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Chemical Synthesis Of Peptides And Peptide Thioesters

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CHEMICAL SYNTHESIS OF PEPTIDES AND PEPTIDE THIOESTERS

by

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DISSERTATION

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Approved by:

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Advisor Date

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DEDICATION

This dissertation is dedicated to my family
for their endless love and support.

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LIST OF ABBREVIATIONS

AA	Amino acid
Ac	Acetyl
All	Allyl
Alloc	Allyloxycarbonyl
aq	Aqueous
Ar	Aryl
BAL	Backbone amide linkage
Bn	Benzyl
Boc	*tert*-Butoxycarbonyl
Bu	Butyl
Bz	Benzoyl
Calcd	Calculated
CAN	Ceric ammonium nitrate
Cbz	Benzyloxycarbonyl
CDI	1,1'-Carbonyldiimidazole
DCM	Dichloromethane
DCC	*N,N'*-Dicyclohexylcarbodiimide
DIAD	Diisopropyl azodicarboxylate
DIBAL-H	Diisobutylaluminium hydride
DIC	*N*,*N'*-Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine

DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DVB	Divinylbenzene
EI-HRMS	Electron impact high resolution mass spectroscopy
ESI-HRMS	Electrospray ionization high resolution mass spectroscopy
equiv.	Equivalent
Et	Ethyl
EDCI	*N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide Hydrochloride
Fm	9-Fluorenylmethyl
Fmoc	9-Fluorenylmethoxycarbonyl
h	Hour
HATU	*O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	*O*-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
HOSu	*N*-hydroxysuccinimide
Hz	Hertz
im	Imidazole
i-Pr	Isopropyl
IR	Infrared
Me	Methyl

min	Minutes
mmol	Millimole
Mp	Melting point
MS	Molecular sieves
NaHMDS	Sodium hexamethyldisilazide
NCS	*N*-Chlorosuccinimide
NCL	Native chemical ligation
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
p	*para*
m	*meta*
PG	Protecting group
Ph	Phenyl
PMB	*p*-Methoxybenzyl
ppm	Parts per million
PTSA	*p*-Toluenesulfonic acid
Py	Pyridine
PyBOP	Benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate
quant.	Quantitative
RP-HPLC	Reverse phase high performance liquid chromatography
UPLC	Ultra pressure liquid chromatography
r.t.	Room temperature

sat.	Saturated
SPPS	Solid phase peptide synthesis
TBAF	Tetrabutylammonium fluoride
TBDPS	*tert*-Butyldiphenylsilyl
temp.	Temperature
Tf	Trifluoromethanesulfonyl
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMOB	2,4,6-Trimethoxybenzyl
TMS	Trimethylsilyl
Trt	Trityl
Ts	*p*-Toluenesulfonyl
TTBP	2,4,6-Tri-*tert*-butylpyrimidine
UV/Vis	Ultraviolate-visible
TIPS	Triisopropyl Silane

CHAPTER 1

INTRODUCTION

1.1 Background and Significance

Proteins are biological macromolecules that play a central role in numerous biological and physiological processes in living organisms, carrying out most biochemical functions of the cell and being widely employed in structural roles.[1] The biological function of a particular protein derives from its unique folded structure, which in turn is defined by the amino acid sequence of its polypeptide chain.[2] The diverse and important roles that proteins play in the biological world, and the correlation between their structure and function have long intrigued scientists.[3] In this context, that of understand the underlying mechanism of these processes at molecular level, the ability to modify structural and functional properties of bioactive proteins is crucial in biological and medical research.[4] The first requirement for the study of proteins is accessibility in terms of purity and quantity. There are three main approaches to consider: (i) native protein isolation, (ii) recombinant techniques for the expression of proteins in microorganisms, and (iii) chemical synthesis.

For the past thirty years, techniques of recombinant DNA-based molecular biology have been used for systematic variation of the amino acid sequence of a polypeptide chain and the resulting changes in behavior of these macromolecules have been correlated to their amino acid sequence. This approach, which is recognized as protein engineering, has provided useful insights into protein function.[3] However, the protein engineering approach to

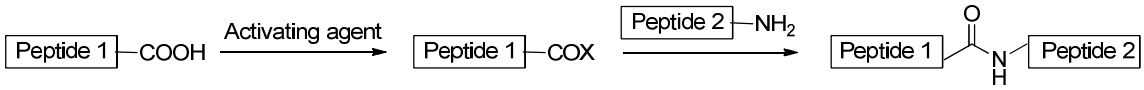
probing the molecular basis of protein function has some limitations, but mainly the fact that only twenty genetically encoded amino acids can be readily incorporated and that site-specific post-translational modifications of the protein molecule are technically difficult to control with precision.[2] Intrepid efforts have been made to overcome these limitations, both in vitro and by the use of synthetic biology in engineered microorganisms but to date, only limited improvements have been made in elucidating the molecular basis of protein function.[5,6]

Chemical synthesis of proteins overcomes these limitations and enables many modifications to the covalent structure of a protein molecule by the introduction of unnatural amino acids, site-specific isotopic labeling, labels for imaging, and the site-specific attachment of affinity tags without limitation as to the number and kind of labels and tags introduced.[7]

1.2 Chemical Synthesis

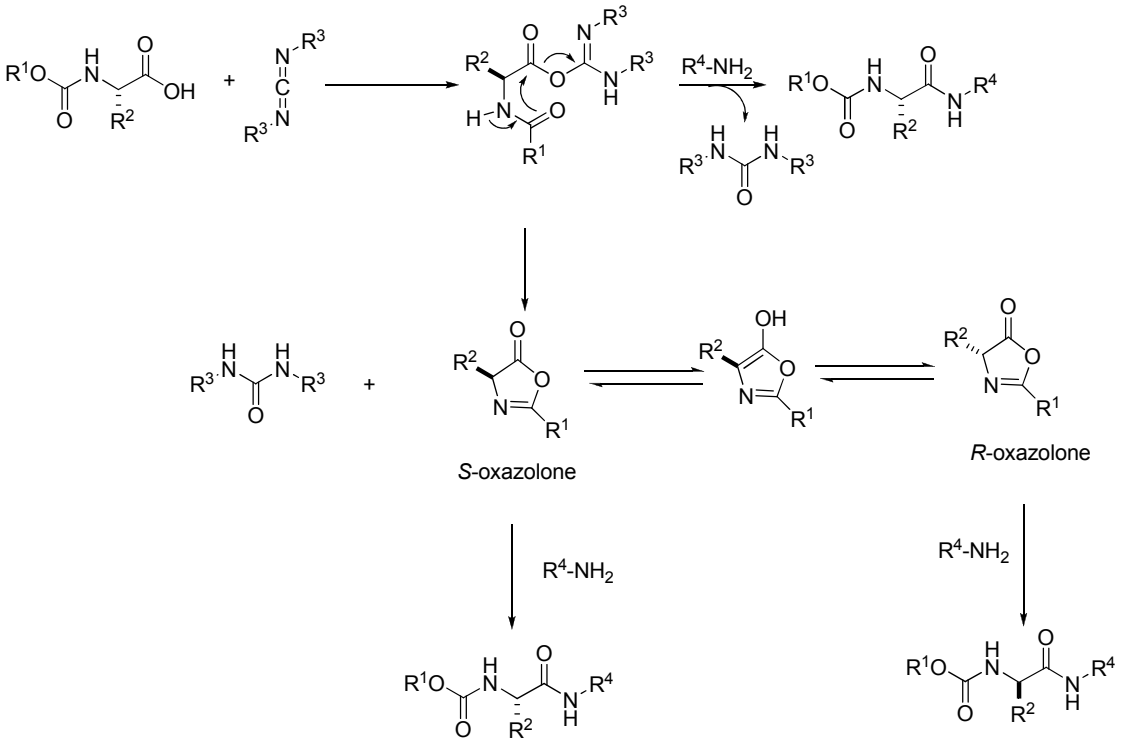
The quest to make enzymes and other protein molecules by chemical synthesis was one of the most ostentatious challenges for organic chemistry in the twentieth century. In the early years of the century, the great German chemist Emil Fischer and his scientific descendants pioneered the field of chemical peptide synthesis that eventually enabled the total chemical synthesis of the complex biologically active peptide hormone oxytocin (with its nine amino acid residues).[8] The total chemical synthesis of proteins remained a major objective of the organic synthesis community in the decades following the Second World War.

The chemical synthesis of a protein involves the formation of an amide bond between a peptide acid and an amine segment. This process involves the activation of a peptidyl acid, which is then subjected to aminolysis with a peptidyl amine to form native amide bond (Scheme 1).



Scheme 1. Amide bond formation

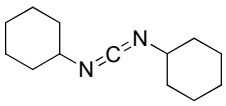
Amide bond formation can often present difficulties such as low yields, epimerization of adjacent stereogenic centers,[9] degradation, difficulties with purification, etc. Activation of the carboxylic group can lead to racemization of C-terminal α-amino acid via the oxazolone intermediate (Scheme 2).[9]



Scheme 2. Mechanism of epimerization via oxazolone formation

However, additives, such as 1-hydroxybenzotriazole **22** suppress racemization by formation of a less-reactive HOBt ester. Over the years, numerous mild coupling reagents and methods have been developed that are not only high yielding, but also help to minimize the racemization of neighboring stereogenic centers, and permit difficult couplings with sterically hindered amino acids. A selection of the most widely used coupling reagents is shown in Figure 1.[10-12]

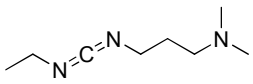
Coupling Reagents:



DCC

N,*N*'-Dicyclohexylcarbodiimide

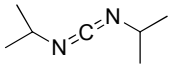
1



EDCI

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

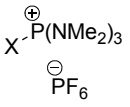
2



DIC

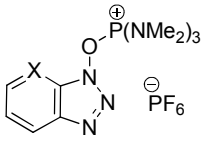
N,*N*'-Diisopropylcarbodiimide

3



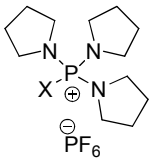
X = Cl, CloP; **4**

X = Br, BroP; **5**



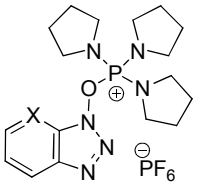
X = CH, BOP; **6**

X = N, AOP; **7**



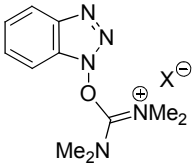
X = Cl, PyCloP; **8**

X = Br, PyBroP; **9**



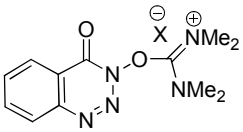
X = CH, PyBOP; **10**

X = N, PyAOP; **11**



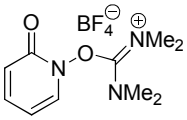
X = PF_6, HBTU; **12**

X = BF_4, TBTU; **13**

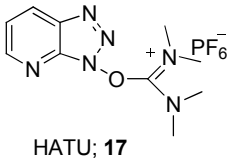


X = PF_6, HDTU; **14**

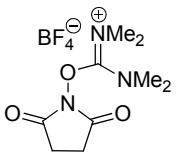
X = BF_4, TBDTU; **15**



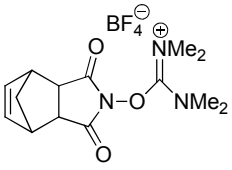
TPTU; **16**



HATU; **17**

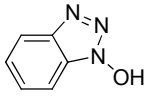


TSTU; **18**



TNTU; **19**

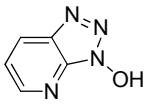
Additives:



HOBt

1-Hydroxybenzotriazole

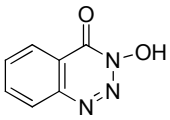
20



HOAt

1-Hydroxy-7-azabenzotriazole

21



HODhbt

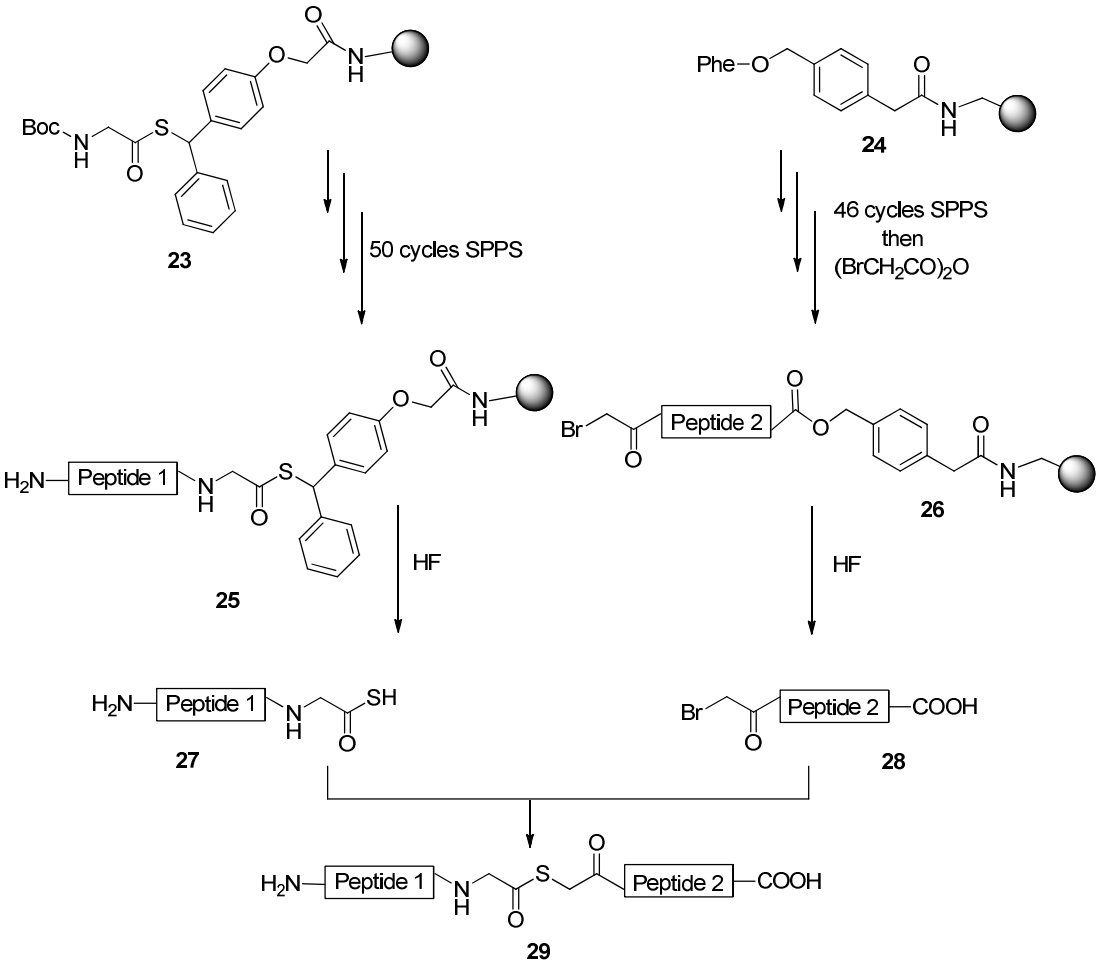
3-Hydroxy-3,4-dihydro 4-oxo-1,2,3-benzotriazine

22

Figure 1. Common coupling reagents and additives

The development of the Merrifield solid-phase method[13] along with the tremendous improvements made in the application of high performance liquid

chromatography for peptide and protein purification has provided the ability to synthesize peptides containing 40-50 amino acid residues with high purity. However, synthesizing long peptide chains beyond 100 amino acid residues remains a very difficult task.[14] The major problems encountered with solid phase synthesis arise from incomplete couplings and deprotection reactions, which create impurities that accumulate in each step ultimately yielding the target peptide in an impure state. In order to circumvent this limitation of solid-phase methodology for the preparation of longer proteins, new approaches have been developed. The most useful and important among these approaches was chemical ligation introduced by Kent and coworkers in 1992, which allowed the coupling of unprotected peptide fragments in aqueous solution.[15] For example, the HIV-1 protease analog containing 99 amino residues **29**, was synthesized by chemical ligation of a 51-residue peptide bearing a C-terminal thioacid **27** and a 48-residue peptide having an *N*-terminal alkyl bromide **28** (Scheme 3).[1]



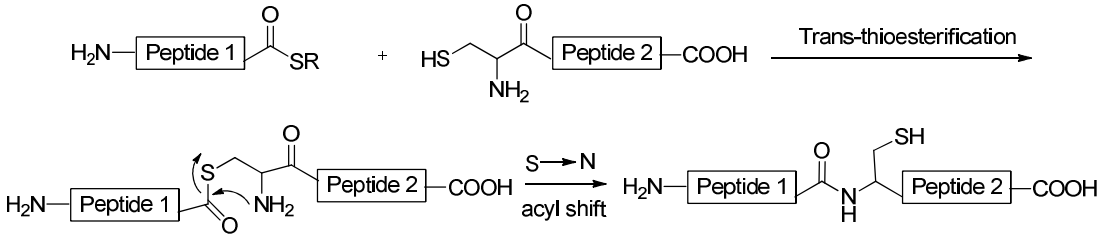
Scheme 3. Chemical ligation of HIV-1 PR analogue

A major disadvantage of this chemical ligation, however, is the formation of an unnatural structure at the ligation site.

1.3 Native Chemical Ligation

In 1994, Kent and coworkers described an important extension of the existing chemical ligation method referred to as the native chemical ligation,[16] which leads to the formation of a native amide bond at ligation site. The original version of this ligation used the chemistry first described by Wieland for coupling cysteine to other amino acids.[17] Thus, the first step of this ligation involves a

chemoselective trans-thioesterification of the unprotected peptide-α-thioester with the sulfhydryl group of a second unprotected peptide segment containing an amino terminal cysteine residue. This gives rise to a thioester linked intermediate as the initial product, which spontaneously rearranges to give the native amide bond at the ligation site joining two unprotected peptide segments and regenerates the cysteine side chain thiol (Scheme 4).



Scheme 4. Native chemical ligation

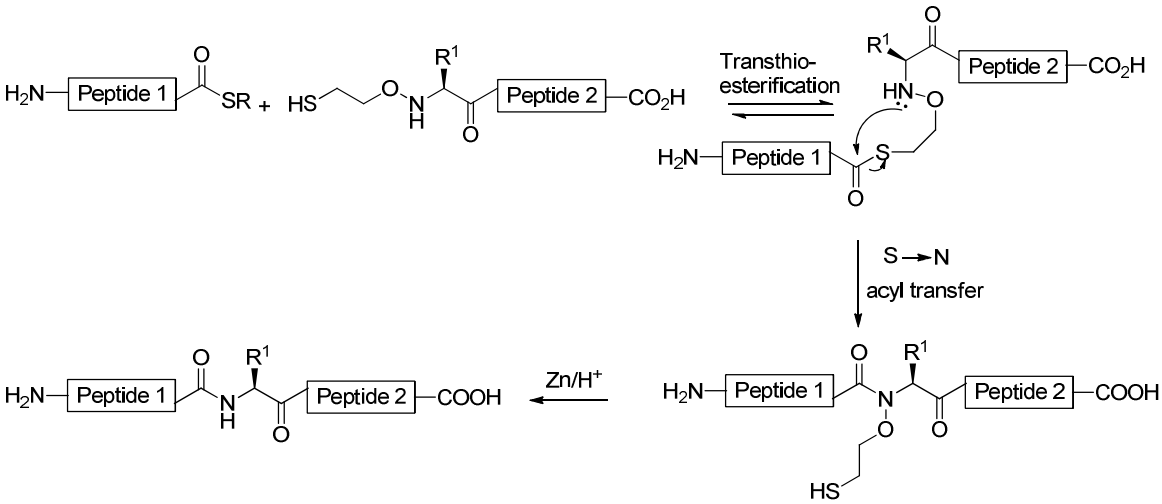
Over the years, the native chemical ligation method introduced by the Kent group has found wide applications in chemical synthesis of hundreds of moderate sized proteins and glycoproteins. By combination with molecular biology methods, site specifically modified proteins of up to 52 kDa (the β-subunit of F1-ATPhase)[18] can be synthesized by expressed protein ligation; a form of protein semi-synthesis that uses NCL to react a peptidyl thiosester generated by recombinant means with a Cys-peptide. In one example, Kent and coworkers have achieved the total synthesis of a covalently coupled HIV-1 protease dimer[19] with the impressive number of 203 amino acids, using modified native chemical ligation methodology. However, NCL strategy has some limitations. First and most important among these is the absence of a truly practical synthesis of peptidyl thioesters, and second is the fact that a cysteine residue is required at the ligation site. This latter is considered to be a serious limitation as naturally

occurring proteins do not always contain a cysteine residue in the right position of their sequences. Therefore, several modifications of the initial method have been introduced to overcome this drawback.

1.4 Native Chemical Ligation without Cysteine

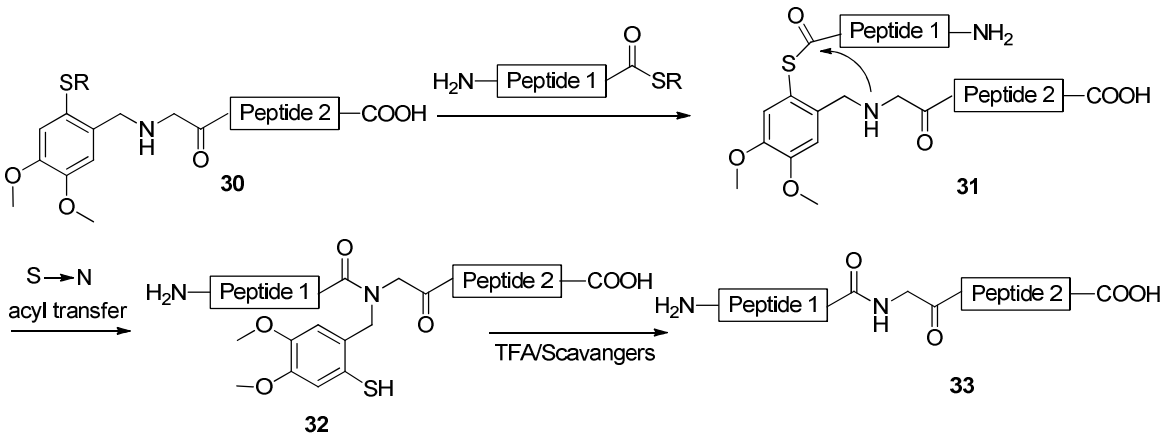
1.4.1 Assistance from N^{α}-Linked Auxiliaries

In principle, reversible attachment of the functional equivalent of a cysteine side chain onto the N-terminus of a peptide would provide universal ligation chemistry. This approach was first tried by Kent and co-workers using a cleavable aminooxy-linked ethanethiol side chain (Scheme 5).[20] However, the slow rearrangement of the thioester via a six membered cyclic intermediate onto the aminooxy group (which is more hindered and less nucleophilic than α-amine used in native chemical ligation) limited the application of this concept.



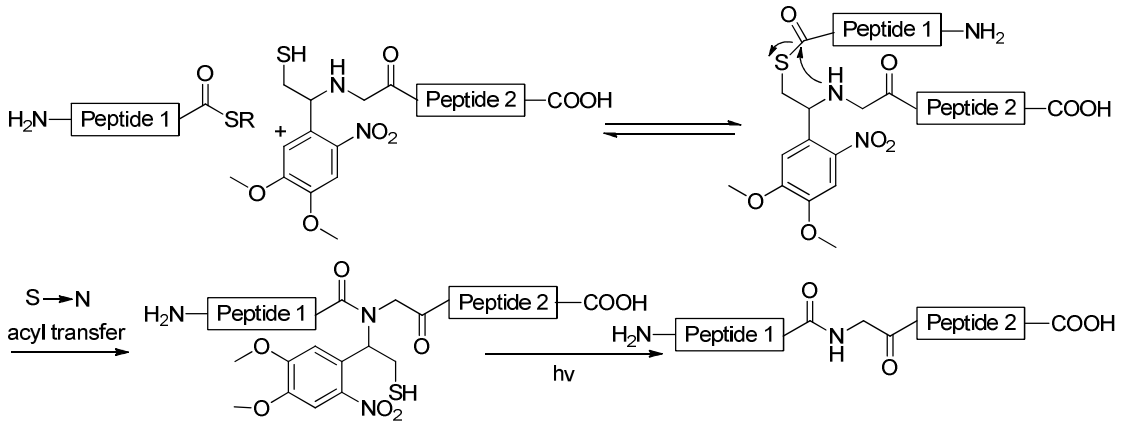
Scheme 5. Auxiliary mediated ligation

In another variation, several groups have applied the 2-mercaptobenzylamine linker **30**[21-24] at the ligation site as a replacement of *N*-terminal cysteine residue. In this system, the intramolecular acyl shift also proceeds through a six membered ring with the linker, which was subsequently removed by treatment with an acid, leading to the native peptides **33** (Scheme 6).[21]



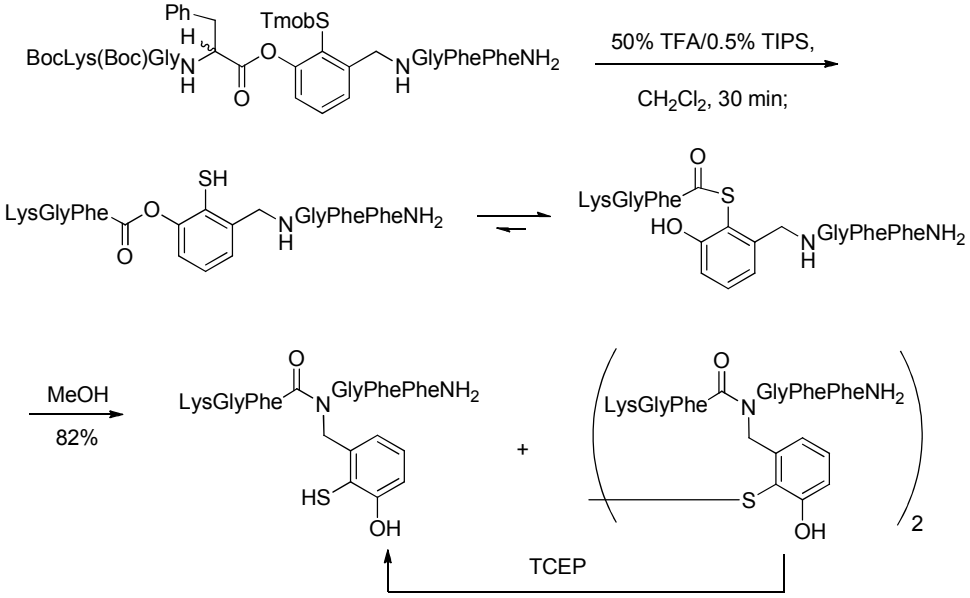
Scheme 6. Ligation through a mercaptobenzylamine linker

A third type of auxiliary for the thioester ligation of unprotected peptide fragments was introduced by Kawakami and Aimoto[25] and shortly thereafter by Marinzi *et al*.[26] Here, the auxiliary attached at the N-terminus of one peptide fragment is based on an *O*-nitrobenzyl scaffold, with which the thioester present in the second peptide fragment can undergo trans-thioesterification as shown in Scheme 7.[25]



Scheme 7. Ligation through a photo removable auxiliary

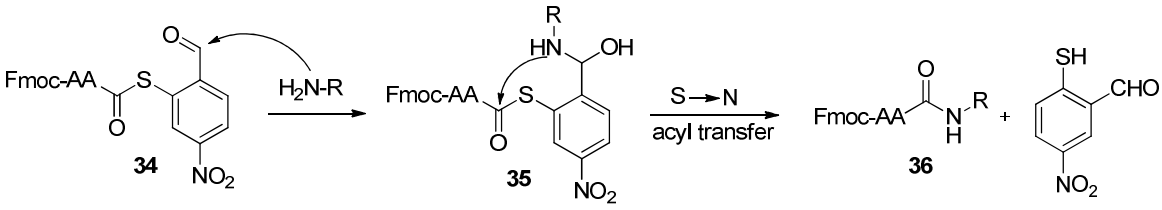
In another pathway, devised by Danishefsky and coworkers, a phenolic ester of a C-terminal peptide is ligated with an N-terminal peptide through two consecutive O→S and S→N acyl transfer (Scheme 8).[27] This method suffers from epimerization in the preparation of phenolic ester and is limited by slow rearrangement steps with sterically hindered amino acids.



Scheme 8. Ligation via O→S and S→N acyl transfer

Owing to the attachment of the auxiliary to the N-terminus, a secondary amine is involved in the *S*→*N* acyltransfer reaction. This increases the steric hindrance at the N-terminus and limits this approach of N^{α}-linked auxiliaries mainly to Gly ligation sites.[7]

In a similar vein, Ito and co-workers introduced an approach, which employs a preformed Fmoc-amino acid 2-formyl-4-nitrophenyl thioester **34** to facilitate S→N acyl transfer by reaction of an amine (Scheme 9).

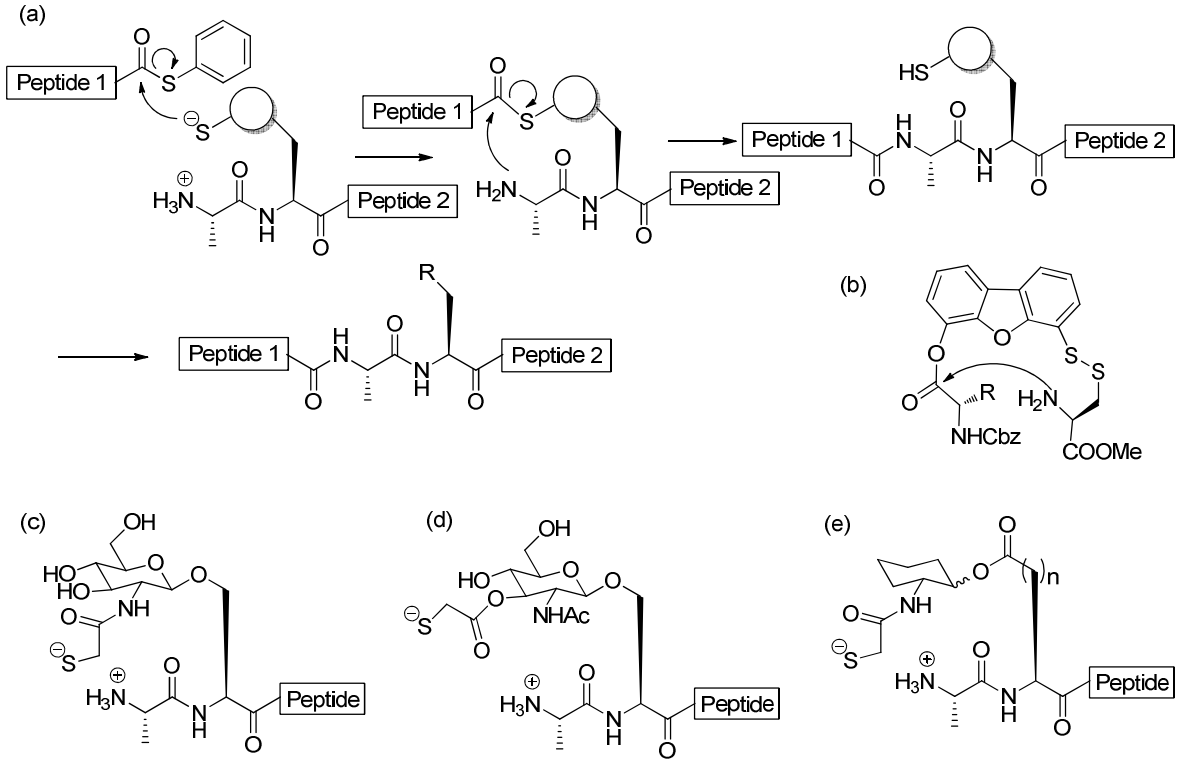


Scheme 9. Ligation via S→N acyl transfer

1.4.2 Side Chain Linked Auxiliaries

Side chain-tethered auxiliaries have also been investigated to facilitate S→N acyl transfer to form a native amide bond (Scheme 10a).[28-31] In this approach, although, the S→N acyl transfer proceeds through a large ring, the N-terminal amine of the peptide is not modified by the auxiliary and is less hindered. This concept is based on the earlier work by Kemp et al. on the prior thiol capture strategy,[32,33] which employs dibenzofuran-based auxiliaries (Scheme 10b) to bring about a high local concentration of each component. Recently, Wong et al. introduced the sugar-mediated ligation[28,29] for glycopeptides in which thiol-modified carbohydrates were attached to the side chain of serine and threonine (Scheme 10c, d). Here, the sugar auxiliary can be converted to a natural glycoform through desulfurization[28] or hydrazinolysis[31] and

can be removed enzymatically.[29] In an analogous approach, a thiol-modified cyclohexane has been attached as an auxiliary to the carboxylate group of aspartic acid or glutamic acid through an ester bond (Scheme 10e).[34] After ligation, this auxiliary can be removed by saponification at pH 10. Still, modulating the efficiency and chemoselectivity of long-range acyl transfer at neutral pH remains a significant challenge in the development of such approaches.[7]

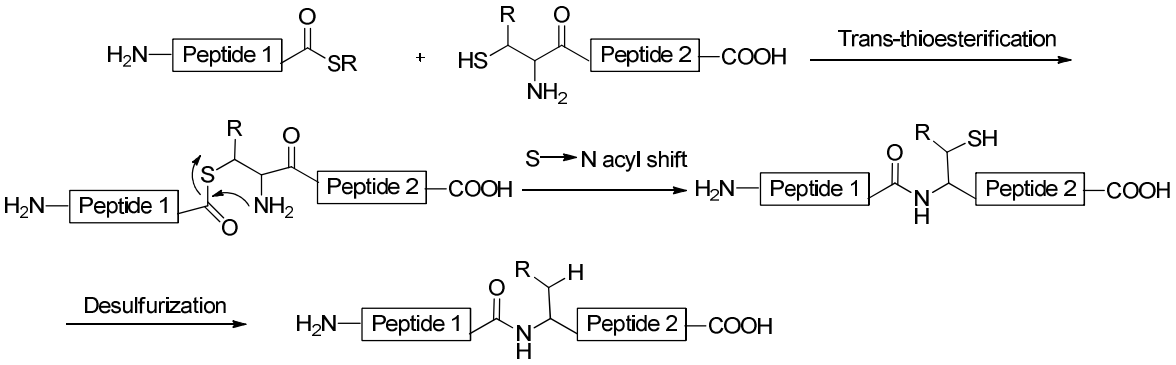


Scheme 10. Ligation through side chain linked auxiliaries

1.4.3 NCL-Desulfurization Combination

In addition to the use of auxiliaries discussed above, considerable efforts have been devoted to removing the restriction to cysteine-containing ligation sites. For example, the use of synthetic β-mercaptoamino acids and their

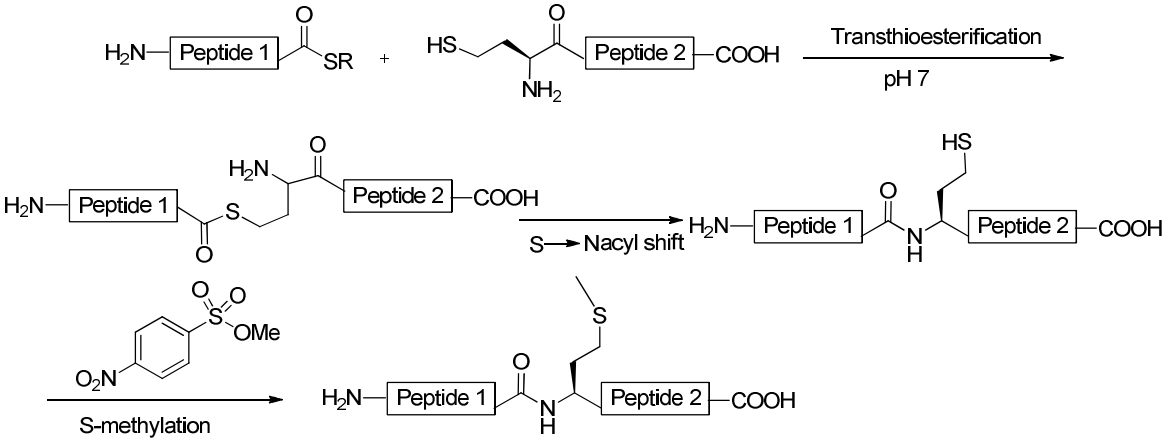
subsequent desulfurization can offer an alternative to the use of auxiliaries.[7,35,36] In this context, the combination of two chemoselective reactions, native chemical ligation, and desulfurization were applied for the synthesis of non-cysteine containing proteins. Dawson applied this approach to chemically modify the cysteine residue, which was converted into an alanine residue after ligation (Scheme 11).[37] Since alanine is one of the most abundant amino acid residues in proteins, the methodology was a significant extension of NCL. The usefulness of the approach was demonstrated by the total chemical synthesis of various polypeptides such as the cyclic antibiotic microcin J25, the 56-amino acid streptococcal protein GB1 domain, and a variant of the 110-amino acid ribonuclease, Barnase.[37] Over a period of time, significant progress has been achieved in the development of new thiolated building blocks that serve as precursors to other amino acids. Crich et al. and Botti et al. extended this strategy to ligation at phenylalanine,[38,39] which was accomplished by means of a synthetic β-mercaptophenylalanine building block and subsequent desulfurization. The building blocks β-mercaptovaline (penicillamine)[40] introduced by Seitz and co-workers and γ-mercaptovaline[41] promoted by Danishefsky and co-workers provide access to hydrophobic ligation sites (Xaa-Val). Danishefsky and co-workers augmented this approach to γ-thiol threonine surrogate to establish ligation at threonine sites.[42] Recently, lysine has been equipped with a δ- and γ-mercapto groups,[43,44] which enables ligations at both the α- and the ε-amino groups.



Scheme 11. NCL followed by desulfurization

1.4.4 Met Ligation: Alkylation of Hcy

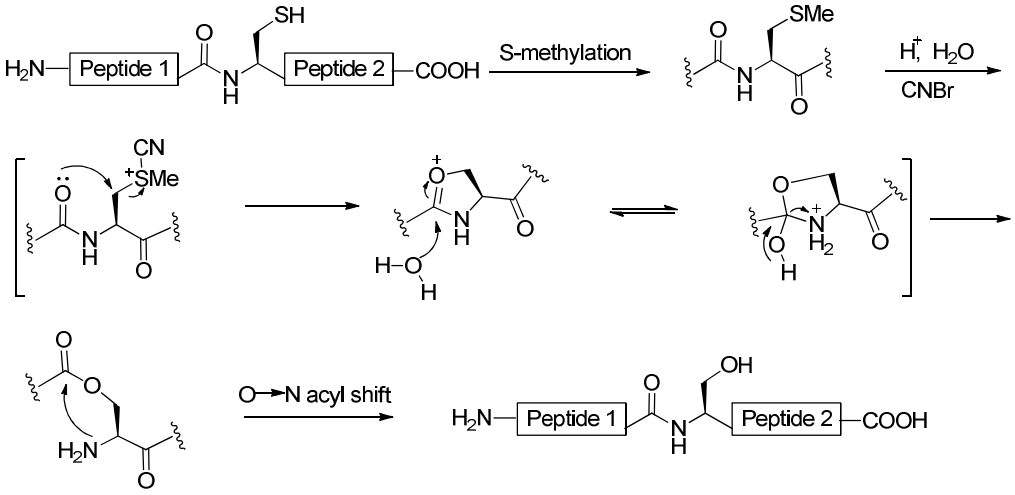
Another important ligation strategy has been developed which involves the alkylation of a homocysteine (Hcy) containing polypeptide with no Cys residues.[45,46] Hcy-containing polypeptides can be obtained, in analogy to NCL, with a homocysteine at the N terminus, where the reaction proceeds via a six-membered transition state (Scheme 12). The final alkylation can be performed under basic, neutral and acidic conditions to yield Met-containing peptides.[47,48]



Scheme 12. NCL at methionine

1.4.5 Ser Ligation

Kajihara and Okamoto made a significant contribution with the introduction of NCL at serine sites.[36,49,50] This strategy depends on the conversion of cysteine to serine after conventional NCL. The conversion reaction consists of three steps; the S-methylation of cysteine, reaction with cyanogen bromide to afford an O-ester linked peptide, and an O→N acyl shift to provide the native peptide linkage with a serine residue (Scheme 13).

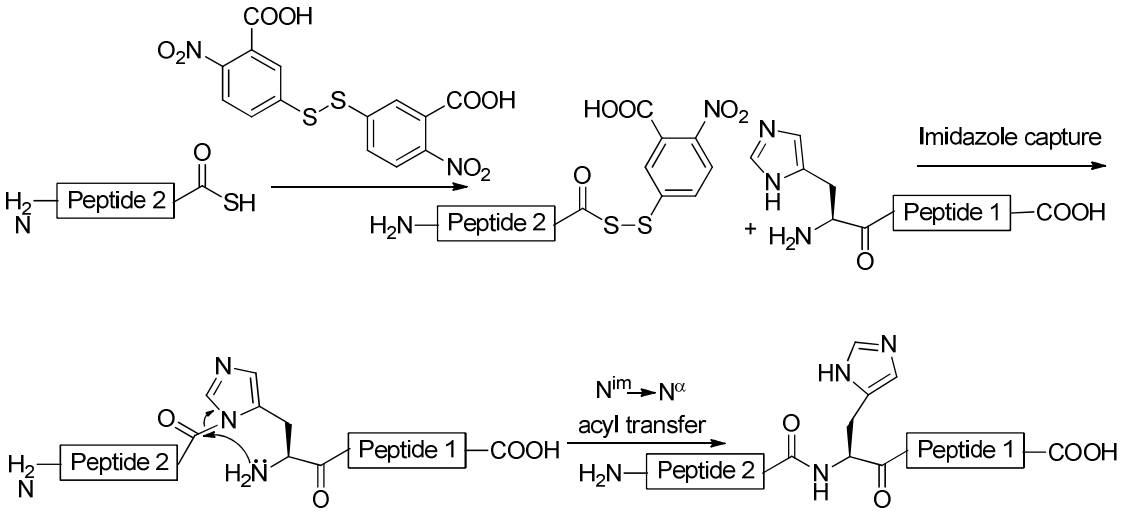


Scheme 13. NCL at serine

1.5 Other Ligation Methods

1.5.1 His Ligation

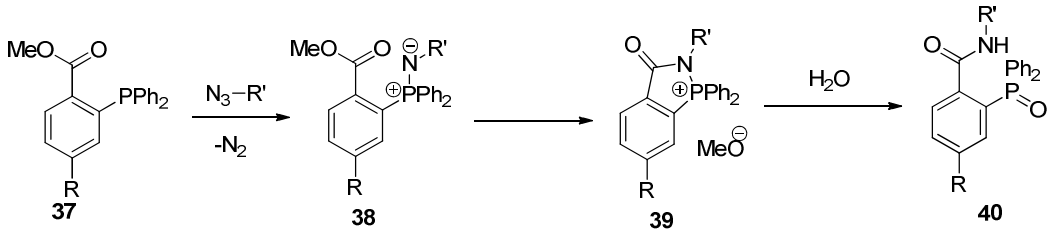
In another modification designed to overcome the drawback of native chemical ligation, homocysteine,[45,47] selenocysteine,[51-53] homoselenocysteine[54] and histidine[55] were used at the N-terminal position as replacements for cysteine. In the case of histidine located at the N-terminus of a peptide, the imidazole side chain nitrogen acts as a nucleophile in the ligation reaction (Scheme 14).[1,55]



Scheme 14. Histidine-mediated ligation of peptidiyl thioacids

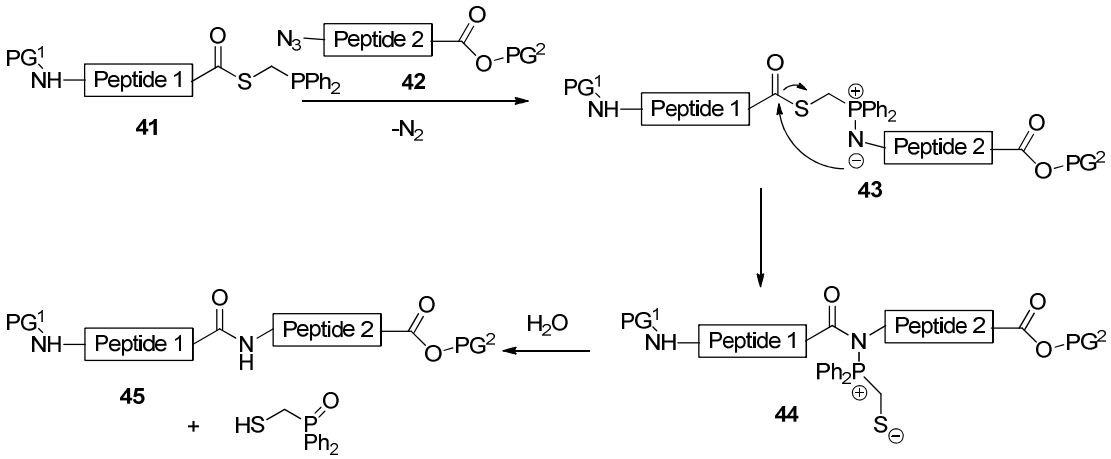
1.5.2 The Staudinger Ligation

An elegant way to overcome the various limitations of the original native chemical ligation was developed independently by two research groups: those of Raines[56-60] and Bertozzi[61,62] and is based on the Staudinger reaction.[1,63] The Staudinger reaction, first reported in 1919, is the reaction of a phosphine **37** with an azide to produce an iminophosphorane **38**. This iminophosphorane intermediate **38** can then be trapped by different electrophiles. In the initial Staudinger Ligation approach, the iminophosphorane intermediate **38** was intramolecularly trapped by an ester leading to an amidophosphonium salt **39**. The amidophosphonium salt **39** was then hydrolyzed to produce an amide bond attached to a phosphine oxide moiety **40** (Scheme 15).[62]



Scheme 15. Staudinger ligation

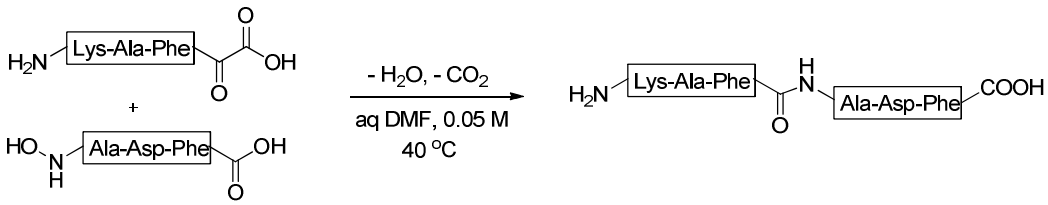
A modification of the Staudinger ligation, in which an amide bond is formed between the two coupling partners to give a product without a triarylphosphane oxide moiety, appears even more attractive. Shortly after their first report, Bertozzi *et al.*[64] and Raines *et al.*[56,57,65] reported such traceless Staudinger ligation, in which the phosphine oxide moiety is cleaved during the hydrolysis step. This reaction can be applied to coupling of two peptide fragments, one bearing a C-terminal phosphinothioester group **41** and another N-terminal azido group **42**. In the first step of this Staudinger ligation, the phosphinothioester **41** reacts with an azide **42** to give an iminophosphorane **43**, which then undergoes an intramolecular S→N acyl shift leading to an amidophosphonium salt **44**. The amidophosphonium salt **44** is then hydrolyzed to produce the amide product **45** and a phosphine oxide (Scheme 16). However, the high reactivity of the aza-ylide does not permit the presence of unprotected side-chain functionalities and therefore the Staudinger ligation is limited to the coupling of fully protected peptide fragments. Owing to the obligatory use of the azide, this ligation is restricted to the formation of secondary amides, furthermore its success depends critically on the choice of the appropriate phosphinothiol.[66]



Scheme 16. Traceless Staudinger ligation

1.5.3 Chemoselective Decarboxylative Amide Ligation

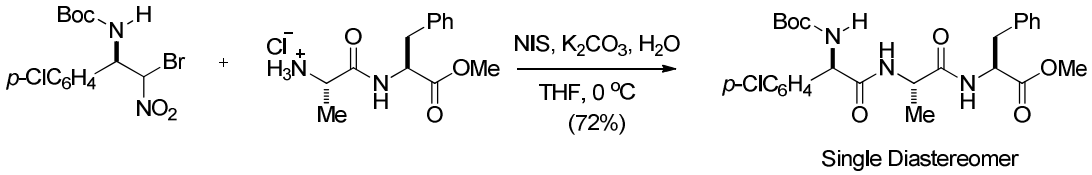
A very promising methodology developed by Bode et al. for the formation of amide bond employs a chemoselective decarboxylative condensation of *N*-alkylhydroxylamines with α-ketoacids.[67,68] This reaction does not involve an addition–elimination reaction of an activated carboxylic acid derivative unlike most other amide bond forming strategies. The reaction proceeds at 40 ^{o}C in polar solvents in good yields without epimerization of the α-ketoacids. Most importantly, peptides containing unprotected Lys and Asp as well as Trp, Tyr, and Arg residues can be ligated as demonstrated in the synthesis of a hexapeptide (Scheme 17).[67] The major limitation of this method, however, is the need to synthesize both peptidyl α-ketoacids and N-hydroxypeptides.



Scheme 17. Decarboxylative amide condensation

1.5.4 Non-conventional Peptide Synthesis

Most recently, Johnston et al. have discovered a non-conventional route for the synthesis of native amide bond via iodonium-promoted nitroalkane-amine coupling (Scheme 18).[69] This method is an uncommon type of reaction wherein the inherent polarity of the acyl donor and amine are reversed (umpolung) from the conventional one. This strategy establishes a novel alternative in peptide synthesis to the long-standing reliance on the activated electrophilic carboxylic acid feedstock.



Scheme 18. Umpolung reactivity in peptide synthesis

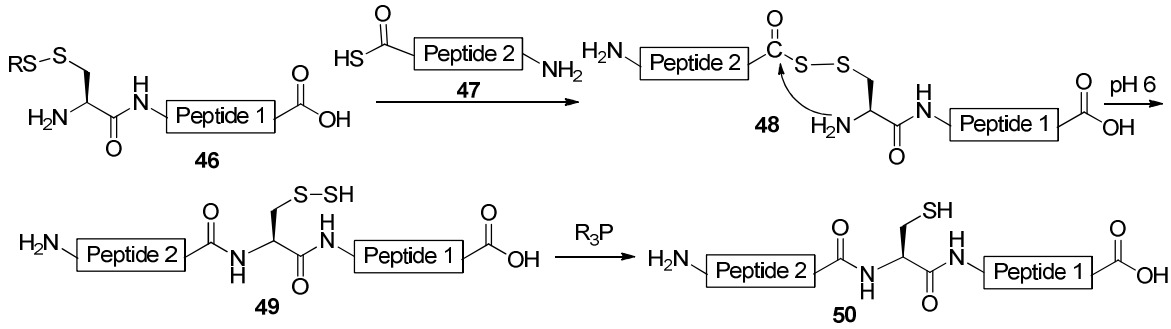
1.6 Thioacid Chemistry

Thioacids present a similar degree of nucleophilicity and selectivity to thiols, but have the additional advantage of incorporating an electrophilic carbonyl group capable of undergoing a number of valuable transformations.[70,71]

1.6.1 Cysteine Acyl Hydrosulfide Strategy

In an alternative approach to peptide ligation at a cysteine junction, the capture of an acyl hydrosulfide derivative was performed at lower pH (Scheme 19). The first step of this sequence involves nucleophilic attack of the thiocarboxylic acid **47** on a disulfide **46** to form a Cys-based acyl hydrosulfide **48**, which then undergoes S→N acyl transfer to give the side chain hydrosulfide **49**

that can be reduced to the thiol **50** to generate the cysteine at ligation junction.[72,73] This methodology takes the advantage of the strong nucleophilicity of the thiocarboxylic acid and can be performed at a pH <2.

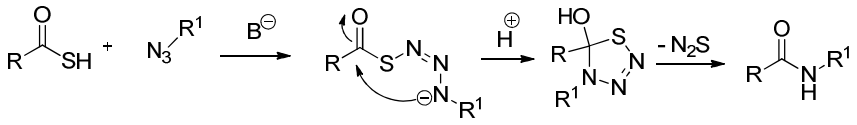


Scheme 19. Thioacid ligation at cysteine junction

1.6.2 Thioacid/Azide Amidation

The reaction of thioacids with azides has recently been developed by the Williams and Liskamp groups as an efficient ligation strategy.[74-77] In contrast to the conventional methods for the chemical synthesis of amides, this method does not need active esters and amines as precursors. Moreover, the application of this methodology for the preparation of several classes of complex amides in nonpolar and polar solvents including water has also been reported (Scheme 20).[77]

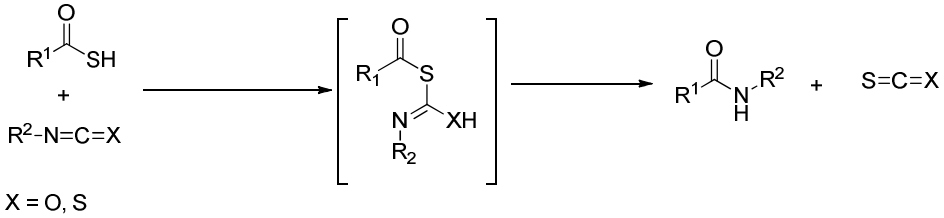
However, these excellent azide-based coupling methods are not without limitations, among which the need to prepare azides and the consequent obligatory restriction to the formation of secondary amides are the most important.



Scheme 20. Coupling of thioacid with azide

1.6.3 Coupling of Thioacids with Isocyanates and Isothiocyanates

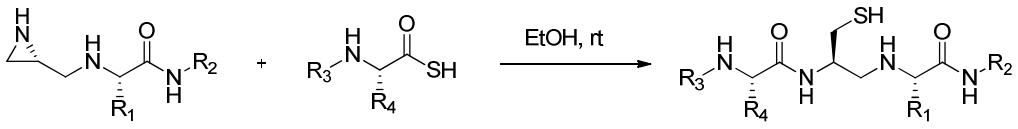
Crich and Sasaki revealed a new methodology based on the reaction of thioacids with more widely available isocyanates and isothiocyanates, which results in the formation of amide bonds (Scheme 21).[78] However, like the azide chemistry described above, this method only permits the formation of secondary amides and requires the synthesis of the electrophilic partner.



Scheme 21. Reaction of thioacids with isocyanates ans isothiocyanates

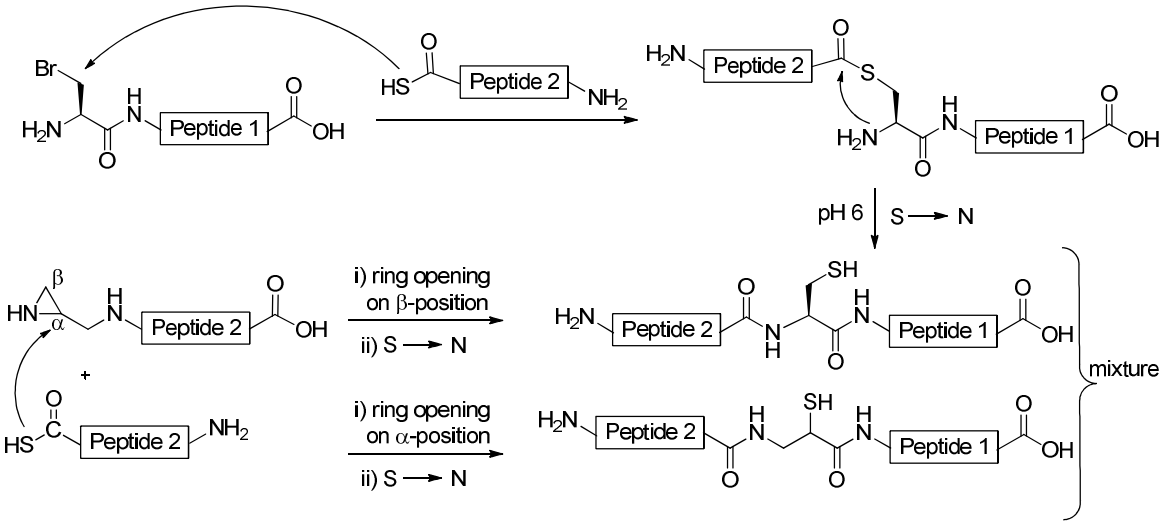
1.6.4 Thioacid/Aziridine Ligation

Recently, Yudin and co-workers have developed a ligation in which peptidyl thioacids react chemoslectively with NH aziridine terminated amino acids and peptides to incorporate a peptidomimetic linkage at the ligation site (Scheme 22).[79]



Scheme 22. Peptidomimetic ligation

The reaction between thioacids and aziridines was first observed by Tam et al. when they performed a chemo-selective reaction between a C-terminal thioacid and an electrophilic β-bromoalanine residue located at the N-terminus of a peptide.[80] At pH 5 or lower, this reaction resulted in the formation of a Cys-thioester which underwent S→N acyl shift to form a native amide bond. However, at pH 6 or higher, the β-bromoalanine residue suffered 1,3-elimination of HBr, which resulted in the formation of an aziridine. Subsequent ring opening of the aziridine by the thioacid led to the formation of an undesired side product (Scheme 23).

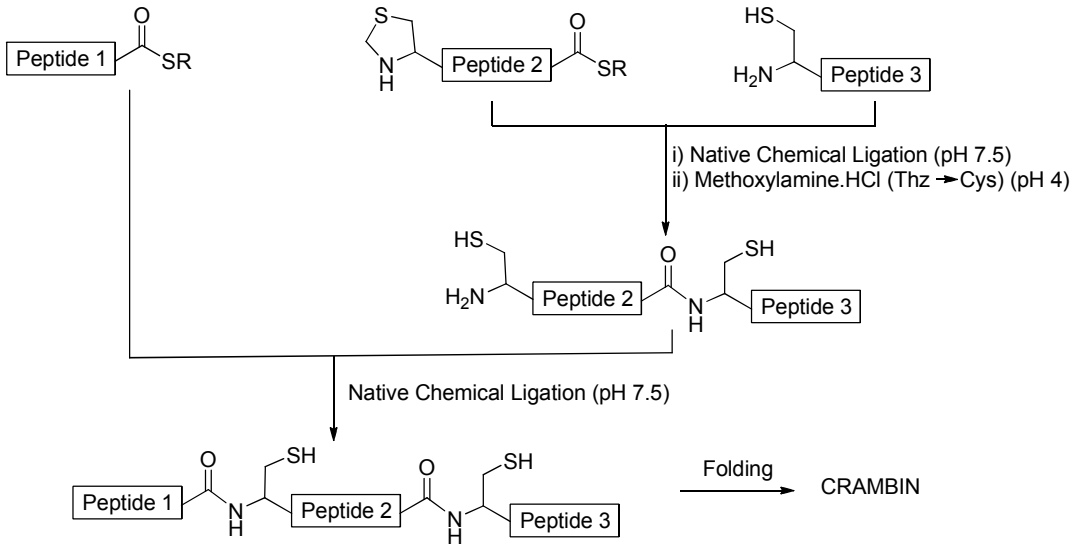


Scheme 23. Thioacid ligation resulting in native peptide bonds

Although, the above chemical ligations have proven to be very useful for peptide coupling, they are restricted to some extent because of the requirement of a cysteine or cysteine type auxiliaries at the ligation site in NCL, and the need to prepare an N-terminal azide or related group in others. Therefore, there is still a need for a general method to perform fast epimerization free ligation with any amino acid at the ligation site.[81-84]

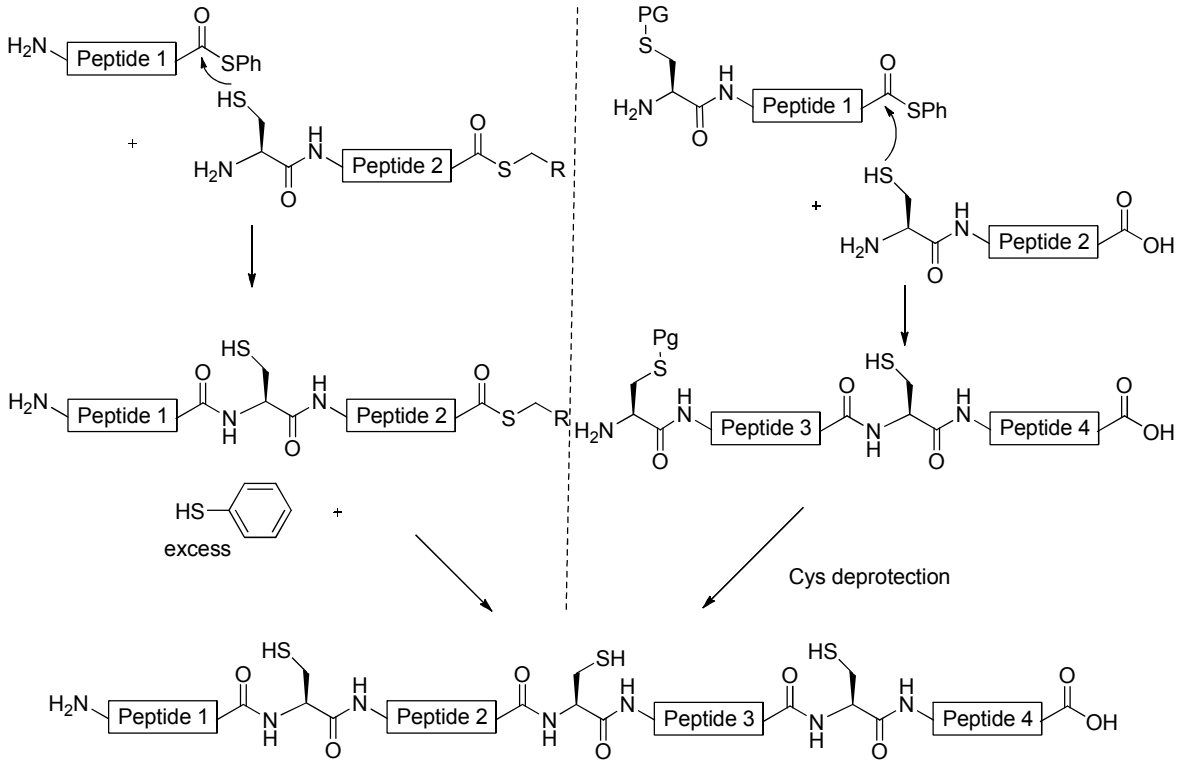
1.7 Convergent Strategies for Multiple Fragment Ligations

All of the strategies presented above require a stepwise congregation of the individual fragments from the C to the N terminus. However, the convergent assembly of multiple fragments in parallel represents a more efficient way for generating whole synthetic proteins.[85,86] Such a convergent synthetic approach making repeated use of the native chemical ligation strategy, would require double protection of a central fragment at the N-terminal Cys as well as at the C-terminal thioester. With this in mind, Kent and co-workers developed a one-pot strategy wherein the N-terminal cysteine of the thioester peptide for the first ligation step was protected as in the form of a thiazolidine group.[87] Once the first reaction reached completion, the N-terminal thiazolidine was converted to a cysteine residue with 0.2 M methoxylamine (pH 4). Subsequently, the pH was readjusted to pH 7 and the next peptidyl thioester was introduced for the second ligation. This methodology was successfully applied in the one-pot synthesis of a 46 amino acid model protein, crambin (Scheme 24).[87,88]



Scheme 24. One pot synthesis of crambin

Kent and co-workers also introduced an alternative strategy, called the kinetically controlled ligation[86,89] in which they exploited the different reactivities of aryl and alkyl thioesters (Scheme 25). Alkyl thioesters are less reactive than and do not compete with aryl thioesters in ligation reactions.[90] They can even tolerate unprotected N-terminal Cys residues without suffering significant amounts of self-ligation. The feasibility of this approach has been demonstrated for in a convergent synthesis of crambin[87] and of lysozyme[91] using judicious Cys(Acm) protection to avoid thiolactone formation.



