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Title:
Peptide Functionalisation Through Isoimide Intermediates

Date:
2020

Persistent Link:
https://hdl.handle.net/11343/241341

Peptide Functionalisation Through Isoimide Intermediates

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Submitted in total fulfilment for the degree of

Doctor of Philosophy

School of Chemistry

The University of Melbourne

2020

Abstract

We have developed new synthetic methods for the modification of peptides, exploiting acyl transfer reactions of isoimides. We have developed a new approach for the chemoselective synthesis of *N*-glycopeptides, using a Ag(I)-promoted coupling. We have successfully applied the Ag(I)-promoted coupling of aspartic acid containing peptide thioamides and glycosyl amines to the chemoselective synthesis of N-glycosylated asparaginyl motifs. The methodology employs peptides possessing a thioamide group adjacent to the aspartic acid residue. Intramolecular Ag(I)-promoted coupling of the aspartic acid side-chain carboxylate with the thioamide generates an isoimide intermediate that is trapped by an external glycosyl amine to form an amide bond and generate an *N*-glycoasparagine. A range of thiopeptides containing unprotected carboxylates undergo Ag(I)-promoted coupling with glycosyl amine derivatives, leading to the preparation of *N*-glycosylated asparaginyl peptides. Moreover, this method enables the site-selective generation of *N*-glycosyl asparagines in peptides possessing multiple unprotected carboxylate side chains groups.

We have also investigated analogous reactions of peptide thioamides possessing a lysine residue, wherein coupling of the aspartate and lysine side chains generates Lys–Asp side-chain lactam-linked peptides. Thiopeptides with *i*,*i*+2, *i*,*i*+3, and *i*,*i*+4-spaced Lys and Asp residues undergo intramolecular Ag(I)-promoted coupling to give lactam bridged peptides.

The final chapter describes initial investigations towards elaborating the Ag(I)-promoted reaction to the synthesis of ^{18}F-radiolabelled peptides. The Ag(I)-promoted method was shown to be effective for the coupling of [^{18}F]-fluorobenzoic acid with thiopeptides, generating^{18}F-fluorobenzoylated peptides.

Declaration

This is to certify that

i. The thesis comprises my original work towards the PhD except;

ii. Due acknowledgment has been made in the text to all other material used;

iii. The thesis is less than 100000 words in length, exclusive of tables, bibliographies and appendices.

Ameer Badri Taresh

April 2020

Acknowledgements

My PhD journey in Craig's group has been thoroughly enjoyable and rewarding. First of all, I would to give my deepest and sincerest thanks to Professor Craig Hutton for his support throughout my post graduate studies (MSc and PhD). His patience, continual mentorship, optimism and understanding have made the last four years feel rather short. I would like also to thank my panel members during my PhD candidature Professor Spencer William and Professor Paul Donnelly for their support, advice and suggestions of improvement.

I would like to thank past and present members of the Hutton group for the great environment to work in. In particular, I would like to thank Sadegh Shabani for making day to day research easy and enjoyable. Your support and friendship have meant a lot to me. Special thanks go to Dr. Mohammad Haskali for his generous help, support, and encouragement, especially in radiochemistry. Thank you also to Dr. Zalihe Hakki for her friendship and teaching me carbohydrate chemistry.

Many thanks go to the University of Melbourne for the award of a Melbourne Research Scholarship. I would also like to thank the support staff in the School of Chemistry and Bio21 Molecular Science and Biotechnology Institute, especially to Dr. Shuai Nie for his assistance with mass spectrometry.

The biggest thanks go to my family for their unconditional support and encouragement throughout my life, especially the last four years. Lastly, to my loving wife, Marwah, and my cheeky kids Yousif and Shahad, I would like to give my greatest thanks for all patience, support and companionship. Without you this thesis would have near impossible to complete, and your love and support has kept me going. Thank you for joining me on my PhD journey.

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List of abbreviations

Ac	acetyl
Alloc	allyloxycarbonyl
Et	ethyl
Bn	benzyl
Boc	*tert*-butoxycarbonyl
BOP	benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate
Bq	becquerel
calc	calculated
Cbz	carboxybenzyl
DABCO	1,4 diazabicyclo[2.2.2]octane
dc	decay corrected
DCC	dicyclohexylcarbodiimide
DCE	dichloroethane
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
dr	diastereomeric ratio
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Equiv.	equivalents
ESI	electrospray ionisation
FBA	fluorobenzoic acid
GlcNAc	*N*-acetylglucosamine
h	hour(s)
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate
HBTU	*O*-(1*H*-benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
Hex	hexane
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HSTU	*O*-(*N*-Succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
IBCF	isobutylchloroformate
iBu	isobutyl
iPr	isopropyl
Man	mannose
Me	methyl
min	minute(s)
MS	mass spectrometry
Mw	microwave
NBS	*N*-bromosuccinimide
NCL	native chemical ligation
ndc	non decay corrected
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidinone
NMR	nuclear magnetic resonance

NOE	nuclear Overhauser effect
OSu	*O*-Succinimide
Ph	phenyl
ppm	parts per million
QMA	quarternary methyl ammonium
RCC	radiochemical conversion
RP-HPLC	reverse phase high performance liquid chromatography
rt	room temperature
RCY	radiochemical yield
SFB	*N*-succinimidyl-4-fluorobenzoate
SPE	solid phase extraction
*t*Bu	tertiary butyl
TBAHS	tetrabutylammonium hydrogen sulfate
TBAOH	tetrabutylammonium hydroxide
TBDPS	tert-Butyldiphenylsilyl
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran
TIPS	2,2,2-triisopropylsilane
TLC	thin layer chromatography
TMSOTF	trimethylsilyl trifluoromethanesulfonate
TPAOH	tetrapropylammonium hydroxide
Trt	trityl
Ts	tosyl
TSTU	N,N,N′,N′-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate

1. Introduction

1.1 Background

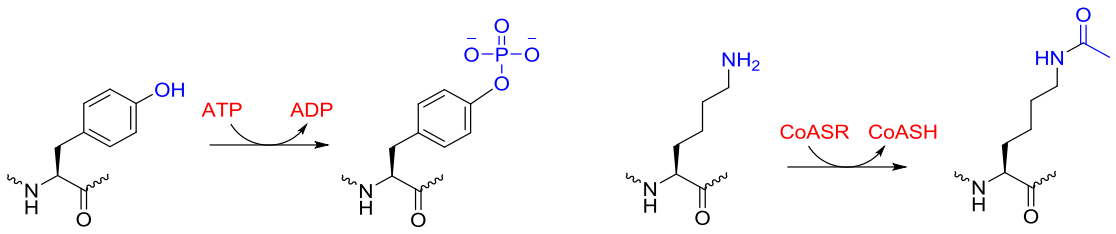
Peptides and proteins are important classes of biomolecules that play essential roles in biological and physiological events, functioning as hormones, antibodies, enzymes, receptors and transport molecules. The biological function of a protein arises from its unique folded structure which is in turn determined by the sequence of the amino acids in the peptide backbone.[1] The amino acid sequence of a protein is defined by the sequence of DNA nucleotides in its encoding gene. The number of genes in higher animals is far fewer than was originally estimated based on the total number of unique proteins in a cell. While the human genome possesses 30,000 genes, the proteome consists of more than 100,000 discrete proteins. The diversity of the structures and functions of proteins produced by a cell is a result of mRNA splicing before translation and by post-translational modification (PTM) of proteins at one or more sites.[2-3]

1.2 Post-translational modification of proteins

Post-translational modification (PTM) of proteins can occur in numerous ways. Modification to the sequence can occur through cleavage of a peptide bond by proteases. Post-translational modification can also occur on amino acid side chains, such as through enzyme-catalysed covalent attachment of a functional group to a specific residue. There are five major categories of post-translational modifications to amino acid side chains: phosphorylation, acylation, alkylation, oxidation, and glycosylation (Scheme 1).[4] Among these different post-translational modifications, glycosylation of amino acid side chains in proteins has emerged as the most common category of post-translational modification.

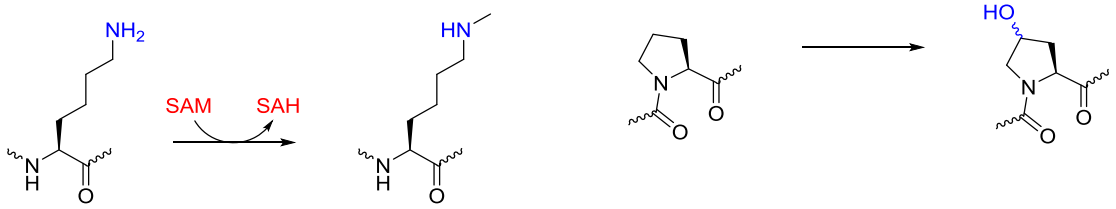
a) Phosphorylation

b) Acylation of



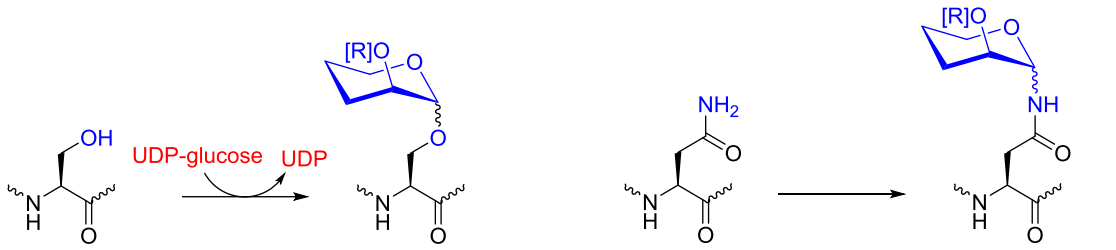
c) Alkylation

d) Oxidation



e) *O*-Glycosylation

f) *N*-Glycosylation



Scheme 1. Major types of covalent modifications of protein side chains

It is estimated that more than 50% of human proteins are post-translationally modified by covalent attachment of carbohydrate components to the side chains of serine (Ser) threonine (Thr) and asparagine (Asn) residues, to form glycoproteins.[5] Modification of proteins by glycosylation leads to higher structural and functional diversity of proteins. Glycoproteins exert a variety of different biological functions such as, hormone activities, immune response, and inflammatory reactions. Also, glycoproteins exhibit pivotal roles in recognition processes, including cell differentiation, cell adhesion and

cell growth.[6-7] In addition, *N*-linked glycoproteins exhibit pivotal roles in quality control of protein synthesis and modulation of the tertiary structure and folding of proteins.[8-9] Changes in glycosylation of proteins lead to a number of serious illnesses including congenital disorders, autoimmune diseases, bacterial and viral invasions and cancer.[10-14] Consequently, disease-linked glycoproteins are important in the drug discovery area, as targets for the development of therapeutic and diagnostic strategies.[15]

1.3 Structures of glycoproteins

There are two major categories of glycoproteins. *N*-Linked glycoproteins have the carbohydrate moieties attached to the amide side chain of asparagine residues.[16] *O*-Linked glycoproteins have carbohydrates linked to the side chain hydroxyl group of serine or threonine residues. In addition, a limited number of unusual glycoproteins exist, including *S*-linked glycoproteins where saccharides are attached to the side chain of cysteine[17] and *C*-linked glycoproteins where saccharides are attached to the indole C-2 of tryptophan.[18]

1.3.1 N-linked glycoproteins

In *N*-linked glycoproteins, the oligosaccharide moieties are post-translationally attached through their reducing end to the amide group of an asparagine residue. In vivo, *N*-glycosylation occurs onto an asparagine residue embedded in the consensus sequence Asn–X–Thr(Ser), where X can be any amino acid except proline.[19] The *N*-linked glycans can be categorised into three main types: high mannose, complex and hybrid (Figure 1).[20] The pentasaccharide structure α-Man(1-6)-α-Man(1-3)-β-Man(1-4)-β-D-GlcNAc(1-4)-β-D-GlcNAc is common to all types with the terminal

N-acetylglucosamine (GlcNAc) moiety attached to the asparagine residue. These three subgroups differ in their antennary structures, with high mannose glycoproteins containing only α-mannosyl residues attached to the core, whereas complex type glycoproteins contain no mannose residue other than those in the core, and hybrid type glycoproteins have the characteristic features of both the high mannose and the complex types.

1.3.2 O-Linked glycoproteins

In *O*-linked glycoproteins, glycans are post-translationally attached to the hydroxyl group of a serine or threonine residue, and infrequently a tyrosine residue.[21-22] In contrast to *N*-glycosylation, *O*-glycosylation of Ser or Thr does not require a consensus sequence for glycan attachment. Also, although *O*-linked glycans do not have a common core structure, most *O*-linked glycans have the initial *N*-acetyl-galactosamine moiety (GalNAc) linked to Ser or Thr *via* an α-linkage. There are at least eight core structures identified (Figure 2). Other *O*-linked glycans have also been observed such as α-fucose and β-GlcNAc.

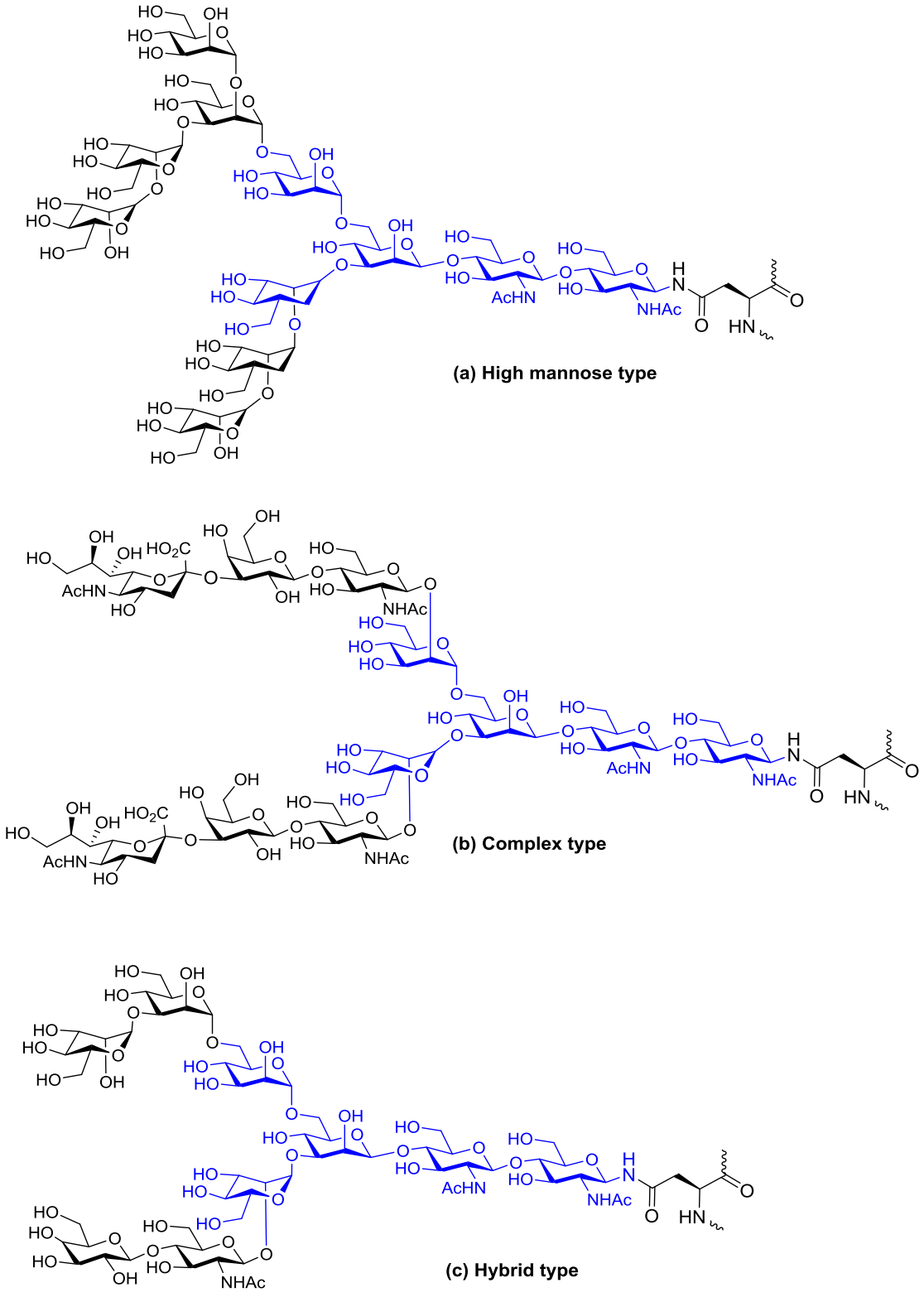


Figure 1. Major types of N-linked glycans

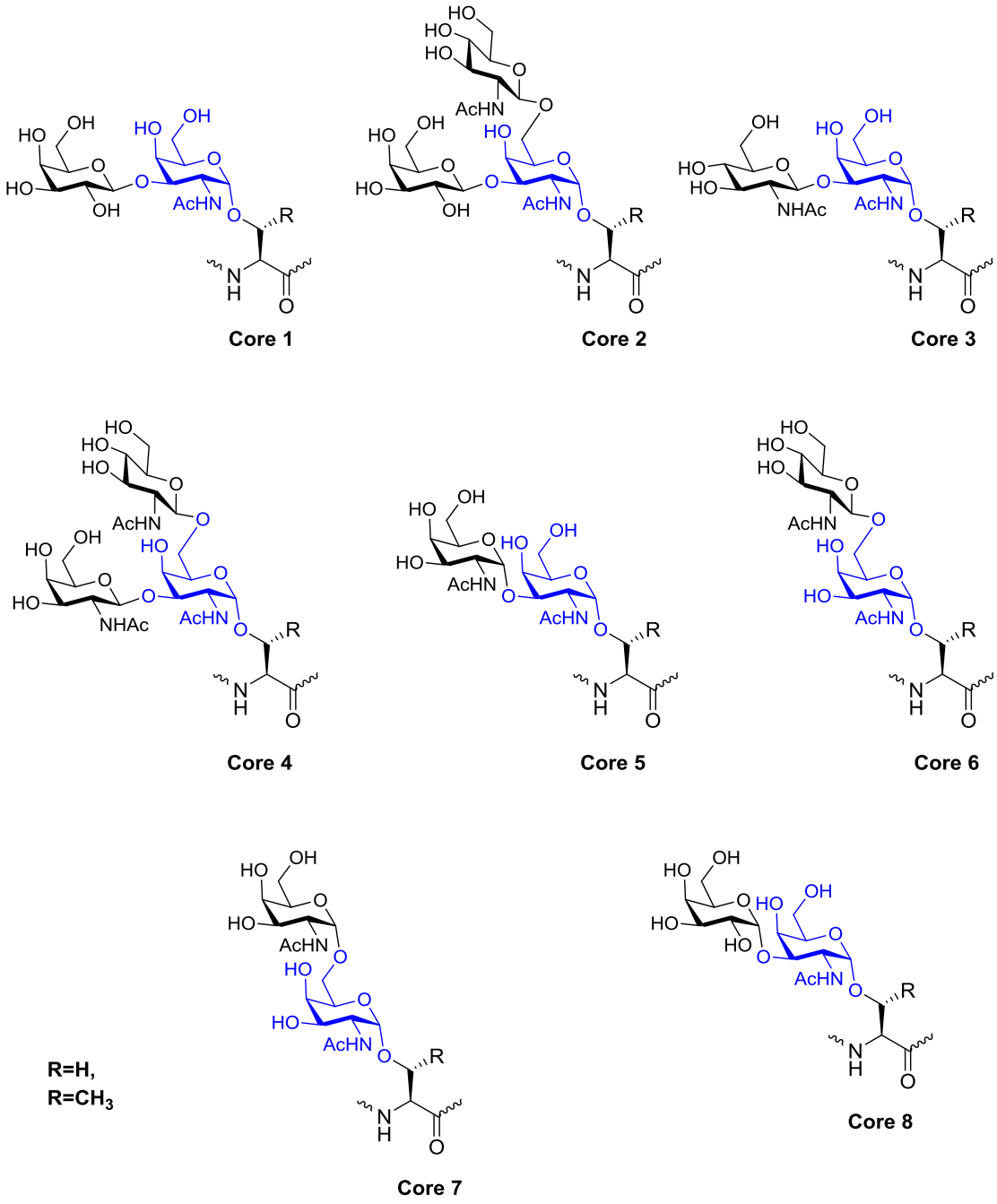


Figure 2. Major types of O-linked glycans

1.4 Homogeneous glycoproteins

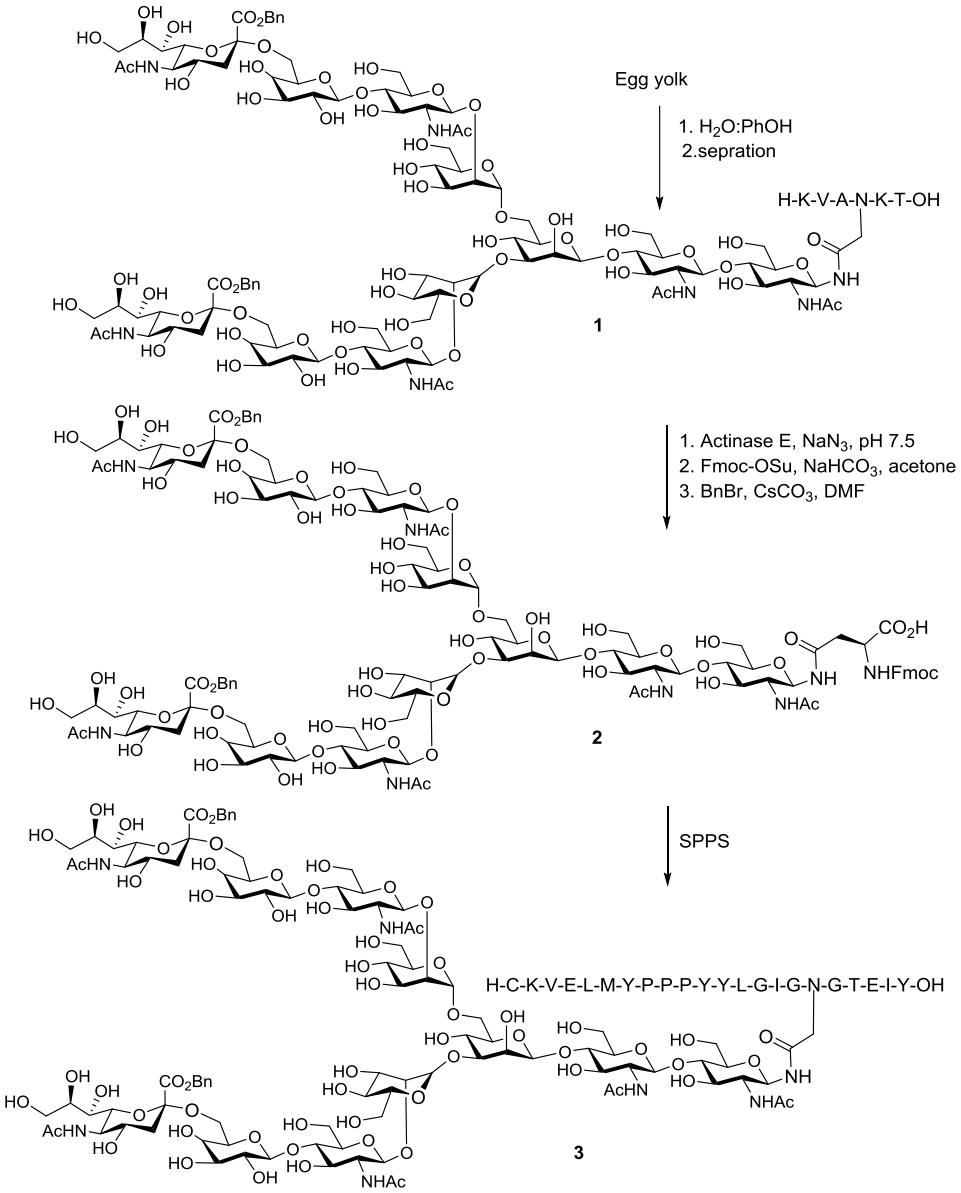
As previously discussed, glycoproteins play vital roles in a wide range of biological activities. However, rigorous investigations of these processes are complicated by obstacles in obtaining homogeneous forms of glycoproteins from natural sources.[23] The heterogeneity of glycoproteins is attributed to the fact that the biosynthesis of the oligosaccharide protein of the glycoprotein is not under direct genetic control, unlike the biosynthesis of the protein component, but rather involves various enzymatic reactions such as glycosidase- and glycosyltransferase-catalysed processes. As a result of these variable enzymatic pathways, glycoproteins are typically produced as mixtures of 'glycoforms', possessing the same protein sequence but differing in the glycan structure. Consequently, the inherent structural complexity and variability of oligosaccharides renders a glycoprotein with various physical and biochemical properties that leads to functional diversity. In order to have a better understanding of the biological roles of glycoproteins, it is imperative to develop alternative strategies towards the construction of homogeneous glycoproteins. Therefore, a wide range of strategies have been developed to enable access to homogenous glycopeptides and glycoproteins, including chemical, chemoenzymatic and biochemical approaches.[24-27]

1.5 Methods of formation of the *N*-glycosidic linkage:

A variety of methods have been developed for formation of the integral glycan–amino acid bond in glycoproteins and glycopeptides. Generation of *N*-glycosylated asparagine motifs involves the coupling of a glycan precursor with an appropriate protected or activated aspartic acid derivative. Numerous synthetic and biosynthetic processes have been developed to furnish *N*-glycosylated asparagines.

1.5.1 Degradation of natural glycoproteins

N-Glycosyl asparagine derivatives can be obtained from natural glycoproteins and glycopeptides through enzymatic degradation. Kajihara and co-workers have applied actinase-mediated transformation of the glycopeptide **1** obtained from egg yolk into Asn-linked building block **2** (Scheme 2). Protection with Fmoc and benzyl groups and subsequent incorporation of **2** into solid phase peptide synthesis gave the glycopeptide CTLA-4 **3**.[28-30]

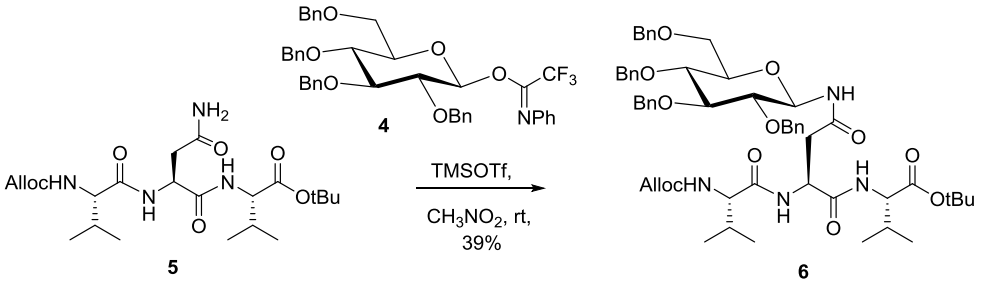


*Scheme2. Synthesis of glycopeptide **3***

1.5.2 Synthesis of *N*-glycosyl asparagines

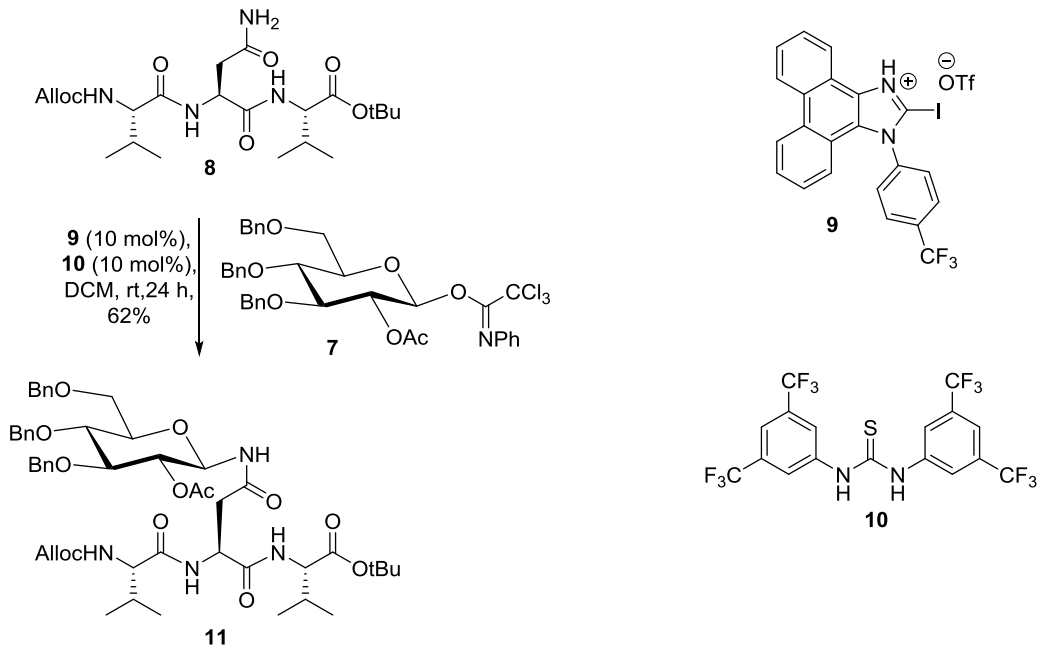
1.5.2.1 *N*-Glycosylation of asparagine

The direct *N*-glycosylation of asparagine is difficult due to the poor nucleophilicity of the nitrogen atom in the carboxyamide group. Several methods have been reported for the direct *N*-glycosylation of asparagine using glycosyl acetimidates as glycosyl donors. Takahashi and co-workers used β-glycosyl-*N*-phenyltrifluoroacetoimidate **4** in the presence of TMSOTf as an activator in nitromethane to generate *N*-glycopeptide **6** in 39% yield (Scheme 39).[31]



*Scheme 3. N-glycosylation of asparagine peptide **6***

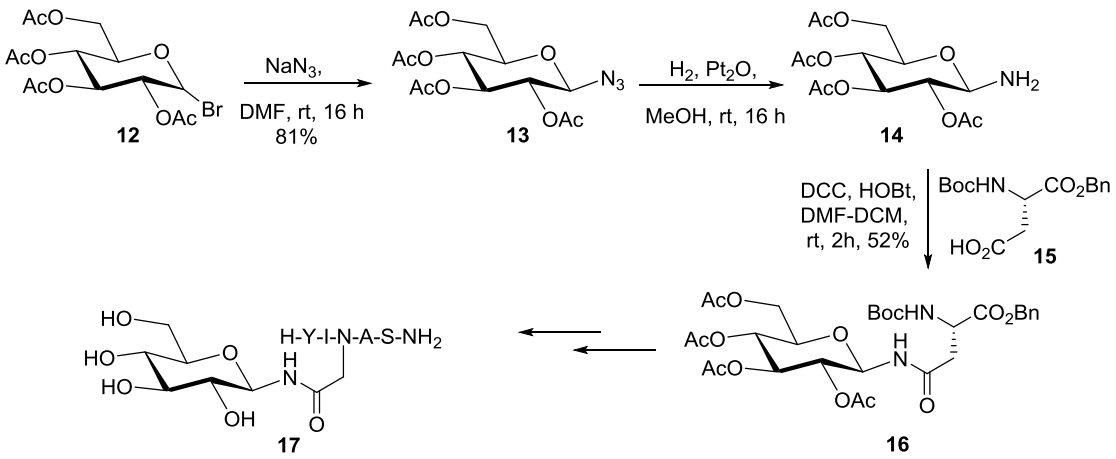
Recently, Takemoto and co-workers have reported a related that method involves coupling of glycosyl trichloroacetimidate **7** and asparagine-containing peptide **8** in the presence of Bronsted acid catalyst **9** and thiourea **10** in DCM (Scheme 4). [32] After 24 h the reaction generated glycopeptide **11** in 62% yield. Both methods suffer from moderate yields and limited scope and generally work only with short peptides.



*Scheme 4. N-glycosylation of asparagine peptide **11***

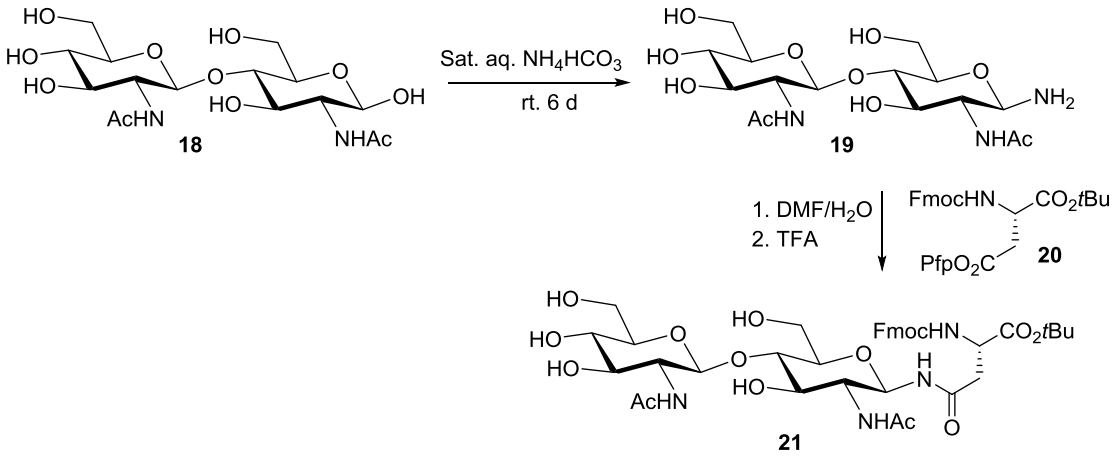
1.5.2.2 Glycosamines

The most common route to *N*-glycosyl asparagines involves coupling of a glycosylamine and an aspartic acid derivative. For example, protected glycosylamine **14** was prepared by converting glycosyl halide **12** to the glycosyl azide **13** followed by reduction (Scheme 5).[33] The resulting glycosylamine **14** was coupled to protected aspartic acid derivative **15** to generate the *N*-linked glucosyl–asparagine building block **16** in good yield. Further elaboration and removal of protecting groups afforded glycopeptide **17**.



*Scheme 5. Synthesis of glycopeptide **17***

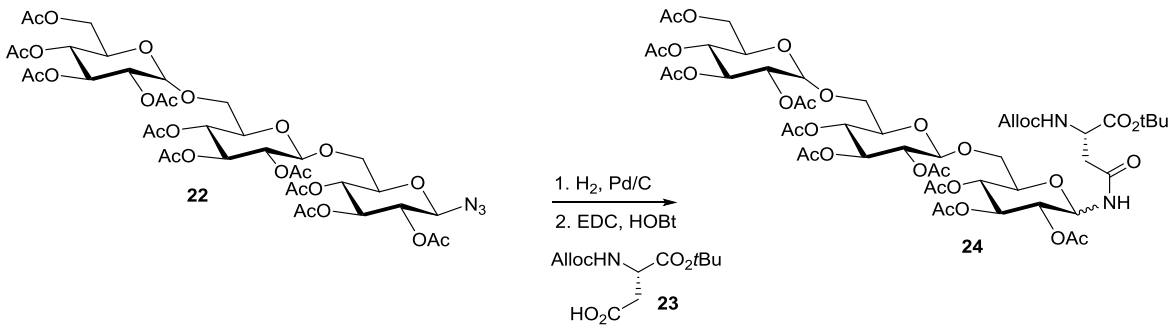
Alternatively, unprotected chitobiosyl amine **19** was obtained by reaction of unprotected chitobiose **18** with ammonium hydrogen carbonate using a method reported by Kochetkov (Scheme 6).[34] Coupling of aspartic acid pentafluorophenyl ester **20** and chitobiosyl amine **19** gave asparagine motif **21**.[35-36]



*Scheme 6. Synthesis of glycosylasparagine **21***

Although these coupling methods have been employed for incorporation of the saccharide onto the asparagine side chain, some disadvantages have also been noted. Glycosylamines are prone to anomerization under reductive conditions. Teshima and co-workers reported that reduction of the α-azide **22** under standard hydrogenation conditions gave an α/β mixture of the glycosylamine (Scheme 7).[37] The resulting

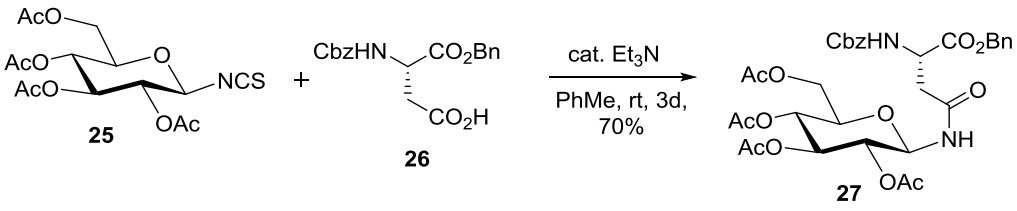
mixture was then coupled with aspartic acid derivative **23** to yield an anomeric mixture of glycopeptide **24**.



*Scheme 7. Synthesis of the anomeric mixture of glycosylasparagine **24***

1.5.2.3 Glycosyl isothiocyanates

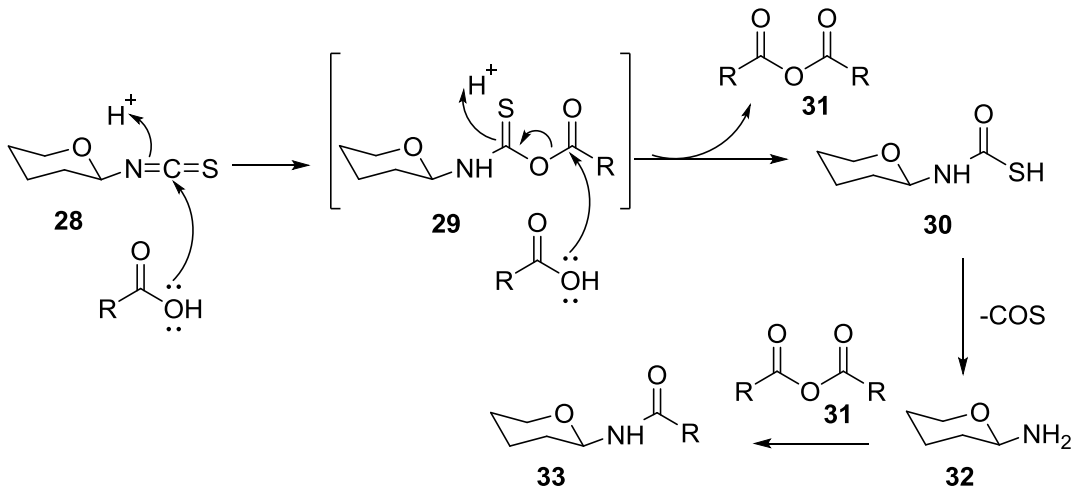
Zurabyan and co-workers used glycosylisothiocyanates to form *N*-glycosidic linkages.[38] In this approach, the isothiocyanate derivative **25** was prepared from the corresponding glucosyl bromide and coupled with the protected aspartic acid **26** to give the *N*-glucosyl asparagine **27** (Scheme 8). However, formation of *N*,*N*-bisglycosyl-thiourea and *N*,*N*-bisglycosylurea was observed.



Scheme 8. Synthesis of glucosylasparagine 27

The proposed mechanism involves carboxylate attack on the isothiocyanate **28** to give the mixed anhydride intermediate **29**, which reacts with another carboxylate to give the glycosyl thiocarbamic acid **30** and symmetrical anhydride **31**. The glycosyl

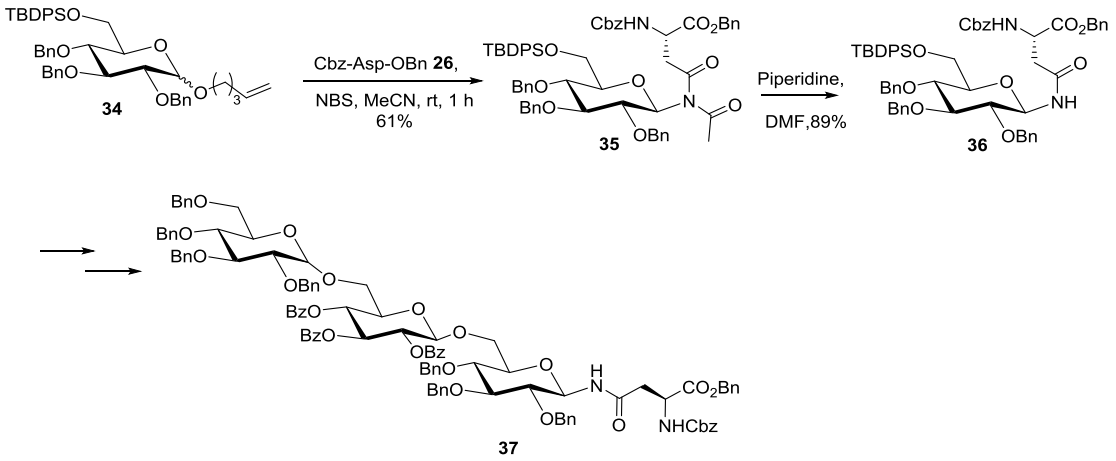
thiocarbamic acid **30** undergoes rearrangement with loss of COS to produce the glycosyl amine **32**, which then reacts with the anhydride **31** to generate the amide **33** (Scheme 9).



Scheme 9. Proposed mechanism for amide formation from isothiocyanate

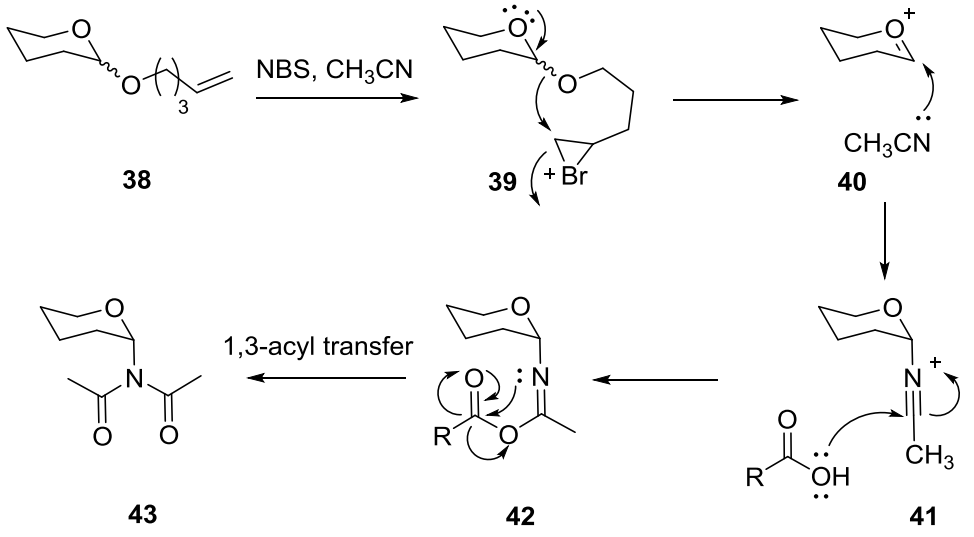
1.5.2.4 Pentenyl glycosides

Fraser-Reid and co-workers introduced pentenyl glycosides as precursors to *N*-α-linked glycosides.[39-40] This method was employed for the preparation of the nephritogenoside core structure **37** (Scheme 10). Coupling of pentenyl glycoside **34** with Cbz-Asp-OBn **26** in acetonitrile gave the protected glycosylated asparagine imide adduct **35** in 61% yield. Removal of the Ac group by treatment with piperidine in DMF furnished **36**, which after elaboration gave the asparagine-linked core trisaccharide **37**.



*Scheme 10. Synthesis of N-glycan **37** from O-pentenyl sugar **34***

This process is proposed to proceed through generation of oxonium ion **40** upon treatment of either anomer of pentenyl glycoside **38** with *N*-bromosuccinimide (NBS) in acetonitrile (Scheme 11). Trapping of intermediate **40** with acetonitrile generates an α-nitrilium ion **41**, which undergoes attack by a carboxylate resulting in generation of a reactive isoimide (or imino anhydride) **42**, which then undergoes 1,3-acyl transfer to afford imide **43**.

