Development of a *Tert*-Butyl Thiol Linker for the Synthesis of Peptide Thioesters and Thioacids for Application in Chemical Ligation Methods

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submitted by

Richard Raz

Los Angeles, CA USA

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Professor Dr. Jörg Rademann

in the period of February 2006 to May 2011 in the department of Biology, Chemistry, and Pharmacy at the Freie Universität Berlin and the Leibniz-Institut für Molekulare Pharmakologie (FMP) Berlin.

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I dedicate this work to my parents, John and Achara

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List of Abbreviations

AA-OH	amino acid
Ac	acetyl
Ac_2O	acetic Anhydride
ACN	acetonitrile
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BTFFH	fluoro-*N*,*N*,*N*',*N*'-bis-(tetramethylen)formamidiniumhexafluorophosphate
Bz	benzoyl
$CDCl_3$	deuterated chloroform
CDI	1,1'-Carbonyl-diimidazole
d	doublet
DBU	1,8-diazabicyclo-[5.4.0]-undec-7-en
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DDQ	2,3-dichloro-5,6-dicyanobenzoquinone
DIC	*N*,*N*'-diisopropylcarbodiimide
DIPEA	*N*-ethyldiisopropylamine
DKP	diketopiperazine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EA	elemental analysis
EDC	*N*-(3-dimethylaminopropyl)-*N*'-ethyl-carbodiimide hydrochloride
EDT	ethanedithiol
Equiv	equivalent
ESI-MS	electrospray ionization-mass spectrometry
Et	ethyl
Et_2O	diethylether

EtOAc	ethyl Acetate
EtOH	ethanol
Fmoc	9-fluorneylmethoxycarbonyl
FT-ATR-IR	fourier transform-attenuated-total-reflection-IR
h	hour
HATU	*N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5b]pyridin-1-ylmethylene] Nmethylmethanaminium, Hexafluorophosphate *N*-Oxide
Hex	hexanes
HF	hydrogen fluoride
HIV	human immunodeficiency virus
HOAt	*N*-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high-Performance-Liquid-Chromatography
IR	infra red
KBr	potassium bromide
$KMNO_4$	potassium permanganate
LC-MS	liquid chromatography-mass spectrometry
LDA	lithium diisopropylamide
M	molarity
m/z	mass to charge ration
Me	methyl
MeOH	methanol
MIC	maleimidocaproyl
MMP	4-mercapto 4-methylpentanol
MSNT	1-(mesitylen-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole
NaOMe	sodium methoxide
NaSMe	sodium thiomethoxide
NCL	native chemical ligation
NMR	nuclear magnetic resonance spectrometry
Pbf	2,2,5,7,8-pentamethyl-dihydrobenzofuran-5-sulfonyl
Pfp	pentraflurophenyl group
PG	protecting group
Ph	phenyl
pH	potentia hydrogenii (pH value)

PP	polypropylene
ppm	parts per million
PS	polystyrene
PTM	post-translational modification
PyBOP	(benzotriazol-1-yloxy)-tripyrollidinophosphonium-hexafluorophosphate
Red-Al	reducing aluminum (Vitride)
rt	room temperature
s	singlet
SiO_2	silicon dioxide
SPPS	solid phase peptide synthesis
t	time
t	triplet
TAL	thioacid/azide ligation
TBAF	tetrabutylammoniumfluoride
TBAI	tetrabutylammoniumiodide
TBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium-tetrafluoroborat
TEA	triethylamine
tert-Bu	*tertiary* butyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIS	triisopropylsilane
TLC	thin layer chromotagrophy
TMS	tetramethylsilane
ToF	time of flight
Trt	trityl
UV	ultraviolet
Z	benzyloxycarbonyl (Cbz)
PEG	poly(ethylene glycol)
MPAA	(4-carboxmethyl)thiophenol

Abstract

The aim of this research project was to apply a base-stable *tert*-butyl thiol linker for the Fmoc SPPS based synthesis of peptide thioesters and thioacids to be used in chemical ligation reactions. The first part of this dissertation was to develop a base-stable linker which would provide the means to synthesize peptide thioesters. The second part of this work was concerned with applying this linker strategy for the synthesis of peptide thioacids. Finally, the newly rediscovered ligation reaction between thioacids and azides was reinvestigated with the addition of metal salts.

Thioesters are generally known to be base labile, which has previously limited their use as linkers in Fmoc-based SPPS. *Tert*-butyl thioesters, however, are special and distinct from from other thioesters: While being stable under under alkaline conditions they are easily cleaved under only slightly basic conditions by primary thiolates. In stability experiments, we have shown that Boc-Phe-S-*t*-butyl has a half life of 6.5 days when treated with 20 % piperidine in DMF being 28 times more stable than Boc-Phe-S-ethyl (half life of 5.5 hours). Accordingly, a *tert*-butyl linker attached to an acid sensitive resin might allow a direct synthesis access to peptide thioesters.

We developed 4-mercapto 4-methylpentanol (MMP) as a *tert*-butyl thiol linker which was coupled to a standard 2-Cl-tritylchloride resin *via* its hydroxyl function. Acylation of the *tert*-butyl thiol with the first amino acid was accomplished using either Fmoc-Amino acid fluorides or the condensation reagent combination EDC/DMAP with yields ranging from 70-90 %. For most dipeptides tested, no diketopiperazine (DKP) formation was observed after coupling of the 2nd amino acid. DKP formation which resulted only in relatively low yield loss, could be fully avoided by quantitative coupling of dipeptides to the first resin bound amino acid using HATU/HOAt. After standard Fmoc-SPPS synthesis, the bound peptide thioesters could be cleaved either with acid to yield fully deprotected or by nucleophilic displacement with thiolate to furnish fully protected peptide thioesters. The yields and puritiesof the synthesized peptide thioesters were good ranging from 50-90 % and could be used directly in further in the Native Chemical Ligation (NCL). As a final test of the robustness of the linker, a 16mer derivative of the cell-penetrating peptide, Penetratin 1, was synthesized using the MMP linker and after acidic cleavage obtained in a 67 % yield after HPLC purification.

Abstract

In the second part of this dissertation, the synthesis of peptide thioacids was investigated using the MMP linker. By displacing the peptide from the resin with ß-eliminable 2-mercaptopropionitrile followed by base treatment, the method was extended to the preparation of peptide thioacids. The thiol is easily obtained from the commercially available disulfide under reducing conditions. Accordingly, an *in-situ* generated pentapeptide thioacid could be transformed *via* addition of tosyl azide to the corresponding sulfonamide. As a result of this, sulfonamides could be prepared from thioester peptides in a two-step one pot reaction.

The final part of this dissertation was concerned with optimizing the ligation reaction between thioacids and electron-rich azides. It has been shown by the group of Williams, that electron deficient azides react rapidly with thioacids under basic conditions to yield sulfonamides in good yields. To date, slightly more electron rich azides, such as peptidyl azides have not found utility in this method due to their lower reactivity. This has left room for improvement since the application of peptide thioacids would be a considerable achievement for this ligation reaction.

The initial investigation was concerned with the effect that metal salts would have on increasing the reaction rate between the thioacids and azides. Surprisingly, upon addition of copper (I) and silver (I) salts, formation of a thioamide product was observed. This reaction was found to occur under a catalytic amount of the metal salt and to be compatible under aqueous conditions. Shifting the electronic nature of the thioacid as well as the azide shifted to a slight loss in chemoselectivity. Nevertheless, Fmoc-Phe-SH was reacted with α-azidoglycine methylester to give a thioamide to amide relationship of 1.6 : 1. This proved to be a promising initial result for a new chemical transformation between thioacids and azides.

Abstract

Ziel dieser Arbeit war die Etablierung eines basenstabilen *tert*-butyl Linkers für die Fmoc-Festphasenpeptidsynthese von Peptidthioestern und Peptidthiosäuren. Auf diese Weise hergestellten Peptide wurden dann weiter verwendet in unterschiedlichen Ligationsreaktionen. Der erste Teil dieses Projektes beschäftigt sich mit der Entwicklung eines basenstabilen Linkers für die Synthese von Peptidthioestern. Der zweite Teil der Arbeit widmete sich der Anwendung dieser Linkerkonzeptes auf die Synthese von Peptidthiosäuren und ihren Einsatz für die Thiosäure/Azid-Ligationsreaktion. Im letzten Teil dieser Arbeit, wurde die Bedeutung von Metalsalzen in der Thiosäure/Azid-Ligation eingehend untersucht.

Auf Grund der Basenlabilität von Thioestern sind bislang wenige Synthesemethoden bekannt, die mit der Fmoc-SPPS kompatibel sind. *Tert*-butyl Thioester unterscheiden sich grundlegend von anderen Alkyl- und Arylthioestern hinsichtlich ihrer Stabilität gegenüber Säuren sowie auch Basen. Trotz dieser erhöhten Stabilität lassen sich T*ert*-butyl-Thioester einfach mit primären Thiolaten abspalten. Mit einer Halbwertszeit von 6,5 Tagen ist der Boc-Phe-S-*t*-butylester gegenüber 20 % Piperidin in DMF 28mal stabiler als der entsprechende Boc-Phe-S-ethylester mit 5,5 Stunden. Es wurde festgestellt, dass auf Grund dieser Ergbnisse ein *tert*-butyl-Linker sehr gut geeignet ist für die Synthese von Peptidthioestern.

Als bester *tert*-butyl-Thiolinker hat sich 4-Mercapto-4-Methylpentanol (MMP) erwiesen. Nach der Kupplung dieser Linkers auf ein säurenlabilen 2-Cl-Tritylchlorid-Harz, erfolgte die Acylierung mit der ersten Aminosäure. Im Acylierungschritt wurden sowohl mit EDC/DMAP als auch mit Fmoc-Aminosäurefluoriden bis zu 90 % Ausbeute erreicht. Bei der Entschützung der zweiten Aminosäure wurde für den Großteil der untersuchten Dipeptid keine Diketopiperazinbildung (DKP) festgestellt. Ein eventueller Ausbeuteverlust durch DKP-Bildung konnte durch die Kupplung von Dipeptiden mit HATU/HOAt komplett ausgeschlossen werden. Die immobilisierten Peptidthioester konnten sowohl durch Abspaltung mit Säure als ungeschütze Peptide als auch nucleophilisch mit Thiolaten als vollgeschützte Peptidthioester in guten Ausbeuten erhalten werden. Die so gewonnenen Thioester konnten direkt in Nativen Chemischen Ligationsreaktionen (NCL) angewendet werden. Um die Robustheit des MMP-Linkers zu testen, wurde ein Analogen von Penetratin 1 synthetiesiert. Nach

saurer Abspaltung und Aufreinigung, konnte das 16 Aminosäure lange Peptid mit einer Ausbeute von 67 % erhalten werden.

Im Zweiten Teil dieser Arbeit wurde der MMP-Linker auf die Synthese von Peptidthiosäuren angewendet. Bei nucleophiler Abspaltung der immobilisierten Peptide mit Thiolen, die zu ß-Eliminierung neigen, erhält man geschützte Peptidthiosäuren. Bei einer Abspaltung eines Pentapeptids mit Aminbasen wurde eine rasche Zersetzung der Thiosäuren beobachtet. Allerdings können diese *in situ* gebildeten Thiosäuren mit Tosylaziden zu den entsprechenden Sulfonamiden reagiert werden. Durch die Kombination von Abspaltung und Thiosäure/Azid-Ligation verkürzt sich das zweistufige Verfahren auf eine Eintopfreaktion.

Der letzte Teil der Arbeit beinhaltete eine Optimierung der Reaktion von Thiosäuren mit elektronenreichen Aziden, die für gewöhnlich erhöhte Temperaturen für die Thiosäure/Azid-Ligation benötigen. Für eine verbesserte Reaktion wurde die Katalyse von Kupfer(I)- und Silber(I)-Salzen untersucht. Dabei wurde eine bislang unbekannte Reaktion der Thiosäure und des Azids zu einem Thioamid festgestellt. Bei genauerer Untersuchung der Reaktion zeigte sich, dass sich das Verhältnis von Thioamid und Amid weiter auf der Seite das Amides liegt, je elektronärmer das Azid war. Bei der Reaktion von Fmoc-Phe-SH mit α-Azidoglycinmethylester ergab sich ein Verhältnis von Thioamid zu Amid von 1.6:1. Die anfänglichen Untersuchungen bieten dabei eine gute Grundlage für einen zukünftigen Zugang zu Thioamiden.

1. Chemical Ligation

1.1. Addressing Protein Structure and Function *via* Chemical Methods

In the field of chemical biology and biomedical research, peptides and proteins are principal players. They are everywhere in living organisms and are involved in almost all processes within the cell. The human genome project has touched off a veritable explosion in the discovery of immense networks of proteins involved in the intracellular machinery of living organisms. There seems to be not a single aspect of life which is not influenced by the workings of proteins. This has been in large part due to the success of genome-sequencing projects. As a result, most of these proteins have been only characterized from their predicted sequence data, the structure and accurate mode of action still remain to be elucidated.[1]

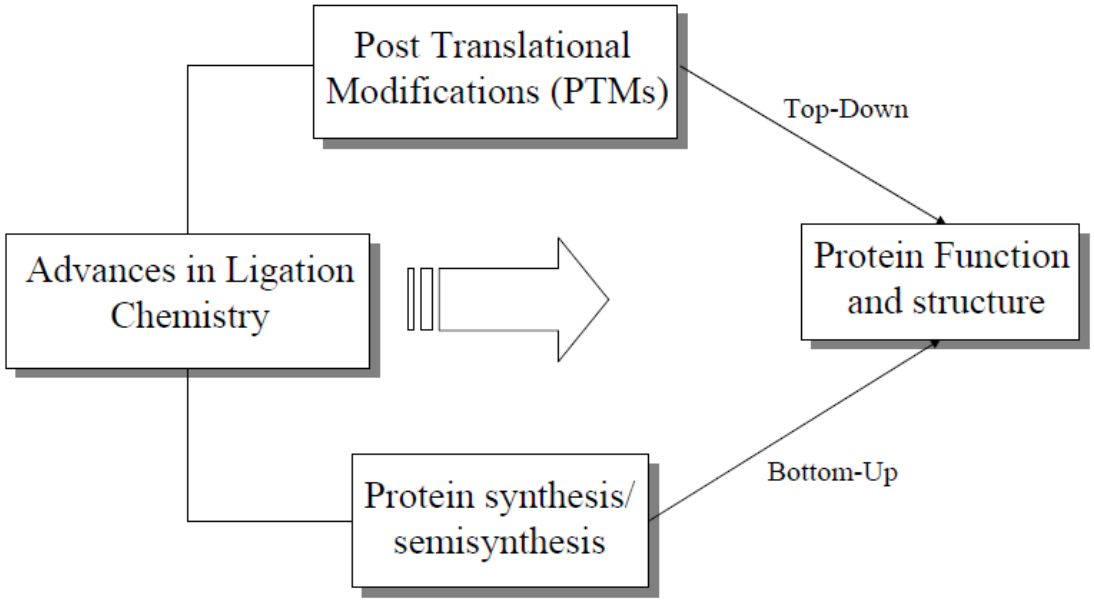


Figure 1-1. Protein structure and function can be ascertained in a number of ways. Here represented are two approaches from either top-down approach, or bottom-up.

Biology has offered perhaps the most powerful tool of the past three decades with the recombinant DNA-based expression of proteins in genetically engineered cells.[2][3] However, with an ever-increasing need to understand the minutae of protein function and form, more sophisticated methods are required. The past decade has seen a sharp increase within the chemistry community in addressing important biological

questions.[4][5][6] There are two directions chemistry has chosen for tackling such complicated questions regarding form and function of proteins. Proteins can either be addressed with chemical modifications—post translational modifications (PTMs) in order to focus on one or multiple types of interactions in a top-down type approach, or their structure can be accessed by hand through chemical synthesis in the laboratory, in a bottom-up type approach (Figure 1). Each of these methods offer significant advantages and disadvantes for the study of protein structure and function.

Considering the first path, it is imperative then that in order to modify the proteins post-translationally the protein must be obtained in an abundant and pure manner of which there are three well-trodden paths. Proteins may be won by either a) native protein isolation or extraction[7] b) recombinant biological experiments for the expression of proteins for example in *Escherichia coli* c) chemical synthesis.[8]

Extraction of native proteins usually involves harsh buffer conditions which may lead to denaturation or partial hydrolysis resulting in the loss of yield and or purity.[9] There are a number of modified techniques which overcome many of these problems, but it is still quite difficult to achieve large amounts of purified protein using this technique.

To date, the science of protein expression is well established and the advantages as well as disadvantages are reasonably well understood. For example, small proteins less than 30 kDa are easier to express than large multi-domain proteins; product heterogeneity is frequently a problem; and overexpression of proteins that are toxic to the cell, including proteases, can be difficult.[10] Furthermore, post translational modifications such as glycosidations are not possible in bacterial cells.[11][12]

1.2. A Bottom-up Approach for the Study of Protein Structure and Function

Proteins have also been synthesized chemically in a bottom-up approach using standard chemical methods. Perhaps the greatest advantage of this method is the capability to introduce unnatural amino acids site-specifically into the protein. This allows pinpoint examination of individual interactions within the protein framework which is not achievable with the other methods. Furthermore, the large scale preparation of peptides is readily accessible. As with the other methods, there are also

limitations to the science of protein synthesis as it exists to date. To a great extent, chemical synthesis requires many laborious and tedious steps as well as the handling of often toxic chemicals. Correct folding of the synthesized peptide is not a trivial matter, and the existing chemistry does not allow complete control of side reactions which occur during synthesis. But perhaps most problematic of all is the limit to the size of protein able to be synthesized on the bench top. Existing chemical methods are normally limited for the synthesis of small proteins and or protein domains (up to ~50 amino acid residues),[13][14] which is not usually sufficient for the complete study of complex systems. The protein domain is not enough to elucidate the function of the fully assembled protein.[15] In other words, it is not sufficient then to simply synthesize protein domains if one truly intends to study its function.

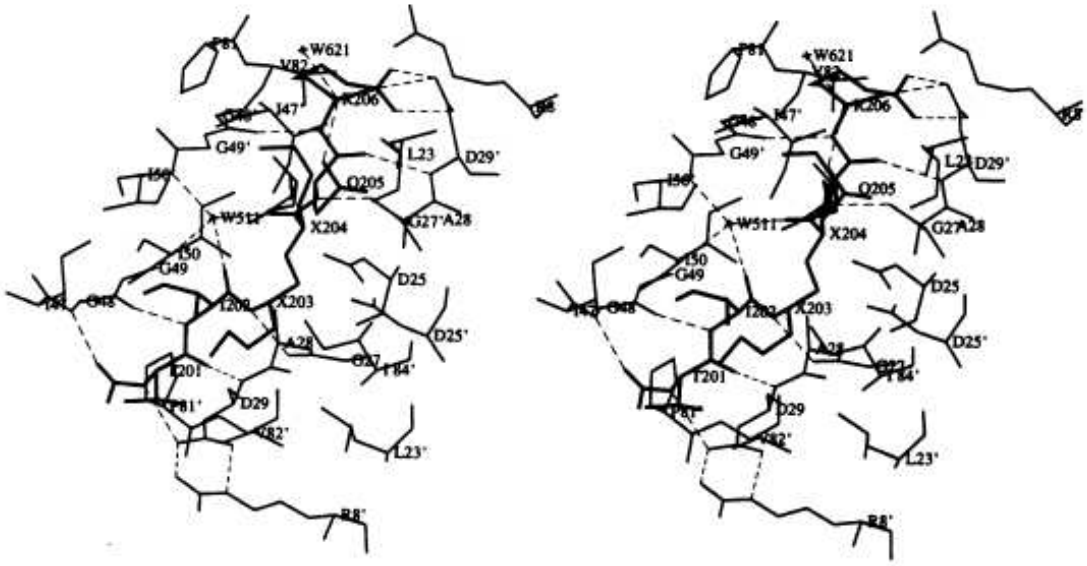


Figure 1-2. Crystal structure of the active site of HIV-I PR with inhibitor MVT-101 (Ac-Thr-Ile-Nle-Ψ[CH_2-NH]-Nle-Gln-Arg-NH_2) resolved at 2.3 Å.

In view of this, it is important that new and facile methods for stitching together large biomacromolecules become available. Accordingly, large proteins can be more readily accessed efficiently in large quantities where other more classical attempts to isolate proteins have failed. A classical example is the synthesis of HIV-I PR from the group of Stephen Kent in 1989.[16] The structure of the ligand-free enzyme updated and corrected erroneous data obtained from a low-resolution structure[17] that had been expressed recombinantly from *E. coli*.

Further work on the synthetic enzyme illicited cocrystal structures of the HIV-I protease complexed with substrate-derived inhibitors (Figure 1-2).[16] This formed the bedrock for the further development of structure-based drug design[18] that led to the development of therapeutic agents against AIDS.[19]

1.3. Protein Structure and Function by Post-Translational Modification (PTM) a Top-Down Approach.

The second route in understanding function of the protein from structure is post-translational modification of proteins, or as previously mentioned as the top-down approach. In this field, a toolbox of known chemoselective reactions can be applied for the modification of proteins thereby gaining insight towards form and function. For this reason, more techniques are required which can introduce alterations to the post-translational protein. Expansion of this chemoselective tool box is a necessity if one wants to address specific questions in and surrounding complex peptides and proteins.

The introduction of natural modifications of proteins is another important application of protein synthesis. Phosphorylation of Ser, Thr, and Tyr residues of proteins represent the most often used method to regulate protein activity.[20] It is often the case that the kinases, which are responsible members for the introduction of phosphates, are often unkown or not always biochemically accessible. Furthermore, the preparation of homogenous phosphorylated proteins using standard approaches is non-trivial.

The group of Muir has examined transforming growth factor ß (TGF-ß) signaling using ligation as well as recombinant techniques to access proteins which are otherwise hard to come by.[21] Briefly, TGF-ß signalling is involved in a myriad of cellular signaling processes including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions. Specifically, the Muir group has investigated the activity of the signaling protein SMAD2. SMAD2 is another signaling protein which is activated in response to the binding of TGF-ß to its cognate receptor.[22][23] In an elegant study, the allowed activity of SMAD2 to be controlled with light by preparing through recombinant as well as synthetic means, an analog of SMAD2 directly caged on the two activating phoshphoserine residues.[21] Using

biochemical and cell biological experiments, they demonstrated that after photolytically removing the caged compounds from the phosphate groups, the semisynthetic protein became fully activated.[21]

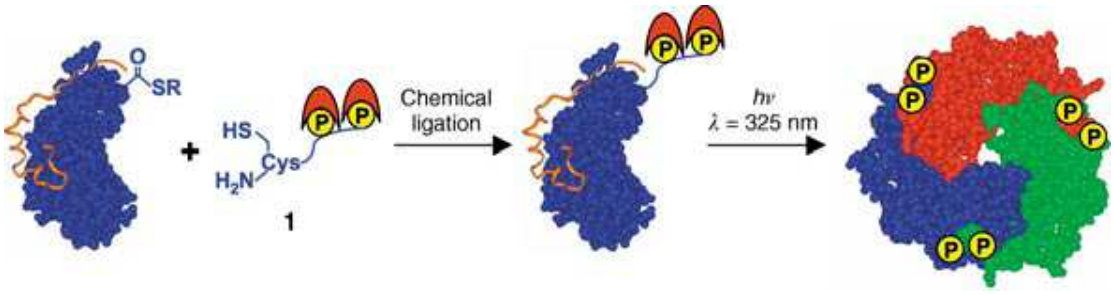


Figure 1-3. Semisynthesis of caged SMAD2-MH2. After photoactivation, SARA-SBD (in orange) is released and the SMAD-MH2 forms a homotrimer.

Extrapolated from the concept of synthetic glycoprotein assembly,[24] Ben Davis has coined the phrase 'Tag-Modify' strategy.[25] The concept is a bit of an off-shoot of post-translational modification which has its conceptual origins from the groups of Bender[26][27] and Koshland.[28] In this strategy, site-selective chemical protein modification is addressed first by insertion of a tag to the protein which can then undergo a chemoselective reaction, or ligation, with some component X. The tag can in this case be almost any kind of modification which will allow for a subsequent addition of another component in a bio-orthogonal manner.[29] Ben Davis' group has exploited these methods in order to expand the diversity of chemical protein modification.

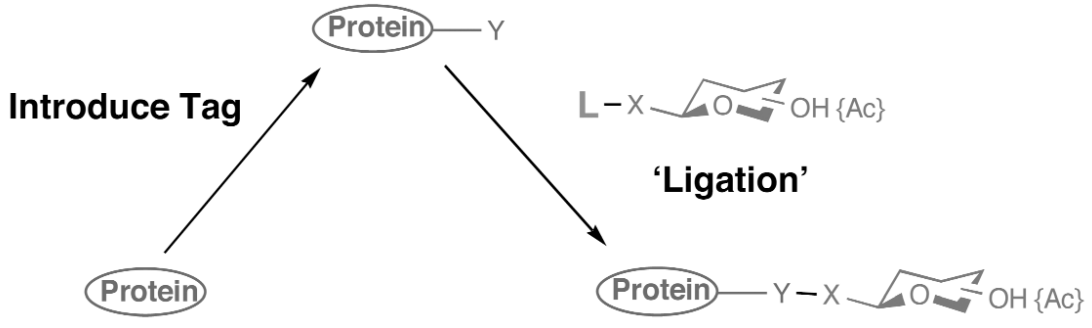


Figure 1-4. The 'tag-modify' strategy consisting of (i) site-controlled introduction of a chemical tag for subsequent reactions (ii) the chemoselective formation of the bond Y-X.

In one example of 'dual tag-modification', his group used a thiol-tag-disulfide as one ligation reaction, and at a second site, an azide functionality was introduced to

take part in Cu(I)-catalyzed cycloaddition 'click' reaction. In this manner, two different sets of modifications were introduced.[30] In a five step process, the wild-type protein is first mutated to convert unwanted Cys and Met residues to Ser and Ile, respectively. The second step is then the introduction of the chemical tags, which are in this case the thiol and azide. After the mutated gene sequence is expressed in step 3, chemoselective and bioorthogonal introduction of the wanted components are introduced in steps 4 and 5 (Figure 1-5).[30]

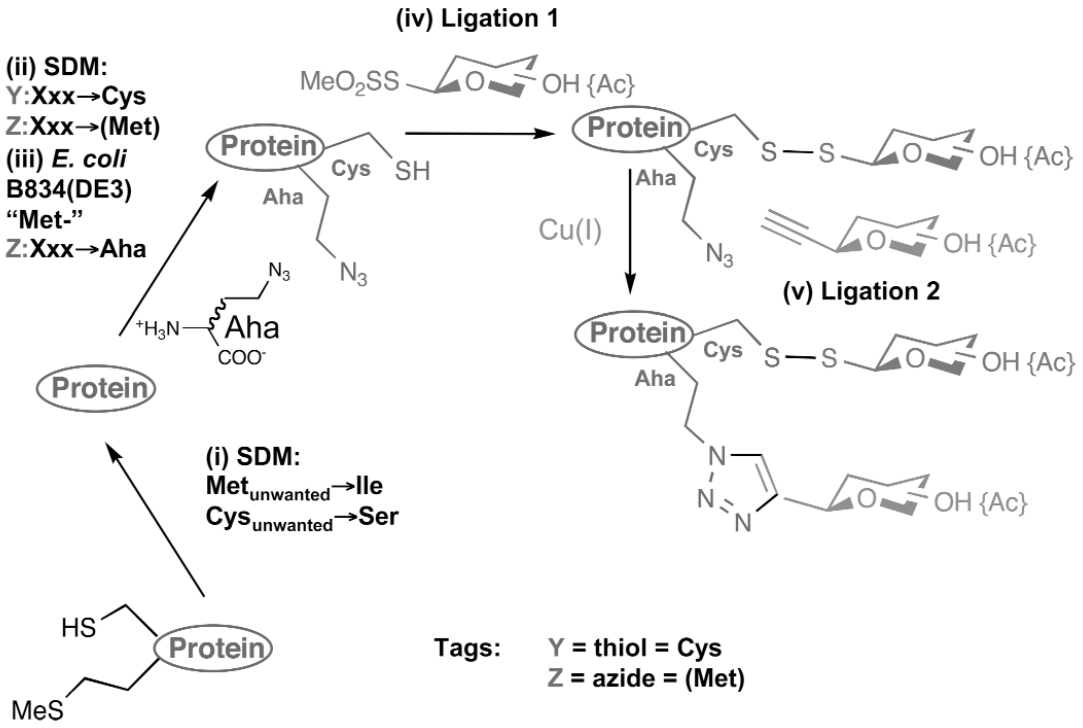


Figure 1-5. Dual-tag modification. In step (ii) the positions of thiol and azide tag anre controlled by the triplet codons for Cys and Met, respectively. In step (iii) gene expression is carried out in an auxotroph deficient in Met biosynthesis.[31]

The total synthesis of complex proteins from the bottom-up in the chemical laboratory using solid phase methods is more or less no longer done, with some notable examples. The advent of new ligation techniques however allow access to larger and larger biomacromolecules and it is becoming quite apparent that with the implementation of newer and more diverse chemical methods to introduce modifications to preformed proteins, the goal of post-translational modification has led to breakthroughs in other fields. With better tools to elucidate structure and function from proteins and their interactions, this greater understanding can also lead to the discovery of new therapeutic agents.[32]

1.4. Chemical Synthesis of Peptides and Proteins, Then and Now

The classical synthesis of peptides matured over many decades starting in the early 20th century from the work of Emil Fischer[33] and also somewhat to Theodor Curtius.[34] Small peptide synthesis was made possible by the introduction of protecting groups such as the benzyloxycarbonyl (Z) group by Bergmann and Zervas[35] in 1931 to mask the functionalities of the reactive parts of the molecules. In 1953,[36] the structure and synthesis of oxytocin, an octapeptide hormone, was elucidated by Vincent du Vigneaud, which would later win him the Nobel Prize.[37] It is important to take note that the initial goal to mask functionality by using protecting groups and the concept of orthogonality which has become essential for organic chemistry was conceived initially for the synthesis of small peptides. Their significance in the history of peptide synthesis and also chemical protein synthesis should not be overlooked.

1.4.1. Solid Phase Peptide Synthesis

The introduction of the polymer or solid phase support revolutionized how chemists were able to synthesize peptides in the laboratory.[38] For the first time, access to larger peptides could be realized in a manner of weeks instead of years. The method which is ingenious in its simplicity relies on the immobilisation of the first amino acid of a peptide sequence starting from the C-terminus to an insoluble polymer support. The amino acid normally carries two types of protecting groups which are orthogonal to one another. After selective removal of the N-terminal protecting group, a second amino acid which has been activated can be added to the first immobilized amino acid. Since the amino acid is immobilized on the solid support, after the deprotection steps and coupling steps any excess material can be simply washed away. Upon completion of the desired peptide sequence, the peptide is normally cleaved from the resin and the side chain groups are deprotected. This may occur in one step or may be carried out in two steps. After precipitation from an organic solvent like Et_2O, the finished peptide is then subjected to chromatographic purification (if necessary) to yield the pure synthesized peptide (Figure 1-6).[38]

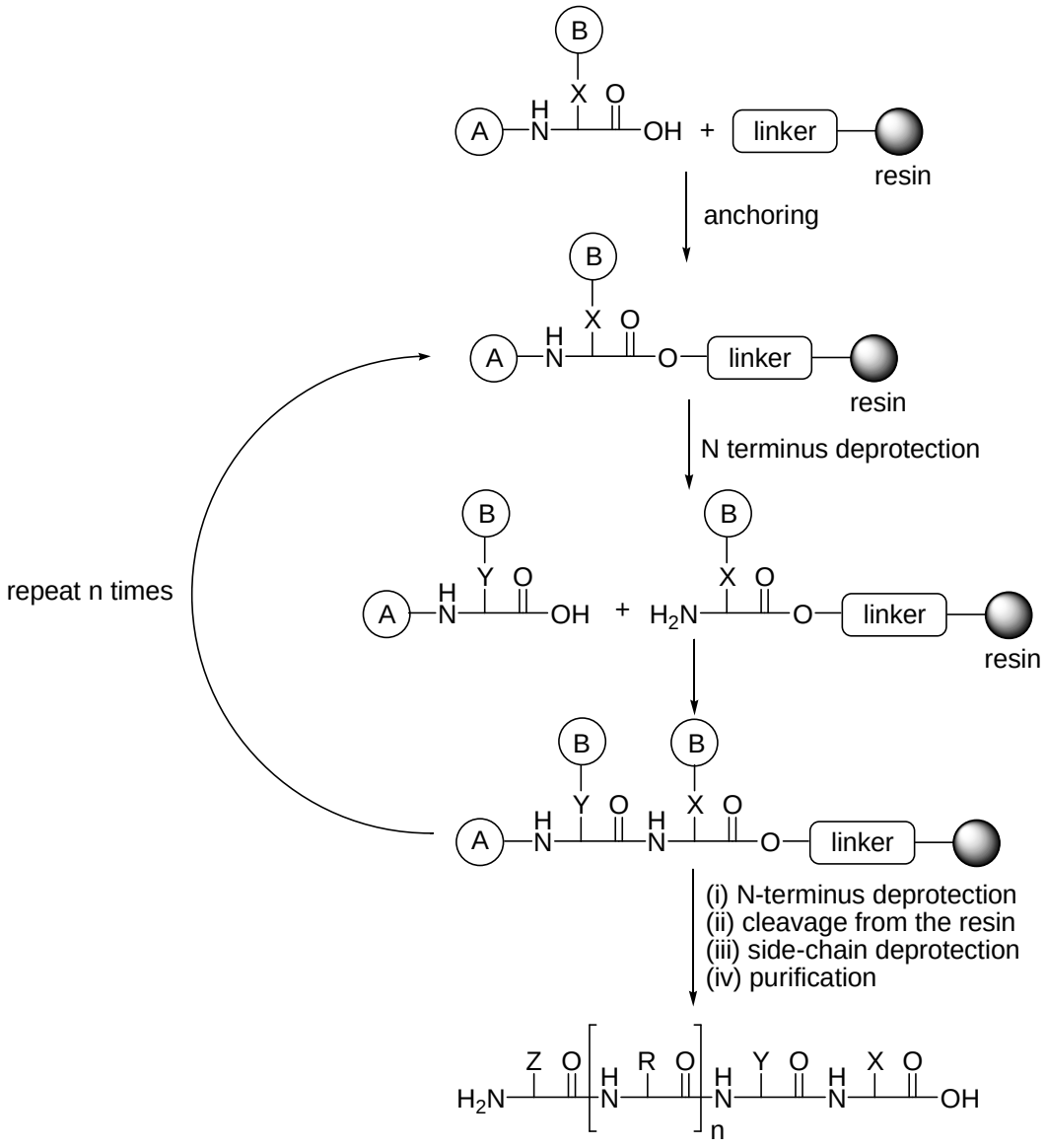


Figure 1-6. Schematic representation of peptide synthesis on the solid phase.

One of the clear advantages of the solid phase method is the ability to remove side products and extra reagents without time-consuming work-up procedures and purification of intermediates. There are, however, a number of limiting factors which have to be considered when performing solid phase reactions. The polymer type used most frequently in the synthesis of peptides is a co-polymer of polystyrene crosslinked to 1 % with divinylbenzene. Originally conceived of for the production of ion-exchange resins,[39] these polymers can swell to a certain extent in organic solvents. The degree of swelling of the polymer is due principally to the extent to which they are crosslinked with higher cross-linking leading generally to a decrease

in swelling capability. The solvent is moreover a greater criterium for the swelling of the polymer and as a result the rate at which reactions can take place.[40]

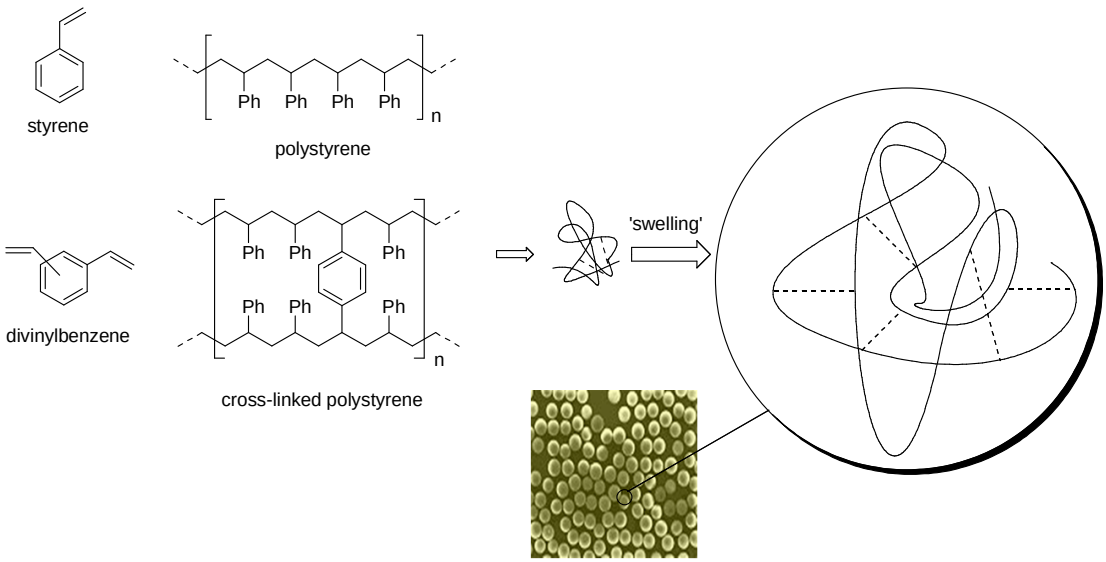


Figure 1-7. Structure and schematic of linear and cross-linked polystyrene.

The most ideally suited solvents for copolymers of styrene and divinylbenzene are are dipolar and aprotic. Since polystyrene is hydrophobic, DCM, THF, pyridine, DMF, NMP, TFA are a few solvents which swell the the polymer matrix particularly well. Poor solvents include alkanes, protic solvents, or water due to their hydrophilic nature.[40] The size of the polymer bead practically expands based on the solvent which is used. Further affecting the size of the polymer resin is the synthetic material which is attached to it.

The size of the resin is affected by the amount of peptide attached to it. As more and more peptidic material is attached to resin the larger the beads become when swollen in solvent. As the beads become more and more filled with the peptidic material, diffusion of new reactants into the swollen bead become more difficult. Sarin and Kent examined how the accessibility of the resin changes, the ‘fuller’ the resin bead becomes.[41] They found that with peptides up to a molecular weight of 5957 g/mol and beads containing up to 81 % peptide and 19 % polystyrene that the efficiency of the resin was still not too limited due to overcrowding, formation of secondary structures, or steric effects. However, the larger the peptide, the more difficult final couplings will become. This may then lead to incomplete couplings and as a result, fail sequences in the synthesis. In this manner, it is common to start with a

low functionalization (between 0.1 mmol/g and 0.3 mmol/g) when synthesizing large peptides.[42][43]

Polystyrene copolymers are functionalized either by chemical transformation of the unfunctionalized polymer, or by copolymerization of functionalized monomers. Popular functional groups for the synthesis of peptides include modifications of chlorobenzyl (the Merrifield resin) group including the amino methyl group. Release of the completed peptide from the resin is accomplished using HF. Advancement of protecting group technology has afforded the Fmoc protecting group for amines which is base labile allowing the construction of peptides on the solid support without the need for TFA cleavage in between steps. This strategy then allows release of the peptide from the solid support with much milder and less toxic acids as HF. More acid sensitive functional groups including the Rink-amide[44][45][46] and the 2-chlorotrityl chloride group.[47]

The advent of polymer resins with poly(ethylene glycol) (PEG) units[48][49] grafted to them has introduced aqueous chemistry to the solid phase. As a result of the PEG units, the polymer is more hydrophilic than pure polystyrene and has the ability to swell in aqueous as well as other protic solvents. The use of PEG resins have their drawbacks as well. The loadings of functionalities to PEG resins is quite low (between 0.15-0.30 mmol/g), and the release of PEG upon treatment with TFA has been reported.[50][51] Additionally, because of the high PEG content, such resins become adherent and as a result are difficult to dry.[50]

The total synthesis of enzymes was able to clarify mechanisms that had either been incorrectly assigned or only generally speculated.[52] The goal of linking the synthesis of larger biomacromolecules to function was however only partially realized. As powerful a method of peptide synthesis as SPPS is, it still lacks the necessary scope to accomplish the total synthesis of even normal sized proteins.

1.5. Current Methods in Chemical Ligation

There are a number of ligation reactions which serve for either the synthesis, semisynthesis or modification of proteins. Hackenberger and Schwarzer gave an excellent overview of different chemical ligation strategies in a 2008 review.[53] A few are notable and related to the present work and thus should be introduced here. They are divided into two categories, and for each category a couple of examples are listed. The first category involves reactions which have two steps; a capture step where the two peptidic fragments are linked followed by a rearrangement step whereby a native amide bond is formed. The second category of ligation reactions involve chemoselective reactions in which the two peptidic components are linked together in a single step reaction where an amide bond is substituted by another type of covalent interaction.

1.5.1. Chemoselective Ligation Reactions Relying on Prior Capture Strategies

1.5.1.1. Native Chemical Ligation

One of the most powerful ligation methods to date, is the so-called native chemical ligation (NCL). Like many other ligation methods, in NCL there is a 'capture step' followed by a rearrangement between the two peptidic fragments. The reaction is based upon the S→N acyl rearrangement first described by Wieland et al. in 1953 in which cysteine reacts with a C-terminal thioester to give a native amide bond.[54] The method came to fruition and was coined native chemical ligation in 1994 with the breakthrough work from the Kent group,interleukin 8 (IL-8) a 77-residue protein was synthesized.[55] In the first step, a transthioesterification occurs between the N-terminal nucleophilic thiol of the cysteine of the first unprotected peptide fragment and the C-terminal thioester of the second unprotected peptide fragment. The second step is a rapid intramolecular S→N acyl shift resulting in a native amide bond at the site of ligation.[56][57]

The speed of the reaction is dependent upon a number of factors including pH and solvent.[58] The reaction is carried out normally under aqueous conditions, although it is possible also to perform the reaction in organic solvents.[59] In an acidic

environment, the reaction proceeds sluggishly due to the protonated thiol group, therefore optimal conditions require a slightly alkaline milieu. Care must be taken to avoid too harsh basic conditions as thioesters are labile under strong basic conditions[60] and unprotected lysine side chains may also react with the thioester leading to aminolysis.[61]

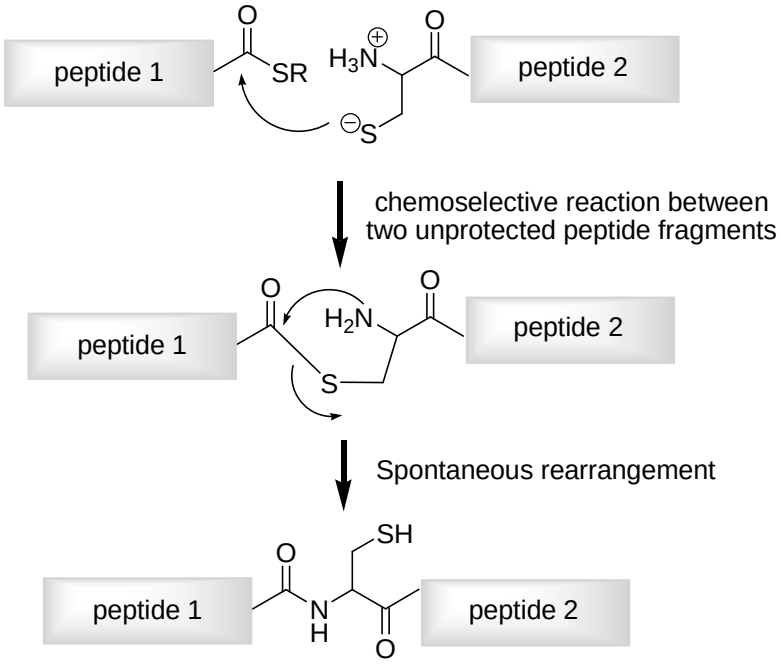


Figure 1-7. The native chemical ligation reaction.

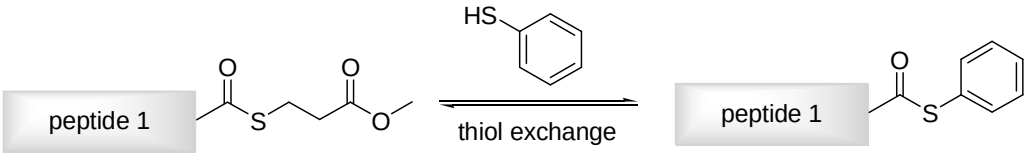
Further affecting the speed of the reaction is the amino acid present at the C-terminal thioester end of the first peptide. Dawson and coworkers investigated this by examining the reaction rates of 20 different pentapeptides containing each of the proteinogenic amino acids at the C-terminus. These experiments showed that all 20 amino acids could undergo the NCL reaction but the different side chains of each amino acid affected the rate at which the amide bond was formed. The fastest reaction rate was observed for the thioester carrying Gly at the C-terminus which reacted quantitatively in less than four hours. More difficult peptides included thioesters with ß-branched amino acids where quantitative conversion was not achieved, even after 48 hours (Table 1-1).[62]

Table 1-1. Influences of C-terminal amino acids on the reactivity of the NCL.

thioester C-terminal amino acid	reaction time
Gly, Cys, His	≤ 4 hours
Phe, Met, Tyr, Ala, Trp	≤ 9 hours
Asn, Asp, Gln, Glu, Ser, Arg, Lys	≤ 12 hours
Leu, Thr, Val, Ile, Pro	≥ 48 hours

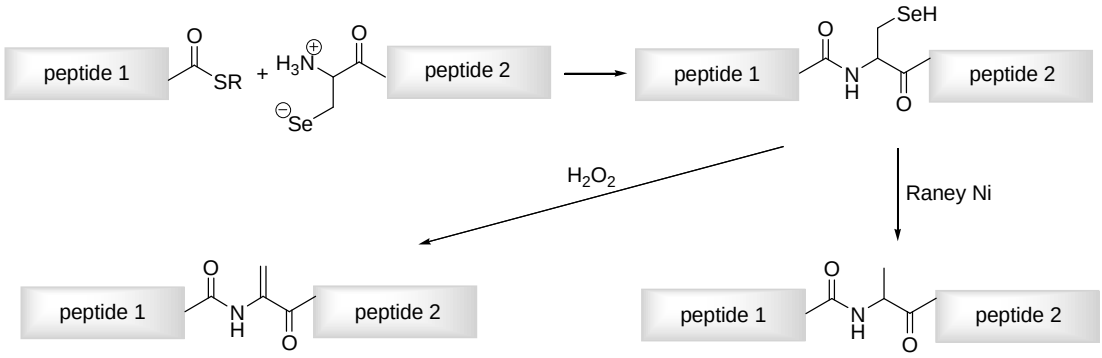
A final consideration regarding the rate of the reaction is the nature of the thioester. Alkyl thioesters are generally more stable than aryl thioesters which is reflected in their handling and synthesis.[62] This added stability, however, also translates to much slower reaction rates in the NCL. The initial thioester exchange is the limiting step in the reaction, and it must proceed as efficiently as possible. Addition of aryl thiols to alkyl thioesters results in an alkyl-aryl thioester exhange to the more reactive aryl form. As such, peptide thioesters are most often synthesized as their alkyl derivatives and then converted into the corresponding aryl thioesters *in situ* by addition of either thiophenol or the water-soluble (4-carboxmethyl)thiophenol (MPAA).[63] The thiol additives also serve to keep the cysteine in a reduced state which is essential for the reaction.

Scheme 1-1. Thioester thiol exchange leading to the more reactive phenylthioester



NCL proceeds without side reactions in high yields. However, it requires a cysteine for the ligation step, which is a bit of a hindrance since Cys residues are barely present in most proteins if at all. This principal ‘capture’ and rearrangment reaction has also been carried out with histidine at the N-terminus instead of cysteine in His-mediated NCL.[64] This method works to some extent, but still limits the reacting partners. As a result, considerable attention has been applied to expand the native chemical ligation reaction to other amino acids and then modify them afterwards.

Scheme 1-2.



A list of recent methods are listed in Table 1-2, notable among them is Sec native chemical ligation. where ligation occurs with selenocysteine which is then followed by deselenization to afford either dehydroalanine at the ligation site or alanine (Scheme 1-2).[65]

Table 1-2. Chemically modified NCL products.

amino acid used for ligation	resulting amino acid	method for modification
penicillamine	valine	desulfurization
β-mercaptophenylalanine	phenylalanine	desulfurization
homocysteine	methionine	methyl alkylation
homoselenocysteine	selenomethionine	methyl alkylation

1.5.1.2. Expressed Protein Ligation

Peptide and protein thioesters are the other principal player in NCL between peptides, however, their synthesis on the size-scale of proteins is not easily accessible. Expressed protein ligation (EPL)[66] has become one of the most powerful methods for the recombinant synthesis of protein thioesters, and has its roots in a naturally occuring process protein splicing.[67] Analogous to RNA splicing, protein splicing occurs where an internal protein domain (an intein) excises itself out of a precursor polypeptide and links the adjacent N and C terminal fragments (exteins) through a native amide bond.[68] The final step in which the intein is excised involves an N→S acyl shift from the N-terminus of the C-extein to the C-terminus of the N-extein.

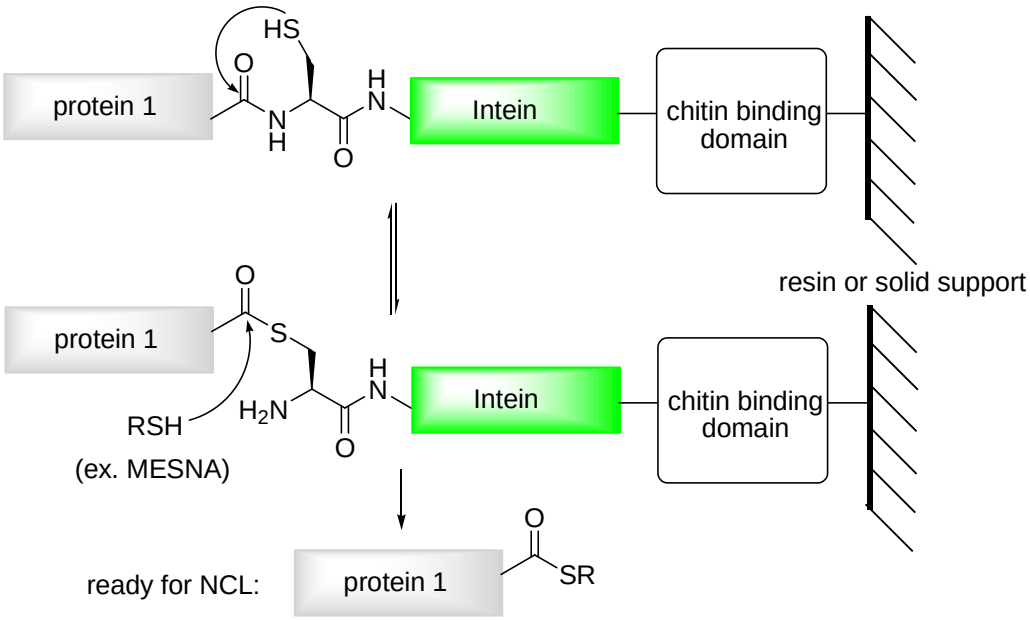


Figure 1-8. The concept of Expressed Protein Ligation (EPL).

For expressed protein ligation, the thioester is obtained using the machinery of the intein in the presence of thiols. More specifically, a protein which should be thioesterified is connected to an intein at the genetic level and represents the N-extein. The C-extein is substituted with a bioaffinity tag such as the chitin binding domain (CBD). Once the intein has performed the N→S acyl shift at its N-terminus to give a thioester linkage, the desired protein is captured as a thioester through a thiol-thioester exchange using an excess of thiols (for instance the water-soluble sodium 2-mercaptoethanesulfonate, MESNA). The resulting protein thioester can be isolated by elution from the resin to which the intein is still bound, and can be modified further by NCL with another protein or other marker. This method was used by Muir and coworkers in order to study the signalling protein SMAD2 (See section 1.3).

1.5.1.3. Traceless Staudinger Ligation

The traceless Staudinger ligation is based upon the same named reaction[69] in which azides are reduced in the presence of phosphines. In the first step of the Staudinger reaction, the phosphine adds to the azide to form an intermediate phosphazide and after release of N_2 forms an aza-ylide that can hydrolize to give the phosphine oxide and an amine.

The reaction requires the presence of a methylene phosphinothioester which in the 'capture' step forms the aza ylide analogous to the Staudinger reaction. In the second step, or rearrangement, the nucleophilicity of the nitrogen of the aza-ylide is exploited attacking the 'electrophilic trap' of the thioester in an S→N acyl shift to produce the amide bond.

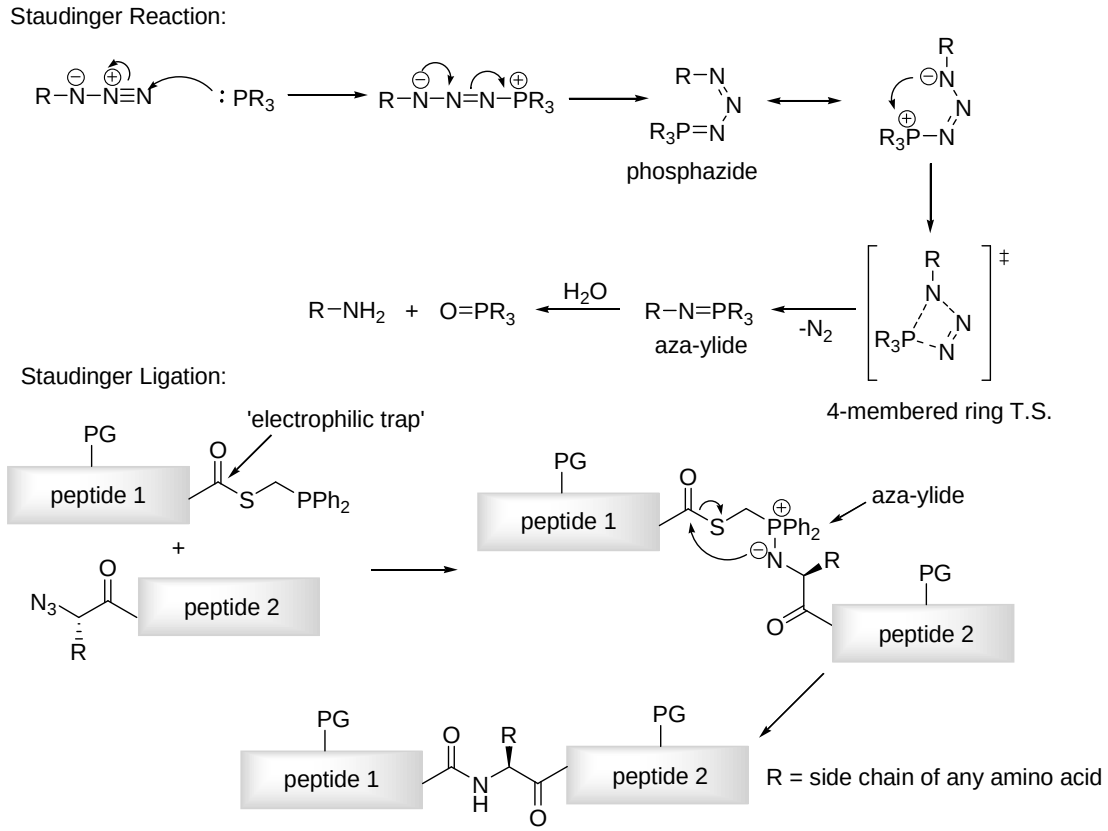


Figure 1-9. The Staudinger reaction and modification for the chemoselective ligation known as the Staudinger ligation.

The traceless Staudinger ligation is a modified version of the originally conceived Staudinger ligation reaction implemented in Bertozzi's group in 2000.[70] In introductary work, a modified triphenyl phosphine carrying a methyl ester ortho to the phosphorus serves as the electrophilic trap to stabilize the aza-ylide bond against hydrolysis. After hydrolysis, the resulting product is an amide bond carrying the phosphinoxide in the ligated scaffold. The seminal application for this reaction and the precursor for the traceless Staudinger reaction involved the selective marking of sialic acid residues. In their initial contribution, Saxon and Bertozzi installed azides within cell surface glycoconjugates by metabolism of a synthetic azidosugar and

reacted them with biotinylated triarylphosphine to produce metabolically stable cell-surface products.[70]

One of the drawbacks of the Staudinger ligation is the requirement for producing peptide thioesters carrying triarylphosphines due to the reactivity of phosphines in the presence of other functional groups. An innovative method from the group of Hackenberger involves the preparation of protected peptide thioester phosphines carrying an azido group at the N-terminus and a borane group to protect the triaryl phosphine. The peptide phosphine thioester is recovered as the phosphonium salt after acidic global deprotection, which is able to cyclize to the cyclic peptide under addition of base. The cyclization occurs in the absence of amino acid side chain protecting groups and has shown promise towards the application in biological systems.[71]

1.5.2. Chemoselective Ligation Reactions Not Relying on a Prior Capture Step

1.5.2.1. 'Click' Coupling of 1,3 Dipoles Mediated by Copper(I) Species.

The cycloaddition of alkynes and azides to triazoles is a chemistry rich in history and has been studied extensively.[72] The triazoles which are formed in this reaction result in 1,4 and 1,5 substitued triazoles.[73] The research groups of Meldal[74] and Sharpless[75] have shown that addition of a Cu (I) source selectively leads to the 1,4-substituted triazole isomer (Scheme 1-3). The reaction has gained traction in ligation chemistry because of its numerous desirable characteristics. It has been shown to be chemoselective to a large number of functional groups, the reaction is fast, with usually excellent yields and perhaps most important for biological systems, it can be carried out in physiological buffer. [76]

The reaction has been extensively reviewed in the literature.[53] The triazole has been examined closely and is proposed as a peptidomimetic in terms of both distance and planarity. The reaction has been used in a number of applications including incorporation into biopolymeric materials. [77] As mentioned in Section 1.3, the reaction has been used for the post-translational modification of proteins and natural products.

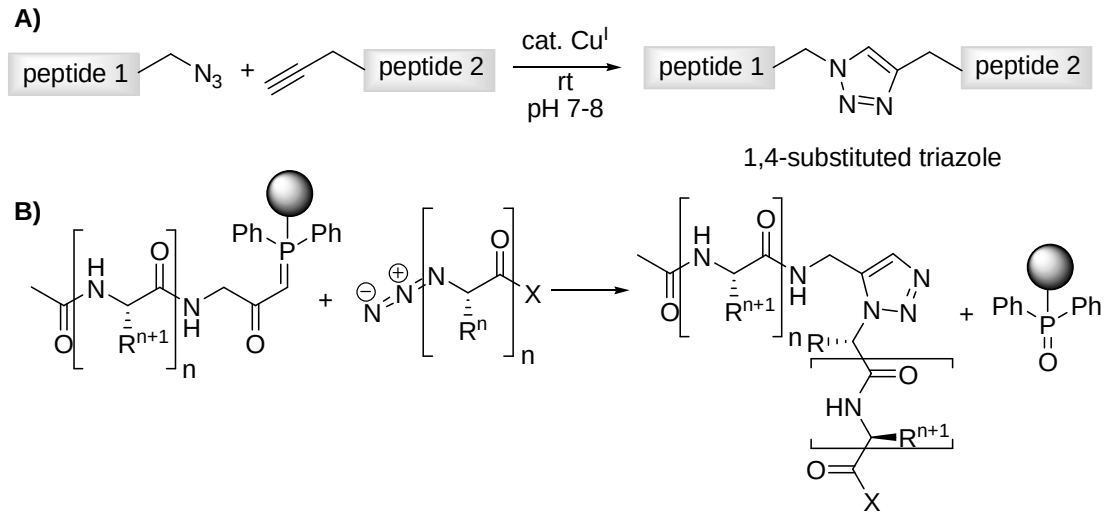


Figure 1-10. The 'Click' reaction has found application in many areas. A) ligation of two peptide fragments in the presence of Cu(I) salts to create 1,4-triazole peptidomimetics. B) A metal-free 'Click' reaction between azidopeptides and peptidylphosphoranes to yield 1,5-triazoles.

In a recent report from the group of Jörg Rademann, peptidyl phosphoranes were reacted with azido peptides to yield 1,5 triazole peptidomimetics (**Figure 1-10**, B).[78] These peptidomimetics are formed in a locked *cis* conformation without the use of metal salts to achieve regioselectivity. Furthermore, by incorporating the azido amino acid at the N-terminus of the peptidyl phosphorane, this method could be used to furnish 1,5-triazole cyclic peptidometics.[79]

1.5.2.2. Michael Addition of Thiol Nucleophiles Using Maleimido Building Blocks

Finally, another quite powerful ligation method is the chemoselective reaction of sulfur nucleophiles to α,ß-unsaturated carbonyl derivatives. This Michael addition has been well established as a conjugation method for linking fluorophores to biological materials under physiological conditions. Ligation techniques exploit the nucleophilicity of the masked thiolate (these reactions may take place at slightly acidic pH where the thiol is most likely to be protonated) in Cys residues to post-translationally mark proteins with maleimidocaproyl (MIC) peptides or biologically relevant materials.[80]

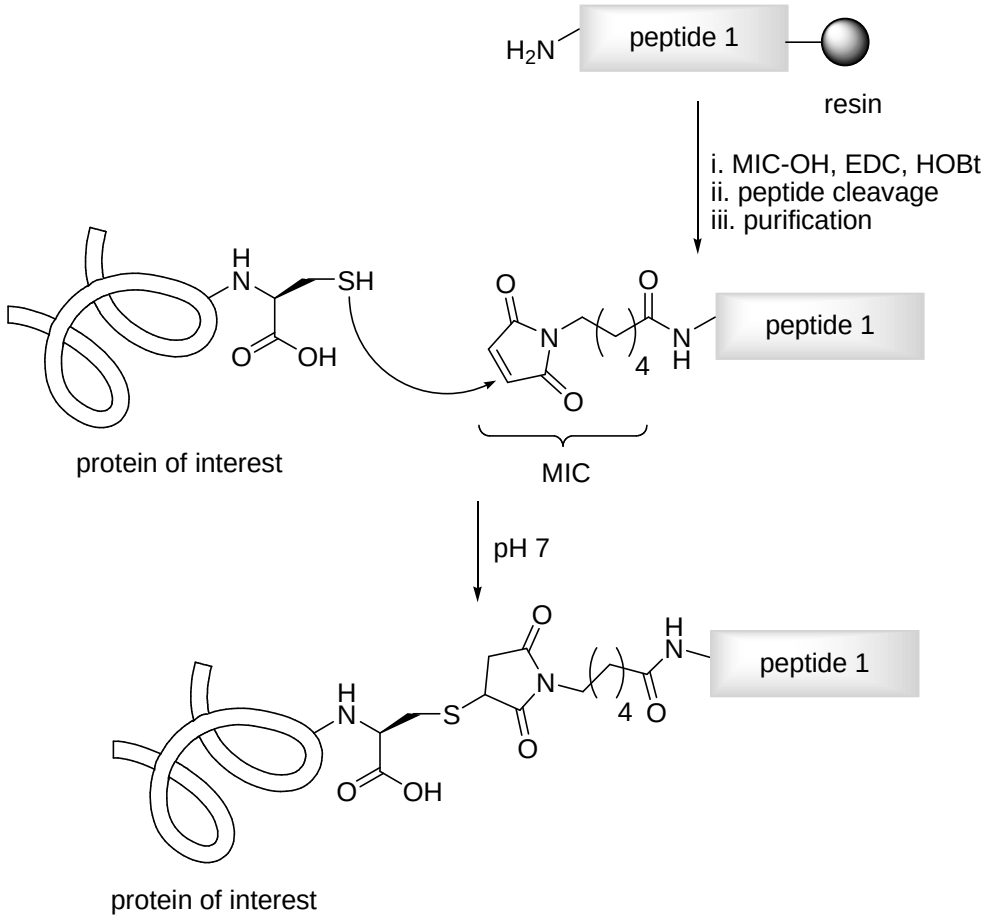


Figure 1-11. MIC strategy for post-translationally modifying proteins containing an active cysteine.