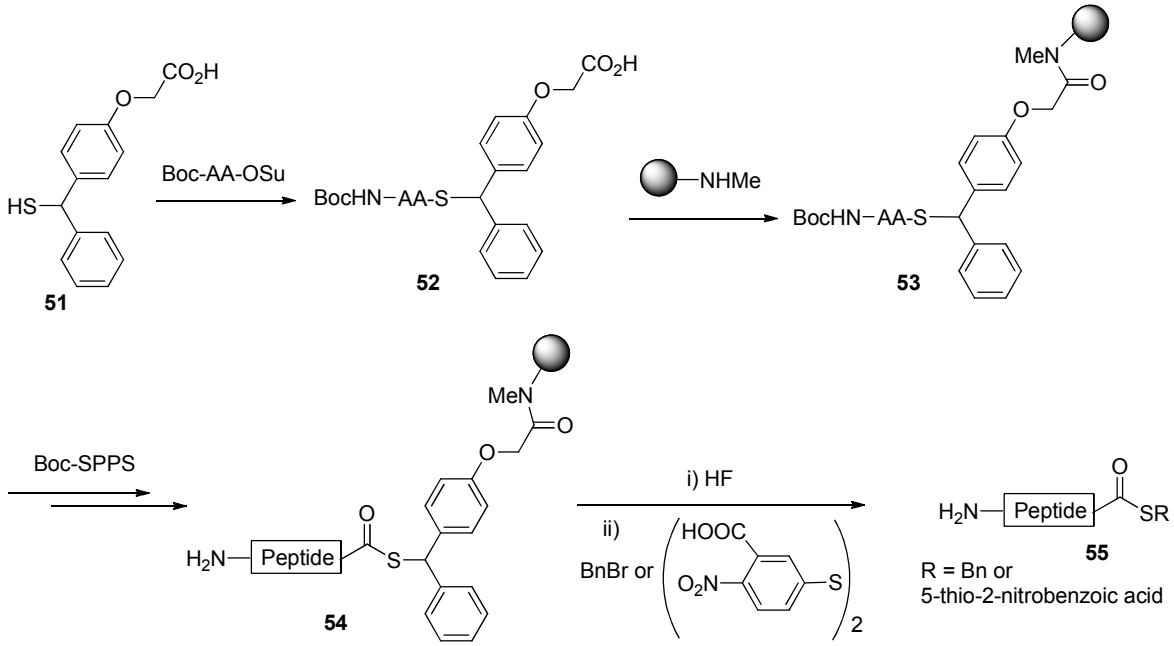
Scheme 25. Kinetically controlled ligation

1.8 Synthesis of C-Terminal Peptidyl Thioesters

Unprotected peptides bearing a C-terminal thioester group are key intermediates in the synthesis and modification of proteins by chemical ligation and its variants.[2,24,85,86] However, synthetic access to unprotected peptidyl thioesters is still a significant bottleneck in this ligation chemistry. Several approaches have therefore been investigateded for their preparation using Boc as well as Fmoc-SPPS strategies.[86]

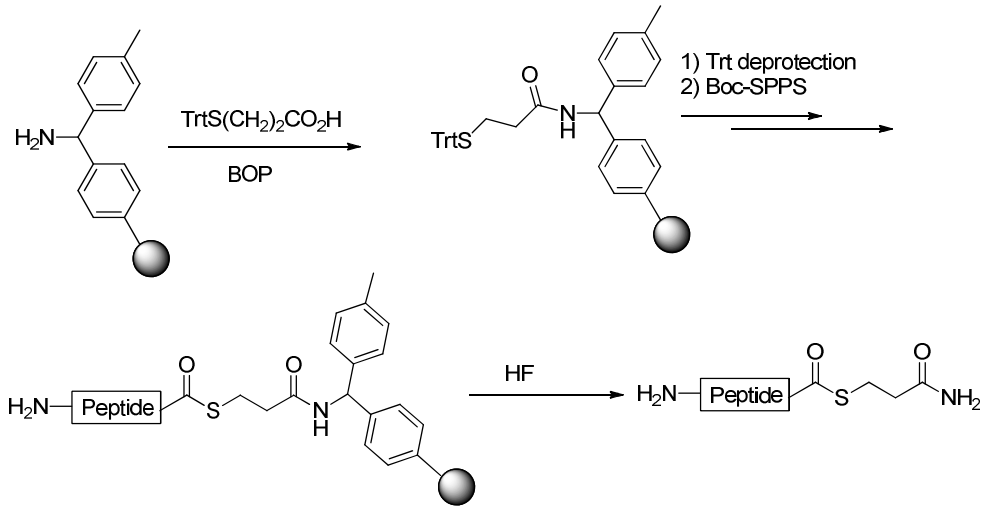
1.8.1 Synthesis of C-Terminal Peptidyl Thioesters on Solid Support using Boc Chemistry

The lability of the carbonyl-sulfur bond in thioesters to the repeated exposure of the nucleophilic base piperidine in standard Fmoc-SPPS protocols led to an initial reliance on Boc-SPPS chemistry for the synthesis of peptidyl thioesters. The first report for the synthesis of unprotected peptidyl thioacid was communicated by Blake *et al*., who employed a preformed benzhydryl thioester linker 4-[α-(Boc–Gly–*S*)benzyl]phenylacetic acid.[92,93] Subsequently, several approaches have flourished based on the use of a modified linker, 2-[4-(mercapto(phenyl)methyl)phenoxy]acetic acid **51**,[94-96] which was derivatized by the first amino acid before hooking it to an aminomethyl polystyrene resin. Standard Boc-SPPS[97] was then applied to the preparation of the desired peptide sequence **54**. Lastly, cleavage of the peptide from the resin with HF led to the formation of the peptidyl thioacid,[95] which was then converted into the thioester **55** in solution phase by the nucleophilic reaction of the thioacid with an alkyl halide such as benzyl bromide or a symmetrical disulfide such as 5, 5'-dithiobis(2-nitrobenzoic acid) as shown in Scheme 26.[16,73,98]



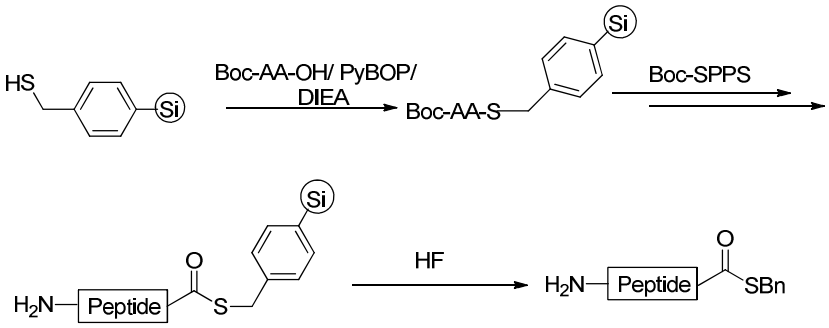
Scheme 26. Dawson's protocol for the synthesis of peptidyl thioesters

Partially protected peptidyl *S*-alkyl thioesters were first prepared by Aimoto *et al*. using the 3-sulfanylpropanoic acid linker[99] or its *tert*-butylsulfanyl derivative,[100] which were coupled to a *p*-methylbenzhydrylamine resin followed by the elongation of peptide chain by Boc-SPPS. In a similar vein, a third protocol for SPPS containing a C-terminal thioester using the Boc strategy was revealed by Tam and co-workers.[80,101,102] This method was based on the usage of a MBHA resin, which was first loaded with *S*-trityl mercaptopropionic acid. After the removal of the trityl-protecting group, the desired polypeptide chain was assembled using standard Boc strategy. Finally, HF cleavage afforded the unprotected peptidyl thioester without the need for subsequent transformations, as are required in the Dawson method (Scheme 27).[101]



Scheme 27. Tam's protocol for the preparation of *C*-terminal thioesters

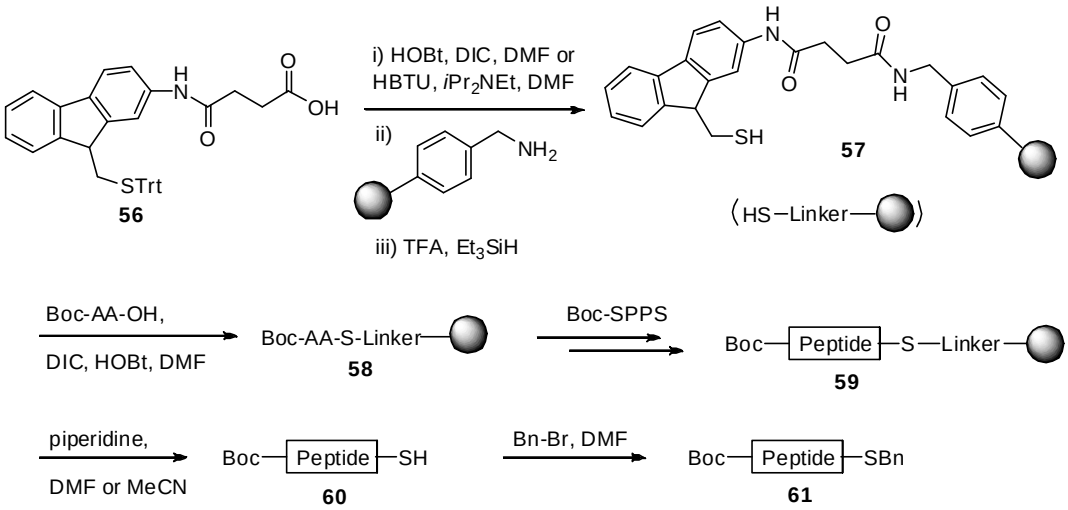
Recently, Yangmei *et al*. described an efficient synthesis of a fully deprotected peptide *S*-benzyl thioester using a volatilizable mercaptomethylphenyl-functionalized silica support.[103] The first amino acid was coupled with the linker followed by the elongation of peptide chain by standard Boc chemistry. Subsequently, cleavage of the peptide from the resin with HF led to the formation of the peptidyl thioester (Scheme 28).[103]



Scheme 28. Synthesis of peptidyl *S*-benzyl thioester on silica support.

Despite the considerable ingenuity showcased in the development of the above methods, none combine the directness that obviously results from the use of a simple C-terminal thioester-based linker with a method for release from the

resin that avoids the use of hazardous HF.[104,105] With this in mind, Crich and Sana introduced the 9-fluorenylmethyl thioester linker **56**,[106] which was fully compatible with the general conditions of Boc-SPPS peptide assembly. Treatment with piperidine, which released the Boc-protected peptide into solution, resulted in the formation of a C-terminal thioacid **60**. This latter could be readily transformed into a thioester **61** by simple alkylation (Scheme 29). This method employs conditions no more forcing than those encountered in standard Boc-SPPS protocols and circumvents the terminal HF treatment that limits most Boc chemistry SPPS methods.



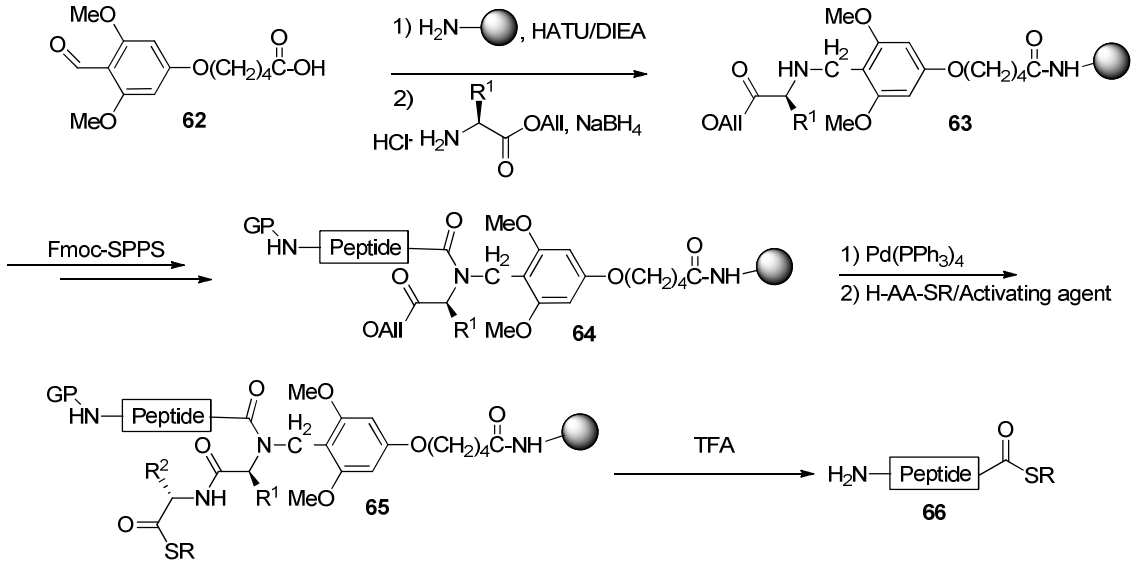
Scheme 29. Thioester synthesis using 9-fluorenylmethyl thioester linker

1.8.2 Synthesis of C-Terminal Peptidyl Thioesters on a Solid Support using Fmoc Chemistry

Despite being a high yielding method, the Boc chemistry SPPS described above requires repeated exposure of the peptide to TFA and typically cleavage/deprotection with HF. These conditions are incompatible with glycoconjugates as they could damage glycosidic linkages. For this reason

glycopeptides and phosphopeptides are constructed primarily using Fmoc based chemistry, which utilizes a mild base (usually piperidine) during synthesis. Since thioesters are sensitive to piperidine, which is required to remove the widely used Fmoc protecting group in Fmoc-SPPS methodology, other non-nucleophilic cleavage cocktails have been explored for the removal of the N-α-Fmoc group. These include the replacement of piperidine by 1-methylpyrrolidine/hexamethylenimine/HOBT or DBU/HOBT mixtures. It has been found, however, that these methods tend to epimerize the stereogenic center present at the thioester position.[107,108]

In 1999, Barany *et al.* revealed the synthesis of peptidyl thioesters following Fmoc-SPPS with BAL strategy, in which the growing peptide was anchored through backbone nitrogen instead of a terminal C^{α}-carboxyl group.[109-111] In the BAL strategy, an acid labile 4-[formyl-3,5-dimethoxyphenoxy]butyric acid-based handle **62** was linked with an amino-functionalized PEG-resin. This was followed by on-resin reductive amination with an allyl ester of amino acid hydrochloride salt and the target peptide was assembled by standard Fmoc chemistry. Selective removal of the C-terminal allyl ester followed by coupling of an amino acid thioester led to the backbone amide anchored C-terminal peptidyl thioester **65**, which was cleaved using TFA to obtain the peptidyl thioester **66** (Scheme 30).[109]

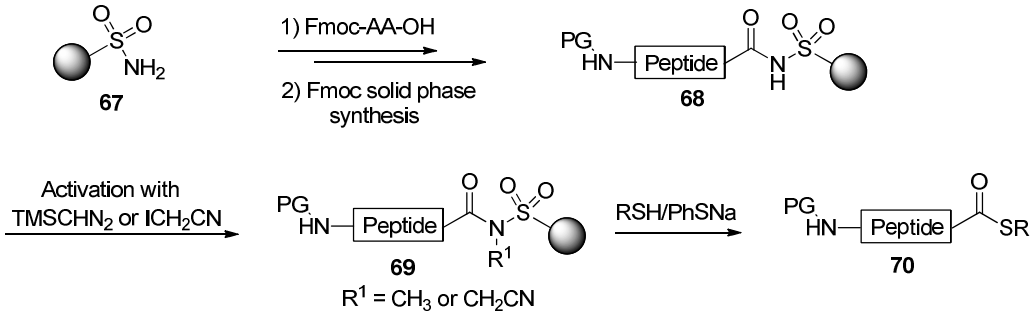


Scheme 30. Preparation of peptidyl thioesters using the BAL approach

Although, this approach was successful in many cases, its scope is limited because of the special precautions required in the coupling of second amino acid to prevent diketopiperazine formation. Another major drawback of this method is the need for careful control in the activation of backbone peptide carboxylic acid to avoid epimerization during introduction of thioester to the C-terminal end of the peptide chain.[112,113] To circumvent these problems, numerous other approaches have been developed using Fmoc chemistry. Most of these were based on the activation of the C-terminal amino acid followed by thiolysis to generate the peptidyl thioester.

In this regard, Ingenito *et al.*[114] developed the use of an acylsulfonamide 'safety-catch linker' **67** for SPPS containing a C-terminal thioester by the Fmoc strategy. This linker, which is stable to both strongly basic and acidic conditions, was first introduced by Kenner *et al.*[115] and later modified by Backes and Ellman.[116] The peptide was assembled using the standard Fmoc protocol to afford solid-phase bound peptides. After the final peptide coupling, the resin was

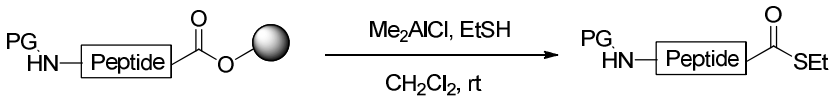
activated by treatment with diazomethane or iodoacetonitrile to yield the secondary sulfonamide **69**. The peptide was then released from the activated resin by nucleophilic displacement involving a thiol group to yield the peptidyl thioester **70** (Scheme 31).[116]



Scheme 31. Synthesis of a peptidyl thioester on Safety-Catch linker

The major drawbacks of this method are low yields of isolated peptidyl thioesters and an undesirable alkylation of unprotected hydroxyl groups on an attached carbohydrate as well as of cysteine and methionine residues.

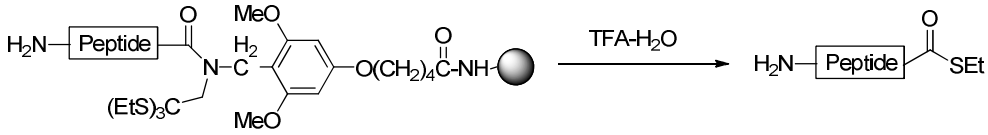
Another strategy[117,118] introduced by Sewing and Hilvert employed a PAM or HMBA resin for the elongation of the peptide chain by Fmoc-SPPS. The cleavage was performed by activation of the ester linkage with $AlMe_2Cl$ in the presence of a large excess of a nucleophilic thiol to yield peptidyl thioester (Scheme 32).[118]



Scheme 32. Lewis acid activation strategy

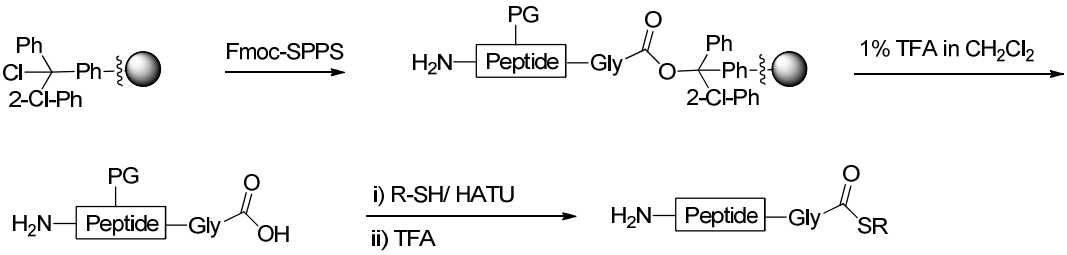
In this method, Sewing and Hilvert observed that treatment with a large excess of oxophilic reagent favored the formation of trithioortho esters and ketone dithioacetals, which on acidic workup yielded peptidyl thioesters, but had

the propensity to racemize at the C-terminal amino acid. However, the need to expose the whole assembled peptide to harsh reagents caused side-reactions to varying degrees, including conversion of side-chain ester groups to their thioesters and the formation of aspartimide units. In this regard, Albericio *et al.* reported a modification of the BAL approach to the synthesis of peptidyl thioesters, wherein they linked a masked thioester, i.e., a trithioortho ester, with a 4-[formyl-3,5-dimethoxyphenoxy]butyric acid handle and assembled the peptide using Fmoc chemistry (Scheme 33).[119] Although the concept of the masked thioester in this strategy can solve the problem of formation of diketopiperazines, its practical utility at present is restricted mainly to the preparation of glycine thioester peptides.



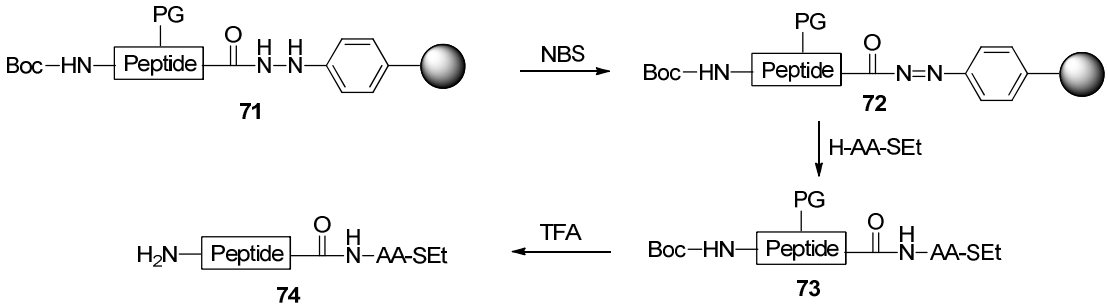
Scheme 33. Synthesis of peptidiyl thioesters by masking of thioesters

Another common approach for the synthesis of thioesters employs a highly acid-sensitive trityl[120] or 2-chlorotrityl resin,[121] which allow cleavage under mild acidic conditions to yield protected peptides.[86] The thioester is introduced by C-terminal activation of the protected peptide in solution after cleavage from the resin and subsequent coupling with a thiol. Global deprotection finally affords an unprotected peptidyl thioester. However, the high risk of racemizing the C-terminal residue by the activation limits this approach to C-terminal Gly residues (Scheme 34).[86]



Scheme 34. Coupling of *C*-terminal acid with thiol

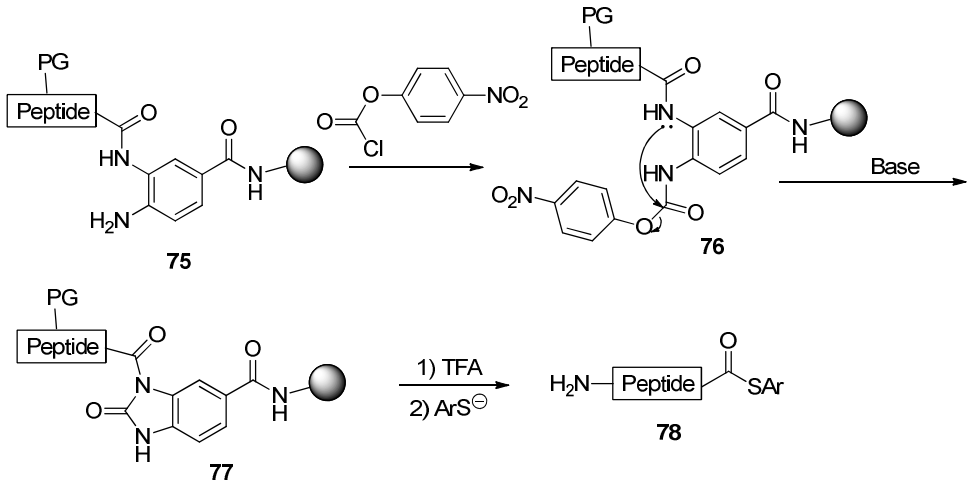
Another protocol for the synthesis of thioesters by Fmoc-SPPS and activation of linker was revealed by Camarero *et al.*[122] who used an aryl hydrazine support **71**, which was completely stable to the conditions of Fmoc- and Boc-SPPS. At the end of the synthesis, the fully protected peptide resin was activated by oxidation with *N*-bromosuccinimide to yield a peptide diazene **72**. The reactive acyl diazene was then cleaved with an α-amino acid *S*-alkyl thioester followed by treatment with TFA to give the peptidyl thioesters **74** (Scheme 35).[122]



Scheme 35. Fmoc-SPPS using an aryl hydrazine support

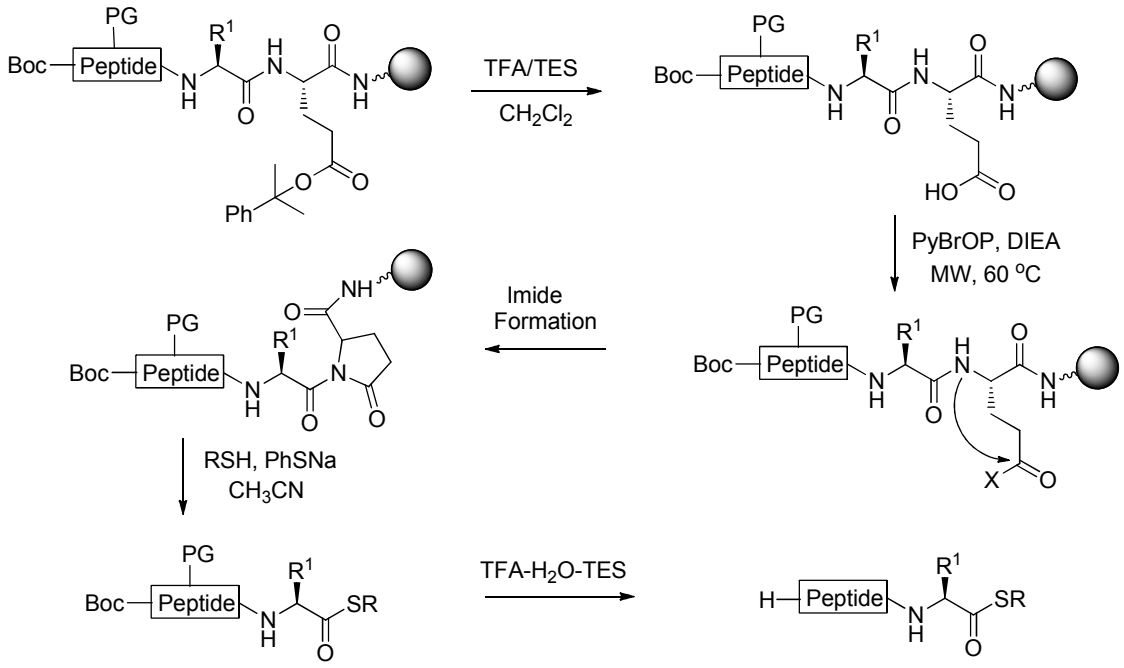
Another elegant way to synthesize petidyl thioesters based on activation of the linker by the formation of active C-terminal aromatic *N*-acylurea functionality was introduced by Dawson and co-workers.[123] The C-terminal carboxylic group was attached to the linker 3,4-diamino benzoic acid, which was first coupled with PEG amine, and then the peptide chain was elongated using

Fmoc chemistry. The resulting peptide was efficiently transformed into an aromatic *N*-acylurea moiety **77**, followed by deprotection and thiolysis to yield peptidyl thioester **78** (Scheme 36).[123]



Scheme 36. Synthesis of peptidiyl thioesters through an *N*-acylurea moiety

Recently, Tofteng et al. communicated a new concept entitled “backbone amide activation” and demonstrated its use for efficient synthesis of peptidyl thioesters.[124] The method relies on a simple glutamic acid linker system, activation of which renders the C─N bond susceptible to thiolysis and provides the formation of peptide thioesters (Scheme 37).

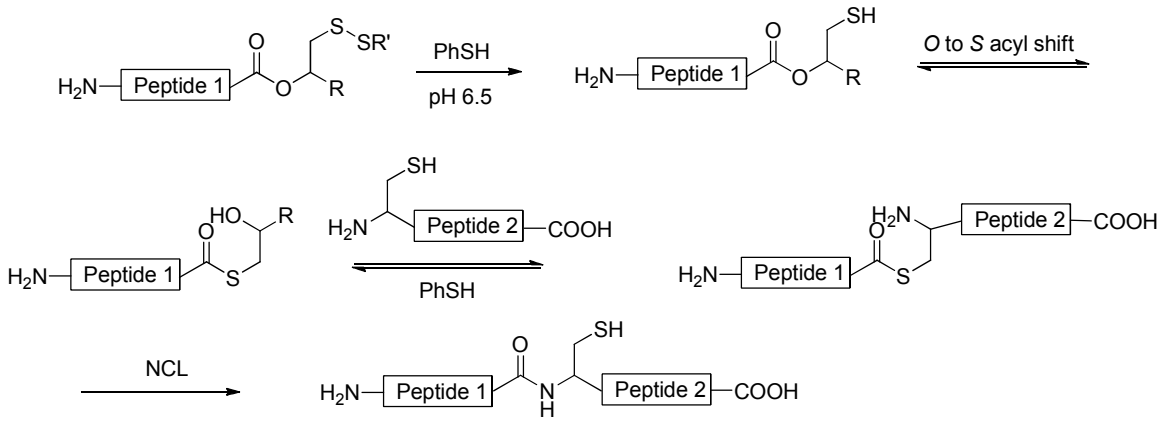


Scheme 37. Thioester synthesis using a pyroglutamyl imide moiety

Although, methods based on the activation of a linker followed by thiolysis leading to the synthesis of peptidyl thioesters have had noticeable success, they also have disadvantages, most pertinently the risk of epimerization on introduction of the thioester to the active C-terminal end of the peptide chain, and the need for additional steps after completion of peptide assembly on the linker.

1.8.2.1 *O*→*S* Acyl Transfer

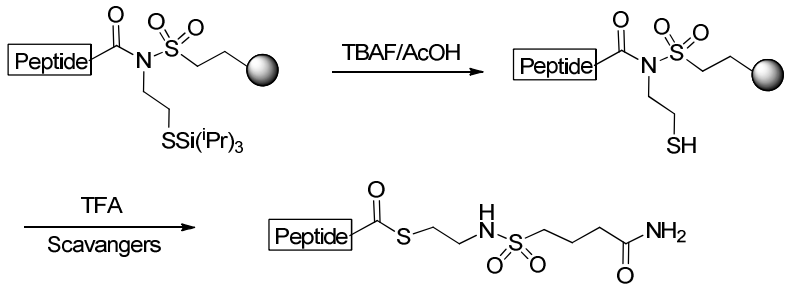
Botti *et al.,*[125] and Danishefsky *et al.*[126] individually developed a novel methodology based on the in situ *O*→*S* acyl shift, which led to the formation of a thioester (Scheme 38).[125] Recently, this *O*→*S* acyl shift protocol[127] was successfully applied by Muir *et al.* to the synthesis a number of naturally occurring cyclic peptidyl thioesters i.e., peptidyl thiolactones.



Scheme 38. In situ formation of thioesters through *O*→*S* acyl shift

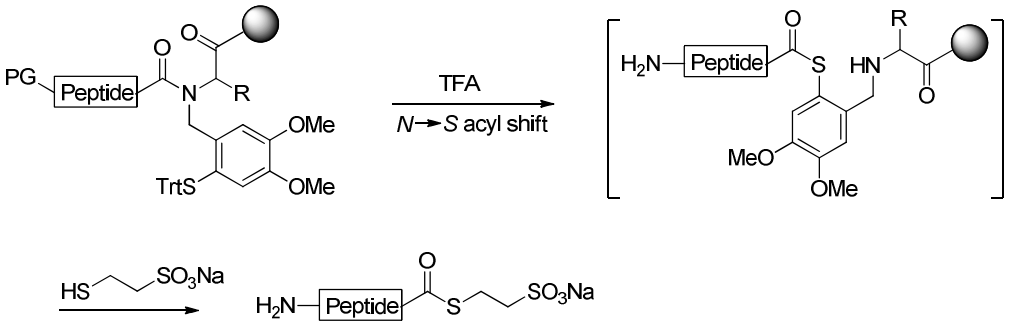
1.8.2.2 N→S Acyl Shift

A second protocol for the synthesis of peptidyl thioesters by Fmoc-SPPS is based on the in situ *N*→*S* acyl shift reaction.[128-134] To this end, Melnyk and co-workers discovered a solid-phase N→S acyl transfer for thioester synthesis in combination with the sulfonamide safety-catch linker, which has been a widely used platform for thioester synthesis.[129] The acylsulfonamide group of the linker underwent an additional Mitsunobu alkylation with a mercaptoethanol derivative, which allowed the introduction of the prerequisite β-amino thiol component that facilitated the subsequent intramolecular N→S acyl shift (Scheme 39). This approach to thioester synthesis is highly dependent on the degree of alkylation.



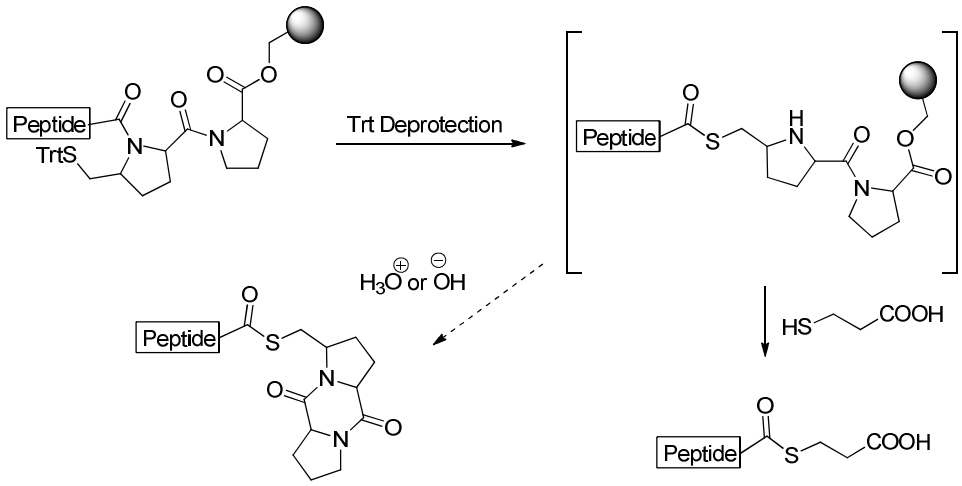
Scheme 39. *N*→*S* acyl shift using the sulfonamide-linker

In another variation, Aimoto *et al.* developed a modified 4,5-dimethoxy-2-mercaptobenzyl protecting group to mediate peptidyl thioester formation via an *N*→*S* acyl shift as shown in Scheme 40.[128]



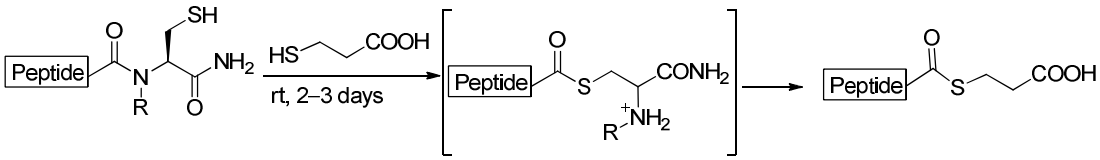
Scheme 40. Thioester synthesis using a solid supported DMMB auxiliary

Hojo and co-workers described a post-peptide chain assembly thioesterification method[131] in which a peptide bearing a C-terminal mercaptomethylated proline derivative was converted into a thioester upon treatment with aqueous 3-mercaptopropionic acid (Scheme 41). A stated drawback of this method was the lengthy preparation of the initial 5-mercaptomethyl proline to be incorporated at the C-terminus of the peptide.



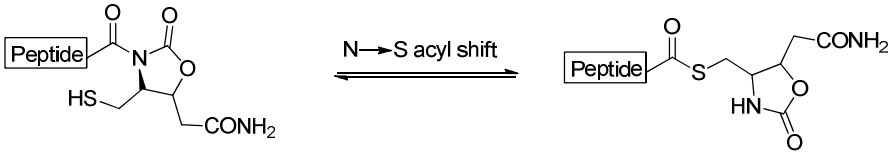
Scheme 41. Thioester through a mercapto-prolyl-prolyl ester motif

Having accredited the N→S acyl shift to the presence of the amino acid proline, Hojo and co-workers diverted their attention to the use of other amino acid residues that might promote thioesterification. For this purpose, N-alkylation of a C-terminal cysteine residue was devised as an alternative to facilitate the intramolecular N→S acyl migration under acidic conditions,[135] followed by the intermolecular trans-thioesterification with MPA to afford the peptide thioester (Scheme 42).



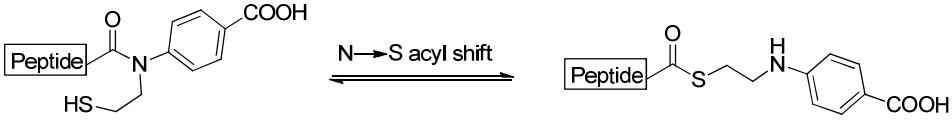
Scheme 42. *N*-Alkyl cysteine-assisted thioesterification

Otaka and co-workers have shown that a modified C-terminal Cys residue, incorporated in the framework of an acyl oxazolidinone, can undergo N→S acyl transfer to yield peptidyl thioesters (Scheme 43).[130]



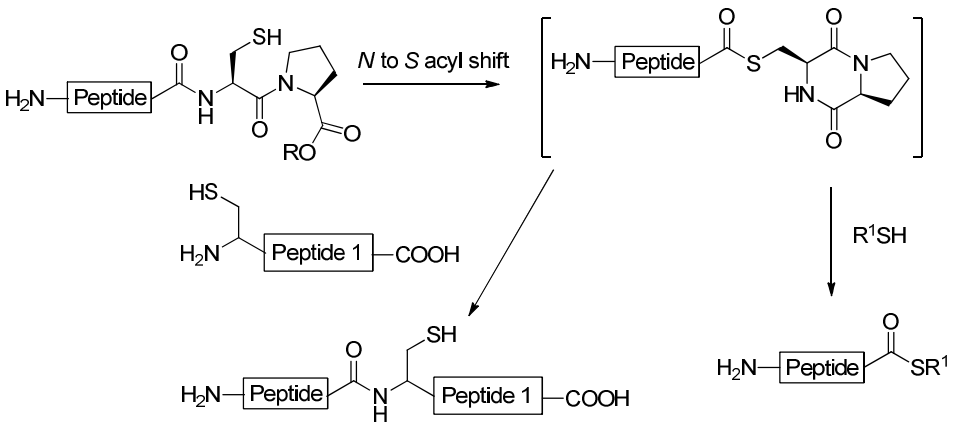
Scheme 43. Thioesterification through *N*-acyloxazolidinones

In another modification, Otaka et al. developed a different device to facilitate intramolecular acyl shift for peptidyl thioester synthesis using an *N*-substituted aniline linker (Scheme 44).[136]



Scheme 44. *N*-Substituted aniline linker mediated thioesterification

Recently, Kawakami and Aimoto proposed that a peptide containing cysteinyl prolyl ester moiety at the C-terminus could spontaneously transform into a diketopiperazine thioester via an intramolecular *N* to *S* acyl shift reaction.[133] Moreover, the CPE peptide could be ligated with a Cys-peptide in a one-pot procedure and could also be transformed into a peptide thioesters by intermolecular thiol–thioester exchange with external thiols (Scheme 45).[133]



Scheme 45. CPE mediated thioesterification/ligation

Despite these notable advances, the synthesis of peptidyl thioesters by Fmoc- SPPS remains significantly more challenging than the synthesis of the corresponding peptidyl acid or amide. Therefore, there is still a need for a direct Fmoc synthesis of peptidyl thioesters.

1.9 Goals of This Thesis

The work described in this thesis was undertaken with two different goals in mind. First, the development of new and alternative methods for peptide synthesis and second, the synthesis of peptidyl thioesters on the solid phase by Fmoc chemistry are presented. Chapter 2 describes the investigations conducted towards the development of a new chemistry for epimerization-free block

synthesis of peptides from thioacids and amines with reagents such as Sanger's and Mukaiyama's reagents. In Chapter 3, studies carried out to probe the reactivity of *N*-terminal sulfonamide towards peptidyl thioacids are described. The goal of these studies was to explore convergent strategies for multiple peptide fragment ligation by tuning the reactivity of peptidyl thioacids and sulfonamides. Continuing this theme studies were undertaken with a goal of developing direct method for the efficient synthesis of peptidyl thioesters by the widely used Fmoc-SPPS chemistry and the results of this work are presented in chapter 4.

CHAPTER 2

BLOCK SYNTHESIS OF PEPTIDES FROM THIOACIDS AND AMINES WITH THE SANGER AND MUKAIYAMA REAGENTS

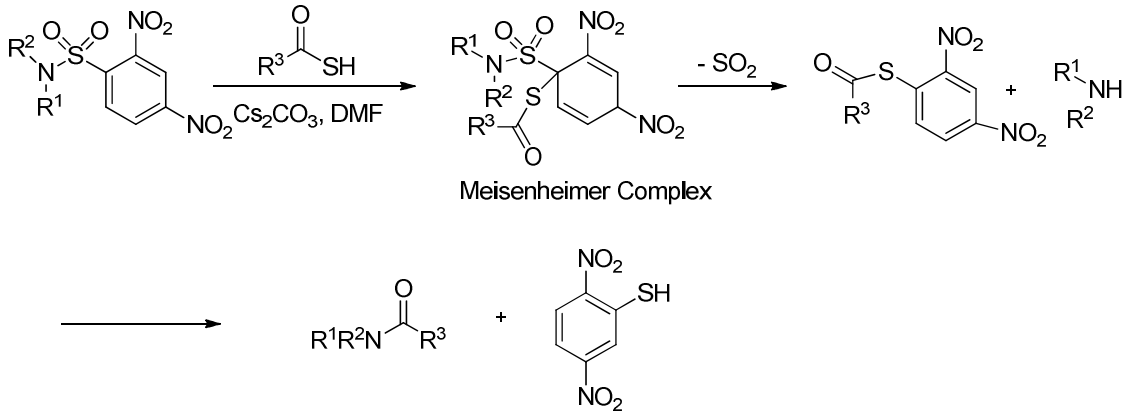
2.1 Introduction

As discussed in chapter 1, a number of elegant approaches have been investigated to meet the challenges present in the synthesis of peptides and proteins; however, these are not without limitations. In this context, the conception and development of a new method for peptide bond formation is outlined in this chapter.

2.2 Amide Bond Formation by the Sanger's Reagent

In 1998, Tomkinson and co-workers described the reaction of 2,4-dinitrobenzenesulfonamides with thioacids leading to the formation of amides at ambient temperature.[137,138] The Crich group extended this reaction, combining amino thioacids and their C-terminal peptide congeners with *N*-terminal sulfonamides, leading to the synthesis of peptides and their glycoconjugates.[139] The Crich group further employed this mild and efficient process to capture thioacids generated *in situ* from nucleophilic ring-opening of a variety of monothio cyclic anhydrides resulting in a useful three-component coupling process.[140] In the peptide-bond forming adaptation of this chemistry, the incompatibility of an unprotected lysine side chain amine, which underwent competing amide bond formation,[139] revealed the mechanism to involve nucleophilic aromatic substitution of the thiocarboxylate on the electron-deficient sulfonamide with release of a highly reactive *S*-(2,4-dinitrophenyl) thioester. This is in contrast to

the alternative mechanism of intramolecular amide bond formation at the level of the intermediate Meisenheimer complex, which would be expected to exclude crossover with external amines. The mechanism of the reaction is outlined in Scheme 46.



Scheme 46. Coupling of a thioacid with a *N*-terminal sulfonamide

The use of nucleophilic aromatic substitution processes to generate active esters for amide and peptide bond formation has been described previously. Notably, Kamiński, Papini, and their coworkers reported the reaction of *N*-triazinyl ammonium salts with protected amino acids in acetonitrile in the presence of *N*-methyl morpholine, leading to the formation of a “superactive ester” followed by coupling with the amine component.[141] Ito and coworkers employed a *S*-(2-formyl-4-nitrophenyl) thioester in peptide coupling reactions assisted by prior condensation of the amine with the formyl group, but generated the thioester in the classical manner by reaction of the 2-formyl-4-nitrothiophenol with a previously activated amino acid.[142] Prior to Crich’s application of the Tomkinson sulfonamide reaction,[139] thioacids had been employed directly in peptide coupling reactions as nucleophilic components in carbodiimide mediated protocols,[143] in the form of their silver salts as electrophiles for direct condensation with

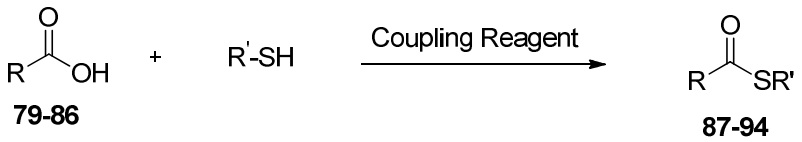
amines,[144] and as the carboxyl component in peptide synthesis catalyzed by papain.[145] Peptide-based thioacids also serve, by alkylation to thioesters, as a point of entry into native chemical ligation methods for chemical protein synthesis.[146]

Sanger's reagent, 2,4-dinitrofluorobenzene, through its reaction with *N*-terminal amines, was traditionally used for the degradation of peptides and proteins, and identification of *N*-terminal amino acids.[147-149] However, Sanger noticed that thiols and other nucleophiles, including the side-chains of histidine and tyrosine, were competing nucleophiles to amines for capture by 2,4-dinitrofluorobenzene.[147] This observation prompted the hypothesis that a thiocarboxylate salt would react significantly more rapidly than an amine with this reagent enabling the formation of the thioester and ultimately peptide synthesis thereby effectively turning a peptide degradation reagent into one for their synthesis.

2.3 Synthesis of Peptidyl Thioesters

A series of thioesters was prepared by coupling suitably-protected amino acids with either 9-fluorenylmethanethiol,[139] 2,4,6-trimethoxybenzylthiol,[150] or triphenylmethanethiol[151] in excellent yield as set out in Table 1.

Table 1. Preparation of Thioesters from Protected Amino Acids and Thiols

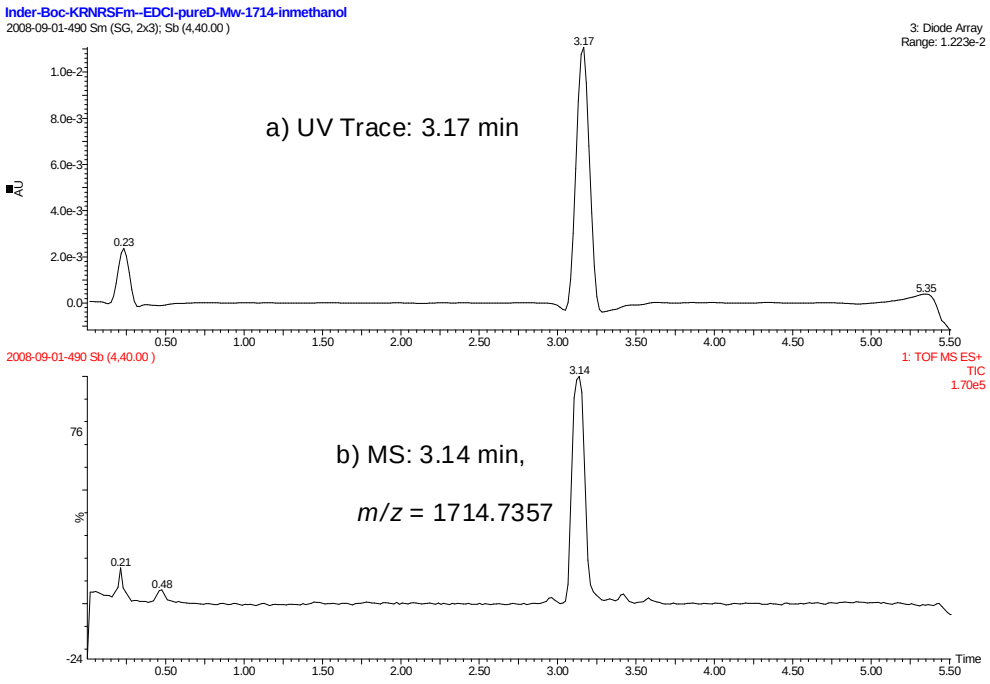


79-86 **87-94**

Entry	Substrate	Thiol/Reagent	Product	% Yield
1	Boc-L-Val-OH (**79**)	FmSH/DCC	Boc-L-Val-SFm (**87**)	99
2	Boc-Aib-OH (**80**)	FmSH/DCC	Boc-Aib-SFm (**88**)	82
3	Boc-L-Asp-(α-OBn)-γ-OH (**81**)	FmSH/DCC	Boc-L-Asp-(α-OBn)-γ-SFm (**89**)	97
4	Fmoc-L-Ala-OH (**82**)	TmobSH/DIC	Fmoc-L-Ala-STmob (**90**)	98
5	Boc-L-Phe-OH (**83**)	TmobSH/DIC	Boc-L-Phe-STmob (**91**)	96
6	Z-L-Val-OH (**84**)	TrtSH/DIC	Z-L-Val-STrt (**92**)	95
7	Z-L-Ala-L-Phe-OH (**85**)	FmSH/PyBop	Z-L-Ala-L-Phe-SFm (**93**)	72
8	Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-OH (**86**)	FmSH/PyBop	Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (**94**)	82

With simple carbamate-protected amino acids, carbodiimide coupling reagents were employed for these condensations. However, for peptides (Table 1, last two entries) the PyBOP reagent recommended by Kajihara and co-workers[152] was used to avoid the widely reputed problem of epimerization in the thioesterification of all but simple amino acids. The final entry in Table 1 is noteworthy both for the sterically hindered nature of the reaction site and for the

fact that no epimerization was observed under the PyBop coupling conditions (Figure 2). LCMS analysis showed only a single peak with retention time of 3.17 min with a molecular weight corresponding to that of the thioester **94** (*m/z* = 1712.7369, Figure 3).



LCMS: [70-90% B (acetonitrile containing 0.1% formic acid) in A (10% CH_3CN/H_2O containing 0.1% formic acid) with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.17 min]

Figure 2. LCMS trace of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (94) obtained with the PyBOP reagent

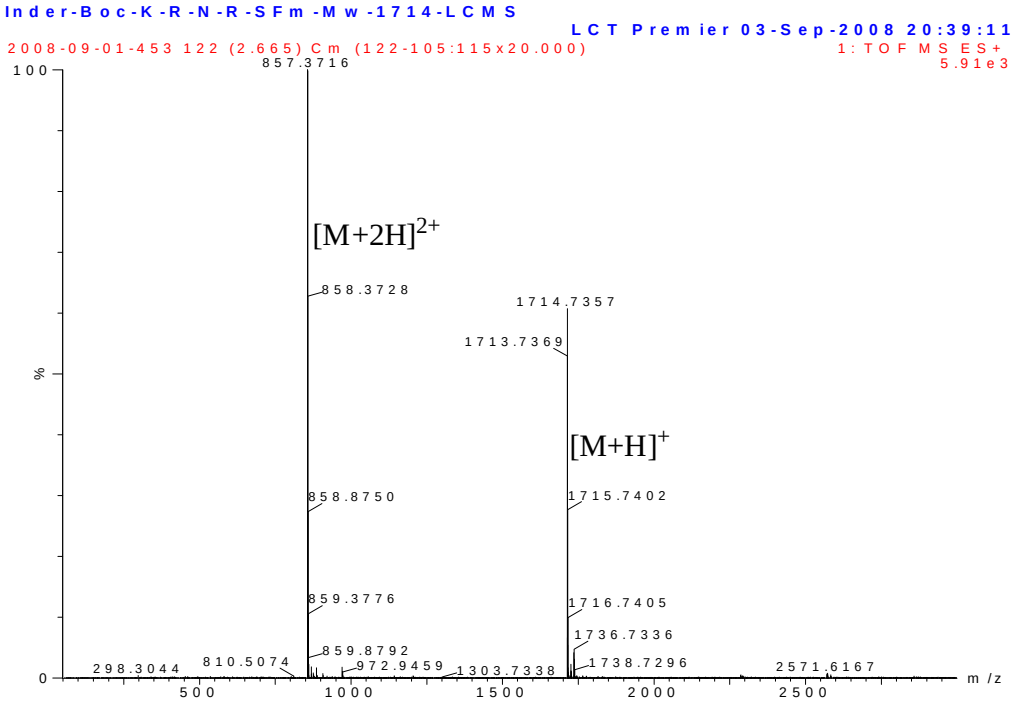
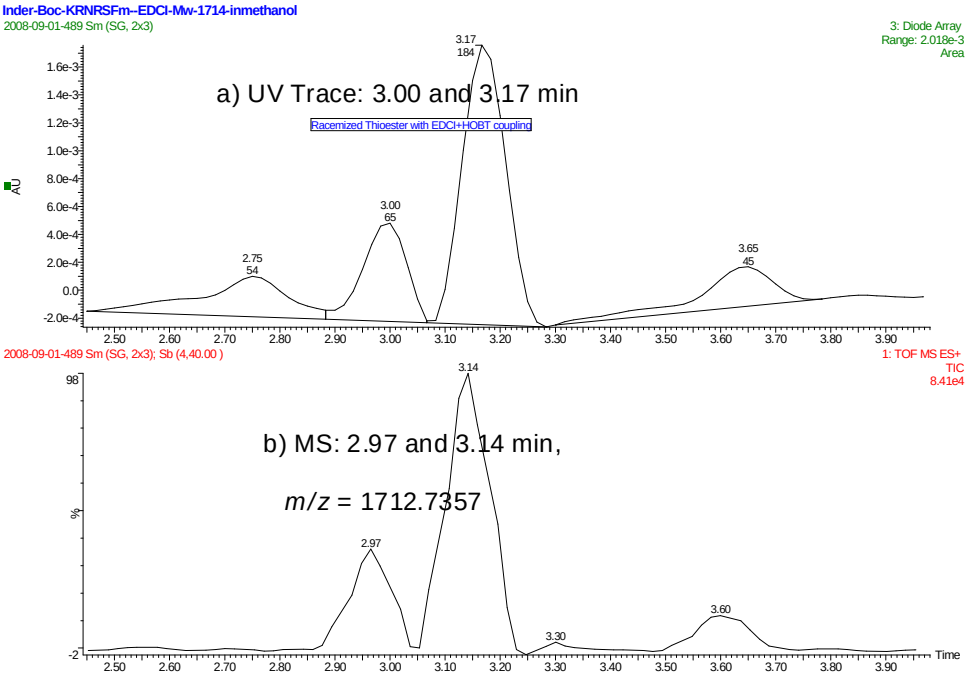


Figure 3. ESI-HRMS spectrum of the peak eluting at 3.14 min [Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (94)]

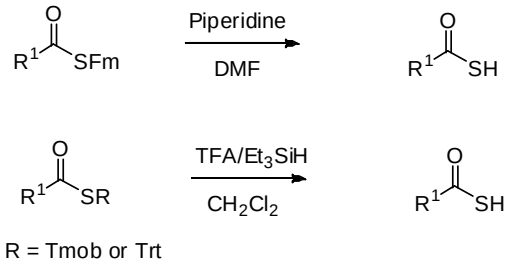
When the same thioester **94** preparation was conducted using the carbodiimide/hydroxybenzotriazole conditions reported[153-155] for epimerization-free thioester synthesis, the product was obtained in excellent yield (77%) but as a 3:1 mixture of epimers (Figure 4).



LCMS: [70-90% B (acetonitrile containing 0.1% formic acid) in A (10% CH_3CN/H_2O containing 0.1% formic acid) with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.00 and 3.17 min]

Figure 4. LCMS trace of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (94) obtained by the EDCI/HOBT coupling

The thioacids needed for the coupling reactions were released cleanly from the thioesters with piperidine[139] in the case of the fluorenylmethyl thioesters, or with trifluoroacetic acid for the trimethoxybenzyl thioesters[151] and triphenylmethyl thioesters,[151] and were used immediately in the peptide bond forming reactions (Scheme 47).

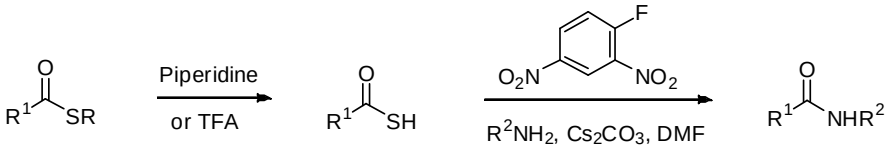


Scheme 47. Preparation of Thioacids

2.4 Peptide Synthesis using the Sanger and Mukaiyama Reagents

The coupling of thioacids with amines in the presence of Sanger's reagent was first investigated with commercially available thioacetic acid, L-tryptophan and its methyl ester (Table 2, entries 1 and 2), and an excellent yield of the acetamide was obtained on mixing of the three components at room temperature in the presence of cesium carbonate. Thus encouraged, a series of dipeptides was accessed by coupling amino thioacids with other amino esters and peptide esters with the yields reported in Table 2. All reactions in Table 2 were conducted at room temperature and involved stirring 0.1 M amine hydrochloride in DMF with thioacid (1.2 equiv) and Cs_2CO_3 (1.5 equiv) as base, followed by the addition of the Sanger's reagent.

Table 2. Coupling of Thioacids with Amines with Sanger's Reagent



Entry	Thioester	Release reagent	Thioacid	Amine	Product	% Yield
1	-	-	AcSH	L-Trp	Ac-L-Trp (**95**)	76
2	-	-	AcSH	L-Trp-OMe.HCl	Ac-L-Trp-OMe (**96**)	99
3	Boc-L-Val-SFm (**87**)	Piperidine	Boc-L-Val-SH	L-Phe-OMe.HCl	Boc-L-Val-L-Phe-OMe (**97**)	84
4	Boc-L-Val-SFm (**87**)	Piperidine	Boc-L-Val-SH	D-Phe-OMe.HCl	Boc-L-Val-D-Phe-OMe (**98**)	82
5	Boc-L-Val-SFm (**87**)	Piperidine	Boc-L-Val-SH	L-Trp-OMe.HCl	Boc-L-Val-L-Trp-OMe (**99**)	76
6	Boc-Aib-SFm (**88**)	Piperidine	Boc-Aib-SH	L-Trp-OMe.HCl	Boc-Aib-L-Trp-OMe (**100**)	95
7	Boc-Aib-SFm (**88**)	Piperidine	Boc-Aib-SH	Gly-OEt.HCl	Boc-Aib-Gly-OEt (**101**)	96
8	Boc-L-Asp-(α-OBn)-γ-	Piperidine	Boc-L-Asp-(α-	L-Phe-OMe.HCl	Boc-L-Asp-(α-OBn)-γ-L-	93

	SFm (**89**)		OBn)-γ-SH		Phe-OMe (**102**)	
9	Fmoc-L-Ala-STmob (**90**)	TFA	Fmoc-L-Ala-SH	Gly-OEt.HCl	Fmoc-L-Ala-Gly-OEt (**103**)	60
10	Fmoc-L-Ala-STmob (**90**)	TFA	Fmoc-L-Ala-SH	L-Phe-OMe.HCl	Fmoc-L-Ala-L-Phe-OMe (**104**)	59
11	Fmoc-L-Ala-STmob (**90**)	TFA	Fmoc-L-Ala-SH	L-Tyr-OMe.HCl	Fmoc-L-Ala-L-Tyr-OMe (**105**)	64
12	Z-L-Ala-L-Phe-SFm (**93**)	Piperidine	Z-L-Ala-L-Phe-SH	Gly-Gly-OMe.HCl	Z-L-Ala-L-Phe-Gly-Gly-OMe (**106**)	66

From Table 2, it is clear that deprotonated thioacids are more nucleophilic toward Sanger's reagent than amines and make, 2,4-dinitrofluorobenzene an effective reagent for the formation of amide bonds between thioacids and amines. In particular, attention is drawn to entries 3 and 4 of Table 1 in which diastereomeric products **97** and **98** were obtained. To verify the possibility of epimerization in the course of thioester deprotection or in the coupling process, a qualitative comparison of the 500 MHz ^{1}H NMR spectra of both diastereomers **97** and **98** was performed as shown in Figure 5. A careful review of the proton NMR spectra of both isomers indicates that each spectrum corresponds to a single diastereomer. The characteristic chemical shifts of α and β protons of the

phenylalanine unit in the diastereomeric dipeptides identify the two diastereomers (Table 3).

