaking in acylation from an incoming protected amino acid, can cause racemization in SPPS.

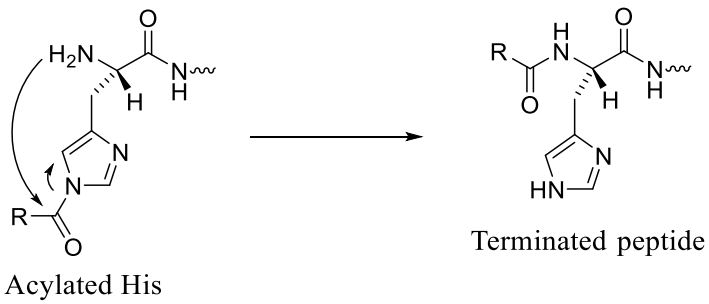
The imidazole group of histidine contains two nitrogen atoms which can participate into the tautomerization of histidine. These nitrogens are labelled π-N (near the alpha carbon) and τ-N (distant from alpha carbon).

Early works conducted by, Fletcher et al[46] and Jones et al[47] have described mechanisms of racemization to be a result of the π-nitrogen. Ways in preventing this racemization to occur is to directly protect the π-nitrogen or by protecting the τ-nitrogen with strong electron-withdrawing group to decrease the basicity of the π-nitrogen. Racemization can occur upon amino acid activation for coupling via an intramolecular base catalyzed reaction of the π nitrogen[47] (scheme 6) forming an enol.



Scheme 6: Possible mechanism of histidine during racemization

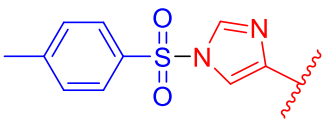
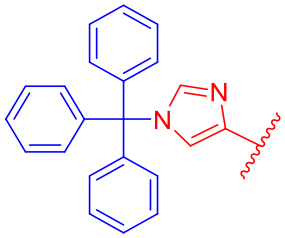
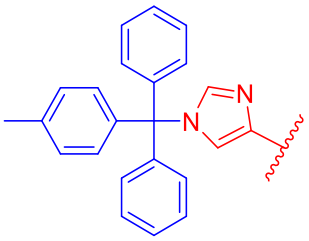
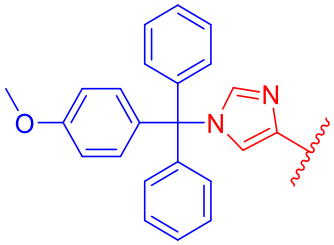
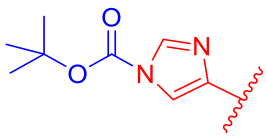
Acylation of the imidazole group can also occur during peptide synthesis which could lead to N^{τ} to α amino group migration (scheme 7).

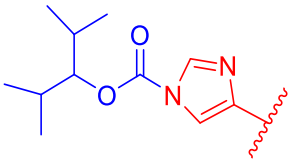
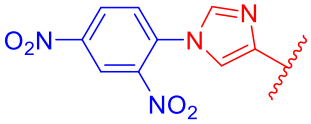
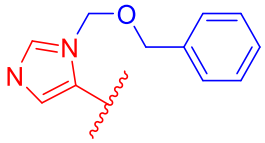
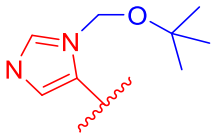


Scheme 7: Acylation of histidine during peptide synthesis with N^{τ} to α amino group migration

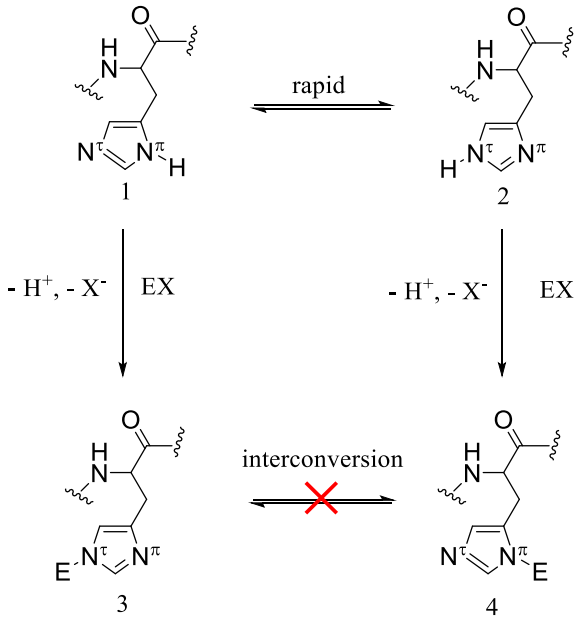
There are many protecting groups developed for histidine amino acid, majority of which protect the τ-nitrogen. Protection at the π-nitrogen is hard to achieve and requires several synthetic steps which can be tedious and expensive to synthesize. The case of racemisation is still not fully resolved, and the development of new protecting groups is essential. Table 2 shows the most common functional groups used for histidine with their strengths and drawbacks in their use a protecting group.

Table 2: Protecting groups used for histidine amino acid

Name and Structure	Strengths	Limitations	References
N^{τ} - tosyl (Tos) 	Bulky, electron withdrawing which minimize racemisation	Removed using harsh HF acid. No full stable to HOBt and other additives used during the coupling	[48]
N^{τ} - trityl (Trt) 	Most commonly used for Fmoc strategy, Bulky	Removed with TFA in 1-2 hr.	[49, 50]
N^{τ} - methyltrityl (Mtt) 	Bulky, electron withdrawing	More labile than Trt	[51]
N^{τ} - monomethoxytrityl (Mmt) 	Bulky, electron withdrawing	More labile than Mmt	[51]
N^{τ} - tert-butyloxycarbonyl (Boc) 	Good for histidine protected peptides on CTC resin	Not stable to prolonged piperidine treatments	[50]

N^{τ} - 2,4-dimethylpent-3-yloxycarbonyl (Doc) 	Resistant to nucleophiles	Removed using liquid HF	[52]
N^{τ} 2,4-dinitrophenyl (Dnp) 	Stable to HF, good for Boc/Bn SPPS, but requires a thiolysis to be removed	Removed by thiolysis Incomplete removal in His-rich peptides	[53, 54]
N^{π} - benzyloxymethyl (Bom) 	Stable to bases and nucleophiles	Releases CH_2O upon deprotection with HF treatment. Expensive to synthesize	[55, 56]
N^{π} - tert-butoxymethyl (Bum) 	Useful in Fmoc SPPS	Releases CH_2O upon deprotection with TFA Expensive to synthesize	[57, 58]

A brief overview of the imidazole group of histidine has been outlined by J. H. Jones in Houben-Weyl Methods of Organic Chemistry[59]. In this, the author explains that the basicities of the two imidazole nitrogens are almost the same (pKa ca. 7) yet their reactivity towards electrophiles is different and that, in most cases interconversion between substituted imidazole nitrogen products does not occur (scheme 8).



Scheme 8: The imidazole nitrogen's reaction towards electrophiles

He also stated that reactions with electrophiles like alkyl halides e.g. benzyl halides with the imidazole side chain of histidine produce mixtures of τ-nitrogen and π-nitrogen derivatives (scheme 8) with the τ-nitrogen product to be dominant.

The most commonly used protecting group for Fmoc peptide synthesis is the Trityl (trt) group. The Trt moiety is attached to the τ-nitrogen of histidine however its electron withdrawing substituents helps in reducing the basicity of the π-nitrogen and its bulkiness helps to minimize the abstraction of the α-proton, but both effects only to an extent. Also, cleavage of the trityl group from histidine occurs in short amount of time, preventing the synthesis of protected histidine peptides useful when carrying out convergent peptide synthesis. The tertiary butoxymethyl (Bum) protecting group is a suitable replacement, however attachment of this group to histidine is a lengthy process which is expensive and during the removal release formaldehyde, which can react with the free amine present in the peptide and therefore form unnecessary side products.

Bearing all of this in mind, the development of inexpensive yet improved protecting group(s) for the synthesis of peptides using Fmoc/t-Bu strategy is the basis of the research work in chapter three.

1.8. References

1. Fischer, E. and E. Fourneau, *Ueber einige Derivate des Glykocolls.* Berichte der deutschen chemischen Gesellschaft, 1901. **34**(2): p. 2868-2877.
2. Merrifield, R.B., *Solid phase peptide synthesis. I. The synthesis of a tetrapeptide.* Journal of the American Chemical Society, 1963. **85**(14): p. 2149-2154.
3. Letsinger, R.L. and M.J. Kornet, *Popcorn Polymer as a Support in Multistep Syntheses.* Journal of the American Chemical Society, 1963. **85**(19): p. 3045-3046.
4. Merrifield, R.B., *Solid-Phase Peptide Synthesis. III. An Improved Synthesis of Bradykinin*.* Biochemistry, 1964. **3**(9): p. 1385-1390.
5. Jaradat, D.s.M.M., *Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation.* Amino Acids, 2018. **50**(1): p. 39-68.
6. Merrifield, B., *The role of the support in solid phase peptide synthesis.* British Polymer Journal, 1984. **16**(4): p. 173-178.
7. Palomo, J.M., *Solid-phase peptide synthesis: an overview focused on the preparation of biologically relevant peptides.* Rsc Advances, 2014. **4**(62): p. 32658-32672.
8. García-Martín, F., et al., *Chlorotrityl Chloride (CTC) Resin as a Reusable Carboxyl Protecting Group.* QSAR & Combinatorial Science, 2007. **26**(10): p. 1027-1035.
9. Meienhofer, J., *3 - Peptide Synthesis: A Review of the Solid-Phase Method*, in *Hormonal Proteins and Peptides*, C.H. Li, Editor. 1973, Academic Press. p. 45-267.
10. Martin, F.G. and F. Albericio, *Solid supports for the synthesis of peptides - From the first resin used to the most sophisticated in the market.* Chimica Oggi-Chemistry Today, 2008. **26**(4): p. 29-34.
11. Atherton, E., D.L.J. Clive, and R.C. Sheppard, *Polyamide supports for polypeptide synthesis.* Journal of the American Chemical Society, 1975. **97**(22): p. 6584-6585.
12. Atherton, E., et al., *The polyamide method of solid phase peptide and oligonucleotide synthesis.* Bioorganic Chemistry, 1979. **8**(3): p. 351-370.
13. Atherton, E., et al., *A physically supported gel polymer for low pressure, continuous flow solid phase reactions. Application to solid phase peptide synthesis.* Journal of the Chemical Society, Chemical Communications, 1981(21): p. 1151-1152.
14. Gutte, B., *Peptides: synthesis, structures, and applications*. 1995: Elsevier.
15. Meldal, M., *Pega: a flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis.* Tetrahedron Letters, 1992. **33**(21): p. 3077-3080.
16. Li, W., X. Xiao, and A.W. Czarnik, *Kinetic comparison of amide formation on various cross-linked polystyrene resins.* Journal of combinatorial chemistry, 1999. **1**(2): p. 127-129.
17. BAYER, E., M. DENGLER, and B. HEMMASI, *Peptide synthesis on the new polyoxyethylene-polystyrene graft copolymer, synthesis of insulin B 21–30.* International Journal of Peptide and Protein Research, 1985. **25**(2): p. 178-186.
18. Zalipsky, S., et al., *Preparation and applications of polyethylene glycol-polystyrene graft resin supports for solid-phase peptide synthesis.* Reactive Polymers, 1994. **22**(3): p. 243-258.
19. Adams, J.H., et al., *A Reinvestigation of the Preparation, Properties, and Applications of Aminomethyl and 4-Methylbenzhydrylamine Polystyrene Resins1.* The Journal of Organic Chemistry, 1998. **63**(11): p. 3706-3716.

20. Renil, M. and V. Rajasekharan Pillai, *Synthesis, characterization and application of tetraethylene glycol diacrylate crosslinked polystyrene support for gel phase peptide synthesis.* Journal of applied polymer science, 1996. **61**(9): p. 1585-1594.
21. Kempe, M. and G. Barany, *CLEAR: A Novel Family of Highly Cross-Linked Polymeric Supports for Solid-Phase Peptide Synthesis1, 2.* Journal of the American Chemical Society, 1996. **118**(30): p. 7083-7093.
22. Côté, S., *Polyether based monomers and highly cross-linked amphiphile resins*. 2010, Google Patents.
23. Wang, S.-S., *p-Alkoxybenzyl Alcohol Resin and p-Alkoxybenzyloxycarbonylhydrazide Resin for Solid Phase Synthesis of Protected Peptide Fragments.* Journal of the American Chemical Society, 1973. **95**(4): p. 1328-1333.
24. Mitchell, A.R., et al., *tert-Butoxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin, a more acid-resistant support for solid-phase peptide synthesis.* Journal of the American Chemical Society, 1976. **98**(23): p. 7357-7362.
25. DeGrado, W.F. and E.T. Kaiser, *Polymer-bound oxime esters as supports for solid-phase peptide synthesis. The preparation of protected peptide fragments.* The Journal of Organic Chemistry, 1980. **45**(7): p. 1295-1300.
26. Pietta, P.G. and G.R. Marshall, *Amide protection and amide supports in solid-phase peptide synthesis.* Journal of the Chemical Society D: Chemical Communications, 1970(11): p. 650-651.
27. Matsueda, G.R. and J.M. Stewart, *A p-methylbenzhydrylamine resin for improved solid-phase synthesis of peptide amides.* Peptides, 1981. **2**(1): p. 45-50.
28. SHEPPARD, R.C. and B.J. WILLIAMS, *Acid-labile resin linkage agents for use in solid phase peptide synthesis.* International Journal of Peptide and Protein Research, 1982. **20**(5): p. 451-454.
29. Mergler, M., et al., *Peptide synthesis by a combination of solid-phase and solution methods I: A new very acid-labile anchor group for the solid phase synthesis of fully protected fragments.* Tetrahedron Letters, 1988. **29**(32): p. 4005-4008.
30. Albericio, F. and G. Barany, *An acid-labile anchoring linkage for solid-phase synthesis of C-terminal peptide amides under mild conditions.* Int J Pept Protein Res, 1987. **30**(2): p. 206-16.
31. Rink, H., *Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin.* Tetrahedron Letters, 1987. **28**(33): p. 3787-3790.
32. Barlos, K., et al., *2-Chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage.* Int J Pept Protein Res, 1991. **37**(6): p. 513-20.
33. Albericio, F. and G. Barany, *Hypersensitive acid-labile (HAL) tris(alkoxy)benzyl ester anchoring for solid-phase synthesis of protected peptide segments.* Tetrahedron Letters, 1991. **32**(8): p. 1015-1018.
34. Bergmann, M. and L. Zervas, *Über ein allgemeines Verfahren der Peptid-Synthese.* Berichte der deutschen chemischen Gesellschaft (A and B Series), 1932. **65**(7): p. 1192-1201.
35. du Vigneaud, V., D.T. Gish, and P.G. Katsoyannis, *A SYNTHETIC PREPARATION POSSESSING BIOLOGICAL PROPERTIES ASSOCIATED WITH ARGININEVASOPRESSIN.* Journal of the American Chemical Society, 1954. **76**(18): p. 4751-4752.
36. Vigneaud, V.d., et al., *THE SYNTHESIS OF AN OCTAPEPTIDE AMIDE WITH THE HORMONAL ACTIVITY OF OXYTOCIN.* Journal of the American Chemical Society, 1953. **75**(19): p. 4879-4880.

37. McKay, F.C. and N.F. Albertson, *New amine-masking groups for peptide synthesis.* Journal of the American Chemical Society, 1957. **79**(17): p. 4686-4690.
38. Carpino, L.A. and G.Y. Han, *9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group.* Journal of the American Chemical Society, 1970. **92**(19): p. 5748-5749.
39. Albericio, F., *Solid-phase synthesis: a practical guide*. 2000: CRC Press.
40. Barany, G. and F. Albericio, *Three-dimensional orthogonal protection scheme for solid-phase peptide synthesis under mild conditions.* Journal of the American Chemical Society, 1985. **107**(17): p. 4936-4942.
41. Barany, G. and R. Merrifield, *A new amino protecting group removable by reduction. Chemistry of the dithiasuccinoyl (Dts) function.* Journal of the American Chemical Society, 1977. **99**(22): p. 7363-7365.
42. Isidro-Llobet, A., M. Álvarez, and F. Albericio, *Amino Acid-Protecting Groups.* Chemical Reviews, 2009. **109**(6): p. 2455-2504.
43. Kavanagh, K. and S. Dowd, *Histatins: antimicrobial peptides with therapeutic potential.* J Pharm Pharmacol, 2004. **56**(3): p. 285-9.
44. Pichon, C., C. Gonçalves, and P. Midoux, *Histidine-rich peptides and polymers for nucleic acids delivery.* Advanced Drug Delivery Reviews, 2001. **53**(1): p. 75-94.
45. Ferrer-Miralles, N., et al., *Biological activities of histidine-rich peptides; merging biotechnology and nanomedicine.* Microbial Cell Factories, 2011. **10**(1): p. 101.
46. Fletcher, A.R., et al., *The use of the N([small pi])-phenacyl group for the protection of the histidine side chain in peptide synthesis.* Journal of the Chemical Society, Perkin Transactions 1, 1979(0): p. 2261-2267.
47. Jones, J.H., W.I. Ramage, and M.J. Witty, *Mechanism of racemisation of histidine derivatives in peptide synthesis.* Int J Pept Protein Res, 1980. **15**(3): p. 301-3.
48. Fujii, T. and S. Sakakibara, *Studies on the synthesis of histidine peptides. I. N Im-Tosylhistidine derivatives as starting materials.* Bulletin of the Chemical Society of Japan, 1974. **47**(12): p. 3146-3151.
49. Barlos, K., D. Papaioannou, and D. Theodoropoulos, *Efficient "one-pot" synthesis of N-tritylamino acids.* The Journal of Organic Chemistry, 1982. **47**(7): p. 1324-1326.
50. Sieber, P. and B. Riniker, *Protection of histidine in peptide synthesis: A Reassessment of the trityl group.* Tetrahedron Letters, 1987. **28**(48): p. 6031-6034.
51. Barlos, K., et al., *Fmoc-His (Mmt)-OH und Fmoc-His (Mtt)-OH. Zwei nue histidin-derivative Nim-geschützt mit säure-hochempfindlichen gruppen. Darstellung, eigenschaften und einsatz in der peptidsynthese.* Tetrahedron letters, 1991. **32**(4): p. 475-478.
52. Karlström, A. and A. Undén, *The N im-(2, 4-dimethylpent-3-yloxycarbonyl)(Doc) group, a new nucleophile-resistant, HF-cleavable protecting group for histidine in peptide synthesis.* Chemical Communications, 1996(8): p. 959-960.
53. Chillemi, F. and R. Merrifield, *Use of Nim-dinitrophenylhistidine in the solid-phase synthesis of the tricosapeptides 124-146 of human hemoglobin. beta. chain.* Biochemistry, 1969. **8**(11): p. 4344-4346.
54. Shaltiel, S. and M. Fridkin, *Thiolysis of dinitrophenylimidazoles and its use during synthesis of histidine peptides.* Biochemistry, 1970. **9**(26): p. 5122-5127.
55. Simone, Z., S. Roger, and Y. Esther, *Application of Nim-2,6-Dimethoxybenzoyl Histidine in Solid-Phase Peptide Synthesis.* European Journal of Organic Chemistry, 2003. **2003**(13): p. 2454-2461.
56. Yoshizawa-Kumagaye, K., et al., *Amino acid deletion products resulting from incomplete deprotection of the Boc group from Nπ-benzyloxymethylhistidine residues*

during solid-phase peptide synthesis. Journal of peptide science: an official publication of the European Peptide Society, 2005. **11**(8): p. 512-515.
57. Colombo, R., F. Colombo, and J.H. Jones, *Acid-labile histidine side-chain protection: the N([small pi])-t-butoxymethyl group.* Journal of the Chemical Society, Chemical Communications, 1984(5): p. 292-293.
58. M., M., et al., *Synthesis and Application of Fmoc-His(3-Bum)-OH.* Journal of Peptide Science, 2001. **7**(9): p. 502-510.
59. Hruby, V.J., *Houben-Weyl Methods of Organic Chemistry. Volume E22A. Synthesis of Peptides and Peptidomimetics. Edited by Murray Goodman, Arthur Felix, Luis Moroder, and Claudio Toniolo. Georg Thieme Verlag, Stuttgart, Germany. 2001. xxvii + 901 pp. 18 × 26 cm. ISBN 3 132 19604 5. 1840 euro.* J. Med. Chem., 2002. **45**(23): p. 5187.

CHAPTER TWO

Fmoc-RinkAmide PEG Octagel Surface Resin – A new PEG-PS based resin

2.1. Introduction

Peptides are continuously growing in demand as new potentials of drugs and therapeutics. Peptide synthesis using the solid-phase approach has become the most commonly used method of synthesizing peptides on a research scale. This approach is seen as a rapid and efficient method of obtaining peptides as it uses resins as a medium to synthesize the peptides. However not all resins are compactable to synthesize certain peptides. This is due to the polymers used in creating the resins as supports. Some polymers interact well with the growing peptide allowing for an easy synthesis, and some have poor solvation and low loading resulting in a difficult synthesis. This chapter introduces a new resin that has entered the market.

OctaGel™ resin (OG resin) is a unique highly uniform surface-active resin. Sites on the interior of the resin beads are capped with PEG, leaving reactive sites only on the surface. This ensures that peptide chain growth is not inhibited by steric factors within the pores of the resin beads and that reagents are easily accessible to all reaction sites. In addition, OG resin has PEG chains of approximately 1500 MW bonded perpendicular to the surface of the beads. The PEG hydrogen bonds with the growing peptide chain to keep even highly hydrophobic peptide sequences solvated and readily accessible for further reactions (Figure 8)[60].

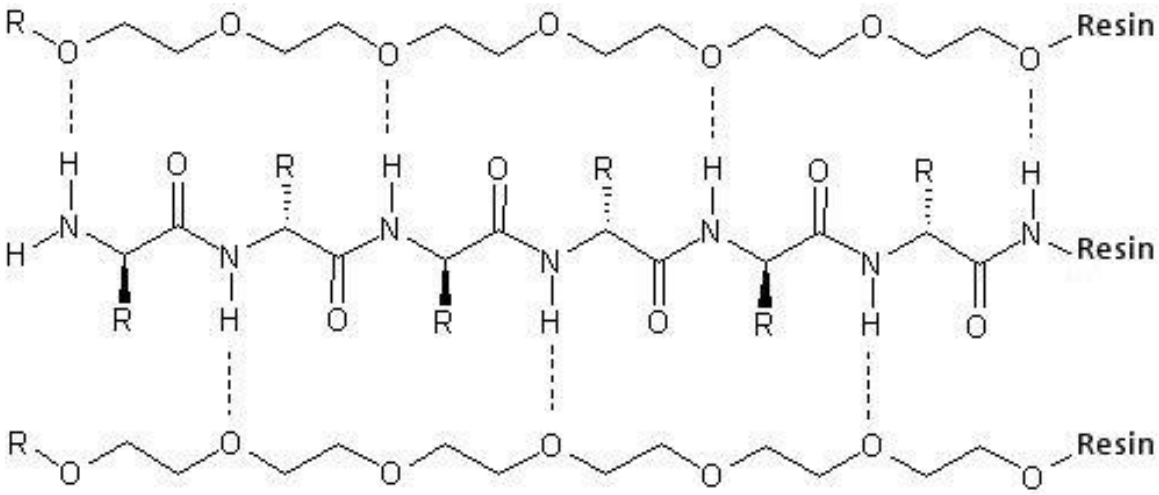
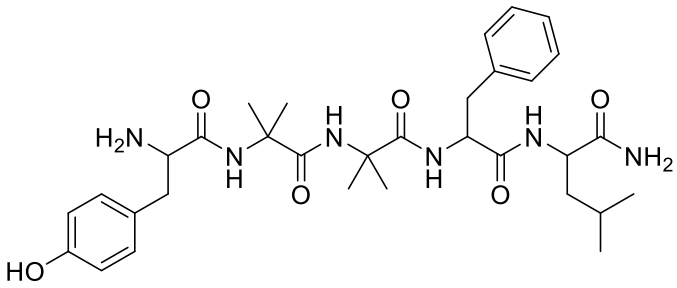


Figure 8: Peptide backbone hydrogen bonding with PEG.

A comparison study was conducted to determine the performance of three different resins towards the synthesis of two model peptide sequences. Two commonly used resins; Fmoc-Rink-Amide Amino-methyl Polystyrene, (Fmoc-Rink-Amide Amino-methyl PS) and H-Rink-Amide ChemMatrix®, (H-Rink-Amide CM) resin was used to compare the performance of a novel Fmoc-Rink-Amide PEG Octagel Surface resin

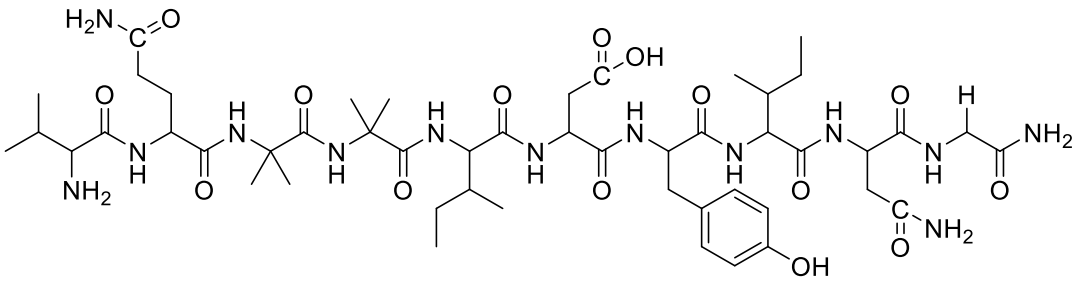
Swelling studies were conducted on each resin using 10 different solvents and swelling calculations were measured (see results). The resin beads were imaged using microscopic techniques. SEM images of each resin was imaged as dry beads before its use in synthesis. Light microscopic images were taken of each resin, when dry and when submersed in solvents like DCM, DMF and 2-MeTHF. The model peptides, Aib-enkephalin pentapeptide and Aib-ACP decapeptide (figure 9) were synthesized on each resin using *N,N'*-Diisopropylcarbodiimide (DIC) and OxymaPure as coupling reagents.

1



Sequence: H-Tyr-Aib-Aib-Phe-Leu-NH_2

2



Sequence: H-Val-Gln-Aib-Aib-Ile-Asp-Tyr-Ile-Asn-Gly-NH_2

Figure 9: Aib-pentapeptide (1) and Aib-ACP decapeptide (2)

2.2. Method

2.2.1. Swelling

Each resin (200 mg) was placed in a 5-mL syringe, treated with enough solvent to swell the resin, and allowed to stand for 10 min with moderate stirring. The swollen resin was compressed with the piston of the syringe until no more solvent could be extracted. The piston was pulled slowly until the resin regained its maximum volume in the syringe, and the volume of the resin was read (the void volume of the tip and the syringe was averaged to 0.15 mL). The swelling was calculated according to the following formula:

$$\frac{(\text{Volume of the swelled resin} + 0.15\ \text{mL})}{0.2\ \text{g}} = \text{X}\ (\frac{\text{mL}}{\text{g}})$$

2.2.2. Optical imaging studies

SEM was conducted on each of the resins at different magnifications (×25 and ×50). Each of the resins were dry when experiment was conducted. Light microscopy was also conducted on each of the resins. Beads of each resin were analysed as dry as well as under solvents of DCM, DMF and 2-MeTHF. A spatula full of each resin were placed onto slides and imaged under the microscope using ×40 magnification. Thereafter, a drop of each solvent was added to each slide and viewed under a microscope.

Images were taken using a Leica ICC50 HD microscope with Leica Microsystems Application Suite version 2.1.0 software.

2.2.3. SPPS

The synthesis was carried out manually in a polypropylene syringe fitted with a polyethylene porous disc. 68 mg of Fmoc-RinkAmide-AM-PS resin (0.74 mmol/g), 113 mg of H-RinkAmide-AM-CM resin (0.45 mmol/g) and 111 mg of Fmoc-RinkAmide-AM-PEG-OG resin (0.44 mmol/g) was used in the synthesis. The swelling of OG and CM resins was achieved as the following follows: MeOH (2×1 min), DMF (2×1 min), DCM (2×1 min), TFA-DCM (1:99) (2×1 min), DIEA-DCM (1:19) (2×1 min), and DCM (2×1 min) (5 mL each)[61].

The resin was treated with deprotection solution (20% piperidine in DMF), then washed by DMF (2×1 min), DCM (2×1 min), and DMF (2×1 min) (5 mL each). Fmoc amino acids derivatives (3 equiv.), OxymaPure (3 equiv.), and DIC (3 equiv.) in minimum amount of DMF were preactivated for 3 min then added to the resin in the first coupling process, and the mixture was shaken for 1 h. After complete coupling, the resin was washed by DMF (2×1 min), DCM (2×1 min), and DMF (2×1 min) (5 mL each) and then de-blocked by treatment with deprotection condition that mentioned above.

The cycle was then repeated to obtain the target peptide with exception of the second Aib residue where double coupling was applied for 1 h in case of Aib-enkephalin pentapeptide and 2 h in case of Aib-ACP decapeptide. The peptide was cleaved from the resin with TFA/TIS/H_2O (95:2.5:2.5) at rt for 2 h. TFA was removed under nitrogen, and the crude peptide was precipitated with cold diethyl ether (Et_2O) (3 × 10 mL). Then, the crude peptide was analyzed by HPLC and LC-MS (electrospray ionization).

2.3. Results and Discussion

2.3.1. Swelling

Table 3. Swelling calculations for the three resins tested under 10 different solvents

Entry	Solvent	Swelling (mL/g)		
		PS	CM	OG
1	DMF	5.5	8.25	6.25
2	DCM	5.75	10.25	7.25
3	MeCN	3.25	6.75	4.25
4	THF	5.75	7.75	5.5
5	2-MeTHF	4	6.25	5.25
6	Dimethyl carbonate	4.25	7	5.25
7	γ – Valerolactone	5.25	6.75	5.5
8	Cyclopentyl methyl ether	3.75	3.75	3.75
9	Propan-2-ol	3.25	3.75	3.75
10	Water	3	6.75	3.75

Swelling is an indication of how well solvents or soluble reagents will interact with the resin to allow an easy synthesis of a peptide. Swelling results indicate that Fmoc-Rink-Amide PEG OG has a good swelling-solvent ratio especially in the standard solvents used for SPPS, (DCM and DMF). Overall, OG resin has a moderate level of swelling amongst the different solvents tested, by comparison.

2.3.2. Scanning electron microscopy

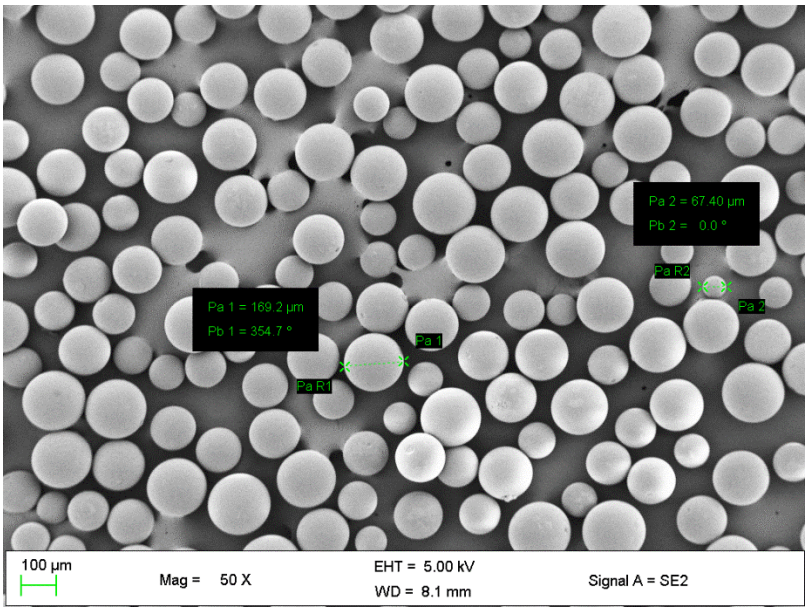


Image 1: SEM image of PS resin with diameter of smallest and largest bead

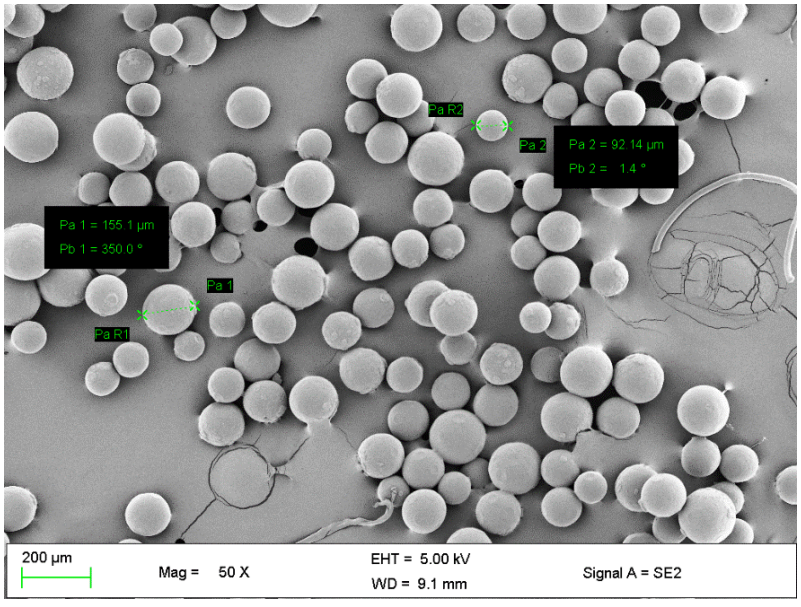


Image 2: SEM image of CM resin with diameter of smallest and largest bead

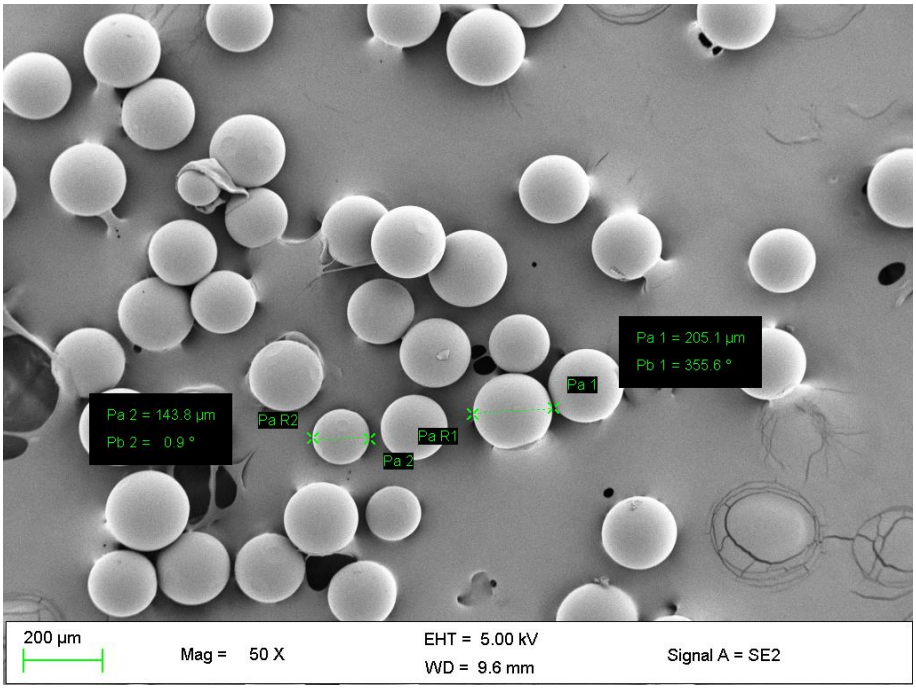


Image 3: SEM image of OG resin with diameter of smallest and largest bead

OG resin beads are of larger particle size (205.1 µm) compared to CM (155.1 µm) and PS (169.2 µm). This property shows its superiority amongst the others as a bigger particle size allows for increased surface area resulting in increased interactions of reagents and solvents with the growing peptide chain on the resin.

OG also shows increased uniformity of its resin bead sizes (compared to CM and PS) providing an ease in filtration and washings in between peptide chain growth (see appendix A for more SEM images). This also helps in the isolation of the peptide from the resin.

2.3.3. Light Microscopy

A light microscope (Leica ICC50 HD) was used to sample each resin beads. Beads were placed on slides as dry samples. Thereafter each solvent was added to the slide and images were taken of the beads using Leica imaging software. Magnification was set at ×40 and images shown below are of individual beads taken from images obtained from the imaging software (see appendix A).

