

Towards an Understanding of the Role of VGF-Derived Peptides

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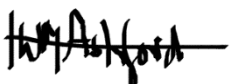
Statement of Student Contribution

This thesis is a summary of work carried out in the School of Chemistry, University of Nottingham, under the supervision of Dr Nicholas Mitchell and Dr Preeti Jethwa between October 2019 and October 2020.

Peptide synthesis was carried out in Dr Nicholas Mitchell's laboratory in the School of Chemistry, University of Nottingham. Peptides were characterised by mass spectrometry and high-performance liquid chromatography. All analytical data was obtained and interpreted by the author.

This thesis is 114 pages in length, with two appendices. All figures have been produced by the author, unless stated otherwise.

I certify that all experimental work presented in this thesis has been carried out by myself.

Signed: 

Toni Winona Marie Ashford

Date: 20/02/2021

Covid-19 Impact Statement

Due to the Covid-19 pandemic, a University of Nottingham research laboratory shutdown was initiated on the 15th March 2020. This precluded the completion of fundamental experimental work, of greatest significance being assessment of a peptide of interest to function as an insulin secretagogue (planned for Spring 2020). In light of this, a review of methods for the ^{18}F -labelling of peptides has been carried out as substitute scholarly activity. This research area is directly related to future work associated with my project. Furthermore, a detailed summary of the aforementioned insulin secretion testing has been included in Chapter 4.

Abstract

Pro-VGF, a product of the *vgf* gene, is expressed in abundance in neuronal and neuroendocrine cells, including the β cells of the islet of Langerhans. Post-translational endoproteolytic processing of pro-VGF results in formation of several biologically active peptides, which are secreted in response to different stimuli and have been shown to have various physiological roles including glycaemic control, memory and learning.

The aim of this project was to synthesise VGF-derived peptides to assess biological function, particularly in relation to diabetes mellitus and neurodegenerative diseases.

Evidence suggests that VGF-derived peptides could function as tissue-specific regulators of glucose homeostasis. It has been hypothesised that the VGF-derived peptide TLQP-62 functions as an insulin secretagogue via direct interaction with pancreatic islet β cells. However, a receptor is yet to be identified and further understanding of the role of this peptide in glucose homeostasis is required. Further insight into which tissue(s) TLQP-62 interacts with, ultimately leading to receptor identification, would be highly impactful. Furthermore, half-life data indicating whether TLQP-62 reaches the tissue(s) in a pharmacologically active concentration would infer whether longer acting analogues are required. Synthesis of TLQP-62 via solid phase peptide synthesis and a rapid and additive-free peptide ligation technique, termed “diselenide-selenoester ligation,” followed by phosphine-mediated deselenisation has successfully been carried out.

Localised change in VGF-derived peptide levels has been attributed to several neurodegenerative diseases, for example Amyotrophic lateral sclerosis, Parkinson’s disease, Alzheimer’s disease and frontotemporal dementia. VGF-derived peptides AQEE-30 and GGEE-45 have been shown to be decreased in the cerebrospinal fluid of patients affected with Alzheimer’s disease. Furthermore, altered expression of AQEE-30 has been observed in the parietal cortex. Synthesis of AQEE-30 and GGEE-8 (a truncated version of GGEE-45) has been successfully achieved using solid phase peptide synthesis.

The aim is that, in the future, which tissue(s) TLQP-62 interacts with and whether longer acting analogues are required will be determined, using N-terminal fluorine as a radionuclide. Furthermore, the biological function of AQEE-30 and GGEE-8 will be evaluated in the future by Dr Preeti Jethwa using *Drosophila melanogaster* flies.

Abbreviations

AD	Alzheimer's disease
ADIBO	Aza-dibenzocyclooctyne
AdoMet	S-adenosyl-L-Met
Ahx	6-Amino hexanoic acid
ALS	Amyotrophic lateral sclerosis
aq.	Aqueous
Ar	Aryl
ATP	Adenosine triphosphate
Ava	5-Aminopentanoic acid
A β	Amyloid- β
Boc	<i>Tert</i> -butyloxycarbonyl
BSA	Bovine serum albumin
Bt	Benzotriazole
Bu	Butyl
ca	Approximately
cAMP	Cyclic adenosine monophosphate
Ci	Curie
Cit	Citrulline
CIDEA-TEG	5'-Chloro-5'-deoxy-2-ethynyladenosine-tetraethylene glycol
CSF	Cerebrospinal fluid
CSY	Cyanosulfurylide
CT	Computed tomography
CTC	2-Chlorotriyl chloride
CuAAC	Copper(I)-catalysed azide-alkyne 1,3-dipolar cycloaddition
d	Doublet
dbcAMP	Dibutryl cyclic adenosine monophosphate
DCM	Dichloromethane
Dde	<i>N</i> -(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)

Dha	Dehydroalanine
DIC	<i>N,N'</i> -diisopropylcarbodiimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DM	Diabetes mellitus
DMAP	4-Dimethylaminopyridine
Dmb	Dimethoxybenzyl
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPDS	Diphenyldiselenide
DTT	Dithiothreitol
DVB	1–2% Divinylbenzene
ELISA	Enzyme-linked immunoassay
eq.	Equivalents
ESI ⁻	Electrospray ionisation (negative mode)
ESI ⁺	Electrospray ionisation (positive mode)
Et	Ethyl
EWG	Electron withdrawing group
FA01010	(4 <i>R</i> ,5 <i>S</i>)-4-Amino-5-methylheptanoic acid
FBS	Fetal bovine serum
FDA	5'-Fluoro-5'-deoxyadenosine
Fmoc	Fluorenylmethoxycarbonyl
Frsk	Forskolin
Gdn	Guanidinium
GSIS	Glucose-stimulated insulin secretion
HA	Hemagglutinin
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	Hexafluoro-2-propanol
Hmb	2-Hydroxy-4-methoxy-benzyl
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase

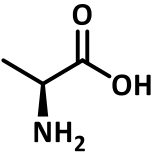
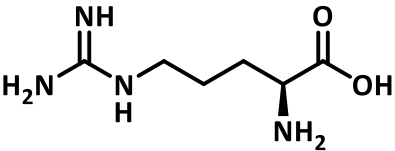
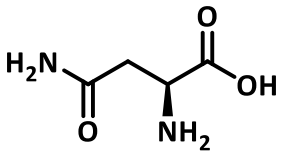
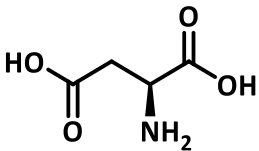
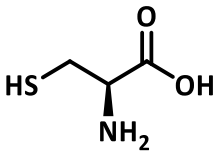
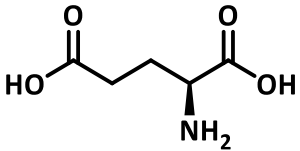
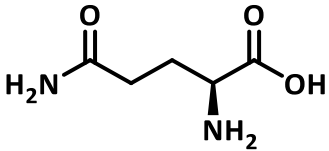
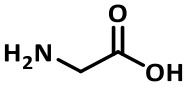
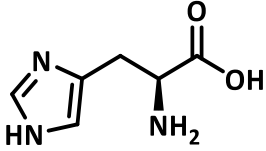
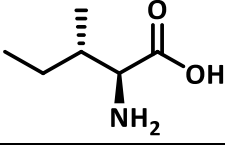
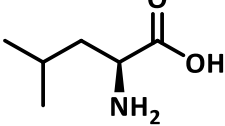
HSG	Histamine-succinyl-glycine
Hz	Hertz
i.p.	Intraperitoneal
IEX	Isotope exchange
IgG	Immunoglobulin
INS1	Insulinoma-derived β cell line
iPr	Isopropyl
IV	Intravenous
<i>J</i>	NMR coupling constant
K _{2.2.2}	Kryptofix® 2.2.2
KAHA	α -Ketoacid-hydroxylamine
kBq	Kilobecquerel
kcal	Kilocalorie
kJ	Kilojoule
KRBH	Krebs-Ringer bicarbonate-HEPES buffer
LC-MS	Liquid chromatography MS
L-SeMet	L-selenoMet
M	Molar
MC4-R	Melanocortin receptor
Me	Methyl
Mhz	Megahertz
mM	Millimolar
mmol	Millimole
mol	Mole
MPAA	Methyl phenylacetic acid
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
m/z	Mass to charge ratio
Nal	Naphthylalanine
NCL	Native chemical ligation

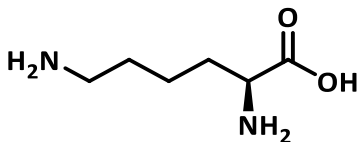
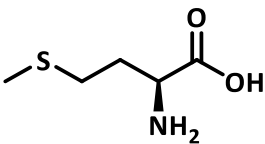
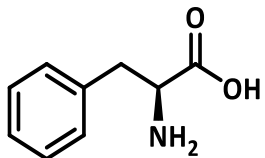
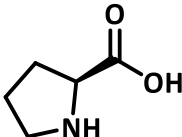
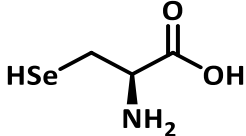
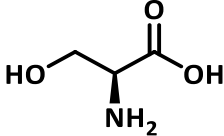
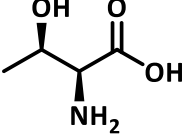
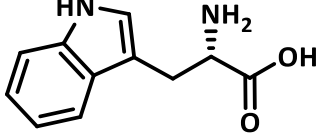
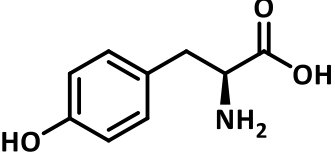
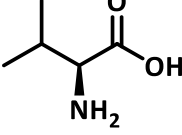
NERP	Neuroendocrine regulatory peptide
ng	Nanogram
NHS	<i>N</i> -hydroxysuccinimide
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance
NODA	1,4,7-Triazacyclononane-1,4-diacetic acid
NOTA	1,4,7-Triazacyclononane-1,4,7-triacetic acid
ONs	Nosylate
OxymaPure®	Ethyl cyanohydroxyiminoacetate
PBS	Phosphate-buffered saline
PC	Prohormone convertase
PD	Parkinson's disease
PEG	Polyethylene glycol
PET	Positron emission tomography
PG	Protecting group
Ph	Phenyl
<i>p</i> IPhe	<i>Para</i> -iodo-L-phenylalanine
ppm	Parts per million
PS	Polystyrene
PSMA	Prostate-specific membrane antigen
PTM	Post-translational modification
PVN	Hypothalamic paraventricular nucleus
q	Quartet
RCC	Radiochemical conversion
RCP	Radiochemical purity
RCY	Radiochemical yield
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase HPLC
rpm	Revolutions per minute
RSA	Rat serum albumin

Rt	Retention time
rt.	Room temperature
s	Singlet
SA	Specific activity
SECIS	Sec insertion sequence
SiFA	Organosilicon-based fluoride acceptor
SN	Substantia nigra
SPAAC	Strain-promoted alkyne-azide cycloaddition
SPE	Solid phase extraction
SPPS	Solid phase peptide synthesis
SPS	Solution phase peptide synthesis
Sta	(3S,4S)-4-Amino-3-hydroxy-6-methylheptanoic acid
t	Triplet
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBA	Tetrabutylammonium
TBAB	Tetrabutylammonium bicarbonate
TBHP	<i>t</i> Bu Hydroperoxide
<i>t</i> Bu	<i>tert</i> -Butyl
TCEP	Tris(2-carboxyethyl)phosphine
TCO	<i>Trans</i> -cyclooctene
TFA	Trifluoroacetic acid
TfO ⁻	Triflate anion
TFP	Tetrafluorophenyl
TGN	<i>Trans</i> -Golgi network
THF	Tetrahydrofuran
TMA	Trimethylammonium
TMSOTf	Trimethylsilyl triflate
TPA	Tetra phorbol myristate
TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol
tRNA	Transfer RNA

Trt	Trityl
UV-Vis	Ultraviolet-visible
VA-044	2,2'-Azobis(2-(2-imidazolin-2-yl)propane)dihydrochloride
δ	Chemical shift
μm	Micrometre
μM	Micromolar
μmol	Micromole
^{18}F	Fluorine-18
^{19}F	Fluorine-19
6- ^{18}F SFPy	^{18}F Nicotinic acid NHS ester
(Sec) ₂	Selenocystine
^{18}F CBT	^{18}F 2-Cyanobenzothiazole
^{18}F FBA	^{18}F Fluorobenzaldehyde
^{18}F FBEM	<i>N</i> -(2-(4- ^{18}F fluorobenzamido)ethyl) maleimide
^{18}F FDG	^{18}F -deoxyglucose
^{18}F FNEM	^{18}F <i>N</i> -(2-(2,5-dioxo-2,5-dihydro-1 <i>H</i> -pyrrol-1-yl)-ethyl)-6-fluoronicotinamide
^{18}F FPBA	^{18}F PEG-benzaldehyde
^{18}F FVSB	^{18}F Fluoro-4-(vinylsulfonyl)benzene
^{18}F HFB	^{18}F Hexafluorobenzene
^{18}F NFSI	<i>N</i> - ^{18}F -fluorobenzenesulfonimide
^{18}F SFB	<i>N</i> -succinidyl-4- ^{18}F fluorobenzoate
^{18}F SiFA-ITC	^{18}F SiFA-isothiocyanate
^{18}F SiFB	<i>N</i> -succinimidyl 3-(di- <i>tert</i> -butyl ^{18}F fluorosilyl) benzoate

Table of Amino Acids

Amino acid	3-letter code	1-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic acid	Asp	D	
Cysteine	Cys	C	
Glutamic acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	

Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Selenocysteine	Sec	U	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

Note: All amino acids are L-configured.

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Chapter 1: Introduction

1.1 The role of proteins in nature

Proteins are essential for life; they act as mediators for the majority of all biological processes.¹ The natural phenomena of transcription-translation and post-translational modification (PTM) result in an incredibly diverse range of naturally expressed proteins.^{2,3} Each of these variants has a unique structure, biological role and physicochemical properties.

In organisms, molecular level events are facilitated, altered or detected by complex protein assemblies.⁴ Soluble, globular proteins are required for small molecule cargo transfer, defence against pathogens, catalysis of metabolic reactions, communication between cells and decoding of genetic information.¹ Fibrous proteins, for example collagen, elastin and silk, have mechanical and architectural roles in nature.^{5,6} As peripheral or integral membrane proteins they also facilitate the transportation of small molecules, function as receptors and participate in the transfer of information between cells.¹ Fundamentally, proteins do not work in isolation; they interact with other molecules in order to effect biological change.

Due the central role of proteins in signalling cascades, structural mutations, such as those affecting a receptor interface, can result in disturbed cell-to-cell communication.³ This can lead to cellular pathogenesis. A wide range of intrinsic and extrinsic factors have the potential to initiate protein misfolding and aggregation.⁷ Such phenomena can induce specific neurotoxic events that underlie a number of neurodegenerative diseases that are currently untreatable.⁸⁻¹²

1.2 Amino acids

An α -amino acid consists of a central carbon atom linked to an amino group, a carboxyl group, a hydrogen atom, and a characteristic side chain (R) group. When four different substituents are connected to the central α -carbon, amino acids are chiral; two optical isomers exist, termed “L” and “D”, as shown in Figure 1. The amino acid R groups have differing physicochemical properties, and are classified as being polar, hydrophobic, charged (acidic) or charged (basic).

By convention, amino acids are denoted by a three and/or one letter code. For example, the simplest α -amino acid “glycine” is also referred to as “Gly” and “G”.

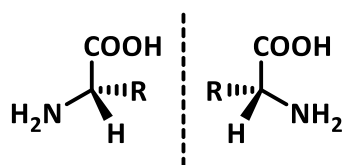


Figure 1: α -amino acid; L (left) and D (right) configurations (R group $\neq$ a hydrogen atom).

Linear polymers of α -amino acid residues are termed “polypeptides”. The carboxyl group of one amino acid joins to the amino group of another, forming a covalent peptide bond via a condensation reaction (loss of water).¹³ The linkage of amino acid monomers in this manner, defines the “primary structure” of a polypeptide.¹

1.2.1 Proteinogenic amino acids

Typically, across all organisms, the ribosomal biosynthesis of peptides and proteins is restricted to the 20 genetically encoded “canonical” amino acids.¹⁴ In eukaryotes, an additional 21st proteinogenic amino acid, “selenocysteine” (Sec, U) (Figure 2), is incorporated via a unique co-translational mechanism. In brief, the stop codon “UGA” is given alternative coding status, facilitated by Sec-binding transfer ribonucleic acid (RNA) (tRNA) and a unique Sec insertion sequence (SECIS).¹⁵ Computational and experimental approaches have identified 25 selenoproteins in the human proteome.¹⁶ These proteins typically exist as oxidised diselenides due to the low redox potential of Sec (-381 mV).^{17,18} All proteinogenic amino acids are L-configured, except Gly, which is achiral. Aside from histidine (His, H), tyrosine (Tyr, Y), tryptophan (Trp, W) and phenylalanine (Phe, F), all proteinogenic R groups are aliphatic (devoid of an aromatic ring).

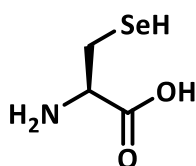


Figure 2: L-selenocysteine (Sec, U).

1.2.2 Expansion of diversity

In eukaryotic systems, a wide variety of enzyme-mediated mechanisms facilitate the reversible and irreversible PTM of canonical amino acids, examples include glycosylation, phosphorylation, acetylation and methylation.³ Post-translational editing is essential for a vast array of cellular processes including maintenance of peptide and protein structure and

integrity, regulation of metabolism and defence, and in cellular recognition events and morphology changes.¹⁹

The ability to expand the diversity of polypeptides is not only valuable in nature but also in a laboratory setting. Peptides represent a unique class of pharmaceutical compounds; molecularly poised between small molecules and proteins, yet biochemically and therapeutically distinct from both.²⁰ The pharmacological properties of peptides can be favourably enhanced using non-proteinogenic amino acids. For example, cytotoxic group linkage and polyethylene glycol (PEG)-ylation has facilitated the development of therapeutics with antineoplastic and non-immunogenic properties, respectively.^{21,22} It is also possible to fine-tune the physicochemical properties of peptide therapeutics in this manner. For example, metabolic stability, lipophilicity and pKa can be favourably altered, resulting in improved *in vivo* performance.^{23–26}

1.3 Polypeptides

Most polypeptides that are found in nature are termed “proteins” and typically contain between 50 and 2000 amino acids.¹³ Shorter chains of 2-50 amino acids are most commonly referred to as “peptides”.

Inside cells, transcription (which describes the role of RNA polymerase in transcribing the genetic information from deoxyribonucleic acid (DNA) to RNA), and translation (where the resultant RNA from transcription is decoded by the ribosome into the unique sequence of proteinogenic amino acids) form the basis of the central dogma of molecular biology.²⁷ Polypeptide biosynthesis is dependent on the pairing of amino acids with their tRNA counterpart. This process, which is catalysed by aminoacyl-tRNA synthetase, results in the formation of aminoacyl-tRNA molecules.^{28,29} During ribosomal translation, messenger RNA (mRNA) triplets are decoded into the corresponding series of amino acids.²⁸

“N-H” and “C=O” groups that constitute the “N-C-C” backbone participate in hydrogen bonding which results in local folding of the polypeptide to form α -helices and β -sheets.¹³ Non-covalent interactions and disulfide bridges between amino acid R groups results in the formation of a stabilised, three-dimensional tertiary structure.¹³ The quaternary structure of a protein refers to the non-covalent interaction of more than one polypeptide subunit.³⁰

1.4 The chemical synthesis of peptides

An increasing demand for a wider range of more clinically effective therapeutics has resulted in the development of chemical approaches for the synthesis of peptides.^{31,32} Peptide therapeutic products are recognised as being highly selective, efficacious and relatively safe and well tolerated by patients.³³ Since the first clinical use of insulin in the 1920s for treatment of diabetes mellitus (DM), peptide therapeutics has emerged as a leading industry.^{20,34} Worldwide sales of peptide drugs in 2017 was USD \$20 billion, and sales are expected to surpass \$50 billion by 2024.³⁵ To date, over 70 therapeutic peptide products have been approved in the United States, Europe and Japan and many more are under global clinical development.^{34,35} Engineering of compounds of interest using peptide (and organic) chemistry, followed by folding (if a three-dimensional structure is required), provides a versatile means to obtain significant yields of high purity product.³⁶ The ability to access peptides in this manner enables circumvention of the limitations associated with the incorporation of non-proteinogenic amino acids via biological means (stop codon reassignment via amber codon suppression).³²

Approaches for the synthesis of peptides have historically centred on the chemical condensation of amino acids.³⁶ At present, primarily one approach is utilised, termed “solid phase peptide synthesis” (SPPS), which involves synthesis on a solid phase. Solution phase peptide synthesis (SPS) is also possible, however, isolation, purification, and characterisation are required to be carried out after coupling of each amino acid.³⁷ Therefore, this technique is only appropriate for synthesis of large batches of very short peptides.

Formation of a new peptide bond is achieved via the coupling of two amino acids; the first is protected at the amino (N)-terminus **1** and the second at the carboxyl (C)-terminus **2**, as shown schematically in Figure 3. Protection of potentially reactive R groups is important for the prevention of unwanted side reactions.