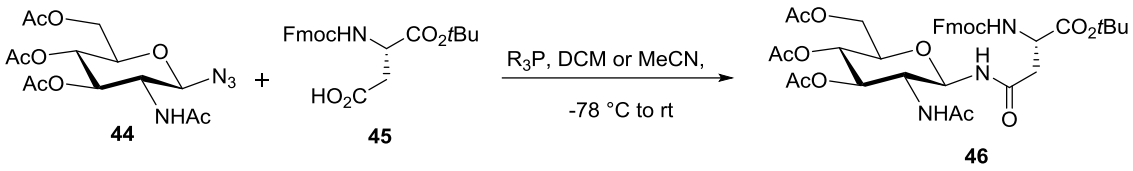


Scheme 11. Proposed mechanism of the Fraser-Reid process

In 1993, Handlon and Fraser-Reid extended their methodology to synthesise GlcNAcβ-Asp motifs, invoking neighbouring group participation.[41]

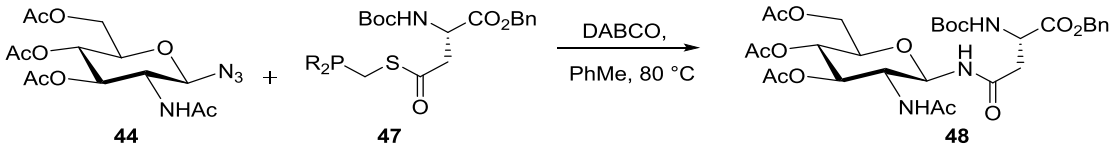
1.5.2.5 Glycosyl azides

Inazu and co-workers described a Staudinger reaction route to construct *N*-Asn linked glycopeptides.[42] Treatment of glycosyl azide **44** with aspartic acid derivative **45** in the presence of trialkylphosphines gave β-Asn *N*-glycoside **46** (Scheme 12).



*Scheme 12. Staudinger of the N-β-linked glycoside **46***

In 2004, a related Staudinger reaction was reported by Kiessling and co-workers for the synthesis of glycosyl amides.[43] Reaction of asparagine-derived phosphinothioester **47** with glycosyl azide **44** gave the *N*-glycosyl asparagine **48** in good yield and stereoselectivity (Scheme 13).



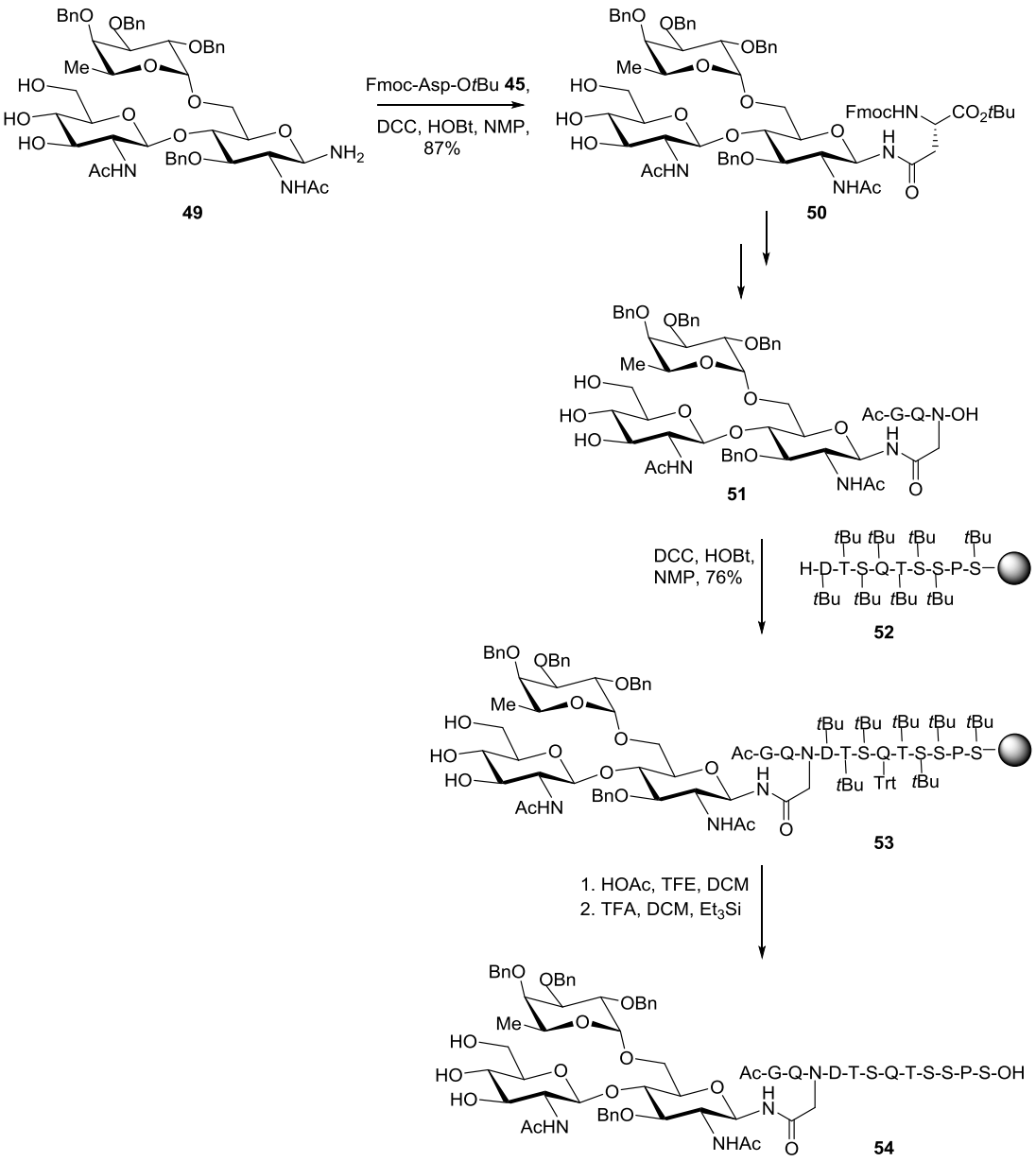
*Scheme 13. Synthesis of The N-β-linked glycoside **48***

With several methods available for the preparation of *N*-glycosyl asparagine derivatives, incorporation of this functionalised amino acid residue into the synthesis of glycopeptide or glycoproteins is required.

1.6 Assembly strategies for glycopeptide synthesis

1.6.1 Linear assembly

The linear assembly of glycopeptides involves generation of the *N*-glycosylated-Asn building block by any of the methods described above, followed by incorporation into solid-phase peptide synthesis (SPPS) to provide the glycopeptide. An example of a linear assembly strategy is the solid-phase synthesis of CD52 glycopeptide **54** containing an *N*-linked core trisaccharide (Scheme 14).[44] The synthesis started with the preparation of the glycosyl amino acid building block **50**. Coupling of aminotrisaccharide **49** with the aspartic acid side chain carboxylate of **45** using DCC and HOBt gave the *N*-glycan **50** in 87% yield. The resulting glycosyl amino acid **50** was then elaborated to the glycopeptide **51** using Fmoc SPPS. Coupling of glycopeptide **51** with nonapeptide **52** on chlorotrityl resin gave the glycopeptide **53**. Cleavage from the resin and removal of the side chain protecting groups furnished the target molecule **54**.

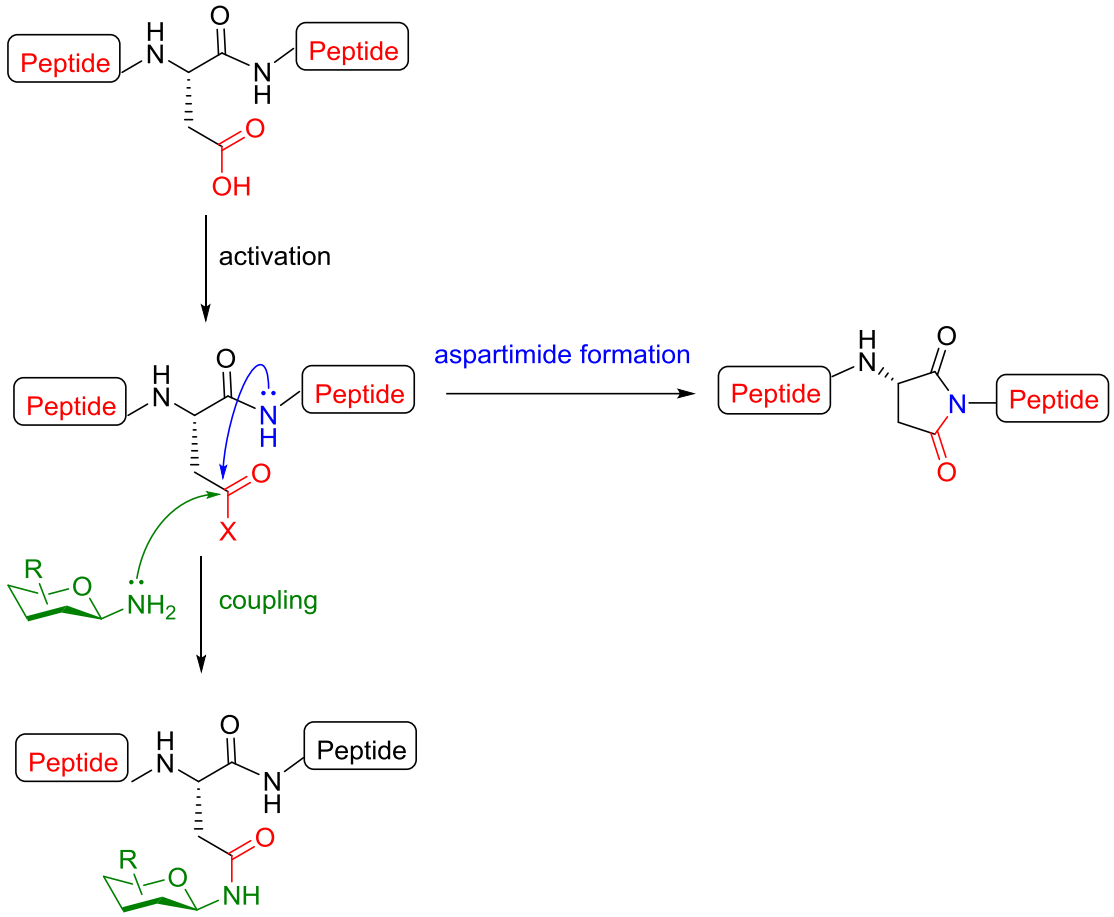


*Scheme 14. Synthesis of a CD-52 glycopeptide **54***

1.6.2 Convergent assembly

A convergent assembly strategy involves the late-stage coupling of a carbohydrate moiety with the side chain carboxyl group of an aspartic acid residue on a partially protected polypeptide to produce a full-length glycopeptide. Lansbury and co-workers devised this method to synthesise *N*-glycopeptides in a convergent approach.[45-46] A major obstacle that limits utility of this approach is the intramolecular reaction to form

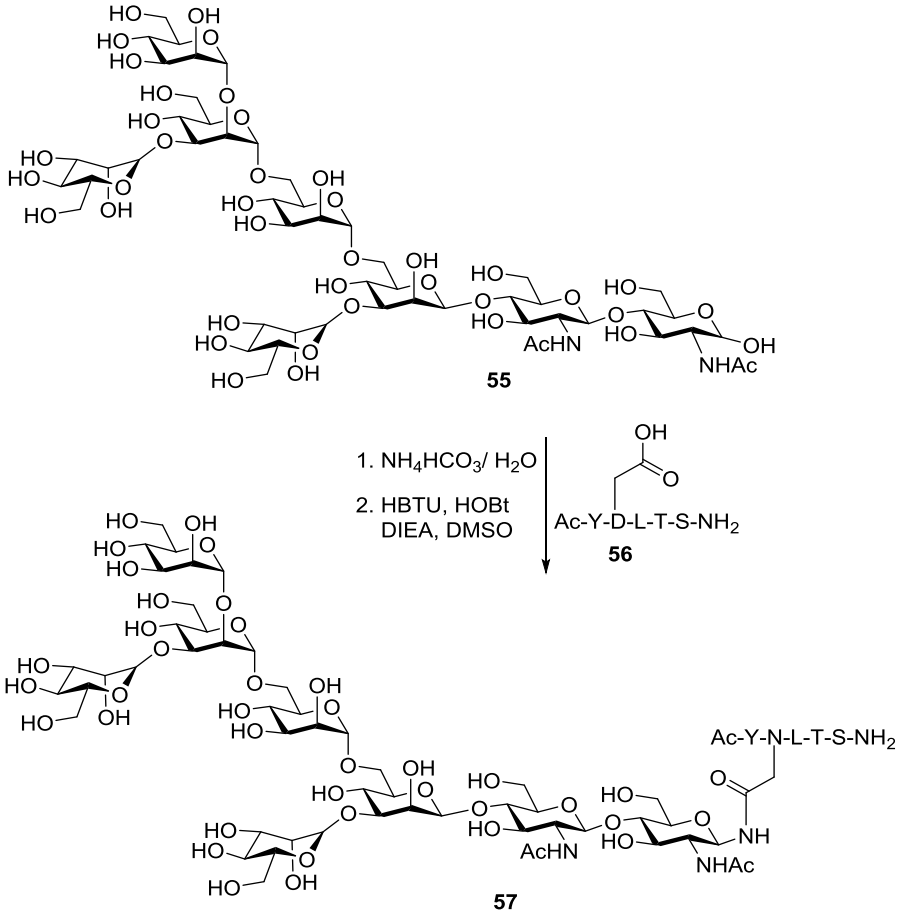
aspartimides during the convergent coupling (Scheme 15). After extensive optimisation, specific conditions such as using unprotected glycosyl amines, minimising the use of base in the reaction and using DMSO as a solvent, were found to minimise the formation of aspartimide.



Scheme 15. Convergent synthesis of N-glycopeptides

The convergent method was applied to the synthesis of the complex glycopeptide **57** containing an heptaoligosaccharide (Scheme 16). The oligosaccharide **55** was converted to the β-glycosylamine by extended treatment with saturated aqueous NH_4HCO_3 using the method developed by Kochetkov.[20] The resulting product was then

coupled with the pentapeptide fragment **56** to give the target glycopeptide **57** in 55% yield.

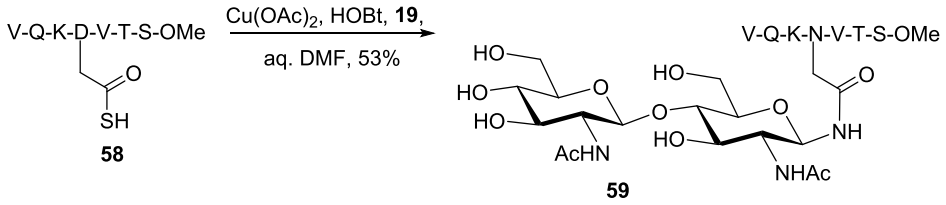


*Scheme 16. Lansbury's approach for convergent synthesis of N-glycopeptide **57***

1.6.3 The use of thioacids

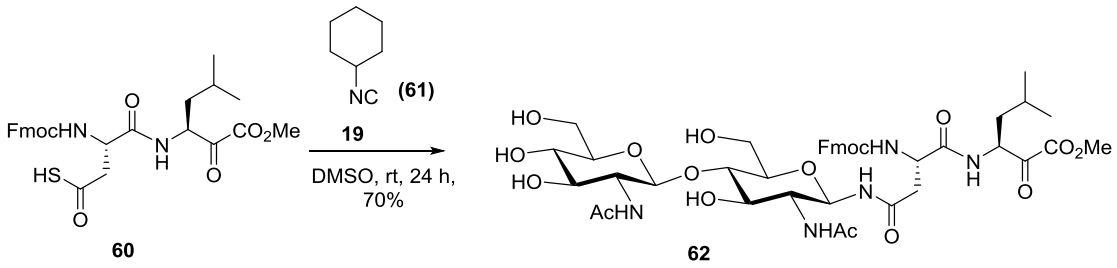
The use of aspartate β-thioacids has been investigated in novel amide bond forming reactions to generate glycopeptides and glycoproteins. Garner and co-workers described a Cu(II)-promoted coupling of glycosylamines with aspartyl-thioacid peptides to generate *N*-glycosylated peptides.[47] The peptide **58**, containing a thioacid on the Asp side chain, was treated with the unprotected disaccharide amine **19** in the presence of $Cu(OAc)_2$ and HOBt to give the glycopeptide **59** in 53% yield (Scheme

17). The disadvantages of this method include the requirement of an excess glycosylamine and in most cases considerable recovery of hydrolysed thioacid is observed.



*Scheme 17. Garner's synthesis of glycopeptide **59***

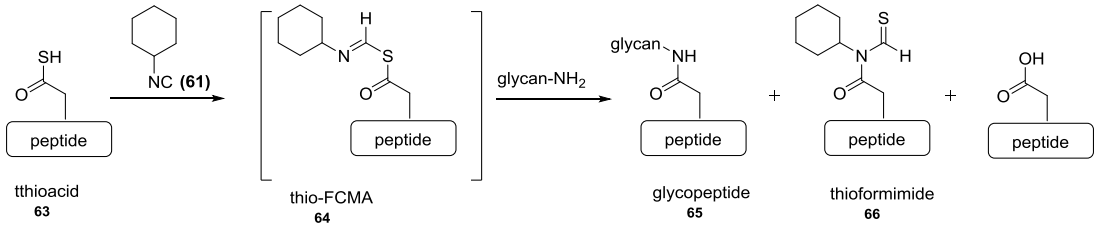
Danishefsky and co-workers have shown that the reaction between thioacid **60** and isonitriles **61** in the presence of a glycosyl amine **19** gives the *N*-glyco-asparagine product **62** under mild conditions (Scheme 18).[48]



*Scheme 18. Convergent synthesis of N-glycopeptide **62***

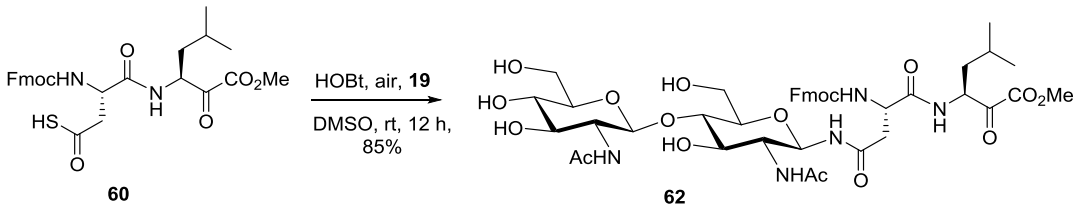
It is proposed that the reaction proceeds through formation of a 'thioformimidate carboxylate mixed anhydride' (thio FCMA) intermediate **64** (Scheme 19).[49-50] In the presence of a glycosyl amine, bimolecular acylation generates an amide product **65**, together with a variety of byproducts, including the aspartate thioformimide **66** and aspartimide systems. Further, this method suffers additional limitations, such as the

requirement of excess of thioacid and difficulties in generating more complex glycopeptides in good yields.



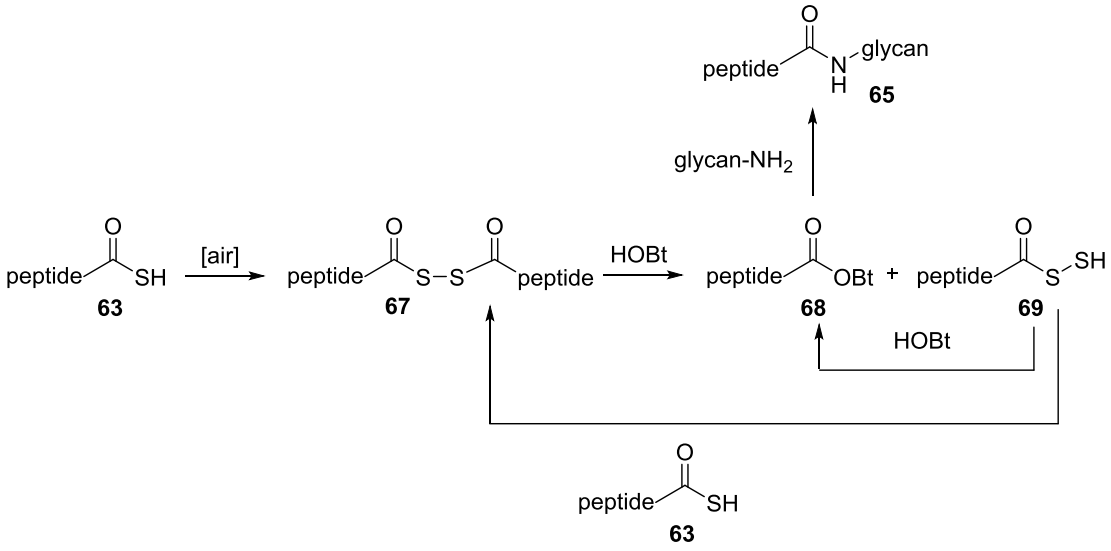
Scheme 19. Reaction between thioacids and isonitrile in the presence of amine

A variation of the reaction is the treatment of peptide thioacid **60** and glycosyl amine **19** in the presence of HOBt under oxidising conditions to generate glycopeptide **62** (Scheme 20).



*Scheme 20. Reaction between thioacid **60** and amine **19** in the presence of HOBt*

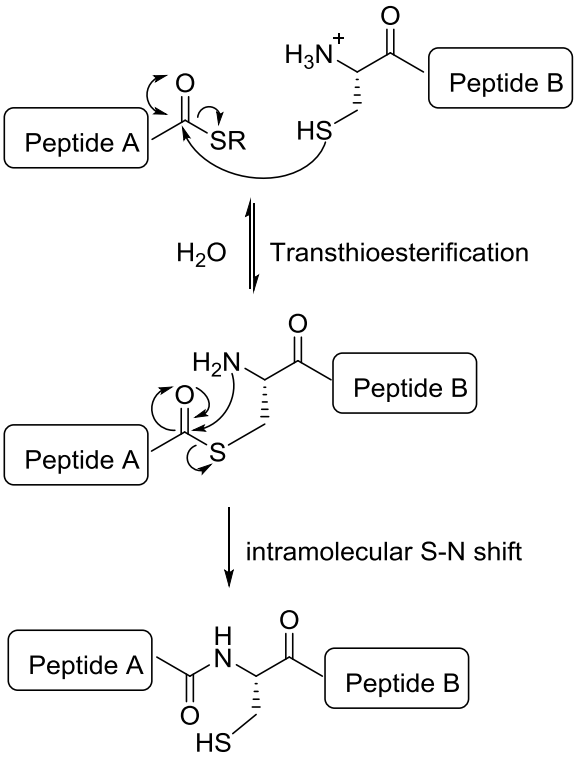
The proposed mechanism proceeds through conversion of the thioacid **63** to intermediate **67** upon exposure to air (Scheme 21). The intermediate **67** reacts with HOBt to give activated ester **68**, along with **69** (which can react with HOBt to give further **68**). Alternatively, intermediate **69** can react with thioacid **63** to give **67**. Activated ester **68** then reacts with a glycosylamine nucleophile to give the desired amide **65**.



Scheme 21. Proposed mechanism of HOBt-mediated N-glycopeptide formation

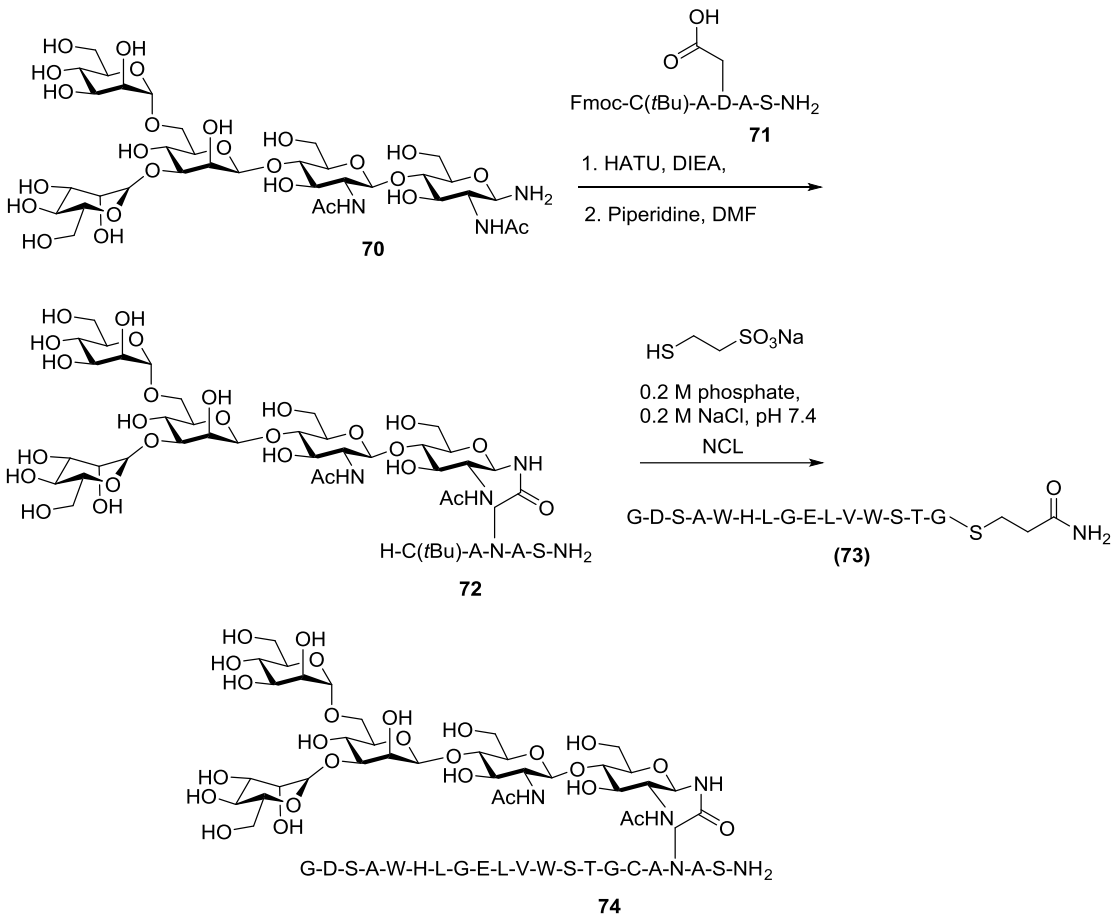
1.6.4 Native chemical ligation

Native chemical ligation (NCL) was developed by the Kent group for the synthesis of native peptides.[51] NCL involves a chemoselective reaction of a peptide containing an *N*-terminal cysteine residue with a peptide containing a *C*-terminal thioester, to form a native amide bond at the ligation site. Native chemical ligation proceeds via transthioesterfication followed by a spontaneous intramolecular *S*→*N* acyl shift to form the amide bond (Scheme 22).



Scheme 22. Mechanism of native chemical ligation

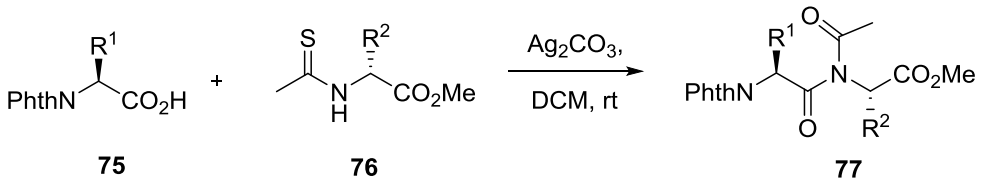
Native chemical ligation has been applied in the synthesis of a range of glycopeptides, as it can be performed in water using unprotected peptides fragments at neutral pH. Danishefsky and co-workers described the synthesis of glycopeptide **74** (Scheme 23).[52] The core unprotected oligosaccharide amine **70** was coupled with pentapeptide **71** bearing a free aspartyl residue to give the glycopeptide **72**. The thioester peptide **72** was linked to glycopeptide **72** through NCL to furnish the glycopeptide **74**. One obvious limitation is the requirement of peptides with only one aspartic acid residue or with other aspartate residues differentially protected.



*Scheme 23. Danishefsky's synthesis of N-linked glycopeptide **74***

1.7 Hutton group's thioamide-based approach towards amide bond formation

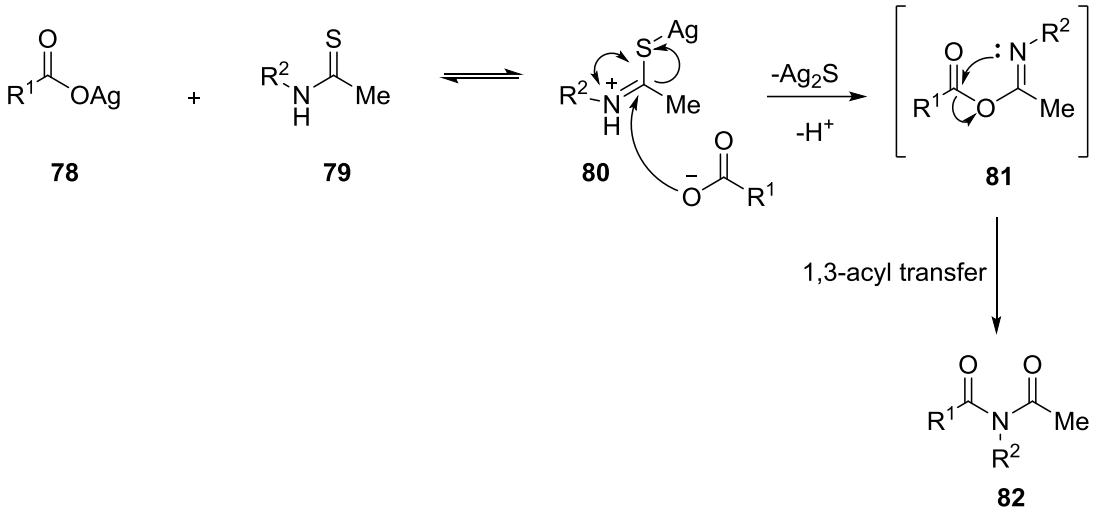
Recent studies in our group have described a Ag(I)-promoted coupling of *N*-phthaloyl-amino acids **75** with thioamide-appended amino acid derivatives **76** to produce dipeptide imides **77** in good yield (Scheme 24).[53]



Scheme 24. Synthesis of dipeptide imides

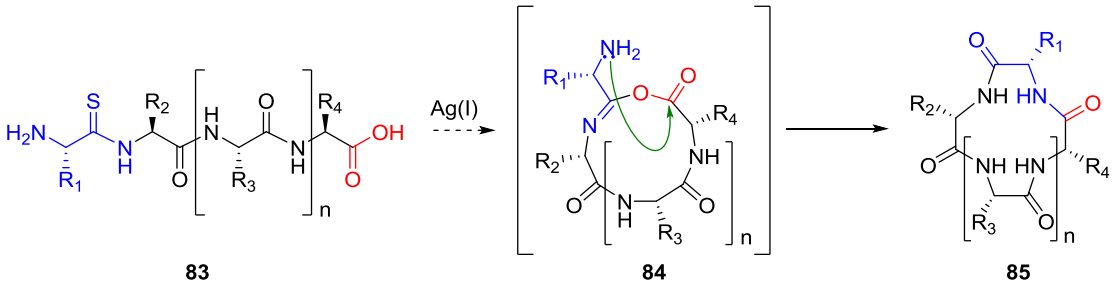
The proposed mechanism involves attack of the thioamide–Ag complex **80** by the carboxylate group to generate a reactive isoimide intermediate **81**, which undergoes

1,3-acyl transfer to afford the corresponding imide **82** (Scheme 25).[54] The amide can be generated by selective imide hydrolysis.



Scheme 25. Proposed mechanism for the formation of peptide imides from thioamides

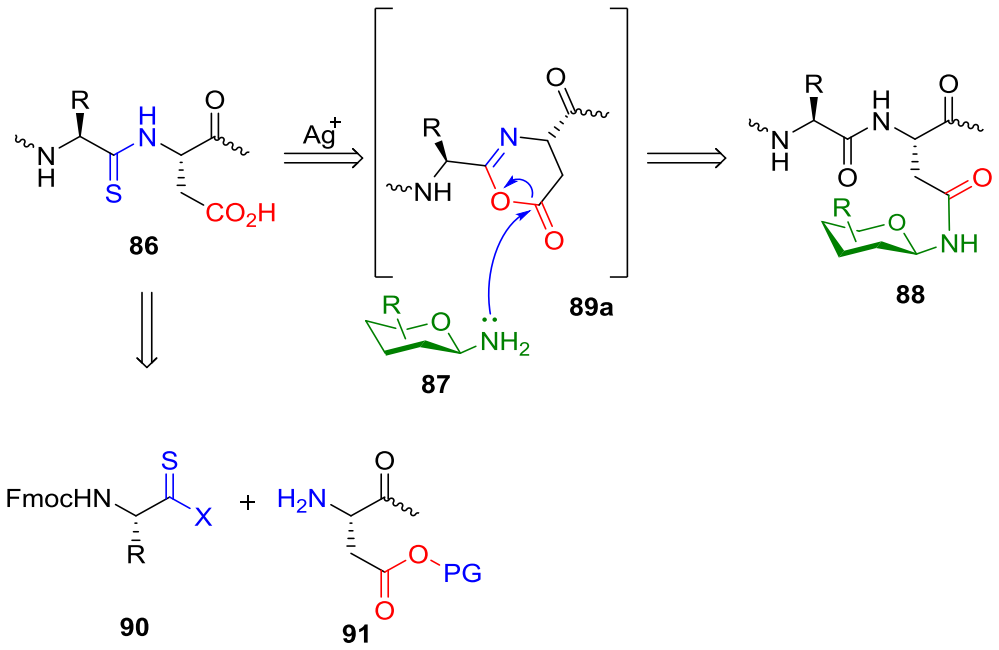
Further studies in the Hutton group have exploited reactions of isoimides in cyclic peptide synthesis (Scheme 26).[55] The method employs a peptide **83** possessing a thioamide at the *N*-terminus. In this process, Ag(I)-promoted intramolecular reaction of the *C*-terminal carboxylate on the thioamide group generates a cyclic isoimide intermediate **84**, which is trapped by the adjacent *N*-terminal amine to generate an amide bond and form the cyclic peptide **85**.



Scheme 26. Proposed mechanism of head-to-tail cyclization

1.8 Aim of the project

The aim of the project is to investigate the application of the Ag(I)-promoted coupling to the chemoselective chemical ligation of a glycosyl amine **87** and an aspartic acid containing peptide thioamide **86** to provide *N*-glycosylated asparagine motifs **88** (Scheme 27). In this approach, it has been proposed that an intramolecular attack of the aspartic acid side chain carboxylate on the backbone thioamide group should afford an intermediate isoimide **89a**. Trapping isoimide intermediate **80** by an external amine should lead to the formation of an amide bond and generate an *N*-alkyl asparagine derivative and also convert the thioamide to an amide. Peptide **86** containing a thioamide at specific site could be assembled using Fmoc-based solid phase peptide synthesis (SPPS) methodology, using thioacylation reagents **90** to incorporate a thioamide unit into a peptide chain.[56] An aspartic acid side-chain protecting group is required that is cleavable under mild conditions that do not affect the protecting groups present on the amino acids side chains. Moreover, this strategy could be used in the site-specific formation of *N*-glycosylated peptide and proteins, where chemoselectivity is directed by the appropriate positioning of the thioamide group adjacent to the aspartic acid to undergo functionalisation. Further, aspartimide formation should be minimised as a conformationally flexible activated aspartate (such as in Scheme 15) is never generated.

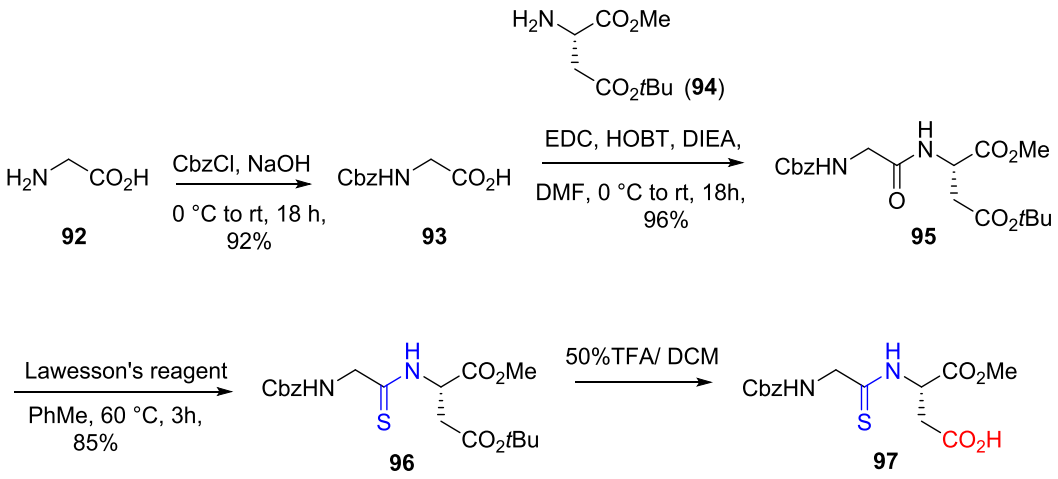


Scheme 27. Proposed method for Ag(I)-promoted N-glycosyl asparagine synthesis from peptide thioamides

2. Results and Discussion: N-Glycosylation of peptides using thioamide-directed coupling reactions

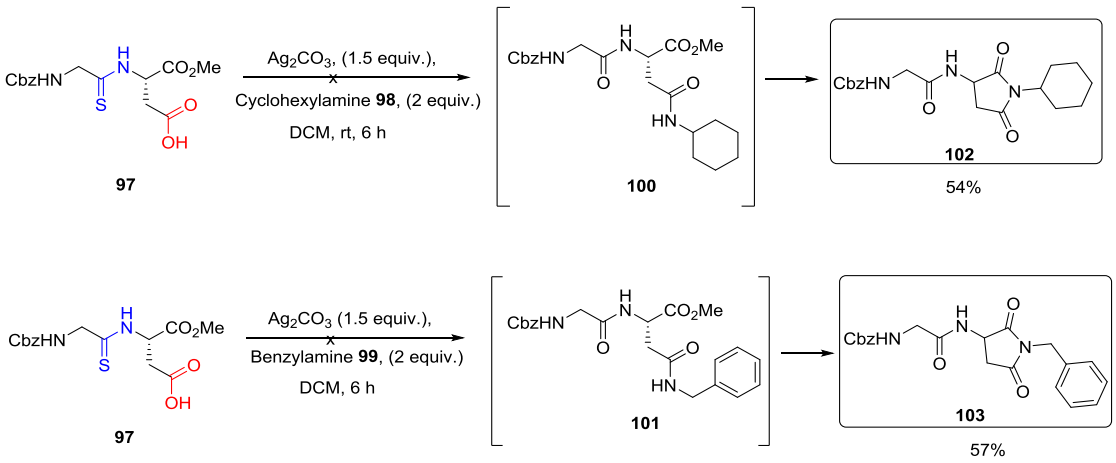
2.1 Synthesis of dipeptide thioamide 97

To test the feasibility of the Ag(I)-promoted coupling of aspartyl thioamides and amines, it was decided to model the reaction with dipeptide thioamide **97** and amines **89** and **90**. The synthesis of the required dipeptide thioamide **97** was initially performed (Scheme 28). Treatment of glycine **92** with CbzCl under basic conditions gave the Cbz-protected amino acid **93**. Coupling of the protected glycine **93** with commercially available H-Asp(O*t*Bu)-OMe **94** afforded the protected dipeptide **95** in excellent yield. Treatment of dipeptide **95** with Lawesson's reagent in toluene at 60 °C gave the dipeptide thioamide **96** in 85% yield. The products **96** was analysed by NMR spectroscopy. The ^{1}H NMR spectrum of the thioamide **96** exhibits a one proton apparent singlet at δ 8.79 ppm corresponding to the thioamide NH group. The mass spectrum contains a pseudo-molecular ion peak at *m/z* 411.1584 corresponding to the molecular formula of the expected product **96**. The *tert*-butyl ester **96** was then removed by treatment with 50% TFA in DCM to give the free carboxylic acid **97**, which was used in the next step without purification.



*Scheme 28. Synthesis of dipeptide thioamide **97***

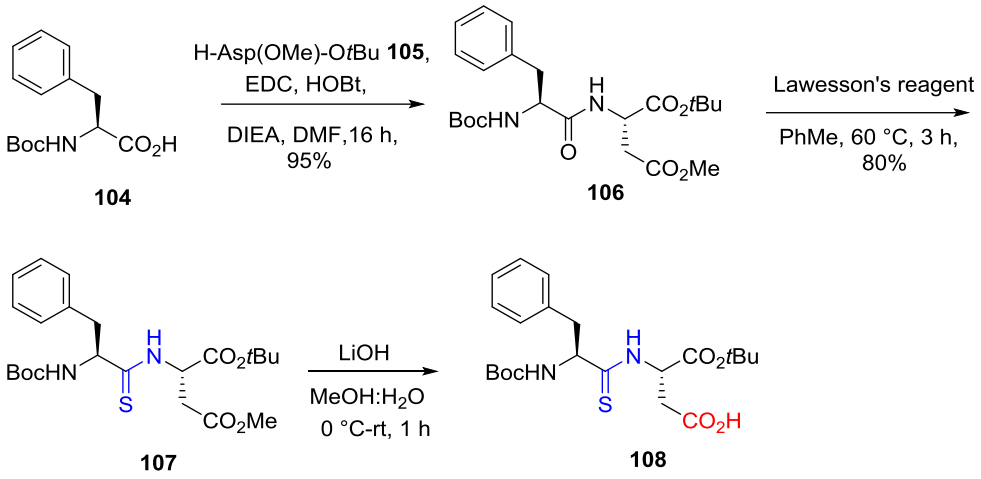
With dipeptide thioamide **97** in hand, the Ag(I)-promoted coupling reactions of dipeptide thioamide **88** with amines **98** and **99** were investigated (Scheme 29). Thioamide **97** was treated with Ag_2CO_3 (1.5 equiv.) for five minutes, then cyclohexylamine **98** or benzylamine **99** (2.0 equiv.) were added at room temperature. Analysis of the reaction mixtures by mass spectrometry showed peaks at *m/z* 420.2 and *m/z* 428.15, corresponding to the molecular formula of the expected products **100** and **101**, respectively. The mass spectra also displayed peaks at *m/z* 388.18 and at *m/z* 396.15 corresponding to the succinimide side products **102** and **103**, respectively. However, following purification by column chromatography only the succinimide products **102** and **103** were isolated. The ^{1}H NMR spectra indicated the absence of the methyl ester peaks at δ ~3.5 in each case. Presumably, the aspartic acid derivative is converted to the N-alkyl asparagine derivatives **100** and **101**, but these are not stable and undergo intramolecular attack of the amide nitrogen at the methyl ester group to form the succinimides **102** and **103**.



*Scheme 29. Attempts to synthesis of amides **100** and **101***

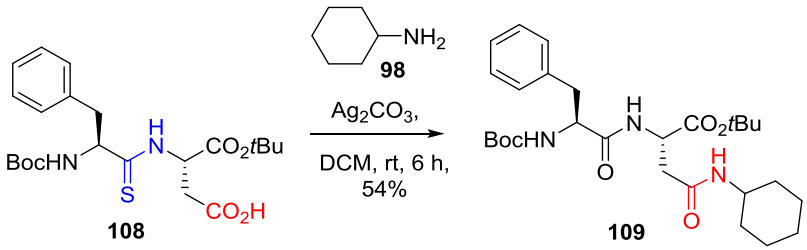
With the asparaginyl esters being prone to succinimide formation, we chose to switch the methyl ester group to a more robust t*ert*-butyl ester protecting group. Therefore, the synthesis of the thioamide derivative **108** was required. Thioamide **107** was prepared from

Boc-phenylalanine **104** over two steps (Scheme 30), employing a similar procedure to that used for preparation of thioamide **96**. Hydrolysis of the side chain methyl ester of **107** under basic conditions provided the required dipeptide thioamide adduct **108**.