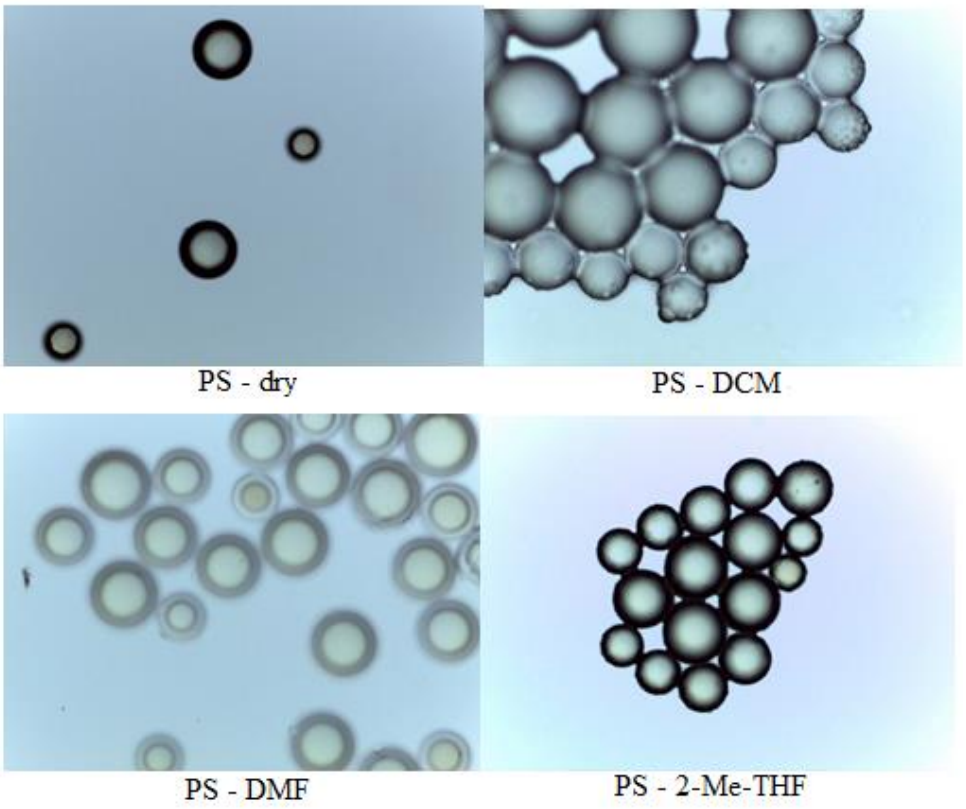


Image 4: PS beads under a light microscope in DCM, DMF and 2-Me-THF

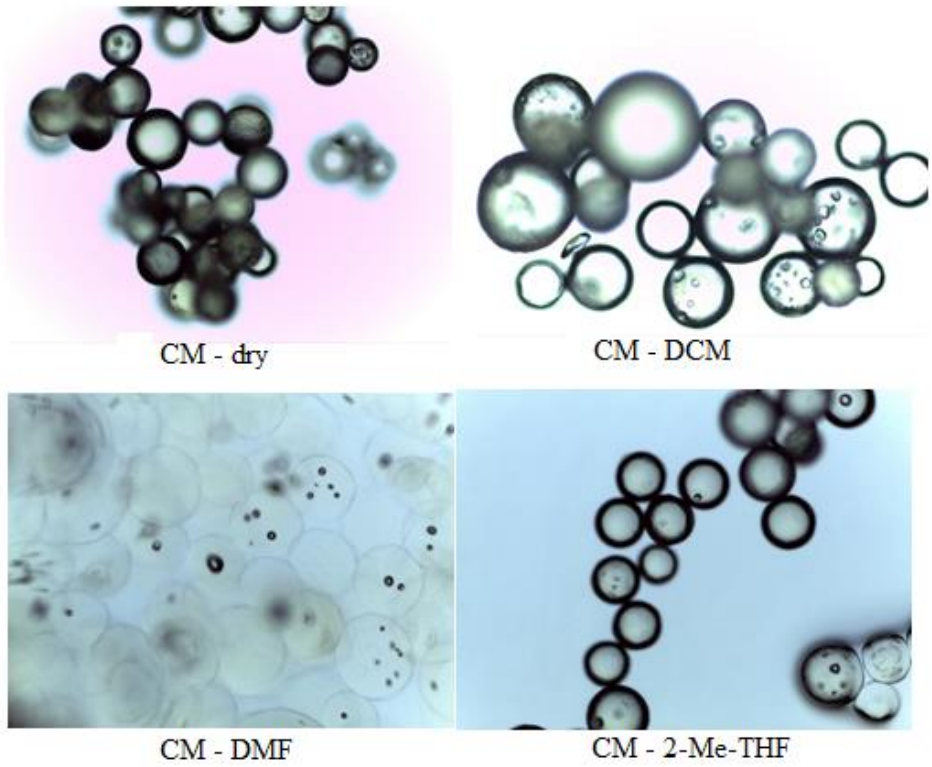


Image 5: CM beads under a light microscope in DCM, DMF and 2-Me-THF

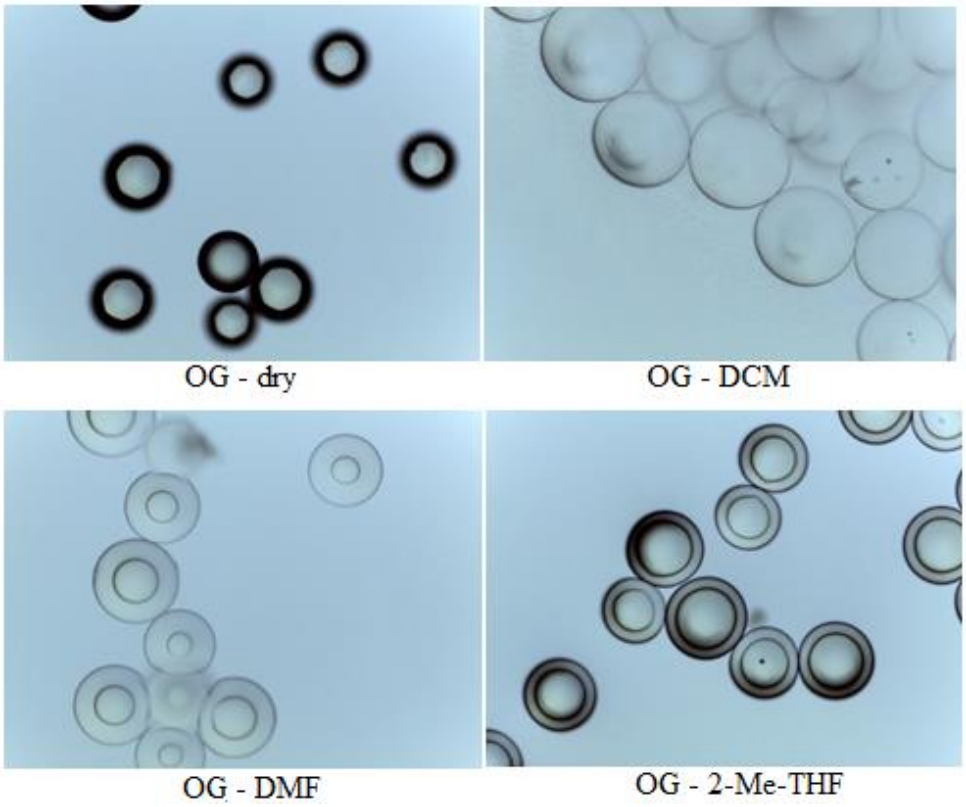


Image 6: OG beads under a light microscope in DCM, DMF and 2-Me-THF

Based on observations using light microscopy, OG beads interacted well with DCM, DMF and 2-MeTHF from the surface towards the interior of the bead (image 6). OG beads also show no damages such as dented tracts, as compared to CM (image 5 and image 6). Each of the beads swells around three times its original size (when dry) under DCM and DMF based on observations. 2-Me THF solvent hardly swells PS and CM beads but shows interaction with OG.

2.3.4. HPLC and LCMS Analysis

Aib-enkephalin pentapeptide:

A linear gradient of 20–40% 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min was applied, with a flow rate of 1 mL/min and detection at 220 nm using an YMC-Triart C18 (3 μm, 3.0× 150 mm) column, t_R(pentapeptide) = 6.7 min, t_R (des-Aib) = 7.01 min. LC–MS showed m/z for the pentapeptide as $[M + H^+]$ = 611.36. and $[M – H^+]$ = 609.14. Mass of peptide = 610.76

Aib-ACP decapeptide:

A linear gradient of 10–50% 0.1% TFA in CH_3CN/0.1% TFA in H_2O over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomex C18 (3 μm, 4.6 ×50 mm) column, t_R(decapeptide) = 6.9 min, t_R(des-Aib) = 7.1 min. LC–MS showed m/z for the decapeptide as [M] = 1090.35 and [M – $2H^+$] = 1088.17. Mass of peptide 1090.25

Table 4. Percentage purity of Aib-enkephalin pentapeptide from HPLC

Entry	**Resin**	**Pentapeptide (%)**	**des-Aib (%)**
1	PS	96.5	3.5
2	CM	95.4	1.7
3	OG	89.0	8.8

Table 5. Percentage purity of Aib-ACP decapeptide HPLC

Entry	**Resin**	**Decapeptide (%)**	**des-Aib (%)**
1	PS	91.1	4.2
2	CM	86.2	4.7
3	OG	95.4	2.0

OG rendered the product in lower purity than PS and CM in case of Aib-enkephalin pentapeptide. The performance of the OG resin was better during synthesis of the longer peptide, Aib-ACP decapeptide. It rendered the product in higher purity than PS and CM resin with a low formation of the des-Aib nonapeptide. See appendix A for the HPLC.

2.4. Conclusion

Fmoc-Rink-Amide PEG OG resin has been successful in the synthesis of Aib-pentapeptide and Aib-ACP decapeptide with high purity of the decapeptide. Swelling results show that OG values falls in between PS and CM resins with synthetic results being similar to that of CM (in terms of yield and purity of the peptide sequence).

The two peptides synthesized were 'difficult' sequences due to the Aib amino acids used in place of glycine residues. This amino acid was left to couple for a longer period, with double coupling on second Aib residue, than the others in the sequence, in all three resins used. Octagel resin gave poor results in the case of Aib-pentapeptide by forming des-Aib tetrapeptide as a side product, based on HPLC analysis. This resulted in a low purity of the desired pentapeptide. This observation also would indicate that the synthesis of Aib-ACP decapeptide, on Octagel, would form the des-Aib nonapeptide more prevalent, however in our case it did not. This may be due to a good and stable interaction of the amino acids in the peptide interaction with the surface of the resin (Octagel).

The resin beads of OG resin are of superior quality based on imaging results obtained. Overall OG resin shows great potential in synthesizing peptide sequences with high purity.

2.5. Future work

Exploring the use of OG resin in the synthesis of longer peptide sequences, as well as green solvent peptide synthesis.

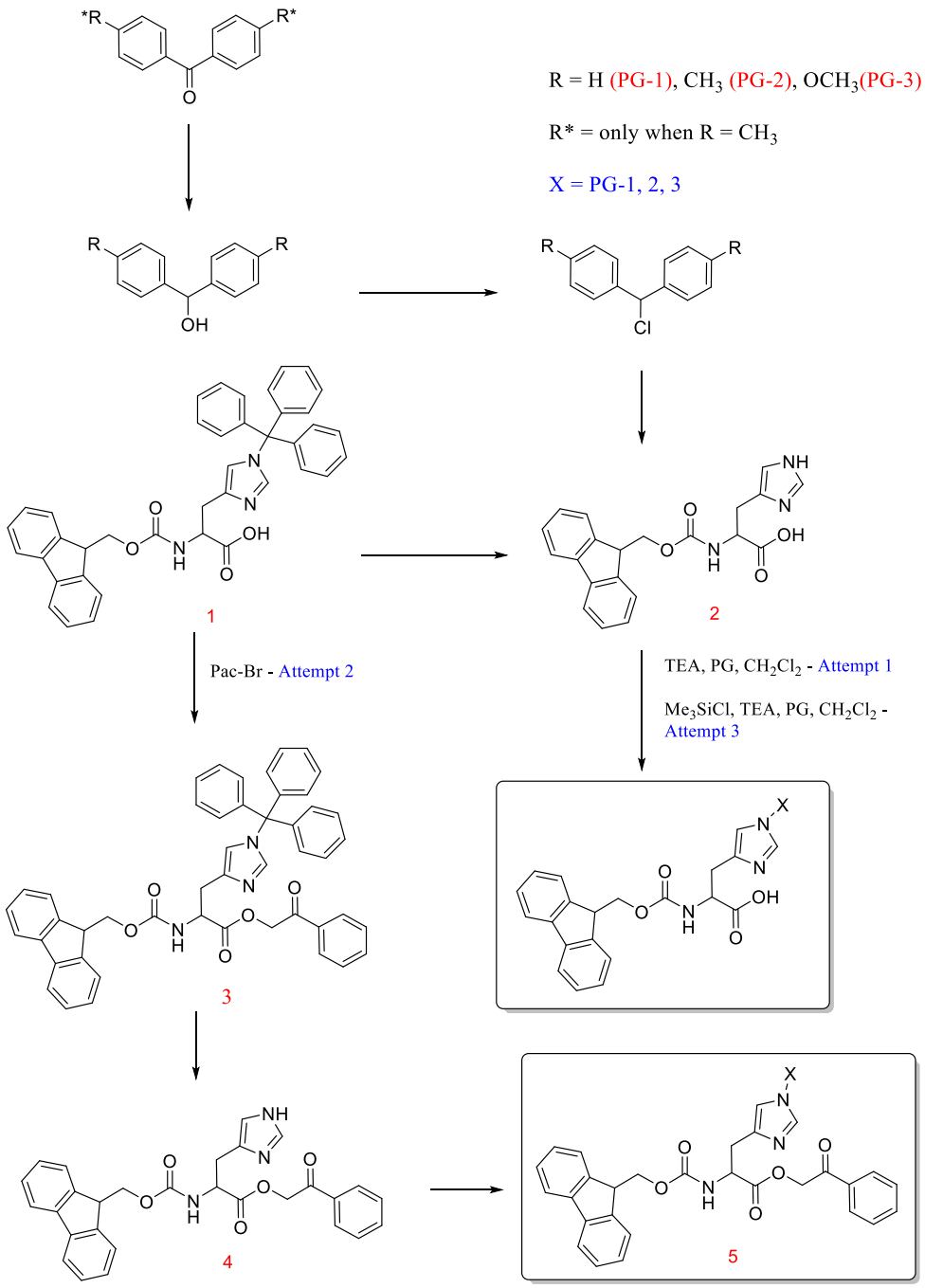
2.6. References

1. Information about Octagel was provided via personal correspondence with Aaptec Biotechnology
2. Garcia-Ramos, Y., et al., *ChemMatrix (R) for complex peptides and combinatorial chemistry.* Journal of Peptide Science, 2010. **16**(12): p. 675-678.

Chapter Three

Novel side chain protecting group of histidine for Fmoc peptide synthesis

3.1. Methodology

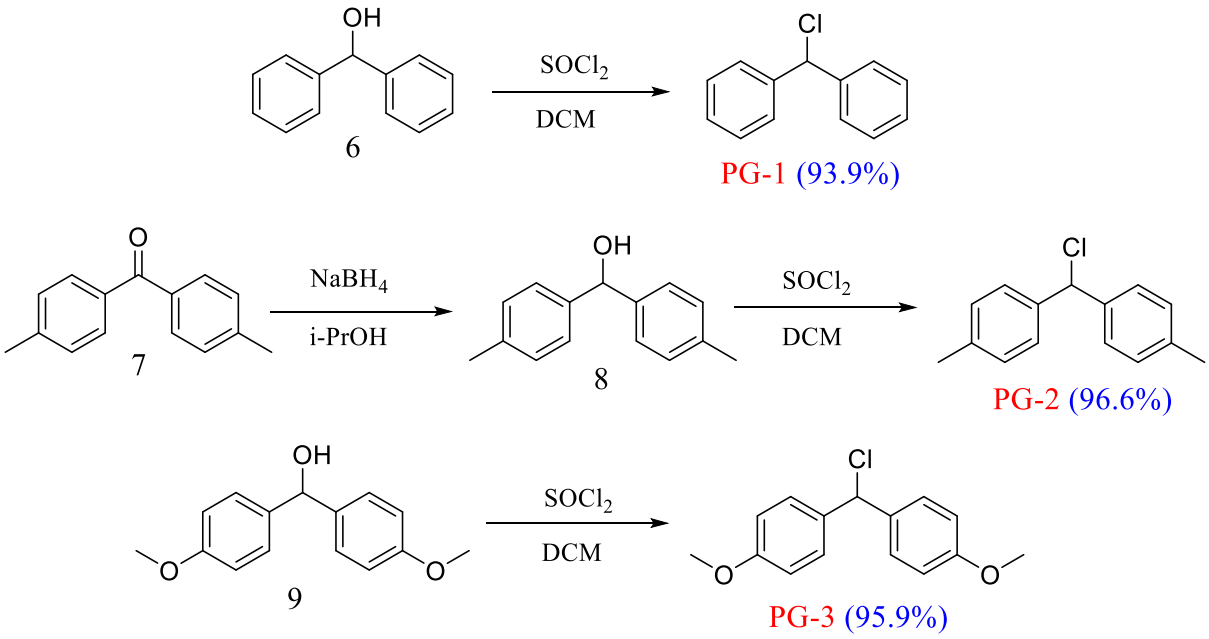


Scheme 9: A representation of different approaches done parallelly in protecting histidine

3.2. Experimental

1. Preparation of protecting groups

Three diphenyl type protecting groups were selected as they are cheap and relatively inexpensive to derivatize. Scheme 10 below shows how modifications to these groups were made to allow easy attachment to the histidine amino acid.



Scheme 10: Synthetic steps used to obtain the necessary protecting group

PG-1 (benzhydryl chloride) and PG-3 (4,4-dimethoxybenzhydryl chloride)

Compounds 1 and 4 from scheme 10 (500 mg, 2.7 mmol) were dissolved in DCM (25 ml) and stirred. Thionyl chloride ($SOCl_2$) (0.3 mL, 3 mmol) was added dropwise to the reaction mixture and was stirred under rt for 5 h. Upon completion, solvent and excess $SOCl_2$ was removed under rotary evaporation to yield PG-1 as a pale-yellow liquid and PG-3 as a pink solid[62].

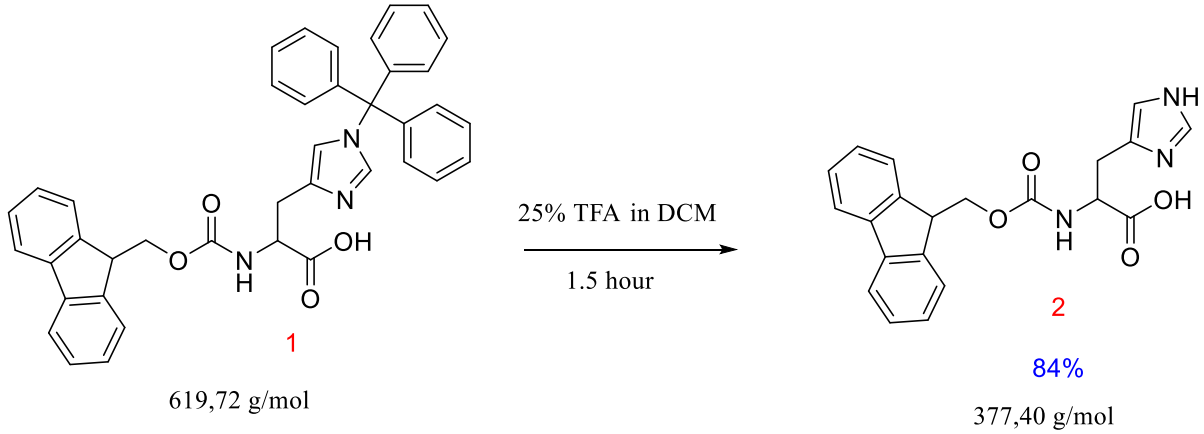
PG-2 (4,4-dimethylbenzhydryl chloride)

Compound 2 (500 mg) was first reduced to 3[63] and then it was converted to PG-2 using the same method above as a pale-yellow crystalline solid.

Three approaches were carried out as attempts to attach the protecting groups to Fmoc-His-OH.

Attempt 1:

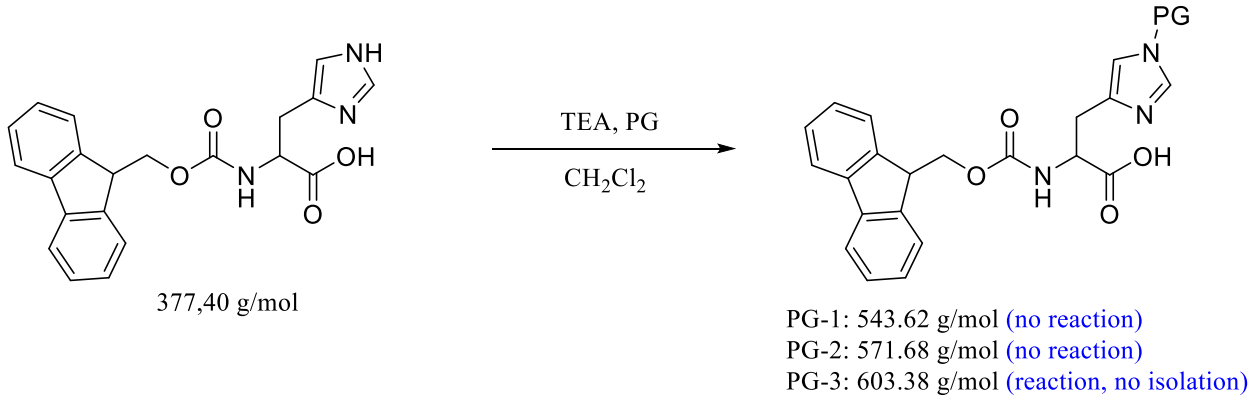
1. Removal of trityl group from Fmoc-His(trt)-OH



Scheme 11: Step 1 in attempt 1

Fmoc-His(trt)-OH (1) (500 mg, 0.81 mmol) was dissolved in DCM (10 mL) and stirred. To this, 2.5 mL TFA were added dropwise and the resulting solution was stirred for 1.5 h (TLC 5% MeOH in DCM). Work up of reaction was done by removal of solvent and acid under rotary evaporation. The concentrated sample was washed with cold Et_2O (15 mL ×5). After decanting of Et_2O, the product obtained was left to dry under bench vacuum to yield (2) (398 mg) as white solid (84% yield).

2. Attaching protecting group to Fmoc-His-OH

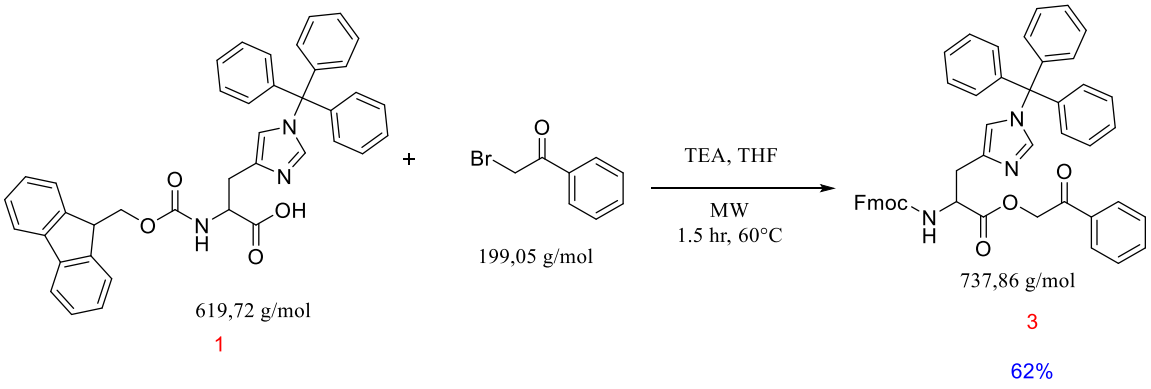


Scheme 12: Step 2 in attempt 1

Fmoc-His-OH (50 mg) (2) (1 equiv.) and the protecting group (PG) (1 equiv.) was dissolved in DCM. Thereafter triethylamine (3 equiv.) was added to the reaction and the mixture was stirred and monitored using TLC.

Attempt 2:

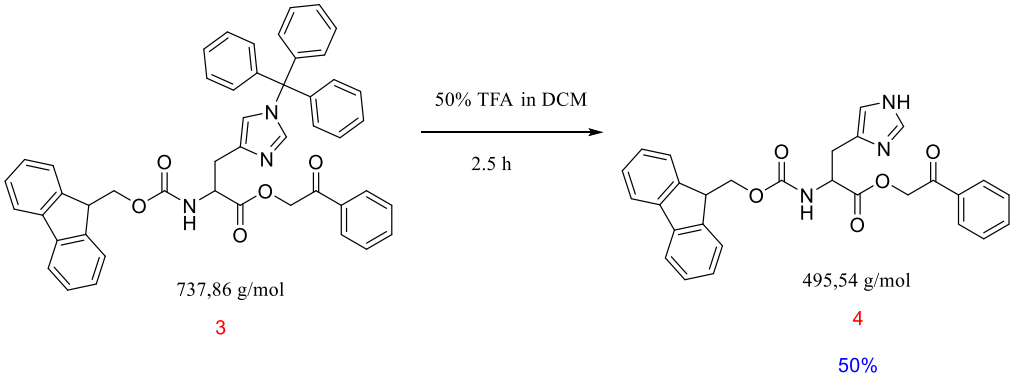
1. Protection of carboxy group using Pac-Br



Scheme 13: Step 1 in attempt 2

Fmoc-His(Trt)-OH (1 equiv.) and PacBr (1.5 equiv.) was dissolved in tetrahydrofuran (THF) in a 50 mL microwave vessel. Thereafter, TEA (3 equiv.) was added to the dissolved solution. The reaction was stirred under microwave conditions for 1.5 h at 60 °C. After completion, work up was done by dissolving in EtOAc and washing with H_2O (×3) and brine solution (×3). The organic layer was collected and dried under $MgSO_4$. The organic layer was filtered and concentrated under rotary evaporation. Purification using neutral alumina column under EtOAc:Hexane (60:40) yielded compound 3 (Fmoc-His(Trt)-O-Pac) as yellow gummy (407 mg) (62% yield)

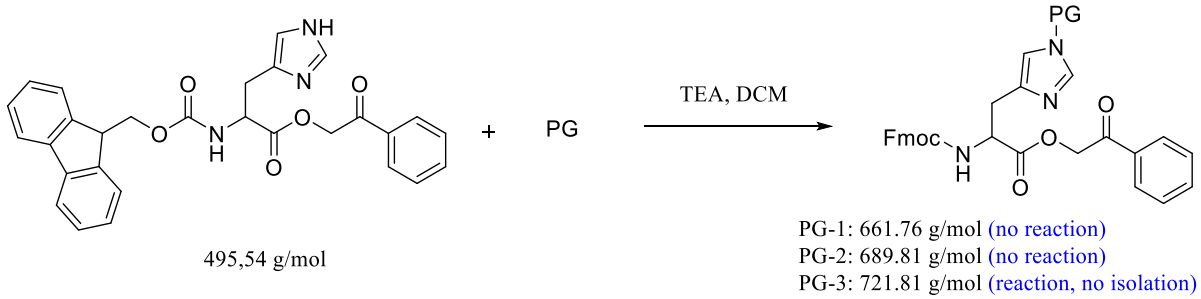
2. Removal of trityl group from Fmoc-His(trt)-O-Pac



Scheme 14: Step 2 in attempt 2

Fmoc-His(Trt)-O-Pac (407 mg) (3) was dissolved in DCM (10 mL) and stirred. To this stirred solution, TFA (5 mL) was added dropwise and the reaction was stirred at rt for 2h (based on TLC analysis: 5% MeOH in DCM). Work up of the reaction is done by removal of solvent (CH_2Cl_2) and TFA under rotary evaporation. The concentrated sample was then washed with cold Et_2O (15mL ×5) until powder was formed. After decanting of Et_2O layer, the sample was dried under bench vacuum to yield compound 4 an off-white powder (50% yield, 258 mg).

3. Attaching protecting group to Fmoc-His-O-Pac



495,54 g/mol

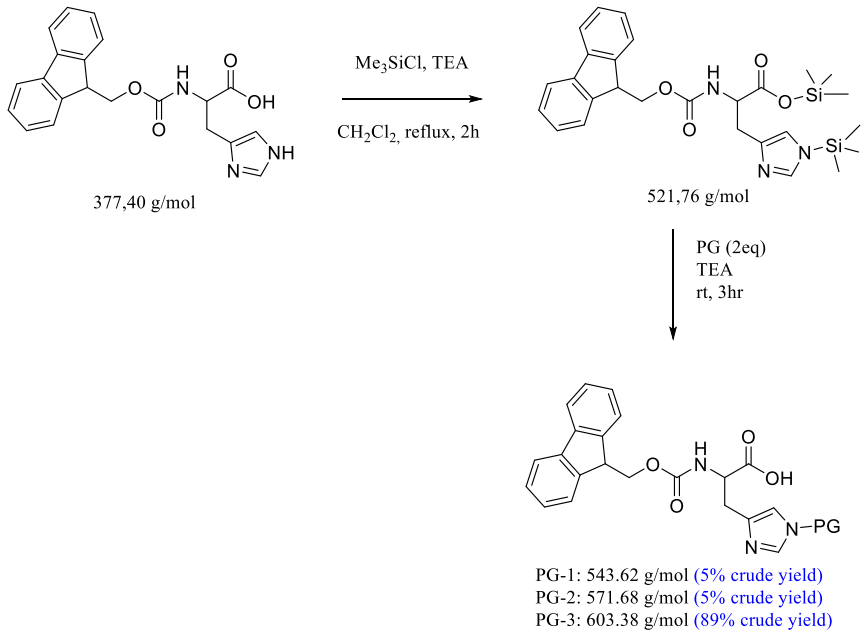
PG-1: 661.76 g/mol (no reaction)
PG-2: 689.81 g/mol (no reaction)
PG-3: 721.81 g/mol (reaction, no isolation)

Scheme 15: Step 3 in attempt 2

Fmoc-His-O-Pac (1 equiv.) and the corresponding protecting group (1 equiv.) was dissolved in DCM. Thereafter, TEA (3 equiv.) was added to the reaction mixture and stirred. The reaction was monitored using TLC.

Attempt 3:

The third attempt was an adaptation of a previous method by Barlos et al[49], in which the reaction was carried out in a one-pot synthesis.



Scheme 16: Reaction mechanism of attempt 3

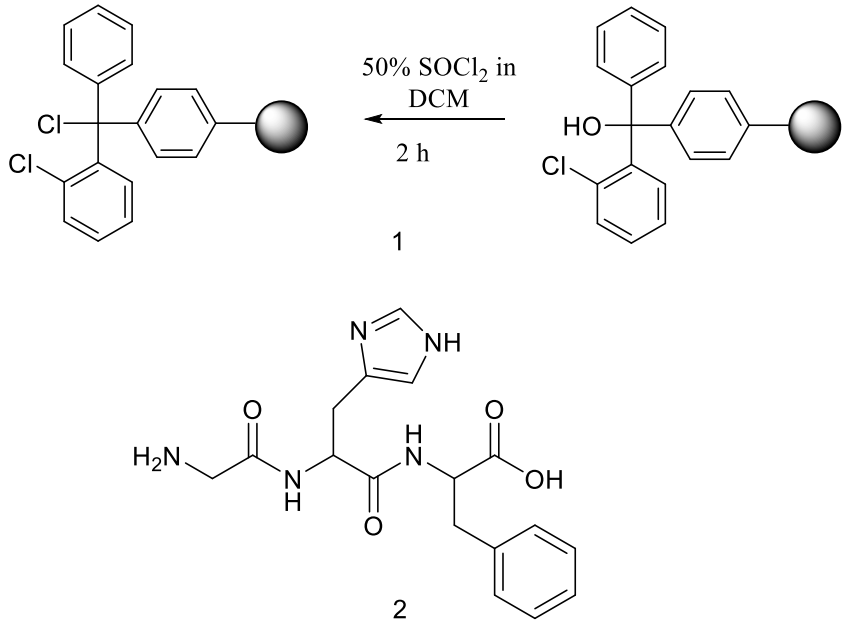
Fmoc-His-OH (1 equiv.) was dissolved in dry DCM and stirred. Me_3SiCl (1 equiv.) was added to mixture and the reaction was refluxed for 2 h. After this time, the reaction was removed from reflux, cooled to rt and added TEA (1 equiv.). The resultant solution was further refluxed for 5 min and removed to cool at room temperature. A solution of the protecting group (1 equiv.) in DCM and TEA (2 equiv.) was added dropwise to the stirred reaction after attaining rt and the solution was stirred for 3 h.

After reaction completion, work up was done as follows. Excess MeOH was added to the reaction mixture and was concentrated under rotary evaporation. The resultant residue was taken up with $CHCl_3$ and was partitioned with a 5% citric acid solution. The organic layer was washed with a brine solution (×3) and collected and dried over $MgSO_4$. The layer was filtered and concentrated to a small volume under rotary evaporation, and n-hexane was added dropwise to precipitate out the product. After decanting hexane, the crude yield obtained was 80% (for PG-3 only)

SPPS

A model peptide was H-Gly-His-Phe-OH was synthesized using 2-CTC resin.

Resin activation:



Scheme 17: Activation of 2-CTC resin (1) and model peptide H-Gly-His-Phe-OH (2)

Peptide synthesis

2-CTC resin was activated as above and washed with dry DCM (5 mL ×5). The coupling of the first amino acid Fmoc-Phe-OH (2 equiv.) with DIEA, 2 equiv. in DCM (0.5 mL) was added to resin and stirred for 1 h. Thereafter, solvent was removed and MeOH (0.75 ml) was added and reaction was stirred for 0.5 h. The Fmoc group was then removed using 20% piperidine in DMF.

Fmoc-amino acid derivatives (3 equiv.), OxymaPure (3 equiv.) and DIC (3 equiv.) in DMF (0.5mL) were preactivated for 3 min and then coupled onto the resin thereafter. At the end, the resin was washed with DMF (2×1 min), DCM (2×1 min) and DMF (2×1 min) (5 mL each) and de-blocked using deprotection solution as above.

3.3. Results and discussion

All protecting groups, while monitoring using TLC, did not show full completion of reaction even when reaction is left for a longer period to stir. Based on proton NMR, product was formed regardless.

Three attempts were carried out to try and attach PG1,2 and 3 onto Fmoc-His-OH. Each attempt was done parallelly as each PG wasn't reacting towards Fmoc-His-OH and trying different experiments would likely improve results. Experiments with PG-1 and PG-2 were unsuccessful however as in each attempt, these groups did not react.

In attempt 1:

The removal of the Trt moiety from Fmoc-His(Trt)-OH in solution required 25% TFA in DCM. This was the optimal percentage used in removing Trt in 500 mg of Fmoc-His(Trt)-OH in 1.5 h. Work-up resulted in a brownish gummy substance being formed. The addition of cold Et_2O slightly precipitates the product out of solution. Mechanically grinding (trituration) of the compound with a spatula can increase the rate of precipitation of the desired product. A by-product of this reaction is the Trt-OH molecule which is soluble in Et_2O and can be removed while triturating the compound. Cold Et_2O is added, triturated and decanted each time to allow for fresh Et_2O to be added to dissolve away the Trt-OH by-product. This continuous process of washing can result in a lower yield of the product therefore it should be done carefully. HPLC analysis of the obtained product at 7.24 min in a linear gradient of 5-95% 0.1%TFA in CH_3CN / 0.1% TFA in H_2O over 15 min gave Fmoc-His-OH in 92% purity but after storing for a few months the purity dropped to 75% with a shift of retention to 8 min due to instrument technical difficulties (appendix B).

When attaching the necessary protecting groups, it was found that PG-1 did not show reactivity towards Fmoc-His-OH even after stirring reaction under heat (water bath 35 °C) and overnight. Same result was obtained when PG-2 was used. The use of different solvents, as well as varying of the equivalent of base and protecting group in the reaction yielded the same result. In my hands, the reactions did not take place. A suspected reason was that the protecting groups had hydrolysed in solution during reaction and did not react.

In the case of PG-3, after 1 h of stirring the reaction, there was a formation of compounds - based on TLC analysis, (4% MeOH in DCM mobile phase) with Rf = 0.26, 0.24, 0,21.

However, several attempts in purifying the crude reaction resulted in no isolation the compound due to mechanical loss during purification. With the goal of attaching a protecting group on the imidazole group I focussed on the other attempts.

In attempt 2:

The aim was to temporarily protect the carboxylic end of Fmoc-His-OH and then to attach the necessary protecting group to the imidazole side chain. The Pac moiety was selected as this group is known to be stable in strong aqueous acids and alkali but is selectively removed with zinc dust in acetic acid (AcOH) or with photolysis[46].

Phenacyl group was attached to Fmoc-His(Trt)-OH under basic conditions to allow the formation of Fmoc-His(Trt)-O-Pac as the major product.

However, with the development of a protecting group for histidine suitable for Fmoc/t-Bu peptide synthesis in mind, this approach is too tedious. It requires the release of the Pac moiety after side chain protection to allow a free carboxyl end to be activated for peptide bond formation. The increase in synthetic steps and the potential use of zinc dust which could leave traces of zinc in peptide synthesis makes this an unreliable approach hence I did not move forward in attaching the protecting groups.