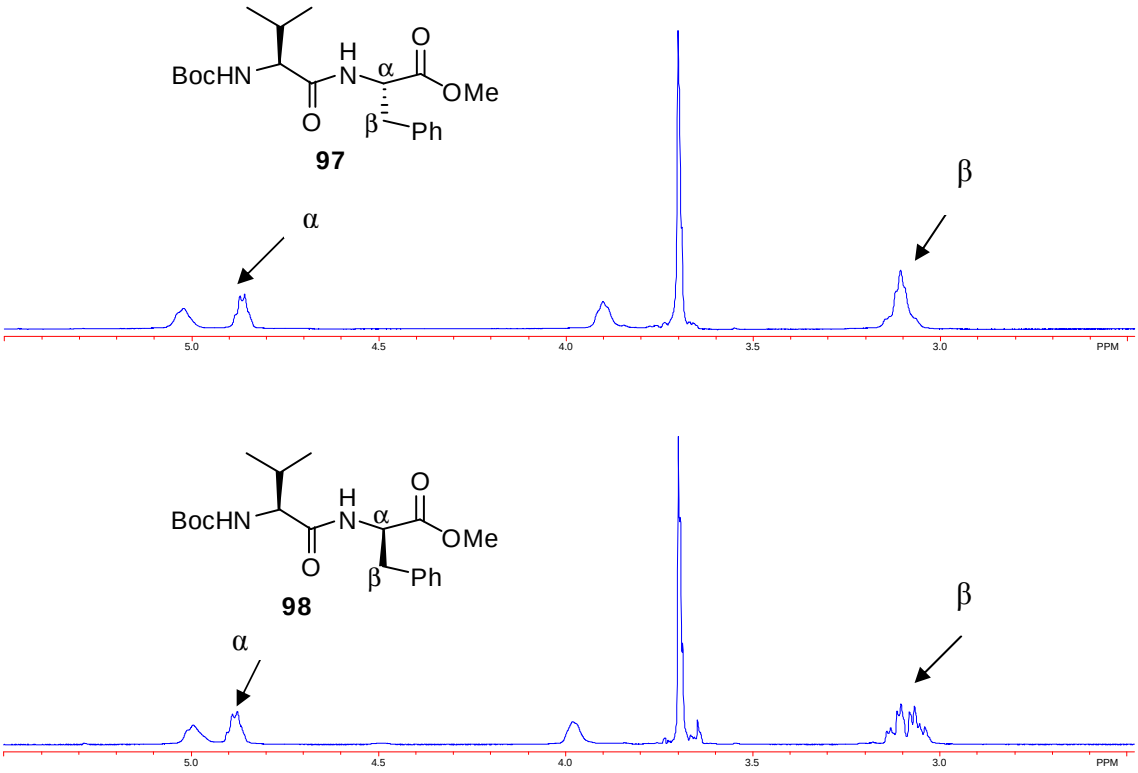


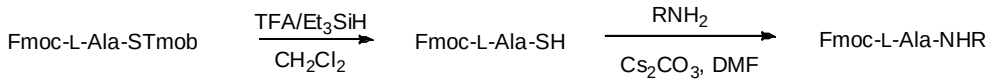
Figure 5. ^{1}H-NMR spectra of dipeptides in $CDCl_3$

Table 3. Selected ^{1}H Chemical Shifts (δ in ppm) and Coupling Constants (*J*) of Dipeptides Boc-L-Val-L-Phe-OMe (97) and Boc-L-Val-D-Phe-OMe (98)

Dipeptide	δ of α-proton (multiplicity, *J*, proton counts)	δ of β- proton (multiplicity, *J*, proton counts)
Boc-L-Val-L-Phe-OMe (**97**)	4.87-4.86 (m, 1H)	3.12-3.06 (m, 2H)
Boc-L-Val-D-Phe-OMe (**98**)	4.91-4.87 (m, 1H)	3.14-3.10 (dd, *J* = 5.5, 13.7 Hz, 1H) 3.08-3.04 (dd, *J* = 5.5, 13.7 Hz, 1H)

The ability to form relatively hindered peptide bonds is evident from entries 5 and 6 (Table 2), where a valine thioacid and an aminoisobutyric thioacid are coupled with the tryptophan amine. The good functional group compatibility of the process is clear from inspection of Table 2 in general. In spite of these promising results the yields with the Fmoc protected thioacids (Table 2, entries 9-12) are generally lower than those obtained with Boc protected thioacids. This was eventually traced to the competing cleavage of the Fmoc group by the fluoride anion released in the course of the nucleophilic aromatic substitution step.[156-158] To circumvent the relative incompatibility of Fmoc groups with fluoride anion, 2,4-dinitroiodobenzene and 2-chloro-1-methylpyridinium iodide (Mukaiyama's reagent)[159] as the condensing agent were investigated. This ruse was successful as shown by the results presented in Table 4. All reactions in Table 4 were conducted at room temperature by stirring of 0.1 M amine hydrochloride in DMF with thioacid (1.2 equiv) and Cs_2CO_3 (1.5 equiv) as base followed by the addition of the coupling reagent.

Table 4. Comparison of Sanger's Reagent with 2,4-Dinitroiodobenzene and with Mukaiyama's Reagent

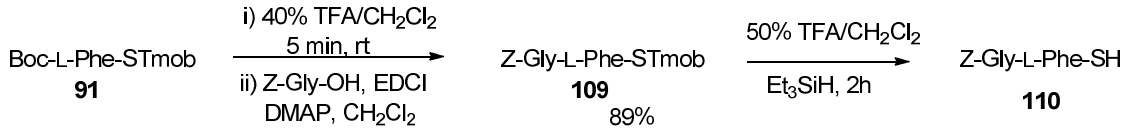


		Coupling Reagent and % Yield		
Amine	Product	Sanger's Reagent	2,4-dinitro-iodobenzene	Mukaiyama's Reagent
Gly-OEt.HCl	Fmoc-L-Ala-Gly-	60	83	80

	OEt (**103**)			
L-Phe-OMe.HCl	Fmoc-L-Ala-L-Phe-OMe (**104**)	59	79	86
L-Tyr-OMe.HCl	Fmoc-L-Ala-L-Tyr-OMe (**105**)	64	86	85
L-Ala-OMe.HCl	Fmoc-L-Ala-L-Ala-OMe (**107**)	-	78	82
L-Cys(Trt)-OEt.HCl	Fmoc-L-Ala-L-Cys(Trt)-OEt (**108**)	-	82	88

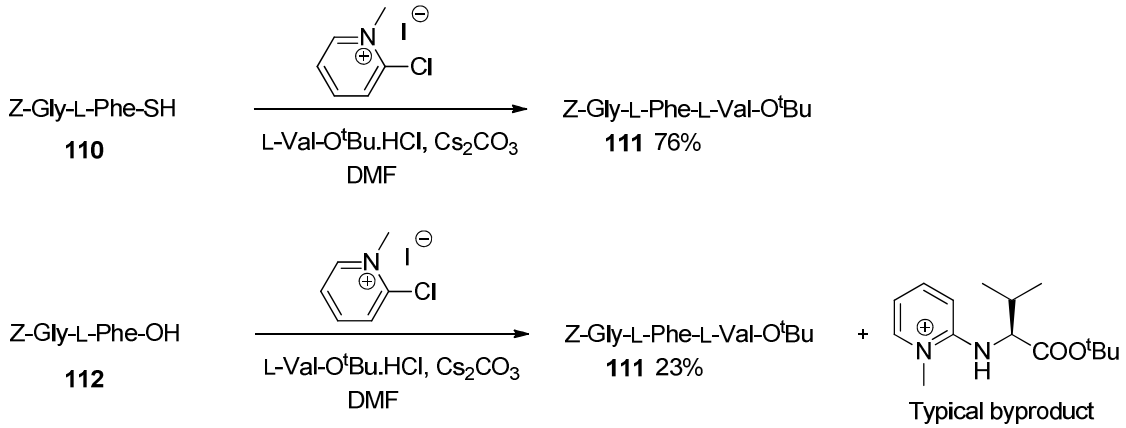
The results presented in Tables 2 and especially 4 are noteworthy because of the high yields obtained in comparison with previous uses of Mukaiyama's reagent to effect peptide coupling reactions between amines and simple carboxylic acids[160,161] where the yields were modest except for secondary and sterically hindered amines.[28,29] As is also implicit in the work of Sanger with peptides, simple peptide based primary amines are more nucleophilic toward electron-deficient arenes than the carboxylic acids. The clear difference between the results reported here and those reported previously in the literature is the use of the more nucleophilic thioacids rather than simple carboxylic acids. To underscore this difference a head to head comparison was undertaken. To this end, the dipeptide thioacid Z-Gly-L-Phe-SH (**110**) was first prepared as described in Scheme 48. This synthesis is noteworthy because it establishes the possibility of removing a Boc group in the presence of the Tmob thioester under acid conditions. In effect, as is clear from the second step of the protocol, removal of

the latter group requires the combination of trifluoroacetic acid and the nucleophilic agent triethylsilane.



Scheme 48. Preparation of a dipeptide thioacid

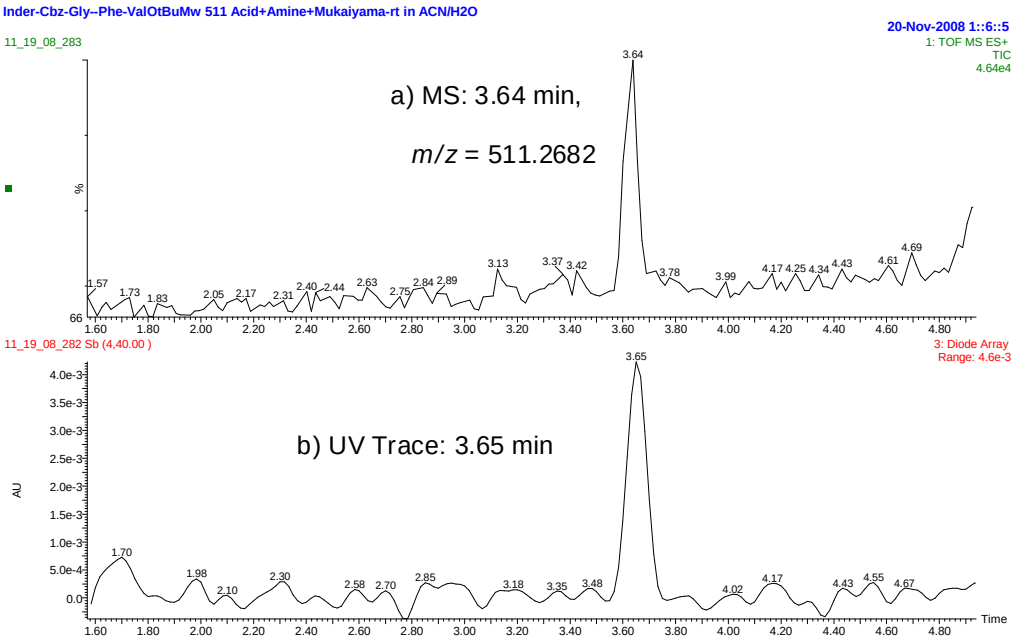
Parallel reactions between Z-Gly-L-Phe-SH (**110**) and Z-Gly-L-Phe-OH (**112**) for coupling to Val-OtBu were conducted with Mukaiyama's reagent. The tripeptide **111** (Z-Gly-L-Phe-L-Val-O^{t}Bu) was achieved in 76% yield from the reaction of the thioacid, while the corresponding carboxylic acid gave only 23% yield under identical conditions (Scheme 49).



Scheme 49. Thioacid vs carboxylic acid

Examination of the reaction mixture from the coupling of the simple dipeptide with Mukaiyama's reagent and L-Val-O^{t}Bu revealed a far more complex reaction mixture than that arising from the use of the corresponding dipeptidyl thioacid. In particular, a major byproduct was identified in this manner as the result of attack of the amine on the coupling reagent. LCMS analysis of the tripeptide **111** produced from the dipeptidyl thioacid with the Mukaiyama's reagent also enabled investigation of the extent of epimerization. A single peak with a retention time of

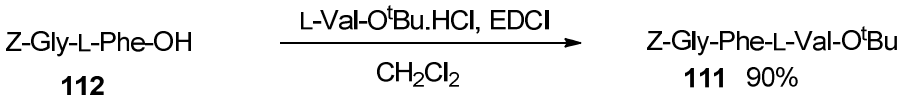
3.65 min was observed with a molecular weight corresponding to the tripeptide **111** (Figure 6), consistent with an epimerization free reaction.



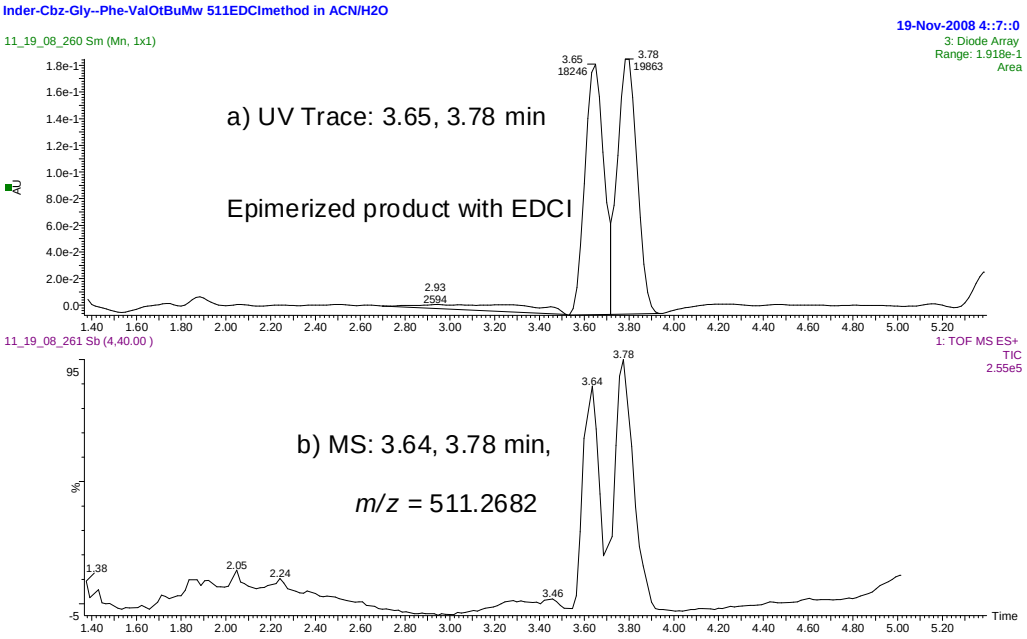
LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

Figure 6. LCMS analysis of Z-Gly-L-Phe-L-Val-O^{t}Bu (111) from the thioacid

To verify that the LC conditions were capable of resolving the epimeric peptides, a further sample of Z-Gly-Phe-L-Val-O^{t}Bu (**111**) was synthesized by EDCI coupling at room temperature (Scheme 49), i.e., under conditions likely to produce epimerization. The LCMS of this crude reaction mixture showed essentially full epimerization (ratio, L/D = 48/52) with two well resolved peaks having retention times 3.65 min and 3.78 min (Figure 7).



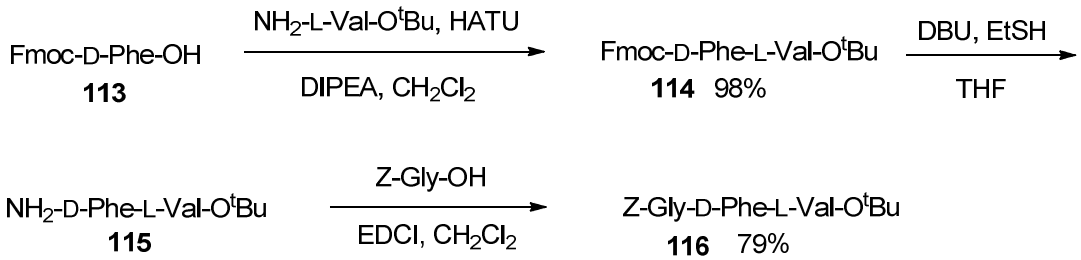
Scheme 49. Coupling with EDCI



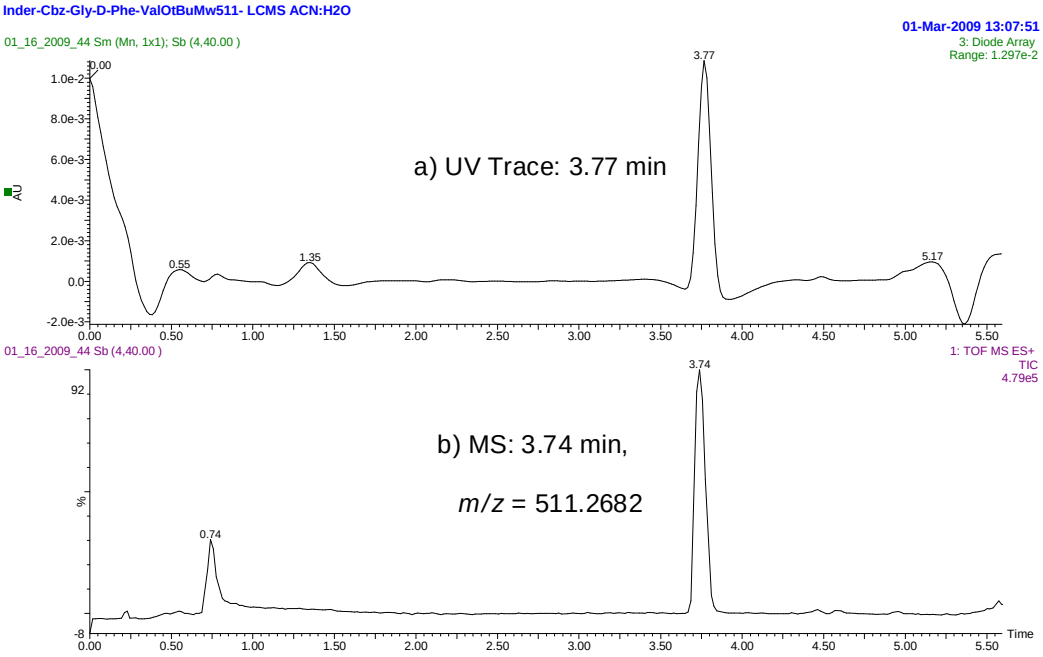
LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min, 3.78 min

Figure 7. LCMS analysis of Z-Gly-Phe-L-Val-O^{t}Bu (111) produced by EDCI coupling

The lack of epimerization in the coupling of the thioacid using Mukaiyama's reagent was further confirmed by synthesis of an authentic sample of Z-Gly-D-Phe-L-Val-O^{t}Bu (**116**) which was shown to have a different retention time (Scheme 50, Figure 8).



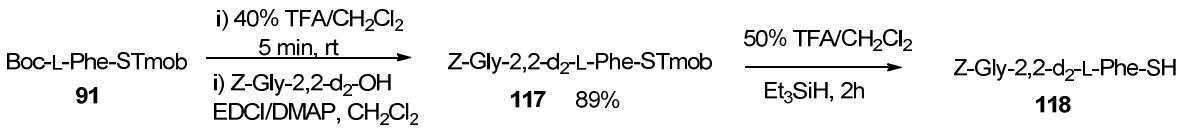
Scheme 50. Synthesis of authentic Z-Gly-D-Phe-L-Val-O^{t}Bu (116)



LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.78 min.

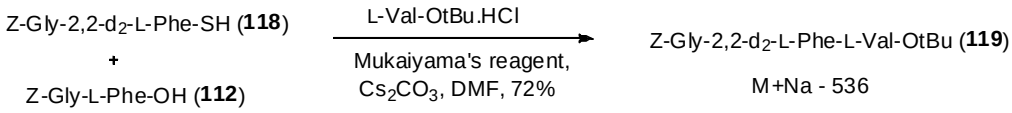
Figure 8: LCMS analysis of an authentic sample of Z-Gly-D-Phe-L-Val-O^{t}Bu (116)

To further probe the superiority of thioacids as nucleophiles over carboxylic acids, a deuterium labeled thioacid **118** was synthesized as outlined in Scheme 51. Here again, the ability to cleave a Boc group under acidic conditions in the presence of the electron-rich S-Tmob ester was a critical component of the synthesis.



Scheme 51. Synthesis of deuterium labelled thioacid 118

This deuterium labeled dipeptidyl thioacid **118** was subjected to a direct competition with an equimolar amount of the corresponding dipeptidyl acid **112**, lacking the label, for coupling by Mukaiyama's reagent to L-valine (Scheme 52).



Scheme 52. Carboxylate/thiocarboxylate competition reaction

The tripeptide product **119** isolated from this reaction mixture was shown by mass spectrometry to be fully deuterium labelled in the glycine residue, thereby unambiguously indicating the superiority of the thioacid in this chemistry (Figure 9). Investigation of the tripeptide by ^{1}H-NMR spectroscopy further confirmed the absence of the isotopomer.

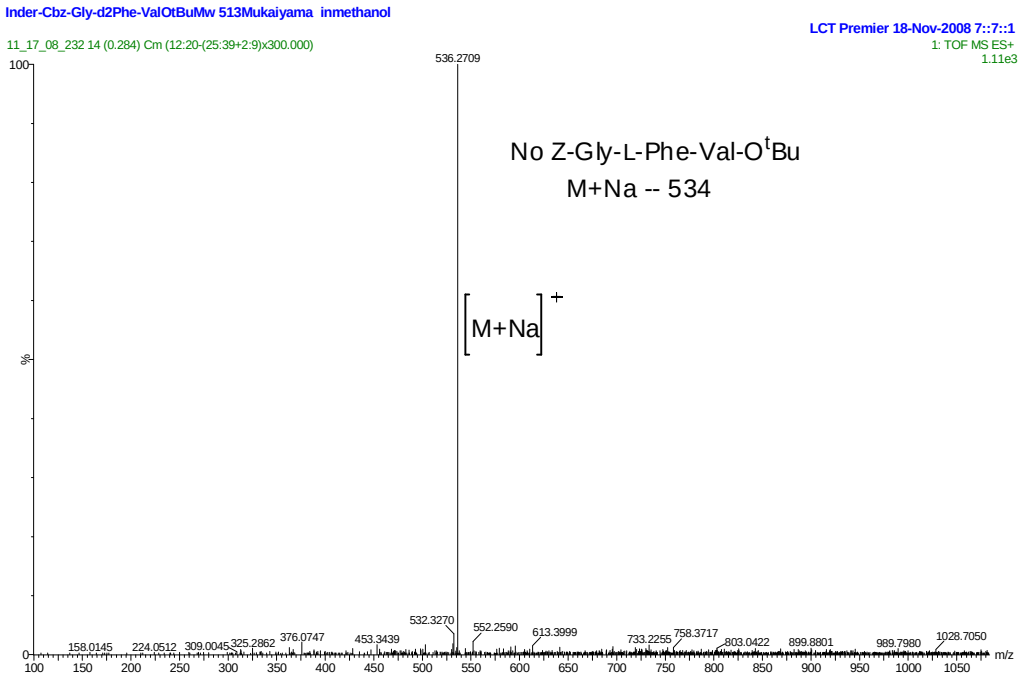
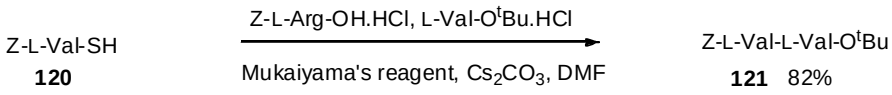


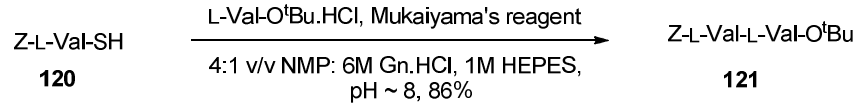
Figure 9. ESI-HRMS spectrum of Z-Gly-2,2-d$_2$-L-Phe-L-Val-O^{t}Bu (119)

In a further reaction the coupling of a valine-derived thioacid **120** with L-valine was conducted in the presence of Z-L-Arg resulting in a high yield of a single dipeptide **121**. This reaction reinforces the superior reactivity of the thioacid over the simple carboxylic acid, while also demonstrating compatibility with the guanidine and carboxylic acids moieties of the arginine derivative (Scheme 53).



Scheme 53. Functional Group Compatibility

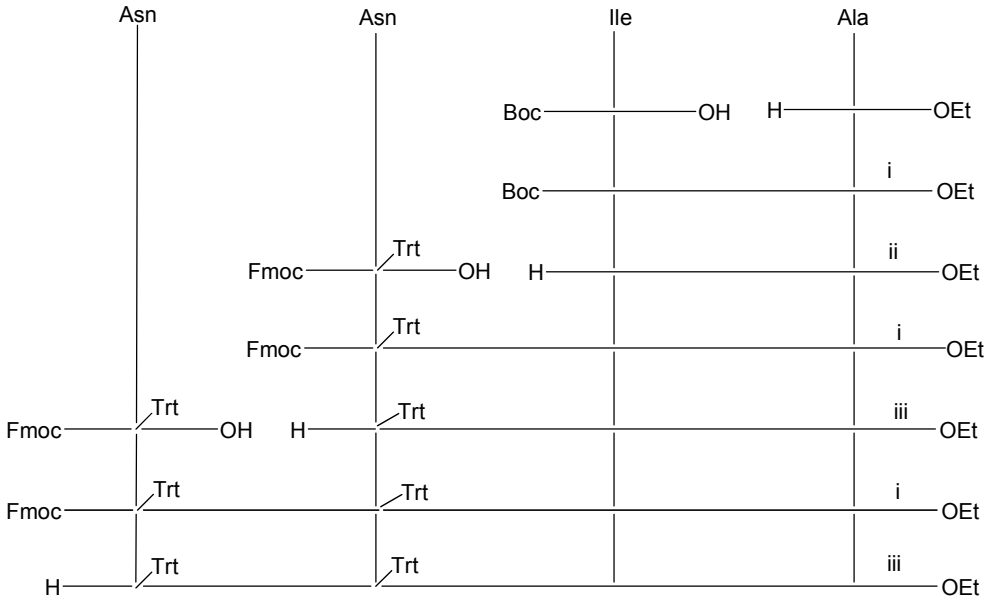
That the coupling reaction with Mukaiyama's reagent could be conducted under aqueous conditions was demonstrated by the example of Scheme 54. A good yield of a dipeptide **121** was achieved in this coupling reaction of a valine-derived thioacid **120** affected with Mukaiyama's reagent in aqueous buffer.[162]



Scheme 54. Coupling under Aqueous Conditions

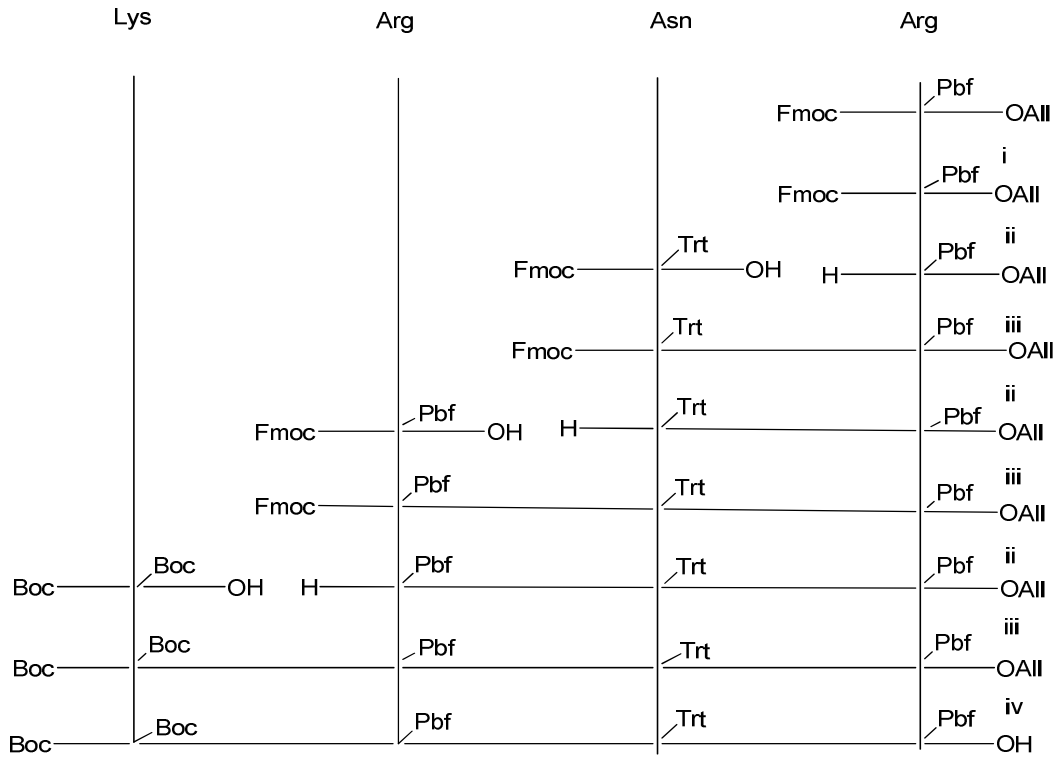
Finally, the application of this new peptide bond forming reaction to the 4+4 block synthesis of an octapeptide L-Lys-L-Arg-L-Asn-L-Arg-L-Asn-L-Asn-L-Ile-L-Ala (**122**) was demonstrated. This octapeptide **122** is the C-terminal sequence of oxyntomodulin from *Porcine jejunoileum* and is responsible for the gastric acid secretion inhibitory properties of an extended version of glucagon.[163] This octapeptide was selected as a target because of the widespread current interest in oxyntomodulin derivatives as appetite suppressants and as potential therapeutics for obesity,[164] and because of its concatenation of multiple functionalized side chains that provides a true test of the methodology.

Two tetrapeptides **123** and **124** were synthesized by standard manual solution phase techniques (Scheme 55 and 56).



i) DIC/HOBt or EDCI/HOBt; ii) TFA/CH_2Cl_2; iii) DBU/EtSH/THF

Scheme 55. Solution phase synthesis of amine segment NH_2-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (123)

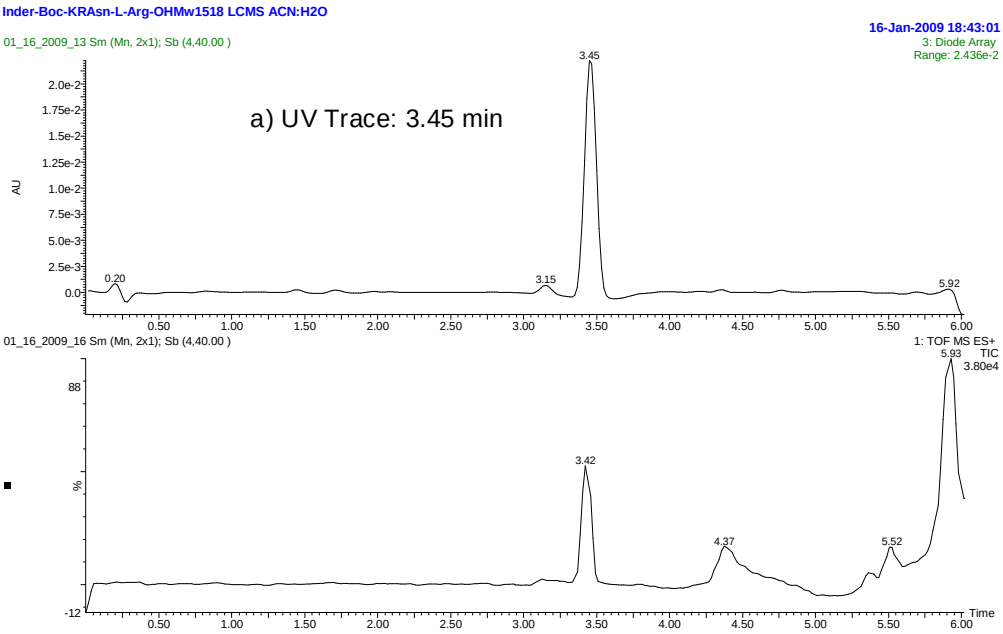


i) $SOCl_2$/allyl alcohol; ii) DBU/EtSH/THF; iii) DIC/HOBt or EDCI/HOBt;

iv) $Pd(PPh_3)_4/PhSiH_3$

Scheme 56. Solution Phase Synthesis of the Peptide Acid Segment Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-OH (124)

At the completion of the second tetrapeptide synthesis an allyl ester protecting group was removed from the C-terminal arginine residue to release the corresponding acid, which was purified by reverse phase HPLC and then subjected to LCMS analysis to check the extent of epimerization at the C-terminus (Figure 10). A single peak in the LCMS trace having a retention time of 3.45 min and a molecular weight of 1518.7357 argues against the possibility of epimerization during synthesis (Figure 11).



LCMS: 50-90% B with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.45 min.

Figure 10. LCMS analysis of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-OH (124)

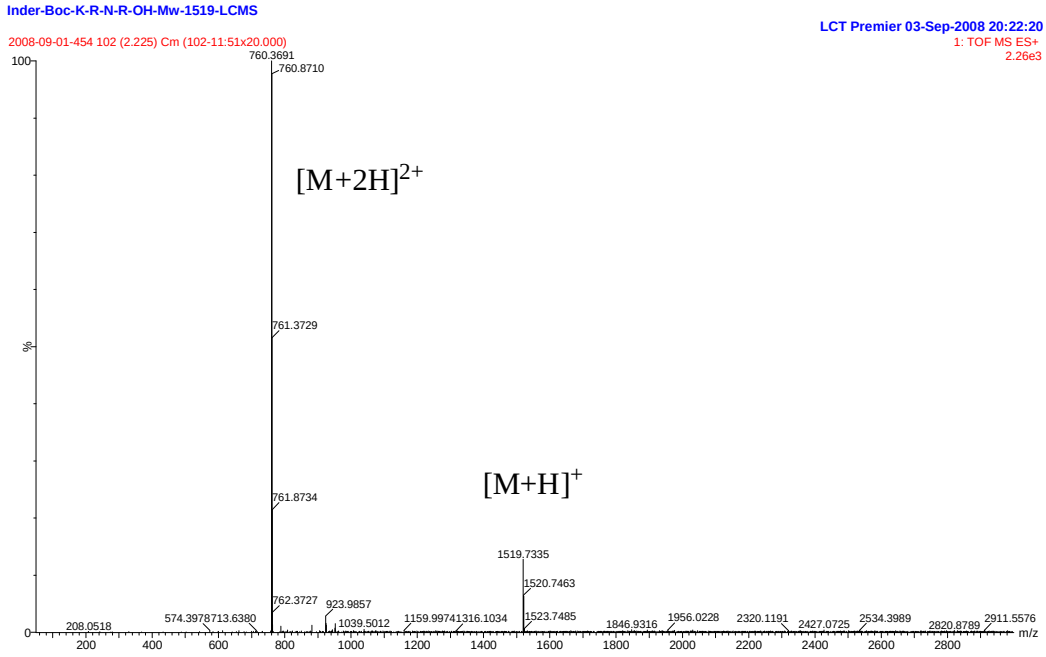
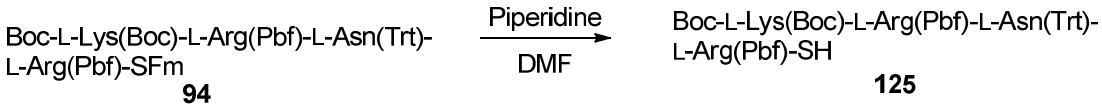


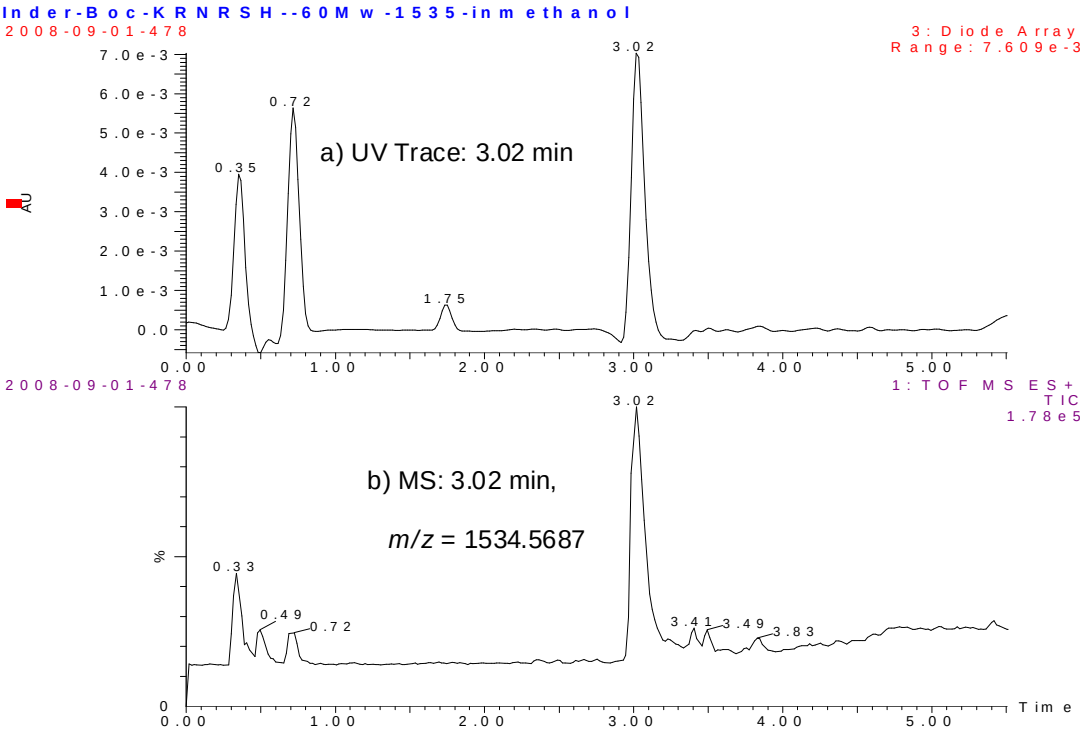
Figure 11. ESI-HRMS spectrum of peak eluting at 3.64 min [Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-OH (124)]

The resulting acid **124** was then converted to the 9-fluorenylmethyl thioester **94** as set out in the final entry of Table 1. Finally, the stage for the 4+4 fragment coupling was set by liberation of the thioacid functionality **125** with piperidine as set out in Scheme 57.



Scheme 57. Synthesis of *C*-terminal thioacid 124

The LCMS trace and ESI-TOF mass spectrum of the resulting crude peptidyl thioacid **125** are presented in Figures 12 and 13 which includes a single peak with a retention time of 3.02 min with a molecular weight corresponding to that of the thioacids **125**.



LCMS: 60-95% B with a flow rate of 0.5 mL/min over 5 min and 254 nm UV detection, retention time = 3.02 min.

Figure 12. LCMS analysis of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SH (125)

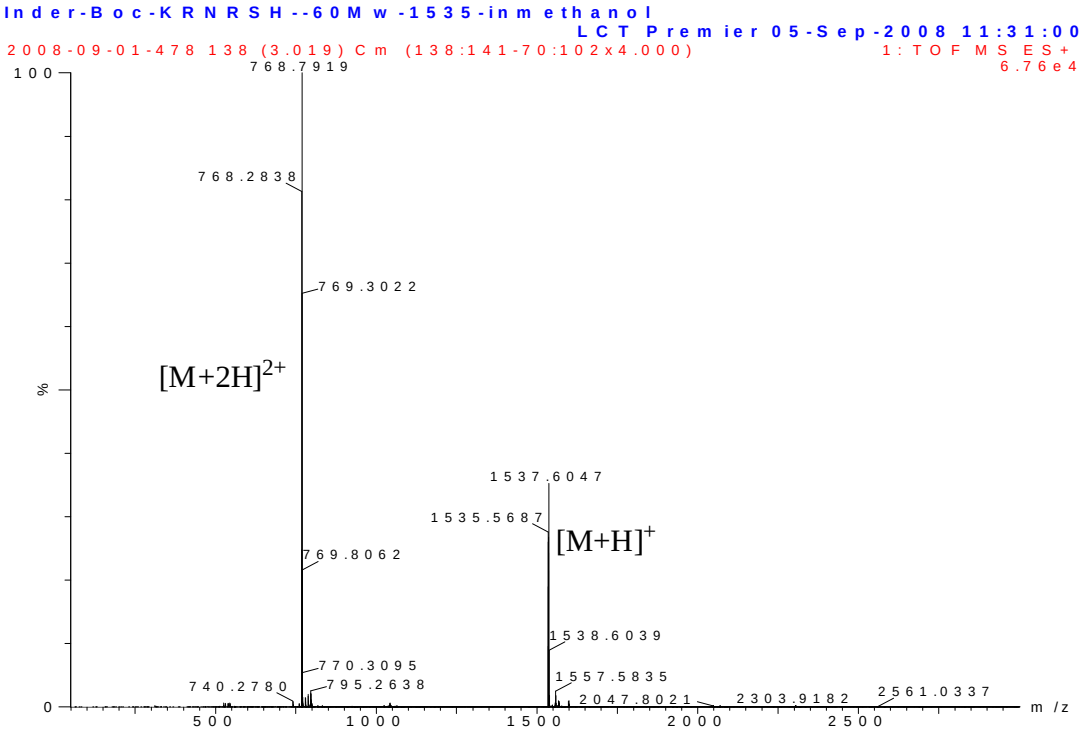
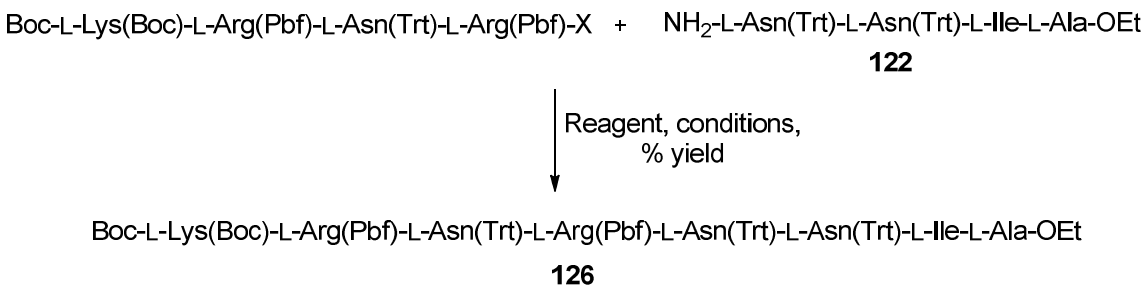


Figure 13. ESI-HRMS spectrum of peak eluting at 3.02 min [Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SH (125)]

The 4+4 fragment coupling was achieved by coupling of *C*-terminal thioacid **125** with the *N*-terminal tetrapeptide **123** in the presence of Mukaiyama's reagent in DMF with cesium carbonate as base. The protected target octapeptide **126** was isolated in 66% yield as single epimer (Scheme 58, Figure 14).

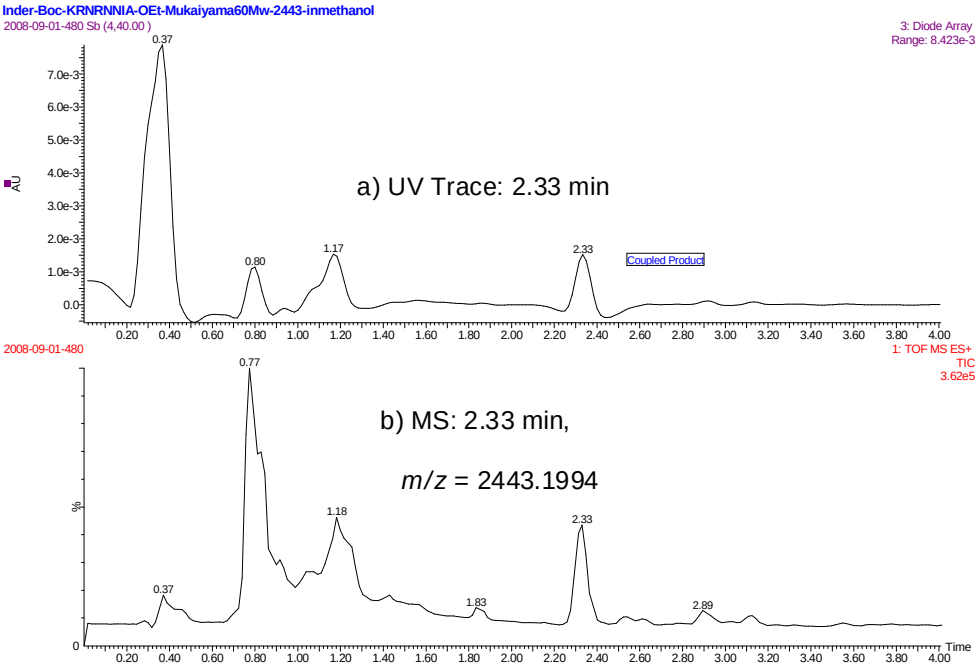
This block synthesis provided an opportunity for the comparison of the thioacid methodology with more common methods. To this end, coupling of the tetrapeptide carboxylic acid was affected with PyBop, HATU, EDCI/HOBT and EDCI[10,11] in DMF under standard conditions of temperature and concentration, and employed epimerization-free, all L-tetrapeptides (Scheme 58). The PyBop EDCI/HOBT and HATU methods gave comparable yields to the thioacid method but required significantly longer reaction times to do so, and were not exempted

from epimerization (Figure 16). The carbodiimide method was not complete even after 14 h and resulted in significantly more epimerization.



X	Coupling Reagent	T (h)	% Yield	Epimer ratio
SH	Mukaiyama‘s	1	66	>99:1
OH	PyBop	8	69	93.7:6.3
OH	HATU	6	70	93.1:6.9
OH	EDCI	14	54	85.8:14.2
OH	EDCI-HOBt	8	63	94.2:5.8

Scheme 58. Comparative block syntheses of an octapeptide 126



LCMS of the crude reaction mixture: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.33 min.

Figure 14. LCMS of octapeptide Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) obtained by coupling of the thioacid with the Mukaiyama's reagent

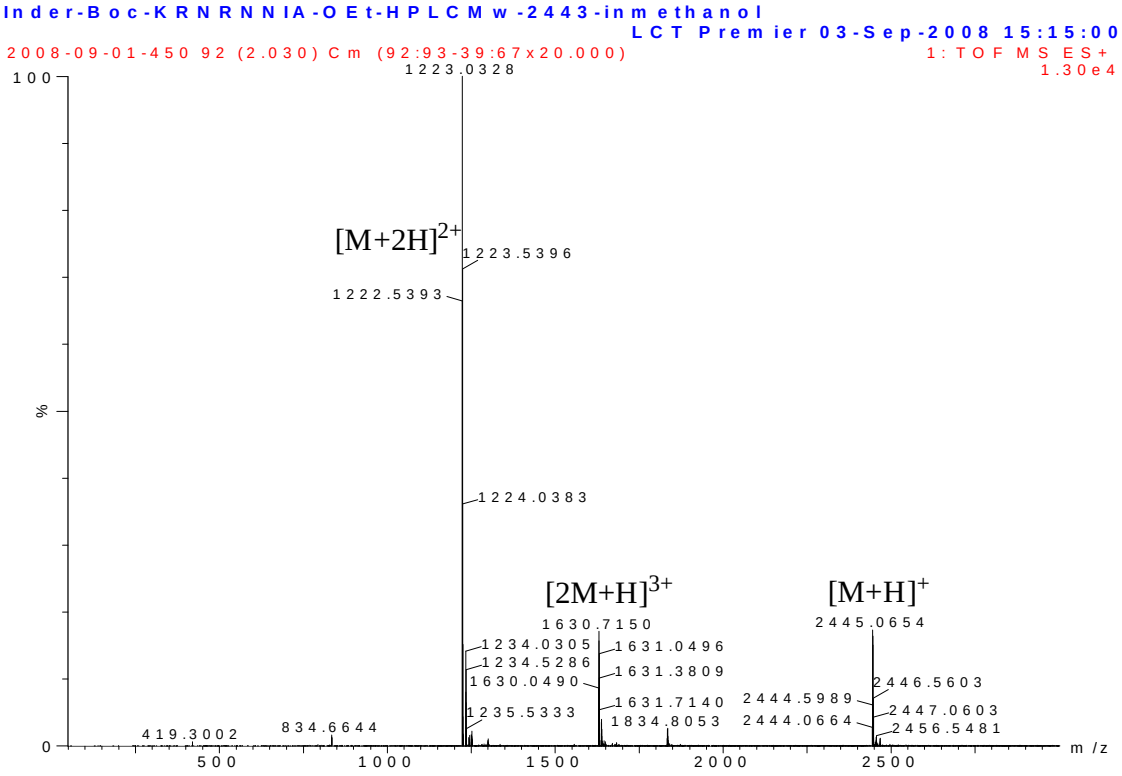
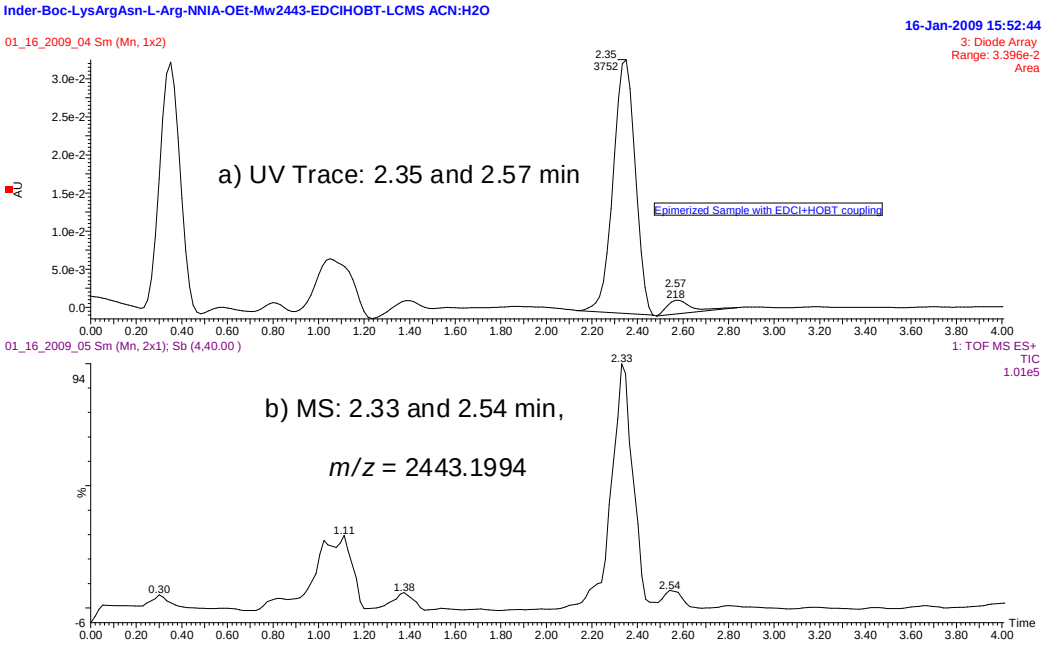


Figure 15. ESI-HRMS spectrum of peak eluting at 2.33 min [Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126)]

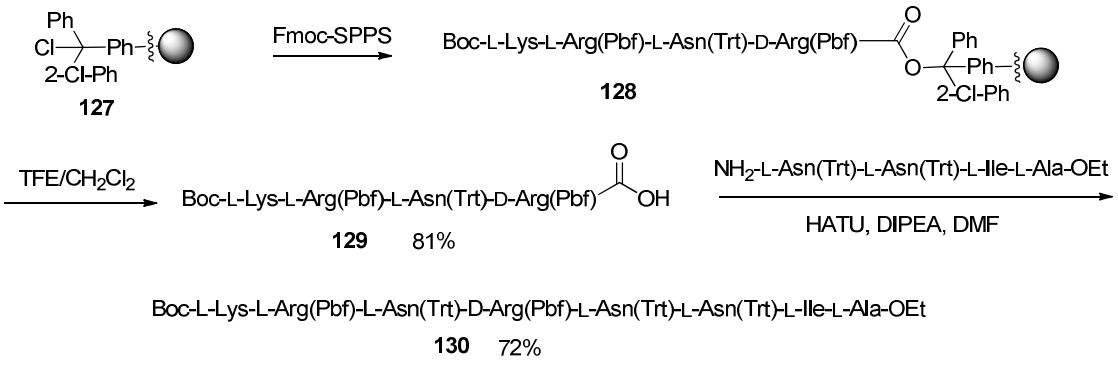


LCMS of the crude reaction mixture showed epimerization: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.35 min and 2.57 min.

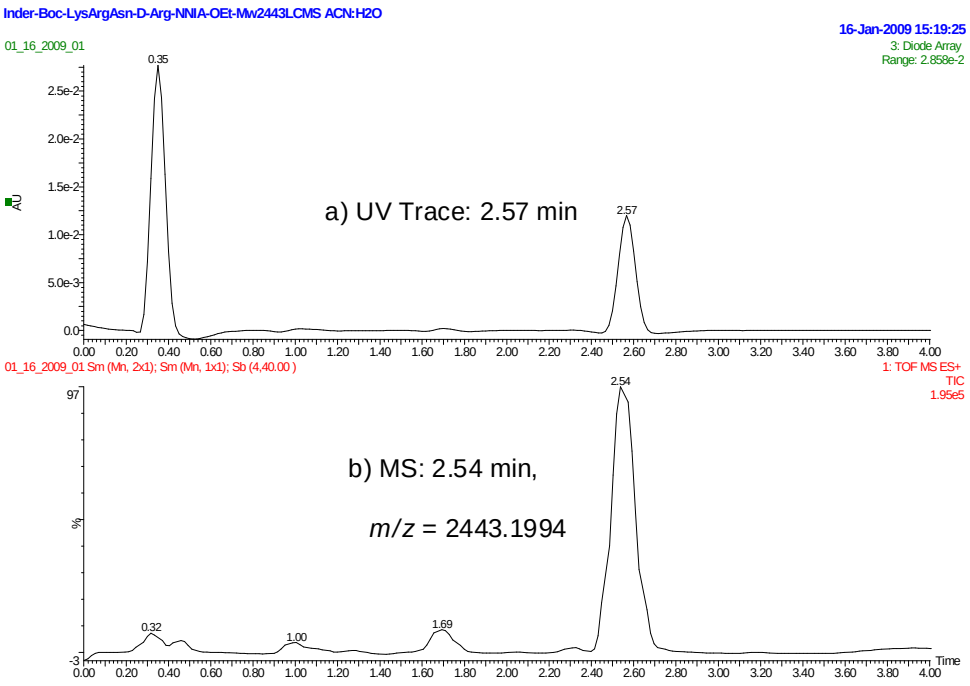
Figure 16. LCMS analysis of octapeptide 126 produced by the EDCI/HOBT method

Epimerization ratios were determined with the help of an authentic sample of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-D-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt **130** (Figure 17). To obtain this isomeric octapeptide **130**, the *C*-terminal tetrapeptide first was synthesized by Fmoc solid phase peptide synthesis on the 2-chloro trityl resin **127** (Scheme 59) and then the coupling of the acid fragment **129** with *N*-terminal tetrapeptide **123** was affected with HATU to afford the octapeptide, which was purified by reverse phase HPLC to provide the pure authentic D-isomer **130**. As expected the retention time of this isomeric

octapeptide corresponded with that of the minor in the couplings set out in Scheme 58.



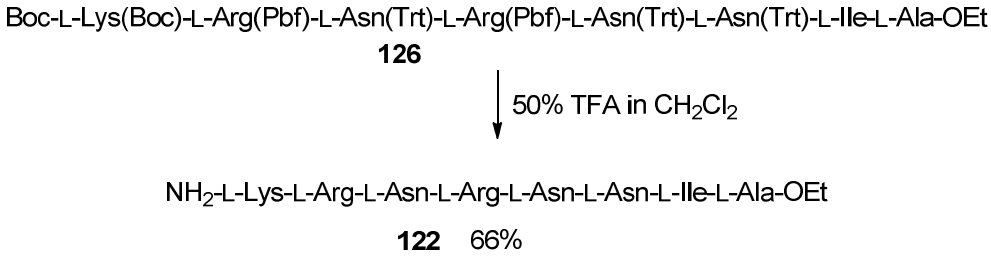
Scheme 59. Synthesis of an authentic D-isomer



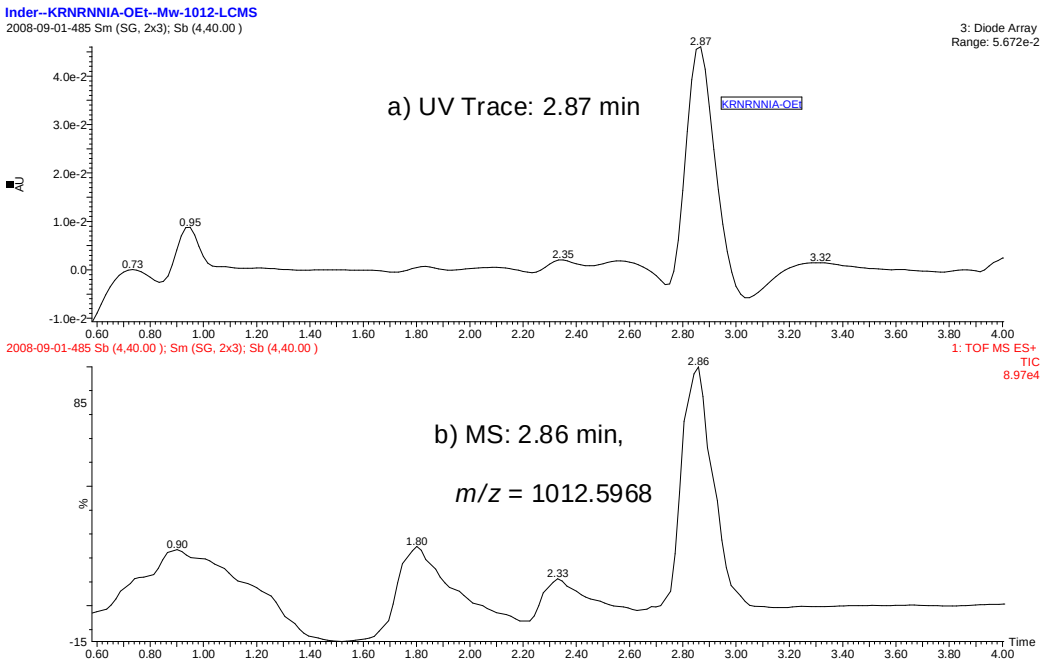
LCMS: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.57 min.

Figure 17. LCMS analysis of authentic D-isomer Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-D-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (130)

Finally, treatment of this octapeptide **126** with trifluoroacetic acid in dichloromethane removed the complete suite of acid-labile protecting groups, and afforded the octapeptide **122** as a single epimer in 66% yield after purification by reverse phase HPLC (Scheme 60, Figure 18 and 19).



Scheme 60. Deprotection of side chains



LCMS: 0-55% A over 3.5 min with a flow rate of 0.5 mL/min and 190 nm UV detection, retention time = 2.87 min.

Figure 18. LCMS analysis of NH_2-L-Lys-L-Arg-L-Asn-L-Arg-L-Asn-L-Asn-L-Ile-L-Ala-OEt (122)

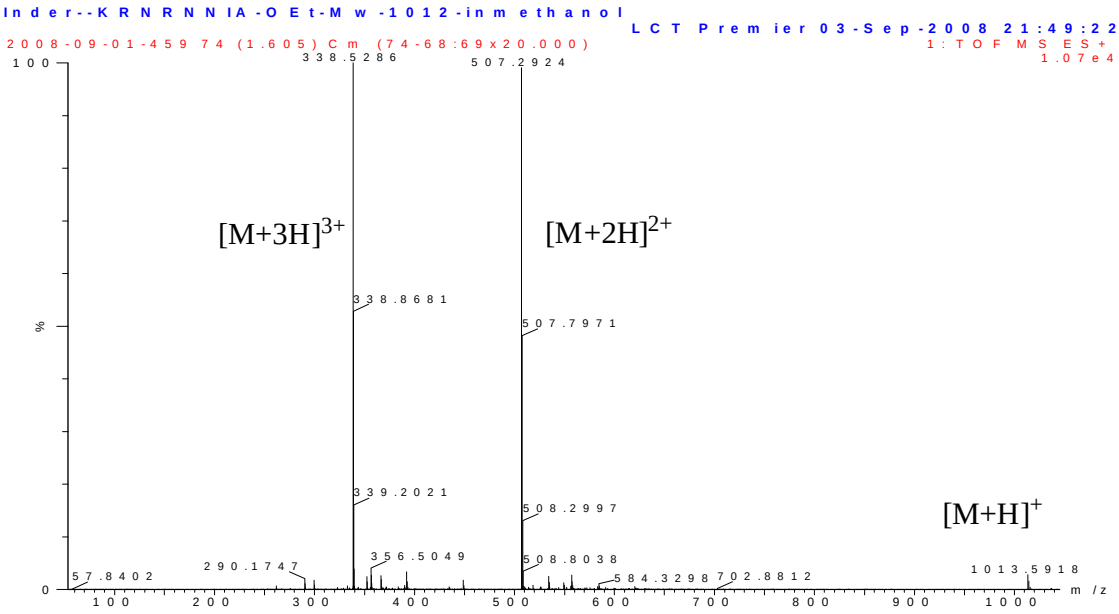


Figure 19. ESI-HRMS spectrum of the peak eluting at 286 min [NH_2-L-Lys-L-Arg-L-Asn-L-Arg-L-Asn-L-Asn-L-Ile-L-Ala-OEt (122)]

2.5. Summary

The high nucleophilicity of thiocarboxylates enables their preferential reaction with electron-deficient aromatic halides by a nucleophilic substitution process in the presence of amines. This method generates highly reactive thioesters *in situ*, which then react directly with the amine to afford peptide bonds. The method tolerates the presence of all proteinogenic amino acid side chains except for cysteine and lysine,[139] which must be protected. This new coupling method does not require the presence of any particular amino acid for coupling, in contrast to native chemical ligation and its variants,[27,146] and is capable of peptide bond forming reactions at hindered residues which makes this methodology ideal for the block synthesis of sterically hindered peptides.

CHAPTER 3

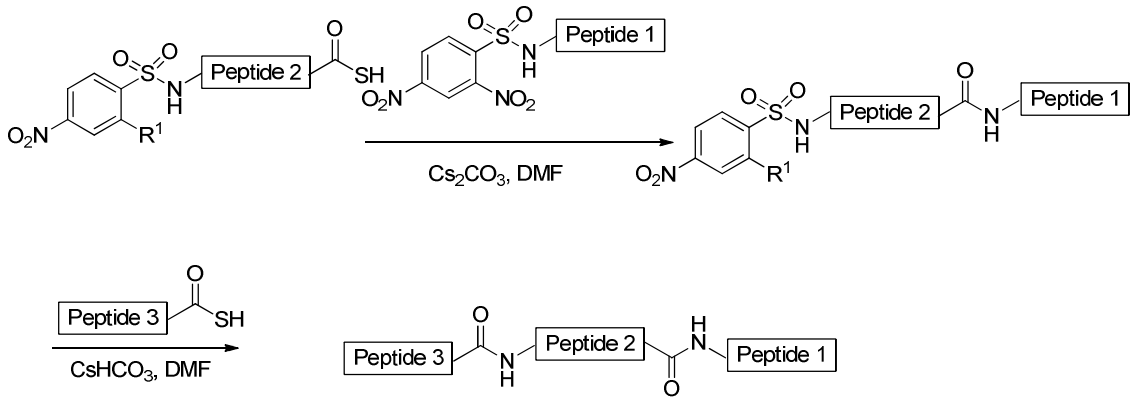
CONVERGENT SYNTHESIS OF PEPTIDES AND PEPTIDE THIOESTERS WITH REACTIVITY-DIFFERINTIATED SULFONAMIDES AND PEPTIDYL THIOACIDS

3.1 Introduction

As discussed in Chapter 1, convergent approaches to peptide synthesis based on NCL have been developed by Kent and co-workers for multiple fragment ligations.[19,87-89,91] These convergent approaches make repeated use of the NCL strategy and require double protection of a central fragment at the *N*-terminal Cys as well as at the *C*-terminal thioester.[91] The development of two approaches for the convergent synthesis for peptides and peptide thioesters is delineated in this chapter.