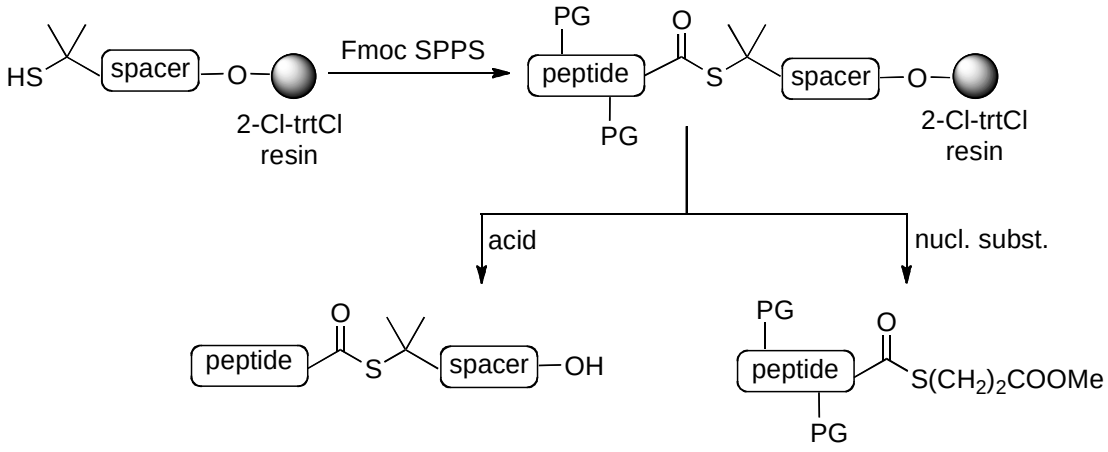
These MIC building blocks can be introduced using SPPS in a straightforward manner and can be used to attach large biomacromolecules with small modifications. A prominent example is the synthesis of lipidated Ras proteins. Bader et al, have shown that Ras proteins can be modified with lipopeptides in large amounts if necessary such that the hybrid lipid-proteins are not sensitive toward degradation and can insert into artificial and biological membranes.[81]

2. Aim of the Work

Ligation chemistry has received incredible attention in the last decade as new technologies emerge which allow more control of chemical reactions in biological systems. This new control has translated directly to the elucidation of structure and function of many new protein targets. Many of the ligation methods require building blocks which are not readily accessible using standard solid phase peptide synthetic methods.

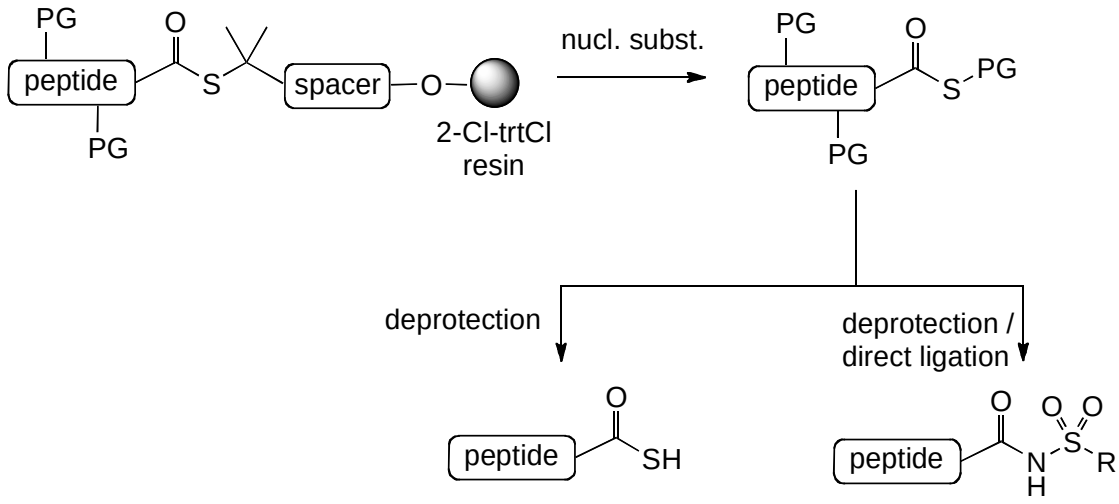
The native chemical ligation is one of the most powerful ligation methods at the moment with a broad range of applications. It is dependent upon two parts: a thioester at the C-terminus of one fragment, and a cysteine at the N-terminus of the second fragment. The synthesis of peptide thioesters for their use in either NCL or in other ligation reactions is a premium concern, and there are decades of research dedicated to their chemical preparation. Thioesters are essentially activated carboxylic acids and their lowered stability makes them susceptible towards degradation and hydrolysis. Their synthesis using Fmoc SPPS methods is challenging and requires optimal handling at best. Futhermore, preparation of large amounts of peptide with high purity remains difficult problems which have not been completely addressed and/or solved. Therefore, it is quite desirable to develop new and alternative methods for their synthesis.

Scheme 2-1



The first part of this work was concentrated on establishing a new method for the synthesis of peptide thioesters using standard Fmoc SPPS methodology. The optimal method is to synthesize peptide thioesters in a direct manner on the resin starting from a thiol linker. The main hindrance is that the formed thioesters are usually unstable toward base treatment. Modified cleavage cocktails have provided good examples and have been cursorily tested in the past by Aimoto and Wade but failed to develop into very robust methods for thioester preparation. Implementation of a *tert* butyl thiol linker would add the necessary stability to the thioester and could then provide a method for the direct synthesis of peptide thiosters on the solid phase using Fmoc methodology. An additional positive aspect to this would be the capability of preparing thioesters either as fully or completely deprotected peptide moieties (Scheme 2-1).

Scheme 2-2



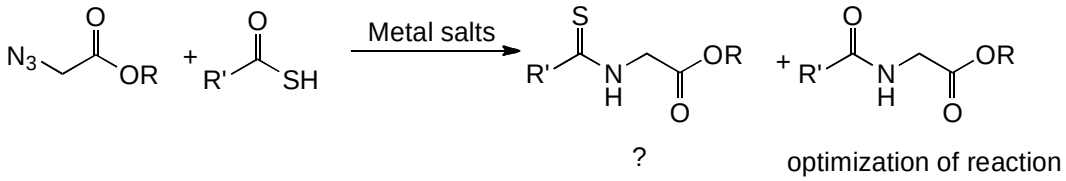
Peptide thioacids have gained interest in previous years. Their preparation has been described in detail using standard Boc SPPS methods, however to date, their reported synthesis using standard Fmoc procedures still leaves room for improvement. The envisioned linker system was expanded to include synthesis of peptide thioacids. By displacing the peptide from the resin with either an hydrosulfide equivalent, or a protected thiol group followed by deprotection should give peptide thioacids also in an elegant manner. Furthermore it may be possible to then take the masked thioacids and unmask them in the presence of electron poor azides in order to implement these

types of thioesters as ‘precursors’ for the thioacid/azide amide forming ligation previously described by Just et al in the 1980s.[82]

The azide/thioacid ligation has received considerable attention in the last years. The reaction between azides and thioacids was first described as a side reaction by Just and coworkers in 1980 and then used by Rosen in 1988 for amidation of azido sugars. The reaction was later reinvestigated in the group Williams in 2003 and has been examined by many groups since.[83][84] Williams found that electron poor azides react rapidly with thioacids to form sulfonamides.[85] However, less electron poor azides such as azido glycine react sluggishly and haven’t found application as of yet for peptidic substrates. This interesting ligation method involving thioacids, has only found utility when electron poor azides such as sulfonyl azides are reacted with thioacids to produce sulfonamides.

The drawbacks mentioned of the thioacid/azide ligation reaction leave room for improvement. It would be of great interest for this ligation method, if conditions could be found which would accelerate or improve the rate of reaction such that even electron rich azides could couple efficiently to thioacids. Such improvements would allow coupling of peptide thioacids to peptide azides in producing native amide bonds.

Scheme 2-3



The starting point of the last topic, revolved around the optimization and brief investigation to this reaction. Another facet was to help elucidate the proposed mechanism in order to further understand the scope and limitations of the reaction. The initial goal was to study the effects that metal salts may have on this reaction specifically the effects of Cu(I) salts and other isoelectronic metals.

3. Fmoc Solid Phase Peptide Thioester Synthesis Using a *Tert*-Butyl Linker

3.1. Current Methodology for the Synthesis of Peptide Thioesters

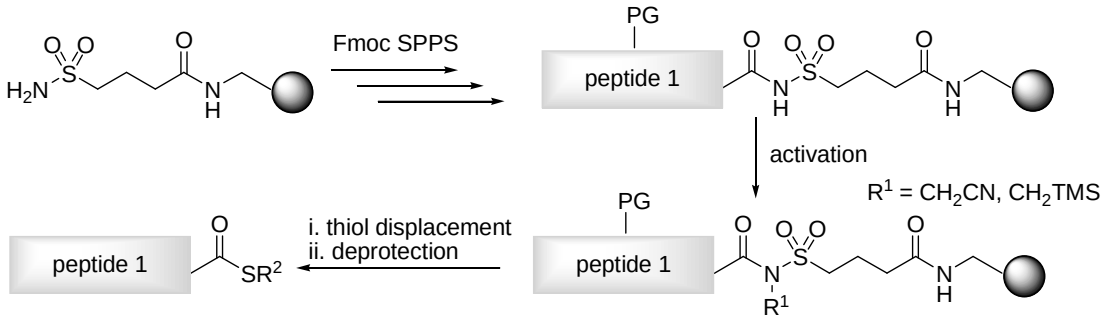
There are many different existing methods for the synthesis of peptide thioesters. Classically, peptide thioesters have been synthesized using Boc solid phase peptide chemistry with thioester linkers.[86] Boc chemistry requires the use of HF as well as special glassware for the cleavage of peptides from the solid support. Aside from this, the Boc method prevents the preparation of many other peptide modifications such as glycosylations due to the harsh nature of the HF cleavage step. Furthermore, with the advent of the Fmoc group for solid phase peptide synthesis which can be removed with the mild base, piperidine, many Boc methodologies are slowly being fazed out of use. Thioesters are generally known to be base-labile, which has hampered their direct preparation in Fmoc-based solid phase peptide synthesis. The few examples which have been cited for the direct preparation of thioesters using Fmoc methodology rely on milder deblocking reagents[87][88] than piperidine/DMF or use the more base labile Fmoc(2-F) group.[89] In this manner peptide thioesters have been accomplished, but the milder deblocking reagents require longer deprotection times and multiple cycles to quantitatively remove the Fmoc group. In addition, fail sequences from incomplete deprotection of the amino acid result in lower yields and impure products.[90]

To avoid the presumed lability of the thioester linker, a number of methods have been established for the preparation of thioesters on the solid phase. The most common approach involves the use of so-called ‘safety-catch linkers’, first introduced by Kenner in 1971,[91] incorporate acyl sulfonamide linkers which can, after the completion of the peptide synthesis be alkylated to activate the sulfonamide bond to be attacked and released as a thioester (Scheme 3-1).

Other examples of safety catch linkers include hydrazides[92] which can be oxidized after completion of the peptide for instance with NBS and then removed in a similar fashion using thiols or amino acid thioesters as the nucleophilic displacing agent.[93] Methionine and also Cys residues however are susceptible toward oxidation with this method. Recently, intramolecular diacylation of backbone

pyroglutamylamides has provided two examples employing the 'safety-catch' principle for the synthesis of peptide thioesters.

Scheme 3-1



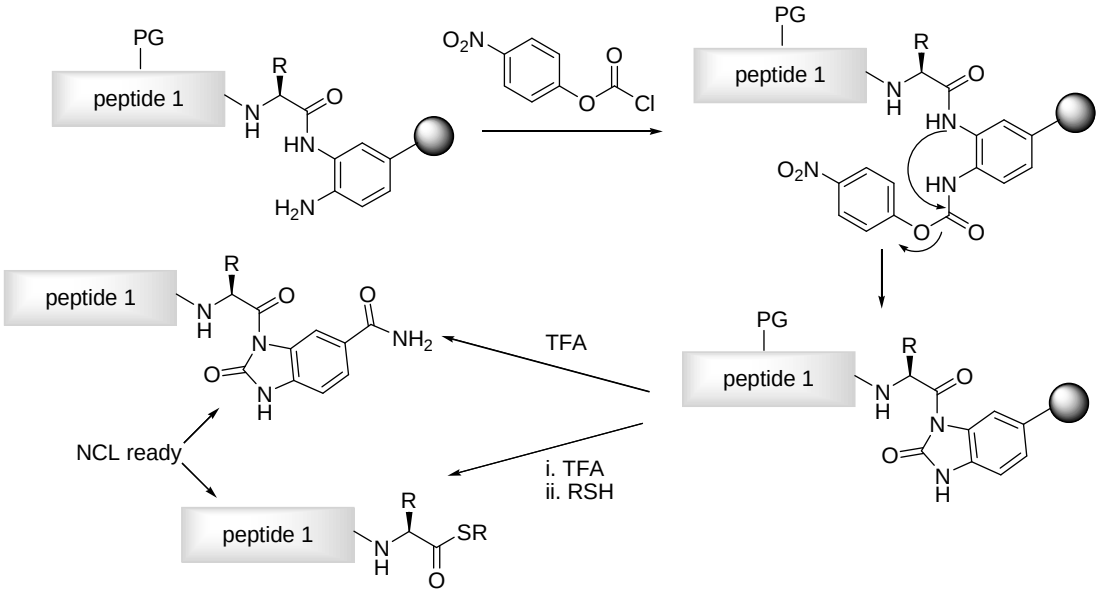
Blanco-Canosa and Dawson applied a 3,4-diaminobenzoyl as the 'safety-catch' linker to which the peptide is assembled. After acylation of the amino group by 4-nitrophenyl chloroformate, in internal spontaneous ring closure occurs resulting in the formatino of an N-acylbenzimidazolone (Nbz) which can be converted into the thioester (Scheme 3-2).[94] Interestingly, they found that the Nbz-peptide can also undergo NCL foregoing the thioesterification step. Unfortunately however, even though the method is robust, yields of larger peptides tend to dip, with the synthesis of a 29mer succeeding in only 36 % yield. The low yield reflects difficulty in the first amino acid acylation of the weakly nucleophilic aniline nitrogen as well as possible difficulty in removing the entire bound peptide from the resin after synthesis.

Similarly, Tofteng et al,[95] used glutamic acid as the first amino acid and after the completion of the peptide, selective deprotection of the glutamic acid residue followed by activation with pyBrop allowed nucleophilic attack of the adjacent amide nitrogen to form the more reactive pyroglutamyl (pGlu) imide moiety (Scheme 3-2). Nucleophilic displacement of the peptide from the resin afforded then the peptide thioester. Though this method is innovative, activation of the Glu residue to form the pGlu imide as well as subsequent thiolytic release of the peptide require extended microwave irradiation to succeed even in moderate yields.

Another prominent example for the synthesis of peptide thioesters is the backbone amide linker (BAL) methodology, originally conceived for the synthesis of small molecules.[96] In this method, the α-amino group of an Alloc ester C-terminal amino acid is loaded to the tris(alkoxy)benzaldehyde resin by reductive amination.

After peptide elongation the alloc group is selectively removed, and the peptide thioester is recovered after aminolysis with an amino acid thioester. However, as with the Dawson method, a limitation to this method are the low yields of the coupling of the second amino acid to the secondary amine of the linker.[97] Further methods include exploiting O→S[98][99] acyl shifts as well as new methodologies surrounding N→S acyl shifts;[100][101] normally these methods are limited to poor yields after the intramolecular rearrangement step.

Scheme 3-2. Benzimidazolones for the synthesis of peptide thioesters



Tert-butyl thioesters are of exceptional stability against nucleophilic bases and strong acids,[102][103][104] which distinguishes them from other alkyl thioesters.[105] In some examples, they were resistant against nucleophilic bases or strong acids. At the same time, *tert*-butyl thioesters remain smoothly cleavable by thiolates.[106] This surprising stability which may result from steric hinderance as well as electronic effects distinguishes *tert*-butyl thioesters from other alkyl and aryl thioesters.

We envisioned that a *tert*-butyl thiol linker immobilized on the solid phase should enable the direct Fmoc-based synthesis of peptide thioesters. This would allow preparation of peptide thioesters in two different ways. Attaching the linker to an acid sensitive followed by acidic cleavage would afford the peptide thioesters as completely deprotected moieties. In contrast to this, nucleophilic displacement under basic conditions would afford the peptide thioesters as completely protected moities.

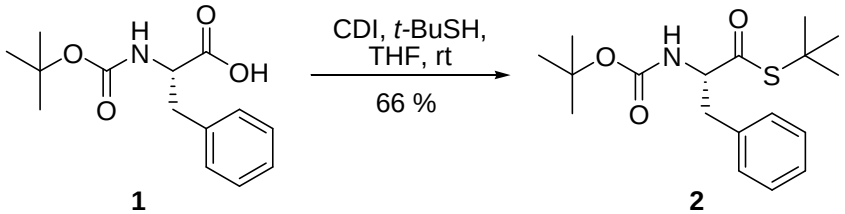
3.2. Synthesis of Polymer Supported Peptide Thioesters

3.2.1. Stability Experiments of Thioesters Under Basic Conditions

In order to validate the strategy, various thioesters were investigated for their stability against standard Fmoc SPPS cleavage conditions. Divined from the previous research, focus was initially placed on the stability of *tert*-butyl thioesters in the presence of the nucleophilic base piperidine. *Tert*-boc protected phenylalanine was a reasonable choice as initial substrate with an aromatic side chain detectable at 254 nm as well as the carbamate group detectable at 220 nm allowing the course of the reaction to be followed either by standard TLC techniques, or LC-MS analysis. Furthermore, aside from the amino terminus, there are no other functional groups.

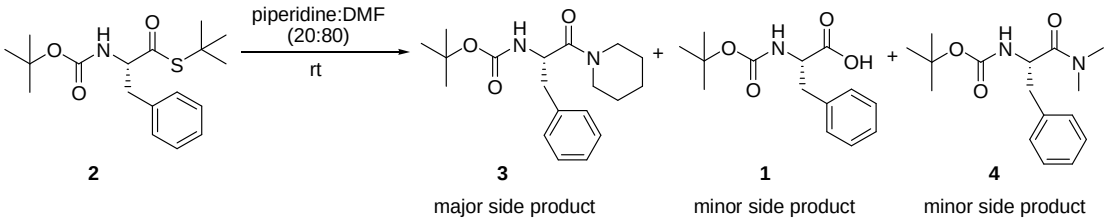
Accordingly, *tert*-Boc-L-Phe-S-*t*-Butyl was synthesized according to literature described protocols from the commercially available *tert*-Boc-L-Phenylalanine.

Scheme 3-1



1 **2**

Treatment of the resulting thioester with 20 % piperidine in DMF was carried out at room temperature under standard Fmoc SPPS conditions and samples were taken periodically throughout the course of the reaction. The thioester **2** showed high stabilityas shown in figure 3-1.



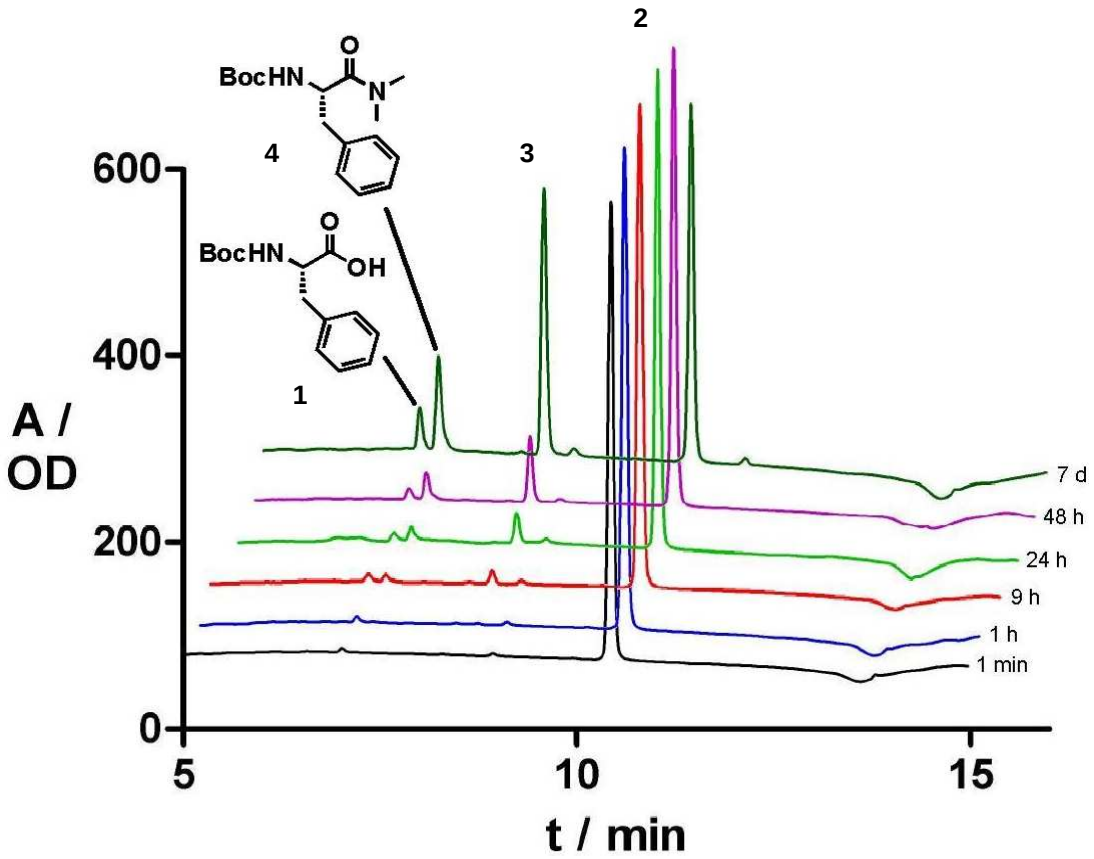


Figure 3-1. Stability profile of the Boc-Phe-*S*-*t*-butyl thioester in the presence of 20 % pip. in DMF.

The major side-product of treatment with piperidine in DMF was the piperidine amide product **2**. Also detectable as side products were the hydrolyzed thioester as well as a DMF derived side-product. The dimethylamide side-product is most likely the result of degradation of DMF under prolonged basic conditions.[107] By analyzing the degradation of the thioester to the amide and side products, a half-life stability was assigned to the *tert*-butyl thioester as being approximately 6.5 days.

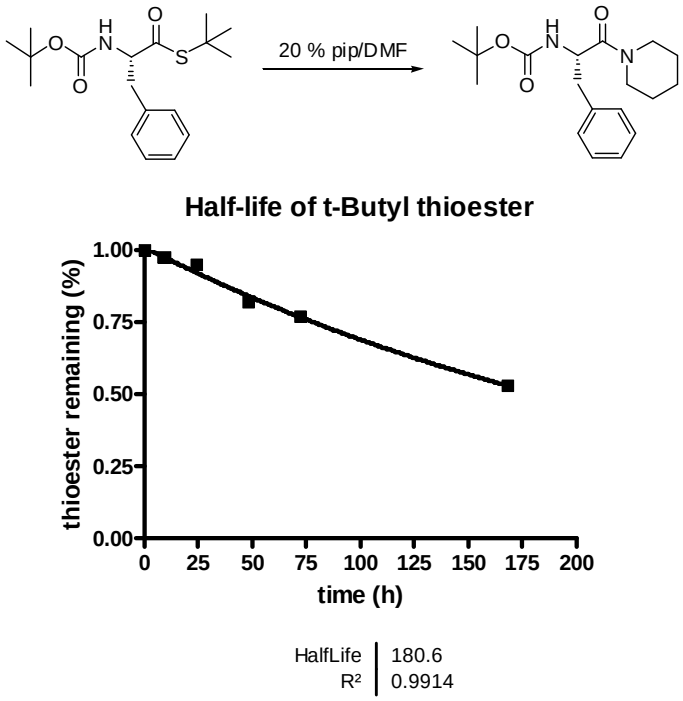


Figure 3-2. The half-life of the *tert*-butyl thioester was determined using LC-MS analysis at 220 nm to be greater than 7 days.

Spurred by this encouraging result, other alkyl and aryl thioesters were tested for their stability. The following thioesters were synthesized following either the previously mentioned protocol with CDI, or using standard DCC/DMAP methods furnishing pure products in high yields.

Table 3-1. Half-life stabilities of various thioesters determined in solution.

entry	thioester	coupling method	yield (%)	half-life
1	-*iso*-propyl (**5**)	CDI	99	8.1 h
2	trityl (**6**)	DCC/DMAP	72	6.5 h
3	ethyl (**7**)	CDI	68	5.5 h
4	benzyl (**8**)	CDI	79	55 min

The final concentration of the thioesters in the piperidine/DMF solution was 62 mM corresponding to a 34 fold excess of base. Half lives were determined by the degradation of the thioesters in 20 % piperidine in DMF identified at 220 nm using LC-MS techniques.

Each thioester was in turn tested against 20 % piperidine in DMF using the same procedure as for the *tert*-butyl thioester. The final concentration of the thioesters in the basic solution was 62 mM which corresponded to a 34 fold excess of base.

Based on the results in **table 3-1.** The following stability pattern could be established: *tert*-butyl > *iso*-propyl > trityl > ethyl > benzyl.

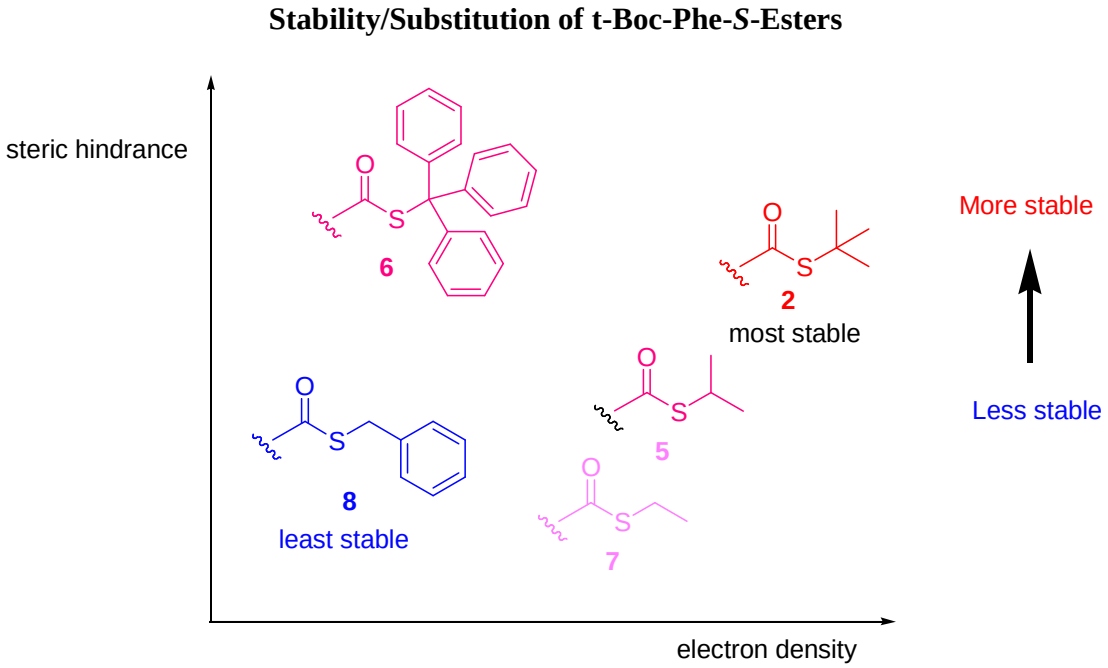


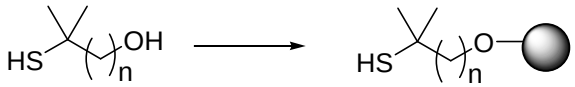
Figure 3-3. Graphical representation of thioester stability.

The results indicate that steric hinderance as well as electronic properties play crucial roles in the stability of thioesters. For example, the sterically most demanding triphenyl methyl (trityl) thioester was cleaved faster than the smaller but more electron-rich isopropyl, the more bulky benzyl thioester faster then ethyl. The stability of the *tert*-butyl thioester phenylalanine strongly support the feasibility of the envisaged linker system for the direct synthesis of peptide thioesters.

3.2.2. Towards the Synthesis of a *Tert*-Butyl Thiol Linker

Attachment of the *tert*-butyl thiol to the solid support was proposed to be attached as easily as possible *via* an ether linkage. In this manner, the linker should be stable to both Fmoc-cleavage as well as standard SPPS coupling conditions. The simplest method of attachment was to take the thiolinker 2-mercapto-2-methylpropan-1-ol as shown in scheme 3-2 and attach it to a solid support where the number of spacer methylene groups (n) is equal to 1.

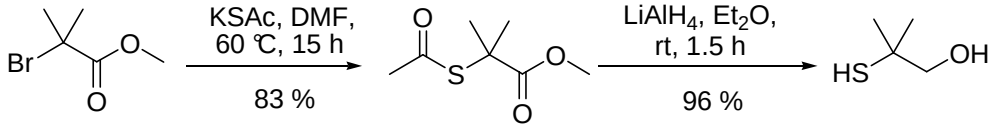
Scheme 3-2



9 **10**

Due to the greater nucleophilicity of the thiol group in the presence of the hydroxyl group, it was necessary to think of orthogonal protection schemes. To this end, disulfides can offer ideal orthogonality in the presence of hydroxyl functional groups.[108] Starting from the commercially available methyl α-bromoisobutyrate, the thiol group was introduced by reaction with potassium thioacetate to give the thioester **12**.

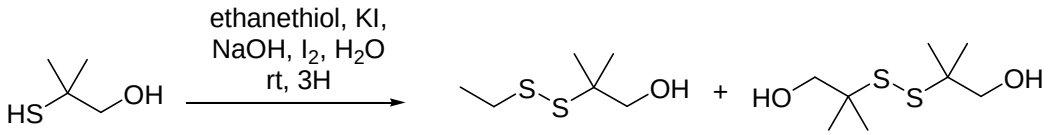
Scheme 3-3



11 **12** **13**

Subsequent reduction of both ester groups gave the desired **13**, 2-mercapto-2-methylpropan-1-ol. Oxidation of the sulfhydryl group to the asymmetric disulfide was accomplished using KI and I_2 in the presence of base.[109] The symmetrical disulfide was also obtained as a side product of the reaction.

Scheme 3-4

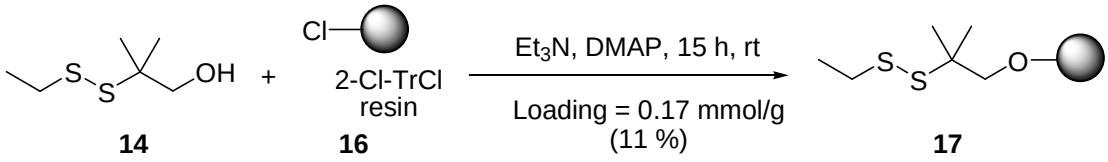


13 **14** **15**

52 % 22 %

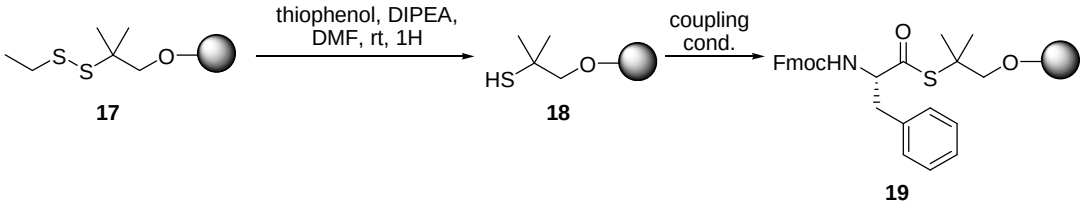
Coupling of the protected linker **14,** led to low coupling yields when reacted with 2-Cl-trityl chloride resin. It is expected that higher yields should be possible for this type of reaction, but optimization was not further investigated. Reduction of the disulfide was first attempted with thiophenol.

Scheme 3-5



Coupling of the reduced thiol was effected immediately after reduction of the disulfide bond. The reduction of the disulfide bond proved to be more challenging than expected and as a result the coupling of the first amino acid to the linker was only marginally successful. The results as shown in Scheme 3-6 left too much room for improvement, and this method was finally abandoned.

Scheme 3-6

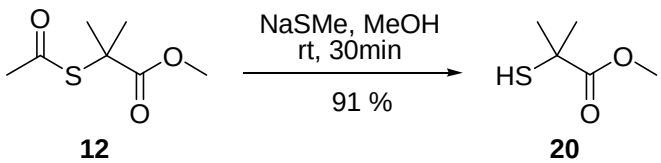


coupling method	time	yield (%)	loading
DIC	18 h	13	0.02 mmol/g
MSNT/ lutidine	18 h	19	0.03 mmol/g

loadings determined by presence of Fmoc via UV-VIS analysis

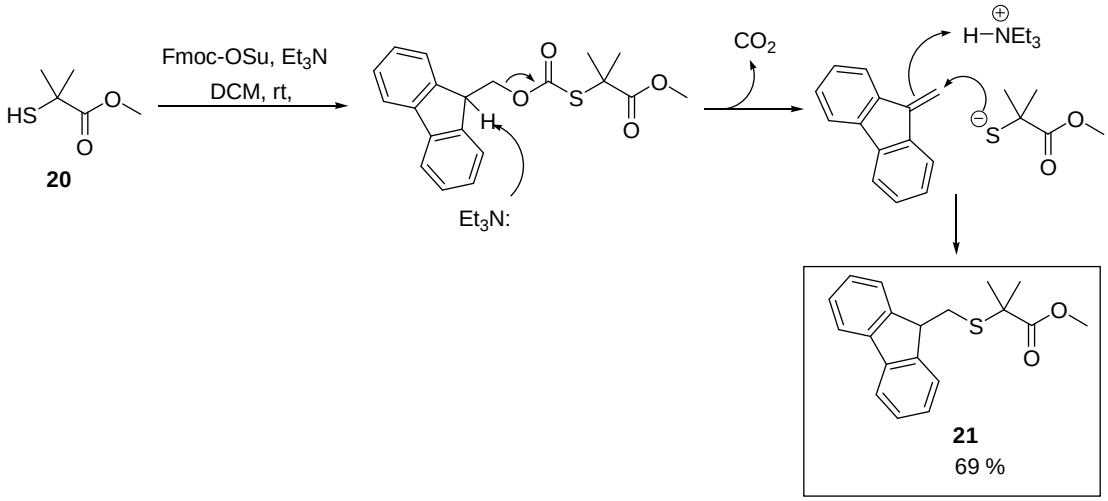
Direction was then focused on different protecting group methods for the introduction of a thiol-protected linker. Base labile protecting groups would allow the continued use of the 2-Cl-tritylchloride resin, and application of such groups with the ability to follow the acylation to the resin were conceived of as a possible alternative. The Fm group is orthogonal to weak base coupling conditions owing to its increased stability over the Fmoc group **12** was selectively deprotected under mild conditions using sodium thiomethoxide.[106]

Scheme 3-7



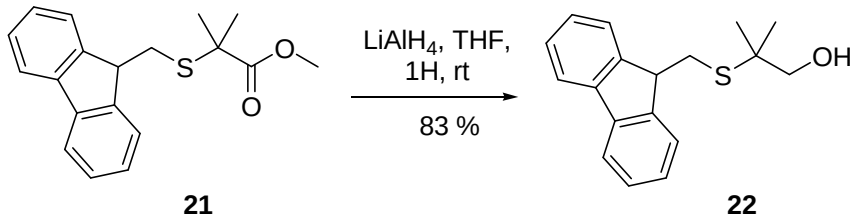
Introduction of the Fm group was accomplished using Fmoc-OSu and triethylamine.[110] After acylation of the Fmoc group to the *tert*-butyl thiol, the product undergoes an elimination/addition to convert the *S*-Fmoc to the *S*-Fm group as shown in Scheme 3-8. According to West et al.[110] when Fmoc-OSu is used in the synthesis, free triethylamine is present in the reaction mixture, and the reaction proceeds to the expected formation of the *S*-Fm product **21**. If Fmoc-Cl is used instead, the resulting Et_3NHCl salt is no longer able to give up a proton for the formation of the S-Fm group.

Scheme 3-8



The reduction of the S-Fm ester **21** using $LiAlH_4$ was subsequently performed, to give the desired product 2-[(9*H*-Fluoren-9-ylmethyl)thio]-2-methylpropan-1-ol, **22**.

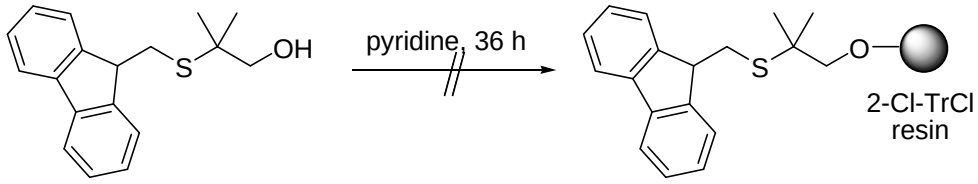
Scheme 3-9



Trityl protection of hydroxyl functionalities require extended reaction times. The Fm group however is not completely stable towards the lengthy treatment with pyridine. As a result, coupling of the of **22** onto a trityl resin resulted in a poor yields

of product **23**, due to the degredation of the linker **22**. As such, this semi-base labile protecting group can be considered not orthogonal to the acylation conditions required for coupling primary hydroxyl groups to trityl chlorides.

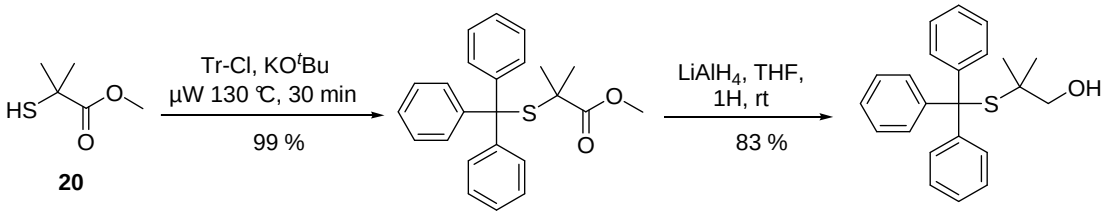
Scheme 3-10



22 **23**

Acid-labile protecting groups for thiols are widely described, however, in order to attach an acid-labile protecting group onto the solid phase, a different, more acid stable solid support had to be introduced. By protecting the **20** with trityl and switching to the classical Merrifield resin, a new method had been realized which afforded a protected thiol linker. Compound **20** was first *S*-tritylated with trityl-chloride in the presence of potassium *tert*-butoxide to give **24** which was in turn reduced with $LiAlH_4$ to give 2-methyl-2-(tritylthio)propan-1-ol **25** in an 83 % yield over 2 steps as shown in Scheme 3-11.

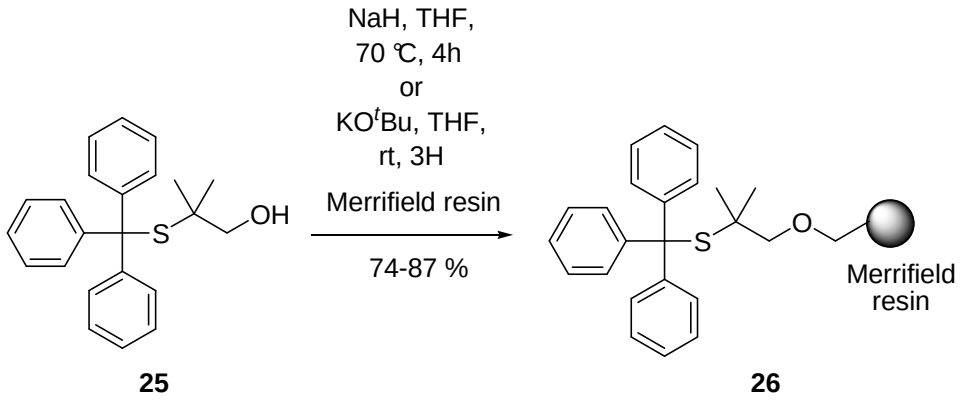
Scheme 3-11



20 **24** **25**

The protected thiol **25** could be acylated to a standard Merrifield resin with NaH in THF or with the application of potassium *tert*-butoxide in a similar fashion to solution phase synthesis. In order to assess the reaction and quantify the outcome, different spectroscopic methods were required.

Scheme 3-12



25 **26**

Characterization of polymer-bound compounds such as **26** can be carried out using AT-IR spectroscopic methods. Carbon-sulfide stretching vibrations are weak and occur in the region of 700-600 cm^{-1} which make it of little value for structural determination due to similar and much stronger vibrations resulting from alkene and phenyl group absorptions. The ether linkage of **26** provides greater structural information for the system. The characteristic response of ethers in the IR is associated with the stretching vibration of the C-O-C system. Owing to greater dipole moment changes than those involving carbon atoms such as C-C-C systems, more intense IR bands are expected for ethers. Strong bands measured at 1220 cm^{-1} which correspond to an asymmetric C-O-C stretch as well as the more characteristic band at 1141 cm^{-1}, allowed qualitative determination of **26**.

Quantitative determination of such trityl bound thioethers was more efficiently characterized by elemental analysis or trityl determination. Both methods do not give extremely sensitive determination of loading of the linker to the solid support, but do offer further evidence of the synthesized molecule. Treatment of the resin with a mixture of TFA/DCM, the trityl cation is released which can be measured quantitatively via UV-Vis techniques. Photometric calibration of the triphenylmethyl cation using triphenylmethanol as substrate has been previously described in the literature using concentrated sulphuric acid.[111] The calibration was repeated, using instead triphenylmethane thiol as substrate to determine the feasibility of this determination with compound **26**.

Although the Lambert-Beer law has been satisfied for the entire studied concentration range, the limiting constraint for using this as a method to quantify the trityl-cation species for solid-phase reactions is the poor swelling character of

divinylbenzene crosslinked polystyrene resins in aqueous media. Using instead a 1:1 mixture of TFA:DCM, the calibration was repeated succefully as shown in figure 3-4.

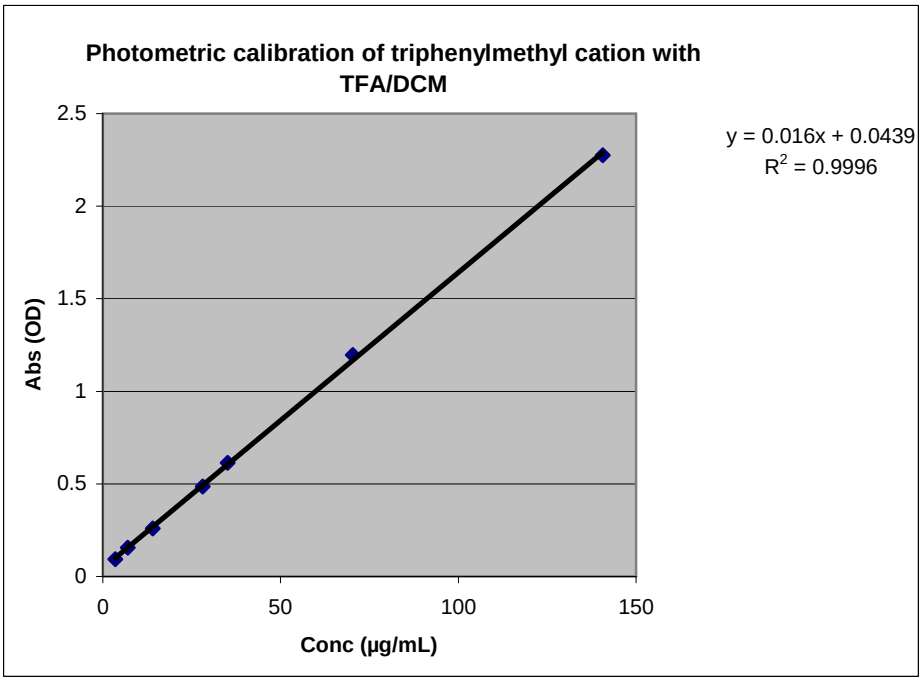


Figure 3-4. Calibration of the triphenylmethyl cation from triphenylmethanethiol.

In both solutions, absorption maxima of the trityl cation were found to be at λ_1 = 408 nm and λ_2 = 430 nm. Agreement of the calibration curve with the Lambert-Beer Law coupled with the better swelling properties of the resin in TFA/DCM allowed implementation of this method to determine more quantitatively the amount of linker bound to the resin. Using equation 1, with molar extinction coefficients of 37,360 and 36,400 for λ_1 and λ_2, respectively,[112] the loading of the resin was determined as an average of the found molar absorption coefficients at λ_1 and λ_2 to be 0.73 mmol/g or a yield of 69 % when compared to the initial substitution of chlorine of the commercially available Merrifield resin.

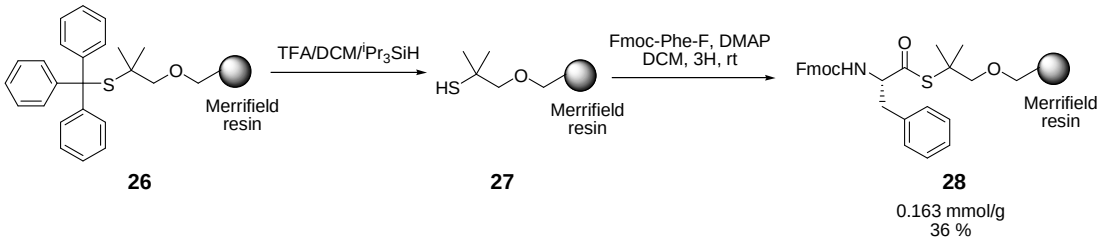
$$x\left[\frac{mmol}{g}\right] = \frac{100000 \cdot E_\lambda}{\varepsilon_\chi \cdot (weighed\ resin)} \qquad (1)$$

Elemental analysis of the same compound gave the following values: Trial 1, Found (%) C: 84.88, S: 2.56, H: 7.335 and Trial 2, Found (%) C: 84.91, S: 2.449, H:

7.219 which corresponded to an average loading of 0.78 mmol/g or 74 % when compared to the starting resin. The slight discrepancy in the loadings may be attributed to inaccurate weighing of the resin before analysis.

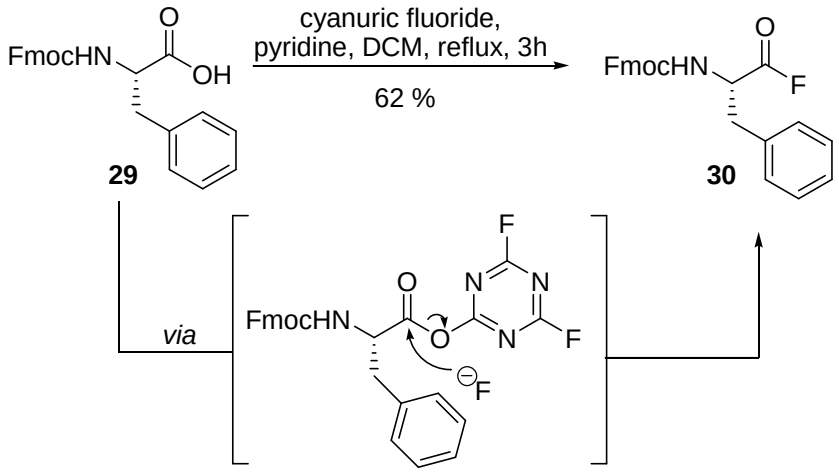
Cleavage of the trityl group was accomplished with TFA in DCM to give the *tert*-butyl thiol which was then directly acylated with Fmoc-Phe-F and DMAP to give the resin bound Fmoc-Phe-*S*-*tert*-butyl ester.

Scheme 3-13



Amino acid fluorides are reasonably stable at room temperature, are synthesized easily from the parent amino acid and avoid the *in situ* geneartion of amino acid fluorides which involves fluoride ion species which may disturb the linker system upon longer reaction times. Synthesis of this building block was performed as described by Carpino et al (Scheme 3-14).[113]

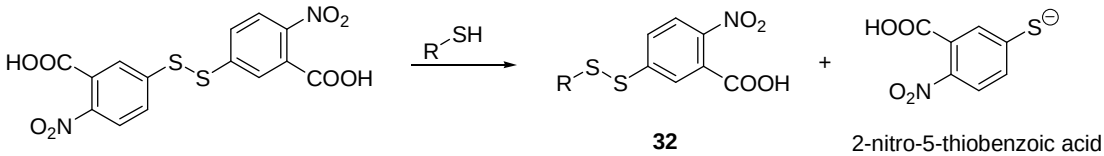
Scheme 3-14



Handling of the resin was crucial for the optimization of the yield. Under aerobic conditions, thiols can be oxidized to their disulfides. On the solid support, intra-bead disulfide formation has been reported.[114] Handling of the resin under

aerobic conditions led to a decrease in free thiols as indicated by a qualitative output from Ellman's test.[115][116][117] This test has been shown to be effective for the qualitative monitoring of free thiols of solid support resins. To date, only a reliable quantitative method has been established with PEG resins which allow application of aqueous solutions of 5,5'-Dithio-bis(2-nitrobenzoic acid) DTNB.[118]

Scheme 3-15. Ellman's test using 5,5'-Dithio-bis(2-nitrobenzoic acid)



5,5'-Dithiobis(2-nitrobenzoic acid)

31

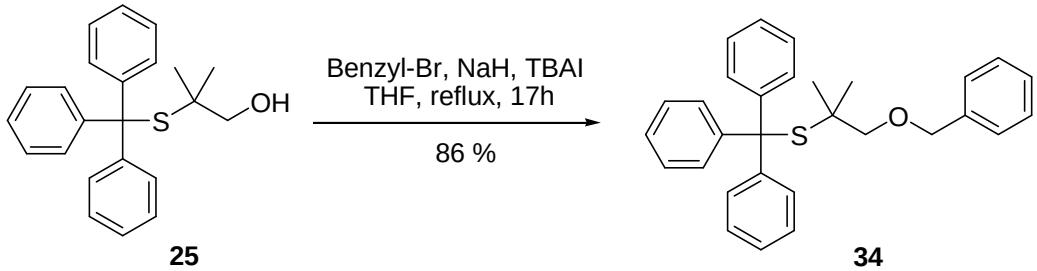
32

2-nitro-5-thiobenzoic acid
brilliant yellow color

33

In order to investigate to what extent oxidation of the sulfhydryl group to the disulfide might hinder the preparation of peptide thioesters on the solid phase, the analogous trityl protected thiol was synthesized in solution.

Scheme 3-16



25

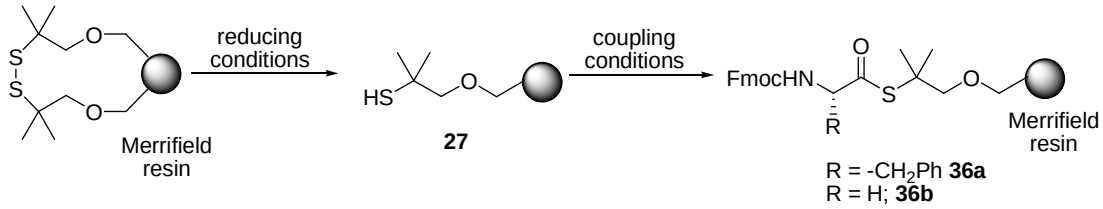
34

Upon treatment of **34** with TFA in DCM (1:1) using Et_3SiH as scavenger the thiol was obtained which rapidly oxidized under atmospheric conditions to the disulfide **35**. The disulfide could be identified via LC-MS giving a m/z value of 391 $[M+H]^+$. Following the reaction onto the solid phase, initial attempts to reduce the disulfide was performed with triethylphosphine or DTT followed by acylation. These attempts afforded only mediocre results; a maximum yield of 25 % after acylation could be obtained (Table 3-2).

The electron rich nature of the *tert*-butyl disulfide bond makes it more stable towards reduction than analogous alkyl disulfide bonds, and as a result, only strong

reducing agents were able to reduce this disulfide bond. For on-resin reductions of the *tert*-butyl disulfide bond, a solution of Red-Al in toluene was the best reagent. After reduction, the resin was carefully washed with dry solvents under an inert atmosphere and the Fmoc amino acid acylation step was carried out immediately thereafter giving much improved yields as shown in Table 3-2.

Table 3-2. Reduction of the disulfide bond followed by acylation of the first amino acid.



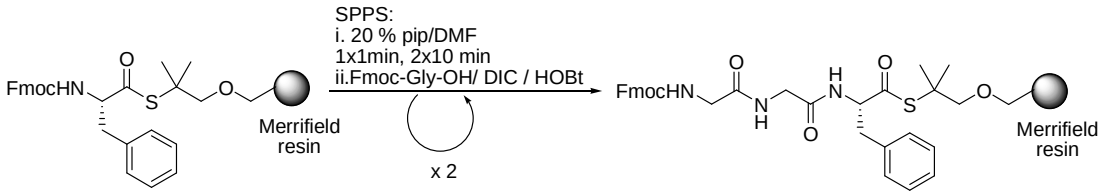
entry	reducing reagent/ reaction time	AA coupling method/ rxn time	amino acid	$loading_{final}$ (mmol/g)	yield (%) of **36a** based on initial loading
1	DTT / 1h rt	Fmoc-Phe-F/ DMAP 3h	Phe	0.13	14
2	Et_3P / 1h rt	Fmoc-Phe-F/DMAP 3h	Phe	0.25	25
3	Red-Al / 3h reflux	EDC/DMAP 14 h	Phe	0.63	73
4	Red-Al / 3h reflux	EDC/DMAP 14 h	Gly	0.53	62

The yields were determined based on the amount of Fmoc cleaved from the acylation step.

Extending the peptide chain from **36a** indicated that the linker was not stable enough against piperidine. Each coupling after the initial first amino acid acylation should be quantitative. The Fmoc deprotection step and subsequent coupling of the next amino acid as previously described should be quantitative. As shown in scheme 3-17, this is not the case. A possible explanation for the low yield stems from the lowered stability of the linker system resulting from the proximity of the benzyl ether moiety to the *tert*-butyl group. As a result, the linker system is too for the preparation longer peptides.

It is conceivable that in this system, the oxygen is near enough to the *tert*-butyl thioester to efficiently withdraw enough electron density thereby making the thiocarbonyl more electropositive and more susceptible toward nucleophilic attack by the piperidine.

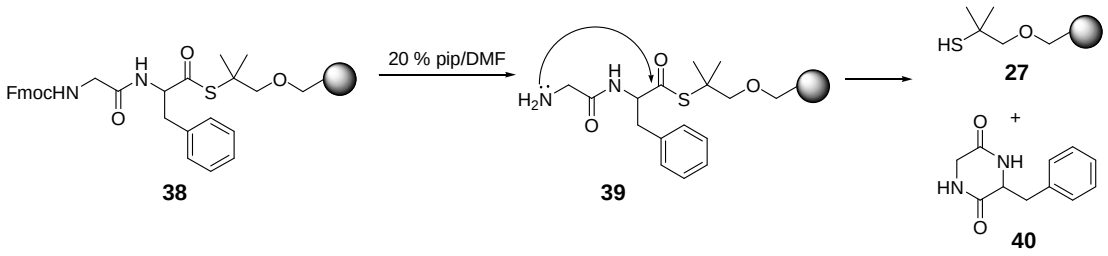
Scheme 3-17. Yields were determined from Fmoc cleavage analysis and based upon the theoretical 100 % yield from step to step.



entry	coupling yield for 2nd amino acid	coupling yield for 3rd amino acid
1	77 %	51 %

The drop in yield from coupling of the second amino acid to the third may be the result of diketopiperazine (DKP) formation after deprotection of the second amino acid from the resin as shown in Scheme 3-18. In the first step, the Fmoc protecting group is removed from the N-terminus of the dipeptide, and in the second step, the liberated amine attacks the thiocarbonyl of the first amino acid and cyclizes it leading to the formation of the DKP and the free thiol.

Scheme 3-18



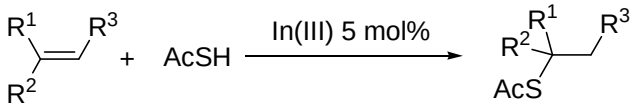
A thiol linker with a longer chain with the number of spacer methylenes (n > 1, Scheme 3-2) should then provide a system with enough stability for longer peptide synthesis.

3.2.3. Towards the Synthesis of a *Tert*-Butyl Thiol Linker Using $InCl_3$ for the Introduction of the Thiol Functionality

In order to provide greater stability to the linker, a longer alkyl spacer between the *tert*-butyl thiol functionality and the hydroxzl group was required. Nucleophilic addition of thiols to substituted olefins is an elegant and straightforward approach. There are several examples which report the addition of thioacetic acid to conjugated Michael systems in basic media,[119] but a more flexible approach would allow site-specific addition to non-activated olefins.

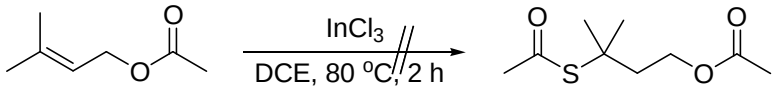
Addition of thioacetic acid using In(III) salts has been described for the introduction of thiols to non activated olefins.[120] The thioacetic acid is introduced selectively to the more substituted carbon in a Markovnikov type addition. The regiochemical outcome is the opposite to that obtained by the radical-type addition with AIBN.

Scheme 3-19



For the synthesis of a an appropriate linker, the reaction was first tested for the production of a thiol linker with two methylene (n = 2) spacer units, as shown in Scheme 3-20. However, the desired thioester product was not recovered.

Scheme 3-20

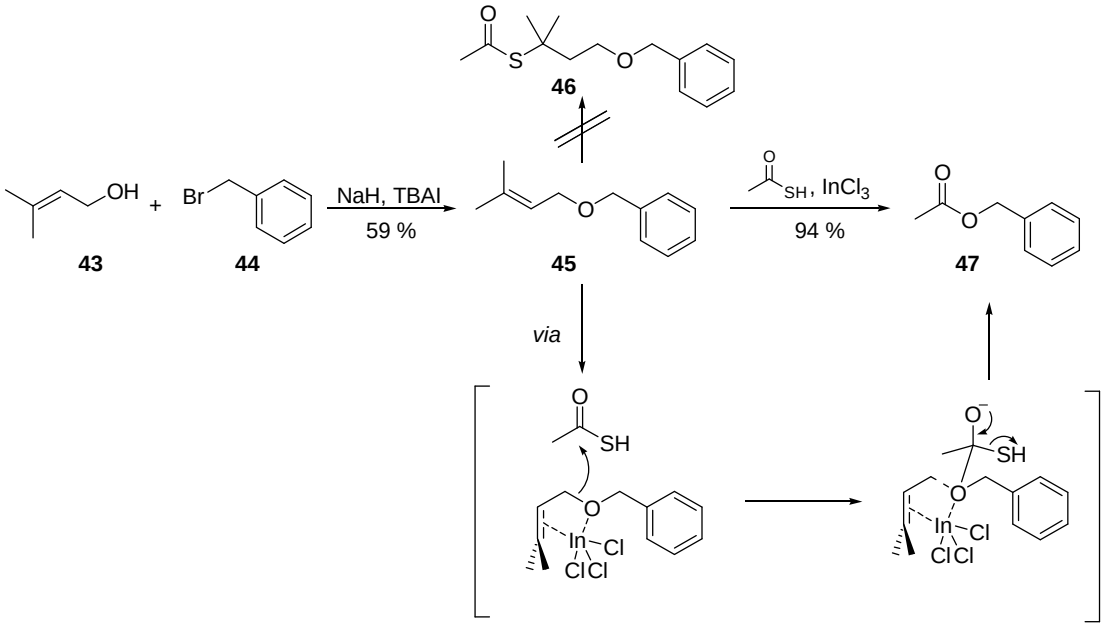


41 **42**

The substrate was altered from an acetyl protecting group to the benzyl group using NaH and tetrabutylammonium iodide (TBAI) to give **45**. TBAI is an excellent reagent for the S_N2 reaction of O-nucleophiles to benzyl bromides. The bromine anion is first nucleophillically replaced by the iodide anion which serves as a better leaving

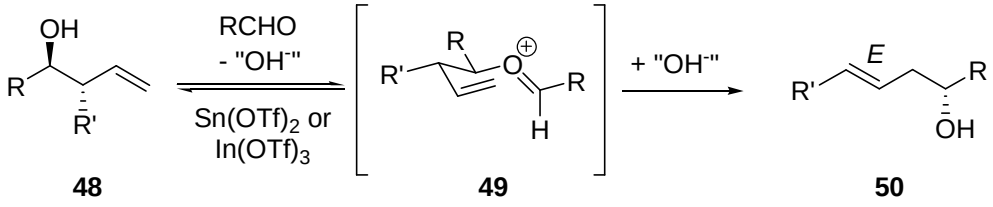
group for the attacking alcoholate. The reaction of **45** with thioacetic acid in the presence of $InCl_3$, however did not give the expected benzyl ether thioester **46**, but instead benzyl acetate **47**. A possible reaction mechanism explanation is shown in Scheme 3-21 where the Indium(III) salt coordinates to the olefin and catalyzes the release of the benzyl oxygen for attack to the thioacetic acid carbonyl in an allyl-type rearrangement.