*Scheme 30. Synthesis of thioamide **108***

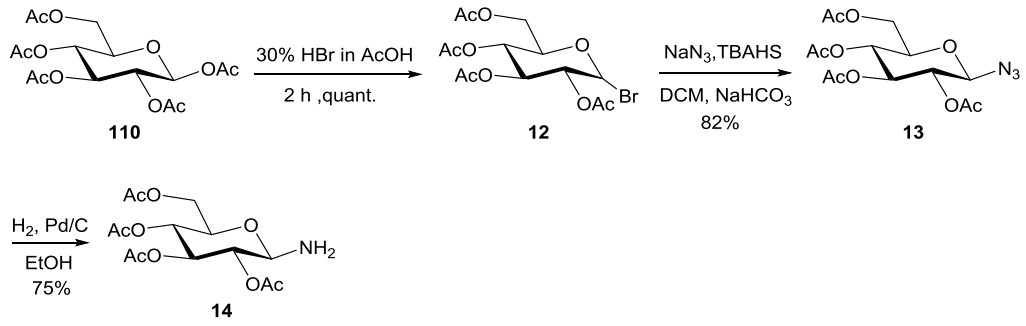
With thioamide **108** in hand, coupling with cyclohexylamine **98** was then investigated (Scheme 31). Thioamide **108** was treated with 1.5 equiv. of Ag_2CO_3 and 2.0 equiv. of cyclohexylamine **98** to give the asparagine derivative **109** in 54% isolated yield. No succinimide byproduct was observed, validating the choice of a more hindered ester protecting group.



*Scheme 31. Synthesis of amide **109***

2.2 Synthesis of the *N*-glycosylated asparagine 111

Given the successful conversion of aspartic acid-containing thioamide **108** to the *N*-cyclohexyl asparagine derivative **109**, we turned to the application of the Ag(I)-promoted coupling in the generation of an *N*-glycosylated asparagine motif. Glucosylamine **14** was chosen as the aminosugar in order to generate an *N*-glucosyl asparagine. Glucosylamine **14** was prepared from the commercially available D-glucose pentaacetate **110** in three steps according to a literature procedure (Scheme 32).[57] D-glucose pentaacetate **101** was treated with 30% HBr in AcOH to provide glucosyl bromide **12**, followed by treatment with sodium azide to give the glucosyl azide derivative **13** in 82% yield. Reduction of the azide **13** using H_2 with Pd/C in ethanol gave the glucosylamine **14** in a good yield.



*Scheme 32. Synthesis of glycosylamine **14***

With the glucosylamine **14** in hand, coupling with thioamide **108** in the presence of Ag_2CO_3 was undertaken. Thioamide **108** was treated with varying amounts of Ag_2CO_3 and aminosugar **14** (Table 1). The *N*-glycosylated asparagine motif **111** was isolated in 32–77% yields, with the amide **112** typically obtained as a minor byproduct. The use of 1.0 equiv. of aminosugar **14** and Ag_2CO_3 generated the amide **111** in 40% yield (entry 1). Use of 2.0 equiv. or 3.0 equiv. of Ag_2CO_3 generated the amide **111** in 51% and 32% yields, respectively (entries 2 and 3). Use of 1.5 equiv. of Ag_2CO_3 generated

the asparagine derivative **111** in 54% yield (entry 4). Reducing the amount of Ag_2CO_3 to 1.2 equiv. gave the glycosylated asparagine **111** in 60% isolated yield (entry 5). Entries 6-7 indicated that an excess of aminosugar **14** is necessary to achieve good yields of the *N*-glycosylated asparagine **111**. Also the use of MeCN instead of DCM as a solvent gave similar results (entry 9). Using a mixture of DCM and MeCN (1:1) led to an improved yield of the asparagine motif **111** (entry 10).

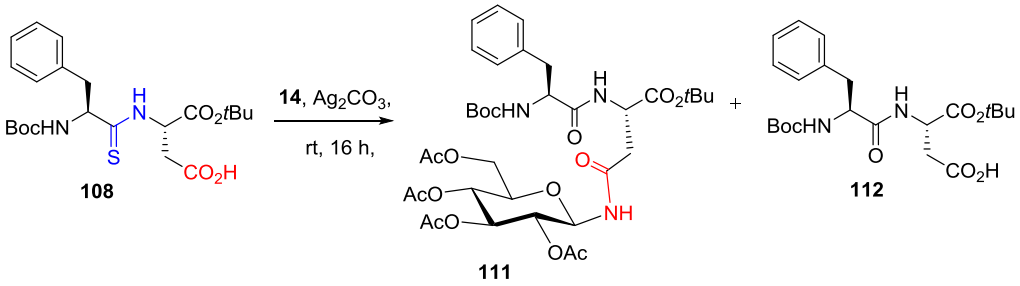
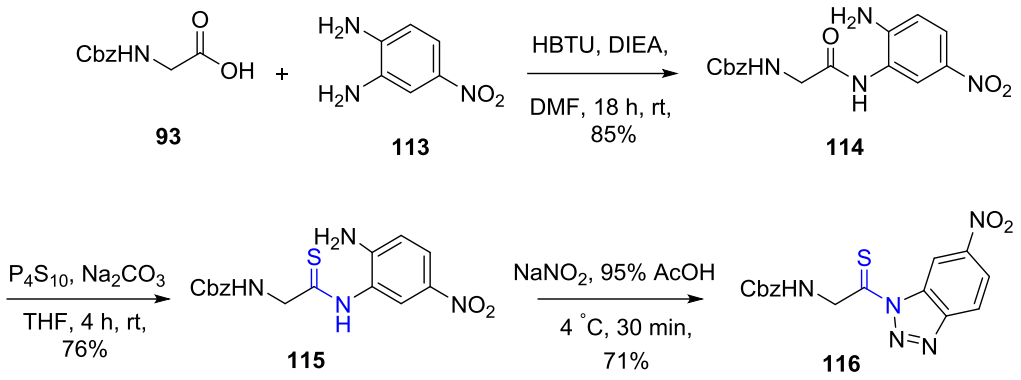


*Table 1. Synthesis of N-glycosylated asaparagine **111***

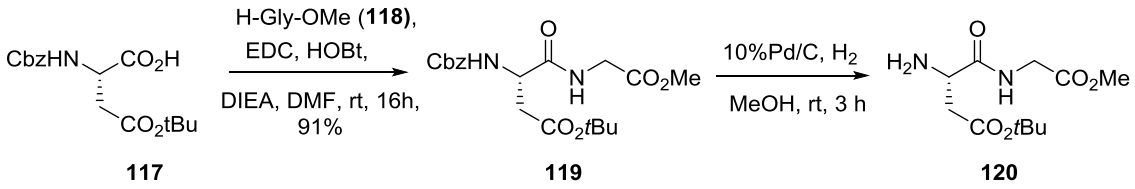
Entry	Equiv. of Ag_2CO_3	Equiv. of **14**	Time	Solvent	Yield% **111**
1	1	2	6 h	DCM	40
2	2	2	6 h	DCM	51
3	3	2	6 h	DCM	32
4	1.5	2	6 h	DCM	54
5	1.2	2	6 h	DCM	60
6	1.2	3	6 h	DCM	68
7	1.2	4	6 h	DCM	74
8	1.2	5	6 h	DCM	70
9	1.2	4	6h	MeCN	73
10	1.2	4	6 h	DCM : MeCN	77

With the generation of the *N*-linked asparagine motifs **109** and **111** optimised, we next investigated the scope of the Ag(I)-promoted coupling of amines with extended thioamide-containing aspartyl peptides. Incorporation of the thioamide linkage into a peptide at specific site cannot be achieved using common thionation reagents such as Lawesson's reagent, as reaction with multiple amide groups would occur to generate a mixture of peptide thioamides. Several methods have been reported towards site-specific incorporation of a thioamide functionality into a peptide chain, including activation of thioacids with phosphonium compounds such as PyBOP[58] or BOPCl[59] or using thioacylating agents such as thioacyl-benzimidazolinones.[60-61] However, these methods are plagued by low yields and racemisation of the thionated residue. Rapoport and co-workers developed an efficient route, employing thioacyl 4-nitrobenzotriazolide of amino acid derivatives as thioacylating agents, which results in higher yields and lower epimerisation levels compared with the previous methods.[62] Thus, we envisaged the application of Rapoport's procedure could be used to introduce thioamide linkage at a specific site of a peptide chain. The glycine thioacyl 4-nitrobenzotriazolide agent **116** was synthesised according to the reported method (Scheme 33). Treatment of *N*-Cbz-glycine **93** with 4-nitro-*O*-phenylenediamine **113** in the presence of HBTU generated Cbz-Gly-nitroanilide **114** in 85% yield. Thionation of anilide **114** with P_4S_{10} in THF gave thioamide **115** in 76% yield. Diazonium formation and cyclization of **115** with sodium nitrite in acetic acid gave benzotriazole **116** in 71% yield. The thioacylation reagent **116** was used directly without further purification due to its instability upon long-term storage.



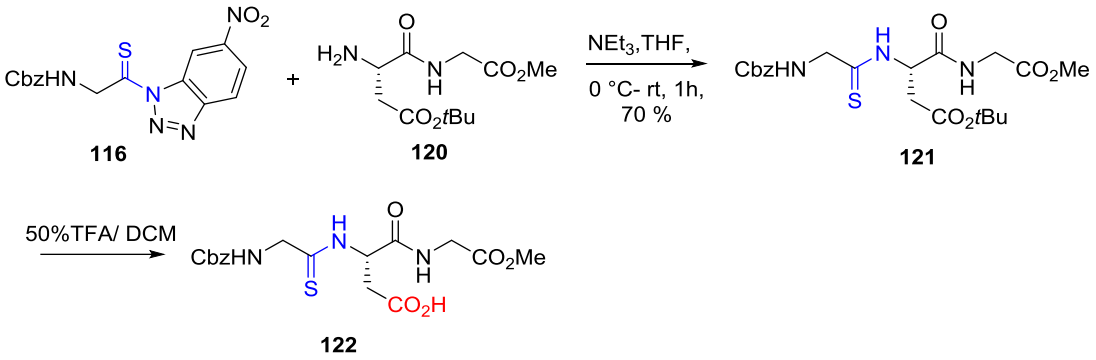
*Scheme 33. Synthesis of thioacyl benzotriazole reagent **116***

We next sought to synthesise the aspartyl–glycine dipeptide **120**. Dipeptide **119** was prepared by standard amino acid coupling procedures using EDC and HOBt in DMF (Scheme 34). Removal of the Cbz protecting group under hydrogenolysis conditions gave the free amine **120**.



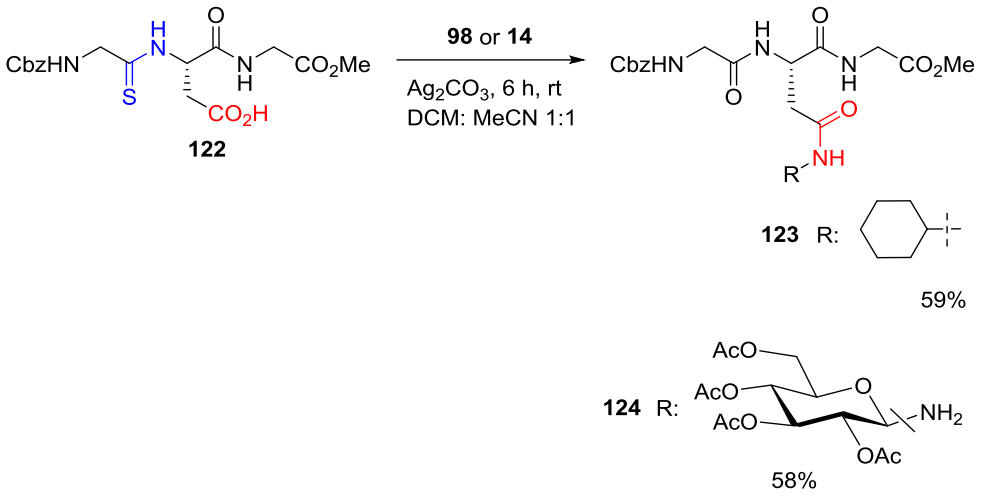
*Scheme 34. Synthesis of dipeptide amine **120***

With both the benzotriazolide **116** and the amine **120** in hand, coupling the two partners under the conditions described by Rapoport was undertaken. Treatment of the amine **120** with glycine benzotriazolide **116** in THF afforded tripeptide thioamide **121** in 70% yield. The *tert*-butyl ester was then removed by treatment with 50%TFA/DCM solution to give the free aspartyl carboxylic acid **122** (Scheme 35).



*Scheme 35. Synthesis tripeptide thioamide **122***

With the tripeptide thioamide **122** in hand, Ag(I)-promoted coupling reactions with cyclohexylamine **98** and aminosugar **14** were performed under identical conditions to those employed for thioamide **111** (Scheme 36). The *N*-linked asparagine motifs **123** and **124** were obtained in good yields after chromatographic purification. The structures of **123** and **124** were confirmed by ^{1}H-NMR spectroscopy. The ^{1}H NMR spectrum of the peptide **123** contains a doublet peak at 7.65 ppm, corresponding to the amide NH on the asparaginyl side chain. The presence of a one-proton doublet peak at 6.81 ppm in the ^{1}H NMR spectrum of the product **124** corresponds to the amide NH on the asparaginyl side chain. The mass spectra contained peaks at *m/z* 477.2348 and at *m/z* 725.2515, corresponding to the molecular formula of the expected products **123** and **124**, respectively. These results indicate that the Ag(I)-induced coupling method is applicable to extended peptide thioamides.



*Scheme 36. Coupling of thiopeptide **122** and amines **98** and **14***

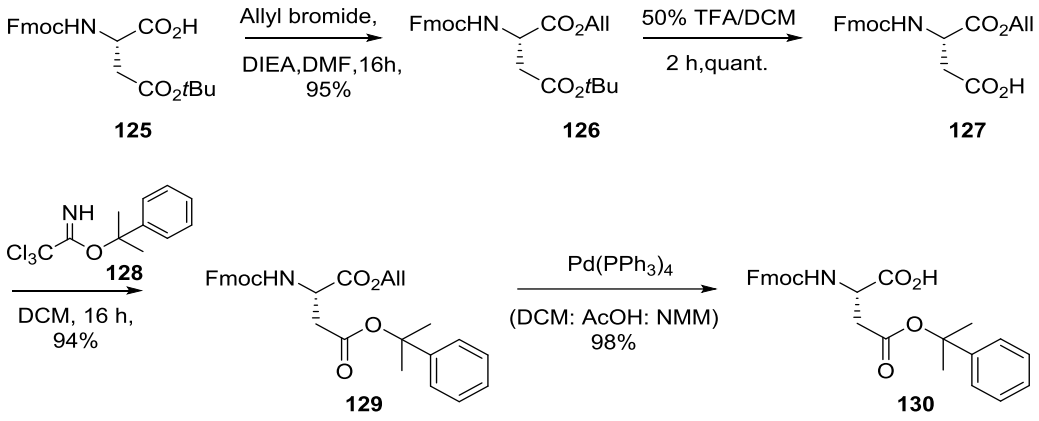
2.3 Synthesis of glycopeptides

2.3.1 Synthesis of thiopeptides on solid phase

With the model studies demonstrating the successful synthesis of the *N*-linked asparaginyl peptides, the scope of the reaction with respect to peptide sequence was investigated. *N*-Glycosylation of peptides and proteins occurs at asparagine residues embedded in the consensus sequence Asn-X-Ser (Thr), where X can be any amino acid except proline. To investigate the efficiency of the Ag(I) coupling with various thionated amino acid residues adjacent to the aspartic acid, synthesis of thioamide-containing peptides AX[S]DAS **138a–f** was required (X = A, L, F, R, K, V). A standard Fmoc-based solid phase peptide synthesis (SPPS) strategy was envisaged for site-specific incorporation of a thioamide linkage into a peptide chain, employing a procedure developed by Petersson and co-workers based on the Rapoport procedure (Scheme 38).[63-64]

In order to provide orthogonal protecting groups, the 2-phenyl isopropyl ester (Ph*i*Pr) was chosen to protect the side chain of the aspartic acid residue. Therefore, the synthesis of the aspartic acid derivative **130** was required. The synthesis of Fmoc-Asp(Pip)-OH

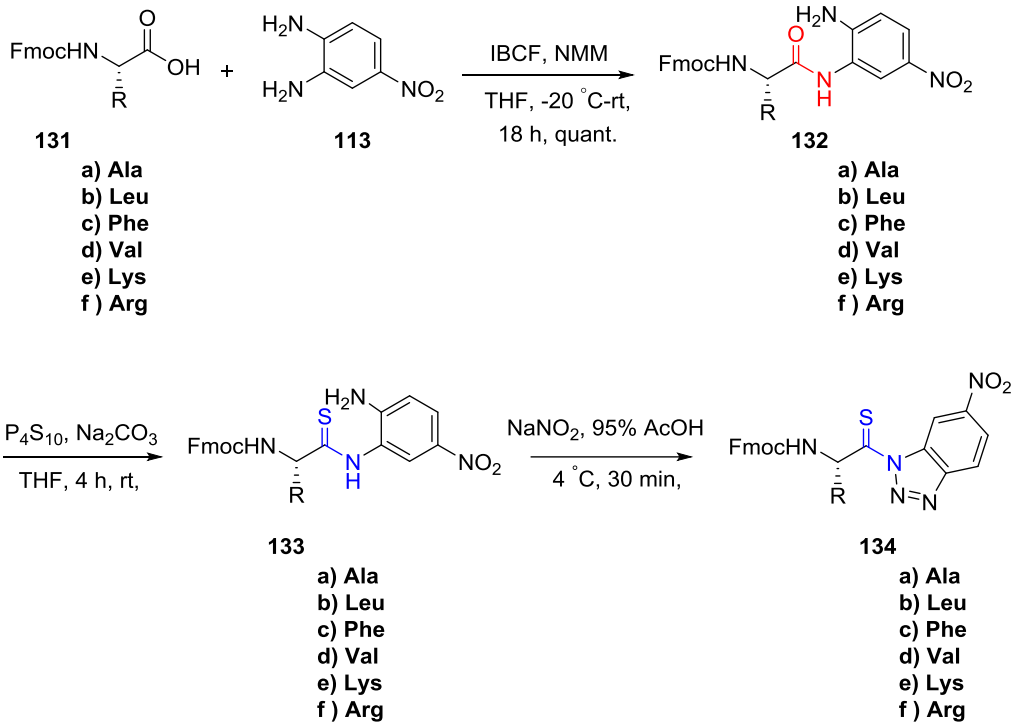
130 was achieved in four steps according to the literature procedure.[65] Accordingly, treatment of aspartic acid **125** with allyl bromide gave the allyl ester **126** in excellent yield. The *tert*-butyl ester **126** was then removed by treatment with TFA to give the carboxylic acid **127**. Reaction of the resulting acid **127** with 2-phenylisopropyl trichloroacetimidate **128** (prepared in one step by treating the sodium salt of 2-phenylisopropanol with trichloroacetamide)[66] provided the fully protected amino acid **129**. Palladium(0)-catalyzed removal of the allyl ester **129** afforded the aspartic acid derivative **130** in 95% yield (Scheme 37).



*Scheme 37. Synthesis of aspartic acid derivative **130***

The synthesis of the required Fmoc-protected thioacyl 4-nitrobenzotriazolide reagents **134a**–**f** was achieved in three steps in a similar manner to that described for the glycine derivative **116** (Scheme 38). Coupling of the commercially available Fmoc-protected amino acids **131** with 4-nitro-1,2-phenyldiamine **113** in the presence of isobutyl chloroformate and *N*-methylmorpholine in THF gave the corresponding aminoanilides **132a**–**f** in quantative yields. Thionation of anilides **132a**–**f** with a mixture of P_4S_{10} and Na_2CO_3 in THF gave the corresponding thioamides **133a**–**f** in good yields. Intramolecular diazonium cyclization of thioanilides **133a**–**f** using *in situ* generated

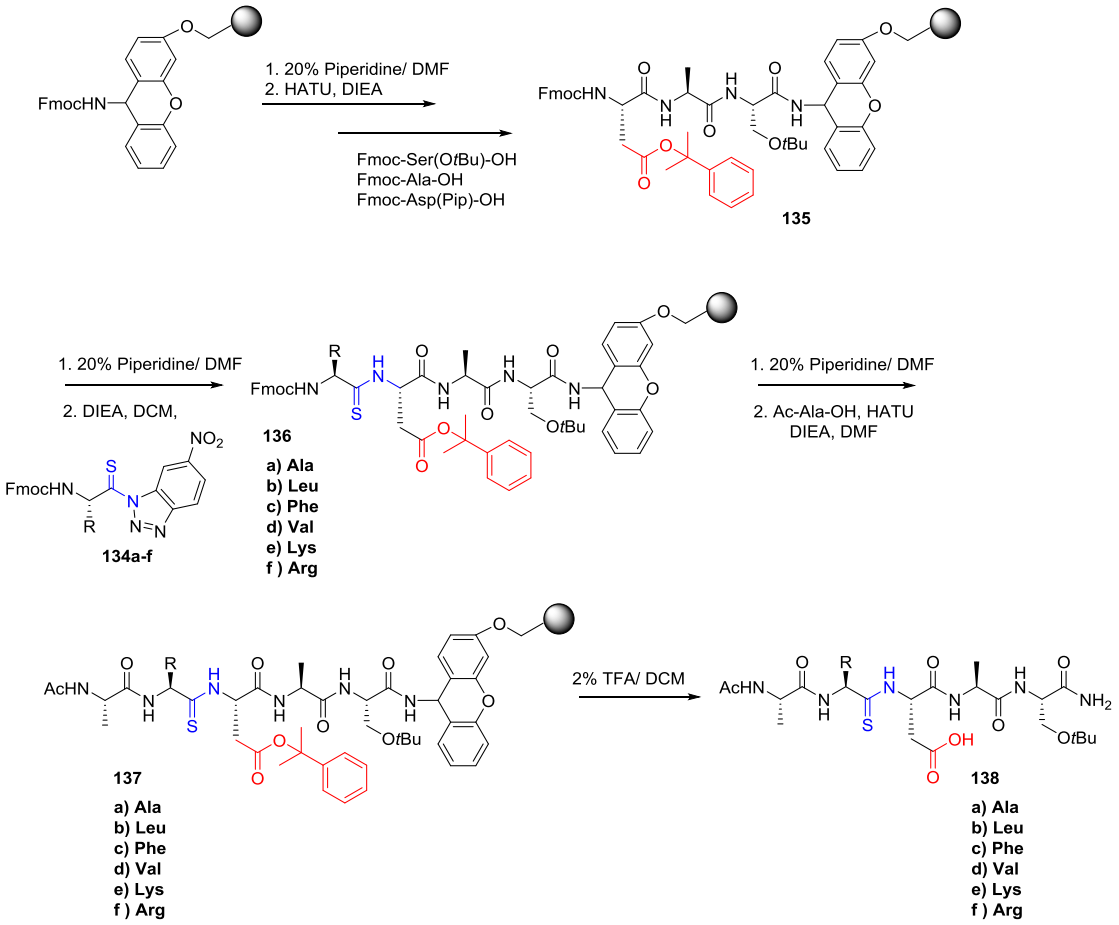
nitrous acid gave the benzotriazole derivatives **134a–f** in good yield. The benzotriazole derivatives **134a–f** were then incorporated into SPPS without purification.



*Scheme 38. Synthesis of the benzotriazole derivatives **134a–f***

With the aspartic acid **130** and benzotriazole derivatives **134a–f** in hand, standard solid phase peptide synthesis (SPPS) protocols were performed to generate peptides thioamides **138a–f** (Scheme 39). Peptide **135** was assembled on acid-sensitive Sieber amide resin to facilitate the synthesis of a sidechain protected peptide possessing a *C*-terminal amide. After Fmoc deprotection of **135** with 20% piperidine in DMF, the peptide was treated with the benzotriazolide derivatives **134a–f** in the presence of DIEA in DCM. Subsequent removal of the Fmoc protecting group and coupling with Ac-Ala gave peptide thioamides **137a–f** on resin. Treatment with 2%TFA in DCM solution resulted in removal of the 2-phenyl isopropyl ester from the aspartate side chain and cleavage of the peptides from solid support to provide crude peptide thioamides **138a–f**, which were purified by preparative RP-HPLC to afford peptide thioamides **138a–f** in reasonable overall yields. The structures of thiopeptides **138a–f** were confirmed by

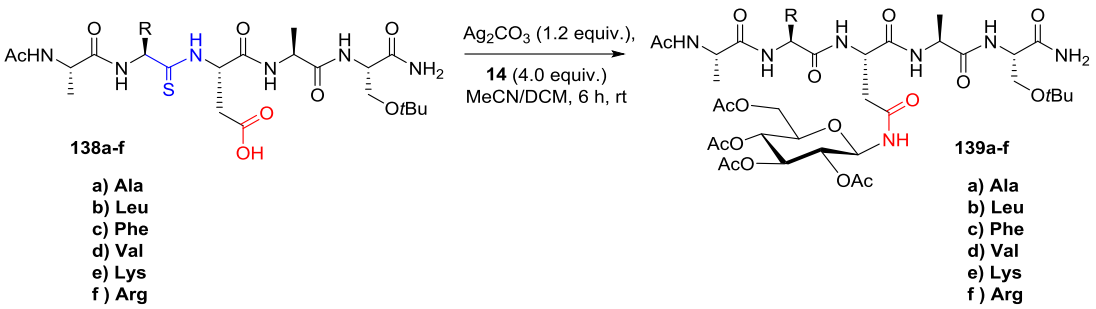
mass spectrometry and ^{1}H NMR spectroscopy. The presence of a one-proton doublet peak at ~10.0 ppm in the ^{1}H NMR spectra of compounds **138a–f** corresponds to the thioamide NH group.



*Scheme 39. Synthesis of peptides thioamide **138a–f***

2.3.2 Synthesis of glycopeptides

With the successful synthesis of pentapeptide thioamides **138a–f**, we next investigated their reactions with glucosylamine **14** to determine whether the scope of the Ag(I)-promoted coupling could be applied to longer peptides with a variety of thionated amino acids residues. The thiopeptides **138a–f** were treated with Ag_2CO_3 and amniosugar **14** under optimised conditions. The *N*-glycosylated asparagine motifs **139a–f** were purified by HPLC and obtained in good overall yields, along with the amides typically obtained as minor byproducts (Scheme 40, Table 2).



*Scheme 40. Coupling of thiopeptides **138a–f** and glucosylamine **14***

*Table 2. Yield and characterisation of glycopeptides **139a–f***

Peptide	Mass		Isolated yield %
	Calculated	Observed	
139a	860.3884	860.3884	29
139b	902.4353	902.4350	30
139c	936.4197	936.4205	33
139d	888.4197	888.4189	27
139e	1017.4986	1017.4988	26
139f	1197.5344	1197.5361	34

The glycopeptides **139a–f** were characterized by NMR spectroscopy and HRMS. ^{1}H NMR analysis of glycopeptides **139a–f** showed that all displayed a doublet peak at ~ 8.7 ppm, corresponding to the NH-glycosidic amide, with a coupling constant ~ 9.4 Hz consistent with a β-linked *N*-glycan. For further confirmation of the glycopeptide structures **139a–f** was provided by 2D NMR spectroscopy. Glycopeptide **139c** displayed a doublet at 8.79 ppm corresponding to the NH-glycosidic amide, and a triplet at 5.35 ppm corresponding to the glycan β-anomeric proton. The H–N–C–H coupling constant was 9.4 Hz, confirmed by COSY NMR. The point of glycan attachment to peptide **139c** was confirmed by means of a NOESY NMR experiment. A correlation between the glycosidic amide proton and the asparagine methylene protons confirms

the glycan attachment to the asparaginyl side chain. The molecular formula of **139a**–**f** were confirmed by HRMS (Table 2).

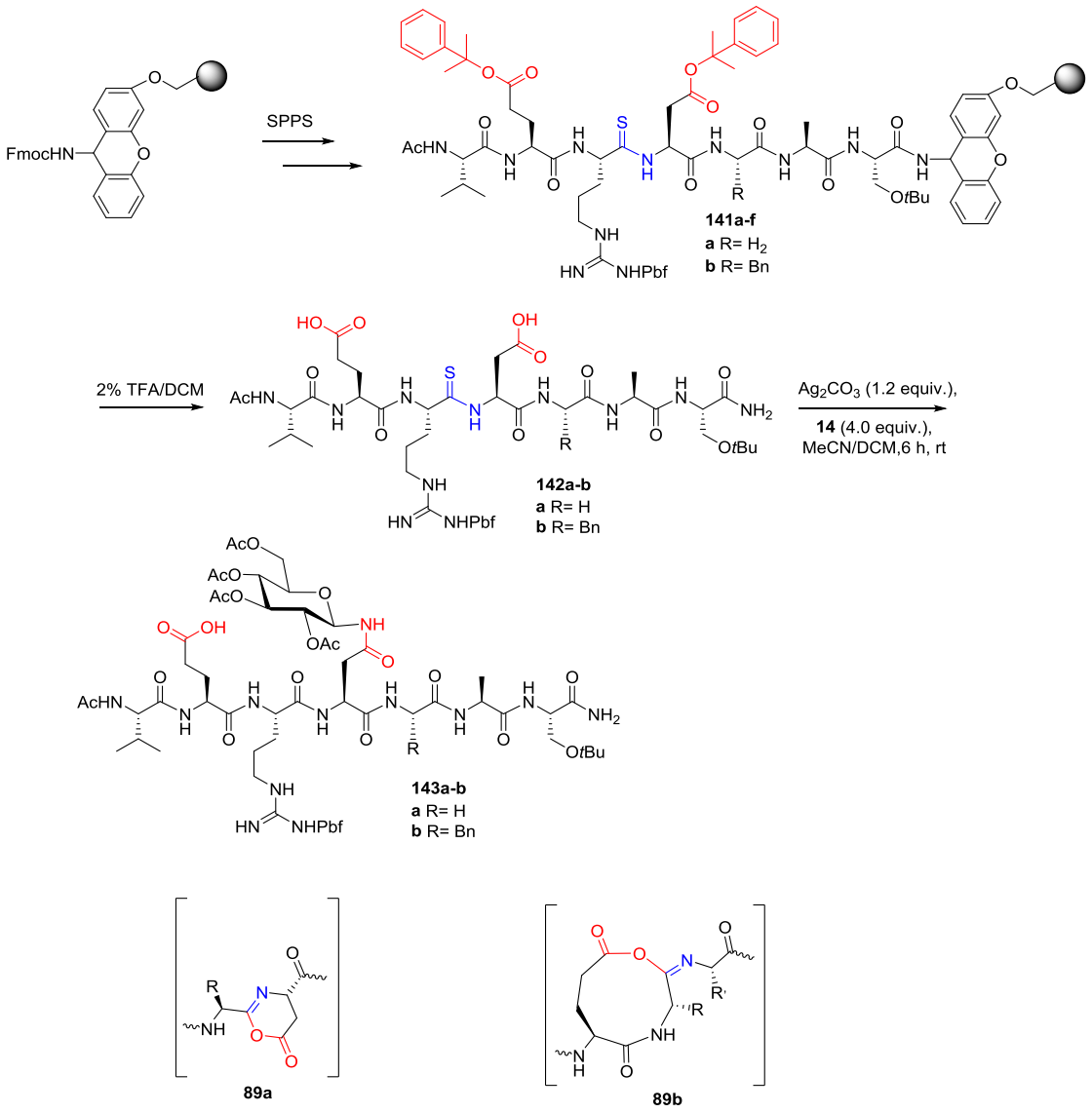
2.4 Site-specific glycosylation

Given the successful coupling of glucosylamine **14** to the thiopeptides **138a**–**f** for the preparation of the *N*-linked glycopeptides **139a**–**f**, it was decided to investigate whether the Ag(I)-promoted coupling could be extended to site-selectively generate *N*-glycosyl asparagines in peptides possessing multiple unprotected carboxylate side chains groups. CSF114(Glc) glycopeptide **140** was chosen as an example: this glycopeptide is comprised of 21 amino acids, and contains one *N*-glucosyl asparagine residue (Figure 3). CSF114(Glc) has been investigated for the diagnosis and monitoring of multiple sclerosis, where it has been used as an antigenic probe to accurately identify specific autoantibodies as disease biomarkers for monitoring disease progression and efficiency of therapeutic treatment.[67-68] We chose to investigate the site-specific Ag(I)-promoted reaction with CSF114 fragment **145** containing an aspartic acid residue at the glycosylation site in the presence of a glutamic acid residue.

Figure 3. Structure of glycopeptide CSF114(Glc) ***140***

To initiate our investigations of the site-specific coupling reaction, it was decided to model the glycosylation reaction with thiopeptides **142a**–**b**, analogues of CSF114 fragment **140**. The synthesis of thiopeptides **142a**–**b** was undertaken using the standard SPPS protocol described for thiopeptides **138a**–**f** (Scheme 40). Thiopeptides **142a**–**b** were generated in good purities after HPLC purification. Treatment of thiopeptides

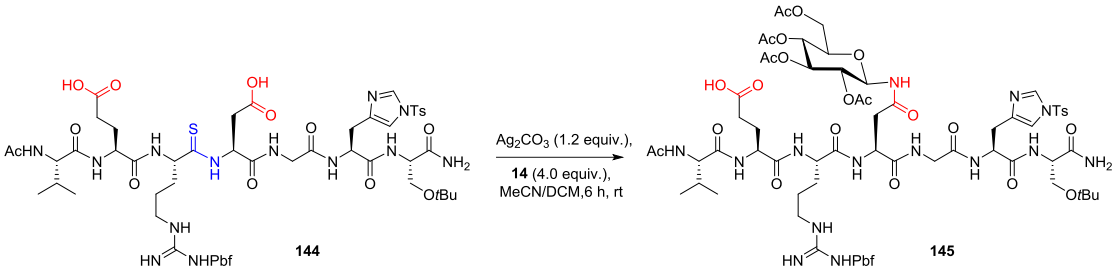
142a–**b** with glucosylamine **14** in the presence of Ag_2CO_3 for 6 h under optimised conditions was undertaken. The *N*-glycosylated asparagine peptides **143a**–**b** were generated in good conversion and as the sole glycosylated products, indicating that the unprotected glutamate carboxylate side chain group does not affect the key Ag(I)-promoted coupling process. The highly chemoselective conversion of the Asp residue to the *N*-Glc Asn in these reactions presumably results from the preferential generation of a six-membered isoimide intermediate **89a**, where the Asp carboxylate attacks the peptide thioamide. The equivalent conversion of the Glu residue to the *N*-Glc Gln would require generation of a less favourable nine-membered isoimide intermediate **89b**.



*Scheme 41. Coupling of thiopeptide **134a-b** and glucosylamine **14***

With this pleasing result in hand, we turned to the application of the Ag(I)-promoted coupling for a site-specific chemical ligation of glucosylamine **14** with the thioamide-containing CSF114 peptide fragment **144** to form the *N*-glycosylated asparagine derivative **145**. The thiopeptide **144** possessing a native histidine residue was treated with glucosylamine **14** and Ag_2CO_3 under optimised conditions. However, the glycosylated peptide **145** was generated in only a trace amount. The mass spectrum contains a pseudo-molecular ion peak at *m/z* 1631.6601 corresponding to the molecular

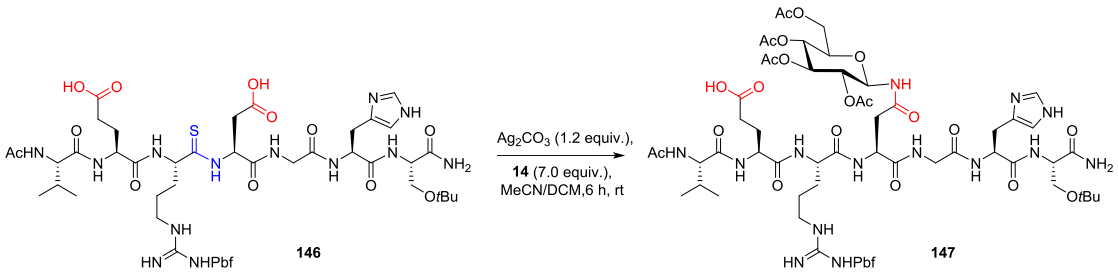
formula of the expected product **145**. The major product isolated was the oxoamide derived from the starting thioamide **144** (Scheme 42).



*Scheme 42. Coupling of thiopeptide **135** and glucosylamine **14***

With difficulties encountered in preparation of the glycosylated peptide **136** from peptide **135** containing and *N*-Ts protected His residue, we investigated the use of an unprotected histidine residue in thiopeptide **146**. Treatment of thiopeptide **146** with glucosylamine **14** in the presence of Ag_2CO_3 under the optimised Ag(I)-promoted conditions (4 equiv. **14**) yielded the corresponding asparagine peptide fragment **147** in moderate conversion, with the oxoamide derived from the starting thioamide **146**. also generated (Scheme 43). Increasing the amount of **14** to 7.0 equiv. resulted in an increase in conversion to the *N*-glucosyl peptide **147** to 39%. The glucosyl peptide **147** was purified using HPLC and analysed by using mass spectroscopy. The mass spectrum of **147** displayed a molecular ion peak at *m/z* 1477.6512 corresponding to the molecular formula of the expected product. The structure of **147** was determined using 1D and 2D NMR techniques. The ^{1}H NMR spectrum displays a doublet at δ 8.81 ppm corresponding to the NH-glycosidic amide and a triplet at δ 5.35 ppm corresponding to the glycan β-anomeric proton. The H–N–C–H coupling constant was 9.4 Hz, confirmed by COSY NMR experiment. The point of glycan attachment to the asparaginyl side-chain was confirmed by means of a NOESY NMR experiment. The NOESY NMR spectrum of **147** showed a correlation between the glycosidic amide proton and the

asparagine methylene protons, confirming formation of the *N*-glucosyl asparagine residue rather than the *N*-glucosyl glutamine.

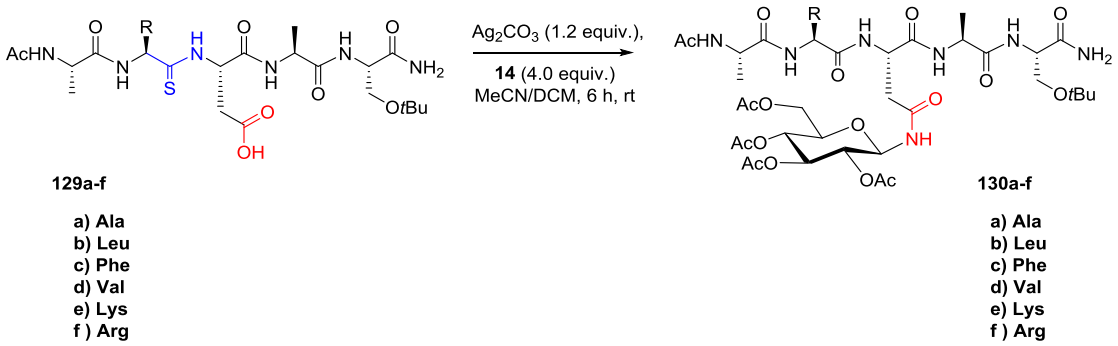


*Scheme 43. Coupling of thiopeptide **146** and amine **14***

2.5 Conclusion

- **Chemoselective synthesis of glycopeptides**

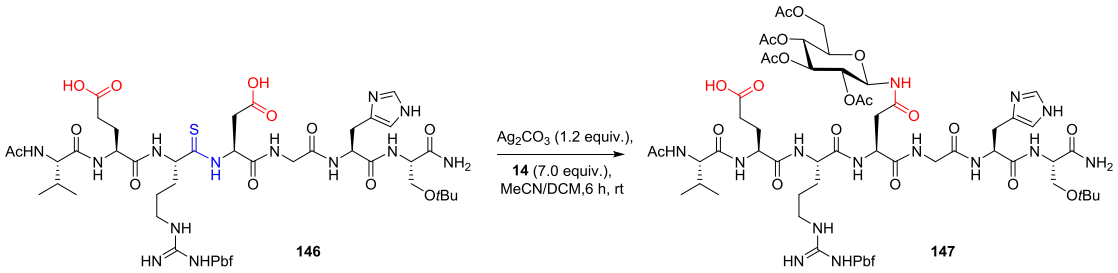
We have successfully developed a new method for the chemoselective synthesis of glycopeptides, using a Ag(I)-promoted coupling reaction. Reaction of glucosylamine **14** with peptide thioamides **138a–f** generated glycopeptides **139a–f** in generally high yields and purities (Scheme 40).



*Scheme 39. Coupling of thiopeptides **129a–f** and glucosylamine **14***

- **Site-specific formation of *N*-glycosylated peptides**

The Ag(I)-promoted coupling reaction was shown to be effective for site-selectively generate *N*-glycosyl asparagines in peptides possessing multiple unprotected carboxylate side chains groups. The Ag(I)-promoted coupling of aminosugar **14** with peptide thioamide **146**, proceeds effectively to generate *N*-glycosyl asparagine-containing peptide **147** (Scheme 43). This example demonstrates that the formation of the *N*-glycopeptides proceed efficiently in peptides possessing a variety of protected and unprotected side-chain functional groups, such as those in arginine, serine, histidine and glutamic acid residues. That is, chemoselective reaction of the adjacent aspartate carboxylate occurs with the peptide thioamide to generate the crucial isoimide intermediate, even in the presence of other nucleophilic functional groups.



*Scheme 43. Coupling of thiopeptide **146** and amine **14***

The Ag(I)-promoted coupling method enables the generation of *N*-glycosyl asparagines in peptides with no evidence of aspartimide formation, highlighting that this method is a major improvement in the formation of *N*-glycopeptides over previous methodologies discussed in Chapter 1.