Carboxyl activating agents, including carbodiimides and phosphonium salts, are used in the chemical synthesis of polypeptides.³⁷ In the case of carbodiimides, such as *N,N'*-diisopropylcarbodiimide (DIC) **4** (Figure 3), a strongly activated *O*-acylisourea **5** intermediate (Figure 4) is primarily formed; carbodiimide-catalysed deprotonation of the amino acid C-terminal carboxyl is followed by attachment of the resultant carboxylate to the electron-

deficient carbon centre of the carbodiimide.³⁸ In the presence of a N-terminal deprotected amino acid or peptide, this highly reactive intermediate undergoes aminolysis to result in formation of a new peptide bond and a resonance-stabilised urea by-product.³⁷ In the second possible pathway, another molecule of the amino acid reacts with **5**, leading to the formation of a reactive symmetric anhydride that participates in peptide bond formation.

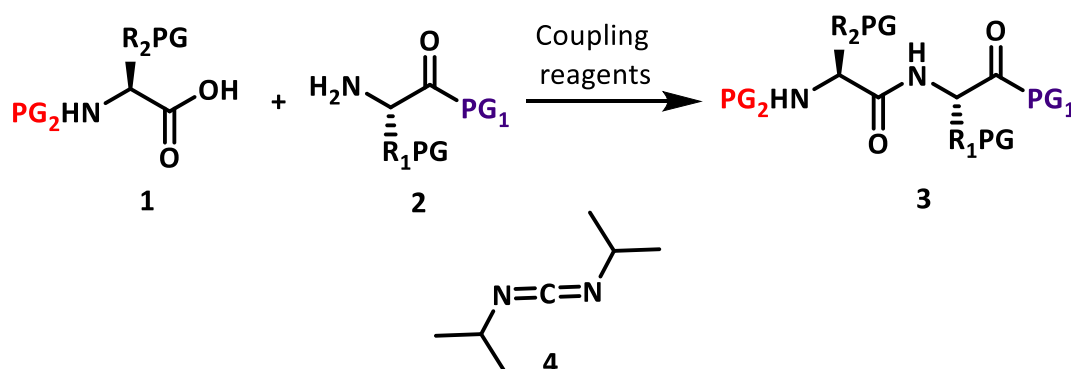


Figure 3: Amino acid coupling during peptide synthesis: PG₁ refers to the C-terminus protecting group (for SPPS, PG₁ is an insoluble resin); PG₂ refers to the N-terminus protecting group; PG refers to a R protecting group.

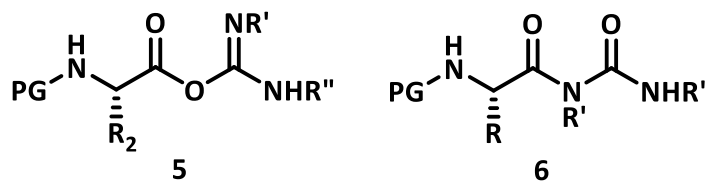


Figure 4: *O*-acylisourea (LHS) and *N*-acylurea (RHS). PG refers to protecting group.

Although it is possible to solely use a carbodiimide as a coupling agent, this approach is associated with significant formation of an unreactive *N*-acylurea **6** (Figure 4) via the spontaneous rearrangement of *O*-acylisourea **5**.³⁸ Furthermore, formation of an epimerisation-prone oxazolone species and the subsequent loss of enantiomeric purity at the α -carbon is a characteristic outcome of carbodiimide use, as highlighted in Figure 5. Such base-catalysed epimerisation is driven by formation of the highly stable urea by-product. Consequently, *N*-hydroxylamine additives such as the hydroxy derivative of benzotriazole (Bt) and ethyl cyanohydroxyiminoacetate (OxymaPure®) **11** are commonly used alongside carbodiimides to suppress the formation of **6** and epimerisation.^{38,39} Such additives function as rapidly reacting nucleophiles, hence formation of the resultant *N*-hydroxylamine active ester from **5** occurs faster than the competing acyl transfer.⁴⁰ A schematic of **5** to *N*-hydroxylamine active ester conversion via use of **11** is shown in Figure 6. The active esters

associated with HOBt and **11** use are still active enough to couple with the amine, in fact HOBt also functions to encourage/stabilise the approach of the amine via hydrogen bonding (thereby accelerating aminolysis).⁴⁰

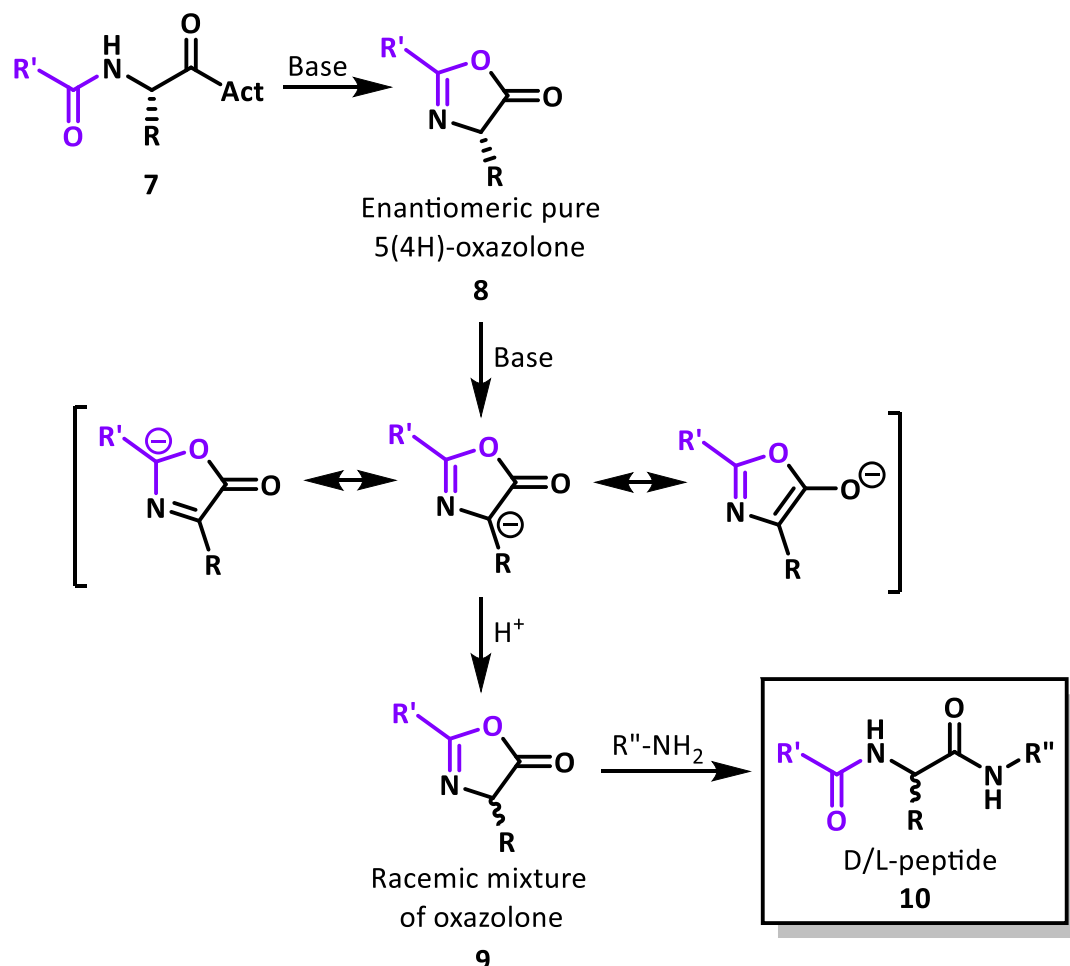


Figure 5: Schematic of base-catalysed epimerisation during activation; formation of an epimerisation-prone oxazolone intermediate that subsequently leads to generation of both D and L-configurations around the α -carbon **9**. Subsequent peptide bond formation results in formation of the D/L product **10**.³⁷ Part of the N-terminal protecting group is shown in purple.

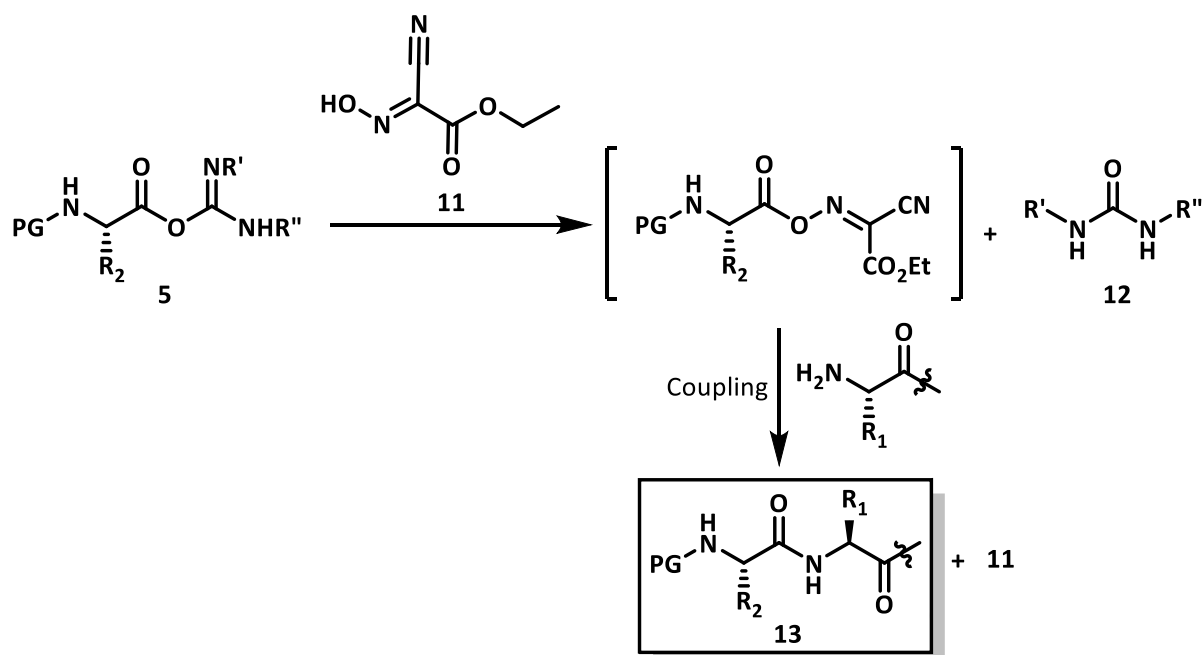


Figure 6: Schematic of *O*-acylisourea to *N*-hydroxylamine active ester conversion via use of OxymaPure®, followed by peptide bond formation.

1.5 Solid phase peptide synthesis (SPPS)

SPPS was first introduced by Robert Merrifield in 1963.⁴¹ His original methodology has since been adapted; the result is the technique that is routinely used today for the chemical synthesis of polypeptides. An orthogonal protecting group strategy is employed for SPPS; temporary terminal and R protecting groups are removed under different conditions.³⁷ Use of such a strategy is important for the prevention of premature R group deprotection.

SPPS involves the stepwise elongation of an initial, “resin”-immobilised amino acid. Resins consist of a polymeric solid support plus a linker moiety. The linker acts as a “bridge” between the polymeric material and the peptide, thereby enabling temporary anchorage.³⁷ Choice of linker not only governs the type of functional group at the amino acid-linker junction, but also the protocol for amino acid loading and for cleavage of the peptide from resin.^{37,42}

1-2% divinylbenzene (DVB)-cross-linked polystyrene (PS) (PS-DVB) **16** beads (Figure 7) of approximately 50 micron in diameter are commonly used as the solid support.^{37,43} Alternative materials also exist, including those that are composed of PEG, PEG-PS, polyamide, PEG-acrylamide, or polyethylene-PS.³⁷ Effective swelling of the resin prior to loading of the first amino acid is essential for efficient chain assembly during SPPS.⁴² For example, cross-linked

PS resins are typically solvated in a polar, aprotic solvent such as dichloromethane (DCM) for at least 30 minutes.

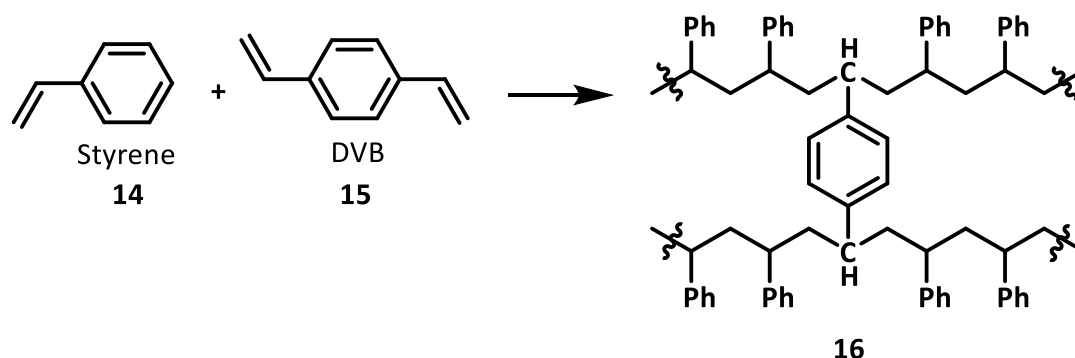


Figure 7: PS-DVB copolymer. Ph refers to a phenyl group.⁴³

Despite the fact that N-terminal, R group and backbone anchoring is possible, C-terminal amino acid linkage and subsequent C- to N- SPPS remains the most favoured approach.³⁷ In brief, an N-terminal protected amino acid (carboxyl-containing or pre-activated) is attached to an insoluble support via a linker moiety. The structures of three resins (2-chlorotrityl chloride (CTC) **17**, Rink Amide **18** and Wang **19**) are highlighted in Figure 8. Resin loading (mmol/g) indicates the amount of functionalisable sites (substitution) per gram of resin. In general: <0.2 mmol/g is low, 0.5-0.8 mmol/g is medium and >1.0 mmol/g is high.

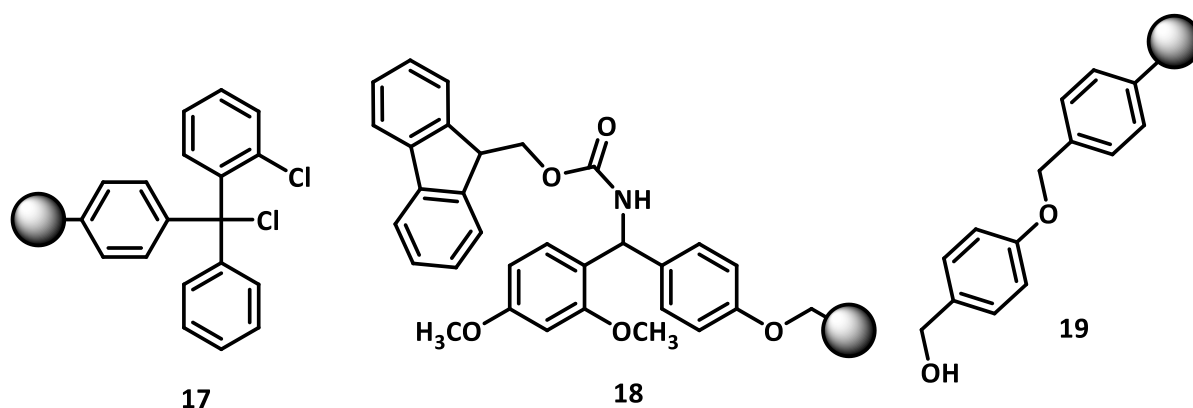


Figure 8: Resins for C-terminal amino acid linkage in SPPS; CTC, Rink Amide and Wang. The linkers and insoluble polymeric solid support are shown.

CTC is one of the most popular linkers used for the synthesis of C-terminal acid peptides due to the fact that activation of the first amino acid is not necessary (as is the case with some C-terminal resins such as Rink Amide). Traditionally, loading of the first amino acid to Wang resin requires the use of 4-dimethylaminopyridine (DMAP) to yield an initial carboxylate

anion, and a carbodiimide activating agent such as DIC **4**.⁴⁴ Use of these reagents can lead to the previously discussed occurrence of base-catalysed epimerisation. Formation of the epimerisation-prone oxazolone species can be circumvented by using a catalytic amount of DMAP and an excess of the amino acid (in relation to the carbodiimide), to promote formation of the symmetric anhydride. Alternatively, a coupling agent such as OxymaPure® **11** is utilised to form an active ester and suppress epimerisation.

A common issue when coupling the first amino acid after loading to a C-terminal resin (via an ester linkage) is the formation of a diketopiperazine **20** (Figure 9) which cleaves the dipeptide from the resin and dramatically lowers the yield.⁴⁵ This problem is most pronounced when Pro is loaded followed by coupling to Gly. To circumvent this, a dipeptide is often coupled to Pro. The steric hindrance of the CTC resin **17** trityl (Trt) linker also prevents diketopiperazine formation, hence this resin is often recommended for synthesis of C-terminal Pro peptides via SPPS.

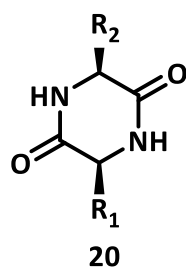


Figure 9: Diketopiperazine; R refers to amino acid R group.

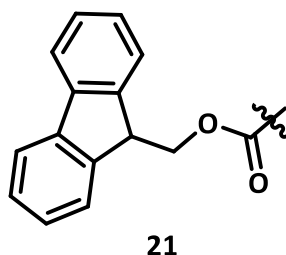


Figure 10: The Fmoc protecting group.

The most commonly used approach for C- to N- SPPS involves the use of the fluorenylmethoxycarbonyl (Fmoc) group **21** (Figure 10) for N-terminal protection of amino acids. R protecting groups, as shown in Figure 11, are trifluoroacetic acid (TFA) labile, whereas Fmoc is removed via treatment with a mild base (an orthogonal protecting group strategy).^{31,37}

After loading of the first amino acid, “end-capping” of the resin is carried out to block any unreacted active sites, followed by washing of the resin with polar, aprotic solvent to remove excess reagents. Removal of the base-labile Fmoc N-terminal protecting group is then carried out using piperidine **28** in dimethylformamide (DMF), as represented in Figure 12, followed by washing of the resin with polar, aprotic solvent. Fmoc removal results in release of a fluorene group with strong ultraviolet-visible (UV-Vis) absorption properties that give a useful indicator of loading/synthesis success; estimation of loading is enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29** (Figure 12).³¹ A second Fmoc protected amino acid is then coupled to the first, followed by further washing of the resin with polar, aprotic solvent. Double couplings are typically performed for sections of repeated residues, hydrophobic regions, coupling to Pro and for all residues after the first 20. Any unreacted amines are then acetylated or “capped” to prevent elongation of any uncoupled sites. Cycles of deprotection-coupling-capping are then repeated until the desired peptide sequence has been achieved, as shown in Figure 13. Acidolytic cleavage of the peptide from the resin is then performed (treatment with TFA); the R group deprotected peptide is isolated as a free acid, amide, thioacid, thioester etc., depending on linker choice.³⁷ A range of commercially available linkers exist that can be used to achieve functionalisation at the C-terminus of a peptide of interest, upon cleavage from resin.⁴² Precipitation of the R group deprotected peptide in cold ether is used as a final step, in advance of purification.

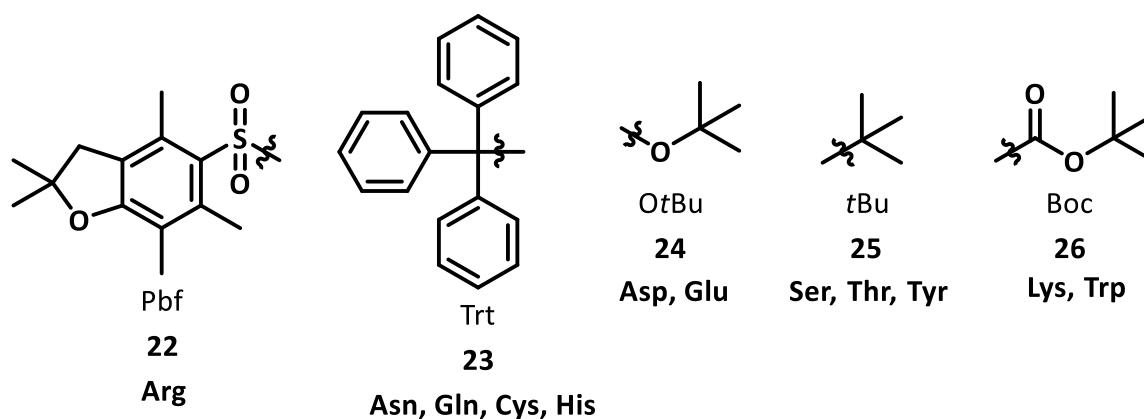


Figure 11: TFA-labile protecting groups for Fmoc-SPPS: Pbf refers to 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl; Trt refers to trityl; OtBu refers to *tert*-butyl ester; *t*Bu refers to *tert*-butyl; Boc refers to *tert*-butyloxycarbonyl. The amino acids are in **bold**.³¹

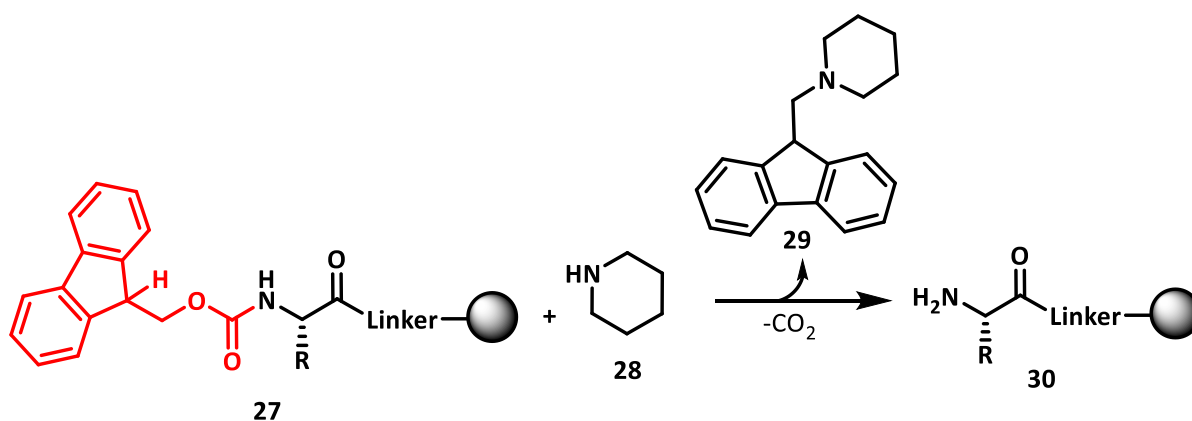


Figure 12: Schematic of Fmoc group removal via piperidine.³⁷ Fmoc is shown in red.