Scheme 3-21



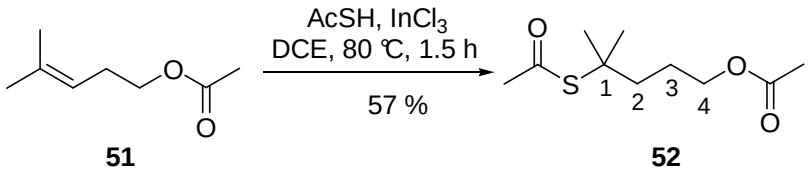
The allyl-transfer reaction catalyzed in the presence of Lewis acids including In(III) salts, has been previously postulated by the group of Teck-Peng Loh[121][122] invoking a 2-oxonia[3,3]-sigmatropic rearrangement mechanism proposed by Samoshin and Nokami (Scheme 3-22).

Scheme 3-22. Reaction pathway postulation of the allyl-transfer reaction.



It was hoped that by adding an extra carbon atom—replacing the protected allylic alcohol with a protected homoallylic alcohol, the allyl transfer reaction could be repressed and successful addition to the desire olefin would result. Acetyl-protected 4-methylpent-3-en-1-ol was reacted with thioacetic acid in the presence of $InCl_3$ to give the thioacid adduct in 57 % yield (Scheme 3-23).

Scheme 3-23



51 **52**

Characteristic ^{13}C signals at δ = 196.6 and 171.2 ppm indicating that both expected carbonyl peaks are present. From this information the following assumptions could be verified: 1) the addition of the thioacid had taken place, 2) an allyl-transfer or elimination had not occurred otherwise the carbonyl peak of the oxoester would not be present. Further NMR analysis of the product was found in the 1D ^{1}H NMR and 2D DQF-Cosy experiments (Figure 3-5).

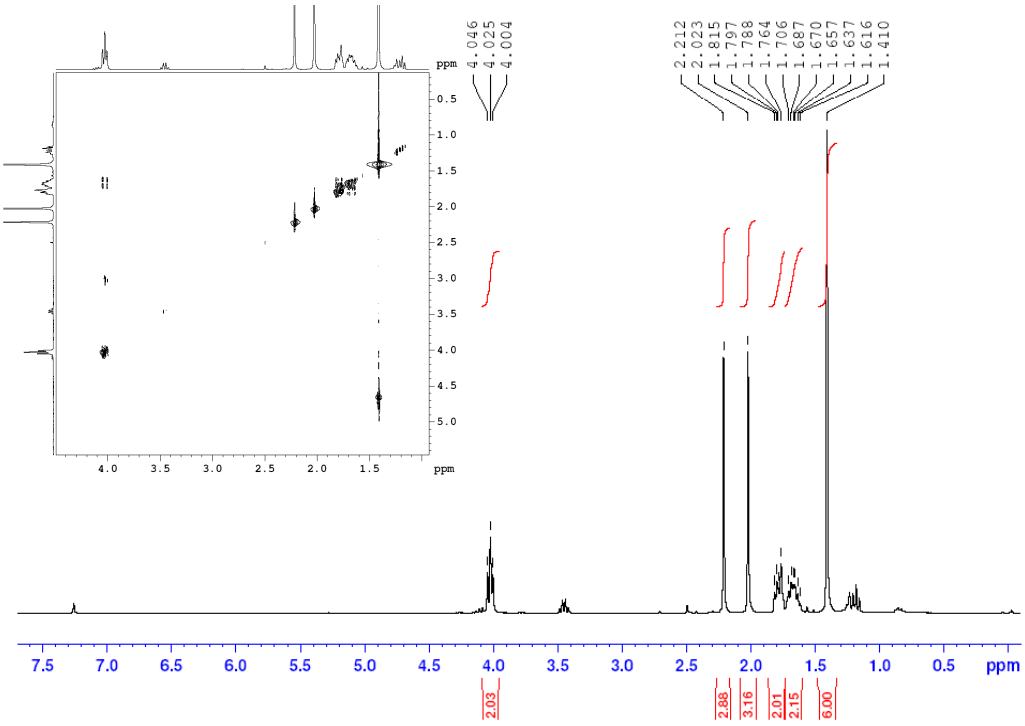
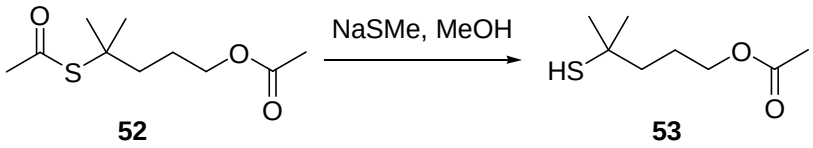


Figure 3-5. NMR spectra of the thioester **52**.

Selective deprotection of the thioacid in the presence of the carboxy ester was carried out with NaSMe as previously described.

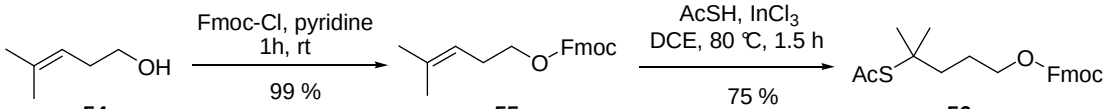
Scheme 3-24



52 **53**

These proof of principle reactions demonstrated the utility of the Indium(III) salts in developing tertiary thiols from inactivated olefins and their orthogonality towards base sensitive protecting groups. In this direction, the alcohol was then *O*-Fmoc protected[123] and the thiol was introduced using thioacetic acid in the presence of $InCl_3$.

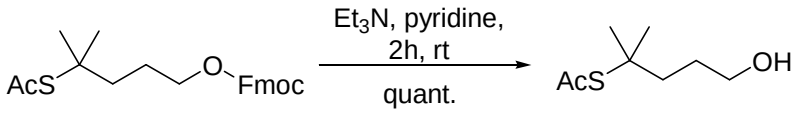
Scheme 3-25



54 **55** **56**

After deprotection of the Fmoc group, the protected mercapto-methylpentanol (MMP) linker **57** could be evaluated for further stability tests.

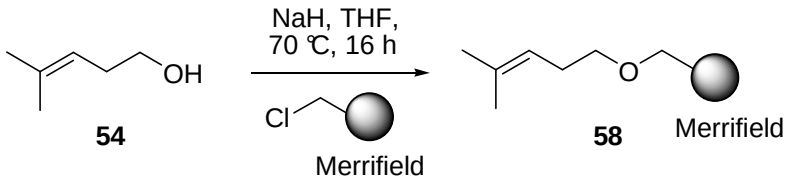
Scheme 3-26



56 **57**

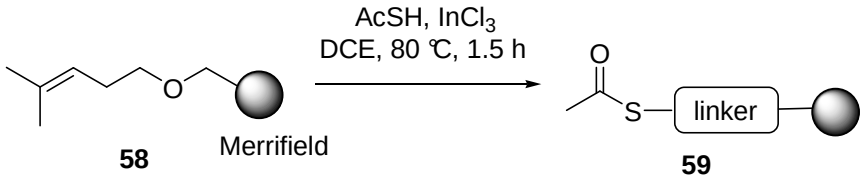
The addition of the thiol to a non-activated olefin using In(III) chloride for the direct synthesis of a thiol linker on the solid support was then attempted for thioacetic acid to acid-stable resins. Using a Merrifield resin as the solid support, **54** was acylated with NaH to give the solid-supported linker precursor (blue overlay, Figure 3-6).

Scheme 3-27



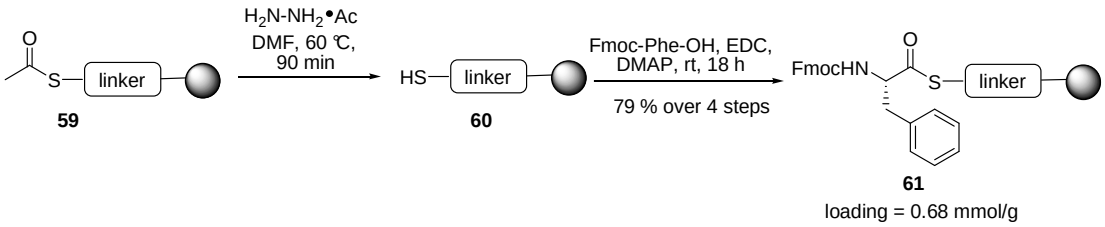
Addition of thioacetic acid to **58** was carried out under similar conditions as performed in solution. A subequimolar amount of indiumtrichloride was used with an excess of thioacetic acid. After heating the reaction to 80 °C and reacting overnight followed by extensive washing of the resin with DMF, THF, and DCM, elemental analysis of the resin showed a significant amount of thiol.

Scheme 3-28



ATR-IR analysis of the solid supported resin shows a clear thioester band at 1690 cm^{-1} (Red overlay, Figure 3-6). After deprotection of the acetate group, the band at 1690 cm^{-1} disappears (Green overlay, Figure 3-6). Acylation of the first amino acid using EDC/DMAP gave the thioester **61** with a loading of 0.68 mmol/g which corresponded to a yield of 79 % over 4 steps.

Scheme 3-29



Coupling of the second amino acid to the resin resulted in non quantitative yield as determined by Fmoc determination (Scheme 3-30). A Kaiser test of the resin after the second coupling however revealed no presence of free primary amines.

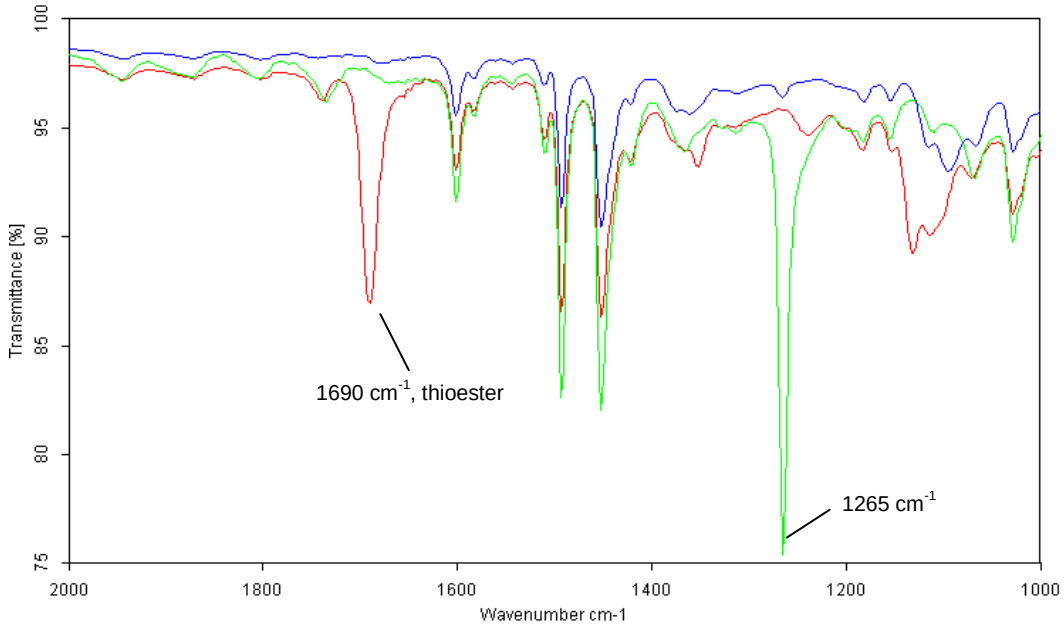
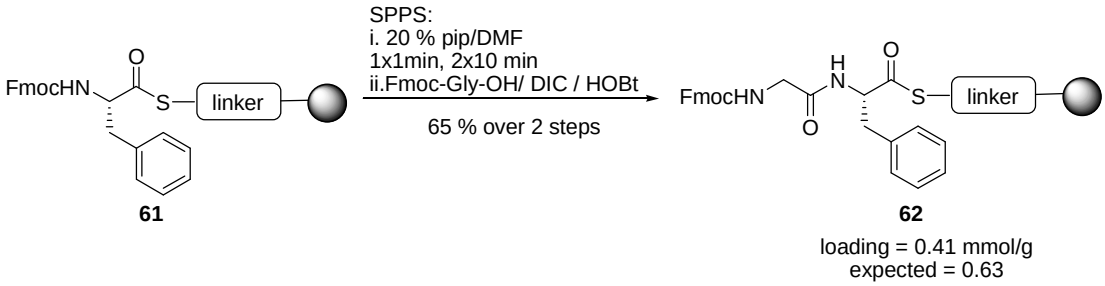


Figure 3-6. Overlay of the resin before (blue), after on-resin activation with thioacetic acid in the presence of $InCl_3$ (red) and after acetyl deprotection (green).

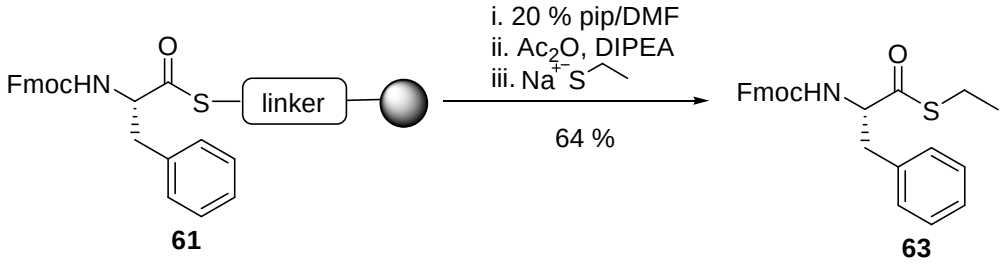
Despite the complete coupling, there was still a drop in yield. Nucleophilic cleavage of a single amino acid loaded to the resin could also give an indication towards the robustness of the linker.

Scheme 3-30



The Fmoc group of **61** was deblocked as previously described. The free amine was then acetylated with acetic anhydride and the amino acid was cleaved from the solid support using sodium ethanethiolate as a proof of principle reaction. The desired Ac-Phe-*S*-Et ester was recovered as a pure product, but the yield was much lower than expected.

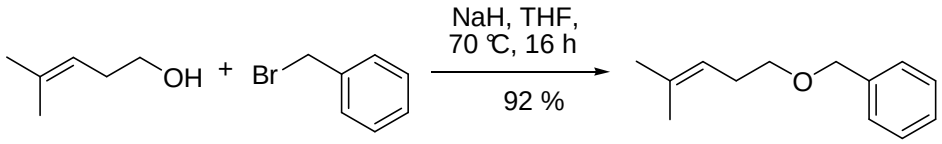
Scheme 3-31



61 **63**

The discrepancy of the yield to the proposed stability of the linker was then invetigated. The on-resin linker synthesis was examined using a solution-phase method to ascertain whether or not the linker was indeed a *tert*-butyl linker. 4-methylpent-3-en-1-ol (**54**) was reacted with benzyl bromide in the presence of NaH and TBAI as catalyst using the same protocol as previously described to give the benzyl ether **65**.

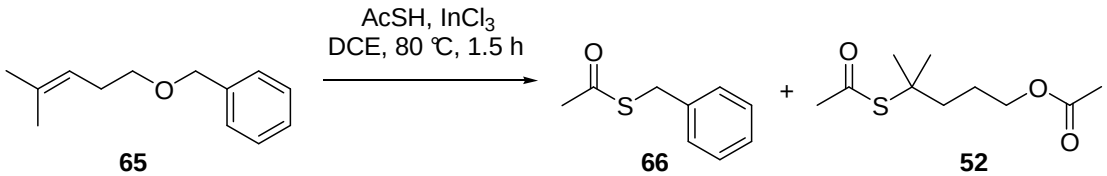
Scheme 3-32



54 **64** **65**

The thiol addition to **65** was examined in solution to model the analogous solid-phase reaction. The aim was to determine whether or not thioacetic acid addition to a non-activated olefin would proceed to the desired product. Unfortunately, addition of the thioacid did not occur as desired. Instead, an O-C cleavage of the benzyl-oxoether resulted in the formation benzylthioacetate and surprisingly the double acetylated product **52** (Scheme 3-33).

Scheme 3-33



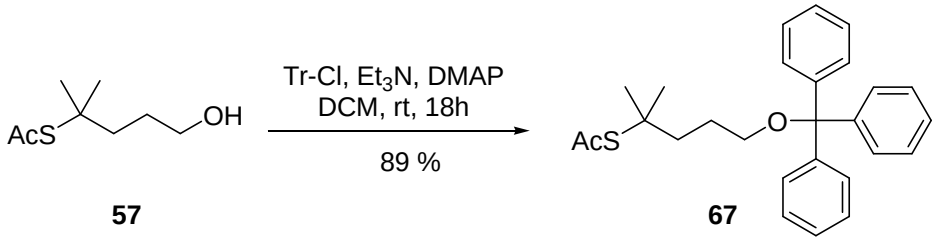
65 **66** 28 % **52** 29 %

The analogous reaction on a standard Merrifield resin would most likely give a resin bound analog of compound **66**. Benzyl thiosters are much more labile toward piperidine base treatment than the other alkyl thioesters (Table 3-1, Entry 4), having a half life of only 55 minutes. This explanation is in agreement with the poor yield obtained after coupling of the second amino acid (Scheme 3-30), and to a lesser extent the recovered yield of the cleaved acetylated amino acid **63**.

3.2.4. Solution Phase Stability Tests of the MMP linker

The alternative method for immobilizing the tert-butyl thio group to the solid phase was to couple the linker **57** to a trityl resin. Again, the stability of the linker system was first tested on a model system in solution by first protecting **57** with trityl chloride. The reaction was finished within 18 hours and gave a yield of 89 % (Scheme 3-34).

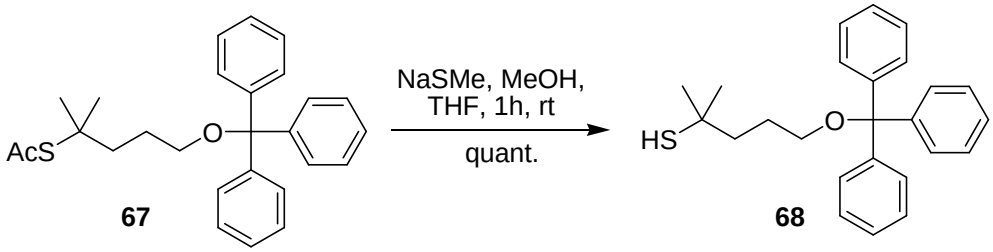
Scheme 3-34



57 **67**

Deprotection of the acetyl group of **67** followed, using sodium thiomethoxide in MeOH to quantitatively give the thiol **68**. The product could be obtained as described without any apparent oxidation of the sulfur to the disulfide bond which could be verified with a singlet at 1.66 ppm corresponding to the lone single proton of the sulfhydryl group.

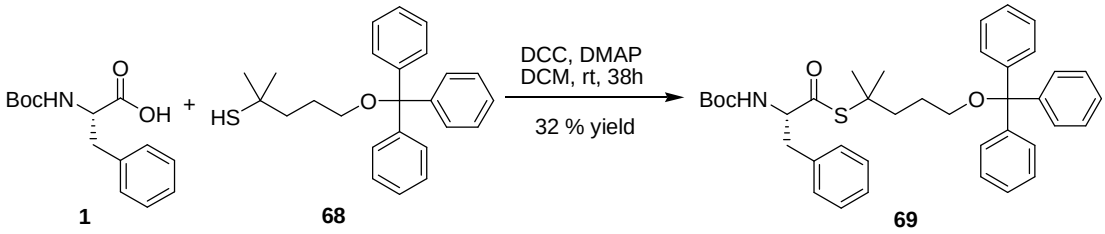
Scheme 3-35



67 **68**

The trityl-protected *tert*-butyl thiol could then be coupled to Fmoc-Phe-OH as previously described for the synthesis of Fmoc-Phe-thioesters using DCC and a catalytic amount of DMAP (Scheme 3-36).

Scheme 3-36



After work-up, the Fmoc-Phe-MMP-Trt, **69** product was obtained in a 32 % yield. The low yield of this reaction may be the result of lowered nucleophilicity of the tertiary thiol. **69** was then treated with 20 % piperidine in DMF to determine the stability. As shown in figure 3-7, the product **69** had exceptional stability with a calculated half-life of 215 hours (9.4 days).

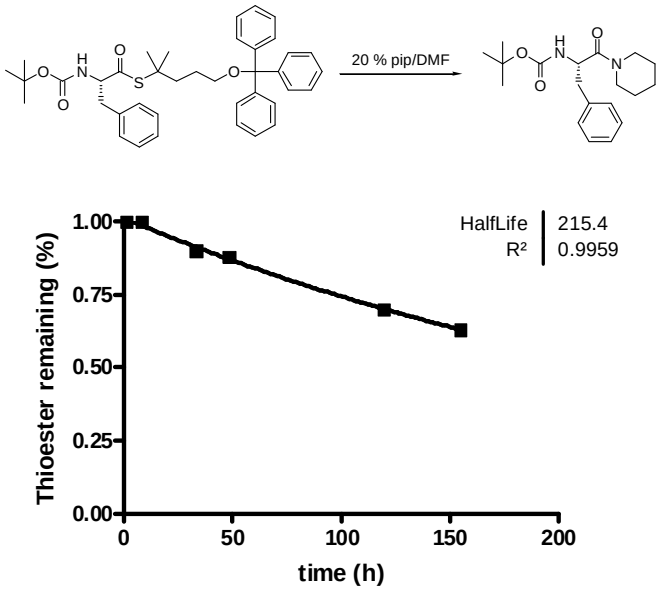
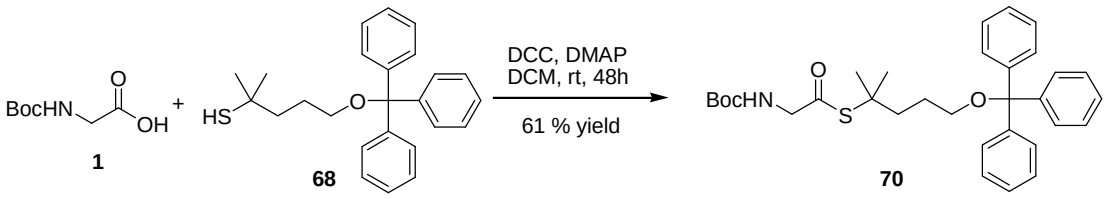


Figure 3-7. Stability test of compound **69** in 20 % piperidine/DMF.

In comparison, the glycine thioester was synthesized in an analogous fashion to compound **69** using Fmoc-Gly-OH in the presence of DCC and DMAP. The yield of the reaction was higher than that of the reaction with Fmoc-Phe-OH. This may be

due to less sterical hindrance of the activated amino acid when encountering the bulky attacking thio-nucleophile.

Scheme 3-37



1 **68** **70**

The increase in yield which may be due to the higher more reactive glycine active ester may explain the marked drop in stability of compound **70** when treated with 20 % piperidine in DMF. Although the Fmoc-Gly-OH is carrying the *tert*-butyl linker, compound **70** has a much lower half-life of 7.5 hours.

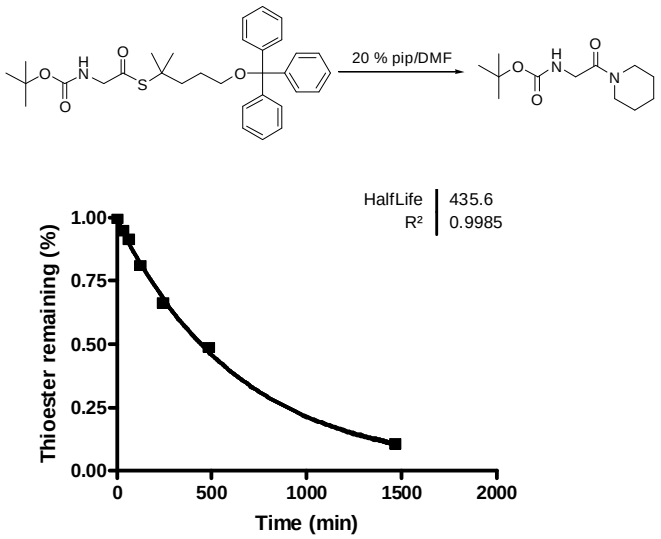


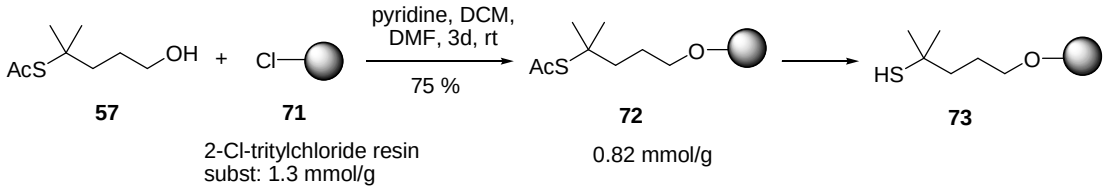
Figure 3-8. Stability test of compound **70** in 20 % piperidine/DMF.

In spite of this observed decrease in stability which may affect the yields of peptide thioesters carrying glycine at the carboxy terminus, *tert*-butyl thioesters should be useful as linkers for all C-terminal amino acids with the highest reactivity for glycine.

3.2.5. Synthesis of Peptide Thioesters *via* the *Tert*-butyl Thiol MMP Linker.

The protected thiol **57** was coupled to a 2-chloro tritylchloride resin using pyridine in a mixture of dry DCM and dry DMF over 3 days to give the solid supported Ac-MMP linker **72**. Elemental analysis of the reaction gave a substitution of sulphur as 2.63 % averaged between two measurements, which corresponded to a loading of 0.82 mmol/g and a yield of 75 %. The yield reflects the stability of the acetyl protected thiol when treated over a long period of time with pyridine.

Scheme 3-38



ATR-IR analysis of the resin showed a carbonyl peak at 1683 cm^{-1} (Figure 3-9, blue overlay) for compound **72** slightly shifted from that of the benzyl thioacetate at 1690cm^{-1}. After deprotection with hydrazine acetate, the carbonyl band disappeared as expected (Figure 3-9, red overlay) to give compound **73**.

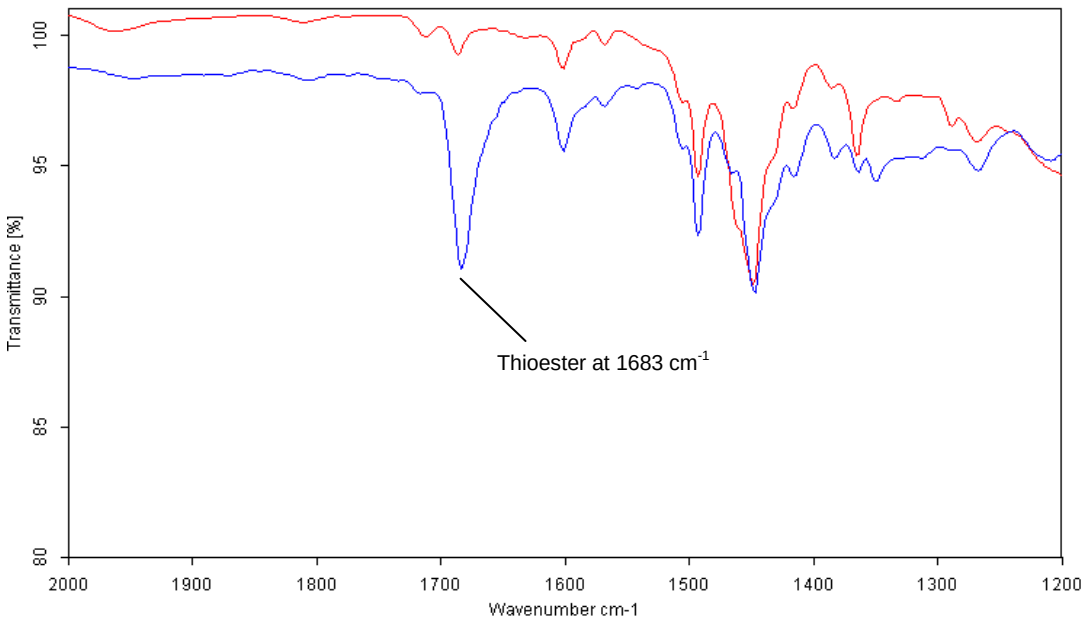
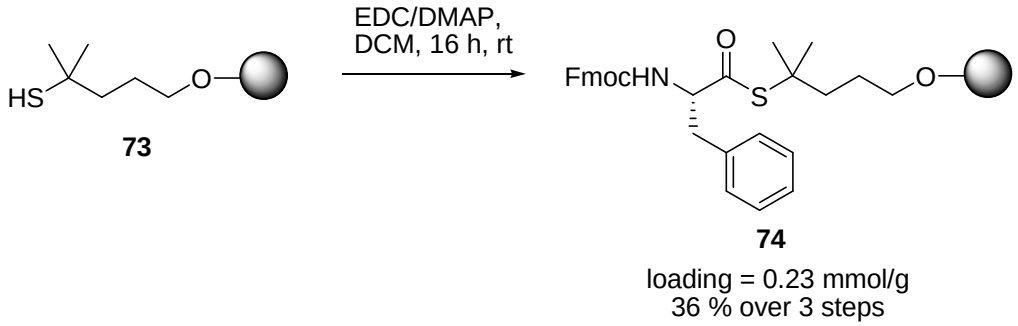


Figure 3-9. Overlay of the solid supported linker **72** (blue) and after deacetylation **73** (red)

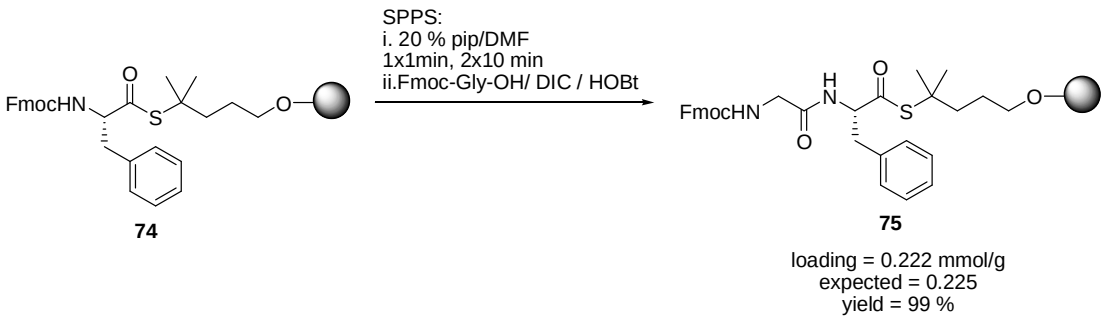
Acylation of the *tert*-butyl thiol supported resin **73** with Fmoc-Phe-OH with EDC in the presence of a catalytic amount of DMAP gave the solid supported Fmoc-Phe-*S*-*tert*-butyl thioester.

Scheme 3-39



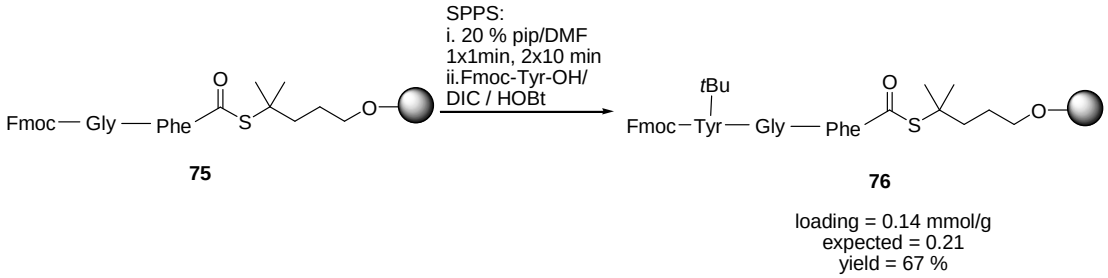
Removal of the Fmoc group followed by coupling of Fmoc-Gly-OH to the free amine under standard DIC/HOBt conditions gave the resin bound dipeptide in an almost quantitative yield (Scheme 3-40).

Scheme 3-40



Deblocking of the second amino acid followed by coupling of the Fmoc-Tyr(O-*tert*-Bu)-OH to the free amine resulted, however, in a drop of expected yield (Scheme 3-41). This drop of yield could be explained once again by DKP formation as described in Scheme 3-18 (*vide supra*).

Scheme 3-41



Solvent from the filtrate of the deblocking step from Scheme 3-41 was removed under reduced pressure and examined *via* LC-MS (Figure 3-10). Along with the Fm-piperidine product expected from the deprotection, the diketopiperazine product was found with a retention time of 1.4 minutes (5-99 % of MeOH for 2.5 minutes).

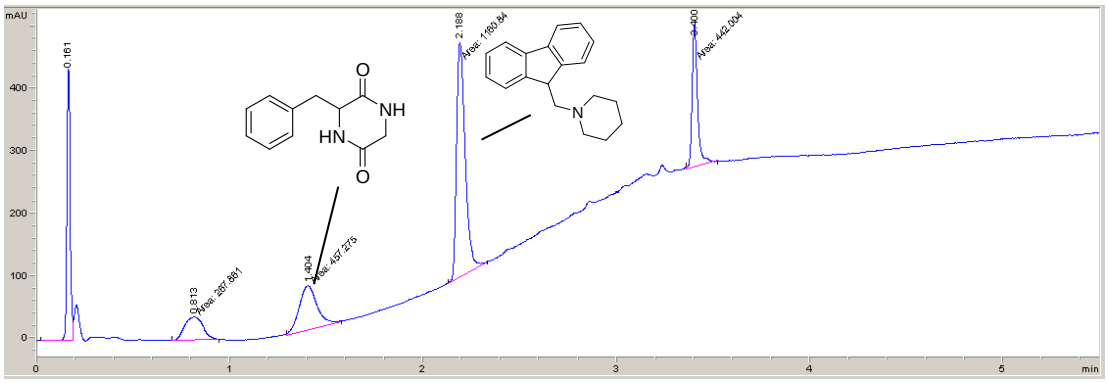
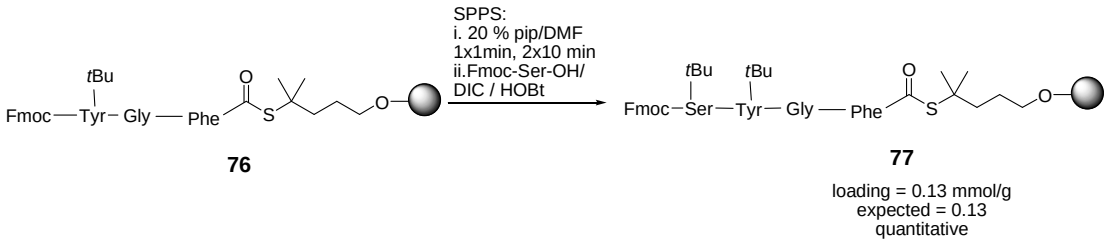


Figure 3-10. LC-MS analysis of the filtrate reveals presence of DKP.

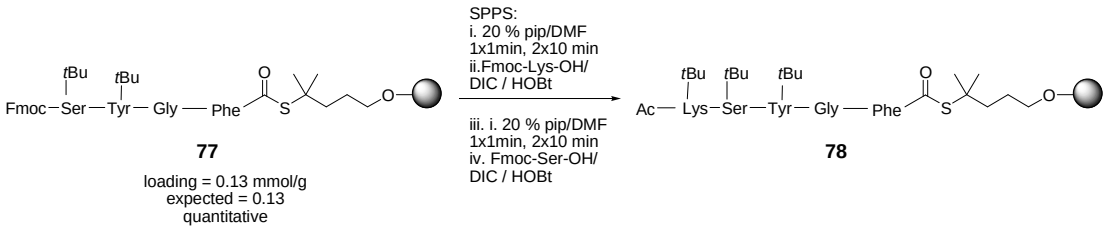
After deblocking the Fmoc group from the growing peptide, Fmoc-Lys(Boc)-OH was then coupled again using DIC and HOBt to give the tetrapeptide Fmoc-Ser(O-*tert*-Bu)-Tyr(O-*tert*-Bu)-Gly-Phe-MMP-resin. After a negative Kaiser test which indicated that the coupling was completed, Fmoc determination from this step revealed quantitative coupling demonstrating the continued stability of the thioester linkage towards piperidine.

Scheme 3-42



The fourth amino acid was deblocked again with piperidine in DMF and coupled with a fifth amino acid, Fmoc-Lys(*t*Boc)-OH. Kaiser test after this coupling step was negative again, indicating complete coupling of the fifth amino acid. After deblocking of the Fmoc group of this step the N-terminus was capped with acetic anhydride, to give the finished resin bound protected peptide Ac-Lys-Ser-Tyr-Gly-Phe-MMP-resin.

Scheme 3-43



The pentapeptide was then cleaved from the resin with TFA in DCM to yield the desired pentapeptide Ac-Lys-Ser-Tyr-Gly-Phe-MMP. The product as shown in Figure 3-11 was not completely deprotected and resulted in a mixture of the completely deprotected peptide **A** and a pentapeptide with the *tert*-butyl group still in place on peptide **B**. Longer cleavage times would prevent this problem. Additionally, there was a small peak which eluted prior to peptide **A**. This product, however, did not have a peptidic mass implying that from the peptide construction and cleavage, no fail or truncated sequences were observed.

The conditions for attaching the linker **57** to the solid phase were optimized. Applying an excess of three equivalents of the linker and 6 equivalents of base reacted for 3 days gave yields of up to 95 % with high loadings around 1.3 mmol/g as confirmed by elemental analysis.

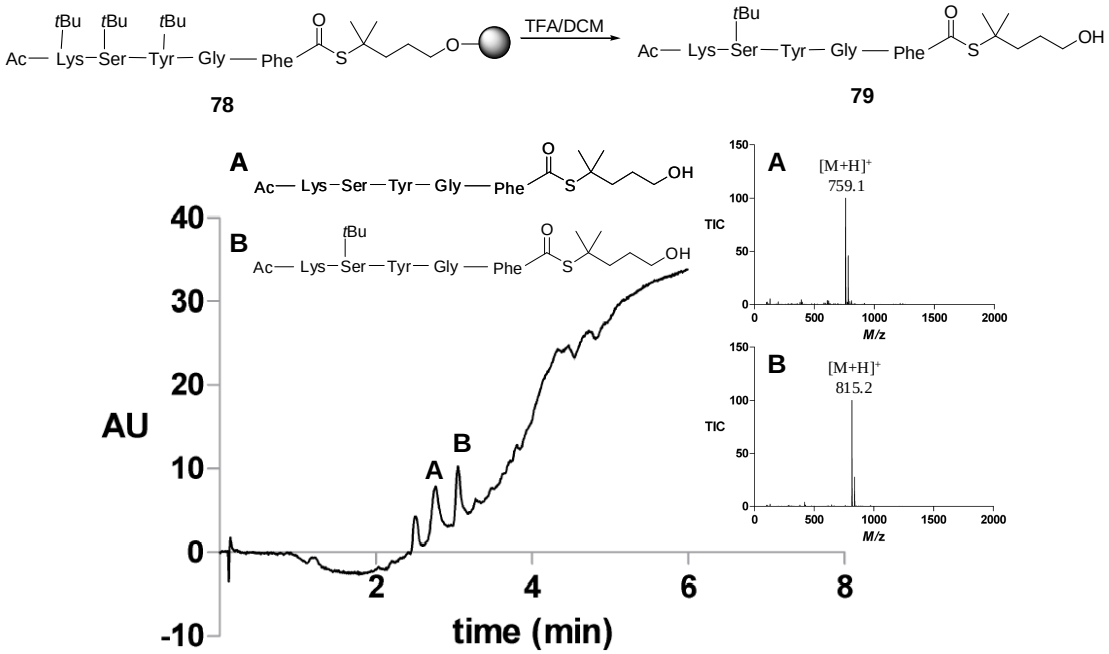


Figure 3-11. Initial test cleavage of the model peptide Ac-KSYGF in TFA.

The synthesis of the peptide was next performed for the sequence SYRGF. The arginine moiety makes the peptide very water soluble, and the neighbouring glycine and tyrosine moieties make the peptide hydrophobic enough to give a good retention time when the peptide is examined *via* LC-MS. This peptide motif serves as a good candidate to evaluate later NCL reactions.

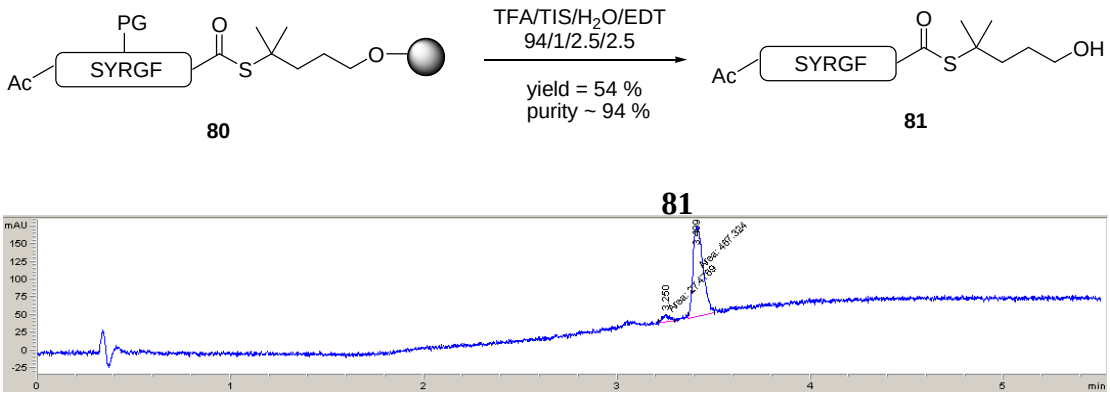


Figure 3-12. Crude chromatogram of the model peptide Ac-SYRGF-MMP, **81**, cleaved acidically with a TFA cocktail.

The peptide Ac-SYRGF-MMP-resin, **80**, was then prepared in the same fashion as the KSYGF peptide. The initial loading of the first amino acid Fmoc-Phe-OH to the solid support was 0.25 mmol/g which reflected a 22 % yield of the first acylation step after loading of the Ac-MMP linker onto the resin. As previously

described, the peptide was then cleaved from the resin, this time with a different cleavage cocktail including water and EDT as nucleophilic scavengers for the cleaved *tert*-butyl cations.

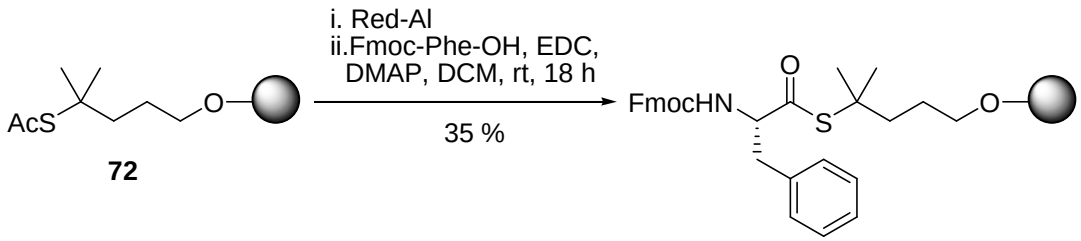
The main peak elutes at around 3.5 minutes (using a gradient of 5-99 % MeOH in 2.5 minutes followed by 3 minutes at 99 %), and is of a high purity with the exception of a small peak which elutes shortly before at 3.25 minutes. The minor peak has a *M*/z of 295.1 which does not correspond to any fail sequence.

3.2.6. Optimization of the First Amino Acid Acylation

It was shown that peptides could be cleanly synthesized using the base stable MMP linker, however, the yield of the first acylation left room for improvement. Initial experiments gave loadings of around 0.30 mmol/g which compares well with the loadings of many commercially available pre-loaded resins. Lower loadings can be advantageous for the synthesis of very large peptides,[42] but in order to optimize the method, higher initial loadings of the first amino acid have to be achieved.

The acylation of the first amino acid was investigated taking at first into consideration the tendency for thiols to undergo intra-bead oxidative cross-linking. Deprotection of the acetyl group under reductive conditions was studied using Red-Al, a reagent which had previously served as a good reagent to reduce the *tert*-butyl-S-S-*tert* butyl bond. After reducing the acetyl group to the *tert*-butyl thiol, Fmoc-Phe-OH was coupled to the linker was immediately with EDC and DMAP (Scheme 3-44).

Scheme 3-44



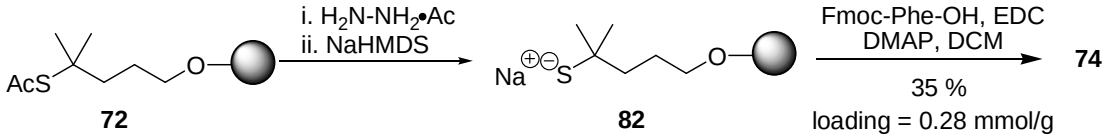
72

loading = 0.29 mmol/g

74

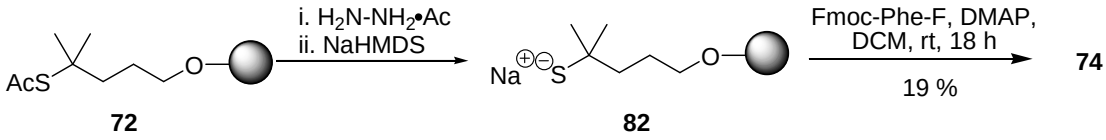
It can be assumed that the thiol is reduced under these conditions and that it should react as a free thiol. The problem then with the low yield might partially have to do with the nature of the *tert*-butyl thiols nucleophilicity. In order to increase the yield of this reaction, either the nucleophile can be made more nucleophilic, or the electrophile can be made more electropositive. Considering the first alternative, after reacting **72** with hydrazine acetate to avoid extensive washings, the free thiol **73** was then treated with NaHMDS to achieve a more reactive nucleophilic sodium thiolate salt, **82** which was then reacted with Fmoc-Phe-OH, EDC and DMAP to give the thioester **74**.

Scheme 3-45



Care was taken to wash the resin **82** with dry solvent to remove the hexamethyldisilazide (bis(trimethylsilyl)amide) anion from the resin without possibly reprotonating the thiolate. The reaction was also repeated using the amino acid fluoride instead of pre-activating the amino acid with EDC. The yield however, did not improve.

Scheme 3-46



There are a variety of activating agents which have proven successful for the *S*-acylation of the thiols. In principle, acylation of even more sterically hindered thiols, should not be a problem due to their increased nucleophilicity as shown above (see the solution phase examples). Different activating agents were tested on the *tert*-butyl thio llinker with only minimal success suggesting the lowered activity of the *tert*-butyl thiol linker.

Table 3-3: Coupling yields of Fmoc-Phenylalanine to the *tert*-butyl thiol linker **73** under different activating conditions.

entry	coupling conditions	solvent	yield (%)[a]
1	5 equiv DIC, 5 equiv HOBt	DMF	13
2	5 equiv MSNT, 4.9 equiv lutidine	DCM	3
3	5 equiv HBTU, 5 equiv DIPEA	DCM	3
4	5 equiv PyBOP, 5 equiv DIPEA	DCM	0
5	5 equiv BTFFH, 5 equiv DIPEA	DMF	2
6	5 equiv HATU, 5 equiv HOAt, 8.3 equiv DIPEA	THF	0
7	4 equiv Dichlorobenzoyl-Cl, 6.6 equiv Pyridine	DMF	16
8	5 equiv EDC, 0.75 equiv DMAP	DCM	37
9	5 equiv Fmoc-Phe-F, 0.75 equiv DMAP	DCM	19

[a] Coupled with 5 equiv of Fmoc-Phe-OH at room temperature for 18 hours unless noted otherwise.

Coupling different amino acids to the linker **73** gave slightly different yields depending on the amino acid used. For instance, Fmoc-Val-OH with EDC/DMAP gave an initial yield of 45 %, with a loading of 0.38 mmol/g. Fmoc-Gly-OH also coupled with a similarly high yield of 37 % (0.32 mmol/g) compared with Fmoc-Phe-OH. The initial coupling results of the first amino acid acylation can be seen in Table 3-4.

Coupling of the linker with Fmoc-Ala-OH (Table 3-4) indicated that exclusion of water is critical for the success of the coupling.[124] Thiols are normally much more nucleophilic than hydroxyl groups, however due to the sterically hindered nature of the *tert*-butyl thiol group, the thiol in this case is much less nucleophilic. Another factor determining the kinetics of the reaction is the nature of the thiol when it is attached to the solid support. It has been found that coupling rates are quite rapid, but are generally 2-3 slower than those in solution.[125] This may be a result of a decrease in productive collisions between the activated amino acid derivative and the bound linker. In contrast, a free-tumbling thiol in solution has a much greater probability to interact with the amino acid derivative. The resulting lowered nucleophilicity of resin-bound *tert*-butyl thiols implies that other nucleophiles such as water can compete for

the activated amino acids. Accordingly, removing water from Fmoc-Phe-OH by azeotropic distillation with toluene increased the coupling yield (Table 3-4, Entry 12).

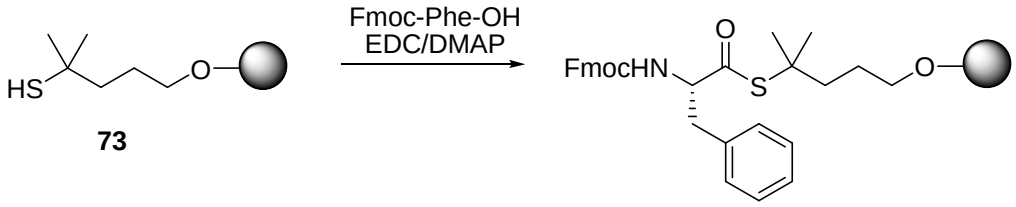
Table 3-4. Primary optimization results of the first amino acid coupling.

entry	Fmoc-amino acid	reaction time	yield[a]
1	Fmoc-Phe-OH	19 h	37
2	Fmoc-Val-OH	21 h	45[b]
3	Fmoc-Val-OH	21 h	61
4	Fmoc-Gly-OH	18 h	37
5	Fmoc-Trp-OH	18 h	24
6	Fmoc-Pro-OH	16 h	3
7	Fmoc-His-OH	18 h	14
8	Fmoc-Gln-OH	18 h	40
9	Fmoc-Arg-OH	18 h	6
10	Fmoc-Ser-OH	18 h	23
11	Fmoc-Ala-OH•H_2O	18 h	0
12	Fmoc-Phe-OH[c]	18 h	46

[a] The yield was determined by Fmoc analysis using UV-Vis techniques based upon elemental analysis of the resin **72**. Deprotection of the acetyl group was carried out each time with hydrazine acetate as previously described. Amino acids were coupled with 5 equiv of EDC, 0.75 equiv of DMAP, DCM, at rt. [b] Coupled in DMF. [c] coupled with azeotroped Fmoc-Phe-OH.

The yield of the first acylation step could be further improved with double couplings of the first amino acid with water-free Fmoc-Phe-OHto 53 % (Table 3-5, Entry 1). Each coupling was performed for 21 hours after which the resin was handled carefully including avoiding pulling air through the resin, and washings with dry solvents. Varying these conditions to double couplings of 2 hours per coupling gave approximately the same yield of 55 % (Table 3-5, Entry 2). The optimal conditions were found to be double couplings of 2 and 20 hours. This gave a yield of up to 74 % (Table 3-5, Entry 3).

Table 3-5. Optimization of the double coupling times for the acylation of Fmoc-Phe-OH to the linker **73**.



73 **74**

entry	double coupling times	yield (%) after 1st coupling[a]	yield (%) after 2nd coupling[a]
1	2 x 21 h	46	53
2	2 x 2 h	41	55
3	1 x 2 h, 1 x 20 h	47	74

[a] Yields were determined from UV-Vis analysis of cleaved Fmoc group following treatment with 20 % piperidine in DMF.

Azeotropic distillation of the Fmoc-Phe-OH improves the first coupling to a certain degree as indicated in Table 3-4. The yield, however, is not quantitative after a second coupling suggesting possibly a competing reaction which somehow blocks or slows the acylation of the free thiol. Entry 3 of Table 3-5 indicates a higher degree of coupling if the second coupling is performed for a longer period of time. This is not completely consistent with a hypothesis that the competing reaction may be due solely to oxidative cross-linking. Nevertheless, it is possible that intra-bead oxidation may occur and resin handling is crucial for the optimization of the reaction, in particular washings between coupling steps.

Accordingly, the first acylation for various amino acids were retested using the optimized double coupling procedure in order to improve the original yields. These results are given in Table 3-6. Amino acids which were not succesfully azeotropically distilled, were used in their commercially available form.

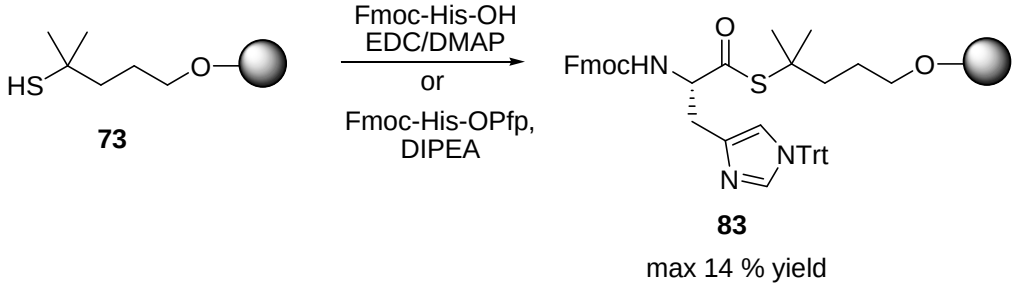
Table 3-6. Optimization of the first amino acid coupling to the thiol linker **73**.

entry	Fmoc-amino acid	loading after coupling (mmol/g)	yield (%)[a]
1	Fmoc-Gly-OH	0.50	54
2	Fmoc-Gln-OH	0.33	43
3	Fmoc-Thr-OH	0.45	59
4	Fmoc-Ser-OH	0.32	34
5	Fmoc-Trp-OH	0.13	20
6	Fmoc-His-OH	0.10	14
7	Fmoc-Val-OH	0.66	83[b]

[a] The reactions were coupled using EDC/DMAP as previously described and carried out in DCM at room temperarture with double couplings of 1 x 2 h and 1 x 18 h unless otherwise noted. [b] This yield was obtained after only one coupling for 17 hours.

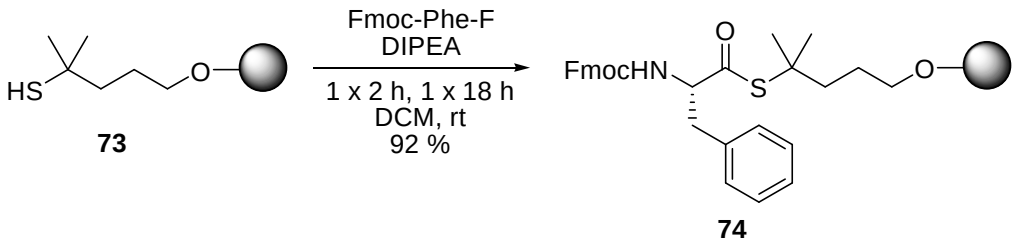
For the most part, the yields increased under the optimized conditions. The loadings of the resin after coupling were also to a large extent acceptable with an average around 0.3-0.5 mmol/g. Fmoc-Trp-OH failed to couple more efficiently than under conditions described in Table 3-6. Furthermore, the acylation of histidine proved to be more difficult. Repeated attempts with activated His residues failed to give completely satisfying yields (Scheme 3-47).

Scheme 3-47



The yield of the reaction for Fmoc-Phe-OH could be optimized yet further with the use of Fmoc amino acid fluorides as the activated amino acid instead of EDC/DMAP for activation. Double couplings of 1 x 2 h and 1 x 18 h with DIPEA as a base to scavenge the liberated proton gave yields of up to 92 % with Fmoc-Phe-F (Scheme 3-48).[126]

Scheme 3-48

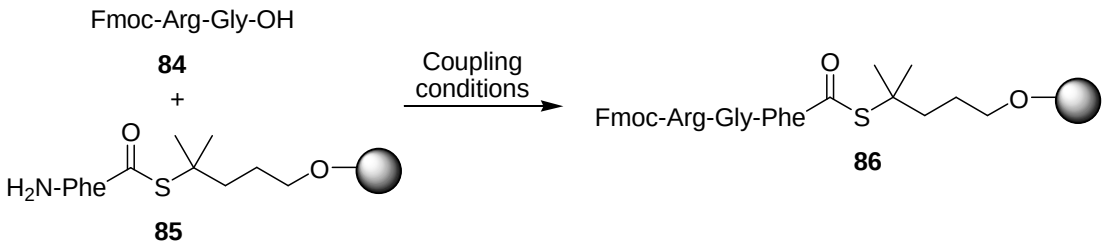


3.2.7. Coupling Dipeptides to Overcome Loss of Yield from DKP Formation

The drop in yield after deblocking the Fmoc group from the second amino acid in the sequence SYRGF was examined in order to increase the final yield. Lowering the deprotection times after the second coupling may increase the final yield slightly, but this is still not a satisfying solution, since a drop in yield would still be expected. An alternative method was found by coupling a dipeptide to the first amino acid, thereby circumventing the DKP forming step altogether. Accordingly, the dipeptide Fmoc-Arg(Pbf)-Gly-OH, **84**, was synthesized on a trityl resin. First, Fmoc-Glycine was coupled to a 2-chlorotrityl chloride resin using an excess of DIPEA as base to give a final loading of 0.68 mmol/g. An excess of the amino acid was used for this reaction since the peptide to be produced is very small. The synthesis of larger peptides can become encumbered by the steric crowding of the growing peptide on the solid support. In those cases, an underscore of peptide will help in the attachment of amino acids to the growing peptide chain and thereby increase yield and purity of the final peptide.

After deblocking the amino group, Fmoc-Arg-OH was again coupled using standard Fmoc SPPS coupling conditions. The dipeptide was cleaved from the resin using 1 % TFA in DCM for 5 x 30 seconds which serves as a milder cleavage procedure to ensure that the Pbf protecting group would also not be cleaved. Using this protocol, the dipeptide could be obtained in a 97 % yield based on the initial loading of glycine to the resin. The dipeptide **84** was obtained as a pure product after removal of solvent and lyophilisation and did not need to be further purified. In order to find couple the dipeptide to the resin, a variety of coupling conditions for coupling to the primary amino acid were attempted.

Table 3-7. Optimization of the dipeptide coupling to the amino acid loaded resin.



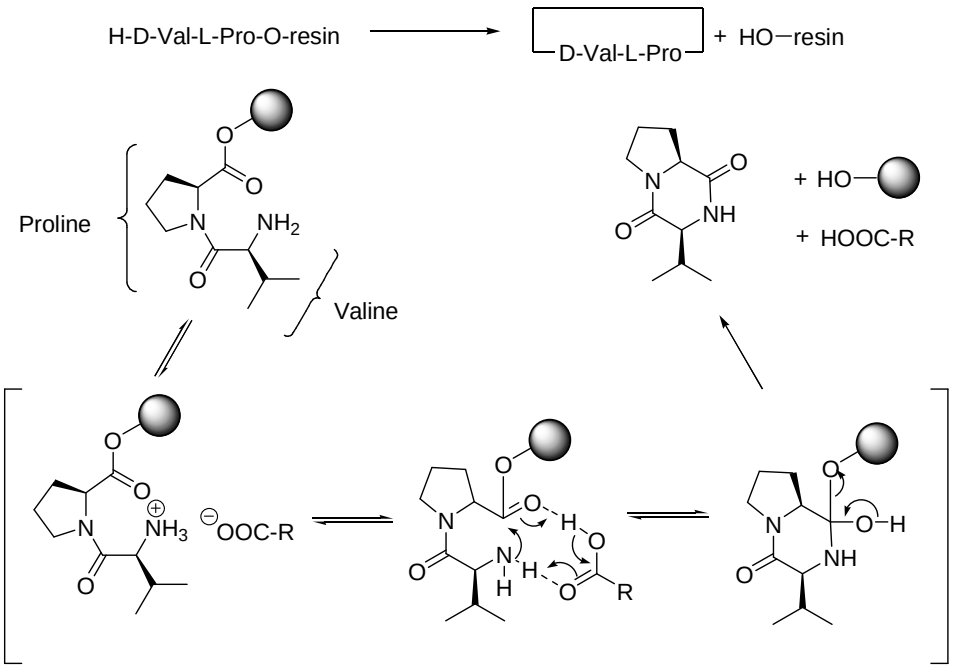
entry	activating agent (equiv)	base / equiv	solvent	time	yield (%)[a]
1	DIC (4.9) / HOBt (5)	-/-	DMF	17 h	18
2	HBTU (3)	DIPEA / 3	DMF	1 h	58
3	HATU (4)	Collidine / 4	DMF	20 h	28
4	HATU (4.4) / HOAt (4.4)	DIPEA / 7.3	THF	3 h	89-99

a) Yield based on Fmoc determination after the coupling step.

After optimization of the coupling of the dipeptide to the solid supported Phe-MMP-resin, the peptide was completed as previously described. The peptide was then cleaved from the resin using the forementioned TFA cocktail and precipitated in MTBE to give again a pure product pentapeptide Ac-SYRGF-MMP, **81** which was obtained in a yield of 83 % based on initial loading.