3.2 Convergent Synthesis of Peptides

As reported in chapter 2, the activation of thioacids by nucleophilic aromatic substitution in the presence of amines is a viable method for the formation of peptide bonds. The application of this method to a convergent approach involving multiple segment couplings and minimal protecting group manipulation would require a set of at least two sulfonamides with differential reactivity toward thiocarboxylates (Scheme 61).



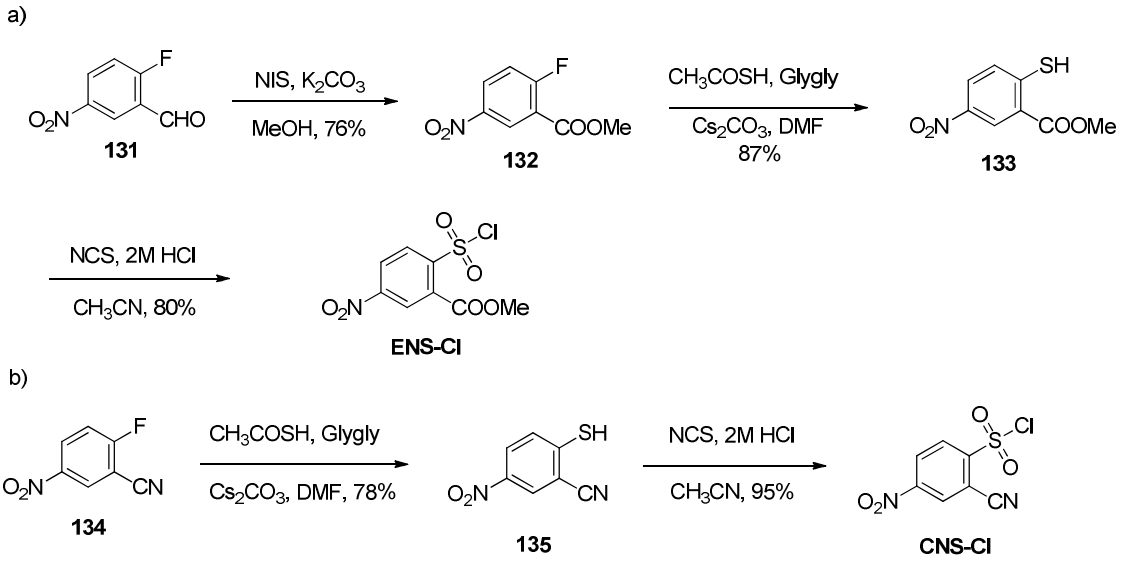
Scheme 61. One pot triblock peptide synthesis

In this proposed sequence a first peptide (peptide 1) representing the *C*-terminal segment of the eventual target is activated as the highly reactive dinitrobenzenesulfonamide. This segment is expected to react in a first step with peptide 2 which carries a *C*-terminal thioacid and an *N*-terminal sulfonamide that is less reactive than the dinitrobenzenesulfonamide. Finally, a second thioacid (peptide 3) is introduced and is caused to react with the remaining sulfonamide. The main challenge in this approach is the reactivity of the sulfonamide on peptide 2, which must be less than that of the dinitrobenzenesulfonamide, but yet sufficient to enable the second coupling to take place. A second challenge is represented by the need to synthesize the second peptide unit without self-condensation.

3.3 Preparation of Sulfonyl Chlorides

As a very first step towards the convergent approach, a series of benzene sulfonyl chlorides having different electron withdrawing groups around the aromatic ring was prepared. All the sulfonyl chlorides in the series were commercially available except two (ENS-Cl and CNS-Cl), which were

synthesized from the commercially available starting materials **131** and **134** as described in Scheme 62.



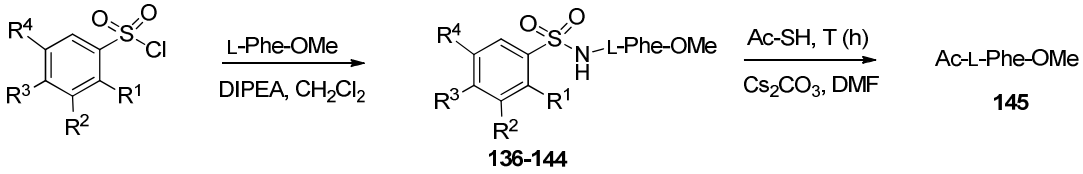
Scheme 62. Synthesis of sulfonyl chlorides

An interesting point to note in this synthesis is the use of nucleophilic aromatic substitution approach described in Chapter 2 for the synthesis of the thiol, which was then subjected to oxidation with NCS[151] to provide the corresponding sulfonyl chloride.

3.4. Screening of *N*-Arenesulfonylphenylalanine Derivatives for Reaction with Thioacetic Acid

With the sulfonyl chlorides in hand, a series of *N*-arenesulfonylphenylalanine derivatives was prepared and then screened for reactivity toward thioacetic acid (Table 5). All the reactions were run under a standard set of conditions and employed 0.15 M sulfonamide in DMF with thioacetic acid (1.5 equiv), and Cs_2CO_3 (1.5 equiv) as base.

Table 5. Reactivity of thioacetic acid toward *N*-arenesulfonylphenylalanines



Entry	R^1	R^2	R^3	R^4	Ar-SO_2-NH-L-Phe-OMe (Yield, %)	T (h)	Ac-Phe-OMe 145 (Yield, %)
1	H	H	Ac	H	**136** (88)	12	-
2	H	H	CN	H	**137** (92)	12	-
3	CF_3	H	H	H	**138** (91)	12	-
4	NO_2	H	H	H	**139** (90)	12	-
5	H	CF_3	H	CF_3	**140** (93)	12	-
6	COOMe	H	NO_2	H	**141** (92)	6	85
7	CN	H	NO_2	H	**142** (91)	6	87
8	NO_2	H	CF_3	H	**143** (90)	4	88
9	NO_2	H	NO_2	H	**144** (71)	0.07	94

It is clear from Table 5 that a single electron-withdrawing group is insufficient to induce the desired reaction, as was the presence of two trifluoromethyl groups in the 3- and 5-positions. However, 2,4-disubstituted systems in which a single nitro group was complemented by a second, but less potent electron-withdrawing group functioned nicely (entry 6, 7 and 8). The ENS and the CNS systems were found to be less reactive than the FNS system (Figure 19). All three such systems (ENS, CNS and FNS) investigated proved significantly less reactive than the 2,4-dinitrobenzenesulfonamide (entry 9) and therefore met the reactivity criteria for the block synthesis approach.

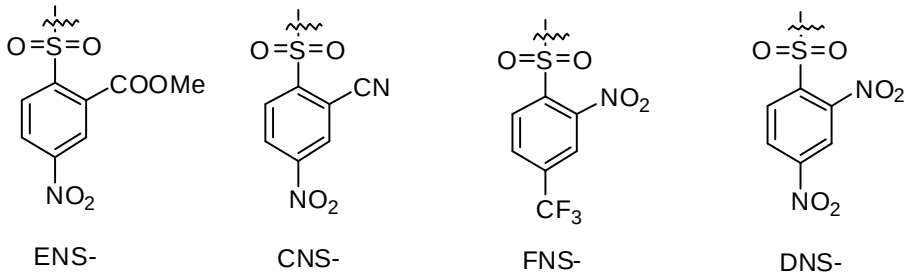
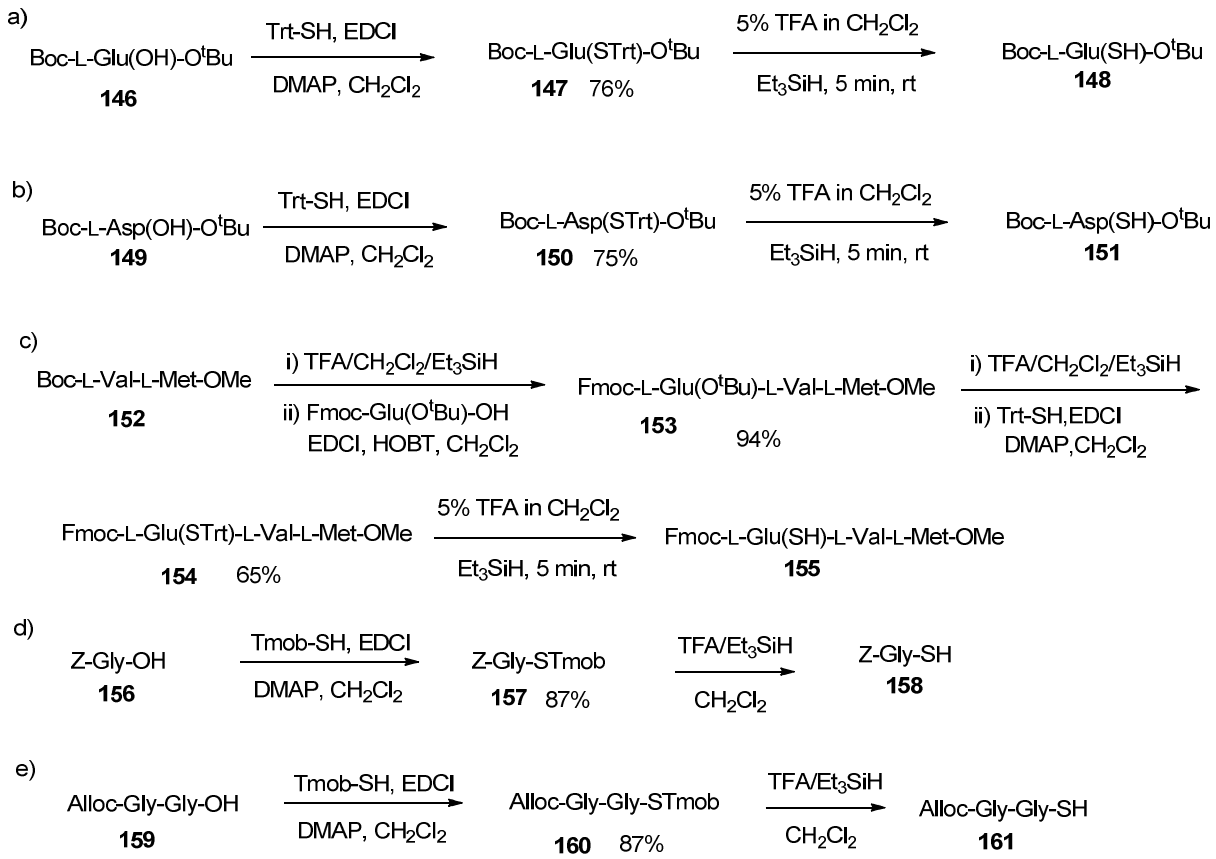


Figure 19. The ENS, CNS, FNS and DNS systems

3.5 Synthesis of Thioacids

For a more elaborate study of the reactivity pattern, side chain peptidyl thioacids of glutamic acid and aspartic acid along with α-amino derived thioacids such as glycine thioacids were prepared as described in Scheme 63. The side chain amino thioacids were obtained in the form of the trityl esters, while glycine derived thioacids were equipped with Tmob groups. The thioacids were released from the corresponding thioesters after treatment with TFA and triethylsilane.



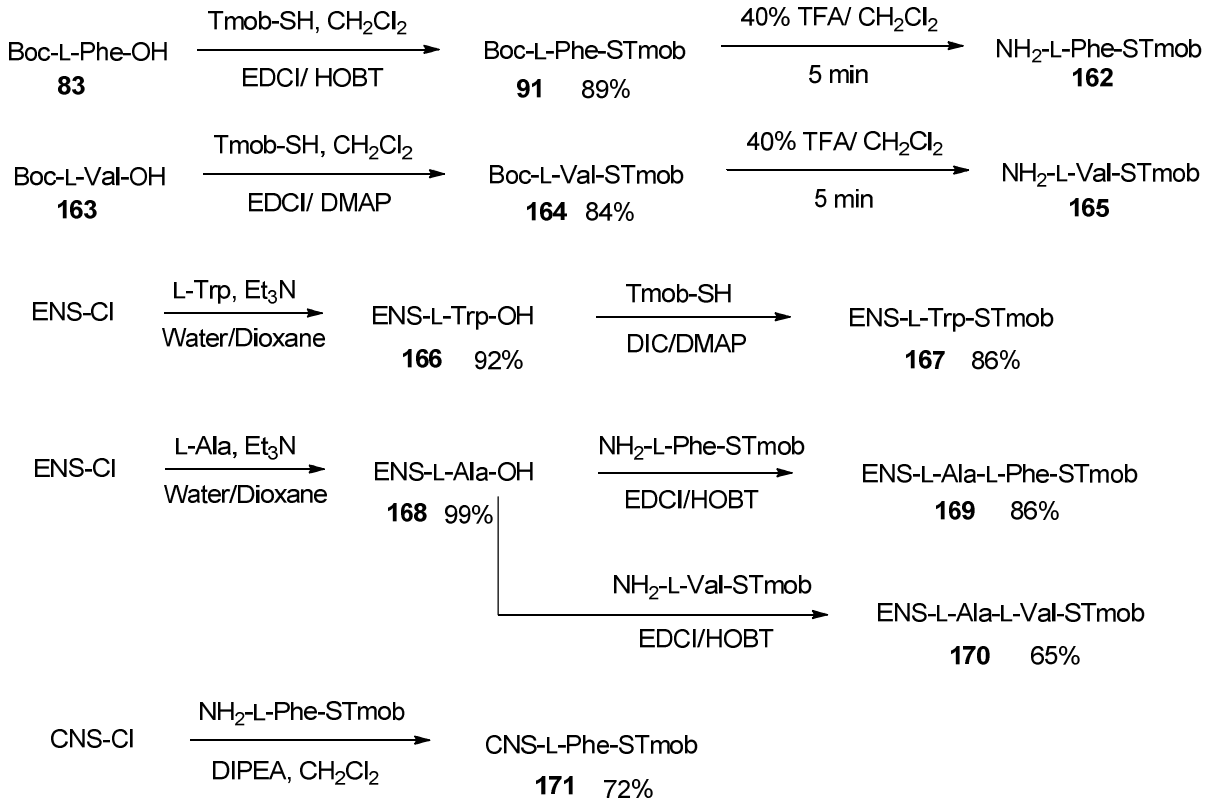
Scheme 63. Synthesis of thioacids

The synthesis of the glutamic and aspartic thioacids is noteworthy in so far as it establishes the possibility of removing a Trt group in the presence of the Boc group under acidic conditions.

3.6 Synthesis of the ENS and the CNS Protected Peptidyl Thioesters

Following the literature, the ENS protected α-amino acids were prepared from the reaction of α-amino acids and the ENS chloride in presence of triethylamine (Scheme 67). These ENS protected α-amino acids were coupled either with the 2,4,6-trimethoxybenzyl thiol to provide the ENS protected α-amino thioester **167** or with an α-amino thioester **165** (Prepared by selective removing of a Boc group in the presence of Tmob thioester under acidic conditions) to

provide the ENS protected di-peptidyl thioesters **169** and **170**. The CNS protected α-amino thioester was directly synthesized by the reaction of the CNS chloride and α-amino thioester in the presence of diisopropylethylamine in methylene chloride in good yield (Scheme 64).

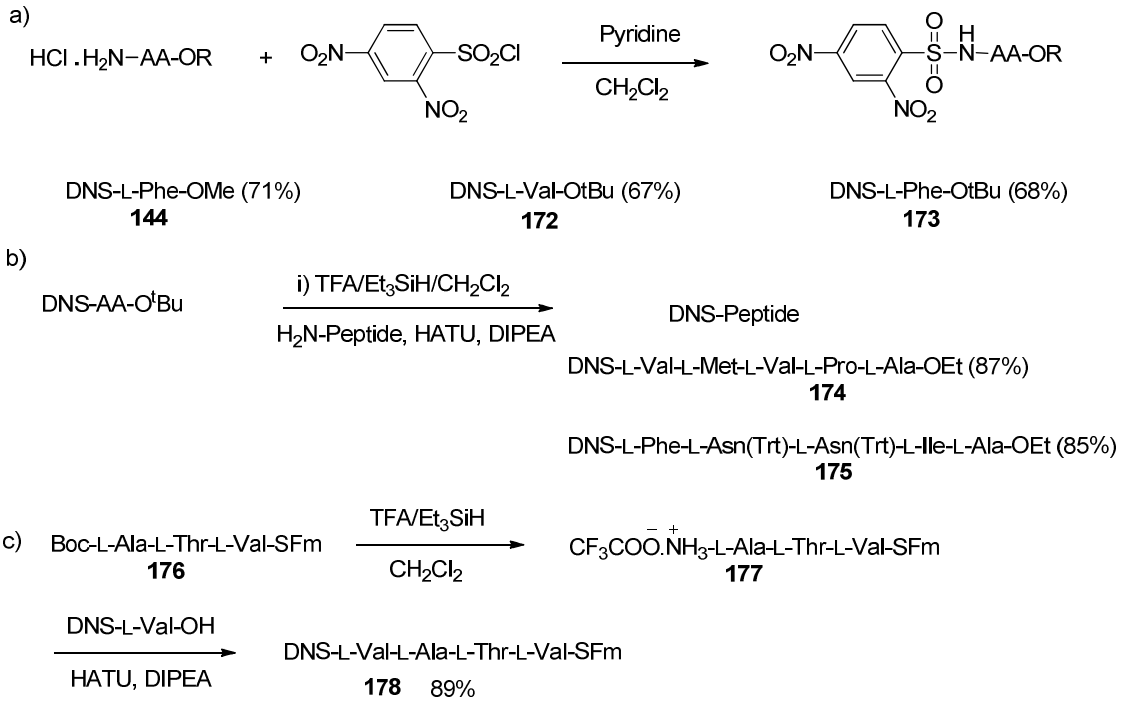


Scheme 64. Synthesis of the sulfonamide protected peptidyl thioesters

3.7 Synthesis of the DNS Peptides

Following the literature,[165] several *N*-[(2,4-dinitrobenzene)sulfonyl]-α-amino acid alkyl esters were prepared from the reaction of the hydrochloride salts of α-amino acid esters and 2,4-dinitrobenzenesulfonyl chloride in presence of pyridine (Scheme 65). However, these conditions were found to be unsuitable for the synthesis of the higher DNS-peptides as the reactions of peptidyl amines and 2,4-dinitrobenzenesulfonyl chloride did not proceed smoothly. To circumvent

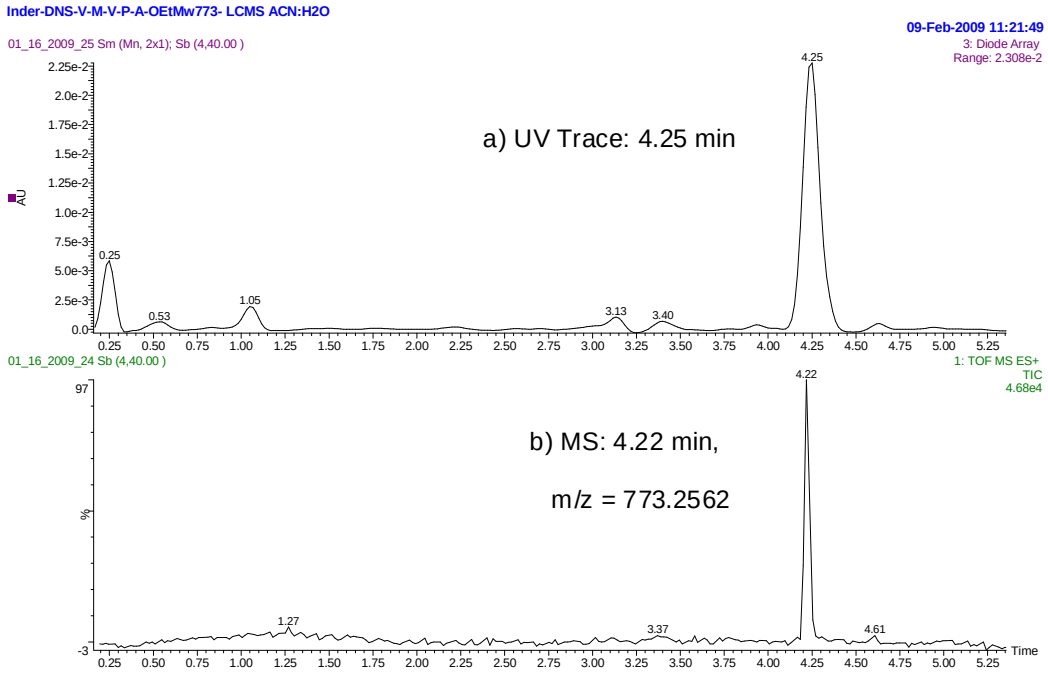
this incompatibility, a different route was investigated, which involved the synthesis a *N*-[(2,4-dinitrobenzene)sulfonyl]-α-amino acid *tert* butyl ester by the reaction of the corresponding hydrochloride salts of α-amino acid *tert* butyl esters and 2,4-dinitrobenzenesulfonyl chloride in presence of pyridine (Scheme 68) followed by the cleavage of the *tert* butyl group under acidic conditions. This route gave access to *N*-[(2,4-dinitrobenzene)sulfonyl]-α-amino acids, that were then coupled with the peptidyl amines by the HATU method. Several DNS peptides were accessed in this way in excellent yield as described in Scheme 65.



Scheme 65. Synthesis of the DNS peptides

The last entry in Scheme 65 is noteworthy as a Boc group was removed from a peptide SFm thioester under acidic conditions to generate a trifluoroacetate salt of peptide thioester lacking protection at the *N*-terminus. As thioesters are vulnerable to cyclization with the free *N*-terminal amine, the salt was first mixed with the *N*-[(2,4-dinitrobenzene)sulfonyl]-α-valine and HATU in

methylene chloride and then was neutralized with DIPEA to provide a DNS protected peptide thioester **178**. The LCMS traces and ESI-TOF mass spectra of all the DNS peptides so-prepared are presented in Figures **20-25**.



LCMS: 5-20% B over 1 min then 20-55% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 4.25 min.

Figure 20. LCMS analysis of DNS-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (174)

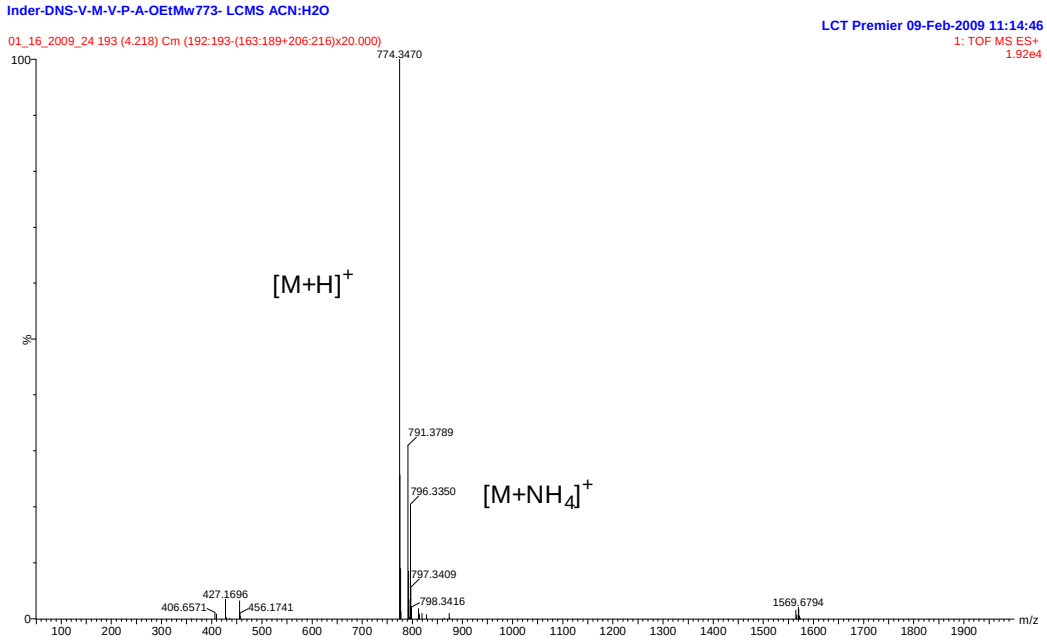
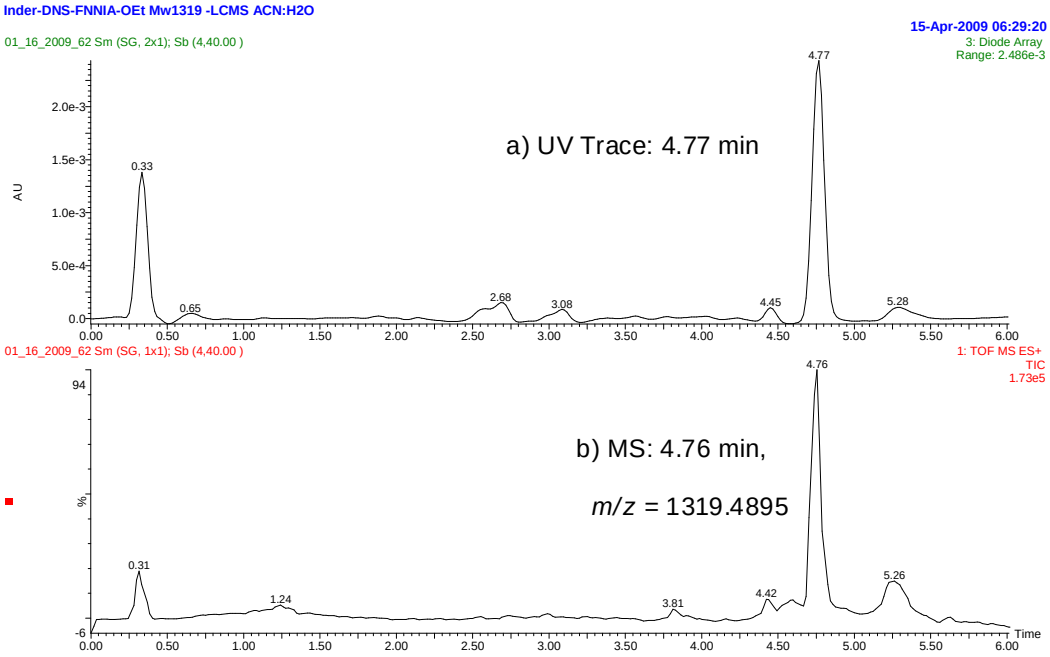


Figure 21. ESI-HRMS spectrum of the peak eluting at 4.22 min [DNS-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (174)]



LCMS: 5-98% B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.77 min.

Figure 22. LCMS analysis of the DNS-L-Phe-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (175)

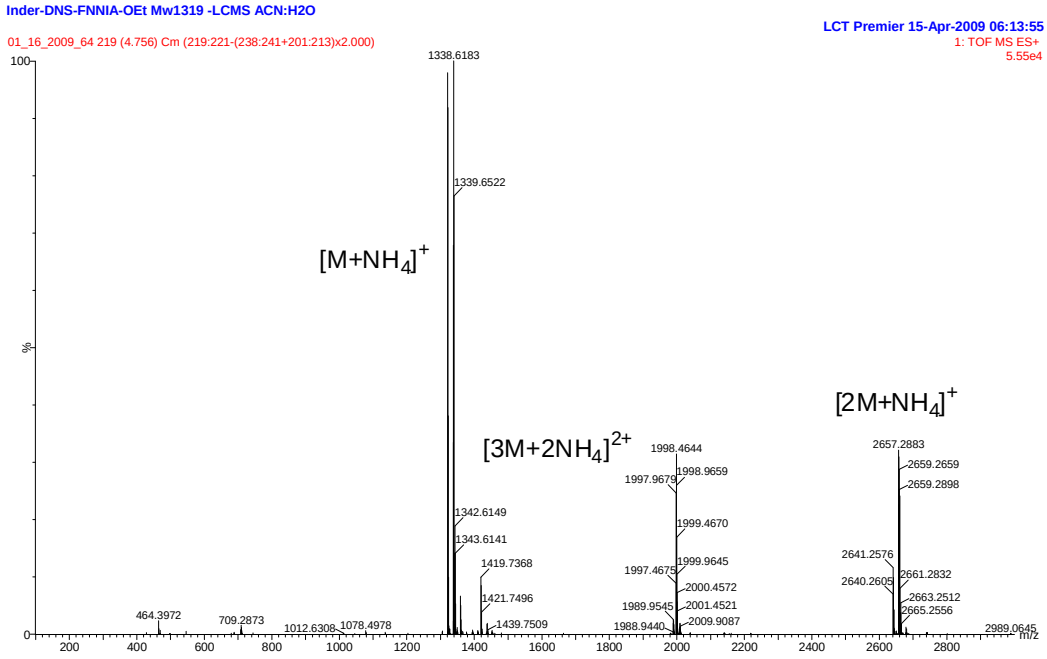
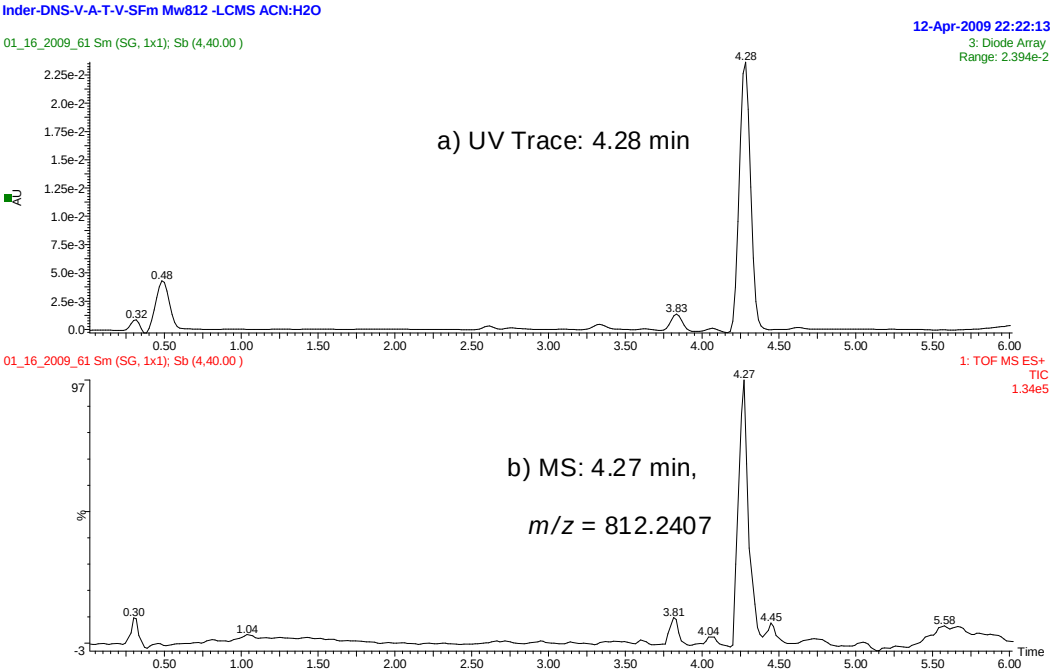


Figure 23. ESI-HRMS spectrum of the peak eluting at 4.76 min [DNS-L-Phe-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (175)]



LCMS: 5-98 % B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.28 min.

Figure 24. LCMS analysis of the DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (178)

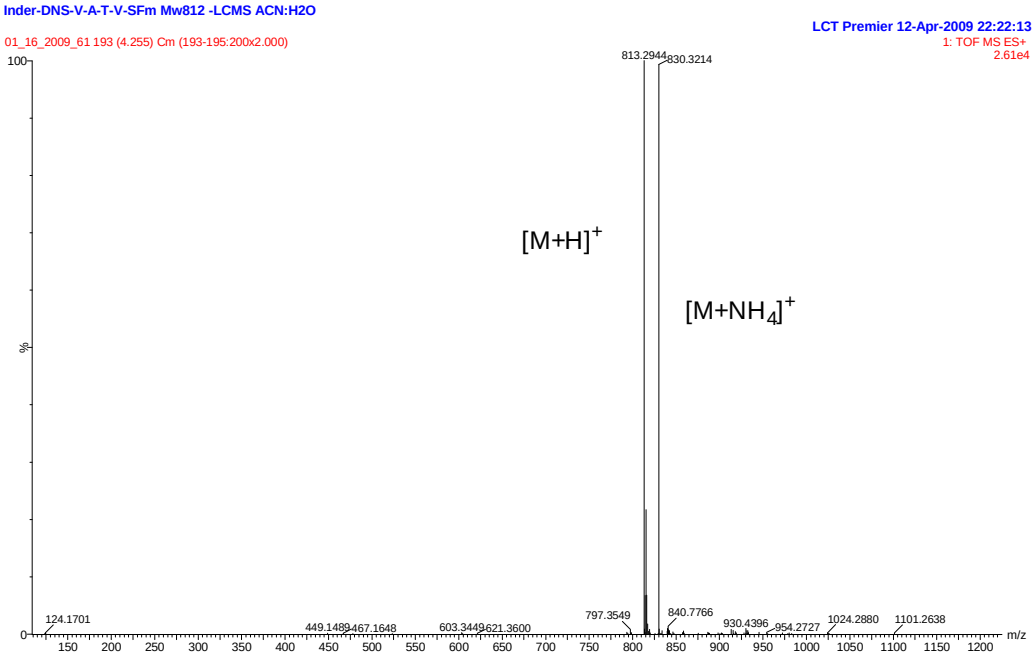
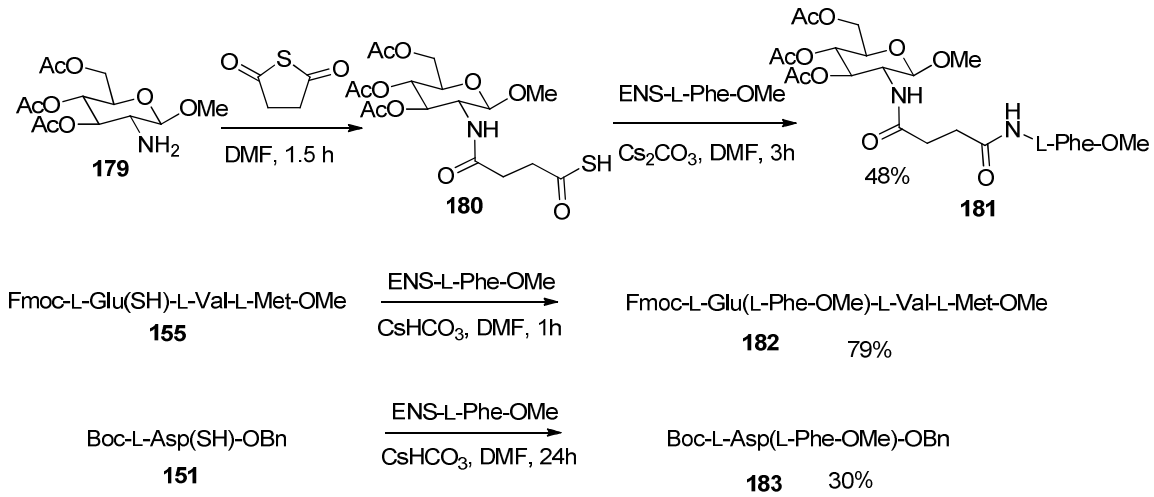


Figure 25. ESI-HRMS spectrum of the peak eluting at 4.27 min [DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (178)]

3.8 Triply Convergent Synthesis

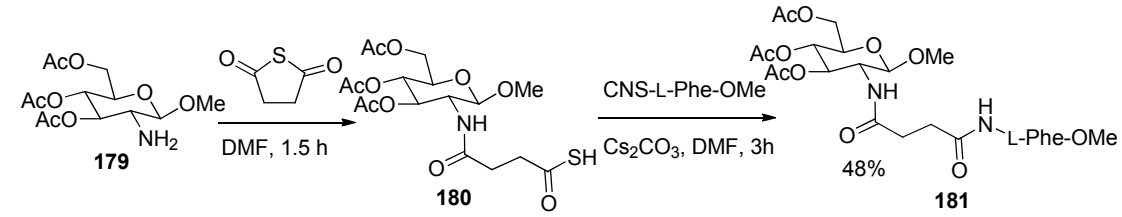
A further series of experiments was conducted with the side chain peptidyl thioacids to probe the reactivates of both the CNS and the ENS sulfonamides. To this end the ENS sulfonamides were first used in a three component coupling sequence, which involved the ring opening of thiosuccinic anhydride with a sugar-based amine **179** to release a primary thioacid **180** followed by trapping with the ENS sulfonamide to provide a glycoconjugate **181** (Scheme 66). Next, the reactivity of the ENS sulfonamides was further checked with the glutamic acid and aspartic side chain thioacids. It was found that the ENS sulphonamides were amenable to reaction with the glutamic side chain thioacids **155** but reacted only slowly with the corresponding aspartic thioacid **151** (Scheme 66). Moreover, the

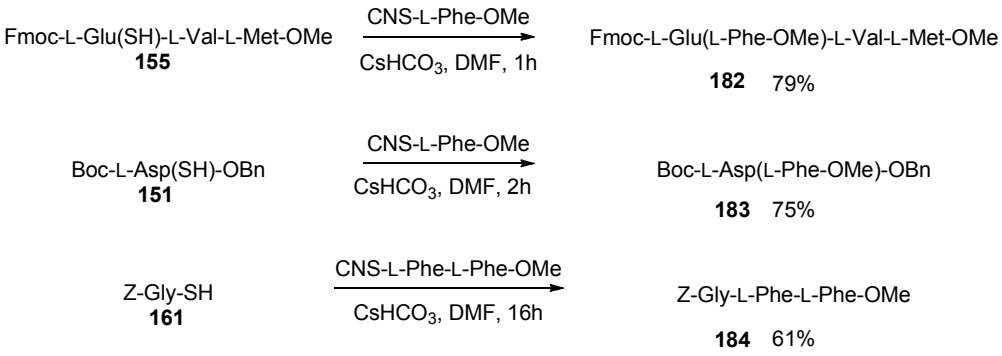
ENS sulfonamides either failed to react or reacted only very slowly with peptide-based thioacids such as Z-Gly-SH (**158**) or Alloc-Gly-Gly-SH (**161**).



Scheme 66. Reactivity of the ENS group

The CNS sulfonamides were also tested in the same three component coupling sequence, and were screened for reactivity toward side chain thioacids and peptide-based thioacids such as Z-gly-SH **158** (Scheme 67). The CNS sulfonamides were found to be more reactive than the corresponding ENS sulfonamides as demonstrated by the synthesis of the tripeptide Z-Gly-L-Phe-L-Phe-OMe **184** (Schemes 67). Nevertheless, the CNS sulfonamides either failed to react or reacted only very slowly with peptide-based thioacids such as Alloc-Gly-Gly-SH (**161**).



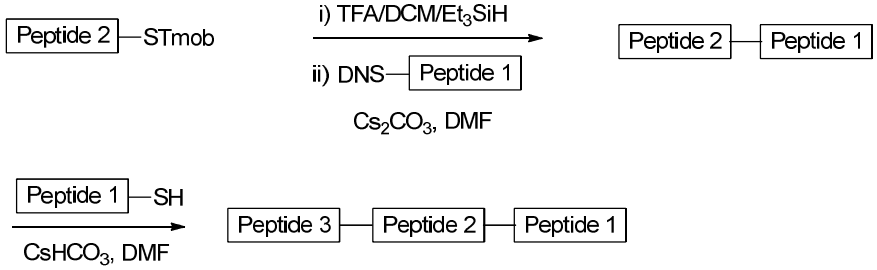


Scheme 67. Reactivity of the CNS group

While not ideal, the overall reactivity pattern of the ENS and the CNS sulfonamides was sufficient to enable a first series of triply convergent reactions employing an amino acid or peptide protected at the *N*-terminus by an ENS or CNS group and carrying a 2,4,6-trimethoxybenzy thioester at the *C*-terminus. These sulfonamide protected Tmob thioesters were first treated with triethylsilane and trifluoroacetic acid to release the *C*-terminal thioacids and were then exposed to a DNS protected peptide followed by addition of a mild base (Table 6). This critical series of experiments established the feasibility of generation of a thioacid in the presence of a moderately electron-deficient sulfonamide though the use of the acid labile Tmob thioesters, and the ability of that thioacid to undergo subsequent and selective condensation with the more reactive DNS-class of sulfonamides on addition of a mild base. This first coupling resulted in the formation of new peptides bearing the ENS or CNS group at the *N*-terminus

ready for subsequent coupling with a further thioacid, albeit necessarily a primary one (Table 6).

Table 6: Triply Convergent Synthesis

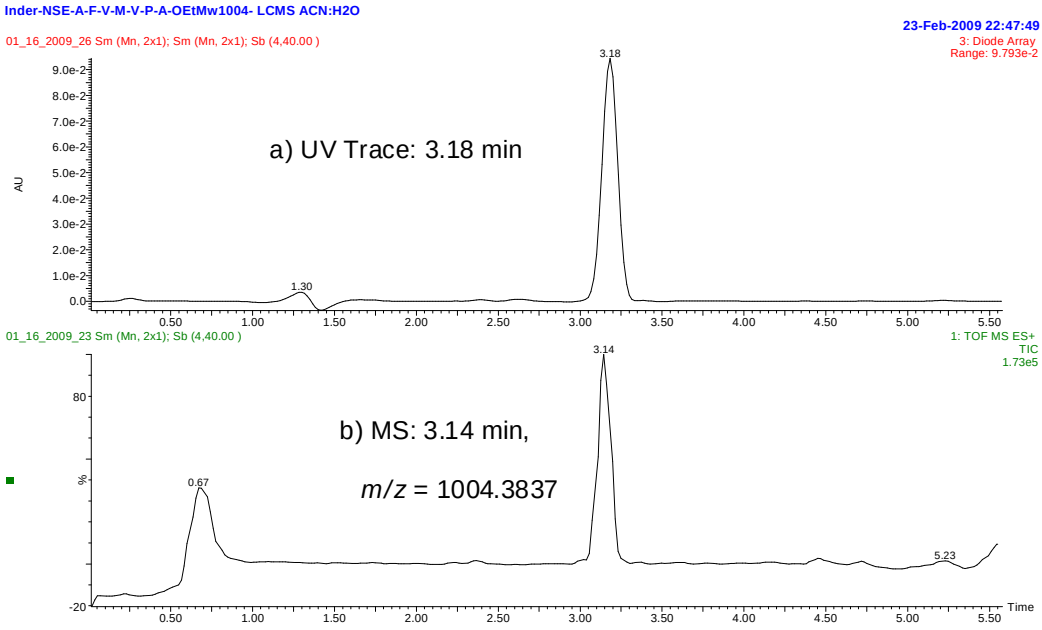


Entry	Peptide 2-STmob	DNS-Peptide 1	Peptide 2-Peptide 1 (Yield, %)	Peptide 3-SH	Peptide 3-Peptide 2-Peptide 1 (Yield, %)
1	ENS-Trp-STmob (**167**)	DNS-Phe-OMe (**144**)	ENS-Trp-Phe-OMe **185**	Ac-SH	Ac-Trp-Phe-OMe **190** (60)
2	CNS-Phe-STmob (**171**)	DNS-Phe-OMe (**144**)	CNS-Phe-Phe-OMe **186** (84)	Boc-Glu(SH)-O^{t}Bu (**148**)	Boc-Glu(Phe-Phe-OMe)-O^{t}Bu **191** (81)
3	CNS-Phe-STmob (**171**)	DNS-Phe-OMe (**144**)	CNS-Phe-Phe-OMe **186** (84)	Boc-Asp(SH) – Obn (**151**)	Boc-Asp(Phe-Phe-OMe)-OBn **192** (78)
4	ENS-Ala-Phe-STmob (**169**)	DNS-Phe-OMe (**144**)	ENS-Ala-Phe-Phe-OMe **187** (81)	Boc-Glu(SH)-O^{t}Bu (**148**)	Boc-Glu(Ala-Phe-Phe-OMe)-O^{t}Bu **193** (83)
5	ENS-Ala-Phe-STmob	DNS-Val-Met-Val-Pro-Ala-	ENS-Ala-Phe-Val-Met-Val-Pro-Ala-OEt	Boc-Glu(SH)-O^{t}Bu (**148**)	Boc-Glu(Ala-Phe-Val-Met-Val-Pro-Ala-OEt)-O^{t}Bu

	(**169**)	OEt (**174**)	**188** (81)		**194** (71)
6	ENS-Ala-Val-STmob (**170**)	DNS-Val-Met-Val-Pro-Ala-OEt (**174**)	ENS-Ala-Val-Val-Met-Val-Pro-Ala-OEt **189** (78)	Boc-Glu(SH)-O^{t}Bu (**148**)	Boc-Glu(Ala-Val-Val-Met-Val-Pro-Ala-OEt)-O^{t}Bu **195** (65)
7	ENS-Ala-Phe-STmob (**169**)	DNS-Phe-OMe (**144**)	ENS-Ala-Phe-Phe-OMe **187** (74)	Boc-Glu(SH)-O^{t}Bu (**148**)	Boc-Glu(Ala-Phe-Phe-OMe)-O^{t}Bu **193** (80)

The feasibility of a one pot triply convergent synthesis by this methodology was demonstrated by the successful synthesis of the dipeptide Ac-Trp-Phe-OMe **190** in moderate yield (Table 6, entry 1). The reaction sequence was also applied successfully to synthesis of simple tri- and tetrapeptides (Table 6, entries 2, 3 and 4) and to the synthesis of model octapeptides (Table 6, entries 5 and 6). The ability to form relatively hindered peptide bonds such as Val-Val is evident from entry 6 (Table 6). The diastereomeric purity of all peptides produced in this way was controlled by LCMS, with examples of the chromatograms given in in Figures 26-29.

Finally, the sequence was shown to be amenable to the use of aqueous buffered media, rather than DMF as solvent, with little loss of yield as is clear from a comparison of entries 4 and 7 of Table 6.



LCMS: 5-30% B over 1 min then 30-60% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 3.18 min.

Figure 26. LCMS analysis of the ENS-L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (188)

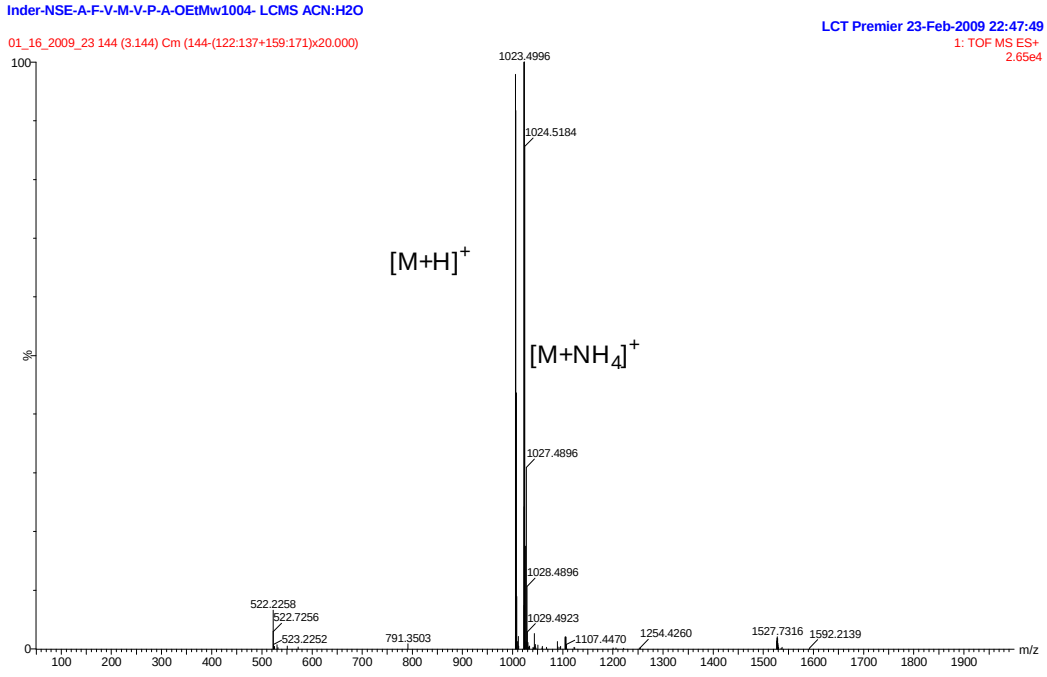
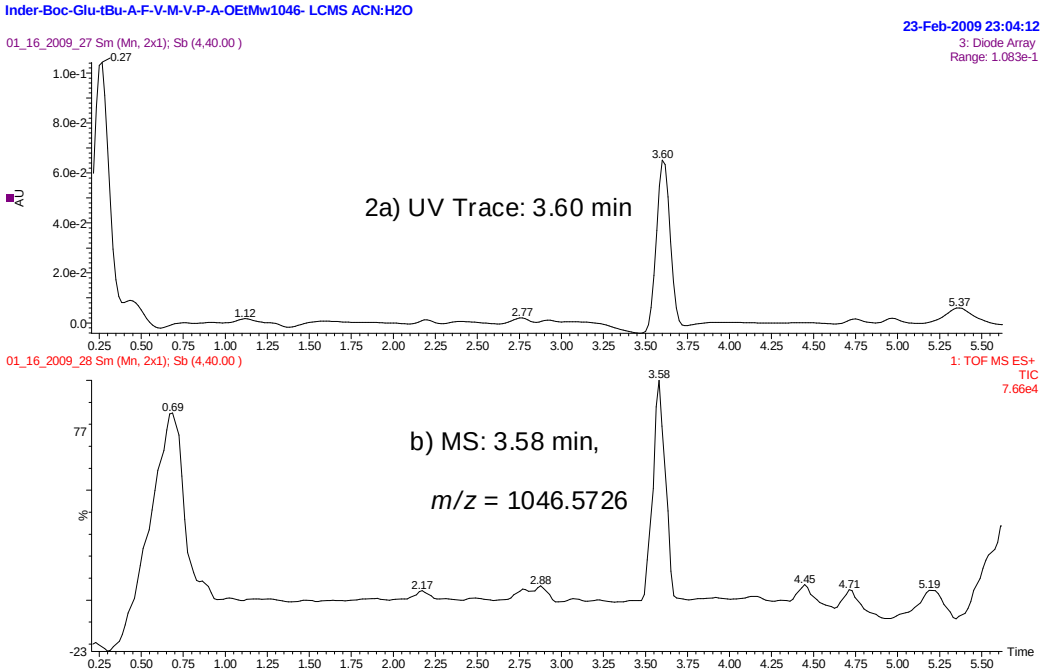


Figure 27. ESI-HRMS spectrum of the peak eluting at 3.14 min[ENS-L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (188)]



LCMS: 5-30% B over 1 min then 30-60% B over 4 min with a flow rate of 0.7 mL/min and 195 nm UV detection, retention time = 3.60 min.

Figure 28. LCMS analysis of Boc-L-Glu(L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt)-OtBu (194)

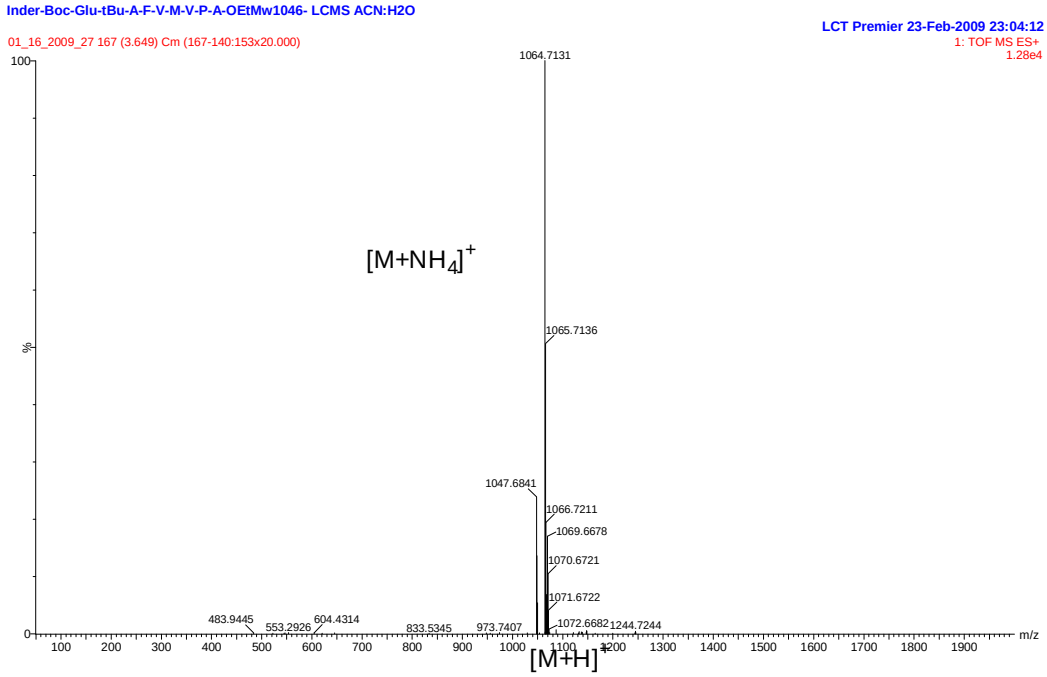
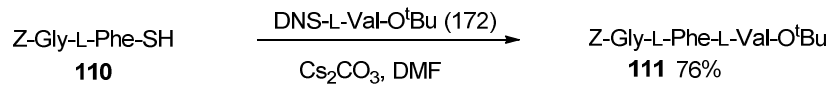


Figure 29. ESI-HRMS spectrum of the peak aluting at 3.58 min (Boc-L-Glu(L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt)-OtBu)

3.9 Verification of the Extent of Epimerization in the DNS Coupling

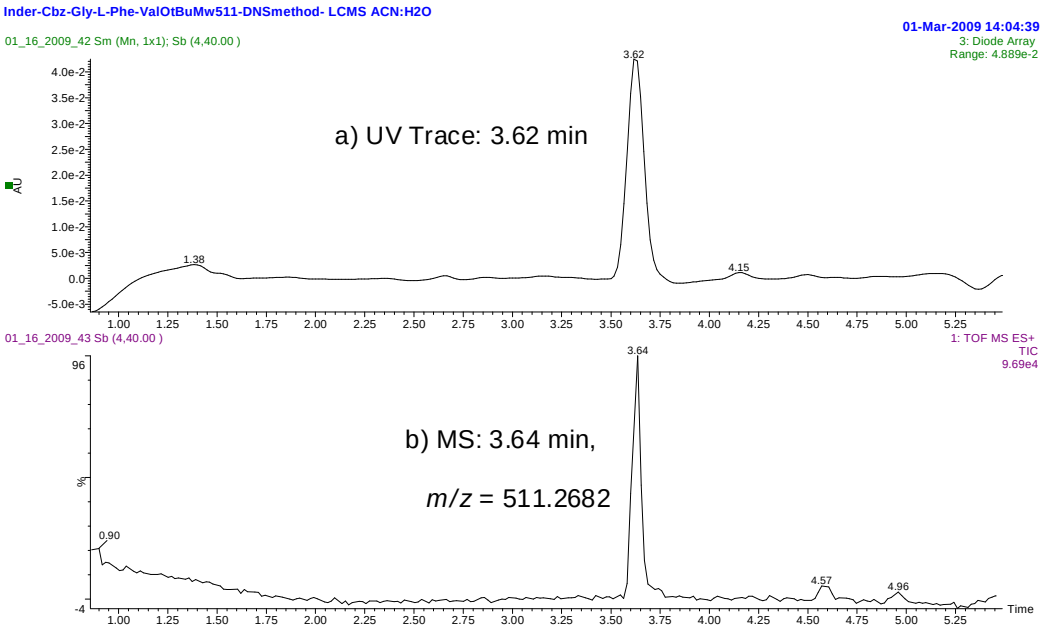
To investigate the extent of epimerization during the thioacid-sulfonamide coupling, a tripeptide Z-Gly-L-Phe-L-Val-O^{t}Bu **111** was synthesized by the coupling of a dipeptide thioacid Z-Gly-L-Phe-SH **110** with the DNS-L-Val-O^{t}Bu **172** in DMF (Scheme 68).



Scheme 68. Synthesis of Z-Gly-L-Phe-L-Val-O^{t}Bu (111) by the DNS coupling method

LCMS analysis of the tripeptide **111** produced by this sequence shows a single peak with a retention time of 3.65 min with a molecular weight

corresponding to the tripeptide **111** (Figure 30), consistent with an epimerization free reaction.



LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

Figure 30. LCMS analysis of Z-Gly-L-Phe-L-Val-OtBu (111) from the DNS coupling

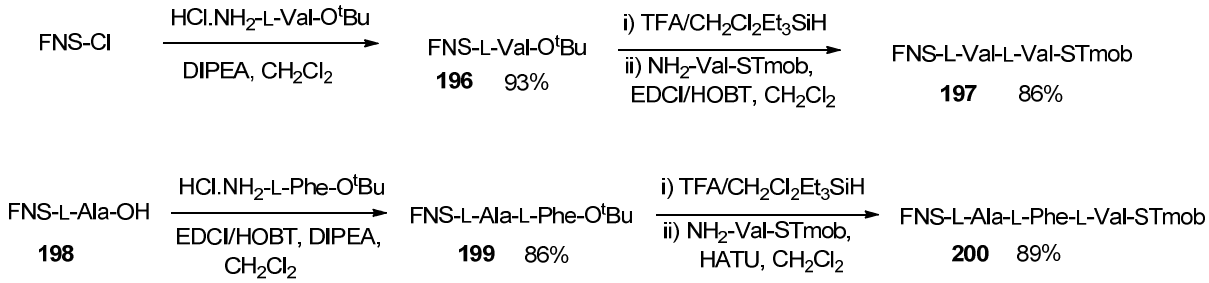
To verify that the LC conditions were capable of resolving the epimeric peptides, a further sample of Z-Gly-L-Phe-Val-OtBu (**111**) was synthesized by EDCI coupling under conditions likely to produce epimerization akin to the ones described in Chapter 2. LCMS of this crude reaction mixture showed essentially full epimerization (ratio, L/D = 48/52) with two well resolved peaks having retention times 3.65 min and 3.78 min (Chapter 2, Figure 7).

The lack of epimerization in the coupling of the thioacid using the DNS coupling method was further confirmed by synthesis of an authentic sample of Z-Gly-D-

Phe-Val-OtBu (**116**) as described in Chapter 2, which was shown to have a different retention time (Chapter 2, Figure 8).

3.10 Synthesis of the FNS Protected Peptidyl Thioesters

Synthesis of the *N*-FNS protected di-peptide thioester FNS-L-Val-L-Val-STmob (**197**) involved the preparation of a FNS α-valine *tert* butyl ester (**196**) by the reaction of the corresponding hydrochloride salts of α-valine *tert* butyl ester and the FNS chloride in presence of diisopropylethyl amine (Scheme 69) followed by the cleavage of the *tert* butyl group under acidic conditions. This route gave access to a FNS protected α-valine acid, that was then coupled with the valine thioester amine **165** (NH_2-L-Val-STmob) by the EDCI/HOBT method to access the FNS protected Val-Val thioester in good yield (Scheme 69).



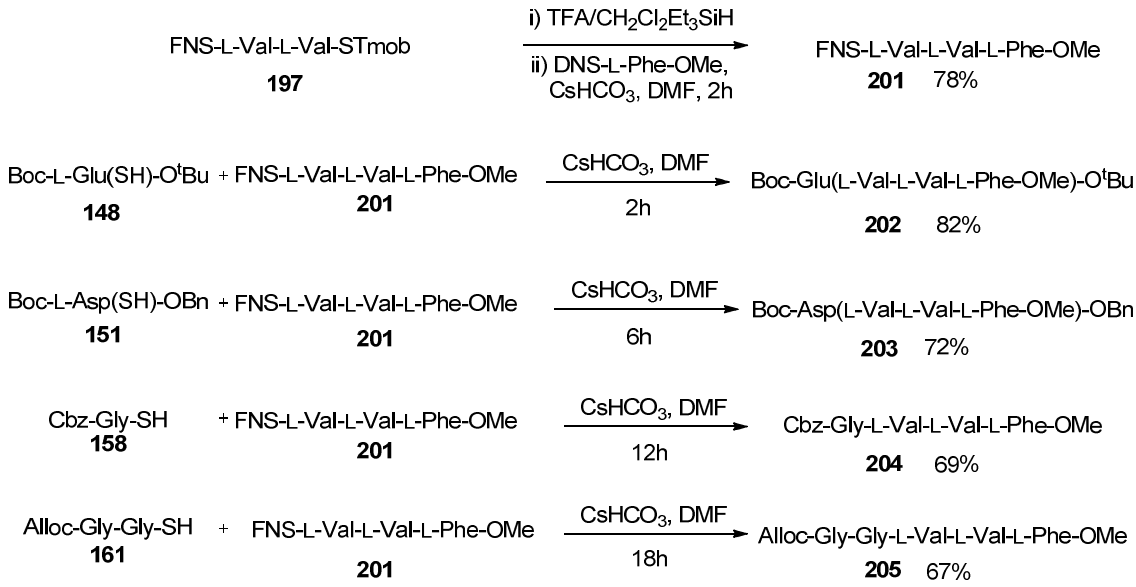
Scheme 69. Synthesis of the FNS thioesters

A left to right strategy was applied to the synthesis of the *N*-FNS protected tri-peptide thioester, which involved the preparation of a FNS protected di-peptide *tert* butyl ester by the reaction of the corresponding hydrochloride salts of α-amino acid *tert* butyl ester **199** and the FNS protected α-amino acid **198** with EDCI/HOBT coupling method (Scheme 69) followed by the cleavage of the *tert* butyl group under acidic conditions. This route gave access to a FNS protected di-peptide acid that was then coupled with the valine thioester amine **165** (NH_2-L-

Val-STmob) by the HATU method to access the FNS protected tri-peptide thioester **200** in excellent yield.