In attempt 3:

This procedure is an adaptation of a "one-pot" synthesis of *N*-Trt-amino acids[4949]. Fmoc-His-OH amino acid was used instead of the free amino acid derivative as per initial procedure and the trityl chloride was replaced with the synthesized protecting groups (PGs). In the case of PG-1 and PG-2, work up of the reaction resulted in precipitation of the compound in low yield ($\leq 5\%$) in a 200 mg reaction. While carrying out the work-up, partitioning the crude with 5% citric acid resulted in precipitate forming in the citric acid wash. Upon further analysis, it was seen based on HPLC analysis that the precipitate formed was that of the unreacted Fmoc-His-OH. This proves that PG-1 and PG-2 have been hydrolysed and did not partake in the reaction.

When PG-3 was used in the synthesis, there was no presence of unreacted Fmoc-His-OH found in the citric acid wash. Furthermore, after work up of the reaction, the crude was obtained in 85% yield. A HPLC analysis of the crude shows that *N* τ-Fmoc-His(PG-3)-OH is the major compound (55%) as the major component(see appendix B).

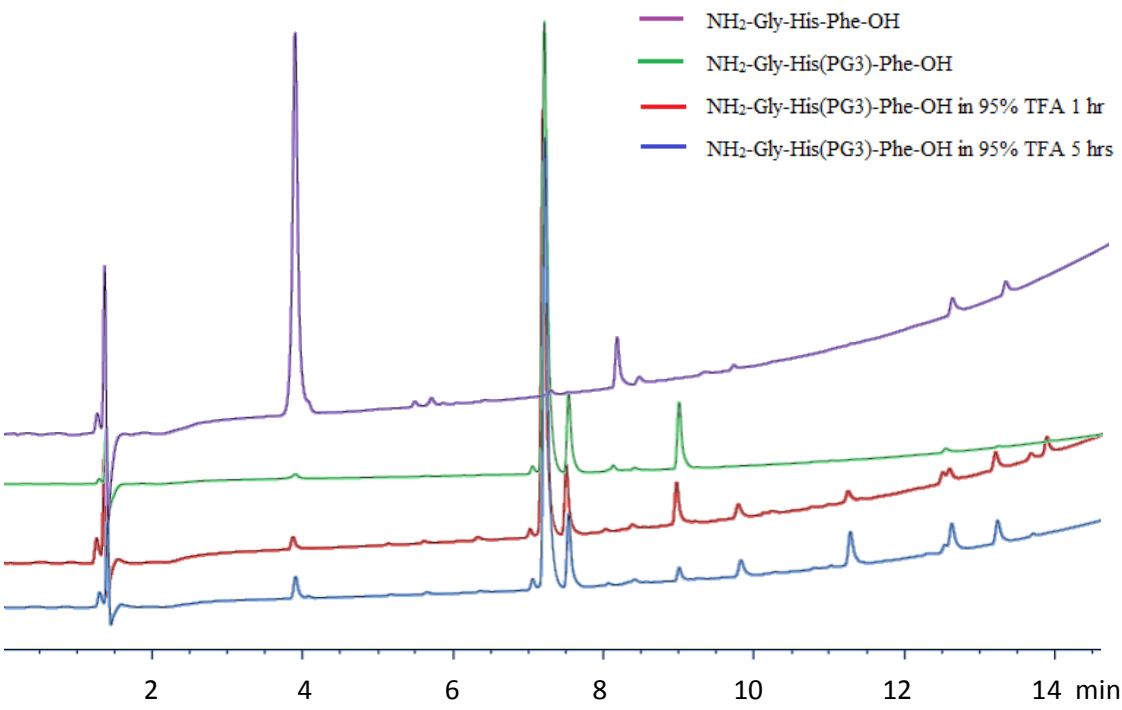
Further purification using silica gel column chromatography of the crude was unsuccessful as a mixture of N-τ Fmoc-His(PG-3)-OH and Fmoc-His(PG-3)-O-PG3 was recovered (60% yield). LC/MS data confirmed the respective masses of these compounds (appendix B).

The crude, Fmoc-His(PG-3)-OH was used in a model peptide H-Gly-His-Phe-OH. The presence of Fmoc-His(PG-3)-O-PG3 in the crude will not interfere in the reaction, because it will not couple to the peptide since its protected at the carboxyl end. The model peptide was first synthesized using Fmoc-His(Trt)-OH on 2-CTC resin. Cleavage of this peptide from CTC was done using a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The same cocktail was used in cleaving the peptide containing Fmoc-His(PG-3)-OH. Cleavage of the peptide containing Fmoc-His(Trt)-OH from resin showed the removal of the Trt group in 15 min after treatment of the cleavage cocktail.

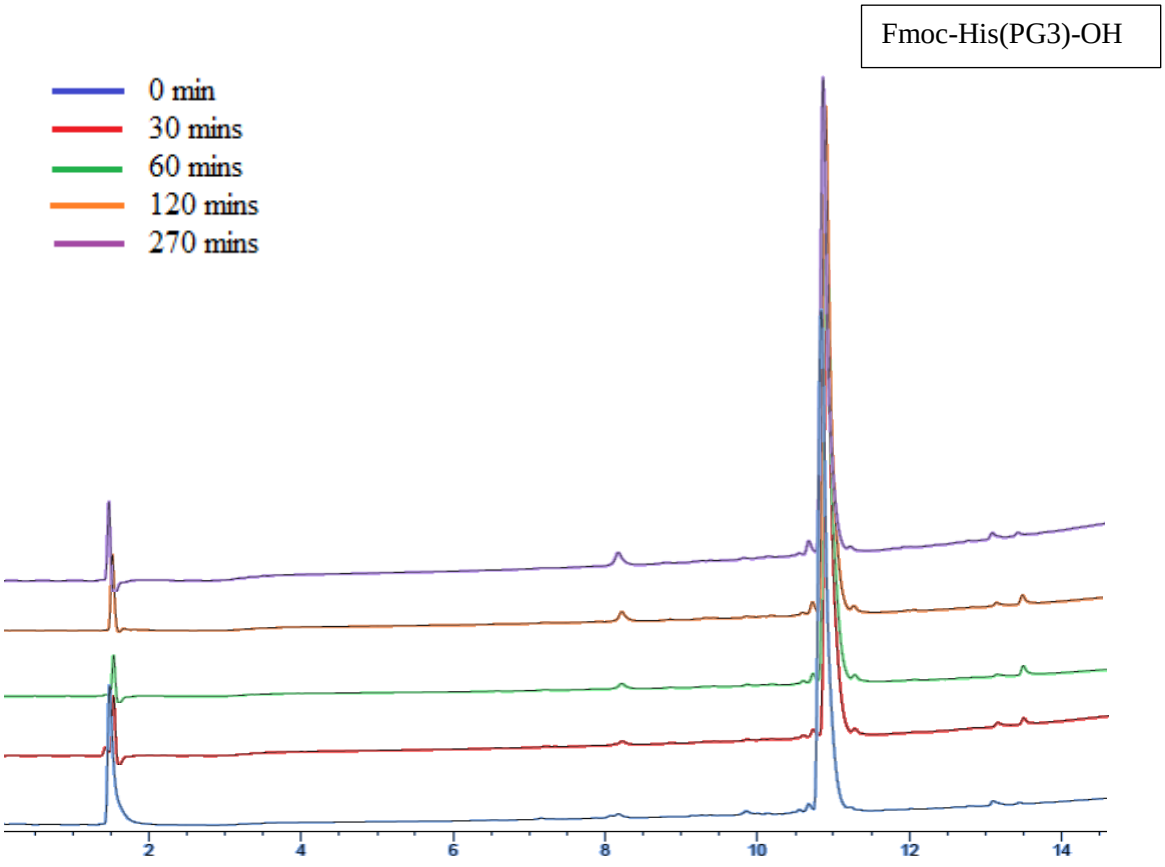
When the same conditions were applied for the tripeptide containing Fmoc-His(PG3)-OH, after 15 min, the peptide still contained PG-3 on the His side chain. Prolonged treatments with the cleavage cocktail (1 h and 5 h) showed very little formation of the desired peptide without any side chain protection (see chromatogram 1). Keeping the peptide in the cleavage cocktail overnight has no significant difference in the results. Cleavage of PG-3 occurs at a low rate when attached to the peptide.

Semi-preparative chromatography purification of the crude compound yielded approx. 10 mg of pure Fmoc-His(PG-3)-OH. NMR (^{1}H and ^{13}C) was obtained for the compound (see appendix B). The product, after NMR analysis was diluted in H_2O and lyophilised to obtain Fmoc-His(PG3)-OH in powder form. Acid lability tests were conducted on the amino acid using three acid cocktails; 100% TFA, 97.5% TFA with 2.5% H_2O and 95% TFA with 2.5% H_2O and 2.5% TIS.

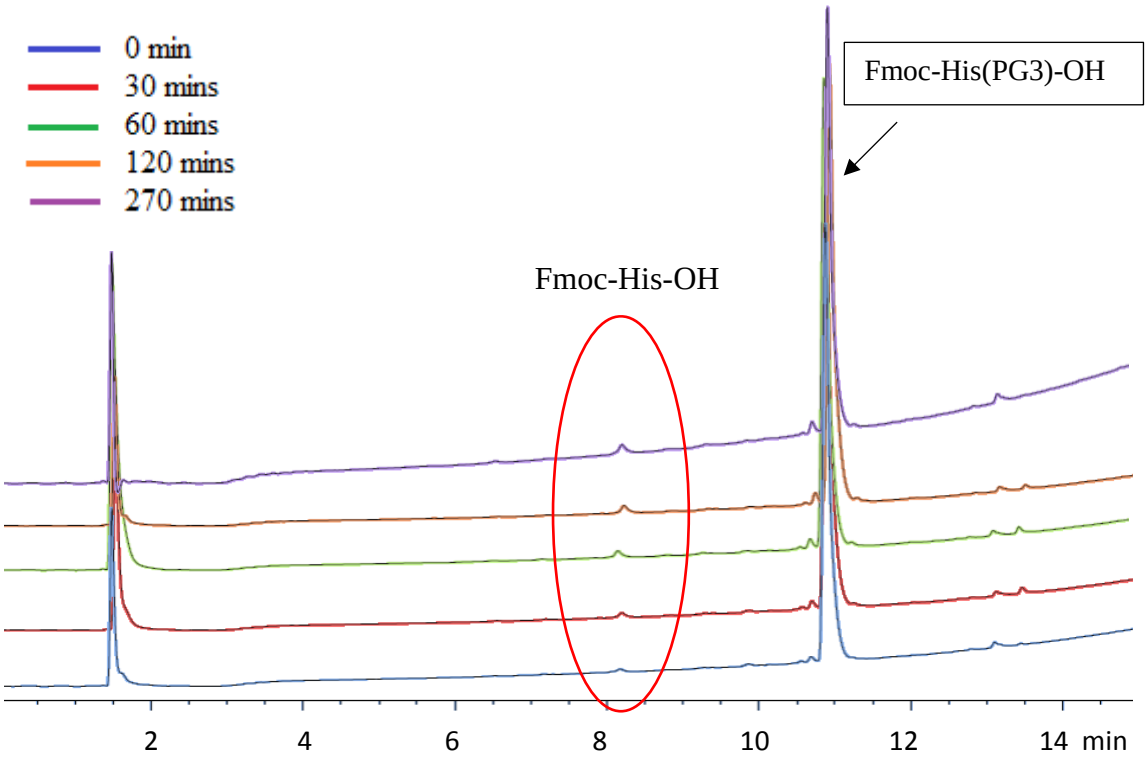
A 3.3 μM stock solution of Fmoc-His(PG3)-OH in DCM was made for analysis. 50 μL of the solution was added to three Eppendorf's and solvent was removed (DCM). Thereafter each cleavage cocktail was added to separate Eppendorf's and the reaction was monitored using HPLC at different time intervals. See chromatograms 2-4. The HPLCs indicate that PG-3 is stable in TFA treatment when attached onto Fmoc-His-OH. See appendix B for all data.



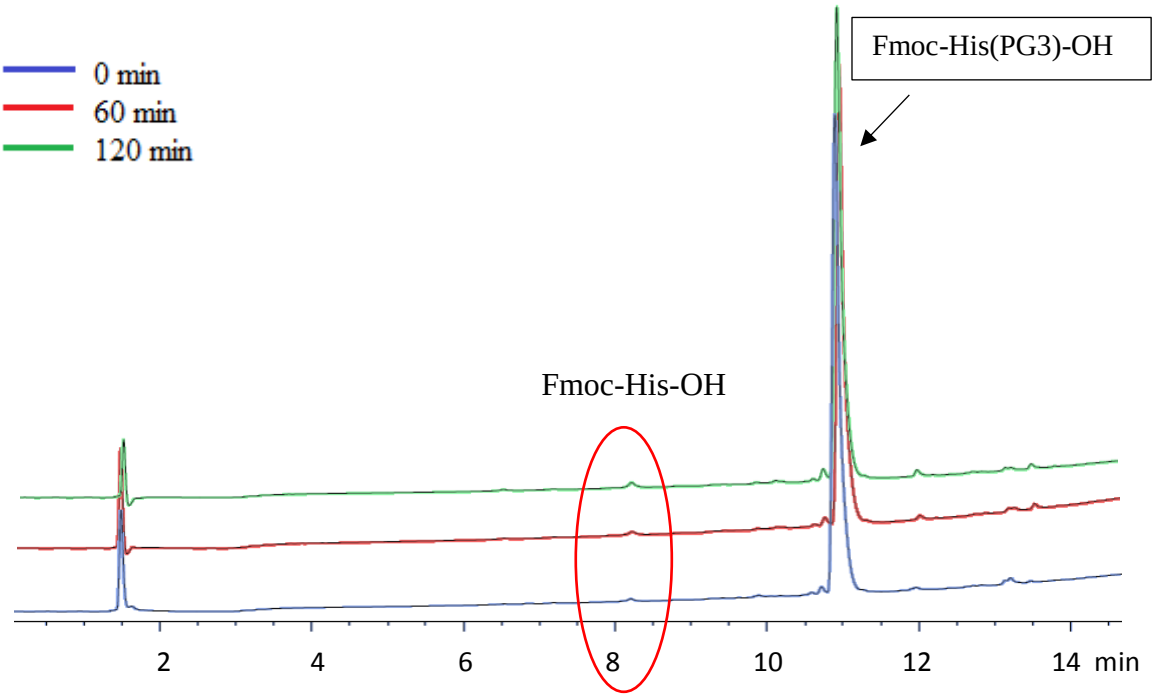
Chromatogram 1: Comparison of model peptide against treatment of TFA at different time interval



Chromatogram 2: Fmoc-His(PG3)-OH in 100% TFA during different time intervals



Chromatogram 3: Fmoc-His(PG-3)-OH in 97.5% TFA and 2.5% H_2O at different time intervals



Chromatogram 4: Fmoc-His(PG-3)-OH in 95% TFA, 2.5% H_2O and 2.5% TIS at different time intervals

3.4. Conclusion

Attempts of attaching a protecting group on Fmoc-His-OH amino acid was successful for only one of the functionalities used, PG-3. The 4,4-dimethoxydiphenyl moiety (PG-3) was suspected to behave like the trityl moiety in terms of acid lability however based on the results obtained, PG-3 showed a high stability towards high percentages of TFA with and without the use of scavenger's H_2O and TIS even after 4 h in the cleavage solution. The crude Fmoc-His(PG3)-OH from attempt three was successfully used to create the model peptide H-Gly-His-Phe-OH. Cleavage of the peptide from CTC resin indicated that PG-3 was still attached to the His residue after cleavage (see appendix B HPLC and LC/MS results).

3.5. Future work

Optimize the reaction and purification of attempt 3 with the use of PG-3 and develop a cleavage condition to remove PG-3 from Fmoc-His-OH

3.6. References

1. Fischer, E. and E. Fourneau, *Ueber einige Derivate des Glykocolls.* Berichte der deutschen chemischen Gesellschaft, 1901. **34**(2): p. 2868-2877.
2. Merrifield, R.B., *Solid phase peptide synthesis. I. The synthesis of a tetrapeptide.* Journal of the American Chemical Society, 1963. **85**(14): p. 2149-2154.
3. Letsinger, R.L. and M.J. Kornet, *Popcorn Polymer as a Support in Multistep Syntheses.* Journal of the American Chemical Society, 1963. **85**(19): p. 3045-3046.
4. Merrifield, R.B., *Solid-Phase Peptide Synthesis. III. An Improved Synthesis of Bradykinin*.* Biochemistry, 1964. **3**(9): p. 1385-1390.
5. Jaradat, D.s.M.M., *Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation.* Amino Acids, 2018. **50**(1): p. 39-68.
6. Merrifield, B., *The role of the support in solid phase peptide synthesis.* British Polymer Journal, 1984. **16**(4): p. 173-178.
7. Palomo, J.M., *Solid-phase peptide synthesis: an overview focused on the preparation of biologically relevant peptides.* Rsc Advances, 2014. **4**(62): p. 32658-32672.
8. García-Martín, F., et al., *Chlorotrityl Chloride (CTC) Resin as a Reusable Carboxyl Protecting Group.* QSAR & Combinatorial Science, 2007. **26**(10): p. 1027-1035.
9. Meienhofer, J., *3 - Peptide Synthesis: A Review of the Solid-Phase Method*, in *Hormonal Proteins and Peptides*, C.H. Li, Editor. 1973, Academic Press. p. 45-267.

10. Martin, F.G. and F. Albericio, *Solid supports for the synthesis of peptides - From the first resin used to the most sophisticated in the market.* Chimica Oggi-Chemistry Today, 2008. **26**(4): p. 29-34.
11. Atherton, E., D.L.J. Clive, and R.C. Sheppard, *Polyamide supports for polypeptide synthesis.* Journal of the American Chemical Society, 1975. **97**(22): p. 6584-6585.
12. Atherton, E., et al., *The polyamide method of solid phase peptide and oligonucleotide synthesis.* Bioorganic Chemistry, 1979. **8**(3): p. 351-370.
13. Atherton, E., et al., *A physically supported gel polymer for low pressure, continuous flow solid phase reactions. Application to solid phase peptide synthesis.* Journal of the Chemical Society, Chemical Communications, 1981(21): p. 1151-1152.
14. Gutte, B., *Peptides: synthesis, structures, and applications*. 1995: Elsevier.
15. Meldal, M., *Pega: a flow stable polyethylene glycol dimethyl acrylamide copolymer for solid phase synthesis.* Tetrahedron Letters, 1992. **33**(21): p. 3077-3080.
16. Li, W., X. Xiao, and A.W. Czarnik, *Kinetic comparison of amide formation on various cross-linked polystyrene resins.* Journal of combinatorial chemistry, 1999. **1**(2): p. 127-129.
17. Bayer, E., M. Dengler, and B. Hemmasi, *Peptide synthesis on the new polyoxyethylene-polystyrene graft copolymer, synthesis of insulin B 21–30.* International Journal of Peptide and Protein Research, 1985. **25**(2): p. 178-186.
18. Zalipsky, S., et al., *Preparation and applications of polyethylene glycol-polystyrene graft resin supports for solid-phase peptide synthesis.* Reactive Polymers, 1994. **22**(3): p. 243-258.
19. Adams, J.H., et al., *A Reinvestigation of the Preparation, Properties, and Applications of Aminomethyl and 4-Methylbenzhydrylamine Polystyrene Resins1.* The Journal of Organic Chemistry, 1998. **63**(11): p. 3706-3716.
20. Renil, M. and V. Rajasekharan Pillai, *Synthesis, characterization and application of tetraethylene glycol diacrylate crosslinked polystyrene support for gel phase peptide synthesis.* Journal of applied polymer science, 1996. **61**(9): p. 1585-1594.
21. Kempe, M. and G. Barany, *CLEAR: A Novel Family of Highly Cross-Linked Polymeric Supports for Solid-Phase Peptide Synthesis1, 2.* Journal of the American Chemical Society, 1996. **118**(30): p. 7083-7093.
22. Côté, S., *Polyether based monomers and highly cross-linked amphiphile resins*. 2010, Google Patents.
23. Wang, S.-S., *p-Alkoxybenzyl Alcohol Resin and p-Alkoxybenzyloxycarbonylhydrazide Resin for Solid Phase Synthesis of Protected Peptide Fragments.* Journal of the American Chemical Society, 1973. **95**(4): p. 1328-1333.
24. Mitchell, A.R., et al., *tert-Butoxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin, a more acid-resistant support for solid-phase peptide synthesis.* Journal of the American Chemical Society, 1976. **98**(23): p. 7357-7362.
25. DeGrado, W.F. and E.T. Kaiser, *Polymer-bound oxime esters as supports for solid-phase peptide synthesis. The preparation of protected peptide fragments.* The Journal of Organic Chemistry, 1980. **45**(7): p. 1295-1300.
26. Pietta, P.G. and G.R. Marshall, *Amide protection and amide supports in solid-phase peptide synthesis.* Journal of the Chemical Society D: Chemical Communications, 1970(11): p. 650-651.
27. Matsueda, G.R. and J.M. Stewart, *A p-methylbenzhydrylamine resin for improved solid-phase synthesis of peptide amides.* Peptides, 1981. **2**(1): p. 45-50.
28. Sheppard, R.C. and B.J. Williams, *Acid-labile resin linkage agents for use in solid phase peptide synthesis.* International Journal of Peptide and Protein Research, 1982. **20**(5): p. 451-454.
29. Mergler, M., et al., *Peptide synthesis by a combination of solid-phase and solution methods I: A new very acid-labile anchor group for the solid phase synthesis of fully protected fragments.* Tetrahedron Letters, 1988. **29**(32): p. 4005-4008.
30. Albericio, F. and G. Barany, *An acid-labile anchoring linkage for solid-phase synthesis of C-terminal peptide amides under mild conditions.* Int J Pept Protein Res, 1987. **30**(2): p. 206-16.

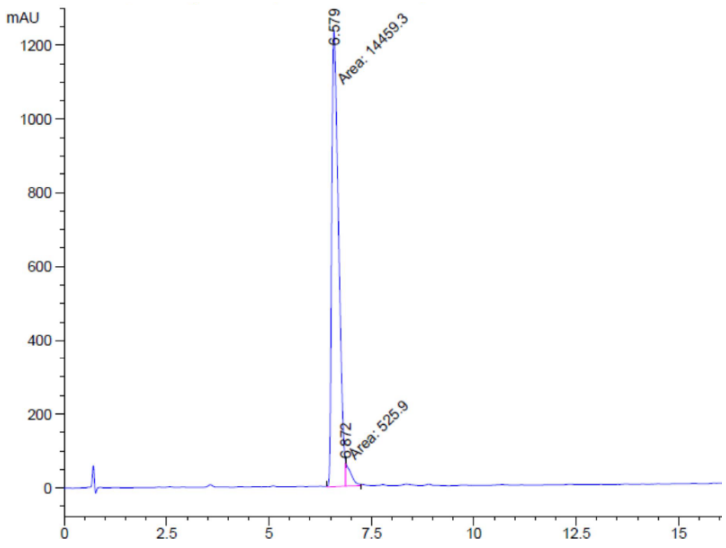
31. Rink, H., *Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin.* Tetrahedron Letters, 1987. **28**(33): p. 3787-3790.
32. Barlos, K., et al., *2-Chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage.* Int J Pept Protein Res, 1991. **37**(6): p. 513-20.
33. Albericio, F. and G. Barany, *Hypersensitive acid-labile (HAL) tris(alkoxy)benzyl ester anchoring for solid-phase synthesis of protected peptide segments.* Tetrahedron Letters, 1991. **32**(8): p. 1015-1018.
34. Bergmann, M. and L. Zervas, *Über ein allgemeines Verfahren der Peptid-Synthese.* Berichte der deutschen chemischen Gesellschaft (A and B Series), 1932. **65**(7): p. 1192-1201.
35. du Vigneaud, V., D.T. Gish, and P.G. Katsoyannis, *A synthetic preparation possssing biological properties associated with arginine vasopressin.* Journal of the American Chemical Society, 1954. **76**(18): p. 4751-4752.
36. Vigneaud, V.d., et al., *The synthesis of an octapeptide amide with the hormonal activity of oxytocin.* Journal of the American Chemical Society, 1953. **75**(19): p. 4879-4880.
37. McKay, F.C. and N.F. Albertson, *New amine-masking groups for peptide synthesis.* Journal of the American Chemical Society, 1957. **79**(17): p. 4686-4690.
38. Carpino, L.A. and G.Y. Han, *9-Fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group.* Journal of the American Chemical Society, 1970. **92**(19): p. 5748-5749.
39. Albericio, F., *Solid-phase synthesis: a practical guide*. 2000: CRC Press.
40. Barany, G. and F. Albericio, *Three-dimensional orthogonal protection scheme for solid-phase peptide synthesis under mild conditions.* Journal of the American Chemical Society, 1985. **107**(17): p. 4936-4942.
41. Barany, G. and R. Merrifield, *A new amino protecting group removable by reduction. Chemistry of the dithiasuccinoyl (Dts) function.* Journal of the American Chemical Society, 1977. **99**(22): p. 7363-7365.
42. Isidro-Llobet, A., M. Álvarez, and F. Albericio, *Amino Acid-Protecting Groups.* Chemical Reviews, 2009. **109**(6): p. 2455-2504.
43. Kavanagh, K. and S. Dowd, *Histatins: antimicrobial peptides with therapeutic potential.* J Pharm Pharmacol, 2004. **56**(3): p. 285-9.
44. Pichon, C., C. Gonçalves, and P. Midoux, *Histidine-rich peptides and polymers for nucleic acids delivery.* Advanced Drug Delivery Reviews, 2001. **53**(1): p. 75-94.
45. Ferrer-Miralles, N., et al., *Biological activities of histidine-rich peptides; merging biotechnology and nanomedicine.* Microbial Cell Factories, 2011. **10**(1): p. 101.
46. Fletcher, A.R., et al., *The use of the N([small pi])-phenacyl group for the protection of the histidine side chain in peptide synthesis.* Journal of the Chemical Society, Perkin Transactions 1, 1979(0): p. 2261-2267.
47. Jones, J.H., W.I. Ramage, and M.J. Witty, *Mechanism of racemisation of histidine derivatives in peptide synthesis.* Int J Pept Protein Res, 1980. **15**(3): p. 301-3.
48. Fujii, T. and S. Sakakibara, *Studies on the synthesis of histidine peptides. I. N Im-Tosylhistidine derivatives as starting materials.* Bulletin of the Chemical Society of Japan, 1974. **47**(12): p. 3146-3151.
49. Barlos, K., D. Papaioannou, and D. Theodoropoulos, *Efficient "one-pot" synthesis of N-tritylamino acids.* The Journal of Organic Chemistry, 1982. **47**(7): p. 1324-1326.
50. Sieber, P. and B. Riniker, *Protection of histidine in peptide synthesis: A Reassessment of the trityl group.* Tetrahedron Letters, 1987. **28**(48): p. 6031-6034.
51. Barlos, K., et al., *Fmoc-His (Mmt)-OH und Fmoc-His (Mtt)-OH. Zwei nue histidin-derivative Nim-geschützt mit säure-hochempfindlichen gruppen. Darstellung, eigenschaften und einsatz in der peptidsynthese.* Tetrahedron letters, 1991. **32**(4): p. 475-478.
52. Karlström, A. and A. Undén, *The N im-(2, 4-dimethylpent-3-yloxycarbonyl)(Doc) group, a new nucleophile-resistant, HF-cleavable protecting group for histidine in peptide synthesis.* Chemical Communications, 1996(8): p. 959-960.

53. Chillemi, F. and R. Merrifield, *Use of Nim-dinitrophenylhistidine in the solid-phase synthesis of the tricosapeptides 124-146 of human hemoglobin. beta. chain.* Biochemistry, 1969. **8**(11): p. 4344-4346.
54. Shaltiel, S. and M. Fridkin, *Thiolysis of dinitrophenylimidazoles and its use during synthesis of histidine peptides.* Biochemistry, 1970. **9**(26): p. 5122-5127.
55. Simone, Z., S. Roger, and Y. Esther, *Application of Nim-2,6-Dimethoxybenzoyl Histidine in Solid-Phase Peptide Synthesis.* European Journal of Organic Chemistry, 2003. **2003**(13): p. 2454-2461.
56. Yoshizawa-Kumagaye, K., et al., *Amino acid deletion products resulting from incomplete deprotection of the Boc group from Nπ-benzyloxymethylhistidine residues during solid-phase peptide synthesis.* Journal of peptide science: an official publication of the European Peptide Society, 2005. **11**(8): p. 512-515.
57. Colombo, R., F. Colombo, and J.H. Jones, *Acid-labile histidine side-chain protection: the N([small pi])-t-butoxymethyl group.* Journal of the Chemical Society, Chemical Communications, 1984(5): p. 292-293.
58. M., M., et al., *Synthesis and Application of Fmoc-His(3-Bum)-OH.* Journal of Peptide Science, 2001. **7**(9): p. 502-510.
59. Hruby, V.J., *Houben-Weyl Methods of Organic Chemistry. Volume E22A. Synthesis of Peptides and Peptidomimetics. Edited by Murray Goodman, Arthur Felix, Luis Moroder, and Claudio Toniolo. Georg Thieme Verlag, Stuttgart, Germany. 2001. xxvii + 901 pp. 18 × 26 cm. ISBN 3 132 19604 5. 1840 euro.* J. Med. Chem., 2002. **45**(23): p. 5187.
60. Information about Octagel was provided via personal correspondence with Aaptec Biotechnology
61. Garcia-Ramos, Y., et al., *ChemMatrix (R) for complex peptides and combinatorial chemistry.* Journal of Peptide Science, 2010. **16**(12): p. 675-678.
62. Srivastava, P., et al., *Selective Naked-Eye Detection of Hg2+ through an Efficient Turn-On Photoinduced Electron Transfer Fluorescent Probe and Its Real Applications.* Analytical chemistry, 2014. **2014 v.86 no.17**(no. 17): p. pp. 8693-8699.
63. Góngora-Benítez, M., et al., *Acid-Labile Cys-Protecting Groups for the Fmoc/tBu Strategy: Filling the Gap.* Organic Letters, 2012. **14**(21): p. 5472-5475.

APPENDIX A

HPLC -Aib enkephalin pentapeptide

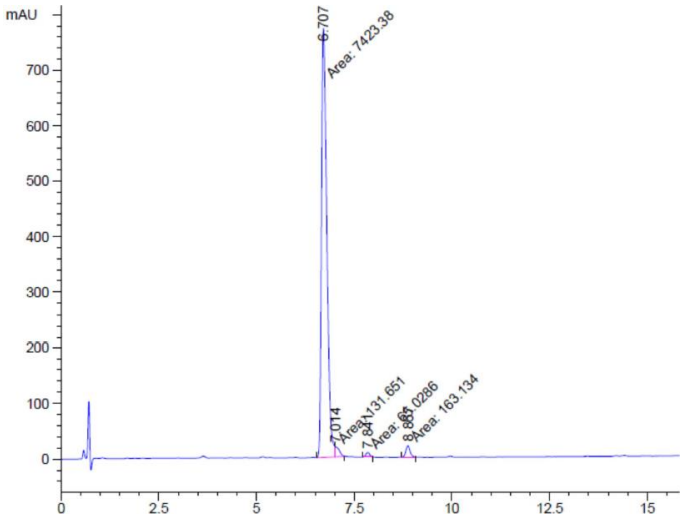
Polystyrene



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %
1	3.593		0.0000	0.00000		0.0000
2	6.579	MF	0.1951	1.44593e4		96.4905
3	6.872	FM	0.1392	525.89954		3.5095

Totals : 1.49852e4

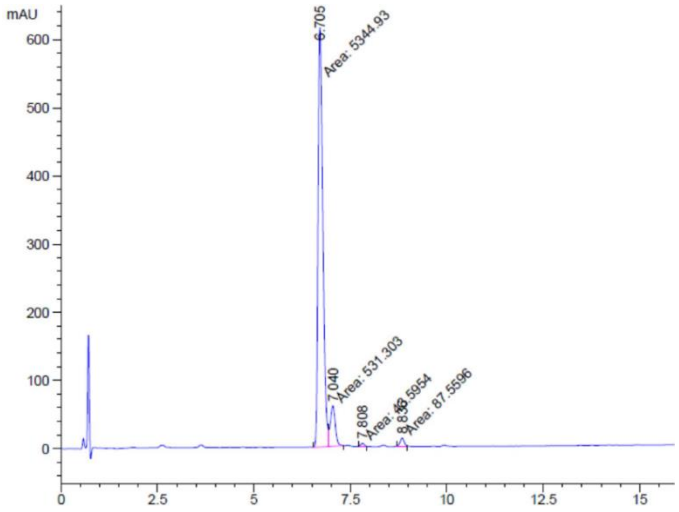
ChemMatrix



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %
1	6.707	MF	0.1600	7423.38281		95.3770
2	7.014	FM	0.1179	131.65147		1.6915
3	7.841	MM	0.1237	65.02863		0.8355
4	8.531		0.0000	0.00000		0.0000
5	8.867	MM	0.1279	163.13434		2.0960

Totals : 7783.19725

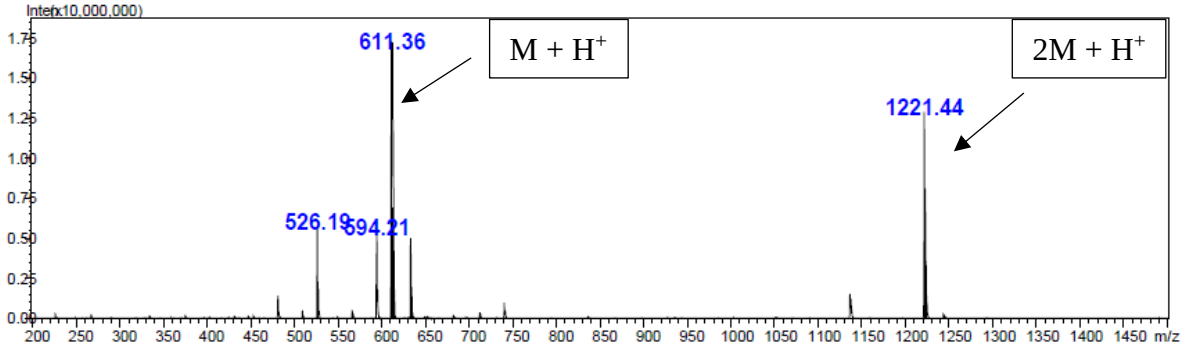
Octagel



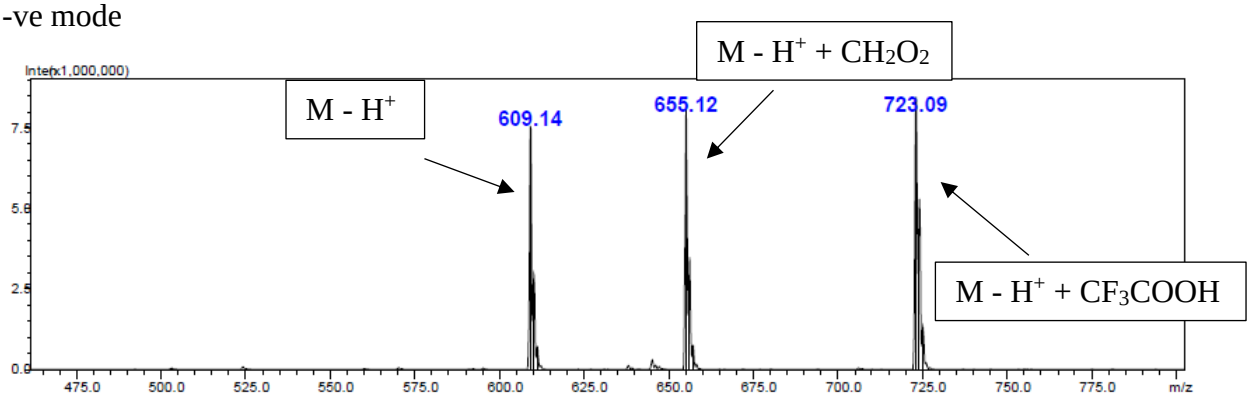
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %
1	3.593		0.0000	0.00000	0.0000
2	6.705	MF	0.1445	5344.92578	88.9726
3	7.040	FM	0.1483	531.30255	8.8442
4	7.808	MM	0.1325	43.59539	0.7257
5	8.836	MM	0.1232	87.55962	1.4575
Totals :				6007.38335	