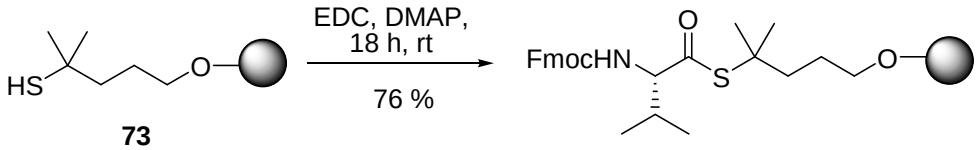
The formation of DKP is expected to be pronounced for sequences which contain proline either at the carboxy terminus or at the second position. A partial explanation to this is the fixed cis amide bond which proline adopts allowing more facile attack of the N-terminal amino acid.[127][128] Interestingly, in the work of Merrifield, DKP formation was observed as a product during Boc solid phase synthesis of the peptide sequence D-Pro-D-Val-L-Pro. However, the loss in yield due to DKP formation was not described as occurring during either the deprotection step with TFA or the neutralization step with DIPEA. Instead, DKP formation has been shown to be carboxyl catalyzed of the next amino acid. The proposed mechanism is shown in Scheme 3-49.[128]

Scheme 3-49



This may also happen to a slightly lesser degree when the proline is coupled as the second amino acid. Less DKP formation is however expected due to lower nucleophilicity of the secondary proline amine than other amino acid primary amines. Nevertheless, it has been reported that when proline is at the second position, DKP formation occurs.[129] Furthermore, this report showed that the DKP formation occurred under basic conditions during neutralization of the amino group after acidic removal of the Boc protecting group. Other studies have shown that the DKP side reaction can be accelerated by addition of tertiary amines.[130]

Scheme 3-50



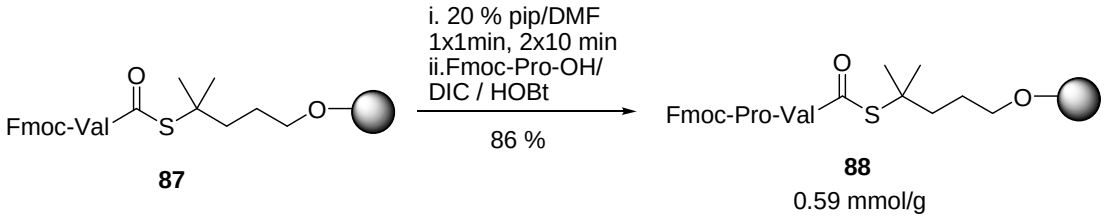
73

87
0.74 mmol/g

The test peptide SYRPV was selected to demonstrate the extent to which DKP formation would limit this system where Pro-Val or Val-Pro motifs are found at the carboxy terminus. Fmoc-Val-OH was coupled to the linker **73** using EDC and

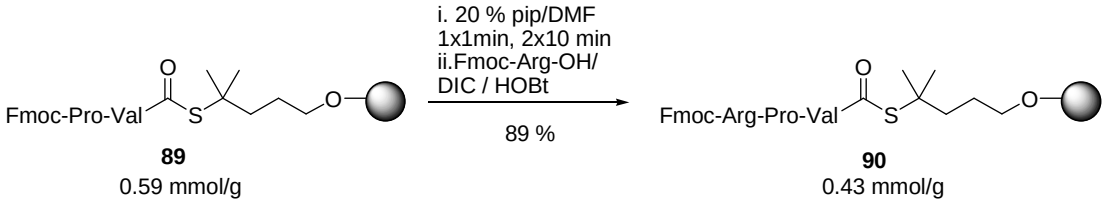
DMAP. Removal of the Fmoc group followed by coupling of the next amino acid Fmoc-Pro-OH gave **88** with a loading of 0.59 mmol/g.

Scheme 3-51



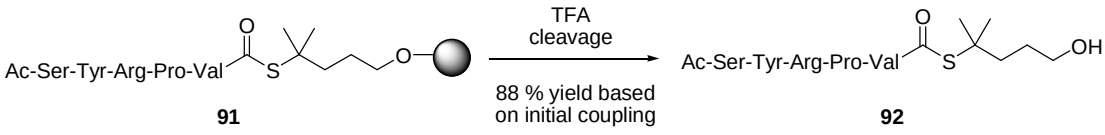
After deblocking the dipeptide **88**, solvent from the filtrate was removed under reduced pressure and analyzed via LC-MS. Formation of the Pro-Val DKP product could not be detected either by LC-MS techniques. The Fmoc group was once again removed as previously described and coupling of the third amino acid gave an 89 % yield as determined by Fmoc analysis.

Scheme 3-52



After completion of the solid supported Ac-SYRPV-MMP **91**, peptide as previously described, the peptide **92** was recovered from the resin using the same TFA cocktail with greater than 90 % purity and a yield of 88 %. The dipeptide motif Pro-Val, was proven to not to be present in the synthesis of the pentapeptide Ac-SYRPV-MMP as the yield indicates.

Scheme 3-53



It can be concluded that DKP formation using MMP linker is not a general problem and occurs for only certain peptide sequences. Furthermore, the yield through application of dipeptides is low, implying that the MMP linker provides suitable yields alone without the aid of circumventing DKP formation.

3.2.8. Variation of the Carboxy Terminal Amino Acid for the Synthesis of Model Thioester Pentapeptides and Their Subsequent Cleavage with TFA

In order to test the applicability of this method with varying peptide sequences, a number of peptides were prepared with the motif Ac-SYRX_{aa}-MMP (X_{aa} = any given natural amino acid). Table 3-8 shows yields of four test peptides and their respective yields after global deprotection with TFA.

Table 3-8. Synthesis of selected test pentapeptides.

entry	compound	peptide sequence	yield (%)[a]
1	81	Ac-SYRGF-MMP	54/83[b]
2	92	Ac-SYRPV-MMP	88
3	93	Ac-SYRGW-MMP	90[b]
4	94	Ac-SYRGQ-MMP	53

[a] Yields were derived from the isolated products and calculated based on the loadings of the first amino acid to the solid support. Purities of the crude products were determined via HPLC at 220 nm via UV/Vis spectroscopy to be greater > 90 % pure. [b] The yields given here were obtained after coupling of the dipeptide Arg(Pbf)-Gly-OH to the first amino acid.

For the sequences where DKP formation was observed, coupling of dipeptides to the initial peptide succeeded in increasing the yield (Table 3-8, entries 1 and 3). Remarkably, the crude peptides that were cleaved from the resin using TFA were obtained as pure peptides without trace of fail or truncated sequences. Furthermore, the purity of the compounds could be assessed at 220 nm via UV-Vis spectroscopy to be ≥ 90 % (Figure 3-13). The yield for each peptide synthesis was satisfactory, and gave quite pure products without need for further HPLC purification.

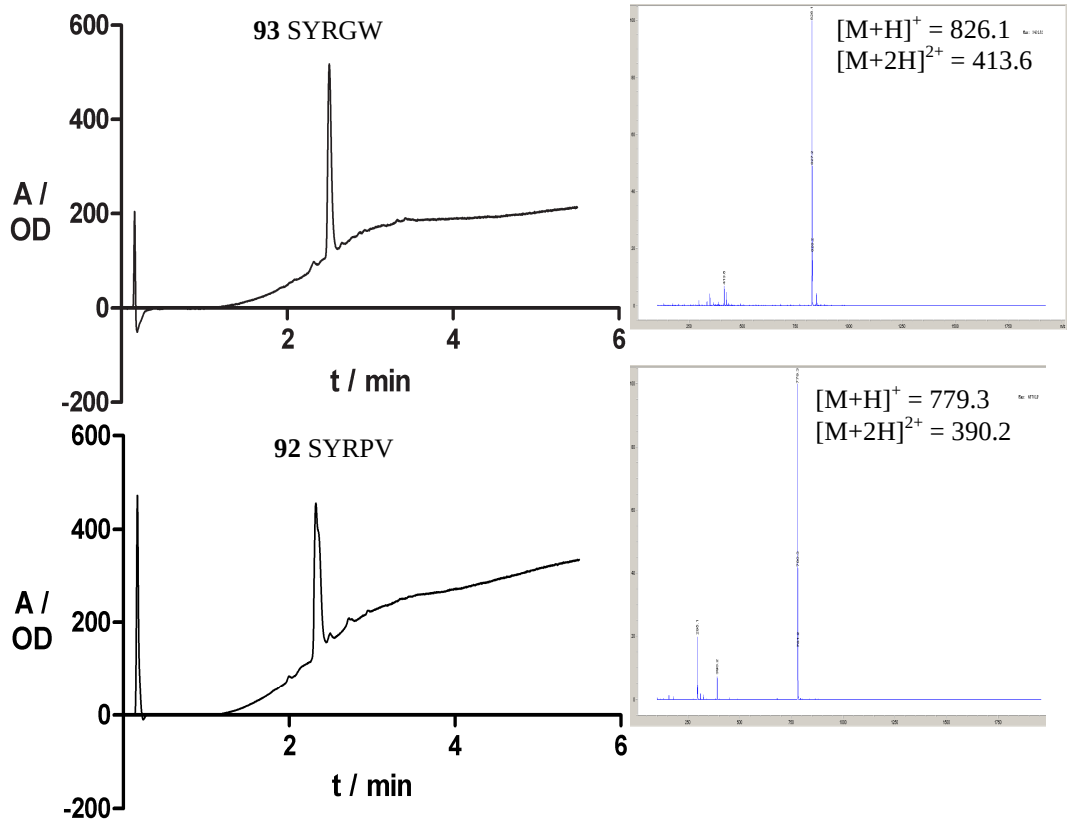
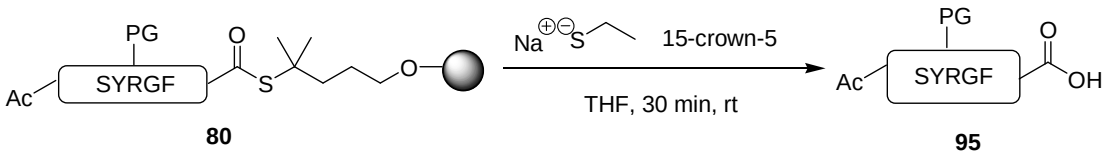


Figure 3-13. Crude Spectra of sample compounds **92** and **93** with their respective mass analysis.

3.2.9. Nucleophilic Displacement *via* Thiolates of Peptide Thioesters Followed by TFA Cleavage to Give Model Thioester Pentapeptides

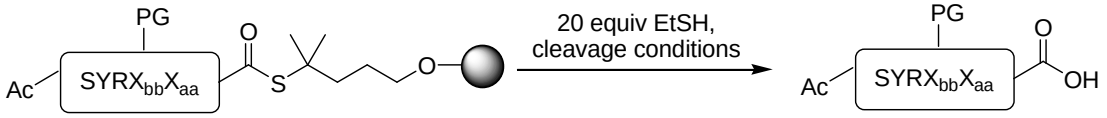
The MMP linker proved to be a robust linker for the direct preparation of on-resin peptide thioesters. It was expected that the *tert*-butyl linker would still remain labile towards thiolate nucleophiles, which could afford protected thioester peptides. Based on the experiments carried out with the previous linker system (Scheme 3-31), displacement of the pentapeptide Ac-SYRGF from the solid support was first attempted with sodium ethanthiolate. This initial trial yielded only hydrolyzed product **95** (Scheme 3-54)

Scheme 3-54



Varying the base and the conditions under which the peptide was cleaved from the resin also resulted only in the hydrolysis product (Table 3-9). Each cleavage was performed under an inert atmosphere with dry solvents.

Table 3-9. Initial nucleophilic cleavage attempts yielding only hydrolyzed material.



entry	base / equiv	additive	solvent	time (min)[a]
1	NaH / 20	15-crown-5	THF	30
2	Na / 100	15-crown-5	DMF	30
3	DBU /20	--	DMF	60
4	DBU / 20	--	DCM	45
5	DBU / 2	--	DMF	10

[a] time required for complete conversion to the acid. Product formation was followed using LC-MS techniques at 220 nm and 254 nm.

Examined more carefully, it was found that the transthioester product was initially formed and then rapidly hydrolyzed to the acid. After 1 minute, there was already a significant degradation of the thioester product; after 45 minutes, the product has been completely degraded leaving the acid and an udidentifiable side-product with a mass of the product –62. Significant amounts of the hydrolysis product may be due in part to excess water which may already be present in the resin before cleavage.

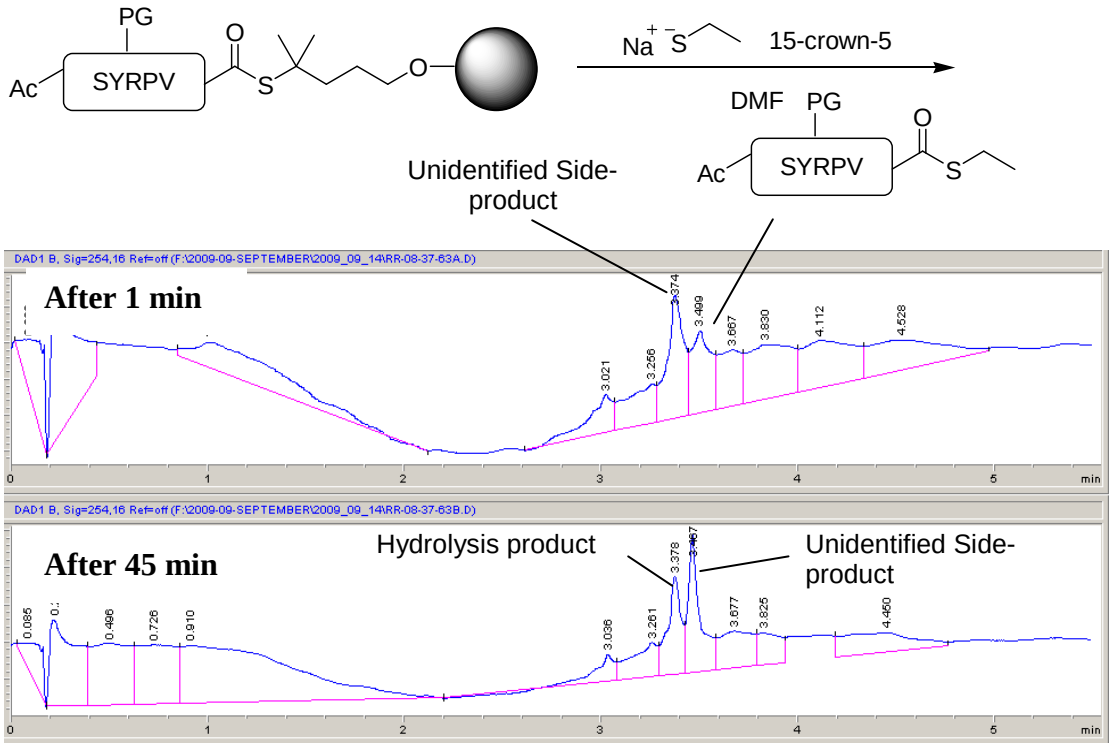
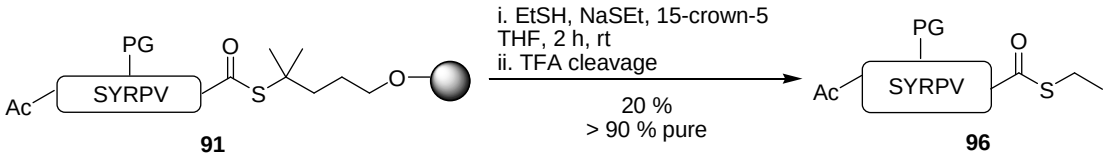


Figure 3-14. Attempted nucleophilic cleavage of the model peptide Ac-SYRPV-Set, **96**, from the solid support.

Successful nucleophilic displacement to the peptide ethylthioester was accomplished by applying an excess of ethanethiol and using sodium thiophenolate for the transthioesterification in the presence of 15-crown-5. In this manner the reaction could be controlled such that the kinetic product, the thioester could be obtained as the sole product (Scheme 3-55). It may be considered that the reaction first occurs *via* two transthioesterifications; at first the thioester is cleaved from the resin as the very active peptide thiophenyl ester which undergoes a second thioesterification to yield the peptide ethylthioester.

Scheme 3-55



Nucleophlic substitution was also attempted on a small array of different pentapeptide substrates and are summarized in Table 3-10.

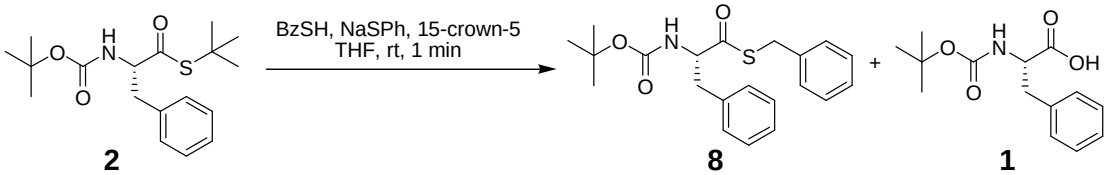
Table 3-10. Synthesized peptides cleaved using thioethanol.

entry	compound	peptide sequence	yield (%)[a]
1	96	Ac-SYRPV-SEt	20[b]
2	97	Ac-SYRGF-SEt	14
3	98	Ac-SYRGQ-SEt	26
4	99	Ac-SYRGS-SEt	16[c]

[a] Yields were derived from the isolated products and calculated based on the loadings of the first amino acid to the solid support. Purities of the crude products were determined *via* HPLC at 220 nm via UV/Vis spectroscopy to be greater > 90 % pure. [b] The yield of this compound was obtained after HPLC purification [c] The purity of this compound was 77 %.

Lengthening the reaction time in order to increase the yield led instead to a significant rise in side product formation. Accordingly, different thiols were screened in order to find optimal cleavage conditions. Applying the more reactive benzylmercaptan to the reaction in place of ethanethiol led to a more rapid conversion of the *tert*-butyl thioester to the benzyl thioester, but also with a significant amount of hydrolysis product.

Scheme 3-56. Transthioesterification of *t*Boc-Phe-S-*t*-Butyl using benzyl mercaptan.



Following the protocol of Ingenito et al.,[131] excess 3-mercaptopropionic acid methyl ester in the presence of sodium thiophenolate as base and the cryptand 15-

crown-5 was able to furnish the desired thioesterified product without trace of hydrolysis.

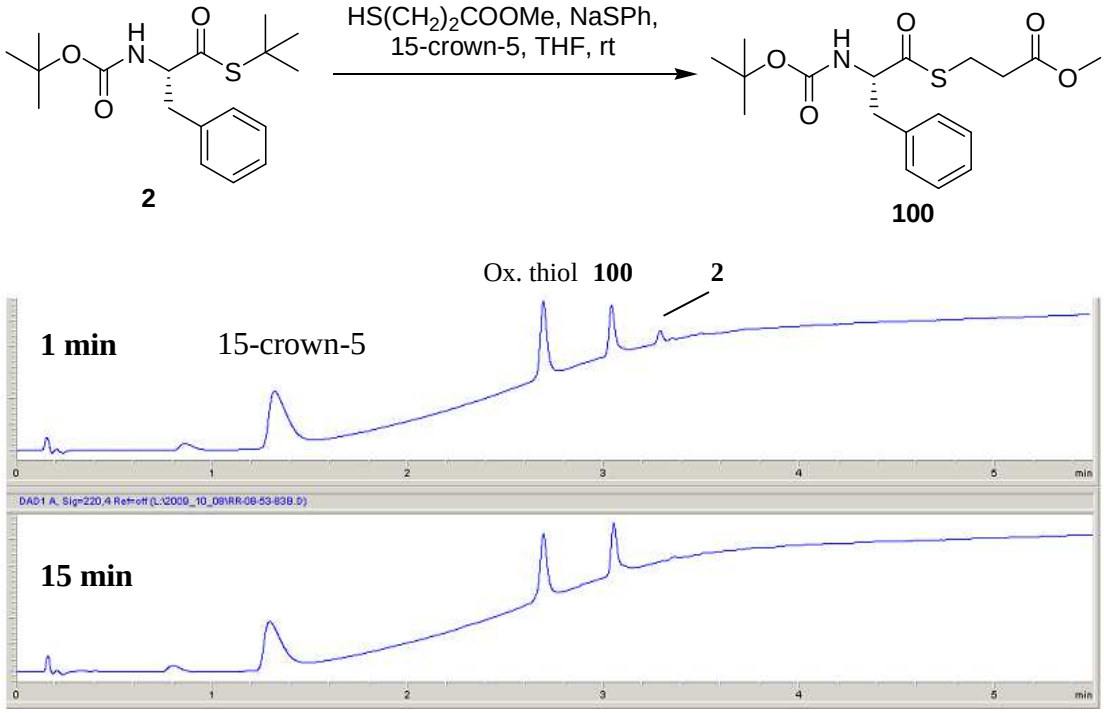


Figure 3-15. Initial test nucleophilic displacement with excess 3-mercaptopropionic acid methyl ester in the presence of sodium thiophenolate.

The reaction in Figure 3-15 was carried out in solution and was finished after 15 minutes. When applying this protocol to the model peptide system Ac-SYRGF, the corresponding peptide thioester, **101** was succesfully obtained in a 69 % yield with a purity > 90 % (Figure 3-16) after TFA cleavage and precipitation from MTBE.

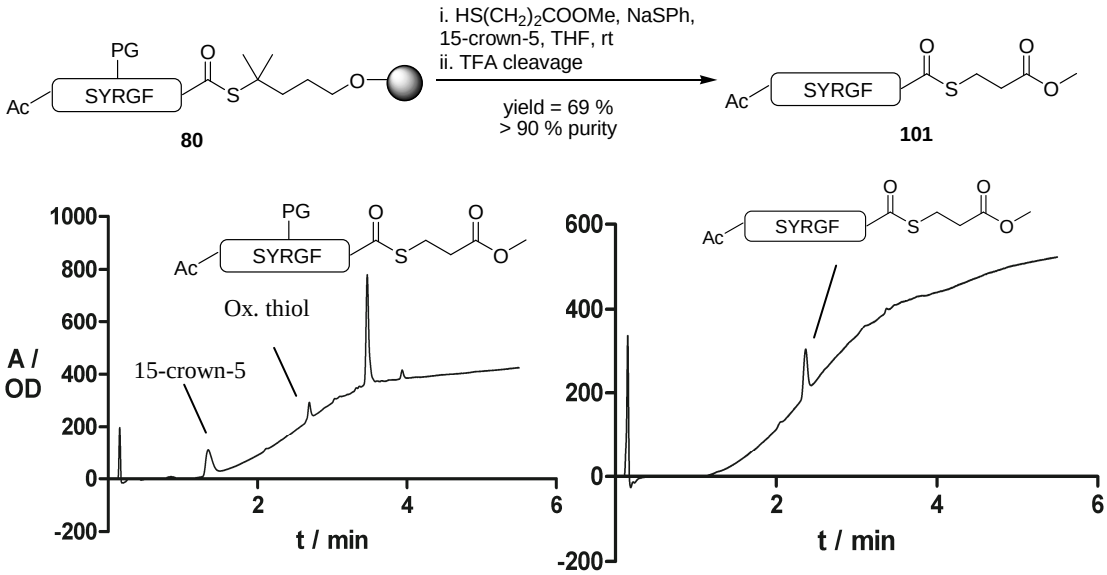


Figure 3-16. Nucleophilic displacement of the protected peptide thioester from the solid support and subsequent global deprotection with TFA.

The yield for the nucleophilic cleavage of the peptide from the resin could be optimized by applying two cycles each for two hours. This kept the cleaving time to a minimum ensuring no side reactions could take place allowing for cleaner products. The viability of nucleophilic cleavage from the linker was tested using the same model peptides previously mentioned, and the results are summarized in Table 3-11.

Table 3-11. Nucleophilic displacement yielding a variety of pentapeptide thioesters.

entry	compound	peptide sequence	yield (%)[a]
1	101	Ac-SYRGF-S$(CH_2)_2$COOMe	69/89[b]
2	102	Ac-SYRGW-S$(CH_2)_2$COOMe	78[b]
3	103	Ac-SYRGQ-S$(CH_2)_2$COOMe	86
4	104	Ac-SYRPV-S$(CH_2)_2$COOMe	67

[a] Yields were derived from the isolated products and calculated based on the loadings of the first amino acid to the solid support. Purities of the crude products were determined via HPLC at 220 nm via UV/Vis spectroscopy to be greater > 90 % pure. [b] The yields given here were obtained after coupling of the dipeptide Fmoc-Arg(Pbf)-Gly-OH to the first amino acid.

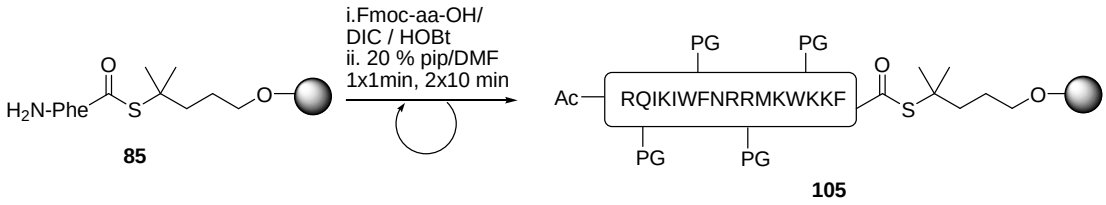
In some cases, for instance in the sequence Ac-SYRPV, the ß-elimination product of the thioester, the thioacid was obtained. This side reaction could be avoided by increasing the amount of thiol added (normally 100 equivalents were enough to suppress the side reaction).

3.2.10 Synthesis of Longer Thioester Peptides

To further test the robustness of the MMP linker, a derivative of the peptide Penetratin 1^{TM} was chosen Ac-RQIKIWFNRRMKWKKF. Penetratin 1^{TM} is a cell-penetrating peptide corresponding to the third helix of the homeodomain of Antennapedia protein.[132][133] The peptide is able to translocate across biological membranes and has been used successfully to internalize covalently attached peptides and oligonucleotides and to convey them to the cytoplasm and nucleus of many cell types.

Starting from the deprotected H_2N-Phe-MMP-resin **85**, the Penetratin 1^{TM} derivative was synthesized in a standard stepwise fashion using DIC and HOBt as condensation reagents to give the resin bound peptide **85**.

Scheme 3-57



Cleavage of the peptide from the resin was accomplished using reagent K (TFA/thioanisole/water/phenol/EDT 82.5:5:5:5:2.5 v/v) and gave a reasonably pure product with a small amount of oxidized methionine. Thioanisole in some cases has been known to suppress methionine oxidation during cleavage as well as accelerate removal of the Pbf group.[134] Observed methionine oxidation (Figure 3-17) most likely resulted during peptide chain assembly which was performed under aerobic conditions.

After HPLC purification, the peptide Ac-RQIKIWFNRRMKWKKF-MMP **106** was obtained in 67 % yield. This yield was was very satisfying since it implies very little loss due to DKP formation. Furthermore, the stability of the linker was well established and the lack of deletion or fail sequences shows how robust this method for the preparation of longer peptide thioesters can be.

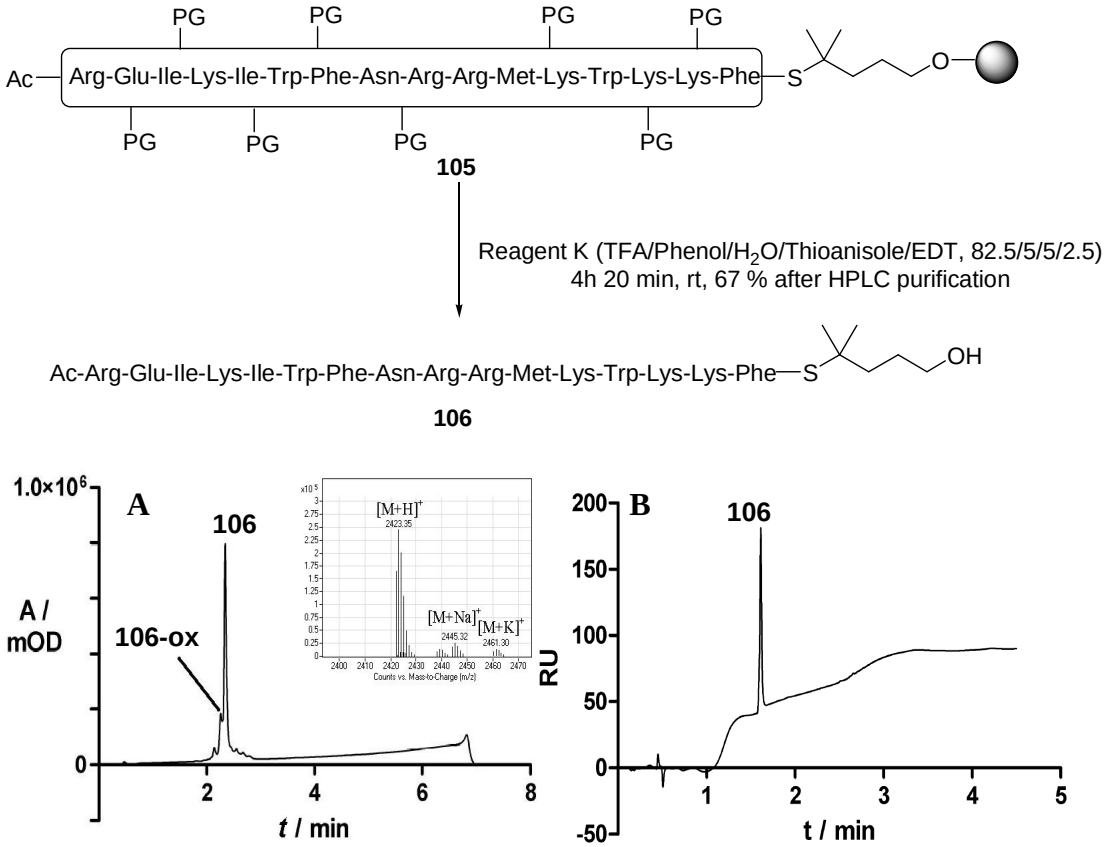
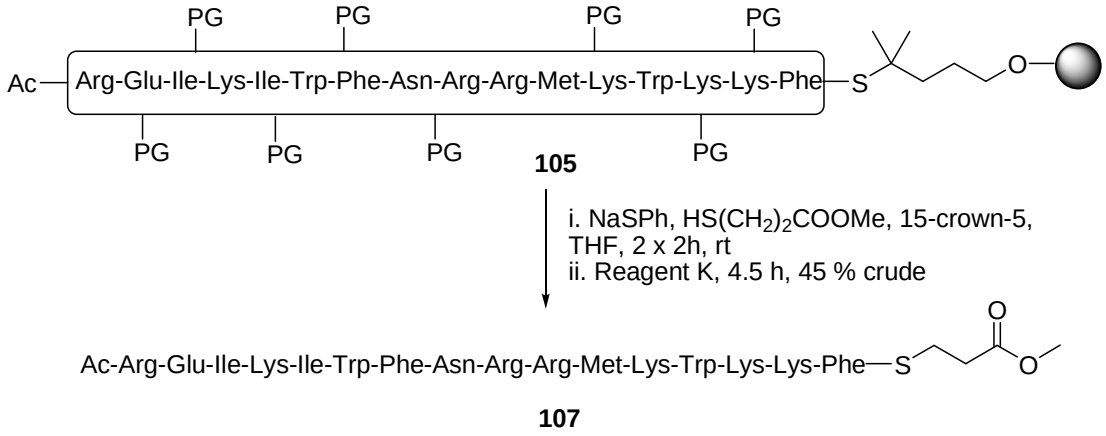


Figure 3-17. Synthesis and acidic cleavage of the Penetratin 1 peptide.A) HPLC with accompanying mass spectrum of the crude 16mer penetratin derivative **106**. A small portion of the peptide, **106-ox**, was oxidized at the methionine residue to give the sulfoxide (+16 *m*/z). B) HPLC purification gave the clean product **106**.

Likewise, the Penetratin 1^{TM} derivative could also be displaced nucleophilically using the same conditions, 3-mercaptopropionic acid methyl ester with sodium thiophenolate as and 15-crown-5. The protected peptide was obtained which was then totally deprotected using Reagent K under the same conditions described for the MMP-thioester.

Scheme 3-58



As with the MMP-thioester, the nucleophilically displaced thioester was obtained in good overall yield and reasonably high purity (Figure 3-18). Also analogous to the MMP-thioester, a small portion of the peptide was oxidized at the methionine residue to give the sulfoxide (+16 *m*/z).

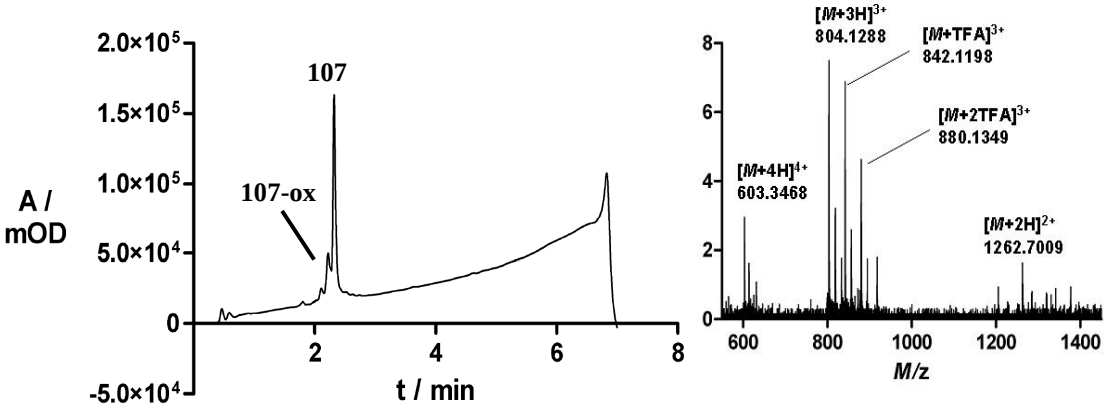


Figure 3-18. Crude chromotgram of the nucleophilically displaced Penetratin 1 peptide **107**.

3.2.11 Investigation of Epimerization of the Carboxy Terminal Amino Acid

For the further development of peptide thioesters using the MMP linker, epimerization of the α-proton at the carboxy terminal amino acid was investigated. Detailed high resolution NMR analysis of the pentapeptide Ac-SYRGF-MMP was carried out with 1H, TOCSY, HMQC, HMBC.

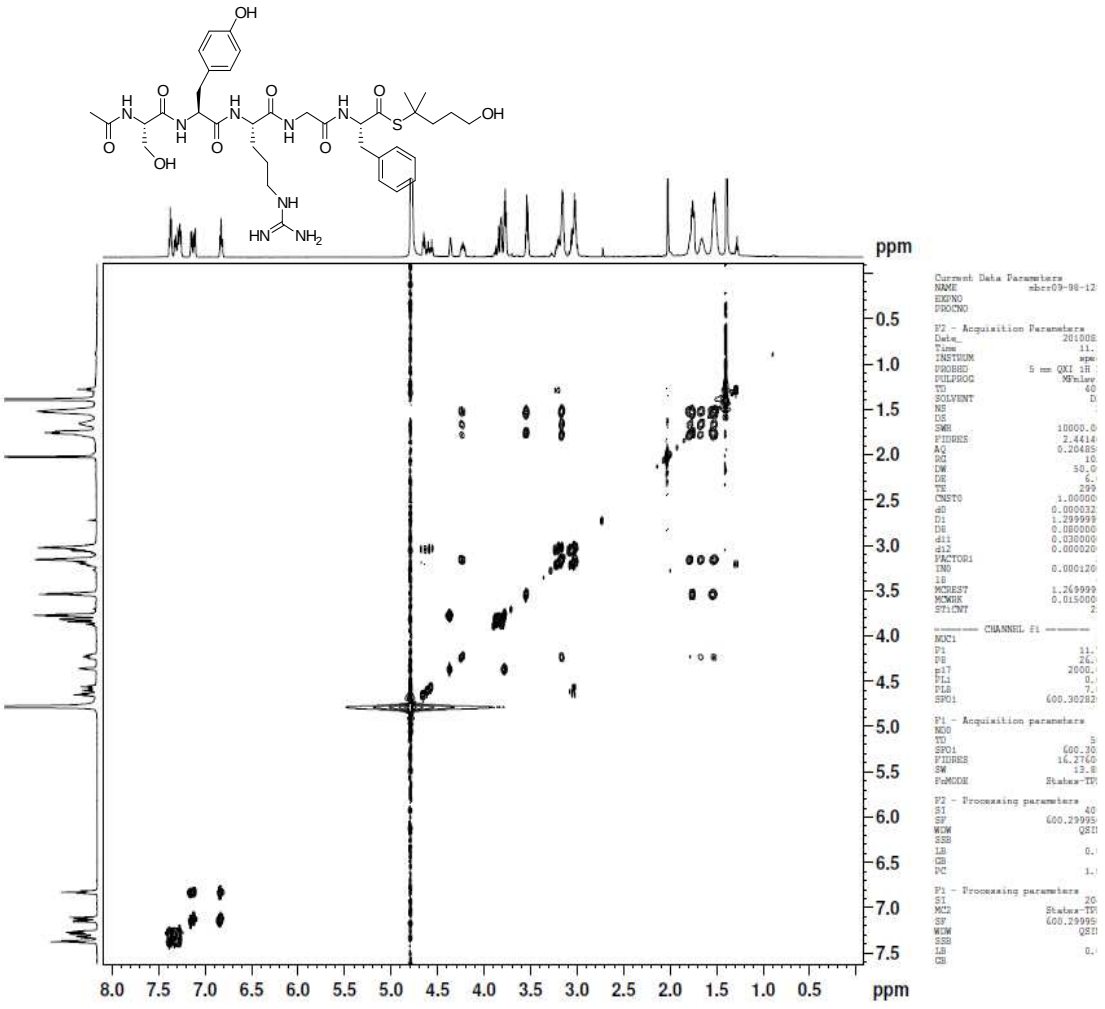


Figure 3-19. TOCSY spectrum of the peptide Ac-SYRGF-MMP **81**.

Proton correlation of all peaks from the pentapeptide could be assigned from the TOCSY experiment. The α-protons of the amino acids were then assigned as follows: the $^{\alpha}$Tyr proton comes at 4.65 ppm, the $^{\alpha}$Ser proton comes as a multiplet between 4.34-4.38 ppm, the $^{\alpha}$Arg proton at 4.23 ppm and the two $^{\alpha}$Phe protons at 4.56 and 4.60 ppm. Integration of the α-protons in Figure 3-20 via 1H NMR gave almost

complete racemization. However, the coupling of the first amino acid to the resin for the synthesis of the displayed peptide was carried out under racemizing conditions using EDC/DMAP.[135]

The synthesis was performed once again coupling the first amino acid as its acid fluoride, Fmoc-Phe-F, along with DIPEA as base. These conditions have been described by Carpino as being non racemizing.[113] ^{1}H NMR analysis of the pentapeptide under the modified conditions also gave rise to strong racemization at the $^{\alpha}$Phe with a ratio of 0.55:0.45 L:D (Figure 3-20).

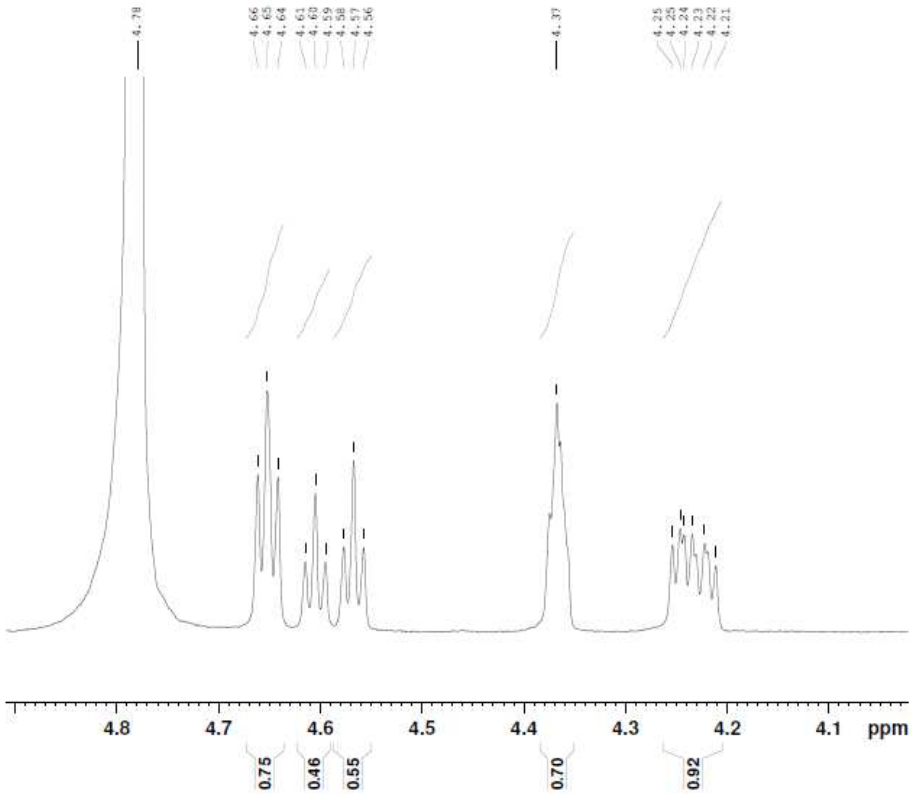


Figure 3-20. NMR racemization studies of the synthesized pentapeptide Ac-SYRGF after cleavage from the solid support.

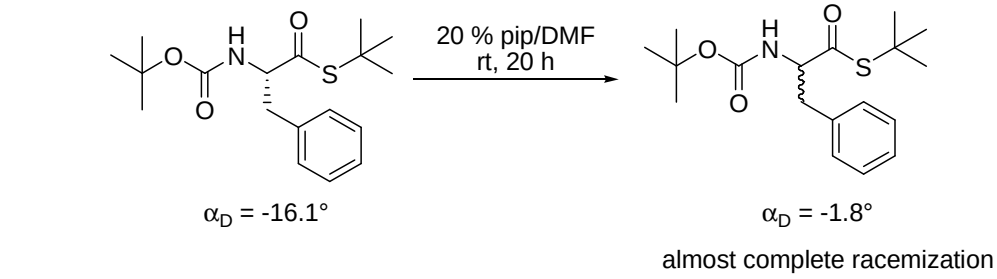
It can be inferred from this data that the racemization occurs during peptide assembly. Thioesters undergo racemization of the α-position during prolonged exposure to base. It is evident from this result that piperidine in DMF represent strong enough basic conditions to induce racemization of the α-position. This result also confirms previously described reports of similar thioesters.[136]

In order to confirm these results, Boc-Phe-*S*-*tert*-Butyl **2**, prepared from commercially available Boc-Phe-OH using CDI, and the thioester was obtained in a

reasonable optically pure yield (α_D = -16.1°). The thioester was then treated with 20 % piperidine in DMF for 20 hours under standard SPPS conditions. After removal of the piperidine and DMF, the compound was analyzed via optical rotation and found to be almost completely racemized with an α_D = -1.8°.

These results indicated that the α-proton of the thioester is considerably more acidic than those of their analogous carboxy esters. Indeed these results are in accordance with dynamic kinetic resolution experiments carried out in the group of Drueckhammer.[137] Regarding the mechanism, upon base treatment of the thioester, the α-proton is deprotonated yielding the enolate which upon reprotonation yields the racemized product (Scheme 3-59).

Scheme 3-59



Complete racemization of the carboxy terminal amino acid will result upon repeated exposure of the growing peptide thioester with piperidine. A possible solution to this problem is to use milder deblocking reagents to remove the Fmoc group without disturbing the carboxy terminal amino acid. The group of Saburo Aimoto[88][138] has reported that racemization levels are reduced when applying a cocktail of 1-methylpyrrolidine, hexamethyleneimine and HOBt in a DMSO-DMF (1:1, v/v) solution. Due to their more mild nature, such cocktails do not always quantitatively remove the Fmoc group.[90][139][87] As a result, longer exposure to the deblocking cocktails are required coupled with repetitive deprotection cycles in order to prevent loss of yield due to deletion sequences. To reduce racemization of the

carboxy terminal amino acid using these milder deblocking conditions, Aimoto and his group had to implement the highly base sensitive Fmoc(2-F) group for α-amino protection.[89] This also limits this method since the established Fmoc-SPPS applies the standard Fmoc group for α-amino protection.

3.2.12. Investigation of S→ O Transesterification at the Carboxy Terminus

Conservation of the thioester after acidic cleavage from the resin was analyzed by high resolution NMR 1H, HMBC, and HMQC experiments. The methylene phenylalanine proton gave a cross signal to a thioester carbonyl ^{13}C peak appearing at 205 ppm, indicating retention of the thioester. The racemized α-protons, however, give a set of extra carbonyl cross peaks indicating the presence of another species (Figure 3-21).

HMQC experiments reveal (Figure 3-21) that the racemized α-protons of the phenylalanine residue couple to a single carbon atom. It is therefore unlikely that a second peptide is present in the sample, for instance a truncated peptide, or the thioacid. This also supports the HPLC trace and mass experiments which show a single isolated product. It is reasonable that the thioester undergoes to a certain degree a transesterification at the C-terminus.

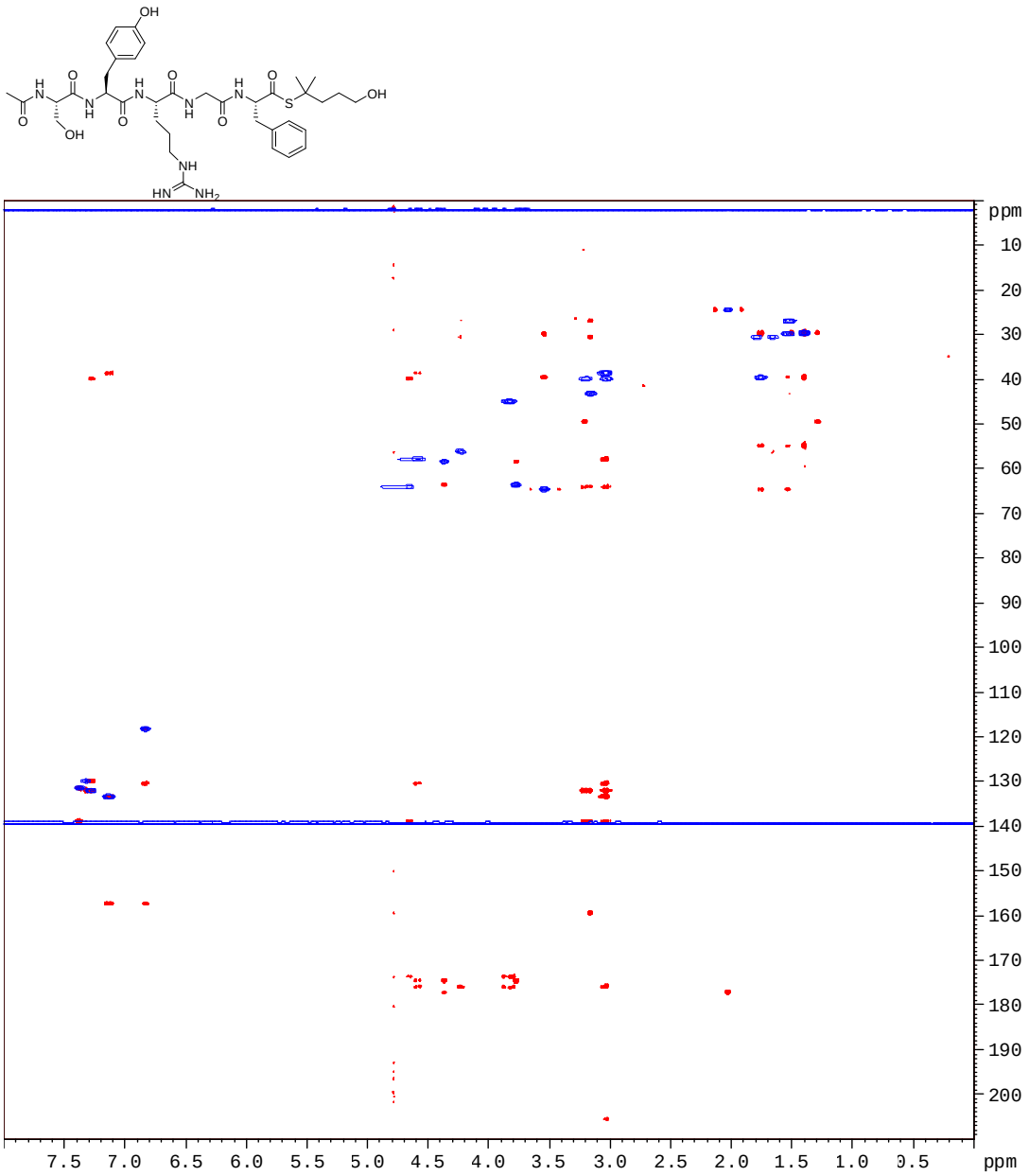


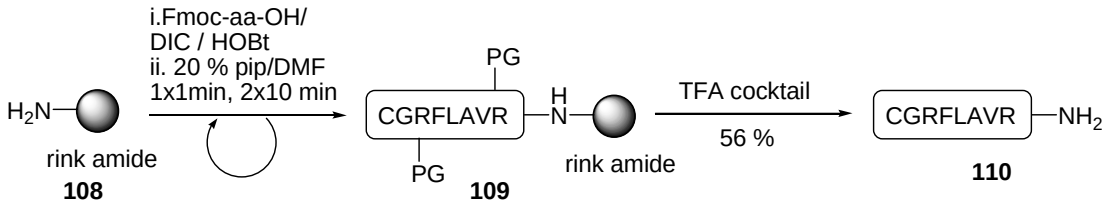
Figure 3-21. Overlapped HMBC and HMQC experiments. 600 MHz spectra of peptide **81**: overlay of HMBC (red) and HMQC (blue) spectra. Diagnostic signals include the cross-signal of the Phe-β-proton at 3.02 ppm coupling to the thiocarbonyl group at 205.5 ppm.

3.3. NCL reactions

3.3.1. NCL of the Nucleophilically Substituted Peptide Thioesters

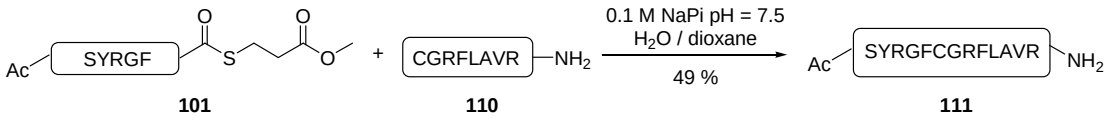
The nucleophilically displaced peptide thieosters were obtained in good yields as previously shown. In order to fortify the MMP linker method for the synthesis of peptide thioesters, the nucleophilically displaced peptide thioester Ac-SYRGF-$S(CH_2)_2COOMe$ **101** was applied in the NCL reaction. As a test peptide substrate, CGRFLAVR-NH_2 **110** was synthesized using a rink amide resin under standard Fmoc SPPS conditions (Scheme 3-60).

Scheme 3-60



The crude model peptide **101** was taken as prepared without purification and then reacted with the cysteine peptide **110** in a sodium phosphate buffer solution (pH = 7.5) mixture with dioxane to completely effect dissoltion of both peptides.

Scheme 3-61



The reaction went to completion, but only after many hours which is in accordance with the slow nature of ligation of peptide thioester with phenylalanine at the carboxy terminus.[114] After purification over a semi-preparative column, the ligated peptide was obtained in 49 % yield (Figure 3-22).

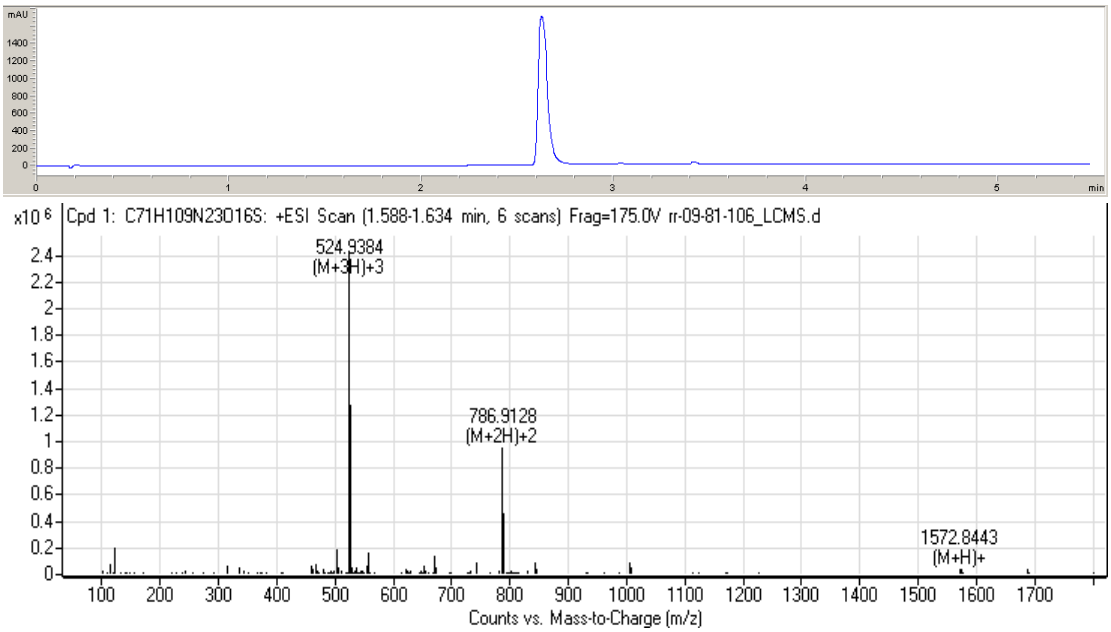
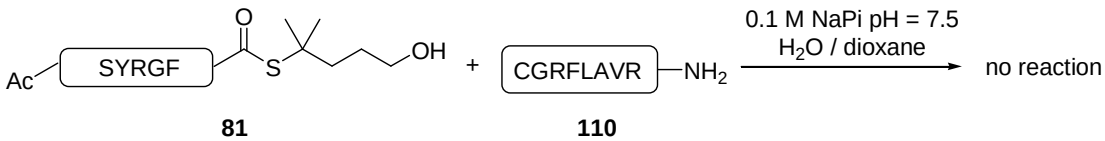


Figure 3-22.-NCL ligation product, **111** of the nucleophilically displaced Ac-SYRGF peptide **101** and cysteine peptide **110**, after HPLC purification.

3.3.2. NCL of the MMP Peptide Thioesters

It was demonstrated that MMP peptide thioesters can be achieved in high yields and purities after acidic cleavage from the solid support. Furthermore, the crude peptides were clean enough for use in other transformations. In this manner, the utility of the MMP peptide thieosters was then tested in the Native Chemical ligation. The ligation between the MMP thioesters was attempted with the model cysteine peptide **110**. Initial attempts of the MMP thioester Ac-SYRGF-MMP **81** with the cysteine peptide **110** were unsuccessful; even after 24 hours of reaction time, only the starting material was recovered.

Scheme 3-62



81 **110**

Even with addition of thiophenol as catalyst, the reaction also did not take place. The aquous milieu at pH 7.5 was not basic enough to effect the first

transthioesterification step to the activated aryl thioester. From this experiment, it was apparent that the *tert*-butyl group which afforded the stability to the thioester, was also responsible for its low reactivity in the transthioesterification step of the NCL reaction. This result also further reinforces the decreased reactivity of alkyl thioesters in comparison to aryl thioesters for the NCL reaction demonstrated by Dawson et al. in 1997.[62]

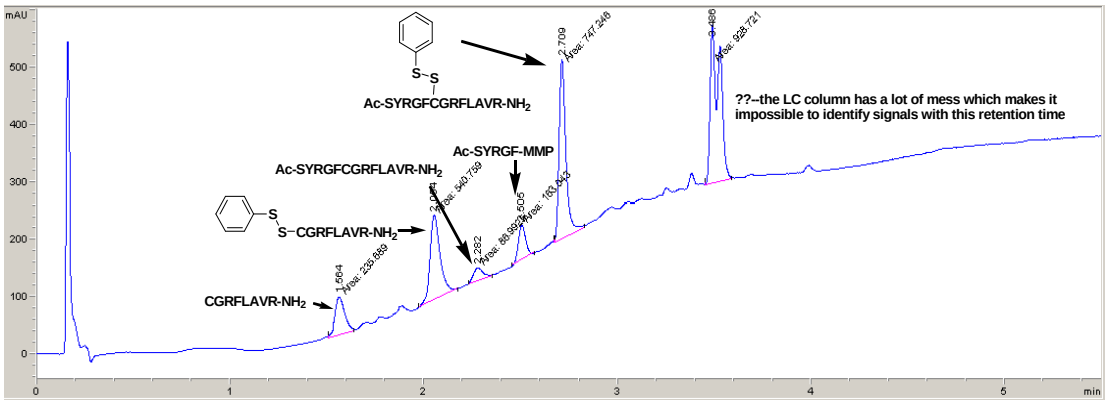
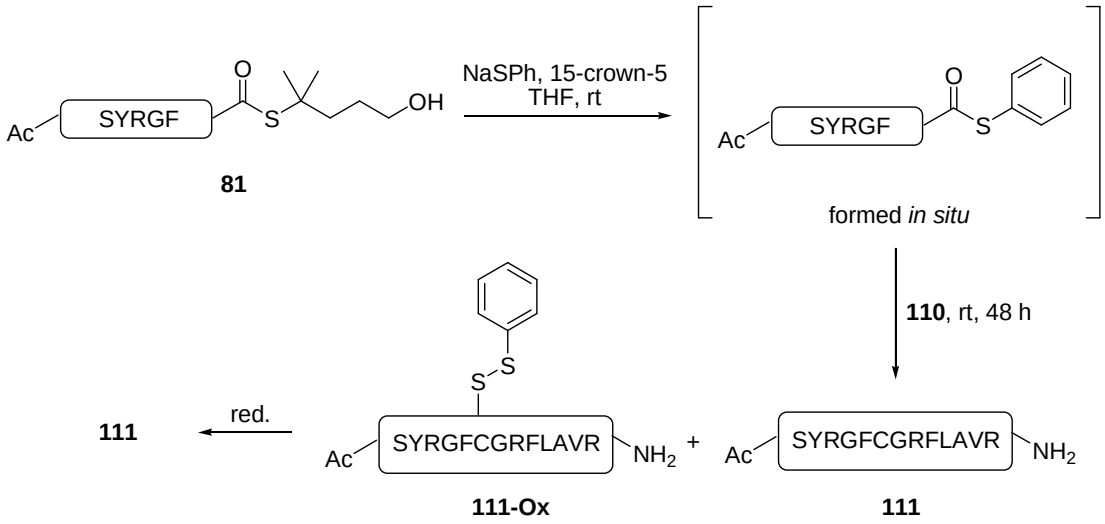


Figure 3-23. Crude spectrum of the NCL reaction between the model peptide Ac-SYRGF-MMP and cysteine peptide CGRFLAVR-NH_2 carried out in dry THF with sodium thiophenolate as activating agent.

In order to make the MMP thioester more active, the same principle for the nucleophilic displacement reaction discussed previously was utilized for this reaction. Using sodium thiophenolate and 15-crown-5 in THF, the MMP thioester **81** was activated *in-situ* to the more reactive phenyl thioester. After addition of an excess of the cysteine peptide **110** the ligation product was obtained after 48 hours as a mixture of the cysteine oxidized ligation and the native ligation product. Reduction of the assymetric disulfide bond with ß-mercaptoethanol[140] and subsequent HPLC purification gave the ligation product **111** in a 44 % yield.

Scheme 3-63



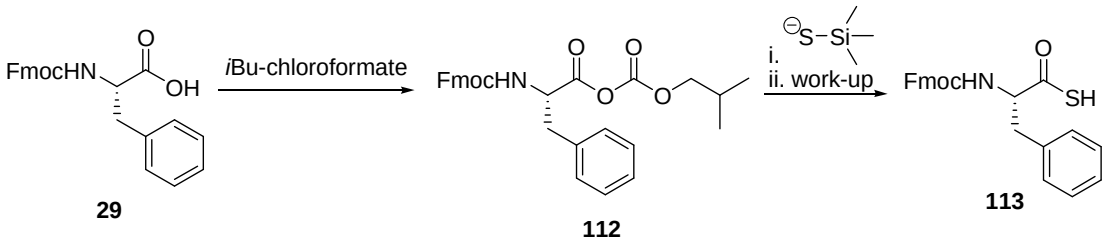
In summary, *tert*-butyl thioesters show a remarkable stability in basic conditions. Extrapolating this idea to the synthesis of a linker, the MMP-linker could be obtained quickly and efficiently in three steps from the commercially available 4-methylpent-3-en-1-ol. The MMP linker proved to be stable in solution when treated with 20 % piperidine/DMF having a half-life when attached to Boc-Phe-OH of greater than 9 days. It could then be used for the preparation of protected and unprotected peptide thioesters displaying a broad range of sequences. The products could be directly used in native chemical ligations. This approach for the synthesis of peptide thioesters may also find utility for building blocks used in other chemical ligation reactions including auxiliary methods and the traceless Staudinger reaction.

4. Solid Phase Synthesis of Peptide Thioacids using the MMP linker

4.1. Current methods for the synthesis of thioacids

Thioacids are an interesting and overlooked class of compounds with a unique reactivity. This stems from the pronounced nucleophilicity of their conjugate base partners which tend to react selectively with α-haloacetic acid derivatives in aqueous media at pH 3-6.[141] Preparation of thioacids has been achieved classically by activating a carboxylic acid to the active ester followed by nucleophilic displacement with H_2S or some other hydrosulfide equivalent such as sodium sulfide, Na_2S. Fmoc protected thioacids have also been prepared by the group of Williams via formation of asymmetric anhydrides followed by nucleophilic displacement with trimethylsilylthiolate (Scheme 4-1).