3. Results and Discussion: Lactam stapling of peptides using a thioamide coupling approach

3.1 Background

Protein–protein interactions (PPIs) play a major role in mediating many important intracellular processes. It is estimated that 62% of PPIs have an α-helix motif at the interface, indicating the vital role of the helical structures in facilitating biological processes.[69] One area of active drug design research is the development of drug molecules which can mimic α-helices found at the binding region between two proteins, thereby acting as inhibitors of PPIs.[70]

Peptides have shown potential activity for modulation of PPIs due to their medium size compared with classical small molecules (molecular weight < 500 Da) or large biologics (molecular weight >5000 Da). Targeting PPIs using small molecules is usually not feasible: the approximate contact surface area of most PPIs is 1500–3000 Å, whereas the contact surface area of small molecule–protein interactions is 300–1000 Å.[71] Furthermore, peptides are favoured over their larger protein biologics counterparts: production of biologics is often expensive and more complex than peptide synthesis due to their larger molecular weight.[72] However, pharmacological applications of short synthetic peptides are plagued by their lack of metabolic stability, poor membrane permeability, and rapid clearance. Also, unbound peptides do not retain their secondary structure and binding capability under physiological conditions. Peptides can also suffer from proteolytic degradation and cannot penetrate cell membranes.[73] In order to develop peptides as potential therapeutic agents for inhibition of PPIs, there is need for modification of promising peptides towards imparting structural rigidity and reinforcing the desired α-helical conformation. Also, such peptide modifications may improve proteolytic stability and cell permeability.[74-78] The most commonly employed strategy for generating α-helices is peptide stapling.[79-80]

3.2 Strategies for peptide stapling

A wide range of peptide stapling strategies have been developed to promote a helical structure: Non-covalent strategies include hydrophobic interactions,[81] salt bridges[82] and cation–π interactions,[83] while covalent techniques include formation of disulfide bridges, ring-closing metathesis, cycloaddition reactions, and lactamisation (Figure 4).[84]

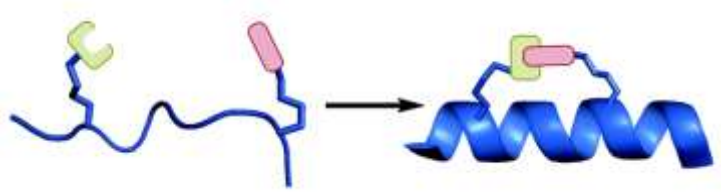
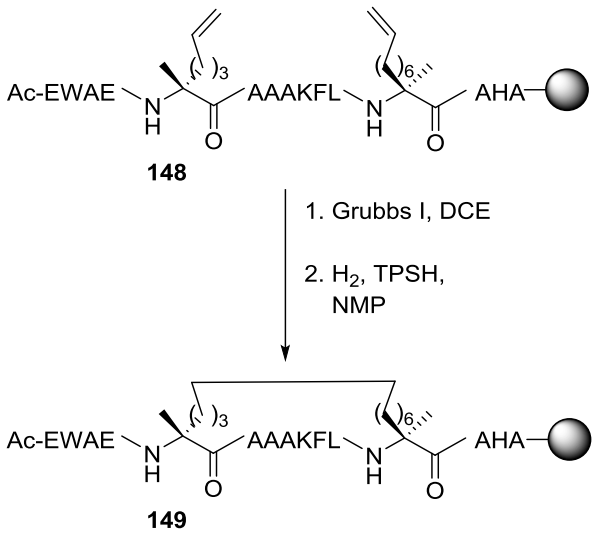


Figure 4. Peptide stapling involves direct cyclisation between two side-chains

3.2.1 Ring closing metathesis

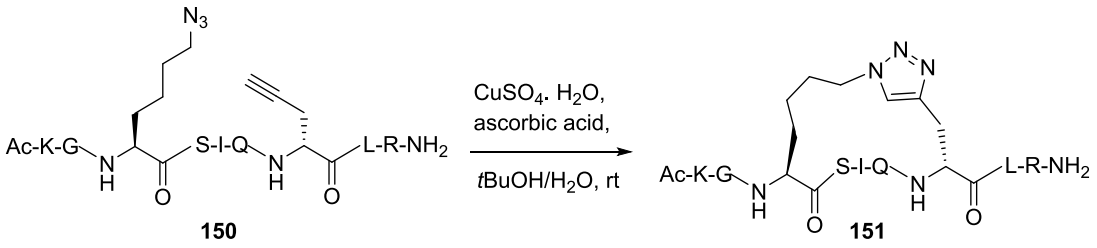
Alkene ring closing metathesis (RCM) has been employed as a peptide stapling strategy for constructing macrocyclic peptides. Ring closing metathesis involves a catalytic carbon–carbon bond formation reaction through combination of two olefinic side-chains to form hydrocarbon-stapled peptides. Verdine and co-workers developed a ring-closing metathesis strategy for the synthesis of constrained peptides using the *C*-terminal sequence of RNAse A.[85] Peptide **148** incorporating α,α-disubstituted amino acids bearing olefinic side-chains was successfully cross linked to furnish the hydrocarbon stapled peptide **149** (Scheme 44). The ruthenium-catalysed ring-closing metathesis and subsequent hydrogenation were conducted on solid phase before cleaving peptides from the resin.



Scheme 44. Peptide stapling using ring-closing metathesis strategy

3.2.2 Copper(I) Azide–Alkyne Cycloaddition (CuAAC)

Chorev and co-workers reported a Cu(I)-catalysed azide–alkyne cycloaddition (CuAAC) 'click' reaction to form stable helical peptides.[86] The copper(I)-catalysed reaction between *i*,*i*+4-spaced azido and alkynyl groups on the side chains of residues results in formation of the the triazolyl-linked peptide **151** (Scheme 45).

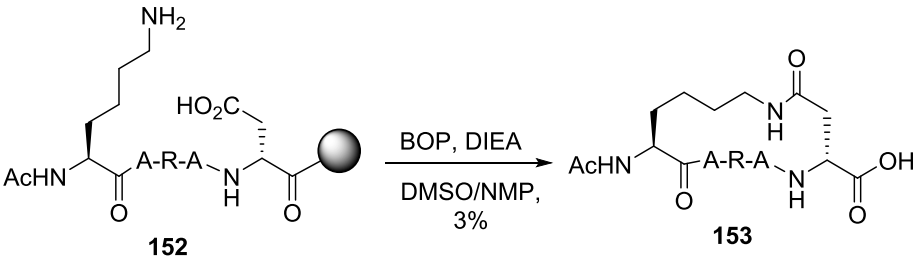


Scheme 45. Peptide stapling using CuAAC

Whilst these methods represent general syntheses of stabled helical peptides, the requirement of incorporation of non-proteogenic modified amino acids can be problematic.

3.2.3 Side-chain lactamisation

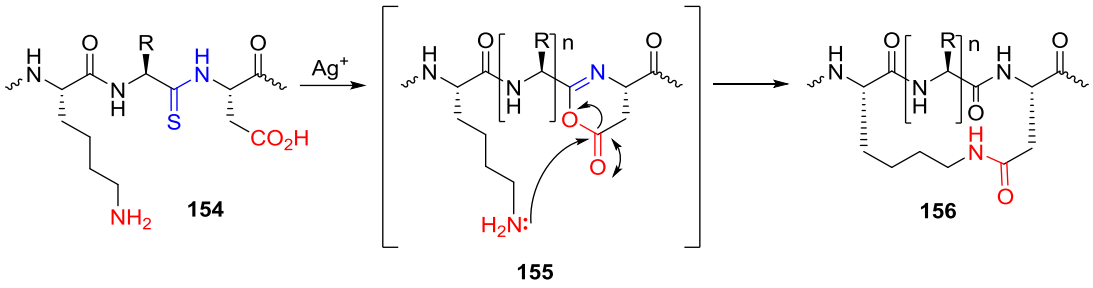
Lactam formation was the earliest and remains one of the most common methods for generation of α-helical peptides. In this method, a lactam linkage is generated through condensation of a side-chain carboxyl group of Asp or Glu with a side-chain amine group of a Lys residue.[87] The intramolecular amide bond formation is usually achieved through initial activation of the carboxylic acid using a coupling reagent, with the activated acid then reacting with the side chain amine. The major advantage of this method is the incorporation of proteinogenic amino acids, exploiting the reactivity of functional groups found on the side chains to form lactam bridges. The side-chain lactamisation method has been applied in the synthesis of a range of helical peptides with potential biological applications.[88] However, there are some limitations of using coupling reagents to effect side-chain macrolactamisation. The major limitation is the need for orthogonal protection and deprotection strategies of the acid and the amine functionalities. Another limitation is that cyclisation of linear peptides often proceeds in poor yield and purity. For example, side-chain lactamisation of linear peptide **152** to give macrocyclic peptide **153** proceeded in only 3% yield in the presence of BOP coupling reagent (Scheme 46).[89]



*Scheme 46. Synthesis of lactam stapled peptide **153***

2.3 Proposed thioamide-based ligation for stapled peptides

Given the previous studies described in Chapter 2 that demonstrate intermolecular trapping of an isoimide intermediate with an external amine is a useful strategy for generating amide bonds on the side chain of an aspartic acid, we envisaged the corresponding reaction with a lysine side chain-amine could generate lactam-stapled peptides (Scheme 47). This process avoids the use of coupling reagents and may improve the yield and selectivity of side-chain lactamisation process.

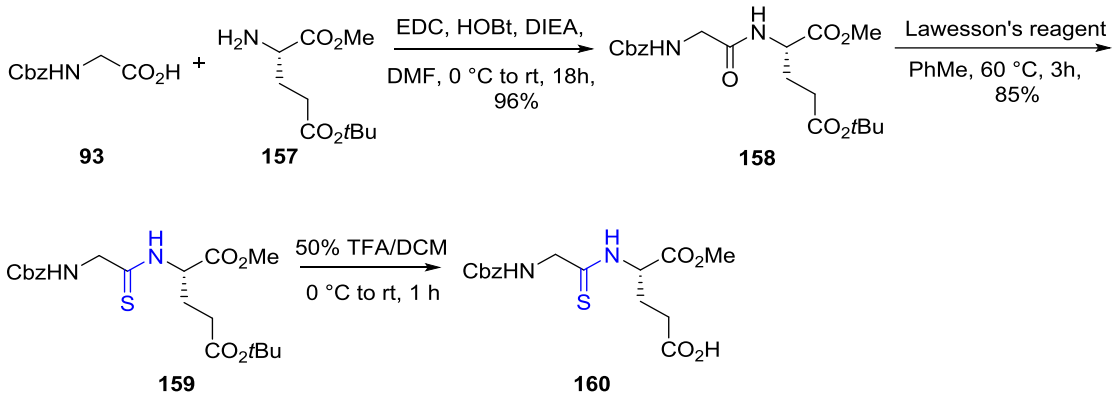


Scheme 47. Proposed mechanism of side-chain lactamisation

3.4 Model study

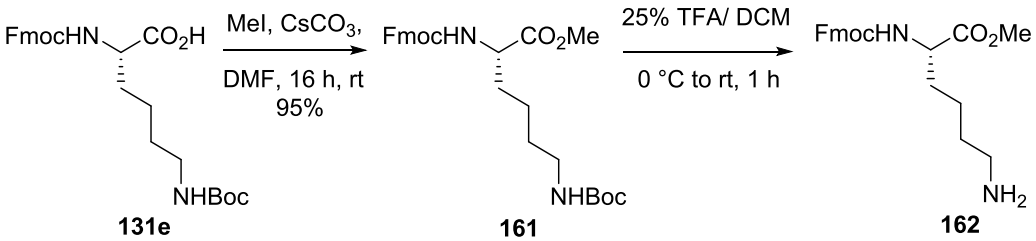
Before investigating the intramolecular Ag(I)-promoted macrolactamisation process, we first needed to determine whether trapping an isoimide intermediate with a lysine derivative proceeded with the same efficiency as demonstrated for alkyl amines. As a model study, the intermolecular Ag(I)-promoted coupling of dipeptide thioamides **97** or **160** and the lysine derivative **162** was investigated. Preparation of the peptide thioamide **160** and the lysine derivative **162**, were required. The Gly–Glu dipeptide thioamide **159** was prepared from glycine **93** and glutamic *tert*-butyl ester **157** over two steps (Scheme 48), employing the same procedure used for preparation of thioamide **96**. The ^{1}H NMR spectrum of the thioamide **159** exhibits a one proton apparent singlet at δ 8.88 ppm corresponding to the thioamide NH group. The mass spectrum of **159**

contains a molecular ion peak at *m/z* 425.1741 corresponding to the molecular formula of the expected product. The *tert*-butyl ester **159** was then removed by treatment with 50% TFA in DCM to give the free carboxylic acid **160**, which was used in the next step without purification.



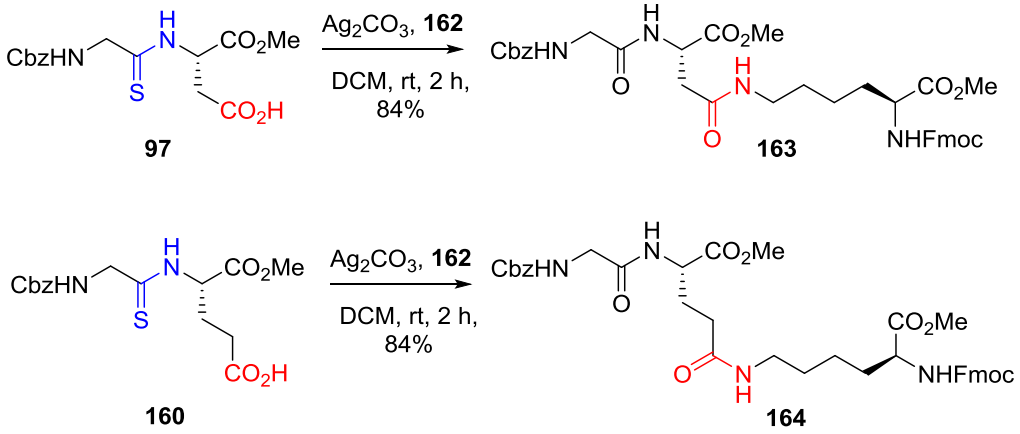
*Scheme 48. Preparation of dipeptide thioamide **160***

Lysine derivative **162** was prepared in two steps from the commercially available lysine **131e** (Scheme 49). Esterification of the lysine **131e** with methyl iodide under basic conditions gave the protected lysine **161**. Removal of the Boc group under acidic conditions afforded the lysine derivative **162**, which was used in the next step without purification. The mass spectrum of the crude amine **162** contains a molecular ion peak at *m/z* 383.1962 corresponding to the molecular formula of the expected product.



*Scheme 49. Preparation of lysine **162***

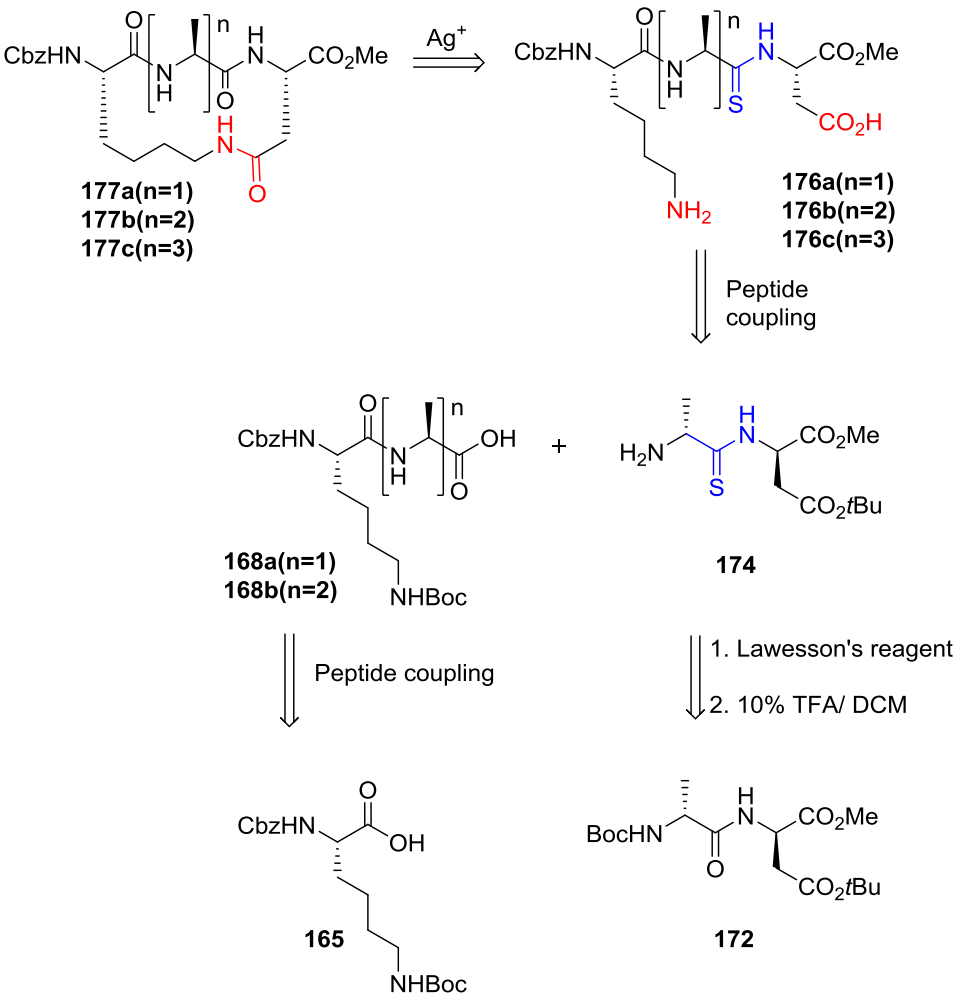
With lysine derivative **162** possessing an unprotected side-chain amine in hand, coupling with thioamides **97** or **160** was investigated to confirm whether the Ag(I)-promoted reaction would proceed. Treatment of dipeptide thioamide **97** with Ag_2CO_3 in the presence of lysine amine **162** in DCM afforded the fully protected isotripeptide **163** in 84% isolated yield (Scheme 50). Similarly, treatment of the glutamate dipeptide thioamide **160** with **162** under the same conditions gave the coupled adduct **164**, albeit in lower yield (47%). Presumably, peptide thioamide **160**, bearing an unprotected glutamyl residue, generates a seven-membered isoimide intermediate with less efficiency than the corresponding reaction of **97** to generate a six-membered isoimide. The ^{1}H NMR spectra of the coupled adducts **163** and **164** contain three-proton singlet peaks at δ 3.60 ppm, corresponding to the presence of the additional methyl ester groups, and characteristic peaks of the Cbz and Fmoc groups between at δ ~ 7.0–8.0 ppm. The mass spectra of **163** and **164** show molecular ion peaks at *m/z* 703.2977 and 717.3136 corresponding to the molecular formula of the expected products **163** and **164**, respectively.



*Scheme 50. Coupling of thioamides **97**, **160** and lysine **162***

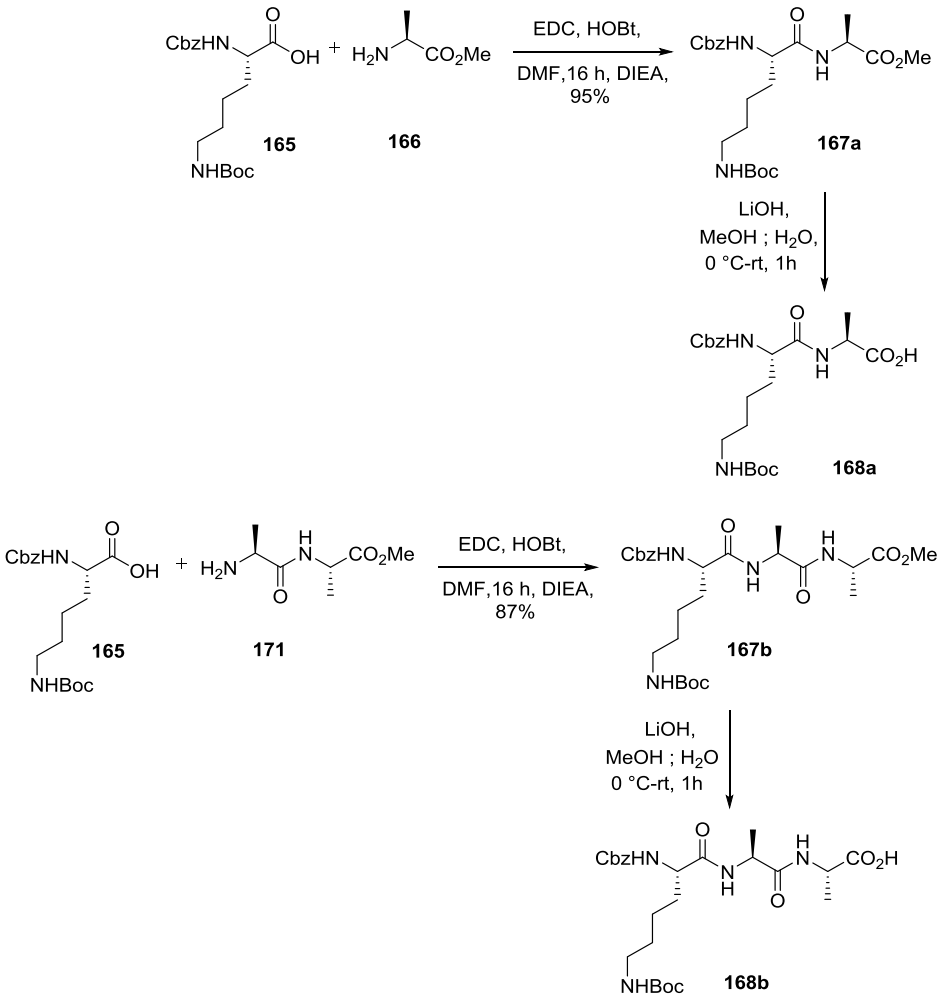
3.5 Side-chain lactamisation

Given the successful intermolecular Ag(I)-promoted side-chain coupling between Lys and Asp or Glu residues, we next investigated whether this method could be applied in an intramolecular process for lactam formation between Lys and Asp residues. Therefore, synthesis of linear thiopeptides containing lysine and aspartic acid residues was required. Thiopeptides **166a-c** were targeted for investigation, representing (*i*,*i*+2), (*i*,*i*+3), and (*i*,*i*+4)-spaced Lys–Asp side-chain lactam bridges. An intramolecular coupling/lactamisation induced by Ag(I) would generate the cyclic peptides **167a-c**. Linear thioamide-containing peptides **166a-c** could be assembled from a coupling reaction of a lysine derivative and dipeptide thioamide **164**. Lysine-containing peptides **159a-b** would be synthesised using standard peptide coupling protocols. Dipeptide peptide thioamide **164** could be obtained from the dipeptide thioamide **163**, which in turn is to be synthesised from dipeptide **162** with Lawesson's reagent (Scheme 51).



*Scheme 51. Retrosynthesis of cyclic peptides **167a-c***

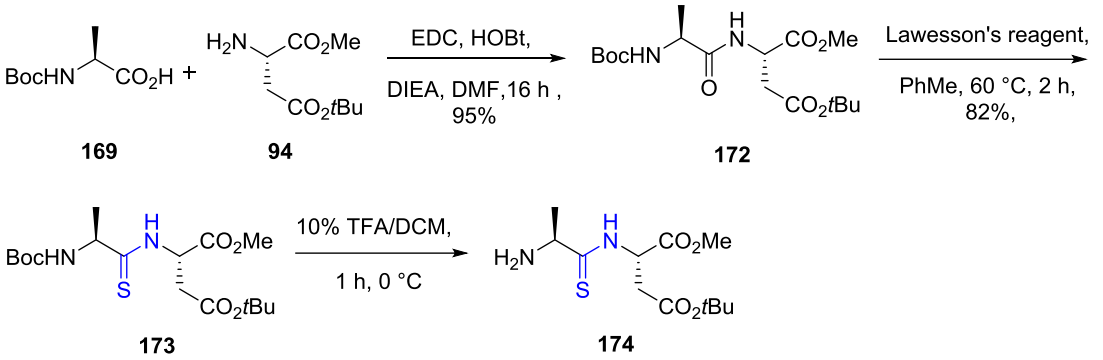
Synthesis of peptides **168a-b** was accomplished using standard peptide coupling reactions (Scheme 52). Dipeptide **168a** was synthesised by coupling of the lysine derivative **165** and alanine methyl ester **166** to give dipeptide **167a** in excellent yield. Tripeptide **168b** was assembled by coupling of the lysine derivative **165** and dipeptide alaninyl-alanine methyl ester **171** in 87% yield. The methyl esters of **167a-b** were then removed under basic conditions to give peptides **168a-b**, which were used in the next step without purification. The mass spectra of **168a** and **168b** show molecular ion peaks at *m/z* 452.2390 and 536.2844 corresponding to the molecular formula of the expected products **168a** and **168b**, respectively.



*Scheme 52. Synthesis of peptides **168a-b***

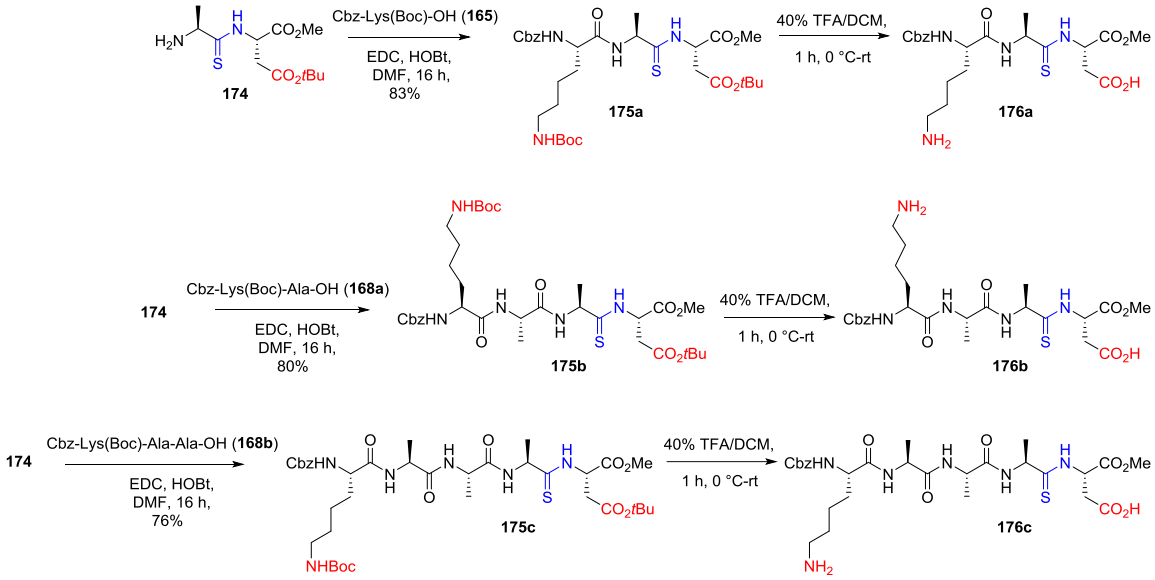
The synthesis of dipeptide thioamide **174** was achieved in three steps (Scheme 53). The protected amino acid *N*-Boc-L-alanine **169** was coupled to H-Asp(O*t*Bu)-OMe **94** under standard peptide coupling conditions to give the dipeptide **172** in excellent yield. Dipeptide **172** was then treated with Lawesson's reagent to give the dipeptide thioamide **173** in 82% yield. The Boc protecting group was then removed selectively by treatment with 10% TFA acid in DCM solution to provide the free amine **174**, which was used in the next step without purification. The mass spectrum of the crude amine **174** shows a

molecular ion peak at *m/z* 291.1372 corresponding to the molecular formula of the expected product.



*Scheme 53. Synthesis of dipeptide thioamide **174***

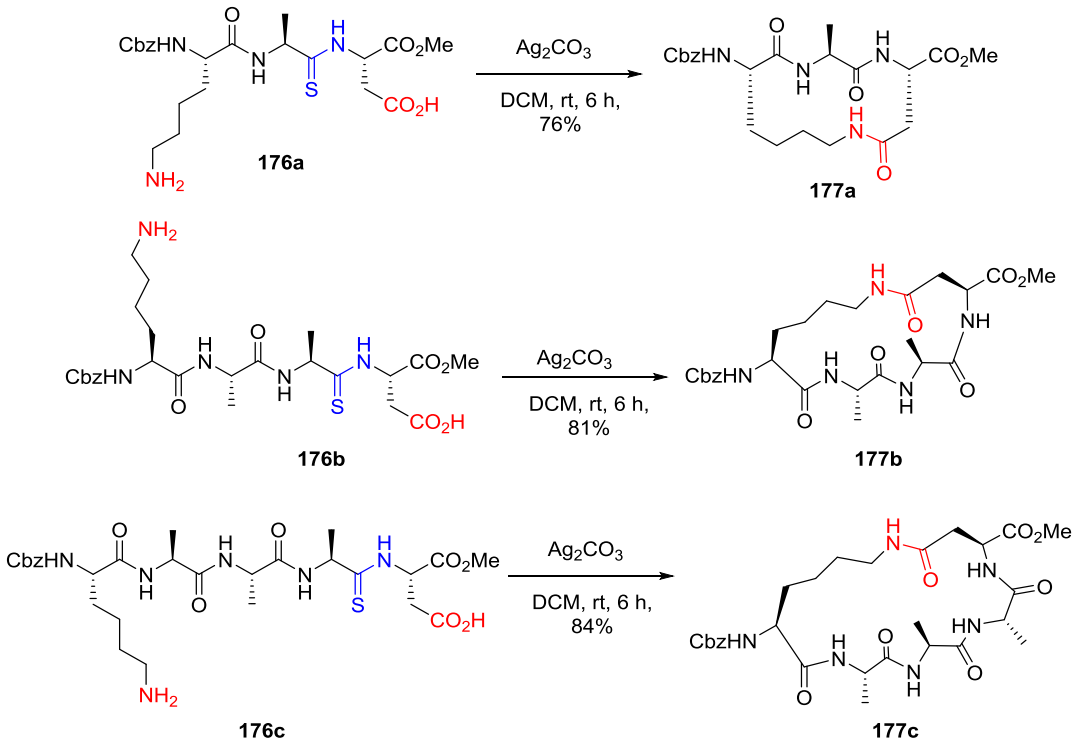
With all starting materials in hand, synthesis of linear thiopeptides **176a-c** was performed (Scheme 54). Coupling of lysine **165** with dipeptide thioamide **174** gave tripeptide **175a** in 83% yield. Linear thiopeptides **175b** and **175c** were generated by coupling of dipeptide thioamide **174** with peptides **168a** and **168b** in 80% and 76% yield, respectively. The structures of linear thiopeptides **175a-c** were confirmed by NMR spectroscopy. The presence of a one-proton doublet peaks at δ ~10.0 ppm in the ^{1}H NMR spectra of compounds **175a-c** correspond to the thioamide NH group. Treatment of peptides **175a-c** with 40% TFA/DCM solution resulted in cleavage of both Boc and tBu groups in one step to give the side-chain unprotected linear thiopeptides **176a-c**.



*Scheme 54. Synthesis of thioamides **176a-c***

With linear thiopeptides **176a-c** possessing unprotected aspartyl and lysinyl side chains in hand, the Ag(I)-promoted lactamisations were investigated (Scheme 55). Treatment of thiopeptide **176a** with Ag_2CO_3 (1.2 equiv.) in DCM afforded the cyclic tripeptide **177a** in 82% isolated yield. Similarly, treatment of linear thiopeptides **176b** and **176c** under the same conditions gave the corresponding cyclic peptides **177b** and **177c** in 79% and 76% yields, respectively. The structures of **177a-c** were confirmed by NMR spectroscopy. The ^{1}H NMR spectrum of the cyclised peptide **177a** contains a triplet at 7.59 with coupling constant 5.7 Hz, corresponding to the amide NH peak of the Lys–Asp side-chain amide bond. The ^{1}H NMR spectra of the cyclised peptides **177b-c** contain triplets at δ 7.73 ppm with a coupling constant of 5.5 Hz and δ 7.45 ppm with a coupling constant of 5.7 Hz, respectively, corresponding to the amide NH peaks of the Lys–Asp side-chain amide bond. The mass spectra of **177a-c** show molecular ion peaks consistent with the expected molecular formulas of the expected products **177a-c**. Lactam-stapled peptides **177a-c** were produced as single products in good yield with no competing formation of cyclodimers observed, demonstrating that the Ag(I)-

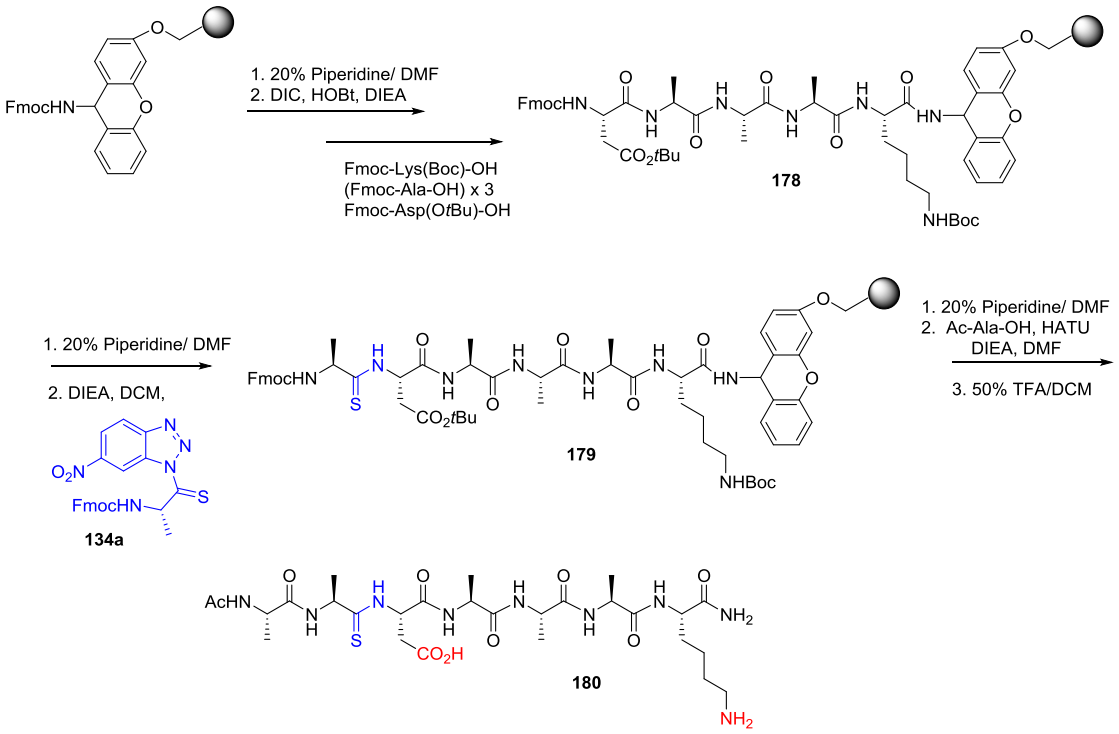
promoted lactamisation proceeds efficiently for various spacings of the Lys and Asp residues in the linear peptide sequences.



Scheme 55. Lactamisation of thiopeptides ***177a-c***

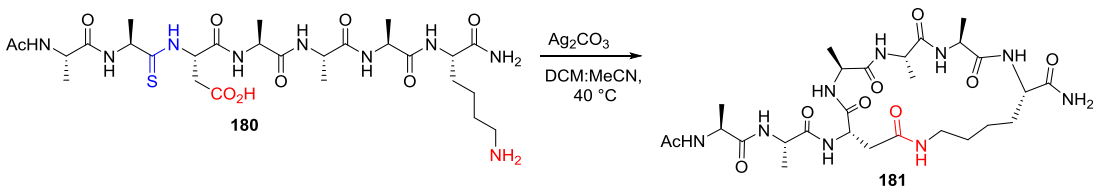
With successful formation of peptides **177a-c**, we next investigated the Ag(I)-promoted macrolactamisation with the positions of the Asp and Lys residues reversed: that is, the aspartic acid residue at the *N*-terminus and the lysine residue at the *C*-terminus. Synthesis of thiopeptide **180** was undertaken using standard SPPS protocols on Sieber amide resin (Scheme 56). The linear peptide **178** was first assembled, then after Fmoc deprotection the peptide was treated with alanine-derived thioacylating agent **134a** to give thiopeptide **179** on resin. Removal of the Fmoc protecting group, followed by coupling with *N*-Ac-Ala completed the SPPS steps. Treatment with 50% TFA/DCM resulted in removal of the Boc and *t*-butyl protecting groups and cleavage of the peptide from the resin, furnishing thiopeptide **180**. Confirmation of the linear thiopeptide

structure was obtained by mass spectrometry (ESI-MS: *m/z* [M+H$^+$] 674.3293 and [M+2H]$^{2+}$ 337.6682), corresponding to the molecular formula of the expected product.



*Scheme 56. Synthesis of thiopeptide **180***

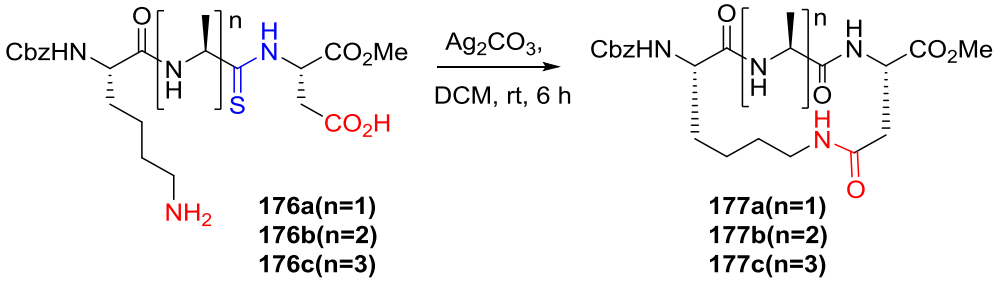
With heptathiopeptide **180** in hand, and without purification, the Ag(I)-promoted intramolecular macrolactamisation was investigated. Thioamide **180** was treated with Ag_2CO_3 under optimised conditions. The reaction was monitored using LC/MS which indicated successful conversion of peptide **180** to the cyclic peptide **181** (Scheme 57). The cyclic peptide **180** was purified using HPLC and analysed using mass spectrometry. Accordingly, the mass spectrum of **181** displays a molecular ion peak at *m/z* 640. 3417 corresponding to the molecular formula of the expected cyclised product.



*Scheme 57. Synthesis of cyclic peptide **181***

2.6 Conclusion

The Ag(I)-promoted coupling reaction has been shown to be an effective method for the synthesis of lactam-stapled peptides. The intramolecular Ag(I)-promoted coupling of the of thiopeptides **176a-c** containing (*i*,*i*+2)-, (*i*,*i*+3)-, and (*i*,*i*+4)-spaced Lys and Asp residues was shown to be an effective method to generate the Lys–Asp side-chain lactam bridged peptides **177a-c** in good yields.



4. Results and Discussion: Application of the Ag(I)-promoted thioamide coupling reaction to the synthesis of ^{18}F-labelled peptides

4.1 Background

Positron Emission Tomography (PET) is a powerful non-invasive molecular imaging technique that used in wide range of clinical diagnosis of diseases.[90-95] The method of PET produces three dimensional images of functional processes in the body through detection of the gamma photons generated upon electron–positron annihilation following spontaneous positron emission from specific radionuclides. Many radionuclides are used for the production of PET radio tracers, of which fluorine-18 is the most widely used (Table 3). Fluorine-18 represents a unique radionuclide candidate for the labelling of biologically-active molecules with a near quantitative decay profile and low energy of emission. In addition, the 110 minute half-life is sufficient to perform complex radiosynthesis while short enough to minimize radiation exposure.[96]

Radionuclide	Half-life (min)	$E_{max}(\beta^+)$ (KeV)	% β^+ Decay
^{15}O	2.1	1732	100
^{13}N	9.97	1190	100
^{11}C	20.3	961	100
^{68}Ga	67.6	1899	89
^{18}F	110	634	97
^{64}Cu	762	635	18

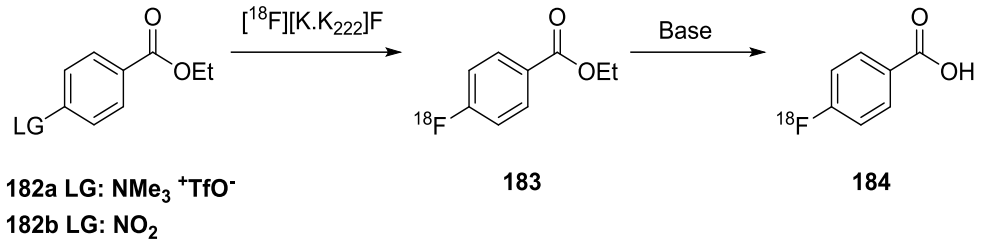
Table 3. Comparison of PET radionuclides

4.2 Strategies for radiolabelling peptides

[^{18}F]-Radiolabelled peptides are important biomolecules for the development of radiotracers used in PET to diagnose and monitor cancer.[97-98] Radiolabelled peptides have been designed to bind to receptors on cancer cells with high affinity and specificity. There are two main strategies for introduction of fluorine-18 into peptides.[99] Direct radiofluorination involves the introduction of fluorine-18 in the final step of radiotracer synthesis. Limitations of this method include the requirements of high temperature to introduce fluorine-18 into tracer molecules, aprotic water-free solvents, and the presence of reactive functional groups such as acidic hydrogens. In contrast, indirect fluorination involves initial radiofluorination of a small organic molecule known as a prosthetic group. This prosthetic group is then incorporated to the peptide *via* a second reaction such as acylation under mild conditions.

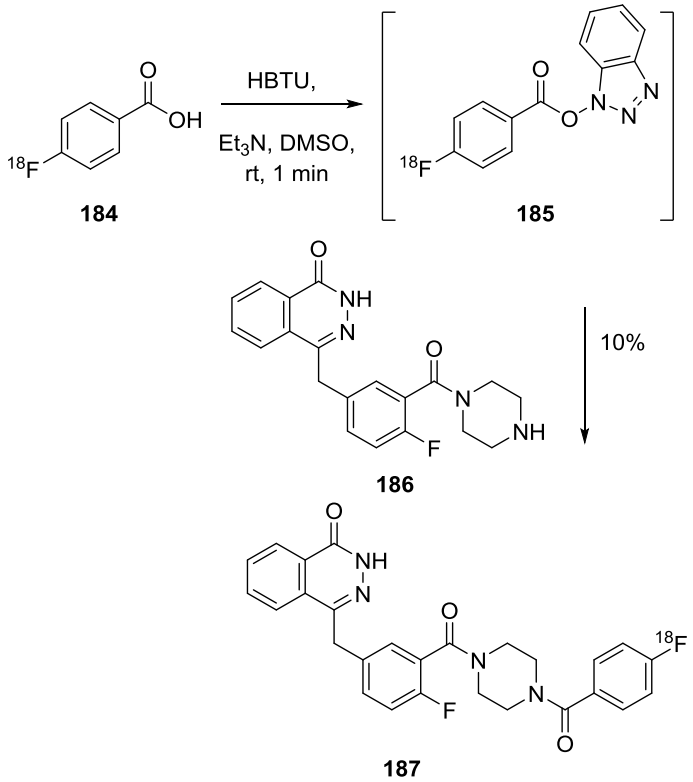
Among the range of acylation prosthetic groups, one of the most commonly used is *N*-succinimidyl-4-[^{18}F]fluorobenzoate [^{18}F]SFB **188**. The main advantage of labelling peptides with [^{18}F]-fluorobenzoic acid derivatives is that the aromatic C–F bond of [^{18}F]SFB **188** is stable towards hydrolysis.[100]

[^{18}F]-Fluorobenzoic acid **184** is synthesised from ethyl 4-nitrobenzoate **182a** or (4-ethoxycarbonylphenyl)-trimethylammonium triflate **182b** (Scheme 58). Nucleophilic aromatic substitution with [^{18}F]-fluoride, followed by hydrolysis of the ethyl ester under basic conditions generates **184**, which is purified by solid-phase extraction (SPE).[101-102]



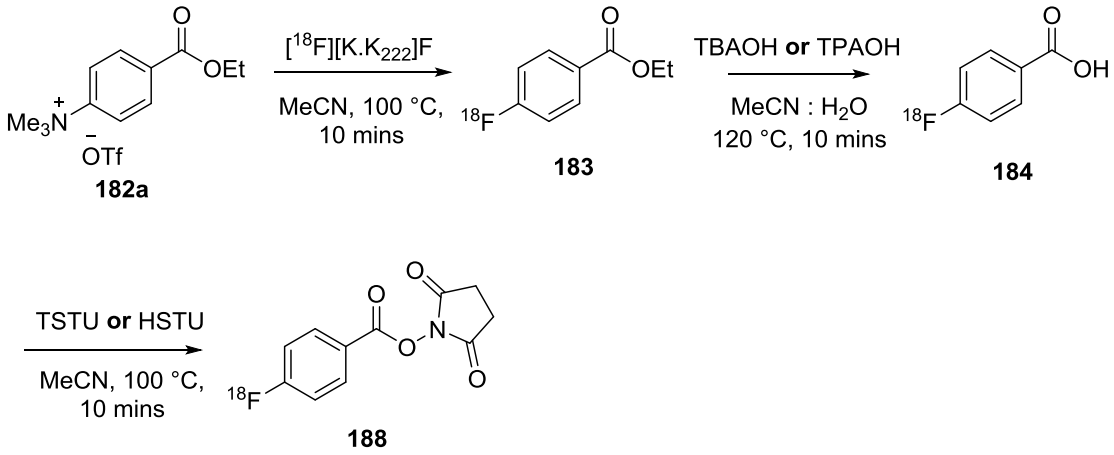
*Scheme 58. Synthesis of [^{18}F]fluorobenzoic acid **184***

The purified [^{18}F]fluorobenzoic acid **184** is then converted into active ester *in situ* using a coupling reagent to form a good leaving group, followed by treatment with an amine to form an amide. For example, activation of [^{18}F]-fluorobenzoic acid **184** with HBTU generated activated ester **185**, which following treatment with amine **186** generated the amide **187** in 10% radiochemical yield (Scheme 59).[103]



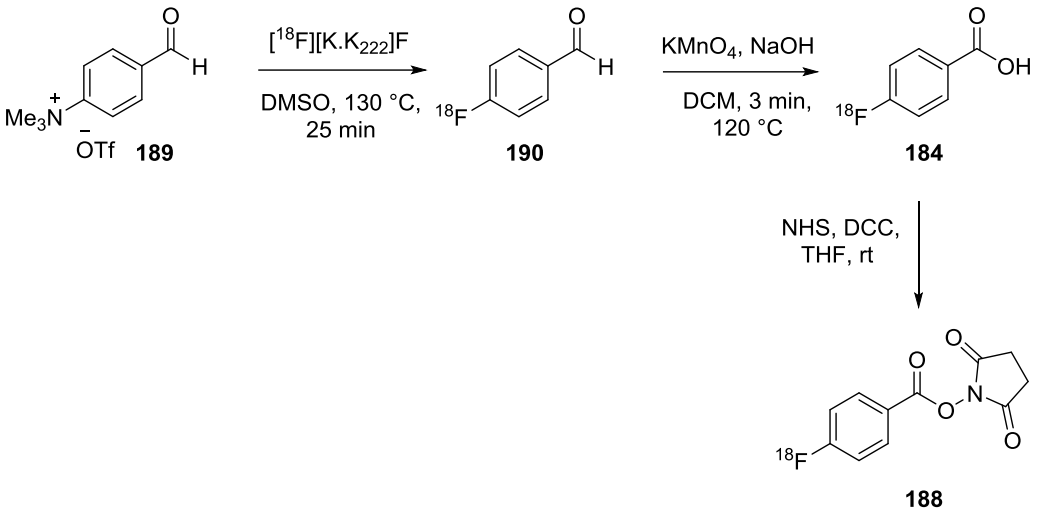
*Scheme 59. Synthesis of the amide **187***

Alternatively, radiolabelled active esters such as [^{18}F]SFB **188** can be prepared and purified prior to the coupling to peptides. There are two common methods to prepare ([^{18}F]SFB) **188**. The first method was first reported by Tang and co-workers and involves a one-pot, three-step procedure (Scheme 60). Following preparation of [^{18}F]fluorobenzoic acid **184**, treatment with TSTU or HSTU generates [^{18}F]SFB **188** that is typically purified by trapping on a C18 SPE cartridge. [^{18}F]SFB **188** is obtained in up to 75% radiochemical yield in approximately 40 mins.