LC/MS -Aib-pentapeptide

+ve mode

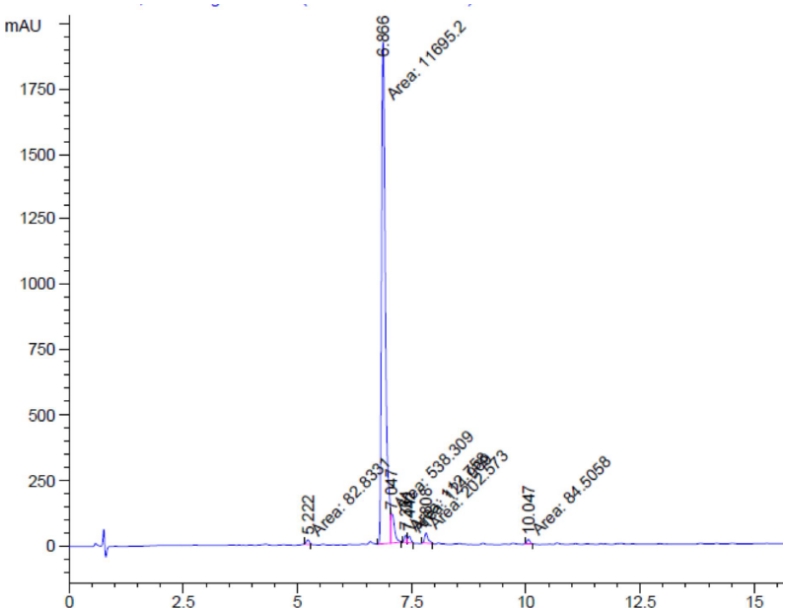


-ve mode



Aib-ACP decapeptide

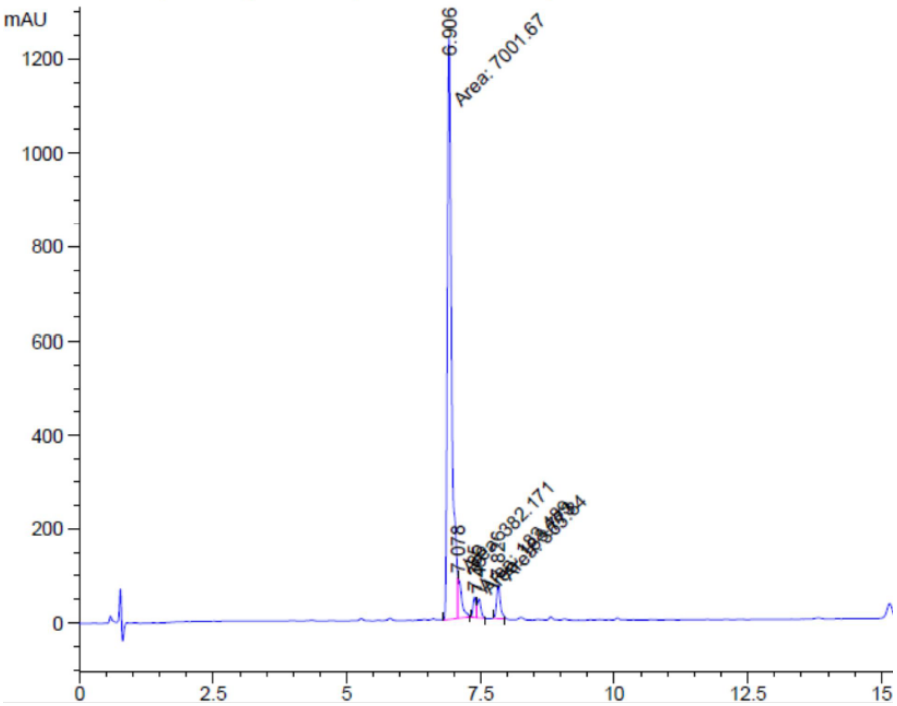
Polystyrene



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %
1	5.222	MM	0.0795	82.83313	0.6452
2	6.866	MF	0.1013	1.16952e4	91.0973
3	7.047	FM	0.0805	538.30902	4.1930
4	7.361	MF	0.0657	112.75774	0.8783
5	7.442	FM	0.0763	121.96915	0.9501
6	7.808	MM	0.0880	202.57278	1.5779
7	8.531		0.0000	0.00000	0.0000
8	10.047	MM	0.0767	84.50584	0.6582

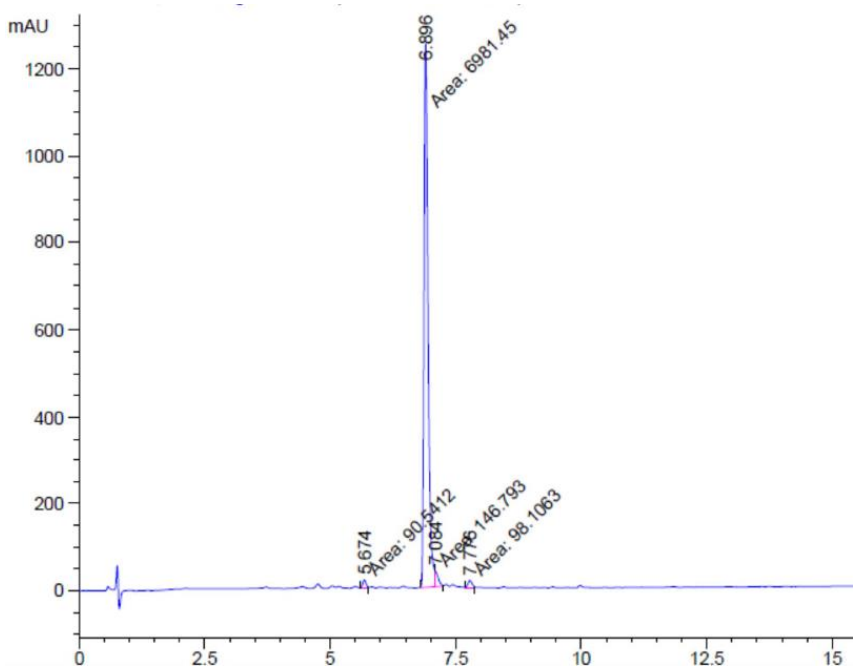
Totals : 1.28382e4

ChemMatrix



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %
1	6.906	MF	0.0942	7001.67383	86.2706
2	7.078	FM	0.0749	382.17130	4.7089
3	7.385	MF	0.0693	182.48874	2.2485
4	7.465	FM	0.0746	183.77330	2.2643
5	7.826	MM	0.0837	365.84015	4.5077
6	8.531		0.0000	0.00000	0.0000
Totals :				8115.94731	

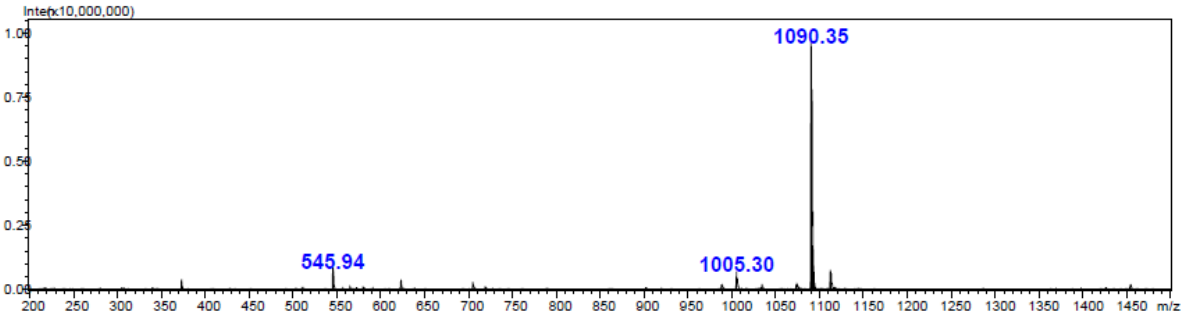
Octagel



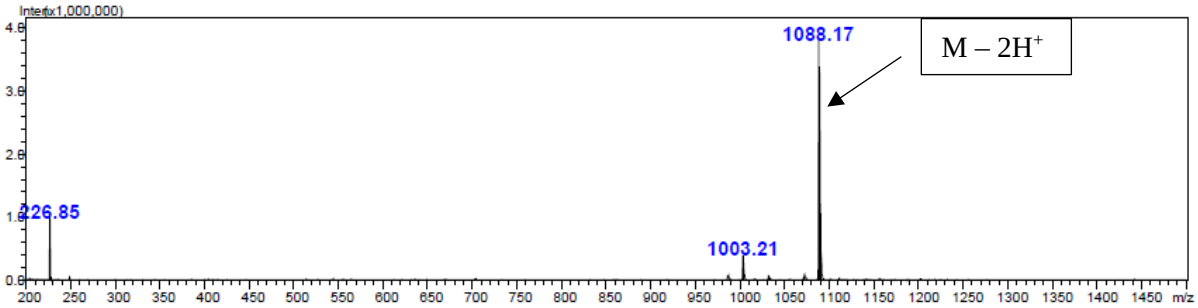
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %
1	5.674	MM	0.0780	90.54121	1.2374
2	6.896	MF	0.0929	6981.45117	95.4155
3	7.084	FM	0.0677	146.79285	2.0062
4	7.776	MM	0.0951	98.10634	1.3408
5	8.531		0.0000	0.00000	0.0000
Totals :				7316.89157	