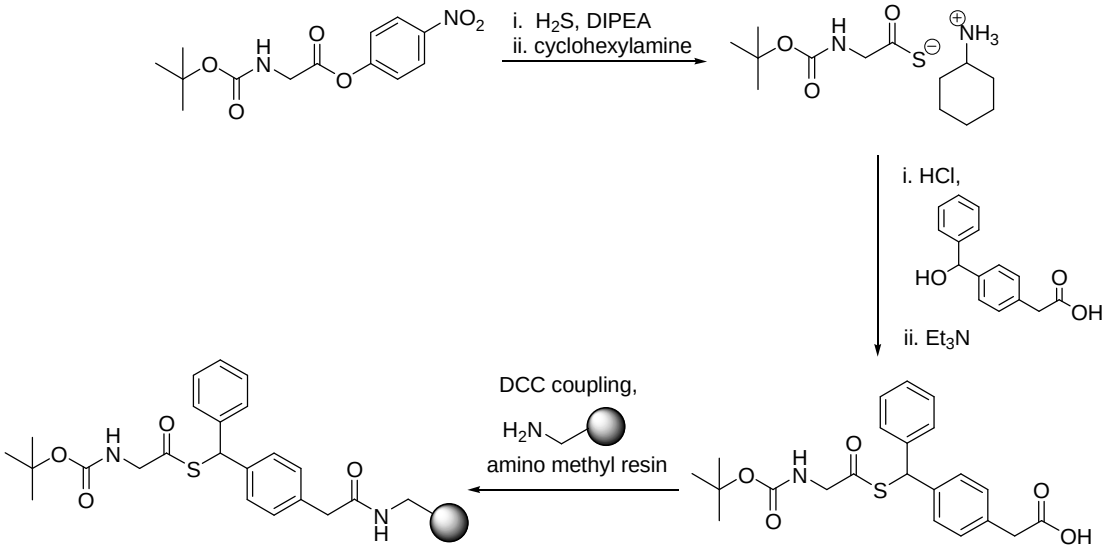
Scheme 4-1



29 **112** **113**

Peptide thioacids did not really come into vogue until their implementation as useful building blocks for the ligation between unprotected peptides with the goal of larger peptide and protein synthesis. In the early 1980s, Blake et al. devised a method for obtaining peptide thioacids by first converting Boc-Gly-OH into its corresponding thioacid and coupling it to an acid labile linker: α- (4-carboxymethylphenyl)-benzenemethanol using concentrated HCl followed by coupling with triethyl amine. The in-solution synthesized amino acid-linker was then attached to an amino methyl resin (Scheme 4-2) giving the 4-[α-(Boc-Gly-S)benzyl]phenoxyacetamidomethyl resin.[142]

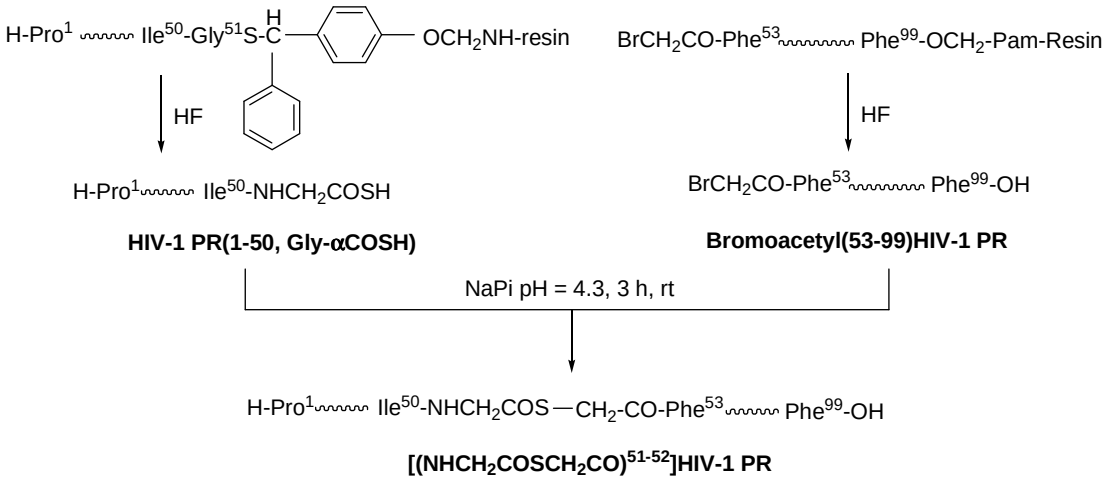
Scheme 4-2



Standard Boc-SPPS methodology followed, and the preparation of a peptide thioacid fragment of an analog of human ß-endorphin (ß-EP) was attained. Final synthesis of the endorphin analog was accomplished using Ag^+ mediated coupling with N-hydroxysuccinimide to link two segments of the endorphin peptide together. The problem, however, with this route is the need to develop a linker resin for each individual amino acid at the carboxyl terminus (Scheme 4-2) which is time consuming and not optimal. This problem was solved in the group of Kent, by introducing the thiol functionality with thiourea followed by hydrolysis with NaOH. After optimizing the linker, the method culminated in the synthesis of an analog of HIV-1 PR by ligating two unprotected peptide fragments: HIV-1 PR (1-50, GlyαCOSH) with [$BrCH_2CO$(53-99)HIV-1 PR forming a thioester bond where normally a Gly-Gly linkage would be in the native protease (Scheme 4-3).[52]

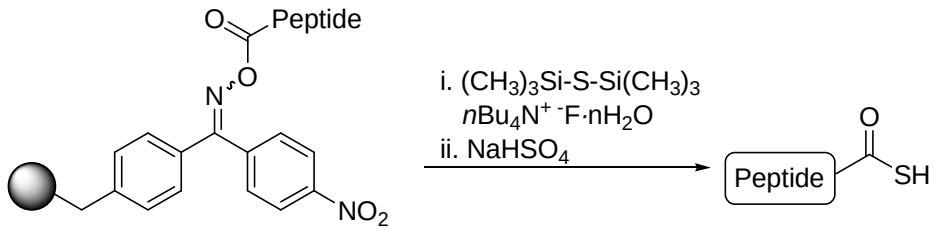
The ligation reaction between a thioacid peptide fragment and the bromoacetyl peptide fragment was conducted in a sodium phosphate buffer of pH 4.3 with 6M guanidine hydrochloride (GuHCl). The ligation reaction was carried out at room temperature and was essentially complete after 3 hours. Despite this slight alteration, the HIV-1 PR analog showed the same activity as the wild-type enzyme.[52]

Scheme 4-3



Another method of circumventing the problem of having to prepare different resins for each C-terminal amino acid involves the use of the Kaiser Oxime resin.[143] This approach showed for the first time preparation of small peptide thioacids by nucleophilic displacement from the solid support using an H_2S equivalent, specifically lithium trimethylsilylsulfide in THF developed by Kraus.[144] Based on earlier work of Kaiser, Schwabacher and Maynard synthesized a variety of single and dipeptide thioacids (Scheme 4-4).[145] Racemziation studies for the preparation of thioacid peptides using this method has not been established.

Scheme 4-4

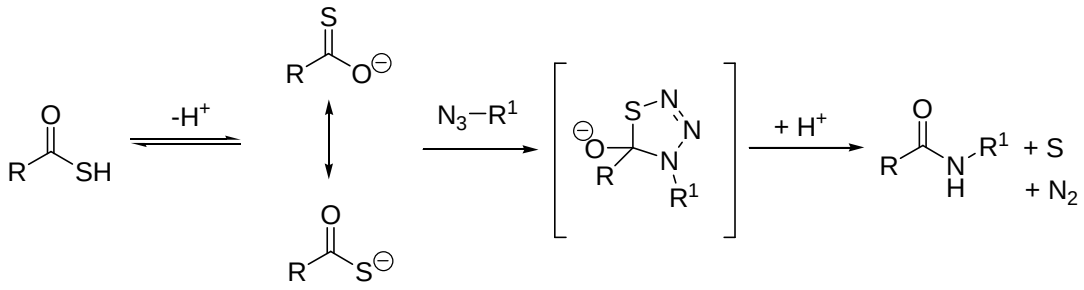


Preparation of peptide thioacids has also been performed by first synthesizing the peptide with a carboxylic acid at the C-terminus using standard Fmoc methods, and then transforming the carboxylic acid into the thioester which can be hydrolyzed with sodium sulfide to the thioacid.[146] Other novel approaches to the synthesis of peptide thioacids include the application of an Fmoc-linker combined with Boc-SPPS

which allows release of peptide thioacids from the solid support by piperidine thereby avoiding the more toxic HF cleavage approach.

Among the many applications for thioacids, the reduction of azides in the presence of thioacids regained attention in the previous years. In 1980 while investigating the reactivity of potassium thioacetate, Just and Hakimelahi found that upon prolonged exposure of potassium thioacetate in the presence of azides, acetamides were produced as a by-product.[82] The reaction was more closely examined eight years later when Rosen et al studied the reduction of azides with an excess of thioacetic acid. Finally, in 2003, the group of Williams and coworkers reinvestigated the reaction of thioacids and azides and found that the formation of the amide results from an anion-accelerated [3+2] cycloaddition followed by a retro-[3+2] cycloaddition.

Scheme 4-5



Since this reexamination and the discovery that thioacids react very rapidly with sulfonylazides to give sulfonamides the reaction has been exploited by many groups interested in using this chemistry for a variety of different ligations.[83][147] Accordingly, it has been given its own ligation terminology ranging from ‘thioacid/azide ligation (TAL)’ to ‘Sulfo-click’.

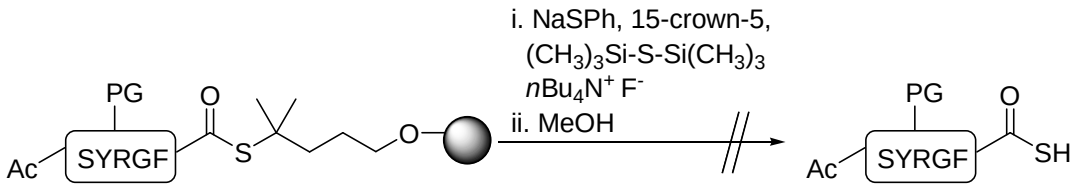
The *tert*-butyl thiolinker proved to be a reliable system for the preparation of peptide thioesters which could be cleaved from the solid support *vida supra*. Following this reasoning, the *tert*-butyl thiolinker should be applicable for the efficient prepparation of peptide thioacids using standard Fmoc SPPS methods.

4.2. Synthesis of peptide thioacids

4.2.1. Initial thiodisplacement experiments

The nucleophilically displaced thioesters from the previous chapter gave encouraging preliminary results implicating that hydrothiolysis of the *tert*-butyl linker should also be a plausible route towards the synthesis of peptide thioacids. The initial conditions for the experiment were adapted from the thioester synthesis in which the sodium thiophenolate and 15-crown-5 are added to effect the initial transthioesterification product. However, the attempt to capture the model peptide thioacid from the activated Ac-SYRGF-MMP-(resin) with hexamethyldisilathiane (as shown for the preparation of peptide thioacids using the Kaiser Oxime resin) did not yield the desired peptide thioacid as shown in Scheme 4-6.

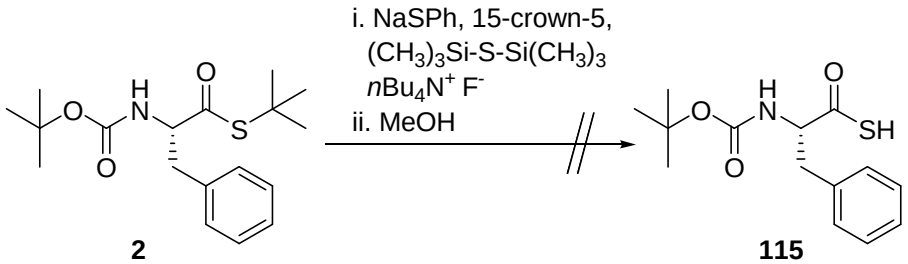
Scheme 4-6



80 **114**

Analogous to previous experiments, in-solution tests were carried out with the model Boc-Phe-*S*-tert-butyl compound and did not give the corresponding thioacid when treated with hexamethyldisilathiane.

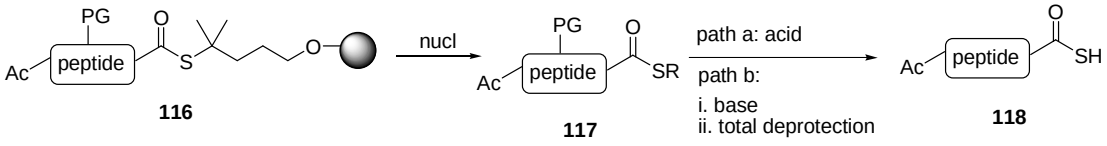
Scheme 4-7



2 **115**

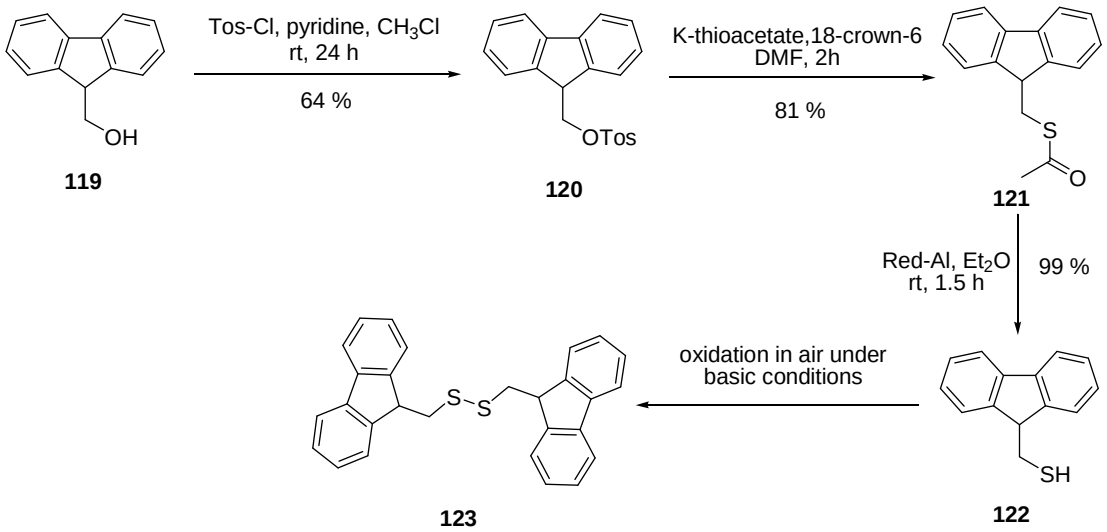
Other H_2S equivalents as mentioned were used in order to effect the thio hydrolysis of thioesters.[146] Na_2S, or NaHS however are not suitable for standard SPPS conditions owing the poor swelling nature of polystyrene in water or alcohols. Instead, a less direct method for preparing peptide thioacids was considered whereby the peptide thioacid could be generated from a thioester. In this route (Scheme 4-8), the C-terminal thioacid protecting group R, can be cleaved either with base or acid.

Scheme 4-8



Cleaving acidically via path a, will give the unprotected peptide in one step. Peptide thioacids however, may be more prone to acidic hydrolysis than their carboxylic acid analogs due to the thiol being a much better leaving group than the alcohol. Deprotection under basic conditions is superior despite the extra step of deprotecting the amino acid side chains with acid, since the thiolate HS^- is a poor leaving group.

Scheme 4-9

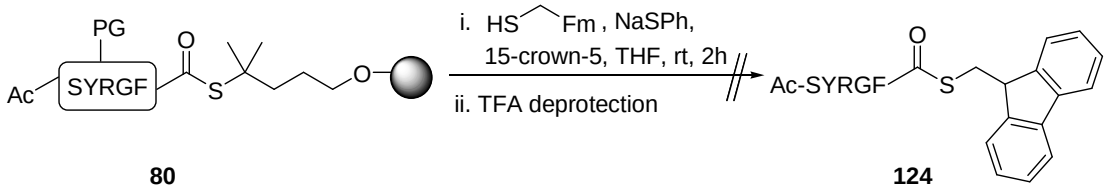


The Fm linker established by Crich for the preparation of peptide thioacids is a good starting point.[141][148] The commercially available Fm alcohol was first tosylated

with pyridine in chloroform to give compound **120**. The thiol was then introduced using potassium thioacetate and 18-crown-6 to furnish the protected Fm-thiol **121** in 81% yield. The thiol was then reduced using Red-Al in Et_2O to give the final compound **122**. The preparation of the Fm thiol, however, has a tendency to readily oxidize to the disulfide (Scheme 4-9).

An initial test using this thiol did not give the desired peptide thioacid using the established nucleophilic displacement conditions (Scheme 4-10). It was assumed that applying the basic conditions necessary for the nucleophilic displacement led to severe oxidation of the S-Fm group to the disulfide. The recovered material did not correspond to either the thioester or desired thioacid.

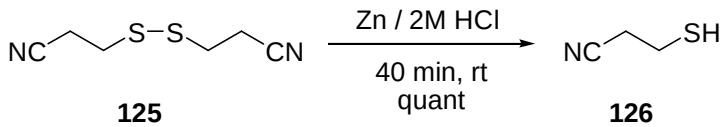
Scheme 4-10



80 **124**

Considering the necessary synthesis steps for the thiol as well as its problem with oxidizing to the disulfide, a new thiol nucleophile was investigated. The cyanoethyl protecting group for carboxylic acids was a useful alternative to introduce the thiol functionality which hopefully should be able to be cleaved under mild conditions after introduction. Mercaptopropionitrile is commercially available as its more stable disulfide form, 3,3'-dithiobis(propionitrile), which can be reduced in the presence of zinc powder and 2M HCl (Scheme 4-11).[149]

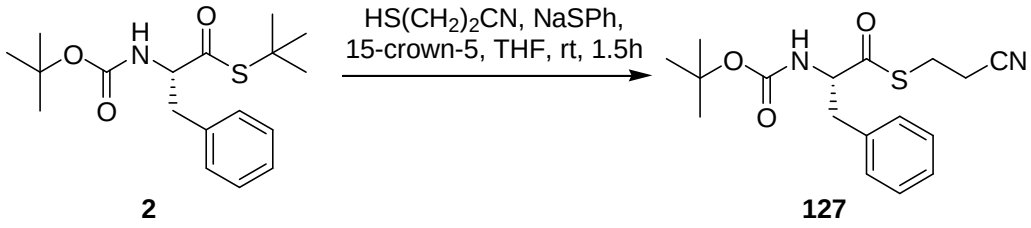
Scheme 4-11



125 **126**

In a solution test, the thiol **126** was then used to transthioesterify Boc-Phe-*S*-*tert*-Bu under the same conditions previously described for nucleophilic displacement of the peptides from the solid support.

Scheme 4-12



2 **127**

The reaction was analyzed qualitatively again via LC-MS to determine the feasibility of the reaction using large peptide substrates. As shown in Figure 4-1 of the crude reaction mixture, the tBu thioester was converted to the cyanoethyl thioester **127**. The first peak eluting at 2.69 minutes has the mass of 221 and 243 which correspond to the cryptand 15-crown-5 and its sodium coordinated salt.

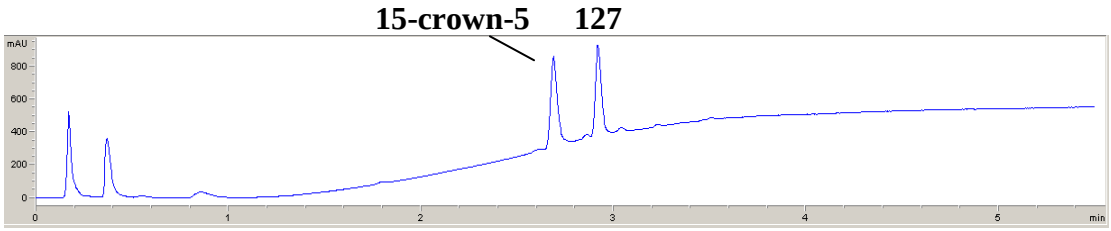


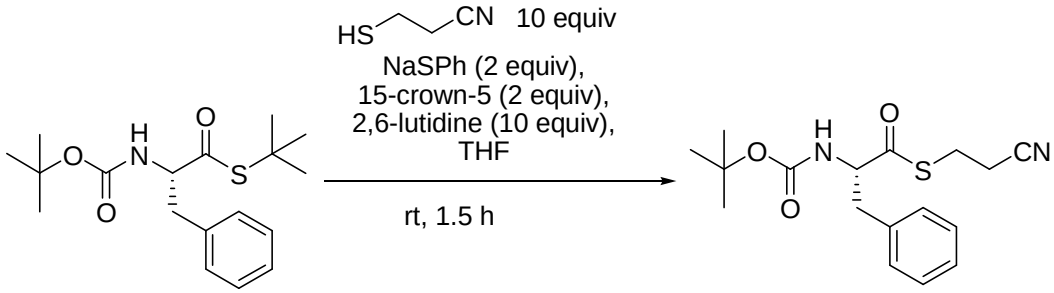
Figure 4-1. Converstion of the *t*-butyl thioester to the ethylcyano thioester.

Deprotection of the 2-cyanoethyl protecting group for carboxylic acids has been reported by many groups in the past. Cleavage has been effected using various reagents including TBAF[150], alkali bases[151] and sulfide bases[152] presumably all following a similar ß-elimination mechanistic pathway. These deprotection methods all afford the carboxylic acid, and this concept was adapted for the preparation of thioacids. Basic deprotection of the cyanoethyl group from the thioacid should as previously mentioned, give a stable thioacid product. Additionally, the thioacid in basic milieu can be directly ligated with azides.

The ligation of thioacids and azides to give sulphonamides was found to proceed optimally under basic conditions using 2,6-lutidine.[153] Unmasking the thioacid under the same conditions as the ligation reaction would elegantly afford the sulphonamides in a one step approach. Furthermore, if the deprotection of the ethylcyano group can be effected with 2,6-lutidine, then it may be possible to achieve first the transthioesterification followed by base elimination to the thioacid.

The Boc-Phe-*S*-*tert*-Bu thioester was reacted with thiopropionitrile under the previous conditions along with 2,6-lutidine to effect ß-elimination to the thioacid product. However, only, the ethylcyano thioester was recovered.

Scheme 4-13



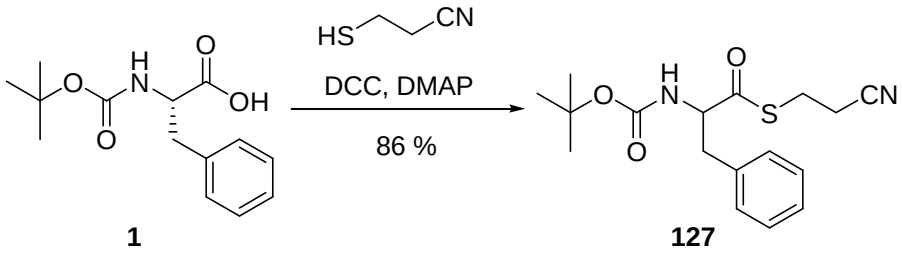
2 **127**

This result was nevertheless encouraging as it indicated that the the transthioesterification can still proceed smoothly with mercaptopropionitrile. This proof of principle experiment showed that at least, such thioesters can be produced which may serve as useful peptide thioacid precursors.

4.2.2. Solution-phase Optimization for the Deprotection of the 2-Mercapto Propionitrile Group

The one-pot multi step procedure to convert a *tert*-butyl thioester to a thioacid left room for improvement. Specifically, it was necessary to examine the ß-elimination procedure more carefully. It was important to Thusly, mercaptopropionitrile was then reacted with Boc-Phe-OH using DCC and DMAP to make the corresponding thioester for further solution tests.

Scheme 4-14



1 **127**

The reaction of **127** with mild bases DIPEA and 2,6-lutidine did not give the ß-elimination to the desired thioacid. Stronger basicity was required to effect elimination of the 2-cyanoethyl group. Reacting **127** with DBU in DMF gave complete conversion of the thioester, according to LC-MS evaluation. Unfortunately, significant hydrolysis to the carboxylic acid product was also observed.

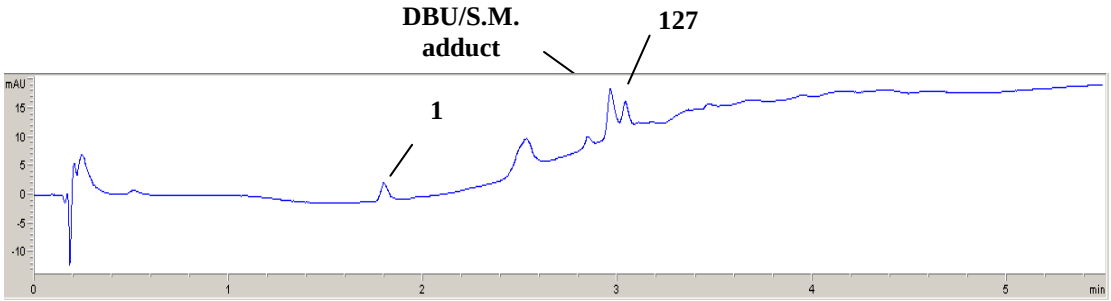
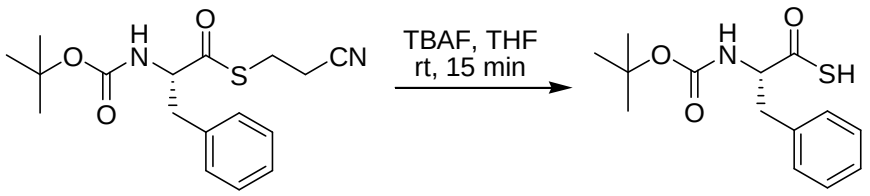


Figure 4-2. Initial deprotection attempt of the 2-cyanoetyl group mediated by DBU.

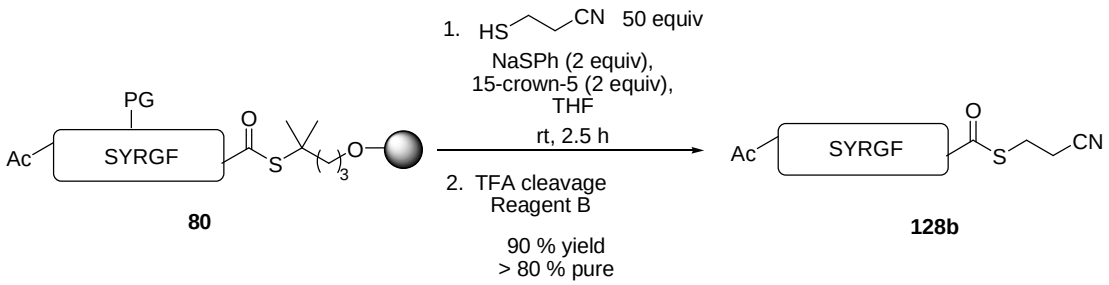
The deprotection of the 2-cyanoethyl group has also been described with TBAF.[150] Kita and coworkers have shown that selective cleavage of the 2-cyanoethyl carboxylic ester was accomplished in the presence of acetate, THP ether,

methoxd-methyl (MOM) ether, benzyl ester, TMSE ester, and benzyl carbamate. Thus, the 2-cyanoethyl group was cleaved with TBAF in THF to give the thioacid with a small amount of impurities. The reaction was carried out in dry DMF and gave the furnished thioacid relatively cleanly after only 15 minutes.

Scheme 4-15



Preparation of the 2-cyanoethyl pentapeptide thioester was accomplished in similar fashion to the previously described. An excess of 50 equivalents of the thiol was reacted together with sodium thiophenolate and 15-crown-5 in THF to afford the protected pentapeptide. A portion of the peptide was then deprotected using TFA to give the unprotected pentapeptide (Figure 4-4).



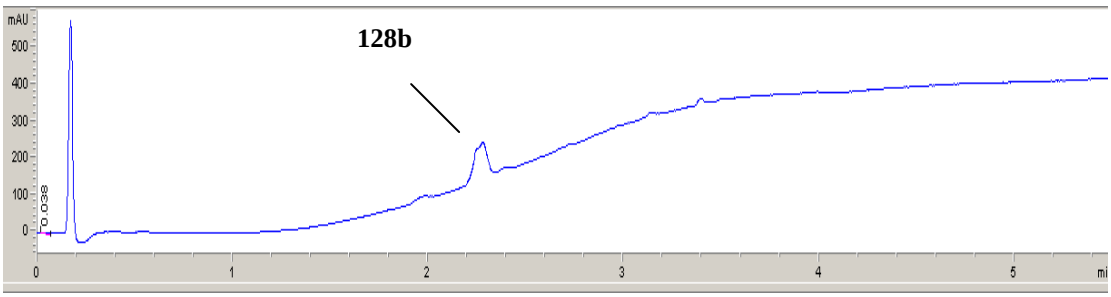
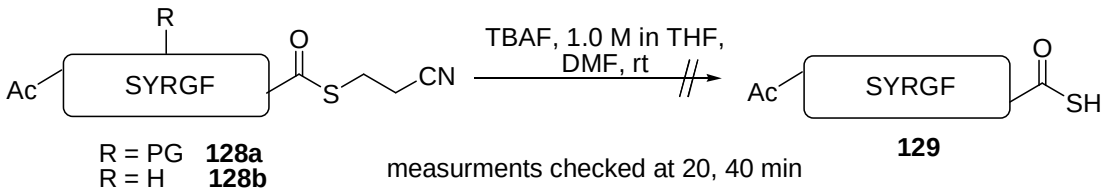


Figure 4-4. Reaction scheme and crude HPLC chromatogram of pentapeptide **128b**.

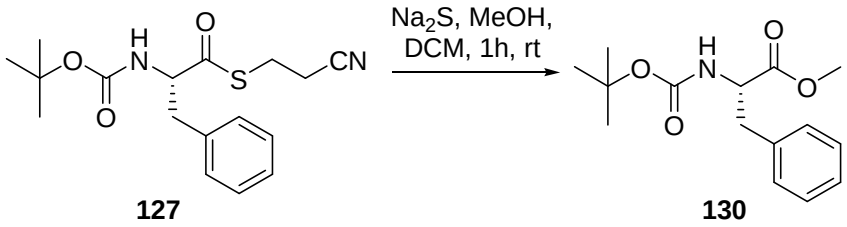
Due to the reactivity of TBAF and possible side reactions involving the fluoride anion, both the protected and unprotected peptides were further reacted. Unfortunately, neither deprotection method gave the peptide thioacid.

Scheme 4-16



Although the fluoride deprotection method worked well with amino acids to deprotect the 2-cyanoethyl group, its failure with peptides underscored the need to apply more standard methods. Sodium sulfide was the first attempt and the reaction was performed with a slight excess of the base in comparison to the Boc-Phe-*S*-cyanoethyl ester in methanol which was used due to low solubility of the sulfide in other organic solvents.

Scheme 4-17



The thioester was converted in one hour, however the recovered product was not the expected thioacid but rather the methyl ester (Scheme 4-17). LC-MS and NMR analysis confirmed the presence of the methyl ester (Figure 4-5) with a characteristic shift at 3.7 ppm and integration of three protons.

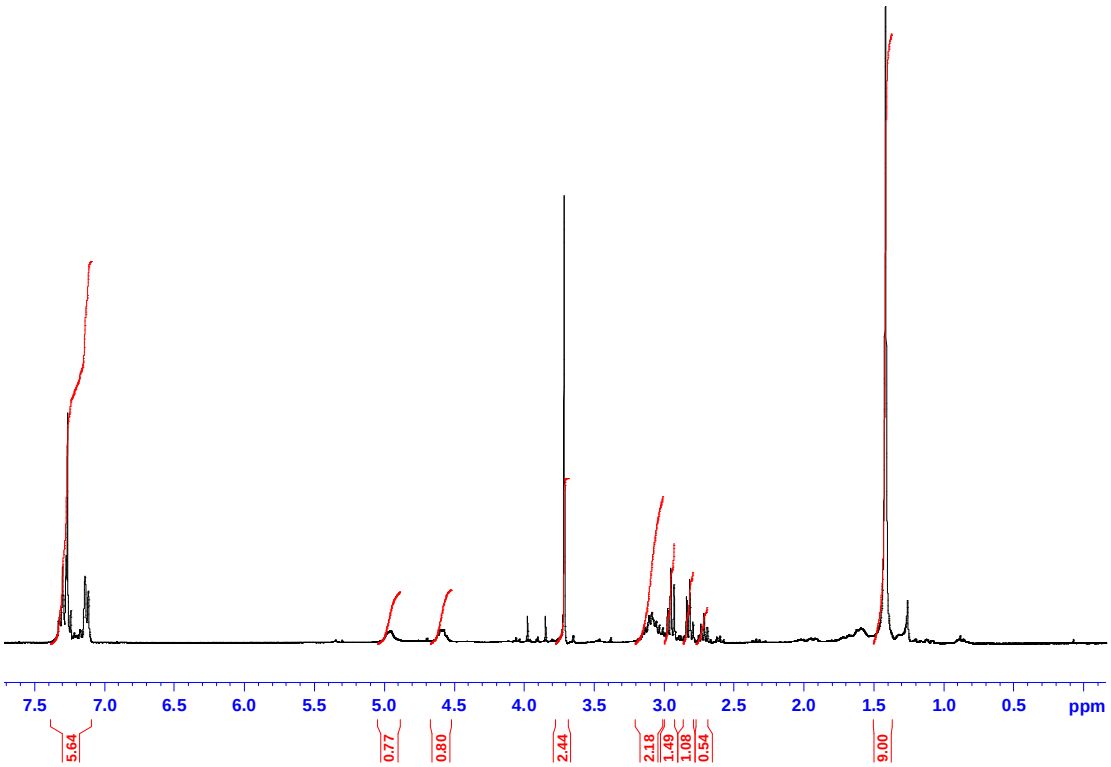
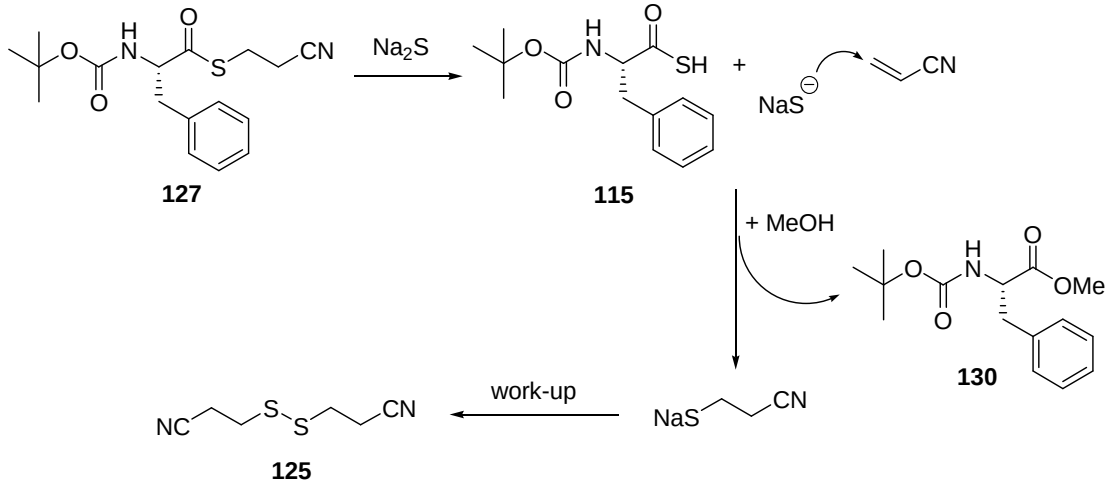


Figure 4-5. Crude spectrum of the methyl ester.

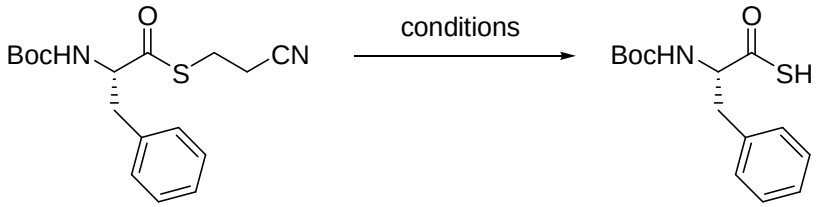
Furthermore, the signals of the methylene protons belonging to the ethyl cyano group are visible between 3.0 and 2.7 ppm. The characteristic signal of the SH, protons are missing at 1.82 ppm indicating that the disulfide was recovered. Most likely, the acrylonitrile which is eliminated is scavenged by excess Na_2S to give the mercaptopropionitrile compound which then oxidizes to the disulfide (Scheme 4-18).

Scheme 4-18



Potassium *tert*-butoxide is another alternative to the sodium bases. It is a stronger base than the alkali metal hydroxides and primary and secondary alkali metal alkoxides.[154] Furthermore, the fact that it is substantially weaker than alkali metal amides and their alkyl derivatives such as lithium diisopropylamide (LDA) makes it more compatible with the peptide structures. The reaction was once again optimized to find the best conditions for the conversion of the thioester to the thioacid.

Table 4-1



127 **115**

entry	base, equivalent/ additive, equivalent	solvent	time	conversion[a]
1	KO*t*Bu, 1.1 / --	*t*-BuOH / DCM (8/2)	40 min	incomplete and slow hydrolysis
2	KO*t*Bu, 1.1 / --	H_2O / DMSO (1/1)	30 min	incomplete
3	KO*t*Bu, 1.1/ 18-crown-6, 1.1	*t*-BuOH / DMSO (8/2)	1 h	complete

[a] Conversion of the thioester was followed by LC-MS

The reaction proceeded very smoothly using a solvent mixture of *t*-butanol and dimethyl sulphoxide in addition to the cryptand 18-crown-6 to complex the potassium cation (Entry 3, Table 4-1). The reaction was finished in one hour and the thioacid could be observed as a pure product via LC-MS analysis.

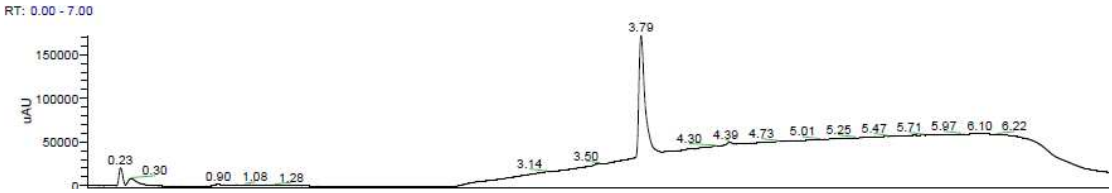


Figure 4-6. HPLC chromatogram of the crude thioacid after conversion from the thioester.

After this promising result, the same reaction conditions failed to give equally good results when applied to the larger pentapeptide. The reaction was not finished after 30 minutes (Figure 4-7), but as can be seen already a significant amount of side

product has formed. Neither longer reaction times or alteration of the base equivalent were able to improve the reaction conversion.

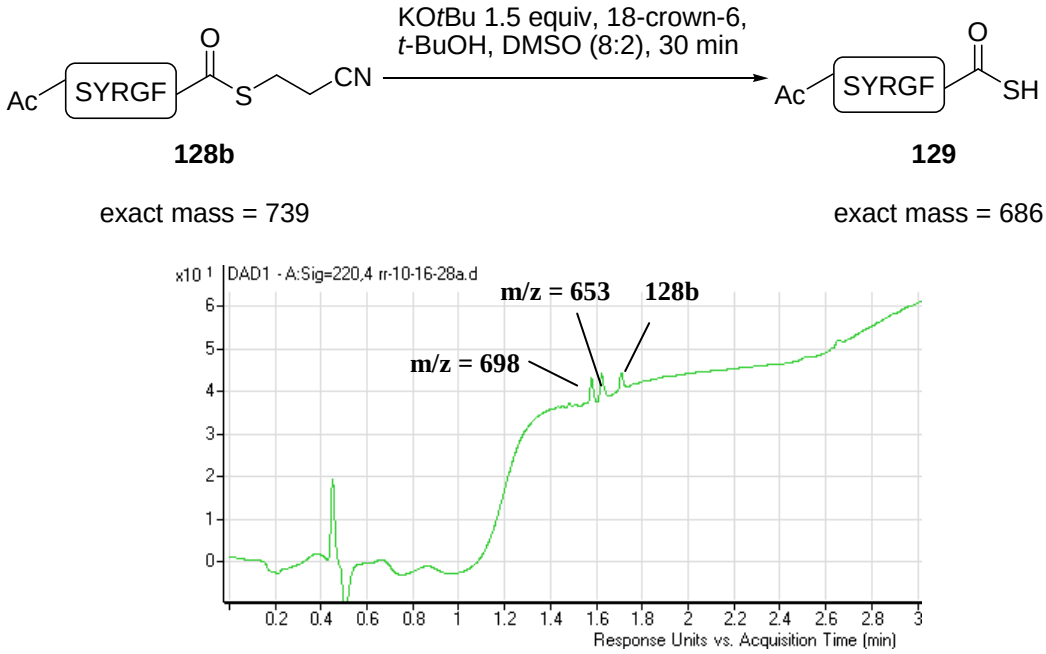
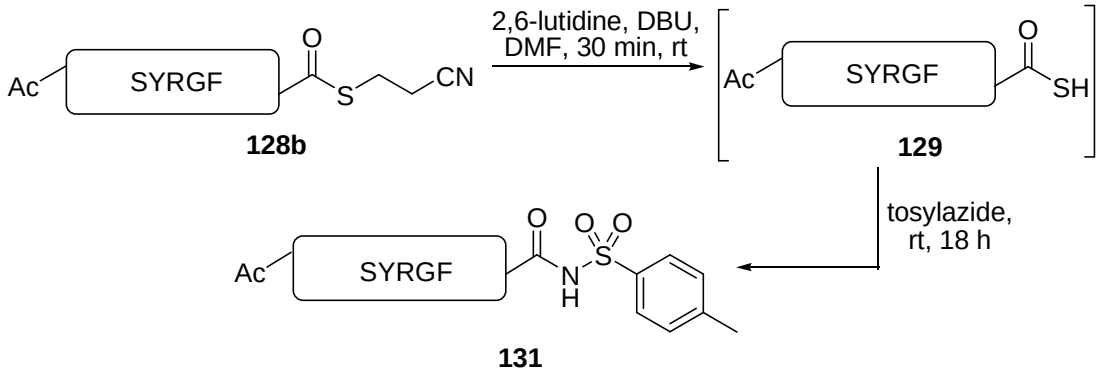


Figure 4-7. Attempted deprotection of the cyanethy group from the pentapeptide thioacid.

As previously mentioned, the 2-cyanoethyl group masks the thioacid functionality which in principle can be used in the direct ligation with tosyl azides. Williams et al. as previously mentioned, reacted the thioacids with electron deficient azides in the presence of 2,6-lutidine to yield sulfonamides. Despite their inability to ß-eliminate to the thioacid in the presence of 2,6-lutidine, the cyanoethyl thioesters did convert to the thioacids with DBU and the presence of 2,6-lutidine should not have deleterious effects on the ß-elimination 'unmasking' of the thioacid. Furthermore, the DBU should not have a negative effect on the thioacid/ azide ligation reaction.

To prove this, the pentapeptide cyanoethyl thioester was reacted with a base cocktail of DBU and 2,6 lutidine in DMF for 30 minutes to generate the thioacid *in situ*. After 30 minutes, tosyl azide was added to this mixture and as expected the pentapeptide sulfonamide was recovered.

Scheme 4-19



The reaction was followed by LC-MS and showed the complete conversion of the thioester peptide. The major peak had a corresponding mass of 822 $[M+H]^+$, the found mass of the sulphonamide product is 824 $[M+H]^+$. Accompanying the two peaks is the hydrolyzed pentapeptide acid which does not take part in the azide thioacid reaction.

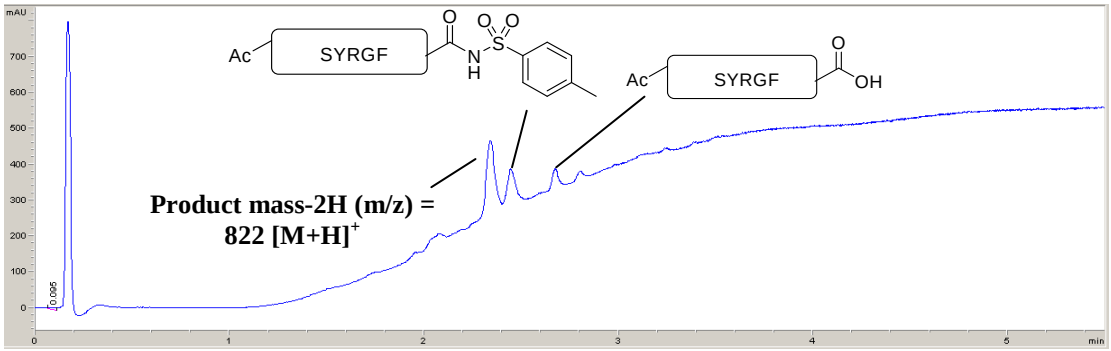


Figure 4-8. HPLC spectrum of the crude sulfonamide reaction between a thioacid formed *in situ* and tosyl azide.

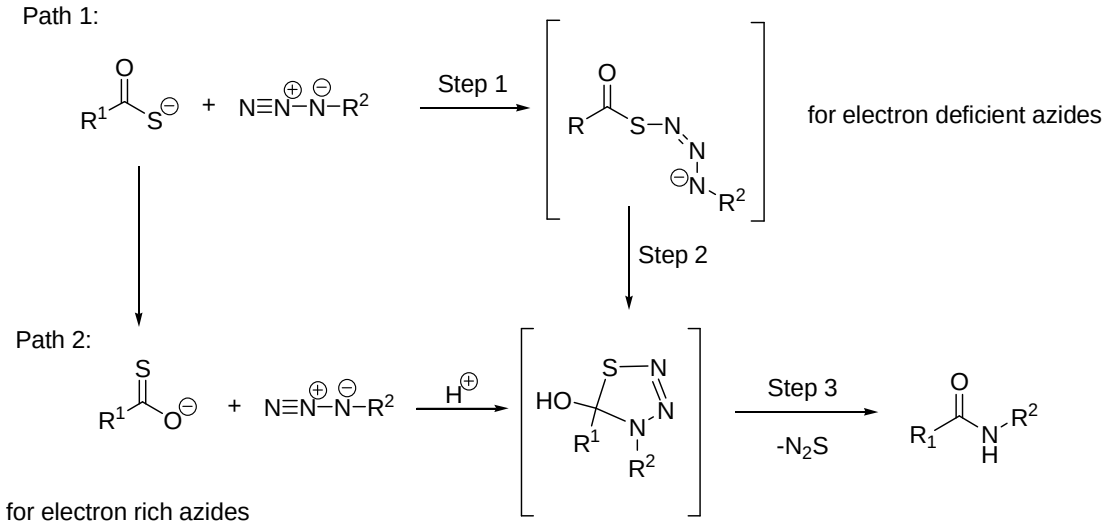
This proof of principle reaction (illustrated with the HPLC chromatogram of the crude product in Figure 4-8) demonstrates the viability of this method to first use the cyanoethyl protecting group to mask the thioacid functionality enabling in situ generation of the thioacid which can then react under DBU/lutidine conditions to the sulfonamide. The cyanoethyl thiol functionality is easily prepared from the commercially available parent disulphide in one quick and easy reducing step and in combination with sodium thiophenolate cleaves the peptide thioester efficiently from the resin.

5. Thioamide formation from thioacids and azides, towards a new ligation reaction

5.1. Introduction

Upon reexamining the amide formation of azides and thioacids, Williams and coworkers found that the reaction proceeds through two possible mechanisms based on the electronic substitution of the azide. For relatively electron rich azides, the reaction is proposed to proceed through an anion-accelerated [3+2] cycloaddition to give a thiatriazoline presumably in a concerted fashion. Highly electron poor azides are also proposed to proceed through a thiatriazoline intermediate, but rather in an unconcerted fashion. The bimolecular union of the terminal nitrogen of the azide with sulfur of the thiocarboxylate first gives rise to a linear adduct and after cyclization of this intermediate a thiatriazoline is formed. Decomposition to the amide is found to proceed via retro-[3+2] cycloaddition of the neutral thiatriazoline intermediates.

Scheme 5-1. Proposed mechanism of the thioacid/azide amidation.



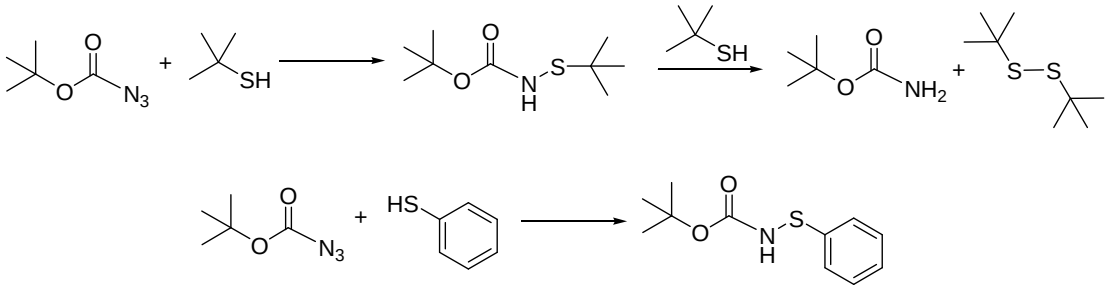
The reaction of electron rich azides with thioacids proceeds quite slowly at room temperature and as such elevated temperatures are required for sufficient conversion. Glycosyl and amino acid derived azides must be warmed to 60 °C before they can react with the thioacids. In contrast, electron poor azides such as sulfonylazides react very quickly with thioacids at room temperature. Different

groups have exploited the much faster reaction of electron poor azides with thioacids as a type of ligation reaction. Liskamp and coworkers have utilized this reaction for the ligation of N-ß-protected sulfonyl azides and dipeptide thioacids.[83] Recently, the reaction has found utility to label purified ubiquitin thiocarboxylate with PEG-sulfonyl azide and biotin-sulfonyl azide.[147] Finally, Krishnamoorthy and Begley have applied this ligation reaction for the detection of protein thiocarboxylates in phosphite dehydrogenase (PdtH) of the *Pseudomonas stutzeri* proteome, a putative protein thiocarboxylate involved in the biosynthesis of the pyridine thiocarboxylic acid siderophore.[155]

The limiting factor of this ligation reaction lies within the poor reactivity of relatively less electron deficient azides with thioacids. To date, there have been no successful examples of azido peptides reacting with thioacid peptides in the formation of large peptide fragments. In order for the reaction to become a more powerful ligation method, the reaction must be optimized to suit this demand. The goal of the final part of this doctoral thesis was to investigate and optimize the reaction between azides and thioacids.

The group of Takeop Saegusa reported in 1970 that when alkyl azides are reacted with thiols in the presence of copper(I) salts sulfenyl amides are produced along with primary amines and disulfides.[156] In their survey of the reaction, the sulfenyl amide was formed after 4-10 hours at room temperature and depended largely on the thiol used. Electron rich thiols such as *tert*-butyl thiol reacted to give 77 % conversion of the azide to the sulfenylamide whereas electron poorer thiols such as thiophenol reacted exclusively to give the amide.

Scheme 5-2

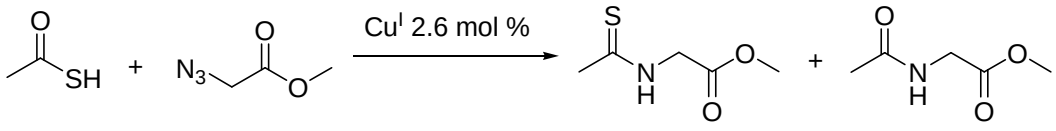


5.2. Optimization of the Thioacid Azide Amidation in the Presence of Copper(I) Salts.

The initial goal was to investigate the effect that copper(I) salts have on the thioacid/azide amidation reaction. Ideally, the reaction would be accelerated by the presence of the metal salt and allow the ligation reaction to occur smoothly at room temperature in a short time. α-Azido glycine methylester **133** was used as the initial azide substrate to test this system. It was prepared efficiently from sodium azide and α-bromoacetate in two and half hours at room temperature and was obtained in a 69 % yield.[157]

The α-azido glycine methylester **133** was then reacted with thioacetic acid in the presence of a catalytic amount of copper(I) chloride. Thioacetic acid is cheap, commercially available, and despite its somewhat unpleasant aroma is easy to handle.

Scheme 5-3



132 **133** **134** **135**

Conversion ratio: 89 11

Upon addition of the thioacid to a solution of the azide with the copper(I) salt, precipitate formation was observed immediately. The reaction was monitored using LC-MS and the conversion was calculated based on the integration of the signals found at 214 nm. Surprisingly, the main product from the addition of the thioacid and azide in the presence of Cu(I) is a thioamide product (Scheme 5-3). Mass analysis identified the thioamide as the correct product. The proposed thioamide product was isolated and characterized by ^{1}H and ^{13}C NMR experiments and are shown in Figure 5-1. It is shown in the ^{13}C NMR experiment that the two different carbonyl signals at 201.6 ppm and 168.6 ppm correspond to the thioamide and ester carbonyl peaks, respectively.

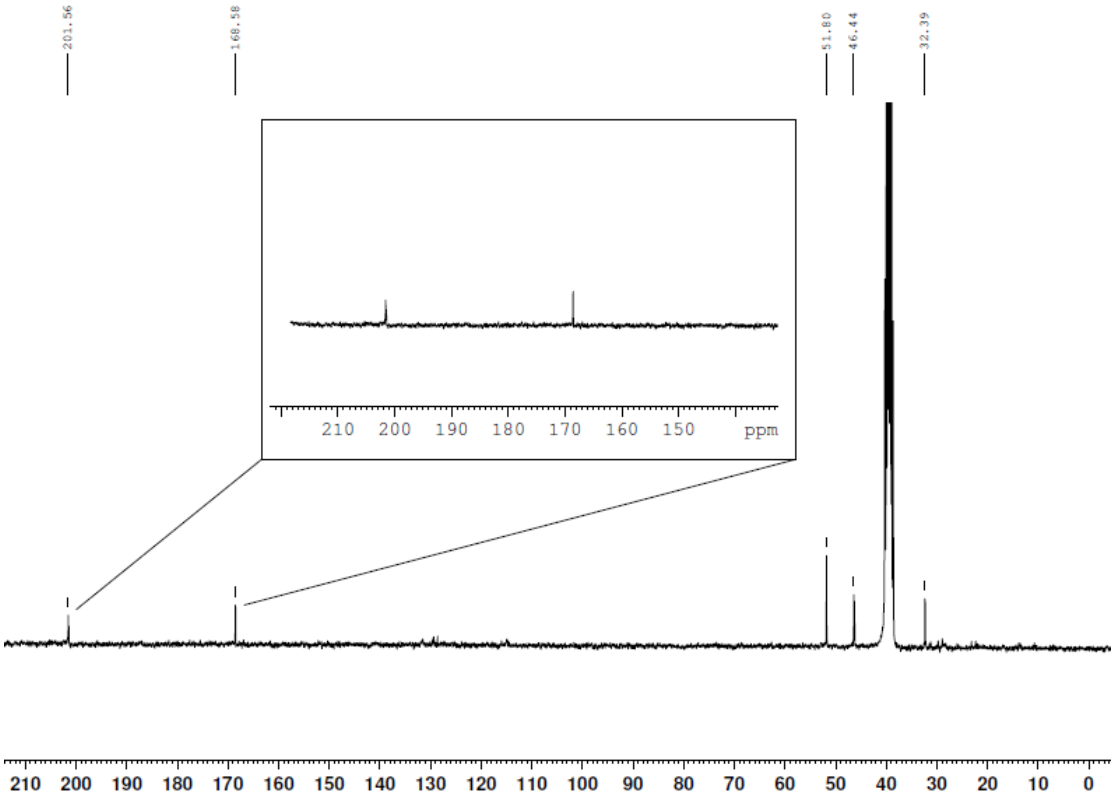
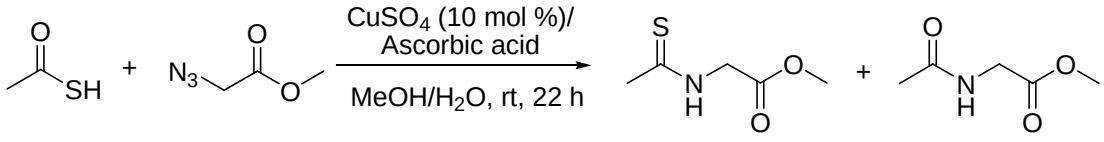


Figure 5-1. ^{13}C NMR spectrum of the thioamide product.

As for the copper source, copper(I) chloride oxidizes readily and a fresh source is required. Thusly, copper(I) chloride is not often used as copper(I) source for such reactions. More common is the copper(II) sulfate/ascorbic acid system in which the copper(I) salt is generated *in situ* upon reduction with the ascorbic acid. This reagent mixture was applied to the same reaction and an even better conversion to the thioamide product was observed.

Scheme 5-4



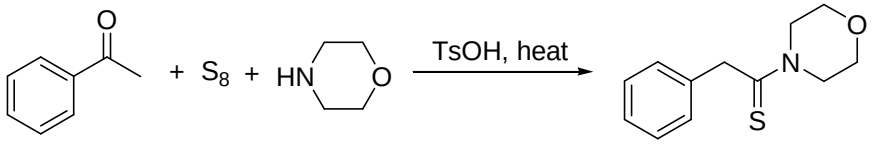
5.3. Existing methods for the synthesis of thioamides

This interesting change in chemoselectivity from the amide to the thioamide sparked an interest in investigating this reaction, and furthermore the preparation of thioamides using thioacids and azides as reacting partners. To our knowledge, the reaction of thioacids and azides in the presence of metal salts has not yet been described.

Thioamides have become an important class of compounds and their synthesis has been studied thoroughly.[158][159] Synthetically, sulfur-sulfur bond formation has been explored using thioamides in the presence of DDQ[160][161] They are less polar than their amide analogs and more soluble in organic solvents. They are more reactive than normal amides but stable enough to be handled under air and in aqueous media. The conformation of peptides with thioamides is influenced by the nature of hydrogen bonding of the functional group; the NH of the thioamide bond is a stronger hydrogen donor whereas the sulfur atom is a weaker hydrogen bond acceptor when compared to the corresponding amides.[162] This results in a weaker longer H-bond from the thiocarbonyl to an opposing NH amide bond.[162]

Classically, thioamides were prepared in a three component reaction of aldehydes or ketones, amines, and elemental sulfur in the Willgerodt-Kindler reaction. A classic example is the reaction of acetophenone and morpholine in the presence of elemental sulfur (Scheme 5-5).[163] In the past, this transformation was a key operation for the initial synthesis of racemic Naproxen.[164]

Scheme 5-5



The mechanism of the movement of a carbonyl group from methylene to methylene has been proposed by Carmack to go through an intricate pathway *via* a highly reactive intermediate with a sulfur-containing heterocyclic ring.[163]

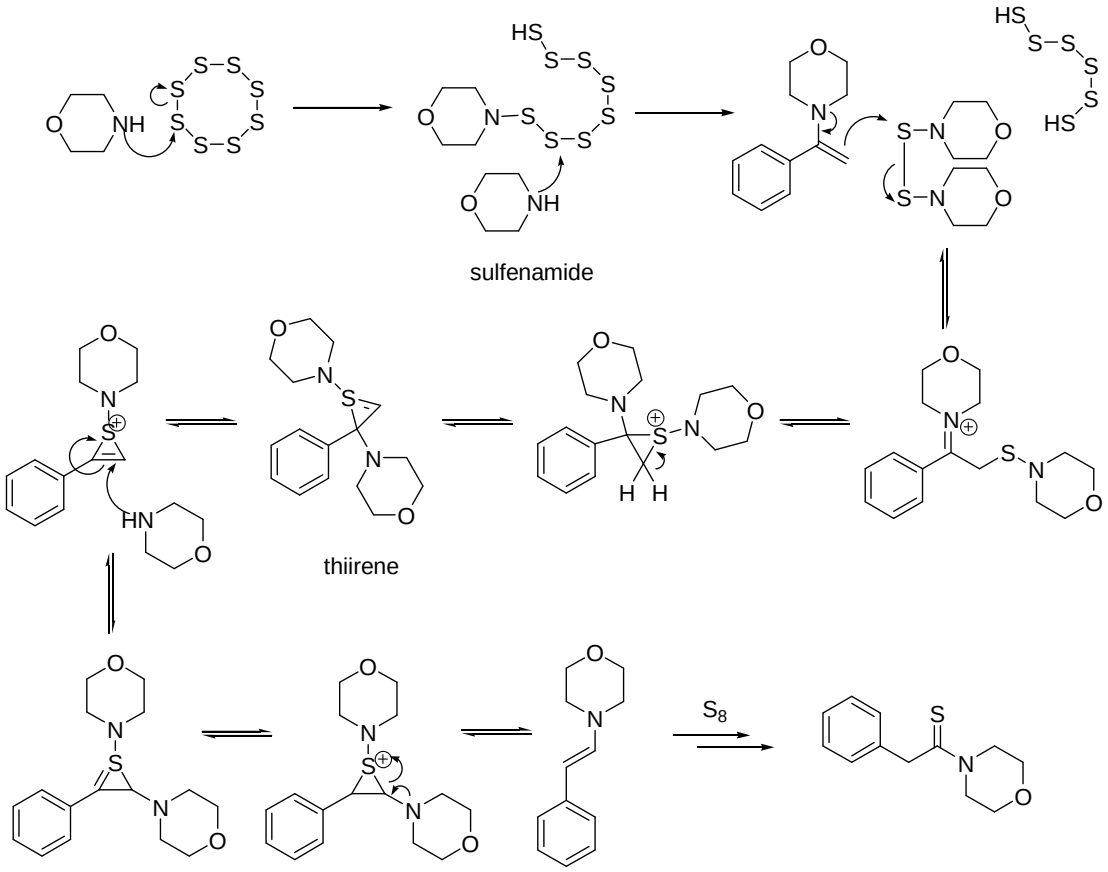
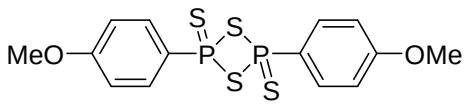


Figure 5-2. Proposed mechanism of thioamide formation between acetophenone, morpholine, and sulfur.

To date there are only a few methods of preparing thioamides or thiocarbonyl compounds. The most popular method to thionate ordinary amides or carbonyls to their respective thio-carbonyl compounds, is the use of 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-adiphosphetane 2,4-disulfide otherwise known as Lawesson's reagent (Scheme 5-6).[165]

Scheme 5-6. Lawesson's Reagent.



Since its introduction, Lawesson's reagent has undergone minor modifications to optimize synthesis. A fluorous Lawesson's reagent has been used for the thionation of amides, 1,4 diketones, *N*-(2-oxoalkyl)amides, *N,N'*-acylhydrazines, and acyl-protected uridines.[166]

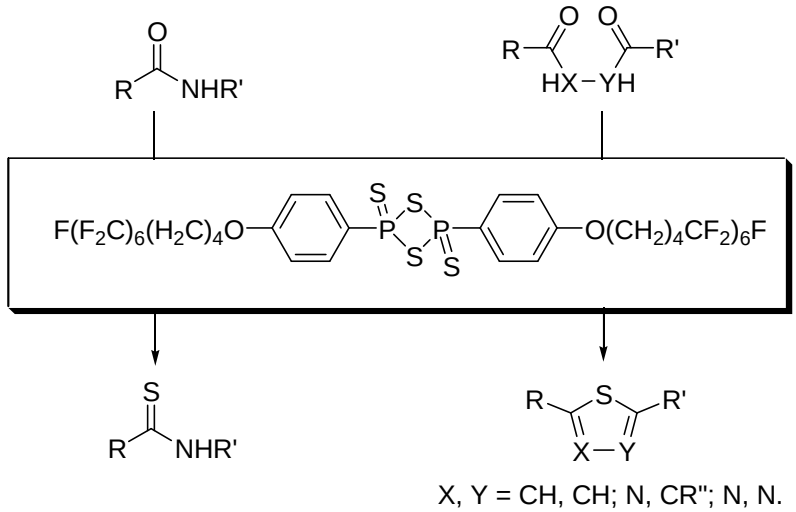


Figure 5-3. Application of a fluorous Lawesson's reagent.