*Scheme 60. Three-step synthesis of [^{18}F]SFB **188***

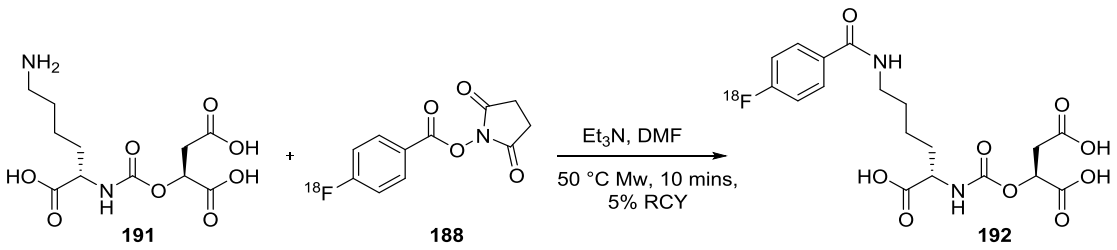
The second method to synthesise [^{18}F]SFB **188** is *via* 4-[^{18}F]fluorobenzaldehyde **190** as reported by Zalutsky and co-workers.[104] 4-(Trimethylammonium)benzaldehyde **189** underwent radiofluorination to give the corresponding 4-[^{18}F]fluorobenzaldehyde **190**. Oxidation of [^{18}F]fluorobenzaldehyde **190** with $KMnO_4$ at 120 °C gave [^{18}F]FBA **184** (Scheme 61). Treatment of **184** with *N*-hydroxysuccinimide (NHS) in the presence of DCC in dry THF for 10–60 min at room temperature gave [^{18}F]SFB **188** in 25% overall radiochemical yield after HPLC purification.



*Scheme 61. Synthesis of [^{18}F]SFB **188***

In order to improve preparation of [^{18}F]SFB **188** from [^{18}F]fluorobenzaldehyde **190**, Glaser and co-workers used (diacetoxyiodo)benzene as an oxidant in the presence of NHS to give [^{18}F]SFB **188** in 49±6% decay-corrected overall radiochemical yield after HPLC purification.[105] However, Ganguly and co-workers recently used Glaser's method and obtained [^{18}F]SFB **188** only 25% decay-corrected overall radiochemical yield using this procedure.[106]

[^{18}F]SFB **188** has been applied in the labelling of a range of peptides used as PET radiotracers.[107] For example, a tracer for visualisation of Prostate-Specific Membrane Antigen **192** (PSMA) was generated by treatment of **191** with [^{18}F]SFB **188** to give **192** in 5% radiochemical yield (Scheme 62).[108]

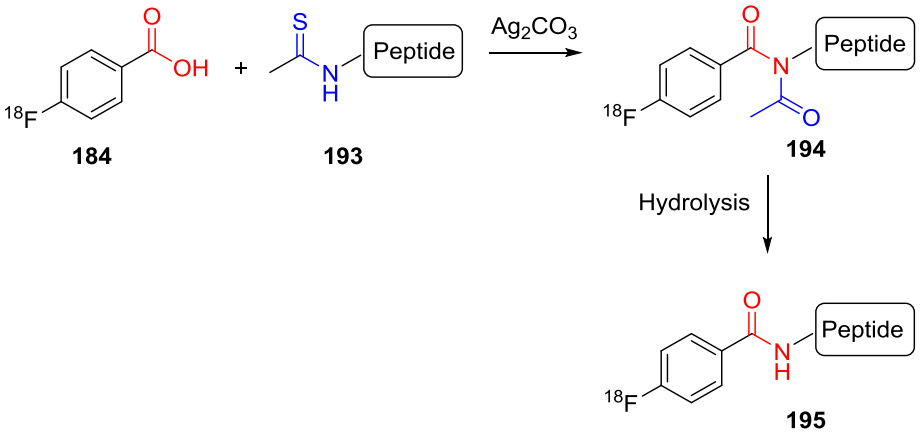


*Scheme 62. Synthesis of ^{18}F-PSMA derivative **192***

Whilst the use of [^{18}F]-fluorobenzoic acid-derived activated esters was demonstrated in the synthesis of a wide range of peptides radiotracers, limitations include hydrolysis of the activated esters and low radiochemical yield obtained due to the lengthy synthetic steps. Direct coupling of [^{18}F]-fluorobenzoic acid **184** with peptides may be able to overcome these issues.

4.3 Proposed thioamide-based synthesis of [^{18}F]-labelled peptides

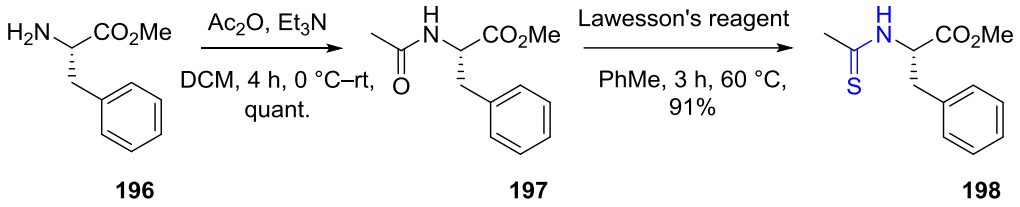
Given our previous work on the synthesis of peptides through the Ag(I)-promoted coupling of peptide acids with thioamides, we envisage that the Ag(I)-promoted coupling of [^{18}F]-fluorobenzoic acid **184** could facilitate its direct attachment to peptides (Scheme 63). We propose that the Ag(I)-promoted coupling of a peptide thioamide **193** and [^{18}F]-fluorobenzoic acid **184** would generate a radiolabelled imide **194** which could undergo hydrolysis to give the amide **195**.



*Scheme 63. Proposal of peptides thioamide-based radiolabelling with [^{18}F]-fluorobenzoic acid **184***

4.4 F-19 standard

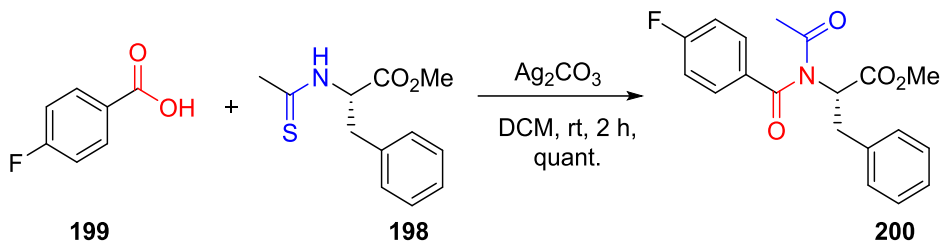
In order to investigate the Ag(I)-promoted coupling of 4-fluorobenzoic acid and thioamides, investigation of a model process using ^{19}F-4-fluorobenzoic acid was first undertaken. *N*-Thioacetyl phenylalanine methyl ester **198** was chosen as a model thioamide substrate, and was prepared in two steps from phenylalanine methyl ester **196**. Treatment of **196** with acetic anhydride gave acetamide **197**, which was treated with Lawesson's reagent to generate thioamide **198** in 91% yield, over two steps (Scheme 64).



196 **197** **198**

*Scheme 64. Synthesis of thioamide **198***

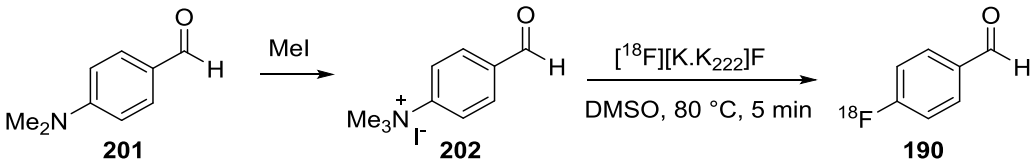
The coupling of model thioamide **198** with 4-fluorobenzoic acid **199** was then performed according to the procedure established by our group. Thioacetamide **198** was treated with 4-fluorobenzoic acid **199** in the presence of Ag_2CO_3 (1.5 equiv.) in DCM to generate the corresponding imide **200** in a quantitative yield (Scheme 65). The structure of **200** was confirmed by NMR spectroscopy. The ^{1}H NMR spectrum of imide 2**00** displayed two multiplets (2H each) at 7.14–7.05 ppm and 7.04–6.94 ppm indicative of a 4-fluorobenzoyl group. Moreover, the presence of a three-proton singlet peak at δ 1.89 ppm corresponds to the *N*-Ac group further confirming the structure of the product. In addition, mass spectrometric analysis showed a molecular ion at *m/z* 344.1292, which corresponds to the molecular formula of the imide **200**.



*Scheme 65. Coupling of thioamide **198** with 4-fluorobenzoic acid **199***

4.5 Synthesis of [^{18}F]-fluorobenzoic acid 184

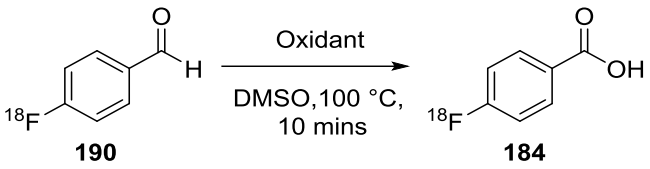
We investigated preparation [^{18}F]-fluorobenzoic acid **184** from [^{18}F]-fluorobenzaldehyde **190**. Accordingly, preparation the ammonium salt **202** was required. The ammonium salt **202** was prepared in 72% yield by treatment of 4-(dimethylamino)benzaldehyde **201** with methyl iodide. Treatment of **202** under typical radiofluorination conditions reaction gave [^{18}F]-fluorobenzaldehyde **190** in 88% decay corrected radiochemical yield (Scheme 66).



*Scheme 66. Synthesis of [^{18}F]-fluorobenzaldehyde **190***

The oxidation of [^{18}F]flurobenzaldehyde **190** to [^{18}F]-fluorobenzoic acid **184** was then investigated (Scheme 67). When aldehyde **190** treated with hydrogen peroxide at 100 $^{\circ}$C for 10 minutes, only trace amount of the oxidised product **184** was detected. When the reaction was repeated with *tert*-butylperoxide, the acid **184** was obtained in 20% RCC (radiochemical conversion). Using alkaline permanganate solution as an oxidant gave the acid **184** in 58% RCC, however, due to problems arising from the intense colour of permanganate solution, it was not explored further in automatic system. When the reaction was repeated using sodium chlorite (10.0 equivalents) in water,

[^{18}F]-fluorobenzoic acid **184** was generated in 85% RCC with >99% radiochemical purity (Figure 5). We found that these oxidation conditions can be applied to automated radiochemical synthesis systems to synthesise [^{18}F]-fluorobenzoic acid **184** in a one-pot, two-step procedure.



Entry	Oxidant	RCC (%)
1	30% H_2O_2/H_2O	3
2	*Tert*-butylperoxide	17
3	$KMnO_4/NaOH$	58
4	$NaClO_2/H_2O$	85

*Scheme 67. Manual optimisation of oxidation of [^{18}F]-fluorobenzaldehyde **190***

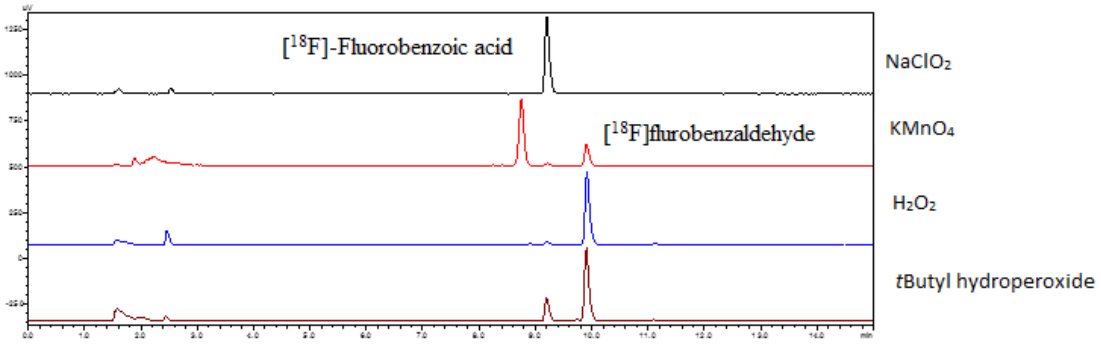
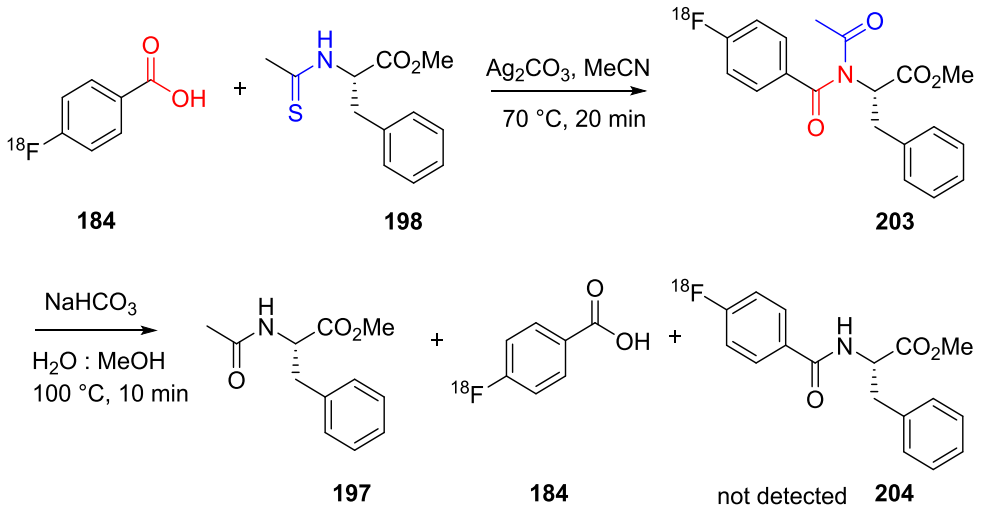


*Figure 5. RP-HPLC radiotraces of oxidation of [^{18}F]-fluorobenzaldehyde **190***

4.6 Attempted synthesis of radiolabelled peptide 204

With a protocol for [^{18}F]-fluorobenzoic acid **184** generation in hand, the Ag(I)-promoted coupling with model thioamide **198** was investigated. The thioamide **198** was treated with [^{18}F]-fluorobenzoic acid **184** in the presence of Ag_2CO_3 in MeCN at 70 ˚C for 20 min (Scheme 68). Analysis of the reaction mixture by radio RP-HPLC showed that the radiolabelled imide **203** formed in 96% RCC yield (Figure 6). Subsequent hydrolysis of the imide was effected by treatment with sodium hydrogen carbonate in MeOH/H_2O, which gave the amide **197** together with [^{18}F]-fluorobenzoic acid **184**. That is, hydrolysis of the imide **203** proceeds selectively at the fluorobenzoyl group, presumably as this is the most electrophilic acyl position. Thus, this approach is not applicable to the generation of [^{18}F]-fluorobenzoylated peptides.



*Scheme 68. Attempted synthesis of radiolabelled peptide **204***

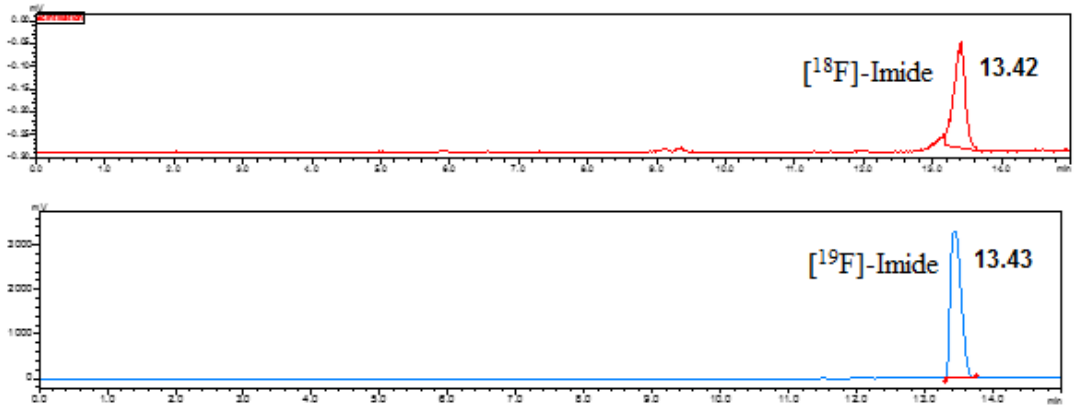
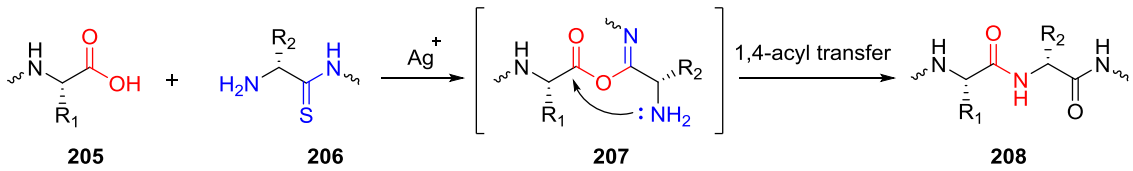


Figure 6. Radiotrace of reaction mixture containing product **203** *(red) and RP-HPLC trace of cold standard* **200** *(blue) (UV=254 nm).*

4.7 Synthesis of radiolabelled peptide 215

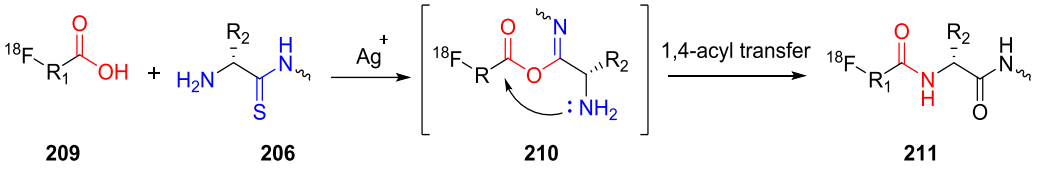
With the unsuccessful generation of radiolabelled amide **204**, we turned our attention to investigation a modified thioamide system that can provide radiolabelled peptides through the Ag(I)-promoted reaction. Previous work in the Hutton group developed a method for peptide synthesis through the Ag(I)-promoted reaction of *N*-terminal peptide thioamides with peptide *C*-terminal carboxylates (Scheme 69).



Scheme 69. Proposed mechanism for the Ag(I)-promoted ligation

Accordingly, we envisaged that this method might be applicable to synthesis of radiolabelled peptides as it results in direct amide bond formation rather than proceeding via an imide. We proposed that a Ag(I)-promoted coupling of a peptide possessing a thioamide **206** adjacent to an amine group with a radiofluorinated

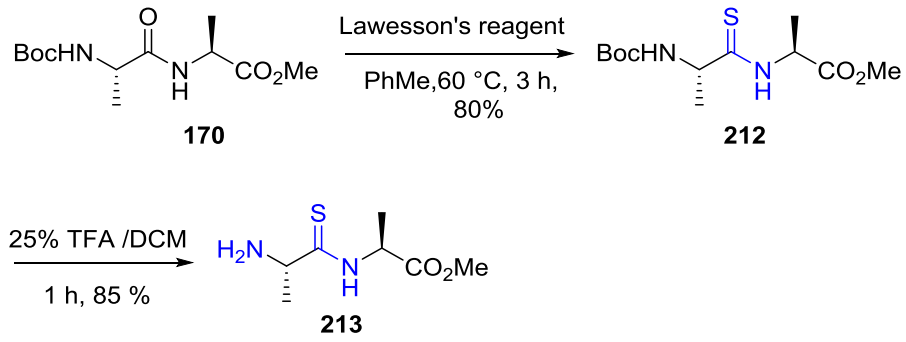
carboxylate **209** would generate an isoimide intermediate **210**, which could undergo 1,4-acyl transfer resulting in conjugation of the fluorinated group to give a radiolabelled peptide **211** (Scheme 70).



Scheme 70. Proposed mechanism of the Ag(I)-promoted coupling

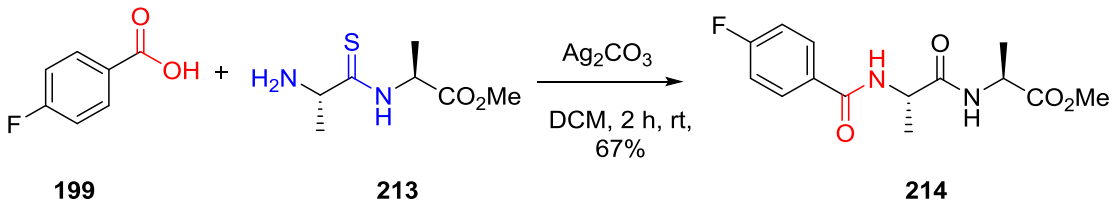
4.8 Preliminary investigation on Ag(I)-promoted coupling/radiolabelling of peptides

In order to investigate the Ag(I)-promoted coupling of [^{18}F]-fluorobenzoic acid **184** with thioamides, investigation of model system **213** was first undertaken. The dipeptide thioamide **213** was prepared in two steps starting from dipeptide **170**. Treatment of dipeptide **170** with Lawesson's reagent gave dipeptide thioamide **212** in high yield. Removal of the Boc-group under acidic conditions gave the amine **213** in good yield (Scheme 71).



*Scheme 71. Preparation of dipeptide thioamide **213***

With dipeptide thioamide **213 –** possessing an unprotected amine – in hand, coupling to 4-fluorobenzoic acid **199** was investigated. Accordingly, treatment of dipeptide thioamide **213** with 4-fluorobenzoic acid **199** in the presence of Ag_2CO_3 under optimised conditions, gave the fluorobenzoylated peptide **214** in 67% yield (Scheme 72). The product **214** was analysed by ^{1}HNMR spectroscopy and two resonances were observed at δ 7.85–7.77 ppm (m, 2H) and 7.15–7.05 ppm (m, 2H) that correspond to the 4-fluorobenzoyl group. The product **214** was also analysed by mass spectrometry and displayed a protonated molecular ion at *m/z* 297.144 corresponding to the molecular formula of the expected product.

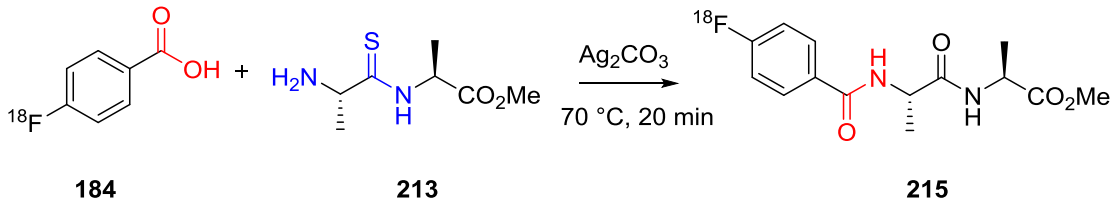


199 **213** **214**

*Scheme 72. F-19 cold standard synthesis of peptide **214***

Given the successful coupling of 4-[^{19}F]fluorobenzoic acid **199** with dipeptide thioamide **213**, we next investigated whether the Ag(I)-promoted coupling was applicable to coupling of the radiolabelled [^{18}F]-fluorobenzoic acid **184** counterpart (Scheme 73). Accordingly, the dipeptide thioamide **213** was treated with [^{18}F]-fluorobenzoic acid **184** in the presence of Ag_2CO_3 (1.5 equiv.) in chloroform at 70 °C. HPLC analysis of the crude reaction mixture showed that the radiolabelled peptide **215** was formed in 44% yield with 56% recovery of the [^{18}F]-fluorobenzoic acid **184**. A co-injection with the previously synthesised cold standard **214** showed product peaks with a matching retention time of 8.37 mins, indicating the peptide had been

radiolabelled to give the [^{18}F]-fluorinated peptide **215** (Figure 7). When the reaction repeated in acetonitrile or DMSO, the yield of the ligated product **215** dropped to 25%.



*Scheme 73. Synthesis of radiolabelled peptide **215***

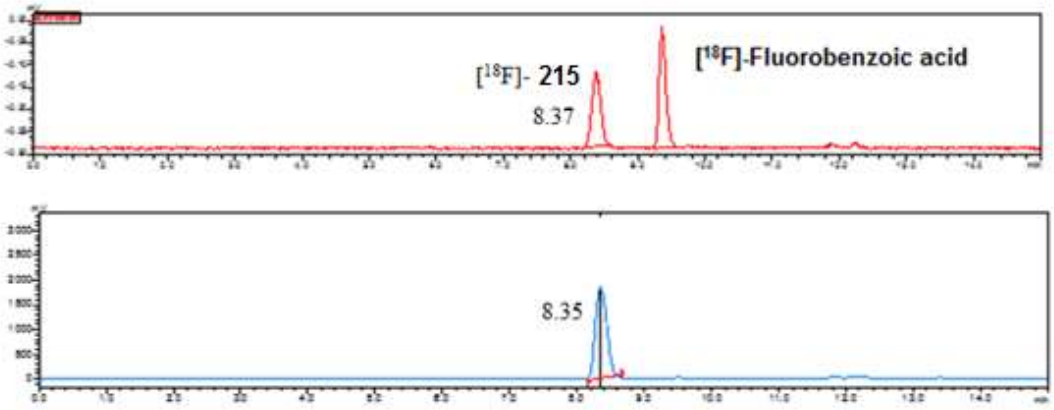


Figure 7. Radiotrace of reaction mixture containing product **215** *(red), and RP-HPLC trace of cold standard* **214** *(blue) (UV=254 nm).*

4.9 Conclusion and future work

The results described in this chapter highlight new methods for the synthesis of ^{18}F-radiolabelled peptides through a Ag(I)-promoted coupling method. We have investigated two approaches in the synthesis of ^{18}F-radiolabelled peptides using the Ag(I)-promoted coupling reaction. Reaction of [^{18}F]-fluorobenzoic acid **184** with thioamide **198** in the presence of Ag_2CO_3 generated imide **203** in 96% RCC. However,

subsequent treatment of the imide **203** under mildly basic conditions did not generate the desired amide **204**: instead hydrolysis of imide **203** favoured cleavage of the [^{18}F]-fluorobenzoyl group due to the increased electrophilicity at this site.

We subsequently demonstrated that the Ag(I)-promoted coupling of a peptide possessing a thioamide group at the *N*-terminal position with [^{18}F]-fluorobenzoic acid is effective for generating [^{18}F]-fluorobenzoylated peptides. Reaction of peptide thioamide **213** with [^{18}F]-fluorobenzoic acid **184** in the presence of Ag_2CO_3 gave the radiolabelled peptide **215** in 44% RCC. Optimisation of the reaction conditions and the application of this method toward synthesis of radiolabelled peptides will be the focus of future endeavours.

5.Experimental

5.1 General Information

Reactions requiring anhydrous conditions were carried out under an atmosphere of dry nitrogen or argon in flame- or oven-dried glassware. Anhydrous dichloromethane (DCM), tetrahydrofuran (THF) and dimethylformamide (DMF) were obtained from a solvent dispensing system where the solvent was dried by passage through two columns of neutral alumina. Most reagents were commercially available reagent grade chemicals and were used without further purification.

Analytical thin layer chromatography (TLC) was performed with aluminium-backed plates precoated with silica gel 60 F254 (0.2 mm), and chromatograms were visualised using short- and long-wave UV light. Compounds were stained with phosphomolybdic acid dip [phosphomolybdic acid (5 g), absolute ethanol (100 ml)]. Column chromatography was performed using silica gel (230–400 mesh); eluting solvents are reported as %volume/volume mixtures.

Analytical and preparative reverse phase HPLC (RP-HPLC) were performed using an Agilent 1200 series LC System. Analytical HPLC employed a Discovery C18 column (4.6 × 150 mm column, 5 μm particle size, flow rate of 1 mL min^{-1}). Preparative RP-HPLC employed a Phenomenex C18 column (21.2 × 150 mm, 5 μm particle size, flow rate 6 mL min^{-1}). The mobile phase consisted of eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). The results were analysed on Agilent ChemStation version B.01.03 software.

^{1}H NMR spectra were recorded using an Agilent 500 (500 MHz) or a Varian Unity Inova 400 (400 MHz) spectrophotometer. Spectra were obtained in $CDCl_3$ (7.26) or d_6-DMSO (2.50). The spectra are reported as: parts per million (ppm) downfield shift, relative to the residual solvent peak; relative integral, multiplicity (s = singlet, br = broad, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, dq doublet of quartets, m = multiplet) and

coupling constant (*J* in Hz). ^{13}C NMR spectra were recorded using an Agilent 500 (125 MHz) or a Varian Unity Inova 400 (101 MHz) spectrophotometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the internal standard of the solvent peak; $CDCl_3$ (77.16), or d_6-DMSO (39.52). Mass spectra were obtained using an MSFP OrbiTRAP infusion mass spectrometer.

5.2 General procedures

5.2.1 General procedure A: Peptide couplings in solution phase

To a solution of an *N*-protected amino acid (1.0 mmol) and an amino acid ester (1.0 mmol) in DMF (10 mL) at 0 °C was added EDC.Cl (1.1 mmol), HOBt (1.1 mmol) and DIEA (2.2 mmol), and stirred for 15 min. The reaction mixture was then stirred at room temperature overnight. The mixture was diluted with EtOAc (30 mL) and water (10 mL). The aqueous layer was extracted with EtOAc (2 × 20 mL) and the combined organic extracts were washed with aqueous HCl (30 mL, 1N), saturated aqueous $NaHCO_3$ (30 mL), brine (30 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (5% MeOH/DCM) to afford the desired peptide.

5.2.2 General procedure B: Formation of thioamides using Lawesson's reagent at 60°C

To a solution of the dipeptide (1.0 mmol) in toluene (10 mL) was added Lawesson's reagent (0.6 mmol). The reaction mixture was heated at 60 °C for 3 h. The solvent was evaporated under reduced pressure. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the thioamide product.

5.2.3 General Procedure C: Formation of thioamides using P_4S_{10} at room temperature

To a solution of phosphorus pentasulfide (0.7 mmol) in dry THF (10 mL) was added anhydrous sodium carbonate (0.7 mmol) and the mixture was stirred at room temperature for 1 h under

argon. The mixture was cooled to 0 °C and the amide (1.0 mmol) was added and the mixture was stirred at room temperature for 3 h. The mixture was filtered through a pad of Celite, and the filtrate was concentrated under reduced pressure. The residue was dissolved in EtOAc (25 mL), and was washed with aqueous $NaHCO_3$ (2 × 15 mL, 5%) and brine (20 mL) and dried with Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (50% EtOAc/Hex) to give the thioamide product.

5.2.4 General Procedure D: Formation of nitrobenzotriazolide

A thioanilide (0.2 mmol) was dissolved in 95% glacial acetic acid (2.0 mL), and cooled to 0 °C. Sodium nitrite (0.3 mmol) was added and the mixture was stirred at 0 °C for 30 min. The reaction was quenched with ice-water (10 mL), and the precipitated product was filtered and washed with cold water (3 × 10 mL). The crude product was dried to give the benzotriazole which was used in the next step without purification.

5.2.5 General Procedure E: Synthesis of amides (132a–f)

To a solution of the Fmoc-amino acid **131** (5.0 mmol) in THF (50 mL), cooled to –20 °C, was added dropwise *N*-methylmorpholine (1.1 mL, 10 mmol) and isobutylchloroformate (0.714 mL, 5.5 mmol), and the mixture was stirred for 15 min under Ar. Diaminonitrobenzene **113** (0.842 g, 5.5 mmol) was added and the solution was stirred at –20 °C for 2 h, then at room temperature overnight under Ar. The solvent was removed under reduced pressure, and the residue was dissolved in DMF (20 mL). Upon addition of brine (100 mL), a yellow solid precipitated. The precipitate was filtered and rinsed with cold water. The crude mixture was purified by column chromatography (5% MeOH/DCM) to give the amide **132**.

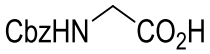
5.2.6 General Procedure F: Synthesis of thiopeptides on solid support

Thiopeptides were synthesised using standard Fmoc SPPS coupling methods on Sieber amide resin (100-200 mesh, 0.51 mmol/g loading) in 12 mL fritted syringes. The resin was swelled in DMF (6 mL) for 1 hour, then the solvent was drained. All peptides were synthesised on 0.1 mmol scale using 4 equiv. of Fmoc-amino acid (0.4 mmol for a 0.1 mmol scale) that were activated using 4 equiv. of HATU in the presence of DIEA (8 equiv.). The coupling reactions were stirred for 1 hour at room temperature. Fmoc removal was performed using 20% v/v piperidine in DMF (2 × 5 min). The peptides were elongated up to the thioamide position. Before thioamide coupling, the resin was washed with dry DCM (2 × 5 mL). Next, the required Fmoc-amino acid thiobenzotriazolide **134** (0.15 mmol. 1.5 equivalents) and DIEA (0.15 mmol,1.5 equivalents) in dry DCM (5 mL) were added and the reaction was stirred for 1 hour. After Fmoc deprotection, the remaining amino acids were coupled using standard SPPS. Peptides were cleaved from the resin using (2% TFA in DCM, 6 mL) solution for 30 min. The solutions were then drained and collected and this process was repeated a further time. The solutions were concentrated to a yellow oil under a stream of nitrogen. The crude peptides were then precipitated by trituration with cold diethyl ether (10 mL) and the mixtures were centrifuged at 3000 rpm for 5 min. The collected precipitates were washed with cold diethyl ether (3 × 10 mL) followed by centrifugation. Thiopeptides were purified by RP-HPLC using gradient elution with buffer B 5–50% over 30 minutes, monitoring at a wavelength of 214 nm.

5.2.7 General Procedure G: Synthesis of glycopeptides peptides 139a–f

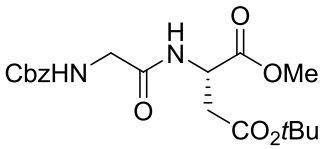
To a solution of the thiopeptide (1.0 equiv.) in DCM:ACN (1:1, 20 mL/mmol), was added Ag_2CO_3 (1.2 equiv.) and aminosugar **14** (4.0 equiv.) The mixture was stirred at room temperature for 6 h. The black Ag_2S precipitate was removed by centrifugation. The glycopeptide was purified by RP-HPLC using gradient elution with buffer B 5–70% over 30 minutes, monitoring at a wavelength of 214 nm.

Cbz-Gly-OH (93)



To a solution of L-glycine (**92**) (1.00 g, 13.3 mmol) in aqueous sodium hydroxide (2 M, 20 mL) at 0 °C was added dropwise benzyl chloroformate (3.2 mL, 22.6 mmol) and the mixture was stirred for 18 h. The mixture was acidified with concentrated hydrochloric acid to pH 1 then was extracted with EtOAc (3 × 50 mL). The organic layers were dried over $MgSO_4$. The solvent was evaporated under reduced pressure to give the product **93** (2.56 g, 92%). The protected amino acid **93** was sufficiently pure to be used in the next step without further purification; **^{1}H NMR** (400 MHz, DMSO-d_6) δ 12.54 (s, 1H), 7.54 (t, *J*=6.0 Hz, 1H), 7.40 – 7.25 (m, 7H), 5.02 (s, 2H), 3.65 (d, *J*=6.2 Hz, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.0, 157.0, 137.5, 128.8, 128.3, 128.2, 65.9, 42.6. **MS** (ESI) *m/z* 210 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{10}H_{12}NO_4$ 210.0761, found 210.0761.

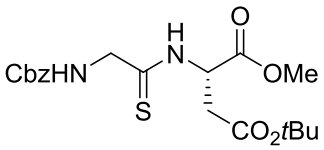
Cbz-Gly-Asp(OtBu)-OMe (95)



Dipeptide **95** was prepared from H-Asp(OtBu)-OMe hydrochloride (**94**) (316 mg, 1.32 mmol) and Cbz-Gly (276 mg, 1.32 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **95** (435 mg, 92%) as colourless crystals; R_f 0.33 (10% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.45–7.26 (m, 5H), 6.95 (d, *J*=8.1 Hz, 1H), 5.46 (m, 1H), 5.12 (s, 2H), 4.83 (dt, *J*=8.6, 4.5 Hz, 1H), 4.01–3.83 (m, 2H), 3.73 (s, 3H), 2.93 (dd, *J*=17.0, 4.5 Hz, 1H), 2.72 (dd, *J*=17.2, 4.5 Hz, 1H), 1.42 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.0, 170.0, 169, 136.1, 128.5, 128.2, 128.1, 82.0, 67.2, 52.7, 48.6, 44.4, 37.3, 28.0.

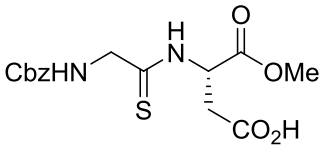
MS (ESI) *m/z* 395 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{19}H_{27}N_2O_7$ 395.1813, found 395.1813.

Cbz-Gly$^{[S]}$-Asp(O*t*Bu)-OMe (96)



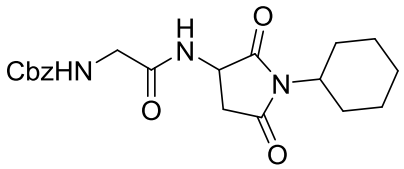
Compound **96** was prepared from dipeptide **95** (193 mg, 0.49 mmol) according to General Procedure B. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the title compound **96** (165mg, 82%) as a light yellow oil; R_f 0.50 (50% EtOAc/Hex).**^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 7.40–7.27 (m, 5H), 5.57 (s, 1H), 5.42 (dd, *J*=10.1, 6.2 Hz, 1H), 5.18–5.09 (m, 2H), 4.31 (dd, *J*=17.3, 6.1 Hz, 1H), 4.22 (dd, *J*=17.3, 6.0 Hz, 1H), 3.76 (s, 3H), 3.0 –2.91 (m, 2H), 1.42 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 199.4, 170.2, 169.8, 156.6, 135.70, 128.6, 128.3, 128.1, 82.2, 67.4, 53.7, 52.9, 52.1, 35.97, 27.9. **MS** (ESI) *m/z* 411 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{19}H_{27}N_2O_6S$ 411.1584, found 411.1584.

Cbz-Gly$^{[S]}$-Asp-OMe (97)



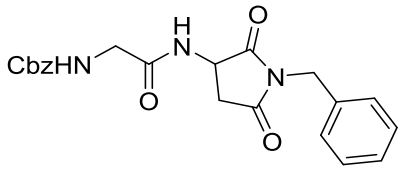
To a solution of **96** (100 mg, 0.24 mmol) in DCM (2 mL) at 0 °C was added TFA (2mL) and the solution was stirred for 1 h. The solvent and TFA were evaporated to afford the title compound **97** (84 mg) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 355 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{15}H_{19}N_2O_6S$ 355.0958, found 355.0959.

Benzyl (2-((1-cyclohexyl-2,5-dioxopyrrolidin-3-yl)amino)-2-oxoethyl)carbamate (102)



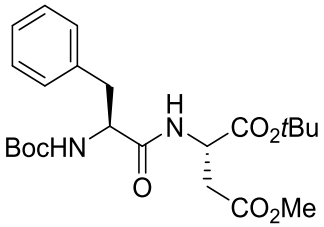
To a solution of **97** (29 mg, 0.08 mmol) in DCM (2 mL) was added Ag_2CO_3 (33 mg, 0.12 mmol) and cyclohexylamine **98** (19 µL 0.16 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue was purified by column chromatography (5–15% MeOH/DCM) to give the title compound **102** (23 mg, 54%) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.39–7.28 (m, 5H), 7.01 (d, *J*=6.2 Hz, 1H), 5.54 (s, 1H), 5.11 (s, 2H), 4.30 (dd, *J*=15.2, 6.4 Hz, 1H), 3.98 (m, 1H), 3.89 (d, *J*=5.5 Hz, 2H), 2.99 (dd, *J*=17.7, 9.1 Hz, 1H), 2.66 (m, 1H), 2.10 (q, *J*=12.5 Hz, 2H), 1.81 (d, *J*=12.3 Hz, 2H), 1.65 (m, 3H), 1.24 (m, 3H). **MS** (ESI) *m/z* 388 [$(M+H)^+$,100%]. **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{20}H_{26}N_3O_5$ 388.1867, found 388.1864.

Benzyl (2-((1-benzyl-2,5-dioxopyrrolidin-3-yl)amino)-2-oxoethyl)carbamate (103)



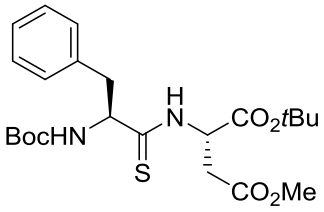
To a solution of **97** (29 mg, 0.08 mmol) in DCM (2 mL) was added Ag_2CO_3 (33 mg, 0.12 mmol) and benzylamine (18 µL 0.16 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by column chromatography (5–15% MeOH/DCM) to give the title compound **103** (18 mg, 57%) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.3 –7.22 (m, 10H), 7.05 (s, 1H), 5.66–5.57 (m, 1H), 5.06 (m, 2H), 4.69–4.59 (m, 2H), 4.33 (d, *J*=5.8 Hz, 1H), 3.84 (d, *J*=4.4 Hz, 2H), 2.96 (dd, *J*=17.4, 8.8 Hz, 1H), 2.68 (m, 1H). **MS** (ESI) *m/z* 396 [$(M+H)^+$,100%]; **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{21}H_{22}N_3O_5$ 396.1554, found 396.1557.