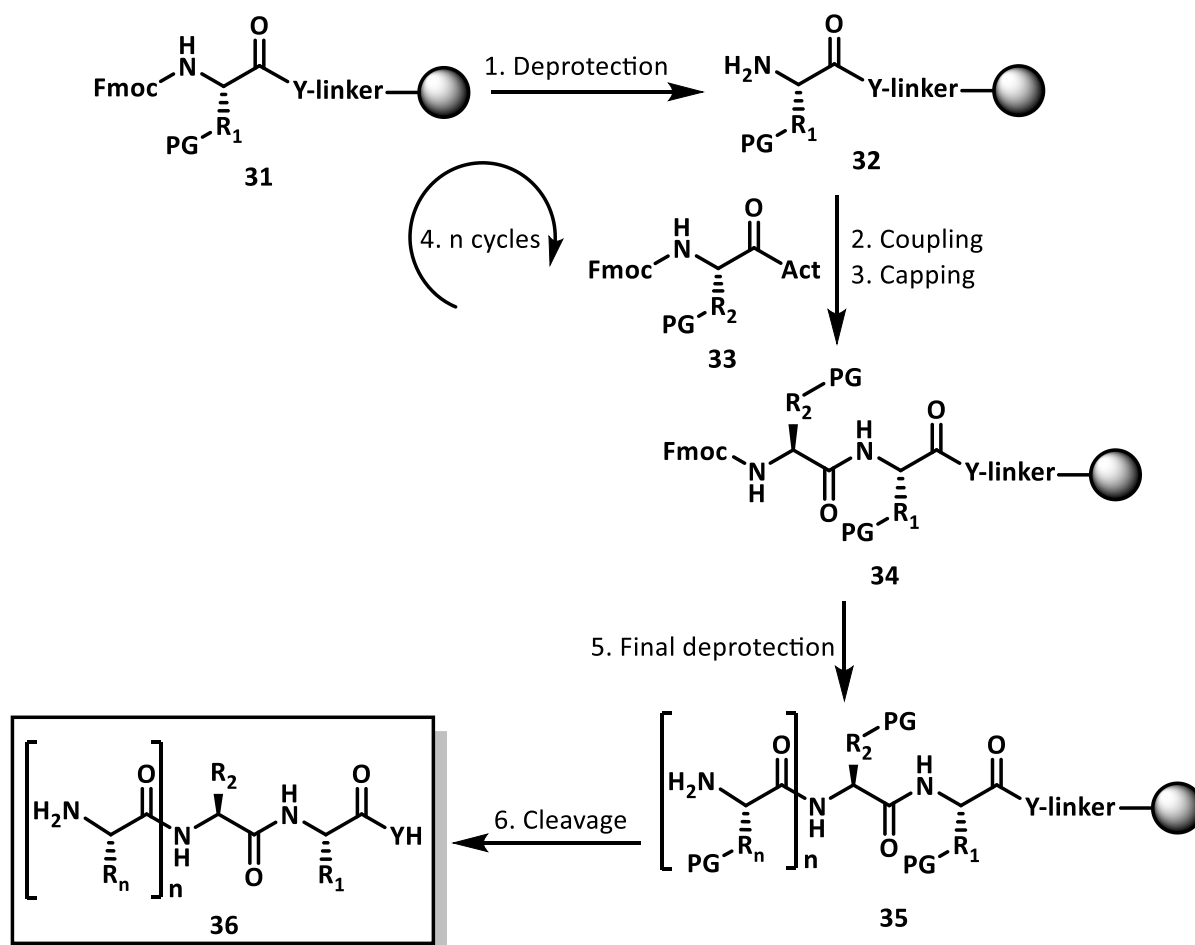


Figure 13: Schematic of Fmoc-SPPS: Y refers to O (ester linkage), NH (amide linkage) or S (thioester linkage) etc.; PG refers to R protecting group; Act refers to activated carboxyl.³⁷

On-bead aggregation via hydrogen bonding during chain elongation is sequence-dependent and associated with side-reaction occurrence, clusters of repeating hydrophobic residues such as Gly or those with sterically hindered R groups such as valine (Val, V).³⁷ Therefore, C-terminal resins with a medium degree of substitution e.g. Rink Amide **18** and Wang **19** are typically selected for use when attempting the synthesis of difficult peptide sequences, since

the lower the resin loading, the lower the peptide concentration on resin. In the case of CTC resin **17** (~1.32 mmol/g), under loading, for example adding 0.5 equivalents (eq.) of the first amino acid to available sites is preferable. Furthermore, application of heat during coupling in Fmoc-SPPS, for example via microwave assistance in an automated approach, can potentially suppress chain aggregation (as well as reduce synthesis time).³¹

Serine (Ser, S) and threonine (Thr, T)-derived oxazolidine dipeptides **39** (termed “pseudo-prolines”) are routinely used to prevent inter-chain association.³¹ Such dipeptides exert a pronounced effect upon the peptide backbone conformation due to their structural similarity with proline (Pro, P) (which induces a “kink” in the peptide).⁴⁶ Pseudo-peptides are readily accessible by cyclisation of Ser or Thr with an aldehyde or ketone (e.g. acetone **37**) and serve as an acid-labile, reversible R protecting group in Fmoc-SPPS, as highlighted in Figure 14.⁴⁶

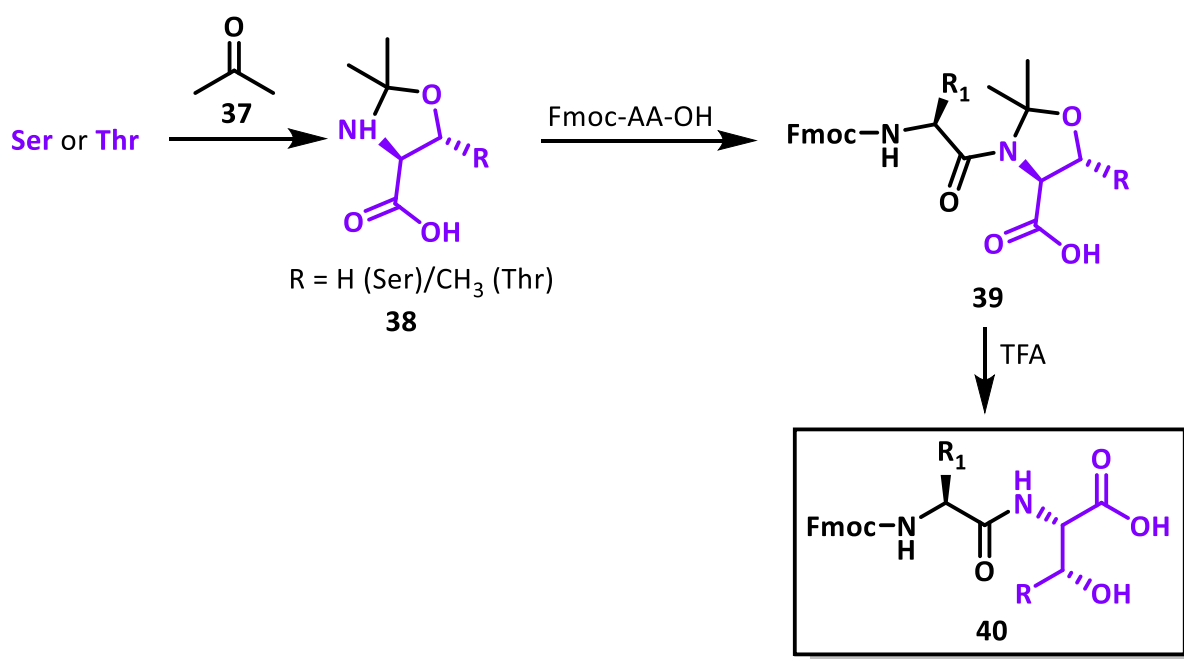


Figure 14: Schematic of pseudo-proline dipeptide synthesis; a Ser or Thr is cyclised with acetone to form an oxazolidine **38**, followed by attachment to a secondary amino acid. Treatment with TFA during cleavage, results in formation of the native residue.⁴⁶

One of the most problematic side-reactions associated with synthesis of longer peptide sequences using Fmoc chemistry is aspartimide **42** formation, as shown in Figure 15.⁴⁷ Cyclisation of an aspartic acid (Asp, D) residue R group to yield a cyclic imide results from exposure to a base during Fmoc-removal or peptide coupling. Formation of **42** is highly sequence-dependent, with -Asp-Gly-, -Asp-Asp-, -Asp-asparagine (Asn, N)-, -Asp-arginine

(Arg, R)-, Asp-Thr- and -Asp-Cys- showing the highest propensity.³¹ The undesired formation of **42** during Fmoc-SPPS results in poor yielding or even inaccessible peptide sequences. Attack by water yields an undesired D/L- β -aspartyl peptide **44** in a ratio of 3:1 to the D/L- α -aspartyl peptide **43**.³¹ Ring opening by piperidine **28** gives a mixture of a D/L- α -piperidide **45** and a D/L- β -piperidide **46**.

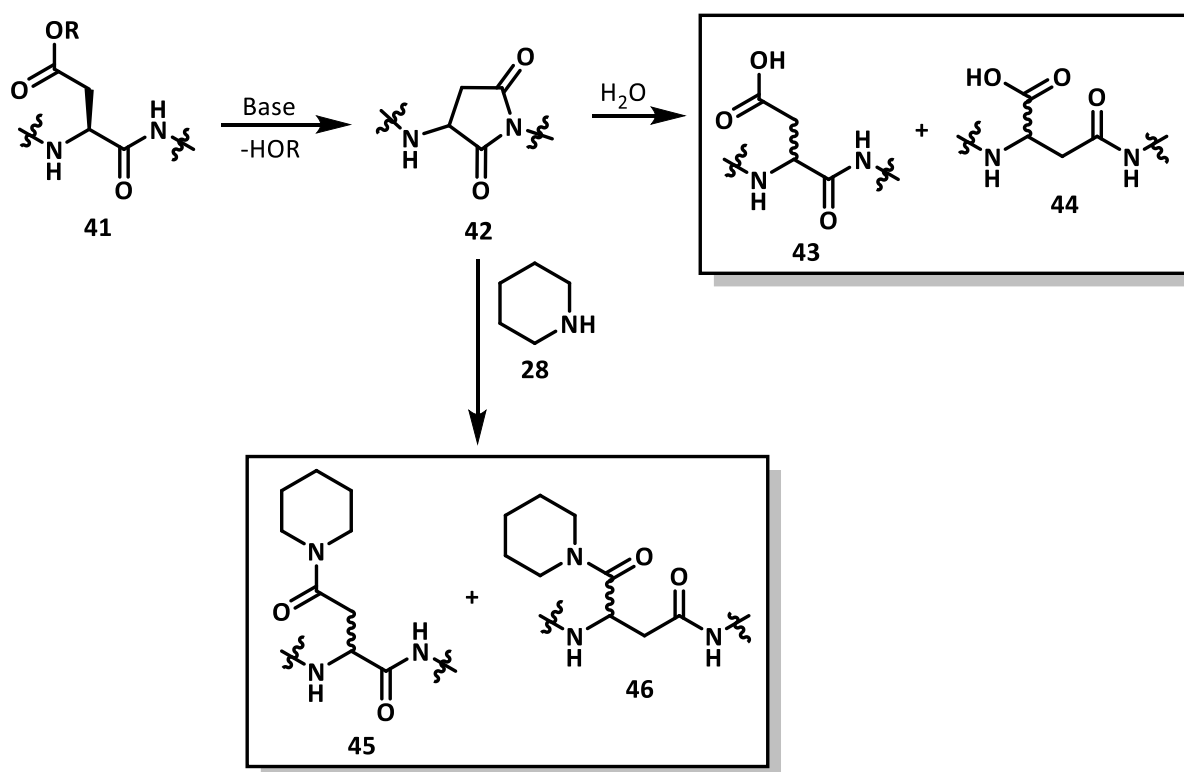


Figure 15: Schematic of base-catalysed aspartimide formation and subsequent by-product formation via reaction with H₂O or piperidine. R refers to variable group.⁴⁷

Use of a pseudoproline dipeptide **39** immediately before an aspartimide-prone residue can minimise the associated side reaction.³¹ Furthermore, a decrease in formation of aspartimide **42** is related to increased steric bulk of the Asp ester moiety; White and co-workers recently highlighted that trialkylcarbinol-based ester derivatives of the standard *tert*-butyl (*t*Bu) group (methyl groups are substituted for longer alkyl chains) offer excellent protection.^{47,48} Unlike more traditional approaches (e.g. use of β -branching alkyl chains) for modification of the Asp ester moiety, installation of substituted tertiary esters bearing linear alkyl chains did not result in poor coupling efficiency.⁴⁸ Bode and co-workers report in their 2020 publication that use of cyanosulfurylide (CSY) as a carboxylic acid protecting group completely suppresses formation of **42** and increases solubility during peptide elongation on resin.⁴⁷ As shown in Figure 16, in contrast to ester-based protecting groups, CSY consists of a stable C-C bond that

can be selectively cleaved under mild, aqueous acidic conditions from peptides with an electrophilic halogen species to regenerate the carboxylic acid **48**. Amide backbone protection via acid-labile groups such as dimethoxybenzyl (Dmb) and 2-hydroxy-4-methoxybenzyl (Hmb) precludes the possibility of formation of **42**.³¹ However, their coupling efficiency is poor and must therefore be coupled as dipeptides, with only Asp-(Dmb/Hmb)Gly (Figure 17) being commonly available.⁴⁷

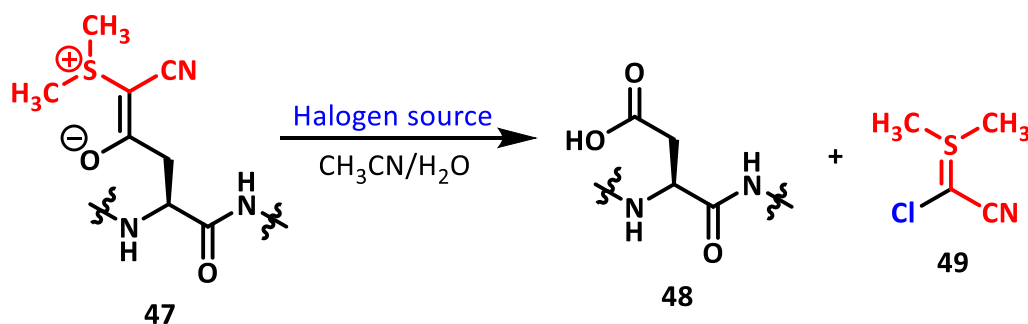


Figure 16: The CSY carboxylic acid protecting group undergoes a rapid reaction with an electrophilic halogen species, leading to the release of the unprotected carboxylic acid and elimination of cyanohalogenylide.⁴⁷ Part of the CSY is shown in red.

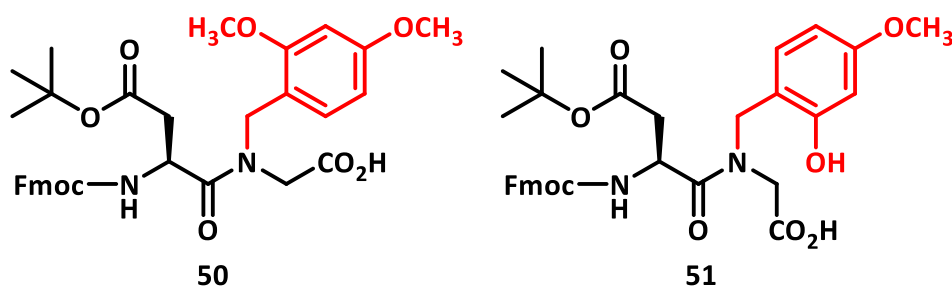


Figure 17: Fmoc-Asp(tBu)-(Dmb)Gly (LHS) and Fmoc-Asp(tBu)-(Hmb)Gly (RHS). The amide backbone protecting groups are shown in red.

The addition of acidic modifiers (such as OxymaPure® **11**, 2,4-dinitrophenol or small amounts of organic acids) to piperidine **28** during Fmoc removal has also been proposed as a solution to formation of aspartimide **42**.^{49,50} Replacement of **28** (pKa of 11.12) with the milder base piperazine (pKa of 9.73) can also hinder this side reaction, however, at the cost of the reaction rate.⁴⁹

Formation of N-pyroglutamic acid **53** (as shown in Figure 18) from N-terminal Gln **52** as a consequence of acid-catalysed Trt sidechain deprotection is another side-reaction that is associated with Fmoc-SPPS.⁵¹

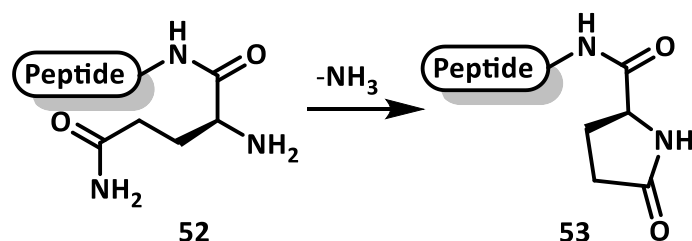


Figure 18: Formation of N-pyroglutamic acid from N-terminal Gln (M-18).

SPPS is immensely valuable for the synthesis of polypeptides in a controlled manner. However, the number of amino acids that can be coupled together in this stepwise manner is somewhat limited; typically a maximum chain length of 40-50 residues can be achieved.³² Merging of chemical synthesis and recombinant engineering has led to the advancement of “semisynthesis”; a technique that can be applied to longer and more complex peptides and proteins.^{32,36} Access to longer polypeptides of interest solely via chemical means requires the synthesis and subsequent ligation of purified peptide fragments. Traditional methods for the chemoselective ligation of deprotected peptide fragments generally result in a non-amide bond being formed (e.g. oxime, thioester etc).^{52–54} A successful and widely utilised method of ligation that affords a native amide at the ligation junction is native chemical ligation (NCL).