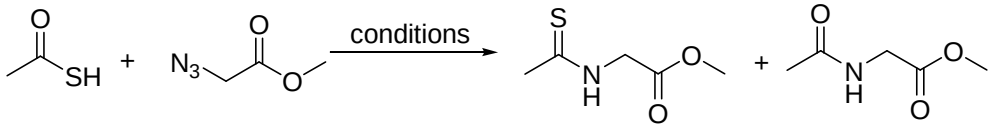
Other methods include reacting Grignard reagents to isothiocyanates, as well as conversion of nitriles with P_4S_{10}, but are not as popular as using Lawesson's reagent. The usage of Lawesson's reagent for the preparation of thioamides does however have its drawbacks. No chemoselectivity for amide carbonyls limits the ability to selectively introduce thioamides into a peptide backbone. Furthermore, Lawesson's reagent is foul-smelling and must be kept under a laboratory hood with good ventilation. In comparison, peptide thioacids are not volatile and a traceless ligation between one peptide carrying a peptide thioacid with another carrying an N-terminal azido function can chemoselectively introduce a thioamide bond at the desired point in the peptide structure.

5.4. Initial Thioamide Formation Studies

The chemoselective thioamidation reaction of azides with thioacids in the presence of metal salts could be a promising new method for the introduction of thioamide functionality. Starting from the initial reaction (Scheme 5-3), the solvent system was screened for the best conversion to get the thioamide product. The reaction was carried out at room temperature, with a slight excess of the thioacid (1.4 equiv to 1) compared with the azide to effect complete conversion.

Under neat conditions without solvent, base or metal catalyst, the predominantly formed product is the amide. It is interesting to note, that even without the addition of base, a small amount of the thioamide is also formed perhaps suggesting a new reaction intermediate which has not been postulated in the literature until now.

Table 5-1. Solvent and catalyst amount screening for the thioamide optimization



132 **133** **134** **135**

entry	$CuSO_4$ /ascorbic acid (mol %)	solvent	rxn time (h)	conversion 134:135[a]
1	--/--	Neat	10	7.4 : 72.8
2	2.6 / 11	MeOH / H_2O	22	89 : 11
3	10 / 33	MeOH / H_2O	22	96 : 2.4
4	10 / 10	MeOH / H_2O	18	88.4 : 11.6
5	5 / 10	DCM	19	1 : 99
6	5 / 10	DMF	23	95 : 5
7	10 / 30	2-propanol	21	14 : 86

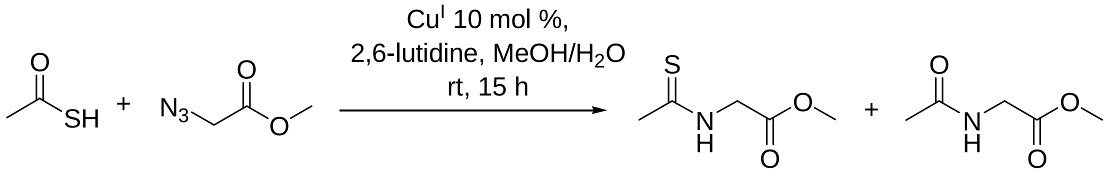
[a] The conversion was determined by LC-MS at 214 nm.

The best results were obtained with methanol and water as co-solvents in an equivolumar amount. The dramatic shift from formation of the thioamide to the amide product using DCM as solvent for the reaction (Table 5-1, Entry 5) may be attributed to the poor solubility of the copper sulphate and ascorbic acid in DCM. This is also

noticeable for 2-proponaol (Table 5-1, Entry 7) where the solubility of catalyst and reductant is also poor.

Surprisingly, with the addition of 2,6-lutidine in the presence of the copper(I), only the amide product was formed. It is apparent from this experiment that the base is a greater determining factor for the chemoselectivity of the amide over the thioamide, than addition of the metal salt.

Scheme 5-7

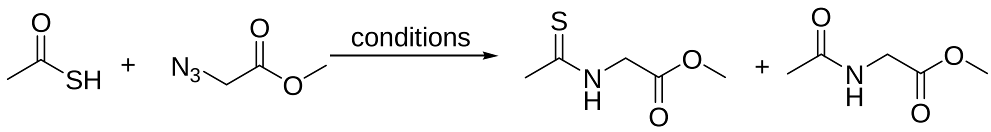


132 **133** **134** **135**

conversion < 1 > 99

The reaction of **132** and **133** was then screened with other metal salts to optimize the conversion to the thioamide. It was found that almost complete conversion was obtained implementing silver(I) salts.

Table 5-2. Catalyst screening for optimizing thioamidation.



132 **133** **134** **135**

entry	catalyst	mol %	solvent	rxn time (h)	conversion 134:135[a]
1	Cu^{II}/Asc. Acid	10 / 33	MeOH / H_2O	22	96 : 2.4
2	$ZnCl_2$	10	MeOH / H_2O	19	59 : 41
3	Au(I)Cl	10	DCM	19	1.5 : 98.5
4	AgOTf	10	MeOH / H_2O	20	97 : 3
5	$AgNO_3$	10	MeOH / H_2O	23	99 : 1

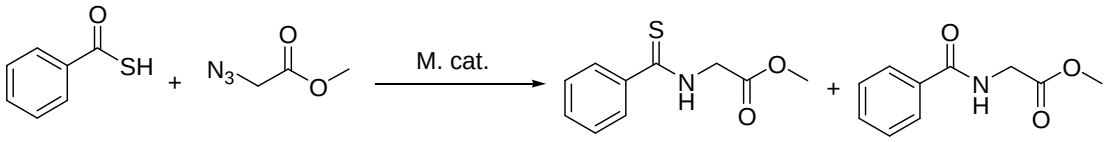
[a] As determined by LC-MS at 214 nm.

It is apparent from these experiments that silver(I) salts worked better than the Zn(II) salt and were slightly better than the copper(I) salt. From these experiments, it

can be reasoned that the chelation of metal salts to the carbonyl or thiocarbonyl works better for silver(I) salts than for zinc(II) or gold(I) salts. The attempt with Au(I) salts (Table 5-2, Entry 3) was performed in DCM due to better solubility; despite this the selectivity led to almost exclusive production of the amide. The activation of thioacids for amide bonding formation has been previously described by Blake in 1981 (see chapter 4), however, their interaction with azides has not been described to date.

The substrates for the reaction were then varied to analyze the scope of chemoselective control exhibited by addition of metal salts. Thiobenzoic acid **136** was reacted with **133** at room temperature under varying conditions (Table 5-3). The reaction was followed again using LC-MS and the results were tabulated as follows.

Table 5-3. Thioamidation between thiobenzoic acid and α-azidoglycine methylester.



136 **133** **137** **138**

entry	equivalents 1 / 5	catalyst	mol %	solvent	rxn time (h)	conversion 137 : 138[a]
1	1 / 1.4	Cu^{II}/Asc. Acid	20/30	MeOH / H_2O	19	43 : 57
2	1 / 1.4	$AgNO_3$	20	MeOH / H_2O	22	36 : 64
3	1 / 1	$AgNO_3$	20	MeOH / H_2O	15	53 : 47
4	1 / 1.4	$AgNO_3$	20	DCM	16	4 : 96
5	1 / 1.4	AgOTf	10	MeOH / H_2O	16	26 : 74
6	1 / 1	$AgNO_3$	20	MeOH	16	63 : 37

[a] The relationship conversion was determined by LC-MS visualized at 214 nm.

The chemoselectivity from the reaction of thiobenzoic acid with **133** varies significantly to reactions carried out with thioacetic acid. Generally, the ability to chemoselectively produce the thioamide is quite suppressed when the thioacid moiety is more electron poor. The reaction between these two substrates is more sensitive to variation in the reaction conditions. For instance, greater amounts of the metal catalyst (20 mol %) were required to generate even a moderate conversion to the thioamide. Furthermore, a 1 to 1 relationship of thioamide to amide could be obtained when equimolar amounts of thioacid and azide were reacted with one another (Table

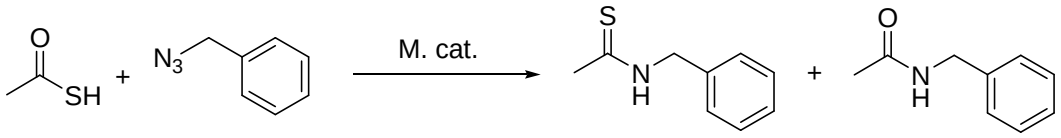
5-3, Entry 3). And finally, solvent effects also play a role, with even better results for the conversion to the thioamide product obtained when water was excluded from the reaction mixture (Table 5-3, Entry 6).

5.4.1. Preliminary Studies of the Thioamidation with Electron Rich Azides.

In order to elucidate more from the reaction, the electronic nature variation of the substituents was further analyzed. The relatively rich benzyl azide **140** was prepared in a 97 % yield from benzyl chloride and sodium azide applying microwave irradiation.[167]

The azide **140** was then reacted with thioacetic acid to determine the effect that a more electron rich azide would have on the system. The reaction was again carried out at room temperature, and the conditions were varied.

Table 5-4. Investigation of the thioamidation reaction with electron rich azides.



132 **140** **141** **142**

entry	equivalents 1 / 5	catalyst	mol %	solvent	rxn time (h)	conversion 141:142[a]
1	1 / 1.4	$AgNO_3$	20/30	MeOH / H_2O	17	34 : 66
2	1 / 1.4	Cu^{II}/ Asc. Acid	20	MeOH / H_2O	20	46 : 54
3	1 / 1	$AgNO_3$	20	MeOH / H_2O	21	55 : 45
4	1 / 1.4	Cu^{II}/ Asc. Acid	20	DCM	21	16.5 : 83.5
5	1 / 1	$AgNO_3$	20	MeOH	21	31 : 69

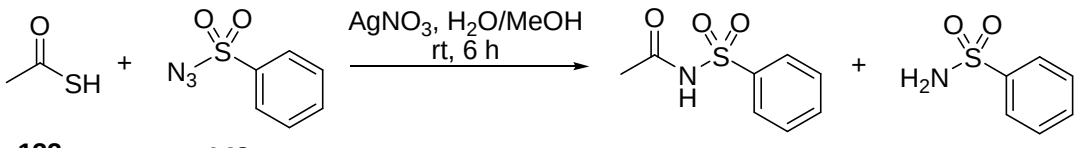
[a] The reaction was followed by LC-MS.

The chemoselectivity is roughly similar to the previous study where at best the reaction can be controlled to a 1 to 1 relationship formation between thioamide and amide. As with the other reactions, the best results were obtained using equal volumes of water and methanol. Also similar to the other analyzed conditions, a slight excess of the thioacid over the azide resulted in predominance of the amide product.

5.4.2. Preliminary Studies of the Thioamidation with Electron Deficient Azides.

Finally, the reaction in the presence of silver(I) was tested with an electron-deficient azide. When reacted with benzenesulfonyl azide, no thioamide product was observed (Scheme 5-8).

Scheme 5-8



132 **143** **144** **145**

conversion: 79 20

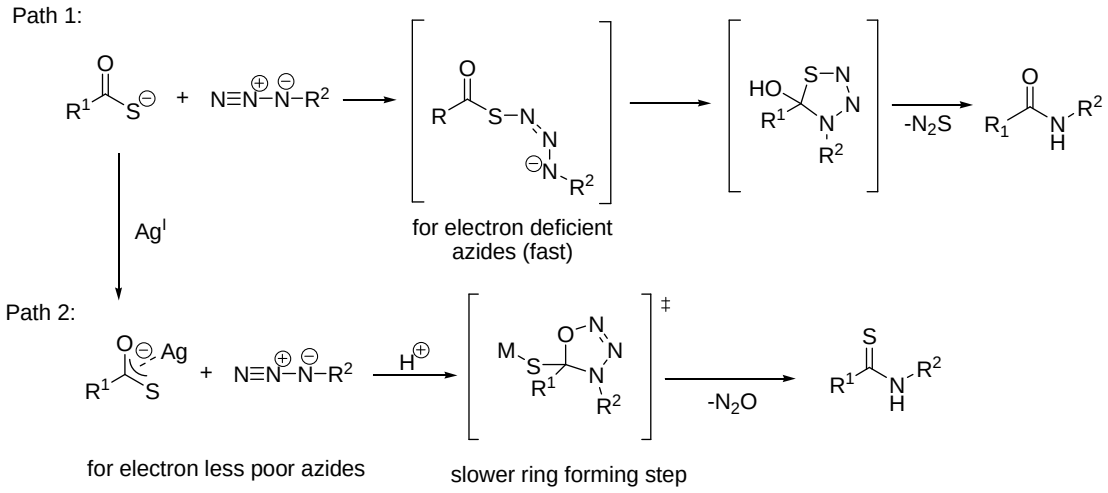
In comparison, in the absence of metal salts, electron deficient azides react with thioacids the quickest to produce amides. In the proposed mechanism by Williams and coworkers, an intermediate thiatriazoline intermediate is formed in two steps in which the first step is the intermolecular coupling between the sulphur of the thioacid and the terminal nitrogen of the azide analogous to a prior-capture reaction. It is apparent from this reaction (Scheme 5-8) that if it occurs through the thiatriazoline intermediate to the final amide product, it is not disturbed by the addition of metal salts.

The other examples show that it is possible another intermediate is formed to produce the thioamide product. If such an intermediate is formed in a similar manner to that described by Williams and coworkers, than it most likely would be an oxotriazoline type intermediate (Scheme 5-9).

The introduction of metal salts to the reaction have been shown to be a requirement for the formation of the thioamide product. Furthermore, it was shown that metal salts which have a higher affinity for sulfur gave a higher proportion of the thioamide product than the amide product (Table 5-2). It can be inferred from these results that the metal salt modifies the reaction pathway proposed by Williams by coordinating strongly to the sulfur thereby acting as a type of protecting group. Once the intermediate oxotriazoline is formed, the elimination of N_2O gives the thioamide product as shown in Scheme 5-9. The driving force for the formation of the thioamide is most likely the formation of N_2O. In comparison, under basic conditions, the

driving force for amidation is the formation of the amide bond which is far more stable than the thioamide bond.

Scheme 5-9



The reaction is however strongly influenced by the electronic nature of the substrates. The best selectivity is reached with slightly ‘electron neutral’ azides and thioacids. As soon as more electron density is introduced into or taken away from the azide, the amide product is formed. The same holds true for the thioacid; if the thioacid is slightly more electron poor, than the selectivity of thioamide over amide product is lost.

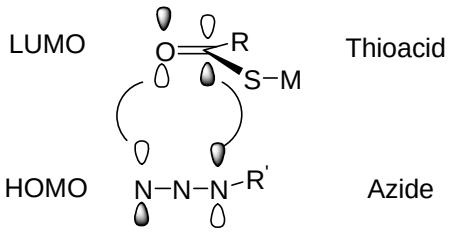


Figure 5-2. Proposed HOMO-LUMO interaction of the thioacid and azide thioamidation.

Since the chemoselectivity of the thioamide to amide formation is also controlled somewhat by the electronic nature of the substituents, HOMO-LUMO interactions should also be considered. The azide is a 1,3-dipole and its mechanism in cycloaddition reactions has been extensively investigated.[168][169] The thioacid in this

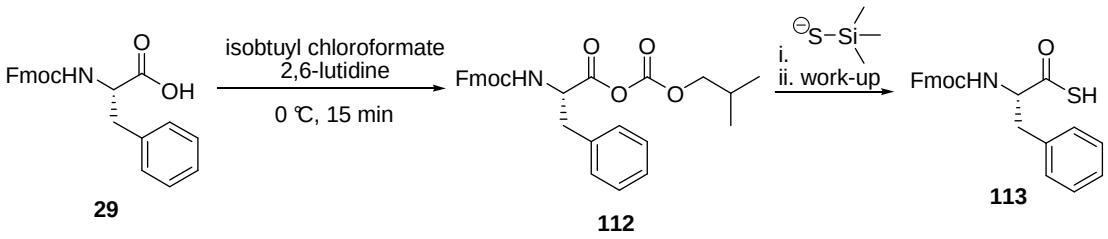
mechanism acts as the dipolarophile and the [3+2] reaction pathway can be considered formally as a [π4s + π2s] cycloaddition followed by a [π4s + π2s] cycloreversion. In this reaction sequence, a possible interaction is between the HOMO of the azide interacts with the LUMO of the thioacid as shown in Figure 5-2.

The addition of metal salts to the reaction however casts doubt to whether such a concerted pathway is actually taking place or whether or not the formation of intermediate species first happens analogous to the mechanism described by Williams.

5.4.3. Preliminary studies for the Thioamidation Between Thioacid Amino Acids and α-azido glycine methylester.

Notwithstanding, the complexities of the reaction mechanism and which path is more or less preferred, the goal of the thioamidation reaction was to carry it out on more complex peptidic or amino acid fragments. In order to test the feasibility of this reaction as a possible ligation reaction, Fmoc-phenylalanine thioacid was prepared according to the method described by Williams.[170]

Scheme 5-10



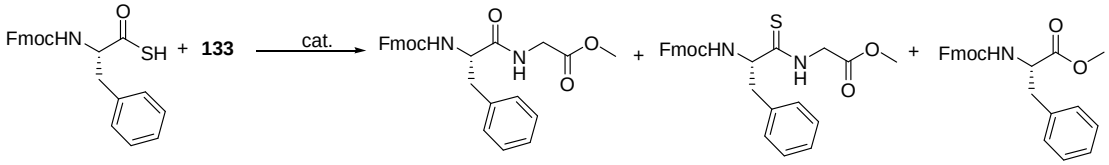
29 **112** **113**

The crude Fmoc-Phe-SH **113** was reacted with azido glycine methyl ester **133** in the presence of Ag(I) and separately in the presence of Cu(I). Both reactions were carried out at room temperature under the same conditions as previously described. No presence of the thioamide product could be detected.

The thioacid was converted mostly to the methyl ester as shown in Table 5-5 with no conversion to the thioamide. The lack of coordination of the silver cation to the thioacid may be due in part to the solvent, but may also be the result of poor availability of the the silver cation in solution. It has been observed in previous

experiments where silver(I) has catalyzed hydroarylation to alkynes that an excess of a protic acid is required to efficiently generate the cationic silver(I) species.[171]

Table 5-5. Initial thioamidation attempts between Fmoc-Phe-SH **113** and α-azidoglycine methylester **133**.



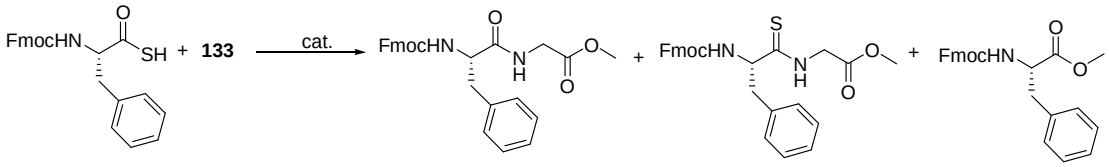
113 **146** **147** **148**

entry	catalyst	mol %	solvent	rxn time (h)	conversion 146 / 147 / 148[a]
1	$AgNO_3$	20	MeOH	20.5	18 / -- / 44[b]
2	$CuSO_4$ / Asc. Acid	20 / 60	MeOH	21	13.5 / -- / 84

[a] Determined by LC-MS at 214 nm. [b] 22 % of the starting material remained unreacted.

The reaction between Fmoc-Phe-SH **113** and **133** was then repeated in the presence of diluted sulfuric acid as a proton source. The final pH of the solution was measured approximately to be around pH 2-3. Under these conditions, the thioamide product was formed as a mixture with the amide product and some methyl ester (Table 5-14). The reaction was also attempted with copper(I) as the metal species at lowered pH, but failed to yield any thioamide product. All other reaction conditions were held the same.

Table 5-6. Thioamidation between Fmoc-Phe-SH **113** and α-azido glycine methylester **133**.



113 **146** **147** **148**

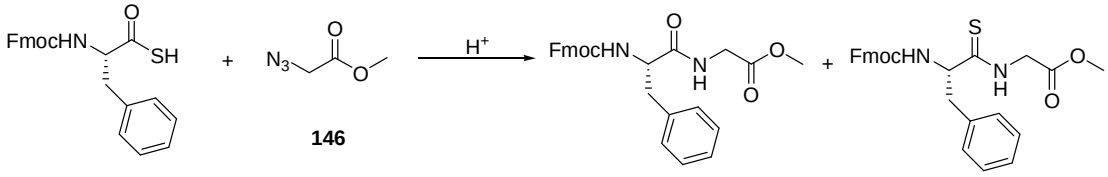
entry	catalyst	mol %	solvent	rxn time (h)	conversion 3 / 4 / 5[a]
1	$AgNO_3$	20	MeOH	20.5	30 / 50 / 6.5
2	$CuSO_4$ / Asc. Acid	20 / 60	MeOH	21	37 / -- / 51.5

[a] Determined by LC-MS at 214 nm.

The acidity or presence of protons in the reaction milieu has a direct correlation on whether or not the thioamide product is formed. This further bolsters

the claim that silver(I) species is responsible for chelating to the thiol and allowing formation of the oxotriazoline intermediate followed by release of N_2O. As a negative control the reaction was carried out again in the absence of silver(I) but in the presence of acid.

Scheme 5-11



113 **146** **147** 10 % **148** 0 %

The reaction was again controlled via LC-MS and as shown, no formation of the thioamide was observed and only a small amount of amide was formed. The absence of any thioamide formation further supports the claim that silver(I) cations are the mediating species for the formation of the thioamide bond, proposed to occur through a five-membered intermediate involving the eventual release of N_2O.

In order to further bolster this mechanistic representation, a positive proof for the formation of N_2O would be required. As of yet, no successful examples have been carried out. The amidation reaction between azides and thioacids can be ‘switched’ to a certain extent to a thioamidation reaction which is mediated either by copper(I) or silver(I) species. However, slight alterations in the electronic nature of the substrate affect the formation of the thioamide product leading to a higher proportion of the more stable amide form. In the end, it is most likely that the more stable amide product is formed especially with electron poor azides and that the metal species serve to disrupt this reaction whereby the thioamide product can be recovered.

In conclusion, through the investigation of the amidation reaction between thioacid and azides in the presence of copper(I) species, it was established that a new byproduct--the thioamide product is formed. This method describes a new process by which thioamides may be formed in a traceless manner completely novel to the previously mentioned methods for creating thioamides. The reaction is, however, limited in scope, since varying the electron substitution of either the azide or thioacid

results in a non-chemoselective fashion affording a mixture of both amide and thioamide.

6. Summary, Conclusion, and Outlook

The first part of this dissertation describes the synthesis of peptide thioesters using a *tert*-butyl thiol linker. It has been previously shown that *tert*-butyl thioesters exhibit remarkable stability in comparison to their alkyl and aryl counterparts. They are stable towards acidic, as well as basic milieu. It was proposed that *tert*-butyl thiols could be used as linkers for polymeric support and synthesis of peptide thioesters.

The *tert*-butyl thioester of phenylalanine was treated under standard SPPS Fmoc cleavage conditions showed an astounding stability of the thioester. The stability, which was dramatically greater than that of other less branched or less electron rich thioesters indicated that these thiols might find utility for the Fmoc SPPS synthesis of peptide thioesters.

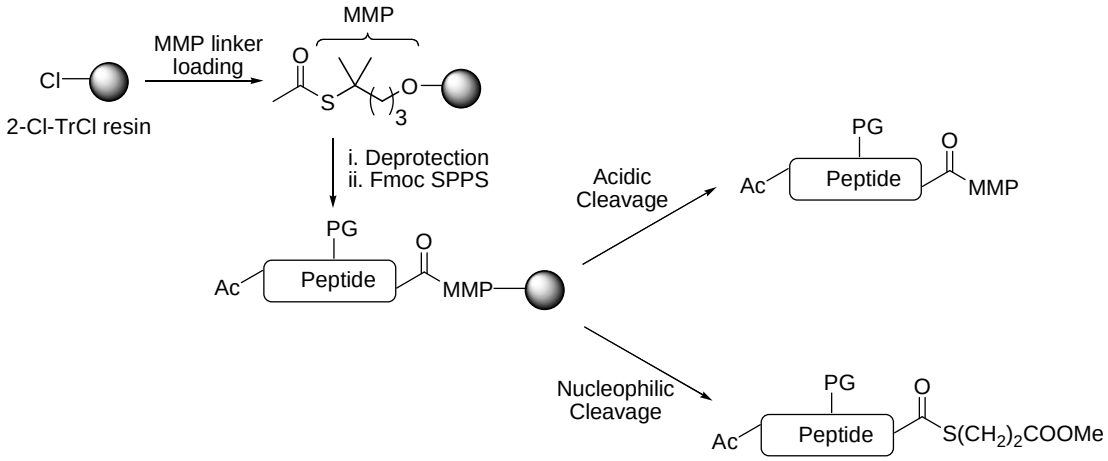
The initial *tert*-butyl thiol linker was attached directly to the resin via an ether linkage. This alteration in the electronic nature of the *tert*-butyl group translated into a system which was much too labile in order to produce peptide thioesters of significant length. The synthesis of a *tert*-butyl thiol linker with enough distance to the attachment point to the linker became another significant hindrance due to the introduction of the thiol functionality. This problem was eventually overcome by using indium trichloride to activate a terminal double bond of an Fmoc-protected alcohol for the introduction of the thiol. Once coupled to the resin, this linker proved to be robust enough for the synthesis of small peptide thioesters. Other problems surfaced which had to be solved in order to optimize the strategy.

After deprotection of the thiol, it was discovered that the thiol undergoes slow oxidative disulfide formation if left under air for extended periods of time. This process is accelerated incredibly when the air is flushed through the resin as is often the case in SPPS. Ellman's test for the presence of free thiols revealed that after deprotection of the thiol and simple washing, the amount of free thiols present in the resin was dramatically decreased. The disulfides are reducible, but require strong reducing agents such as Red-Al to accomplish this. This problem of oxidation was solved simply with the use of dry solvents and a nitrogen atmosphere for performing the deprotection and subsequent coupling step.

Acylation of activated amino acids to thiols proceeded quickly in high yields under normal conditions due to the excellent nucleophilic nature of the thiol group.

This nucleophilicity was lessened to a great extent when a *tert*-butyl thiol was used instead of a less-hindered thiol. This reduced reactivity translated to lower first acylation yields and poor results after peptide synthesis. Removal of the competing nucleophile, water, from the amino acid had a remarkable effect on the yield. The appropriate activation agent was also critical, with preparation of amino acid fluorides giving the best yields. Finally, double couplings of first 2 hours, then 20 hours gave yields up to 90 % for the first acylation. In this manner it became possible to synthesize peptide bound resins in good to excellent yields. Cleavage of the peptides from the resin could be accomplished following two different routes. Either with TFA, or by nucleophilic displacement which initially gave only problems.

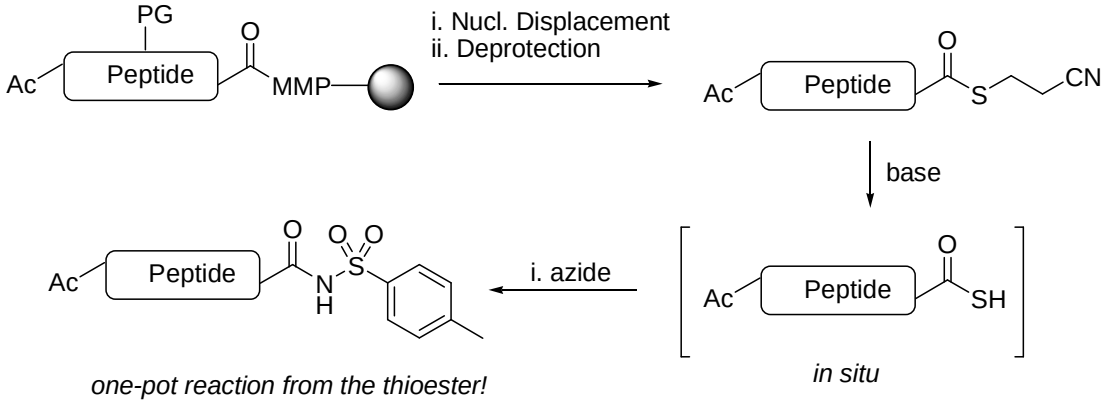
Scheme 6-1



The optimized conditions in order to release the peptide thioesters from the resin nucleophilically and a row of different thioesters with varying amino acids at the C-terminus were synthesized. Thioesters were further applied in the NCL without further purification. In this manner, thioesters were synthesized cleanly and in good yields without trace of fail sequences, one carrying the *tert*-butyl linker, and the other as the nucleophilically displaced thioester. The former could be activated *in situ* with sodium thiophenolate in organic solvents to a more reactive thioester in order to be carried out in NCL reactions. Ultimately, the real test of this linker was the synthesis of larger peptide thioesters. Synthesis of a 15mer Penetratin peptide derivative in excellent yields and purities confirmed the robustness of this methodology. It is still being investigated whether racemization of the first amino acid can be reduced as well as even further optimization of the first amino acid coupling to the solid support.

Considering the robustness of the linker under Fmoc SPPS conditions, we considered that this method would be an excellent starting point also for the synthesis of other moieties including peptide thioacids. This was the goal for the second part of this dissertation. Thioacids are versatile molecules, but to date no satisfying method for their synthesis on the solid phase using Fmoc methodology has been reported. Using the *tert*-butyl method protected thioacids were obtained by nucleophilic displacement with the ß-eliminable thiol, 3-mercaptopropionitrile. After total deprotection with acid, the thioesters were obtained did not require further purification for following steps. It was found that these thioesters could be deprotected to form the thioacids *in situ* which could then further go on to react with electron poor azides in the amidation ligation reaction in a one-pot 2 step synthesis. The optimization of these reactions are still ongoing. Isolating the peptide thioacids would forego the requirement to perform the following ligation reaction in situ thus improving yields and also reducing the number of possible side reactions.

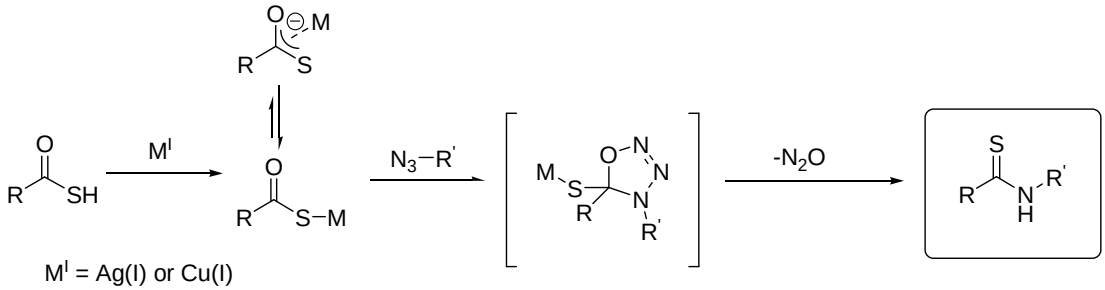
Scheme 6-2



The final topic of this dissertation concerned the exploration of the thioacid/azide ligation reaction. The reaction was first described by Just in 1980, and then used by Rosen in 1988 for the amidation of glycosides. After its rediscovery in 2003 by the group of Williams, it has gathered speed as a new and powerful ligation reaction. However, it is limited to a large extent by its requirement for electron poor azides such as tosyl azides for the necessary speed for the ligation. The introduction of metal salts in the coupling of this reaction were investigated starting with copper(I) salts. Remarkably, with the test system that was used, copper(I) effected the almost complete conversion of the thioacid and azide into the thioamide product. The

preparation of thioamides has not been extensively reported in the literature. They have found utility as intermediate building blocks for a myriad of different chmical transformations. Accordingly, it is important to investigate new methods for their synthesis.

Scheme 6-3



The chemoselectivity of the reaction was even better when silver(I) salts were used. The results of the intial tests seemed to indicate that the metal salt is required to chelate to the thiocarbonyl moiety acting as a protecting group so that the oxygen can be eliminated with two nitrogens of the azide as N_2O. Selectivity is shifted from thioamide to a mixture of thioamide and amide as soon as the electronic nature of the azide and or thioacid is altered. The result is a near equal formation of thioamide and amide products. Nonetheless, this reaction was tested with Fmoc-Phe-SH and could be shown that under relatively optimized conditions the thioamide could be obtained with 50 % conversion in comparison to the amide product, obtained in around 30 %. The reaction conditions for this ligation are also still currently being investigated.

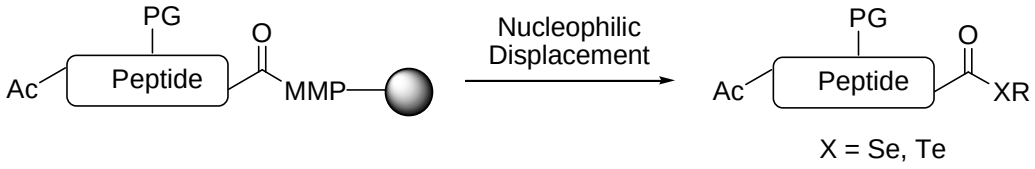
6.1. Outlook.

6.1.1. Continuing Application of the MMP Linker

The *tert*-butyl thiolinker has been shown to be a versatile linker for the synthesis of peptide thioesters and acids. This is due to its stability but also because it is smoothly cleavable by thio nucleophiles. This technique may be further expanded for the preparation of other moitieis for instance selenoesters. Selenoesters are another class of compound which have gained interest recently due to their enhanced reactivity in the NCL. The ester could be prepared in an analogous fashion to the nucleophilic displacement to the thioester. In principle the only limiting factor should be the preparation of the selenohydryl from its respective diselenide.

In this manner, the synthesis of telluro-carboxylic acid esters (tellurol esters) should also be able to be realized. Synthesis of tellurol esters are limited in comparsion to thiol and selenol esters. This lack of investigation makes them possibly an interesting class of molecules to investigate. They can be obtained classically by transesterification of esters with *i*-$Bu_2AlTeBu$[172] which implies that their synthesis should also be possible using the MMP linker.

Scheme 6-4

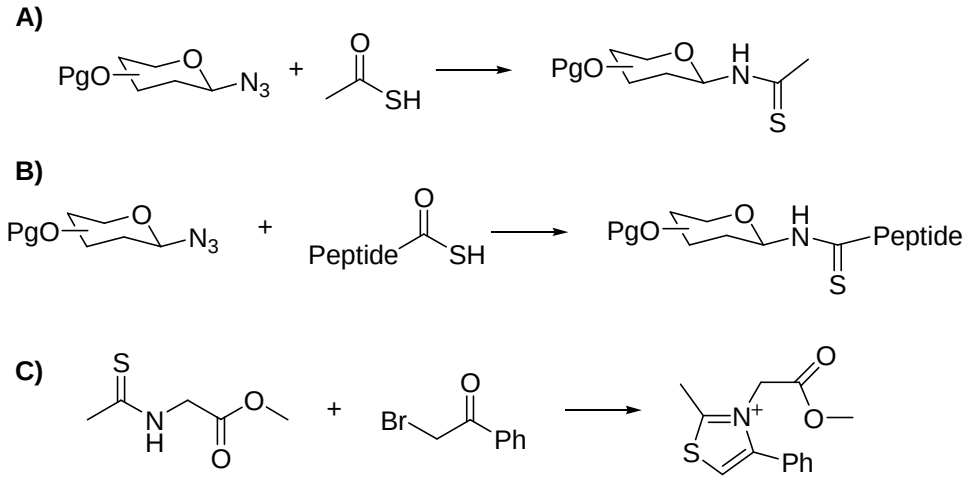


It was shown that it is possible to synthesize thioacid peptides *in situ* and then to react them further in the thioacid/azide amidation reaction. In this elegant manner, the amidation reaction can occur in a one-pot synthesis from the thioester. Despite this, there is still room for improvement regarding this reaction. Isolation of the thioacid in good yields would expand the number of different transformations, the thioacid peptide building block could take part in. In order to better isolate the thioacid peptide, it may be necessary to perform the ethylcyano deprotection in an excess of Na_2S in an aqueous environment, thereby ensuring greater hydrothiolysis.

Nucleophilic displacement can also be extrapolated in the same manner for the synthesis of thioacid derivatives. For example, using the same methodology, it may also be possible to prepare selenoacids using the MMP linker. The major difficulty to overcome is the isolation of selenoacids due to their increased reactivity over thioacids. For instance, selenocarboxylic acids are immediately oxidized in air to afford the corresponding diacyl selenides.[173] Their ability to add to aryl isocyanates to give the corresponding acyl carbamoyl selenides[174] make them an interesting class of compounds.

The final topic showed promising introductory results for a new type of transformation of thioacids and azides to yield thioamides. There is room for improvement within this topic, first and foremost improving the chemoselectivity for the thioamide product over the amide product. The reaction can also be expanded and with the thioamidation of different substrates (Scheme 6-5).

Scheme 6-5



One of the first possibilities to vary the substrate would be the implementation of azido sugars for the production of glycothioamides, and peptide thioamide glycosides (Scheme 6-5, A and B). Thioamides may also be used as precursors and building blocks for subsequent reactions, for instance in the synthesis of different thiazoles (Scheme 6-5, C) which has received interest recently.[175][176]

The methodology shown in this work has utilized a mixture of solid phase and solution phase chemistry for the synthesis of peptide thioesters and thioacids. These thioesters and thioacids are building blocks for a number of established ligation

methods. This research underscores the ever growing interest and importance for different and new ligation chemical techniques. Furthermore, the discovery of a new ligation-type reaction gives a promising look into possible future methods. As the complexity of differing ligation techniques grows to meet the complex biological world, the greater will be the ability to answer new and important questions regarding protein function and chemistry.

7. Experimental Part

7.1. General Procedures

Compounds were named according to their IUPAC rules, and are not always numbered accordingly. All moisture sensitive reactions were performed in flame dried Schlenck flasks or tubes and were carried out under a Nitrogen atmosphere which had been dried over a silica gel rubin/P_4O_{10}/$CaCl_2$ drying column. The dry solvents DCM, THF, DMF, toluene, diethylether, were obtained commercially from Fluka, and Aldrich as super-dry, stored over molecular sieves and were used without further distillation. Other non-dry solvents were obtained from Riedel de Haen, Acros, Bernd Kraft GmbH, VWR and Aldrich. Acetonitrile for use in HPLC as well as MS analysis was obtained from Fisher Scientific as HPLC grade. Methanol also for use in HPLC as well as MS analysis was obtained as extra pure HPLC-grade from VWR. Water for HPLC and MS was obtained through a Milli Q machine from the company Millipore. Deuterated solvents for NMR measurements were obtained commercially either from Deutero or Euriso-top. All other chemicals and reagents were obtained commercially from Sigma-Aldrich, Fluka, Riedel de Haen, Acros, ABCR, Bachem and Merck and were used without further purification unless otherwise noted. Some compounds were purified with Kugelrohr distillation using a Büchi Glass Oben B-585.

7.2. Instrumentation and analytical methods

7.2.1. Thin layer and column chromatography

TLC chromatographie was carred out with TLC silica gel 60 F_{254} on aluminium sheets. Column chromatography was carried out either over silica gel 60 (0.04-0.06 mm) or aluminium oxide using glass columns of diameter 10, 20, 30, and 40 mm. Compounds were visualized either spectroscopically at 254 nm or stained with the following reagents:

Vanillin: 6 g vanillin in EtOH (250 mL) and conc. H_2SO_4 (2.5 mL).

Anisaldehyde: 6 g anisaldehyde in EtOH (250 mL) and conc. H_2SO_4 (2.5 mL).
Permanganate: $KMnO_4$ (3 g), K_2CO_3 (20 g), 5 % aqueous NaOH (5 ml) and water (300 mL).
MoStain: $Ce(IV)(SO_4)_2 \cdot 4H_2O$ (2 g), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (100 g), conc. H_2SO_4 (115 mL), 1885 mL H_2O

7.2.2. NMR spectroscopy

^1H, ^{13}C, DEPT, DQF-COSY, HMQC, HMBC, and TOCSY NMR experiments were recorded on a Bruker AVANCE 300 MHz or 600 MHz instruments and chemical shifts (δ) were measured in parts per million (ppm) relative to tetramethylsilane (TMS).

7.2.3. UV/Vis and IR spectroscopy

Loadings of peptide resins as well as calibrations for colorimetric tests were quantitatively determined using a Jasco V-550 UV/Vis spectrophotometer. For Fmoc-determinations, samples of 2-3 mg of resin were added to a 10 mL volumetric flask and measured exactly. To this, 20 % piperidine in DMF was added to the calibration line and allowed to stand for 45-60 minutes with occasional swirling. From this solution, approximately 300 µL were added to Hellma Suprasil cuvettes with a path length of 1 mm, a blank sample was prepared in the same manner with only 20 % piperidine in DMF.

For the Fmoc determinations, a wavelength range of 260-320 nm was used and the Molar absorptivity coefficients were given at λ_1 = 267 nm, λ_2 = 289 nm and λ_3 = 301 nm and calculated using the following equation:

$$x\left[\frac{mmol}{g}\right] = \frac{100000 \cdot E_\lambda}{\varepsilon_\lambda \cdot (weighedsample)}$$

x = resin loading
E_λ = molar absorptivity value

ε_λ = molar absorptivity coefficients
weighedsample = the weighed resin in mg

For this analysis, the following molar absorptivity coefficients were used:

$\varepsilon_{267} = 17500\ cm^{-1}$
$\varepsilon_{289} = 5800\ cm^{-1}$
$\varepsilon_{301} = 7800\ cm^{-1}$

For the trityl determination, a wavelength range of 340-500 nm was used and the Molar absorptivity coefficients were given at λ_1 = 408 nm and λ_2 = 430 nm and calculated using the same equation as for the Fmoc determination. For the analysis, the following molar absorptivity coefficients were used.[112]

$\varepsilon_{408} = 37360\ cm^{-1}$
$\varepsilon_{430} = 36400\ cm^{-1}$

The loading of the samples was determined by averaging the measured values at both wavelengths.

Solid phase reactions were monitored by FTR-IR spectrometry of the resin using a Bruker Tensor 27 FTR-IR equipped with a Pike MIRacle™ ATR fitted with a ZnSe crystal plate. Samples were measured between 4000 and 515 cm^{-1} with 32 scans at a resolution of 2 cm^{-1}.

7.2.4. Kaiser Test

Solid phase reactions were monitored after coupling protected amino acids to primary amines using the Kaiser test. The Kaiser test is a test to determine the presence of primary amines and thusly the extent to which the reaction has gone to completion. For the Kaiser test, the following solutions were used:

Solution I: 0.01 M KCN(aq) (2 mL) is diluted with pyridine (98 mL).

Solution II: Ninhydrin (500 mg) is dissolved in EtOH (10 mL).

A very small portion of the resin is taken (no more than 20 resin particles are needed) and added to a 1.5 mL eppendorf tube. To this, 0.1 mL of each of the solutions is added and the suspension is heated to 110 °C for 4-5 minutes. The presence of blue beads or a strong blue color of the solution indicates the presence of free amines.

7.2.5. Ellman's Test

The procedure used was performed as described by Kay et al[177] adapted from the original literature from Ellman.[115] A solution of 5,5'-dithiobis(2-nitrobenzoic acid) (40 mg) in a NaPi buffer (pH = 8) (10 mL). To test the presence of free thiols on the resin, between 3 and 5 drops of the solution was added to the resin beads swollen in dioxane or THF. In our hands, the resin beads turned a yellow-orange color indicating the presence of free thiol groups on the resin.

7.2.5. Elemental Analysis

Elemental analysis was performed with a varioEL elemental analyser, Elementar Analysensysteme GmbH.

7.2.6. Microwave supported synthesis

Microwave-supported syntheses were carried out in a Biotage Initiator with set power programs. The scale of the reactions were carried out in 2, 5, and 20 mL glass vials fitted with crimped Teflon septa.

7.2.7. Liquid Chromatography Mass Spectrometry (LC-MS)

LC-MS data were recorded on an Agilent 1100 series chromatography workstation (Agilent Technologies) equipped with a single quadrupole mass spectrometer and

electrospray ionization (ES). Eluents A (0.1 % TFA in Millipore water), B (0.1 % TFA in acetonitire), and C (0.1% TFA in MeOH) were used in a linear gradient (5-99 % B or C in 5 min or 30 min for preparative runs).

7.2.8. High Performance Liquid Chromatography (HPLC)

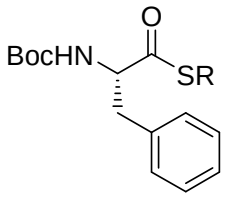
Peptides were purified either on a preparative HPLC column (10 µm, 250 x 20 mm, Grom-SIL 300 ODS-5-ST RP-C18) or on a semipreparative HPLC column (VP 250/10 Nucleodor 100-5 C18 ec Machery-Nagel) employing individual gradients derived from analytical runs (eluents A, B, and C).

7.2.9. Liquid Chromatography Mass Spectrometry Time of Flight (LC-MS-TOF)

HRMS measurements were conducted with an Agilent 6220 ESI-TOF mass spectrometer. Eluents A (0.1 % TFA in Millipore water), B (0.1 % TFA in acetonitire), and C (0.1% TFA in MeOH) were used in a linear gradient (5-99 % B or C in 2.5 min).

7.3. Synthetic Procedures for the Synthesis of Peptide Thioesters

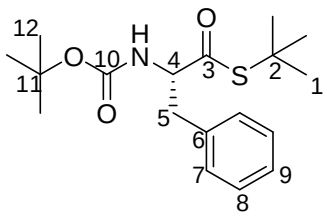
General protocol for tBoc-thioester synthesis for solution stability tests:



Method A: To a flame-dried round bottom Schlenck flask filled and evacuated with N_2, was added under a cross current of N_2, *t*Boc-AA-OH (3.77 mmol, 1 equiv). The amino acid was dissolved in dry THF (5 mL) and CDI (0.672 g, 4.146 mmol, 1.1 equiv) was added in portions to the flask under a cross-current of N_2. The thiol (11.31 mmol, 3 equiv) was then added and the reaction was allowed to stir overnight at r.t. The reaction was controlled via TLC using 100 % DCM as eluent. Once the reaction was determined finished, 4 N NaOH (10 mL) was added and immediately extracted with DCM (4 x 7 mL). The organic phases were then combined and dried over $MgSO_4$, the solvent removed *in vacuo* and purified over silica gel using 100 % DCM as eluent to give the thioesters as white solids in 65-99 % yield.

Method B: To a flame-dried round bottom Schlenck flask filled and evacuated with N_2 was added the tBoc amino acid (1.0 mmol, 1 equiv) and dissolved in dry DCM (6 mL). To this was added DMAP (0.1 mmol, 0.1 equiv) and the thiol (1.05 mmol, 1.05 equiv) after which the reaction was cooled to 0 °C. DCC (1.05 mmol, 1.05 equiv) was then added and the stirring solution was allowed to warm to room temperature. After 3-18 hours, depending on the thiol, the precipitated thiol urea was then filtered through a pad of celite and the filtrate evaporated down *in situ*. The residue was taken up in DCM , washed twice with 0.5 N HCl, saturated $NaHCO_3$ solution, and dried over $MgSO_4$. The product was then purified via flash column chromatography over silica gel (18 cm) using DCM as eluent.

***S*-(*Tert*-butyl) (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropanethioate (2)**

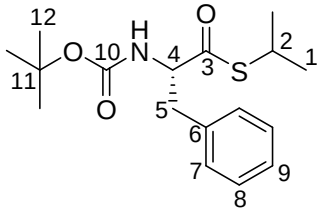


2

Using **method A**, the product was obtained and obtained as a white solid in a 0.834 g (66 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.40 (s, 9H, CH-1), 1.45 (s, 9H, CH-12), 2.96-3.16 (m, 2H, CH-5), 2.96-3.17 (m, 2H, CH-5), 4.46-4.60 (m, 1H, CH-4), 4.86-5.01 (m, 1H, NH), 7.12-7.20 (m, 1H, CH-9), 7.21-7.33 (m, 2H, CH-8,9).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 28.2 (C-12), 29.6 (C-1), 38.8 (C-5), 48.0 (C-11), 61.1 (C-4), 79.9 (C-11),126.9 (C-9), 128.4 (C-7), 129.4 (C-8), 135.8 (C-6), 154.8 (C-10), 200.7 (C-2).
HRMS (ESI-TOF): *m/z* calcd for $C_{18}H_{28}NO_3S$, 338.1784 $[M+H]^+$. Found, 338.1786 $[M+H]^+$.

***S*-Isopropyl (2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropanethioate (5)**



5

Using **method A**, the product was obtained as a white solid in a 1.219 g (99 %) yield.

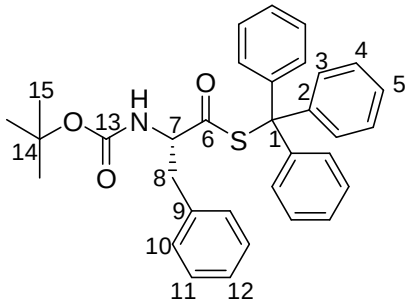
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.28 (d, *J* = 6.73 Hz, 3H, CH, CH-1), 1.40 (s, 9H, CH-12), 2.96-3.18 (m, 2H, CH-5), 2.96-3.17 (m, 2H, CH-5), 3.61 (septuplet, *J*

= 6.81 Hz, 1H, CH-2), 4.51-5.65 (m, 1H, CH-4), 5.00 (d, *J* = 8.23 Hz, NH), 7.11-7.18 (m, 1H, CH-9), 7.19-7.32 (m, 2H, CH-8,9).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 22.8 (C-1), 28.2 (C-11), 34.7 (C-2), 38.4 (C-5), 61.0 (C-4), 80.2 (C-11),127.0 (C-9), 128.5 (C-7), 129.3 (C-8), 135.8 (C-6), 155.0 (C-10), 200.9 (C-3).

HRMS (ESI-TOF): *m/z* calcd for $C_{17}H_{26}NO_3S$, 324.1628 $[M+H]^+$. Found, 324.1629 $[M+H]^+$.

_S_-Trityl (2_S_)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropanethioate (6)



6

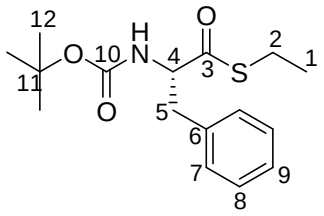
Using **method B**, the product was obtained from 1.0 mmol of tBoc-Phe-OH and 1.05 mmol of tritylthiol as a white solid in a 0.379 g (72 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.40 (s, 9H, CH-15), 2.84-3.01 (m, 2H, CH-8), 4.54-4.63 (m, 2H, CH-7), 4.77-4.82 (m, 1H, NH), 6.96-7.04 (m, 2H, CH-10), 7.18-7.31 (m, 18H, CH2-5,11-12).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 28.3 (C-15), 37.9 (C-8), 61.0 (C-7), 70.3 (C-1), 80.2 (C-14), 127.1 (Ar),127.7 (Ar), 128.5 (Ar), 129.5 (Ar), 129.8 (Ar), 135.6 (C-9), 143.5 (C-2), 155.0 (C-13), 197.6 (C-6).

HRMS (ESI-TOF): *m/z* calcd for $C_{33}H_{33}NNaO_3S$, 546.2073 $[M+Na]^+$. Found: 546.2061 $[M+Na]^+$.

_S_-Ethyl (2_S_)-2-[(_tert_-butoxycarbonyl)amino]-3-phenylpropanethioate (7)



7

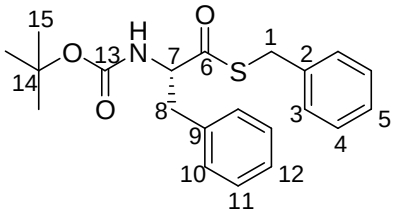
Using **method A**, the product was obtained as a white solid in a 0.791 g (68 %) yield.

1H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.23 (t, *J* = 7.44 Hz, 3H, CH, CH-1), 1.41 (s, 9h, CH-12), 2.87 (q, *J* = 7.39 Hz, 2H, CH-2), 2.96-3.17 (m, 2H, CH-5), 4.56-4.68 (m, 1H, CH-4), 4.93 (d, *J* = 8.23 Hz, NH), 7.13-7.19 (m, 1H, CH-9), 7.20-7.33 (m, 2H, CH-8,9).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 14.0 (C-1), 22.8 (C-2), 27.8 (C-12), 37.9 (C-5), 60.6 (C-4), 79.8 (C-11),126.6 (C-9), 128.1 (C-7), 128.9 (C-8), 135.3 (C-6), 154.6 (C-10), 200.6 (C-2).

HRMS (ESI-TOF): *m/z* calcd for $C_{16}H_{23}NO_3S$, 310.1471 $[M+H]^+$. Found: 310.1471 $[M+H]^+$.

_S_-Benzyl (2_S_)-2-[(_tert_-butoxycarbonyl)amino]-3-phenylpropanethioate (8)



8

The product was obtained using **method A** as a white solid in a 1.101 g (79 %) yield.

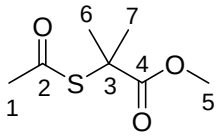
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.43 (s, 9H, CH-15), 3.05-3.21 (m, 2H, CH-8), 4.05-4.16 (m, 2H, CH-1), 4.14-4.24 (m, 2H, CH-7), 4.63-4.74 (m, 1H, CH-7),

4.91-5.01 (m, 1H, NH), 7.07-7.15 (m, 2H, CH-5,12), 7.20-7.38 (m, 8H, CH-3,4,10,11).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 28.2 (C-15), 33.2 (C-1), 38.3 (C-8), 60.8 (C-7), 80.2 (C-14), 127.2 (Ar),128.5 (Ar), 128.9 (Ar), 129.3 (Ar), 135.5 (C-9), 137.1 (C-2), 154.9 (C-13), 200.3 (C-6).

MS (ESI): *m/z* calcd for $C_{21}H_{25}NO_3S$ 394.1 $[M+Na]^+$. Found 394.1.

Methyl 2-(acetylthio)-2-methylpropanoate (12)



12

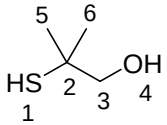
To a stirring mixture of potassium thioacetate (10.0 g, 66.3 mmol, 1 equiv) and DMF (10 mL) in a round bottom 50 mL Schlenck flask evacuated and filled with N_2 was added dropwise via canula, methyl α-bromoisobutyrate (10.0 g, 7.148 mL, 55.2 mmol, 1 equiv). The reaction mixture was then stirred at 60 °C for 15 hours whereupon it was quenched with H_2O (25 mL). The product was extracted Et_2O (4 x 25 mL). After removal of the solvent under reduced pressure, and the pure product was distilled *via* kugel-rohr distillation between 120 and 150 °C at 0.14 mbar to give a yellow liquid in an 8.098 g (83 %) yield.

R_f: 0.45 (4:1, Hex:EtOAc)

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.57 (s, 6H, CH-6,7), 2.26 (s, 3H, CH-1), 3.72 (s, 3H, CH-4).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 25.9 (C-6,7), 30.1 (C-1), 51.2 (C-5), 52.8 (C-3), 174.2 (C-4), 194.9 (C-2).

2-Mercapto-2-methylpropan-1-ol (13)

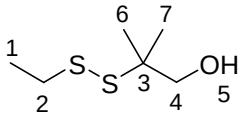


13

To a stirring mixture of $LiAlH_4$ (0.883g, 0.0233 mmol, 2 equiv) and dry Et_2O (10 mL) in a flame dried 3-neck round bottom flask fitted with a condenser and flushed thrice with N_2 was added under a cross-current of N_2 at 0 °C, **12** (2.05g, 11.6 mmol, 1 equiv) in dry Et_2O (10 mL). The reaction mixture was allowed to warm to room temperature and stirred for 1.5 hours and checked via TLC for completion of the reaction. Upon completion, the reaction mixture was quenched with H_2O and then acidified with 30 mL of 10% HCl. The product was extracted with DCM (3 x 50 mL) and the organic phases were collected and successively washed with sat. $NaHCO_3$, H_2O, and brine and dried over $MgSO_4$. The solvent was removed under reduced pressure to give the title compound as a colorless oil in a 1.190 g (96 %) yield.

R_f: 0.33 (4:1, Hex:EtOAc)
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.34 (s, 6H, CH-5,6), 1.62 (s, 1H, SH, H-1), 2.99 (br, 1H, OH), 3.42 (s, 2H, CH-3).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 24.7 (C-5,6), 51.5 (C-2), 69.5 (C-3).

2-(Ethyldithio)-2-methylpropan-1-ol (14)



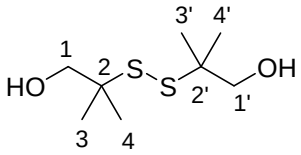
14

To a solution of NaOH (0.075 g, 1.884 mmol, 2 equiv) and potassium iodide (0.005 g, 0.028 mmol, 0.03 equiv) in H_2O (2 mL), was added ethanethiol (0.059 g, 0.07 mL, 0.942 mmol, 1.25 equiv) and **13** (0.08 g, 0.75 mmol, 1 equiv) at 0 °C. After stirring for 1 hour, I_2 (0.239 g, 0.942 mmol, 1.25 equiv) was added in small portions at room temperature and allowed to stir for an additional 2 hours whereupon a deep red color evolved signalling the completion of the reaction. The reaction was allowed to stir for an additional 10 minutes and then immediately extracted with Et_2O (3 x 10 mL). The organic phases were collected and the solvent was removed under reduced pressure to give the crude product as a reddish oil. Flash column chromatography with hexanes

and ethyl acetate (10:1) gave the title compound as an amber oil in a 0.065 g (52 %) yield.

R_f: 0.19 (10:1, Hex:EtOAc), stained with MoStain.
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.30 (s, 6H, CH-6,7), 1.31 (t, *J* = 7.29 Hz 3H, CH-1), 1.93 (br, 1H, OH), 2.72 (q, *J* = 7.43Hz, 2H, CH-2), 3.49 (s, 2H, CH-4).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 14.3 (C-1), 24.3 (C-6,7), 34.3 (C-2), 52.5 (C-3), 68.8 (C-4).
MS (ESI): *m/z* calcd for $C_6H_{14}OS_2$ 184.1 $[M+H_2O]^+$. Found 184.0.

2-Mercapto-2-methylpropan-1-ol disulfide (15)

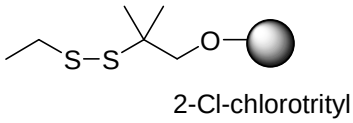


15

15 was obtained from the same reaction and eluted with MeOH from the column as described above. After removal of silica gel, the title compound was obtained as a yellowish oil in a 0.022 g (22 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.29 (s, 12H, CH-3, 4, 3', 4'), 2.18 (br, 2H, OH) 3.47 (s, 4H, CH-1, 1')

2-(Ethyldithio)-2-methylpropan-1-ol-polystyrene-copolymer (17)



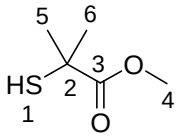
17

To a 5 mL microwave vial filled and evacuated with N_2 was added under a slight cross-current of N_2, 2-cl-tritylchloride resin (0.300 g, 0.42 mmol (1.829 mmol/g), 1 equiv). The vial was then capped and evacuated and filled with N_2 once more, and

then a solution of **14** (0.284 g, 1.708 mmol, 4.06 equiv), Et_3N (0.237 mL, 1.708 mmol, 4 equiv), DMAP (0.038 g, 0.315 mmol, 0.75 equiv) in dry DCM (5 mL). The reaction was allowed to stir 15 hours and then arrested by the addition of MeOH (0.8 mL/g) for 1 hour to cap any remaining free chloride functionalities. The resin was then poured into a 5 mL syringe fitted with a polypropylene filter disc and washed successively with DMF, THF, and DCM (5 x 5 mL).

Elemental analysis (Found (%)): S-Analysis: 1.074. Loading = 0.17 mmol/g (11 %).

Methyl 2-mercapto-2-methylpropanoate (20)

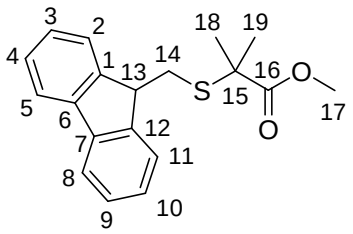


20

To a stirring solution of **12** (0.881g, 5.0 mmol, 1 equiv) in dry MeOH (30 mL) in a flame dried 100 mL round bottom flask flushed and evacuated with N_2, was added under a cross current of N_2, a solution of sodium thiomethoxide (0.350g, 5 mmol, 1 equiv) in dry MeOH (5 mL). The reaction was stirred at room temperature for 30 minutes and monitored via TLC using Hex:EtOAc (10:1). Upon completion, the reaction was quenched with 0.1 M HCl (20 mL) and extracted with DCM (3 x 25 mL). The organic phases were collected and washed with brine and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the title compound was obtained as a pure yellowish oil in a 0.609 g (91 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.58 (s, 6H, CH-5,6), 2.43 (s, 1H, H-1) 3.73 (s, 3H, CH-4).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 29.0 (C-5,6), 44.7 (C-2), 52.6 (C-4), 175.4 (C-5).

Methyl 2-[(9*H*-fluoren-9-ylmethyl)thio]-2-methylpropanoate (21)



24

To a stirring solution of **20** (0.5g, 3.726 mmol, 1 equiv) in dry DCM (20 mL) in a flame dried round bottom flask filled and evacuated with N_2 was added in one portion Fmoc-Osu (1.37g, 4.06 mmol, 1.09 equiv) under a cross current of N_2, followed by Et_3N (0.4g, 0.551 mL, 3.95 mmol, 1.06 equiv) and allowed to stir at room temperature for 4 hours. The reaction was controlled via TLC and upon completion the volatiles were removed under reduced pressure. The crude oil was then purified via flash column chromatography using Hex:EtOAc (30:1) as eluent to give the title compound **24** as a yellow oil in a 0.804 g (69 %) yield.