Boc-Phe-Asp(OMe)-O*t*Bu (106)



Dipeptide **106** was prepared from *N*-Boc-Phe **104** (266 mg, 1.0 mmol) and H-Asp(OMe)-OtBu hydrochloride **105** (240 mg, 1.0 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **106** (415 mg, 92%) as colourless crystals; R_f 0.33 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.34–7.10 (m, 5H), 6.82 (d, *J*=7.5 Hz, 1H), 4.93 (d, *J*=8.2 Hz, 1H), 4.63 (m, 1H), 4.39 (m, 1H), 3.65 (s, 3H), 3.08 (m, 2H), 2.93 (dd, *J*=16.9, 4.3 Hz, 1H), 2.79 (dd, *J*=16.8, 5.0 Hz, 1H), 1.43 (s, 9H), 1.39 (s, 9H).**^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.0, 170.9, 169.0, 155.2, 136.3, 129.3, 128.6, 126.9, 82.6, 80.1, 55.4, 51.8, 49.3, 38.3, 36.3, 28.2, 27.8.**MS** (ESI) *m/z* 451 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{23}H_{35}N_2O_7$ 451.2439, found 451.2441.

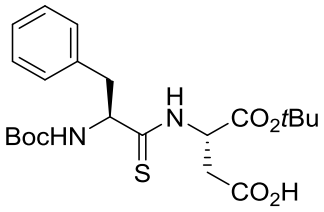
Boc-Phe$^{[S]}$-Asp(OMe)-O*t*Bu (107)



Compound **107** was prepared from dipeptide **106** (193 mg, 0.49 mmol) according to General Procedure B. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the title compound **107** (165mg, 82%) as a light yellow oil; R_f 0.50 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.44 (d, *J*=7.2 Hz, 1H), 7.34–7.09 (m, 5H), 5.16 (m, 1H), 5.07 (m, 1H), 4.73–4.52 (m, 1H), 3.63 (s, 3H), 3.17 (m, 2H), 3.08 (dd, *J*=17.1, 4.9 Hz, 1H), 2.99 (dd, *J*=17.1, 4.0 Hz, 1H), 1.41 (s, 9H), 1.38 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 202.8, 170.8, 168.1, 154.9, 136.2, 129.2, 128.6, 127.0, 83.2, 80.4, 62.6, 54.1, 51.9, 41.5, 34.7, 28.2, 27.8.

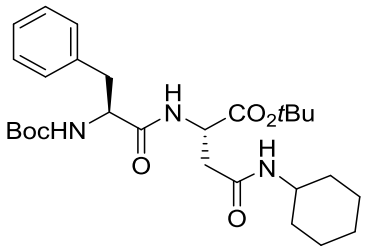
MS (ESI) *m/z* 467 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$)calcd. for $C_{23}H_{35}N_2O_6S$ 467.2210, found 467.2202.

Boc-Phe$^{[S]}$-Asp-O*t*Bu (108)



To a solution of **107** (150 mg, 0.32 mmol) in a mixture of MeOH:H_2O (1:1, 4 mL) cooled to 0 °C, was added LiOH.H_2O (40 mg,0.96 mmol). The mixture was stirred for 10 min at 0 °C and 1 h at room temperature. The solution was concentrated, diluted with water (10 mL), then acidified to pH 4 with aqueous HCl (1 M). The solution was extracted with EtOAc (3 × 10 mL) and washed with water (20 mL), brine (20 mL) and dried with Na_2SO_4. The solvent was evaporated under reduced pressure to give the crude acid **108** which was used in the next step without further purification. **MS** (ESI) *m/z* 453 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{22}H_{33}N_2O_6S$ 453.2054, found 453.2057.

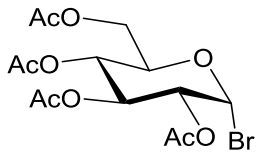
Boc-Phe-Asn(cyclohexyl)-O*t*Bu (109)



To a solution of **108** (36 mg, 0.08 mmol) in DCM (2 mL) was added Ag_2CO_3 (33 mg, 0.12 mmol) and cyclohexylamine **98** (19 µL, 0.16 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **109** (22 mg, 54%) as a white solid.

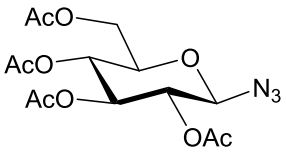
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31–7.17 (m, 5H), 7.04 (d, *J*=7.9 Hz, 1H), 5.70 (d, *J*=8.1 Hz, 1H), 4.95 (d, *J*=8.0 Hz, 1H), 4.60 (m, 1H), 4.35 (m, 1H), 3.69 (m, 1H), 3.14 (dd, *J*=13.9, 5.6 Hz, 1H), 2.95 (m, 1H), 2.74 (dd, *J*=15.5, 4.7 Hz, 1H), 2.63 (dd, *J*=15.4, 4.5 Hz, 1H), 1.94–1.80 (m, 2H), 1.69 (dt, *J*=13.0, 4.0 Hz, 3H), 1.59 (m, 1H), 1.45 (s, 9H), 1.37 (s, 9H), 1.26 (m, 1H), 1.19–1.03 (m, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 171.2, 169.4, 168.5, 155.2, 136.4, 129.3, 128.6, 126.9, 82.5, 80.0, 55.6, 49.8, 48.4, 38.5, 38.3, 33.0, 33.0, 28.2, 27.9, 25.4, 24.9. **MS** (ESI) *m/z* 518 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{28}H_{44}N_3O_6$ 518.6745, found 518.6745.

2,3,4,6-Tetra-*O*-acetyl-*α*-D-glucopyranosyl bromide (12)



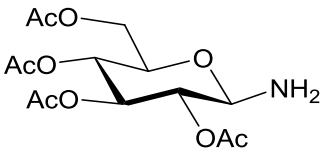
β-D-Glucose pentaacetate **110** (1.0 g, 2.56 mmol) was added to HBr (30% in acetic acid, 5 mL) at 0 °C and the mixture was stirred for 15 min, then at room temperature overnight. Ice-water (50 mL) was added and the mixture was extracted with EtOAc (2 × 25 mL). The organic layers were washed with saturated aqueous $NaHCO_3$ (4 × 30 mL) and water (4 × 30 mL) and dried over $MgSO_4$. The solvent was evaporated under reduced pressure to give the crude product **12** (990 mg, 94%), as an orange oil which was used in the next step without further purification. R_f 0.35, (50% EtOAc/Hex). **^{1}H NMR** (500 MHz, $CDCl_3$) δ 6.60 (d, *J* = 4.0 Hz, 1H), 5.55 (m, 1H), 5.15 (dd, *J*=10.2, 9.4 Hz, 1H), 4.83 (dd, *J*=10.0, 4.0 Hz, 1H), 4.34–4.27 (m, 2H), 4.11 (dd, *J*=4.4, 2.7 Hz, 1H), 2.09 (s, 3H), 2.09 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H). All spectroscopic data were consistent with that reported in the literature.[57]

2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosyl azide (13)



2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide **12** (980 mg, 2.38 mmol), sodium azide (619 mg, 9.52 mmol) and tetrabutylammonium hydrogen sulfate (808 mg, 2.38 mmol) were added to a mixture of DCM/aq. $NaHCO_3$ (5 mL, 1:1). The reaction mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with DCM (20 mL) and was then washed with saturated aqueous $NaHCO_3$ (3 × 20 mL) and dried over $MgSO_4$. The solvent was evaporated and the residue purified by column chromatography (25% EtOAc/Hex) to give the title compound **13** (730 mg, 82%) as a white solid. R_f 0.33 (25% EtOAc/Hex).**^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.22 (t, *J*=9.5 Hz, 1H), 5.10 (t, *J*=9.7 Hz, 1H), 4.95 (t, *J*=9.2 Hz, 1H), 4.64 (d, *J*=8.8 Hz, 1H), 4.27 (dd, *J*=12.5, 4.8 Hz, 1H), 4.17 (dd, *J*=12.4, 2.3 Hz, 1H), 3.79 (ddd, *J* = 10.1, 4.8, 2.3 Hz, 1H), 2.10 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.6, 170.1, 169.3, 169.2, 87.9, 74.0, 72.6, 70.6, 67.9, 61.6, 20.7, 20.5, 20.5, 20.5. **MS** (ESI) *m/z* 391 [$(M+NH_4)^+$,100%]. **HRMS** (ESI, $[M+NH_4]^+$) calcd. for $C_{14}H_{18}N_3O_9$ 391.1460, found 391.1459. All spectroscopic data were consistent with that reported in the literature.[57]

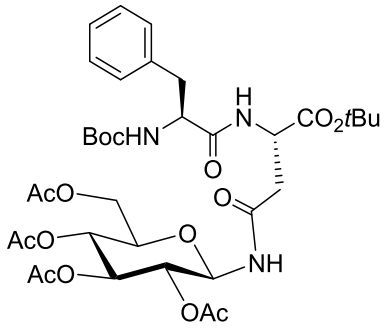
2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosylamine (14)



To a solution of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl azide **13** (100 mg, 0.27 mmol) in MeOH (4 mL) was added 10% palladium on carbon (20 mg). The mixture was stirred under an atmosphere of hydrogen for 2 h. The mixture was filtered through a pad of Celite and the filtrate

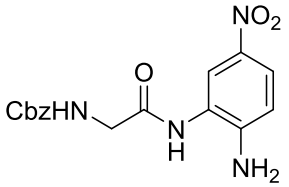
was concentrated under reduced pressure. The residue was purified by column chromatography (5% MeOH/DCM) to give the title compound **14** (70 mg, 75%) as a white solid; R_f 0.3 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.24 (t, *J*=9.5 Hz, 1H), 5.03 (dd, *J*=10.0, 9.2 Hz, 1H), 4.82 (dd, *J*=9.7, 8.9 Hz, 1H), 4.25–4.15 (m, 2H), 4.10 (dd, *J*=12.3, 2.3 Hz, 1H), 3.69 (ddd, *J*=10.1, 4.9, 2.3 Hz, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H). **MS** (ESI) *m/z* 348 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{14}H_{22}NO_9$ 348.3275, found $$. All spectroscopic data were consistent with that reported in the literature.[57]

Boc-Phe-Asn(Glc(Ac)$_4$)-O*t*Bu (111)



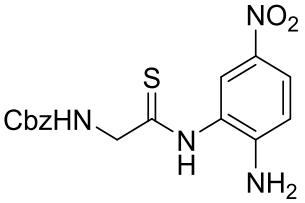
To a solution of **108** (46 mg, 0.1 mmol) in DCM/ACN (1:1, 2 mL) was added Ag_2CO_3 (33 mg, 0.12 mmol) and aminosugar **14** (139 mg, 0.4 mmol) and the mixture was stirred at room temperature for 6 h. The solvents were evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **111** (59 mg, 77%) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.36–7.17 (m, 6H), 7.14 (d, *J*=7.5 Hz, 1H), 6.79 (d, *J*=9.4 Hz, 1H), 5.28 (t, *J*=9.4 Hz, 1H), 5.18 (t, *J*=9.3 Hz, 1H), 5.11 (m, 1H), 5.03 (t, *J*=9.7 Hz, 1H), 4.95 (t, *J*=9.5 Hz, 1H), 4.62 (m, 1H), 4.52–4.30 (m, 2H), 4.02 (d, *J*=12.2 Hz, 1H), 3.77 (m, 1H), 3.17 (dd, *J*=14.0, 5.2 Hz, 1H), 2.96 (m, 1H), 2.85–2.65 (m, 2H), 2.05 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.44 (s, 9H), 1.37 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.8, 170.8, 170.0, 169.6, 169.0, 155.4, 136.4, 129.3, 129.2, 128.6, 127.2, 127.0, 82.8, 78.1, 73.8, 72.8, 70.5, 68.1, 61.5, 55.6, 49.6, 38.2, 38.0, 28.2, 27.8, 20.7, 20.7, 20.6. **MS** (ESI) *m/z* 766 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{36}H_{52}N_3O_{15}$ 766.3393, found 766.3393.

Cbz-Gly 2-amino-5-nitroanilide (114)



A solution of Cbz-Gly **93** (251 mg, 1.2 mmol) in DMF (12 mL) was cooled to 0 °C then HBTU (910 mg, 2.4 mmol), diaminonitrobenzene **113** (184 mg, 1.2 mmol) and DIEA (419 µL, 2.4 mmol) were added. The mixture was warmed to room temperature and was stirred for 18 h. The mixture was poured into saturated aqueous KCl (25 mL) and extracted with EtOAc (3 × 25 mL), and the combined organic extracts were washed brine (25 mL) and dried over $MgSO_4$. Purification of the residue by column chromatography (80% EtOAc /Hex) gave the title compound **114** (351 mg, 85%) as a yellow solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.29 (s, 1H), 8.13 (d, *J*=2.5 Hz, 1H), 7.85 (dd, *J*=9.0, 2.6 Hz, 1H), 7.55 (t, *J*=5.8 Hz, 1H), 7.39–7.26 (m, 5H), 6.74 (d, *J*=9.1 Hz, 1H), 6.49 (s, 2H), 5.05 (s, 2H), 3.86 (d, *J*=5.9 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO) δ 169.2, 157.1, 149.9, 137.4, 135.9, 128.8, 128.3, 128.2, 123.6, 122.3, 121.5, 114.0, 66.0, 44.4.**MS** (ESI) *m/z* 345 [$(M+H)^+$],100%]; **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{16}H_{17}N_4O_5$ 345.1193, found 345.1192.

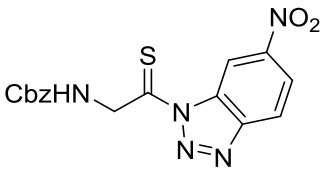
Cbz-$^{[S]}$Gly-2-amino-5-nitroanilide (115)



Thioamide **115** was prepared from amide **114** (102 mg, 0.3 mmol) according to General Procedure C. Column chromatography (20-80% EtOAc/Hex) gave the title product **115** (82 mg, 76%) as a yellow solid; R_f 0.4 (80% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.04 (s, 1H), 7.94 (dd, J=9.1, 2.6 Hz, 1H), 7.88 (d, J=2.4 Hz, 1H), 7.67 (t, J=5.6 Hz, 1H), 7.41–7.25

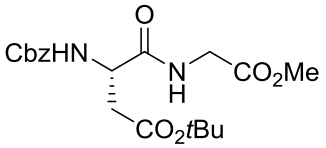
(m, 5H), 6.77 (d, J=9.1 Hz, 1H), 6.53 (s, 2H), 5.06 (s, 2H), 4.18 (d, J=5.8 Hz, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 203.1, 156.9, 151.3, 137.4, 135.6, 128.8, 128.3, 128.2, 125.5, 125.4, 122.8, 114.4, 109.7, 66.3, 66.2, 52.0. **MS** (ESI) *m/z* 361[$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{16}H_{17}N_4O_4S$, 361.0965 found 361.0965.

1-(*N*-Cbz-thioglycinyl)-6-nitrobenzotriazolide (116)



Nitrobenzotriazolide **116** was prepared from thioamide **115** (50 mg, 0.14 mmol) according to General Procedure D. The title compound **116** was obtained as a light orange solid (37 mg, 71%), **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.66 (s, 1H), 8.47 (dd, *J*=8.9, 2.1 Hz, 1H), 8.33 (d, *J*=8.9 Hz, 1H), 7.45–7.34 (m, 5H), 5.69 (t, *J*=6.4 Hz, 1H), 5.23 (d, *J*=6.2 Hz, 2H), 5.21–5.17 (m, 2H). **MS** (ESI) *m/z* 372 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{16}H_{14}N_5O_4S$, 372.0761found 372.0760.

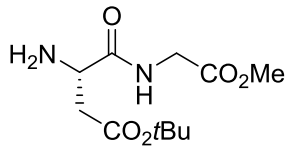
Cbz-Asp(O*t*Bu)-Gly-OMe (119)



Dipeptide **119** was prepared from Cbz-Asp(O*t*Bu)-OH **117** (97 mg, 1.0 mmol) and H-Gly-OMe .HCl **118** (38 mg, 0.3 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **119** (108 mg, 91%) as colourless crystals. R_f 0.4 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.41–7.26 (m, 5H), 7.05 (t, *J*=5.6 Hz, 1H), 6.01 (d, *J*=8.6 Hz, 1H), 5.12 (m, 2H), 4.66 – 4.49 (m, 1H), 4.58 (m, 2H), 3.72 (s, 3H),

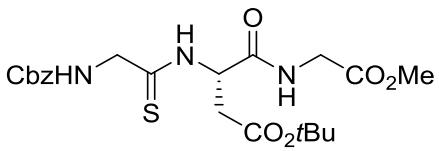
2.88 (dd, *J*=17.0, 4.7 Hz, 1H), 2.64 (dd, *J*=17.0, 6.5 Hz, 1H), 1.42 (s, 9H). **MS** (ESI) *m/z* 396 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$)calcd. for $C_{19}H_{27}N_2O_7$, 395.1813 found 395.1812.

H-Asp(O*t*Bu)-Gly-OMe (120)



To a solution of dipeptide **110** (79 mg, 0.2 mmol) in MeOH (4 mL) was added 10% palladium on carbon (15 mg). The mixture was stirred under an atmosphere of hydrogen for 3 h. The mixture was filtered through a pad of Celite and the filtrate was concentrated under reduced pressure to give the crude amine **120** (50 mg, 0.19 mmol) which was used in the next step without purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.00 (t, *J* 5.6 Hz, 1H), 4.11–3.96 (m, 2H), 3.87 (m, 1H), 3.73 (s, 3H), 3.01 (brs, 2H), 2.86 (m, 1H), 2.61 (dd, *J*=16.9, 7.5 Hz, 1H), 1.43 (s, 9H). **MS** (ESI) *m/z* 261 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$)calcd. for $C_{11}H_{21}N_2O_5$, 261.1445 found 261.1448.

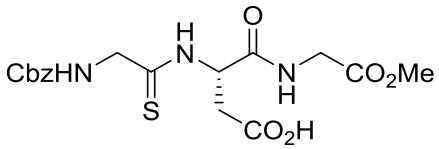
Cbz-$^{[S]}$Gly-Asp(O*t*Bu)-Gly-OMe (121)



A solution of the amine **120** (50 mg, 0.19 mmol) and Et_3N (30 µL, 0.21 mmol) in dry THF (2 mL) was cooled to 0 °C was added a solution of **116** (71 mg, 0.19 mmol) in dry THF (2 mL) . The resulting mixture was stirred at room temperature for 1 h. The solvent was concentrated under reduced pressure and the residue was purified by column chromatography (5% MeOH/ DCM) to give the thiopeptide **121** (62 mg, 70%) as a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.27 (d, *J*=7.9 Hz, 1H), 7.40–7.30 (m, 6H), 5.53 (m, 1H), 5.42 (m, 1H), 5.19–5.10 (m, 2H),

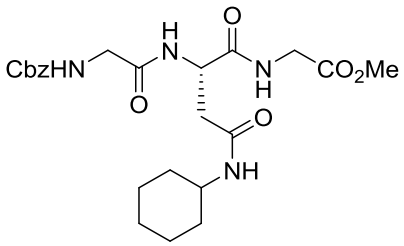
4.27 (m, 2H), 4.00 (m, 2H), 3.72 (s, 3H), 3.04 (m, 1H), 2.62 (m, 1H), 1.47 (s, 9H). **MS** (ESI) *m/z* 468 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$)calcd. for $C_{21}H_{30}N_3O_7S$, 468.1799 found 468.1797.

Cbz-$^{[S]}$Gly-Asp(OH)-Gly-OMe (122)



A solution of **121** (50 mg, 0.11 mmol) in DCM (1 mL) at was added 0 °C then TFA (1 mL). The resulting mixture was stirred at room temperature for 1 h. The solvent and TFA were evaporated under reduced pressure to give the acid **122** (42 mg) as colourless oil which was used in the next step without further purification. **MS** (ESI) *m/z* 412 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{17}H_{22}N_3O_7S$ 412.1173, found 412.1171.

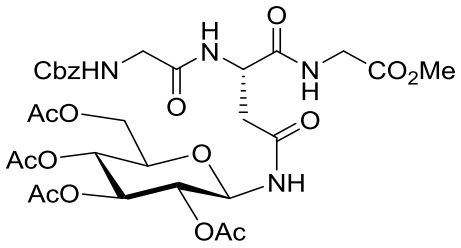
Cbz-Gly-Asn-*N*-(cyclohexyl)-Gly-OMe (123)



A solution of **122** (21 mg, 0.05 mmol) in (DCM: ACN) (1:1) (2 mL) was added Ag_2CO_3 (17 mg, 0.06 mmol) and cyclohexylamine **98** (24 µL, 0.2 mmol) and stirred at room temperature for 6 h. The solvents were evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **123** (14 mg, 59%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.18 (t, *J*=5.9 Hz, 1H), 8.08 (d, *J*=8.1 Hz, 1H), 7.65 (d, *J*=7.8 Hz, 1H), 7.44 (t, *J*=6.0 Hz, 1H), 7.38–7.23 (m, 5H), 5.01 (s, 2H), 4.59 (m, 1H), 3.86–3.72 (m, 2H), 3.71–3.60 (m, 2H), 3.59 (s, 3H), 3.47 (m, 1H), 2.46–2.29 (m, 2H), 1.74–1.57 (m, 4H), 1.56–1.43 (m, 1H),

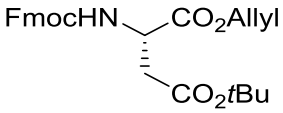
1.28–1.00 (m, 5H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.9, 170.5, 169.3, 168.6, 156.9, 137.4, 128.8, 128.2, 128.1, 66.0, 52.1, 50.0, 47.9, 44.0, 41.2, 38.0, 32.8, 25.7, 25.0. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{23}H_{33}N_4O_7$ 477.2344, found 477.2343.

Cbz-Gly-Asn-*N*-(Glc(Ac)$_4$)-Gly-OMe (124)



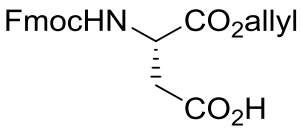
A solution of **122** (21 mg, 0.05 mmol) in (DCM: MeCN) (1:1) (2 mL) was added Ag_2CO_3 (17 mg, 0.06 mmol) and aminosugar **14** (70 mg, 0.2 mmol) and stirred at room temperature for 6 h. The solvents were evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **124** (21 mg, 58%) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.87 (d, *J*=8.1 Hz, 1H), 7.35 (m, 6H), 6.81 (d, *J*=8.8 Hz, 1H), 5.54 (t, *J*=5.7 Hz, 1H), 5.30 (t, *J*=9.5 Hz, 1H), 5.23 (t, *J*=9.2 Hz, 1H), 5.13 (s, 2H), 5.07 (t, *J*=9.7 Hz, 1H), 4.95 (t, *J*=9.6 Hz, 1H), 4.82 (m, 1H), 4.26 (dd, *J*=12.5, 4.1 Hz, 1H), 4.09 (m, 1H), 3.98–3.91 (m, 2H), 3.89 (d, *J*=6.0 Hz, 2H), 3.77 (d, *J*=9.8 Hz, 1H), 3.72 (s, 3H), 2.79 (dd, *J*=16.1, 3.6 Hz, 1H), 2.53 (dd, *J*=15.7, 5.5 Hz, 1H), 2.11 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 172.2, 171.7, 170.7, 170.5, 169.9, 169.9, 169.5, 136.0, 128.6, 128.3, 128.1, 78.1, 73.6, 72.6, 70.2, 68.0, 67.4, 61.5, 52.4, 49.5, 44.7, 41.2, 36.4, 20.7, 20.7, 20.6. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{31}H_{41}N_4O_{16}$ 725.2512, found 725.2511.

Fmoc-Asp(OtBu)-OAlly (126)



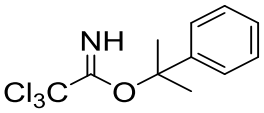
To a solution of **125** (3 g, 7.29 mmol) in DMF (50 mL) was added allyl bromide (945 µL, 10.94 mmol) and DIEA (1.91 mL, 10.94 mmol). The reaction mixture was stirred at room temperature for 16 h. The mixture was diluted with water (50 mL), was extracted with EtOAc(3 × 50 mL). The combined organic extracts were washed with an aqueous HCl solution (50 mL, 1N), saturated aqueous NaHCO3 (350 mL), brine (50 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (50% EtOAc/Hex) to afford the desired peptide **126** (3.127 g, 95%) as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.76 (d, *J*=7.5 Hz, 2H), 7.60 (dd, *J*=7.6, 4.2 Hz, 2H), 7.40 (t, *J* =7.5 Hz, 2H), 7.31 (t, *J*=7.4 Hz, 2H), 5.91 (m, 1H), 5.82 (d, *J* = 8.7 Hz, 1H), 5.34 (dd, *J*=17.2, 1.7 Hz, 1H), 5.25 (dd, *J* = 10.3, 1.4 Hz, 1H), 4.73–4.58 (m, 3H), 4.43 (dd, *J*=10.5, 7.2 Hz, 1H), 4.34 (dd, *J*=10.5, 7.3 Hz, 1H), 4.25 (t, *J*=7.2 Hz, 1H), 2.97 (dd, *J*=16.9, 4.7 Hz, 1H), 2.78 (dd, *J*=16.9, 4.5 Hz, 1H), 1.45 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.6, 170.0, 156.0, 143.9, 143.7, 141.3, 131.5, 127.7, 127.1, 125.2, 125.1, 120.0, 118.8, 81.9, 67.3, 66.3, 50.6, 47.1, 37.8, 28.0. **MS** (ESI) *m/z* 452 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{26}H_{30}NO_6$ 452.2068, found 452.2068.

Fmoc-Asp-OAllyl (127)



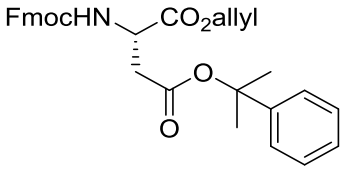
To a solution of **126** (3.0 g, 6.64 mmol) in DCM (10 mL) at 0 °C was added TFA (10 mL) and the solution was stirred for 2 h. The solvent and TFA were evaporated to afford the title compound **127** (2.626 g) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 396 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{22}H_{22}NO_6$ 396.1442, found 396.1440.

2-Phenylisopropyl 2,2,2-trichloroacetimidate (128)



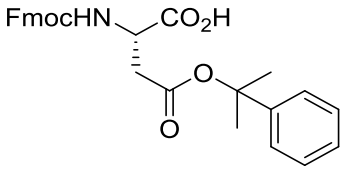
To a suspension of NaH (60% in oil) (120 mg, 3.0 mmol) in dry Et_2O (10 mL) was added dropwise a solution of 1-methyl-1-phenylethanol (4.86 g, 30 mmol) in dry Et_2O (10 mL). The reaction mixture was stirred at room temperature for 20 min. The mixture was cooled to 0 °C followed by a dropwise addition of trichloroacetonitrile (3.0 mL, 30 mmol). The reaction was warmed to room temperature and stirred for a further 1 h. The reaction mixture was concentrated under reduced pressure, and the resulted oil was dissolved in pentane (10 mL). The crude mixture was filtered, and the filtrate was concentrated under reduced pressure to give the crude product **128** (7.6 g, 90%) as a light brown oil, which was used in the next step without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.21 (s, 1H), 7.51–7.32 (m, 5H), 1.90 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 159.4, 145.1, 128.3, 127.2, 124.3, 112.4, 85.0, 28.1. **MS** (ESI) *m/z* 280 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{11}H_{13}Cl_3NO$ 280.0057, found 280.0059.

Fmoc-Asp(2-PhiPr)-OAllyl (129)



To a solution of **128** (3.285 g, 11.77 mmol) in dry DCM (20 mL) was added to a solution of **127** (2.326 g, 5.86 mmol) in dry DCM (20 mL), and the reaction mixture was stirred at room temperature for 16 h. The precipitate was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (30% EtOAc/Hex) to give the compound **129** (2.83 g, 94%) as a colourless oil. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, *J*=7.5 Hz, 2H), 7.59 (d, *J*=7.6 Hz, 2H), 7.41 (t, *J*=7.5 Hz, 2H), 7.37–7.24 (m, 7H), 5.92–5.76 (m, 2H), 5.31 (m, 1H), 5.22 (m, 1H), 4.71–4.58 (m, 3H), 4.44 (dd, *J*=10.5, 7.2 Hz, 1H), 4.34 (dd, *J*=10.5, 7.4 Hz, 1H), 4.24 (t, *J*=7.2 Hz, 1H), 3.09 (dd, *J*=17.1, 4.7 Hz, 1H), 2.88 (dd, *J*=17.1, 4.4 Hz, 1H), 1.78 (s, 6H).**MS** (ESI) *m/z* 514 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{31}H_{32}NO_6$ 514.2224, found 514.2222.

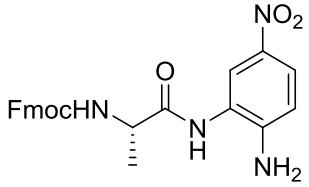
Fmoc-Asp(2-PhiPr)-OH (130)



To a degassed solution of **129** (2.820 g, 5.49 mmol) in DCM/AcOH/NMM (37:2:1) (40 mL), was added $Pd(PPh_3)_4$ (635 mg, 0.55 mmol) and the mixture was stirred for 2 h. The mixture was diluted with DCM (60 mL), and was washed with HCl solution (3 × 50 mL, 1N), then dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography (5% MeOH/DCM) to give the product **130** (2.55 g, 98%) as a yellow solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.76 (d, *J*=7.5 Hz, 2H), 7.58 (d, *J*=7.1 Hz,

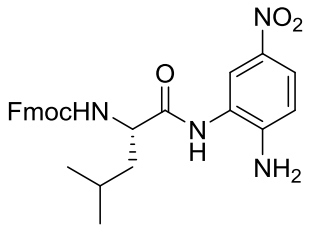
3H), 7.40 (t, *J*=7.5 Hz, 2H), 7.34–7.22 (m, 7H), 5.80 (d, *J*=8.4 Hz, 1H), 4.64 (m, 1H), 4.43 (dd, *J*=10.5, 7.2 Hz, 1H), 4.35 (dd, *J*=10.6, 7.2 Hz, 1H), 4.22 (t, *J*=7.1 Hz, 1H), 3.08 (dd, *J*=17.3, 4.4 Hz, 1H), 2.87 (dd, *J*=17.0, 4.6 Hz, 1H), 1.78 (s, 3H), 1.76 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 174.5, 170.0, 156.1, 145.1, 143.6, 141.3, 128.4, 127.7, 127.2, 127.1, 125.1, 124.2, 120.0, 83.3, 67.4, 50.3, 47.1, 37.3, 28.7, 28.4. **MS** (ESI) *m/z* 514 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{28}H_{28}NO_6$ 474.1911, found 474.1913.

Fmoc-Ala 2-amino-5-nitroanilide (132a)



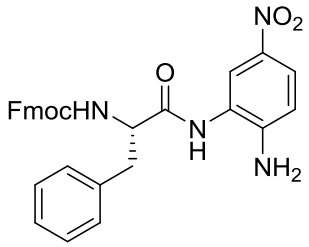
The title compound **132a** was prepared from **131a** (1.557 g, 5.0 mmol) according to General Procedure E. After purification the title compound **132a** was isolated as a yellow solid (2.05 g, 92%); R_f 0.45 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.35 (s, 1H), 8.16 (d, *J*=2.7 Hz, 1H), 7.91–7.80 (m, 3H), 7.77–7.67 (m, 3H), 7.40 (t, *J*=7.5 Hz, 2H), 7.32 (t, *J*=7.4 Hz, 2H), 6.75 (d, *J*=9.1 Hz, 1H), 6.42 (brs, 2H), 4.35–4.27 (m, 2H), 4.26–4.16 (m, 2H), 1.32 (d, *J*=7.0 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.6, 156.5, 149.9, 144.3, 144.2, 141.2, 135.9, 128.1, 127.5, 125.8, 125.7, 123.6, 122.3, 121.6, 120.6, 114.0, 66.2, 51.1, 47.1, 18.1. **MS** (ESI) *m/z* 447 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{24}H_{23}N_4O_5$ 447.1663, found 447.1667.

Fmoc-Leu 2-amino-5-nitroanilide (132b)



The title compound **132b** was prepared from **131b** (1.767 g, 5.0 mmol) according to General Procedure E. After purification the title compound **132b** was isolated as a yellow solid (2.247 g, 92%); R_f 0.42 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.39 (s, 1H), 8.15 (d, *J*=2.6 Hz, 1H), 7.91–7.82 (m, 3H), 7.75–7.66 (m, 3H), 7.44–7.35 (m, 2H), 7.35–7.26 (m, 2H), 6.75 (d, *J*=9.1 Hz, 1H), 6.40 (brs, 2H), 4.37–4.14 (m, 4H), 1.68 (m, 1H), 1.62–1.50 (m, 2H), 0.92 (d, *J*=6.5 Hz, 3H), 0.90 (d, *J*=6.5 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.5, 156.7, 149.8, 144.3, 144.2, 141.2, 135.9, 128.1, 127.5, 125.7, 123.6, 122.3, 121.6, 120.6, 114.1, 71.0, 66.1, 54.0, 47.1, 24.7, 23.5, 22.0, 19.4. **MS** (ESI) *m/z* 489 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{27}H_{29}N_4O_5$ 489.2132, found 489.2135.

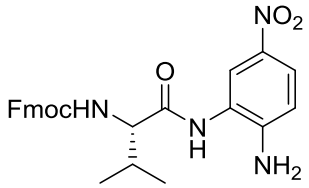
Fmoc-Phe 2-amino-5-nitroanilide (132c)



The title compound **132c** was prepared from **131c** (1.767 g, 5 mmol) according to General Procedure E. After purification the title compound **132c** was isolated as a yellow solid (2.22 g, 85%); R_f 0.32 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.41 (s, 1H), 8.04 (d, *J*=2.6 Hz, 1H), 7.92–7.79 (m, 4H), 7.66 (t, *J*=6.5 Hz, 2H), 7.39 (t, *J*=7.2 Hz, 2H), 7.35–7.19 (m, 7H), 6.73 (d, *J*=9.1 Hz, 1H), 6.32 (brs, 2H), 4.42 (m, 1H), 4.27–4.10 (m, 3H), 3.09 (dd, *J*=13.7, 5.7 Hz, 1H), 2.93 (dd, *J*=13.7, 9.3 Hz, 1H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.5,

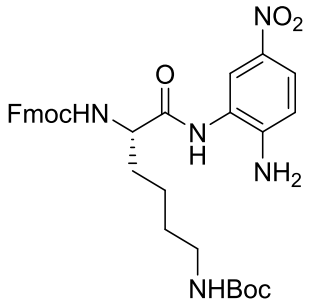
156.5, 149.9, 144.2, 144.2, 141.1, 138.2, 135.9, 129.8, 128.6, 128.1, 127.5, 126.9, 125.8, 125.7, 123.7, 122.4, 121.4, 120.6, 114.0, 66.2, 57.0, 47.0, 40.6, 40.4, 40.2, 40.0, 39.8, 39.5, 39.3, 37.6. **MS** (ESI) *m/z* 523 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{30}H_{27}N_4O_5$ 523.1976, found 523.1972.

Fmoc-Val 2-amino-5-nitroanilide (132d)



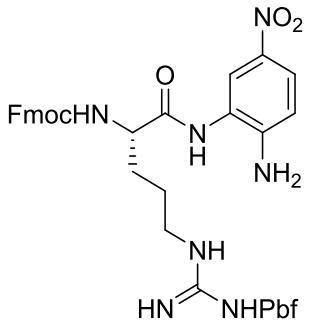
The title compound **132d** was prepared from **131d** (1.697 g, 5 mmol) according to General Procedure E. After purification the title compound **132d** was isolated as a yellow solid (2.11 g, 89%); R_f 0.41 (5% MeOH/DCM).**^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.40 (s, 1H), 8.22 (d, *J*=2.6 Hz, 1H), 7.92–7.82 (m, 3H), 7.79–7.67 (m, 3H), 7.44–7.35 (m, 2H), 7.35–7.27 (m, 2H), 6.76 (d, *J*=9.0 Hz, 1H), 6.41 (brs, 2H), 4.35–4.18 (m, 3H), 4.01 (t, *J*=7.8 Hz, 1H), 2.07 (h, *J*=6.9 Hz, 1H), 0.95 (d, *J*=1.9 Hz, 3H), 0.94 (d, *J*=1.9 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.5, 157.0, 149.4, 144.3, 144.2, 141.2, 136.0, 128.1, 127.5, 125.8, 123.5, 121.7, 121.6, 120.6, 114.2, 66.3, 61.4, 47.1, 30.3, 19.7, 19.1. **MS** (ESI) *m/z* 475 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{26}H_{27}N_4O_5$ 475.1976, found 475.1977.

Fmoc-Lys(Boc) 2-amino-5-nitroanilide (132e)



The title compound **132e** was prepared from **131e** (2.343 g, 5 mmol) according to General Procedure E. After purification the title compound **132e** was isolated as a yellow solid (2.75 g, 91%); R_f 0.38 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.36 (s, 1H), 8.18 (d, *J*=2.7 Hz, 1H), 7.91–7.81 (m, 3H), 7.76–7.66 (m, 3H), 7.44–7.36 (m, 2H), 7.35–7.28 (m, 2H), 6.81–6.71 (m, 2H), 6.40 (brs, 2H), 4.33–4.18 (m, 3H), 4.12 (m, 1H), 2.91 (d, *J* = 6.6 Hz, 2H), 1.80–1.57 (m, 2H), 1.44–1.31 (m, 13H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.1, 156.8, 156.0, 149.7, 144.3, 144.2, 141.2, 135.9, 128.1, 127.5, 125.8, 123.6, 122.1, 121.6, 120.6, 114.1, 77.8, 66.2, 55.7, 47.1, 31.6, 29.7, 28.7, 23.4. **MS** (ESI) *m/z* 604 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{32}H_{38}N_5O_7$ 604.2766, found 604.2768.

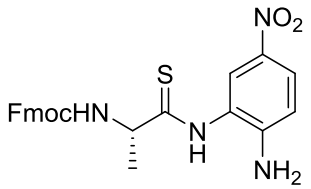
Fmoc-Arg(Pbf) 2-amino-5-nitroanilide (132f)



The title compound **132f** was prepared from **131f** (3.244 g, 5 mmol) according to General Procedure E. After purification the title compound **132f** was isolated as a yellow solid (3.724 g, 95%); R_f 0.38 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.39 (s, 1H), 8.18 (d,

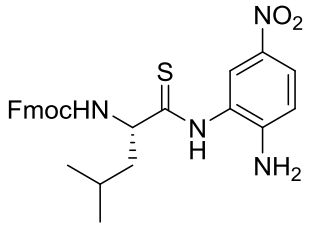
J=2.6 Hz, 1H), 7.93–7.80 (m, 3H), 7.79–7.65 (m, 3H), 7.39 (t, *J*=7.5 Hz, 2H), 7.31 (t, *J*=7.4 Hz, 2H), 6.75 (d, *J*=9.1 Hz, 1H), 6.42 (brs, 2H), 4.34–4.26 (m, 2H), 4.22 (m, 1H), 4.14 (m, 1H), 3.11–3.01 (m, 2H), 2.90 (s, 2H), 2.46 (s, 3H), 2.40 (s, 3H), 1.96 (s, 3H), 1.74 (m, 1H), 1.59 (m, 1H), 1.48 (m, 2H), 1.37 (s, 6H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.9, 157.9, 156.7, 156.5, 149.7, 144.3, 144.2, 141.2, 137.7, 135.9, 131.9, 128.1, 127.5, 125.7, 124.8, 123.6, 122.2, 121.5, 120.6, 116.7, 114.1, 86.7, 66.2, 55.3, 47.1, 42.9, 29.3, 28.7, 19.4, 18.0, 12.7. **MS** (ESI) *m/z* 784 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{40}H_{46}N_7O_8S$ 784.3123, found 784.3124.

Fmoc-Ala$^{[S]}$ 2-amino-5-nitroanilide (133a)



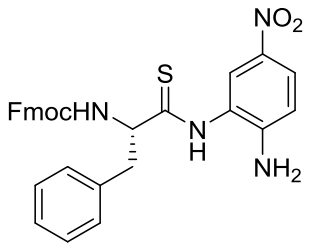
The title compound **133a** was prepared from **132a** (447 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133a** was isolated as a yellow solid (379 mg, 82%); R_f 0.34 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.23 (s, 1H), 7.97–7.91 (m, 2H), 7.90–7.88 (m, 2H), 7.87 (s, 1H), 7.74 (d, *J*=7.5 Hz, 1H), 7.71 (d, *J*=7.5 Hz, 1H), 7.45–7.36 (m, 2H), 7.35–7.27 (m, 2H), 6.77 (d, *J*=9.1 Hz, 1H), 6.37 (brs, 2H), 4.51 (m, 1H), 4.30 (m, 1H), 4.27–4.15 (m, 2H), 1.43 (d, *J* = 6.8 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 208.7, 156.6, 151.1, 144.3, 141.2, 144,1, 135.7, 128.1, 127.6, 125.8, 125.7, 125.4, 125.2, 122.9, 120.6, 114.3, 66.3, 57.4, 47.1, 20.7. **MS** (ESI) *m/z* 463 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{24}H_{23}N_4O_4S$ 463.1435, found 463.1433.

Fmoc-Leu[S] 2-amino-5-nitroanilide (133b)



The title compound **133b** was prepared from **132b** (489 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133b** was isolated as a yellow solid (429 mg, 85%); R_f 0.33 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.34 (s, 1H), 7.97–7.91 (m, 2H), 7.88 (d, *J*=7.5 Hz, 2H), 7.83 (d, *J*=2.6 Hz, 1H), 7.72 (dd, *J*=12.1, 7.5 Hz, 2H), 7.40 (t, *J*=7.4 Hz, 2H), 7.30 (t, *J*=7.4 Hz, 2H), 6.77 (d, *J*=9.1 Hz, 1H), 6.34 (brs, 2H), 4.49 (m, 1H), 4.31 (m, 1H), 4.27–4.15 (m, 2H), 1.81 – 1.60 (m, 3H), 0.95 (d, *J*=5.9 Hz, 3H), 0.92 (d, *J*=6.0 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 208.8, 157.0, 151.1, 144.3, 144.0, 141.2, 135.8, 128.1, 127.5, 127.5, 125.9, 125.7, 125.4, 125.1, 122.9, 120.6, 114.4, 66.3, 60.1, 47.1, 43.2, 24.8, 23.3, 22.4. **MS** (ESI) *m/z* 505 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{27}H_{29}N_4O_4S$ 505.1904, found 505.1903.

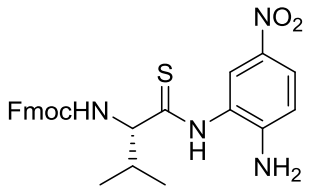
Fmoc-Phe[S] 2-amino-5-nitroanilide (133c)



The title compound **133c** was prepared from **132c** (523 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133c** was isolated as a yellow solid (469 mg, 87%); R_f 0.33 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.25 (s, 1H), 8.13 (d, *J*=6.6 Hz, 1H), 7.87 (d, *J*=7.6 Hz, 2H), 7.69 (t, *J*=7.7 Hz, 2H), 7.55 (d, *J*=2.7 Hz, 1H), 7.43–

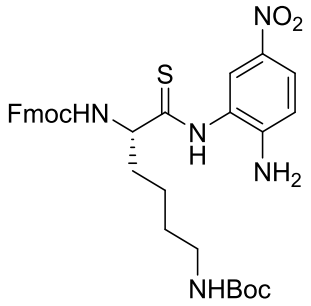
7.21 (m, 10H), 6.73 (d, *J*=9.2 Hz, 1H), 6.18 (brs, 2H), 4.67 (m, 1H), 4.28 (m, 1H), 4.18 (m, 2H), 3.20–3.02 (m, 2H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 207.3, 156.7, 151.0, 144.2, 144.0, 141.1, 137.8, 135.7, 130.0, 128.6, 128.1, 128.1, 127.6, 127.5, 127.0, 125.9, 125.7, 125.4, 125.0, 122.6, 120.6, 114.3, 66.3, 63.0, 47.0. **MS** (ESI) *m/z* 539 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{30}H_{27}N_4O_4S$ 539.1748, found 539.1747.