1.6 Native chemical ligation (NCL) and subsequent advances

Introduced by Kent and co-workers in 1994, NCL (Figure 19) is a chemoselective approach that exploits the unique nucleophilicity of the Cys R group (thiol, R-SH).⁵⁴ This technique is based on the ligation of two SPPS-generated peptides in aqueous denaturing buffer to yield a polypeptide of up to ~250 amino acids in length. The first peptide contains a C-terminal thioester moiety **54** (typically synthesised via cleavage of a peptide from CTC resin **17** using hexafluoro-2-propanol (HFIP) and thioesterification of the resultant activated carboxyl group followed by acidolytic deprotection of the R groups) and the second an N-terminal Cys **55** (obtained via standard Fmoc SPPS).^{53,55} The initial step involves a thiol-thioester exchange reaction. The generated thioester-linked intermediate spontaneously rearranges by intramolecular sulfur to nitrogen acyl transfer to yield a Cys residue at the ligation junction.⁵⁶ Ligation is not only chemoselective, but also site-selective, even in the presence of internal Cys residues and when both peptides are fully deprotected; the irreversible intramolecular sulfur to nitrogen shift can only occur at the unique N-terminal Cys residue and any internal

Cys residues that participate in the formation of the intermediate thioester rapidly exchange backwards.^{54,55}

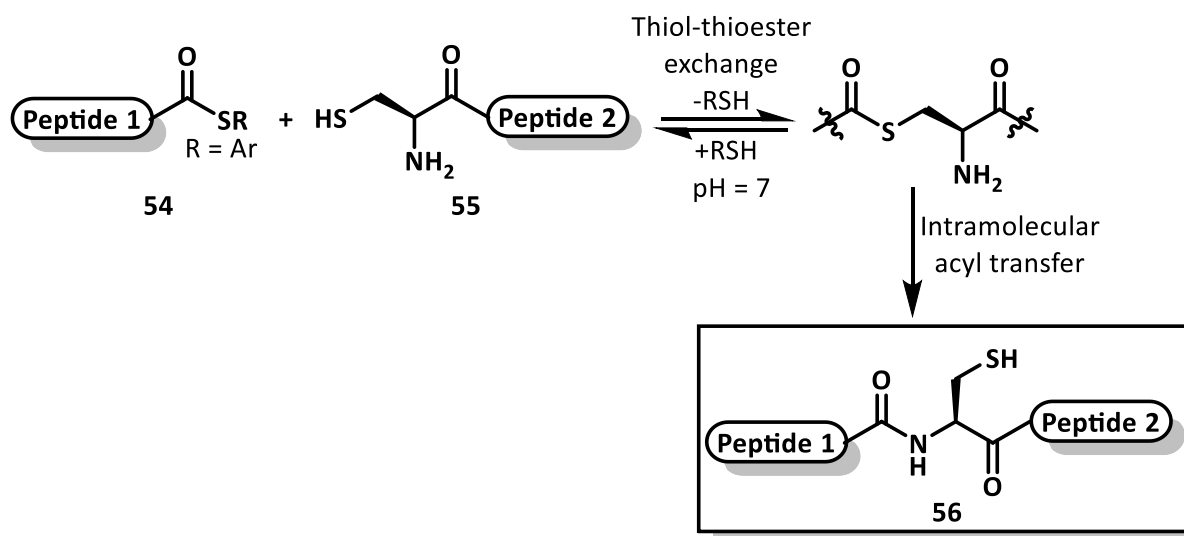


Figure 19: Schematic of NCL. The chemoselective capture step is mediated by a reversible thiol-thioester exchange between a C-terminal electrophilic (aryl) thioester and a nucleophilic thiol of an N-terminal Cys residue. The resultant Cys-thioester undergoes a rapid intramolecular sulfur to nitrogen transfer to form a native peptide bond.⁵⁶

NCL is undoubtedly a valuable technique; ligation can be carried out in aqueous buffer and under mild conditions. However, there are a number of shortcomings associated with this method for peptide ligation. For example, a large excess of an aryl thiol catalyst e.g. 4-mercaptophenylacetic acid is often required for optimisation of NCL due to the stable but relatively unreactive alkyl thioester at neutral pH.¹⁸ Mercaptophenylacetic acid-dependent conversion of an alkyl thiol to an aryl thiol yields a better leaving group at the C-terminal thioester site, thereby promoting successful ligation. Rate of ligation is also dependent on the C-terminal amino acid of the thioester moiety **54**; sterically hindered residues e.g. isoleucine (Ile, I) and Val are associated with slower ligation reactions. Prolonged reactions times can result in thioester hydrolysis.

The relatively low natural abundance of Cys in proteins (ca 1.5% across all organisms) is somewhat detrimental to NCL; peptide ligation is dependent on presence of a native Cys at a suitable position or installation of Cys.^{54,56,57} A residual, non-native Cys is often undesirable; Cys is the most reactive amino acid toward disulfide bond formation, is highly reactive with O₂(g) and other electrophiles and is prone to conversion to dehydroalanine (Dha) which can undergo further reaction.⁵⁷

In an effort to expand the scope of NCL beyond Cys-containing peptides and proteins, Dawson and co-worker developed an NCL-desulfurisation approach for peptide synthesis; participation of a temporary Cys residue in NCL followed by Pd/Al₂O₃ or Raney nickel catalysed hydrogenolytic desulfurisation of Cys to alanine (Ala, A).^{56,58} The research group recognised that Ala is one of the most highly abundant residues in proteins and proposed that their technique had the potential to significantly extend the applicability of NCL. Whilst they successfully used their approach to synthesise a number of linear and cyclic proteins, it was deemed to be compatible only with those that are devoid of native Cys residues (desulfurisation is not selective; all unprotected Cys residues are affected).^{54,58,59} Furthermore, the use of Raney nickel can cause epimerisation of secondary alcohols and the reduction of unprotected thioethers and thioesters.⁵⁹ Various alternative metal-based desulfurisation protocols have since been proposed. However, generally the desulfurisation of Cys with metal reagents continues to be associated with unfavourable side-reactions.

A protocol for the metal-free desulfurisation of Cys using a radical initiator e.g. 2,2'-azobis(2-(2-imidazolin-2-yl)propane)dihydrochloride (VA-044) or light and a phosphine, e.g., tris(2-carboxyethyl)phosphine (TCEP) **59** was published by Danishefsky and co-worker in 2007 (Figure 20).⁵⁹ It is expected that radical-induced hydrogen atom abstraction yields a thiyl radical which attacks **59** and results in formation of Ala at the ligation junction in the presence of a suitable hydrogen atom donor species (e.g. a thiol additive such as *tert*-butylthiol **60**).⁵⁶ This desulfurisation method is tolerant of a wide range functional groups including a range of sulfur-containing groups, but is non-site selective in the presence of unprotected native Cys.⁵⁹

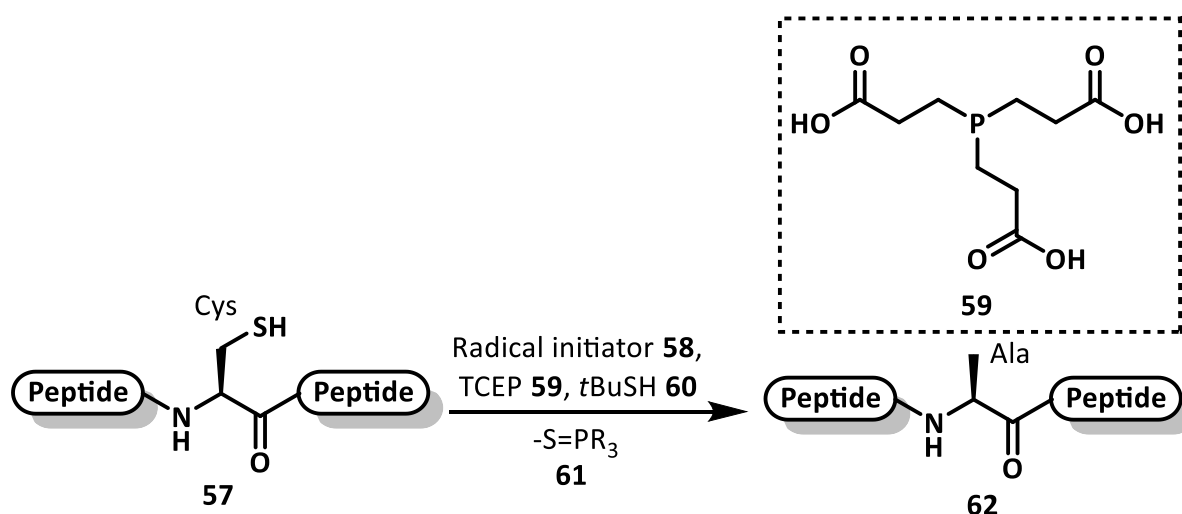


Figure 20: TCEP-facilitated radical desulfurisation of Cys to Ala. R=C₂H₄CO₂H.⁵⁹

A number of research groups have developed β - or γ -thiol amino acid variants. These non-proteinogenic residues are temporarily installed at the N terminus of the C-terminal peptide for participation in an NCL-like reaction. Subsequent TCEP **59**/radical initiator-facilitated desulfurisation serves to restore the native amino acid at the ligation junction.⁶⁰ The major challenge associated with such an approach is that the precursor amino acids are not commercially available; laborious, multi-step synthesis protocols are associated with their use.^{54,61} Furthermore, the method relies on radical desulfurisation; protection of native Cys residues is required.¹⁷

Recent alternatives to traditional NCL also include those that can be carried out in the absence of Cys. For example, chemoselective coupling of an α -ketoacid **63** to a hydroxylamine **64** (termed α -ketoacid-hydroxylamine (KAHA) ligation) affords an amide bond under aqueous, acidic conditions without the need for protecting groups or coupling agents (Figure 21).⁶² It is possible to generate **63** via use of a suitable acid-labile Fmoc-SPPS linker sequence. Use of a 4-membered cyclic amino acid as the hydroxylamine (prepared via a straightforward route) to afford Ser at the ligation junction has been demonstrated.⁶³

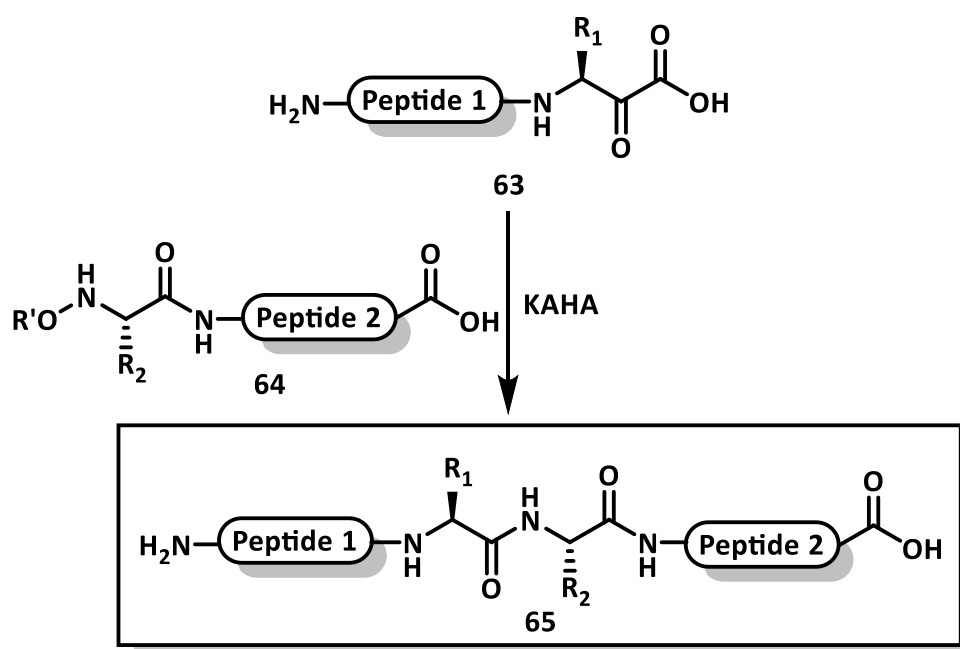


Figure 21: Chemoselective coupling of an unprotected peptide α -ketoacid to a peptide hydroxylamine via KAHA ligation.⁶² R' refers to H or variable R group (free, *O*-substituted or cyclic hydroxylamine).

1.7 Diselenide-selenoester ligation (DSL)

Diselenide-selenoester ligation (DSL) was introduced in 2015 by Payne and co-workers; a deprotected, Fmoc-SPPS generated C-terminal phenylselenoester peptide **66** and peptide dimer bearing an N-terminal selenocystine [(Sec)₂] **67** were ligated in a site-selective manner, as highlighted in Figure 22.¹⁸ **66** was synthesised via cleavage of a peptide from CTC resin **17** using HFIP and selenoesterification of the resultant activated carboxyl group via treatment with diphenyldiselenide (DPDS) followed by acidolytic deprotection of the R groups.

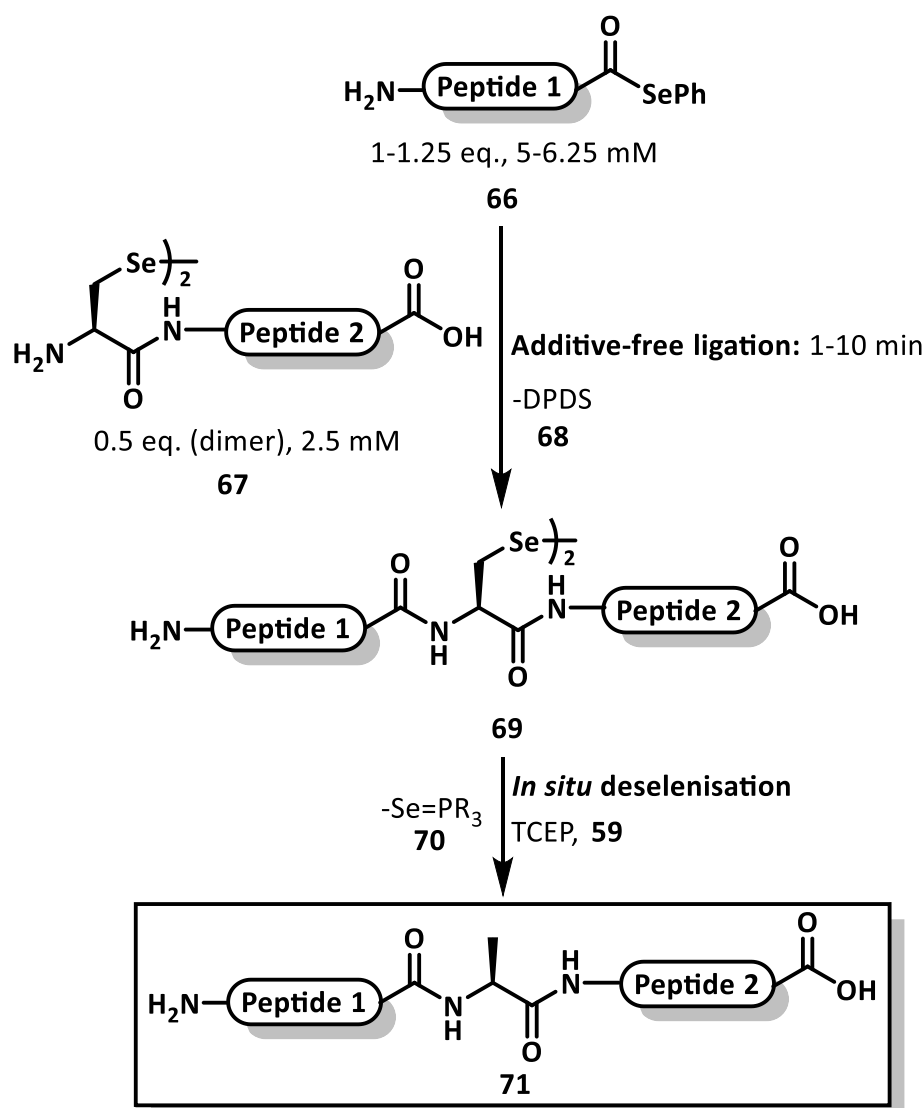


Figure 22: Schematic of rapid additive-free DSL between a peptide phenylselenoester and a N-terminal (Sec)₂ dimer peptide, followed by *in situ* deselenisation. R=C₂H₄CO₂H. Conditions: additive-free ligation: 6 M guanidinium (Gdn) hydrochloride, 0.1 M Na₂HPO₄, pH 6.2; *in situ* deselenisation: (i) hexane extraction, (ii) TCEP (50 eq.), dithiothreitol (DTT) (5 eq.), 16 h.¹⁸

It was proposed that generation of a peptide selenolate anion, followed by direct attack onto the peptide selenoester **66** acyl donor results in formation of an intermediate. The intermediate spontaneously rearranges by intramolecular selenium to nitrogen acyl transfer to yield the desired ligation product **69**. Payne and co-workers presented a reversible redox ligation cycle in their paper; it was suggested that precipitation of DPDS was the driving force behind the forward reaction. It is expected that initiation of ligation occurs via deselenide and selenoester attack (referred to hereafter as pathways A and B, respectively). Initiation via pathway A (direct peptide diselenide dimer **67** attack onto the acyl donor of **66**) results in formation of a phenylselenolate anion which facilitates turnover of the redox ligation cycle through oxidation to insoluble DPDS. Initiation via pathway B involves attack of **66** onto **67**, which affords the peptide selenolate anion. Using this approach, ligation can be completed rapidly (1-10 minutes) in aqueous buffer and under mild conditions, even at sterically hindered junctions. Payne and co-workers successfully demonstrated that use of a thiol or reductive additive e.g. TCEP **59** for generation of a nucleophilic peptide N-terminal selenol moiety was not required. These additives are associated with a slow rate of reduction and deselenisation of **67**, respectively.

Coupling of this ligation technique with phosphine-mediated *in situ* deselenisation (use of excess TCEP **59** and DTT **73** as the hydrogen atom source) can be used to afford the highly abundant Ala at the ligation junction; unprotected native Cys residues are unaffected (Figure 23).⁵⁶ The chemoselectivity of this reaction is attributed to the ability of peptide diselenides to homolyse readily under ambient conditions (hence use of a radical initiator is not required), the lability of the polarised C-Se bond towards homolysis and the relatively low bond enthalpy of C-Se vs. C-S (Cys thiol). A mechanism for the radical deselenisation of Sec-containing peptides was first proposed by Dawson and co-workers in 2010; a selenyl radical attacks TCEP **59** to yield a phosphoranyl radical which evolves by homolysis of the C-Se bond into an alanyl radical (formation of a stable phosphine selenide by-product) (Figure 23).⁶⁴ Hydrogen atom abstraction from a suitable donor species (e.g. **73**) results in Ala at the ligation junction.

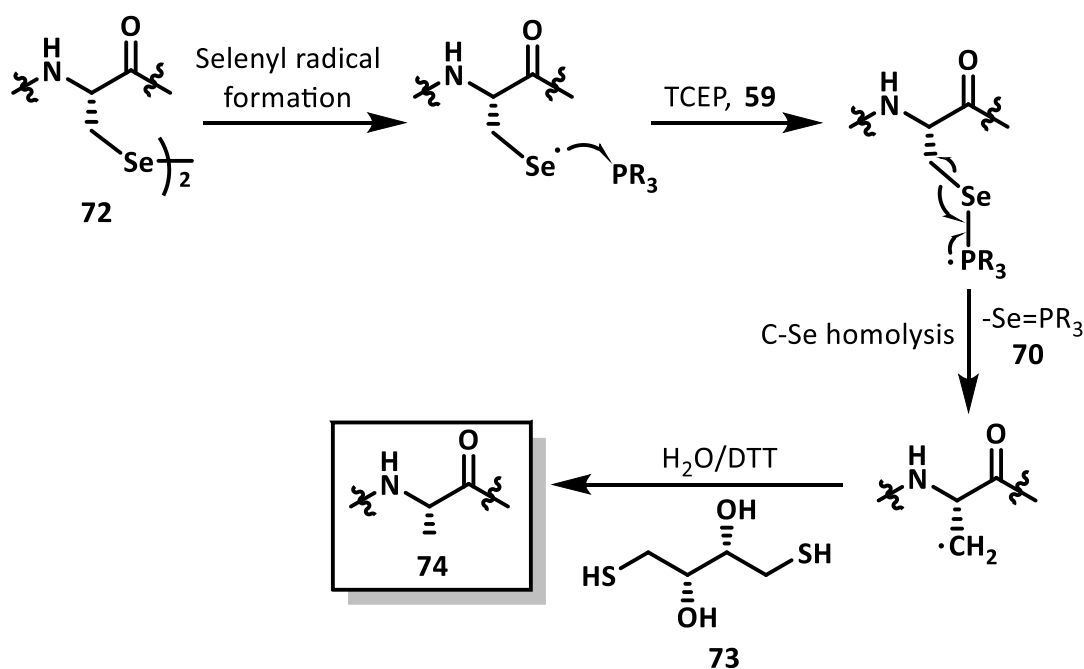


Figure 23: Mechanism of radical deselenisation of Sec-containing peptides with TCEP (PR_3 ; $\text{R}=\text{C}_2\text{H}_4\text{CO}_2\text{H}$) in aqueous solution (proposed by Dawson and co-workers).⁶⁴

1.8 VGF-derived peptides

The coding region of the *vgf* single copy gene is conserved between mammalian species and has a relatively simple organisation; a single exon encodes a single peptide.⁶⁵ The product of this gene is a 68 kDa VGF (non-acronymic) precursor peptide (termed “pro-VGF”) that is expressed in neurones of the central nervous system (including the cerebral cortex, hypothalamus and hippocampus) and the peripheral nervous system (including the dorsal root and sympathetic ganglia). Furthermore, *vgf* expression occurs in neuroendocrine tissues such as the adrenal medulla, pituitary gland, gastrointestinal tract and pancreas.^{66,67}

Pro-VGF undergoes endoproteolytic cleavage as part of the secretory pathway (catalysed by neuroendocrine-specific prohormone convertases (PCs) 1/3 and 2) generating a family of peptides that differ in length and biological activity, as shown in Figure 24.^{65,66,68} Cleavage sites typically consist of a short section of charged, basic residues.⁶⁹ Upon processing, pro-VGF has the potential to yield a number of peptides that are stored in dense core granules and secreted through the regulated pathway; secretion of VGF-derived peptides occurs in response to different stimuli, such as membrane depolarisation and intracellular signalling activation.^{66,70} These peptides have been shown to play a role in regulation of appetite and glucose homeostasis, energy metabolism, pain, memory, learning, mood and reproduction.⁷¹

TLQP-21 and TLQP-62 (21 and 62 residues in length, respectively) are characterised by the N-terminal “Thr-Leu-Gln-Pro” sequence. AQEE-30 (30 mer) is also a C-terminal VGF-derived peptide, with an “AQEE” motif at its N-terminus. Minamino and co-workers recently identified two novel biologically active peptides, designated neuroendocrine regulatory peptide (NERP) 1 and 2, via secretome analysis of human medullary thyroid carcinoma TT cells.⁷² NERP-1 and NERP-2 are C-terminally amidated peptides of 25 and 38 residues, respectively.⁷³ The 18 amino acids at the extreme C-terminus of the VGF precursor constitute a peptide that induces penile erection upon injection into the hypothalamic paraventricular nucleus (PVN) of male rats.⁷⁴

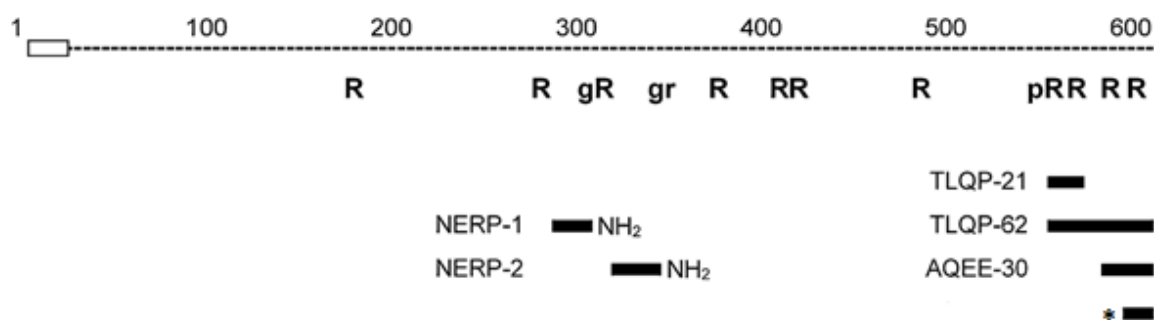


Figure 24: The biologically active VGF-derived peptides resulting from proteolytic post-translational processing of the VGF precursor peptide at the indicated cleavage sites: R refers to Arg-AA/lysine(Lys,K)-Arg/Arg-Arg or Arg-(AA)-Arg; gR refers to Gly-Arg-Arg (C-terminal amidated peptide); gr refers to Gly-Arg (C-terminal amidated peptide); pR refers to Pro-Arg-Pro. The C-terminal peptide is indicated (*) (18 residues in length). *Adapted from Cocco and co-workers.*⁶⁹

1.9 Glycaemic control and diabetes mellitus (DM)

1.9.1 The pancreas

The endocrine portion of the pancreas makes up 2% of the total mass and consists of small semi-spherical clusters of about 1500 cells, termed the islet of Langerhans.⁷⁵ The functional role of pancreatic islets in glucose homeostasis was first demonstrated in 1890 when Joseph von Mering and co-workers highlighted that dogs developed DM following a pancreatectomy.⁷⁶

1.9.2 Insulin

Insulin is a 51 residue, 5.8 kDa peptide hormone that is synthesised by the β cells of the pancreatic islets.⁷⁷ Containing two chains connected by disulfide bonds, the mature hormone is the post-translational product of a single-chain precursor, termed proinsulin.^{78,79} Packaging of proinsulin into immature secretory granules (proinsulin sorting for entry) in the *trans*-Golgi network (TGN), followed by granule maturation to yield a readily releasable pool of mature, insulin-containing secretory granules occurs; hexameric insulin is stored in the cytosol of pancreatic islet β cells in advance of regulated secretion into the bloodstream.^{80,81} The role of this hormone is to maintain normal blood glucose levels by facilitating cellular glucose uptake.