R_f: 0.38 (10:1, Hex:EtOAc)

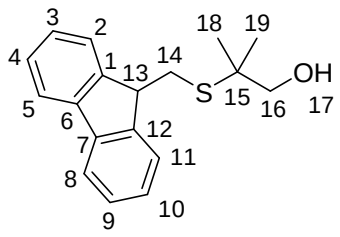
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.52 (s, 6H, CH-18,19), 3.14 (d, *J* = 6.71 Hz, 2H, CH-14), 3.65 (s, 3H, CH-17), 4.04 (t, *J* = 6.72 Hz, 1 H, CH-13), 7.29-7.43 (m, 4H, CH-9,10,3,4), 7.65-7.68 (m, 2H, CH-11,2), 7.71-7-74 (m, 2H, CH-8,5).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 25.8 (C-18,19), 33.7 (C-14), 46.9 (C-13), 47.1 (C-15), 52.4 (C-17), 120.0 (C-5,8), 127.5 (C-3,10), 127.1 (C-4,9), 127.7 (C-2,11), 141.0 (C-1,12), 146.1 (C-6,7), 174.5 (C-16).

MS (ESI): *m/z* calcd for $C_{19}H_{20}O_2S$ 313.1 $[M+H]^+$. Found 313.0, 330.1 $[M+H_2O]^+$, 335.2 $[M+Na]^+$.

R_t: 10.41 min (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

2-[(9*H*-Fluoren-9-ylmethyl)thio]-2-methylpropan-1-ol (22)



22

To a 0° C cooled suspension a suspension of $LiAlH_4$ (0.014g, 0.359 mmol, 1.1equiv) in dry THF (1 mL) in a 2 neck round bottom flask equipped with a reflux condenser and N_2 valve which had been flame dried and evacuated and filled with N_2, was added dropwise a solution of **24** in dry THF (3.5 mL). The reaction mixture was allowed to stir for 1.5 hours and checked periodically for completion *via* TLC after which 10 % HCl (3 mL) was slowly added at 0 °C to quench the reaction. The quenched suspension was allowed to stir for an additional 1 hour after which the slurry was then extracted with DCM (3 x 10 mL). The organic phases were collected and washed with H_2O and brine and finally dried over $MgSO_4$. The solvent was removed under reduced pressure to afford a light yellow oil in a 0.072 g (77 %) yield.

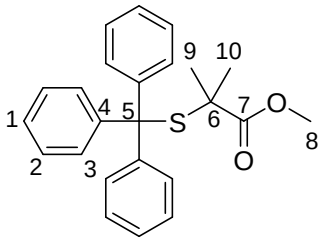
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.25 (s, 6H, CH-18,19), 2.09 (br, 1H, OH), 3.02 (d, *J* = 6.1 Hz, 2H, CH-14), 3.34 (s, 2H, CH-16), 4.10 (t, J = 6.11 Hz,1H, CH-13), 7.29-7.43 (m, 4H, CH-9,10,3,4), 7.65-7.70 (m, 2H, CH-11,2), 7.73-7-78 (m, 2H, CH-8,5).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]:

MS (ESI): *m/z* calcd for $C_{18}H_{20}OS$ 285.1 $[M+H]^+$. Found 285.1, 307.0 $[M+Na]^+$, 323.2 $[M+K]^+$.

R_t: 9.07 min (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

Methyl 2-methyl-2-(tritylthio)propanoate (24)



24

To a stirring suspension of triphenyl methane thiol (2.519 g, 9.11 mmol, 1.1 equiv) and potassium *tert*-butoxide (1.022 g, 9.11, 1.1 equiv) in dry THF (17 mL) in a 20 mL microwave vial was added methyl α-bromoisobutyrate (1.50 g, 1.072 mL, 8.285 mmol, 1 equiv) and capped. The reaction vessel was then irradiated in a microwave oven reactor to 130 °C for 30 minutes upon which the product was extracted with DCM (3 x 10mL) and washed successively with NH_4Cl, H_2O, and brine. After removal of the solvent under reduced pressure, the product was purified via flash column chromatography to give the title compound as a white solid in a 3.119 g (99 %) yield.

R_f: 0.34 (10:1, Hex:EtOAc)

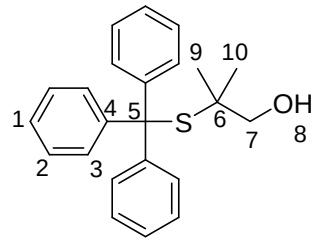
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.26 (s, 6H, CH-9,10), 3.34 (s, 3H, CH-8), 7.09-7.30 (m, 9H, CH-1,2), 7.49-7.57 (m, 6H, CH-3).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 26.8 (C-9,10), 54.3 (C-6), 67.4 (C-5), 68.7 (C-1), 126.6 (C-1), 127.7 (C-2), 129.7 (C-3), 145.0 (C-4).

MS (ESI): *m/z* calcd for $C_{24}H_{24}OS$ 399.1 $[M+Na]^+$. Found 399.1, 415.0 $[M+K]^+$.

R_t: 11.21 min (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

2-Methyl-2-(tritylthio)propan-1-ol (25)

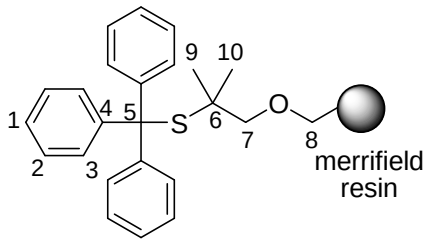


25

To a suspension of $LiAlH_4$ (0.064 g, 1.689 mmol, 1.1 equiv) in dry THF (10 mL) in a flame dried 3-neck flask equipped with reflux condenser and septen, filled and evacuated with N_2, was added under a cross-current of N_2, **24** (0.578 g, 1.535 mmol, 1 equiv) in dry THF (5 mL) and stirred at room temperature for 1 hour during which time the color of the suspension turned to bright yellow/green. Upon completion the reaction was quenched with H_2O (10 mL) and then extracted with DCM (4 x 25 mL). The organic phases were collected and then washed successively with NH_4Cl (sat), H_2O, brine and then dried over $MgSO_4$. The solvent was removed under reduced pressure to yield an off-white solid in a 0.445 g (83 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.14 (s, 6H, CH-9,10), 1.99 (br, 1H, OH-8), 2.40 (s, 2H, CH-7), 7.19-7.33 (m, 9H, CH-1,2), 7.65-7.70 (m, 6H, CH-3).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 26.8 (C-9,10), 54.3 (C-6), 67.4 (C-5), 68.7 (C-7), 126.6 (C-1), 127.7 (C-2), 129.7 (C-3), 145.0 (C-4).
MS (ESI): *m/z* calcd for $C_{23}H_{24}OS$ 371.1 $[M+Na]^+$. Found 371.1, 387.1 $[M+K]^+$.
R_t: 5.05 (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

2-Methyl-2-(tritylthio)propan-1-ol-polystyrene copolymer (26)



26

To a suspension of sodium hydride (0.060 g of a 60 % dispersion in mineral oil, 1.5 mmol, 3 equiv) in dry THF (1.5 mL) at 0 °C in an evacuated 5 mL microwave vial filled with N_2 was added *via* canula a solution of **25** (0.523 g, 1.5 mmol, 3 equiv) in dry THF (3.5 mL) and stirred for 15 minutes at 0 °C and then allowed to warm to room temperature. After stirring for 1 hour at room temperature, the chloro-Merrifield copolymer resin (0.319 g, 0.5 mmol, 1 equiv) was added in portions and the mixture was then heated to 70 °C for 48 hours. The reaction was then arrested with the addition of MeOH (0.255 mL, 0.8 mL/g) and stirred for an additional 2 hours. The

resin was finally washed with DMF, THF, and DCM (5 x 5 mL per solvent), shrunk with MeOH (5 x 5 mL) and washed a final time with Et_2O (5 x 5 mL) and dried under high vacuum.

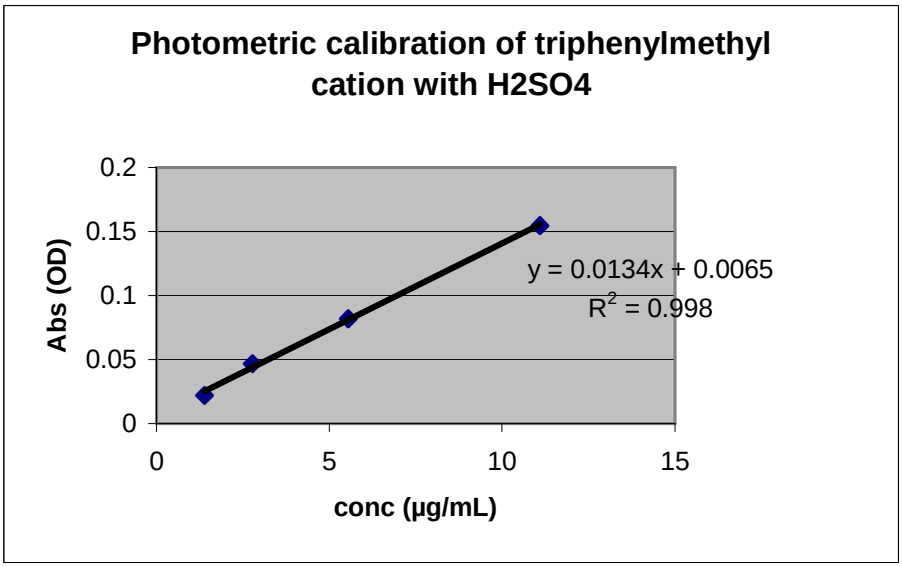
ATR-IR: ν = 3025, 2922, 1782, 1679, 1601, 1492, 1452, 1341, 1219, 1169, 1140, 1028, 906, 819, 756 cm^{-1}.
Elemental analysis (Found (%)): S-Analysis: 2.944. Loading = 0.918 mmol/g (87 %).
UV-Vis absorption maxima of trityl cation upon acidic treatment (1:1, DCM:TFA, 300 µL): λ_1 = 408 nm, λ_2 = 430 nm.

Trityl determination:

For the calibration of the the triphenylmethyl cation in sulfuric acid, triphenyl methanethiol (3.15 mg) was taken and dissolved in concentrated sulfuric acid (25 mL) and allowed to stand for 3.5 hours. After this time, the solution was diluted four times to give concentrations of: 0.126, 0.0063, 0.00315, and 0.001575 mg/ mL. These varying concentrations gave the following absorbance values (respectively, in OD): 0.1546, 0.0816, 0.0468, 0.0220. These data were then fitted and gave a linear relationship as shown in Figure 7-1, A. From this data, it was determined that the absorption of the trityl cation of triphenylmethanethiol could be applied to the Lambert-Beer relationship previously described.

The calibration of the triphenyl methyl cation in DCM/TFA was conducted in a similar fashion. Triphenyl methanethiol (7.99 mg) was dissolved in a 1:1 mixture of DCM and TFA (25 mL total) to give a concentration of 0.3196 mg/mL). This solution was diluted to give concentrations of: 140.6, 70.3, 35.15, 28.12, 14.06, 7.03, 3.515 µg/mL. These varying concentrations gave the following absorbance values (respectively, in OD): 2.2756, 1.1969, 0.6134, 0.4859, 0.2602, 0.1552, 0.0933. This data was then plotted and gave a linear relationship for all concentrations as can be seen in Figure 7-1, B

(A)



(B)

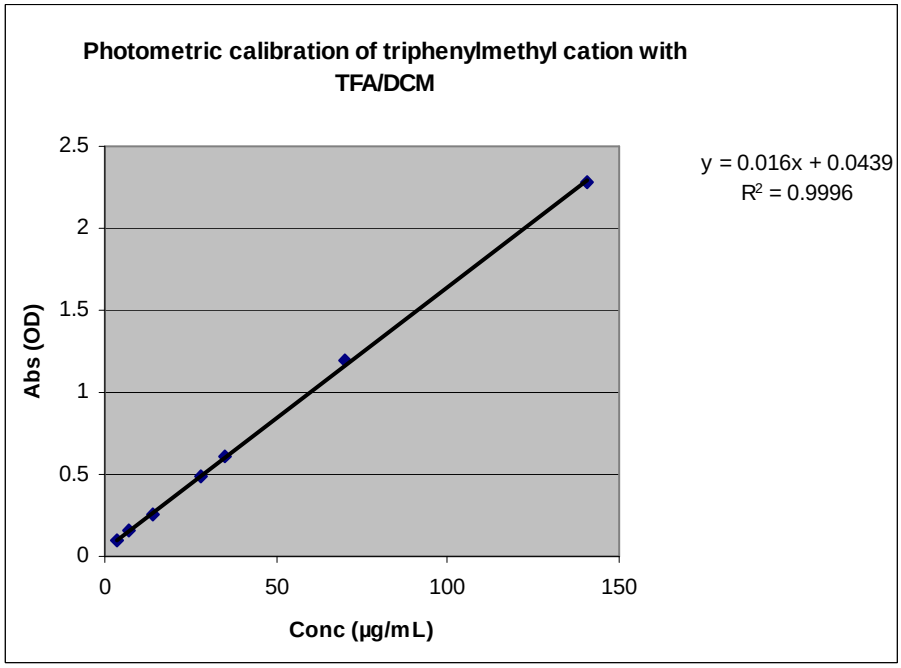
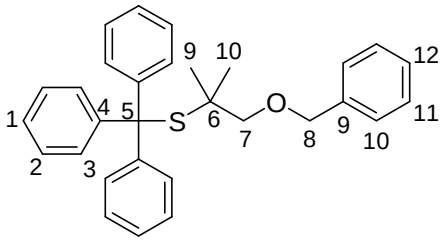


Figure 7-1. Photometric calibration of the triphenylmethyl cation from triphenylmethane thiol in **(A)**concentrated H_2SO_4 and **(B)** TFA/DCM.

Benzyl 2-methyl-2-(tritylthio)propyl ether (34)

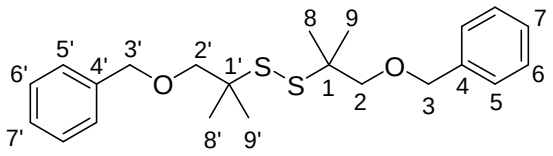


34

To a suspension of sodium hydride (0.202 g of a 60 % dispersion in mineral oil, 5.06 mmol, 1.1 equiv) in dry THF (5 mL), in a flame dried 50 mL round bottom Schlenck flask equipped with a reflux condenser and evacuated and filled with N_2, was slowly added at 0 °C **25** in dry THF (10 mL). The reaction was stirred at 0 °C for 30 minutes and warmed to room temperature for 15 minutes to which benzyl bromide (0.826 g, 0.574 mL, 4.83 mmol, 1.05 equiv) and tetrabtuylammonium iodide (0.17 g, 0.46 mmol, 0.1 equiv) were added and the reaction was refluxed for 17 hours. Water (10 mL) was added slowly and the reaction was diluted with DCM (10 mL). The aqueous layer was extracted with DCM (3 x 15 mL), the combined organic layers were washed with water (20 mL), brine (20 mL), and finally dried over $MgSO_4$. After removal of the solvent under reduced pressure, the crude product was precipitated from hexanes in EtOAc (30:1) and filtered over a short silica gel column with DCM to yield the title compound as a white solid in a 1.738 g (86 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.08 (s, 6H, CH-9,10), 2.79 (s, 2H, CH-7), 4.33 (s, 2H, CH-8), 7.16-7.39 (m, 14H, CH-1,2,10,11,12), 7.59-7.70 (m, 6H, CH-1).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 26.6 (C-9,10), 51.4 (C-6), 67.5 (C-5), 72.9 (C-8), 78.1 (C-4), 126.3 (C-13), 127.2 (C-10), 127.3 (C-11), 127.4 (C-12), 128.1 (C-12), 130.0 (C-11).
MS (ESI): *m/z* calcd for $C_{30}H_{30}OS$ 461.2 $[M+Na]^+$. Found 461.2, 477.2 $[M+K]^+$.
R_t: 13.09 (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

Bis[2-(benzyloxy)-1,1-dimethylethyl] disulfide (35)

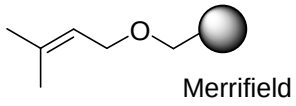


35

To a flame dried round bottom Schlenck flask evacuated and filled with nitrogen was added **34** (1.316 g, 3.0 mmol, 1 equiv). To this was added equal volumes of DCM and TFA (10 mL, 10 mL) and Et_3SiH slowly at 0 °C. The color of the solution at the start of the reaction was orange and rapidly became colorless. The reaction was allowed to stir for 1 hour and monitored via TLC. After completion, the reaction was quenched with $NaHCO_3$ and water. After washing twice with $NaHCO_3$, water, and then brine, the DCM was removed under reduced pressure. Most of the product was determined to be the disulfide, and remaining free thiol rapidly oxidized to the disulfide under air.

MS (ESI): *m/z* calcd for $C_{22}H_{30}O_2S_2$ 391.2 $[M+H]^+$. Found 391.4, 413.4 $[M+Na]^+$.
R_t: 3.99 (linear gradient 1-99% C, 12 min, 99% C for 3 min.)

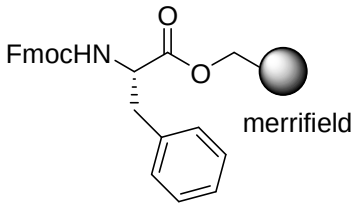
Benzyl 3-methylbut-2-enyl ether-polystyrene copolymer



To a 5 mL microwave vial equipped with a stir bar and evacuated and filled with N_2, was added under a slight cross-current of N_2, Cl-Merrifield resin (0.20 g, 0.296 mmol, 1 equiv) and sodium hydride (0.036 g, 1.481 mmol, 5 equiv). A solution of 3-Methylbut-2-en-1-ol (0.128 g, 1.481 mmol, 5 equiv) in dry THF (2 mL), was then slowly added and the vial was capped and fitted with a N_2 balloon. The reaction was then heated to 80 °C and slowly stirred overnight. The reaction was arrested with the addition of MeOH (0.8 mL/g) for one hour and was then filtered into a 5 mL syringe fitted with a polypropylene filter disc. The resin was finally washed with DMF, THF,

and DCM (5 x 5 mL per solvent), shrunk with MeOH (5 x 5 mL) and washed a final time with Et_2O (5 x 5 mL) and dried under high vacuum.

Fmoc-Phe-carboxymethyl polystyrene-copolymer

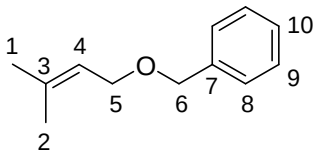


To a suspension of the Benzyl 3-methylbut-2-enyl ether-polystyrene copolymer (0.50 g, 0.784 mmol, 1 equiv) in dry DCE (5 mL) in a glass tube under inert conditinos at room temperature was added $InCl_3$ (0.13 g, 0.59 mmol, 0.75 equiv) followed by dropwise addition thioacetic acid (0.298 g, 3.92 mmol, 5 equiv). The tube was capped and a nitrogen balloon was attached and the reaction was heated to 80 °C for 22 h. After the reaction was finished, resin was washed with DMF, THF, and DCM (5 x 5 mL per solvent), shrunk with MeOH (5 x 5 mL) and washed a final time with Et_2O (5 x 5 mL) and dried under high vacuum.

The acetyl protecting group was then removed with hydrazine acetate (0.217 g, 2.352 mmol, 3 equiv) in DMF (5 mL) and reacted for 90 minutes at 60 °C. After cooling to room temperature, the resin was briefly washed as previously described and a solution of the activated phenylalanine (0.152 g, 0.392 mmol, 5 equiv) with BTFFH (0.124 g, 0.392 mmol, 5 equiv) and DIPEA (0.137 mL, 0.784 mmol, 10 equiv) in DMF (2 mL) was added. The reaction was then shaken for 24 hours at room temperature, filtered and then washed as previously described.

Fmoc determination: 0.63 mmol/g (expected 1.063 mmol/g) = 59 % yield.

Benzyl 3-methylbut-2-enyl ether (45)

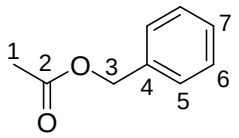


45

To a suspension of sodium hydride (0.153 g of a 60 % dispersion in mineral oil, 6.386 mmol, 1.1 equiv) in dry THF (10 mL), in a flame dried 25 mL round bottom Schlenck flask equipped with a reflux condenser and evacuated and filled with N_2, was slowly added at 0 °C, 3-Methyl-2-buten-1-ol (0.50 g, 0.581 mL, 5.805 mmol, 1 equiv). The reaction was stirred for 40 minutes and then benzyl bromide (1.042 g, 0.725 mL, 6.095 mmol, 1.05 equiv) and tetrabtuylammonium iodide (0.214 g, 0.581 mmol, 0.1 equiv) were added and the reaction was refluxed for 19 hours. Water (5 mL) was added slowly and the reaction was diluted with Et_2O (10 mL). The aqueous layer was extracted with Et_2O (3 x 20 mL), the combined organic layers were washed with water (20 mL), brine (20 mL), and finally dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was purified *via* column chromatography using hexanes and EtOAc (25:1) as eluent to give a colorless oil in 0.602 g (59 %) yield.

R_f = 0.22 (25:1, Hex:EtOAc).
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.65 (s, 3H, CH-1), 1.75 (s, 3H, CH-2), 3.99 (d, *J* = 7.33 Hz, 2H, CH-5), 4.49 (s, 2H, CH-6), 5.40 (t, *J* = 7.33 Hz, 1H, CH-4), 7.22-7.39 (m, 6H, CH-8-10).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 18.0 (C-1), 25.7 (C-2), 66.5 (C-5), 72.0 (C-6), 121.1 (C-4), 127.4 (C-10), 127.7 (C-8), 128.3 (C-9), 137.1 (C-3), 138.6 (C-7).

Benzyl acetate (47)



47

To a solution of **45** (0.20 g, 1.135 mmol, 1 equiv) in dry DCE (3 mL) in a flame dried 10 mL Schlenck flask evacuated and filled with N_2, was added in one portion under a cross- current of indiumtrichloride (0.013 g, 0.057 mmol, 5 mol %) followed by dropwise addition of thioacetic acid (0.095 g, 0.089 mL, 1.248 mmol, 1.1 equiv) and allowed to stir at 80 °C for 20 hours. After complete conversion of the starting material, the reaction was quenched with 1M HCl (5 mL) and extracted with Et_2O (3

x 10 mL). The organic phases were collected and washed with sat. Aq. $NaHCO_3$ and dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was purified via flash column chromatography using hexane:EtOAc (60:1) to give benzyl acetate as the sole product in 0.18 g (95 %) yield.

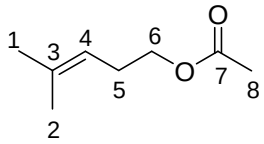
ATR-IR: ν = 1739 (C=O), 1689, 1633 cm^{-1}

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 2.10 (s, 3H, CH-1), 5.10 (s, 2H, CH-3), 7.33-7.37 (m, 5H, CH-5-7).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 20.5 (C-1), 65.8 (C-3), 127.8 (Ar), 128.1 (Ar), 128.5 (Ar), 135.5 (C-4), 170.4 (C-2).

The physical data are in agreement with the literature values.[178]

4-Methylpent-3-enyl acetate (51)



51

To a neat solution of 4-Methyl-3-penten-1-ol (0.200 g, 0.233 mL, 2.0 mmol, 1 equiv) in acetic anhydride (0.952 mL, 10.0 mmol, 5 equiv) was added LiOTf (0.062 g, 0.4 mmol, 0.2 equiv) and the reaction was allowed to stir 16 hours. After the reaction was complete, water was added (25 mL), and the product was extracted with DCM (2 x 30 mL). The organic phases were collected and then washed with $NaHCO_3$ (2 x 25 mL) and once with water (15 mL) and then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was purified *via* flash column chromatography using 20:1 hexanes and EtOAc (20:1) to give **51** as a colorless oil in a 0.218 g (77 %) yield.

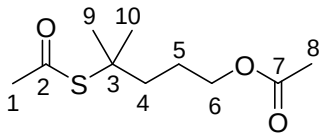
Boiling point: 170-180 °C (0.1 Mbar)

R_f = 0.57 (Hex: EtOAc, 10:1)

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.62 (s, 3H, CH-1). 1.70 (s, 3H, CH-2), 2.03 (s, 3H, CH-8), 2.30 (dd, *J* = 7.24 Hz, 2H, CH-5), 4.01 (t, *J* = 7.26 Hz, 2H, CH-6), 5.09 (t, *J* = 7.26 Hz, 1H, CH-4).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 17.9 (C-1), 21.1 (C-8), 25.8 (C-2), 27.7 (C-5), 64.3 (C-6), 119.4 (C-4), 134.7 (C-3), 171.3 (C-2).

4-(Acetylthio)-4-methylpentyl acetate (52)



52

To a suspension of **51** (0.151 g, 1.06 mmol, 1 equiv) and $InCl_3$ (0.012 g, 0.053 mmol, 5 mol %) in dry DCE (3 mL) in a flame-dried 25 mL round bottom Schlenck flask equipped with a reflux condenser and evacuated and filled with N_2 was added under a cross-current of N_2, thioacetic acid (0.089 g, 0.083 mL, 1.168 mmol, 1.1 equiv). The reaction was then heated to 80 °C for 1.5 hours and then quenched with 1M HCl (5 mL) and extracted with Et_2O (3 x 10 mL). The organic phases were collected and then washed with $NaHCO_3$ (15 mL) and then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was then purified *via* flash column chromatography using silica gel and hexanes and EtOAc (10:1) as eluent to afford **52** as a light yellow oil in 0.131 g, (57 %) yield.

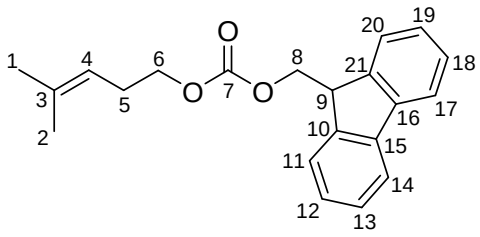
R_f = 0.19 (Hex:EtOAc, 10:1)

ATR-IR: ν = 1738 (C=O), 1683 (thio C=O), 1636, 1534, 1470, 1451, 1388, 1365, 1239, 1112, 1063, 1038, 946, 639 cm^{-1}.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.41 (s, 6H, CH-9,10), 1.61-1.71 (m, 2H, CH-5), 1.73-1.82 (m, 2H, CH-4), 2.02 (s, 3H, CH-8), 2.21 (s, 3H, CH-1), 4.02 (t, *J* = 6.54 Hz, 2H, CH-6).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 21.1 (C-8), 24.4 (C-5), 27.7 (C-9,10), 31.5 (C-1), 37.5 (C-4), 51.3 (C-3), 64.6 (C-6), 171.2 (C-7), 196.6 (C-2).

9H-Fluoren-9ylmethyl 4-methylpent-3-enyl carbonate (55)



55

To a round bottom flask containing Fmoc-chloride (0.798 g, 3.08 mmol, 1.2 equiv) was added pyridine (25 mL, 0.31 mole) and stirred at room temperature. To this stirring suspension was added 4-methylpent-3-en-1-ol (0.257 g, 2.57 mmol, 1 equiv) via canula. The resulting mixture was stirred for one hour and controlled via TLC (Hex:EtOAc, 10:1). The stirring mixture was quenched with H_2O (20 mL) upon completion of the reaction, and then extracted with Et_2O (3 x 20 mL). The organic phases were collected and then washed sequentially with NH_4CO_3 and brine. After removal of the solvent *in vacuo* the crude material was purified over silica gel (18 cm) with Hex:EtOAc (40:1) to yield a colorless oil **55** (0.817 g, 99 % yield).

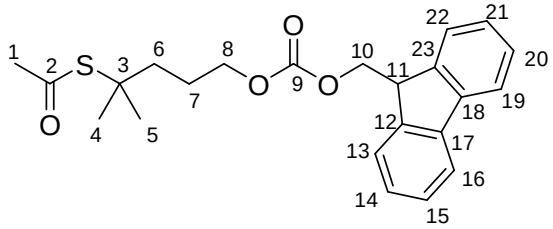
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.69 (s, 3H, CH, CH-1), 1.76 (s, 3H, CH, CH-2), 2.44 (q, *J* = 7.16, 2H, CH-5), 4.19 (t, *J* = 7.17, 2H, CH-6), 4.30 (t, *J* = 7.38, 1H, CH-9), 4.44 (d, *J* = 7.16, 2H, CH-8), 5.13-5.23 (m, 1H, CH-4), 7.33 (t, *J* = 7.45, 2H, CH-19,12), 7.42 (t, *J* = 7.43, 2H, CH-18,13), 7.65 (d, *J* = 7.51, 2H, CH-11,20), 7.79, (d, *J* = 7.51, 2H, CH-17,14).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 17.9 (C-2), 25.8 (C-1), 27.8 (C-5), 46.9 (C-9), 67.8 (C-6), 69.8 (C-8), 118.7 (C-4), 120.2 (C-14,17), 125.3 (C-11,20), 127.3 (C-12,19), 128.0 (C-13,18), 135.2 (C-3), 141.4 (C-15,16), 143.6 (C-10,21), 155.4 (C-7).

ESI-MS: *m/z* calcd for $C_{21}H_{22}O_3Na$, 345.1 $[M+Na]^+$. Found 345.1 $[M+Na]^+$.

R_t 3.99 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

_S_-(4-{[9_H_-Fluoren-9-ylmethoxy)carbonyl]oxy}-1,1-dimethylbutyl) ethanethioate (56)



56

To a nitrogen purged, flame-dried, round bottom Schlenk flask equipped with a reflux condensor was added $InCl_3$ (0.051 g, 0.23 mmol, 15 mol %) under a cross-current of nitrogen. To this, **55** (0.50 g, 1.55 mmol, 1 equiv) in dry DCE (5 mL) was added in one portion followed by thioacetic acid (0.121 mL, 0.171 mmol, 1.1 equiv) dropwise. The mixture was then heated to 80 °C and stirred for 17 h. The reaction was controlled via TLC (Hex:EA, 10:1) and upon completion was quenched with 1M HCl (6 mL) and extracted with Et_2O (3 x 7 mL). The organic layers were collected and washed with $NaHCO_3$ and then dried over $MgSO_4$. After removal of the solvent *in vacuo* the crude product was purified over silica gel (18 cm) with Hex:EtOAc (30:1) to give **56** (0.462 g, 75 % yield) as an amber colored oil.

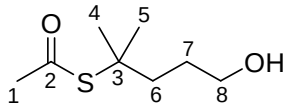
^{1}H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.44 (s, 6H, CH-4,5), 1.82-1.73 (m, 2H, CH-6,7), 1.92-1.83 (m, 2H, CH-6,7), 2.23 (s, 3H, CH-1), 4.16 (t, *J* = 6.50 Hz, 2H, CH-8), 4.28 (t, *J* = 7.38 Hz, 1H, CH-11), 4.40 (d, *J* = 7.51, 2H, CH-10), 7.32 (t, *J* = 7.37 Hz, 2H, CH-21,14), 7.40 (t, *J* = 7.37 Hz, 2H, CH-20,15), 7.62 (d, *J* = 7.51 Hz, 2H, CH-22,13), 7.76 (d, *J* = 7.51 Hz, 2H, CH-19,16).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 24.6 (C-6 or 7), 27.7 (C-3,4), 31.6 (C-1), 37.3 (C-6 or 7), 46.9 (C-11), 51.2 (C-5), 68.3 (C-8), 69.9 (C-10), 120.2 (C-19,16), 125.3 (C-22,13), 127.3 (C-21,14), 128.0 (C-20,15), 141.4 (C-17,18), 143.6 (C-12,23), 155.3 (C-9), 196.6 (C-2).

MS (ESI): *m/z* calcd for $C_{23}H_{26}O_4$, 421.1 $[M+Na]^+$. Found 421.0 $[M+Na]^+$.

R_t 4.01 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

S-(4-Hydroxy-1,1-dimethylbutyl) ethanethioate (57)

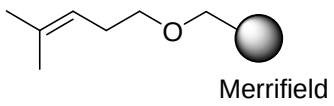


57

To a solution of **56** (0.200 g, 0.502 mmol, 1 equiv) in dry pyridine (5 mL) was added Et_3N (0.696 mL, 5.02 mmol, 10 equiv). The reaction mixture was then stirred for 2 hours and controlled via TLC (Hex:EtOAc, 5:1) using an anisaldehyde or vanillin solution to develop the TLC plates. After completion of the reaction, the solvents were removed under reduced pressure and the crude was purified over silica gel (18 cm) Hex:EtOAc (2:1) to give a colorless oil **57** (0.088 g, quantitative yield).

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.42 (s, 6H, CH-4,5), 1.57-1.65 (m, 2H, CH-7), 1.76-1.82 (m, 2H, CH-6), 2.21 (s, 3H, CH-1), 3.61 (t, *J* = 6.47 Hz, 2H, CH-8).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 27.7 (C-4,5), 28.3, (C-7), 31.6 (C-1), 37.6 (C-6), 51.5 (C-3), 63.1 (C-8), 197.0 (C-2).
MS (ESI): *m/z* calcd for $C_{23}H_{26}O_4$, 177.1 $[M+H]^+$. Found 177.1 $[M+H]^+$, 199.2 $[M+Na]^+$.
R_t 2.53 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Benzyl 4-methylpent-3-enyl ether-polystyrene copolymer (58)



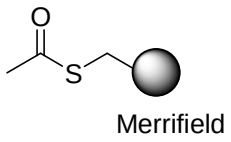
58

To a 20 mL microwave vial equipped with a stir bar and evacuated and filled with N_2, was added under a slight cross-current of N_2, chloro-Merrifield resin (1.594 g, 2.5 mmol, 1 equiv) and sodium hydride (0.20 g, 5 mmol, 2 equiv). A solution of 4-Methyl-3-penten-1-ol (0.50 g, 0.582 mL, 5.0 mmol, 2 equiv) in dry THF (15 mL), was then slowly added and the vial was capped and fitted with a N_2 balloon. The reaction was then heated to 70 °C and slowly stirred overnight. The reaction was

arrested with the addition of MeOH (0.8 mL/g) for one hour and was then filtered into a 5 mL syringe fitted with a polypropylene filter disc. The resin was finally washed with DMF, THF, and DCM (5 x 5 mL per solvent), shrunk with MeOH (5 x 5 mL) and washed a final time with Et_2O (5 x 5 mL) and dried under high vacuum.

ATR-IR: ν = 3025, 2920, 1601, 1510, 1492, 1451, 1093, 1028, 906, 820, 756, 697, 536 cm^{-1}.

_S_-Benzyl ethanethioate-polystyrene copolymer (59)

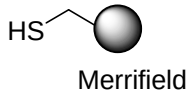


59

To a suspension of the resin **58** (0.319 g, 0.5 mmol, 1 equiv) in dry DCE (4 mL) in a purged 20 mL glass vial with a stir bar, was added under a slight cross current of N_2, indium trichloride (0.083 g, 0.375 mmol, 0.75 equiv) using dry DCE (1 mL) to wash. Thioacetic acid (0.190 g, 0.178 mL, 2.5 mmol, 5 equiv) was then added to this mixture and the reaction mixture was slowly stirred and heated to 80 °C overnight. After 18 hours, the resin was filtered into a 5 mL syringe fitted with a polypropylene filter disc. After washing the resin with DMF, THF, and DCM (5 x 5 mL per solvent), the resin was then shrunk with MeOH (5 x 5 mL) and washed a final time with Et_2O (5 x 5 mL) and dried under high vacuum.

ATR-IR: ν = 3025, 2921, 1690 (thioester C=O),1601, 1513, 1492, 1451, 1352, 1131, 1028, 956, 839, 756, 696, 624.
Elemental analysis (Found (%)): S-Analysis: 5.096. Loading = 1.589 mmol/g (95 %).

Mercaptomethyl-polystyrene-copolymer (60)

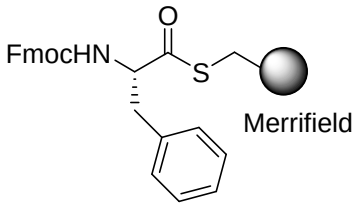


60

In a glass tube equipped with a stir bar was added resin **59** (0.126 g, 0.198 mmol, 1 equiv) and hydrazine acetate (0.055 g, 0.593 mmol, 3 equiv). The glass tube was then capped and successively evacuated and filled with nitrogen (3x). Under a crosscurrent of nitrogen, dry DMF (3 mL) was added to the vial. The reaction was then allowed to stir very slowly so as to not destroy the resin particles and heated to 60 °C for 90 minutes. Upon completion, the glass tube was cooled to room temperature and the resin was subsequently washed multiple times with dry DMF (4 x 3 mL) and Dry DCM (4 x 3 mL) taking care that air was not pulled through the resin. Ellman's test of the resin gave a positive result for free thiols.

FT-IR (ATR) ν (cm^{-1}): disappearance of 1690 (Thioester C=O).

Fmoc-Phe-mercaptomethyl polystyrene-copolymer (61)



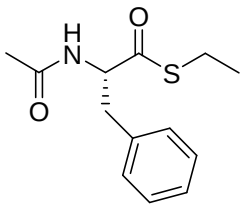
61

The resin **60** was resuspended in dry DCM (0.5 mL), and a preactivated mixture of Fmoc-Phe-OH (0.384 g, 0.99 mmol, 5 equiv), EDC (0.19 g, 0.99 mmol, 5 equiv) , DMAP (0.018 g, 0.149 mmol, 0.75 equiv) in dry DCM (5 mL) was added to the resin and shaken for 16 hours at room temperature. The resin was then filtered into a polypropylene syringe fitted with a filter frit and washed with DCM/THF/DMF (5 x 30 s each) (10 mL/g resin). Excess thiols were capped with a solution Ac_2O, DIPEA, and DMF (0.2 mL / 0.4 mL / 1.4 mL), followed by repeated washings with

DCM/THF/DMF. Air was then pulled through the resin to dry, and a second Ellman's test was negative, indicating the absence of free thiols.
The loading of the amino acid onto the resin was determined by a quantitative Fmoc-determination (see corresponding write-up).

Loading (as determined by UV-Vis Fmoc determination): 0.68 mmol/g (79 %) over 4 steps.

_S_-ethyl (2_S_)-2-(acetylamino)-3-phenylpropanethioate (63)



63

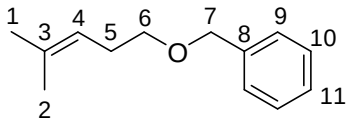
To the resin **61** in a 5 mL syringe fitted with a polypropylene filter disk was added 20 % piperidine in DMF (3 mL) and shaken for 1 x 1min and 2 x 10 minutes with washings of DMF (5 x 3 mL DMF each for 30 seconds) in between. After complete deprotection of the Fmoc group, the N-terminus was acetylated with acetic anhydride (0.2 mL), DIPEA (0.4 mL), in DMF (1.4 mL) for 15 minutes. After completion, the resin was washed as previously described and a Kaiser test revealed quantitative coupling of the N-terminal groups.

The resin was then transferred to a glass tube (2 mL), and then suspended in dry THF (1 mL). To this was added sodium thioethanolate (0.024 g, 0.255 mmol, 5 equiv)and the suspension was shaken for 45 minutes. Upon completion the resin was filtered into a 5 mL syringe fitted with a polypropylene filter disk and cotton wool. Dry THF (8 mL) was used to wash the product through the frit/wool and after removal of the solvent under reduced pressure, the title compound was obtained as a yellow oil in an 8 mg (64 %) yield.

MS (ESI): *m/z* calcd for $C_{13}H_{17}NO_2S$ 252.1 $[M+H]^+$. Found 252.1, 274.1 $[M+Na]^+$, 525.0 $[2xM+Na]^+$.

R_t: 2.73 min (linear gradient 5-99% B, 2.5 min, 99% B for 3 min).

Benzyl 4-methylpent-3-enyl ether (65)



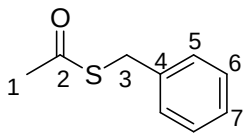
65

To a suspension of sodium hydride (0.220 g of a 60 % dispersion in mineral oil, 5.0 mmol, 1.1 equiv) in dry THF (10 mL), in a flame dried 50 mL round bottom Schlenck flask equipped with a reflux condenser and evacuated and filled with N_2, was slowly added at 0 °C, 4-Methyl-3-penten-1-ol (0.50 g, 0.583 mL, 5.0 mmol, 1 equiv). The reaction was stirred for 40 minutes and then benzyl bromide (0.897 g, 0.623 mL, 5.24 mmol, 1.05 equiv) and tetrabtuylammonium iodide (0.185 g, 0.5 mmol, 0.1 equiv) were added and the reaction was refluxed for 18 hours. Water (20 mL) was added slowly and the reaction was diluted with Et_2O (20 mL). The aqueous layer was extracted with Et_2O (3 x 25 mL), the combined organic layers were washed with water (20 mL), brine (20 mL), and finally dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was purified *via* column chromatography using 100 % hexanes as eluent to give **65** as a colorless oil in 0.879 g (92 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.64 (s, 3H, CH-1), 1.71 (s, 3H, CH-2), 2.33 (q, *J* = 6.97 Hz, 2H, CH-5), 3.46 (t, J = 7.06 Hz), 4.51 (s, 2H, CH-7), 5.15 (t, J = 7.06 Hz, 1H, CH-4), 7.24-7.39 (m, 5H, CH-9,10,11).

^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 17.9 (C-1), 25.9 (C-2), 28.9 (C-5), 70.3 (C-6), 73.0 (C-7), 120.5 (C-4), 127.6 (C-11), 127.8 (C-9), 128.5 (C-10), 133.7 (C-3), 138.8 (C-8).

***S*-Benzyl ethanethioate (66)**



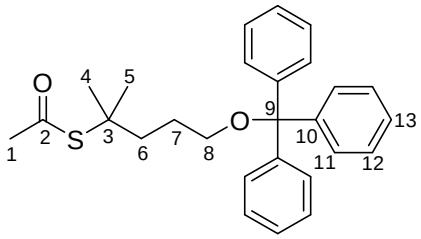
66

To a suspension of **65** (0.845 g, 4.44 mmol, 1 equiv) and $InCl_3$ (0.098 g, 0.444 mmol, 10 mol %) in dry DCE (5 mL) in a flame-dried 25 mL round bottom Schlenck flask equipped with a reflux condenser and evacuated and filled with N_2 was added under a cross-current of N_2, thioacetic acid (0.372 g, 0.347 mL, 4.884 mmol, 1.1 equiv). The reaction was then heated to 80 °C for 1.5 hours and then quenched with 1M HCl (15 mL) and extracted with Et_2O (3 x 10 mL). The organic phases were collected and then washed with $NaHCO_3$ (15 mL) and then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the product was then purified *via* flash column chromatography using silica gel and hexanes and EtOAc (20:1) as eluent to afford **66** as a yellow oil in 0.208 g, (28 %) yield.

R_f =0.27 (Hex: EtOAc, 20:1)
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 2.36 (s, 3H, CH-1), 4.14 (s, 2H, CH-3), 7.21-7.35 (m, 5H, CH-5,6,7).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 33.4, 127.0, 128.6, 128.8, 137.6, 195.0

Also recovered from this reaction was compound **52** as previously described in 0.153 g, (29 %) yield. For these two products, 3.83 mmol of thioacetic acid was consumed (78 % of the reacted amount).

_S_-[1,1-Dimethyl-4-(trityloxy)butyl] ethanethioate (67)

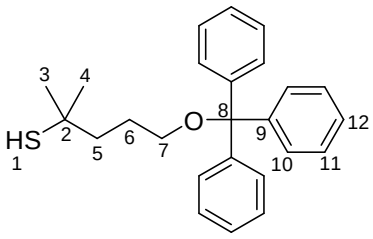


67

To a solution of trityl chloride (0.139 g, 0.498 mmol, 1.1 equiv) in dry DCM (4 mL) in a flame-dried round bottom Schlenck flask evacuated and filled with nitrogen, was added Et_3N (0.113 mL, 0.815 mmol, 1.8 equiv) and DMAP (0.003 g, 0.023 mmol, 0.05 equiv). To this mixture was then added **57** in dry DCM (1 mL) and the reaction mixture was stirred at room temperature for 18 hours. The reaction was monitored via TLC (20:1 Hex/EtOAc) and upon completion, was quenched with 10 mL of H_2O. The crude product was then extracted with DCM (3 x 15 mL), the organic phases combined and washed with a saturated NH_4Cl solution, water, and dried over Na_2SO_4. The resulting residue was purified over a silica gel (18 cm) and eluted with Hex:EtOAc (50:1) to give the title compound (0.17g, 89 %) as a white solid.

^{1}H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.43 (s, 6H, CH-4,5), 1.62-1.73 (m, 2H, CH-7), 1.74-1.82 (m, 2H, CH-6), 2.21 (s, 3H, CH-1), 3.05 (t, *J* = 6.35 Hz, 2H, CH-8), 7.20-7.34 (m, 9H, CH-12,13), 7.41-7.48 (m, 6H, CH-11).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 25.7 (C-7), 27.7 (C-4,5), 31.6 (C-1), 38.1 (C-6), 51.7 (C-5), 63.9 (C-8), 86.6 (C-9), 127.0 (C-13), 127.9 (C-11), 128.8 (C-12), 144.5 (C-10), 196.7 (C-2).

1,1-Dimethyl-4-(trityloxy)butyl thiol hydrosulfide (68)

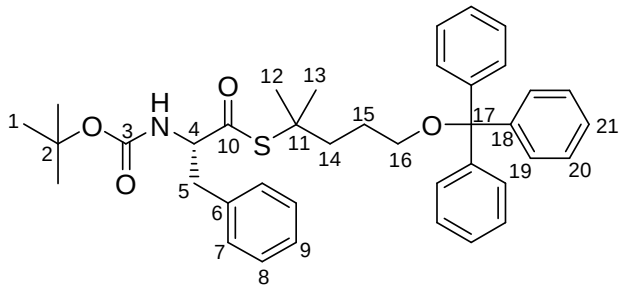


68

In a flame dried round bottom Schlenck flask evacuated and filled with N_2 was added **67** (0.12 g, 0.287 mmol, 1 equiv) and dry MeOH (3 mL). To this was added via canula NaSMe (0.020 g, 0.287 mmol, 1 equiv) in dry MeOH (1 mL). To effect complete dissolution of the compound in the solvent, dry THF (1 mL) was added. The reaction was then allowed to stir for 1h at room temperature, during which it was periodically controlled via TLC (Hex:EtOAc, 20:1). After completion of the reaction, the mixture was diluted with DCM (20 mL) and quenched with H_2O (10 mL). The solution was then acidified with 0.1 M HCl (5 mL) and the product was extracted with DCM (3 x 15 mL). The organic phases were collected and washed with a saturated NH_4Cl/brine and then dried over Na_2SO_4. Removal of the slovent under reduced pressure gave 0.108 g (quantitative) of white solid **68**.

^{1}H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.37 (s, 6H, CH-3,4), 1.56-1.64 (m, 2H, CH-6), 1.66 (s, 1H, SH-1), 1.68-1.81 (m, 2H, CH-5), 3.08 (t, *J* = 6.36 Hz, 2H, CH-7), 7.18-7.33 (m, 9H, CH-11,12), 7.40-7.48 (m, 6H, CH-10).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 26.3 (C-6), 32.9 (C-3,4), 43.2 (C-5), 44.7 (C-2), 64.0 (C-7), 86.6 (C-8), 127.0 (C-12), 127.9 (C-10), 128.8 (C-11), 144.5 (C-9).

_S_-[1,1-Dimethyl-4-(trityloxy)butyl] (_2S_)-2-{[9_H_-fluoren-9-ylmethoxy)-carbonyl]-amino}-3-phenylpropanethioate (69)



69

To a flame dried round bottom Schlenck flask evacuated and filled with N_2 was added under a cross-current of N_2, tBOC-Phe-OH (0.028g, 0.101 mmol, 1 equiv) followed by addition of dry DCM (1 mL). DMAP (3.3 mg, 0.027 mmol, 0.25 equiv) was then added followed by addition of **68** (0.040 g, 0.106 mmol, 1.05 equiv) in dry DCM (1 mL). The reaction vessel was cooled to 0 °C and DCC (0.021 g, 0.101 mmol, 1 equiv)

was added under a cross-current of N_2. The reaction was then allowed to warm to r.t. and stirred for a total of 38 hours. After 38 hours, the reaction mixture was filtered through a celite pad washed with copious amounts of DCM. The organic phase was then washed twice with a saturated aq. NH_4Cl, once with a saturated aqueous $NaHCO_3$ and finally dried over Na_2SO_4. The product was then dried in vacuo and purified over silica gel using hexane:EtOAc as eluent (10:1) to give 0.020g (32 %) of a white solid **69**. Unreacted thiol **68** (23 mg, 0.06 mmol) was also salvaged from the column.

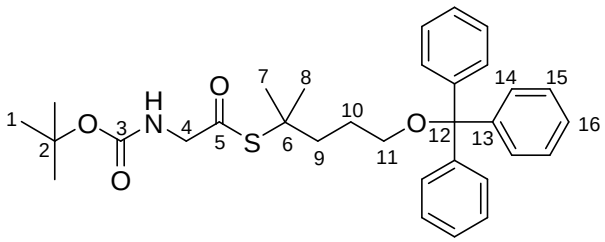
^{1}H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.39 (s, 9H, CH-1), 1.42 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.52-1.70 (m, 2H, CH-15), 1.71-1.84 (m, 2H, CH-14), 2.93-3.13 (m, 4H, CH-5,16), 4.46-4.61 (m, 1H, CH-5), 4.88 (d, *J* = 8.93 Hz, NH) 7.10-7.17 (m, 2H, Ar), 7.18-7.34 (m, 12H, Ar), 7.40-7.46 (m, 6H, CH-19).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 26.7 (C-15), 27.6 (C-12,13), 27.7 (C-1), 38.2 (C-15), 39.0 (C-5), 52.0 (C-11), 61.4 (C-4), 63.9 (C-16), 80.2 (C-2), 86.6 (C-17), 127.0 (C-21), 127.1 (C-9), 127.9 (C-19), 128.6 (C-7), 128.8 (C-20), 129.6 (C-8), 136.0 (C-6), 144.5 (C-18), 155.0 (C-3), 200.7 (C-10).

MS (ESI): *m/z* calcd for $C_{39}H_{45}NO_4S$ 646.3 $[M+Na]^+$. Found 646.1.

R_t 4.25 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

***S*-[1,1-Dimethyl-4-(trityloxy)butyl] {[9*H*-fluoren-9-ylmethoxy)-carbonyl]-amino}-ethanethioate (70)**



70

The reaction protocol was performed exactly as **69**. 0.012 g of tBoc-Gly-OH (0.063 mmol, 1 equiv), 0.027 g of **68** (0.066 mmol, 1.05 equiv), 0.013 g of DCC (0.063 mmol, 1.05 equiv), 0.88 mg DMAP (6.3 µmol, 0.1 equiv) were reacted in 1 mL of dry DCM at r.t. for 48 hours. The product **70** was purified over a silica gel column (18 cm x 20 mm) using hexane:EtOAc (10:1) (rf value of 0.13) to give the title compound (22 mg, 61 %) as a white solid.

^{1}H-NMR: (300 MHz, $CDCl_3$) δ [ppm]: 1.44-1.47 (m, 15H, CH-1,7-8), 1.59-1.69 (m, 2H, CH-10), 1.75-1.84 (m, 2H, CH-9), 2.98 (t, *J* = 6.4 Hz, 2H, CH-11), 3.88 (d, *J* = 5.3 Hz, 2H, CH-4), 4.93 (b, 1H, NH) 7.19-7.35 (m, 9H, Ar), 7.40-7.48 (m, 6H, CH-19).

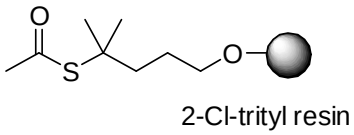
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 25.7 (C-10), 27.9 (C-1), 28.0 (C-7,8), 38.4 (C-9), 38.6 (C-6), 50.8 (C-4), 63.9 (C-11), 80.2 (C-2), 86.6 (C-12), 127.0 (C-16), 127.9 (C-15), 128.8 (C-14), 144.5 (C-13), 151.7 (C-3), 197.7 (C-12).

MS (ESI): *m/z* calcd for $C_{32}H_{39}NO_4S$ 556.2 $[M+Na]^+$. Found 556.1.

R_t 3.70 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Functionalization of the solid support with the MMP linker and loading of the first amino acid

Coupling of 4 to a standard 2-Cl-chlorotrityl resin:
***S*-(4-Hydroxy-1,1-dimethylbutyl) ethanethioate-polystyrene-copolymer (72)**

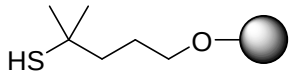


72

A solution of linker **57** (0.086 g, 0.489 mmol, 3 equiv) in dry DCM (1 mL), dry DMF (1 mL), and dry pyridine (0.079 mL, 0.978 mmol, 6 equiv) was added to 2-Cl-tritylchloride resin (0.125 g, 0.163 mmol, 1 equiv) in a glass tube equipped with a septum. The tube was flushed briefly with N_2, capped, shaken for 3 days at room temperature and controlled via FTIR (ATR) spectroscopy. After the allotted time, MeOH (4 mL) was added to the resin and shaken for 45 minutes to arrest the reaction and to cap the remaining free chloride functional groups. The resin was then filtered and washed repeatedly with DCM (5 x 30 sec), THF (5 x 30 sec), and DMF (5 x 30 sec), then briefly with MeOH and Et_2O to expunge the resin of remaining solvent for drying. The resin was then dried under high vacuum for several hours.
Elemental analysis of sulphur from the dried resin gave a substitution of 1.03 mmol/g (94 % yield) of the functional groups.

FT-IR (ATR) ν (cm^{-1}): 1683 (Thioester C=O).

(4-Mercapto-4-methylpentan-1-ol)-2-chlorotrityl-polystyrene-copolymer (73)



73

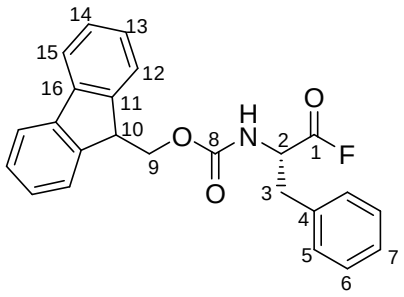
In a glass tube equipped with a stir bar was added resin **72** (0.066g, 0.068 mmol, 1 equiv) and hydrazine acetate (0.019g, 0.204 mmol, 3 equiv). The glass tube was then capped and successively evacuated and filled with nitrogen (3x). Under a cross-

current of nitrogen, dry DMF (1.5 mL) was added to the vial. The reaction was then allowed to stir very slowly so as to not destroy the resin particles and heated to 60 °C for 90 minutes. Upon completion, the glass tube was cooled to room temperature and the resin was subsequently washed multiple times with dry DMF (4 x 3 mL) and Dry DCM (4 x 3 mL) taking care that air was not pulled through the resin. Ellman's test of the resin gave a positive result for free thiols. It was determined that pulling air through the resin was sufficient enough to significantly oxidize the free thiols to intra-bead disulfide bridges, as based on subsequent coupling steps.

FT-IR (ATR) ν (cm^{-1}): disappearance of 1683 (Thioester C=O).

Representative procedure for the synthesis of Fmoc amino acid fluorides:

Fmoc-Phe-F (30)



30

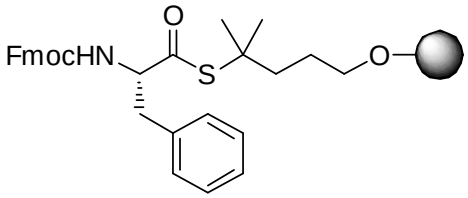
The procedure employed was performed as described by Carpino et al.[113] To a solution of Fmoc-Phe-OH (0.1.550 g, 4.0 mmol, 1 equiv) in dry DCM (15 mL), and dry pyridine (0.322 mL, 4.0 mmol, 1 equiv) in a flame-dried round bottom flask equipped with a reflux condenser, evacuated and flushed with N_2 was added dropwise cyanuric fluoride (0.686 mL, 8.0 mmol, 2 equiv). The reaction was then heated to reflux for 3h during which time a white precipitate evolved from the solution mixture. After cooling, the crude reaction slurry was poured into DCM (30 mL) and wased with cold H_2O (3 x 15 mL). The organic layer was then dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give a white glassy solid. Recrystallization from DCM/Hex gave **30** (0.965 g, 62 %) as a powdery white solid.

FT-IR (ATR): ν = 3314, 3032, 2957, 1842, 1704, 1537, 1448 1262 cm^{-1}.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 3.14-3.25 (m, 2H, CH-3), 4.22 (t, 1H, *J* = 6.4 Hz, CH-10), 4.38-4.48 (m, 2H, CH-9), 4.77-4.87 (m, 1H, NH), 5.24 (d, *J* = 8.1 Hz, 1H,), 7.14-7.21 (m, 2H), 7.24-7.45 (m, 7H), 7.49-7.57 (m, 2H), 7.76-7.80 (m, 2H).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 37.1 (C-3),47.2 (C-11), 53.5, 54.3, 67.5 (C-9), 120.2 (C-15), 125.1 (C-12), 127.2 (C-13), 127.4, 127.9 (C-14), 129.4, 129.5, 134.4 (C-4), 141.5 (C-16), 143.8 (C-11), 155.6 (C-8), 164.3 (C-1).

Fmoc-Phe-[4-mercapto-4-methylpentan-1-ol]-2-chlorotrityl-polystyrene-copolymer (74)



74

EDC/DMAP method: The resin was resuspended in dry DCM (0.5 mL), and a preactivated mixture of Fmoc-Phe-OH (0.124 g, 0.32 mmol, 5 equiv), EDC (0.061 g, 0.32 mmol, 5 equiv) , DMAP (0.006 g, 0.048 mmol, 0.75 equiv) in dry DCM (1.5 mL) was added to the resin and stirred for 2 h at room temperature. The filtrate was carefully decanted from the resin via canula and the resin was washed twice with dry DCM. To the washed resin was then added a fresh mixture using the exact same proportions as aforementioned and stirred at room temperature for an additional 18 h. The resin was then filtered into a polypropylene syringe fitted with a filter frit and washed with DCM/THF/DMF (5 x 30 s each) (10 mL/g resin). Excess thiols were capped with a solution Ac_2O, DIPEA, and DMF (0.2 mL / 0.4 mL / 1.4 mL), followed by repeated washings with DCM/THF/DMF. Air was then pulled through the resin to dry, and a second Ellman's test was negative, indicating the absence of free thiols.

The loading of the amino acid onto the resin was determined by a quantitative Fmoc-determination (see corresponding write-up). With Fmoc-Phe-OH a loading of 0.69 mmol/g (74 %) was determined.

Amino acid fluoride method: The resin was resuspended in dry DMF (0.5 mL), and a solution of **19** (0.14 g, 0.36 mmol, 5 equiv) in DMF (1 mL) was added to the resin and stirred for 2 h at room temperature. The filtrate was carefully decanted from the resin via canula and the resin was washed twice with dry DMF. To the washed resin was then added a fresh mixture using the exact same proportions as aforementioned and stirred at room temperature for an additional 18 h. The resin was then filtered into a polypropylene syringe fitted with a filter frit and washed with DCM/THF/DMF (5 x 30 s each) (10 mL/g resin). Excess thiols were capped with a solution Ac_2O, DIPEA, and DMF (0.2 mL / 0.4 mL / 1.4 mL), followed by repeated washings with DCM/THF/DMF. Air was then pulled through the resin to dry, and a second Ellman's test was negative, indicating the absence of free thiols.

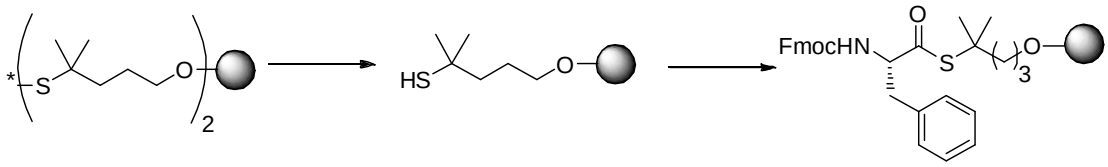
The loading of the first amino acid onto the resin was determined by a quantitative Fmoc-determination (see corresponding write-up) with a loading of 0.71 mmol/g (90 %) was determined.

Table 7-1. Coupling yields to the *tert*-butyl thiol linker determined by spectroscopic quantification of Fmoc groups.

entry	amino Acid	coupling conditions	reaction time	yield[a]
1	Fmoc-Phe-OH	5 equiv AA, 5 equiv EDC, 0.75 equiv DMAP, DCM, RT	18 h	41 %
2	Fmoc-Phe-OH	5 equiv AA, 5 equiv DIC, 5 equiv HOBt•H_2O, DMF, RT	18 h	8 %
3	Fmoc-Phe-F	5 equiv AA, 0.75 equiv DMAP, DCM, RT	18 h	36 %
4	Fmoc-Phe-F	5 equiv AA, 5 equiv DIPEA, DMF, RT	1 x 2 h, 1 x 18 h	92 %
5	Fmoc-Val-OH	5 equiv AA, 5 equiv EDC, 0.75 equiv DMAP, DCM, RT	18 h	76 %
6	Fmoc-Phe-OH	5 equiv AA, 5 equiv EDC, 0.75 equiv DMAP, DCM, RT	1 x 2 h, 1 x 18 h	73 %
7	Fmoc-Ala-OH•H_2O	5 equiv AA, 5 equiv EDC, 0.75 equiv DMAP, DCM, RT	18 h	0 %

[a] as determined by UV-Vis quantification of cleaved Fmoc groups relative to the linker loading as determined by elemental analysis.

Reduction of intrabead disulfide bridges followed by acylation



To a flame-dried round bottom flask equipped with reflux condenser and stir bar, and evacuated and filled with N_2 was added under a light cross current of N_2 the resin **10** (0.078g, 0.064 mmol, 1 equiv). The resin was then suspended in dry toluene (3 mL) and Red-Al (65 % in toluene, 0.058 mL, 0.192 mmol, 3 equiv) was added dropwise via canula. The reaction was then heated to reflux for 3 hours while stirring very slowly to inhibit resin destruction. After the allotted time, the reaction was cooled to room temperature and washed carefully with copious amounts of dry DCM (7 x 3 mL) then dry THF (7 x 3 mL). The solvents were removed via decanting, and the resin was dried under high vacuum. A sample was taken and after the Ellman's test (see corresponding write-up) was administered the beads turned a brilliant yellow indicating free thiols.