LC/MS – Aib ACP decapeptide

+ve mode

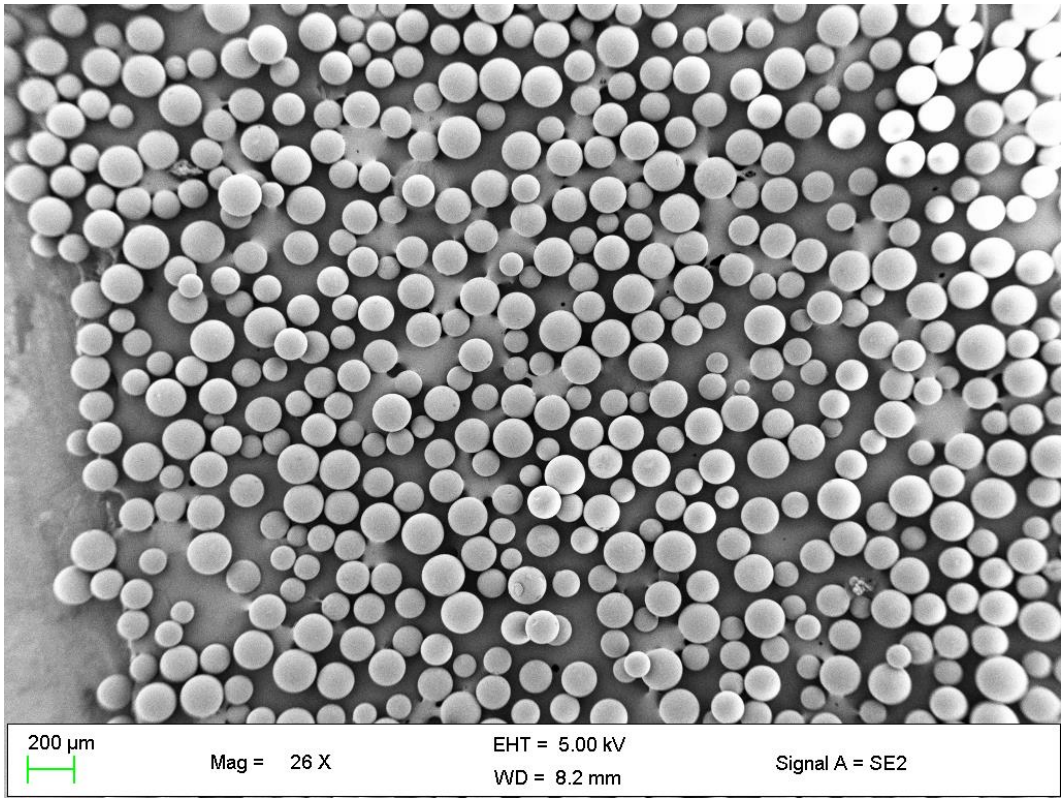


-ve mode

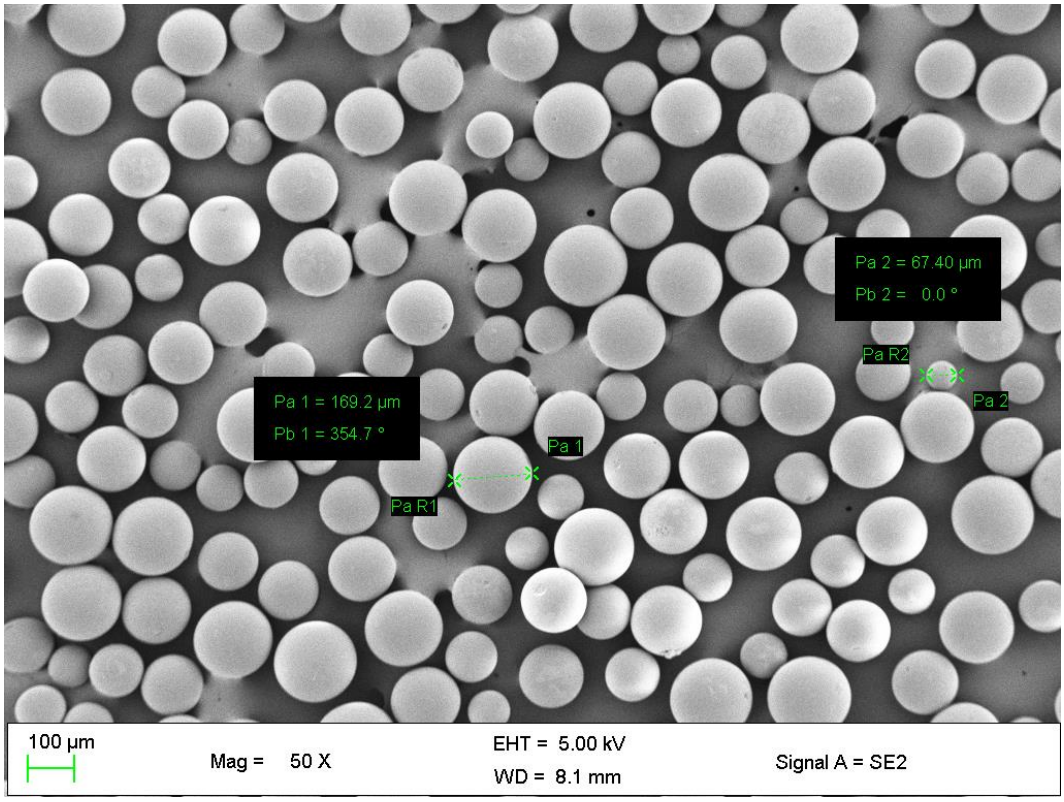


SEM images

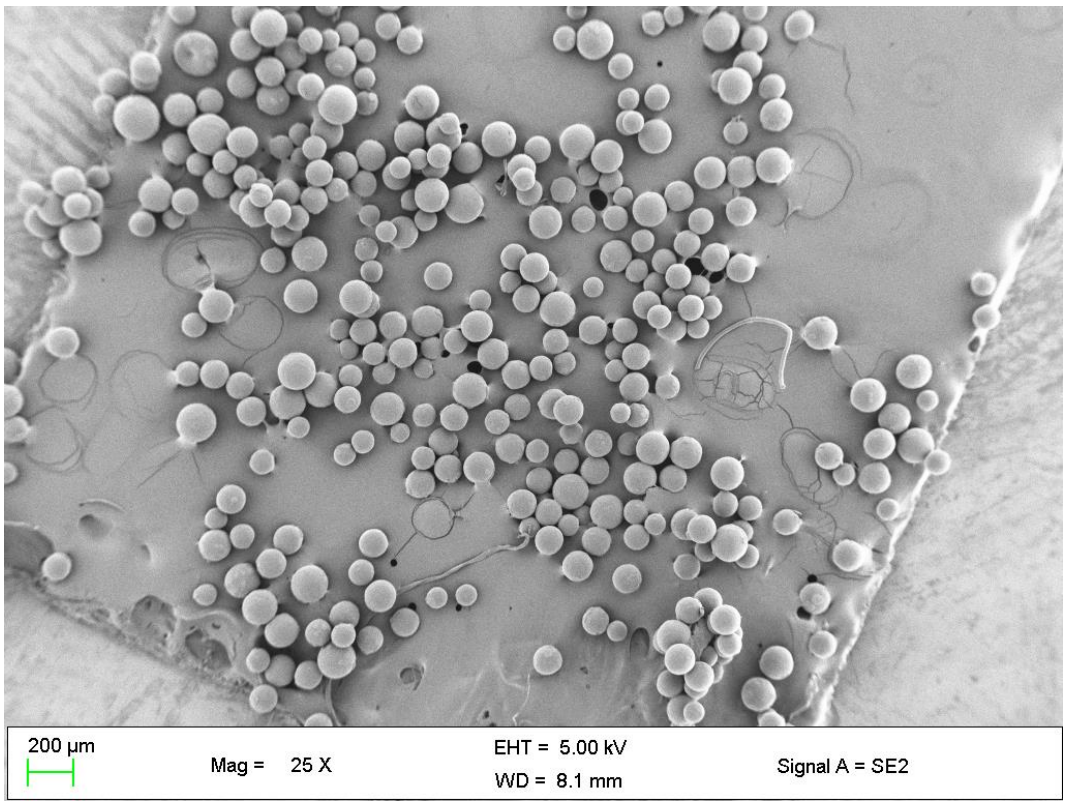
Polystyrene ×25



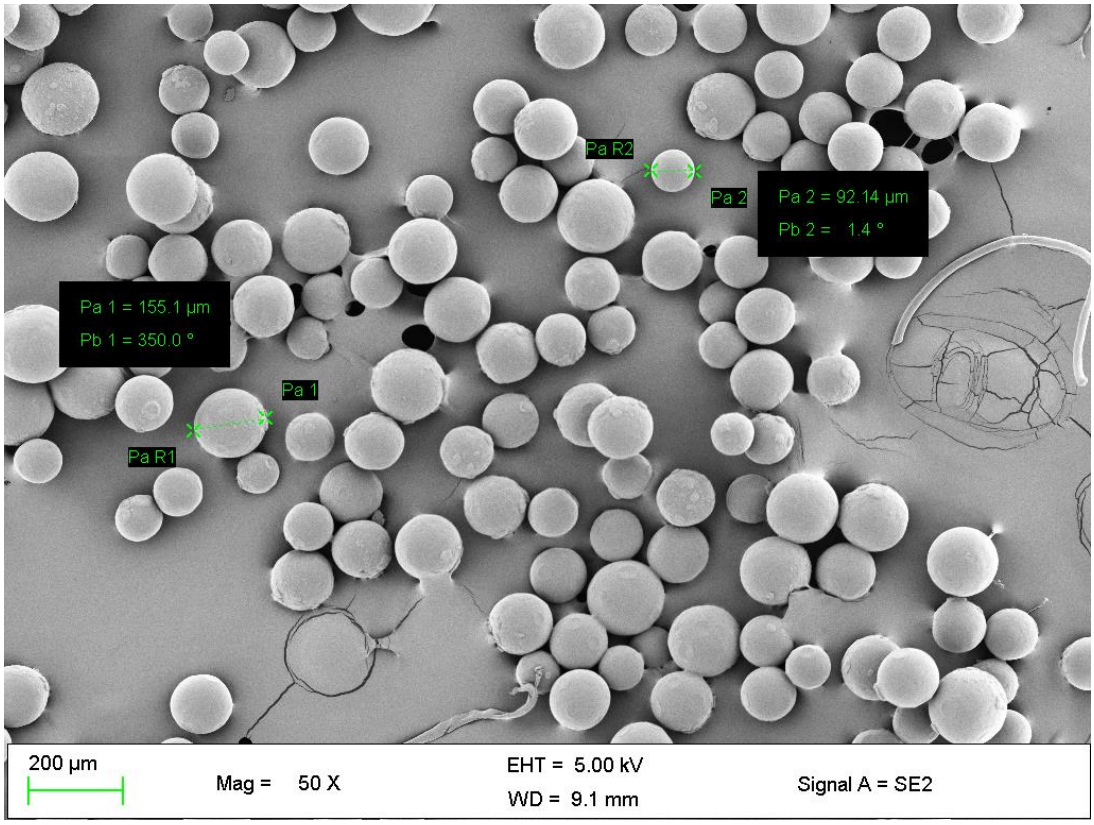
Polystyrene ×50



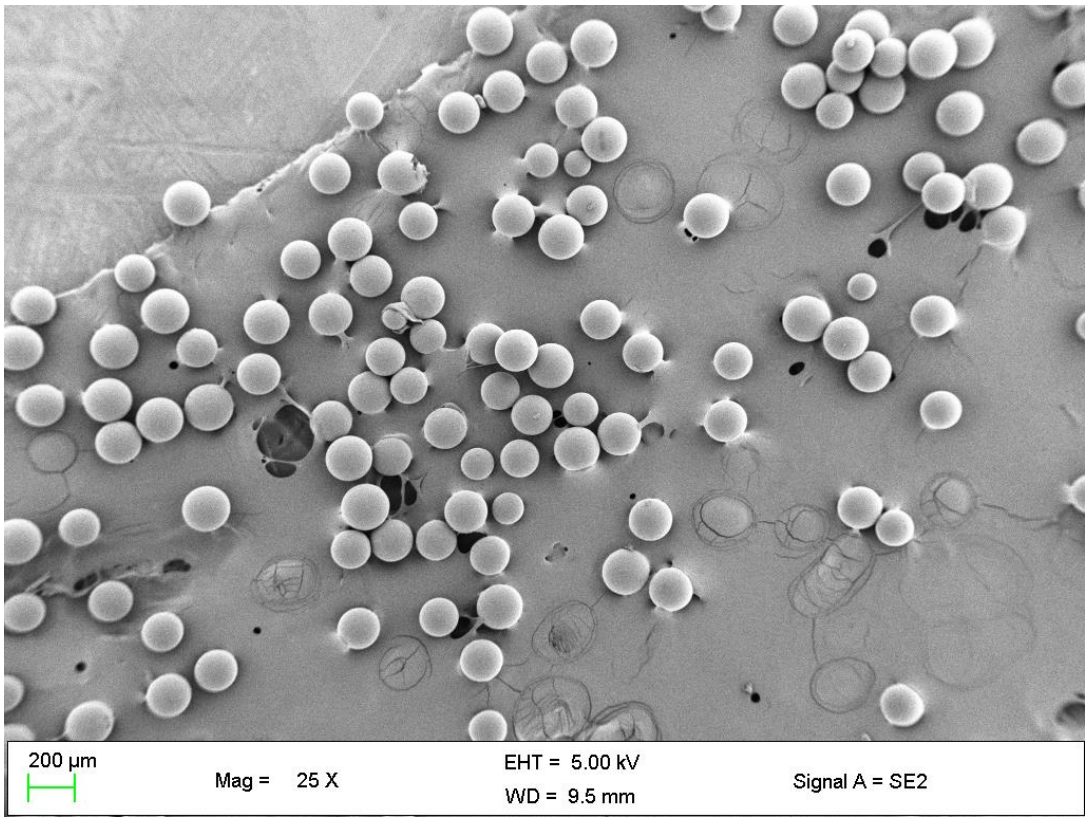
ChemMatrix × 25



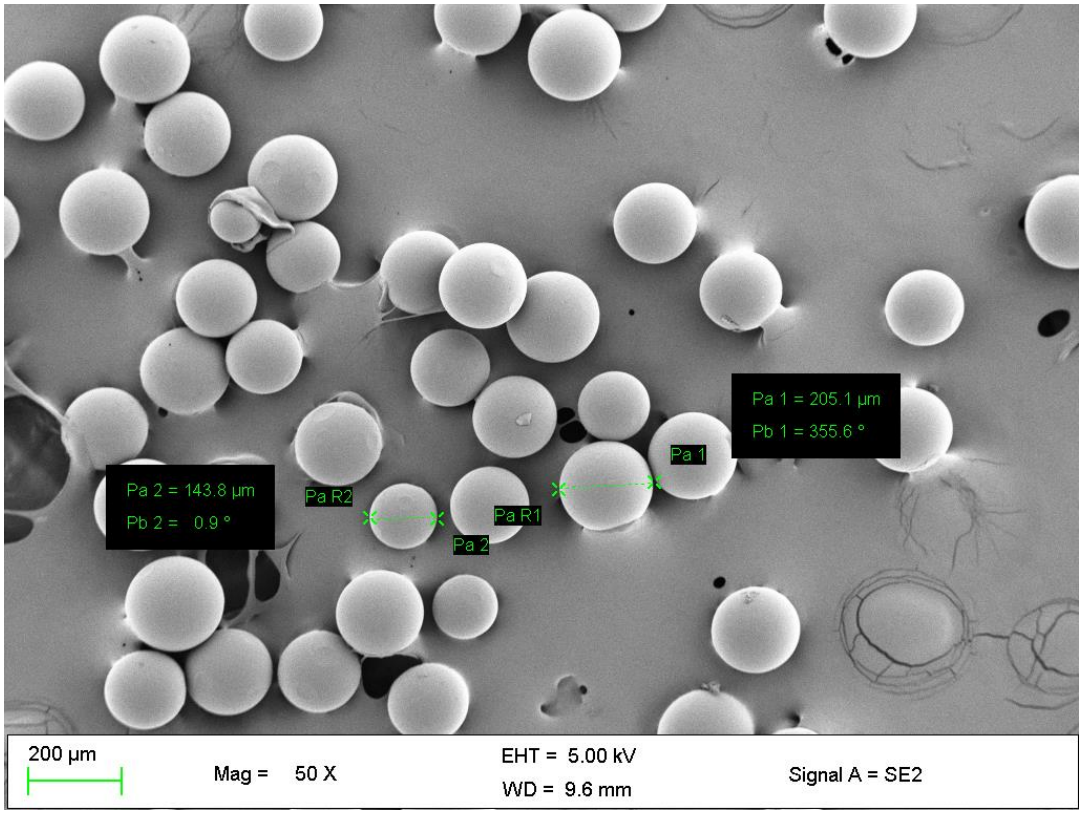
ChemMatrix × 50



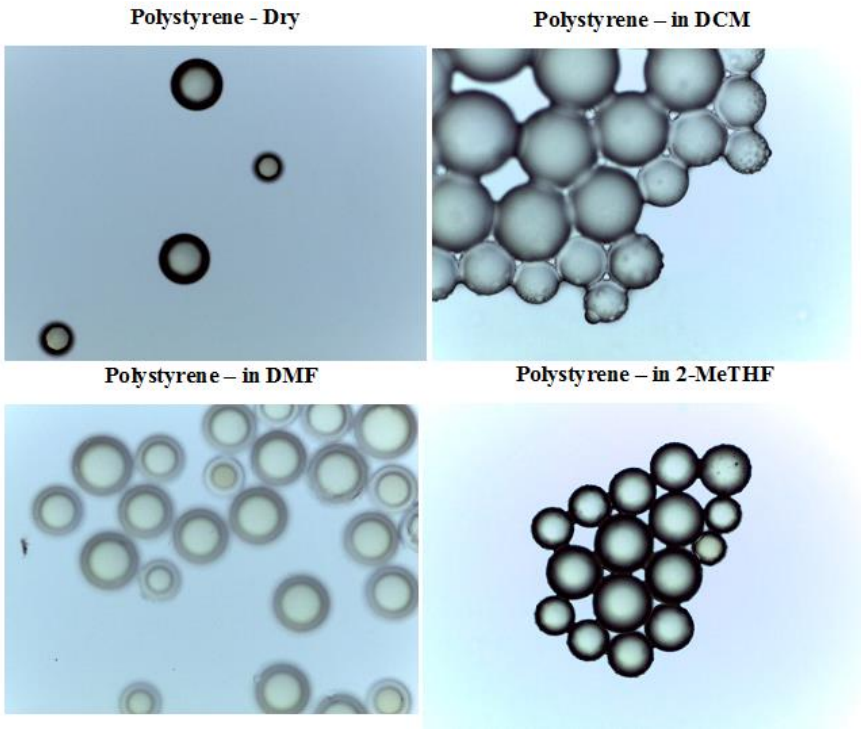
Octagel × 25



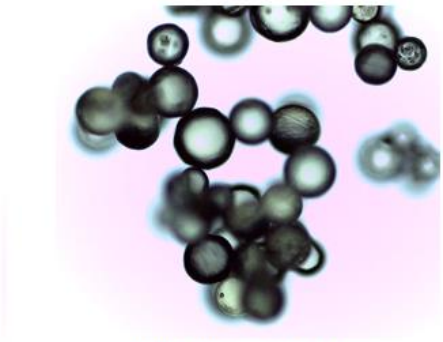
Octagel × 50



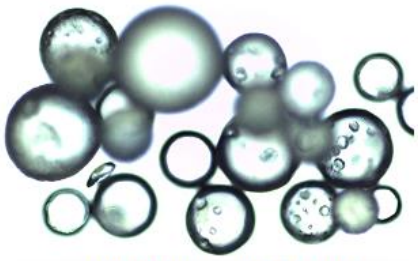
Light Microscope Images



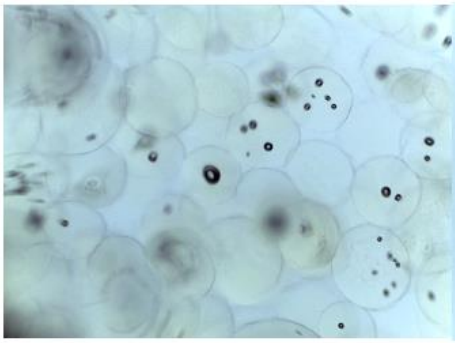
ChemMatrix - Dry



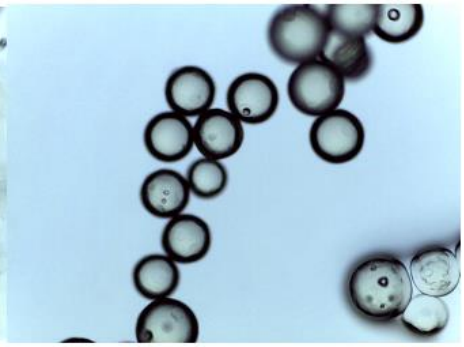
ChemMatrix – in DCM



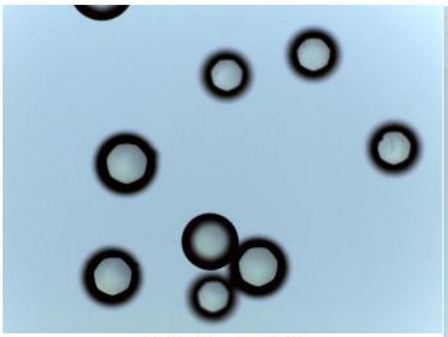
ChemMatrix – in DMF



ChemMatrix – in 2-MeTHF

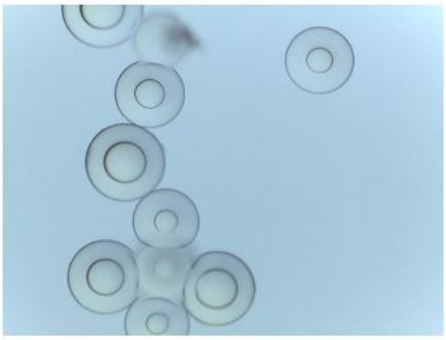


Octagel – Dry

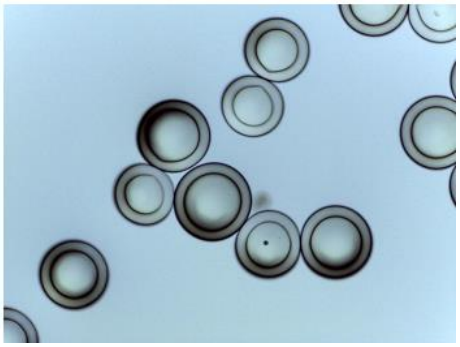


Octagel – in DCM

Octagel – in DMF



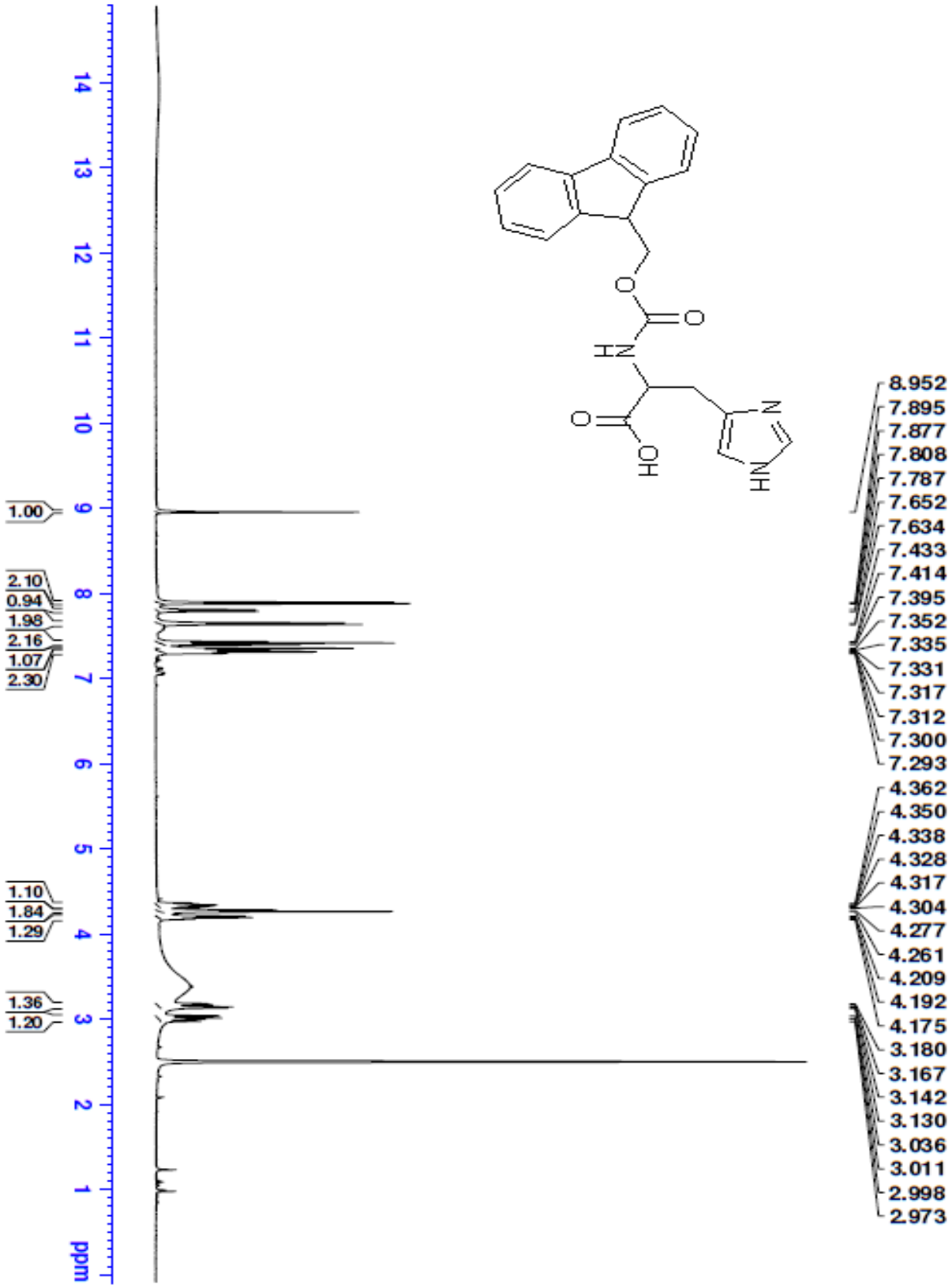
Octagel – in 2-MeTHF



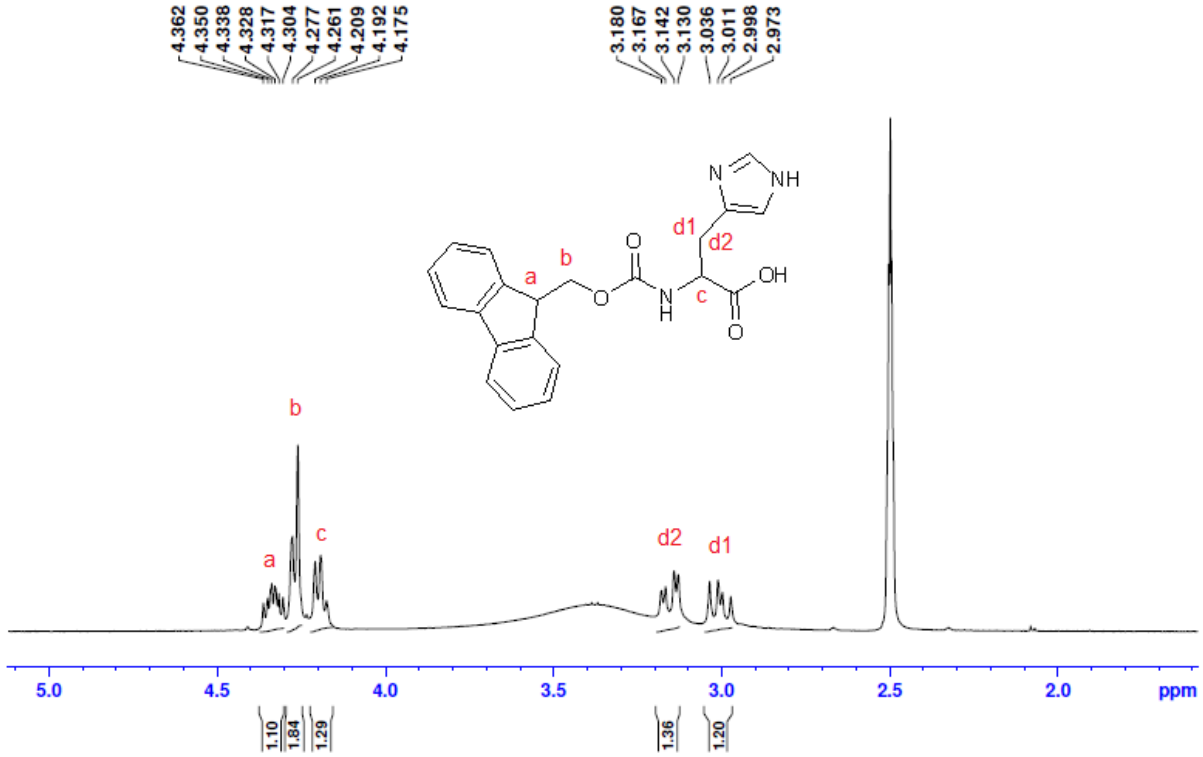
APPENDIX B

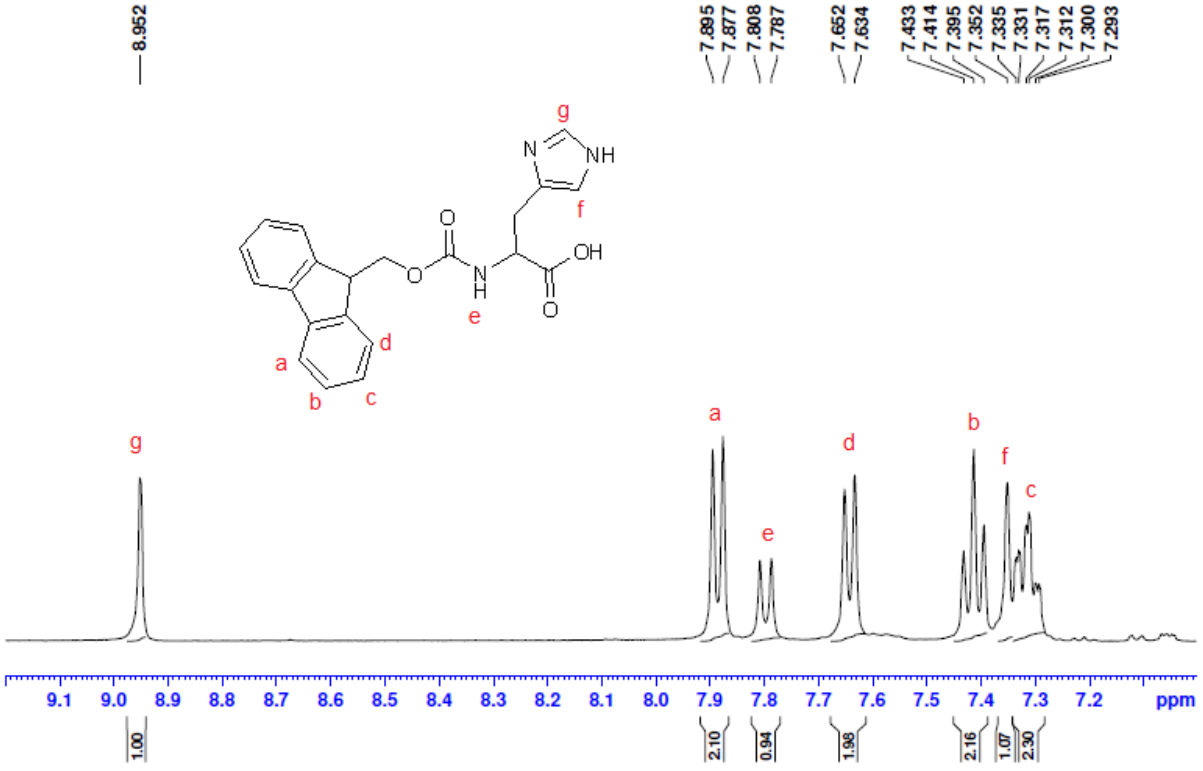
NMR data

Fmoc-His-OH: ^{1}H NMR in DMSO

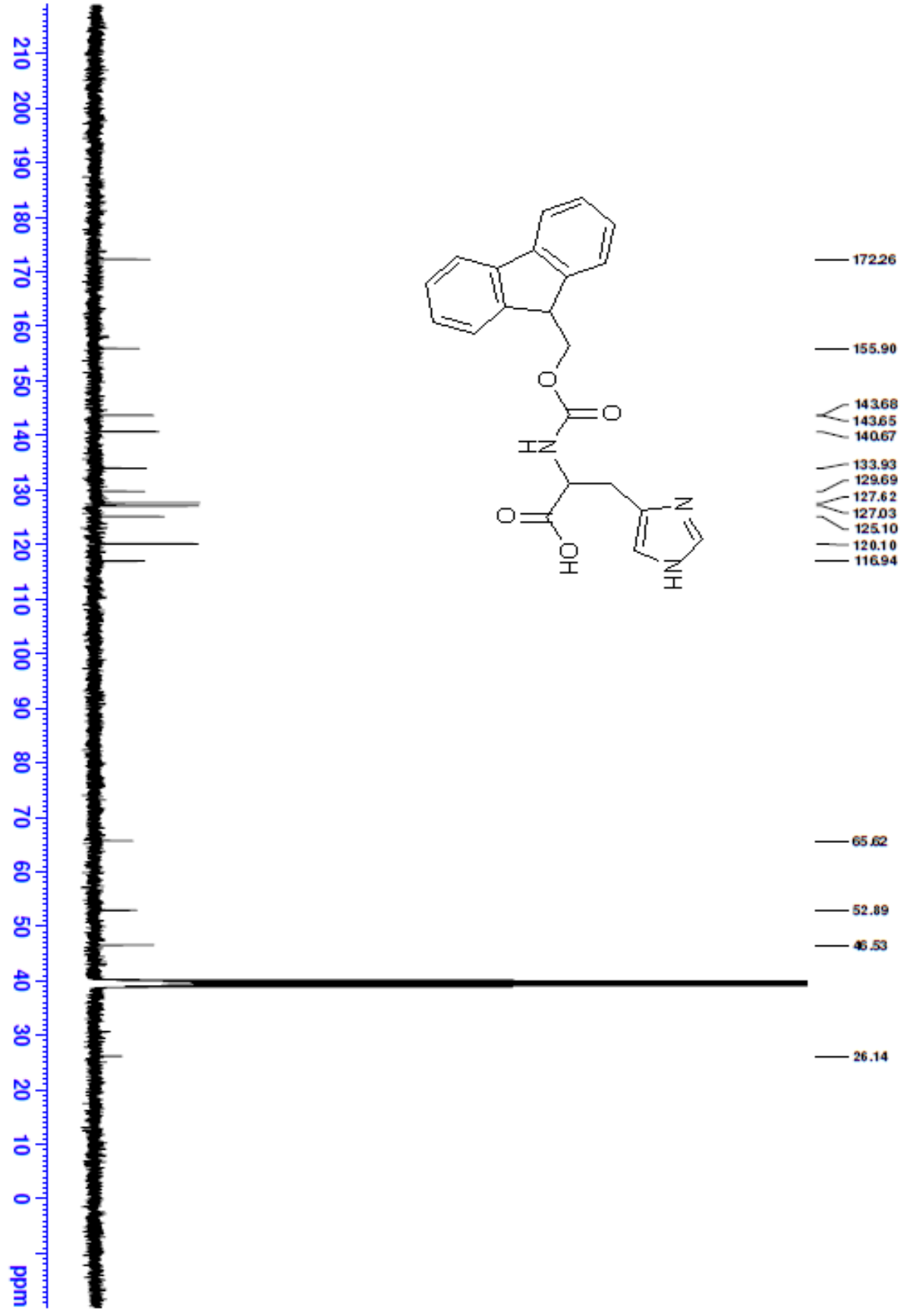


Fmoc-His-OH: ^{1}H NMR in DMSO expanded

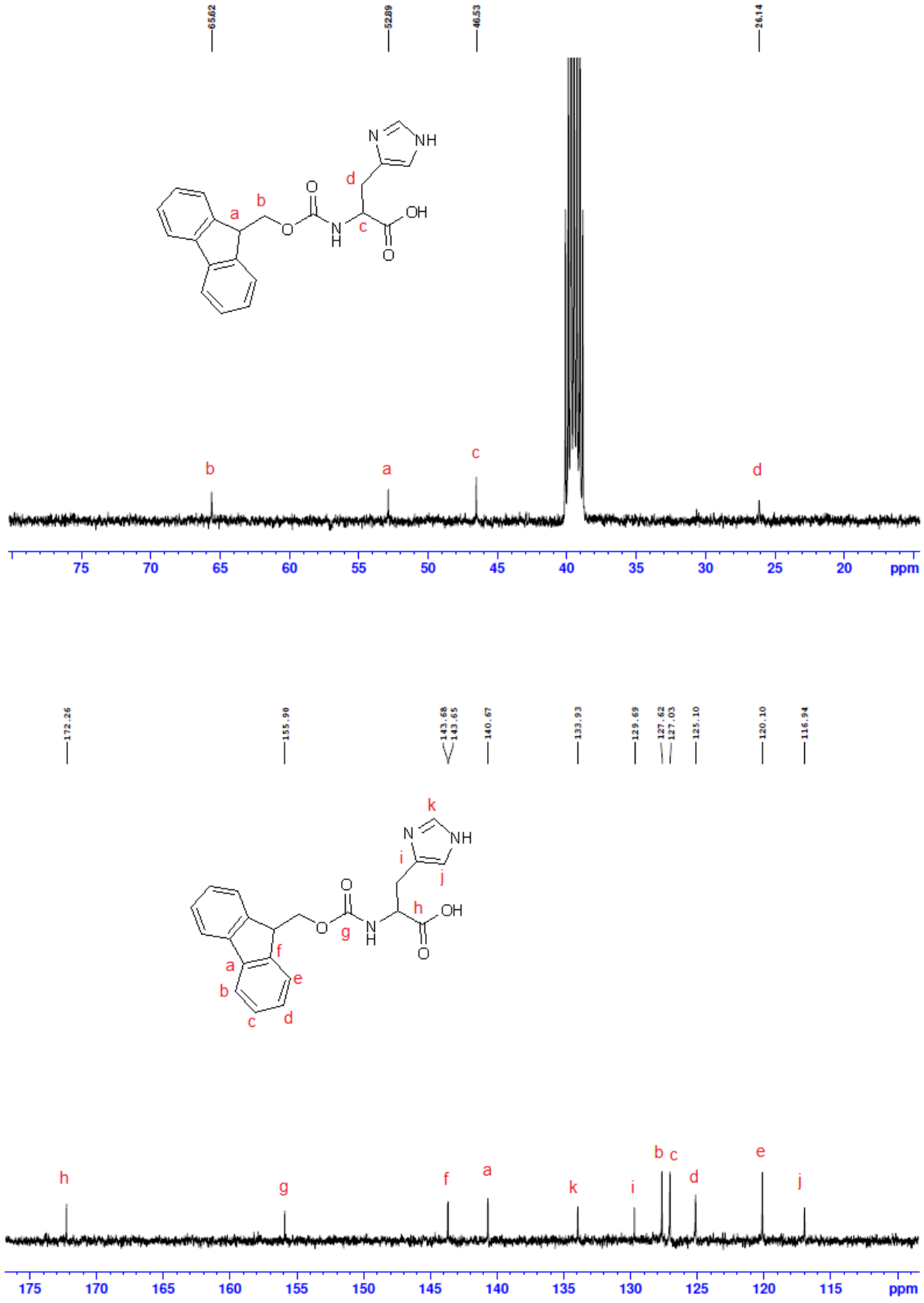




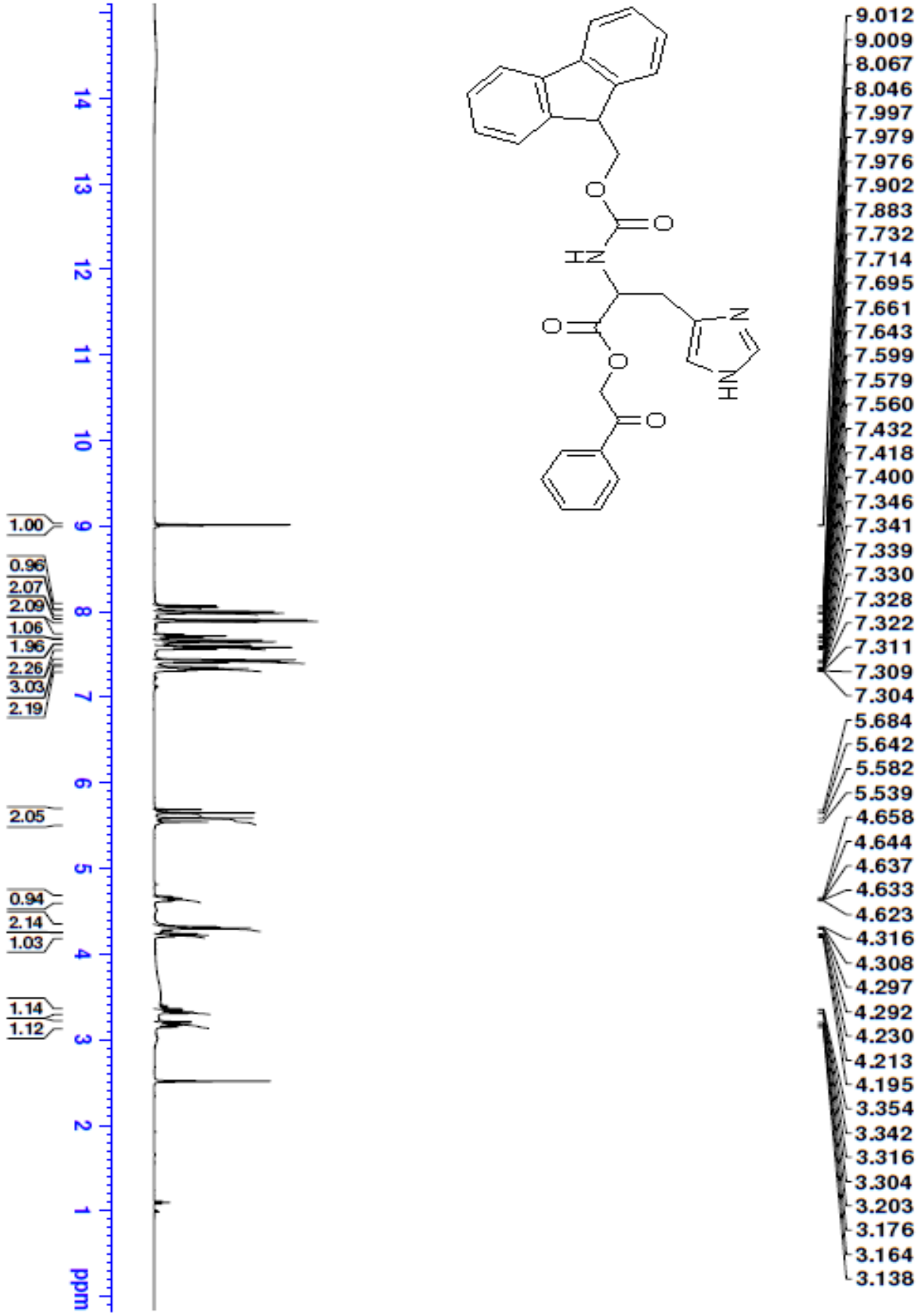
Fmoc-His-OH: ^{13}C NMR in DMSO



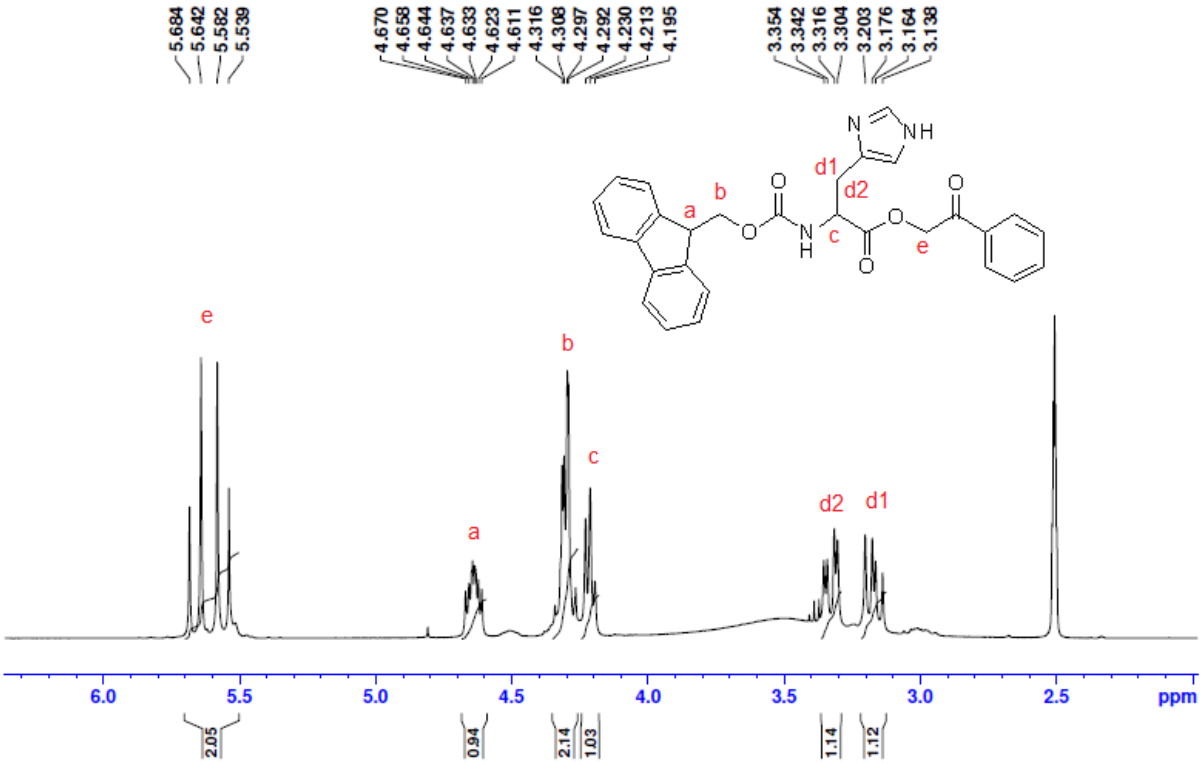
Fmoc-His-OH: ^{13}C NMR in DMSO expanded