1.9.3 Insulin and glucose homeostasis

The pancreatic islet β cells respond to many nutrients (and non-nutrient secretagogues in the context of environmental stimuli and interplay of hormones) in the blood circulation.⁷⁷ Glucose is evolutionarily the primary stimuli for insulin release in some animal species because it is a principal food component and can accumulate immediately after food ingestion.⁸² The role of pancreatic islet β cells is to recognise rises in extracellular glucose concentration and rapidly secrete insulin in response. In the case of glucose-regulated insulin secretion, entry of glucose into β cells occurs via a glucose transporter (GLUT2) on the plasma membrane. Glucose is then phosphorylated by glucokinase to form glucose-6-phosphate and adenosine triphosphate (ATP). ATP is a key signalling molecule for insulin secretion because the β cell is equipped with ATP-sensitive K^+ channels which close in response to elevated cytoplasmic ATP levels.⁸³ Closure of K^+ -ATP-dependent channels results in membrane depolarisation and activation of voltage-dependent calcium ion channels; the intracellular calcium ion concentration increases and insulin secretion occurs via exocytosis. Augmentation of this response occurs by both a K^+ -ATP channel-independent calcium ion-dependent pathway and a K^+ -ATP channel-independent calcium ion-independent pathway.^{77,84}

1.9.4 Diabetes mellitus (DM)

The disease burden related to DM is high and rising in every country, fuelled by the global rise in the prevalence of obesity.⁸⁵ Estimates indicate that the global prevalence of DM in adults will rise to 9.9% by 2045 (628.6 million cases by 2045).⁸⁶ Type 1 and type 2 diabetes, (T1D)

and (T2D) respectively, are the two main types, T2D accounting for the majority (>85%) of cases.⁸⁵ Persistently high blood glucose levels can lead to serious life-changing and life-threatening complications; both forms of DM are associated by a wide variety of complications, including retinopathy cardiomyopathy, nephropathy and neuropathy.^{85,87,88} The premature morbidity, mortality, reduced life expectancy and financial costs of DM make it a major public health problem worldwide.^{85,87}

At present there are different treatments, both oral and injectable, available for the management of DM; as previously highlighted, insulin therapy is a well-established approach.^{85,89} Pharmacological interventions, used in conjunction with lifestyle changes, are designed to reduce the development or progression of the complications of DM by glycaemic control.⁹⁰ As a cure is unlikely, significant efforts are currently focussed on the development of novel therapeutic agents that improve the quality of life of DM patients.

Both T1D and T2D are characterised by loss of pancreatic islet β cell insulin secretory function and loss of mass/progressive β cell destruction, of which apoptosis is the main form.^{82,91} T1D is a T-cell-mediated autoimmune disease resulting from selective destruction of pancreatic β cells.⁸² Both in experimental animals and people, obesity is a leading pathogenic factor for developing chronic insulin resistance (associated with the prediabetic state associated with T2D). β cell failure during progression to T2D is a consequence of chronic exposure to glucose and fatty acids, known as “glucotoxicity” and “lipotoxicity”, respectively. Maintenance of functional β cells is recognised as a key strategy for delaying and/or preventing the development of DM.⁸⁰

1.10 VGF-derived peptides and glucose homeostasis

Deletion of the *vgf* gene (termed *vgf* “knockout”), resulted in mice that were hypermetabolic, hyperactive, had a reduced body mass and reduced peripheral fat stores.⁹² Furthermore, double mutant (*A^y/a, vgf-/vgf-*) mice, i.e. *A^y/a* mice (variants with defective signalling in the melanocortin satiety inducing pathway via overexpression of the melanocortin receptor (MC4-R) antagonist agouti) crossed with homozygous *vgf* mutants, were completely free from the obese agouti phenotype.⁹³ Interestingly, deletion of the *vgf* gene did not block the effects of the *A^y* allele on coat colour, hence it was hypothesised that pro-VGF functions downstream of MC4-R to effect development of agouti-induced obesity. *Vgf* deficiency also led to lowered

circulating levels of glucose and insulin and reduced pancreatic islet size in murine models also susceptible to obesity and adult onset DM (including *A^{y/a}* and *MC4-R^{-/-}* mice), which was attributed to increased insulin sensitivity.⁹⁴

Conditional knockout of *vgf* in pancreatic islet β cells resulted in decreased glucose-stimulated insulin secretion (GSIS) *in vitro* and *in vivo*.⁸⁰ Furthermore, conditional knockout of *vgf in vivo* impaired glycaemic excursion and insulin response; it was hypothesised that loss of pro-VGF or a pro-VGF-regulated pathway is linked to development of pancreatic islet β cell dysfunction. This contradictory evidence, in relation to the aforementioned global *vgf* knockout studies, most likely reflects the complexity of the cleavage of pro-VGF into multiple tissue-specific peptides with differing biological activities. Newgard and co-workers suggested that pro-VGF functions to regulate secretory granule formation in pancreatic islet β cells, replenish insulin reserve pools following nutrient stimulation and in the processing of proinsulin. This ties in with findings published by Levi and co-workers in which they showed that pro-VGF is present in the insulin secretory granules of insulinoma-derived β cell line (INS1) cells and VGF-derived peptides are secreted via a regulated pathway in response to stimulation.⁹⁵ C-terminal, VGF-derived peptides have been shown to be reduced in the circulation of patients with T2D when compared to euglycaemic individuals, thereby suggesting a role of these peptides in maintenance of glucose homeostasis.⁹⁶

Studies have shown that C-terminal VGF-derived peptides can enhance pancreatic islet β cell function.^{70,96–98} Newgard and co-workers demonstrated that TLQP-21 potentiates GSIS in isolated pancreatic islet β cells and *in vivo*, and promotes β cell survival through a cyclic adenosine monophosphate (cAMP)-mediated pathway.⁹⁷ Whilst Possenti and co-workers demonstrated that incubation of INS1 cells in medium containing TLQP-62 results in strong, dose-dependent induction of GSIS, TLQP-21 had only a very weak effect under low-glucose concentration (but it nevertheless potentiated GSIS, which is consistent with results obtained by Newgard and co-workers).⁷⁰ A slight increase in cell trophism and proliferation after a 24 hour stimulation with TLQP-62 was observed, but this data was deemed not to be statistically significant. Furthermore Possenti and co-workers observed an improvement in glucose tolerance within 30 minutes following glucose load in mice pre-treated with TLQP-62. Prior to this, Nakazato and co-workers revealed that N-terminal NERP-2 increases GSIS from the β cell line MIN6, as well as from isolated mouse pancreatic islets.⁹⁸ Additionally, plasma insulin

significantly increased 10 minutes after intraperitoneal (i.p.) administration of NERP-2 to rodents and response to NERP-2 was higher than for glucose alone.

1.11 VGF-derived peptides and neurodegenerative diseases

Biological markers (termed “biomarkers”) are characteristics that can be objectively measured and evaluated as an indicator or surrogate endpoint of a biological process, pathophysiological process, or a response to pharmacotherapeutic intervention.⁹⁹ Detection of biomarkers in blood or urine is a powerful tool for early diagnosis and treatment of disease; identification of novel biomarkers has the potential to lead to significant advancements.¹⁰⁰

Localised change in VGF-derived peptide levels has been attributed to a number of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinson’s disease (PD), Alzheimer’s disease (AD) and frontotemporal dementia.⁹⁹ Consequently, efforts have recently been focussed on determination of the scope of VGF-derived peptides as biomarkers. Furthermore, VGF-derived peptides have been highlighted as being potentially significant for the treatment of neurodegenerative diseases.

1.11.1 Amyotrophic lateral sclerosis (ALS)

ALS is a progressive neurodegenerative disease that affects motor neurones in the brain and spinal cord leading to muscle wasting, paralysis and eventual death from respiratory failure within 3 to 5 years.^{101,102} The lifetime risk is as high as 1 in 300, with likelihood of disease increasing with age; a 69% increase in the number of ALS cases worldwide is expected by 2040 due to the ageing population.¹⁰² Despite many clinical trials for treatments, there has been little success in the search for neuroprotective agents.¹⁰³

Proteomic analysis has revealed that the cerebrospinal fluid (CSF) content of VGF(398-411) can be used to successfully distinguish patients with ALS from healthy individuals.⁹⁹ Furthermore, Pasinetti and co-workers used quantitative enzyme-linked immunoassay (ELISA) studies to highlight that a negative correlation exists between the level of the VGF precursor peptide in the CSF and clinical severity of ALS; there is potential for monitoring of disease progression using this peptide.¹⁰¹

In their 2010 publication, Hara and co-workers present that a lower abundance of pro-VGF is found in the spinal cords of sporadic ALS patients vs. control patients.¹⁰³ Furthermore,

administration of a small molecule inducer of *vgf* (termed “SUN N8075”) resulted in slower disease progression and prolonged survival in rodent models of ALS. Pasinetti and co-workers demonstrated that viral-mediated exogenous *vgf* expression in spinal cord neurone cells attenuates ALS-associated excitotoxic injury.¹⁰¹ It was concluded by both Hara and co-workers and Pasinetti and co-workers that restoration of pro-VGF in spinal cord neurone cells via administration of this peptide has potential for the prevention/treatment of ALS.

1.11.2 Parkinson’s disease (PD)

PD is one of the fastest growing non-curable neurological disorders, due to the global ageing population; 6.2 million patients currently live with PD, which will double by 2040.^{104,105} PD is characterised by a progressive loss of dopaminergic neurones in the substantia nigra (SN) of the mid-brain and the resultant motor impairments (including bradykinesia, rigidity, and resting tremor).¹⁰⁶ In spite of recent improvements in the quality of life of PD patients (due to advances in dopaminergic medications), mortality rates continue to be increased in those with the disease, ranging from 1.5 to 2.3 times higher than for those without PD. Precise diagnosis at the time of disease onset is currently not possible, hence significant efforts are being focussed on the development of novel diagnostic approaches for early diagnosis of PD.¹⁰⁷

Using post-mortem parietal brain cortex from PD patients, Ferri and co-workers initially identified a reduction in the N-terminal NERP-1 (C-terminal VGF-derived peptides were expressed but their levels were unchanged).¹⁰⁸ Furthermore, Dunckley and co-workers used RNA sequencing to assess gene expression in the cortex; *vgf* downregulation was associated with PD.¹⁰⁹ Ferri and co-workers subsequently showed a linear relationship between pro-VGF immunoreactivity and disease duration and that replenishing dopamine via long-term treatment with Levodopa restored *vgf* expression.¹⁰⁷ Therefore, it was concluded that plasma levels may represent a useful biomarker, especially in the early stages of PD.

1.11.3 Dementia

The clinical syndrome of dementia, characterised by progressive cognitive decline, can be due to a variety of underlying non-curable pathophysiological processes.¹¹⁰ The most common of these is AD (50-75%) followed by vascular dementia (20%), dementia with Lewy bodies (5%) and frontotemporal dementia (5%). Age is the main risk factor for dementia, and the global

population is aging; the number of people affected by dementia is expected to increase to 131 million in 2050, resulting in huge social and economic costs.¹¹¹ Therefore, in recent years a dramatic increase in research focussed on the discovery of drugs for prevention of the diseases associated with dementia has occurred.¹¹²

AD is characterized by progressive memory and orientation loss and other cognitive deficits, including impaired judgment and decision making, apraxia and language disturbances.¹¹² Despite all scientific efforts, at the moment there are no effective pharmacotherapeutic options for prevention and treatment of AD.^{112,113}

An emerging picture suggests that glial cells' loss of beneficial roles or gain of toxic functions can contribute to neurodegenerative conditions.¹¹⁴ It is recognised that phagocytic dysfunction of glial cells can result in the accumulation of amyloid- β (A β) plaques in the brain which is associated with AD.¹¹³ Among glial cells, microglia have been shown to play phagocytic roles by engulfing synapses, apoptotic cells, cell debris and released toxic proteins.¹¹⁴ Microglial phagocytic activity and directed process motility is regulated by purinergic signalling, which is impaired in mouse models of AD.¹¹⁵ Semtner and co-workers recently observed stimulatory effects of TLQP-21 on primary cultured microglia via the C3aR1 receptor; key purinergic signalling-dependent functions were evoked.¹¹⁵ Furthermore, Salton and co-workers demonstrated that chronic intracerebroventricular administration of TLQP-21 to 5xFAD mice (a mouse model of AD), resulted in reduced amyloid pathology and microgliosis.¹¹³ It was therefore concluded that TLQP-21 may be a potential therapeutic agent for treatment of AD and other neurodegenerative diseases.

Furthermore, VGF-derived peptides have been shown to be decreased in the CSF of patients affected with forms of dementia, suggesting their potential utility as biomarkers and a possible role for pro-VGF in disease pathogenesis and progression.^{99,113} Indeed, GGEE-45 (VGF(373-417)), VGF(378-397), VGF(26-62), AQEE-30 and NERP-1 have been shown to be reduced in patients with AD or frontotemporal dementia.^{11,108,116-118} Interestingly, differentiated expression depending on the type of dementia was observed, potentially offering scope for more effective diagnosis and targeted treatment.¹¹

Project Aims

The aim of this project is to synthesise VGF-derived peptides to assess biological function, particularly in relation to DM and neurodegenerative diseases.

1. Synthesis of TLQP-62 peptide

Pilot studies conducted at the School of Biosciences, University of Nottingham, indicated that systemic infusion of TLQP-62 30 minutes prior to an i.p. glucose tolerance test leads to enhanced glucose clearance in both mice and Siberian hamsters (a model of adiposity) (Jethwa and co-workers, unpublished). No effect was observed with prior treatment with TLQP-21.

Despite there being a proposed intracellular model for the action of TLQP-62 on pancreatic cells, a receptor for this peptide is yet to be identified.⁷⁰ Therefore, it has not been determined whether this peptide functions as an insulin secretagogue via direct interaction with islet β cells, or if it binds to insulin sensitive tissues such as adipose tissue to effect glucose uptake; further understanding of the role of this peptide in glucose homeostasis and hence in relation to DM is required. Further insight into which tissue(s) TLQP-62 interacts with, ultimately leading to receptor identification, would be highly impactful. Furthermore, half-life data indicating whether TLQP-62 reaches the tissue(s) in a pharmacologically active concentration would infer whether longer acting analogues are required.

Two fragments of TLQP-62 will be synthesised then ligated together; the goal will be to obtain the native peptide in a purified form. Incorporation of a well characterised 9 residue hemagglutinin (HA) immunoreactive tag (YPYDVPDVA) at the N-terminus will enable identification of TLQP-62 in various excised tissues following *in vivo* infusion in mice (carried out by Dr Preeti Jethwa). Furthermore, HA tag-inclusion will be used to infer whether N-terminal modification of TLQP-62 precludes biological activity (as the receptor for this peptide is unknown), in preparation for future work; determination of which tissues(s) TLQP-62 interacts with and whether longer acting analogues are required, using N-terminal fluorine as a radionuclide.

2. Synthesis of AQEE-30 and GGEE-8 peptides

VGF-derived peptides AQEE-30 and GGEE-45 have been shown to be decreased in the CSF of patients affected with AD.^{108,116} Furthermore, altered expression of AQEE-30 has been observed in the parietal cortex.¹⁰⁸ AQEE-30 and GGEE-8 (a truncated version of GGEE-45) will be synthesised and then submitted for testing at the School of Biosciences, University of Nottingham. Once again, peptides will contain an N-terminal HA tag for visualisation purposes. Both VGF-derived peptides will be administered to *Drosophila melanogaster* flies by Dr Preeti Jethwa (via food and microinjection into the thorax); optimisation of the route (and dose) for adequate expression in the brain for subsequent distribution analysis will occur via immunohistochemical studies.

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Chapter 2: Results and Discussion

2.1 Synthesis of HA-TLQP-62 peptide via diselenide-selenoester ligation (DSL)

The primary aim of this project was to synthesise a suitable peptide selenoester and peptide dimer bearing an N-terminal (Sec)₂ for ligation, followed by deselenisation. HA-tag inclusion at the N-terminus of the selenoester resulted in HA-TLQP-62 **75**, as shown in Figure 25. Use of the corresponding anti-HA tag antibody and a secondary, labelled antibody would enable detection of TLQP-62 in excised tissue in a specific and sensitive manner.¹¹⁹

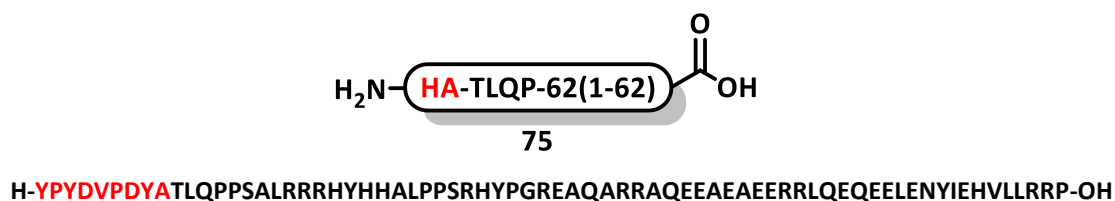


Figure 25: HA-TLQP-62 peptide; 8587.43 Da. The HA tag is shown in red.