The resin was then resuspended in dry DCM (5 mL), and a mixture of Fmoc-Phe-OH (0.124 g, 0.32 mmol, 5 equiv), EDC (0.061 g, 0.32 mmol, 5 equiv) , DMAP (0.006 g, 0.048 mmol, 0.75 equiv) was added to the resin and stirred for 18 h. The resin was then filtered into a polypropylene syringe fitted with a filter frit and washed with DCM/THF/DMF (5 x 30 s each). Air was then pulled through the resin to dry, and a second Ellman's test was performed indicating the absence of free thiols.

The loading of the amino acid onto the resin was determined by a quantitative Fmoc-determination (see general procedures). With Fmoc-Phe-OH a loading of 0.30 mmol/g (35 %) was determined.

Synthesis Fmoc-protected dipeptides

General procedure:

To a 10 mL polypropylene syringe fitted with a polyethylene filter disk was added Cl-TrtCl-resin (0.5 g, 1.3 mmol/g). To this was added a solution of the first amino acid Fmoc-Pro-OH (0.658 g, 1.95 mmol, 3 equiv) or Fmoc-Gly-OH (0.581 g, 1.95 mmol, 3 equiv) and DIPEA (1.07 mL) in DCM (2.5 mL), and the mixture was stirred for 15 min. Extra DIPEA (2.15 mL, total 19.5 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was arrested by adding MeOH (0.4 mL) and stirring for 10 min. The resin was then washed with DCM (5 x 5 mL), followed by subsequent washings with THF (5 x 5 mL), DMF (5 x 5 mL), DCM (5 x 5 mL) and dried *in vacuo*. The Fmoc was deprotected with adding piperidine-DMF 2:8, (v/v) and stirring for 1 x 1min, 2 x 10 min), with washings between each deprotection step with DMF (5 x 5mL). After complete Fmoc deprotection, the resin was washed as previously described with DMF, THF, DCM and dried *in vacuo*.

A solution of Fmoc-Arg-OH (2.109 g, 3.25 mmol, 5 equiv), HOBt (0.439 g, 3.25 mmol, 5 equiv) and DIC (0.493 mL, 3.18 mmol, 4.9 equiv) in DMF (3 mL) was added to the resin and shaken for 90-120 minutes. A negative ninhydrin test determined that the coupling was complete. The resin was then filtered and washed as previously described and finally dried *in vacuo*.

The protected peptide was then cleaved from the resin by TFA-DCM (1:99) (5 x 30s) (10 mL / g resin). The filtrate was dropped into a 250 mL flask containing H_2O (8 mL) and the DCM as well as a partial amount of H_2O was removed via rotoevaporation. MeCN was then added to dissolve the solid that formed during rotoevaporation and the solution was lyophilized to give the title compound.

Fmoc-Arg-Gly-OH. 84. 469 mg (97 % based on the initial loading) of the title compound with a purity > 95 % as confirmed by LC-MS at 220 nm.

MS (ESI): *m/z* calcd for $C_{36}H_{43}N_5O_8S$, 706.3 $[M+H]^+$. Found 706.1 $[M+H]^+$, 728.0 $[M+Na]^+$.

R_t 3.38 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Fmoc-Arg-Pro-OH. 84b. This dipeptide was synthesized although it was deemed unnecessary for the sequence SYRPV. The product was isolated as a white powder (0.40 g, 87%).

MS (ESI): *m/z* calcd for $C_{39}H_{47}N_5O_8S$, 746.3 $[M+H]^+$. Found 746.1 $[M+H]^+$, 768.0 $[M+Na]^+$.
R_t 3.31 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Stability-determinations of thioesters in solution

General procedure:

The stabilities of the different thioesters under Fmoc-cleavage conditions were determined in solution as follows. The thioesters (0.062 mmol, 1 equiv) were stirred in a solution of piperidine : DMF (2:8) (v:v) (1mL, 38 equiv of piperidine) in round bottom flasks at room temperature with a final concentration of the thioester at 62 mM. The reactions were followed by LC-MS at 220 nm. At this wavelength the thioesters as well as the piperidine amide and other side products were detectable well. Half life stabilities of the thioesters were measured as the rate of breakdown of the thioesters where at time (t = 0 minutes) the thioester was considered to be 100 % intact, and as time (t > 0) increases, the thioester begins to degrade to either the piperidine adduct as shown, or other side products. At each time interval given, a sample (10 µL) was diluted with 300 µL of ACN/H_2O containing 0.1 % formic acid and measured immediately.

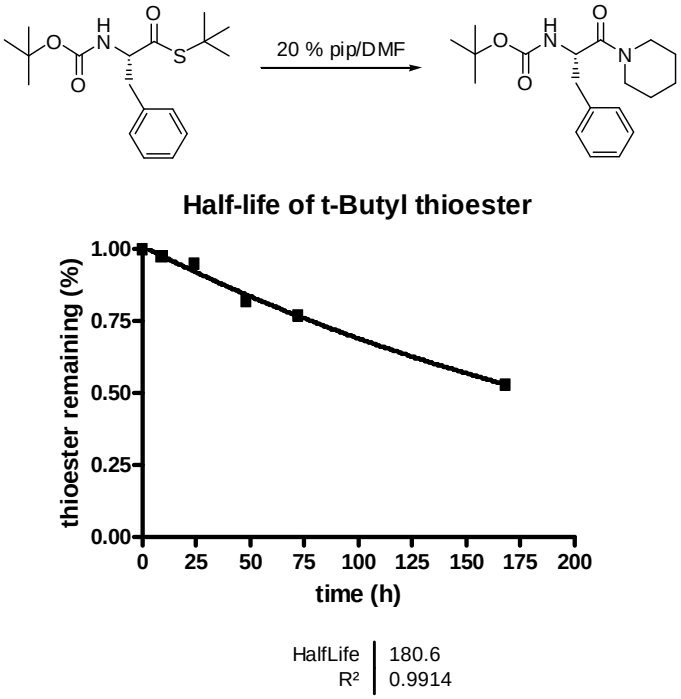


Figure 7-1: Half-life of thioester **2.**

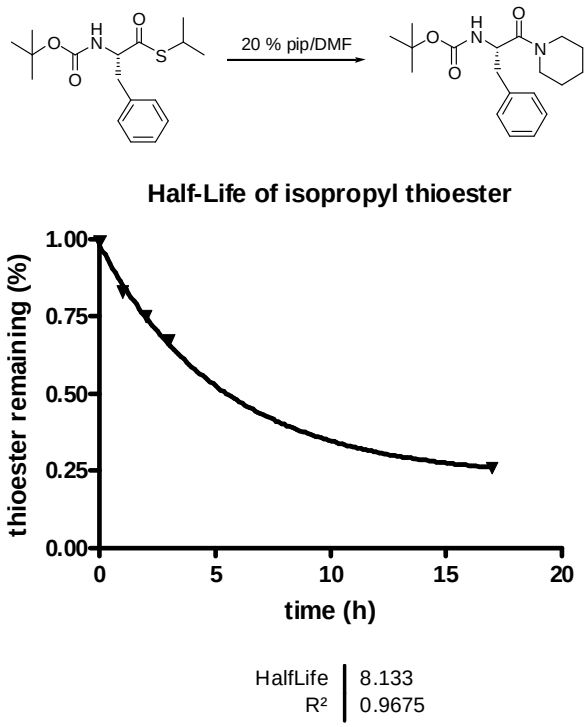


Figure 7-2: Half-life of thioester **5.**

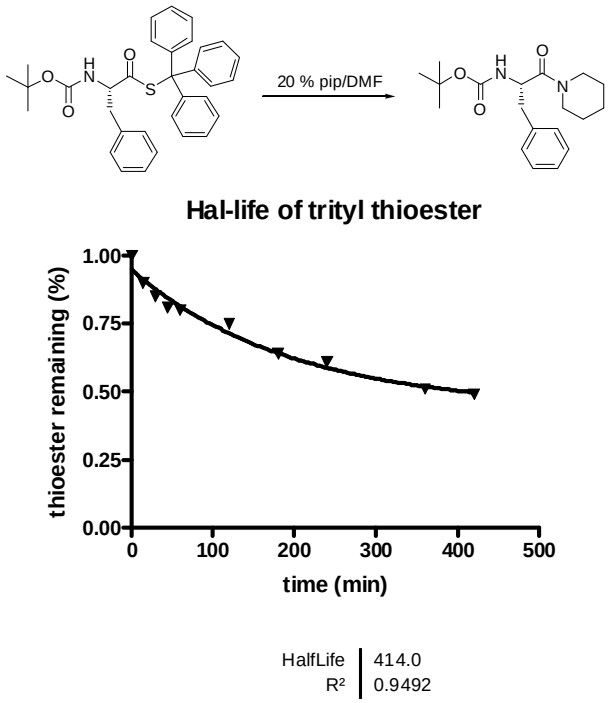


Figure 7-3: Half-life of thioester **6.**

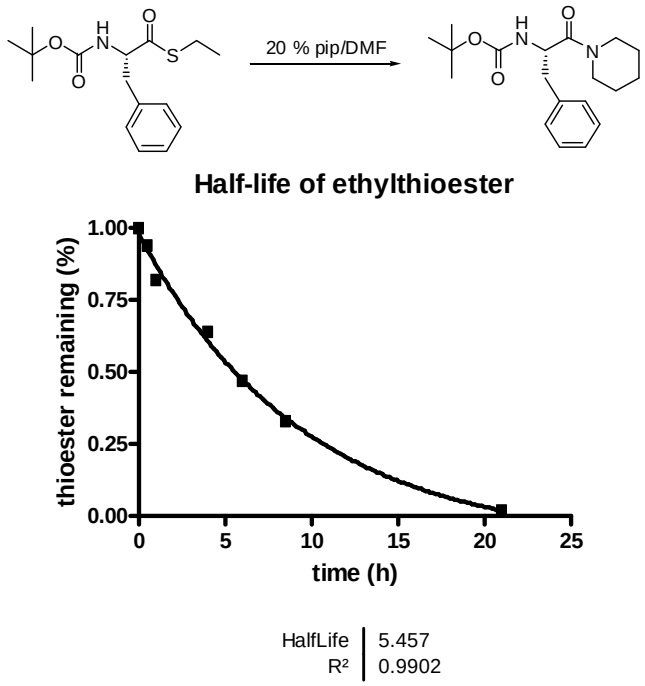


Figure 7-4: Half-life of thioester **7.**

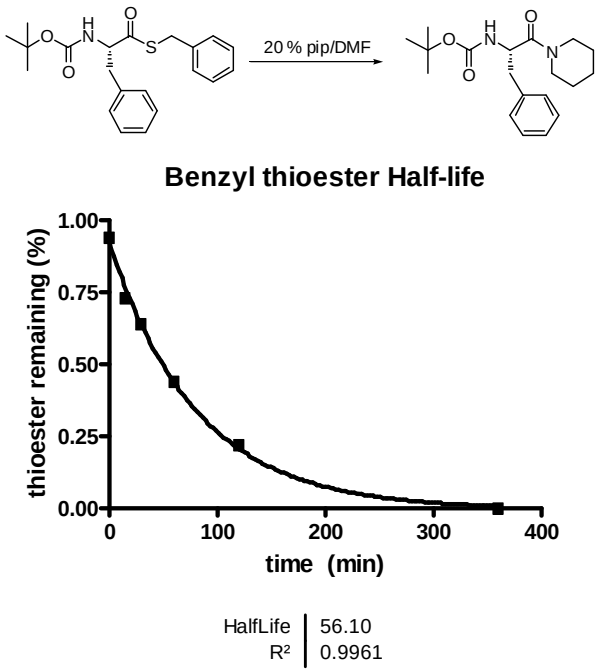


Figure 7-5: Half-life of thioester **8.**

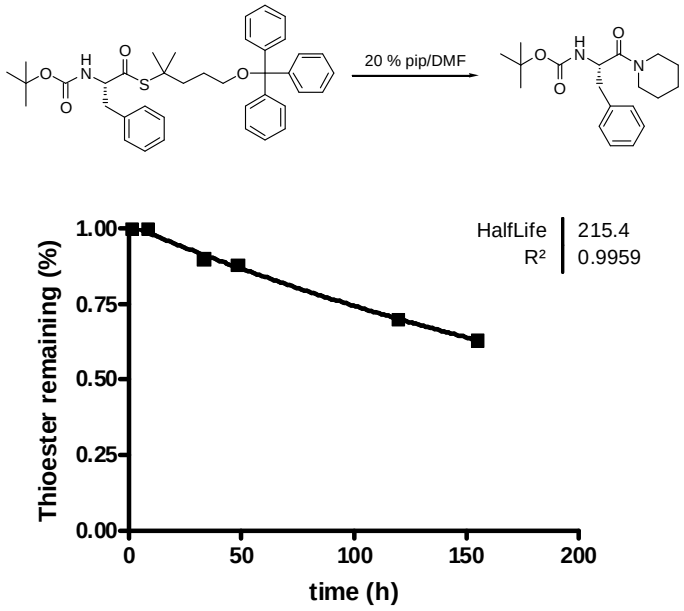


Figure 7-6: Half life of thioester **69.**

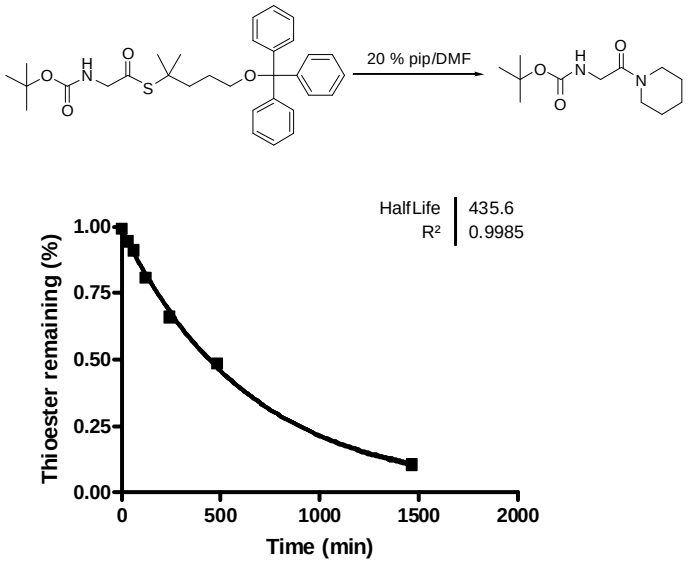
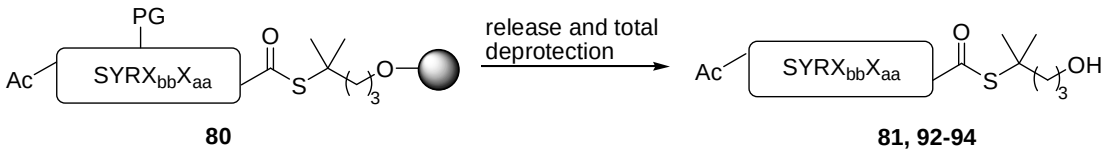


Figure 7-7: Half-life of thioester **70.**

Release and total deprotection of peptide thioesters 81, 92-94 from the solid support:



80 **81, 92-94**

A TFA/TIS/H_2O/EDT cocktail (94/1/2.5/2.5, 4 mL) was added to the resin 8 in a 5 mL polypropylene syringe equipped with a filter frit and shaken at room temperature for 2.5 hours. The resin was then filtered into a round bottom flask (50 mL) and washed with neat TFA (2 x 4 mL). The volatiles were then removed under a stream of N_2 and the peptide thioester was triturated with cold ether (3 x 2 mL) decanting each time. The peptide was then taken up in H_2O/ACN and lyophilized to give a white powder **81** (7 mg, 54 % yield based on initial coupling).

Peptide **81** Ac-SYRGF-MMP. ESI-MS-TOF: *m/z*: calcd for $C_{37}H_{54}N_8O_9S$: m/z 787.3807 $[M+H]^+$. Found, 787.3811 $[M+H]^+$.

^{1}H-NMR: (600 MHz, D_2O) δ [ppm]: 1.39 (s, 6H, 2xCH_3), 1.48-1.59 (m, 4H, CH_2 (Arg) CH_2 (MMP)), 1.61-1.69 (m, 2H, CH_2 (Arg)), 1.72-1.78 (m, 2H, CH_2 (MMP)),

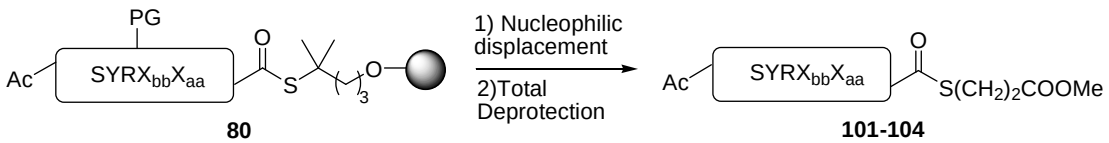
2.02 (s, 3H, CH_3 (Acetyl)), 2.99-3.04 (m, 2H, CH_2 (Phe)), 3.04-3.08 (m, 1H, CH_2 (Tyr)), 3.13-3.18 (m, 2H, -CH-$CH_2CH_2\underline{CH_2}$-NH-C(NH)-NH_2 (Arg)), 3.18-3.23 (m, 1H, CH_2 (Tyr)), 3.54 (dd, *J* = 6.5 Hz, CH_2 (MMP)), 3.77 (t, *J* = 6.1 Hz, CH_2 (Ser)), 3.80-3.83 (m, 2H, CH_2 (Gly)), 4.23 (quintet, *J* = 7.3 Hz, 1H, α-CH (Arg)), 4.34-4.38 (m, 1H, α-CH (Ser)), 4.56 (t, *J* = 7.3 Hz, α-CH (Phe)), 4.60 (t, *J* = 7.3 Hz, α-CH (Phe)), 4.65 (t, 7.3 Hz, α-CH (Tyr)), 6.80-6.85 (m, 2H, Ar (Tyr)), 7.09-7.16 (m, 2H, Ar (Tyr)), 7.24-7.34 (m, 3H, Ar (Phe)), 7.34-7.40 (m, 2H, Ar (Phe)).

Peptide **92** Ac-SYRPV-MMP, recovered as a white powder (8 mg, 88 % yield based on initial coupling) which was determined to be 91 % pure by LC-MS measurement at 220 nm: HRMS, ESI-MS-TOF: *m/z*: calcd for $C_{39}H_{55}N_9O_9S$, 779.4120 $[M+H]^+$. Found, 779.4128 $[M+H]^+$.

Peptide **93** Ac-SYRGW-MMP, recovered as a white powder (2.2 mg, 90 % yield based on initial coupling) which was determined to be 84 % pure by LC-MS measurement at 220 nm. ESI-MS-TOF: *m/z*: calcd for $C_{39}H_{55}N_9O_9S$, 826.3916 $[M+H]^+$. Found, 826.3936 $[M+H]^+$.

Peptide **94** Ac-SYRGQ-MMP, recovered as a white powder (3.4 mg, 81 % yield based on initial coupling) which was determined to be 79 % pure by LC-MS at 220 nm: HRMS, ESI-MS-TOF: *m/z*: calcd for $C_{33}H_{54}N_9O_{10}S$, 768.3709 $[M+H]^+$. Found, 768.3728 $[M+H]^+$.

Nucleophilic displacement of the resin bound peptide thioesters with HS-$(CH_2)_2$-COOMe followed by total deprotection (101-104):



80 **101-104**

In a standard experiment, 25 mg of resin **80** (5.25 µmoles, 1 equiv) was added to a Shlenck tube equipped with a stir bar. The tube was then evacuated and flushed with nitrogen and to this was added HS-$(CH_2)_2$COOMe (28 µL, 262.5 µmole, 50 equiv) in

THF (0.5 mL). In a separate flask, sodium thiophenolate (1.4 mg, 10.5 µmoles, 2 equiv) was stirred with 15-crown-5 (2 µL, 10.5 µmoles, 2 equiv) in dry THF (0.5 mL). Upon dissolution of the sodium thiophenolate, this mixture was then added via canula to the resin **80** and stirred vigorously for 2-3 hours at room temperature. The resin was then filtered and washed twice with dry THF (5 mL total). If necessary, this cleavage was repeated for an additional 2 hours. After removal of the volatile materials *in vacuo* or under a stream of N_2, the protected peptide was directly cleaved with Reagent B (TFA/Phenol/TIS/H_2O 88/5/2/5, 1.5 mL) for 2-3 hours. The deprotected peptide in cleavage cocktail was then precipitated with MTBE (15 mL, 10 volumes) and then centrifuged at 3200 rpm for 30 minutes. The supernatant was then decanted and the peptide was taken up in H_2O/ACN (1/1) and lyophilized to yield a white powder **101** (2.8 mg, 69 % based on initial coupling) which was determined by LC-MS at 220 nm to be greater than 95 % pure.

Ac-SYRGF-S$(CH_2)_2$COOMe **(101)**: ESI-MS-TOF: *m/z*: calcd for $C_{35}H_{48}N_8O_{10}S$, 773.3287 $[M+H]^+$. Found: 773.3302 $[M+H]^+$.

From the resin bound Ac-SYRGW (0.033g, 4.75 µmol), peptide Ac-SYRGW-S$(CH_2)_2$COOMe **(102)** was recovered as a white powder (3.2 mg, 78 % based on initial coupling) and determined by LC-MS at 220 nm after lyophilisation to be 93 % pure. ESI-MS-TOF: *m/z*: calcd for $C_{37}H_{50}N_9O_{10}S$, 812.3396 $[M+H]^+$. Found: 773.3389 $[M+H]^+$.

From resin bound Ac-SYRGQ (0.0256 g, 6.66 µmol), peptide Ac-SYRGQ-S$(CH_2)_2$COOMe **(103)** was recovered as a white powder (4.3) mg, 86 % based on initial coupling) and determined by LC-MS at 220 nm after lyophilisation to be 95 % pure. ESI-MS-TOF: *m/z*: calcd for $C_{31}H_{48}N_9O_{11}S$, 754.3189 $[M+H]^+$. Found: 754.3193 $[M+H]^+$.

From resin bound Ac-SYRPV (0.018 g, 8.39 µmol), peptide Ac-SYRPV-S$(CH_2)_2$COOMe **(104)** was recovered as a white powder (4.3 mg, 80 % based on initial coupling), using 100 equivalents of HS-$(CH_2)_2$COOMe (0.101 g, 0.839 mmol)

which was determined by LC-MS at 220 nm after lyophilisation to be 95 % pure: ESI-MS-TOF: *m/z*: calcd for $C_{34}H_{52}N_8O_{10}S$, 765.3600 $[M+H]^+$. Found: 765.3611 $[M+H]^+$.

From the resin bound Ac-SYRPV (0.025g, 10 µmol), peptide Ac-SYRPV-SEt **(96)** was from obtained cleavage with thioethanol (0.5 mmol, 0.036 mL, 50 equiv) (all other cleavage reagents remaining the same) as a white powder, 1.4 mg (20 %), which was determined by LC-MS at 220 nm after lyophilisation to be 95 % pure. ESI-MS-TOF: *m/z*: calcd for $C_{32}H_{50}N_8O_8S$, 707.3 $[M+H]^+$. Found: 707.2 $[M+H]^+$.

From the resin bound Ac-SYRGF (0.020g, 6.0 µmol), peptide Ac-SYRGF-SEt **(97)** was recovered as an impure white powder from cleavage with thioethanol (0.3 mmol, 0.022 mL, 50 equiv) (all other cleavage reagents remaining the same) and subsequently purified over a semipreparative HPLC column to give 0.6 mg (14 %). ESI-MS-TOF: *m/z*: calcd for $C_{33}H_{46}N_8O_8S$, 715.3 $[M+H]^+$. Found: 715.3 $[M+H]^+$.

From the resin bound Ac-SYRGQ (0.030g, 6.7 µmol), peptide Ac-SYRGQ-SEt **(98)** was obtained from cleavage with thioethanol (0.33 mmol, 0.025 mL, 50 equiv) (all other cleavage reagents remaining the same) as a white powder, 1.2 mg (26 %). ESI-MS-TOF: *m/z*: calcd for $C_{29}H_{45}N_9O_9S$, 696.3 $[M+H]^+$. Found: 696.2 $[M+H]^+$.

From the resin bound Ac-SYRGS (0.023g, 3.7 µmol), peptide Ac-SYRGS-SEt **(98)** was obtained from cleavage with thioethanol (0.183 mmol, 0.012 mL, 50 equiv) (all other cleavage reagents remaining the same) as a white powder, 0.4 mg (16 %). ESI-MS-TOF: *m/z*: calcd for $C_{27}H_{42}N_8O_9S$, 654.3 $[M+H]^+$. Found: 654.2 $[M+H]^+$.

Synthesis of the cell penetrating peptide Penetratin™ 1 derivative Ac-RQIKIWFNRRMKWKKF-MMP (106).

The resin **85** (0.034 g, 0.71 mmol/g) was placed in a 2 mL polypropylene syringe fitted with a polyethylene filter disk. The Fmoc group was removed as previously described and Fmoc-Lys(Boc)-OH (57 mg, 0.12 mmol, 5 equiv), Fmoc-Trp(Boc)-OH (64 mg, 0.12 mmol, 5 equiv), Fmoc-Met-OH (45 mg, 0.12 mmol, 5 equiv), Fmoc-Arg(Pbf)-OH (78 mg, 0.12 mmol, 5 equiv), Fmoc-Arg(Pbf)-OH (78 mg, 0.12 mmol, 5

equiv), Fmoc-Asn(Trt)-OH (72 mg, 0.12 mmol, 5 equiv), Fmoc-Phe-OH (47 mg, 0.12 mmol, 5 equiv), Fmoc-Trp(Boc)-OH (64 mg, 0.12 mmol, 5 equiv), Fmoc-Ile-OH (43 mg, 0.12 mmol, 5 equiv), Fmoc-Lys(Boc)-OH (57 mg, 0.12 mmol, 5 equiv), Fmoc-Ile-OH (43 mg, 0.12 mmol, 5 equiv), Fmoc-Gln-OH (74 mg, 0.12 mmol, 5 equiv), Fmoc-Arg(Pbf)-OH (78 mg, 0.12 mmol, 5 equiv), Fmoc-Arg(Pbf)-OH (78 mg, 0.12 mmol, 5 equiv) were added sequentially using DIC (18 μL, 0.19 mmol, 4.9 equiv) and HOBt (16 mg, 0.12 mmol, 5 equiv) in DMF (1 mL). In most cases, after 120 minutes of coupling, the ninhydrin test was negative, the coupling was repeated either for an extra 2 hours or overnight for couplings which were not quantitatively achieved. After the final coupling, the Fmoc group was removed as described and the N-terminus was capped with Ac_2O (0.2 mL) and DIPEA (0.4 mL) in DMF (1.4 mL) for 15 minutes. A portion of the resin (0.032g, 4.4 µmol based on the Fmoc determination of the last Arg coupling) was taken and cleaved with Reagent K (TFA/phenol/H_2O/Thioanisole/EDT; 82.5/5/5/2.5, 2 mL) for 4 hours and 20 minutes. The solution was filtered into a round bottom flask and the cleaved resin was then washed with neat TFA (2 x 1 mL). The volatiles were then removed under a current of N_2 until 1 mL of solution remained which was then transferred to an eppendorf tube containing 10 mL of MTBE (10:1 volumes with the TFA cleavage mixture) whereupon crude peptide precipitated out of solution. After centrifugation at 3200 rpm for 30 min the supernatant was carefully decanted and the solute was redissolved in ACN/H_2O (1:1) with 0.1 % formic acid and lyophilized. The crude material (~14 mg by dry weight) was then taken up in ACN/H_2O and purified on a semipreparative HPLC column (VP 250/10 Nucleodor 100-5 C18 ec Machery-Nagel) using a linear gradient between 15 and 45 % B over 30 min to yield the peptide **106** in 7.1 mg (67 %) yield.

ESI-MS-TOF: *m/z* calcd for $C_{116}H_{183}N_{33}O_{20}S_2$, 2422.3759 $[M+H]^+$. Found 2422.3692 $[M+H]^+$, 1212.6858 $[M+2H]^{2+}$, 808.7924 $[M+3H]^{3+}$, 606.8449 $[M+4H]^{4+}$, 485.6767 $[M+5H]^{5+}$.

R_t 1.62 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Compound **107** was synthesized using the exact same protocol as previously described for the nucleophilic displacement of peptides from the resin. 18 mg of the

peptide bound resin **105** (2.5 µmoles, 1 equiv) was added to a Shlenck tube equipped with a stir bar. The tube was then evacuated and flushed with nitrogen and to this was added HS-$(CH_2)_2$COOMe (27 µL, 247 µmole, 100 equiv) in THF (1 mL). In a separate flask, sodium thiophenolate (1.4 mg, 10.5 µmoles, 2 equiv) was stirred with 15-crown-5 (1 µL, 5.0 µmoles, 2 equiv) in dry THF (1 mL). Upon dissolution of the sodium thiophenolate, this mixture was then added via canula to the resin **105** and stirred vigorously for 2-3 hours at room temperature. The resin was then filtered and washed twice with dry THF (5 mL total). This cleavage was repeated for an additional 2 hours. After removal of the volatile materials *in vacuo* or under a stream of N_2, the protected peptide was directly cleaved with Reagent K (TFA/phenol/H_2O/Thioanisole/EDT; 82.5/5/5/2.5, 2 mL) for 2 hours. The deprotected peptide in cleavage cocktail was then precipitated with MTBE (15 mL, 10 volumes) and then centrifuged at 3200 rpm for 30 minutes. The supernatant was then decanted and the peptide was taken up in H_2O/ACN (1/1) and lyophilized to yield a white powder **101** (2.7 mg, 45 % based on initial coupling) which was determined by LC-MS at 220 nm to be greater than 80 % pure.

ESI-MS-TOF: *m/z* calcd for $C_{114}H_{177}N_{33}O_{21}S_2$, 2409.3317 $[M+H]^+$. Found 1262.7009 $[M+2xTFA]^{2+}$, 804.1288 $[M+3H]^{3+}$, 603.3468 $[M+4H]^{4+}$.
R_t 1.79 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Synthesis of *N*-terminal cysteine segment H-CGRFLAVR-NH_2 (110)

The synthesis of **110** was carried out on a standard Rink-amide PS-resin (100-200 mesh) (0.323 g, 0.239 mmol) using Fmoc-chemistry as described under general methods for SPPS. A portion of the peptide (0.216 g, 0.085 mmol) was cleaved from the resin with TFA/TIS/H_2O/EDT (94/1/2.5/2.5, 5 mL) and precipitated with ether as described under the general methods for SPPS. The peptide was dissolved in MeOH-H_2O (1:1) and purified by RP-HPLC on a preparative column (10 µm, 250 x 20 mm, Grom-SIL 300 ODS-5-ST RP-C18) using a linear gradient of 5 and 99 % C over 30 min. Excess MeOH was removed via rotoevaporation and the product was lyophilized to give a white powder (0.044g, 0.048 mmol, 56 % yield).

ESI-MS-TOF: *m/z* calcd for $C_{40}H_{69}N_{15}O_8S$, 920.5247 $[M+H]^+$. Found 920.5174 $[M+H]^+$, 460.7637 $[M+2H]^{2+}$

R_t 1.50 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Native Chemical Ligation:

For the Native Chemical Ligation of peptide thioesters 101-104. The procedure was adapted using the protocol given by Ingenito et al.[131] In a 1.5 mL polypropylene eppendorf vial, peptide 13a (2.04 mg, 2.63 µmol) was dissolved in 720 µL of 0.1 M NaPi, pH 7.5, and 360 µL dioxane to a final concentration of (clear solution). This solution was then transferred to a 10 mL round bottom flask containing the cysteine peptide 16 to a final concentration of 2.43 mM. The ligation was initiated by the addition of 1 % (v/v) thiophenol (10.8 µL) to promote conversion of the less reactive 2-mercaptopropionate thioester to the more reactive phenyl α-thioester. The reaction was stirred at room temperature for several hours and was monitored by LC-MS at 220 nm. After the reaction was complete, the mixture was treated with 0.1 % TFA (in Millipore H_2O) (5 mL) and lyophilized. The lyophilized powder was then redissolved in TFA (1 mL) and precipitated in MTBE (9 mL). After centrifugation at 3200*g* (30 min), the supernatant was removed and the precipitate was dissolved in ACN/H_2O and lyophilized. The peptide was redissolved in ACN/H_2O (1:1) and then purified on a semipreparative HPLC column (VP 250/10 Nucleodor 100-5 C18 ec Machery-Nagel) using a linear gradient between 15 and 45 % B over 30 min to yield the peptide **111** in 2.04 mg (49 %) yield.

MS (ESI): *m/z* calcd for $C_{71}H_{109}N_{23}O_{16}S$, 1572.8216 $[M+H]^+$, 768.9144 $[M+2H]^{2+}$, 524.9454 $[M+3H]^{3+}$. Found 1572.8443 $[M+H]^+$, 768.9128 $[M+2H]^{2+}$, 524.9384 $[M+3H]^{3+}$

R_t 2.48 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Native chemical ligation of peptide thioesters carrying the MMP linker 81,92-94.

To a 10 mL round bottom flask containing peptide **81** (0.8 mg, 1.01 µmol, 1 equiv) and the cysteine peptide **110** (9.35 mg, 10.16 µmol, 10 equiv) was added a solution of

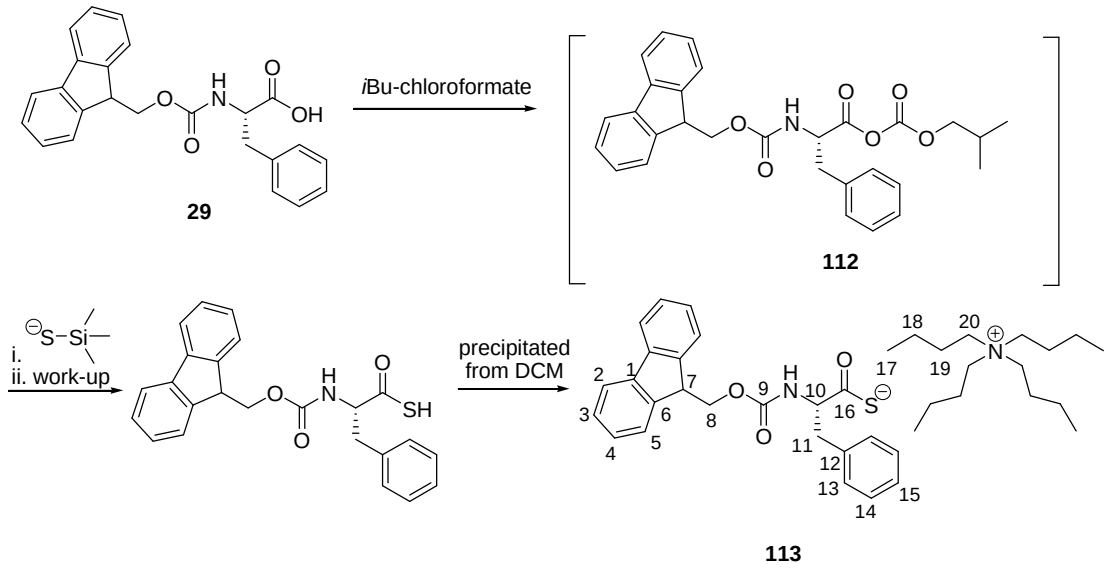
sodium thiophenolate (0.267 mg, 2.02 µmol, 2 equiv) and 15-crown-5 (0.4 mL, 2.02 µmol, 2 equiv) in dry THF (1 mL). The mixture was stirred overnight and checked periodically (15 min, 1h, 1.5 h, 2.5 h, 4h, 24h) via LC-MS. After completion of the reaction the solvent was removed under a stream of nitrogen, taken up in TFA (1 mL) and precipitated in MTBE (10 mL). The lyophilized product was then redissolved in 0.1 mL NH_4HCO_3 flushed with nitrogen and ß-mercaptoethanol (50 µmol, 5 equiv relative to the number of thiols to be reduced) was added to the solution and allowed to stir for 2 hours. The solution was then acidified with 10 % AcOH (aq) and then lyophilized. The peptide was redissolved in ACN/H_2O (1:1) and then purified on a semipreparative HPLC column (VP 250/10 Nucleodor 100-5 C18 ec Machery-Nagel) using a linear gradient between 15 and 45 % B over 30 min to yield the peptide **111** in a 0.7 mg (44 %) yield.

MS (ESI): *m/z* calcd for $C_{71}H_{109}N_{23}O_{16}S$, 768.9 $[M+2H]^{2+}$. Found 768.8 $[M+2H]^{2+}$, 525.0 $[M+3H]^{3+}$.

R_t 2.48 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

7.4. Synthetic Procedures for Synthesis of Peptide Thioacids and Subsequent Ligation

Fmoc-Phe-SH (**113**)



Under an inert atmosphere, a round bottom flask was charged with dry THF (2.5 mL) and bis(trimethylsilyl)sulfide (1.5 equiv relative to carboxylic acid, here: 0.139 g, 0.164 mL). To this solution was added anhydrous TBAF (1.8 equiv relative to the carboxylic acid, here: 0.936 mL of a 1.0M solution) was added dropwise at -78 °C and the reaction was allowed to warm to rt (approx. 30 min).

To a round bottom shlenck flask charged with dry DCM under a nitrogen atmostphere was added Fmoc-Phe-OH (0.200g, 0.52 mmol, 1 equiv) (azeotroped with toluene prior to addition), followed by the addition of 2,6-lutidine (0.166 g, 1.548 mmol, 3.0 equiv) and isobutylchloroformate (0.0813 mL, 0.624 mmol, 1.2 eq) at 0 °C. The reaction mixture was stirred for 30 min then trimethylsilyl thiolate (prepared above) was added via canula. The reaction mixture was then stirred until TLC analysis indicated complete consumption of the mixed anhydride (2.5 h). The reaction was then quenched with MeOH, dried *in vacuo*, and then azeotroped with MeOH (2 x 10 mL) to remove volatiles. The crude thioacid was then used as prepared without further purification. The thioacid could be isolated as its n-Bu amine salt by precipiatation in DCM

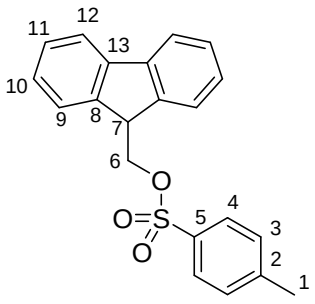
^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 0.92 (t, *J* = 7.25 Hz, 9H, CH-17), 1.20-1.37 (m, 6H, CH-18), 1.47-1.64 (m, 6H, CH-19), 2.76-2.90 (t, 2H, CH-11), 3.05-3.22 (m, 8H, CH-20), 4.06-4.40 (m, 12H, CH-7,8,10,17-19), 7.14-7.34 (m, 7.36-7.49 (m, 5H, Ar), 7.56-7.71 (m, 4H, Ar), 7.79-7.95 (m, 4H, Ar).

^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 13.5 (C-17), 19.2 (C-18), 23.1 (C-19), 36.2 (C-11), 46.6 (C-7), 57.6 (C-20), 62.5 (C-10), 65.8 (C-8), 120.1 (C-2), 125.2 (C-5), 126.5 (), 127.0 (Ar), 127.6 (Ar), 128.2 (Ar), 129.1 (Ar), 137.2 (C-12), 140.7 (C-1), 143.6 (C-6), 155.9 (C-9), 200.7 (C-16).

MS (ESI): *m/z* calcd for $C_{24}H_{20}NO_3S$ $[M-H]^-$ 402.1. Found 402.3.

R_t 12.56 min (linear gradient 5 to 99% C, 12.5 min, 99 % for 3 min).

9*H*-fluoren-9-ylmethyl 4-methylbenzenesulfonate (120)



120

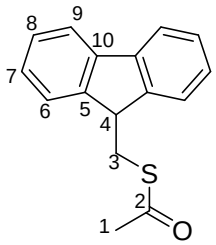
To a solution of 9-fluorenemethanol 2.034g, 10.36 mmol, 1 equiv) and tosyl chloride (2.96g, 15.55mmol, 1.5 equiv) in dry DCM under inert atmosphere was added pyridine (1.667 mL, 20.72 mmol, 2 equiv) via canula. The reaction was then allowed to stir at room temperature for 24 hours and was monitored via TLC. After completion, the reaction was quenched with 1M HCl and then extracted three times with DCM (100 mL total). The organic phases were collected and then washed with H_2O and brine and then dried over sodium sulphate. After removal of the solvent under reduced pressure the pure material was either precipitated with a mixture of Hex/EA/Et_2O or was purified over a silica gel column using hex/EtOAc as elluent (10:1) to give the title compound as a white powder in a 2.319 g (64 %) yield.

R_f = 0.49 (10: 3, Hex : EtOAc)

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 2.43 (s, 3H, CH-1), 4.23-4.30 (m, 3H, CH-6,7), 7.25-7.33 (m, 4H, CH-9,12), 7.37-7.43 (m, 2H, CH-3), 7.51-7.57 (m, 2H, CH-4), 7.71-7.80 (m, 4H, CH-10,11).

MS (ESI): *m/z* calcd for $C_{21}H_{25}NO_3S$ 373.1 $[M+Na]^+$. Found 373.0.

_S_-(9_H_-fluoren-9-ylmethyl) ethanethioate (121)



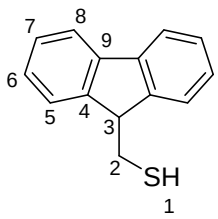
121

To a flame dried round bottom Schlenck flask flushed with nitrogen was added a solution of **120** (0.1.2 g, 3.42 mmol, 1 equiv) in dry DMF (5 mL). To this mixture was added potassium thioacetate (0.469 g, 4.11 mmol, 1.2 equiv) and 18-crown-6 (0.905 g, 3.42 mmol, 1 equiv) and allowed to stir at room temperature for 2 hours and monitored via TLC. After completion of the reaction, the mixture was diluted with EtOAc (~ 20 mL) and then quenched with water (20 mL). The organic layer was separated and then washed with water and brine and finally dried over Na_2SO_4. After removal of the solvent under reduced pressure, the product was purified over a silica gel column using EtOAc and hex (30:1) as elluent. The product was recovered as a white solid in a 0.708 g (81 %) yield.

R_f = 0.15 (Hex:EtOAc, 30:1)

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 2.27 (s, 3H, CH-1), 3.52 (d, *J* = 5.9 Hz 2H, CH-3), 4.17 (t, *J* = 5.9 Hz, 1H, CH-4), 7.28-7.34 (m, 2H, CH-7), 7.35-7.40 (m, 2H, CH-8), 7.62 (m, 2H, CH-6),
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 30.7 (C-1), 32.6 (C-3), 46.8 (C-4), 120.0 (C-9), 124.7 (C-7), 127.2 (C-8), 127.8 (C-6), 141.2 (C-5), 145.5 (C-10), 195.4 (C-2).

9*H*-fluoren-9-ylmethanethiol (122)



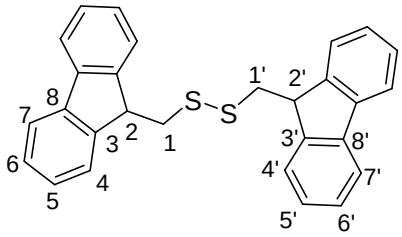
122

To a flame dried round bottom Schlenck flask evacuated and flushed with nitrogen was added a solution of **121** (0.678 g, 3.9 mmol, 1 equiv) in dry Et_2O (17 mL). To this was added dropwise Red-Al (65 % in toluene, 2.593 mL, 8.63 mmol, 2.2 equiv) and the reaction was stirred at room temperature for 1 hour, and monitored via TLC. After completion, the reaction was cooled to 0 °C and then quenched slowly with dropwise addition of 2M HCl (20 mL). The reaction mixture was then extracted with Et_2O (30 mL total) and the organic fractions were combined and then washed with brine. After drying the organic layer of Na_2SO_4, the solvent was removed first under reduced pressure and finally dried to a viscous oil under high vacuum. The product was recovered as a yellow oil and determined to be the product and used without further purification.

R_f = 0.48 (Hex:EtOAc, 19:1)

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.21 (t, *J* = 8.24 Hz, 1H, SH), 3.19 (t, *J* = 7.2 Hz, 2H, CH-2), 4.17 (t, *J* = 5.43 Hz, 1H, CH-3), 7.31-7.43 (m, 4H, CH 6-7), 7.59-7.63 (m, 2H, CH-5), 7.75-7.79 (m, 2H, CH-8).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 28.0 (C-2), 49.3 (C-3), 120.1 (C-8), 124.5 (C-6), 127.2 (C-7), 127.8 (C-5), 141.6 (C-4), 145.2 (C-9).

bis(9*H*-fluoren-9-ylmethyl) disulfide (123)

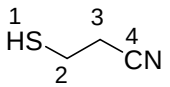


123

Oxidation of **122** to its disulfide occurred under a number of conditions. Attempting to purify the thiol by column chromatography resulted in mostly oxidized thiol. Deprotecting **121** with sodium thiomethoxide resulted in oxidation to the disulfude. Additionally, allowing the thiol to stand at room temperature in solvent under atmospheric conditions over a prolonged period of time resulted in the disulfide. Treatment of the disulfide with Ellman's reagent, did not give a positive test for free thiols.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 3.07 (d, *J* = 6.65 Hz, 4H, CH-1,1'), 4.06 (t, *J* = 6.58 Hz, 2H, CH-2,2'), 7.28-7.36 (m, 4H, CH 5,5'), 7.36-7.44 (m, 4H, CH-6,6') 7.62-7.68 (m, 4H, CH-7,7'), 7.74-7.80 (m, 4H, CH-4,4').
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 37.7 (C-1,1'), 47.0 (C-2,2'), 120.0 (C-7,7'), 125.0 (C-5,5'), 127.1 (C-6,6'), 127.7 (C-4,4'), 141.2 (C-3,3'), 146.2 (C-8,8').

3-Mercaptopropionitrile (126)



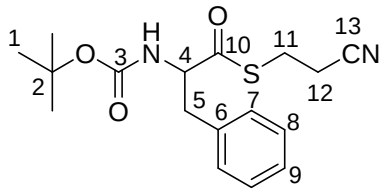
126

To a stirring solution of 3,3'-Dithiobis(propionitrile) (1.723 g, 0.01 mmol, 1 equiv) in 2 M HCl (25 mL) in a round bottom flask, was gradually added zinc powder (1.308 g, 0.02 mmol, 2 equiv) in multiple portions at 40 °C. The reaction was stirred for 30 minutes and then allowed to cool, whereupon the product was extracted with DCM (4 x 10 mL). The organic layers were combined and dried over $MgSO_4$. After removal of

the solvent under reduced pressure, the title compound was obtained as a clear liquid in 1.730 g (99 %) and stored at –20 °C until further use.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.82 (t, J = 8.6 Hz, 1H, SH), 2.67-2.73 (m, 2H, CH-3), 2.76-2.84 (m, 2H, CH-2).

_S_-(2-cyanoethyl) (2_S_)-2-[(_tert_-butoxycarbonyl)amino]-3-phenylpropanethioate (127)

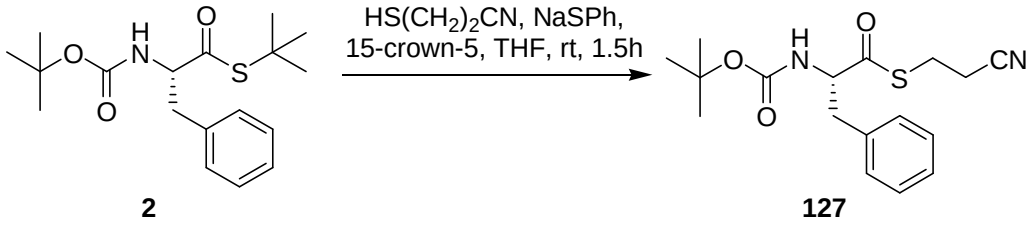


127

To a flame-dried round bottom Schlenck flask filled and evacuated with N_2 was added tBoc-Phe-OH (0.265 g, 1.0 mmol, 1 equiv) and dissolved in dry DCM (6 mL). To this was added DMAP (0.012 g, 0.1 mmol, 0.1 equiv) and the thiol (0.087 g, 1.2 mmol, 1.2 equiv) after which the reaction was cooled to 0 °C. DCC (0.206 g, 1.2 mmol, 1.2 equiv) was then added and the stirring solution was allowed to warm to room temperature. After 3-18 hours, depending on the thiol, the precipitated thiol urea was then filtered through a pad of celite and the filtrate evaporated down *in situ*. The residue was taken up in DCM , washed twice with 0.5 N HCl, saturated $NaHCO_3$ solution, and dried over $MgSO_4$. The product was then purified via flash column chromatography over silica gel (18 cm) using DCM as eluent to give a white powder in a 0.287 g (86 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 1.41 (s, 9H, CH-1), 2.62 (t, J = 6.99 Hz, 2H, CH-12), 3.06-3.17 (m, 4H, CH-5,11), 4.59-4.66 (m, 1H, CH-4), 4.90 (d, *J* = 8.17 Hz 1H, NH), 7.15-7.19 (m, 1H, CH-7), 7.24-7.33 (m, 2H, CH-8,9).
^{13}C-NMR: (75.5 MHz, $CDCl_3$) δ [ppm]: 18.4 (C-12), 24.7 (C-11), 28.4 (C-1), 38.1 (C-5), 61.2 (C-4), 80.9 (C-2), 117.9 (C-13), 127.4 (C-9), 128.9 (C-7), 129.4 (C-8), 135.4 (C-6), 155.1 (C-2), 200.7 (C-10).
ESI-MS: *m/z* calcd for $C_{17}H_{22}N_2O_3S$, 335.1 $[M+H]^+$. Found, 335.1 $[M+H]^+$.

Conversion of the *tert*-butyl thiol ester to the cyanoethylester



2 **127**

To a solution of **2** (0.02g, 0.06 mmol, 1 equiv) in THF (1.5 mL), was added a solution of sodium thiophenolate (0.016 g, 0.12 mmol, 2 equiv), 15-crown-5 (0.026 g, 0.12 mmol, 2 equiv), and **126** (0.098 mL, 1.2 mmol, 20 equiv) in THF (0.5 mL). The reaction was allowed to stir at room temperature for 1.5 hours after which it was determined by LC-MS analysis at 220 nm that the *tert*-butyl thioester had completely trans-thioesterified to the cyanoethyl thioester.

Peptide Ac-SYRGF-S$(CH_2)_2$CN (128b)

128b

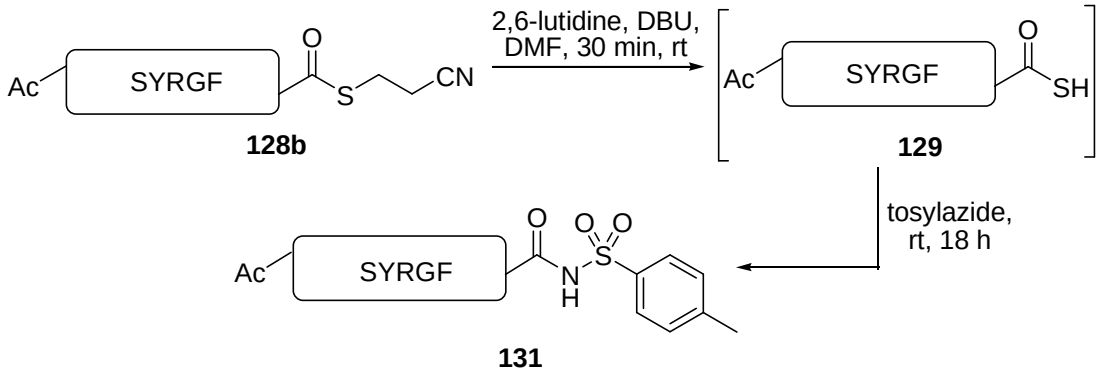
In a standard experiment, 20 mg of resin 11 (7.6 µmoles, 1 equiv) was added to a Shlenck tube equipped with a stir bar. The tube was then evacuated and flushed with nitrogen and to this was added HS-$(CH_2)_2$CN (43 µL, 380 µmole, 50 equiv) in THF (0.5 mL). In a separate flask, sodium thiophenolate (2 mg, 15.2 µmoles, 2 equiv) was stirred with 15-crown-5 (3.3 mg, 15.2 µmoles, 2 equiv) in dry THF (0.5 mL). Upon dissolution of the sodium thiophenolate, this mixture was then added via canula to the resin 11 and stirred vigorously for 2-3 hours at room temperature. The resin was then filtered and washed twice with dry THF (5 mL total). If necessary, this cleavage was repeated for an additional 2 hours. After removal of the volatile materials *in vacuo* or under a stream of N_2, the protected peptide was directly cleaved with Reagent B (TFA/Phenol/TIS/H_2O 88/5/2/5, 1.5 mL) for 2-3 hours. The deprotected peptide in cleavage cocktail was then precipitated with MTBE (15 mL, 10 volumes) and then centrifuged at 3200 rpm for 30 minutes. The supernatant was then decanted and the

peptide was taken up in H_2O/ACN (1/1) and lyophilized to yield a white powder 13a (5.0 mg, 77 % based on initial coupling) which was determined by LC-MS at 220 nm to be 88 % pure.

Ac-SYRGF-S$(CH_2)_2$CN (**128b**): ESI-MS-TOF: *m/z*: calcd for $C_{34}H_{45}N_9O_8S$, 740.3 $[M+H]^+$. Found: 740.2 $[M+H]^+$.

R_t 2.28 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

Conversion of peptide Ac-SYRGF-S$(CH_2)_2$CN to the thioacid *in situ* and subsequent ligation to the sulfonamide



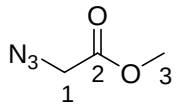
To a solution of the **128b** peptide (2 mg, 2.7 µmol, 1 equiv) in DMF (0.8 mL) was added 2,6-lutidine (0.0031 mL, 27.03µmol, 10 equiv). The reaction was allowed to stir for 20 minutes upon which DBU (0.004 mL, 27.03µmol, 10 equiv) was added. The reaction was allowed to react for another 30 minutes after which tosyl azide (0.005 g, 2.7 µmol, 10 equiv) was added and the reaction was allowed to stir for 18 hours and monitored via LC-MS. After completion, the solvent was removed in vacuo, and the peptide was precipitated from the obtained residue in cold Et_2O.

Ac-SYRGF-$NHSO_2(C_6H_4)CH_3$ (**131**): ESI-MS-TOF: *m/z*: calcd for $C_{38}H_{49}N_9O_{10}S$, 824.3 $[M+H]^+$. Found: 824.2 $[M+H]^+$.

R_t 2.44 min (linear gradient 5 to 99% C, 2.5 min, 99 % for 3 min).

7.5. Synthetic Procedures for Thioamidation of Thioacids and Azides

Methyl azidoacetate (133)

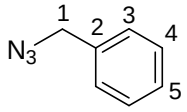


133

To a solution of sodium azide (2.93 g, 0.045 mole, 1.06 equiv) in dry DMF (10 mL) under a nitrogen atmosphere in a flame-dried, evacuated round bottom schlenck flask was added under a cross current of nitrogen, methyl bromocetate (3.91 mL, 0.0425 mole, 1 equiv) dropwise over 10 minutes at room temperature. Soon after addition, a white precipitate formed indicating formation of sodium bromide. The reaction was allowed to stir for 2.5 hours and was then quenched with an equivalent volume of water (~14 mL). The product was then extracted with Et_2O (3 x 50 mL), and the organic layers were combined and washed with water (6 x 20 mL) and then dried over $MgSO_4$. Solvent was removed under reduced pressure to give the title compound as a colorless oil in a 3.586 g (73 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 3.77 (s, 3H, CH-3), 3.85 (s, 2H, CH-1).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 50.2 (C-1), 52.5 (C-3), 168.8 (C-2).
FT-IR (ATR) ν (cm^{-1}): 2101 (azide).

Benzyl azide (140)

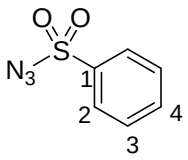


140

A suspension of benzyl chloride (0.823 mL, 10.0 mmol, 1 equiv) and sodium was azide (1.625 g, 25.0 mmol, 2.5 equiv) in water (19 mL) was irradiated using microwave irradiation to 150 °C for 120 minutes. After completion, the reaction mixture was poured into water (50 mL) and diluted with DCM (10 mL). The organic layer was separated and washed with water (2 x 10 mL) and then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the title compound was obtained as a colorless oil in a 0.810 g (61 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 4.34 (s, 2H, CH-1), 7.30-7.44 (m, 5H, CH-3-5).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 54.8 (C-1), 128.1 (C-4), 128.2 (C-5), 128.8 (C-3), 135.3 (C-2).
FT-IR (ATR) *ν* (cm^{-1}): 2092 (azide), 3299, 1634, 1534.

Benzenesulfonyl azide (143)

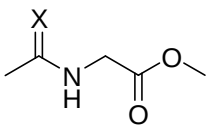


143

To a solution of benzenesulfonyl chloride (17.7 g, 0.10 mol, 1 equiv) in EtOH (150 mL) was added a solution of sodium azide (7.8 g, 0.121 mol, 1.207 equiv) in water (30 mL) and stirred at room temperature for one hour. After completion of the reaction, the mixture was poured over water (100 mL). The product was extracted with DCM then washed with water (30 mL) and dried over $MgSO_4$. The solvent was reduced in vacuo to give the title compound as a white solid in a 8.98 g (49 %) yield.

^{1}H-NMR: (300.1 MHz, $CDCl_3$) δ [ppm]: 7.68 (t, *J* = 7.6 Hz, 2H, CH-3), 7.80 (t, *J* = 7.6 Hz, 1H, CH-4), 7.98 (d, *J* = 7.6 Hz, 2H, CH-2).
^{13}C-NMR (75.5 MHz, $CDCl_3$) δ [ppm]: 128.2 (C-2), 130.7 (C-3), 135.9 (C-4), 139.4 (C-1).
FT-IR (ATR) *ν* (cm^{-1}): 2129(Azide), 1371 (SO_2), 1168 (SO_2).

Glycinate synthesis

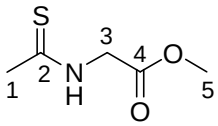


X = S, O

General method: To a suspension of methylazido acetate (0.100g, 0.869 mmol, 1 equiv) and $AgNO_3$ (0.015 g, 0.0869 mmol, 0.1 equiv) in a water / methanol mixture

(2 mL: 2 mL) was added thioacetic acid and the reaction was stirred for 18 hours. After the reaction had reached completion, the mixture was diluted with methanol (10 mL) and then filtered over a celite pad. The solvent was then removed under reduced pressure and the product was purified over a short silica gel column using 10 % methanol in DCM to give an off-white oil in 0.030 g (23 %) yield.

Methyl *N*-ethanethioylglycinate (134)



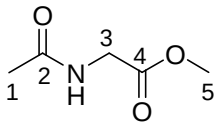
134

^{1}H-NMR: (300.1 MHz, DMSO-d_6)) δ [ppm]: 2.44 (s, 3H, CH-1), 3.63 (s, 3H, CH-5), 4.30-4.34 (m, 2H, CH-3).

^{13}C-NMR (75.5 MHz, DMSO-d_6)) δ [ppm]: 32.4 (C-5), 46.5 (C-3), 51.8 (C-1), 168.6 (C-5), 201.5 (C-2).

ESI-MS: *m/z* calcd for $C_5H_9NO_2S$, 148.0 $[M+H]^+$. Found, 148.0 $[M+H]^+$.

Methyl *N*-acetylglycinate (135)



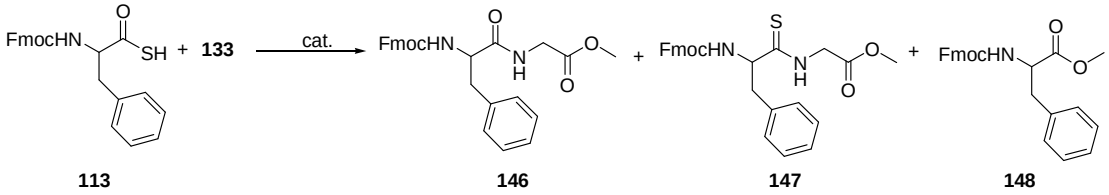
135

^{1}H-NMR: (300.1 MHz, DMSO-d_6) δ [ppm]: 1.68 (s, 3H, CH-1), 3.61 (s, 3H, CH-5), 3.80 (d, *J* = 8.01 Hz, 2H, CH-3).

^{13}C-NMR (75.5 MHz, DMSO-d_6)) δ [ppm]: 22.2 (C-5), 40.5 (C-3), 51.6 (C-1), 169.6 (C-5), 171.4 (C-2).

ESI-MS: *m/z* calcd for $C_{17}H_{22}N_2O_4$, 132.0 $[M+H]^+$. Found, 132.1 $[M+H]^+$.

Methyl-*N*-((2*S*)-2-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-3-phenylpropanethioyl)glycinate



To a solution of the Fmoc-Phe-SH (0.071 g, 0.18 mmol, 1 equiv) in methanol and H_2O (1:1, 2 mL total) was added a diluted amount of H_2SO_4 (0.5 mL of a mixture of 2 drops of acid in 2 mL of MeOH) to lower the pH of the mixture to between 2-3. To this solution was then added a solution of the methylazido acetate (0.024, 0.21 mmol, 1.2 equiv) in MeOH (1 mL)after which was added silver nitrate (0.007 g, 0.044 mmol, 0.25 equiv) in H_2O (1ml, final amount of water to methanol, 2 mL: 2 mL) upon which salt precipitation occurred immediately. To this stirring suspension was then added methylazido acetate and the reaction was allowed to stir for 17 hours. After completion, the product was worked up in a similar fashion as previously mentioned; the product mixture was diluted with methanol and filtered over a celite pad. The product was analyzed via LC-MS and gave a relationship of thioamide: amide in 1.63: 1 ratio.

Amide (**146**):
ESI-MS: *m/z* calcd for $C_{27}H_{26}N_2O_5$, 459.1 $[M+H]^+$. Found, 459.2 $[M+H]^+$.

Thioamide (**147**):
ESI-MS: *m/z* calcd for $C_{27}H_{26}N_2O_4S$, 475.1 $[M+H]^+$. Found, 475.0 $[M+H]^+$.

Methyl Ester (**148**):
ESI-MS: *m/z* calcd for $C_{25}H_{23}NO_4$, 402.1 $[M+H]^+$. Found, 402.2 $[M+H]^+$.

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