Fmoc-Val$^{[S]}$ 2-amino-5-nitroanilide (133d)



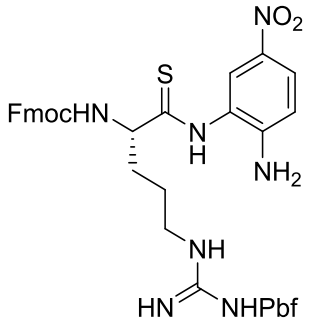
The title compound **133d** was prepared from **132d** (475 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133d** was isolated as a yellow solid (392 mg, 82%); R_f 0.32 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.39 (s, 1H), 8.02 (d, *J*=6.9 Hz, 1H), 7.95 (dd, *J*=9.1, 2.7 Hz, 1H), 7.88 (d, *J*=7.5 Hz, 2H), 7.81 (d, *J*=2.6 Hz, 1H), 7.73 (dd, *J*=14.4, 7.4 Hz, 2H), 7.40 (t, *J*=7.4 Hz, 2H), 7.31 (t, *J*=7.4 Hz, 2H), 6.77 (d, *J*=9.1 Hz, 1H), 6.34 (brs, 2H), 4.37–4.26 (m, 2H), 4.26–4.15 (m, 2H), 4.10 (m, 1H), 2.13 (m, 1H), 1.04 (d, *J* = 6.5 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H). **MS** (ESI) *m/z* 491 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{26}H_{27}N_4O_4S$ 491.1748, found 491.1749.

Fmoc-Lys[S](Boc) 2-amino-5-nitroanilide (133e)



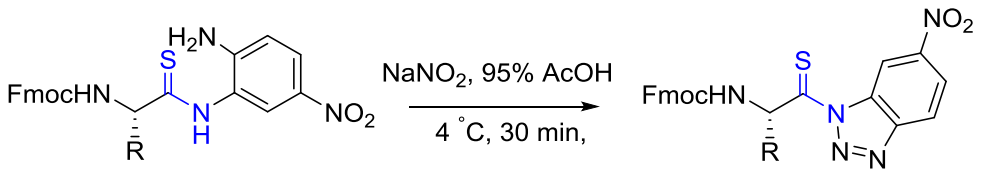
The title compound **133e** was prepared from **132e** (604 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133e** was isolated as a yellow solid (527 mg, 85%); R_f 0.3 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 8.00–7.91 (m, 2H), 7.89 (s, 1H), 7.86 (d, *J*=4.1 Hz, 2H), 7.74 (d, *J*=7.5 Hz, 1H), 7.71 (d, *J*=7.5 Hz, 1H), 7.40 (t, *J*=7.5 Hz, 2H), 7.31 (t, *J*=7.4 Hz, 2H), 6.82 (6.82 (t, *J*=5.9, 5.3 Hz, 1H), 6.76 (d, *J*=9.2 Hz, 1H), 6.36 (brs, 2H), 4.38 (m, 1H), 4.30 (m, 1H), 4.26–4.16 (m, 2H), 2.92 (t, *J*=6.5 Hz, 2H), 1.90–1.65 (m, 2H), 1.45–1.30 (m, 13H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 208.4, 157.0, 156.1, 151.1, 144.3, 144.0, 141.1, 135.7, 128.1, 127.6, 127.5, 125.9, 125.7, 125.5, 125.2, 122.8, 120.6, 114.3, 77.8, 66.3, 61.8, 60.2, 47.0, 33.9, 29.7, 28.7, 23.3, 21.2, 14.5. **MS** (ESI) *m/z* 620 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{32}H_{38}N_5O_6S$ 620.2537, found 620.2536.

Fmoc-Arg[S](Pbf) 2-amino-5-nitroanilide (133f)



The title compound **133f** was prepared from **132f** (784 mg, 1.0 mmol) according to General Procedure E. After purification the title compound **133f** was isolated as a yellow solid (648 mg, 81%); R_f 0.3 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.99–7.91 (m, 2H), 7.88 (s, 1H), 7.86 (m, 2H), 7.73 (d, *J*=7.5 Hz, 1H), 7.70 (d, *J*=7.4 Hz, 1H), 7.40 (t, *J*=7.5 Hz, 2H), 7.30 (t, *J*=7.5 Hz, 2H), 6.77 (d, *J*=9.1 Hz, 1H), 6.36 (brs, 2H), 4.40 (q, *J*=6.9 Hz, 1H), 4.36–4.27 (m, 1H), 4.23 (d, *J*=6.1 Hz, 2H), 3.09 (q, *J*=6.7 Hz, 2H), 2.92 (s, 2H), 2.48 (s, 3H), 2.42 (s, 3H), 1.98 (s, 3H), 1.90–1.67 (m, 2H), 1.64–1.42 (m, 2H), 1.37 (s, 6H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 207.9, 170.8, 157.9, 156.9, 156.5, 151.1, 144.3, 144.0, 141.2, 137.7, 135.8, 131.9, 128.1, 127.6, 127.5, 125.9, 125.7, 125.5, 125.1, 124.8, 122.7, 120.6, 116.7, 114.4, 86.7, 66.4, 61.5, 60.2, 47.1, 42.9, 31.7, 28.7, 21.2, 19.4, 18.1, 14.5, 12.7; **MS** (ESI) *m/z* 800 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{40}H_{46}N_7O_7S_2$ 800.2895, found 800.2893.

Synthesis of thiobenzotriazolides (134a–f)



133

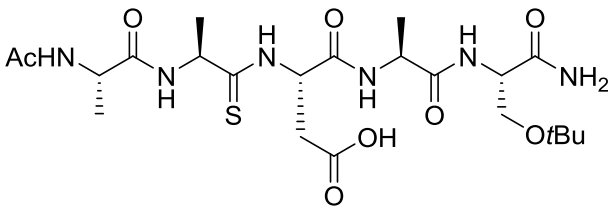
a) Ala
b) Leu
c) Phe
d) Val
e) Lys
f) Arg

134

a) Ala
b) Leu
c) Phe
d) Val
e) Lys
f) Arg

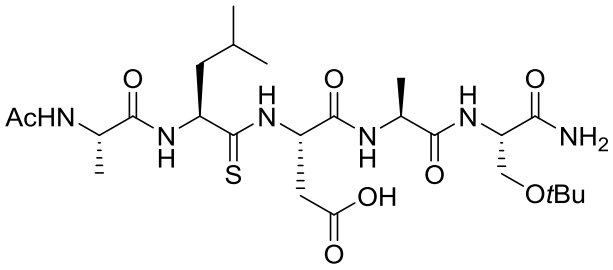
Thiobenzotriazolides **134a**–**f** were prepared from thioamides **133a**–**f** on 0.4 mmol scale according to General Procedure D. The title compounds **134a**–**f** were obtained as light orange solids which were used in SPPS without further purification.

Ac-Ala-Ala$^{[S]}$-Asp-Ala-Ser(*t*Bu)-NH$_2$ (138a)



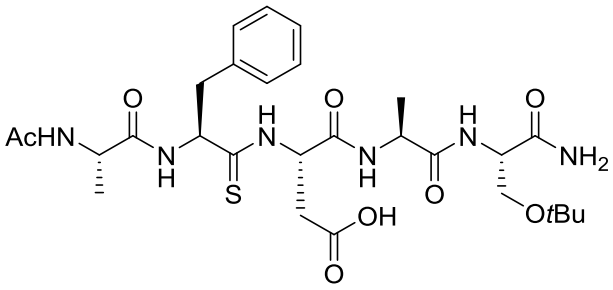
The title compound **138a** was prepared according to General Procedure F. After HPLC purification, the title compound **138a** was obtained as a white solid (14 mg, 25%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.48 (brs, 1H), 10.02 (d, *J*=7.2 Hz, 1H), 8.12 (d, *J*=4.0 Hz, 1H), 8.11 (d, *J*=4.0 Hz, 1H), 8.02 (d, *J*=7.1 Hz, 1H), 7.62 (d, *J*=8.0 Hz, 1H), 7.18 (s, 1H), 7.11 (s, 1H), 5.12 (m, 1H), 4.62 (m, 1H), 4.29–4.16 (m, 3H), 3.47–3.41 (m, 2H), 2.82 (dd, *J*=17.0, 5.3 Hz, 1H), 2.73 (dd, *J*=16.9, 8.0 Hz, 1H), 1.83 (s, 3H), 1.26 (d, *J*=6.9 Hz, 3H), 1.20 (s, 3H), 1.18 (s, 3H), 1.10 (s, 9H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 206.1, 172.3, 172.0, 171.9, 169.7, 169.1, 73.2, 62.1, 55.2, 54.6, 53.6, 49.1, 40.6, 48.7, 35.5, 27.7, 22.9, 21.7, 18.4, 18.2. **MS** (ESI) *m/z* 547 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{22}H_{39}N_6O_8S$ 547.2545, found 547.2547.

Ac-Ala-Leu[S]-Asp-Ala-Ser(*t*Bu)-NH_2 (138b)



The title compound **138b** was prepared according to General Procedure F. After HPLC purification, the title compound **138b** was obtained as a white solid (9 mg, 16%).**^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.48 (brs, 1H), 10.07 (d, *J*=7.0 Hz, 1H), 8.58 (dd, *J*=4.3, 1.3 Hz, 1H), 8.08 (d, *J*=7.3 Hz, 1H), 8.04 (d, *J*=7.2 Hz, 1H), 7.91 (d, *J*=8.2 Hz, 1H), 7.64 (d, *J*=8.0 Hz, 1H), 7.20 (s, 1H), 7.11 (s, 1H), 5.10 (m, 1H), 4.67 (m, 1H), 4.29–4.20 (m, 2H), 4.19 (m, 1H), 3.52–3.47 (m, 2H), 2.82 (dd, *J*=17.0, 5.3 Hz, 1H), 2.72 (dd, *J*=17.1, 8.2 Hz, 1H), 1.82 (s, 3H), 1.61 (m, 1H), 1.49 (m, 2H), 1.19 (s, 3H), 1.17 (s, 3H), 1.10 (s, 9H), 0.88–0.82 (m, 6H **MS** (ESI) *m/z* 589 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{25}H_{45}N_6O_8S$ 589.3014, found 589.3012.

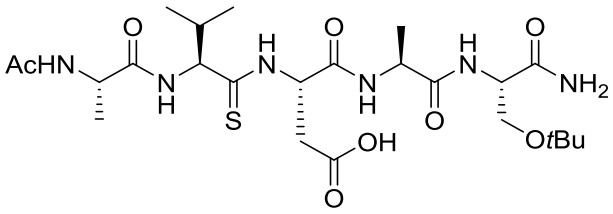
Ac-Ala-Phe[S]-Asp-Ala-Ser(*t*Bu)-NH_2 (138c)



The title compound **138c** was prepared according to General Procedure F. After HPLC purification, the title compound **138c** was obtained as a white solid (14 mg, 23%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.49 (brs, 1H), 10.14 (d, *J*=7.2 Hz, 1H), 8.34 (d, *J*=8.3 Hz, 1H), 8.06 (d, *J*=7.0 Hz, 1H), 7.93 (d, *J*=7.0 Hz, 1H), 7.57 (d, *J*=8.0 Hz, 1H), 7.30–7.12 (m, 7H), 5.21–5.14 (m, 1H), 4.87–4.80 (m, 1H), 4.28–4.16 (m, 3H), 3.45–3.41 (m, 3H), 3.13 (dd, *J*=13.9, 3.4

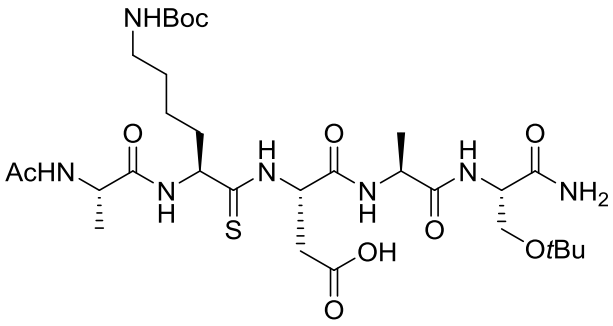
Hz, 1H), 2.87–2.73 (m, 3H), 1.78 (s, 3H), 1.20 (d, *J*=7.0 Hz, 3H), 1.10 (s, 9H), 0.91 (d, *J*=7.0 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 204.71, 172.99, 172.03, 171.90, 171.87, 169.81, 169.18, 138.15, 129.69, 128.41, 126.78, 73.17, 62.14, 60.58, 55.54, 53.61, 49.18, 48.76, 40.98, 35.65, 27.68, 22.78, 18.21. **MS** (ESI) *m/z* 623 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{28}H_{43}N_6O_8S$ 623.2858, found 623.2858.

Ac-Ala-Phe$^{[S]}$-Asp-Ala-Ser(*t*Bu)-NH$_2$ (138d)



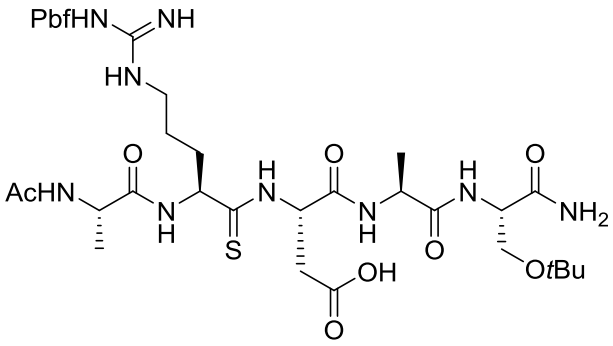
The title compound **138d** was prepared according to General Procedure F. After HPLC purification, the title compound **138d** was obtained as a white solid (10 mg, 17%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.45 (brs, 1H), 9.85 (d, *J*=7.4 Hz, 1H), 8.37 (d, *J*=7.4 Hz, 1H), 8.25 (d, *J*=6.2 Hz, 1H), 7.69 (d, *J*=7.0 Hz, 1H), 7.47 (d, *J*=8.2 Hz, 1H), 7.12 (m, 2H), 5.16 (m, 1H), 4.56 (m, 1H), 4.28–4.15 (m, 3H), 3.49 (dd, *J*=9.2, 5.5 Hz, 1H), 3.43 (m, 1H), 2.88–2.83 (m, 2H), 1.82 (s, 3H), 1.61 (m, 1H), 1.21–1.15 (m, 6H), 1.10 (s, 9H), 0.87 (d, *J*=6.2 Hz, 3H), 0.83 (d, *J*=6.1 Hz, 3H). **MS** (ESI) *m/z* 623 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{24}H_{43}N_6O_8S$ 575.2858, found 575.2856.

Ac-Ala-Lys[S](Boc)-Asp-Ala-Ser(*t*Bu)-NH_2 (138e)



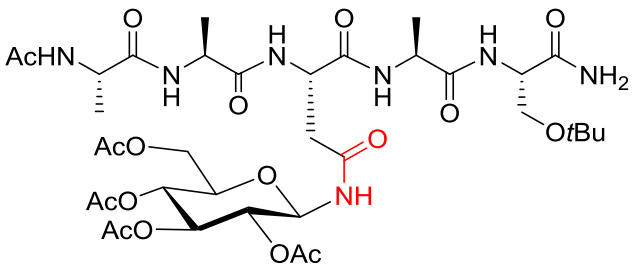
The title compound **138e** was prepared according to General Procedure F. After HPLC purification, the title compound **138e** was obtained as a white solid (17 mg, 24%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.48 (brs, 1H), 10.08 (d, *J*=7.1 Hz, 1H), 8.14–8.04 (m, 2H), 7.94 (d, *J*=8.0 Hz, 1H), 7.63 (d, *J*=8.0 Hz, 1H), 7.15 (d, *J*=38.4 Hz, 2H), 6.75 (t, *J*=5.7 Hz, 1H), 5.18–5.02 (m, 1H), 4.62–4.50 (m, 1H), 4.29–4.21 (m, 2H), 4.21–4.16 (m, 1H), 3.51–3.48 (m, 2H), 2.89–2.77 (m, 3H), 2.72 (dd, *J*=17.0, 8.2 Hz, 1H), 1.83 (s, 3H), 1.73–1.60 (m, 1H), 1.54–1.47 (m, 1H), 1.35 (s, 9H), 1.3–1.27 (m, 2H), 1.28–1.21 (m, 2H), 1.19 (s, 3H), 1.18 (s, 3H), 1.10 (s, 9H); **^{13}C NMR** (126 MHz, DMSO-d_6) δ 205.3, 172.4, 172.1, 171.9, 169.6, 169.1, 156.0, 77.8, 73.2, 62.2, 58.6, 55.2, 53.6, 49.1, 48.7, 35.5, 29.7, 28.7, 27.7, 23.1, 22.9, 18.4, 18.2; **MS** (ESI) *m/z* 704 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd for $C_{30}H_{53}N_7O_{10}S$ 704.3647, found 704.3650.

Ac-Ala-Arg[S](Pbf)-Asp-Ala-Ser(*t*Bu)-NH_2 (138f)



The title compound **138f** was prepared according to General Procedure F. After HPLC purification, the title compound **138f** was obtained as a white solid (16 mg, 18%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.48 (s, 1H), 10.11 (d, *J*=7.0 Hz, 1H), 8.11–8.07 (m, 2H), 7.94 (d, *J*=8.0 Hz, 1H), 7.63 (d, *J*=8.0 Hz, 1H), 7.19 (s, 1H), 7.11 (s, 1H), 6.61 (s, 1H), 6.53–6.20 (m, 2H), 5.09 (m, 1H), 4.59 (m, 1H), 4.22 (m, 3H), 3.0 (m, 2H), 2.96 (s, 2H), 2.81 (dd, *J*=17.1, 5.3 Hz, 1H), 2.71 (dd, *J*=17.1, 8.2 Hz, 1H), 2.50–2.48 (m, 2H), 2.47 (s, 3H), 2.41 (s, 3H), 2.00 (s, 3H), 1.83 (s, 3H), 1.68 (m, 1H), 1.53 (m, 1H), 1.46–1.38 (m, 8H), 1.19 (s, 3H), 1.18 (s, 3H), 1.10 (s, 9H). **MS** (ESI) *m/z* 884 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd for $C_{38}H_{62}N_9O_{11}S_2$ 884.4005, found 884.4009.

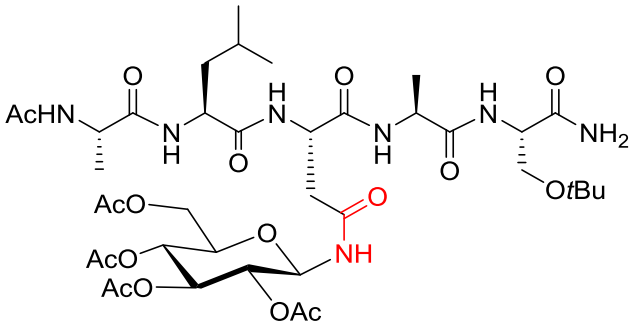
Ac-Ala-Ala-Asn(Glc(Ac)$_4$)-Ala-Ser(tBu)-NH_2 (139a)



The title compound **139a** was prepared from thiopeptide **138a** (9 mg, 0.017mmol) according to General Procedure G. After HPLC purification, the title compound **139a** was obtained as a

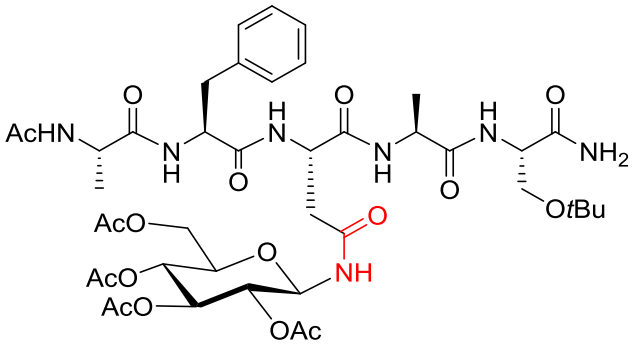
white solid (4.2 mg, 29%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.77 (d, *J*=9.3 Hz, 1H), 8.09 (d, *J*=7.8 Hz, 1H), 8.03–8.00 (m, 2H), 7.78 (d, *J*=7.1 Hz, 1H), 7.73 (d, *J*=8.2 Hz, 1H), 7.14 (brs,1H), 7.07 (brs,1H), 5.36–5.29 (m, 2H), 4.88 (t, *J*=9.8 Hz, 1H), 4.80 (t, *J*=9.4 Hz, 1H), 4.52 (m, 1H), 4.30–4.10 (m, 5H), 4.03 (m, 1H), 3.96 (dd, *J*=12.4, 2.2 Hz, 1H), 3.48–3.45 (m, 2H), 2.62 (m, 1H), 2.45 (m, 1H), 1.99 (s, 3H), 1.98 (s, 3H), 1.94 (s, 3H), 1.92 (s, 3H), 1.82 (d, *J*=2.7 Hz, 3H), 1.21–1.16 (m, 9H), 1.11 (s, 9H). **MS** (ESI) *m/z* 860 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{36}H_{58}N_7O_{17}$ 860.3884, found 860.3884.

Ac-Ala-Leu-Asn(Glc(Ac)$_4$)-Ala-Ser(*t*Bu)-NH$_2$ (139b)



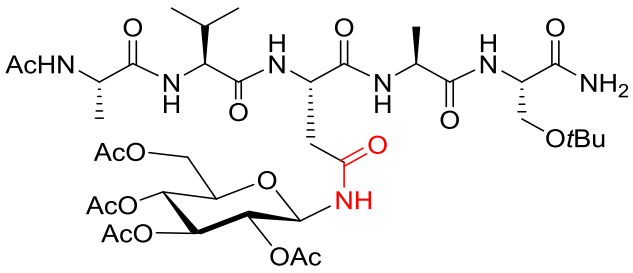
The title compound **139b** was prepared from thiopeptide **138b** (7 mg, 0.012 mmol) according to General Procedure G. After HPLC purification, the title compound **139b** was obtained as a white solid (3.2 mg, 30%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.77 (d, *J*=9.4 Hz, 1H), 8.12 (d, *J*=7.9 Hz, 1H), 8.01 (d, *J*=7.3 Hz, 1H), 7.89 (d, *J*=7.9 Hz, 1H), 7.76 (d, *J*=7.2 Hz, 1H), 7.74 (d, *J*=8.1 Hz, 1H), 7.13 (s, 1H), 7.08 (s, 1H), 5.36–5.28 (m, 2H), 4.89 (t, *J*=9.8 Hz, 1H), 4.80 (t, *J*=9.4 Hz, 1H), 4.52 (m, 1H), 4.30–4.18 (m, 4H), 4.15 (dd, *J*=12.4, 4.2 Hz, 1H), 4.03 (m, 1H), 3.96 (dd, *J*=12.4, 2.2 Hz, 1H), 3.50–3.46 (m, 4H)2.64 (dd, *J*=16.4, 6.4 Hz, 1H), 2.44 (dd, *J*=16.2, 6.4 Hz, 1H), 1.99 (s, 3H), 1.98 (s, 3H), 1.95 (s, 3H), 1.92 (s, 3H), 1.82 (s, 3H), 1.58 (hept, *J*=6.7 Hz, 1H), 1.47–1.40 (m, 2H), 1.21–1.14 (m, 6H), 1.11 (s, 9H), 0.86 (d, *J*=6.6 Hz, 3H), 0.82 (d, *J*=6.5 Hz, 3H). **MS** (ESI) *m/z* 902 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{39}H_{64}N7O_{17}$ 902.4353, found 902.4350.

Ac-Ala-Phe-Asn(Glc(Ac)$_4$)-Ala-Ser(*t*Bu)-NH$_2$ (139c)



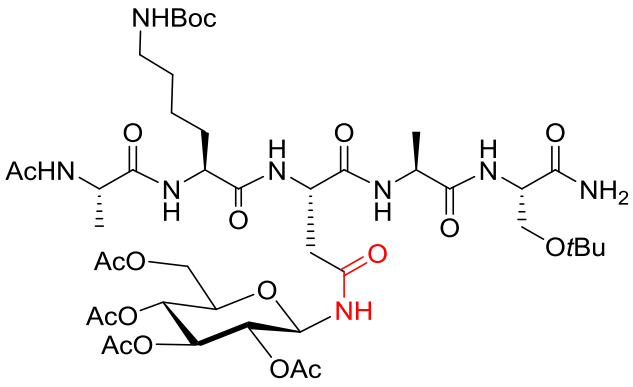
The title compound **139c** was prepared from thiopeptide **138c** (10 mg, 0.016 mmol) according to General Procedure G. After HPLC purification, the title compound **139c** was obtained as a white solid (5 mg, 33%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.79 (d, *J*=9.5 Hz, 1H), 8.29 (d, *J*=8.3 Hz, 1H), 7.97 (d, *J*=7.4 Hz, 1H), 7.90 (d, *J*=7.1 Hz, 1H), 7.85 (d, *J*=7.8 Hz, 1H), 7.75 (d, *J*=8.3 Hz, 1H), 7.25–7.15 (m, 5H), 7.13 (s, 1H), 7.08 (s, 1H), 5.35 (t, *J*=9.4 Hz, 1H), 5.31 (t, *J*=9.6 Hz, 1H), 4.89 (t, *J* = 9.8 Hz, 1H), 4.81 (t, *J*=9.4 Hz, 1H), 4.55 (m,1H), 4.43 (m, 1H), 4.27–4.14 (m, 4H), 4.03 (m, 1H), 3.97 (dd, *J*=12.5, 2.2 Hz, 1H), 3.54–3.43 (m, 2H), 3.01 (dd, *J*=14.0, 4.6 Hz, 1H), 2.78 (dd, *J*=13.8, 9.0 Hz, 1H), 2.65 (m, 1H), 2.46–2.43 (m, 1H), 1.98 (d, *J*=3.5 Hz, 6H), 1.94 (s, 3H), 1.92 (s, 3H), 1.79 (s, 3H), 1.20 (d, *J*=7.1 Hz, 3H), 1.14–1.07 (m, 12H). **MS** (ESI) *m/z* 936 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{42}H_{62}N_7O_{17}$ 936.4197, found 936.4205.

Ac-Ala-Val-Asn(Glc(Ac)$_4$)-Ala-Ser(*t*Bu)-NH$_2$ (139d)



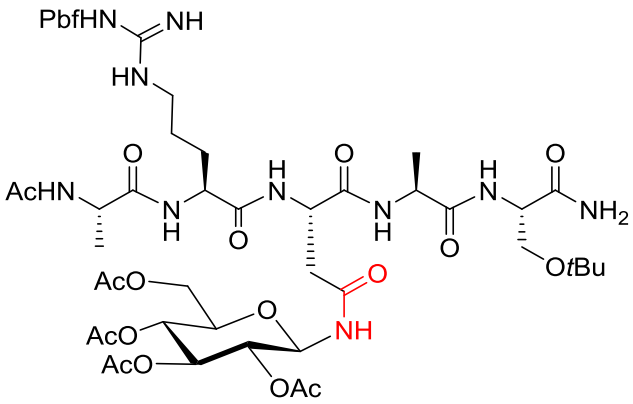
The title compound **139d** was prepared from thiopeptide **138d** (7 mg, 0.012 mmol) according to General Procedure G. After HPLC purification, the title compound **139d** was obtained as a white solid (2.9 mg, 27%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.77 (d, *J*=9.4 Hz, 1H), 8.21 (d, *J*=7.8 Hz, 1H), 8.04 (d, *J*=7.5 Hz, 1H), 7.82 (d, *J*=7.2 Hz, 1H), 7.77 (d, *J*=8.2 Hz, 1H), 7.69 (d, *J*=8.5 Hz, 1H), 7.11 (d, *J*=31.7 Hz, 2H), 5.38–5.27 (m, 2H), 4.89 (t, *J*=9.8 Hz, 1H), 4.80 (t, *J*=9.4 Hz, 1H), 4.56 (m, 1H), 4.31 (m, 1H), 4.27–4.18 (m, 2H), 4.18–4.09 (m, 2H), 4.03 (m, 1H), 3.96 (m, 1H), 3.52–3.44 (m, 2H), 2.65 (dd, *J*=16.3, 6.4 Hz, 1H), 2.43 (dd, *J*=16.2, 6.7 Hz, 1H), 1.99 (s, 3H), 1.9 –1.95 (m, 4H), 1.94 (s, 3H), 1.92 (s, 3H), 1.82 (s, 3H), 1.21–1.14 (m, 6H), 1.11 (s, 9H), 0.81 (d, *J*=6.8 Hz, 3H), 0.79 (d, *J*=6.8 Hz, 3H). **MS** (ESI) *m/z* 888 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{38}H_{62}N_7O_{17}$ 888.4197, found 888.4195.

Ac-Ala-Lys(Boc)-Asn(Glc(Ac)$_4$)-Ala-Ser(*t*Bu)-NH$_2$ (139e)



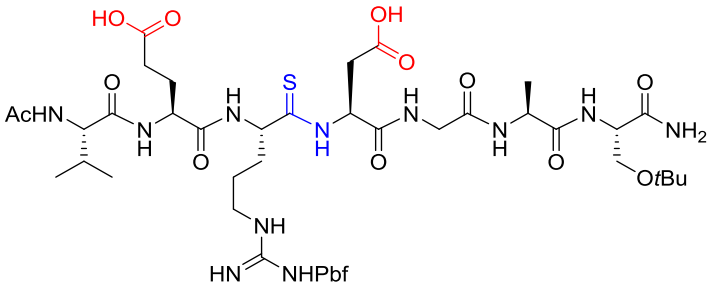
The title compound **139e** was prepared from thiopeptide **138e** (8 mg, 0.017 mmol) according to General Procedure G. After HPLC purification, the title compound **139e** was obtained as a white solid (4.5 mg, 26%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.77 (d, *J*=9.4 Hz, 1H), 8.13 (d, *J*=7.9 Hz, 1H), 8.02 (d, *J*=7.3 Hz, 1H), 7.88 (d, *J*=7.6 Hz, 1H), 7.81 (d, *J*=7.2 Hz, 1H), 7.73 (d, *J*=8.1 Hz, 1H), 7.12 (s, 1H), 7.08 (s, 1H), 6.71 (t, *J*=5.6 Hz, 1H), 5.24–5.40 (m, 2H), 4.89 (t, *J*=9.8 Hz, 1H), 4.80 (t, *J*=9.5 Hz, 1H), 4.53 (m, 1H), 4.31–4.07 (m, 5H), 4.06–3.99 (m, 1H), 3.96 (dd, *J*=12.4, 2.2 Hz, 1H), 3.52–3.44 (m, 2H), 2.85 (m, 2H), 2.63 (dd, *J*=16.1, 6.5 Hz, 1H), 2.44 (dd, *J*=16.1, 6.5 Hz, 1H), 1.99 (s, 3H), 1.97 (s, 3H), 1.94 (s, 3H), 1.91 (s, 3H), 1.83 (s, 3H), 1.61(m, 1H), 1.47 (m, 1H), 1.36 (s, 9H), 1.35–1.26 (m, 2H), 1.26–1.20 (m, 2H), 1.21–1.13 (m, 6H), 1.11 (s, 9H). **MS** (ESI) *m/z* 1017 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{44}H_{73}N_8O_{19}$ 1017.4986, found 1017.4988.

Ac-Ala-Arg(Pbf)-Asn(Glc(Ac)$_4$)-Ala-Ser(*t*Bu)-NH$_2$ (139f)



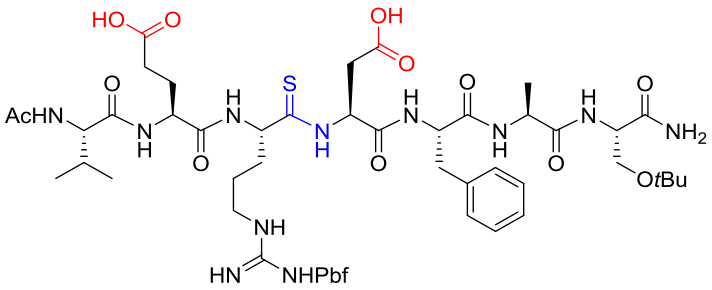
The title compound **139f** was prepared from thiopeptide **138f** (11 mg, 0.012 mmol) according to General Procedure G. After HPLC purification, the title compound **139f** was obtained as a white solid (4.9 mg, 34%). **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.77 (d, *J*=9.5 Hz, 1H), 8.14 (d, *J*=7.8 Hz, 1H), 8.03 (d, *J*=7.2 Hz, 1H), 7.93 (d, *J*=7.7 Hz, 1H), 7.84 (d, *J*=7.2 Hz, 1H), 7.75 (d, *J*=8.1 Hz, 1H), 7.13 (s,1H), 7.08 (s,1H), 6.94–6.46 (m, 2H), 6.37 (s, 1H), 5.38–5.26 (m, 2H), 4.89 (t, *J*=9.8 Hz, 1H), 4.80 (t, *J*=9.5 Hz, 1H), 4.53 (m, 1H), 4.30–4.10 (m, 5H), 4.02 (m, 1H), 3.96 (dd, *J*=12.5, 2.3 Hz, 1H), 3.00 (q, *J*=6.6 Hz, 2H), 2.96 (s, 2H), 2.62 (dd, *J*=16.2, 6.4 Hz, 1H), 2.49–2.43 (m, 4H), 2.41 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.93 (s, 3H), 1.92 (s, 3H), 1.82 (s, 3H), 1.65 (m, 1H), 1.48 (m, 1H), 1.45–1.28 (m, 8H), 1.18 (m, 6H), 1.10 (s, 9H). **MS** (ESI) *m/z* 1197 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{52}H_{81}N_{10}O_{20}S$ 1197.5344, found 1197.5361.

Ac-Val-Glu-Arg[S](Pbf)-Asp-Gly-Ala-Ser(*t*Bu)-NH$_2$ (142a)



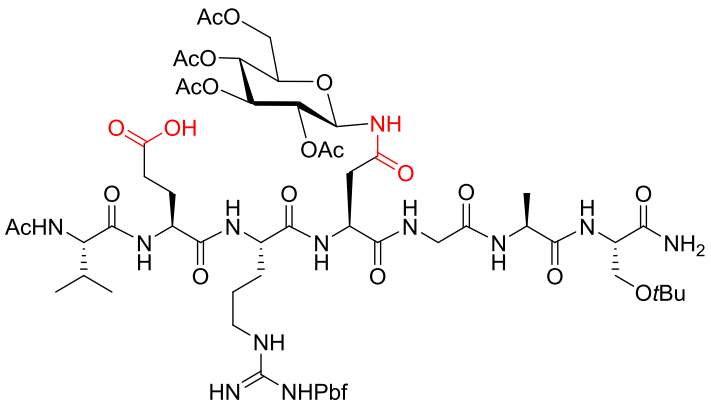
The title compound **142a** was prepared according to General Procedure F (0.05 mmol scale). After HPLC purification, the title compound **142a** was obtained as a white solid (6 mg, 11%). **MS** (ESI) *m/z* 1098 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{47}H_{76}N_{11}O_{15}S_2$ 1098.4958, found 1098.4958.

Ac-Val-Glu-Arg[S](Pbf)-Asp-Phe-Ala-Ser(*t*Bu)-NH$_2$ (142b)



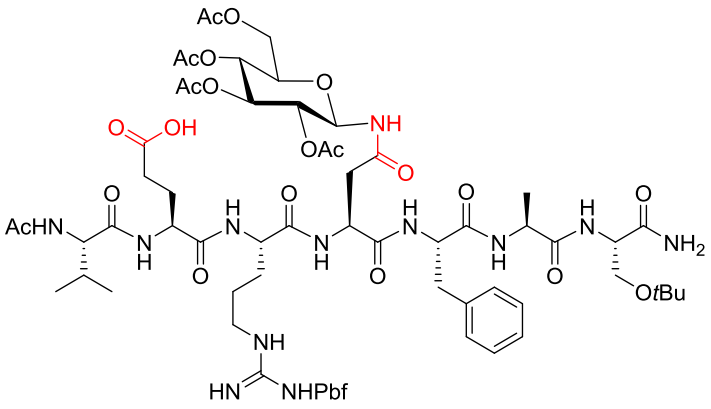
The title compound **142b** was prepared according to General Procedure F on (0.05 mmol) scale. After HPLC purification, the title compound **142b** was obtained as a white solid (7 mg, 13%). **MS** (ESI) *m/z* 1188 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{54}H_{82}N_{11}O_{15}S_2$ 1188.5428, found 1188.5428.

Ac-Val-Glu-Arg(Pbf)-Asn(Glc(Ac)$_4$)-Gly-Ala-Ser(*t*Bu)-NH$_2$ (143a)



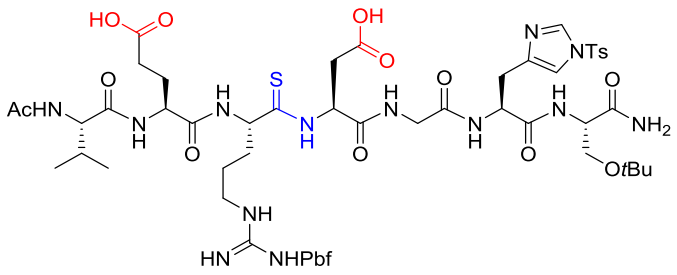
The title compound **143a** was prepared from thiopeptide **142a** (5 mg, 0.005 mmol) according to General Procedure G. After HPLC purification, the title compound **143a** was obtained as a white solid (1.1 mg, 16%). **MS** (ESI) *m/z* 1411 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{61}H_{95}N_{12}O_{24}S$ 1411.6297, found 1411.6297.

Ac-Val-Glu-Arg(Pbf)-Asn(Glc(Ac)$_4$)-Phe-Ala-Ser(*t*Bu)-NH$_2$ (143b)



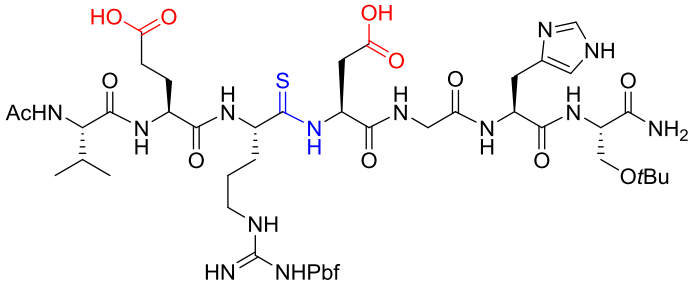
The title compound **143b** was prepared from thiopeptide **142b** (6 mg, 0.005 mmol) according to General Procedure G. After HPLC purification, the title compound **143b** was obtained as a white solid (1.5 mg, 29%). **MS** (ESI) *m/z* 1411 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{68}H_{101}N_{12}O_{24}S$ 1501.6767, found 1411.6767.

Ac-Val-Glu-Arg[S](Pbf)-Asp-Gly-His(Ts)-Ser(*t*Bu)-NH_2 (144)



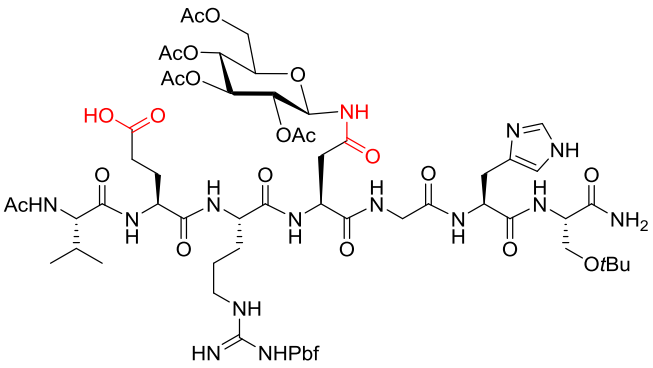
The title compound **144** was prepared according to General Procedure F on (0.05 mmol) scale. After HPLC purification, the title compound **144** was obtained as a white solid (6.6 mg, 10%). **MS** (ESI) *m/z* 1318 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{57}H_{84}N_{13}O_{17}S_3$ 1318.5265, found 1318.5262.

Ac-Val-Glu-Arg[S](Pbf)-Asp-Gly-His-Ser(tBu)-NH_2 (146)



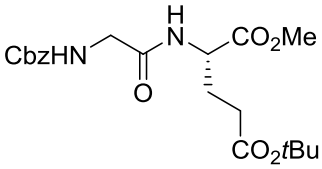
The title compound **146** was prepared according to General Procedure F on (0.05 mmol) scale. After HPLC purification, the title compound **146** was obtained as a white solid (7 mg, 12%). **MS** (ESI) *m/z* 1164 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{50}H_{78}N_{13}O_{15}S_2$ 1164.5176, found 1164.5175.

Ac-Val-Glu-Arg(Pbf)-Asn(Glc(Ac)$_4$)-Gly-His-Ser(tBu)-NH$_2$ (147)



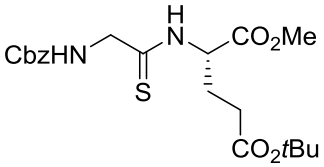
The title compound **147** was prepared from thiopeptide **146** (6 mg, 0.005 mmol) and aminosugar **14** (12.5 mg, 0.036 mmol) according to General Procedure G. After HPLC purification, the title compound **147** was obtained as a white solid (2.9 mg, 39%). **MS** (ESI) *m/z* 1501 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{64}H_{97}N_{14}O_{24}S$ 1477.6515, found 1477.6512.

Cbz-Gly-Glu(tBu)-OMe (158)



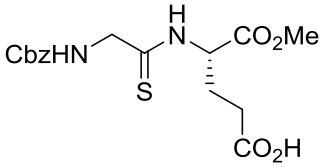
Dipeptide **158** was prepared from Glu(OtBu)-OMe hydrochloride **157** (254 mg, 1.0 mmol) and Cbz-Gly **93** (209 mg, 1.0 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **158** (392 mg, 96%) as colourless crystals; R_f 0.34 (10% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.44–7.20 (m, 5H), 6.79 (d, *J*=7.5 Hz, 1H), 5.41 (m, 1H), 5.13 (s, 2H), 4.60 (m, 1H), 3.99–3.84 (m, 2H), 3.74 (s, 3H), 2.39–2.20 (m, 2H), 2.14 (m, 1H), 1.95 (m, 1H), 1.43 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 172.1, 169.0, 156.5, 136.1, 128.5, 128.2, 128.0, 81.0, 67.2, 52.5, 51.8, 44.3, 31.3, 28.0, 27.1. **MS** (ESI) *m/z* 409 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{20}H_{29}N_2O_7$ 409.1969, found 409.1969.

Cbz-Gly[S]-Glu(tBu)-OMe (159)



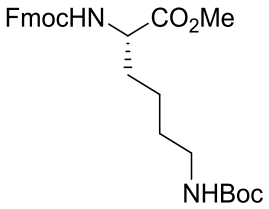
Compound **159** was prepared from dipeptide **158** (204 mg, 0.5 mmol) according to General Procedure B. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the title compound **159** (180 mg, 85%) as a light yellow oil; R_f 0.45 (50% EtOAc/Hex. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 7.47–7.21 (m, 5H), 5.59 (m, 1H), 5.15 (s, 2H), 5.10 (m, 1H), 4.34–4.18 (m, 2H), 3.75 (s, 3H), 2.43–2.30 (m, 2H), 2.26 (m, 1H), 2.11 (m, 1H), 1.43 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 200.1, 172.4, 171.0, 156.7, 136.0, 128.5, 128.2, 128.1, 81.3, 67.4, 57.2, 52.7, 51.9, 31.3, 28.0, 26.0. **MS** (ESI) *m/z* 409 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{20}H_{29}N_2O_6S$ 425.1741, found 425.1741.

Cbz-Gly[S]-Glu-OMe (160)



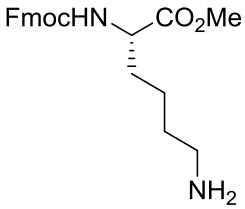
To a solution of **159** (100 mg, 0.24 mmol) in DCM (2 mL) was added TFA (2mL) and the solution was stirred for 1 h at room temperature. The solvent and TFA were evaporated to afford the title compound **160** (85 mg) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 369 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{16}H_{21}N_2O_6S$ 369.1115, found 369.1113.