Peptides **76** and **77**, as shown in Figure 26, were initially selected for synthesis. A suitably protected Fmoc-Arg(Pbf)-OH was immobilised on CTC resin **17** in the presence of DCM and *N,N*-diisopropylethylamine (DIPEA).¹⁸ Within 30 minutes of commencement of the loading reaction, the solution was purple/red in colour. Any unreacted sites on the resin were subsequently capped to afford the resin-bound Arg. Fmoc-deprotection was carried out via treatment with 20 vol.% piperidine **28** in DMF. Estimation of loading was enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29**. Loading had not been successful; a value of 0.1 mmol, 50% was recorded (>80% was considered to be acceptable). It was hypothesised that the purple/red colour was an indication that acid-catalysed cleavage of Arg from **17** had occurred, resulting in the presence of the red Trt cation. The procedure was repeated using 8 eq. of base, as oppose to 4. Additionally, a 0.4 mmol scale loading reaction was attempted, in the presence of 4 eq. of base. For both test reactions, the purple/red colour was observed after 30 minutes. As a result of this, the standard procedure for loading was amended to incorporate a pre-reaction base wash of **17** (4 eq. of DIPEA in DCM (1 mL)). This approach was carried out using a 0.05 mmol scale loading reaction (75 mg resin). In this instance, after 3 hours, no colour change had occurred. Estimation of residue loading was performed (0.05 mmol, 93%). When the reported procedure was scaled up, insufficient amino acid loading was once again observed. This was the case even in the

presence of 8 and 16 eq. of base (loading values of 0.07 mmol (38%) and 0.09 mmol (45%), respectively). As a result of this, the initially suggested peptide sequences were amended (to **78** and **79**, Figure 27). As well as enabling circumvention of Arg loading to **17**, the new approach meant that the precursor to the peptide dimer bearing an N-terminal (Sec)₂ would no longer contain an N-terminal Gln which, as has been previously discussed, is associated with a (M-18) rearrangement. Furthermore, according to the results published by Payne and co-workers in 2015, use of a peptide containing a C-terminal Gln phenylselenoester had the potential to result in a good yield of the desired product (53% yield after additive-free ligation and *in-situ* deselenisation using model peptides).¹⁸

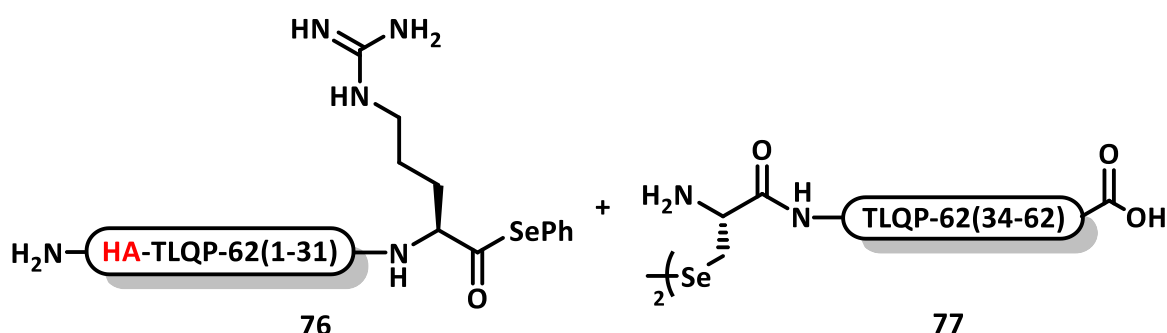


Figure 26: Initial peptides selected for synthesis; a selenoester **76** and N-terminal (Sec)₂ dimer peptide **77**. Unsuccessful loading of Arg in the case of **76** resulted in alteration of these peptides to **78** and **79** (Figure 27).

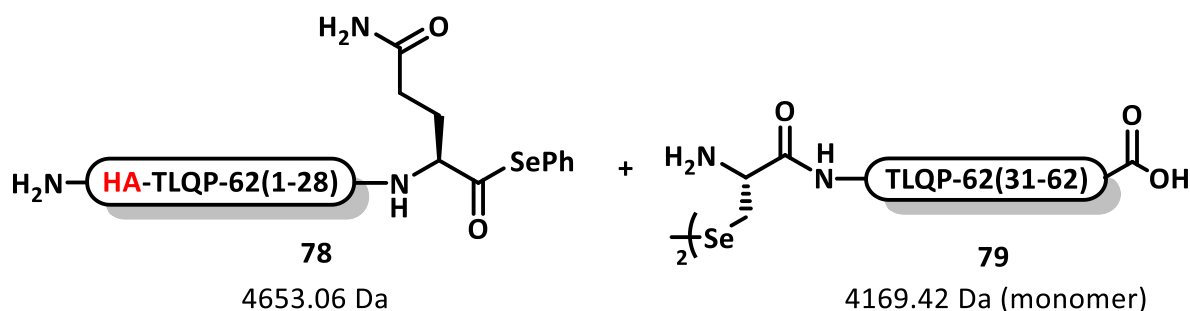


Figure 27: HA-TLQP-62 peptides **78** and **79** (a selenoester and N-terminal (Sec)₂ dimer peptide respectively) for ligation.

2.1.1 Synthesis of HA-TLQP-62 peptide selenoester **78**

A suitably protected Fmoc-Gln(Trt)-OH was immobilised on CTC resin **17** in the presence of DCM and DIPEA.¹⁸ Any unreacted sites on the resin were subsequently capped to afford the resin-bound Gln. Fmoc-deprotection was carried out via treatment with 20 vol.% piperidine **28** in DMF. Estimation of loading was enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29** (0.15 mmol, 98%). Submission to peptide assembly

(Fmoc-SPPS) via amino acid coupling, capping and Fmoc-deprotection afforded the resin-bound peptide HA-TLQP-62(1-29) **80**, as shown in Figure 28.

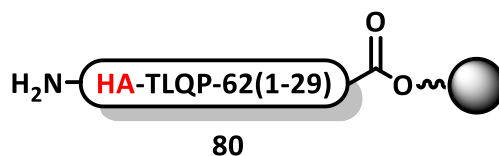


Figure 28: HA-TLQP-62(1-29) peptide; 4513.98 Da.

A mini-cleavage of the fully elongated peptide using TFA, TIS and water, followed by mass spectrometric analysis was used to determine that successful synthesis of peptide **80** had been carried out; the desired mass was observed.¹⁸

Cleavage of peptide **80** from the CTC resin **17** was effected using a mildly acidic solution of 30 vol.% HFIP in DCM, as shown in Figure 29.¹⁸ This solution effectively cleaved the peptide of the resin whilst preserving the acid labile R protecting groups. The peptide was treated with a solution of excess tributylphosphine (Bu₃P) (to activate the carboxylic acid) and DPDS in DMF for 3 hours at 0 °C to form the corresponding phenylselenoester **78**. The selenoesterification reaction (Figure 29) was conducted at low temperature to prevent base-catalysed epimerisation during carboxyl activation (via the previously discussed epimerisation-prone oxazolone species) and subsequent loss of enantiomeric purity at the α-carbon. The R protecting groups of the peptide were then removed via treatment with a mixture of TFA, TIS and water for 2 hours. Following ether precipitation and centrifugation, purification of the pellet by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and lyophilisation afforded HA-TLQP-62 peptide **78** (1% overall yield based on original loading). Analytical data is shown in Figures 30 and 31.

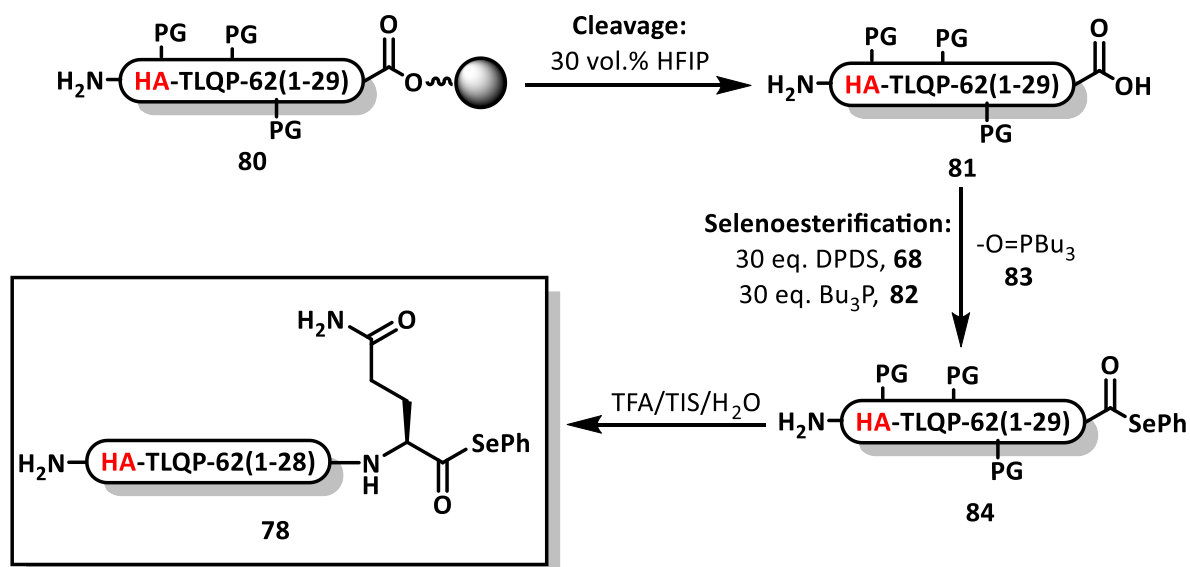


Figure 29: Selenoesterification of peptide **80** (on resin) to yield HA-TLQP-62 peptide **78**; peptide cleavage from resin using 30 vol% HFIP in DCM followed by treatment with DPDS and Bu_3P . R group protection group removal is effected with a 90:5:5 (v/v/v) mixture of TFA, TIS and H_2O .¹⁸

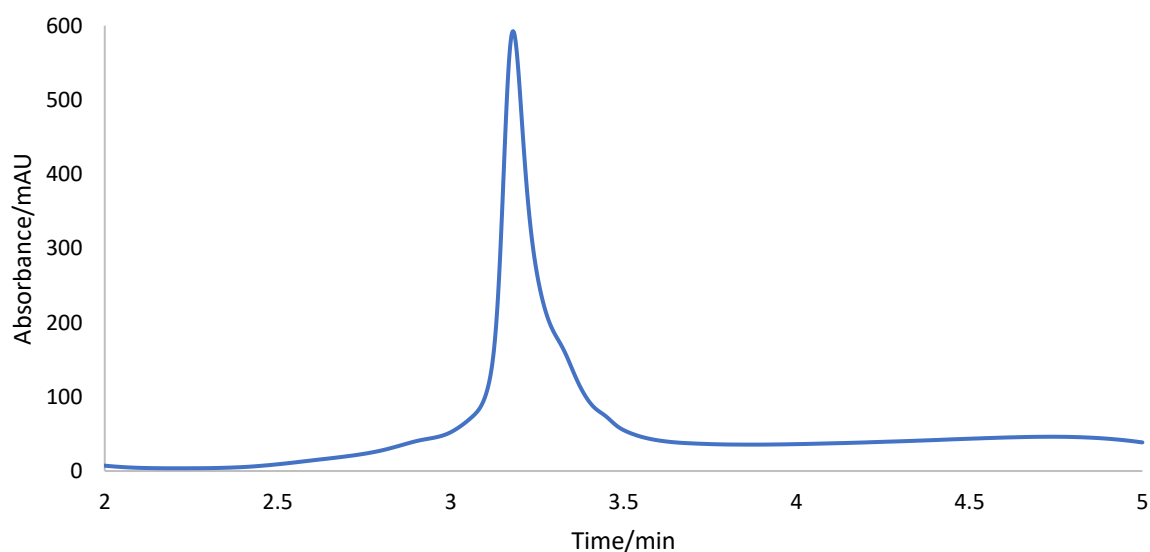


Figure 30: HPLC data for HA-TLQP-62 peptide **78**: Rt 3.18 min (20-80% B over 5 min, 0.6 mL/min).

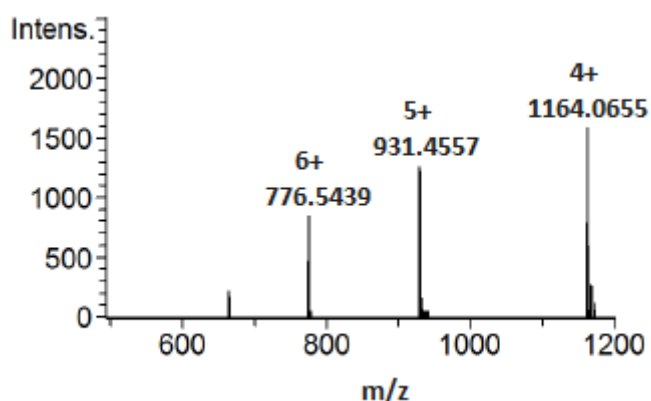


Figure 31: MS data for HA-TLQP-62 peptide **78**. *Calculated Mass* $[M+4H]^{4+}$: 1164.27, $[M+5H]^{5+}$: 931.61 and $[M+6H]^{6+}$: 776.51; *Mass Found (ESI⁺)*: $[M+4H]^{4+}$: 1164.07 (100%), $[M+5H]^{5+}$: 931.46 (80%) and $[M+6H]^{6+}$: 776.54 (54%).

2.1.2 Synthesis of HA-TLQP-62 N-terminal (Sec)₂ dimer peptide 79

Fmoc-Pro-OH was immobilised on CTC resin **17** in the presence of DCM and DIPEA.¹⁸ Any unreacted sites on the resin were subsequently capped to afford the resin-bound Pro. Fmoc-deprotection was carried out via treatment with 20 vol.% piperidine **28** in DMF. Estimation of loading was enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29** (0.02 mmol, 94%). Submission to peptide assembly (Fmoc-SPPS) via amino acid coupling, capping and Fmoc-deprotection afforded the resin-bound peptide HA-TLQP-62(31-62) **85**, as highlighted in Figure 32.

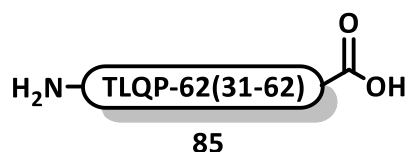


Figure 32: HA-TLQP-62(31-62) peptide; 4020.37 Da.

A mini-cleavage of the fully elongated peptide using TFA, TIS and water, followed by mass spectrometric analysis was used to determine that successful synthesis of peptide **85** had been carried out; the desired mass was observed.¹⁸

Tert-butoxycarbonyl (Boc)-protection of (Sec)₂ (Figure 33) using excess Boc₂O **86** was performed in advance of a manual Fmoc-SPPS coupling reaction between the resultant (Boc-Sec-OH)₂ **87** and the resin-immobilised peptide **85**.^{18,120} 2 eq. (relative to original loading of amino acid) of **87** was initially trialled. After 16 hours, the coupling solution was dark brown

in colour. This was not consistent with the red colour associated with Trt cation formation. Mass-spectrometric analysis indicated that the desired product was not present. The coupling was reattempted using 1 equivalent of **87** over 4 hours. In this instance, no colour change was observed. A mini-cleavage of the product was carried out using TFA, TIS and water. Mass-spectrometric analysis of the crude peptide was performed to determine presence of the asymmetrical diselenide **89** (Figure 34). During ligation, it is likely that this peptide diselenide would afford a non-peptide linked selenolate anion. This reactive species would potentially attack the peptide selenoester acyl donor, thereby reducing the yield of the desired ligation product. Fortunately, only the desired symmetrical peptide diselenide dimer **88** was identified. If the asymmetrical variant had been present, then 1 equivalent of TCEP **59** could have been introduced to the sample immediately before purification via preparative RP-HPLC. This step would have resulted in reduction of **89** to the corresponding selenol for use in the subsequent ligation, and a Sec by-product which could have been removed via purification.

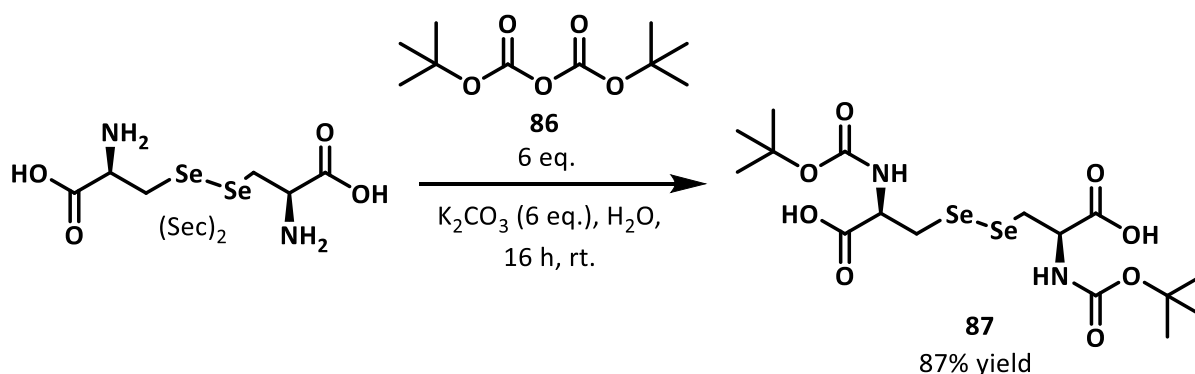


Figure 33: Synthesis of (Boc-Sec-OH)₂ via Boc-protection of (Sec)₂.¹²⁰

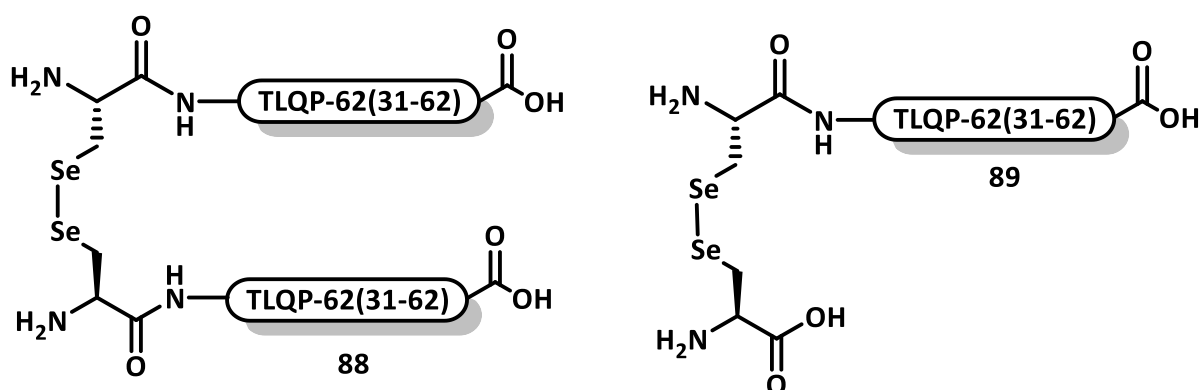


Figure 34: N-terminal (Sec)₂ dimer peptide **79**: symmetrical **88** and asymmetrical **89** diselenide.

Acidolytic Boc/R protecting group removal and cleavage from the resin was carried out using a mixture of 90:5:5 v/v/v TFA, TIS and water, followed by precipitation of the peptide in cold ether.¹⁸ The peptide was submitted to preparative RP-HPLC purification; peaks were collected in multiple fractions and analysed by mass spectrometry (MS) to ascertain which contained desired product. High-performance liquid chromatography (HPLC) analysis of several of the product-containing fractions revealed two peaks at 3.75 and 3.90 minutes, as shown in Figure 35. It was hypothesised that these correspond to the reduced and oxidised forms of peptide **79**, respectively. Immediately prior to RP-HPLC column injection, 1 equivalent of TCEP **59** had been added to the sample, to ensure that the selenopeptide was only present as the reduced monomer (to aid in the purification process). It was suggested that the reduced selenopeptide was spontaneously oxidising in solution due to the low redox potential of Sec. This was confirmed using a TCEP treatment test. Excess **59** was added to the peptide sample 16 hours prior to HPLC column injection to effect *in situ* deselenisation and result in the presence of N-terminal Ala **90** (Figure 36) (analysis by MS gave the desired mass; a single HPLC peak was observed, as highlighted in Figure 37).

Purification was followed by peptide lyophilisation to afford the HA-TLQP-62 peptide **79** (5% overall yield based on original loading).¹⁸ Analytical data is shown in Figures 38 and 39.

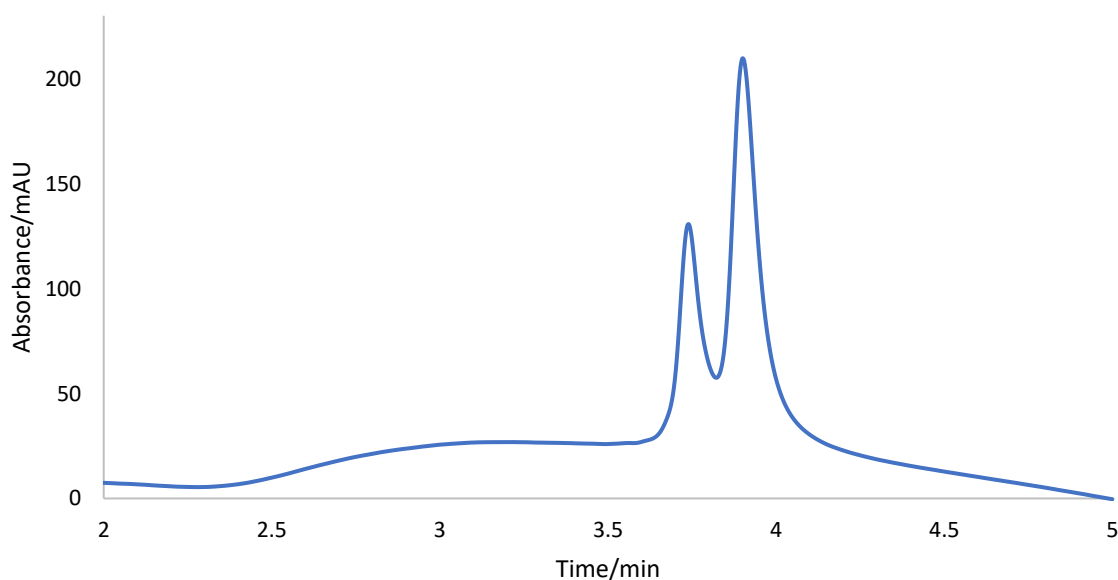


Figure 35: HPLC data for HA-TLQP-62 peptide **79**; Rt 3.75 (red.) and 3.90 (ox.) min (20-80% B over 5 min, 0.60 ml/min).

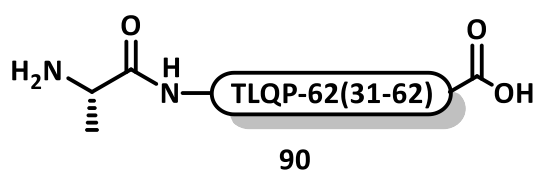


Figure 36: N-terminal Ala peptide.