3.11 The Left to Right Strategy using the FNS group

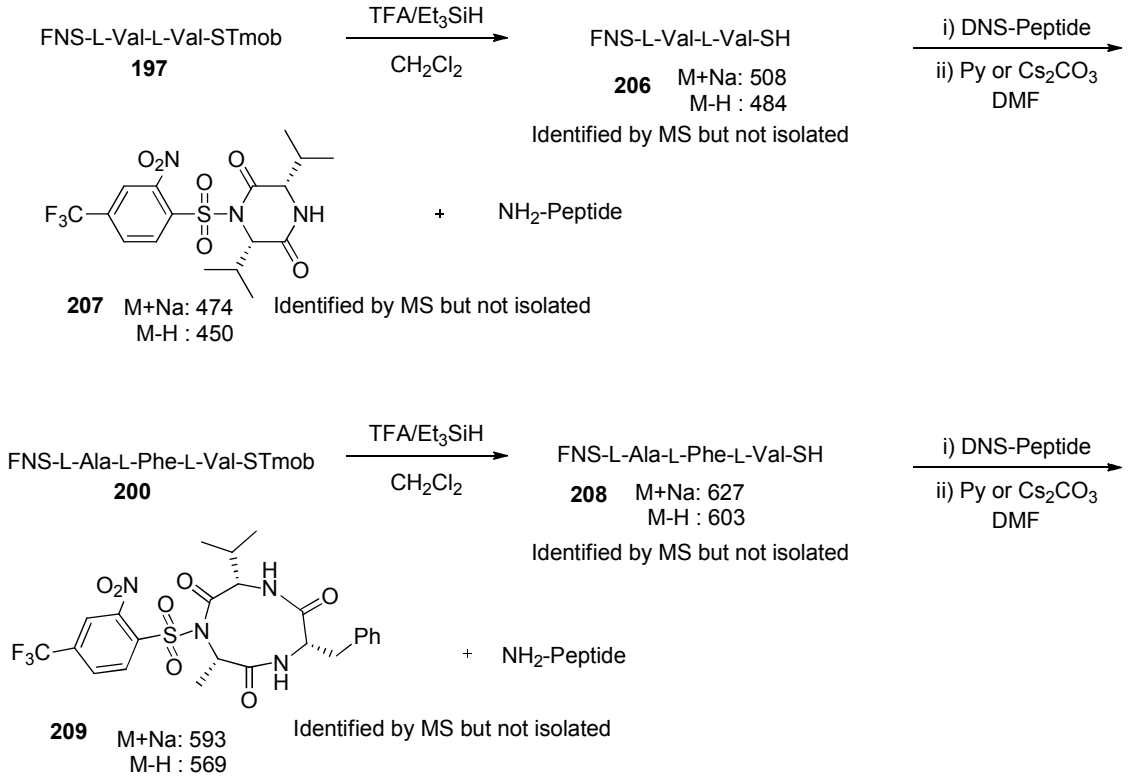
Further investigation revealed the FNS group to be somewhat more reactive than the CNS and the ENS groups toward the thiocarboxylates as illustrated by the examples in Scheme 70. These reactions proceeded in good yield with α-amino-derived thioacids unlike the case of the CNS and the ENS derivatives, but still took significantly more time than the side chain amino thioacids.



Scheme 70. Reactivity of the FNS group toward thiocarboxylates

A critical point in the use of the FNS group was the switch from cesium carbonate to cesium bicarbonate for the coupling reaction. With cesium carbonate as base the *N*-FNS protected di- and higher peptides were observed by mass spectrometry to undergo cyclization, with retention of the FNS group, rather than condensation when exposed to a DNS peptide and cesium

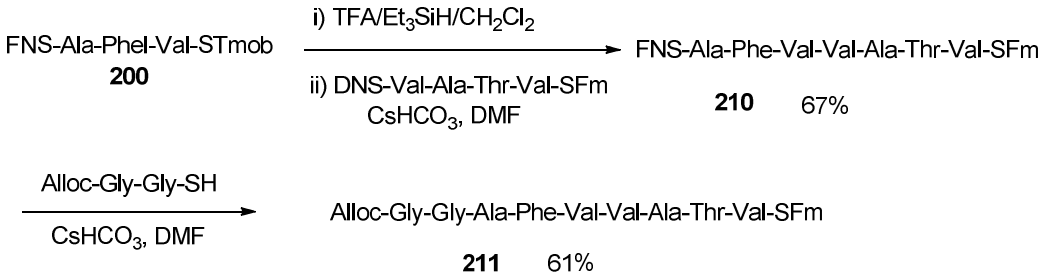
carbonate. This was attributed to the acidity of the FNS sulfonamide NH group and so was circumvented by the use of the milder base. The use of pyridine as base also afforded a similar result to cesium carbonate (Scheme 71).



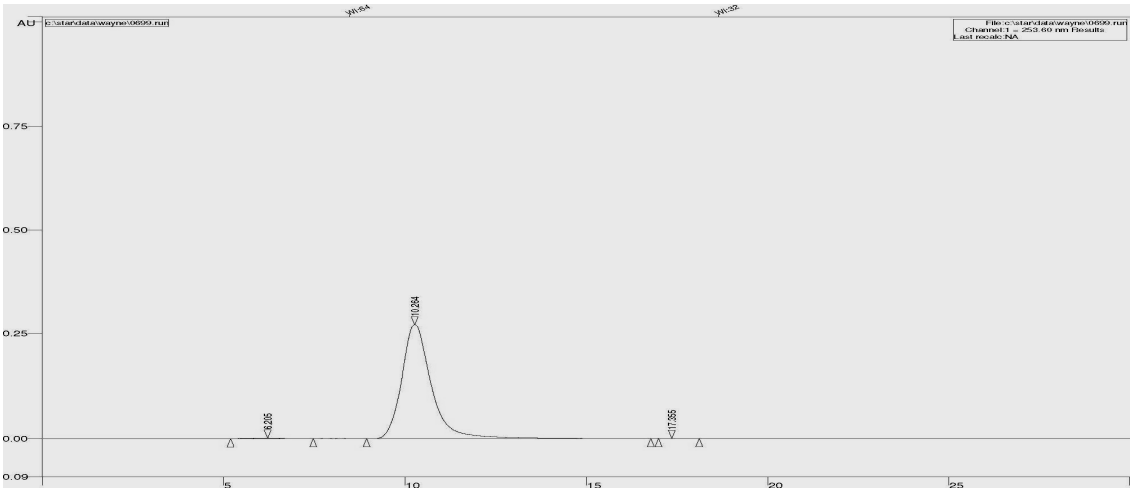
Scheme 71. Rationalization of problems encountered with the use of Cs_2CO_3 and pyridine in the activation of *N*-FNS peptide thioacids

This block synthesis strategy based on the thiocarboxylate-sulfonamide coupling is complementary to the methods developed by Kent based on native chemical ligation. However, for maximum flexibility in approaching future targets, compatibility of the thioester functionality with the thiocarboxylate-sulfonamide coupling approach was required. Thus, building on the promising results presented in Scheme 70, the FNS protected Tmob thioester **200** was first treated under acidic conditions to release the corresponding thioacids **208**, which was

then exposed to a DNS protected peptide **178** carrying a Fm thioester at the *C*-terminal position followed by the treatment with cesium hydrogen carbonate to provide the FNS protected peptide Fm thioester **210** (Figures 31 and 32) in good yield (Scheme 72). The FNS protected peptide Fm thioester **210** was then trapped with Alloc-Gly-Gly-SH to afford the nonapeptide Fm thioester **211**. This triblock synthesis in which a fluorenylmethyl thioester is carried through two coupling steps nicely illustrates the compatibility of simple thioester moieties with the thiocarboxylate-sulfonamide approach and opens the door for further chain extension by the well-established native chemical ligation method.



Scheme 72. Compatibility of the Fm Thioesters with the Thioacid-Sulfonamide Block Synthesis



HPLC: 40 -100% acetonitrile in water over 25 min with a flow rate of 1.0 mL/min and 254 nm UV detection, retention time = 10.264 min

Figure 31. HPLC trace of FNS-L-Ala-L-Phe-L-Val-L-Val-L-Ala-L-Thr-L-Val-SFm (210)

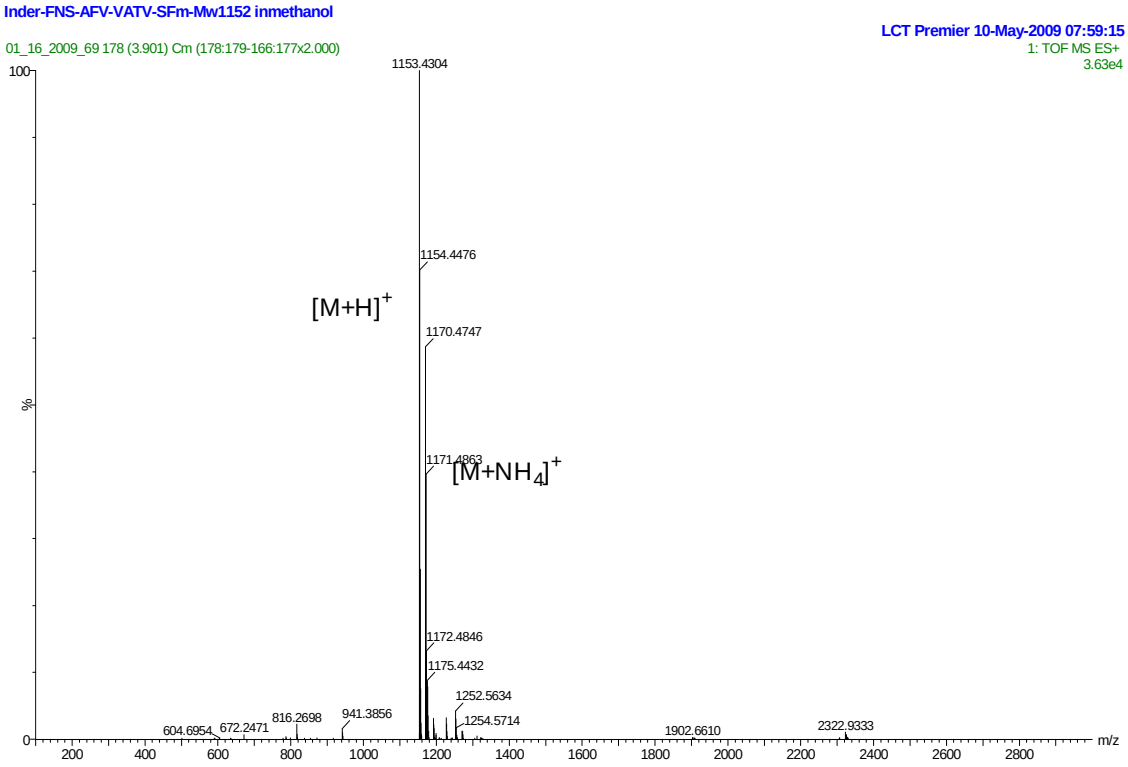
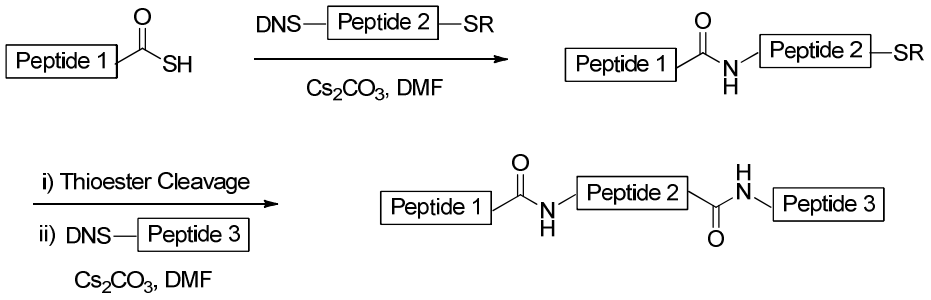


Figure 32. ESI-HRMS spectrum of the peak eluting at 10.264 min [FNS-L-Ala-L-Phe-L-Val-L-Val-L-Ala-L-Thr-L-Val-SFm (210)]

3.12 The Left to Right Strategy

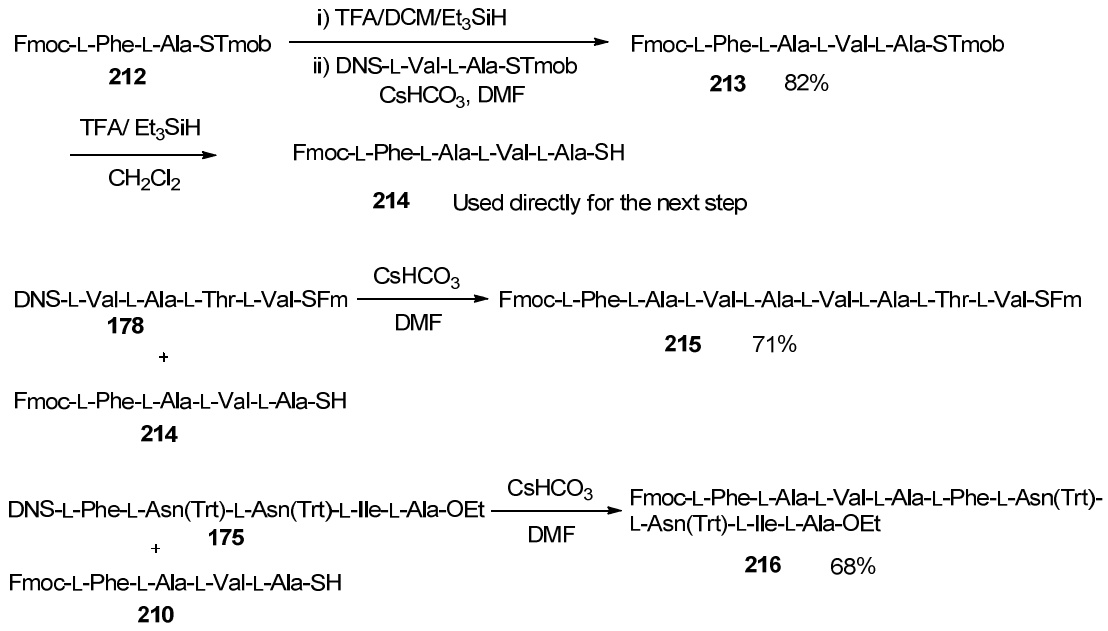
While successful, the right to left strategy for block peptide synthesis described above suffered from a major limitation, namely its necessary reliance on the use of a series of sulfonamides of decreasing reactivity. This design requirement essentially means that a first fast reaction is followed up by a second inherently slower one, and ultimately limits the application of the method. In view of this limitation an alternative left to right strategy was envisaged that employs the more reactive DNS sulphonamide in all coupling steps (Scheme 73).



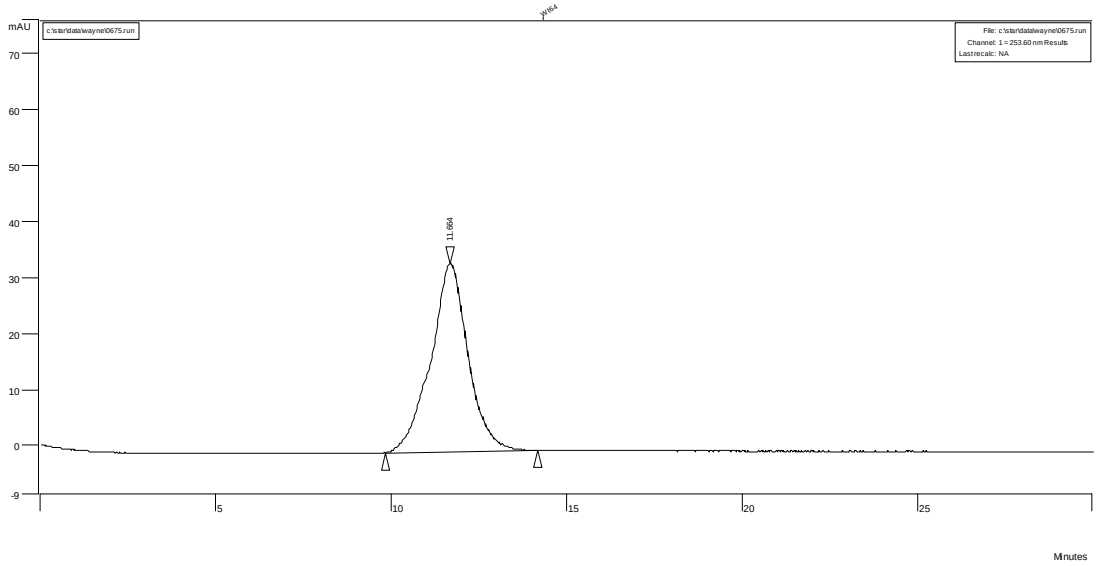
Scheme 73. Convergent synthesis by the left to right strategy

As is apparent from Scheme 73, this new sequence necessitates the compatibility of the Tmob thioester functionality with the sulfonamide coupling reaction. While this was a reasonable expectation on the basis of the tolerance of the thioacid-sulfonamide coupling for the Fm thioesters as presented above, corroboration was required. To this end, the Fmoc protected di-peptide Tmob thioester **212** was treated under acidic conditions to release the corresponding thioacid, which was then exposed to a DNS protected di-peptide Tmob thioester followed by the treatment with cesium carbonate to provide the Fmoc protected tetra-peptide Tmob thioester **213** in good yield (Scheme 74). The Tmob group in this tetrapeptide was then cleaved off to release the thioacids **214**, which was then reacted with the DNS protected tetra-peptide Fm thioester **178** to afford an Fmoc protected octapeptide Fm thioester **215**, whose diastereomeric purity was established in the usual manner (Figure 33). This last sequence of reactions confirms the stability of both the Fm and Tmob thioesters, thereby again setting the stage for the application of this chemistry in the preparation of thioesters for native chemical ligation sequences. The octapeptide Fm thioesters are also important building blocks for cyclic peptide synthesis as has been demonstrated very recently.[154]

In another variation on the general theme of left to right block synthesis, the Fmoc protected tetra-peptide thioacid **214** was treated with a DNS protected penta-peptide **175** to afford a nona-peptide **216** (Scheme 74).



Scheme 74. Left to right strategy showing compatibility with thioesters



HPLC: 40 -100% acetonitrile in water over 25 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 11.66 min

Figure 33. HPLC trace of Fmoc-L-Phe-L-Ala-L-Val-L-Ala-L-Val-L-Ala-L-Thr-L-Val-SFm (215)

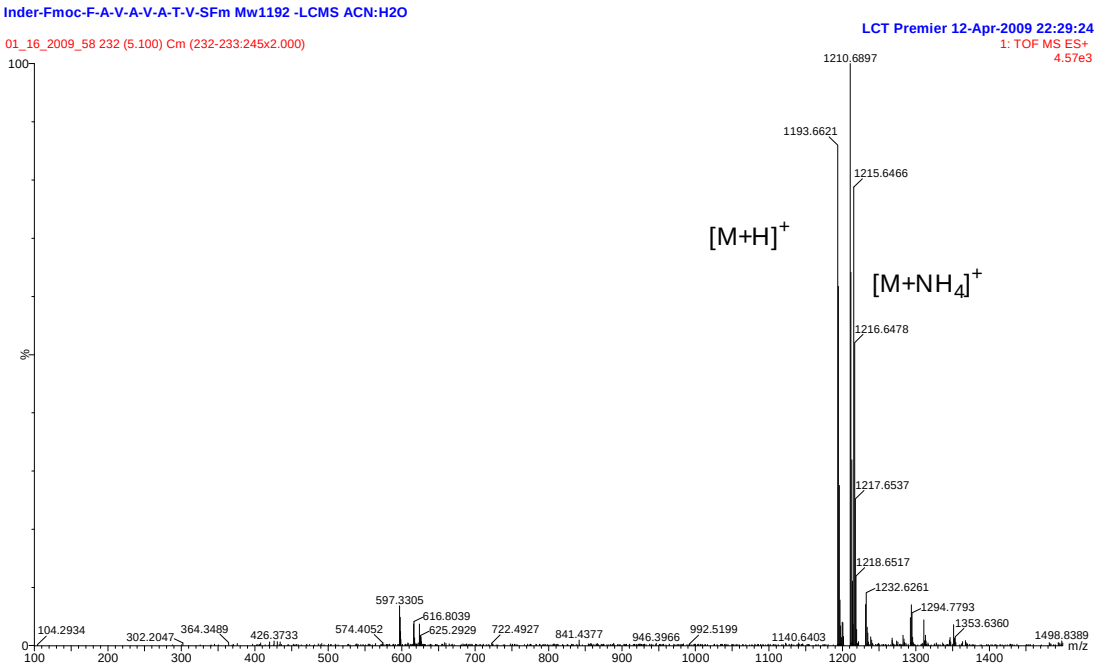
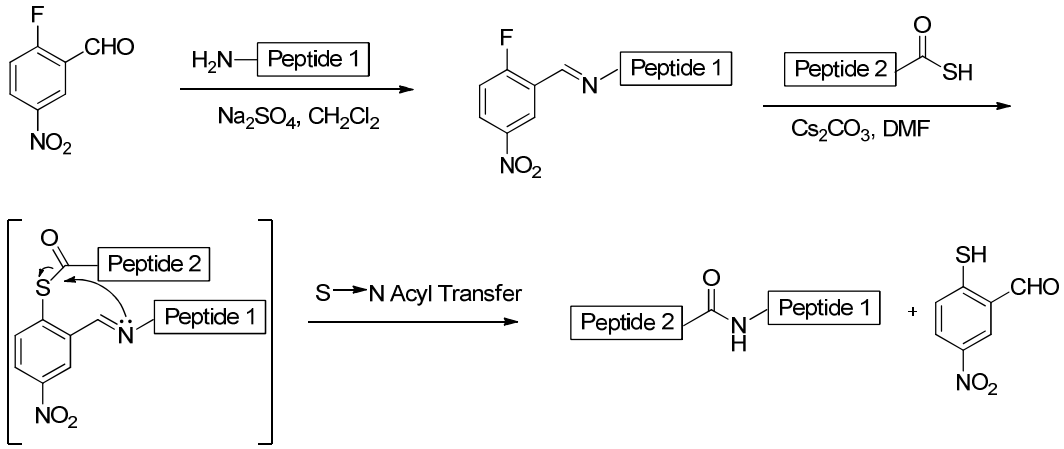


Figure 34. ESI-MS spectrum of the peak eluting at 11.66 min [Fmoc-L-Phe-L-Ala-L-Val-L-Ala-L-Val-L-Ala-L-Thr-L-Val-SFm (215)]

3.13. Attempted Redesign of the System to Intramolecular Amine Transfer to the Active Thioester

While successful, the strategies delineated above for block peptide synthesis involve the intermolecular coupling for the formation of an amide bond and suffer from the usual limitations of intermolecular reactions, namely slower reactions for the coupling of larger segments. In native chemical ligation Kent overcame this problem by using the more nucleophilic thiol in the intermolecular step, and thereby rendering the amide bond forming step intramolecular. Taking a leaf from Kent's book, a coupling sequence was envisaged in which the *N*-terminal amine of one peptide segment would be captured in the form of a Schiff's base with the 2-fluoro-5-nitrobenzaldehyde. Subsequent intermolecular nucleophilic substitution by a *C*-terminal thioacid on this moiety would then give a

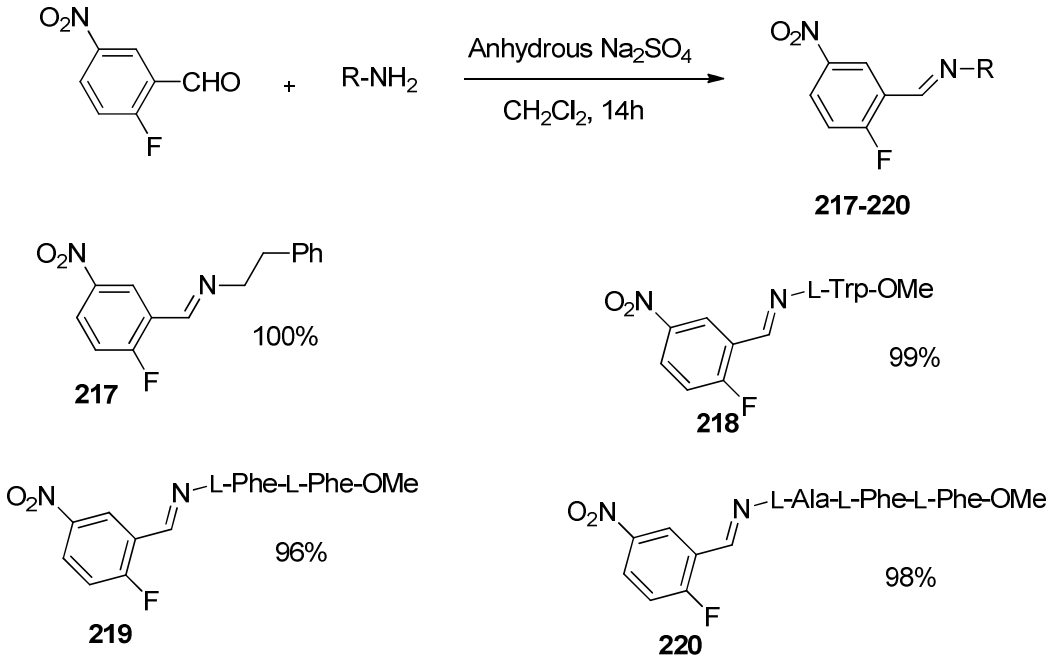
thioester, suitably place to accept the nitrogen nucleophile in an intramolecular manner (Scheme 75). This reaction scheme intercepts the previous work of Ito and coworkers who accessed essentially the same key intermediate for S-N acyl transfer by reaction of an amine with a preformed 2-formyl-4-nitrophenyl thioester.[142]



Scheme 75. Potential S-N acyl transfer

3.14 Synthesis of the Schiff Bases

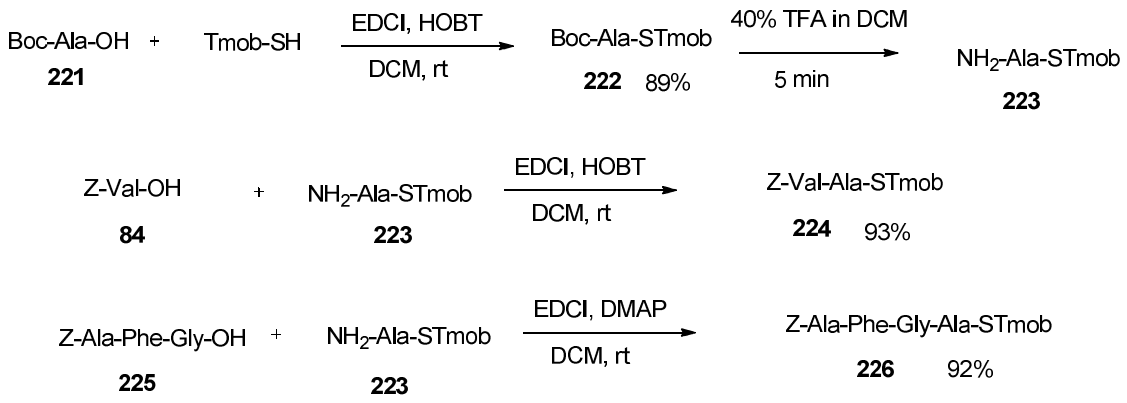
Following the literature,[153] several Schiff bases **217-220** were prepared in excellent yields from the reaction of the corresponding primary amine and 2-fluoro-5-nitrobenzaldehyde in presence of anhydrous sodium sulfate in methylene chloride (Scheme 76).



Scheme 76. Synthesis of the Schiff bases

3.15 Synthesis of Thioesters

The synthesis of the α-amino thioesters was performed as described in Chapter 2. However, for the synthesis of higher peptidyl thioesters, an α-amino thioester was coupled with the corresponding peptidyl acid by the EDCI coupling method as outlined in Scheme 77.

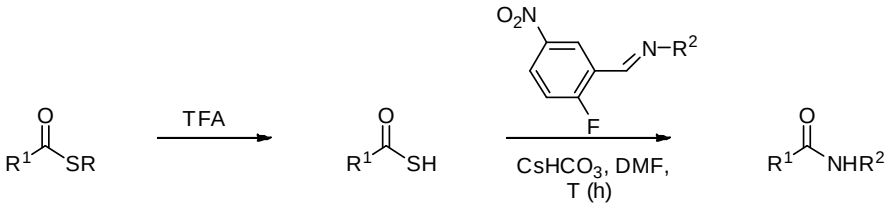


Scheme 77. Synthesis of peptidyl thioesters employing EDCI as reagent

3.16 Peptide Synthesis using the Thioacids and the Schiff Bases

The coupling of thioacids with the Schiff bases was first investigated with commercially available thioacetic acid, and the Schiff bases of phenylethylamine and L-tryptophan methyl ester (Table 7, entries 1 and 2), and a good yield of the acetamide was obtained on mixing of the both components in DMF at room temperature in the presence of cesium carbonate. Thus encouraged, a series of oligopeptides was accessed by coupling amino thioacids with other Schiff bases with the yields reported in Table 7. All reactions in Table 7 were conducted at room temperature and involved 0.1 M Schiff base in DMF with thioacid (1.2 equiv) and Cs_2CO_3 (1.5 equiv) as base.

Table 7. Coupling of Thioacids with Schiff Bases

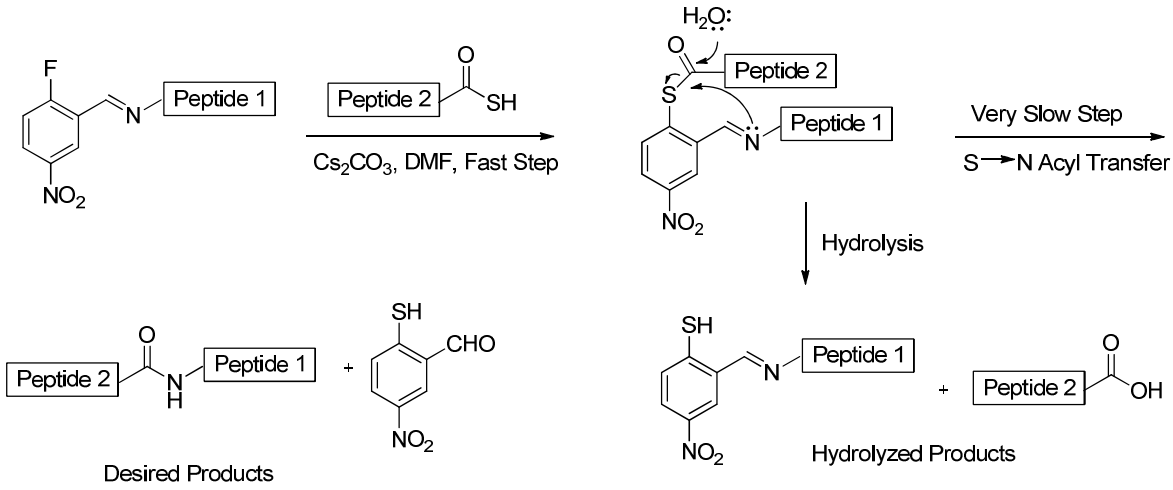


Entry	Thioester	Thioacid	Schiff Base	T (h)	Product	% Yield
1	-	AcSH	**217**	4	$AcNHCH_2CH_2Ph$ (**227**)	75
2	-	AcSH	**218**	4	Ac-L-Trp-OMe (**96**)	78
3	Z-L-Val-STrt (**92**)	Z-L-Val-SH	**218**	8	Z-L-Val-L-Trp-OMe (**228**)	57
4	Alloc-Gly-Gly-STmob (**160**)	Alloc-Gly-Gly-SH	**218**	6	Alloc-Gly-Gly-L-Trp-OMe (**229**)	83

5	Z-L-Val-L-Ala-STmob (**224**)	Z-L-Val-L-Ala-SH	**218**	6	Z-L-Val-L-Ala-L-Trp-OMe (**230**)	81
6	Z-L-Val-STrt (**92**)	Z-L-Val-SH	**219**	14	Z-L-Val-L-Phe-L-Phe-OMe (**231**)	54
7	Z-L-Val-L-Ala-STmob (**224**)	Z-L-Val-L-Ala-SH	**219**	10	Z-L-Val-L-Ala-L-Phe-L-Phe-OMe (**232**)	71
8	Alloc-Gly-Gly-STmob (**160**)	Alloc-Gly-Gly-SH	**219**	10	Alloc-Gly-Gly-L- Phe-L-Phe-OMe (**233**)	74
9	Z-L-Val-L-Ala-STmob (**224**)	Z-L-Val-L-Ala-SH	**220**	12	Z-L-Val-L-Ala-L-Ala-L-Phe-L-Phe-OMe (**234**)	55
10	Z-L-Ala-L-Phe-Gly-L-Ala-STmob (**226**)	Z-L-Ala-L-Phe-Gly-L-Ala-SH	**220**	14	Z-L-Ala-L-Phe-Gly-L-Ala-L-Ala-L-Phe-L-Phe-OMe (**235**)	51

The ability to form relatively hindered peptide bonds is evident from entries 3 and 6 (Table 7), where a valine thioacid is coupled with the Schiff bases of tryptophan and phenylalanine. The good functional group compatibility of the process is clear from inspection of Table 7 in general. In spite of these promising results the yields for the formation of the sterically and electronically more demanding junctions (Table 7, entries 3 and 6) are lower than those obtained in less demanding cases (Table 7, entries 4, 5, 7 and 8). S→N Acyl transfer is also substrate dependent in this protocol and becomes slow with the increase chain length of the peptide fragments as evident from the entries 9 and 10.

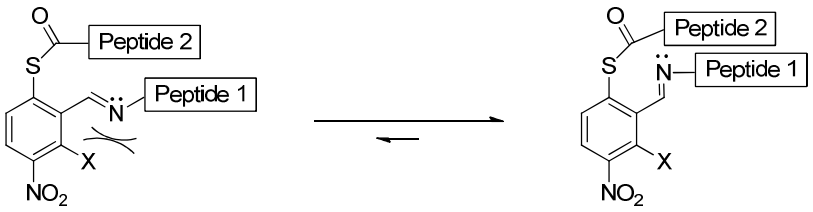
Monitoring of this reaction sequence by mass spectrometry revealed that the intermolecular nucleophilic aromatic substitution step to be rapid and to provide the thioester intermediate within an hour for all the examples presented in Table 7. The second step in the sequence, the S→N acyl transfer with release of the peptide, however, was found by mass spectrometric monitoring to be highly substrate dependent. Thus, for small segments the desired amide functionality was formed in good yield in a few hours. On the other hand , larger fragments were found to afford the peptide only very slowly with the initial adduct continuing to be observed as a major product after several hours. Because of this slower transfer, hydrolysis competes and the overall yields are correspondingly lowered (Scheme 78). In view of this dependence on the size of the units to be coupled it is interesting to note that the Ito group only reported the synthesis of di and tripeptides in their closely related approach.[142]



Scheme 78. Hydrolysis of the thioester intermediate

It is conceivable that the problem of slow transfer might be circumvented by the introduction of a group X at the 3-position of the aromatic linker such that would bias the conformation about the Ar-imine bond by steric buttressing[166-168] in such a

way as to predispose it toward cyclization onto the thioester (Scheme 82). This possibility remains to be tested.



Scheme 79. Potential application of steric buttressing to accelerate S→N acyl transfer

3.17. Conclusion

Overall, a combination of powerful new methods for the block synthesis of peptides based on the reaction of thioacids with electron-deficient sulfonamides is presented in this chapter. The assembly of the various blocks was conducted in a right to left or left to right manner and arranged in such a way as to provide a peptide thioester ready for incorporation in to a native chemical ligation sequence.

CHAPTER 4

Direct Fmoc-SPPS of Peptidyl Thioesters

4.1 Background and Significance

As discussed in Chapter 1, over the years several useful, elegant and indirect methods have been developed for the synthesis of *C*-terminal peptidyl thioesters by Fmoc-SPPS. Despite these notable advances, the synthesis of peptidyl thioesters remains significantly more challenging than the synthesis of the corresponding peptidyl acids or amides. In this regard, the development of a general and direct approach for solid-phase peptidyl α-thioesters synthesis was undertaken, the results of which are described in this chapter.

4.2 Fmoc-SPPS of Peptidyl Thioesters from Thioamides

Thioamides are analogs of amides and are versatile building blocks in organic chemistry.[169-171] They have also been incorporated into peptides as isosteres for the amide bond in the design of peptidase and/or protease inhibitors.[172-174] The small difference in electronegativity between carbon and sulfur and the larger size of sulfur allow greater charge transfer from nitrogen to sulfur in thioamides, which makes them more nucleophilic than amides and enables their selective alkylation in the presence of amides (Figure 35).[175]

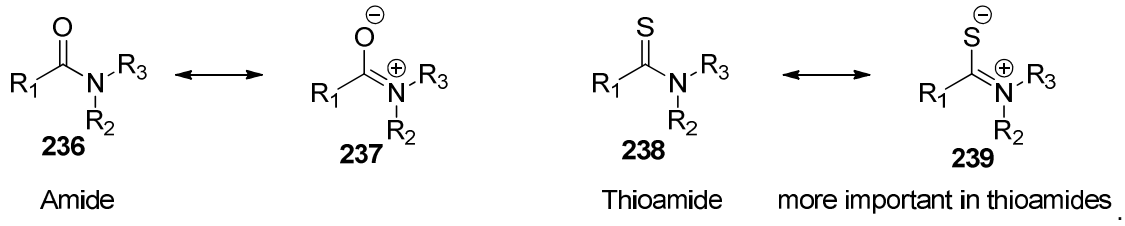
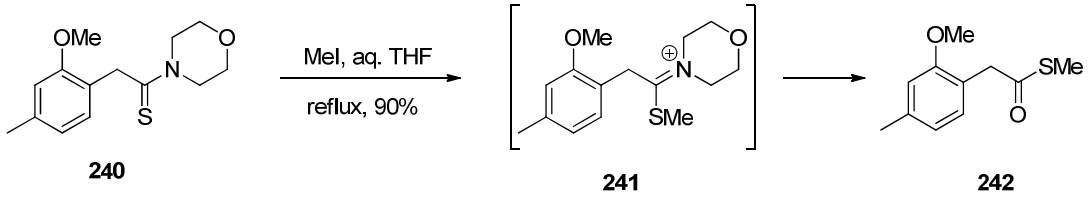


Figure 35. Amide vs thioamide resonance

Thioamides can be converted to thioesters as described previously by several groups.[155,160,161,176-179] For example, thioamides have been converted to thioesters by simply warming in an aqueous solution containing an alkylating agent, which implies thioimide formation followed by hydrolysis (Scheme 80).

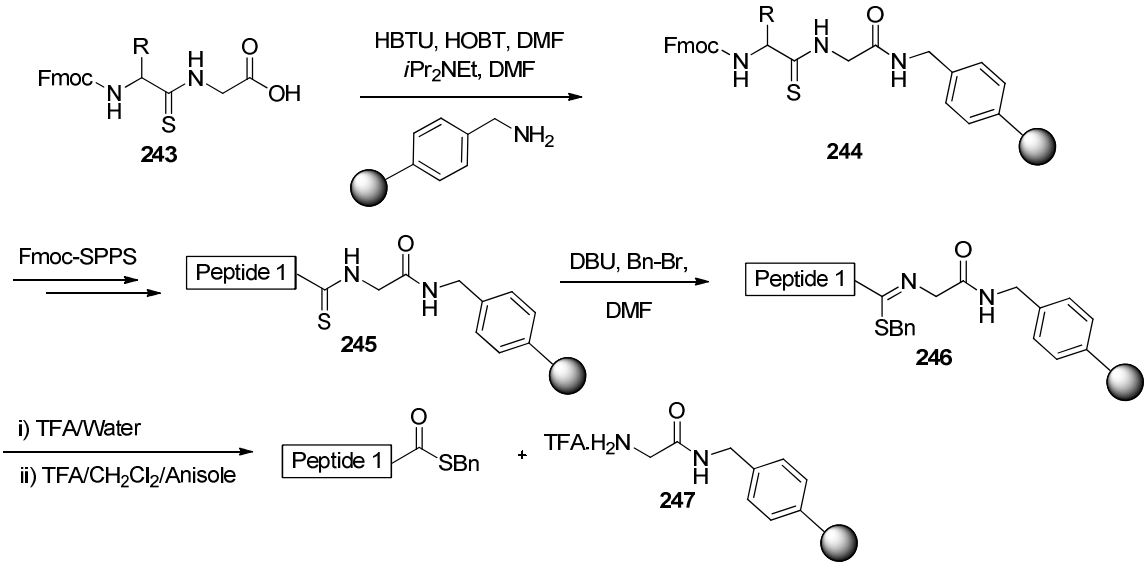


Scheme 80. Thioamide to thioester

Peptidyl thioesters have also been generated from the corresponding thioamides by sequential *S*-alkylation with isolation of the thioimidates followed by subsequent acidic hydrolysis.[176,178] The thioamide moiety was also found to be completely stable under the cleavage conditions[180-184] used to remove the *N*-Fmoc group in peptide chemistry.[185,186]

Based on these observations, Fmoc-SSPS of the peptidyl thioesters was envisaged by a process in which an Fmoc protected thioamide derivative would be attached to the resin by HBTU/HOBT coupling followed by the elongation of the peptide chain by Fmoc-SPPS. Subsequent *S*-benzylation of this resin bound

peptide **245** would then give a thioimidate **246** that could be easily hydrolyzed to provide the peptidyl thioester (Scheme 81).

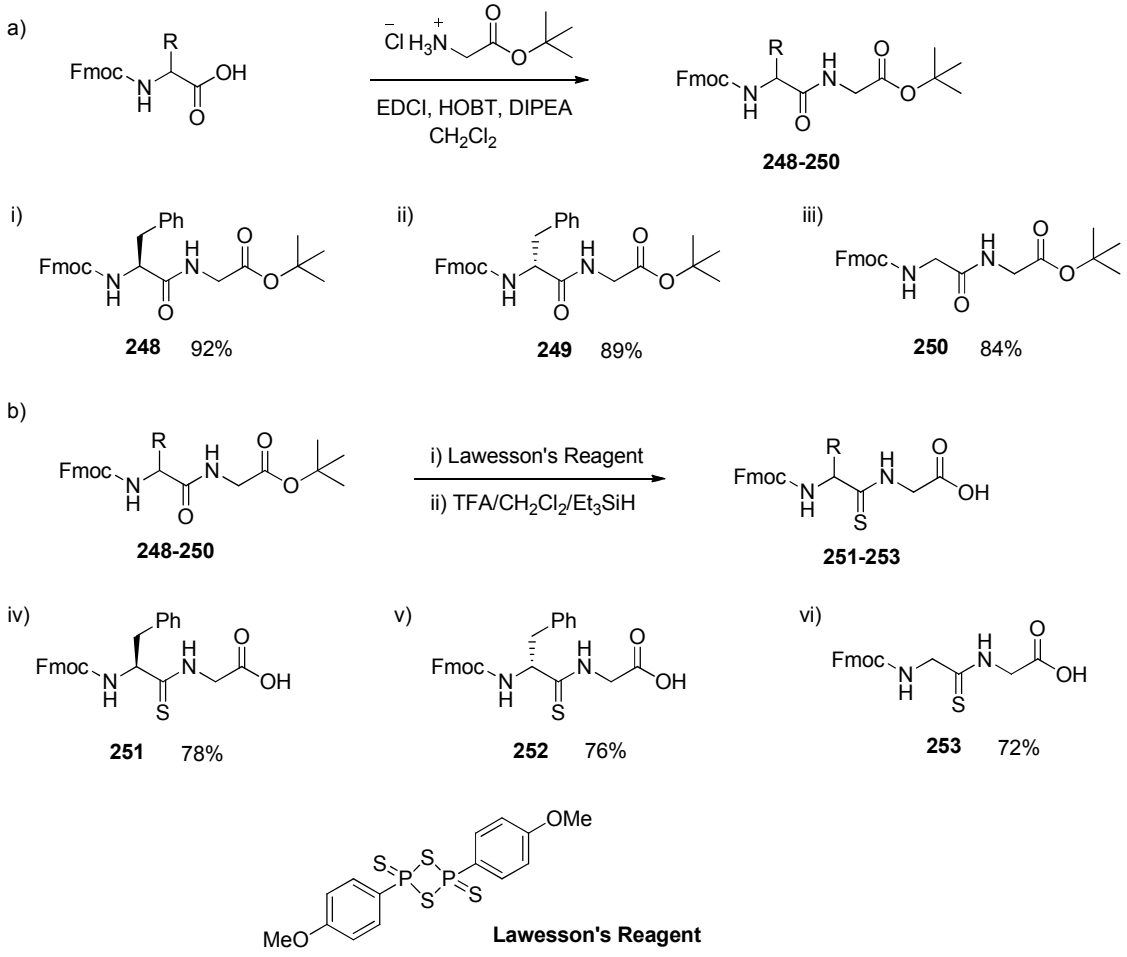


Scheme 81. Projected Fmoc solid-phase synthesis of peptidyl thioesters

4.3 Synthesis of the Thioamides Linkers

Following the literature,[10,11] several Fmoc-protected dipeptides were prepared from the corresponding Fmoc-protected α-amino acids and the hydrochloride salt of glycine *tert* butyl ester by coupling with EDCI/HOBT in excellent yields (Scheme 82). These dipeptides were converted to the corresponding thioamides by the well documented reaction of Lawesson's reagent[187,188] in refluxing toluene followed by the removal of *tert* butyl group under acidic conditions to afford the corresponding Fmoc protected thioamide acids in good yields. In these thionation reactions, the Lawesson reagent was completely selective for the transformation of the amide bond in the presence of the carbamate and ester bonds. This is clear from the IR and ^{13}C NMR spectra.

Thus, in the IR spectra of the products which only the amide carbonyl at 1660 cm^{-1} was lost, with the carbamate and ester absorptions at 1705 and 1740 cm^{-1} being retained; the new thioamide thiocarbonyl absorption was typically seen at 1248 cm^{-1}. In the ^{13}C-NMR spectrum the amide carbonyl at δ 172 was converted to the thiocarbonyl resonance at δ 203, while the carbamate and ester carbonyl resonances remained unchanged.



Scheme 82. Synthesis of the thioamide linkers

4.4 Amino Acid Side-Chain Protection Strategies in Fmoc-SPPS

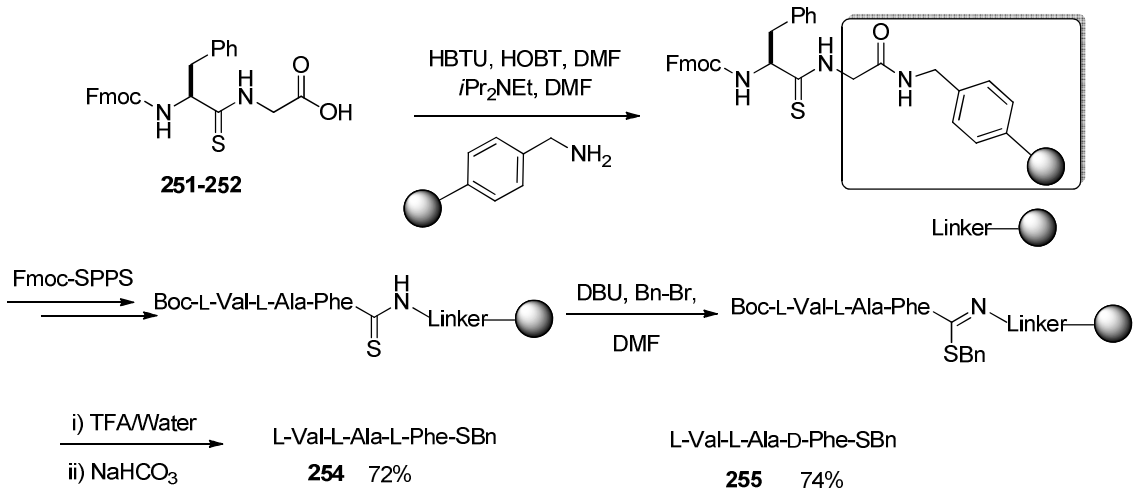
More than half of the amino acids commonly encountered in proteins have side chains that contain reactive functional groups. One of the more demanding parts of solid phase peptide synthesis is the necessity to mask these reactive functional groups to prevent their interference in peptide bond formation. Self-evidently any side chain protecting groups employed must be stable to the conditions employed for removal of the *N*-terminal protecting group and for peptide chain extension.

The Fmoc-SPPS approach developed in this chapter involves an orthogonal protecting group strategy, known as the Fmoc/*t*-Butyl strategy, which involves the use of base-labile N^{α}-Fmoc group and acid-labile side chain protecting groups. All the side chain protecting groups were selected based on their orthogonality to Fmoc-SPPS and were removed during the final cleavage of peptidyl thioesters from the linker. Thus, side-chain alcohols and acids were protected as ^{t}Bu ethers and esters, whereas amines and guanidines were protected as ^{t}Bu carbamates and Pbf sulfonamides, respectively. In addition, thioether function of methionine was further protected as sulfoxides to avoid any undesired alkylation.[36,49,50] All the amino acids used in this Fmoc-SPPS approach were commercially available.

4.5. Synthesis of the both L- and D-Diastereomers of L-Val-L-Ala-Phe-SBn

With all the building blocks in hand attention was turned to solid-phase synthesis of peptidyl thioesters on the aminomethyl polystyrene resin, using manual techniques (Scheme 83). Accordingly, the first N^{α}-Fmoc protected

thioamide acid was activated with HBTU and HOBt in DMF and the pre-activated amino acid was then allowed to react with the aminomethyl polystyrene resin to yield the resin bound thioamide derivative. Subsequently, the desired peptide chain was elongated using standard Fmoc techniques, which consisted of the following steps in each cycle: (i) removal of the N^{α}-Fmoc protecting group of the last coupled amino acid by treatment with piperidine; (ii) washing of the resin with isopropyl alcohol, hexane, methylene chloride and, (iii) coupling of the next pre-activated appropriate side-chain protected N^{α}-Fmoc amino acid. Between each of these steps a thorough flow wash with DMF was carried out to remove excess reagents. The final amino acid was introduced with Boc protection rather than the standard Fmoc group. Finally after completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (10 equiv) and benzyl bromide (9 equiv) in DMF to provide the resin bound thioimidate, that was subjected to hydrolysis with TFA/water to afford the desired peptidyl thioesters as shown in Scheme 83.



Scheme 83. Synthesis of the diastereomers

An unprotected tri-peptide thioester L-Val-L-Ala-L-Phe-SBn was obtained in this manner in good yield as set out in Scheme 84. 500 MHz ^{1}H NMR analysis of the tripeptide thioester in $CDCl_3$ produced from this method also enabled investigation of the extent of epimerization. To verify that the ^{1}H NMR was capable of resolving the epimeric peptide thioesters, the diastereomeric tripeptide L-Val-L-Ala-D-Phe-SBn was also synthesized in the same manner. Both the isomers were subjected to 500 MHz ^{1}H NMR analysis in $CDCl_3$. A qualitative comparison of the 500 MHz ^{1}H NMR spectra of both diastereomers was performed as shown in Figure 36. A careful review of the proton NMR spectra of both isomers indicates that each spectrum corresponds to a mixture of diastereomers. The characteristic chemical shifts of benzyl protons of the thioester unit and the methyl protons of alanine and valine in the diastereomeric tripeptides identify the two diastereomers as shown in Table 8. It is evident from the ^{1}H NMR integration analyses that the tripeptide thioester sequence proceeds with ~ 22% epimerization under standard conditions.

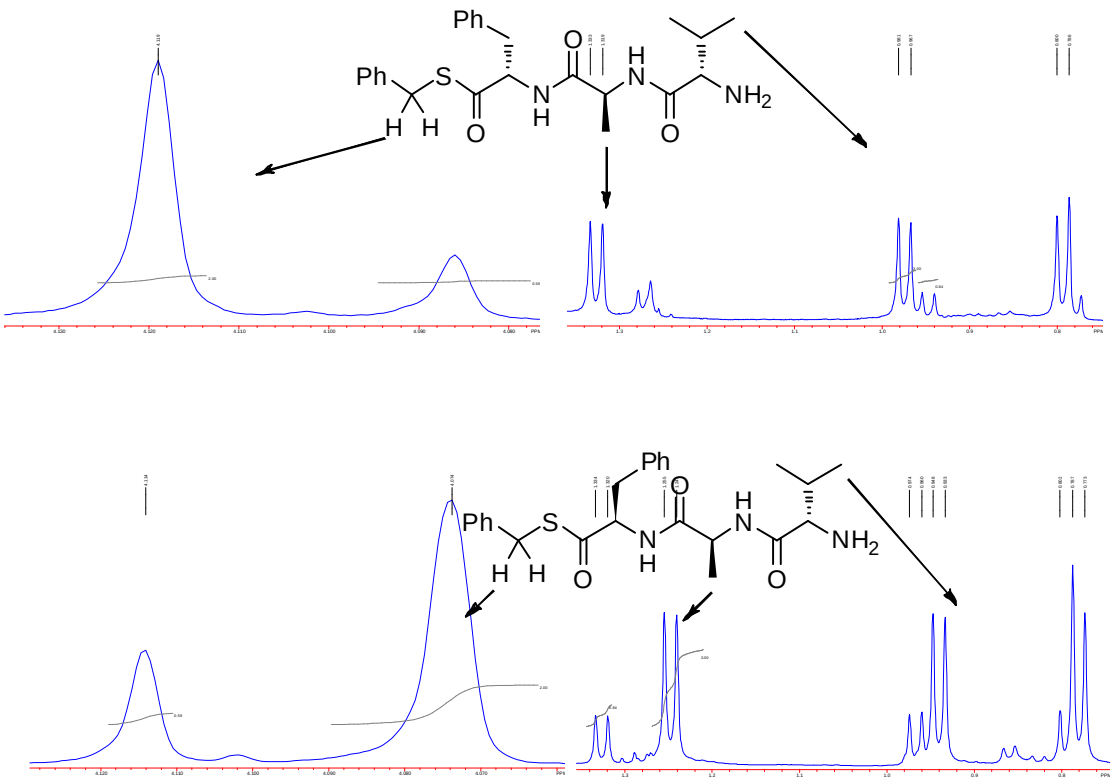


Figure 36. ^{1}H-NMR spectra of tripeptide thioesters in $CDCl_3$.

Table 8. Selected ^{1}H Chemical Shifts (δ in ppm) and Coupling Constants (*J*) of Tripeptide Thioesters L-Val-L-Ala-L-Phe-SBn (254) and L-Val-L-Ala-D-Phe-SBn (255)

Tripeptide Thioesters	δ of benzyl proton (multiplicity, *J*, proton counts)	δ of methyl proton of Ala and Val (multiplicity, *J*, proton counts)	Epimer Ratio % (L:D)
L-Val-L-Ala-L-Phe-SBn (**254**)	4.11 (s, 1H)	1.33 (d, *J* = 7.0 Hz, 3H), 0.97 (d, *J* = 7.0 Hz, 3H), 0.79 (d, *J* = 7.0 Hz, 3H)	77.5:22.5
L-Val-L-Ala-D-Phe-SBn (**255**)	4.07 (s, 1H)	1.25 (d, *J* = 7.0 Hz, 3H), 0.94 (d, *J* = 7.0 Hz, 3H), 0.78 (d, *J* = 7.0 Hz, 3H)	21.9:78.1

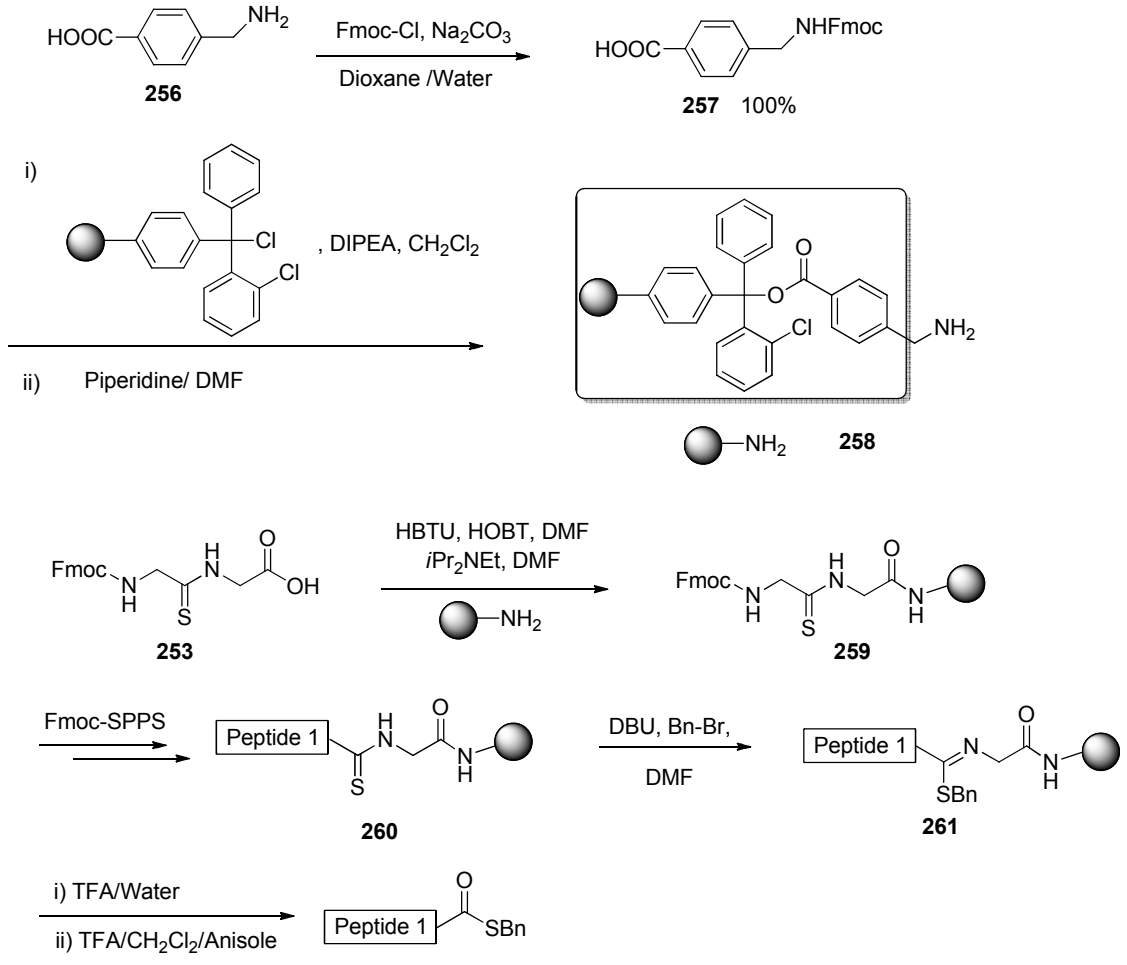
4.6. Fmoc-SPPS of Peptidyl Thioesters on the 2-Chlorotrityl Resin

Thus encouraged, a series of unprotected *C*-terminal oligo-peptide thioesters was accessed using Fmoc-SPPS on 2-chlorotrityl resin,[121] which could be cleaved under exceptionally mild acidic conditions leaving all the acid sensitive protecting groups intact. After each coupling step, the formation of the desired sequence was verified by cleavage of a small amount (~ 5 mg) of 2-chlorotrityl resin using a solution of 2% TFA in methylene chloride, followed by examination by ESI-TOF mass spectrometry.

To avoid the risk of racemization, a Gly residue was introduced at the *C*-terminal position in all peptide sequences. To probe the scope of the method, a number of peptides were synthesized employing various amino acid residues (Ala, Thr, Val, Ser, Tyr, Arg, Asn, Trp, Ile, Phe) at different positions of the sequences, thereby providing a true test of this Fmoc-SPPS approach.

The synthetic work commenced with the commercially available 4-(aminomethyl)benzoic acid **256**, of which Fmoc protection of the amine was achieved in quantitative yield. The acid **257** was then attached to the 2-chlorotrityl resin by the reaction with resin bound 2-chlorotrityl chloride and diisopropylethylamine in methylene chloride as illustrated in Scheme 84. The Fmoc group was then removed and the resulting amine **258** was coupled with the glycine thioamide linker **253** using the HBTU/HOBT method to provide the resin bound thioamide derivative. Subsequently, the peptide chain was elongated on the 2-chlorotrityl resin using standard Fmoc techniques as described previously in this chapter. The sequence was capped by introduction of a Boc-protected

amino acid at the *N*-terminus. Finally after completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (10 equiv) and benzyl bromide (9 equiv) in DMF to provide the resin bound thioimidate **261** that was subjected to hydrolysis with TFA/water with concomitant side chain deprotection to provide the protecting group free peptidyl thioesters in the form of their trifluoroacetate salts, as shown in Scheme 84. All the crude peptide thioesters were purified by reverse phase HPLC with 0.1% TFA in water/ acetonitrile as eluent and obtained in the yields presented in Scheme 84.



i) TFA.L-Thr-L-Ala-L-Ser-L-Phe-L-Ser-L-Leu-Gly-SBn
262 57%

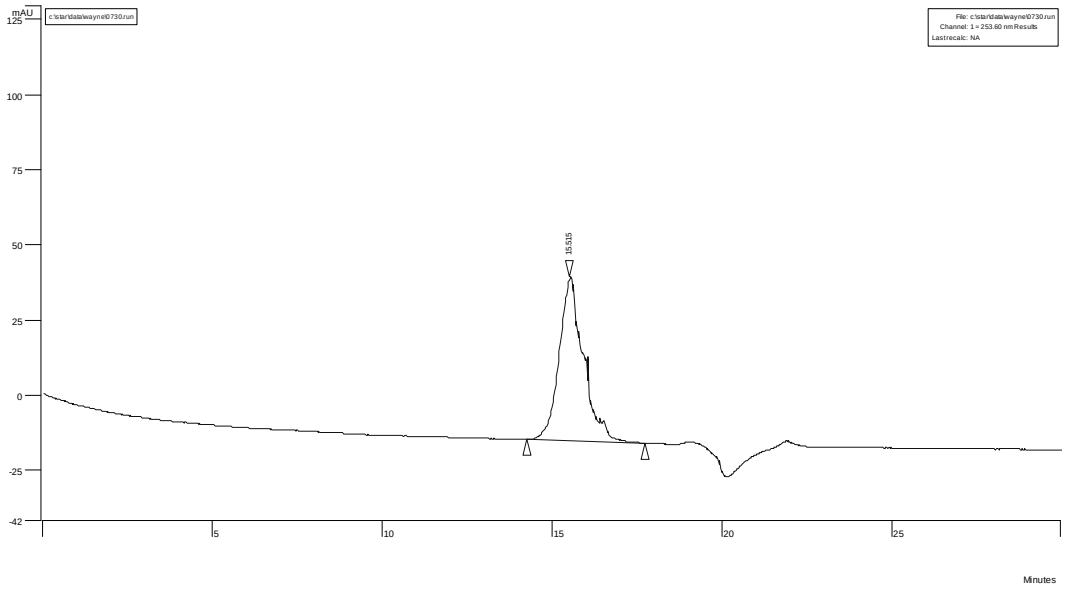
ii) TFA.L-Thr-L-Phe-L-Tyr-L-Ser-L-Ala-L-Tyr-Gly-SBn
263 56%

iii) TFA.L-Asn-L-Trp-L-Arg-L-Tyr-L-Ile-L-Ser-L-Thr-L-Phe-Gly-SBn
264 48%

Scheme 84. Functional group compatibility

The good functional group compatibility of the process is clear from inspection of Scheme 84 in general. In particular, the last entry in Scheme 84 places special emphasis on the robustness of the method, as it illustrates the synthesis of a functionally diverse nonapeptide thioester containing several types of side chain functionality such as the guanidine of arginine, the primary amide of

asparagine, the indole of tryptophan, the phenol of tyrosine, and the hydroxyl group of serine and threonine, which had suitable protection throughout the synthesis. The analytical RP-HPLC traces and ESI-TOF mass spectra of the peptidyl thioesters prepared in this manner are presented in Figures 37-42.