Fmoc-Lys(Boc)-OMe (161)



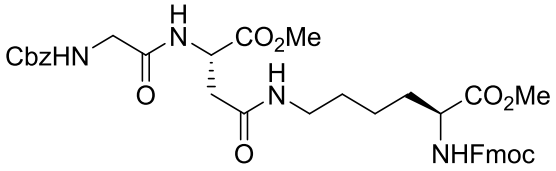
To a suspension of **131e** (937 mg, 2.0 mmol) and Cs_2CO_3 (978 mg, 3.0 mmol) in DMF (20 mL) at 0 °C was added methyl iodide (187 µL, 3.0 mmol). The reaction mixture was stirred at room temperature for 16 h. The mixture was diluted with EtOAc (25 mL) and water (10 mL). The aqueous layer was extracted with EtOAc (2 × 25 mL) and the combined organic extracts were washed with water (30 mL), brine (30 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (30% EtOAc/Hex) to give the title compound **161** as a white solid (917 mg, 95%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, *J*=7.5 Hz, 2H), 7.65–7.56 (m, 2H), 7.40 (t, *J*=7.5 Hz, 2H), 7.32 (dd, *J*=8.1, 6.6 Hz, 2H), 5.37 (d, *J*=8.3 Hz, 1H), 4.55 (s, 1H), 4.47–4.31 (m, 3H), 4.23 (t, *J*=7.0 Hz, 1H), 3.75 (s, 3H), 3.11 (q, *J*=6.6 Hz, 2H), 1.86 (m, 1H), 1.71 (m, 1H), 1.53–1.32 (m, 13H);^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9, 156.1, 156.0, 143.9, 143.8, 141.3, 127.7, 127.1, 125.1, 120.0, 120.0, 67.0, 53.7, 52.4, 47.2, 40.0, 32.1, 29.6, 28.4, 22.4. MS (ESI) *m/z* 483 [$(M+H)^+$,100%]; HRMS (ESI, $[M+H]^+$ calcd. for $C_{27}H_{35}N_2O_6$ 483.2490, found 483.2487.

Fmoc-Lys-OMe (162)



To a solution of **161** (200 mg, 0.4 mmol) in DCM (3.0 mL) at 0 °C, was added TFA (1.0 mL). The mixture was stirred for 1 h at room temperature. The solution was brought to pH 8.0 by adding saturated aqueous $NaHCO_3$. The aqueous layer was extracted with DCM (3 × 10 mL) and the combined organic extracts were dried with Na_2SO_4. The solvent was evaporated under reduced pressure to give **162** as colourless oil (155 mg), which was used in the next step without purification. **MS** (ESI) *m/z* 383 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{22}H_{27}N_2O_4$ 383.1965, found 383.1962.

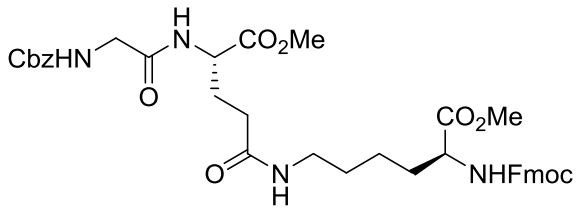
Cbz-Gly-Asp(Fmoc-Lys-OMe)-OMe (163)



To a solution of **97** (35 mg, 0.1 mmol) in dichloromethane (3.0 mL) was added Ag_2CO_3 (41 mg, 0.15 mmol) and **162** (77 mg, 0.2 mmol) and the mixture was stirred at room temperature for 2 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **163** (59 mg, 84%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.20 (d, *J*=7.8 Hz, 1H), 7.94–7.83 (m, 3H), 7.74 (d, *J*=7.7 Hz, 1H), 7.69 (dd, *J*=7.5, 2.6 Hz, 2H), 7.45 (t, *J*=6.2 Hz, 1H), 7.40 (t, *J*=7.4 Hz, 2H), 7.37 – 7.21 (m, 7H), 5.01 (s, 2H), 4.60 (m, 1H), 4.34–4.24 (m, 2H), 4.20 (m, 1H), 3.97 (m, 1H), 3.66–3.53 (m, 2H), 3.60 (s, 3H), 3.57 (s, 3H), 3.06–2.93 (m, 2H), 2.60–2.49 (m, 2H), 1.75–1.51 (m, 2H), 1.47–1.18 (m,

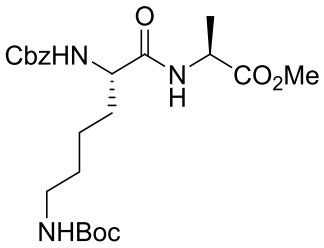
4H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 173.4, 172.1, 169.5, 169.0, 156.9, 156.6, 144.2, 141.2, 137.5, 128.8, 128.2, 128.1, 127.5, 125.7, 120.6, 66.1, 65.9, 54.3, 52.3, 49.2, 47.1, 43.7, 38.7, 37.4, 30.8, 29.0, 23.4. **MS** (ESI) *m/z* 703 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{37}H_{43}N_4O_{10}$ 703.2974, found 703.2977.

Cbz-Gly-Glu(Fmoc-Lys-OMe)-OMe (164)



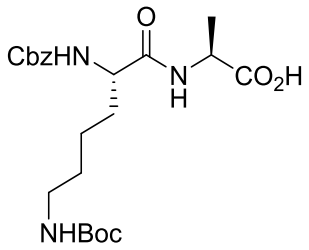
To a solution of **160** (37 mg, 0.1 mmol) in DCM (3.0 mL) was added Ag_2CO_3 (41 mg, 0.15 mmol) and **162** (77 mg, 0.2 mmol) and the mixture was stirred at room temperature for 2 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH/DCM) to give the compound **164** (34 mg, 47%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.29 (d, *J*=7.4 Hz, 1H), 7.88 (d, *J*=7.5 Hz, 2H), 7.80–7.72 (m, 2H), 7.70 (dd, *J*=7.5, 2.4 Hz, 2H), 7.40 (m, 3H), 7.36–7.21 (m, 7H), 5.01 (s, 2H), 4.29 (m, 1H), 4.25– 4.16 (m, 2H), 4.0 –3.97 (m, 2H), 3.6 –3.62 (m, 2H), 3.60 (s, 3H), 3.59 (s, 3H), 3.04–2.93 (m, 2H), 2.17–2.00 (m, 2H), 1.92 (m, 1H), 1.78 (m, 1H), 1.71–1.50 (m, 2H), 1.43–1.13 (m, 4H).**^{13}C NMR** (101 MHz, DMSO-d_6) δ 173.4, 172.7, 171.3, 169.7, 156.9, 156.6, 144.2, 141.2, 137.5, 128.8, 128.2, 128.1, 128.1, 127.5, 125.7, 120.6, 66.1, 65.9, 54.3, 52.3, 52.0, 47.1, 43.6, 38.7, 31.9, 30.8, 29.1, 27.4, 23.4. **MS** (ESI) *m/z* 717 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{38}H_{45}N_4O_{10}$ 717.3130, found 717.3136.

Cbz-Lys(Boc)-Ala-OMe (167a)



Dipeptide **167a** was prepared from Cbz-Lys(Boc)-OH (**165**) (380 mg, 1.0 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **167a** (442 mg, 95%) as a white solid; R_f 0.34 (10% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.40–7.27 (m, 5H), 6.62 (d, *J*=7.5 Hz, 1H), 5.50 (d, *J*=7.8 Hz, 1H), 5.10 (s, 2H), 4.69 (m, 1H), 4.55 (m, 1H), 4.17 (m, 1H), 3.74 (s, 3H), 3.20–2.99 (m, 2H), 1.86 (m, 1H), 1.65 (m, 1H), 1.53–1.46 (m, 2H), 1.45–1.37 (m, 14H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.2, 171.4, 156.2, 136.2, 128.5, 128.2, 128.1, 79.1, 67.0, 54.6, 52.5, 48.0, 39.7, 32.1, 29.5, 28.4, 22.2, 18.1.**MS** (ESI) *m/z* 466 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{23}H_{36}N_3O_7$ 466.2548, found 466.2548.

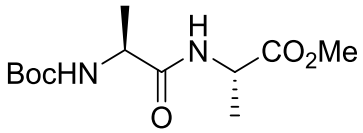
Cbz-Lys(Boc)-Ala-OH (168a)



To a solution of **167a** (100 mg, 0.22 mmol) in a mixture of MeOH/H_2O (1:1, 5 mL) cooled to 0 °C, was added LiOH.H_2O (28 mg, 0.66 mmol). The mixture was stirred for 10 min at 0 °C and 2 h at room temperature. The solution was concentrated and was diluted with water (10 mL) then acidified to pH 4 with aqueous HCl (1 M). The solution was extracted with EtOAc (3 ×10 mL) and washed with water (20 mL), brine (20 mL) and dried with Na_2SO_4. The solvent was evaporated under reduced pressure to give the crude acid **168a** as colourless oil (90 mg),

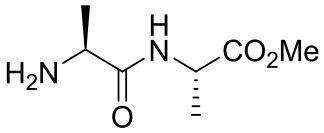
which was used in the next step without further purification. **MS** (ESI) *m/z* 452 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{22}H_{34}N_3O_7$ 452.2391, found 452.2390.

Boc-Ala-Ala-OMe (170)



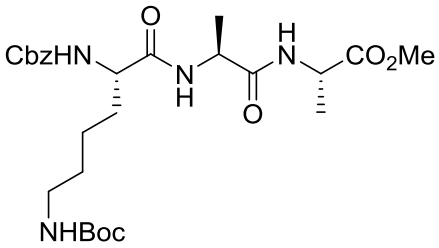
Dipeptide **170** was prepared from Boc-Ala-OH **169** (189 mg, 1.0 mmol) and H-Ala-OMe (140 mg, 1.0 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **170** (252 mg, 92%) as a white solid; R_f 0.34 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.60 (d, *J*=7.5 Hz, 1H), 5.11–4.84 (m, 1H), 4.57 (p, *J*=7.2 Hz, 1H), 4.27–4.02 (m, 1H), 3.75 (s, 3H), 1.45 (s, 9H), 1.40 (d, *J*=7.1 Hz, 3H), 1.36 (d, *J*=7.1 Hz, 3H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.2, 172.2, 52.4, 49.9, 48.0, 28.3, 18.3. **MS** (ESI) *m/z* 275 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_{12}H_{23}N_2O_5$ 275.1601, found 275.1601.

H-Ala-Ala-OMe (171)



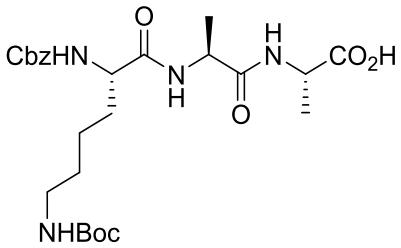
To a solution of **170** (200 mg, 0.73 mmol) in DCM (1.5 mL) at 0 °C, was added TFA (0.5 mL). The mixture was stirred for 1 h at room temperature. The solvent was evaporated under reduced pressure to give **171** as colourless oil (205 mg), which was used in the next step without purification. **MS** (ESI) *m/z* 383 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$ calcd. for $C_7H_{15}N_2O_3$ 175.1077, found 175.1075.

Cbz-Lys(Boc)-Ala-Ala-OMe (167b)



Tripeptide **167b** was prepared from Cbz-Lys(Boc)-OH **165** (278 mg, 0.73 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **167b** (341 mg, 87%) as a white solid; R_f 0.3 (10% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.44–7.24 (m, 5H), 6.87 (d, *J*=8.0 Hz, 1H), 6.77 (d, 1H), 5.79–5.67 (m, 1H), 5.09 (s, 2H), 4.75 (t, *J*=5.9 Hz, 1H), 4.52 (h, *J*=7.7 Hz, 2H), 4.23–4.09 (m, 1H), 3.73 (s, 3H), 3.18–2.98 (m, 2H), 1.90–1.62 (m, 3H), 1.4 (s, 9H), 1.51–1.34 (m, 10H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.1, 171.7, 171.5, 156.4, 136.1, 128.5, 128.2, 128.1, 79.2, 67.1, 54.9, 52.5, 48.8, 48.1, 39.5, 31.8, 29.5, 28.4, 22.2, 18.1. **MS** (ESI) *m/z* 537 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{26}H_{41}N_4O_8$ 537.2919, found 537.2922.

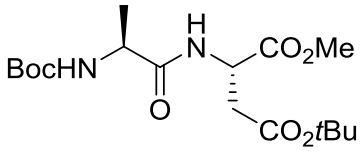
Cbz-Lys(Boc)-Ala-Ala-OH (168b)



To a solution of **167a** (118 mg, 0.22 mmol) in a mixture of MeOH/H_2O (1:1, 5 mL) cooled to 0 °C, was added LiOH.H_2O (28 mg, 0.66 mmol). The mixture was stirred for 10 min at 0 °C and 2 h at room temperature. The solution was concentrated and was diluted with water (10 mL) then acidified to pH 4 with aqueous HCl (1 M). The solution was extracted with EtOAc (3 ×10 mL) and washed with water (20 mL), brine (20 mL) and dried with Na_2SO_4. The solvent

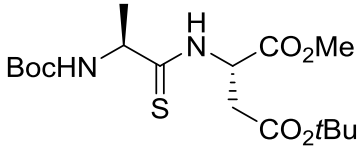
was evaporated under reduced pressure to give the crude acid **168b** as colourless oil (105 mg) which was used in the next step without further purification. **MS** (ESI) *m/z* 536 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{26}H_{40}N_4O_8$ 536.2846, found 536.2844.

Boc-Ala-Asp(*t*Bu)-OMe (172)



Dipeptide **172** was prepared from Boc-Ala-OH **169** (189 mg, 1.0 mmol) and H-Asp(tBu)-OMe hydrochloride (**94**) (240 mg, 1.0 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **172** (356 mg, 95%) as a white solid; R_f 0.38 (10% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 6.93 (d, *J*=8.3 Hz, 1H), 5.05 (s, 1H), 4.78 (dt, *J*=8.7, 4.6 Hz, 1H), 4.26–4.07 (m, 1H), 3.73 (s, 3H), 2.92 (dd, *J*=17.0, 4.6 Hz, 1H), 2.70 (dd, *J*=17.0, 4.6 Hz, 1H), 1.43 (s, 9H), 1.42 (s, 9H), 1.36 (d, *J*=7.0 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 172.5, 171.1, 170.0, 81.8, 52.6, 48.6, 37.3, 28.3, 28.0, 18.6. **MS** (ESI) *m/z* 375 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{17}H_{31}N_2O_7$ 375.2126, found 375.2126.

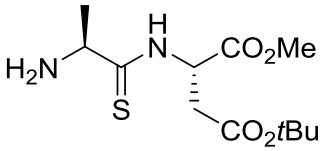
Boc-Ala$^{[S]}$-Asp(*t*Bu)-OMe (173)



Compound **173** was prepared from dipeptide **172** (337 mg, 0.9 mmol) according to General Procedure B. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the title compound **173** (288 mg, 82%) as a light yellow oil; R_f 0.47 (50% EtOAc/Hex. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.63 (d, *J*=7.8 Hz, 1H), 5.38 (dt, *J*=8.1, 4.2 Hz, 1H), 5.20 (s, 1H), 4.46 (m, 1H), 3.78 (s, 3H), 3.07–2.94 (m, 2H), 1.46 (d, *J*=6.9 Hz, 3H), 1.44 (s, 9H), 1.43 (s,

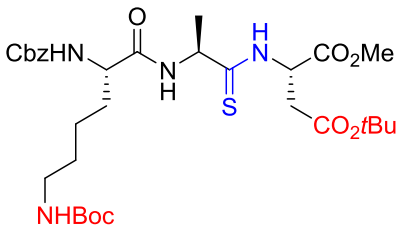
9H).^{13}**C NMR** (101 MHz, $CDCl_3$) δ 205.4, 170.2, 170.0, 154.9, 82.2, 53.7, 52.8, 35.9, 28.3, 28.0, 21.9. **MS** (ESI) *m/z* 391 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{17}H_{31}N_2O_6S$ 391.1897, found 391.1899.

H-Ala$^{[S]}$-Asp(*t*Bu)-OMe (174)



To a solution of **174** (234 mg, 0.6 mmol) in DCM (9.0 mL) at 0 °C, was added TFA (1.0 mL). The mixture was stirred for 1 h at room temperature. The solution was brought to pH 8.0 by adding saturated aqueous $NaHCO_3$. The aqueous layer was extracted with DCM (3 × 15 mL) and the combined organic extracts were dried with Na_2SO_4. The solvent was evaporated under reduced pressure to give **174** as a yellow oil (144 mg) which was used in the next step without purification. **MS** (ESI) *m/z* 291 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{12}H_{23}N_2O_4S$ 291.1373, found 291.1372.

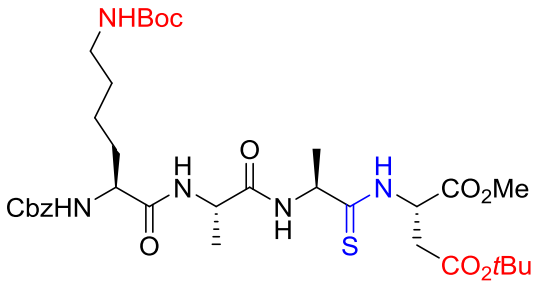
Cbz-Lys(Boc)- Ala$^{[S]}$-Asp(tBu)-OMe (175a)



Tripeptide **175a** was prepared from Z-Lys(Boc)-OH **165** (19 mg, 0.05 mmol) and amine **174** (15 mg, 0.05 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **175a** (27 mg, 83%) as a white solid; R_f 0.35 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.71 (d, *J*=9.4 Hz, 1H), 7.4 –7.27 (m, 5H), 7.12 (d, *J*=7.4 Hz, 1H), 5.60 (s, 1H), 5.39 (m, 1H), 5.15–5.07 (m, 2H), 4.78 (m, 1H), 4.67 (s, 1H),

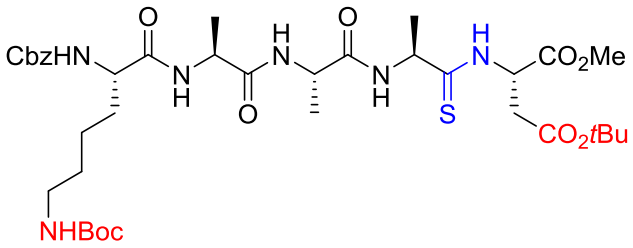
4.19 (m, 1H), 3.76 (s, 3H), 3.19–3.05 (m, 2H), 3.00 (dd, *J*=17.2, 4.4 Hz, 1H), 2.93 (dd, *J*=17.1, 4.3 Hz, 1H), 1.96–1.76 (m, 2H), 1.75–1.60 (m, 2H), 1.51–1.45 (m, 5H), 1.43 (s, 9H), 1.41 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 204.9, 171.9, 171.6, 171.1, 170.2, 170.1, 170.0, 156.3, 136.2, 128.5, 128.1, 82.2, 81.9, 79.2, 67.1, 54.8, 54.7, 53.9, 52.8, 48.9, 48.7, 37.3, 36.0, 29.5, 28.4, 28.0, 22.4, 22.0. **MS** (ESI) *m/z* 291 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{31}H_{49}N_4O_9S$ 653.3215, found 653.3217.

Cbz-Lys(Boc)-Ala-Ala$^{[S]}$-Asp(tBu)-OMe (175b)



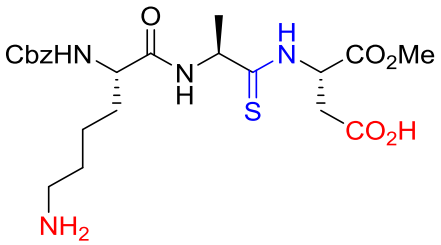
Tetrapeptide **175b** was prepared from Cbz-Lys(Boc)-Ala-OH **168a** (90 mg, 0.2 mmol) and amine **174** (58 mg, 0.2 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **175b** (116 mg, 80%) as a white solid; R_f 0.31 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.23 (d, *J*=7.7 Hz, 1H), 8.05 (d, *J*=7.6 Hz, 1H), 8.00 (d, *J*=7.4 Hz, 1H), 7.44–7.24 (m, 5H), 6.74 (t, *J*=5.8 Hz, 1H), 5.25 (m, 1H), 5.01 (s, 2H), 4.70 (m, 1H), 4.28 (m, 1H), 3.95 (m, 1H), 3.63 (s, 3H), 2.95–2.82 (m, 2H), 2.82–2.70 (m, 2H), 1.70–1.44 (m, 2H), 1.38 (s, 9H), 1.36 (s, 9H), 1.26 (d, *J*=6.9 Hz, 3H), 1.36–1.08 (m, 2H), 1.21 (d, *J*=7.1 Hz, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 206.9, 172.2, 171.7, 170.1, 168.9, 156.4, 156.0, 137.5, 128.8, 128.2, 128.1, 81.3, 77.8, 65.8, 55.0, 54.4, 54.1, 52.8, 48.3, 36.4, 32.1, 29.7, 28.7, 28.1, 23.3, 21.8, 18.4. **MS** (ESI) *m/z* 724 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{34}H_{54}N_5O_{10}S$ 724.3586, found 724.3589.

Cbz-Lys(Boc)-Ala-Ala-Ala[S]-Asp(tBu)-OMe (175c)



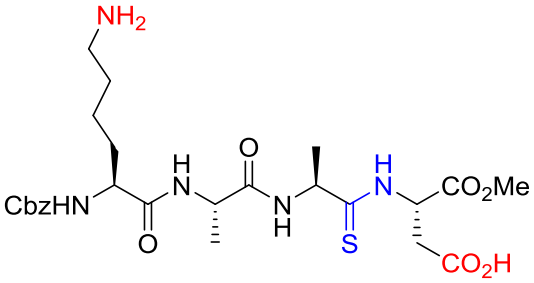
Pentapeptide **176c** was prepared from Cbz-Lys(Boc)-Ala-Ala-OH **168b** (52 mg, 0.1 mmol) and amine **174** (29 mg, 0.1 mmol) according to General Procedure A. Purification by column chromatography gave the title compound **175c** (61 mg, 76%) as a white solid; R_f 0.3 (5% MeOH/DCM). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.23 (d, *J*=7.5 Hz, 1H), 8.10–7.90 (m, 3H), 7.45–7.23 (m, 6H), 6.74 (t, *J*=5.8 Hz, 1H), 5.25 (m, 1H), 5.01 (s, 2H), 4.69 (m, 1H), 4.33–4.20 (m, 2H), 3.94 (m, 1H), 3.63 (s, 3H), 2.91–2.82 (m, 2H), 2.84–2.72 (m, 2H), 1.63–1.45 (m, 2H), 1.38 (s, 9H), 1.36 (s, 9H), 1.42-125(m, 6H), 1.27 (d, *J*=6.8 Hz, 3H), 1.22 (s, 3H), 1.20 (s, 3H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 206.9, 172.3, 172.1, 171.6, 170.1, 168.9, 156.4, 137.5, 128.8, 128.2, 128.1, 81.3, 77.8, 65.8, 55.0, 54.4, 54.1, 52.8, 48.4, 36.4, 32.0, 29.6, 28.7, 28.1, 23.3, 21.8, 18.7, 18.3. **MS** (ESI) *m/z* 795 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{37}H_{59}N_6O_{11}S$ 795.3957, found 795.3960.

Cbz-Lys-Ala[S]-Asp-OMe (176a)



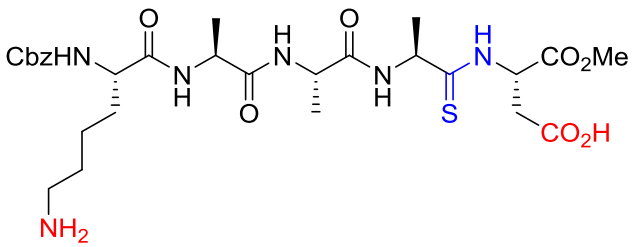
To a solution of **175a** (25 mg, 0.038 mmol) in DCM (1 mL) was added TFA (1 mL) and the solution was stirred for 1 h. The solvent and TFA were evaporated to afford the title compound **176a** (16 mg) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 496 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{22}H_{33}N_4O_7S$ 497.5865, found 497.5863.

Cbz-Lys-Ala-Ala[S]-Asp-OMe (176b)



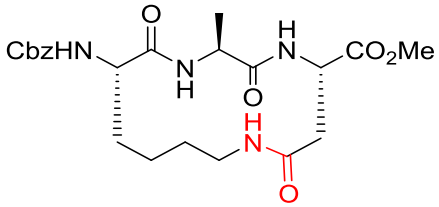
To a solution of **176b** (100 mg, 0.14 mmol) in DCM (1 mL) was added TFA (1 mL) and the solution was stirred for 1 h. The solvent and TFA were evaporated to afford the title compound **176b** (60 mg) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 568 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{25}H_{38}N_5O_8S$ 568.2436, found 568.2433.

Cbz-Lys-Ala-Ala-Ala[S]-Asp-OMe (176c)



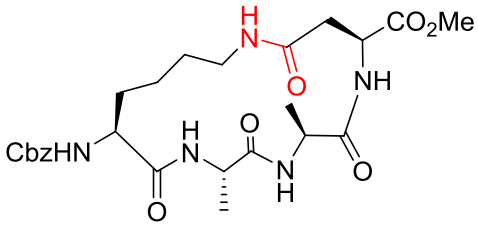
To a solution of **176c** (50 mg, 0.063 mmol) in DCM (1 mL) was added TFA (1 mL) and the solution was stirred for 1 h. The solvent and TFA were evaporated to afford the title compound **176c** (40 mg) as colourless oil which was used without further purification. **MS** (ESI) *m/z* 639 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{28}H_{43}N_6O_9S$ 639.2807, found 639.2805.

Cbz-(cyclo-1,3)-[Lys-Ala-Asp]-OMe (177a)



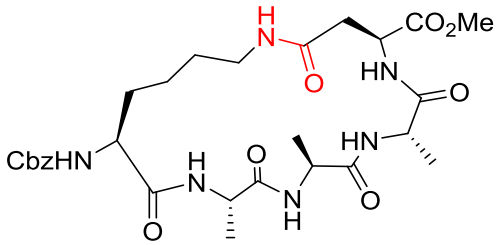
To a solution of **176a** (16 mg, 0.0.032 mmol) in DCM (10 mL) was added Ag_2CO_3 (11 mg, 0.039 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH /DCM) to give the stapled peptide **177a** (11 mg, 76%) as a white solid. **^{1}H NMR** (500 MHz, DMSO-d_6) δ 8.38 (d, *J*=8.4 Hz, 1H), 7.90 (d, *J* = 7.5 Hz, 1H), 7.59 (t, *J*=5.7 Hz, 1H), 7.39–7.28 (m, 6H), 5.04–4.93 (m, 2H), 4.53 (m, 1H), 4.43 (m, 1H), 3.94 (m, 1H), 3.61 (s, 3H), 3.29–3.13 (m, 2H), 2.56–2.51 (m, 2H), 1.56–1.39 (m, 2H), 1.31–1.20 (m, 2H), 1.19–1.02 (m, 5H). **MS** (ESI) *m/z* 463 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H] $^+$) calcd. for $C_{22}H_{31}N_4O_7$ 463.5105, found 463.5103.

Cbz-(cyclo-1,4)-[Lys-Ala-Ala-Asp]-OMe (177b)



To a solution of **176b** (50 mg, 0.09 mmol) in DCM (10 mL) was added Ag_2CO_3 (30 mg, 0.11 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH /DCM) 3to give the stapled peptide **177b** (38 mg, 81%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.19 (d, *J*=7.7 Hz, 1H), 8.02 (d, *J*=8.3 Hz, 1H), 7.73 (t, *J*=5.5 Hz, 1H), 7.56 (d, *J*=6.7 Hz, 1H), 7.40–7.24 (m, 6H), 4.99 (s, 2H), 4.66 (m, 1H), 4.25–4.11 (m, 2H), 3.97 (m, 1H), 3.61 (s, 3H), 3.12–2.89 (m, 2H), 2.48–2.39 (m, 2H), 1.46 (m, 2H), 1.34–1.11 (m, 10H).**^{13}C NMR** (101 MHz, DMSO-d_6) δ 172.5, 172.0, 171.6, 169.0, 137.5, 128.8, 128.2, 128.2, 65.8, 54.7, 52.6, 49.6, 49.5, 48.9, 38.8, 38.1, 32.3, 29.2, 22.4, 18.6, 17.9. **MS** (ESI) *m/z* 534 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{25}H_{36}N_5O_8$ 534.2558, found 534.2559.

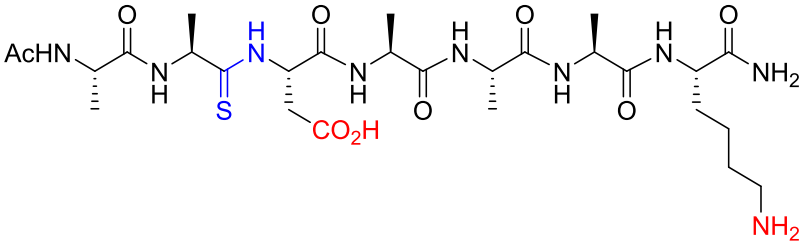
Cbz-(cyclo-1,5)-[Lys-Ala-Ala-Ala-Asp]-OMe (177c)



To a solution of **176c** (40 mg, 0.063 mmol) in DCM (10 mL) was added Ag_2CO_3 (21 mg, 0.076 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by column chromatography (10% MeOH /DCM) to give the stapled peptide **177c** (30 mg, 81%) as a white solid. **^{1}H NMR** (400 MHz, DMSO-d_6) δ 8.40 (d, *J*=8.1 Hz, 1H), 8.17 (d, *J*=5.4 Hz, 1H), 7.93 (d, *J*=8.0 Hz, 1H), 7.46 (t, *J*=5.7 Hz, 1H), 7.39 (d, *J*=7.5

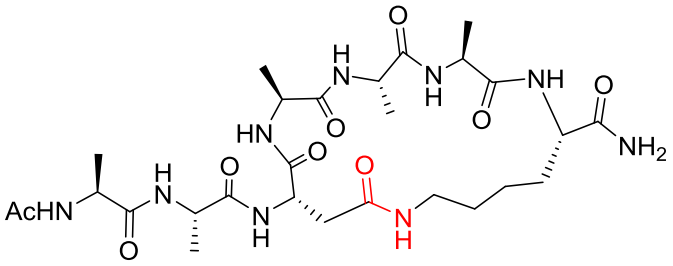
Hz, 1H), 7.32 (m, 5H), 7.26 (d, *J*=7.2 Hz, 1H), 4.98 (s, 2H), 4.53 (m, 1H), 4.22–4.10 (m, 2H), 3.99 (m, 1H), 3.91 (m, 1H), 3.60 (s, 3H), 3.23 (m, 1H), 2.81 (m, 1H), 2.44–2.39 (m, 2H), 1.50–1.35 (m, 4H), 1.24–1.11 (m, 11H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 206.9, 172.3, 172.1, 171.6, 170.1, 168.9, 156.4, 137.5, 128.8, 128.2, 128.1, 81.3, 77.8, 65.8, 55.0, 54.4, 54.1, 52.8, 48.4, 36.4, 32.0, 29.6, 28.7, 28.1, 23.3, 21.8, 18.7, 18.3. **MS** (ESI) *m/z* 605 [(M+H)$^+$,100%]; **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{28}H_{41}N_6O_9$ 605.2930, found 605.2934.

Ac-Ala-Ala$^{[S]}$-Asp-Ala-Ala-Ala-Lys-NH$_2$ (180)



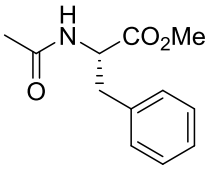
The title compound **180** was prepared according to General Procedure F on (0.05 mmol) scale. Peptide thioamide on resin was treated with TFA in DCM (50%) for 30 min. The solvent was drained and this process was repeated a further 2 times. The solvent was evaporated and the residue (3.4 mg) was used in the next step without further purification. **MS** (ESI) *m/z* 674 [(M+H)$^+$,100%]. **HRMS** (ESI, [M+H]$^+$) calcd. for $C_{27}H_{48}N_9O_9S$ 674.3290, found 674.3293.

Ac-Ala-Ala-(cyclo-3,7)-[Asp-Ala-Ala-Ala-Lys]-NH_2 (181)



To a solution of peptide thioamide **180** (3.4 mg) in DCM/MeCN (1:1, 5 mL) was added Ag_2CO_3 (1.7 mg, 0.006 mmol) and the mixture was stirred at room temperature for 6 h. The solvent was evaporated and the residue purified by HPLC to give the product **181** (0.4 mg) as a white solid. **MS** (ESI) *m/z* 640 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{27}H_{44}N_9O_9$ 640.3413, found 640.3417.

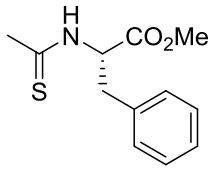
Ac-Phe-OMe (197)



Acetic anhydride (284 µL, 3.0 mmol) was added dropwise to a cooled solution at 0 °C of **196** (431 mg, 2mmol) and Et_3N (555 µL, 4.0 mmol) in DCM (10 mL) and the solution was stirred for 4 h. The solution was diluted with DCM (10 mL) and partitioned with 1 N HCl (20 mL). The aqueous phase was extracted with DCM (2 × 20 mL). The combined organic fractions were washed with aqueous HCl (30 mL, 1N), saturated aqueous $NaHCO_3$ (30 mL), brine (30 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure to provide the crude amide **197** (439 mg) which was used in the next step without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.35–7.20 (m, 3H), 7.14–7.02 (m, 2H), 5.92 (d, *J*=7.8 Hz, 1H), 4.89 (m, 1H), 3.72 (s, 3H), 3.1 –3.04 (m, 2H), 1.98 (s, 3H).**^{13}C NMR** (101 MHz, $CDCl_3$) δ

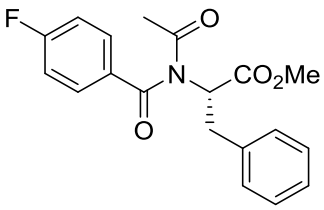
173.2, 172.3, 137.1, 131.1, 131.0, 129.5, 128.6, 126.9, 60.4, 52.8, 35.1, 26.7. **MS** (ESI) *m/z* 222 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{12}H_{16}NO_3$ 222.1125, found 222.1124.

Ac-Phe$^{[S]}$-OMe (198)



Thioamide **198** was prepared from the amide **197** (430 mg, 1.94 mmol) according to General Procedure B. Purification of the residue by column chromatography (30% EtOAc/Hex) gave the title compound **198** (419 mg, 82%) as a white solid; R_f 0.34 (30% EtOAc/Hex). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.54 (s, 1H), 7.32–7.23 (m, 3H), 7.12–7.02 (m, 2H), 5.40 (m, 1H), 3.77 (s, 3H), 3.43 (dd, *J*=13.9, 6.1 Hz, 1H), 3.21 (dd, *J*=14.0, 4.4 Hz, 1H), 2.56 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 200.9, 171.3, 135.4, 129.2, 128.7, 127.3, 58.6, 52.6, 36.1, 34.2. **MS** (ESI) *m/z* 238 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{12}H_{16}NO_2S$ 238.0896, found 238.0896.

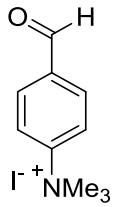
Ac-*N*-(4-Fluorobenzoyl)-Phe-OMe (200)



To a solution of 4-fluorobenzoic acid **199** (20 mg, 0.1 mmol) and thioamide **198** (52 mg, 0.22 mmol) in DCM (3.0 mL) was added Ag_2CO_3 (61 mg, 0.15 mmol). The mixture was stirred for 2 h at room temperature, then the solvent was evaporated and the residue purified by column chromatography (50% EtOAc/Hex) to afford the peptide **200** (35 mg, 100 %), as a white solid.

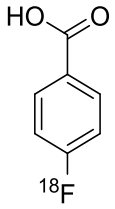
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28–7.18 (m, 5H), 7.14–7.05 (m, 2H), 7.04–6.94 (m, 2H), 5.24 (dd, *J*=11.1, 5.2 Hz, 1H), 3.81 (s, 3H), 3.50 (dd, *J*=14.3, 5.2 Hz, 1H), 3.40 (dd, *J*=14.2, 11.1 Hz, 1H), 1.89 (s, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.2, 172.3, 170.2, 166.4, 163.9, 137.1, 131.6, 131.1, 131.0, 129.5, 128.6, 126.9, 116.0, 115.8, 60.4, 52.8, 52.7, 35.1, 26.7. **MS** (ESI) *m/z* 344 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{19}H_{19}FNO_4$ 344.1293, found 344.1292.

4-*N,N,N*-Trimethylaminobenzaldehyde (202)



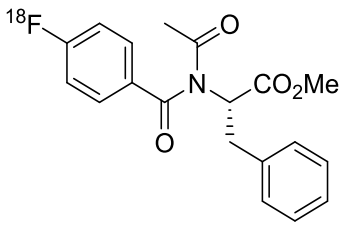
To a solution of 4-*N,N*-dimethylaminobenzaldehyde **201** (200 mg, 1.34 mmol) in acetone (3 mL), was added MeI (250 µL, 4.02 mmol), and the mixture was heated at reflux for 16 h. The precipitate was isolated by filtration, washed with acetone (10 mL) and dried to give the title compound **202** (280 mg, 72%) as a white solid, which was used in the next step without purification.**^{1}H NMR** (400 MHz, DMSO-d_6) δ 10.10 (s, 1H), 8.20 (m, 2H), 8.14 (m, 2H), 3.63 (s, 9H). **MS** (ESI) m/z 164 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{10}H_{14}NO$ 164.1070, found 164.1069.

[^{18}F]-4-Fluorobenzoic acid (184)



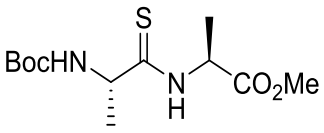
A solution of K_{222} (10 mg), $KHCO_3$ (2–3 mg) in MeCN/H_2O (8:2, 1.0 mL, vial 1) was eluted through a QMA cartridge containing $^{18}F^-$ (503 MBq) into the reaction vessel. $K_{222}.K^{18}F^-$ complex was azeotropically dried, then precursor **202** (5.0 mg, 0.017 mmol, vial 3) in anhydrous DMSO (0.6 mL) was added. The mixture was heated for 5 min at 80 °C. $NaClO_2$ (15 mg, 0.17 mmol, vial 2) in water (1.0 mL) was then added, and the mixture was heated for 5 mins at 80 °C. After cooling to room temperature, the mixture was diluted with 0.1% TFA in H_2O (5 mL). The mixture was then loaded on C18 SPE-plus cartridges, then [^{18}F]-fluorobenzoic acid **184** was eluted using anhydrous MeCN (1.0 mL) (141 MBq, 28% ndc yield).

Ac-*N*-(4-^{18}F-fluorobenzoyl)-Phe-OMe (203)



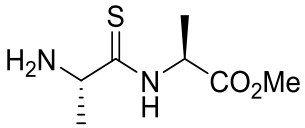
[^{18}F]-fluorobenzoic acid **184** (80.29 MBq) isolated on a C18 SPE cartridge was eluted with MeCN (0.1 ml) into a vial charged with **198** (2.0 mg, 0.008 mmol) and Ag_2CO_3 (4.6 mg, 0.016 mmol). After heating for 20 min at 70 °C, the material was purified by RP-HPLC (0.1% TFA in 25–100% MeCN:H_2O over 10 min) to afford product **203** in 96% RCC yield. The analytical trace of the radiolabeled material **193** matched that of the fully characterised cold material **200**.

Boc-Ala[S]-Ala-OMe (212)



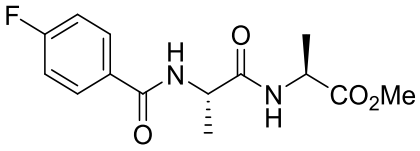
Compound **212** was prepared from dipeptide **170** (274 mg, 1.0 mmol) according to General Procedure B. Purification of the residue by column chromatography (50% EtOAc/Hex) gave the title compound **202** (232 mg, 80%) as a light yellow solid; R_f 0.45 (50% EtOAc/Hex). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.43 (s, 1H), 5.08 (m,1H), 4.46 (m, 1H), 3.78 (s, 3H), 1.51 (d, *J*=7.1 Hz, 3H), 1.46 (d, *J*=6.9 Hz, 3H), 1.44 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 205.4, 172.4, 155.3, 80.4, 56.4, 53.1, 52.6, 28.3, 21.6, 16.9. **MS** (ESI) *m/z* 291 [$(M+H)^+$,100%]; **HRMS** (ESI, $[M+H]^+$) calcd. for $C_{12}H_{23}N_2O_4S$ 291.1373, found 291.1372.

H-Ala[S]-Ala-OMe (213)



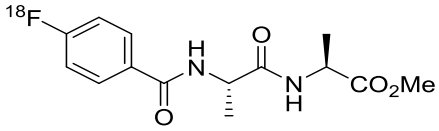
To a solution of **212** (200 mg, 0.69 mmol) in DCM (6 mL) at 0 °C, was added TFA (2 mL). The mixture was stirred for 1 h at room temperature. The solution was brought to pH 8.0 by addition of saturated aqueous $NaHCO_3$. The aqueous layer was extracted with DCM (3 × 10 mL) and the combined organic extracts were dried with Na_2SO_4. The solvent was evaporated under reduced pressure to give **213** (122 mg) as a yellow oil, which was used in the next step without purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.97 (s, 1H), 5.11 (q, *J*=7.2 Hz, 1H), 3.99 (q, *J*=6.9 Hz, 1H), 3.78 (s, 3H), 1.52 (d, *J* 6.5 Hz, 3H), 1.45 (d, *J*=7.6 Hz, 3H). **MS** (ESI) *m/z* 191 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$ calcd. for $C_7H_{15}N_2O_3$ 191.0849, found 191.0847.

***N*-(4-Fluorobenzoyl)-Ala-Ala-OMe (214)**



To a solution of 4-fluorobenzoic acid **199** (13 mg, 0.09 mmol) and thioamide **213** (25 mg, 0.13 mmol) in DCM (2.0 mL) was added Ag_2CO_3 (36 mg, 0.13 mmol). The mixture was stirred for 2 h at room temperature, then the solvent was evaporated and the residue purified by column chromatography (50% EtOAc/Hex) to afford the peptide **214** (18 mg, 67%), as a white solid. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.85–7.77 (m, 2H), 7.1–7.05 (m, 2H), 6.93 (d, *J*=7.3 Hz, 1H), 6.75 (d, *J*=7.4 Hz, 1H), 4.74 (m, 1H), 4.57 (m, 1H), 3.77 (s, 4H), 1.51 (d, *J*=7.0 Hz, 3H), 1.42 (d, *J*=7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.0, 171.9, 165.9, 163.6, 129.9, 129.5, 129.4, 115.7, 115.5, 52.7, 49.1, 48.2, 18.8, 18.2. **MS** (ESI) *m/z* 297 [$(M+H)^+$,100%]. **HRMS** (ESI, $[M+H]^+$ calcd. for $C_{14}H_{18}FN_2O_4$ 297.1245, found 297.1244.

***N*-(4-^{18}F-Fluorobenzoyl)-Ala-Ala-OMe (215)**



[^{18}F]-Fluorobenzoic acid **184** (177.6 MBq) isolated on a C18 SPE cartridge was eluted by MeCN (0.1 ml) into a vial charged with **213** (5.6 mg, 0.03 mmol), Ag_2CO_3 (10 mg, 0.036 mmol). After heating for 20 min at 70 °C, the material was purified by RP-HPLC (0.1 % TFA in 25–100% MeCN:H_2O over 10 min) to afford product **215** in 44% RCC. The analytical trace of the radiolabeled material **215** matched that of the fully characterised cold material **214**.

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