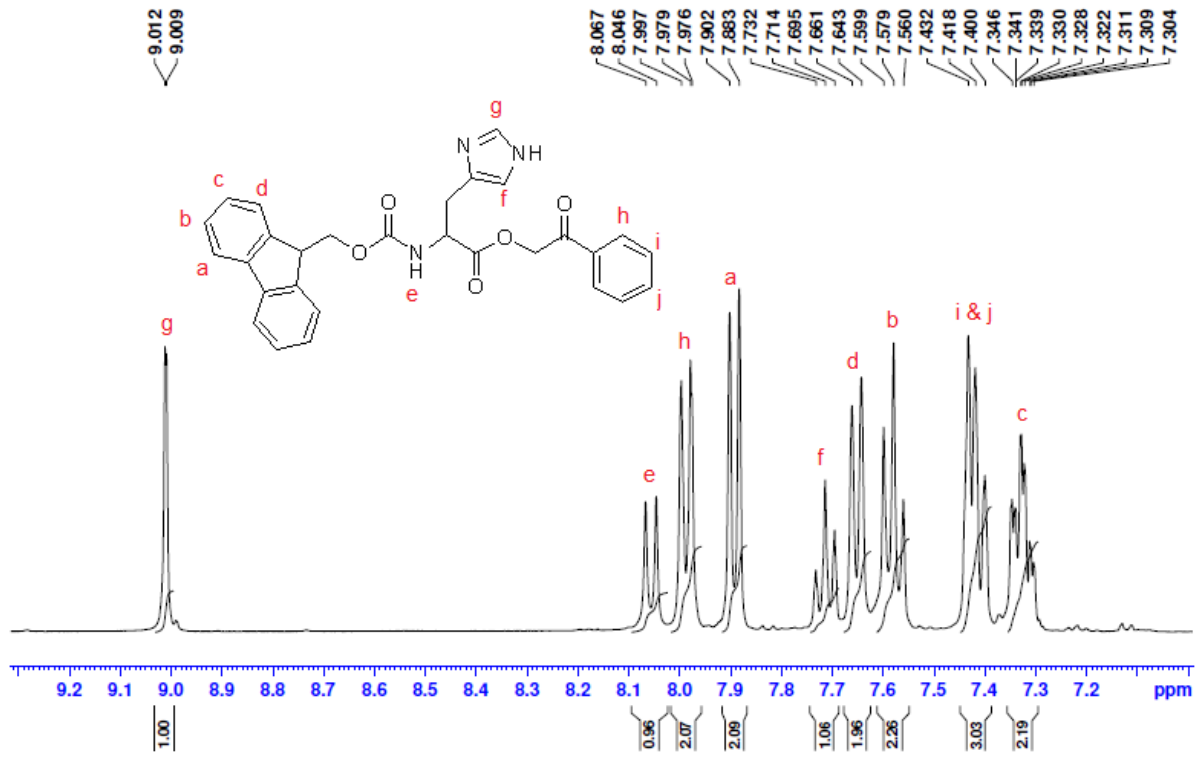


Fmoc-His-O-Phenacyl: ^{1}H NMR in DMSO

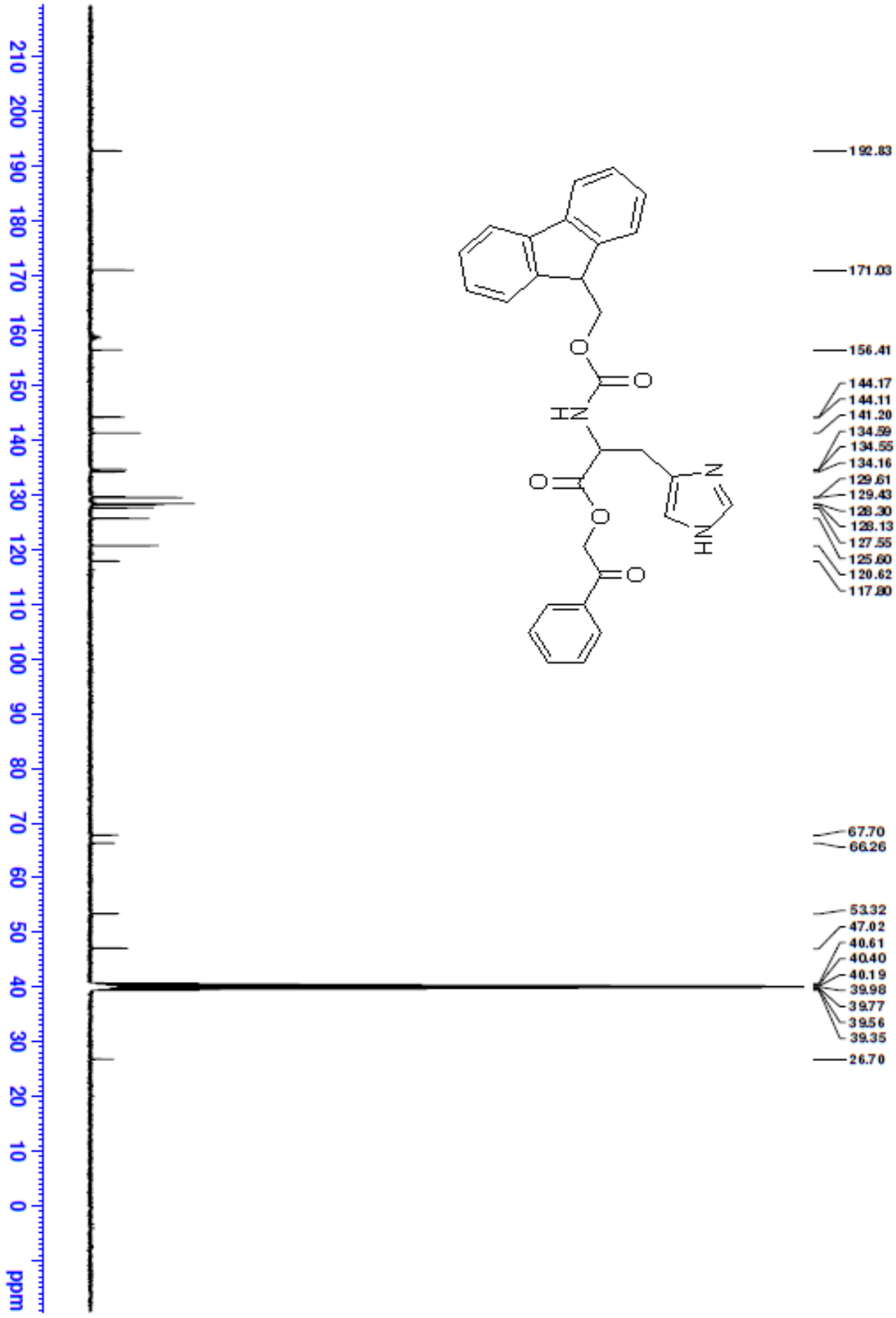


Fmoc-His-O-Phenacyl: ^{1}H NMR in DMSO expanded

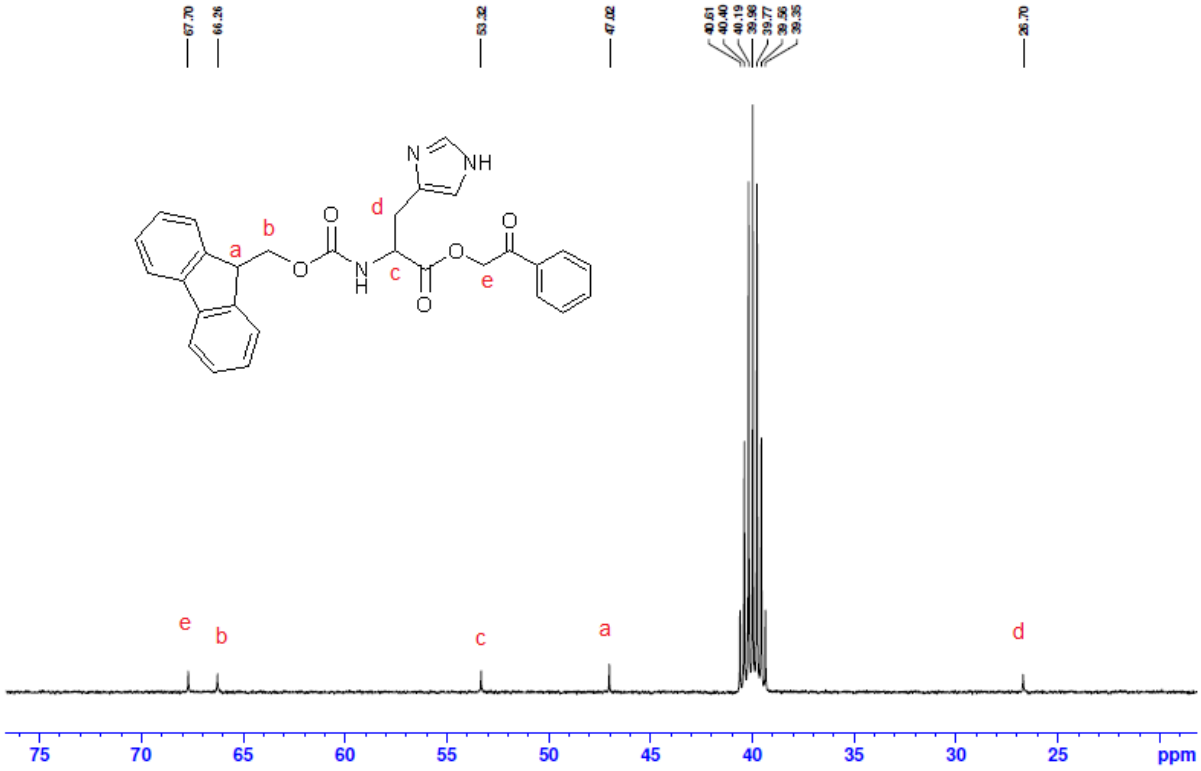


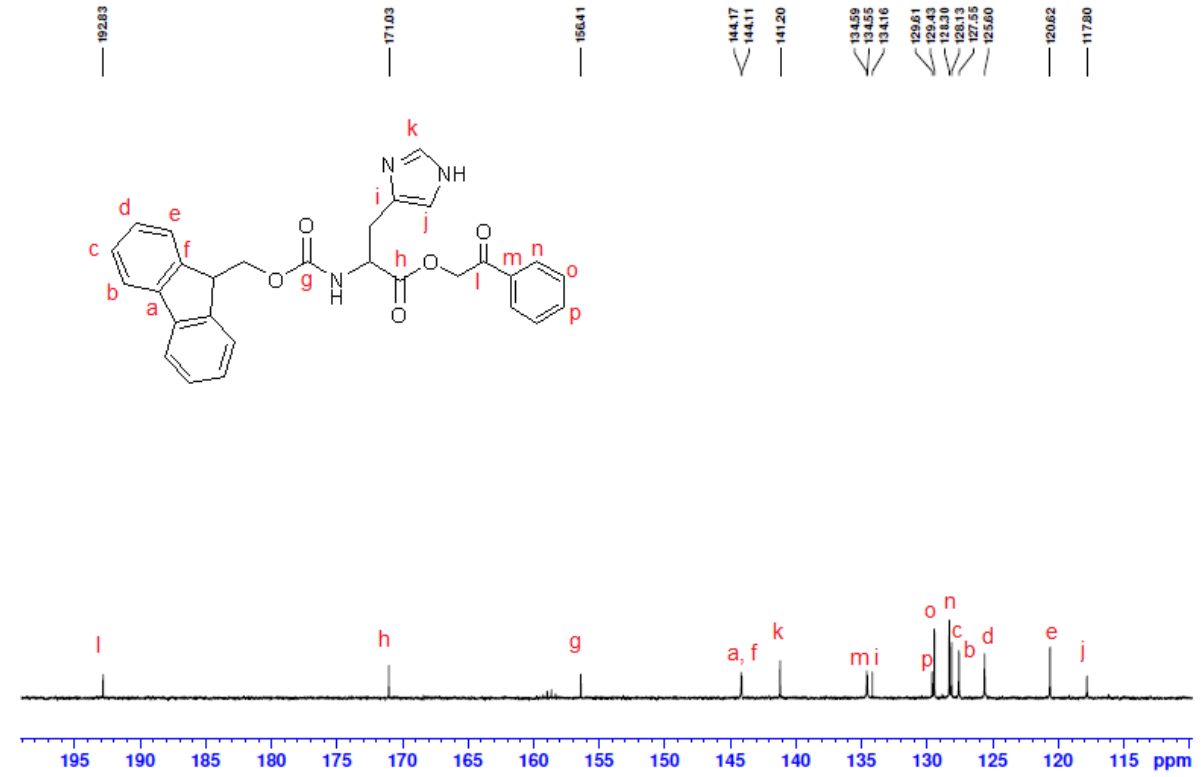


Fmoc-His-O-Phenacyl: ^{13}C NMR in DMSO

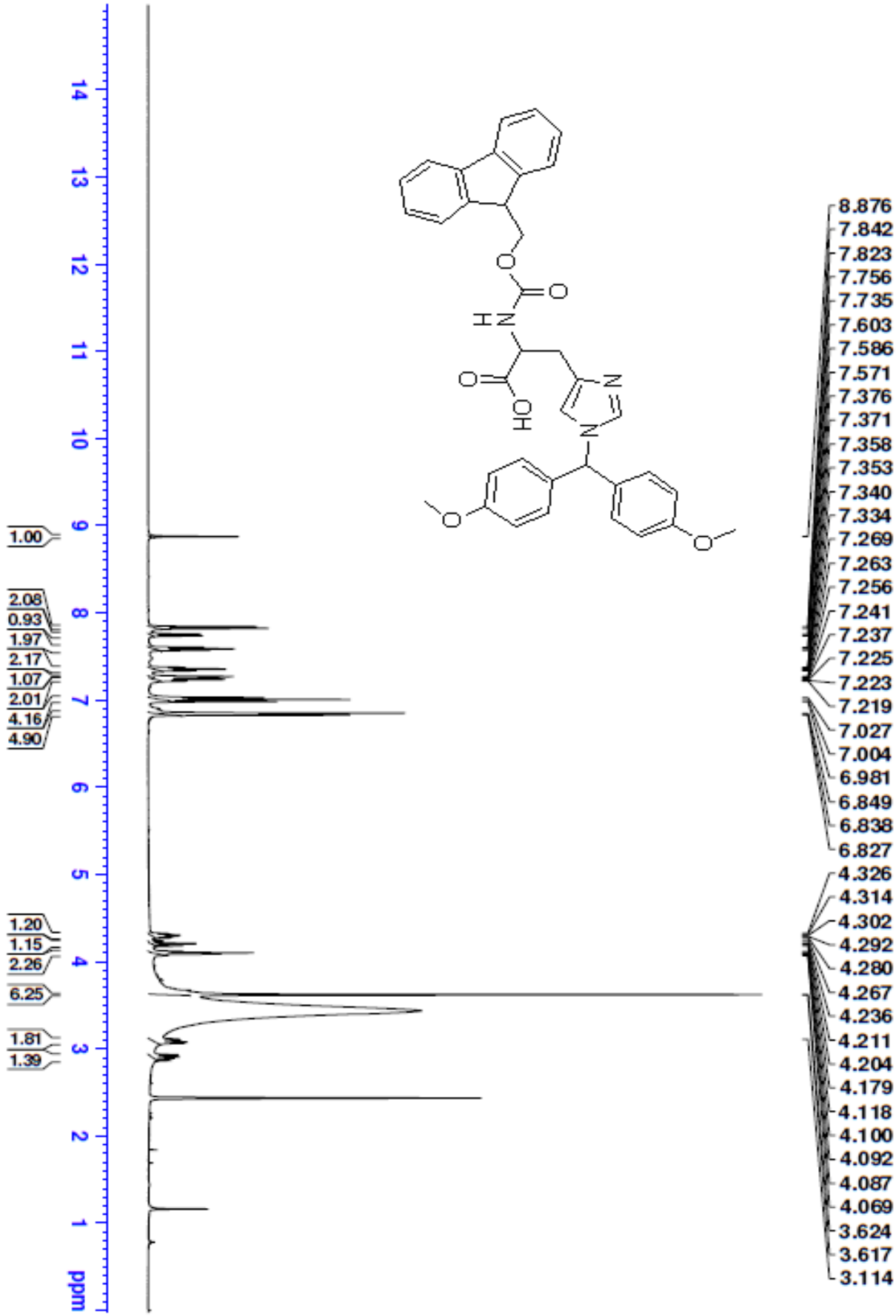


Fmoc-His-O-Phenacyl: ^{13}C NMR in DMSO expanded

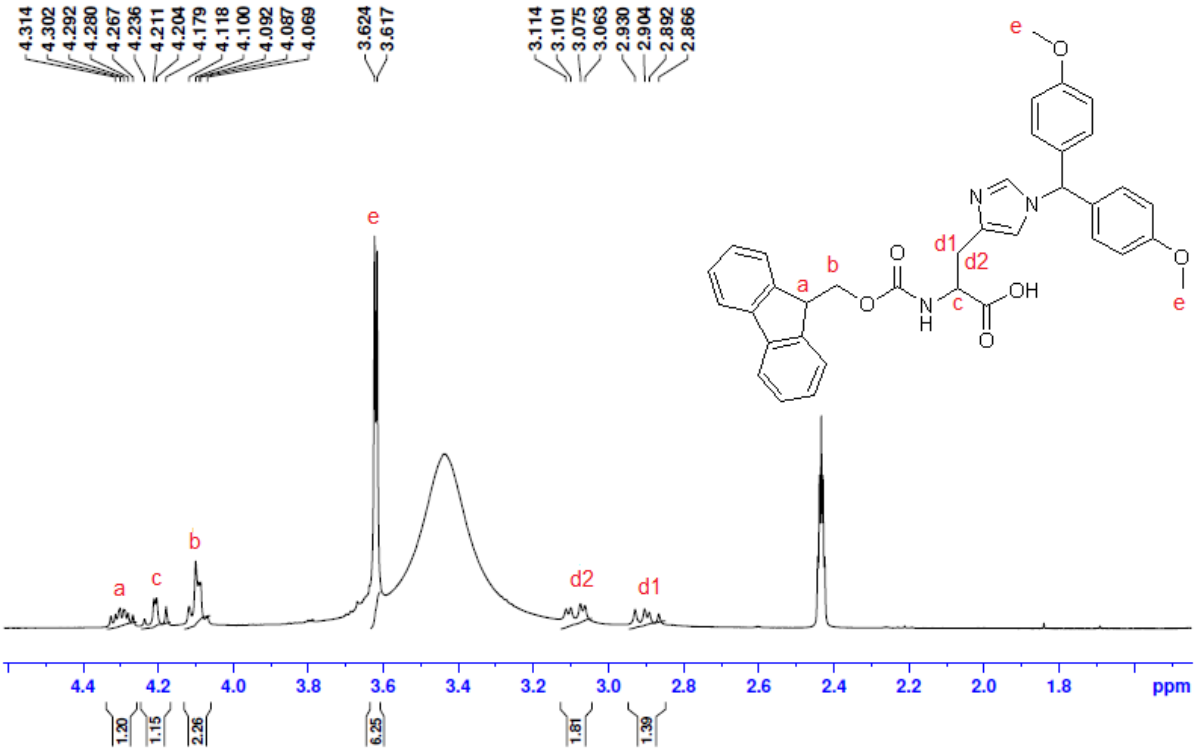


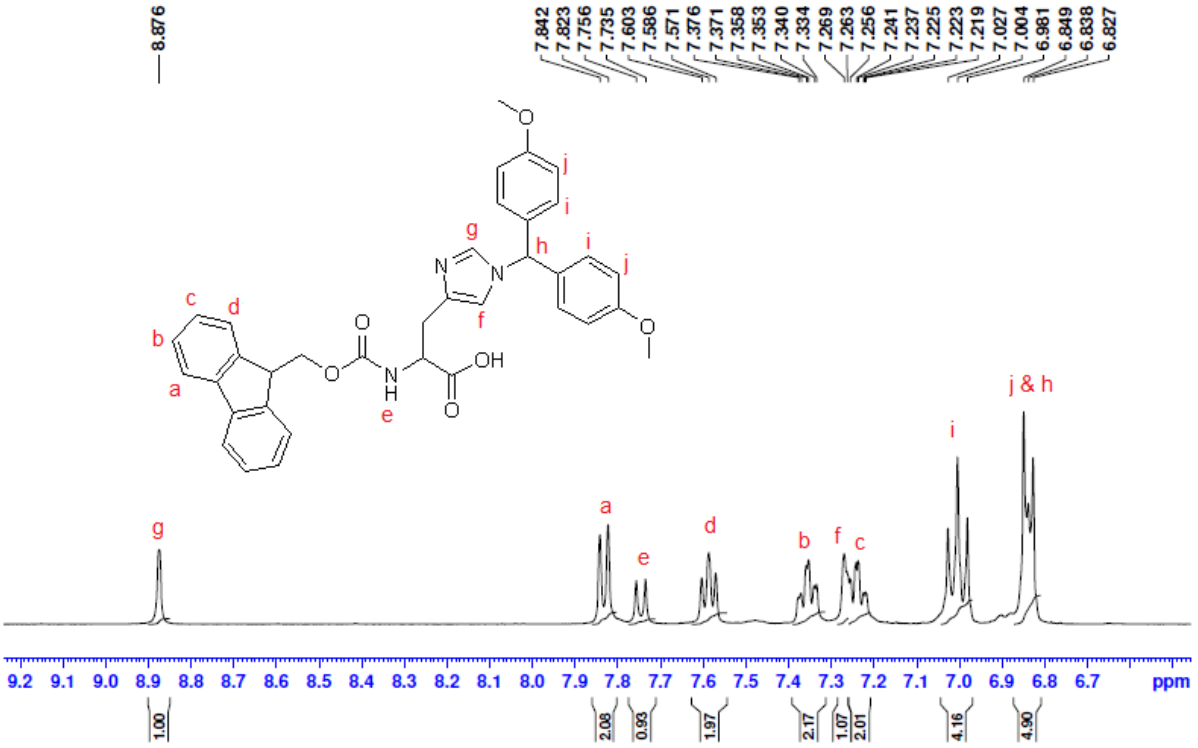


Fmoc-His-(PG3)-OH: ^{1}H NMR in DMSO

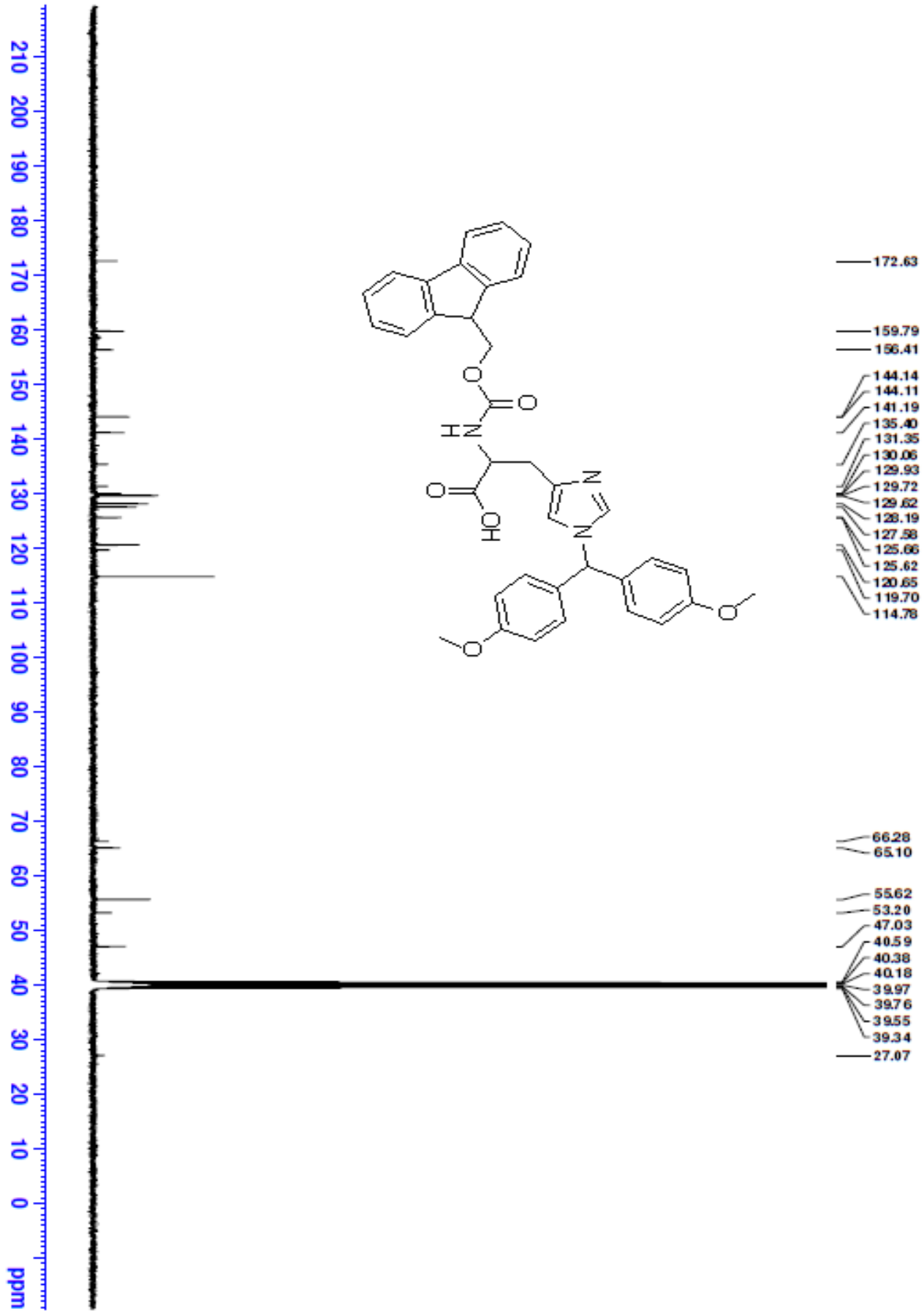


Fmoc-His-(PG3)-OH: ^{1}H NMR in DMSO expanded

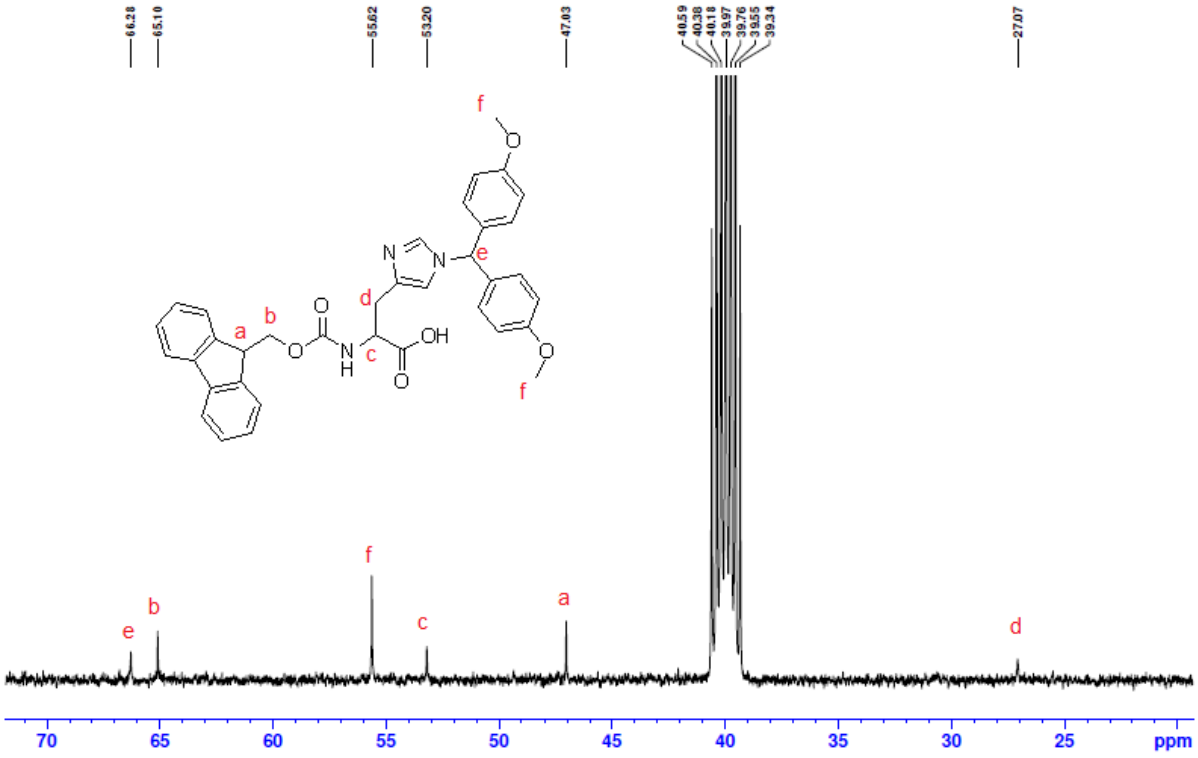


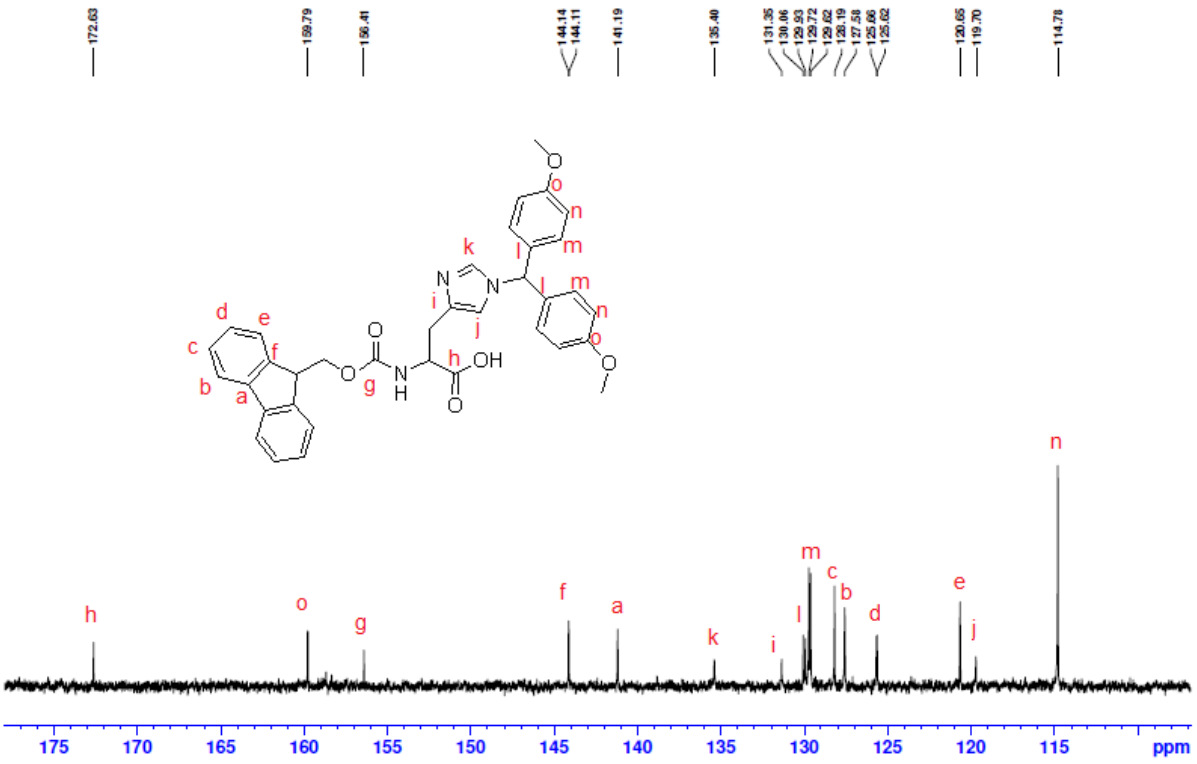


Fmoc-His-(PG3)-OH: ^{13}C NMR in DMSO

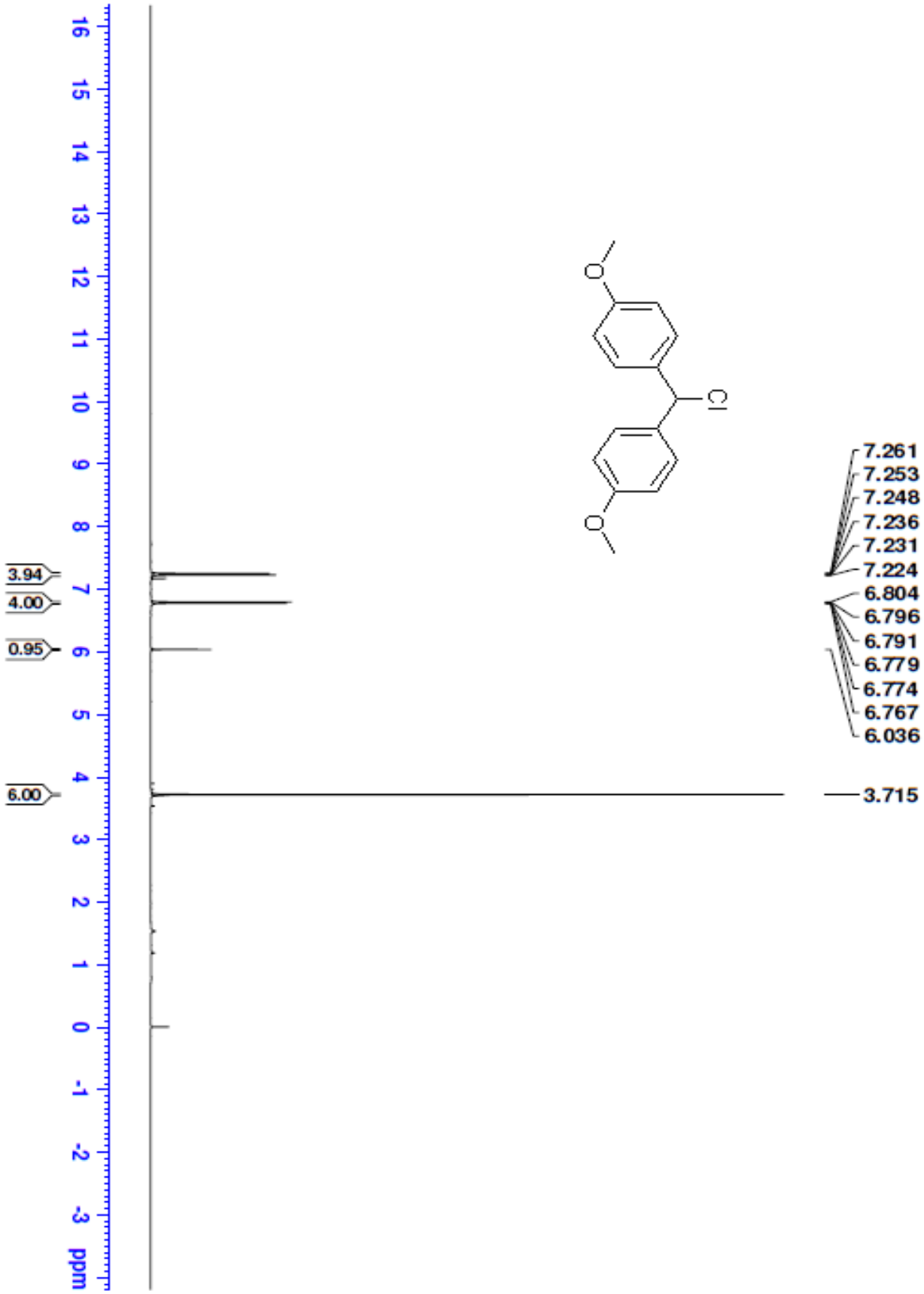


Fmoc-His-(PG3)-OH: ^{13}C NMR in DMSO expanded

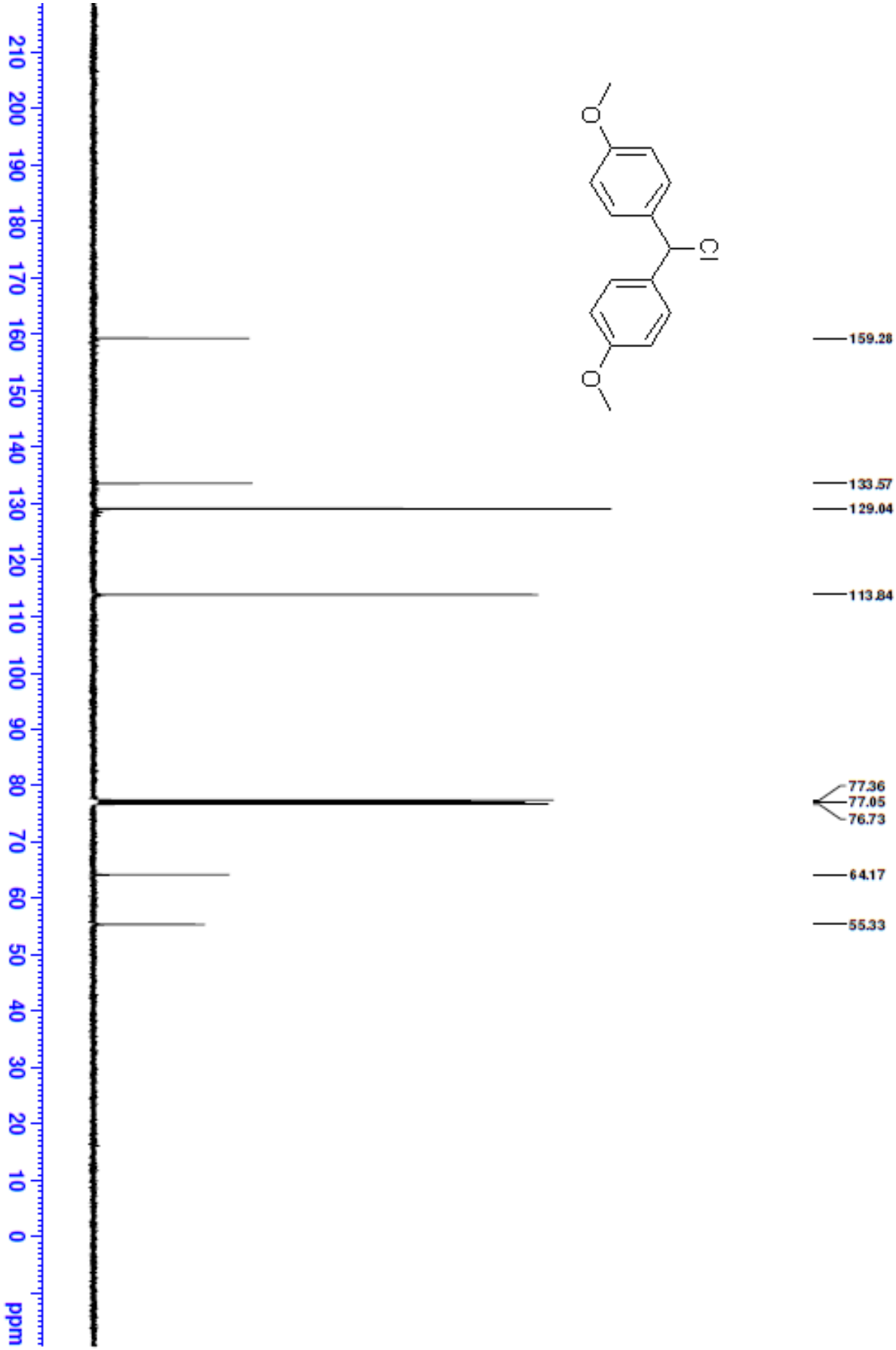




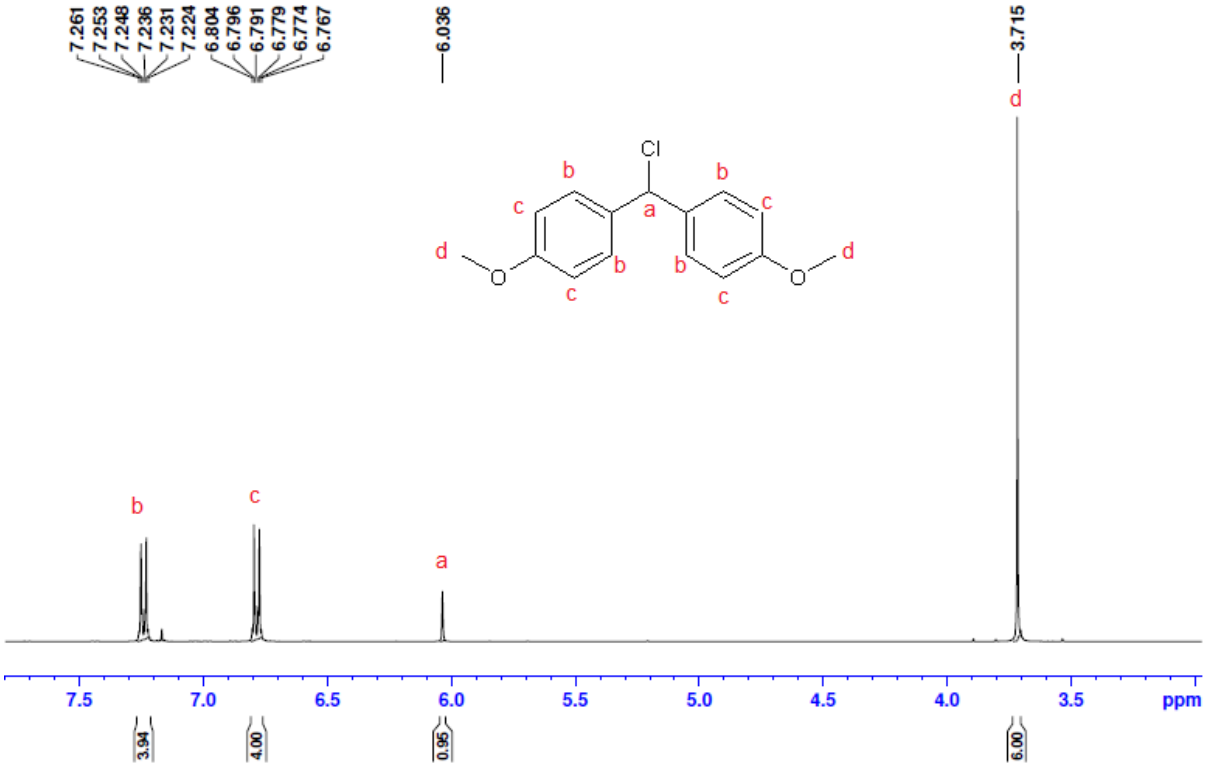
4,4-dimethoxydipenyl chloride (PG3): ^{1}H NMR in $CDCl_3$



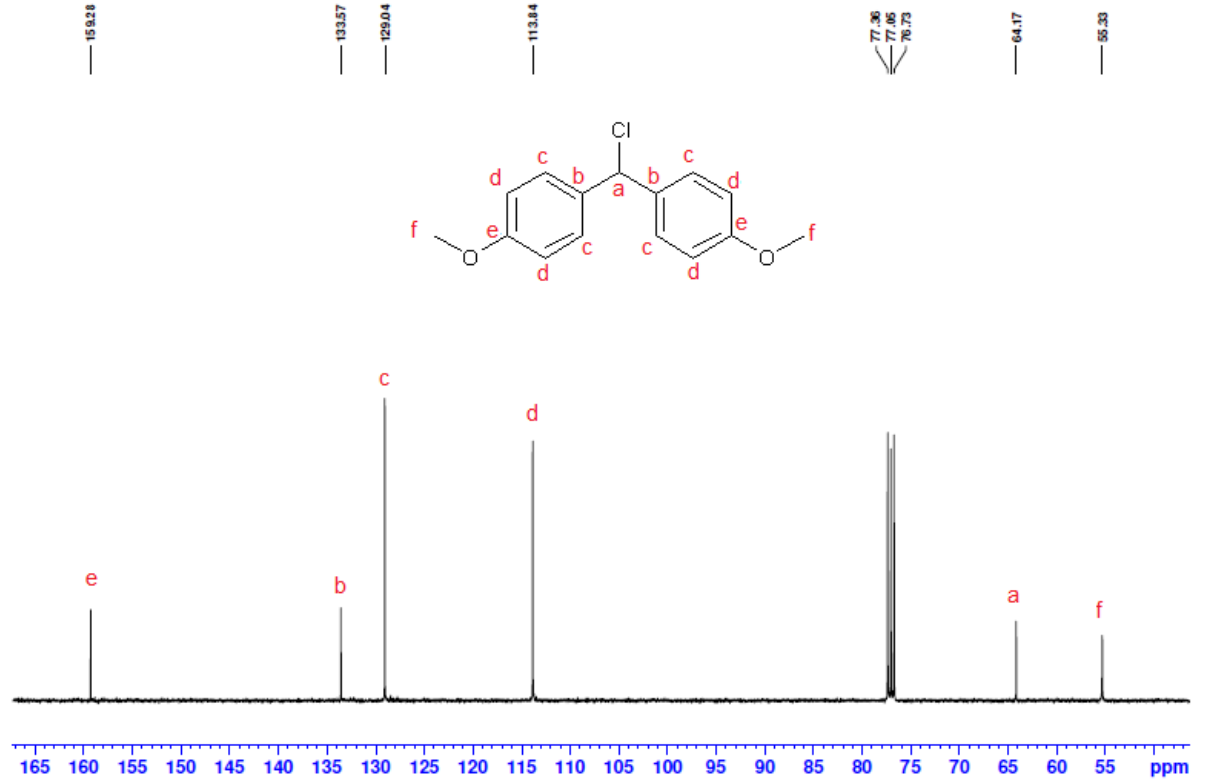
4,4-dimethoxydipenyl chloride (PG3): ^{13}C NMR in $CDCl_3$



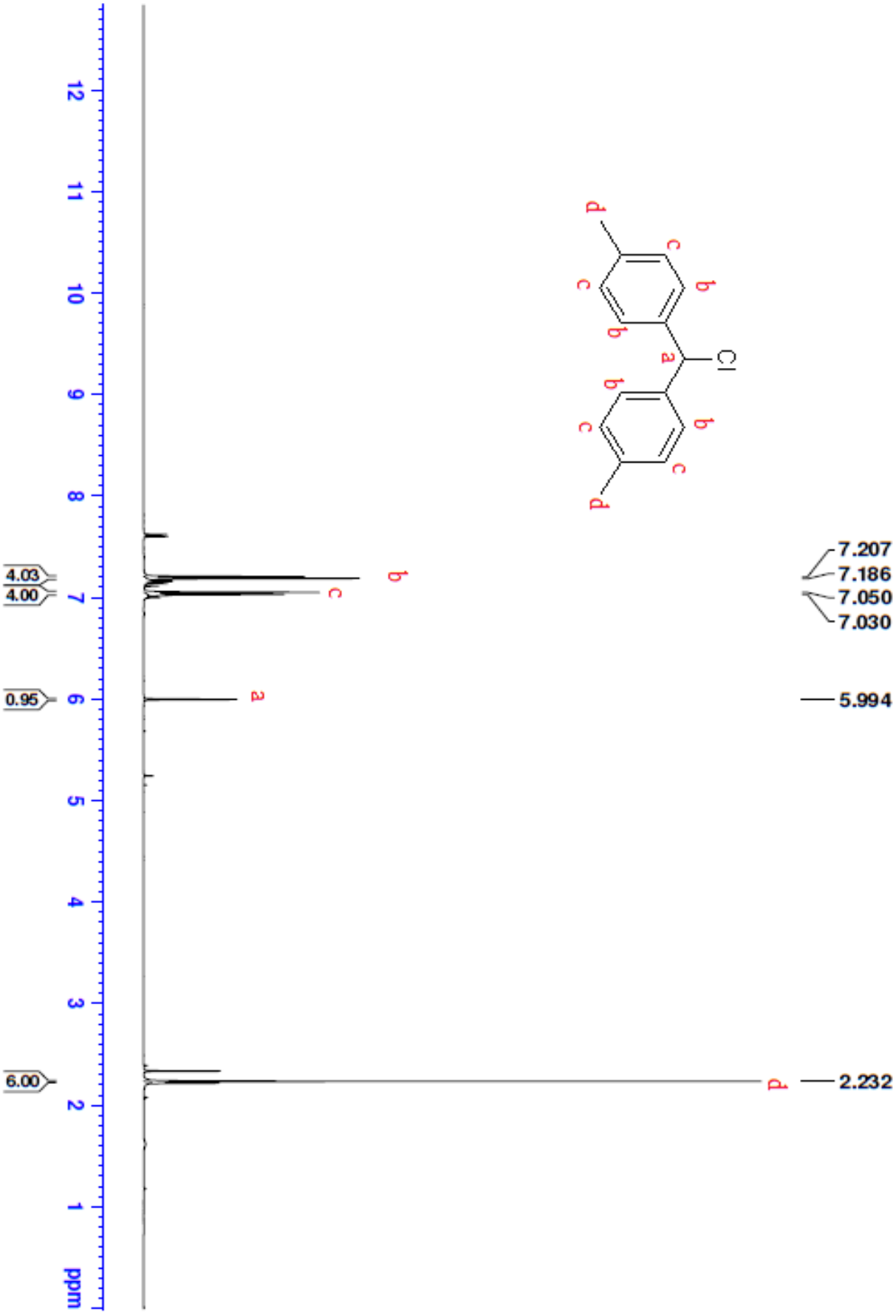
4,4-dimethoxydipenyl chloride (PG3): ^{1}H NMR in $CDCl_3$ expanded



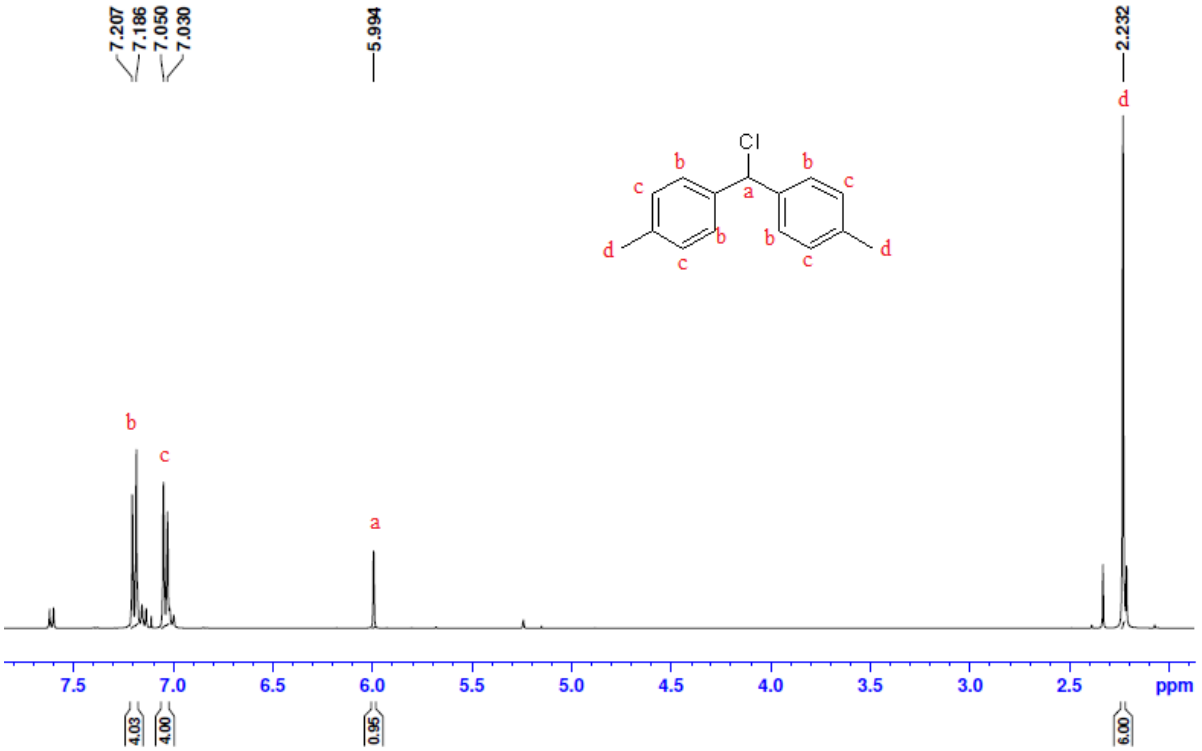
4,4-dimethyoxydipenyl chloride (PG3): ^{13}C NMR in $CDCl_3$ expanded



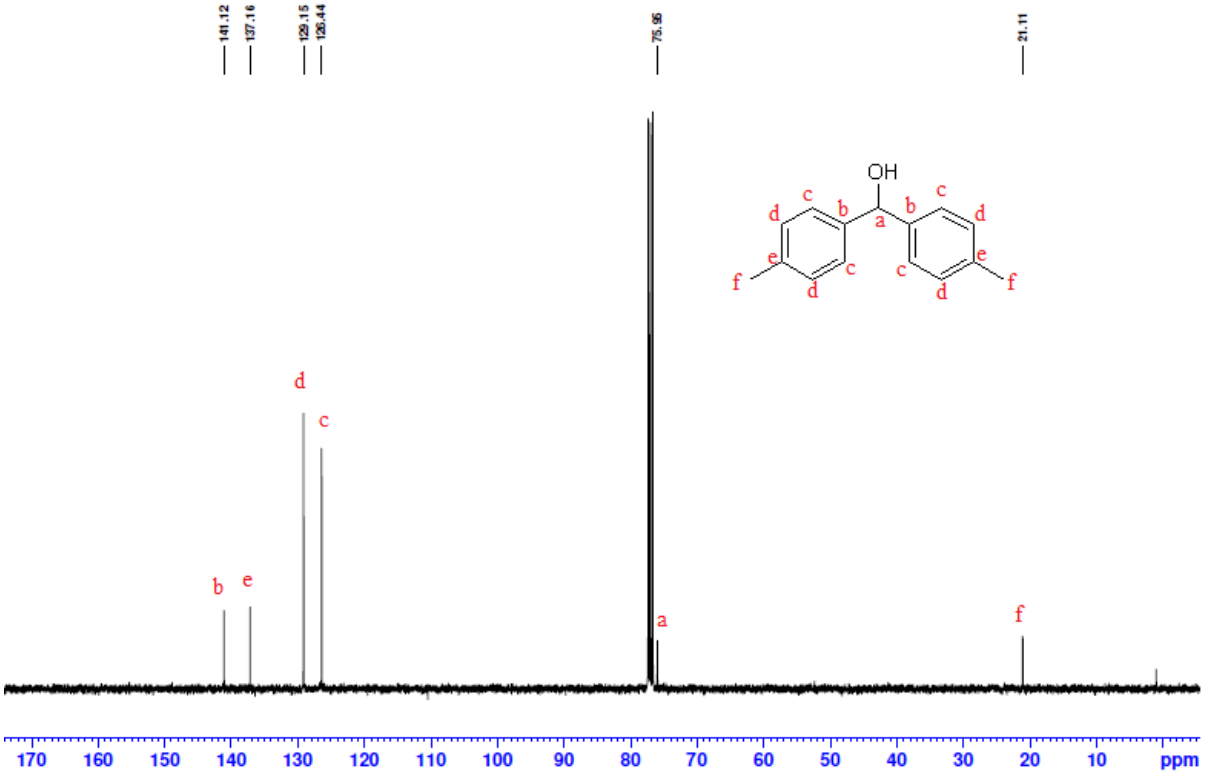
4,4-dimethyl diphenyl chloride (PG-2) ^{1}H NMR in $CDCl_3$