HPLC: 10 -90% acetonitrile in water over 30 min with a flow rate of 1 mL/min and 215 nm UV detection, retention time = 15.52 min

Figure 37. HPLC trace of L-Thr-L-Ala-L-Ser-L-Phe-L-Ser-L-Leu-Gly-SBn (262)

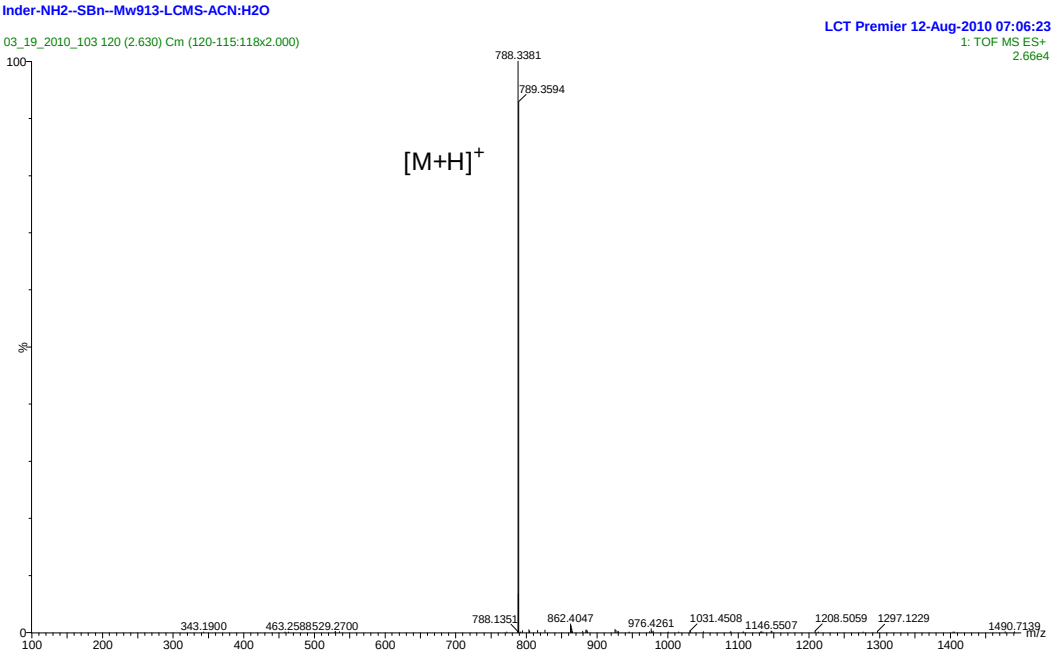
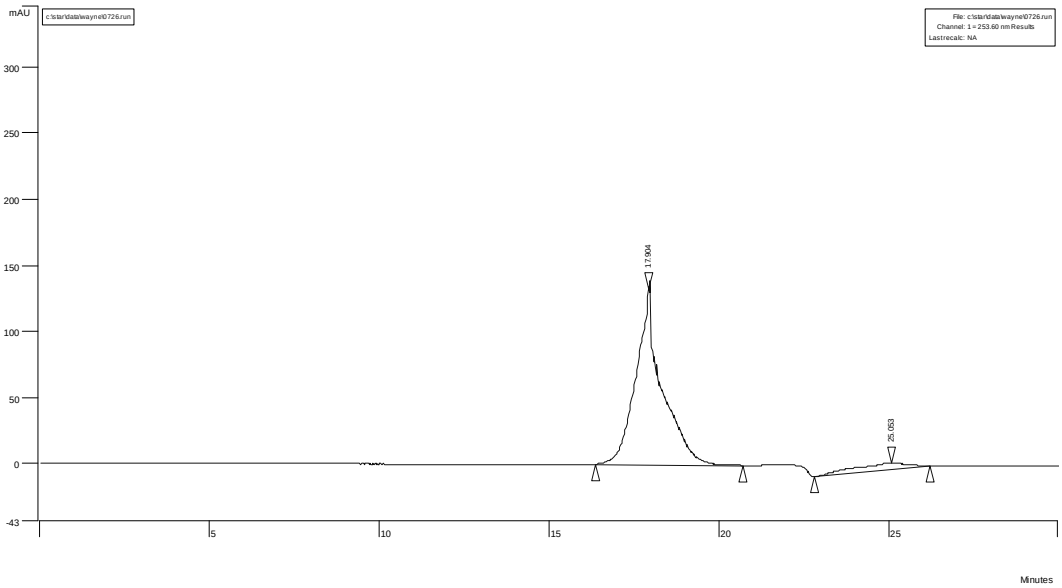


Figure 38. ESI-HRMS spectrum of the peak eluting at 15.52 min [L-Thr-L-Ala-L-Ser-L-Phe-L-Ser-L-Leu-Gly-SBn (262)]



HPLC: 10 -90% acetonitrile in water over 30 min with a flow rate of 1 mL/min and 215 nm UV detection, retention time = 17.90 min

Figure 39. HPLC trace of L-Thr-L-Phe-L-Tyr-L-Ser-L-Ala-L-Tyr-Gly-SBn (263)

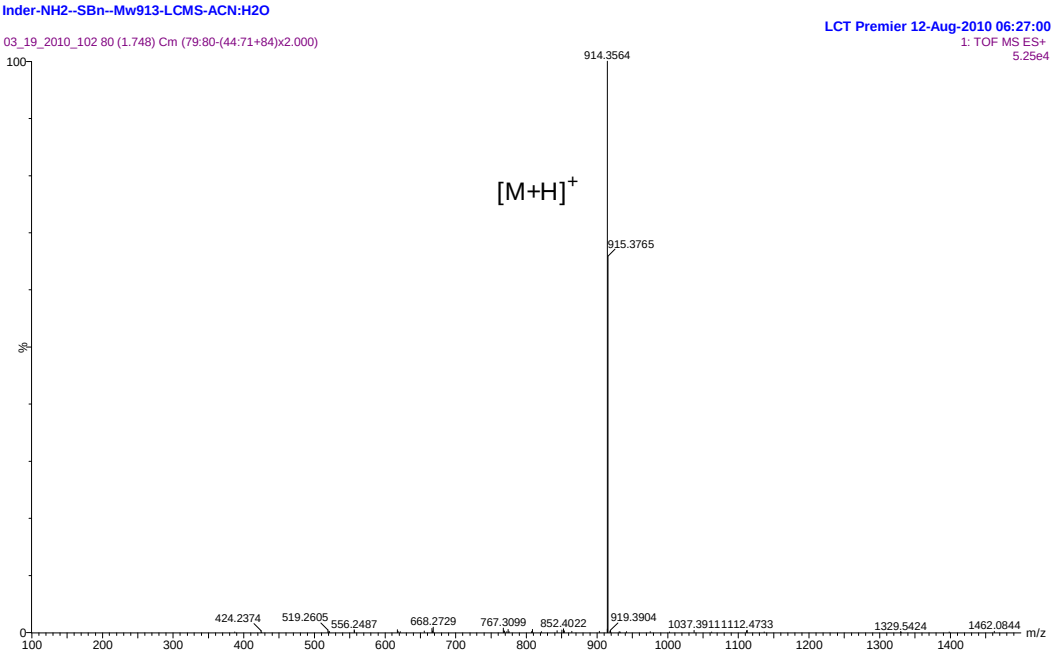
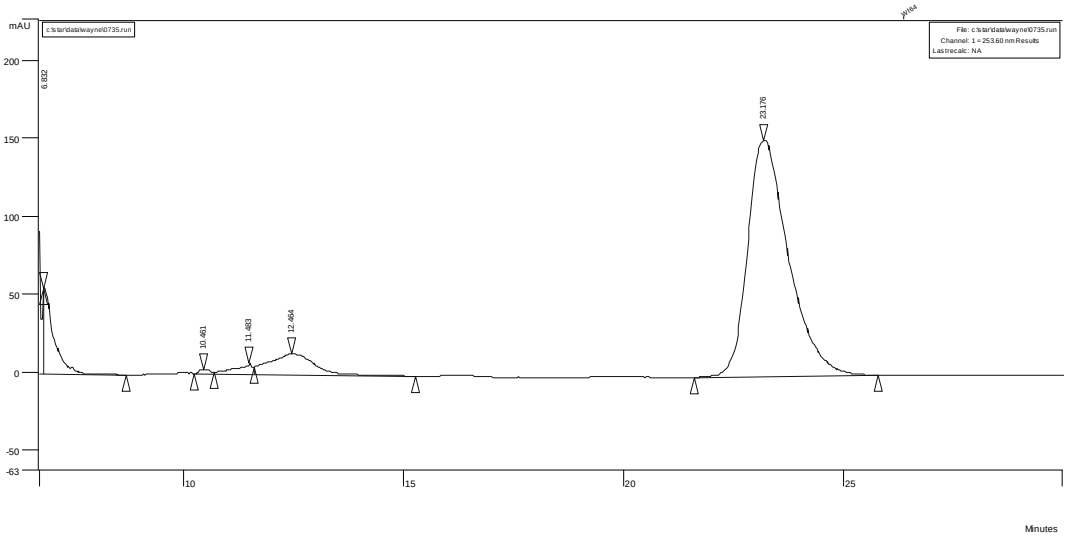


Figure 40. ESI-HRMS spectrum of the peak eluting at 17.90 [L-Thr-L-Phe-L-Tyr-L-Ser-L-Ala-L-Tyr-Gly-SBn (263)]



HPLC: 10 -90% acetonitrile in water over 30 min with a flow rate of 1 mL/min and 215 nm UV detection, retention time = 23.18 min

Figure 41. HPLC trace of L-Asp-L-Trp-L-Arg-L-Tyr-L-Ile-L-Ser-L-Thr-L-Phe-Gly-SBn (264)

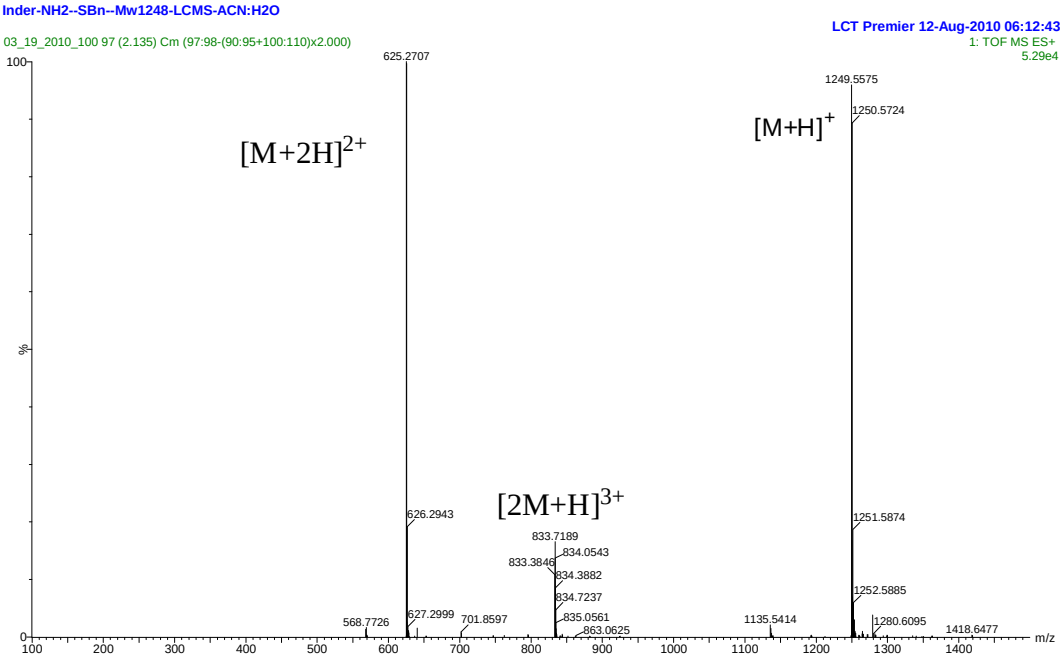
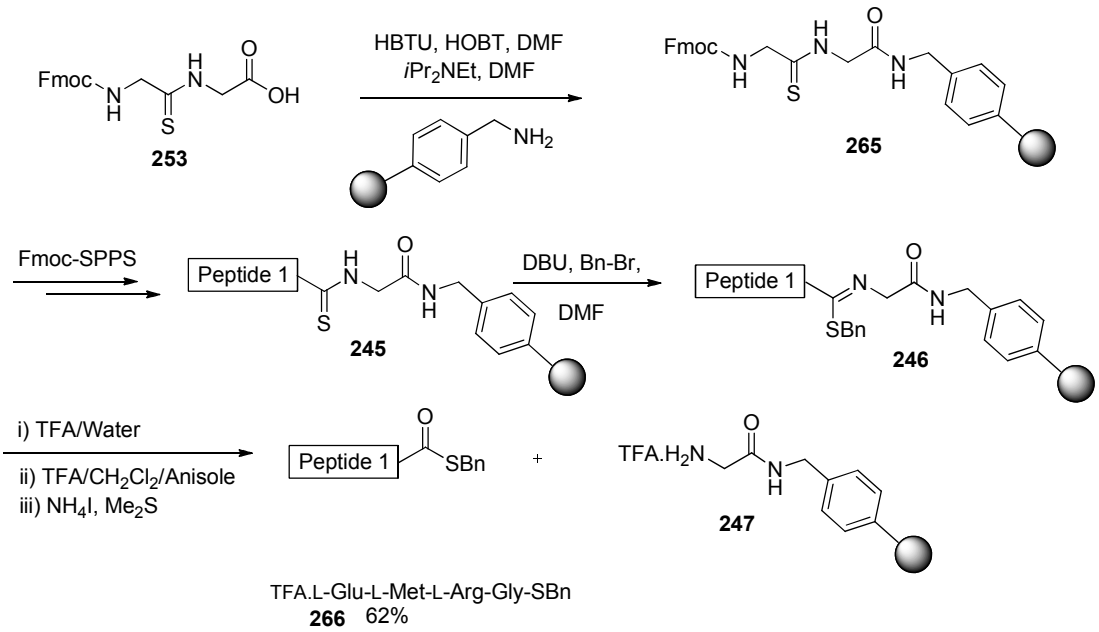


Figure 42. ESI-HRMS spectrum of the peak eluting at 23.18 min of [L-Asp-L-Trp-L-Arg-L-Tyr-L-Ile-L-Ser-L-Thr-L-Phe-Gly-SBn (264)]

4.7 Synthesis of the Thioester Containing Methionine Residue

The compatibility of the method with cysteine and methionine residues was next tested. To this end, solid-phase synthesis of tetra-peptides containing cysteine and methionine residues was performed on the aminomethyl polystyrene resin (Scheme 85). The first N^{α}-Fmoc protected thioamide acid was activated with HBTU and HOBt in DMF and the pre-activated amino acid **253** was then allowed to react with the aminomethyl polystyrene resin to yield the resin bound thioamide derivative **265**. As discussed above the methionine was introduced in the form of sulfoxide to obviate the possibility of their alkylation on introduction of benzyl bromide in the cleavage step. Subsequently, the desired peptide chain was elongated using standard Fmoc techniques. The peptide chain was terminated with a Boc-protected amino acid (Boc-L-Glu(O^{t}Bu)-OH). Finally

after completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (10 equiv) and benzyl bromide (9 equiv) in DMF to provide the resin bound thioimidate **246**, that was subjected to hydrolysis with TFA/water. The final step of the sequence was the *in situ* reduction of the sulfoxide groups with NH_4I/Me_2S[49] followed by reverse phase HPLC purification to access the unprotected peptidyl thioester **266** as shown in Scheme 85.



Scheme 85. Peptides containing methionine residue

The reduction of the sulfoxide was followed by mass spectrometry presented in Figures 43 and 44 for the case of the methionine-containing tetrapeptide. Thus, in the mass spectrum of the product, peaks with mass 614 (M+H) and 636 (M+Na) corresponding to the the sulfoxide were converted to the mass 598 (M+H) and 620 (M+Na) respectively, which clearly indicate the complete conversion of methionine sulfoxide to methionine.

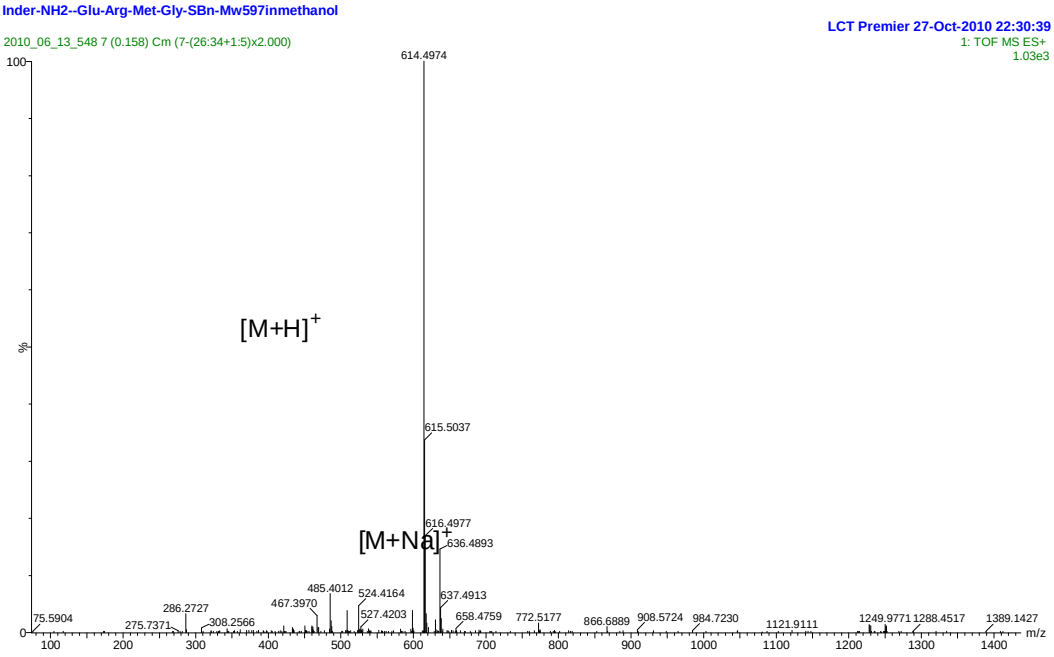


Figure 43. ESI-HRMS spectrum of L-Glu-L-Met(O)-L-Arg-Gly-SBn

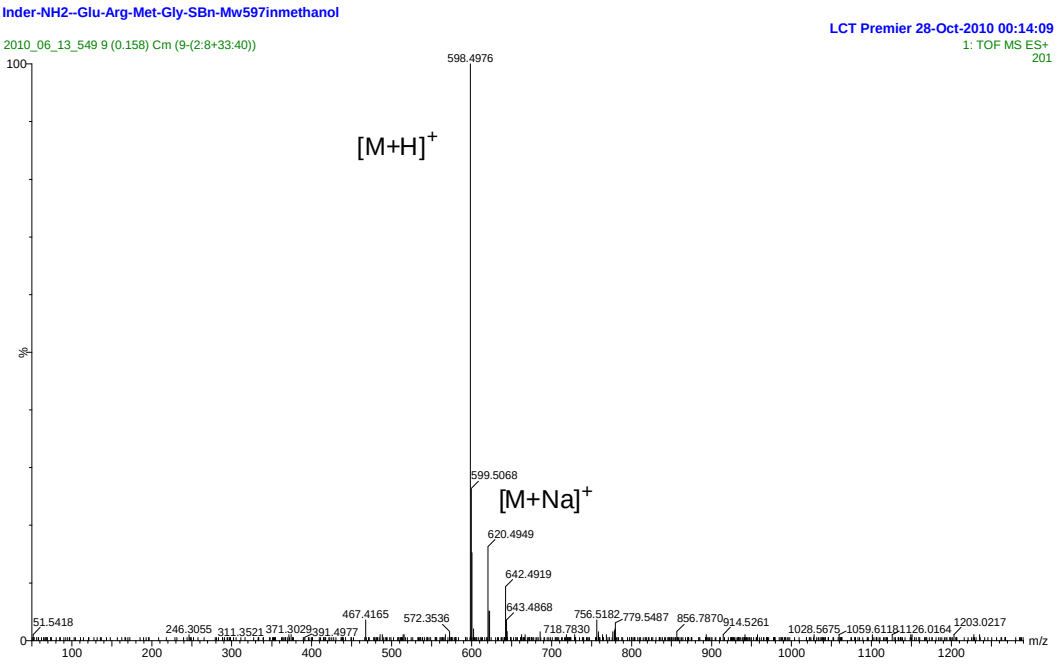


Figure 44. ESI-HRMS spectrum of L-Glu-L-Met-L-Arg-Gly-SBn (266)

The results presented in Scheme 85 support the earlier work from several groups on the use of sulfoxides as protecting groups in advanced peptide

synthesis.[189-192] In particular it is demonstrated that the sulfoxide group is compatible with the thioamide to thioester transformation and thereby enables the methionine residue in peptidyl thioesters to be prepared for native chemical ligation by the methodology of this chapter.

4.8 Conclusion

The work presented in this chapter has resulted in the development of a novel, direct approach to the solid phase synthesis of peptidyl glycyl thioesters based on the use of a thioamide linker in conjunction with standard Fmoc chemistry, followed by an alkylative procedure and simple acidic hydrolysis. This methodology is fully compatible with the Fmoc-SPPS approach and does not require any special linkers, resins or complicated protocols. This chemistry provides a very convenient and mild means to access unprotected peptidyl benzyl thioesters that could be use directly in native chemical ligation and its variants. While issue of the racemization at the *C*-terminal remains to be solved, the new strategy is viewed as a promising platform for the future initiatives in peptide chemistry.

CHAPTER 5

CONCLUSIONS

The applicability of novel methodologies to the block synthesis of peptides and peptidyl thioesters was demonstrated and a direct approach for the synthesis of peptidyl thioesters with standard Fmoc chemistry was developed.

A new chemistry for the epimerization-free block peptide synthesis has been developed. This chemistry involves the *in situ* formation of highly active thioesters by nucleophilic aromatic substitution of *C*-terminal thioacids on electron-deficient halogenoarenes followed by trapping with an *N*-terminal amine. This epimerization-free methodology is superior to available methods and does not require the presence of any particular amino acid for coupling, in contrast to native chemical ligation and its variants. The method is capable of peptide bond forming reactions at hindered residues.

The reactivity pattern of *N*-terminal sulfonamides towards peptidyl thioacids was optimized. Convergent strategies for multiple peptide fragment ligation, based on the reactivity differences of various sulfonamides, were developed and employed in triply convergent synthesis. Simple peptidyl thioesters are compatible with these reaction sequences, which enables the block synthesis of peptidyl thioesters ready for incorporation into a native chemical ligation sequence.

A novel and direct approach to the solid phase synthesis of peptidyl thioesters was established based on the use of a thioamide linker in conjunction with standard Fmoc chemistry. This methodology is fully compatible with the Fmoc-

SPPS approach and has been applied to access unprotected peptidyl benzyl thioesters that could be use directly in native chemical ligation and its variants.

CHAPTER 6

EXPERIMENTAL SECTION

General:

All solvents were dried and distilled by standard protocols. All reactions were conducted under an inert atmosphere of argon or nitrogen unless otherwise stated. All organic extracts were dried over sodium sulfate, and concentrated under aspirator vacuum. Chromatographic purifications were carried out over silica gel. All peptide syntheses were carried out employing 1% DVB cross linked aminomethyl polystyrene resin or 2-chlorotrityl resin in a 10 mL manual synthesizer glass reaction vessel with a Teflon-lined screw cap. Unless otherwise stated optical rotations were recorded on an Autopol® III automatic polarimeter in $CHCl_3$ solution and ^{1}H and ^{13}C spectra were recorded in $CDCl_3$ solution. Melting points were measured on a Barnstead electrothermal (9100) instrument and are uncorrected. Chromatographic purifications were carried out over silica gel. Reverse phase HPLC (RP-HPLC) was performed with 215 and 254 nm UV detection, using a C-18 analytical and preparative columns (250 × 4.6) and (250 × 21.4), respectively. All runs used linear gradients of A in B (A: CH_3CN containing 0.1% TFA and B: 5% CH_3CN/H_2O containing 0.1% TFA). LCMS chromatographic analyses were performed using a Waters Acquity UPLC with a Waters TUV detector equipped with an Acquity UPLC BEH C18 analytical column (2.1 x 50 mm) at a flow rate of 0.5–0.7 mL/min using linear gradients buffer B in A (B: CH_3CN containing 0.1% formic acid, A: 10% CH_3CN/H_2O containing 0.1% formic acid). Electrospray mass spectroscopic and LCMS analyses were conducted on a Waters Micromass LCT premier XE mass

spectrometer in conjunction with the Waters Acquity UPLC. Data is only provided here for new compounds. Epimerization ratios given are those of the integrated HPLC peaks. All yields refer to isolated, chromatographically homogeneous materials.

Fmoc-L-Ala-STmob (90)

To a solution of Fmoc-L-Ala-OH (2.33 mmol), 2,4,6-trimethoxybenzylthiol (2.8 mmol) and DMAP (0.23 mmol) in methylene chloride (20 mL) was added DIC (2.8 mmol) at 0 ºC. The suspension was stirred for 1 h at 0 ºC and overnight at room temperature. The suspension was filtered to remove the resulting white solid, which was washed with methylene chloride (10 mL**)** repeatedly. The filtrate was concentrated, after which chromatographic purification afforded a white solid (1.2 g, 98%). Mp 152-153 ºC; $[\alpha]^{22}_{D}$ –8.4 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.61 (dd, *J* = 6.0, 7.0 Hz, 2H), 7.40–7.27 (m, 4H), 6.10 (s, 2H), 5.36 (d, *J* = 8.0 Hz, 1H), 4.52 (t, *J* = 7.5 Hz, 1H), 4.45 (m, 1H), 4.34 (m, 1H), 4.24 (s, 2H), 4.22 (d, *J* = 7.5 Hz, 1H), 3.80 (s, 3H), 3.78 (s, 6H), 1.45 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 201.9, 161.2, 159.5, 155.8, 144.2, 144.0, 141.5, 127.9, 127.3, 125.4, 125.3, 120.2, 104.6, 90.7, 67.3, 56.8, 56.0, 55.6, 47.4, 22.5, 19.6; ESI-HRMS calcd for $C_{28}H_{29}NO_6SNa$ $[M + Na]^+$, 530.1624; found, 530.1613.

Boc-L-Phe-STmob (91)

To a solution of Boc-L-Phe-OH (1.5 mmol), 2,4,6-trimethoxybenzylthiol (1.5 mmol) and DMAP (0.15 mmol) in methylene chloride (12 mL) was added DIC (1.8 mmol) at 0 ºC. The suspension was stirred for 1 h at 0 ºC and overnight at

room temperature. The suspension was concentrated after which chromatographic purification afforded a white solid (0.67 g, 96%). $[\alpha]^{23}_{D}$ –36.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29–7.17 (m, 5H), 6.11 (s, 2H), 4.95 (d, *J* = 9 Hz, 1H), 4.67 (d, *J* = 7 Hz, 1H), 4.23 (s, 2H), 3.81 (s, 9H), 3.19 (dd, *J* = 5.5, 5.0 Hz, 1H), 3.05 (dd, *J* = 7.0, 7.0 Hz, 2H), 1.39 (s, 9H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 201.48, 161.1, 159.5, 155.2, 136.3, 129.7, 128.7, 127.1, 104.8, 90.7, 80.2, 61.2, 56.0, 55.6, 39.0, 28.5, 28.3, 22.6; ESI-HRMS calcd for $C_{24}H_{31}NO_6SNa$ $[M + Na]^+$, 484.1770; found, 484.1770.

Z-L-Val-STrt (92)

To a solution of Z-L-Val-OH (1.99 mmol), triphenylmethanethiol (2.19 mmol) and DMAP (0.4 mmol) in methylene chloride (15 mL) was added DIC (2.59 mmol) at 0 ºC. The suspension was stirred for 1 h at 0 ºC and overnight at room temperature. The suspension was concentrated, after which chromatographic purification afforded a white solid (0.96 g, 95%). $[\alpha]^{23}_{D}$ –45.5 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.35 (m, 5H), 7.33–7.27 (m, 15H), 5.24–5.16 (m, 2H), 5.15 (d, *J* = 12.5 Hz, 2H), 4.41–4.38 (m, 1H), 2.20 (q, *J* = 6.5 Hz, 1H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.74 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 197.6, 156.4, 143.7, 136.6, 130.4, 130.1, 128.9, 128.8, 128.5, 128.3, 128.2, 128.2, 128.0, 127.4, 71.0, 67.4, 65.9, 31.7, 19.7, 17.1; ESI-HRMS calcd for $C_{32}H_{31}NO_3SNa$ $[M + Na]^+$, 530.1922; found, 530.1920.

General procedure 1. Synthesis of peptides with Sanger's Reagent

To a stirred solution of thioacid (0.33 mmol) in DMF (2 mL) was added Cs_2CO_3 (0.47 mmol). After the solution was stirred for 5 min at room

temperature, a solution of Sanger's reagent (0.31 mmol) in DMF (1 mL) was added to the above solution followed by the addition of amine hydrochloride salt (0.28 mmol) such that the overall concentration of the reaction mixture became ~0.1 M in amine. The reaction mixture was stirred for 3 h at room temperature then was concentrated and the concentrate was diluted with ethyl acetate. The organic layer was washed with water and brine, dried, concentrated. Chromatographic purification afforded the desired peptides.

General procedure 2. Synthesis of peptides with Mukaiyama's Reagent or 2,4-dinitroiodobenzene

To a solution of thioacid (0.36 mmol) in DMF (3 mL) was added Cs_2CO_3 (0.44 mmol). After the solution was stirred for 5 min at room temperature, Mukaiyama's reagent (0.33 mmol) was added to the above solution followed by the addition of amine hydrochloride salt (0.3 mmol). The reaction mixture was stirred for 3 h at room temperature then was concentrated and the concentrate was diluted with ethyl acetate. The organic layer was washed with water and brine, dried, concentrated. Chromatographic purification afforded the desired peptides.

Fmoc-L-Ala-L-Tyr-OMe (105)

Prepared by the general procedure 2 with a yield of 222 mg (86%).Colorless solid, Mp 161-162 ºC; $[\alpha]^{22}_{D}$ +27.6 (*c* = 0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.58(d, *J* = 5.0 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.30 (t, *J* = 7.5 Hz, 2H), 6.90 (d, *J* = 7.5 Hz, 2H), 6.64 (d, *J* = 7.0 Hz, 2H), 6.55 (d, *J* = 7.0 Hz, 1H), 5.97 (s, 1H), 5.40 (d, *J* = 6.5 Hz, 1H), 4.82 (dd, *J* =

6.0, 6.5 Hz, 1H), 4.40 (m, 1H), 4.33 (m, 1H), 4.19 (m, 1H), 3.71 (s, 3H), 3.07(dd, *J* = 5.0, 5.0 Hz, 1H), 2.97(dd, *J* = 6.0, 5.5 Hz, 1H), 1.34 (d, *J* = 6.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 172.6, 172.1, 156.4, 155.7, 144.0, 143.9, 141.5, 141.5, 130.6, 128.0, 127.4, 127.1, 125.4, 120.2, 115.8, 67.5, 53.7, 52.7, 50.6, 47.3, 47.2, 37.2, 18.8; ESI-HRMS calcd for $C_{28}H_{28}N_2O_6Na$ $[M + Na]^+$, 511.1845; found, 511.1864.

Fmoc-L-Ala-L-Cys(Trt)-OEt (108)

Prepared by the general procedure 2 with a yield of 57 mg (88%). White solid, Mp 69-70 ºC; $[\alpha]^{23}_D$ +7.3 (*c* = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 2H), 7.57(d, *J* = 7.2 Hz, 2H), 7.41–7.17 (m, 19H), 6.23 (d, *J* = 6.8 Hz, 1H), 5.38 (bs, 1H), 4.52 (d, *J* = 5.6 Hz, 1H), 4.39 (d, *J* = 5.6 Hz, 2H), 4.23–4.14 (m, 4H), 2.72 (dd, *J* = 5.6, 6.4 Hz, 1H), 2.60 (dd, *J* = 4.0, 4.0 Hz, 1H), 1.37 (d, *J* = 6.8 Hz, 3H), 1.24 (t, *J* = 6.4 Hz, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 172.0, 170.2, 156.0, 144.4, 144.1, 144.0, 141.6, 141.5, 129.7, 128.3, 128.0, 127.3, 127.2, 125.3, 120.2, 67.3, 67.2, 62.1, 51.5, 50.5, 47.4, 33.8, 19.2, 14.3; ESI-HRMS calcd for $C_{46}H_{50}N_4O_{10}S_2Na$ $[M + Na]^+$, 707.2556; found, 707.2552.

Z-Gly-Phe-STmob (109)

To a stirred solution of Z-Gly-OH (1.43 mmol), NH_2-L-Phe-STmob (1.3 mmol) and DMAP (0.39 mmol) in methylene chloride (10 mL) was added EDCI (1.69 mmol) at rt and the reaction mixture was stirred for 1h. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (0.65 g, 91%). $[\alpha]^{24}_D$ –38.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.35 (m, 5H), 7.25–7.22 (m, 3H), 7.12 (d, *J* = 6.5 Hz, 2H), 6.67 (d, *J* = 8.0 Hz, 1H), 6.11 (s,

2H), 5.29 (s, 1H), 5.11 (s, 2H), 5.02–4.97 (m, 1H), 4.24 (dd, *J* = 12.0, 12.5 Hz, 2H), 3.84–3.77 (m, 11H), 3.19 (dd. *J* = 5.5 Hz, 1H), 3.10 (dd. *J* = 7.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 200.1, 168.9, 161.2, 159.5, 156.7, 136.4, 135.9, 129.7, 128.8, 128.8, 128.5, 128.3, 127.3, 104.4, 90.7, 67.4, 59.7, 56.0, 55.6, 44.7, 38.7, 31.2, 22.6; ESI-HRMS calcd for $C29H_{32}N_2O_7SNa$ $[M + Na]^+$, 575.1828; found, 575.1799.

LCMS: 5-50% B over 1 min then 50-70% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 2.08 min.

Z-Gly-L-Phe-SH (110)

Z-Gly-L-Phe-STmob (200 mg, 0.36 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (4 mL) with triethylsilane (300 µL) and stirred at rt for 1 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Z-Gly-L-Phe-SH which was applied immediately to the next step

Z-Gly-L-Phe-L-Val-OtBu (111) by the thioacid coupling with Mukaiyama's reagent

To a stirred solution of Z-Gly-Phe-SH (0.27 mmol), L-valine t-butyl ester hydrochloride (0.23 mmol) in DMF (3 mL) was added cesium carbonate (0.27 mmol) at 0 ºC. After 5 min, Mukaiyama's reagent (0.25 mmol) was added to the reaction mixture at 0 ºC. The reaction mixture was stirred at 0 ºC for 10 min and then warmed up to rt and stirred for 30 min. The organic layer was extracted with ethyl acetate followed by washing with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (50%

ethyl acetate: hexane) afforded the desired product (89 mg, 76%). $[\alpha]^{23}_{D}$ +2.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.31 (m, 5H), 7.25–7.15 (m, 5H), 6.90 (d, *J* = 8.0 Hz, 1H), 6.63 (d, *J* = 8.0 Hz, 1H), 5.65 (s, 1H), 5.11 (s, 2H), 4.80 (d, *J* = 7.0 Hz, 1H), 4.35–4.32 (m, 1H), 3.87 (d, *J* = 5.0 Hz, 2H), 3.07–3.04 (m, 2H), 2.1–2.07 (m, 1H), 1.45 (s, 9H), 0.86 (d. *J* = 6.5 Hz, 3H), 0.84 (d. *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 170.6, 169.1, 156.8, 136.4, 129.6, 128.8, 128.7, 128.4, 128.3, 128.2, 127.3, 82.3, 67.4, 57.9, 54.7, 44.7, 38.6, 31.6, 28.3, 19.0, 17.9; ESI-HRMS calcd for $C_{28}H_{37}N_3O_6Na$ $[M + Na]^+$, 534.2580; found, 534.2552.

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

Z-Gly-L-Phe-L-Val-OtBu (111) by acid coupling with the Mukaiyama's reagent

To a stirred solution of Z-Gly-Phe-OH (0.27 mmol), L-valine t-butyl ester hydrochloride (0.23 mmol) in DMF (3 mL) was added cesium carbonate (0.27 mmol) at 0 ºC. After 5 min, Mukaiyama's reagent was added to the reaction mixture at 0 ºC. The reaction mixture was stirred at 0 ºC for 10 min and then warmed up to rt and stirred overnight. The organic layer was extracted with ethyl acetate followed by washing with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (50% ethyl acetate: hexane) afforded the desired product (35 mg, 23%).

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

An epimerized sample of Z-Gly-L-Phe-L-Val-OtBu (111) by the EDCI coupling method

To a stirred solution of Z-Gly-L-Phe-OH (0.14 mmol), L-valine t-butyl ester hydrochloride (0.14 mmol) in methylene chloride (3 mL) was added EDCI (0.17 mmol) followed by the addition of DIPEA (0.17 mmol) at rt and the reaction mixture was stirred for 4 h. Chromatographic purification (50% ethyl acetate: hexane) afforded the desired product (56 mg, 78%).

LCMS of the crude reaction mixture shows epimerization (ratio, L/D = 48/52): 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min, 3.78 min.

Z-Gly-L-Phe-L-Val-OtBu (111) by the EDCI+HOBT method

To a stirred solution of Z-Gly-L-Phe-OH (0.14 mmol), L-valine t-butyl ester hydrochloride (0.14 mmol) and HOBT (0.17 mmol) in methylene chloride (4 mL) was added EDCI (0.17 mmol) at 0 ºC. The reaction mixture was stirred at 0 ºC for 10 min and then warmed up to rt and stirred for 5 h. Chromatographic purification (50% ethyl acetate in hexane) afforded the desired product (60 mg, 84%).

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

Fmoc-D-Phe-L-Val-O^{t}Bu (114)

To a stirred solution of Fmoc-D-Phe-OH (0.52 mmol) and L-valine t-butyl ester hydrochloride (0.52 mmol) in methylene chloride (4.5 mL) was added HATU (0.88 mmol) followed by the addition of DIPEA (0.88 mmol) at 0 ºC. The reaction mixture was stirred at 0 ºC for 30 min and then warmed up to rt and stirred for 6

h. Chromatographic purification (30% ethyl acetate in hexane) afforded the desired product (274 mg, 98%). $[\alpha]^{23}_{D}$ +9.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.55(t, *J* = 7.0 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.33–7.23 (m, 7H), 6.30 (d, *J* = 7.0 Hz, 1H), 5.48 (d, *J* = 5.5 Hz, 1H), 4.53 (d, *J* = 5.5 Hz, 1H), 4.43–4.33 (m, 3H), 4.21 (d, *J* = 7.0 Hz, 1H), 3.13 (br. s, 2H), 2.06–2.03 (m, 1H), 1.45 (s, 9H), 0.79–0.76 (m, 6H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 170.8, 156.1, 144.0, 143.9, 141.5, 136.7, 129.5, 129.0, 128.0, 127.3, 125.3, 120.2, 82.4, 67.4, 57.8, 56.6, 47.3, 39.1, 31.6, 28.3, 18.9, 17.9; ESI-HRMS calcd for $C_{33}H_{38}N_2O_5Na$ $[M + Na]^+$, 565.2678; found, 565.2628.

NH_2-D-Phe-L-Val-O^{t}Bu (115)

To a stirred solution of Fmoc-D-Phe-L-Val-O^{t}Bu (0.28 mmol) in THF (5 mL), was added ethanethiol (2.8 mmol) followed by DBU (0.08 mmol) at rt after which the reaction mixture was stirred for 1 h. The solvents were removed and the chromatographic purification afforded the free amine, which was immediately taken forward to the next step.

Synthesis of an authentic sample of Z-Gly-D-Phe-L-Val-O^{t}Bu (116)

To a stirred solution of Z-Gly-OH (0.42 mmol), NH_2-D-Phe-L-Val-O^{t}Bu in methylene chloride (4 mL) was added EDCI (0.42 mmol) at rt .The reaction mixture was stirred at rt for 1h before it was subjected to chromatographic purification (50% ethyl acetate in hexane) which afforded the desired product (112 mg, 79%). $[\alpha]^{23}_{D}$ +15.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.31 (m, 5H), 7.27–7.18 (m, 5H), 6.97 (d, *J* = 8.0 Hz, 1H), 6.63 (d, *J* = 8.5 Hz, 1H), 5.73 (t, *J* = 5.5 Hz, 1H), 5.11 (d, *J* = 2.0 Hz, 2H), 4.87 (d, *J* = 7.0 Hz, 1H),

4.35–4.32 (m, 1H), 3.88 (d, *J* = 5.5 Hz, 2H), 3.09–3.06 (m, 2H), 2.0–1.97 (m, 1H), 1.42 (s, 9H), 0.75 (t. *J* = 7.0 Hz, 6H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 170.8, 170.7, 169.2, 156.8, 136.6, 136.5, 129.6, 128.9, 128.8, 128.4, 128.3, 127.2, 82.3, 67.4, 58.0, 54.7, 44.7, 39.1, 31.5, 28.3, 18.9, 17.9; ESI-HRMS calcd for $C_{28}H_{37}N_3O_6Na$ $[M + Na]^+$, 534.2580; found, 534.2542.

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.78 min.

Z-Gly-L-Phe-L-Val-O^{t}Bu (111) by the HATU method

To a stirred solution of Z-Gly-L-Phe-OH (0.28 mmol) and L-valine t-butyl ester hydrochloride (0.28 mmol) in methylene chloride (4 mL) was added HATU (0.36 mmol) followed by the addition of DIPEA (0.36 mmol) at 0 ºC. The reaction mixture was stirred at 0 ºC for 30 min and then warmed up to rt and stirred for 2 h. Chromatographic purification (50% ethyl acetate: hexane) afforded the desired product (117 mg, 82%).

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.65 min.

Z-Gly-2,2-d_2-OH

To a stirred solution of glycine-2,2-d_2 (6.49 mmol) in 1N NaOH solution in water (6.5 mL, pH~10) was added benzyl chloroformate (7.79 mmol) at room temperature. The resulting reaction mixture was stirred at rt for 4h before it was extracted with methyl *t*-butyl ether and the aqueous layer was acidified (pH~1) with 2M HCl solution. The product was extracted with ethyl acetate from aqueous layer, dried over sodium sulfate and concentrated under reduced pressure to

give a white solid (0.96 g, 72%). Mp 110-111 ºC, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (s, 5H), 5.04(s, 2H); ESI-HRMS calcd for $C_{10}H_9D_2NO_4Na$ $[M + Na]^+$, 234.0711; found, 234.0691.

NH_2-L-Phe-STmob (162)

Boc-L-Phe-STmob (0.65 mmol) was dissolved in a mixture of 40% TFA in CH_2Cl_2 (7 mL) and stirred at rt for 5 min. then solvents were removed and the the organic layer was extracted with ethyl acetate followed by washing with 20% aqueous sodium carbonate solution. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to afford the free amine which was applied immediately to the next step.

Z-Gly-2,2-d_2-L-Phe-STmob (117)

To a stirred solution of Z-Gly-2,2-d_2-OH (0.72 mmol), NH_2-L-Phe-STmob (0.65 mmol) and DMAP (0.2 mmol) in methylene chloride (8 mL) was added EDCI (0.85 mmol) at rt and the reaction mixture was stirred for 1h. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (0.32 g, 89%). $[\alpha]^{23}_D$ –28.9 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.32 (m, 5H), 7.25–7.20 (m, 3H), 7.12 (d, *J* = 7.0 Hz, 2H), 6.66 (d, *J* = 8.5 Hz, 1H), 6.10 (s, 2H), 5.46 (s, 1H), 5.09 (s, 2H), 5.01–4.97 (m, 1H), 4.24 (dd, *J* = 12.5 Hz, 2H), 3.80 (s, 3H), 3.79 (s, 6H), 3.19 (dd. *J* = 5.0 Hz, 1H), 3.05 (dd. *J* = 7.5 Hz, 1H); ESI-HRMS calcd for $C29H_{30}D_2N_2O_7SNa$ $[M + Na]^+$, 577.1953; found, 577.1928.

LCMS: 5-50% B over 2 min then 50-70% B over 3 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 2.93 min.

Z-Gly-2,2-d_2-L-Phe-L-Val-O^{t}Bu (119)

To a stirred solution of Z-Gly-2,2-d_2-Phe-SH (0.22 mmol), Z-Gly-L-Phe-OH (0.22 mmol), L-valine t-butyl ester hydrochloride (0.15 mmol) in DMF (3 mL) was added cesium carbonate (0.3 mmol) at 0 ºC. After 5 min, a solution of Mukaiyama's reagent (0.15 mmol) in DMF (0.5 mL) was added to the reaction mixture dropwisely at 0 ºC over 10 min. The reaction mixture then was stirred at 0 ºC for further 30 min before the organic layer was extracted with ethyl acetate. The extracts were washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (50% ethyl acetate: hexane) afforded the desired product (55 mg, 72%). $[\alpha]^{23}_{D}$ –1.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.31 (m, 5H), 7.25–7.15 (m, 5H), 6.89 (d, *J* = 7.5 Hz, 1H), 6.61 (d, *J* = 8.0 Hz, 1H), 5.62 (s, 1H), 5.11 (s, 2H), 4.79 (d, *J* = 7.0 Hz, 1H), 4.35–4.32 (m, 1H), 3.07–3.05 (m, 2H), 2.1–2.07 (m, 1H), 1.45 (s, 9H), 0.85 (d. *J* = 7.0 Hz, 3H), 0.84 (d. *J* = 7.0 Hz, 3H); ESI-HRMS calcd for $C_{28}H_{35}D_2N_3O_6Na$ $[M + Na]^+$, 536.2706; found, 536.2695.

LCMS: 5-40% B over 1 min then 40-50% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.60 min.

Z-L-Val-L-Val-O^{t}Bu (121)

To a stirred solution of Z-L-Val-SH (0.39 mmol), L-valine t-butyl ester hydrochloride (0.33 mmol), and Z-L-Arg-OH hydrochloride (0.33 mmol) in DMF (3 mL) was added cesium bicarbonate (1.31 mmol) at rt. After 5 min, Mukaiyama's reagent (0.33 mmol) was added to the reaction mixture at rt and the reaction mixture was stirred for 1 h. The organic layer was extracted with ethyl acetate

followed by washing with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification afforded the desired product with a yield of 110 mg (82%). $[\alpha]^{23}_{D}$ +1.7 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34–7.27 (m, 5H), 6.53 (d, *J* = 9.0 Hz, 1H), 5.55 (d, *J* = 8.5 Hz, 1H), 5.12 (d, *J* = 3.5 Hz, 2H), 4.44–4.42 (m, 1H), 4.11 (t, *J* = 7.5 Hz, 1H), 2.16–2.11 (m, 2H), 1.45 (s, 9H), 0.98–0.88 (m, 12H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.5, 171.0, 156.7, 136.6, 128.7, 128.3, 128.2, 82.2, 67.2, 60.6, 57.8, 31.5, 28.3, 19.5, 19.1, 18.1, 18.0; ESI-HRMS calcd for $C_{22}H_{34}N_2O_5Na$ $[M + Na]^+$, 429.2365; found, 429.2330.

Z-L-Val-L-Val-OtBu 121 (Reaction in aqueous buffer)

To a stirred solution of Z-L-Val-SH (0.39 mmol), L-valine t-butyl ester hydrochloride (0.33 mmol) in ligation buffer (3 mL, [4:1 v/v *N*-methyl-2-pyrrolidinone (NMP): 6 M guanidine hydrochloride, 1 M HEPES, pH = 8][a]) was added Mukaiyama's reagent (0.33 mmol) at 0 ºC and then the reaction mixture was stirred at rt for 1 h. The organic layer was extracted with ethyl acetate followed by washing with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification afforded the desired product with a yield of 115 mg (86%).

[a] An aqueous buffer containing 6 M Gn.HCl and 1 M HEPES was prepared and adjusted to pH 8 using 25% cesium carbonate solution in water. The resulting solution (1 mL) was diluted with NMP (4 mL) to produce the final buffer for use in peptide coupling reaction.

NH_2-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (123)

The N-terminal tetrapeptidyl amine was prepared by stepwise solution phase synthesis according to the scheme below, beginning with L-Ala-OEt. Fmoc protected amino acid building blocks were coupled using standard conditions EDCI/HOBT in either CH_2Cl_2 or DMF. Fmoc groups were removed using DBU/EtSH in dry THF. ESI-HRMS calcd for $C_{57}H_{63}N_6O_7$ $[M + H]^+$, 943.4758; found, 943.4761.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-OH (124)

The C-terminal tetrapeptidyl acid was prepared by stepwise solution phase synthesis according to the Scheme below, beginning with L-Arg(Pbf)-OAll. Fmoc protected amino acid building blocks were coupled using standard conditions EDCI/HOBT in either CH_2Cl_2 or DMF. Fmoc groups were removed using DBU/EtSH in dry THF. Allyl deprotection was conducted with $Pd(PPh_3)_4/PhSiH_3$ in CH_2Cl_2. ESI-HRMS calcd for $C_{77}H_{107}N_{12}O_{16}S_2$ $[M + H]^+$, 1519.7369; found, 1519.7335.

LCMS: 50-90% B with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.45 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (94) by EDCI+HOBT coupling

To a stirred solution of the peptidyl acid **124** (0.01 mmol), 9-fluorenylmethanethiol (0.25 mmol) and HOBT (0.05 mmol) in dry DMF (0.5 mL) was added EDCI (0.05 mmol) at rt and the reaction mixture was stirred at rt for 4 h before it was diluted with ethyl acetate. The organic layer was washed with

water and brine, dried and concentrated and subjected to reversed-phase HPLC purification (50-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, rt = 59 min) to provide the protected peptidyl thioester with a yield of 13 mg (77%).

LCMS: 70-90% B with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.00, 3.17 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SFm (94) by PyBOP method

To a solution of the peptidyl acid **124** (~0.01 mmol) in DMF (0.5 mL) was added 4Å molecular sieves (~ 0.01 g) and 9-fluorenylmethanethiol (0.25 mmol) and the mixture was stirred at -20 ^{o}C. After 15 min., PyBOP (0.05 mmol) and DIPEA (0.05 mmol) were added and the reaction mixture was stirred at -20 ^{o}C for 1.5 h, before it was filtered, quenched with a saturated solution of NH_4Cl, and diluted with ethyl acetate. The organic layer was washed with water and brine, dried and concentrated. Reversed-phase HPLC purification (50-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, rt = 60 min) provided the protected peptidyl thioester with a yield of 14 mg (82%) ESI-HRMS calcd for $C_{91}H_{117}N_{12}O_{15}S_3$ $[M + H]^+$, 1713.7924; found, 1713.7991. LCMS: 70-90% B with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.17 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-SH (125)

The peptidyl thioester **94** (0.008 mmol) was dissolved in a mixture of 50% piperidine: DMF (0.5 ml) and stirred at rt for 1.5 h. The reaction mixture was

diluted with ethyl acetate (40 ml) and acidified with 1M HCl. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. LCMS: 60-95% B with a flow rate of 0.5 mL/min over 5 min and 254 nm UV detection, retention time = 3.02 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) by the thioacid coupling with Mukaiyama's Reagent Method

To a stirred solution of the peptidyl thioacid **125** (0.008 mmol, 1.1 equiv.) and the peptide amine **123** (0.007 mmol, 1 equiv.) in dry DMF (0.5 ml) was added Cs_2CO_3 (0.009 mmol, 1.2 equiv.) at 0 ºC. After the reaction mixture was stirred at 0 ºC for 5 min, Mukaiyama's reagent (0.008 mmol, 1.1 equiv.) was added to the reaction mixture and stirred at rt for 1 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, retention time = 31 min) provided the octapeptide with a yield of 12 mg (66%). ESI-HRMS calcd for $C_{134}H_{167}N_{18}O_{22}S_2$ $[M + H]^+$, 2444.1944; found, 2444.1365. LCMS of the crude reaction mixture: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.33 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) by the PyBOP Method

To a stirred solution of the peptidyl acid **124** (0.011 mmol) and the peptidyl amine (0.011 mmol) in dry DMF (0.5 mL) was added PyBOP (0.013 mmol) followed by DIPEA (0.013 mmol) at 0 ºC and the reaction mixture was stirred at rt

for 8 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, rt = 30 min) provided the octapeptide with a yield of 18 mg (69%). LCMS of the crude reaction mixture shows epimerization: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.35 min and 2.57 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) by the EDCI+HOBT Method

To a stirred solution of the peptidyl acid **124** (0.013 mmol), the peptidyl amine (0.013 mmol) and HOBT (0.017 mmol) in dry DMF (0.7 ml) was added EDCI (0.017 mmol) 0 ºC. After 5 min, the reaction mixture was warmed up to rt and stirred at rt for 8 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 12 mL/min over 72 min and 254 nm UV detection, retention time = 29 min) provided the octapeptide with a yield of 20 mg (63%). LCMS of the crude reaction mixture showed epimerization: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.35 min and 2.57 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) by the EDCI Method

To a stirred solution of the peptidyl acid **124** (0.01 mmol) and the peptidyl amine (0.01 mmol) in dry DMF (0.5 ml) was added EDCI (0.013 mmol) followed by DIPEA (0.013 mmol) at 0 ºC and the reaction mixture was stirred at rt for 14 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, retention time = 29 min) provided the octapeptide with a yield of 14 mg (54%). LCMS of the crude reaction mixture showed epimerization: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.33 min and 2.54 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-L-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (126) by the HATU Method

To a stirred solution of the peptidyl acid **124** (0.012 mmol) and the peptidyl amine (0.012 mmol) in dry DMF (0.5 ml) was added HATU (0.013 mmol) followed by DIPEA (0.013 mmol) at 0 ºC and the reaction mixture was stirred at rt for 6 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, and concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 10 mL/min over 72 min and 254 nm UV detection, retention time = 30 min) provided the octapeptide with a yield of 20 mg (70%). LCMS of the crude reaction mixture shows epimerization: 80-92% B

over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.35 min and 2.57 min.

Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-D-Arg(Pbf)-OH (129)

The C-terminal tetrapeptidyl acid **129** was prepared by solid phase peptide synthesis in a manual solid phase peptide synthesis vessel beginning with Fmoc-D-Arg(Pbf)-OH. 2-Chlorotrityl chloride resin (0.2 mmol) was suspended in methylene chloride (5 mL) shaken for 5 min and filtered. A solution of Fmoc-D-Arg(Pbf)-OH (0.4 mmol) and DIPEA (1.0 mmol) in methylene chloride (4 mL) was added to the resin, shaken for 30 min at rt, and filtered. The resin was washed with DMF (2x 5 mL) and capped with 5.0 mL of a mixture of methylene chloride/MeOH/DIPEA (80:15:5).

Iterative peptide assembly: The Fmoc group was removed with 25% piperidine in DMF. After Fmoc removal, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL), isopropanol (2x5 mL) and hexane (2x5 mL). All couplings were carried out by adding a preactivated solution of protected amino acid (0.8 mmol), HOBT (1.0 mmol), DIPEA (0.8 mmol), and DIC (1.0 mmol) in dry DMF (4.0 mL) to the resin. After 3 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL).

Cleavage from the resin: The peptide-resin was suspended in a 6 mL mixture of trifluoroethanol/methylene chloride (2:8) and stirred magnetically for 45 min at rt. The resin was filtered and washed twice with 10 mL of cleavage solution. The combined filtrates were concentrated and a mixture of methyl t-butyl ether (15 mL) and hexane (15 mL) was added to the concentrate and the

resulting solid was isolated by centrifugation and washed with the methyl t-butyl ether and hexane (246 mg, 81%). ESI-HRMS calcd for $C_{77}H_{106}N_{12}O_{16}S_2Na$ [M + Na]$^+$, 1541.7189; found, 1541.7253.

LCMS: 50-90% B with a flow rate of 0.7 mL/min over 5 min and 254 nm UV detection, retention time = 3.38 min.

An authentic sample of Boc-L-Lys(Boc)-L-Arg(Pbf)-L-Asn(Trt)-D-Arg(Pbf)-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (130)

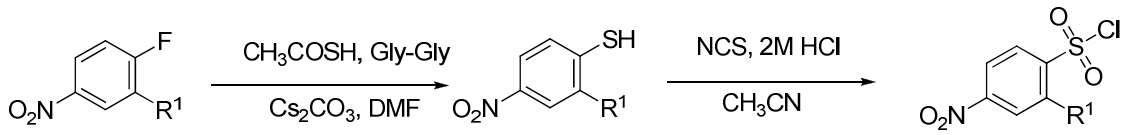
To a stirred solution of the peptidyl acid **129** (0.017 mmol) and the peptidyl amine **123** (0.017 mmol) in dry DMF (0.7 mL) was added HATU (0.033 mmol) followed by DIPEA (0.033 mmol) at 0 ºC. After 30 min, the reaction mixture was warmed up to rt and stirred for 4 h. The reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried, and concentrated. Reversed-phase HPLC purification (80-100% A with a flow rate of 12 mL/min over 60 min and 254 nm UV detection, retention time = 31 min) provided the octapeptide with a yield of 29 mg (72%). ESI-HRMS calcd for $C_{134}H_{166}N_{18}O_{22}S_2Na$ [M + Na]$^+$, 2466.1763; found, 2466.2322.

LCMS: 80-92% B over 0.5 min then 92-98% B over 3 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 2.57 min.

NH_2-L-Lys-L-Arg-L-Asn-L-Arg-L-Asn-L-Asn-L-Ile-L-Ala-OEt (122)

Octapeptide **126** (0.012 mmol) was dissolved in a solution of 50% TFA/ 10%Et_3SiH/ CH_2Cl_2 (1 ml) at room temperature. The reaction mixture was stirred at room temperature for 3 h and then solvents were removed and the crude was washed with diethyl ether 2-3 times and purified by reversed-phase HPLC (11

mg, 92%). ESI-HRMS calcd for $C_{41}H_{77}N_{18}O_{12}$ $[M + H]^+$, 1013.5968; found, 1013.5918.LCMS: 0-55% A over 3.5 min with a flow rate of 0.5 mL/min and 190 nm UV detection, retention time = 2.87 min.



Scheme 1: Preparation of Sulfonyl Chlorides

General Procedure 3 for the Preparation of Sulfonyl Chlorides

To a stirred solution of thioacetic acid (4.51 mmol) and glycineglycine (3.9 mmol) in dry DMF (10 mL) was added cesium carbonate (9.03 mmol) at rt. After 10 min, a solution of the corresponding 1-fluoro-4-nitro compound (3.01 mmol) in dry DMF (5 mL) was added slowly after which the reaction mixture and stirred for 12h. The reaction mixture was diluted with ethyl acetate and washed with 1 M HCl, water and brine, dried over sodium sulfate and concentrated. Chromatographic purification gave the corresponding thiol.

To a stirred solution of *N*-chlorosuccinimide (6.67 mmol) in 2M HCl (0.8 mL) and acetonitrile (4 mL) was added the aboce prepared thiol (1.67 mmol) slowly at 10 ºC after which the reaction mixture was stirred for 30 min keeping the temperature below 20 ºC. The reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over sodium sulfate and concentrated. Chromatographic purification gave the corresponding sulfonyl chlorides.

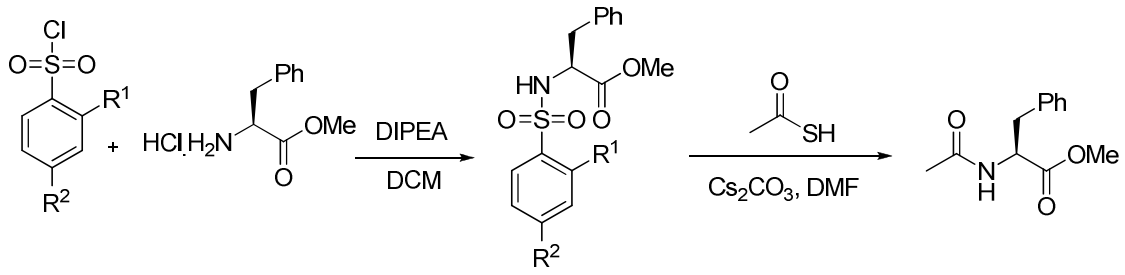
Methyl 2-mercapto-5-nitrobenzoate (133): Prepared by the general procedure 3 with a yield of 3.40 g (87%). Pale yellow solid, Mp 104–105 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.90 (d, *J* = 3.0 Hz, 1H), 8.16(dd, *J* = 3.0, 9.0 Hz, 1H),

7.47 (d, *J* = 8.5 Hz, 1H), 5.40 (s, 1H), 4.00 (s, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 165.9, 148.5, 145.0, 131.6, 127.1, 126.6, 126.0, 53.2; ESI-HRMS calcd for $C_8H_6NO_4S$ $[M-H]^-$, 212.0018; found, 212.0007.

2-Methoxycarbonyl-4-nitrobenzenesulfonyl chloride (ENS-Cl): Prepared by the general procedure 3 with a yield of 3.0 g (80%). White solid, Mp 108–112 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.58 (d, *J* = 2.5 Hz, 1H), 8.54(dd, *J* = 2.5, 9.0 Hz, 1H), 8.41 (d, *J* = 8.5 Hz, 1H), 4.06 (s, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 164.4, 151.0, 146.0, 134.4, 131.1, 126.3, 125.6, 54.3; ESI-HRMS calcd for $C_8H_6NO_6SClNa$ $[M + Na]^+$, 301.9510; found, 301.9502.