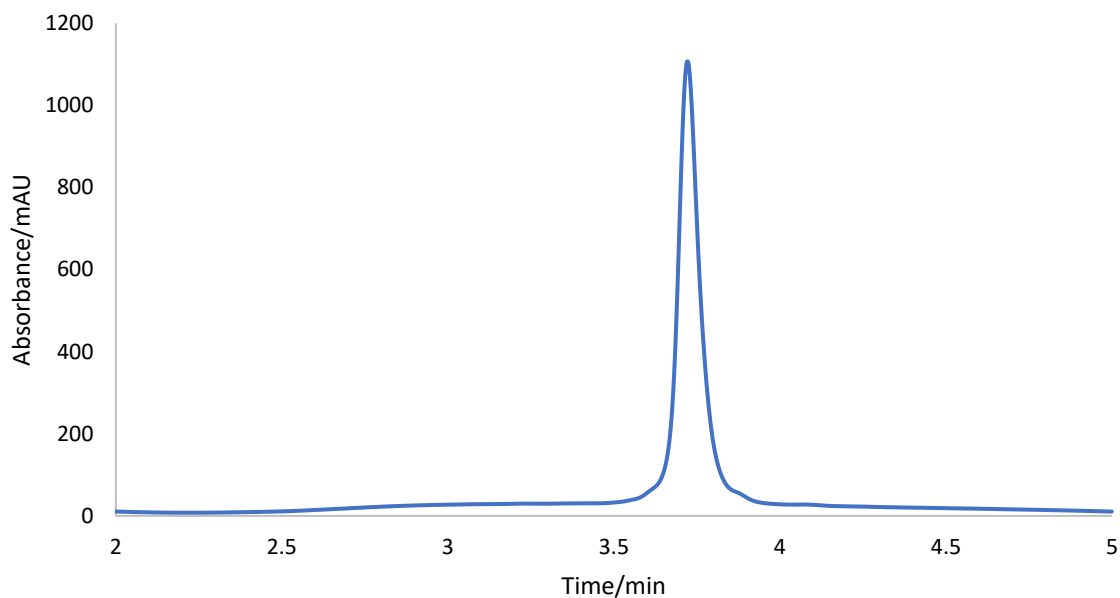


Figure 37: HPLC data for N-terminal Ala peptide **90**: Rt 3.73 (20-80% B over 5 min, 0.60 ml/min).

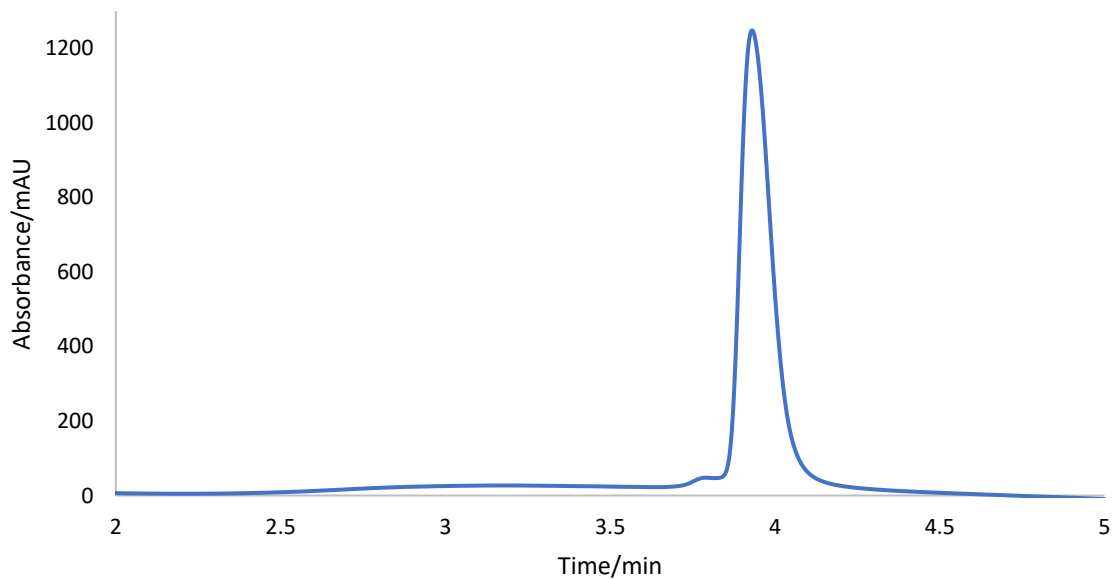


Figure 38: HPLC data for HA-TLQP-62 peptide **79**: Rt 3.94 min (20-80% B over 5 min, 0.6 mL/min).

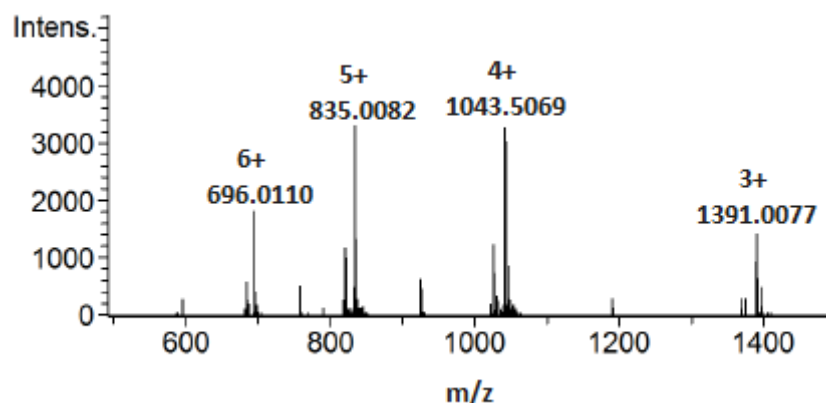


Figure 39: MS data for HA-TLQP-62 peptide **79**. *Calculated Mass* $[M+3H]^{3+}$: 1390.81, $[M+4H]^{4+}$: 1043.36, $[M+5H]^{5+}$: 834.88 and $[M+6H]^{6+}$: 695.90; *Mass Found* (ESI⁺): $[M+3H]^{3+}$: 1391.01 (43%), $[M+4H]^{4+}$: 1043.51 (99%), $[M+5H]^{5+}$: 835.01 (100%) and $[M+6H]^{6+}$: 696.01 (55%).

2.1.3 Ligation of peptides 78 and 79 to form HA-TLQP-62 peptide 75

Additive-free ligation (Figure 40) began by dissolving the peptide selenoester **78** and N-terminal (Sec)₂ dimer peptide **79** into ligation buffer to a final concentration of 6.25 mM with respect to **78** and 2.5 mM with respect to **79**.¹⁸ After ensuring that the pH of the solution was between 5.0 and 7.7, the reaction was allowed to proceed to completion (<10 minutes, DPDS precipitate (characteristic smell)). Extraction of DPDS with hexane was then carried out to prevent quenching of the subsequent deselenisation reaction via radical trapping.

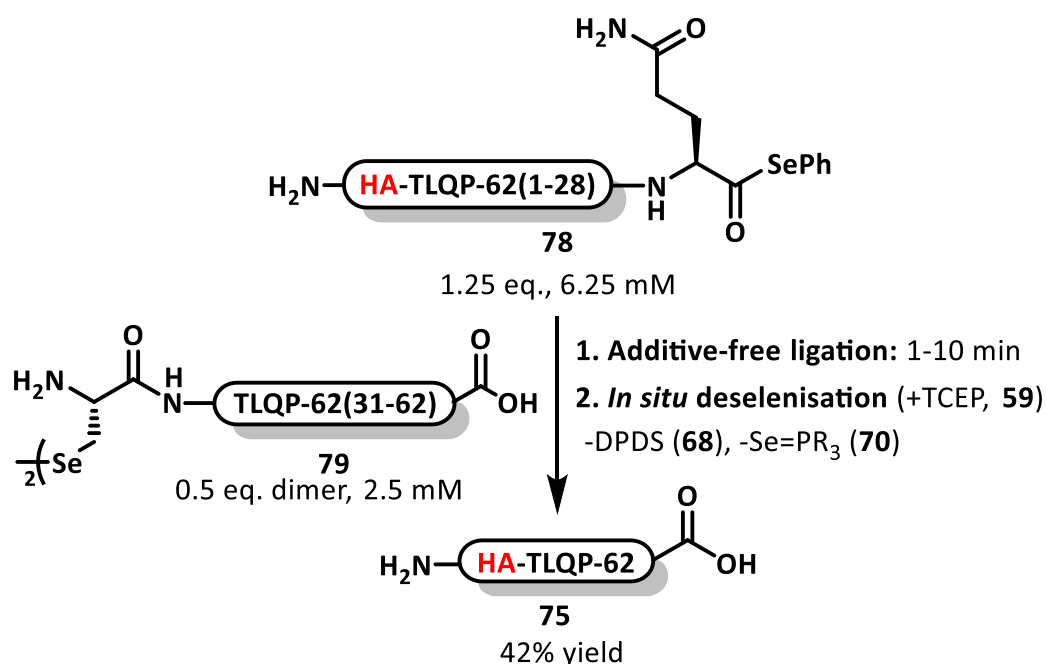


Figure 40: Rapid additive-free DSL between peptide **78** (1.25 eq., 6.25 mM) and peptide **79** (0.5 eq., 2.5 mM), followed by *in situ* deselenisation to yield HA-TLQP-62 peptide **75**. R=C₂H₄CO₂H. Conditions: additive-free ligation: 6 M Gdn-HCl, 0.1 M Na₂HPO₄, pH 6.2-6.5; *in situ* deselenisation: (i) hexane extraction, (ii) TCEP (50 eq.), DTT (5 eq.), 16 h.¹⁸

2 μl of the peptide ligation solution was diluted to 10 μl with acetonitrile/water containing 0.1 vol.% TFA, then analysed via HPLC; successful ligation was indicated by lack of peptide **79** (retention time (Rt) of 3.94 minutes). It was likely that the ligation solution contained some unreacted peptide **79** (present in excess), which was eluting at 3.18 minutes. Based upon the HPLC data, it was suggested that the ligation had yielded multiple products. Mass spectrometric analysis was attempted to characterise the ligation products, however, the concentration of the analytes in the diluted ligation solution was too low to obtain any useable data. Liquid chromatography-mass spectrometry (LC-MS) was also trialled, however, once again the concentration of peptide in the sample was too low. It was hypothesised that the HPLC peaks correspond to the oxidised HA-TLQP-62 ligation product **91**, a peptide selenoester **92** (via selenolate anion attack onto the selenoester **78** acyl donor) and an unsymmetrical peptide diselenide **93** (re-oxidation of the selenolate in the presence of ⁻SePh), as highlighted in Figure 41. Deselenisation of the ligated peptide was carried out over 16 hours using an excess of TCEP **59** and DTT **73** (as the hydrogen atom source) to result in Ala at the ligation junction.¹⁸ The mixture was then subjected to purification via preparative RP-HPLC; the obtained fractions were analysed by MS. Purification was followed by peptide

lyophilisation to afford HA-TLQP-62 peptide **75** (42% yield over two steps). Analytical data for this peptide is shown in Figures 42 and 43. Peptide **75** was submitted for insulin secretion studies testing using rat pancreatic islet β cells (as described in Chapter 4).

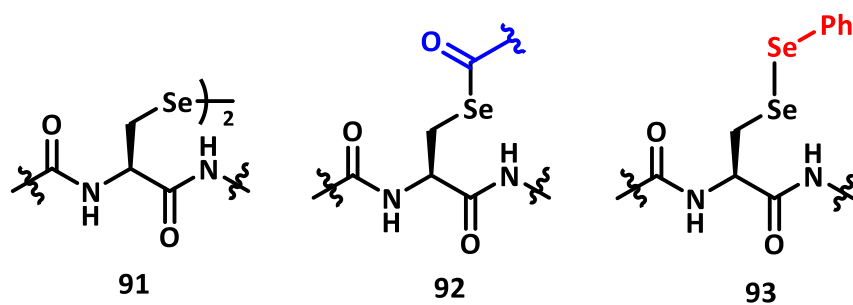


Figure 41: Potential rapid additive-free ligation products: the desired HA-TLQP-62 ligation product **91**, a peptide selenoester **92** and an unsymmetrical peptide diselenide **93**. Part of the peptide selenoester **78** is shown in blue. The re-oxidised phenylselenolate anion is shown in red.

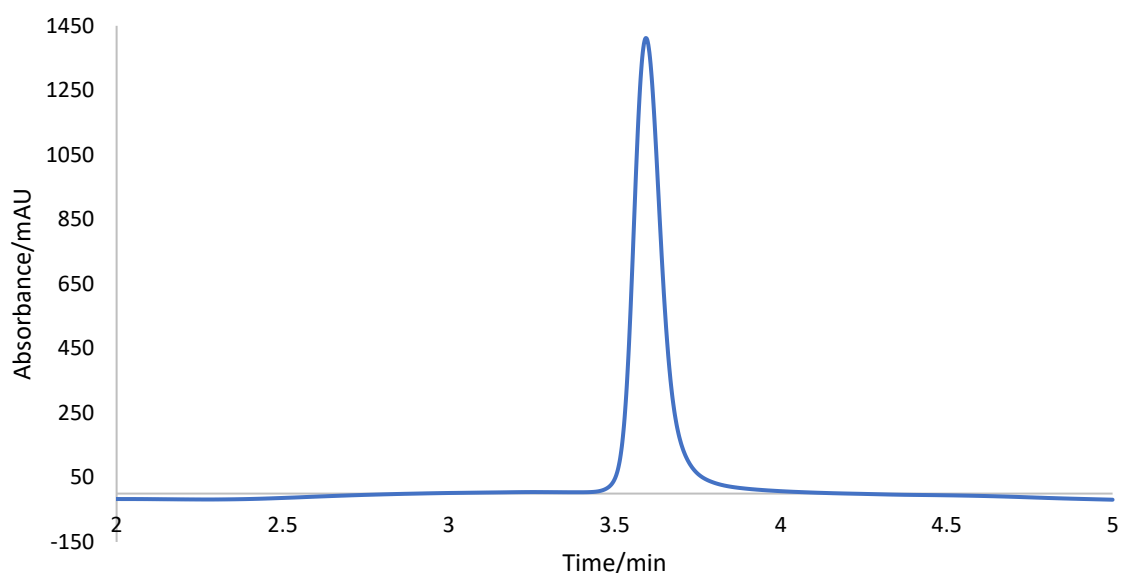


Figure 42: HPLC data for HA-TLQP-62 peptide **75**: Rt 3.6 min (20-80% B over 5 min, 0.6 mL/min).

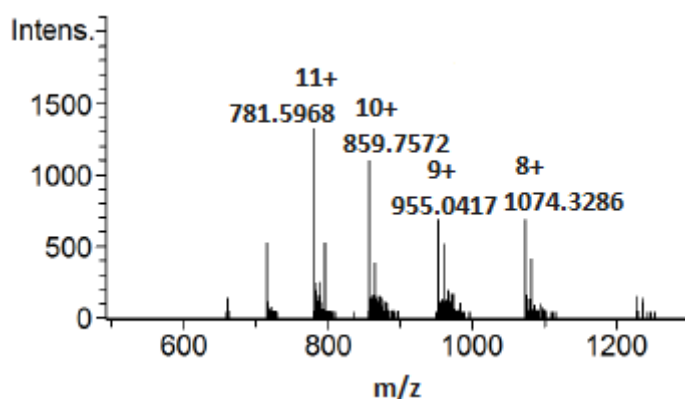


Figure 43: MS data for HA-TLQP-62 peptide **75**. *Calculated Mass* $[M+8H]^{8+}$: 1074.43, $[M+9H]^{9+}$: 955.16, $[M+10H]^{10+}$: 859.74 and $[M+11H]^{11+}$: 781.68; *Mass Found* (ESI⁺): $[M+8H]^{8+}$: 1074.33 (52%), $[M+9H]^{9+}$: 955.04 (53%), $[M+10H]^{10+}$: 859.76 (83%) and $[M+11H]^{11+}$: 781.60 (100%).

2.2 Synthesis of HA-AQEE-30 peptide **94**

As for TLQP-62, the HA tag was selected for inclusion at the N-terminus of AQEE-30 to obtain HA-AQEE-30 **94**, as shown in Figure 44. Use of the corresponding anti-HA tag antibody and a secondary, labelled antibody would enable detection of AQEE-30 in excised *Drosophila melanogaster* fly brain tissue in a specific and sensitive manner.¹¹⁹

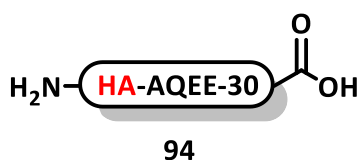


Figure 44: HA-AQEE-30 peptide; 4792.14 Da. The HA tag is shown in red.

For synthesis of peptide **94**, Fmoc-Pro-OH was immobilised on CTC resin **17** in the presence of DCM and DIPEA.¹⁸ Any unreacted sites on the resin were subsequently capped to afford the resin-bound Pro. Fmoc-deprotection was carried out via treatment with 20 vol.% piperidine **28** in DMF. Estimation of loading was enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29** (0.08 mmol, 81%). Submission to peptide assembly (Fmoc-SPPS) via amino acid coupling, capping and Fmoc-deprotection afforded a resin-bound intermediate peptide. A mini-cleavage of the peptide using TFA, TIS and water, followed by mass spectrometric analysis was carried out; analysis by MS revealed a M-18

mass difference which was attributed to the formation of N-pyroglutamic acid **53** from N-terminal Gln **52** as a consequence of acid-catalysed Trt sidechain deprotection.⁵¹

Extension of the peptide with 10 residues to form HA-AQEE-30 peptide **94** was subsequently carried out.¹⁸ Acidolytic Boc/R protecting group removal and cleavage from the resin was carried out using a mixture of 90:5:5 v/v/v TFA, TIS and water, followed by precipitation of the peptide in cold ether. Following precipitation and centrifugation, purification of the pellet by preparative RP-HPLC and lyophilisation afforded peptide **94** (1% overall yield based on original loading). Analytical data for this peptide is shown in Figures 45 and 46. Peptide **94** was submitted for testing (as described in the Project Aims section).

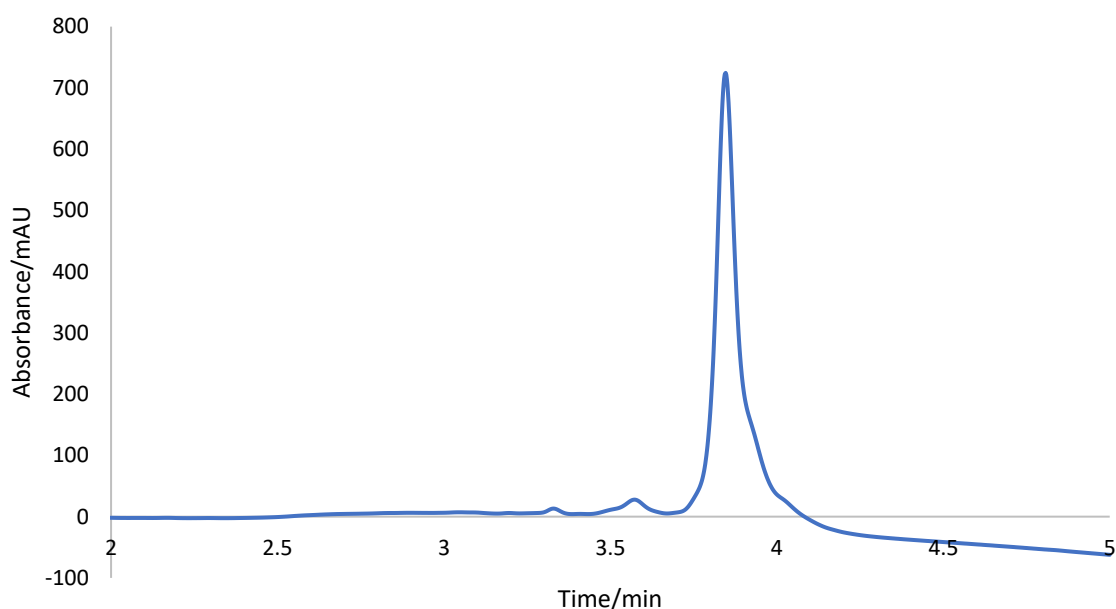


Figure 45: HPLC data for HA-AQEE-30 peptide **94**: Rt 3.85 min (20-80% B over 5 min, 0.6 mL/min).

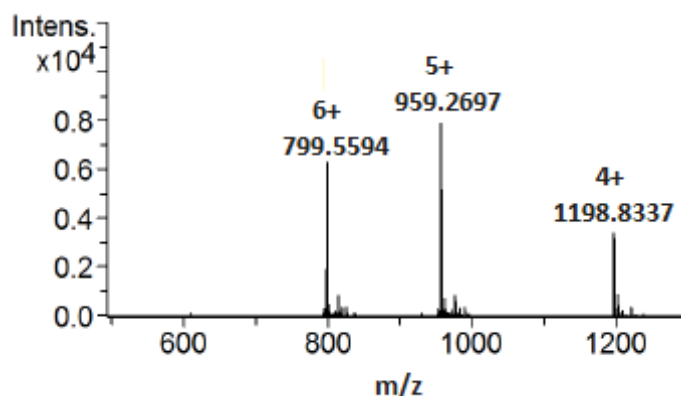


Figure 46: MS data for HA-AQEE-30 peptide **94**. *Calculated Mass* $[M+4H]^{4+}$: 1199.04, $[M+5H]^{5+}$: 959.43 and $[M+6H]^{6+}$: 799.69; *Mass Found* (ESI⁺): $[M+4H]^{4+}$: 1198.83 (44%), $[M+5H]^{5+}$: 959.27 (100%) and $[M+6H]^{6+}$: 799.56 (80%).