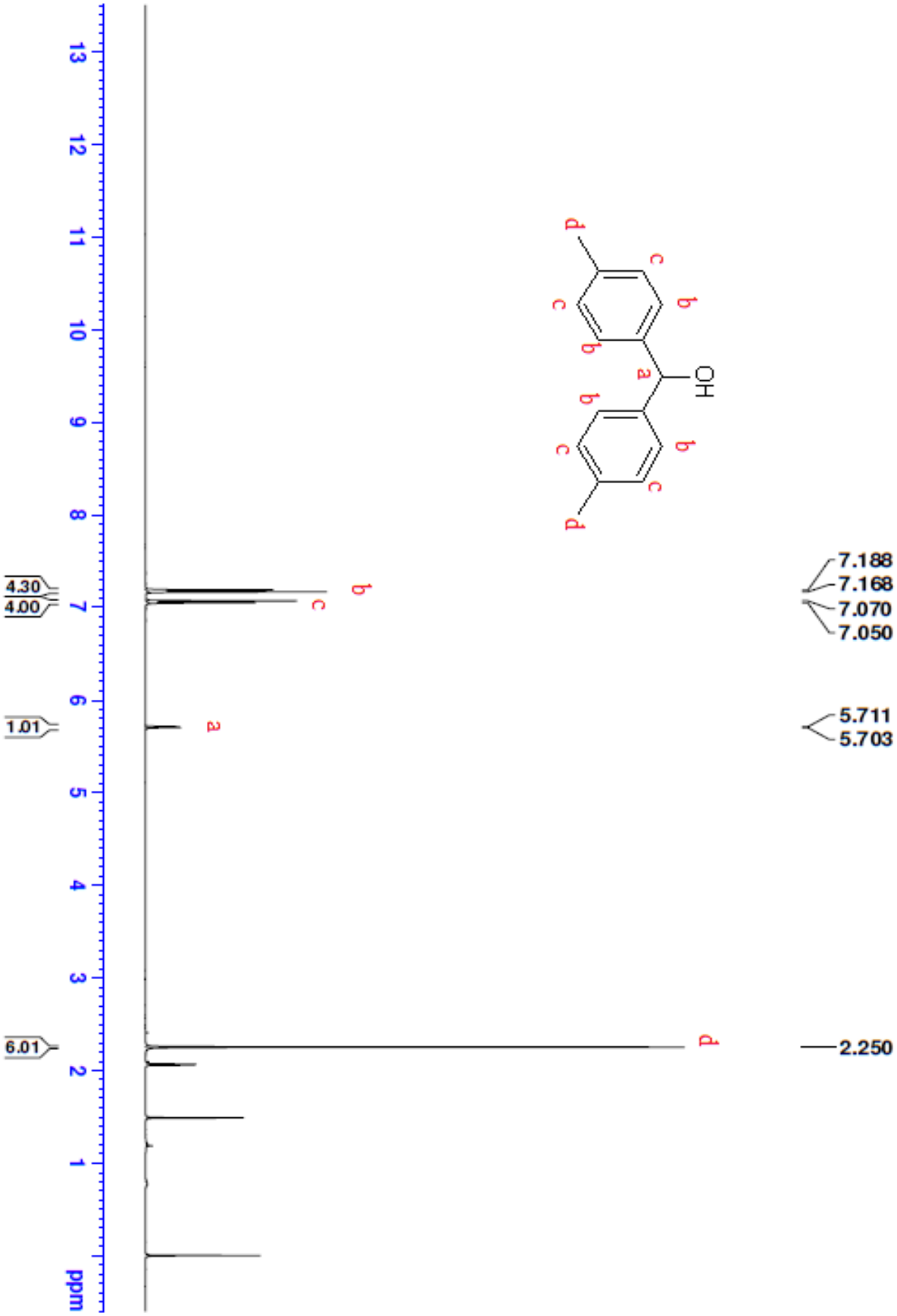
4,4-dimethyl diphenyl chloride (PG-2) ^{1}H NMR in $CDCl_3$ expanded



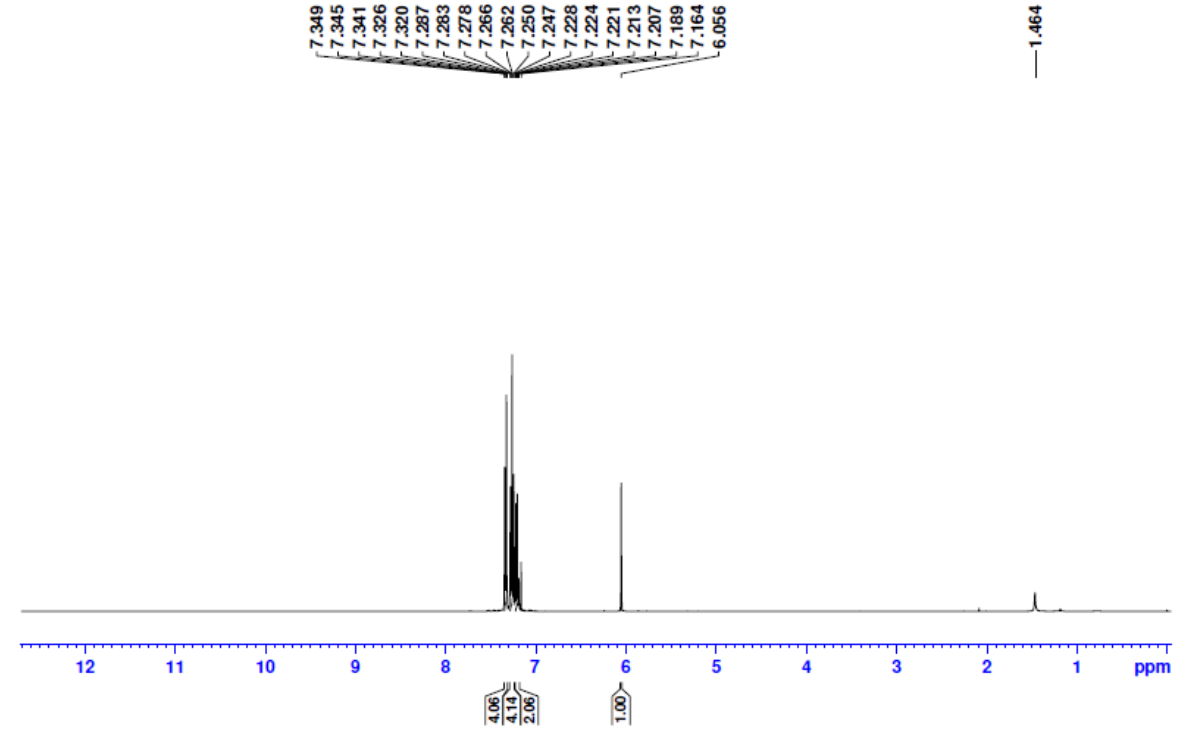
4,4-dimethylbenzhydrol (PG-2) [OH] ^{13}C NMR in $CDCl_3$



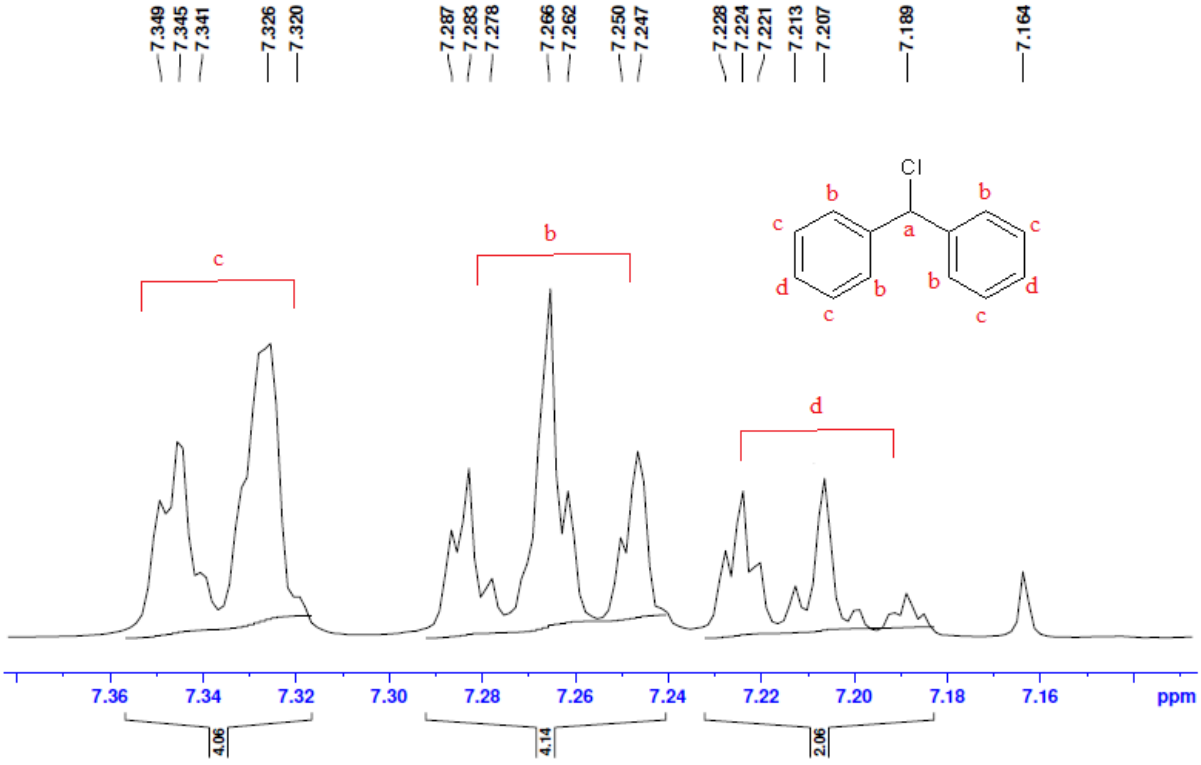
4,4-dimethylbenzhydrol (PG-2) [OH] ^{1}H NMR in $CDCl_3$



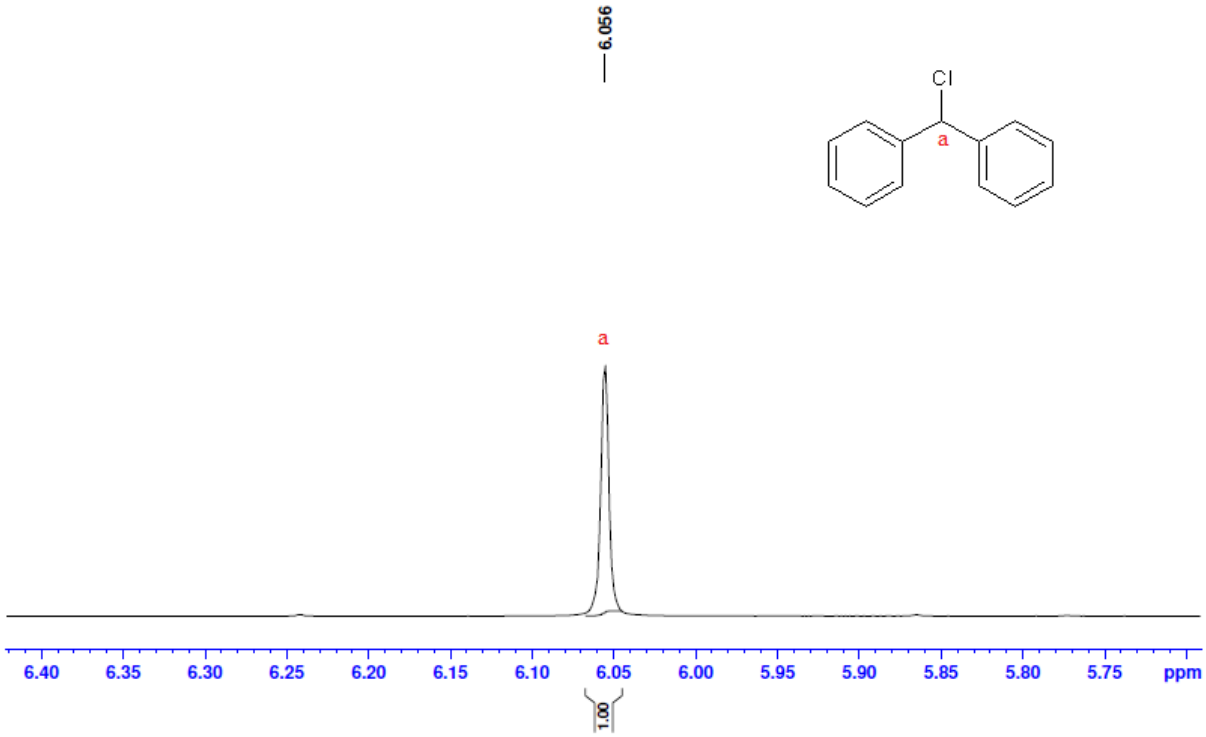
Chlorodiphenylmethane (PG-1) ^{1}H NMR in $CDCl_3$



Chlorodiphenylmethane (PG-1) ^{1}H NMR expanded 1



Chlorodiphenylmethane (PG-1) ^{1}H NMR expanded 2

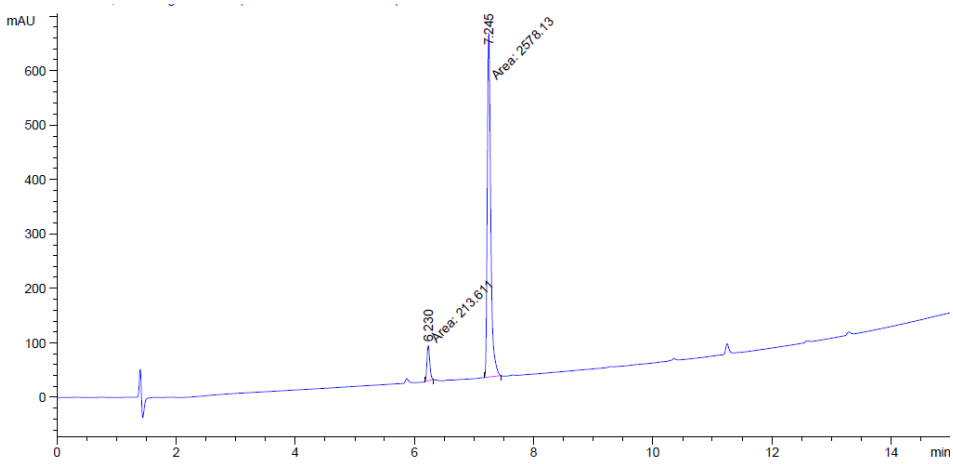


HPLC data

Gradient elution: 5 – 95% of solvent A to solvent B in 15 min

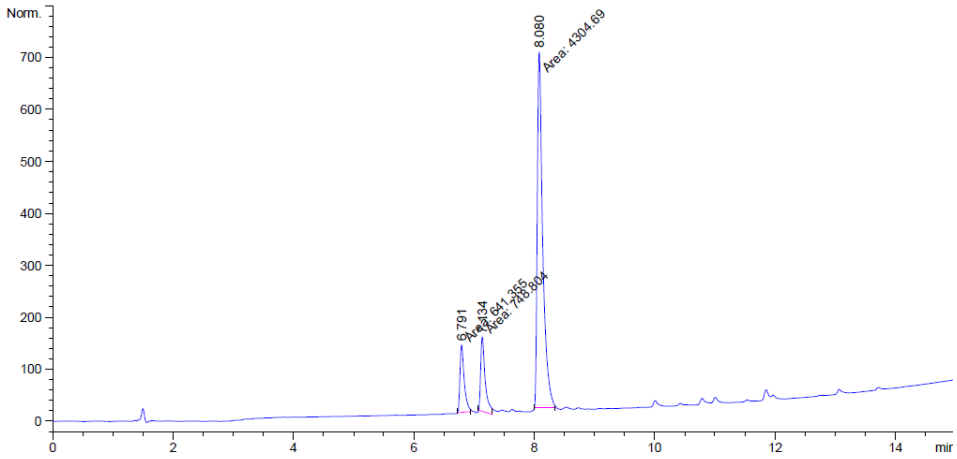
Solvent A: 0.1% TFA in MilliQ H_2O Solvent B: 0.1% TFA in acetonitrile

Fmoc-His-OH (first isolation)



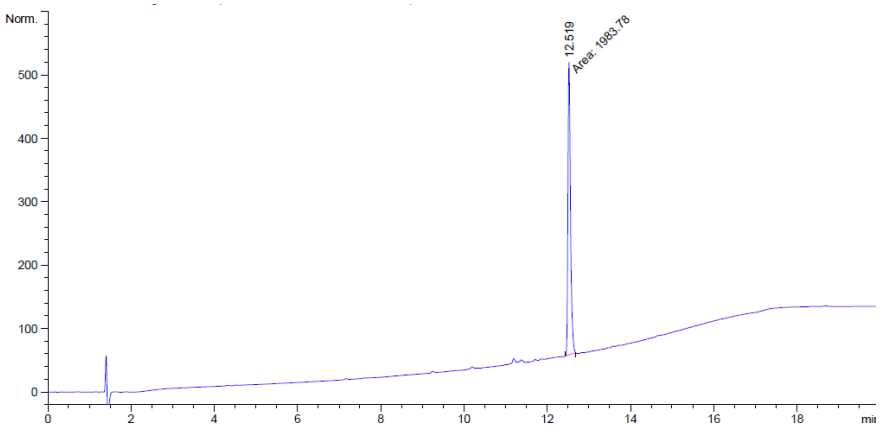
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	6.230	MM	0.0543	213.61055	65.50529	7.6515
2	7.245	MM	0.0673	2578.12524	638.08173	92.3485
Totals :				2791.73579	703.58702	

Fmoc-His-OH (storage 6months)



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	6.791	MM	0.0818	641.35522	11.2620	?
2	7.134	MM	0.0869	748.80408	13.1488	?
3	8.080	MM	0.1048	4304.68555	75.5892	?
4	8.531		0.0000	0.00000	0.0000	
Totals :				5694.84485		

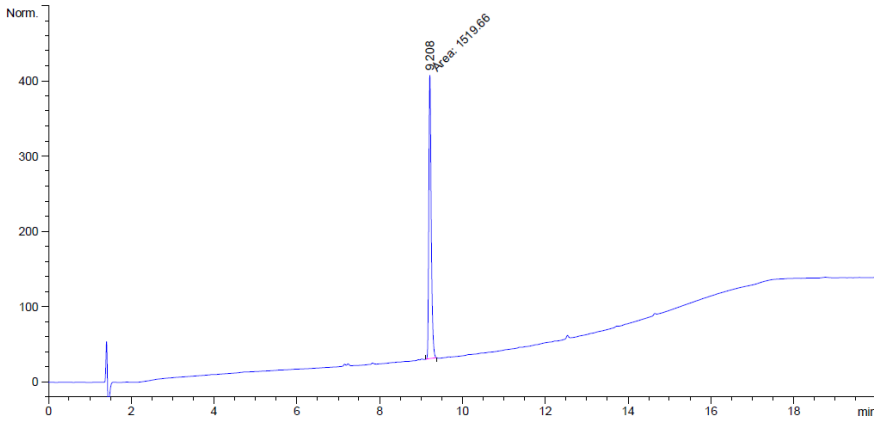
Fmoc-His(trt)-O-Pac after purification



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %	Name
1	8.531		0.0000	0.00000	0.0000		
2	12.519	MM	0.0713	1983.77625	100.0000	?	

Totals : 1983.77625

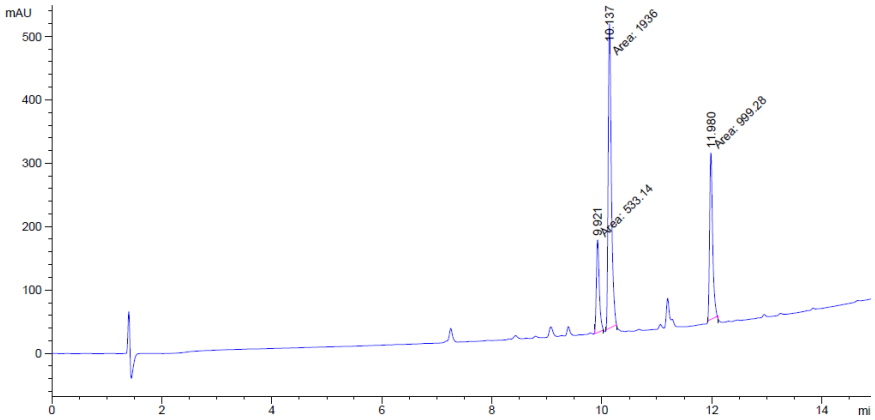
Fmoc-His-O-Pac after purification



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %	Name
1	8.531		0.0000	0.00000	0.0000		
2	9.208	MM	0.0670	1519.65588	100.0000	?	

Totals : 1519.65588

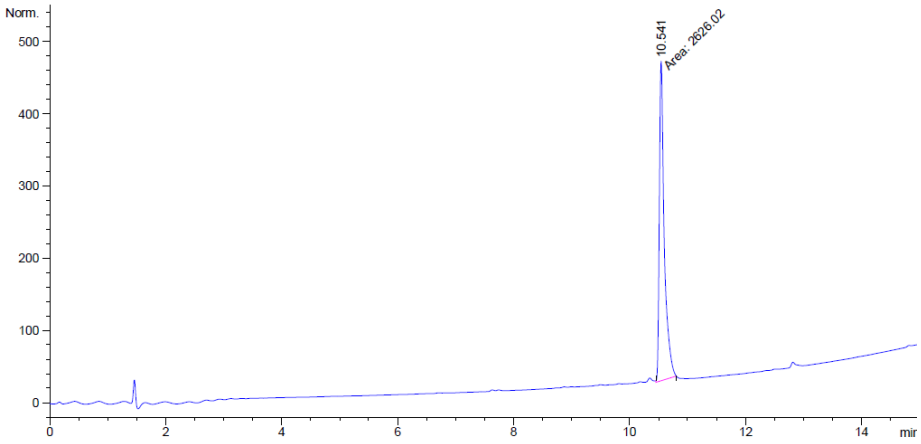
Fmoc-His-(PG3)-OH crude analysis



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.531		0.0000	0.00000	0.0000	
2	9.921	MM	0.0606	533.14044	15.3713	?
3	10.137	MM	0.0669	1935.99829	55.8179	?
4	11.980	MM	0.0629	999.27954	28.8108	?

Totals : 3468.41827

Fmoc-His-(PG3)-OH after semi-preparative HPLC



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.531		0.0000	0.00000	0.0000	
2	10.541	MM	0.0988	2626.01587	100.0000	?

Totals : 2626.01587

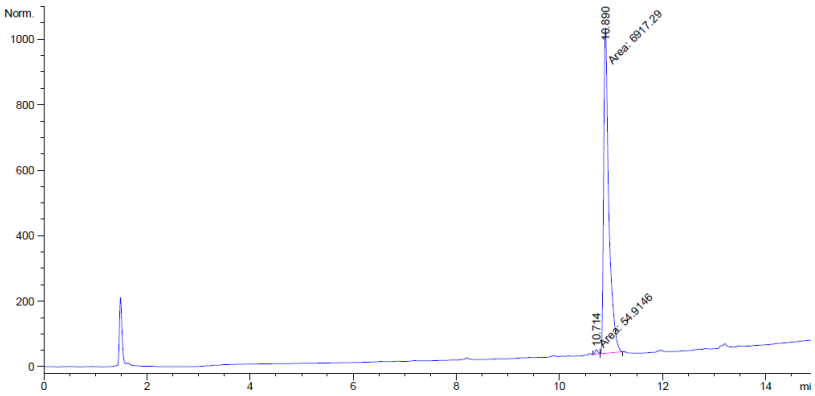
Acid lability study

HPLC conditions: 5 – 95% gradient elution of solvent B to solvent A in 15 min

Solvent A: 0,1% TFA in MilliQ H_2O Solvent B: 0.1% TFA in Acetonitrile

95% TFA, 2.5% H_2O, 2.5% TIS cleavage cocktail

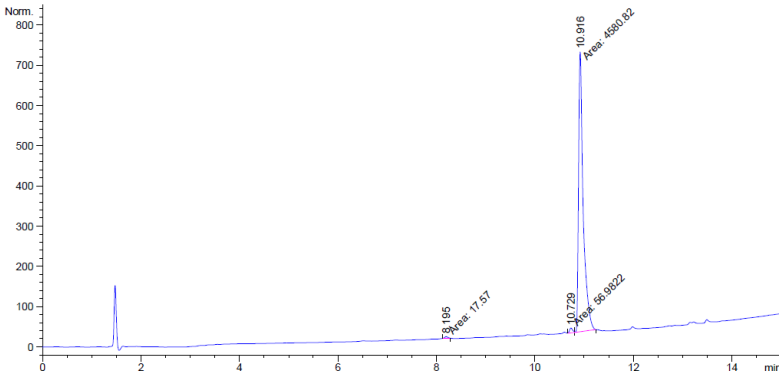
0 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	10.714	MF	0.0660	54.91459	0.7876	?
2	10.890	FM	0.1158	6917.29395	99.2124	?

Totals : 6972.20853

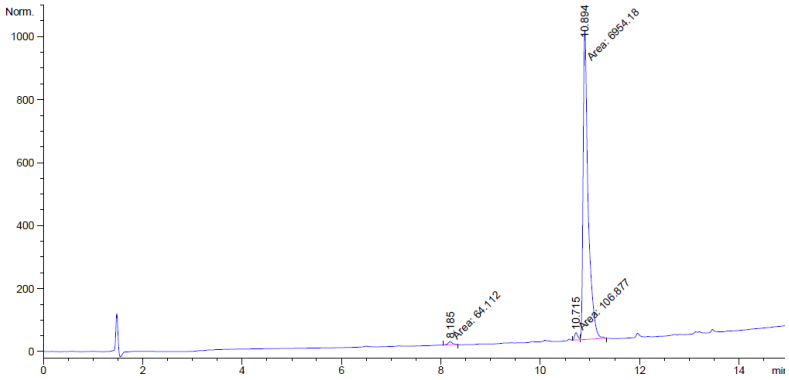
60 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.195	MM	0.0742	17.56999	0.3774	?
2	10.729	MF	0.0760	56.98220	1.2240	?
3	10.916	FM	0.1095	4580.81787	98.3986	?

Totals : 4655.37006

120 min

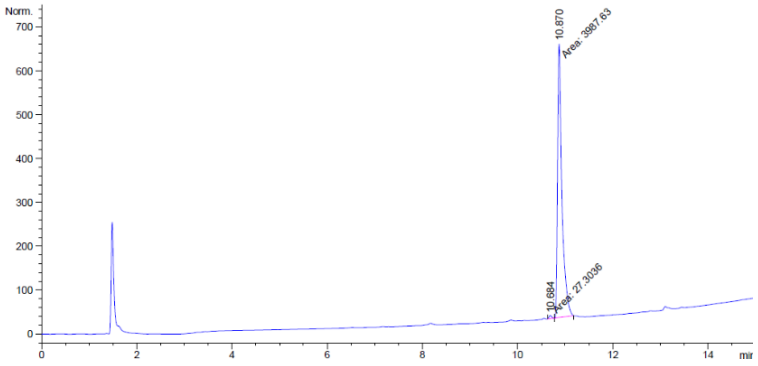


Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.185	MM	0.1038	64.11202	0.8998	?
2	10.715	MF	0.0756	106.87664	1.5000	?
3	10.894	FM	0.1177	6954.18115	97.6002	?

Totals : 7125.16982

97.5% TFA, 2.5% H_2O cleavage cocktail

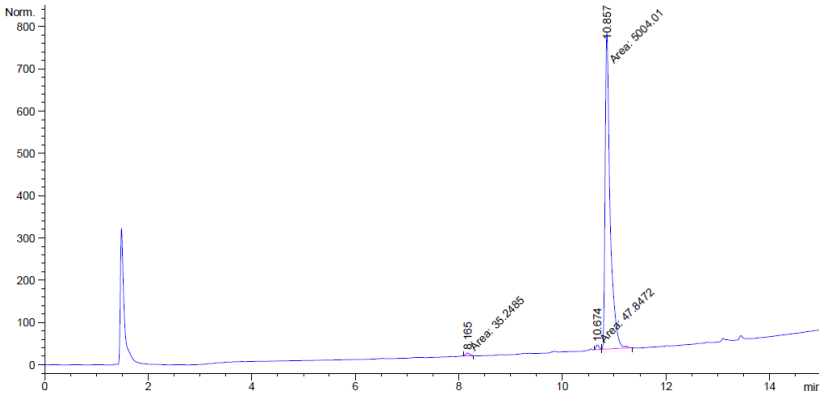
0 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	10.684	MF	0.0642	27.30364	0.6801	?
2	10.870	FM	0.1063	3987.62915	99.3199	?

Totals : 4014.93279

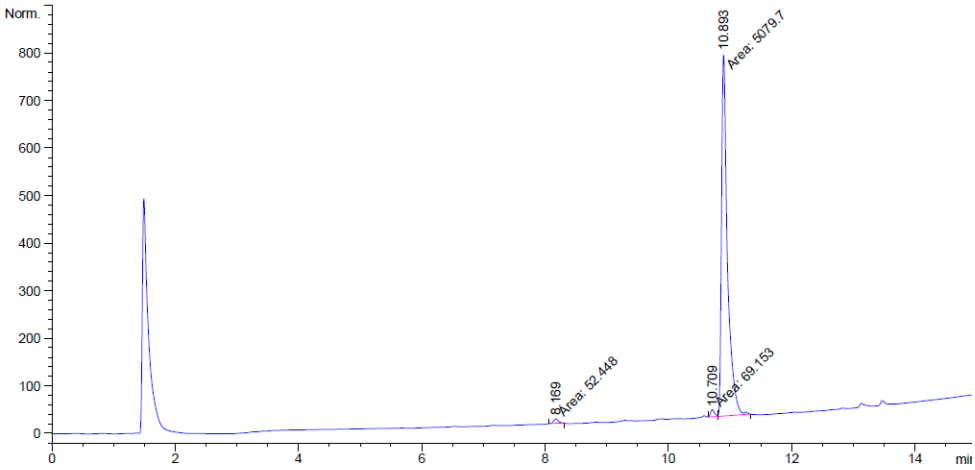
30 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.165	MM	0.0898	35.24848	0.6929	?
2	10.674	MF	0.0703	47.84723	0.9406	?
3	10.857	FM	0.1113	5004.01465	98.3665	?

Totals : 5087.11036

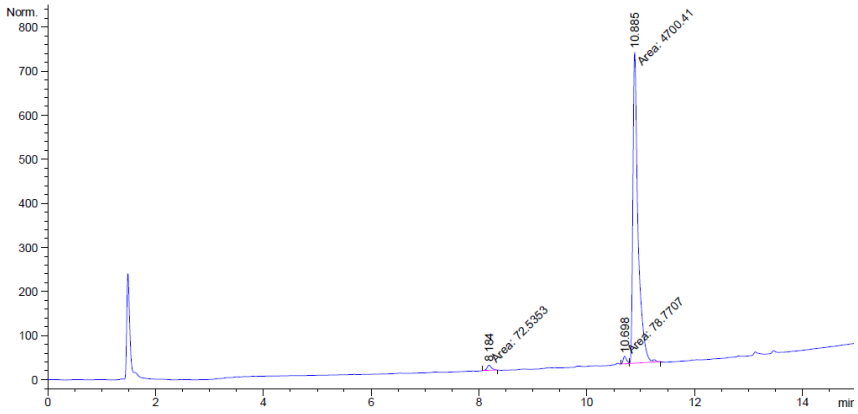
60 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.169	MM	0.0908	52.44798	1.0084	?
2	10.709	MF	0.0752	69.15295	1.3295	?
3	10.893	FM	0.1112	5079.69873	97.6621	?

Totals : 5201.29967

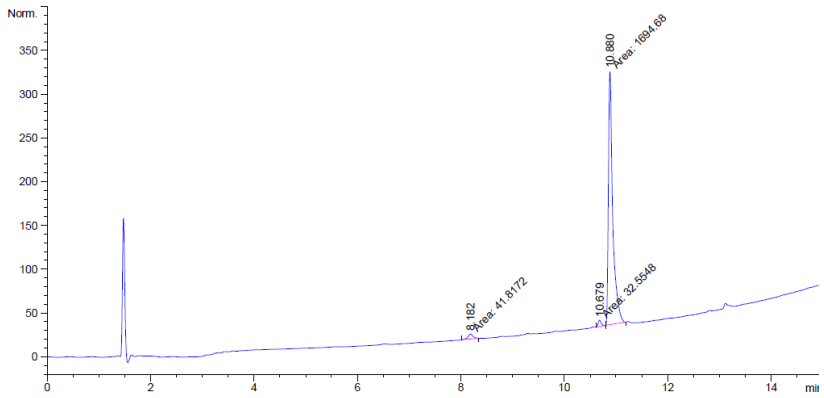
120 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.184	MM	0.1030	72.53529	1.4950	?
2	10.698	MF	0.0739	78.77071	1.6236	?
3	10.885	FM	0.1107	4700.41016	96.8814	?

Totals : 4851.71616

270 min

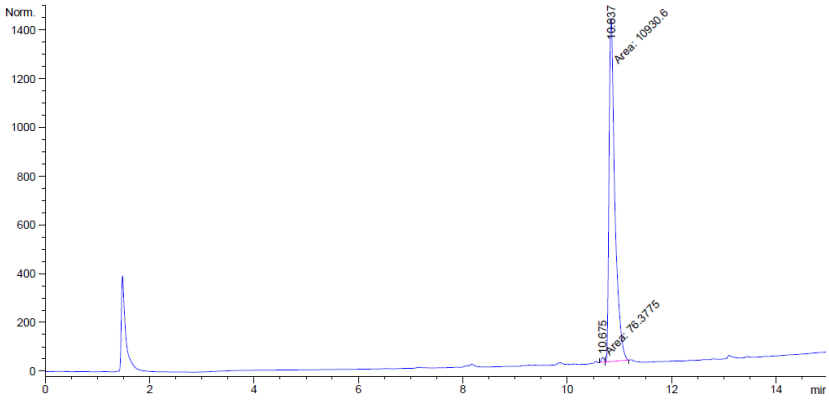


Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.182	MM	0.1163	41.81718	2.3638	?
2	10.679	MF	0.0712	32.55477	1.8402	?
3	10.880	FM	0.0974	1694.67651	95.7959	?

Totals : 1769.04846

100% TFA cleavage cocktail

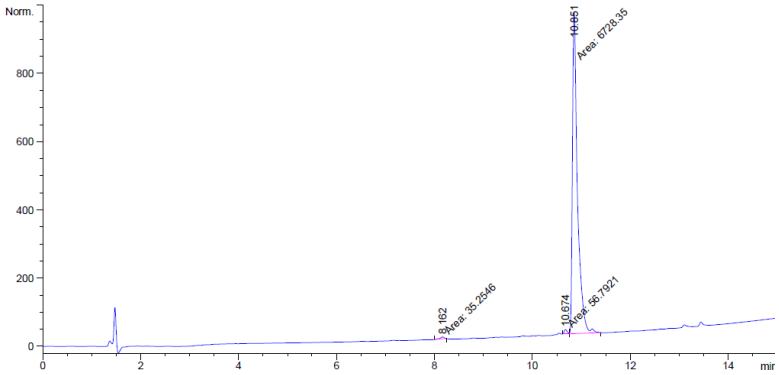
0 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %	Name
1	10.675	MF	0.0648	76.37747	0.6939	?	
2	10.837	FM	0.1291	1.09306e4	99.3061	?	

Totals : 1.10070e4

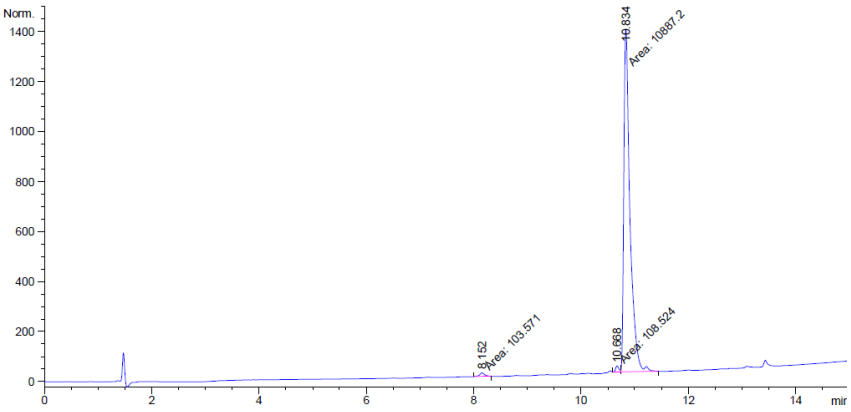
30 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU	Area *s	Area %	Name
1	8.162	MM	0.0957	35.25460	0.5169	?	
2	10.674	MF	0.0722	56.79211	0.8327	?	
3	10.851	FM	0.1189	6728.35059	98.6504	?	

Totals : 6820.39730

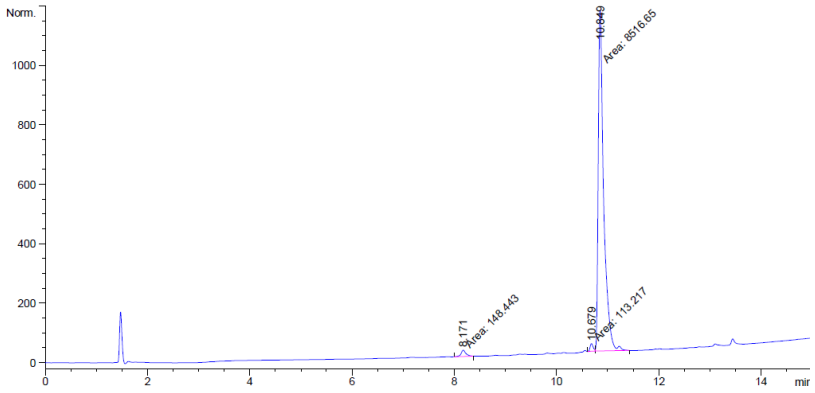
60 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.152	MM	0.1147	103.57052	0.9331	?
2	10.668	MF	0.0736	108.52397	0.9778	?
3	10.834	FM	0.1322	1.08872e4	98.0891	?