2-Mercapto-5-nitrobenzonitrile (135): Prepared by the general procedure 3 with a yield of 423 mg (78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, *J* = 2.4 Hz, 1H), 8.27(dd, *J* = 2.4, 8.8 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 4.49 (s, 1H); ^{13}C NMR (100.9 MHz, $CDCl_3$) δ 147.5, 129.5, 128.7, 127.5, 115.7, 111.9; ESI-HRMS calcd for $C_7H_3N_2O_2S$ $[M-H]^-$, 178.9871; found, 178.9915.

2-Cyano-4-nitrobenzenesulfonyl chloride (CNS-Cl): Prepared by the general procedure 3 with a yield of 394mg (95%). Pale yellow solid, Mp 108-109 ºC; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.82 (d, *J* = 2.5 Hz, 1H), 8.70 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.48 (d, *J* = 8.5 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 150.9, 149.0, 131.2, 128.6, 113.0, 112.6; EI-HRMS calcd for $C_8H_6NO_6SCl$ $[M]^+$, 245.9508; found, 245.9502.



Scheme2: Preparation of Sulfonamides and their Reaction with Thioacetic Acid

General Procedure 4 for the Preparation of Sulfonamides and their Reaction with Thioacetic Acid

To a stirred solution of L-phenylalanine methyl ester hydrochloride (0.4 mmol) and the corresponding sulfonyl chloride (0.48 mmol) in methylene chloride (4 mL) was added DIPEA (0.96 mmol) at rt. The reaction mixture was stirred for 4h before chromatographic purification afforded the desired sulfonamides.

To a stirred solution of thioacetic acid (0.24 mmol) and corresponding sulfonamide (0.15 mmol) in dry DMF (1 mL) was added cesium carbonate (0.24 mmol) at rt. The reaction mixture was stirred for 4-12 h before the it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification afforded the desired products.

***N*-[(4-Methylcarbonylphenyl)sulfonyl]–L-phenylalanine methyl ester (136):** Prepared by the general procedure 4 with a yield of 600 mg (88%). $[\alpha]^{23}_{D}$ +1.9 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.0 Hz, 2H), 7.81(d, *J* = 8.0 Hz, 2H), 7.22 (s, 3H), 7.06 (d, *J* = 3.5 Hz, 2H), 5.35 (d, *J* = 9.5 Hz, 1H), 4.27–4.23 (m, 1H), 3.54 (s, 3H), 3.08 (dd, *J* = 5.5, 14 Hz, 1H), 3.01 (dd, *J* =

6.0, 13.5 Hz, 1H), 2.64 (s, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 197.0, 171.4, 143.9, 140.2, 135.1, 129.6, 129.0, 128.9, 127.6, 57.1, 52.8, 39.5, 27.1; ESI-HRMS calcd for $C_{18}H_{19}NO_5SNa$ $[M + Na]^+$, 384.0914; found, 384.0882.

***N*-[(4-Cyanophenyl)sulfonyl]–L-phenylalanine methyl ester (137):** Prepared by the general procedure 4 with a yield of 130 mg (92%). $[\alpha]^{22}_D$ –2.9 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 2.0, 7.0 Hz, 2H), 7.66 (dd, *J* = 2.0, 6.5 Hz, 2H), 7.23–7.20 (m, 3H), 7.06–7.04 (m, 2H), 5.65 (d, *J* = 9.5 Hz, 1H), 4.25–4.21 (m, 1H), 3.61 (s, 3H), 3.10 (dd, *J* = 5.5, 14 Hz, 1H), 2.96 (dd, *J* = 7.5, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.5, 144.2, 135.2, 133.0, 129.6, 129.0, 127.8, 127.6, 117.6, 116.4, 57.4, 53.0, 39.3; ESI-HRMS calcd for $C_{17}H_{16}N_2O_4SNa$ $[M + Na]^+$, 367.0712; found, 367.0728.

***N*-[(2-Trifluoromethylphenyl)sulfonyl]–L-phenylalanine methyl ester (138):** Prepared by the general procedure 4 with a yield of 121 mg (91%). $[\alpha]^{22}_D$ +10.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (dd, *J* = 1.5, 8.25 Hz, 1H), 7.82(dd, *J* = 1.0, 8.0 Hz, 1H), 7.68–7.62 (m, 2H), 7.24–7.21 (m, 3H), 7.07–7.05 (m, 2H), 5.32 (d, *J* = 9.0 Hz, 1H), 4.33–4.29 (m, 1H), 3.49 (s, 3H), 3.10 (dd, *J* = 6.0, 14 Hz, 1H), 3.03 (dd, *J* = 6.5, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.1, 138.6, 135.0, 133.0, 132.5, 131.2, 129.5, 128.9, 128.8, 128.7, 128.3, 128.0, 127.7, 127.6, 127.5, 123.0 (q, *J* = 274.2), 57.5, 52.6, 39.4; ESI-HRMS calcd for $C_{17}H_{16}NO_4SF_3Na$ $[M + Na]^+$, 410.0660; found, 410.0650.

***N*-[(3,5-Trifluoromethylphenyl)sulfonyl]–L-phenylalanine methyl ester (140):** Prepared by the general procedure 4 with a yield of 196 mg (93%); $[\alpha]^{23}_D$ –47.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (s, 2H), 8.0 (s, 1H),

7.21–7.16 (m, 3H), 7.05–7.03 (m, 2H), 5.49 (d, *J* = 9.0 Hz, 1H), 4.35–4.31 (m, 1H), 3.63 (s, 3H), 3.13 (dd, *J* = 5.5, 14 Hz, 1H), 3.0 (dd, *J* = 6.0, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.3, 143.0, 134.8, 133.4, 133.1, 132.8, 132.5, 129.4, 128.9, 127.8, 127.5, 126.4, 125.9, 123.7, 121.5, 119.3, 57.5, 53.0, 39.4; ESI-HRMS calcd for $C_{18}H_{15}NO_4SF_6Na$ $[M + Na]^+$, 478.0514; found, 478.0524.

***N*-[(2-Methoxycarbonyl-4-nitrophenyl)sulfonyl]–L-phenylalanine methyl ester (141):** Prepared by the general procedure 4 with a yield of 250 mg (92%), Mp 128–129 ºC; $[\alpha]^{23}_D$ –3.3 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.62 (s, 1H), 8.35 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.07 (d, *J* = 8.0 Hz, 1H), 7.21–7.18 (m, 3H), 7.12–7.10 (m, 2H), 6.76 (d, *J* = 8.5 Hz, 1H), 4.49–4.45 (m, 1H), 4.01 (s, 3H), 3.54 (s, 3H), 3.17 (dd, *J* = 5.5, 14 Hz, 1H), 3.06 (dd, *J* = 7.5, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.2, 165.6, 149.4, 145.7, 135.3, 131.6, 130.5, 129.5, 128.9, 127.6, 126.5, 126.2, 58.1, 54.2, 52.7, 39.4; ESI-HRMS calcd for $C_{18}H_{18}N_2O_8SNa$ $[M + Na]^+$, 445.0656; found, 445.0682.

***N*-[(2-Cyano-4-nitrophenyl)sulfonyl]–L-phenylalanine methyl ester (142):** Prepared by the general procedure 4 with a yield of 131 mg (91%), Mp 164–165 ºC; $[\alpha]^{23}_D$ –46.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.44 (d, *J* = 2.0 Hz, 1H), 8.39 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.07 (d, *J* = 8.5 Hz, 1H), 7.17–7.13 (m, 3H), 7.07–7.06 (m, 2H), 5.86 (s, 1H), 4.40 (dd, *J* = 5.0, 8.5 Hz, 1H), 3.75 (s, 3H), 3.20 (dd, *J* = 4.5, 14 Hz, 1H), 2.96 (dd, *J* = 9.0, 14.5 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.1, 149.3, 148.3, 135.1, 130.5, 129.9, 129.5, 129.0, 127.6, 127.5, 114.4, 112.3, 58.1, 53.3, 39.1; ESI-HRMS calcd for $C_{18}H_{18}N_2O_8SNa$ $[M + Na]^+$, 412.0549; found, 412.0579.

***N*-[(2-Nitro-4-trifluoromethylphenyl)sulfonyl]–L-phenylalanine methyl ester (143):** Prepared by the general procedure 4 with a yield of 36 mg (90%); $[\alpha]^{23}_{D}$ –25.2 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 1.0 Hz, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.86 (dd, *J* = 1.0, 8.0 Hz, 1H), 7.19–7.15 (m, 3H), 7.10–7.08 (m, 2H), 5.99 (d, *J* = 8.5 Hz, 1H), 4.50 (dd, *J* = 3.0, 8.0 Hz, 1H), 3.64 (s, 3H), 3.21 (dd, *J* = 5.0, 14 Hz, 1H), 3.04 (dd, *J* = 8.0, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.0, 147.6, 138.0, 135.0, 131.4, 129.9, 129.5, 129.0, 127.8, 123.1, 58.4, 52.9, 39.3; ESI-HRMS calcd for $C_{17}H_{15}N_2O_6SF_3Na$ $[M + Na]^+$, 455.0494; found, 455.0501.

Boc-L-Glu(STrt)-O^{t}Bu (147)

To a stirred solution of Boc-L-Glu(OH)-O^{t}Bu (260 mg, 0.86 mmol), triphenylmethanethiol (236 mg, 0.86 mmol) and DMAP (21 mg, 0.17 mmol) in dry methylene chloride (5 mL) was added EDCI (192 mg, 1.03 mmol) at rt. The reaction mixture was stirred for 3 h and then subjected to chromatographic purification (30% ethyl acetate: hexane) which afforded the desired product (365 mg, 76%). $[\alpha]^{24}_{D}$ +15.4 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.31–7.22 (m, 15H), 4.99 (d, *J* = 8.0 Hz, 1H), 4.14 (d, *J* = 7.5 Hz, 1H), 2.66–2.54 (m, 2H), 4.25–4.21 (m, 2H), 2.10–2.05 (m, 1H), 1.88–1.81 (m, 1H), 1.45 (s, 18H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 196.2, 171.4, 155.7, 144.0, 130.0, 128.0, 127.4, 82.5, 80.1, 70.7, 53.5, 40.0, 28.6, 28.2; ESI-HRMS calcd for $C_{33}H_{39}NO_5SNa$ $[M + Na]^+$, 584.2479; found, 584.2447.

Boc-L-Glu(SH)-O^{t}Bu (148)

Boc-L-Glu(STrt)-O^{t}Bu (63 mg, 0.11 mmol) was dissolved in a mixture of 5% TFA in methylene chloride (2 mL) with triethylsilane (100 µL) and stirred at rt for 5 min. Toluene (5 mL) was added to the reaction mixture and then the solvents were removed. The residue was diluted with ethyl acetate and washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure to give the crude thioacid.

Boc-L-Asp(STrt)-OBn (150)

To a stirred solution of Boc-L-Asp(OH)-OBn (100 mg, 0.31 mmol), triphenylmethanethiol (85 mg, 0.31 mmol) and DMAP (8 mg, 0.06 mmol) in dry methylene chloride (3 mL) was added EDCI (71 mg, 0.37 mmol) at rt. After stirring for 3 h, chromatographic purification (20% ethyl acetate: hexane) afforded the desired product (137 mg, 76%). $[\alpha]^{23}_{D}$ –1.5 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.20 (m, 20H), 5.30 (d, *J* = 8.0 Hz, 1H), 5.10 (q, *J* = 12.5 Hz, 2H), 4.51–4.48 (m, 1H), 3.22 (dd, *J* = 5.0, 16.5 Hz, 1H), 3.07 (dd, *J* = 4.5, 16.5 Hz, 1H), 1.43 (s, 9H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 195.2, 170.7, 155.5, 143.7, 135.5, 130.0, 128.8, 128.6, 128.4, 128.1, 127.5, 80.3, 71.4, 67.7, 51.0, 44.9, 28.5; ESI-HRMS calcd for $C_{35}H_{35}NO_5SNa$ $[M + Na]^+$, 604.2172; found, 604.2134.

Boc-L-Asp(SH)-OBn (151)

Boc-L-Asp(STrt)-OBn (65 mg, 0.11 mmol) was dissolved in a mixture of 5% TFA in methylene chloride (2 mL) with triethylsilane (100 µL) and stirred at rt for 5 min. Toluene (5 mL) was added to the reaction mixture and then solvents were removed. The residue was diluted with ethyl acetate followed by washing

with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure to give the crude thioacid.

Fmoc-L-Glu(OtBu)-L-Val-L-Met-OMe (153)

Boc-L-Val-L-Met-OMe (425 mg, 1.18 mmol) was dissolved in a mixture of 40% TFA in methylene chloride (6 mL) with triethylsilane (200 µL) and stirred at rt for 15 min. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give a crude ammonium salt, which was neutralized with triethylamine to give crude NH_2-L-Val-L-Met-OMe which was applied immediately to the next step.

To a stirred solution of Fmoc-L-Glu(O^tBu)-OH (500 mg, 1.18 mmol), the above prepared NH_2-L-Val-L-Met-OMe and HOBT (190 mg, 1.42 mmol) in dry methylene chloride (7 mL) was added EDCI (270 mg, 1.42 mmol) at 0 ºC after which the reaction mixture was stirred at rt for 6 h. Chromatographic purification (55% ethyl acetate: hexane) afforded the desired product (740 mg, 94%). $[\alpha]^{23}_D$ –7.2 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 5.5 Hz, 2H), 7.39 (t, *J* = 7.0 Hz, 2H), 7.28 (s, 2H), 7.14 (d, *J* = 6.5 Hz, 2H), 6.13 (d, *J* = 6.0 Hz, 1H), 4.71 (d, *J* = 4.0 Hz, 1H), 4.37–4.33 (m, 4H), 4.21 (d, *J* = 6.5 Hz, 1H), 3.72 (s, 3H), 2.49–2.40 (m, 4H), 2.17–1.99 (m, 8H), 1.46 (s, 9H), 0.95 (dd, *J* = 10.0, 7.0 Hz, 6H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 173.3, 172.4, 172.0, 171.2, 156.7, 144.1, 144.0, 141.5, 128.0, 127.3, 125.4, 120.2, 81.4, 67.5, 58.9, 54.9, 52.7, 51.8, 47.3, 32.1, 31.6, 31.0, 30.2, 28.3, 19.4, 18.2, 15.6; ESI-HRMS calcd for $C_{35}H_{47}N_3O_8SNa$ $[M + Na]^+$, 692.2902; found, 692.2982.

Fmoc-L-Glu(STrt)-L-Val-L-Met-OMe (154)

Fmoc-L-Glu(O^{t}Bu)-L-Val-L-Met-OMe (200 mg, 0.30 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (200 µL) and stirred at rt for 1 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid Fmoc-L-Glu(OH)-L-Val-L-Met-OMe which was applied immediately to the next step.

To a stirred solution of Fmoc-L-Glu(OH)-L-Val-L-Met-OMe, triphenylmethanethiol (83 mg, 0.30 mmol) and DMAP (4 mg, 0.03 mmol) in dry methylene chloride (3 mL) was added EDCI (69 mg, 0.36 mmol) at rt. After stirring for 4 h, the reaction mixture was subjected to chromatographic purification (55% ethyl acetate: hexane) to give the desired product (169 mg, 65%). $[\alpha]^{24}_{D}$ +1.9 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.0 Hz, 2H), 7.58 (t, *J* = 7.0 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31–7.22 (m, 17H), 6.83 (d, *J* = 7.0 Hz, 2H), 6.55 (d, *J* = 7.5 Hz, 1H), 5.57 (d, *J* = 7.5 Hz, 1H), 4.68 (dd, *J* = 7.5, 12.5 Hz, 1H), 4.43–4.36 (m, 2H), 4.25–4.21 (m, 2H), 4.07 (d, *J* = 6.5 Hz, 1H), 3.72 (s, 3H), 2.77–2.74 (m, 1H), 2.66–2.63 (m, 1H), 2.43 (t, *J* = 7.5 Hz, 2H), 2.16–1.91 (m, 4H), 0.91 (d, *J* = 7.0 Hz, 3H), 0.87 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 197.8, 172.3, 171.3, 170.8, 156.3, 144.0, 143.8, 141.5, 130.1, 128.1, 128.0, 127.5, 127.3, 125.4, 125.3, 120.2, 71.1, 67.5, 59.0, 54.1, 52.7, 51.7, 47.3, 39.9, 31.5, 30.8, 30.2, 28.6, 19.3, 18.0, 15.7; ESI-HRMS calcd for $C_{50}H_{53}N_3O_7S_2Na$ $[M + Na]^+$, 894.3131; found, 894.3223.

Fmoc-L-Glu(SH)-L-Val-L-Met-OMe (155)

Fmoc-L-Glu(STrt)-L-Val-L-Met-OMe (18 mg, 0.02 mmol) was dissolved in a mixture of 25% TFA in methylene chloride (1 mL) with triethylsilane (100 µL) and stirred at rt for 5 min. Toluene (5 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Fmoc-L-Glu(SH)-L-Val-L-Met-OMe which was applied immediately to the next step.

Z-Gly-STrt (158)

To a stirred solution of Z-Gly-OH (150 mg, 0.71 mmol), triphenylmethanethiol (197 mg, 0.71 mmol) and DMAP (17 mg, 0.14 mmol) in dry methylene chloride (5 mL) was added EDCI (164 mg, 0.85 mmol) at rt. The reaction mixture was stirred for 2 h then subjected to chromatographic purification (20% ethyl acetate: hexane) which afforded the desired product (307 mg, 92%); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36–7.25 (m, 20H), 5.32–5.30 (m, 1H), 5.14 (s, 1H), 4.08 (d, *J* = 5.5 Hz, 2H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 194.7, 156.3, 143.7, 136.5, 130.0, 128.8, 128.4, 128.3, 128.1, 127.5, 80.0, 67.4, 50.9; ESI-HRMS calcd for $C_{29}H_{25}NO_3SNa$ $[M + Na]^+$, 490.1453; found, 490.1457.

Boc-L-Val-STmob (164)

To a stirred solution of Boc-L-Val-OH (500 mg, 2.30 mmol), 2,4,6-trimethoxybenzylthiol (490 mg, 2.3 mmol) and DMAP (28 mg, 0.23 mmol) in dry methylene chloride (8 mL) was added DIC (422 µL, 2.76 mmol) at 0 ºC. The reaction mixture was stirred at 0 ºC for 2 h and then at rt over night. Chromatographic purification (20% ethyl acetate: hexane) afforded the desired product (874 mg, 92%). $[\alpha]^{23}_{D}$ –15.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ

6.10 (s, 2H), 5.02 (d, *J* = 9.5 Hz, 1H), 4.30 (dd, *J* = 4.5, 9.5 Hz, 1H), 4.21 (s, 2H), 3.80 (s, 3H), 3.79 (s, 6H), 2.30–2.67 (m, 1H), 1.44 (s, 9H), 0.98 (d. *J* = 7.0 Hz, 3H), 0.86 (d. *J* = 6.5 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 201.6, 161.0, 159.4, 155.8, 105.0, 90.7, 80.1, 65.4, 56.0, 55.6, 31.6, 28.6, 28.5, 22.3, 19.6, 16.9; ESI-HRMS calcd for $C_{20}H_{31}NO_6SNa$ $[M + Na]^+$, 436.1742; found, 436.1770.

NH_2-L-Val-STmob (165)

Boc-L-Val-STmob (186 mg, 0.45 mmol) was dissolved in a mixture of 40% TFA in CH_2Cl_2 (4 mL) and stirred at rt for 5 min. The solvents were removed and the organic layer was extracted with ethyl acetate followed by washing with 20% aqueous sodium carbonate solution. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to afford the free amine which was applied immediately to the next step.

ENS-L-Trp-STmob (167)

To a stirred solution of L-tryptophan (80 mg, 0.39 mmol) in dioxane/water (5mL/5mL) was added triethylamine (1 mL, 7.80 mmol) at 0 ºC. After 10 min, a solution of ENS-Cl (131 mg, 0.47 mmol) in dioxane (4 mL) was added slowly to the reaction mixture, which was then stirred for 30 min at 0 ºC and then at rt for 1.5 h. The reaction mixture was concentrated and basified with 10% sodium bicarbonate solution (50 mL). The aqueous layer was extracted with methyl t-butyl ether and the aqueous layer was acidified with 1M HCl and extracted with ethyl acetate (2x 100 mL). The combined organic layer was washed with water and brine, dried and concentrated.

To a stirred solution of ENS-L-Trp-OH (160 mg, 0.36 mmol), 2,4,6-trimethoxybenzylthiol (77 mg, 0.36 mmol) and HOBT (58 mg, 0.43 mmol) in dry methylene chloride (4 mL) was added DIC (66 µL, 0.43 mmol) at 0 ºC. Stirring was continued at rt for 6 h before chromatographic purification (60% ethyl acetate: hexane) afforded the desired product (211 mg, 93%). $[\alpha]^{23}_{D}$ –61.6 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (d, *J* = 2.0 Hz, 1H), 7.92 (s, 1H), 7.78 (dd, *J* = 2.0, 8.5 Hz, 1H), 7.66 (d, *J* = 8.5 Hz, 1H), 7.31 (d, *J* = 7.5 Hz, 1H), 7.01–6.93 (m, 3H), 6.89 (d, *J* = 2.0 Hz, 1H), 6.69 (d, *J* = 7.0 Hz, 1H), 6.10 (s, 2H), 4.50–4.48 (m, 1H), 4.23 (s, 2H), 3.94 (s, 3H), 3.82 (s, 3H), 3.81 (s, 6H), 3.46 (dd, *J* = 3.5, 15.0 Hz, 1H), 2.93 (dd, *J* = 11.0, 15.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 201.3, 165.6, 161.2, 159.5, 148.6, 144.2, 136.1, 129.8, 129.5, 126.6, 125.5, 125.2, 124.2, 122.5, 119.9, 118.9, 111.2, 109.7, 104.6, 90.7, 64.2, 56.1, 55.6, 54.0, 29.6, 22.9; ESI-HRMS calcd for $C_{29}H_{29}N_3O_{10}S_2Na$ $[M + Na]^+$, 666.1151; found, 666.1192.

ENS-L-Trp-SH

ENS-L-Trp-STmob (110 mg, 0.17 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (300 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid ENS-L-Trp-SH which was applied immediately to the next step.

ENS-L-Ala-OH (168)

To a stirred solution of L-alanine (147 mg, 1.65 mmol) in dioxane/water (5mL/5mL) was added triethylamine (4.6 mL, 33.0 mmol) at 0 ºC. After 10 min, a

solution of ENS-Cl (600 mg, 2.15 mmol) in dioxane (4 mL) was added slowly to the reaction mixture which was then stirred for 30 min at 0 ºC and then at rt for 1.5 h. The reaction mixture was concentrated and basified with 10% sodium bicarbonate solution (50 mL). The aqueous layer was extracted with methyl t-butyl ether and then the aqueous layer was acidified with 1M HCl and extracted with ethyl acetate (2x 100 mL). The combined organic layer was washed with water and brine, dried and concentrated (543 mg, 99%). $[\alpha]^{24}_{D}$ –24.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 (d, *J* = 2.5 Hz, 1H), 8.45 (dd, *J* = 2.5, 8.5 Hz, 1H), 8.24 (d, *J* = 8.5 Hz, 1H), 6.80 (d, *J* = 8.0 Hz, 1H), 4.29–4.26 (m, 1H), 4.08 (s, 3H), 1.51 (d, *J* = 7.5 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 177.0, 166.1, 149.7, 145.7, 131.9, 130.7, 126.6, 126.2, 54.3, 52.3, 19.5; ESI-HRMS calcd for $C_{11}H_{12}N_2O_8SNa$ $[M + Na]^+$, 355.0179; found, 355.0212.

ENS-L-Ala-L-Phe-STmob (169)

To a stirred solution of ENS-L-Ala-OH (108 mg, 0.33 mmol), the above prepared NH_2-L-Phe-STmob and HOBT (58 mg, 0.43 mmol) in dry methylene chloride (4 mL) was added EDCI (82 mg, 0.43 mmol) at 0 ºC. The reaction mixture was stirred at rt for 6 h before chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (189 mg, 86%). $[\alpha]^{23}_{D}$ –33.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.66 (d, *J* = 2.0 Hz, 1H), 8.29 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.06 (d, *J* = 8.5 Hz, 1H), 7.3–7.25 (m, 3H), 7.12–7.11 (m, 2H), 6.61 (d, *J* = 8.0 Hz, 1H), 6.46 (d, *J* = 8.5 Hz, 1H), 6.10 (s, 2H), 4.75–4.73 (m, 1H), 4.19 (dd, *J* = 12.5, 23.0 Hz, 2H), 4.07 (s, 3H), 3.81 (s, 3H), 3.80 (s, 6H), 3.16 (dd, *J* = 5.5, 14.0 Hz, 1H), 2.95 (dd, *J* = 7.5, 14.0 Hz, 1H), 1.26 (d. *J* = 7.5 Hz, 3H);

^{13}C NMR (125.6 MHz, $CDCl_3$) δ 199.5, 170.5, 166.2, 161.3, 159.5, 149.7, 145.3, 135.9, 132.0, 131.0, 129.6, 128.8, 127.4, 126.6, 126.4, 104.3, 90.7, 60.0, 56.1, 55.6, 54.3, 53.6, 38.9, 22.6, 19.4; ESI-HRMS calcd for $C_{30}H_{33}N_3O_{11}S_2Na$ [M + Na]$^+$, 698.1443; found, 698.1454.

ENS-L-Ala-L-Val-STmob (170)

To a stirred solution of ENS-L-Ala-OH (100 mg, 0.30 mmol), the above prepared NH_2-L-Val-STmob and HOBT (53 mg, 0.39 mmol) in dry methylene chloride (4 mL) was added EDCI (75 mg, 0.39 mmol) at 0 ºC. The reaction mixture was stirred at rt for 3 h, then was subjected to chromatographic purification (40% ethyl acetate: hexane) which afforded the desired product (123 mg, 65%). $[\alpha]^{23}_D$ –47.7 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.69 (d, *J* = 2.5 Hz, 1H), 8.40 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.25 (d, *J* = 8.5 Hz, 1H), 6.79 (s, 1H), 6.51 (d, *J* = 8.5 Hz, 1H), 6.09 (s, 2H), 4.45 (dd, *J* = 5.0, 9.0 Hz, 1H), 4.18 (dd, *J* = 12.5, 25.5 Hz, 2H), 4.07 (s, 3H), 3.80 (s, 3H), 3.78 (s, 6H), 2.18–2.17 (m, 1H), 1.40 (d. *J* = 7.5 Hz, 3H), 0.80 (dd, *J* = 3.5, 7.0 Hz, 6H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 199.6, 171.0, 166.0, 161.2, 159.4, 149.7, 145.4, 132.1, 131.1, 126.6, 126.4, 104.5, 90.7, 63.9, 56.0, 55.6, 54.3, 53.7, 31.8, 22.4, 19.8, 19.5, 17.1; ESI-HRMS calcd for $C_{26}H_{33}N_3O_{11}S_2Na$ [M + Na]$^+$, 650.1496; found, 650.1454.

ENS-L-Ala-L-Phe-SH

ENS-L-Ala-L-Phe-STmob (220 mg, 0.33 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (300 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the

solvents were removed to give the crude thioacid ENS-L-Ala-L-Phe-SH which was applied immediately to the next step.

CNS-L-Phe-STmob (171)

To a stirred solution of NH_2-L-Phe-STmob (78 mg, 0.22 mmol) and 2-cyano-4-nitrobenzenesulfonyl chloride (65 mg, 0.26 mmol) in methylene chloride (2 mL) was added DIPEA (45 µL, 0.26 mmol) at rt. The reaction mixture was stirred for 2h then was subjected to chromatographic purification (30% ethyl acetate: hexane) to give the desired product (89 mg, 72%). $[\alpha]^{24}_{D}$ –37.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 (d, *J* = 2.0 Hz, 1H), 8.30 (d, *J* = 1.0 Hz, 1H), 8.03 (dd, *J* = 0.5, 8.5 Hz, 1H), 7.13–7.11 (m, 3H), 7.08–7.06 (m, 2H), 6.09 (s. 2H), 5.82 (s, 1H), 4.37 (s, 1H), 4.17 (s, 2H), 3.83 (s, 3H), 3.80 (s, 6H), 3.26 (dd, *J* = 5.0, 14 Hz, 1H), 2.92 (dd, *J* = 9.0, 14.0 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 198.6, 161.4, 159.3, 149.1, 148.1, 135.1, 130.6, 129.8, 129.6, 129.0, 127.4, 127.3, 114.4, 112.3, 104.1, 90.7, 64.6, 56.1, 55.6, 39.7, 23.0; ESI-HRMS calcd for $C_{26}H_{25}N_3O_8S_2Na$ $[M + Na]^+$, 594.1009; found, 594.0981.

DNS-L-Val-O^{t}Bu (172)

To a stirred solution of L-valine methyl ester hydrochloride (880 mg, 4.2 mmol) and pyridine (1.36 mL, 16.8 mmol) in dry methylene chloride (9 mL) was added 2,4-dinitrobenzenesulfonyl chloride (1.34 g, 5.05 mmol) slowly at 0 ºC. The reaction mixture was stirred at 0 ºC for 1h and over night at rt before it was diluted with ethyl acetate. The organic layer was washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (20% ethyl acetate: hexane) afforded the desired

product (1.22 g, 72%). $[\alpha]^{23}_{D}$ –179.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.74 (d, *J* = 2.0 Hz, 1H), 8.51 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.28 (d, *J* = 8.5 Hz, 1H), 6.08 (s, 1H), 3.96 (s. 1H), 2.23–2.19 (m, 1H), 1.25 (s, 9H), 1.05 (d, *J* = 7.0 Hz, 3H), 0.92 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 169.8, 150.0, 148.2, 140.1, 132.3, 127.3, 121.2, 83.2, 63.1, 31.6, 27.9, 19.3, 17.3; ESI-HRMS calcd for $C_{15}H_{21}N_3O_8SNa$ $[M + Na]^+$, 426.0937; found, 426.0947.

DNS-L-Phe-O^{t}Bu (173)

To a stirred solution of L-phenylalanine methyl ester hydrochloride (200 mg, 0.78 mmol) and pyridine (191 µL, 2.34 mmol) in dry methylene chloride (7 mL) was added 2,4-dinitrobenzenesulfonyl chloride (247 mg, 0.93 mmol) slowly at 0 ºC. The reaction mixture was stirred at 0 ºC for 1h and 6 h at rt before it was diluted with ethyl acetate. The organic layer was washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (25% ethyl acetate: hexane) afforded the desired product (238 mg, 68%). $[\alpha]^{30}_{D}$ –53.6 (*c* = 0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.65 (d, *J* = 2.0 Hz, 1H), 8.41 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.24–7.14 (m, 5H), 6.01 (d, *J* = 9.0 Hz, 1H), 4.43–4.39 (m, 1H), 3.16 (dd, *J* = 6.0, 14.0 Hz, 1H), 3.05 (dd, *J* = 6.5, 14.0 Hz, 1H), 1.32 (s, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 169.5, 149.8, 140.1, 135.2, 132.1, 129.6, 128.9, 127.7, 127.2, 121.0, 83.6, 58.8, 39.4, 28.0; ESI-HRMS calcd for $C_{19}H_{21}N_3O_8SNa$ $[M + Na]^+$, 474.0947; found, 474.0955.

DNS-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (174)

DNS-L-Val-O^{t}Bu (134 mg, 0.33 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (200 µL) and stirred at rt for 1 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid DNS-L-Val-OH which was applied immediately to the next step.

Boc-L-Met-L-Val-L-Pro-L-Ala-OEt (150 mg, 0.28 mmol) was dissolved in a mixture of 40% TFA in methylene chloride (3 mL) with triethylsilane (100 µL) and stirred at rt for 15 min. Toluene (5 mL) was added to the reaction mixture and then solvents were removed to give a crude ammonium salt, which was neutralized with triethylamine to give the crude amine NH_2-L-Met-L-Val-L-Pro-L-Ala-OEt, which was applied immediately to the next step.

To a stirred solution of the above prepared DNS-L-Val-OH and the above prepared NH_2-L-Met-L-Val-L-Pro-L-Ala-OEt in dry methylene chloride (4 mL) was added HATU (160 mg, 0.42 mmol) followed by DIPEA (73 µL, 0.42 mmol) at 0 ºC. The reaction mixture was stirred at rt for 6 h before it was diluted with ethyl acetate. The organic layer was washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (75–100% ethyl acetate: hexane) afforded the desired product (186 mg, 87%). ESI-HRMS calcd for $C_{31}H_{47}N_7O_{12}S_2Na$ $[M + Na]^+$, 796.2562; found, 796.2622.

LCMS: 5-20% B over 1 min then 20-55% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 4.25 min.

DNS-L-Phe-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (175)

DNS-L-Phe-O^{t}Bu (72 mg, 0.16 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (3 mL) with triethylsilane (200 µL) and stirred at rt for 1 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid DNS-L-Phe-OH which was applied immediately to the next step.

To a stirred solution of the above prepared DNS-L-Phe-OH and NH_2-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (150 mg, 0.16 mmol) in dry methylene chloride (4 mL) was added HATU (91 mg, 0.24 mmol) followed by DIPEA (70 µL, 0.40 mmol) at 0 ºC. The reaction mixture was stirred at rt for 6 h before it was diluted with ethyl acetate and the organic layer was washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (75–100% ethyl acetate: hexane) afforded the desired product (179 mg, 85%). ESI-HRMS calcd for $C_{72}H_{73}N_9O_{14}SNa$ $[M + Na]^+$, 1342.4895; found, 1342.4955.

LCMS: 5-98% B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.77 min.

DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (178)

DNS-L-Val-O^{t}Bu (140 mg, 0.35 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (4 mL) with triethylsilane (200 µL) and stirred at rt for 1 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid DNS-L-Val-OH which was applied immediately to the next step.

Boc-L-Ala-L-Thr-L-Val-SFm (202 mg, 0.35 mmol) was dissolved in a mixture of 40% TFA in methylene chloride (4 mL) with triethylsilane (100 µL) and stirred at rt for 15 min. Toluene (5 mL) was added to the reaction mixture and then the solvents were removed to give the crude ammonium salt, which was applied immediately to the next step.

To a stirred solution of the above prepared DNs-L-Val-OH and crude ammonium salt in dry methylene chloride (4 mL) was added HATU (200 mg, 0.53 mmol) followed by DIPEA (152 µL, 0.88 mmol) at 0 ºC. The reaction mixture was stirred at rt for 6 h before it was diluted with ethyl acetate and the organic layer was washed with 1M HCl, water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (75–100% ethyl acetate: hexane) afforded the desired product (251 mg, 89%). ESI-HRMS calcd for $C_{37}H_{44}N_6O_{11}S_2Na$ $[M + Na]^+$, 835.2407; found, 835.2429.

LCMS: 5-98 % B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.28 min.

Methyl *N*-[(methyl 3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside)-2-ylsuccinamyl]-L-phenylalaninate (181) from ENS-L-Phe-OMe

To a stirred suspension of 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (25 mg, 0.078 mmol) in dry DMF (100 µL) was added a solution of thiosuccinic anhydride (9 mg, 0.078 mmol) in dry DMF (250 µL) at rt over 30 min. The reaction mixture was stirred for 1 h before cesium carbonate (25 mg, 0.078 mmol) was added to the reaction mixture followed by ENS-L-Phe-OMe (25 mg, 0.058 mmol), after which stirring was continued for further 3 h before the

reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification afforded the desired product (16 mg, 48%).

Methyl *N*-[(methyl 3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside)-2-ylsuccinamyl]-L-phenylalaninate (181) from CNS-L-Phe-OMe

To a stirred suspension of 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (25 mg, 0.078 mmol) in dry DMF (100 µL) was added a solution of thiosuccinic anhydride (9 mg, 0.078 mmol) in dry DMF (250 µL) at rt over 30 min. The reaction mixture was stirred for further 1 h before cesium carbonate (25 mg, 0.078 mmol) was added to the reaction mixture followed by CNS-L-Phe-OMe (23 mg, 0.058 mmol). The reaction mixture was stirred for further 3 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification afforded the desired product (17 mg, 51%).

Fmoc-L-Glu(L-Phe-OMe)-L-Val-L-Met-OMe (182) from ENS-L-Phe-OMe

To a stirred solution of Fmoc-L-Glu(SH)-L-Val-L-Met-OMe (0.02 mmol) and ENS-L-Phe-OMe (8 mg, 0.0189 mmol] in dry DMF (200 µL) was added cesium bicarbonate (11 mg, 0.05 mmol) at rt. The reaction mixture was stirred for 1 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Radial Chromatographic purification (70% ethyl acetate: hexane) afforded the

desired product (12 mg, 79%). ESI-HRMS calcd for $C_{41}H_{50}N_4O_9SNa$ $[M + Na]^+$, 797.3285; found, 797.3196.

LCMS: 5-30% B over 1 min then 30-70% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 4.03 min.

Fmoc-L-Glu(L-Phe-OMe)-L-Val-L-Met-OMe (182) by CNS-L-Phe-OMe

To a stirred solution of Fmoc-L-Glu(SH)-L-Val-L-Met-OMe (0.017 mmol) and CNS-L-Phe-OMe (6 mg, 0.015 mmol] in dry DMF (200 µL) was added cesium bicarbonate (9 mg, 0.05 mmol) at rt. After stirring for 1 h, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Radial Chromatographic purification (70% ethyl acetate: hexane) afforded the desired product (10 mg, 81%).

Boc-L-Asp(L-Phe-OMe)-OBn (183) from CNS-L-Phe-OMe

To a stirred solution of Boc-L-Asp(SH)OBn (0.12 mmol) and CNS-L-Phe-OMe (30 mg, 0.077 mmol] in dry DMF (0.4 mL) was added cesium bicarbonate (75 mg, 0.39 mmol) at rt. The reaction mixture was stirred for 2 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (28 mg, 75%).

Boc-L-Asp(L-Phe-OMe)-OBn (183) from ENS-L-Phe-OMe

To a stirred solution of Boc-L-Asp(SH)OBn (0.12 mmol) and ENS-L-Phe-OMe (33 mg, 0.077 mmol] in dry DMF (0.4 mL) was added cesium bicarbonate

(75 mg, 0.39 mmol) at rt. The reaction mixture was stirred for 24 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (11mg, 30%).

CNS-L-Phe-L-Phe-OMe (186)

CNS-L-Phe-STmob (175 mg, 0.31 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (4 mL) with triethylsilane (200 µL) and stirred at rt for 1.5 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid which was applied immediately to the next step.

To a stirred solution of the above prepared crude thioacid CNS-L-Phe-SH and DNS-L-Phe-OMe (85 mg, 0.21 mmol) in dry DMF (1 mL) was added cesium carbonate at rt. After stirring for 45 min the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (93 mg, 84%). $[\alpha]^{23}_{D}$ – 37.4 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.31 (dd, *J* = 2.0, 8.5 Hz, 1H), 8.27 (d, *J* = 2.0 Hz, 1H), 8.01 (d, *J* = 8.5 Hz, 1H), 7.30–7.20 (m, 3H), 7.13 (d, *J* = 7.0 Hz, 2H), 7.06–7.01 (m, 5H), 6.83 (d, *J* = 7.5 Hz, 1H), 6.59 (d, *J* = 7.0 Hz, 1H), 4.86 (q, *J* = 7.0 Hz, 1H), 4.13 (q, *J* = 7.0 Hz, 1H), 3.74 (s, 3H), 3.21 (dd, *J* = 5.5, 14.0 Hz, 1H), 3.12–3.08 (m, 2H), 2.81 (dd, *J* = 10.5, 14.5 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.7, 169.8, 149.1, 147.6, 135.8, 130.7, 130.3, 129.6,

128.9, 127.5, 127.2, 114.4, 111.8, 59.5, 53.9, 52.8, 38.6, 38.0; ESI-HRMS calcd for $C_{26}H_{24}N_4O_7SNa$ $[M + Na]^+$, 559.1254; found, 559.1263.

ENS-L-Ala-L-Phe-L-Phe-OMe (187)

To a stirred solution of ENS-L-Ala-L-Phe-SH (0.33 mmol) and DNS-L-Phe-OMe (89 mg, 0.22 mmol in dry DMF (1 mL) was added cesium carbonate (106 mg, 0.33 mmol) at 0 ºC. The reaction mixture was stirred at rt for 45 min before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (113 mg, 81%). $[\alpha]^{23}_D$ –10.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.38 (d, *J* = 6.4 Hz, 1H), 8.19 (d, *J* = 8.0 Hz, 1H), 7.30–7.20 (m, 6H), 7.15–7.13 (m, 2H), 7.05–7.03 (m, 2H), 6.70 (d, *J* = 8.4 Hz, 1H), 6.55 (d, *J* = 6.0 Hz, 1H), 6.43 (d, *J* = 7.2 Hz, 1H), 4.76–4.71 (m, 1H), 4.54 (dd, *J* = 8.0, 14.8 Hz, 1H), 4.07 (s, 3H), 3.80–3.77 (m, 1H), 3.68 (s, 3H), 3.07 (dt, *J* = 6.4, 14.4 Hz, 2H), 2.94 (dd, *J* = 7.6, 14.0 Hz, 1H), 2.81 (dd, *J* = 8.0, 13.6 Hz, 1H), 1.15 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (100.9 MHz, $CDCl_3$) δ 171.6, 170.8, 170.1, 166.2, 150.0, 144.0, 136.4, 136.1, 132.2, 131.9, 129.4, 129.0, 128.8, 127.4, 127.3, 126.7, 126.5, 76.9, 54.5, 54.4, 53.8, 53.7, 52.5, 38.1, 38.0, 19.1; ESI-HRMS calcd for $C_{30}H_{32}N_4O_{10}SNa$ $[M + Na]^+$, 663.1691; found, 663.1737.

ENS-L-Ala-L-Phe-L-Phe-OMe (187) (Reaction in aqueous buffer)[a]

To a stirred solution of ENS-L-Ala-L-Phe-SH (0.18 mmol) in ligation buffer (0.5 mL, [4:1 v/v *N*-methyl-2-pyrrolidinone (NMP): 6 M guanidine hydrochloride, 1 M HEPES, pH = 8][a]) was added DNS-L-Phe-OMe (49 mg, 0.12 mmol) at 0 ºC.

The reaction mixture was stirred at rt for 45 min before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (57 mg, 74%).

[a] An aqueous buffer containing 6 M Gn.HCl and 1 M HEPES was prepared and adjusted to pH 8 using 25% cesium carbonate solution in water. The resulting solution (1 mL) was diluted with NMP (4 mL) to produce the final buffer for use in the peptide coupling reaction.

ENS-L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (188)

To a stirred solution of ENS-L-Ala-L-Phe-SH (0.099 mmol) and DNS-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (45 mg, 0.058 mmol in dry DMF (0.5 mL) was added cesium carbonate (32 mg, 0.099 mmol) at 0 ºC under a nitrogen atmosphere. The reaction mixture was stirred at 0 ºC for 1 h and then at rt for 2.5 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (40–70% A with a flow rate of 10 mL/min over 50 min and 254 nm UV detection, retention time = 22 min) provided the heptapeptide with a yield of 47 mg (81%). ESI-HRMS calcd for $C_{45}H_{64}N_8O_{14}S_2Na$ $[M + Na]^+$, 1027.3838; found, 1027.3881.

LCMS: 5-30% B over 1 min then 30-60% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 3.18 min.

ENS-L-Ala-L-Val-SH

ENS-L-Ala-L-Val-STmob (62 mg, 0.097 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (3 mL) with triethylsilane (400 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid ENS-L-Ala-L-Val-SH which was applied immediately to the next step.

ENS-L-Ala-L-Val-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (189)

To a stirred solution of ENS-L-Ala-L-Val-SH (0.099 mmol) and DNS-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (45 mg, 0.058 mmol in dry DMF (0.5 mL) was added cesium bicarbonate (34 mg, 0.174 mmol) at 0 ºC under a nitrogen atmosphere. The reaction mixture was stirred at 0 ºC for 1 h and then stirred at rt for 30 min before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (35–60% A in B with a flow rate of 10 mL/min over 40 min and 254 nm UV detection, retention time = 23 min) provided the heptapeptide with a yield of 43 mg (78%). ESI-HRMS calcd for $C_{41}H_{64}N_8O_{14}S_2$ [M + H]$^+$, 979.4000; found, 979.3881.

LCMS: 5-30% B over 1 min then 30-55% B over 4 min with a flow rate of 0.7 mL/min and 254 nm UV detection, retention time = 2.78 min.

Ac-L-Trp-L-Phe-OMe (190)

To a stirred solution of ENS-L-Trp-SH (0.17 mmol) and DNS-L-Phe-OMe (58 mg, 0.14 mmol] in dry DMF (1.5 mL) was added cesium carbonate (68 mg, 0.21 mmol) at rt. After stirring for 2.5 h, thioacetic acid (13 µL, 0.17 mmol) was

added to the reaction mixture followed by cesium carbonate (68 mg, 0.21 mmol). Stirring was continued for a further 2.5 h before the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (5% methanol: methylene chloride) afforded the desired product (35 mg, 60%). $[\alpha]^{23}_{D}$ +11.6 (*c* = 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.22–7.13 (m, 6H), 7.06 (d, *J* = 2.0 Hz, 1H), 6.88 (d, *J* = 7.0 Hz, 2H), 6.27 (d, *J* = 7.0 Hz, 1H), 6.11 (d, *J* = 7.5 Hz, 1H), 4.73–4.69 (m, 1H), 3.65 (s, 3H), 3.31 (dd, *J* = 5.0, 14.0 Hz, 1H), 3.11 (dd, *J* = 8.0, 14.0 Hz, 1H), 3.01 (dd, *J* = 5.5, 14.5 Hz, 1H), 2.93 (dd, *J* = 6.5, 14.0 Hz, 1H), 1.98 (s, 3H); ^{13}C NMR (100.9 MHz, $CDCl_3$) δ 171.5, 171.1, 170.2, 136.4, 135.8, 129.4, 128.8, 127.7, 127.3, 123.6, 122.6, 120.1, 119.1, 111.5, 110.8, 54.0, 53.6, 52.5, 38.0, 29.9, 28.5, 23.5; ESI-HRMS calcd for $C_{23}H_{25}N_3O_4Na$ $[M + Na]^+$, 430.1729; found, 430.1743.

Boc-L-Glu(L-Phe-L-Phe-OMe)-OtBu (191)

To a stirred solution of Boc-L-Glu(SH)OtBu (0.11 mmol) and CNS-L-Phe-L-Phe-OMe (40 mg, 0.075 mmol] in dry DMF (0.5 mL) was added cesium carbonate (36 mg, 0.11 mmol) at rt. After stirring for 4 h, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (60% ethyl acetate: hexane) afforded the desired product (37 mg, 81%). $[\alpha]^{23}_{D}$ +6.3 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27–7.20 (m, 8H), 7.03 (d, *J* = 6.5 Hz, 2H), 6.79 (d, *J* = 7.0 Hz, 1H), 6.48 (d, *J* =

7.0 Hz, 1H), 5.23 (d, *J* = 7.5 Hz, 1H), 4.71 (q, *J* = 6.5 Hz, 1H), 4.66 (q, *J* = 6.5 Hz, 1H), 3.89 (s, 1H), 3.69 (s, 3H), 3.13–3.08 (m, 2H), 3.04–2.97 (m, 2H), 2.22–2.17 (m, 2H), 2.08–2.02 (m, 1H), 1.75–1.72 (m, 1H), 1.48–1.46 (m, 18H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 172.5, 171.6, 171.5, 170.9, 156.1, 136.9, 136.0, 129.6, 129.4, 128.8, 127.3, 127.1, 82.6, 80.3, 54.6, 53.6, 53.3, 52.6, 38.1, 37.7, 32.7, 29.9, 28.6, 28.2; ESI-HRMS calcd for $C_{33}H_{45}N_3O_8Na$ $[M + Na]^+$, 634.3148; found, 634.3104.

Boc-L-Asp(L-Phe-L-Phe-OMe)-OBn (192)

To a stirred solution of Boc-L-Asp(SH)OBn (0.11 mmol) and CNS-L-Phe-L-Phe-OMe (40 mg, 0.075 mmol] in dry DMF (1 mL) was added cesium bicarbonate (88 mg, 0.45 mmol) at rt. The reaction mixture was stirred for 4 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (70% ethyl acetate: hexane) afforded the desired product (37 mg, 78%). $[\alpha]^{23}_D$ +64.4 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34–7.20 (m, 11H), 7.15 (d, *J* = 7.0 Hz, 2H), 7.01 (d, *J* = 7.0 Hz, 2H), 6.39 (d, *J* = 7.0 Hz, 1H), 6.26 (d, *J* = 7.0 Hz, 1H), 5.77 (d, *J* = 8.5 Hz, 1H), 5.15 (s, 2H), 4.74 (dd, *J* = 6.5, 13.5 Hz, 1H), 4.6–4.53 (m, 2H), 3.67 (s, 3H), 3.08 (dd, *J* = 5.5, 14 Hz, 1H), 3.00–2.95 (m, 2H), 2.86–2.83 (m, 1H), 2.68 (dd, J = 4.5, 16 Hz, 1H), 1.43 (s, 9H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.6, 171.5, 170.4, 170.0, 155.9, 136.5, 135.9, 135.7, 129.6, 129.4, 128.9, 128.8, 128.5, 128.4, 127.4, 127.3, 80.3, 67.5, 54.6, 53.7, 52.6, 50.8, 38.4, 38.1, 28.5; ESI-HRMS calcd for $C_{35}H_{41}N_3O_8Na$ $[M + Na]^+$, 654.2834; found, 654.2791.

Boc-L-Glu(L-Ala-L-Phe-L-Phe-OMe)-OtBu (193)

To a stirred solution of Boc-L-Glu(SH)-OtBu (0.094 mmol) and ENS-L-Ala-L-Phe-L-Phe-OMe (40 mg, 0.063 mmol) in dry DMF (300 µL) was added cesium carbonate (31 mg, 0.094 mmol) at rt. The reaction mixture was stirred for 5 h before the it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (10% Methanol: methylene chloride) afforded the desired product (35 mg, 83%). ESI-HRMS calcd for $C_{36}H_{50}N_4O_9Na$ $[M + Na]^+$, 705.3538; found, 705.3475.

LCMS: 5-30% B over 1 min then 30-70% B over 4 min with a flow rate of 0.7 mL/min and 195 nm UV detection, retention time = 3.38 min.

Boc-L-Glu(L-Ala-L-Phe-L-Phe-OMe)-O^{t}Bu 193 (Reaction in aqueous buffer)[a]

To a stirred solution of Boc-L-Glu(SH)-OtBu (0.094 mmol) in ligation buffer (0.3 mL, [4:1 v/v *N*-methyl-2-pyrrolidinone (NMP): 6 M guanidine hydrochloride, 1 M HEPES, pH = 8][a]) was added ENS-L-Ala-L-Phe-L-Phe-OMe (40 mg, 0.063 mmol) at rt. After stirring for 6 h the reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (10% methanol: methylene chloride) afforded the desired product (34 mg, 80%).

Boc-L-Glu(L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt)-O^{t}Bu (194)

To a stirred solution of Boc-L-Glu(SH)-O^{t}Bu (0.018 mmol) and ENS-L-Ala-L-Phe-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (9 mg, 0.009 mmol) in dry DMF (100 µL)

was added cesium carbonate (6 mg, 0.018 mmol) at rt under a nitrogen atmosphere. After stirring for 6 h the reaction mixture was diluted with ethyl acetate and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (40–80% A with a flow rate of 10 mL/min over 50 min and 215 nm UV detection, retention time = 21 min) provided the octapeptide with a yield of 6.6 mg (71%). ESI-HRMS calcd for $C_{51}H_{82}N_8O_{13}SNa$ $[M + Na]^+$, 1069.5726; found, 1069.5620.

LCMS: 5-30% B over 1 min then 30-60% B over 4 min with a flow rate of 0.7 mL/min and 195 nm UV detection, retention time = 3.60 min.

Boc-L-Glu(L-Ala-L-Val-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt)-O^{t}Bu (195)

To a stirred solution of Boc-L-Glu(SH)-O^{t}Bu (0.062 mmol) and ENS-L-Ala-L-Val-L-Val-L-Met-L-Val-L-Pro-L-Ala-OEt (30 mg, 0.031 mmol) in dry DMF (150 µL) was added cesium bicarbonate (24 mg, 0.124 mmol) at rt under a nitrogen atmosphere. The reaction mixture was stirred for 8 h before it was diluted with ethyl acetate and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (40–60% A with a flow rate of 10 mL/min over 40 min and 215 nm UV detection, retention time = 22 min) provided the octapeptide with a yield of 20 mg (65%). ESI-HRMS calcd for $C_{47}H_{82}N_8O_{13}S$ $[M + H]^+$, 1021.5589; found, 1021.5620.

LCMS: 5-30% B over 1 min then 30-60% B over 4 min with a flow rate of 0.7 mL/min and 195 nm UV detection, retention time = 3.05 min.

Reaction of Z-Gly-L-Phe-SH (110) + DNS-L-Val-O^{t}Bu (172) to give Z-Gly-L-Phe-L-Val-O^{t}Bu (111)

To a stirred solution of Z-Gly-L-Phe-SH (0.36 mmol) in DMF (1.0 mL) was added cesium carbonate (117 mg, 0.36 mmol) at 0 ºC. After 5 min, a solution of DNS-L-Val-O^{t}Bu (97 mg, 0.24 mmol) in DMF (0.3 mL) was added to the reaction mixture at 0 ºC which was then stirred for 30 min at 0 ºC before the reaction mixture was quenched with 1 M HCl (4 mL). The organic layer was extracted with ethyl acetate followed by washing with water and brine, dried and concentrated. Chromatographic purification (50% ethyl acetate: Hexane) gave the desired product (87 mg, 71%). The product was analyzed for the presence of Z-Gly-D-Phe-L-Val-OtBu by HPLC with the aid of an authentic sample.

LCMS: 5-36.5% B over 1 min then 36.5-55% B over 4 min with a flow rate of 0.7 mL/min and 215 nm UV detection, retention time = 3.62 min.

Z-Gly-D-Phe-L-Val-OtBu: retention time = 3.77 min.

FNS-L-Val-L-Val-STmob (197)

To a stirred solution of L-valine t-butyl ester hydrochloride (200 mg, 0.95 mmol) and the 2-nitro-4-trifluoromethylbenzenesulfonyl chloride (332 mg, 1.14 mmol) in methylene chloride (7 mL) was added DIPEA (396 µL, 2.28 mmol) at rt. The reaction mixture was stirred for 4h before it was subjected to chromatographic purification to give the desired sulfonamide FNS-L-Val-OtBu (380 mg, 93%).

FNS-L-Val-OtBu (380 mg, 0.89 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (200 µL) and stirred at rt for 1

h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid FNS-L-Val-OH which was applied immediately to the next step.

To a stirred solution of the above prepared FNS-L-Val-OH, NH_2-Val-STmob (294 mg, 0.94 mmol) and HOBT (153 mg, 1.13 mmol) in dry methylene chloride (6 mL) was added EDCI (217 mg, 1.13 mmol) at 0 ºC. The reaction mixture was stirred at rt for 6 h, after which chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (537 mg, 86%). $[\alpha]^{23}_{D}$ –74.2 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (d, *J* = 8.5 Hz, 1H), 8.16 (d, *J* = 1 Hz, 1H), 7.92 (dd, *J* = 1.0, 8.0 Hz, 1H), 6.39 (d, *J* = 9.0 Hz, 1H), 6.33 (d, *J* = 9.0 Hz, 1H), 6.09 (s, 2H), 4.46–4.43 (m, 1H), 4.18 (dd, *J* = 12.5, 23.0 Hz, 2H), 3.97 (dd, *J* = 5.0, 9.0 Hz, 1H), 3.80 (s, 3H), 3.77 (s, 6H), 2.24–2.20 (m, 1H), 2.10–2.05 (m, 1H), 1.03 (d, *J* = 6.5 Hz, 3H), 0.91 (d, *J* = 7.0 Hz, 3H), 0.69 (d, *J* = 7.0 Hz, 3H), 0.65 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 199.5, 169.8, 161.3, 159.4, 148.1, 137.7, 135.9, 135.6, 131.8, 129.9, 123.3, 123.2, 121.1, 104.4, 90.7, 63.9, 63.3, 56.0, 55.6, 31.9, 31.8, 22.4, 19.6, 19.3, 17.2, 17.0; ESI-HRMS calcd for $C_{27}H_{34}N_3O_9F_3S_2Na$ $[M + Na]^+$, 688.1586; found, 688.1697.

FNS-L-Ala-L-Phe-O^{t}Bu (199)

To a stirred solution of FNS-L-Ala-OH (380 mg, 1.11 mmol), L-phenylalanine methyl ester hydrochloride (286 mg, 1.11 mmol) and HOBT (194 mg, 1.44 mmol) in dry methylene chloride (7 mL) was added EDCI (277 mg, 1.44 mmol) at 0 ºC followed by DIPEA (289 µL, 1.67 mmol) and the reaction mixture was stirred at rt for 5 h and then subjected to chromatographic purification (40%

ethyl acetate: hexane) which afforded the desired product (521 mg, 86%). $[\alpha]^{22}_{D}$ –2.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, *J* = 8.0 Hz, 1H), 8.13 (s, 1H), 7.87 (d, *J* = 8.0 Hz, 1H), 7.30–7.10 (m, 5H), 6.54 (d, *J* = 7.5 Hz, 1H), 6.20 (d, *J* = 8.0 Hz, 1H), 4.56 (dd, *J* = 6.5, 14.0 Hz, 1H), 4.13–4.10 (m, 1H), 3.00 (dd, *J* = 6.0, 14.0 Hz, 2H), 2.93 (dd. *J* = 6.5, 14.0 Hz, 1H), 1.39–1.37 (m, 12H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 170.3, 170.2, 148.1, 137.6, 136.1, 136.0, 135.7, 132.0, 130.0, 129.9, 129.6, 128.7, 127.4, 123.3, 123.2, 123.2, 121.1, 83.0, 54.0, 53.7, 38.2, 28.1, 19.9; ESI-HRMS calcd for $C_{23}H_{26}N_3O_7F_3SNa$ $[M + Na]^+$, 568.1341; found, 568.1355.

FNS-L-Ala-L-Phe-L-Val-STmob (200)

FNS-L-Ala-L-Phe-OtBu (300 mg, 0.55 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (7 mL) with triethylsilane (200 µL) and stirred at rt for 1 h. Toluene (15 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid FNS-L-Ala-L-Phe-OH which was applied immediately to the next step.

To a stirred solution of the above prepared FNS-L-Ala-L-Phe-OH and NH_2-L-Val-STmob (172 mg, 0.55 mmol) in dry methylene chloride (5 mL) was added HATU (316 mg, 0.83 mmol) at 0 ºC followed by DIPEA (239 µL, 1.38 mmol) and the reaction mixture was stirred at rt for 5 h before chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (515 mg, 89%). $[\alpha]^{23}_{D}$ –13.4 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.18 (d, *J* = 8.0 Hz, 1H), 8.08 (d, *J* = 1.0 Hz, 1H), 7.82 (dd, *J* = 8.0, 1.0 Hz, 1H), 7.25–7.12 (m, 6H), 6.71 (d, *J* = 9.0 Hz, 1H), 6.37 (d, *J* = 7.5 Hz, 1H), 6.10 (s, 2H), 4.70 (dd, *J* = 7.5, 14.5 Hz,

1H), 4.57 (dd, *J* = 5.0, 9.0 Hz, 1H), 4.24 (dd, *J* = 12.0, 18.0 Hz, 2H), 4.13 (t, *J* = 7.5 Hz, 1H), 3.80 (s, 3H), 3.79 (s, 6H), 3.10 (dd, *J* = 6.5, 14.0 Hz, 1H), 2.83 (dd, *J* = 7.5, 14.0 Hz, 1H), 2.20–2.16 (m, 1H), 1.31 (d. *J* = 7.0 Hz, 3H), 0.90 (d, *J* = 7.0 Hz, 3H), 0.80 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 199.6, 171.0, 170.9, 161.2, 159.5, 148.1, 137.4, 136.3, 135.9, 135.6, 132.2, 129.9, 129.6, 128.9, 127.3, 123.3, 123.0, 121.1, 104.5, 90.7, 67.3, 64.2, 58.4, 56.0, 55.6, 54.8, 53.5, 38.4, 31.7, 22.5, 22.1, 19.4, 17.5; ESI-HRMS calcd for $C_{34}H_{39}N_4O_{10}F_3S_2Na$ $[M + Na]^+$, 807.1957; found, 807.1927.

FNS-L-Val-L-Val-L-Phe-OMe (201)

FNS-L-Val-L-Val-STmob (350 mg, 0.53 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (300 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid FNS-L-Val-L-Val-SH which was applied immediately to the next step.

To a stirred solution of FNS-L-Val-L-Val-SH (0.15 mmol) and DNS-L-Phe-OMe (41 mg, 0.10 mmol in dry DMF (0.5 mL) was added cesium bicarbonate (98 mg, 0.05 mmol) at 0 ºC. The reaction mixture was stirred at 0 ºC for 30 min and then at rt for 2h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (49 mg, 78%). $[\alpha]^{23}_D$ –12.7 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, *J* = 8.5 Hz, 1H), 8.15 (s, 1H), 7.92 (d, *J* = 8.0 Hz, 1H), 7.26–7.22 (m, 3H), 7.07 (d, *J* = 6.5 Hz, 2H), 6.64 (d, *J* = 9.0 Hz, 1H), 6.40

(d, *J* = 8.5 Hz, 2H), 4.83 (dd, *J* = 6.5, 14.0 Hz, 1H), 4.16 (dd, *J* = 6.0, 8.5 Hz, 1H), 3.87 (dd, *J* = 5.5, 8.5 Hz, 1H), 3.73 (s, 3H), 3.11 (dd, *J* = 5.5, 14.0 Hz, 1H), 3.03 (dd, *J* = 6.5, 14.0 Hz, 1H), 2.55–2.11 (m, 1H), 1.86–1.22 (m, 1H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 6.5 Hz, 3H), 0.70 (d, *J* = 7.0 Hz, 3H), 0.63 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 171.8, 170.3, 169.9, 148.1, 137.4, 135.7, 132.0, 129.9, 129.3, 128.9, 127.5, 123.2, 63.4, 58.5, 53.5, 52.7, 38.0, 31.9, 31.4, 19.5, 19.0, 17.8, 17.5; ESI-HRMS calcd for $C_{27}H_{33}N_4O_8SF_3Na$ $[M + Na]^+$, 653.1869; found, 653.1863.