Totals : 1.10993e4

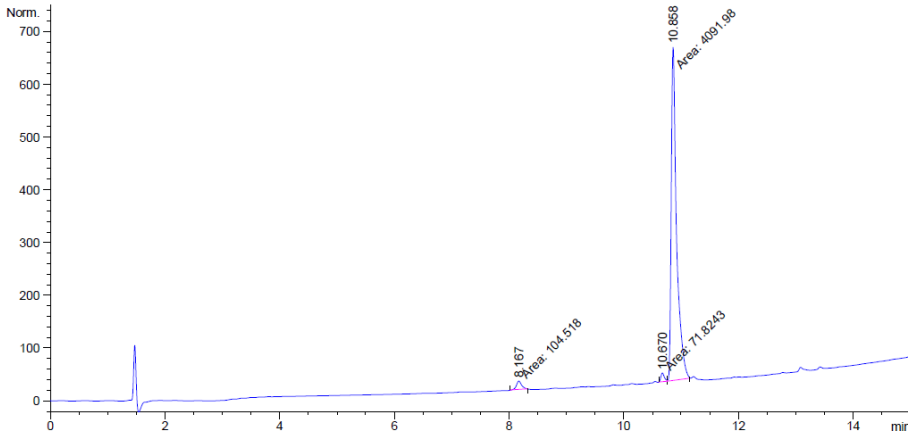
120 min



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.171	MM	0.1135	148.44250	1.6910	?
2	10.679	MF	0.0714	113.21732	1.2897	?
3	10.849	FM	0.1235	8516.65137	97.0192	?

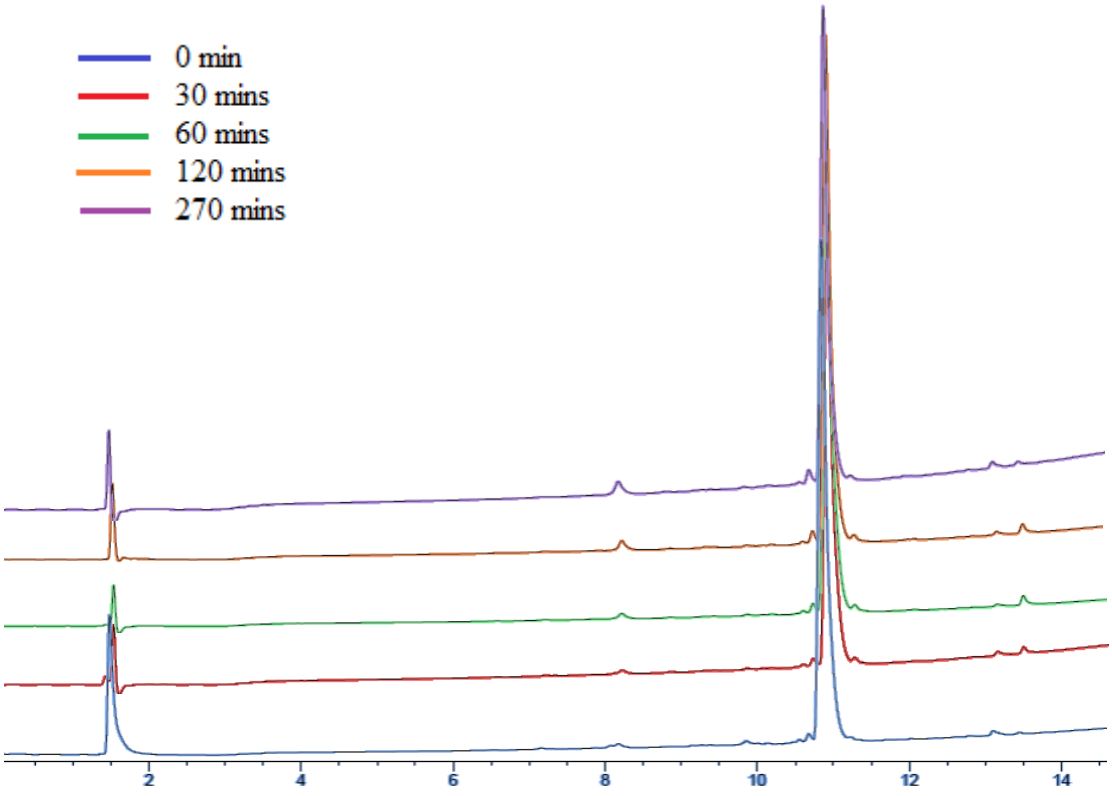
Totals : 8778.31120

270 min

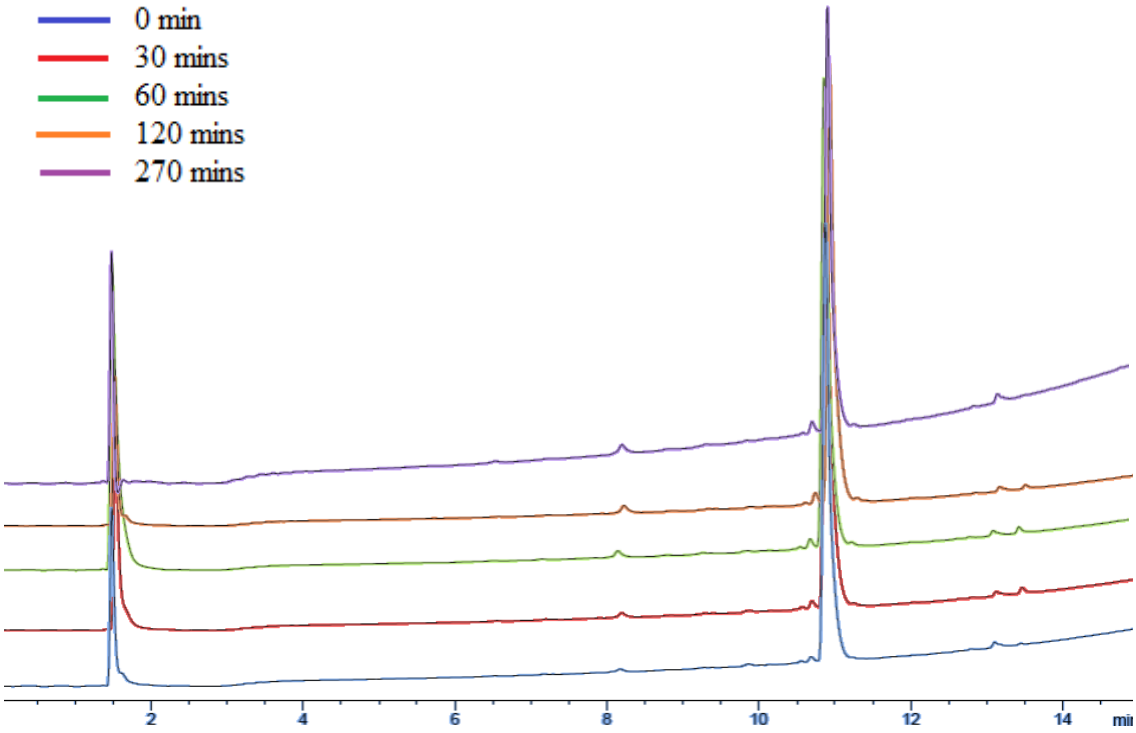


Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	8.167	MM	0.1070	104.51786	2.4487	?
2	10.670	MF	0.0704	71.82434	1.6827	?
3	10.858	FM	0.1078	4091.98145	95.8686	?
Totals :				4268.32365		

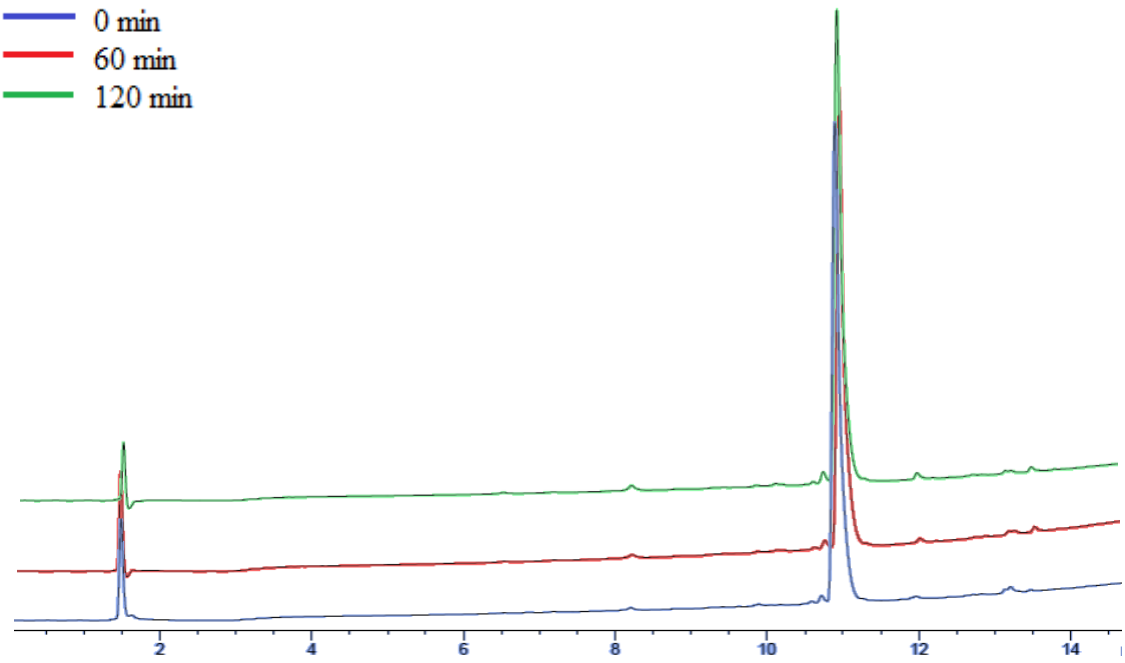
Fmoc-His(PG3)-OH in 100% TFA during different time intervals



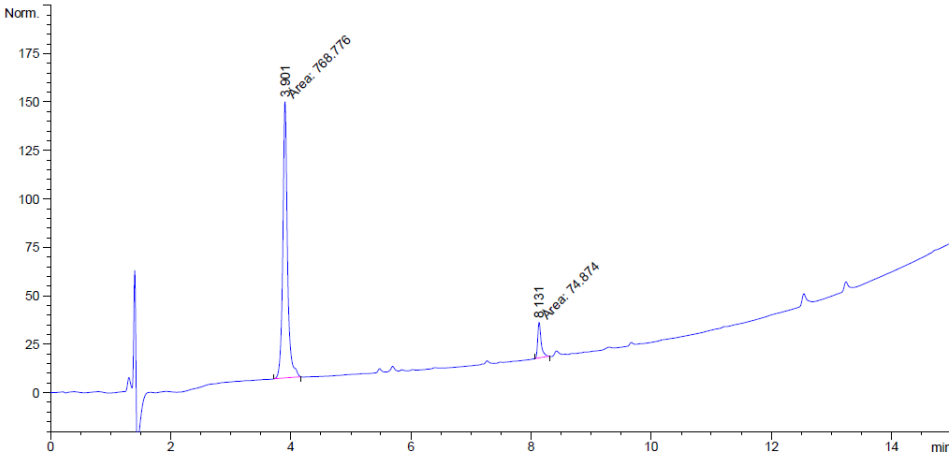
Fmoc-His(PG3)-OH in 97.5% TFA + 2.5% H_2O during different time intervals



Fmoc-His(PG-3)-OH in 95% TFA, 2.5% H_2O and 2.5% TIS at different time intervals



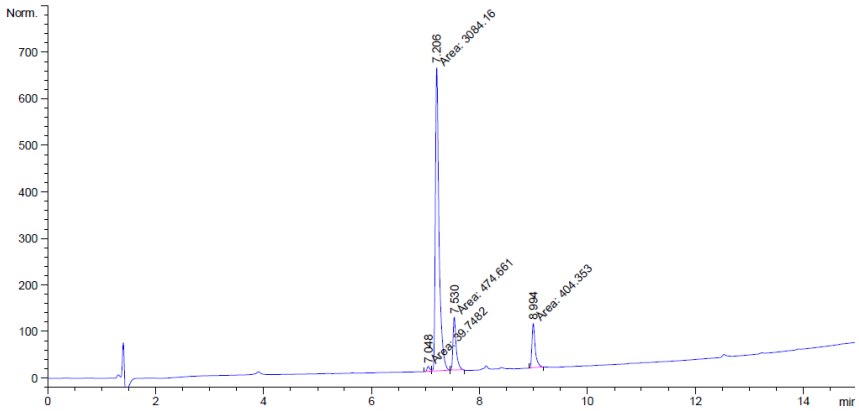
NH_2-Gly-His-Phe-OH tripeptide using Fmoc-His(trt)-OH



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	3.901	MM	0.0896	768.77588	91.1250	?
2	8.131	MM	0.0674	74.87404	8.8750	?
3	8.531		0.0000	0.00000	0.0000	

Totals : 843.64992

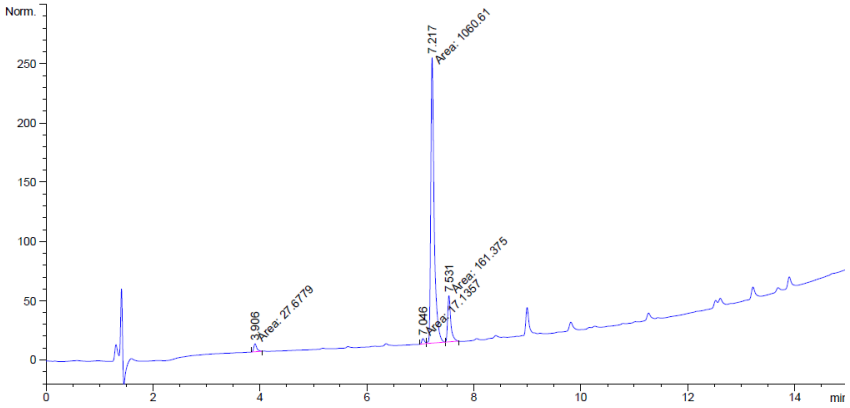
NH_2-Gly-His-Phe-OH tripeptide using Fmoc-His(PG3)-O-PG3 crude



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	7.048	MF	0.0605	39.74821	0.9930	?
2	7.206	FM	0.0786	3084.15674	77.0477	?
3	7.530	MM	0.0690	474.66129	11.8579	?
4	8.531		0.0000	0.00000	0.0000	
5	8.994	MM	0.0705	404.35327	10.1015	?

Totals : 4002.91950

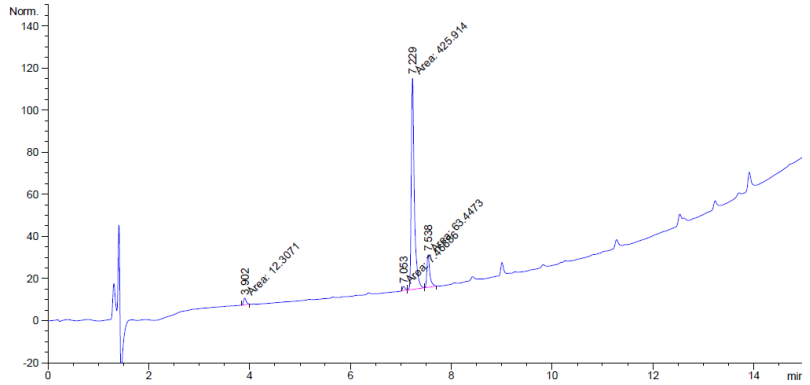
Tripeptide with Fmoc-His(PG3)-O-PG3 in 95% TFA 2,5% TIS, 2.5% H_2O for 1 hour



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	3.906	MM	0.0679	27.67790	2.1849	?
2	7.046	MF	0.0615	17.13570	1.3527	?
3	7.217	FM	0.0731	1060.61194	83.7237	?
4	7.531	FM	0.0683	161.37495	12.7388	?
5	8.531		0.0000	0.00000	0.0000	

Totals : 1266.80049

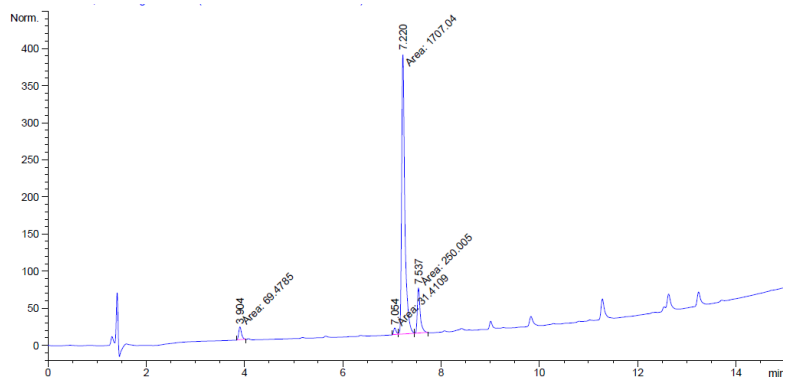
Tripeptide with Fmoc-His(PG3)-O-PG3 in 95% TFA 2,5% TIS, 2.5% H_2O for 2 hours



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	3.902	MM	0.0633	12.30708	2.4173	?
2	7.053	MF	0.0613	7.46686	1.4666	?
3	7.229	MF	0.0704	425.91388	83.6544	?
4	7.538	FM	0.0677	63.44729	12.4618	?
5	8.531		0.0000	0.00000	0.0000	

Totals : 509.13511

Tripeptide with Fmoc-His(PG3)-O-PG3 in 95% TFA 2,5% TIS, 2.5% H_2O for 5 hours



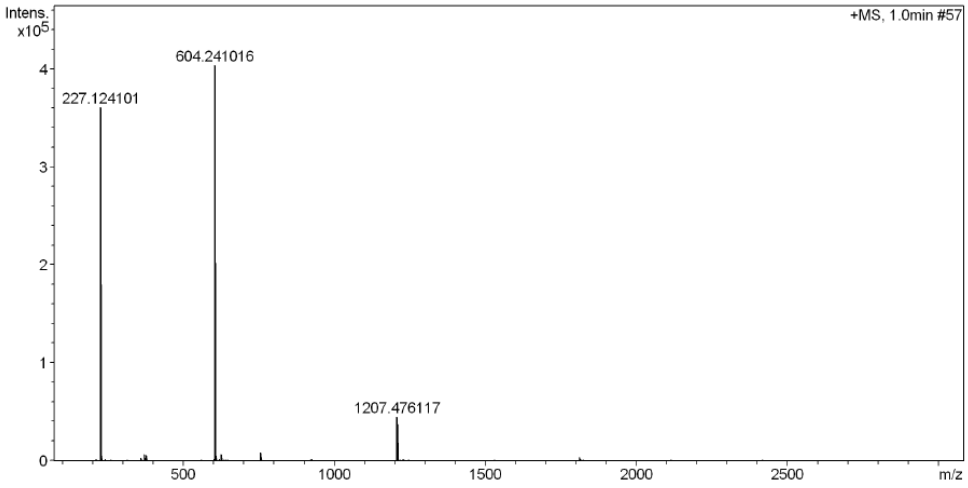
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Area %	Name
1	3.904	MM	0.0662	69.47849	3.3761	?
2	7.054	MF	0.0590	31.41088	1.5263	?
3	7.220	FM	0.0752	1707.04041	82.9492	?
4	7.537	FM	0.0680	250.00481	12.1483	?
5	8.531		0.0000	0.00000	0.0000	
Totals :				2057.93458		

High Resolution Mass Spectrometry (HRMS):

Fmoc-His(PG3)-OH

Theoretical mass: $C_{36}H_{33}N_3O_6$ [M + H] = 604.240190

Mass measured: [M + H] = 604.241016

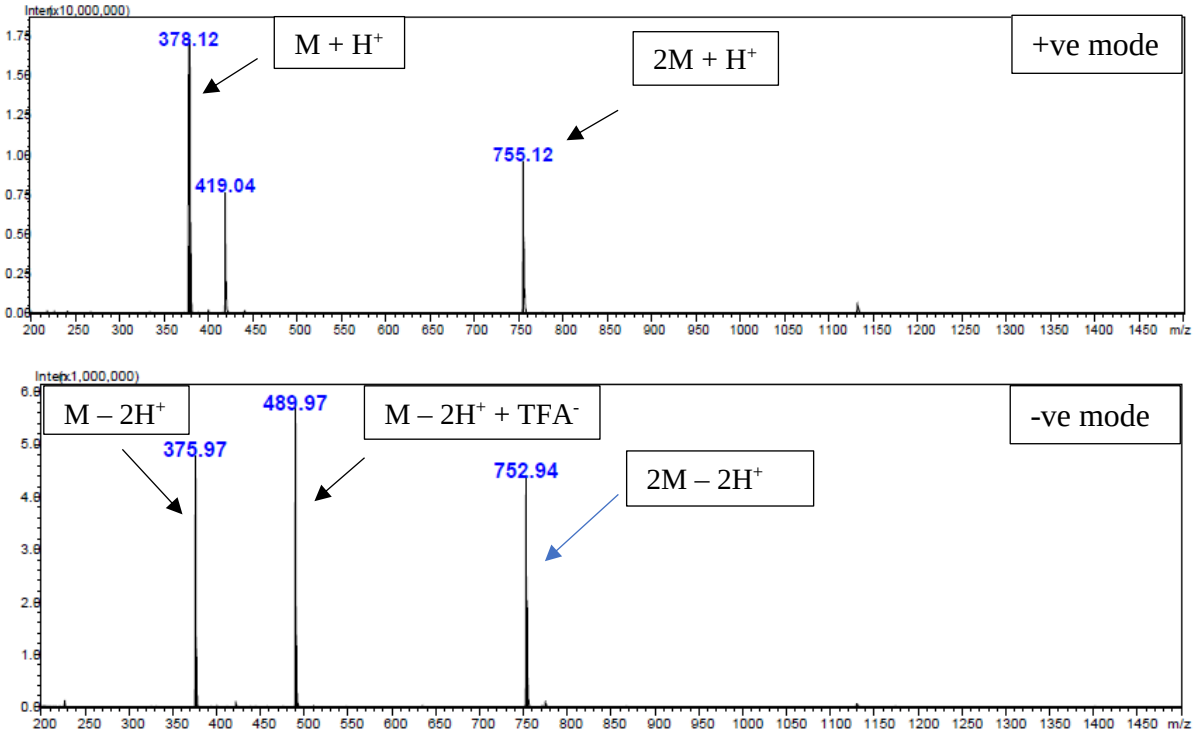


LC/MS data

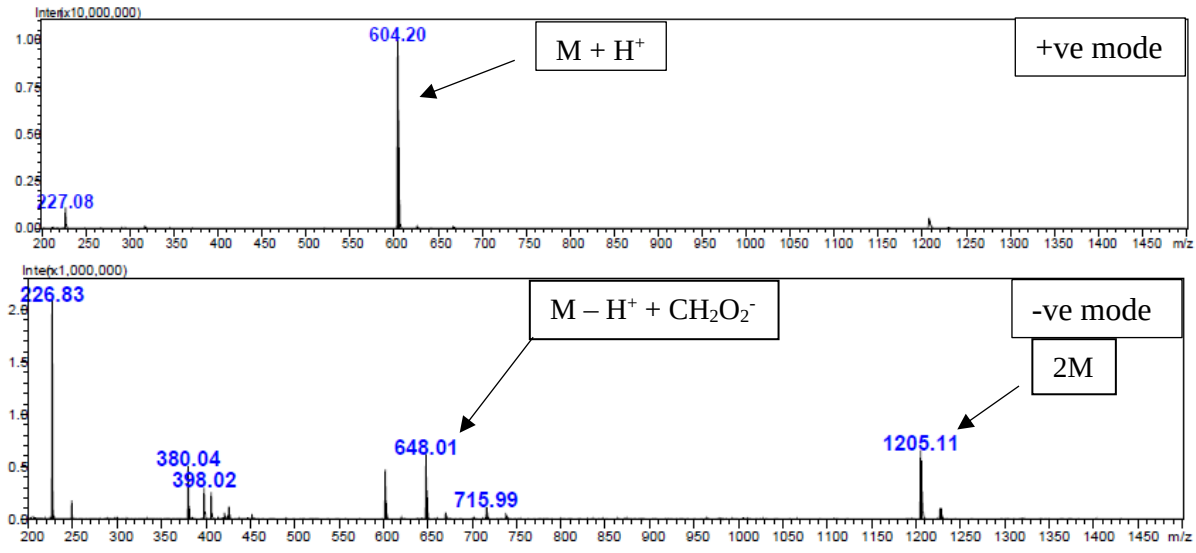
Mobile phase: 5-95 % of solvent B to solvent A

Solvent A: 0.1% Formic acid in MilliQ H_2O Solvent B: 0.1% Formic acid in ACN

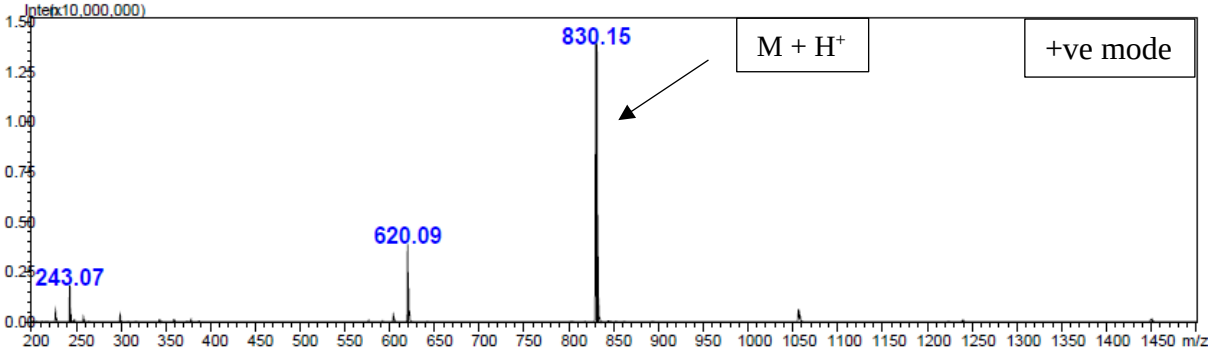
Fmoc-His-OH

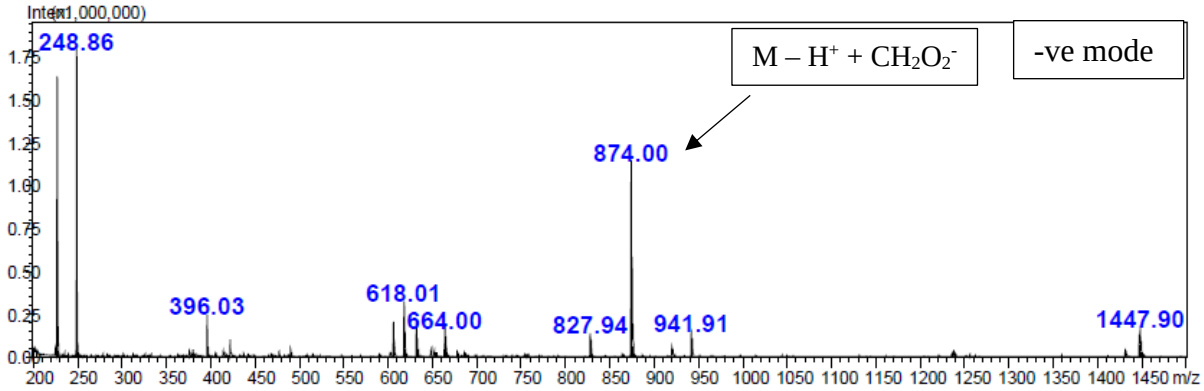


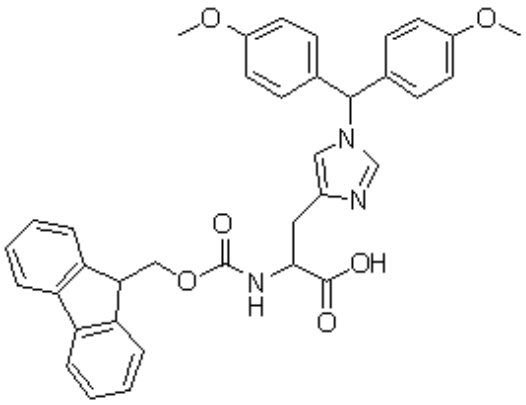
Fmoc-His(PG3)-OH



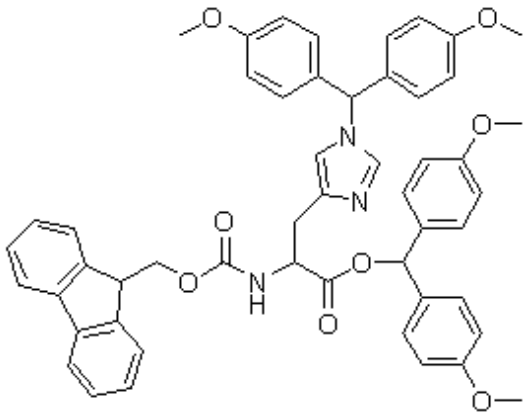
Fmoc-His-(PG3)-O-PG3





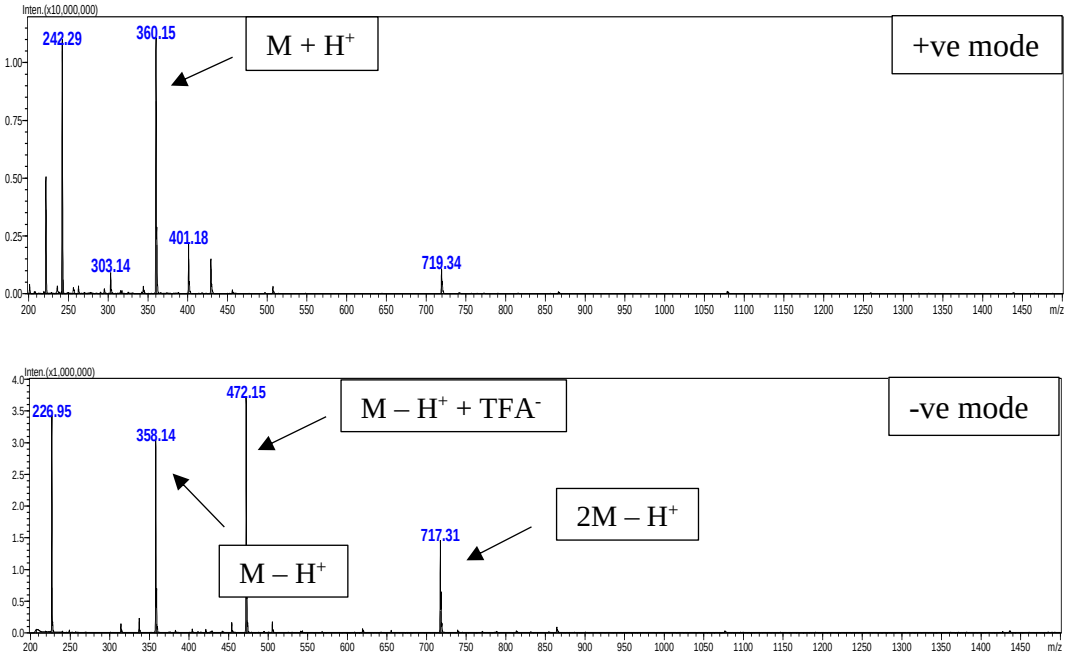


Chemical Formula: $C_{36}H_{33}N_3O_6$
Molecular Weight: 603,68

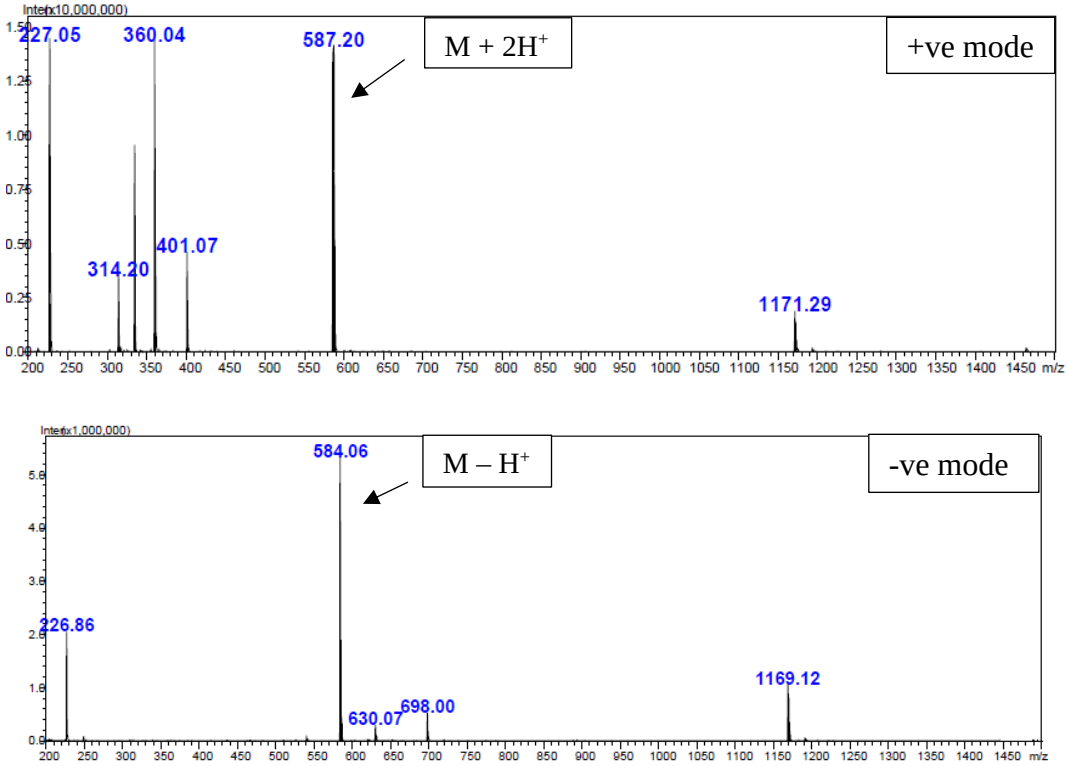


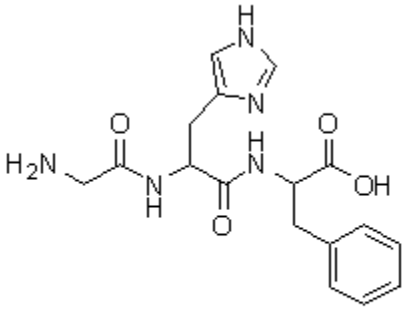
Chemical Formula: $C_{51}H_{47}N_3O_8$
Molecular Weight: 829,95

H-Gly-His-Phe-OH

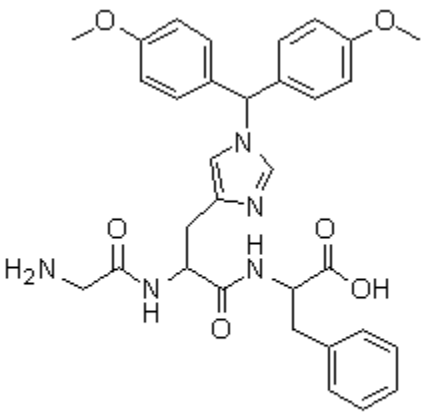


H-Gly-His(PG3)-Phe-OH



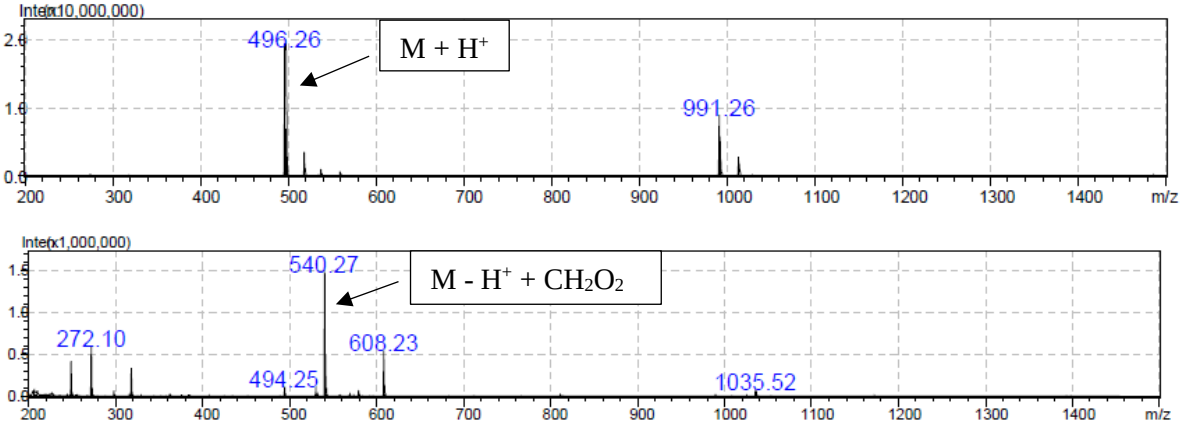


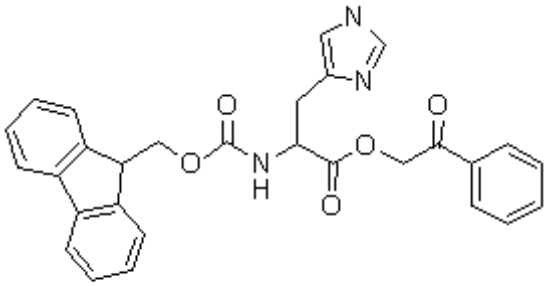
Chemical Formula: $C_{17}H_{21}N_5O_4$
Molecular Weight: 359,39



Chemical Formula: $C_{32}H_{35}N_5O_6$
Molecular Weight: 585,66

Fmoc-His-O-Pac





Chemical Formula: $C_{29}H_{24}N_3O_5$
Molecular Weight: 494,53