Z-Gly-L-Val-L-Val-L-Phe-OMe (204)

Z-Gly-STrt (50 mg, 0.11 mmol) was dissolved in a mixture of 10% TFA in methylene chloride (3 mL) with triethylsilane (100 µL) and stirred at rt for 10 min. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Z-Gly-SH which was applied immediately to the next step.

To a stirred solution of Z-Gly-SH (0.11 mmol) in dry DMF (200 µL) was added cesium bicarbonate (70 mg, 0.36 mmol) followed by a solution of FNS-L-Val-L-Val-L-Phe-OMe (45 mg, 0.071 mmol) in dry DMF (100 µL) rt. The reaction mixture was stirred for 12 h before it was diluted with ethyl acetate and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (5% methanol: methylene chloride) afforded the desired product. (28 mg, 69%).ESI-HRMS calcd for $C_{30}H_{40}N_4O_7Na$ $[M + Na]^+$, 591.2795; found, 591.2833.

Alloc-Gly-Gly-L-Val-L-Val-L-Phe-OMe (205)

Alloc-Gly-Gly-STmob (30 mg, 0.073 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (4 mL) with triethylsilane (200 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Alloc-Gly-Gly-SH which was applied immediately to the next step.

To a stirred solution of the above prepared Alloc-Gly-Gly-SH in dry DMF (200 µL) was added cesium bicarbonate (47 mg, 0.24 mmol) followed by a solution of FNS-L-Val-L-Val-L-Phe-OMe (31 mg, 0.048 mmol) in dry DMF (100 µL) at rt. The reaction mixture was stirred for 18 h before it was diluted with ethyl acetate and the organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (45–100% A in B with a flow rate of 8 mL/min over 40 min and 215 nm UV detection, retention time = 18 min) provided the pentapeptide with a yield of 19 mg (67%). ESI-HRMS calcd for $C_{28}H_{41}N_5O_8Na$ $[M + Na]^+$, 598.2853; found, 598.2897.

FNS-L-Ala-L-Phe-L-Val-L-Val-L-Ala-L-Thr-L-Val-SFm (210)

FNS-L-Ala-L-Phe-L-Val-STmob (100mg, 0.13 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (4 mL) with triethylsilane (300 µL) and stirred at rt for 2 h. Toluene (10 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid FNS-L-Ala-L-Phe-L-Val-SH which was applied immediately to the next step.

To a stirred solution of the above prepared FNS-L-Ala-L-Phe-L-Val-SH (0.13 mmol) and DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (52 mg, 0.064 mmol in dry DMF (300 µL) was added cesium bicarbonate (63 mg, 0.32 mmol) at 0 ºC. After stirring at 0 ºC for 30 min and then at rt for 2h the reaction mixture was diluted with ethyl acetate and the organic layer was washed with 1M HCl solution, water and brine, dried over sodium sulfate and concentrated under reduced pressure. HPLC purification (40–100% A with a flow rate of 6-9 mL/min over 40 min and 254 nm UV detection, retention time = 24 min) provided the heptapeptide with a yield of 49 mg (67%). ESI-HRMS calcd for $C_{55}H_{67}N_8O_{12}S_2F_3Na$ $[M + Na]^+$, 1175.4170; found, 1175.4211.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical column (4.6 × 250 mm). 40-100% A over 25 min with a flow rate of 1.0 mL/min. Retention time = 10.264 min.

Alloc-Gly-Gly-L-Ala-L-Phe-L-Val-L-Val-L-Ala-L-Thr-L-Val-SFm (211)

To a stirred solution of Alloc-Gly-Gly-SH (0.078 mmol) and FNS-L-Ala-L-Phe-L-Val-L-Val-L-Ala-L-Thr-L-Val-SFm (30 mg, 0.026 mmol) in dry DMF (300 µL) was added cesium bicarbonate (31 mg, 0.156 mmol) at rt. The reaction mixture was stirred for 10 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (40–100% A with a flow rate of 6-9 mL/min over 40 min and 254 nm UV detection, retention time =

23 min) provided the nonapeptide with a yield of 17 mg (61%). ESI-HRMS calcd for $C_{56}H_{75}N_9O_{12}SNa$ $[M + Na]^+$, 1120.5161; found, 1120.5154.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical column (4.6 × 250 mm). 40-100% A over 25 min with a flow rate of 1.0 mL/min. Retention time = 10.261 min.

Fmoc-L-Phe-L-Ala-STmob (212)

To a stirred solution of Fmoc-L-Phe-OH (387 mg, 1.0 mmol), NH_2-L-Ala-STmob (285 mg, 1.0 mmol) and HOBT (162 mg, 1.20 mmol) in dry methylene chloride (8 mL) was added EDCI (230 mg, 1.20 mmol) at 0 ºC. The reaction mixture was stirred at rt for 4 h, after which chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (602 mg, 92%). $[\alpha]^{23}_D$ –10.1 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.53 (d, *J* = 7.5 Hz, 2H), 7.41 (t, *J* = 7.5 Hz, 2H), 7.31 (t, *J* = 7.5 Hz, 2H), 7.27–7.19 (m, 5H), 6.51 (d, *J* = 5.5 Hz, 1H), 6.10 (s, 2H), 5.43 (d, *J* = 6.0 Hz, 1H), 4.71–4.68 (m, 1H), 4.22–4.13 (m, 3H), 3.80 (s, 9H), 3.10 (d. *J* = 3.5 Hz, 2H), 1.36 (d, *J* = 6.5, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 200.7, 170.6, 161.2, 159.5, 156.2, 144.0, 141.5, 136.4, 129.7, 129.0, 128.0, 127.4, 125.4, 125.3, 120.2, 104.4, 90.7, 67.3, 56.3, 56.0, 55.6, 55.1, 47.3, 38.7, 22.6, 19.5; ESI-HRMS calcd for $C_{37}H_{38}N_2O_7SNa$ $[M + Na]^+$, 677.2297; found, 677.2295.

DNS-L-Val-L-Ala-STmob

DNS-L-Val-OtBu (380 mg, 0.94 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (9 mL) with triethylsilane (200 µL) and stirred at rt for 1

h. Toluene (15 mL) was added to the reaction mixture and then the solvents were removed to give the crude acid DNS-L-Val-OH which was applied immediately to the next step.

To a stirred solution of DNS-L-Val-OH (0.94 mmol), NH_2-L-Ala-STmob (269 mg, 0.94 mmol) and HOBT (153 mg, 1.13 mmol) in dry methylene chloride (7 mL) was added EDCI (217 mg, 1.13 mmol) at 0 ºC. The reaction mixture was stirred at rt for 5 h before chromatographic purification (40% ethyl acetate: hexane) afforded the desired product (515 mg, 89%). $[\alpha]^{23}_{D}$ –66.0 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.68 (d, *J* = 2.5 Hz, 1H), 8.47 (dd, *J* = 2.5, 8.5 Hz, 1H), 8.28 (d, *J* = 8.5 Hz, 1H), 6.61 (d, *J* = 8.0 Hz, 1H), 6.35 (d, *J* = 9.0 Hz, 1H), 6.08 (s, 2H), 4.49–4.46 (m, 1H), 4.15 (d, *J* = 1.5 Hz, 2H), 3.95 (dd, *J* = 5.0, 9.0 Hz, 1H), 3.80 (s, 3H), 3.78 (s, 6H), 2.21–2.19 (m, 1H), 1.23 (d. *J* = 7.0 Hz, 3H), 1.01 (d, *J* = 6.5 Hz, 3H), 0.94 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 200.5, 169.3, 161.3, 159.4, 150.0, 148.2, 139.6, 132.4, 127.3, 121.2, 104.0, 90.7, 63.6, 56.0, 55.6, 55.2, 31.8, 22.6, 19.6, 19.5, 17.4; ESI-HRMS calcd for $C_{24}H_{30}N_4O_{11}S_2Na$ $[M + Na]^+$, 637.1250; found, 637.1215.

Fmoc-L-Phe-L-Ala-SH

Fmoc-L-Phe-L-Ala-STmob (210 mg, 0.32 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (5 mL) with triethylsilane (300 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Fmoc-L-Phe-L-Ala-SH which was applied immediately to the next step.

Fmoc-L-Phe-L-Ala-L-Val-L-Ala-STmob (213)

To a stirred solution of Fmoc-L-Phe-L-Ala-SH (0.32 mmol) in dry DMF (0.5 mL) was added cesium bicarbonate (159 mg, 0.81 mmol) at 0 ºC followed by a solution of DNS-L-Val-L-Ala-STmob (166 mg, 0.27 mmol in dry DMF (1 mL). The reaction mixture was stirred at 0 ºC for 1 h and then at rt for 3 h before the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Chromatographic purification (85% ethyl acetate: hexane) afforded the desired product (183 mg, 82%). ESI-HRMS calcd for $C_{45}H_{52}N_4O_9SNa$ $[M + Na]^+$, 847.3353; found, 847.3425.

LCMS: 5-98% B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.00 min.

Fmoc-L-Phe-L-Ala-L-Val-L-Ala-SH (214)

Fmoc-L-Phe-L-Ala-L-Val-L-Ala-STmob (10 mg, 0.012 mmol) was dissolved in a mixture of 70% TFA in methylene chloride (3 mL) with triethylsilane (100 µL) and stirred at rt for 1.5 h. Toluene (8 mL) was added to the reaction mixture and then the solvents were removed to give the crude thioacid Fmoc-L-Phe-L-Ala-L-Val-L-Ala-SH which was applied immediately to the next step.

Fmoc-L-Phe-L-Ala-L-Val-L-Ala-L-Val-L-Ala-L-Thr-L-Val-SFm (215)

To a stirred solution of Fmoc-L-Phe-L-Ala-L-Val-L-Ala-SH (0.012 mmol) in dry DMF (50 µL) was added cesium bicarbonate (8 mg, 0.04 mmol) at 0 ºC followed by a solution of DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (6.5 mg, 0.008 mmol in dry DMF (100 µL). The reaction mixture was stirred at 0 ºC for 30 min and then

at rt for 2 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (40–100% A with a flow rate of 8 mL/min over 45 min and 215 nm UV detection, retention time = 31 min) provided the octatapeptide with a yield of 7 mg (71%). ESI-HRMS calcd for $C_{66}H_{80}N_8O_{11}SNa$ $[M + Na]^+$, 1215.5565; found, 1215.5696.

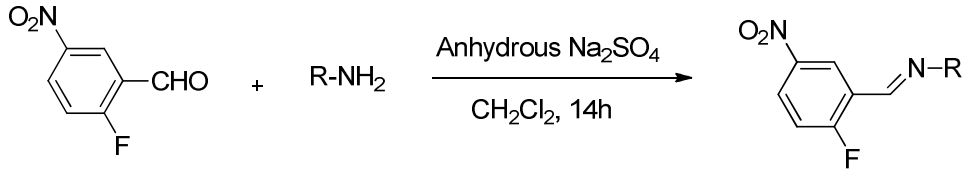
HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Varian Microsorb 300-5C18 analytical column (4.6 × 250 mm). 40-100% A over 25 min with a flow rate of 0.7 mL/min. Retention time = 11.66 min.

Fmoc-L-Phe-L-Ala-L-Val-L-Ala-L-Phe-L-Asn(Trt)-L-Asn(Trt)-L-Ile-L-Ala-OEt (216)

To a stirred solution of Fmoc-L-Phe-L-Ala-L-Val-L-Ala-SH (0.012 mmol) in dry DMF (100 µL) was added cesium bicarbonate (8 mg, 0.04 mmol) at 0 ºC followed by a solution of DNS-L-Val-L-Ala-L-Thr-L-Val-SFm (11 mg, 0.008 mmol) in dry DMF (50 µL). The reaction mixture was stirred at 0 ºC for 30 min and then at rt for 2 h before it was diluted with ethyl acetate. The organic layer was washed with water and brine, dried over sodium sulfate and concentrated under reduced pressure. Reversed phase HPLC purification (45–100% A with a flow rate of 7 mL/min over 40 min and 215 nm UV detection, retention time = 32 min) provided the octatapeptide with a yield of 10 mg (68%). ESI-HRMS calcd for $C_{101}H_{109}N_{11}O_{14}Na$ $[M + H]^+$, 1722.8053; found, 1722.8152.

LCMS: 5-98% B over 5 min with a flow rate of 0.5 mL/min and 254 nm UV detection, retention time = 4.40 min.

General Procedure 5 for the Preparation of Schiff Bases



To a stirred solution of free amine (0.92 mmol) and 2-fluoro-5-nitrobenzaldehyde (0.61 mmol) in dry methylene chloride (6 mL) was added sodium sulfate (2 g) at rt. After 14 h, the reaction mixure was filtered and washed with more methylene chloride. The filterate was concentrated and subjected to chromatographic purification to afford the desired products.

***N*-(2-fluoro-5-nitrobenzylidene)-2-phenylethylamine (217)**

Prepared by the general procedure 5 with a yield of 544 mg (100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.89 (dd, *J* = 2.4, 5.7 Hz, 1H), 8.42 (s, 1H), 8.30–8.24 (m, 1H), 7.33–7.19 (m, 6H), 3.95 (t, *J* = 7.5 Hz, 2H), 3.06 (t, *J* = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 152.6 (d, *J* = 4.5 Hz), 139.6, 129.2, 128.7, 127.3, 127.2, 126.5, 124.2 (d, *J* = 5.0 Hz), 117.3 (d, *J* = 23 Hz), 63.5, 37.4. ESI-HRMS calcd for $C_{15}H_{13}N_2O_2FNa$ $[M + Na]^+$, 272.0961; found, 272.0967.

Methyl 2-((2-fluoro-5-nitrobenzylidene)amino)-3-(1H-indol-3-yl)propanoate (218)

Prepared by the general procedure 5 with a yield of 625 mg (99%), Mp 109-110 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.90 (dd, *J* = 2.5, 5.5 Hz, 1H), 8.26–8.23 (m, 1H), 8.14 (s, 1H), 8.04 (s, 1H), 7.64 (d, *J* = 7.5 Hz, 1H), 7.32 (d, *J* = 8.5 Hz, 1H), 7.18–7.09 (m, 3H), 6.99 (d, *J* = 2 Hz, 1H), 4.36 (dd, *J* = 4.5, 8.5 Hz, 1H),

3.79 (s, 3H), 3.58 (dd, *J* = 4.5, 14.5 Hz, 1H), 3.33 (dd, *J* = 9.0, 14.5 Hz, 1H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 172.1, 166.2, 164.1, 154.8 (d, *J* = 14.5 Hz), 144.8, 136.4, 127.7, 127.6, 127.5, 124.8, 124.7, 124.6, 123.5, 122.4, 119.8, 119.1, 117.2 (d, *J* = 96.5 Hz), 111.4 (d, *J* = 33 Hz), 74.2, 52.7, 29.6. ESI-HRMS calcd for $C_{19}H_{16}N_3O_4FNa$ $[M + Na]^+$, 369.1125; found, 369.1135.

Boc-L-Ala-STmob (222)

To a stirred solution of Boc-L-Ala-OH (500 mg, 2.64 mmol), 2,4,6-trimethoxybenzylthiol (563 mg, 2.64 mmol) and HOBT (428 mg, 3.17 mmol) in dry methylene chloride (8 mL) was added EDCI (608 mg, 3.17 mmol) at 0 ºC. Stirring was continued at rt over night before chromatographic purification (30% ethyl acetate: hexane) afforded the desired product (886 mg, 87%). $[\alpha]^{23}_D$ –55.8 (*c* = 1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.09 (s, 2H), 5.08 (d, *J* = 7.5 Hz, 1H), 4.40 (t, *J* = 7.0 Hz, 1H), 4.18 (d, *J* = 1 Hz, 2H), 3.79 (s, 3H), 3.78 (s, 6H), 1.43 (s, 9H), 1.37 (d. *J* = 7.0 Hz, 3H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 202.5, 161.1, 159.5, 155.2, 104.8, 90.7, 80.1, 56.4, 56.0, 55.6, 28.6, 22.4, 19.5; ESI-HRMS calcd for $C_{18}H_{27}NO_6SNa$ $[M + Na]^+$, 408.1457; found, 408.1484.

NH_2-L-Ala-STmob (223)

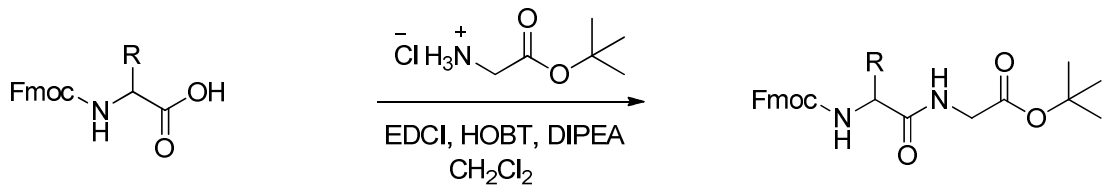
Boc-L-Ala-STmob (186 mg, 0.45 mmol) was dissolved in a mixture of 40% TFA in methylene chloride (4 mL) and stirred at rt for 5 min. The solvents were removed and the the organic layer was extracted with ethyl acetate followed by washing with 20% aqueous sodium carbonate solution. The organic layer was dried over sodium sulfate, concentrated under reduced pressure and purified by

column chromatography to afford the free amine which was applied immediately to the next step.

General Procedure 6 for the Coupling of Thioacids with the Schiff Bases

To a stirred solution of thioacid (0.41 mmol) in dry DMF (1.5 mL) was added cesium bicarbonate (1.35 mmol) followed by the addition of Schiff base (0.27 mmol) at rt. After stirring of 4-12 h at rt, the reaction mixture was diluted with ethyl acetate. The organic layer was washed with water and brine, dried, concentrated. Chromatographic purification afforded the desired peptides.

General Procedure 7 for the Synthesis of Fmoc Protected Peptide *tert* Butyl Esters



To a stirred solution of Fmoc protected amino acid (1 mmol), glycine *tert* butyl ester hydrochloride (1 mmol) and HOBT (1.2 mmol) in dry methylene chloride (10 mL) was added EDCI (1.2 mmol) followed by the addition of DIPEA (3 mmol) at 0 ^{o}C. After stirring of 30 min at 0 ^{o}C, the reaction mixture was warmed up to rt and stirred for 2 h. The solvents were removed and the product was purified by column chromatography over silica gel.

Fmoc-D-Phe-Gly-O^{t}Bu (249)

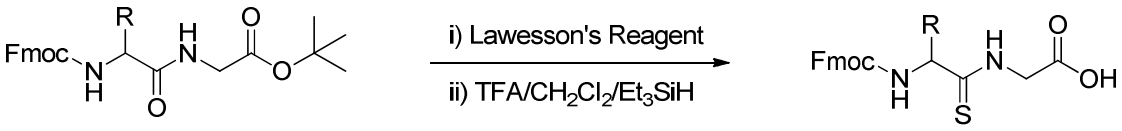
Prepared by the general procedure 7 with a yield of 1.15 g (89%), $[\alpha]^{22}_{D}$ +16.1 (in $CHCl_3$); IR ($CHCl_3$) 1740, 1705, 1660 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 8.0 Hz, 2H), 7.53 (dd, *J* = 8.0, 10.5 Hz, 2H), 7.41 (t, *J* = 7.5 Hz,

2H), 8.33–7.22 (m, 7H), 6.32 (s, 1H), 5.37 (d, *J* = 5.5 Hz, 1H), 4.50–4.33 (m, 3H), 4.19 (t, *J* = 7.0 Hz, 1H), 3.96–3.82 (m, 2H), 3.12 (s, 2H), 1.46 (s, 9H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 172.0, 168.7, 156.2, 144.0, 141.5, 136.6, 129.5, 129.0, 128.0, 127.4, 127.3, 125.3, 120.2, 82.7, 67.3, 56.3, 47.3, 42.3, 38.7, 28.3. ESI-HRMS calcd for $C_{30}H_{32}N_2O_5Na$ $[M + Na]^+$, 523.2209; found, 523.2217.

Fmoc-Gly-Gly-O^{t}Bu (250)

Prepared by the general procedure 7 with a yield of 4.23 g (84%), IR ($CHCl_3$) 1739, 1704, 1660 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (dt, *J* = 7.0, 0.5 Hz, 2H), 6.65 (s, 1H), 5.73 (t, *J* = 5.5 Hz, 1H), 4.43 (d, *J* = 7.0 Hz, 2H), 4.22 (t, *J* = 7.0 Hz, 1H), 3.95 (t, *J* = 6.0 Hz, 4H), 1.47 (s, 9H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 169.4, 169.1, 156.9, 144.0, 141.5, 128.0, 127.3, 125.3, 120.2, 82.8, 67.5, 47.3, 44.6, 42.2, 28.3. ESI-HRMS calcd for $C_{23}H_{26}N_2O_5Na$ $[M + Na]^+$, 433.1739; found, 433.1732.

General Procedure 8 for the Synthesis of Thioamide Linkers



A solution of Fmoc protected peptide *tert* butyl esters (4 mmol) and Lawesson's reagent (2.4 mmol) in dry toluene (40 mL) was heated to reflux under nitrogen for 2 h. After cooling down to rt, toluene was removed and the crude was subjected to column chromatography.

The resulting thioamides *tert* butyl esters were dissolved in a mixture of TFA/CH_2Cl_2/Et_3SiH (15.0/5.0/2.0 mL). After 2 h of stirring at rt, the solvents were

removed and the product was washed with 40% ethyl acetate/hexanes to afford the desired products.

Fmoc-L-Phe-Gly-OH Thioamide Linker (251)

Prepared by the general procedure 8 with a yield of 1.20 g (78%), $[\alpha]^{24}_{D}$ -12.1 (*c* = 0.75, CH_3OH); IR ($CHCl_3$) 3305, 1720, 1705, 1247 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.56 (t, *J* = 9.0Hz, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.31–7.19 (m, 7H), 4.76 (dd, *J* = 5.0, 9.5 Hz, 1H), 4.35–4.10 (m, 5H), 3.36–3.31 (m, 1H), 2.94 (dd, *J* = 10.0, 13.0 Hz, 1H); ^{13}C NMR (125.6 MHz, CD_3OD) δ 205.3, 170.2, 156.8, 144.0, 141.3, 137.7, 129.2, 128.2, 127.6, 127.0, 126.5, 125.2, 125.1, 119.7, 71.2, 66.9, 62.8, 48.7, 47.5, 47.3, 47.1, 46.3, 41.4. ESI-HRMS calcd for $C_{26}H_{24}N_2O_4SNa$ $[M + Na]^+$, 483.1354; found, 483.1346.

Fmoc-D-Phe-Gly-OH Thioamide Linker (252)

Prepared by the general procedure 8 with a yield of 1.15 g (76%), $[\alpha]^{24}_{D}$ +10.5 (c = 0.5, CH_3OH); IR ($CHCl_3$) 3305, 1720, 1705, 1247 cm^{-1}; 1H NMR (500 MHz, CD_3OD) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.56 (t, *J* = 9.0Hz, 2H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.31–7.19 (m, 7H), 4.76 (dd, *J* = 5.0, 9.5 Hz, 1H), 4.35–4.10 (m, 5H), 3.36–3.31 (m, 1H), 2.94 (dd, *J* = 10.0, 13.0 Hz, 1H); ^{13}C NMR (125.6 MHz, CD_3OD) δ 205.3, 170.2, 156.8, 144.0, 141.3, 137.7, 129.2, 128.2, 127.6, 127.0, 126.5, 125.2, 125.1, 119.7, 71.2, 66.9, 62.8, 48.7, 47.5, 47.3, 47.1, 46.3, 41.4. ESI-HRMS calcd for $C_{26}H_{24}N_2O_4SNa$ $[M + Na]^+$, 483.1354; found, 483.1359.

Fmoc-Gly-Gly-OH Thioamide Linker (253)

Prepared by the general procedure 8 with a yield of 2.60 g (72%), IR ($CHCl_3$) 3295, 1725, 1714, 1247 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 7.75 (d, *J* =

7.5 Hz, 2H), 7.65 (d, *J* = 7.5 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.26 (t, *J* = 7.0 Hz, 2H), 4.38–4.34 (m, 4H), 4.20–4.19 (m, 3H); ^{13}C NMR (125.6 MHz, CD_3OD) δ 201.4, 170.5, 157.9, 144.1, 141.4, 127.7, 127.1, 125.1, 119.8, 67.1, 51.3, 48.8, 48.4, 47.4, 47.2, 46.3. ESI-HRMS calcd for $C_{19}H_{18}N_2O_4SNa$ $[M + Na]^+$, 393.0885; found, 393.0891.

Synthesis of the both L- and D-Diastereomers of L-Val-L-Ala-Phe-SBn

Derivatization of aminomethyl polystyrene resin with Fmoc-Phe-Gly-OH thioamides linkers (254 and 255): In a 10 mL glass reaction vessel, aminomethyl polystyrene resin (244 mg, 0.1 mmol) was swelled in DMF (4 mL) for 30 min, after which the solvent was removed by filtration. To a stirred solution of thioamides linker **254** or **255** (184 mg, 0.4 mmol), HOBT (54 mg, 0.4 mmol) and HBTU (152 mg, 0.4 mmol) in dry DMF (3 mL) was added DIPEA (70 µL, 0.4 mmol) at room temperature. The reaction mixture was stirred for 4 min before the activated HOBt ester of **254** or **255** was added to the reaction vessel with an additional DMF (1 mL) which was then shaken for 2 h before the resin was washed thoroughly using DMF (3 × 2 mL) and DCM (3 × 2 mL).

Iterative peptide assembly: The Fmoc group was removed with 20% piperidine in DMF. After Fmoc removal, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL), isopropanol (2x5 mL) and hexane (2x5 mL). All couplings were carried out by adding a preactivated solution of protected amino acid (0.4 mmol), HOBT (0.4 mmol), DIPEA (0.4 mmol), and HBTU (0.4 mmol) in dry DMF (4.0 mL) to the resin. After 2 h, the resin was washed with

DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL). The peptide chain was terminated with the Boc protected amino acid (Boc-L-Val-OH).

Alkylation of resin bound peptide thioamides: After completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (150 µL, 1 mmol) and benzyl bromide (107 µL, 0.9 mmol) in DMF (4 mL). After 2 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL) and dried to provide the resin bound thioimidate.

Cleavage from the resin: The resin bound thioimidate was suspended in a 10 mL mixture of TFA/water (1:1) and stirred magnetically for 2 h at rt. The resin was filtered and washed twice with 10 mL of acetonitrile. The combined filtrates were concentrated and the concentrate was neutralized with a saturated solution of sodium bicarbonate (40 mL) followed by the extraction with chloroform 150 mL (3x50 mL). Then the organic layer was washed with brine and dried. Evaporation of the solvent in a rotovapor afforded the crude peptide thioester, which was subjected to chromatographic purification over silica gel.

L-Val-L-Ala-L-Phe-SBn (254)

Following the above described procedure with a yield of 32 mg (72%). ^{1}H NMR of the **241** shows epimerization (ratio, L/D = 78.1/21.9). ESI-HRMS calcd for $C_{24}H_{32}N_3O_3S$ $[M + H]^+$, 442.2164; found, 442.2168.

L-Val-L-Ala-D-Phe-SBn (255)

Following the above described procedure with a yield of 33 mg (74%). ^{1}H NMR of the **241** shows epimerization (ratio, L/D = 22.5/77.5). ESI-HRMS calcd for $C_{24}H_{32}N_3O_3S$ $[M + H]^+$, 442.2164; found, 442.2158.

Fmoc-SPPS of Peptidyl Thioesters on the 2-Chlorotrityl Resin

Derivatization of 2-chlorotrityl resin with 257: In a 10 mL glass reaction vessel, 2-Chlorotrityl chloride resin (117 mg, 0.1 mmol) was suspended in methylene chloride (5 mL) shaken for 5 min and filtered. A solution of **257** (149 mg, 0.4 mmol) and DIPEA (174 µL, 1.0 mmol) in methylene chloride (4 mL) was added to the resin, shaken for 2 h at rt, and filtered. The resin was washed with DMF (2x 5 mL) and capped with 5.0 mL of a mixture of methylene chloride/MeOH/DIPEA (80:15:5).

Iterative peptide assembly: The Fmoc group was removed with 20% piperidine in DMF. After Fmoc removal, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL), isopropanol (2x5 mL) and hexane (2x5 mL). All couplings were carried out by adding a preactivated solution of protected amino acid (0.4 mmol), HOBT (0.4 mmol), DIPEA (0.4 mmol), and HBTU (0.4 mmol) in dry DMF (4.0 mL) to the resin. After 2 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL). The peptide chain was terminated with the Boc protected amino acid.

Alkylation of resin bound peptide thioamides: After completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (150 µL, 1 mmol) and benzyl bromide (107 µL, 0.9 mmol) in DMF (4 mL). After 2 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL) and dried to provide the resin bound thioimidate.

Cleavage from the resin: The resin bound thioimidate was suspended in a 12 mL mixture of TFA/water/Anisole (6/5/1 mL) and stirred magnetically for 2 h

at rt. 20 mL of additional TFA/DCM/Et_3SIH (15/4/1 mL) was added to the reaction mixture and stirred for 4 h. The resin was filtered and washed twice with 10 mL of acetonitrile. The combined filtrates were concentrated and the concentrate was subsequently dissolved in acetonitrile/water (v/v 1:1, 5 mL) and subjected to RP-HPLC purification (10 - 100% A in B with a flow rate of 12 mL/min over 40 min and 215 nm UV detection) to afford the peptide thioester.

L-Thr-L-Ala-L-Ser-L-Phe-L-Ser-L-Leu-Gly-SBn (262)

Reversed phase HPLC purification (10–100% A with a flow rate of 12 mL/min over 40 min and 215 nm UV detection, retention time = 22 min) provided the heptapeptide thioester with a yield of 51 mg (57%). ESI-HRMS calcd for $C_{37}H_{54}N_7O_{10}S$ $[M + H]^+$, 788.3653; found, 788.3661.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical column (4.6 × 250 mm). 10–90% A over 30 min with a flow rate of 1.0 mL/min. Retention time = 15.52 min.

L-Thr-L-Phe-L-Tyr-L-Ser-L-Ala-L-Tyr-Gly-SBn (263)

Reversed phase HPLC purification (10–100% A with a flow rate of 12 mL/min over 40 min and 215 nm UV detection, retention time = 26 min) provided the heptapeptide thioester with a yield of 57 mg (56%). ESI-HRMS calcd for $C_{46}H_{56}N_7O_{11}S$ $[M + H]^+$, 914.3759; found, 914.3768.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical

column (4.6 × 250 mm). 10–90% A over 30 min with a flow rate of 1.0 mL/min. Retention time = 17.90 min.

L-Asn-L-Trp-L-Arg-L-Tyr-L-Ile-L-Ser-L-Thr-L-Phe-Gly-SBn (264)

Reversed phase HPLC purification (10–100% A with a flow rate of 12 mL/min over 40 min and 215 nm UV detection, retention time = 31 min) provided the nonapeptide thioester with a yield of 76 mg (48%). ESI-HRMS calcd for $C_{61}H_{81}N_{14}O_{13}S$ $[M + H]^+$, 1249.5828; found, 1249.5849.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical column (4.6 × 250 mm). 10–90% A over 30 min with a flow rate of 1.0 mL/min. Retention time = 23.18 min.

L-Glu-L-Met-L-Arg-Gly-SBn (266)

Derivatization of aminomethyl polystyrene resin with Fmoc-Gly-Gly-OH thioamides linker (253): In a 10 mL glass reaction vessel, aminomethyl polystyrene resin (244 mg, 0.1 mmol) was swelled in DMF (4 mL) for 30 min, after which the solvent was removed by filtration. To a stirred solution of thioamides linker **253** (154 mg, 0.4 mmol) and HBTU (152 mg, 0.4 mmol) in dry DMF (3 mL) was added DIPEA (70 µL, 0.4 mmol) at room temperature. The reaction mixture was stirred for 4 min before the activated HOBt ester of **253** was added to the reaction vessel with an additional DMF (1 mL) which was then shaken for 2 h before the resin was washed thoroughly using DMF (3 × 2 mL) and DCM (3 × 2 mL).

Iterative peptide assembly: The Fmoc group was removed with 20% piperidine in DMF. After Fmoc removal, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL), isopropanol (2x5 mL) and hexane (2x5 mL). All couplings were carried out by adding a preactivated solution of protected amino acid (0.4 mmol), HOBT (0.4 mmol), DIPEA (0.4 mmol), and HBTU (0.4 mmol) in dry DMF (4.0 mL) to the resin. After 2 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL). The peptide chain was terminated with the Boc protected amino acid (Boc-L-Glu(O^tBu)-OH).

Alkylation of resin bound peptide thioamides: After completion of the on-resin procedure, the resin-bound peptide was treated with a solution of DBU (150 µL, 1 mmol) and benzyl bromide (107 µL, 0.9 mmol) in DMF (4 mL). After 2 h, the resin was washed with DMF (2x5 mL), methylene chloride (2x5 mL) and DMF (2x5 mL) and dried to provide the resin bound thioimidate.

Cleavage from the resin: The resin bound thioimidate was suspended in a 12 mL mixture of TFA/water/Anisole (6/5/1 mL) and stirred magnetically for 2 h at rt. 20 mL of additional TFA/DCM/Et_3SIH (15/4/1 mL) was added to the reaction mixture and stirred for 4 h. The reaction mixture was now treated with NH_4I/Me_2S (25 mg/100 µL) to reduce the sulfoxide moiety. The resin was filtered and washed twice with 10 mL of acetonitrile. The combined filtrates were concentrated and the concentrate was subsequently dissolved in acetonitrile/water (v/v 1:1, 5 mL) and subjected to RP-HPLC purification (10 - 100% A in B with a flow rate of 12 mL/min over 40 min and 215 nm UV detection,

retention time 22 min) to afford the tetrapeptide thioester with a yield of 51 mg (62%). ESI-HRMS calcd for $C_{25}H_{40}N_7O_6S_2$ $[M + H]^+$, 598.2481; found, 598.2492.

HPLC Analysis was performed on a Varian Prep Star 218 HPLC system with 254 nm UV detection, using a Waters Cosmosil SC18-AR-300 analytical column (4.6 × 250 mm). 10–90% A over 30 min with a flow rate of 1.0 mL/min. Retention time = 15.44 min.

REFERENCES

(1) Kimmerlin, T.; Seebach, D. *J. Pept. Res.* **2005**, *65*, 229.

(2) Kent, S. B. H. *Chem. Soc. Rev.* **2009**, *38*, 338.

(3) Wells, J. A.; Estell, D. A. *Trends Biochem Sci* **1988**, *13*, 291.

(4) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 6705.

(5) Mendel, D.; Cornish, V. W.; Schultz, P. G. *Annu Rev Biophys Biomol Struct* **1995**, *24*, 435.

(6) Xie, J.; Schultz, P. G. *Curr. Opin. Chem. Biol.* **2005**, *9*, 548.

(7) Dirksen, A.; Dawson, P. E. *Curr. Opin. Chem. Biol.* **2008**, *12*, 760.

(8) Kent, S. *J. Pept. Sci.* **2003**, *9*, 574.

(9) Goodman, M.; McGahren, W. J. *Tetrahedron* **1967**, *23*, 2031.

(10) Han, S.-Y.; Kim, Y.-A. *Tetrahedron* **2004**, *60*, 2447.

(11) Montalbetti, C. A. G. N.; Falque, V. *Tetrahedron* **2005**, *61*, 10827.

(12) Kimmerlin, T.; Seebach, D. *J. Pept. Res.* **2005**, *65*, 229.

(13) Barany, G.; Merrifield, R. B. In *Peptides* 1980; Vol. 2, p 1.

(14) Kaiser, E. T. *Acc. Chem. Res.* **1989**, *22*, 47.

(15) Schnoelzer, M.; Kent, S. B. H. *Science* **1992**, *256*, 221.

(16) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.

(17) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H.; Schafer, W. *Justus Liebigs Ann. Chem.* **1953**, *583*, 129.

(18) Muralidharan, V.; Muir, T. W. *Nature Methods* **2006**, *3*, 429.

(19) Torbeev Vladimir, Y.; Kent Stephen, B. H. *Angew. Chem. Int. Ed.* **2007**, *46*, 1667.

(20) Canne, L. E.; Bark, S. J.; Kent, S. B. H. *J. Am. Chem. Soc.* **1996**, *118*, 5891.

(21) Offer, J.; Dawson, P. E. *Org. Lett.* **2000**, *2*, 23.

(22) Kawakami, T.; Akaji, K.; Aimoto, S. *Org. Lett.* **2001**, *3*, 1403.

(23) Vizzavona, J.; Dick, F.; Vorherr, T. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1963.

(24) Wu, B.; Chen, J.; Warren, J. D.; Chen, G.; Hua, Z.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2006**, *45*, 4116.

(25) Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **2003**, *44*, 6059.

(26) Marinzi, C.; Offer, J.; Longhi, R.; Dawson, P. E. *Bioorg. Med. Chem.* **2004**, *12*, 2749.

(27) Chen, G.; Warren, J. D.; Chen, J.; Wu, B.; Wan, Q.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 7460.

(28) Brik, A.; Yang, Y.-Y.; Ficht, S.; Wong, C.-H. *J. Am. Chem. Soc.* **2006**, *128*, 5626.

(29) Brik, A.; Ficht, S.; Yang, Y.-Y.; Bennett, C. S.; Wong, C.-H. *J. Am. Chem. Soc.* **2006**, *128*, 15026.

(30) Yang, Y.-Y.; Ficht, S.; Brik, A.; Wong, C.-H. *J. Am. Chem. Soc.* **2007**, *129*, 7690.

(31) Ficht, S.; Payne, R. J.; Brik, A.; Wong, C.-H. *Angew. Chem. Int. Ed.* **2007**, *46*, 5975.

(32) Kemp, D. S.; Galakatos, N. G.; Bowen, B.; Tan, K. *J. Org. Chem.* **1986**, *51*, 1829.

(33) Kemp, D. S.; Galakatos, N. G. *J. Org. Chem.* **1986**, *51*, 1821.

(34) Lutsky, M.-Y.; Nepomniaschiy, N.; Brik, A. *Chem. Commun.* **2008**, 1229.

(35) Rohde, H.; Seitz, O. *Biopolymers* **2010**, *94*, 551.

(36) Okamoto, R.; Izumi, M.; Kajihara, Y. *Int. J. Pept. Res. Ther.* **2010**, *16*, 191.

(37) Yan, L. Z.; Dawson, P. E. *J. Am. Chem. Soc.* **2001**, *123*, 526.

(38) Crich, D.; Banerjee, A. *J. Am. Chem. Soc.* **2007**, *129*, 10064.

(39) Botti, P.; Tchertchian, S.; (Geneprot Inc., Switz.). Application: WO WO, 2006, p 37pp.

(40) Haase, C.; Rohde, H.; Seitz, O. *Angew. Chem. Int. Ed.* **2008**, *47*, 6807.

(41) Chen, J.; Wan, Q.; Yuan, Y.; Zhu, J.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2008**, *47*, 8521.

(42) Chen, J.; Wang, P.; Zhu, J.; Wan, Q.; Danishefsky, S. J. *Tetrahedron* **2010**, *66*, 2277.

(43) Yang, R.; Pasunooti, K. K.; Li, F.; Liu, X.-W.; Liu, C.-F. *J. Am. Chem. Soc.* **2009**, *131*, 13592.

(44) Pasunooti, K. K.; Yang, R.; Vedachalam, S.; Gorityala, B. K.; Liu, C.-F.; Liu, X.-W. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6268.

(45) Tam, J. P.; Yu, Q. *Biopolymers* **1998**, *46*, 319.

(46) Dose, C.; Seitz, O. *Org. Biomol. Chem.* **2004**, *2*, 59.

(47) Heinrikson, R. L. *J. Biol. Chem.* **1971**, *246*, 4090.

(48) Naider, F. R.; Becker, J. M. *Biopolymers* **1997**, *43*, 3.

(49) Okamoto, R.; Kajihara, Y. *Angew. Chem. Int. Ed.* **2008**, *47*, 5402.

(50) Okamoto, R.; Souma, S.; Kajihara, Y. *J. Org. Chem.* **2009**, *74*, 2494.

(51) Quaderer, R.; Sewing, A.; Hilvert, D. *Helv. Chim. Acta* **2001**, *84*, 1197.

(52) Quaderer, R.; Hilvert, D. *Chem. Commun.* **2002**, 2620.

(53) Gieselman, M. D.; Xie, L.; van der Donk, W. A. *Org. Lett.* **2001**, *3*, 1331.

(54) Gieselman, M. D.; Zhu, Y.; Zhou, H.; Galonic, D.; Van der Donk, W. A. *ChemBioChem* **2002**, *3*, 709.

(55) Zhang, L.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 3.

(56) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939.

(57) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9.

(58) Soellner Matthew, B.; Nilsson Bradley, L.; Raines Ronald, T. *J Org Chem* **2002**, *67*, 4993.

(59) Nilsson Bradley, L.; Hondal Robert, J.; Soellner Matthew, B.; Raines Ronald, T. *J. Am. Chem. Soc.* **2003**, *125*, 5268.

(60) Soellner, M. B.; Dickson, K. A.; Nilsson, B. L.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 11790.

(61) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141.

(62) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007.

(63) Staudinger, H.; Meyer, J. *Helv. Chim. Acta* **1919**, *2*, 635.

(64) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141.

(65) Soellner, M. B.; Nilsson, B. L.; Raines, R. T. *J. Org. Chem.* **2002**, *67*, 4993.

(66) Koehn, M.; Breinbauer, R. *Angew. Chem. Int. Ed.* **2004**, *43*, 3106.

(67) Bode, J. W.; Fox, R. M.; Baucom, K. D. *Angew. Chem. Int. Ed.* **2006**, *45*, 1248.

(68) Carrillo, N.; Davalos, E. A.; Russak, J. A.; Bode, J. W. *J. Am. Chem. Soc.* **2006**, *128*, 1452.

(69) Shen, B.; Makley, D. M.; Johnston, J. N. *Nature* **2010**, *465*, 1027.

(70) Niyomura, O.; Kato, S. *Top. Curr. Chem.* **2005**, *251*, 1.

(71) Kato, S.; Murai, T. **1992**, *2*, 803.

(72) Liu, C.-F.; Rao, C.; Tam, J. P. *Tetrahedron Lett.* **1996**, *37*, 933.

(73) Tam, J. P.; Yu, Q.; Miao, Z. *Biopolymers* **2000**, *51*, 311.

(74) Hakimelahi, G. H.; Just, G. *Tetrahedron Lett.* **1980**, *21*, 2119.

(75) Rosen, T.; Lico, I. M.; Chu, D. T. W. *J. Org. Chem.* **1988**, *53*, 1580.

(76) Rakotomanomana, N.; Lacombe, J. M.; Pavia, A. A. *Carbohydr. Res.* **1990**, *197*, 318.

(77) Shangguan, N.; Katukojvala, S.; Greenberg, R.; Williams, L. J. *J. Am. Chem. Soc.* **2003**, *125*, 7754.

(78) Crich, D.; Sasaki, K. *Org. Lett.* **2009**, *11*, 3514.

(79) Assem, N.; Natarajan, A.; Yudin, A. K. *J. Am. Chem. Soc.* **2010**, *132*, 10986.

(80) Tam, J. P.; Lu, Y.-A.; Liu, C.-F.; Shao, J. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 12485.

(81) Coltart, D. M. *Tetrahedron* **2000**, *56*, 3449.

(82) Clive, D. L. J.; Hisaindee, S.; Coltart, D. M. *J. Org. Chem.* **2003**, *68*, 9247.

(83) Wu, B.; Chen, J.; Warren, J. D.; Chen, G.; Hua, Z.; Danishefsky, S. J. *Angew. Chem. Int. Ed.* **2006**, *45*, 4116.

(84) Offer, J.; Boddy, C. N. C.; Dawson, P. E. *J. Am. Chem. Soc.* **2002**, *124*, 4642.

(85) Macmillan, D. *Angew. Chem. Int. Ed.* **2006**, *45*, 7668.

(86) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem. Int. Ed.* **2008**, *47*, 10030.

(87) Bang, D.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2004**, *43*, 2534.

(88) Bang, D.; Chopra, N.; Kent, S. B. H. *J. Am. Chem. Soc.* **2004**, *126*, 1377.

(89) Bang, D.; Pentelute, B. L.; Kent, S. B. H. *Angew. Chem. Int. Ed.* **2006**, *45*, 3985.

(90) Johnson, E. C. B.; Kent, S. B. H. *J. Am. Chem. Soc.* **2006**, *128*, 6640.

(91) Durek, T.; Torbeev Vladimir, Y.; Kent Stephen, B. H. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 4846.

(92) Blake, J. *Int. J. Peptide Protein Res* **1981**, *17*, 273.

(93) Blake, J.; Li, C. H. *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 4055.

(94) Yamashiro, D.; Li, C. H. *Int. J. Peptide Protein Res.* **1988**, *31*, 322.

(95) Canne, L. E.; Walker, S. M.; Kent, S. B. H. *Tetrahedron Lett.* **1995**, *36*, 1217.

(96) Rajagopalan, S.; Radke, G.; Tomich, J. *Synth. Commun.* **1997**, *27*, 187.

(97) Koch, T. In *Peptide Nucleic Acids* 2nd ed.; Nielsen, P. E., Ed.; Horizon Bioscience: Wymondham, UK, 2004, p 37.

(98) Dawson, P. E.; Churchill, M.; Ghadiri, M. R.; Kent, S. B. H. *J. Am. Chem. Soc.* **1997**, *119*, 4325.

(99) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111.

(100) Hojo, H.; Kwon, Y.; Kakuta, Y.; Tsuda, S.; Tanaka, I.; Hikichi, K.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2700.

(101) Zhang, L.; Tam, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 2363.

(102) Zhang, L.; Tam, J. P. *J. Am. Chem. Soc.* **1999**, *121*, 3311.

(103) Li, Y.; Yu, Y.; Giulianotti, M.; Houghten, R. A. *J. Comb. Chem.* **2008**, *10*, 613.

(104) Anon *Oil Gas J.* **1988**, *86*, 58.

(105) Segal, E. B. *Chemical Health & Safety* **2000**, *7*, 18.

(106) Crich, D.; Sana, K. *J. Org. Chem.* **2009**, *74*, 7383.

(107) Li, X.; Kawakami, T.; Aimoto, S. *Tetrahedron Lett.* **1998**, *39*, 8669.

(108) Hasegawa, K.; Sha, Y. L.; Bang, J. K.; Kawakami, T.; Akaji, K.; Aimoto, S. *Lett. Pept. Sci.* **2002**, *8*, 277.

(109) Alsina, J.; Yokum, T. S.; Albericio, F.; Barany, G. *J. Org. Chem.* **1999**, *64*, 8761.

(110) Alsina, J.; Yokum, T. S.; Albericio, F.; Barany, G. *Tetrahedron Lett.* **2000**, *41*, 7277.

(111) Jensen, K. J.; Alsina, J.; Songster, M. F.; Vagner, J.; Albericio, F.; Barany, G. *J. Am. Chem. Soc.* **1998**, *120*, 5441.

(112) McDermott, J. R.; Benoiton, N. L. *Can. J. Chem.* **1973**, *51*, 2562.

(113) Davies, J. S.; Mohammed, A. K. *J. Chem. Soc., Perkin Trans. 1* **1981**, 2982.

(114) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369.

(115) Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc. Chem. Commun.* **1971**, 636.

(116) Backes, B. J.; Ellman, J. A. *J. Org. Chem.* **1999**, *64*, 2322.

(117) Swinnen, D.; Hilvert, D. *Org. Lett.* **2000**, *2*, 2439.

(118) Sweing, A.; Hilvert, D. *Angew. Chem. Int. Ed.* **2001**, *40*, 3395.

(119) Brask, J.; Albericio, F.; Jensen, K. J. *Org. Lett.* **2003**, *5*, 2951.

(120) Mezo, A. R.; Cheng, R. P.; Imperiali, B. *J. Am. Chem. Soc.* **2001**, *123*, 3885.

(121) Kitagawa, K.; Aida, C.; Fujiwara, H.; Yagami, T.; Futaki, S. *Tetrahedron Lett.* **1997**, *38*, 599.

(122) Camarero, J. A.; Hackel, B. J.; De Yoreo, J. J.; Mitchell, A. R. *J. Org. Chem.* **2004**, *69*, 4145.

(123) Blanco-Canosa, J. B.; Dawson, P. E. *Angew. Chem. Int. Ed.* **2008**, *47*, 6851.

(124) Tofteng, A. P.; Soerensen, K. K.; Conde-Frieboes, K. W.; Hoeg-Jensen, T.; Jensen, K. J. *Angew. Chem. Int. Ed.* **2009**, *48*, 7411.

(125) Botti, P.; Villain, M.; Manganiello, S.; Gaertner, H. *Org. Lett.* **2004**, *6*, 4861.

(126) Warren, J. D.; Miller, J. S.; Keding, S. J.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 6576.

(127) George, E. A.; Novick, R. P.; Muir, T. W. *J. Am. Chem. Soc.* **2008**, *130*, 4914.

(128) Kawakami, T.; Sumida, M.; Nakamura, K. i.; Vorherr, T.; Aimoto, S. *Tetrahedron Lett.* **2005**, *46*, 8805.

(129) Ollivier, N.; Behr, J.-B.; El-Mahdi, O.; Blanpain, A.; Melnyk, O. *Org. Lett.* **2005**, *7*, 2647.

(130) Ohta, Y.; Itoh, S.; Shigenaga, A.; Shintaku, S.; Fujii, N.; Otaka, A. *Org. Lett.* **2006**, *8*, 467.

(131) Nagaike, F.; Onuma, Y.; Kanazawa, C.; Hojo, H.; Ueki, A.; Nakahara, Y.; Nakahara, Y. *Org. Lett.* **2006**, *8*, 4465.

(132) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 25.

(133) Kawakami, T.; Aimoto, S. *Tetrahedron* **2009**, *65*, 3871.

(134) Kang, J.; MacMillan, D. *Org. Biomol. Chem.* **2010**, *8*, 1993.

(135) Hojo, H.; Onuma, Y.; Akimoto, Y.; Nakahara, Y.; Nakahara, Y. *Tetrahedron Lett.* **2007**, *48*, 1299.

(136) Tsuda, S.; Shigenaga, A.; Bando, K.; Otaka, A. *Org. Lett.* **2009**, *11*, 823.

(137) Messeri, T.; Sternbach, D. D.; Tomkinson, N. C. O. *Tetrahedron Lett.* **1998**, *39*, 1673.

(138) Messeri, T.; Sternbach, D. D.; Tomkinson, N. C. O. *Tetrahedron Lett.* **1998**, *39*, 1669.

(139) Crich, D.; Sana, K.; Guo, S. *Org. Lett.* **2007**, *9*, 4423.

(140) Crich, D.; Bowers Albert, A. *Org. Lett.* **2007**, *9*, 5323.

(141) Kaminski, Z. J.; Kolesinska, B.; Kolesinska, J.; Sabatino, G.; Chelli, M.; Rovero, P.; Blaszczyk, M.; Glowka, M. L.; Papini, A. M. *J. Am. Chem. Soc.* **2005**, *127*, 16912.

(142) Ishiwata, A.; Ichiyanagi, T.; Takatani, M.; Ito, Y. *Tetrahedron Lett.* **2003**, *44*, 3187.

(143) Yamashiro, D.; Blake, J. *Int. J. Pept. Prot. Res.* **1981**, *18*, 383.

(144) Blake, J. *Int. J. Pept. Prot. Res.* **1981**, *17*, 273.

(145) Mitin Yu, V.; Zapevalova, N. P. *Int. J. Pept. Prot. Res.* **1990**, *35*, 352.

(146) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776.

(147) Sanger, F. *Biochem. J* **1945**, *39*, 507.

(148) Bioconjugate Techniques (Ed.: G. T. Hermanson), Academic Press, New York, 1995, p. 786.

(149) Eisen, H. N.; Belman, S.; Carsten, M. E. *J. Am. Chem. Soc.* **1953**, *75*, 4583.

(150) Vetter, S. *Synth. Commun.* **1998**, *28*, 3219.

(151) Nishiguchi, A.; Maeda, K.; Miki, S. In *Synthesis* **2006**; *24*, 4131.

(152) Hogenauer, T. J.; Wang, Q.; Sanki, A. K.; Gammon, A. J.; Chu, C. H. L.; Kaneshiro, C. M.; Kajihara, Y.; Michael, K. *Org. Biomol. Chem.* **2007**, *5*, 759.

(153) De Coster, G.; Vandyck, K.; Van der Eycken, E.; Van der Eycken, J.; Elseviers, M.; Roper, H. *Tetrahedron Asymmetry* **2002**, *13*, 1673.

(154) Sasaki, K.; Crich, D. *Org Lett* **2010**, *12*, 3254.

(155) Yazaki, R.; Kumagai, N.; Shibasaki, M. *J. Am. Chem. Soc.* **2010**, *132*, 10275.

(156) Rew, Y.; Shin, D.; Hwang, I.; Boger, D. L. *J. Am. Chem. Soc.* **2004**, *126*, 1041.

(157) Li, W. R.; Jiang, J.; Joullie, M. M. *Synlett* **1993**, 362.

(158) Ueki, M.; Amemiya, M. *Tetrahedron Lett.* **1987**, *28*, 6617.

(159) Mukaiyama, T. *Angew. Chem. Int. Ed.* **1979**, *18*, 707.

(160) Boeini, H. Z.; Kashan, M. E. *Green Chem.* **2009**, *11*, 1987.

(161) Suzuki, Y.; Yazaki, R.; Kumagai, N.; Shibasaki, M. *Angew. Chem. Int. Ed.* **2009**, *48*, 5026.

(162) Payne, R. J.; Ficht, S.; Greenberg, W. A.; Wong, C.-H. *Angew. Chem. Int. Ed.* **2008**, *47*, 4411.

(163) Carles-Bonnet, C.; Martinez, J.; Jarrousse, C.; Aumelas, A.; Niel, H.; Bataille, D. *Peptides* **1996**, *17*, 557.

(164) Wynne, K.; Bloom, S. R. *Nat. Clin. Pract. Endocrinol. Metab.* **2006**, *2*, 612.

(165) Fukuyama, T.; Cheung, M.; Jow, C.-K.; Hidai, Y.; Kan, T. *Tetrahedron Lett.* **1997**, *38*, 5831.

(166) Amsberry, K. L.; Borchardt, R. T. *J. Org. Chem.* **1990**, *55*, 5867.

(167) Sammes, P. G.; Weller, D. J. *Synthesis* **1995**, 1205.

(168) Crich, D.; Cai, F. *Org. Lett.* **2007**, *9*, 1613.

(169) Murai, T. *Top. Curr. Chem.* **2005**, *251*, 247.

(170) Lebel, H. *Science of Synthesis* **2005**, *22*, 141.

(171) Hoeg-Jensen, T. *Phosphorus, Sulfur Silicon Relat. Elem.* **1996**, *108*, 257.

(172) Reiner, A.; Wildemann, D.; Fischer, G.; Kiefhaber, T. *J. Am. Chem. Soc.* **2008**, *130*, 8079.

(173) Raddatz, P.; Jonczyk, A.; Schmitges, C. J.; Sombroek, J. *Adv. Exp. Med. Biol.* **1991**, *306*, 387.

(174) Gupta, S.; Payne, J. W. *J. Pept. Res.* **2001**, *58*, 546.

(175) Wiberg, K. B.; Rablen, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 2201.

(176) Oberhauser, B.; Baumann, K.; Grohmann, B.; Sperner, H. *Synlett* **1999**, 893.

(177) May, P. J.; Bradley, M.; Harrowven, D. C.; Pallin, D. *Tetrahedron Lett.* **2000**, *41*, 1627.

(178) Eberle, M. K.; Jutzi-Eme, A.-M.; Nuninger, F. *J. Org. Chem.* **1994**, *59*, 7249.

(179) Coates, R. M.; Firsan, S. J. *J. Org. Chem.* **1986**, *51*, 5198.

(180) Mezzato, S.; Unverzagt, C. *Carbohydr. Res.* **2010**, *345*, 1306.

(181) Lu, J.-Y.; Riedrich, M.; Mikyna, M.; Arndt, H.-D. *Angew. Chem. Int. Ed.* **2009**, *48*, 8137.

(182) Dawson, R. E.; Hennig, A.; Weimann, D. P.; Emery, D.; Ravikumar, V.; Montenegro, J.; Takeuchi, T.; Gabutti, S.; Mayor, M.; Mareda, J.; Schalley, C. A.; Matile, S. *Nature Chem.* **2010**, *2*, 533.

(183) Bremner, J. B.; Keller, P. A.; Pyne, S. G.; Boyle, T. P.; Brkic, Z.; David, D. M.; Robertson, M.; Somphol, K.; Baylis, D.; Coates, J. A.; Deadman, J.; Jeevarajah, D.; Rhodes, D. I. *Bioorg. Med. Chem.* **2010**, *18*, 2611.

(184) Barra, M.; Roy, O.; Traikia, M.; Taillefumier, C. *Org. Biomol. Chem.* **2010**, *8*, 2941.

(185) Liang, F.; Li, Y.; Li, D.; Cheng, X.; Liu, Q. *Tetrahedron Lett.* **2007**, *48*, 7938.

(186) Beslin, P.; Marion, P. *Tetrahedron Lett.* **1992**, *33*, 935.

(187) Haydon, D. J.; Bennett, J. M.; Brown, D.; Collins, I.; Galbraith, G.; Lancett, P.; Macdonald, R.; Stokes, N. R.; Chauhan, P. K.; Sutariya, J. K.; Nayal, N.; Srivastava, A.; Beanland, J.; Hall, R.; Henstock, V.; Noula, C.; Rockley, C.; Czaplewski, L. *J. Med. Chem.* **2010**, *53*, 3927.

(188) Das, B.; Rajarao, A. V. S.; Rudra, S.; Yadav, A.; Ray, A.; Pandya, M.; Rattan, A.; Mehta, A. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6424.

(189) Vilaseca, M.; Nicolas, E.; Capdevila, F.; Giralt, E. *Tetrahedron* **1998**, *54*, 15273.

(190) Taboada, L.; Nicolas, E.; Giralt, E. *Tetrahedron Lett.* **2001**, *42*, 1891.

(191) Hackenberger, C. P. R. *Org. Biomol. Chem* **2006**, *4*, 2291.

(192) Fujiwara, Y.; Kimura, T.; Akaji, K.; Kiso, Y. *Peptide Science* **1999**, *35th*, 129.

ABSTRACT

CHEMICAL SYNTHESIS OF PEPTIDES AND PEPTIDE THIOESTERS

by

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Major: Chemistry

Degree: Doctor of Philosophy

This dissertation describes investigations toward the development of a new chemistry for the block synthesis of peptides and peptidyl thioesters. A direct approach for the Fmoc-SPPS of peptidyl thioesters is also delineated in this thesis.

In the first part of chapter one, the difficulty and importance of chemical synthesis of peptides is explained, and a brief survey of available methods is given. The second part of chapter one presents the challenges inherent in the synthesis of peptidyl thioesters by Fmoc-SPPS.

The second chapter outlines the investigations conducted towards the development of a new chemistry for epimerization-free block synthesis of peptides from thioacids and amines by a process involving the *in situ* formation of highly active thioesters by nucleophilic aromatic substitution of *C*-terminal thioacids on electron-deficient halogenoarenes. The superiority of this new chemistry over available methods is illustrated through direct comparisons in the block synthesis of an octapeptide.

In chapter three, studies carried out to probe the reactivity of *N*-terminal sulfonamides towards peptidyl thioacids are presented. Studies directed at determining the optimal sulfonamide for use in convergent multiple peptide fragment ligation are presented as is the application of these sulfonamides in both left to right and right to left block synthesis of peptides.. Model studies on the development of an intramolecular variant of the thioacid/sulfonamide coupling are also presented in this chapter.

Continuing the theme, Chapter 4 presents studies undertaken with the goal of developing a direct method for the efficient synthesis of peptidyl thioesters by the widely used Fmoc-SPPS chemistry. A method to construct the thioester functionality from thioamides is described based on alkylation to give an intermediate thioimide and its subsequent hydrolysis. The application of this methodology to the synthesis of functionality diverse oligo-peptide thioesters is also presented.

In chapter five, the overall conclusions of the dissertation are presented, while in chapter six, the experimental procedures and characterization data for the synthesized compounds are documented.

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Publications

1. David Crich and **Indrajeet Sharma** "Influence of the O3 Protecting Group on Stereoselectivity in the Preparation of C-Mannopyranosides with 4,6-O-Benzylidene Protected Donors." *J.Org. Chem.* **2010,** *75*, 8383-8391

2. David Crich and **Indrajeet Sharma**, "Triblock Peptide and Peptide Thioester Synthesis With Reactivity-Differentiated Sulfonamides and Peptidyl Thioacids." *Angew. Chem. Int. Ed.* **2009,** *48***,** 7591-7594

3. David Crich and **Indrajeet Sharma**, "Epimerization-Free Block Synthesis of Peptides from Thioacids and Amines with Sanger's and Mukaiyama's Reagents." *Angew. Chem. Int. Ed.* **2009,** *48*, 2355-2358

4. David Crich and **Indrajeet Sharma**, "Is Donor-Acceptor Hydrogen Bonding Necessary for 4,6-*O*-Benzylidene Directed β-Mannopyranosylation. Stereoselective Synthesis of β-*C*-Mannopyranosides and α-*C*-Glucopyaronosides." *Org. Lett.* **2008**, *10*, 4731-4734

5. Dipakranjan Mal, Sutapa Ray and **Indrajeet Sharma**, "Direct Access to 1,4-Dihydroxyanthraquinones: The Hauser Annulation Reexamined with *p-Quinones." J. Org. Chem.* **2007***, 71,* 4981-4984

6. **Indrajeet Sharma**, Rhitankar Pal, Xiao Cheng Zeng Luis Bohé, and David Crich, "Influence of Remote Protecting Groups on the Magnitude of the Anomeric Effect in Carbohydrate Chemistry. A Hypothesis for the Origin of the a-Directing Effect of a 3-O-Carboxylate Ester in Mannopyranosyl Donors." *J. Am. Chem. Soc.* **2011** (Manuscript in Preparation).

7. **Indrajeet Sharma and** David Crich, " Direct Fmoc-Chemistry-Based Solid Phase Synthesis of Peptidyl Thioesters." *Angew. Chem. Int. Ed.* **2011** (Manuscript in Preparation).