2.3 Synthesis of HA-GGEE-8 peptide 95

Once again, the HA tag was selected for inclusion at the N-terminus of GGEE-8 to obtain HA-GGEE-8 **95**, as shown in Figure 47.

For synthesis of HA-GGEE-8 peptide **95**, a suitably protected glutamic acid (Glu, E) amino acid (Fmoc-Glu(OtBu)-OH) was immobilised on CTC resin **17** in the presence of DCM and DIPEA.¹⁸ Any unreacted sites on the resin were subsequently capped to afford the resin-bound Pro. Fmoc-deprotection was carried out via treatment with 20 vol.% piperidine **28** in DMF. Estimation of loading was enabled via the use of UV-Vis absorbance analysis of the formed fulvene-piperidine adduct **29** (0.19 mmol, 93%). Submission to peptide assembly (Fmoc-SPPS) via amino acid coupling, capping and Fmoc-deprotection afforded the resin-bound peptide **95**.

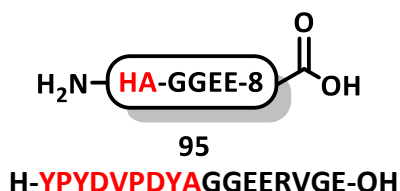


Figure 47: HA-GGEE-8 peptide; 1915.97 Da. The HA tag is shown in red.

Acidolytic Boc/R protecting group removal and cleavage from the resin was carried out using a mixture of 90:5:5 v/v/v TFA, TIS and water, followed by precipitation of the peptide in cold ether.¹⁸ Following centrifugation, HA-GGEE-8 peptide **95** was stored in preparation for

purification and submission for testing (as described in the Project Aims section). Analytical data for the crude peptide **95** is shown in Figures 48 and 49.

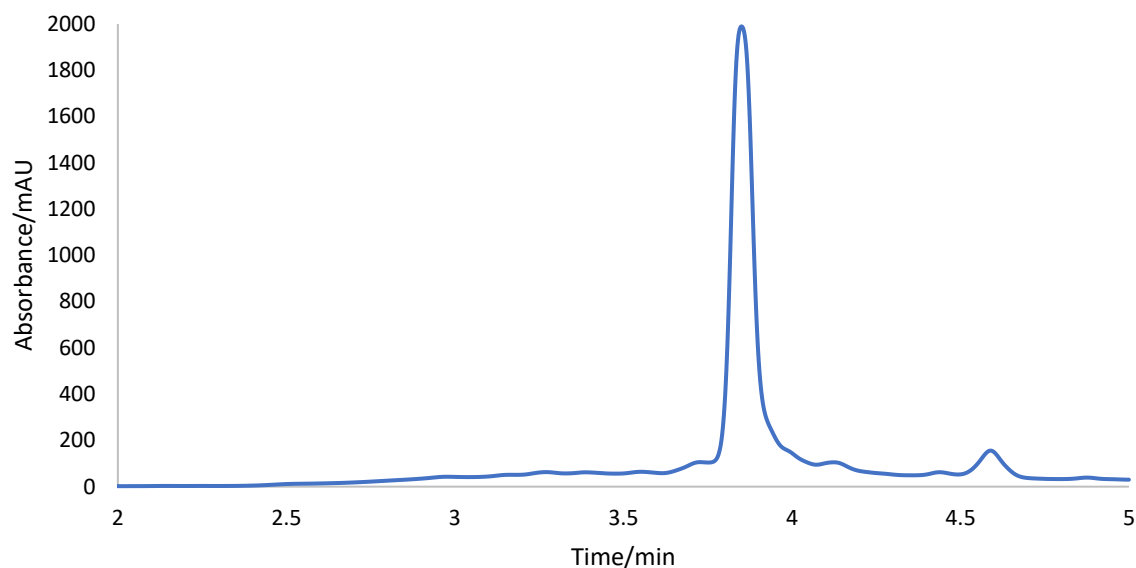


Figure 48: HPLC data for the crude HA-GGEE-8 peptide **95**: Rt 3.86 min (10-70% B over 5 min, 0.6 mL/min).

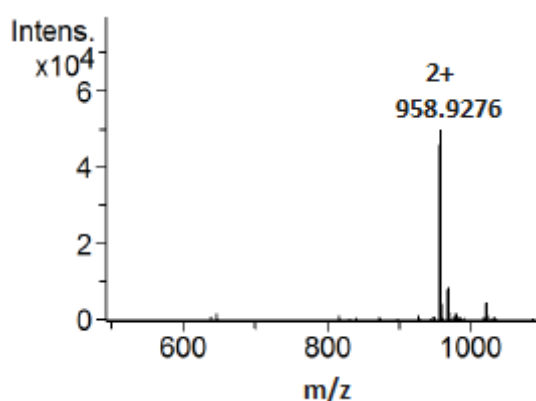


Figure 49: MS data for the crude HA-GGEE-8 peptide **95**. *Calculated Mass* $[M+2H]^{2+}$: 958.99; *Mass Found* (ESI⁺): $[M+2H]^{2+}$: 958.93 (100%).

2.4 Conclusion

In summary, two peptides for HA-tagged TLQP-62 have been successfully synthesised then ligated together via rapid additive-free DSL.¹⁸ *In situ* deselenisation of the formed peptide was subsequently carried out to yield the native HA-TLQP-62 peptide. Furthermore, both HA-AQEE-30 and HA-GGEE-8 have been successfully synthesised in preparation for future studies.

~

Chapter 3: Experimental

3.1 Materials

Fmoc protected amino acids and CTC resin were obtained from Novabiochem® or Sigma-Aldrich. (Boc-Sec-OH)₂ **87** was synthesised from commercially available (H₂N-Sec-OH)₂ (95% pure).

3.2 Instrumentation

MS: High-resolution, electrospray ionisation mass spectra were recorded using a Bruker micrOTOFII, operating in positive mode. Samples were dissolved in a suitable solvent, typically acetonitrile/water containing 0.1 vol.% TFA.

Analytical HPLC: The instrument used was a Thermo Ultimate 3000 uHPLC. A SunFire® C18 5 µm column with column dimensions of 2.1 x 150 mm was selected. The column oven was set to 45 °C. The solvent system was 0.1 vol.% TFA in water (solvent A) and 0.1 vol.% TFA in acetonitrile (solvent B). The instrument had a PDA detector, set to analyse at 210, 254, 280 and 450 nm. The gradient used was 20-80% B over 5 minutes with a flow rate of 0.6 mL/min in the case of all peptides except HA-GGEE-8 **95** where a gradient of 10-70% B over 5 minutes with a flow rate of 0.6 mL/min was selected.

Preparative HPLC for peptide purification: A Waters 1525 binary pump HPLC was used. The column used was a SunFire® Prep C18 OBDTM column, with 5-µm particle size and a 19 x 150 mm column. The solvent system was 0.1 vol.% TFA in water (solvent A) and 0.1 vol.% TFA in acetonitrile (solvent B). The flow rate used was 6 mL/min. The dual wavelength detector was set to 210 and 280 nm. Fractions were collected manually. The gradients used for each individual peptide has been highlighted in section 3.3.3.

Nuclear magnetic resonance (NMR): At least 10 mg of each sample was dissolved in 1 mL of deuterated solvent to achieve the desired sample concentration. This sample was then analysed on a Bruker AVIII 400 NMR system. The frequency for ¹H NMR was 400 MHz, and for ¹³C NMR was 101 MHz. Each chemical shift (δ) is reported in parts per million (ppm). ¹H NMR data is reported as multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, or combinations of these splittings), relative integral, coupling constant (J, Hz) and assignment.

3.3 Peptide synthesis

3.3.1 Manual

CTC resin loading: CTC resin (1.32 mmol/g loading, 1 eq.) was swollen in dry DCM for 30 minutes then washed with DCM (5 x 3 mL). A solution of Fmoc-AA-OH (0.5 eq.) and DIPEA (2 eq.) in DCM (final concentration of amino acid >0.1 M) was added to the resin and left to shake at room temperature for 16 hours.

The resin was washed with DMF (5 x 3 mL) and DCM (5 x 3 mL). The resin was treated with a solution of DCM/methanol/DIPEA (17:2:1 v/v/v, 3 mL) for 1 hour and washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Fmoc removal: The resin was washed with DMF (5 x 3 mL) and DCM (5 x 3 mL) prior to Fmoc removal. The resin was then treated with 20 vol.% piperidine in DMF (3 mL, 2 x 3 minutes). The resin was washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Loading determination: 25 μ L of the 20 vol.% piperidine washings (known volume, approximately 6 mL) was diluted to 10 mL with 20 vol.% piperidine in DMF. The absorbance of the fulvene-piperidine adduct (formed as a by-product during Fmoc-removal) was measured using a UV-Vis spectrophotometer at 301 nm. The molar concentration of the sample (c) was determined using the Beer-Lambert equation ($A = \epsilon \cdot c \cdot l$, A = absorbance, ϵ = molar extinction coefficient ($7800 \text{ M}^{-1} \text{ cm}^{-1}$), l = path length (1 cm)). This value was multiplied by the dilution factor of 400, then the number of moles of amino acid present in the initial volume of the 20 vol.% piperidine washings was determined.

General amino acid coupling: Fmoc-AA-OH (4 eq.) and OxymaPure® (4 eq.) were dissolved in DMF (final concentration of amino acid >0.1 M). To this solution DIC (4 eq.) was added and left to activate for 5 minutes. The solution was then added to the resin and left to shake at room temperature for 1 hour. The resin was then washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL).

Capping: Acetic anhydride/pyridine (1:9 v/v, ~3 mL) was then added to the resin and shaken at room temperature for 3 minutes. The resin was then washed with DMF (5 x 3 mL), DCM (5 x 3 mL) and DMF (5 x 3 mL). The peptide was then subjected to Fmoc removal.

Coupling conditions for (Boc-Sec-OH)₂ 87: A solution of (Boc-Sec-OH)₂ **87** (0.03 mmol, 13.7 mg, 1 eq. based on the dimer and relative to original loading of amino acid), OxymaPure® (2 eq.) and DIC (2 eq.) in DMF (final concentration of **87** = 0.1 M) was added to the resin and shaken for 4 hours at room temperature. The resin was then washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and DMF (5 x 3 mL).

Cleavage: The resin was prepared by washing with DCM (x 10) and dried under vacuum. A solution of TFA/TIS/water (90:5:5) was added to the dried resin (~3 mL) and shaken at room temperature for 2 hours. The cleavage solution was then plunged into a 50 mL falcon tube and the resin washed with more cleavage solution. The combined solutions were concentrated under a stream of nitrogen to <5 mL. Cold diethyl ether (~30 mL) was added to precipitate the peptide. The suspension was centrifuged at 3500 revolutions per minute (rpm) for 5 minutes and the supernatant decanted off. The resultant precipitate was dissolved in acetonitrile/water containing 0.1 vol.% TFA and purified using preparative RP-HPLC.

Purification: Purification was performed using preparative RP-HPLC. The HPLC and column used is described in section 3.2. The peaks were collected in multiple fractions and analysed by MS to ascertain which contained desired product. Fractions of desired purity (>95%) were combined and lyophilised so the mass and yield could be calculated.

3.3.2 Automated

Automated Fmoc-SPPS was carried out on a Biotage Initiator⁺ Alstra microwave assisted peptide synthesiser. The required quantity of pre-loaded resin was placed into a reaction vessel. Each Fmoc removal included a 30 second and 5-minute reaction at 50 °C. Couplings were performed with microwave assistance at 75 °C for 5 minutes (0.5 M concentration for amino acids and coupling reagents). Double couplings were performed for sections of repeated residues, hydrophobic regions, coupling to Pro and for all residues after the first 20 amino acids. Capping was performed for 5 minutes using acetic anhydride and pyridine at room temperature. Cleavages were performed manually, as described in section 3.3.1.

3.3.3 Peptides synthesised

The following is a list of peptides that were synthesised, along with the respective purification gradient, elution time and percentage yield.

HA-TLQP-62 peptide 78

H-YPYDVDPDYATLQPPSALRRRHYYHALPPSRHYPGREAQ-SePh

Synthesised via Fmoc-SPPS using CTC resin (225 mg) followed by selenoesterification. The cleaved peptide was purified using a gradient of 30-95% B over 30 minutes. The desired product eluted at 14.1 minutes. The fractions were lyophilised to yield the desired product: >95% pure, 1.3 µmol, 6.2 mg, 1% overall yield based on original loading.

HA-TLQP-62 peptide 79

H-URRAQEEAAEERRLQEQEELNYIEHVLLRRP-OH

Synthesised via Fmoc-SPPS using CTC resin (37.5 mg) followed by coupling of (Boc-Sec-OH)₂ **87**. The cleaved peptide was purified using a gradient of 10-90% B over 30 minutes. The desired product eluted at 23.3 minutes. The fractions were lyophilised to yield the desired product: >95% pure, 1.2 µmol, 4.8 mg, 5% overall yield based on original loading.

HA-TLQP-62 peptide 75

H-YPYDVDPDYATLQPPSALRRRHYYHALPPSRHYPGREAQARRAQEEAAEERRLQEQEELNYIEHVLLRRP-OH

Synthesised via ligation of the HA-TLQP-62 peptide selenoester **78** And N-terminal (Sec)₂ dimer peptide **79**. The cleaved peptide was purified using a gradient of 35-95% B over 30 minutes. The desired product eluted at 14.9 minutes. The fractions were lyophilised to yield the desired product: >95% pure, 0.2 µmol, 1.8 mg, 42% yield (over two steps).

HA-AQEE-30 peptide 94

H-YPYDVDPDYAAQEEAAEERRLQEQEELNYIEHVLLRRP-OH

Synthesised via Fmoc-SPPS using CTC resin (150 mg). The cleaved peptide was purified using a gradient of 15-95% B over 30 minutes. The desired product eluted at 21.1 minutes. The fractions were lyophilised to yield the desired product: >95% pure, 0.8 µmol, 3.8 mg, 1% overall yield based on original loading.

HA-GGEE-8 peptide 95

H-YPYDVDPYAGGEERVGE-OH

Synthesised via Fmoc-SPPS using CTC resin (300 mg). The crude product was >95% pure: 12.1 μ mol, 23.1 mg, 6% overall yield based on original loading.

3.4 Selenoesterification

Cleavage of peptide **80** (0.15 mmol, based on original loading, 1 eq.) from the resin was effected by treating with 30 vol.% HFIP in DCM for 2 hours before concentrating under vacuum. The crude peptide was dissolved in DCM and cooled to 0 °C. DPDS (4.62 mmol, 1.44 g, 30 eq. in DCM) was added to the solution followed by Bu₃P (4.62 mmol, 1.14 mL, 30 eq.). The reaction was allowed to proceed at 0 °C for 3 hours, after which time the solvent was removed under vacuum. The crude material was put on ice and the protecting groups removed via treatment with TFA:TIS:water (90:5:5 v/v/v) for 2 hours. The cleavage solution was then transferred into a 50 mL falcon tube and concentrated under a stream of nitrogen to <5 mL. Cold diethyl ether (~30 mL) was added to precipitate the pellet. The suspension was centrifuged at 3500 rpm for 5 minutes and the supernatant decanted off. The resultant precipitate was dissolved in acetonitrile/water containing 0.1 vol.% TFA. Purification was performed using preparative RP-HPLC. The HPLC and column used is described in section 3.2. The peaks were collected in multiple fractions and analysed by MS to ascertain which contained desired product **78**. Fractions of desired purity were combined and lyophilised so the mass and yield could be calculated.

3.5 Synthesis of Boc-protected selenocystine 87

Potassium carbonate (4.50 mmol, 0.62 g, 6 eq.) and Boc₂O (4.50 mmol, 0.98 g, 6 eq.) were added to (H₂N-Sec-OH)₂ (0.75 mmol, 0.25 g, 1 eq.) in water (~25 mL). The solution was left stirring for 16 hours at room temperature.

Water (20 mL) and diethyl ether (15 mL) were added to the solution. The aqueous layer was collected and the organic layer extracted with a further 20 mL of water. The combined aqueous layers were acidified with 1 M hydrochloric acid and washed with ethyl acetate (3 x 25 mL). The organic layers were combined, washed with brine, dried over magnesium sulfate, filtered and concentrated. The product was a yellow oil which was dried under vacuum for 24

hours. The resultant product **87** was a yellow crystalline solid, used without further purification (0.66 mmol, 0.35 g, 87% yield). MS: *Calculated Mass* $[M+H]^{1+}$: 535.33 and $[M-H]^{1-}$: 533.33, *Mass Found* (ESI⁺): $[M+H]^{1+}$: 535.03 (87%) and (ESI⁻) $[M-H]^{1-}$: 533.01 (90%). ¹H NMR (400 MHz, MeOD): δ 4.43 (dd, J = 9.0, 4.8 Hz, 1H, CH _{α}), 3.47 (dd, J = 12.6, 4.9 Hz, 1H, CH _{β 1}) 3.23 (dd, J = 12.6, 9.0 Hz, 1H, CH _{β 2}), 1.47 (s, 9H, Boc). ¹³C NMR (101 MHz, MeOD): δ 172.88, 156.41, 79.36, 54.20, 31.05, 27.37.

3.6 Rapid additive-free diselenide-selenoester ligation (DSL)

Peptide selenoester **78** (0.6 μ mol, 2.9 mg, 1.25 eq.) and peptide diselenide dimer **79** (0.3 μ mol, 2.1 mg, 0.5 eq.) were dissolved separately in ligation buffer (6 M Gdn·HCl, 100 mM sodium phosphate dibasic, pH 7.2-7.4) to a concentration of 12.5 mM with respect to **78** and 5 mM with respect to **79**. The solutions were combined to give an overall concentration of 6.25 mM with respect to **78** and 2.5 mM with respect to **79** (pH 6.2-6.5). The ligation reaction was allowed to proceed for 5-10 minutes. To the peptide ligation solution was added an equal volume of hexane. The solution was agitated, then the hexane was removed.

3.7 *In situ* deselenisation

Solutions of TCEP (0.25 M) and DTT (25 mM) in ligation buffer (6 M Gdn·HCl, 100 mM sodium phosphate dibasic, pH 7.2-7.4) were separately prepared. The peptide ligation solution (prepared as described in section 3.6) was treated with an equal volume of each of the TCEP and DTT solutions (50 eq. of TCEP and 5 eq. of DTT). The resultant solution was agitated on an orbital shaker at room temperature for 30 seconds, then left at room temperature for 16 hours. Following completion of the deselenisation reaction, purification was performed using preparative RP-HPLC. The HPLC and column used is described in section 3.2. The peaks were collected in multiple fractions and analysed by MS to ascertain which contained desired product. Fractions of desired purity were combined and lyophilised so the mass and yield could be calculated.

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Chapter 4: Insulin Secretion Studies

4.1 Overview

Synthesis of HA-TLQP-62 **75** was successfully carried out under the supervision of Dr Nicholas Mitchell, with the aim of completing testing on this peptide at the University of Nottingham in the laboratory of Dr Preeti Jethwa. The goal was for the ability of peptide **75** to function as an insulin secretagogue to be assessed in relation to human TLQP-62, using rat INS1 cells. These cells display a number of important characteristics, including high responsiveness to glucose.⁷⁰ Cultured INS1 cells were to be incubated with TLQP-62 and subsequent study of insulin secretion was to be carried out using immunodiagnostics for quantification.

Due to the Covid-19 pandemic, a University of Nottingham research laboratory shutdown was initiated on the 15th March 2020. This precluded completion of the insulin secretion studies. Section 4.3 highlights the experimental work that would have taken place in the Spring of 2020.

4.2 The sandwich enzyme-linked immunoassay (ELISA)

An immunoassay is a type of bioanalytical technique; quantification of an analyte is based upon the interaction of an antigen (analyte) with a specific and selective antibody. Detection of the antigen-antibody complex depends upon measurement of label activity; a radioisotope, fluorescent tag or an enzyme is typically linked to one of the immunoanalytical reagents.¹²¹ Use of these labels enables high sensitivity and a low limit of detection to be achieved. Application of immunoassays in the clinic and in drug discovery programs is key and has facilitated advancements in the diagnosis and treatment of disease, respectively.

A sandwich ELISA requires the use of two antibodies; a highly antigen-specific monoclonal capture antibody (which is attached to a solid surface) and a detection antibody (typically polyclonal in nature). Binding of the antigen to the capture antibody is followed by addition of the detection antibody, which recognises the antigen at a different epitope to the capture antibody. Consequently, the antigen is “sandwiched” between the two antibodies.¹²²

To quantify the extent of capture antibody-antigen binding, an enzyme reporter system is used, as shown in Figure 50. The detection antibody can be directly coupled to the enzyme,

or it can be labelled with a small molecule (e.g. biotin) that facilitates indirect linkage of an enzyme (e.g. horseradish peroxidase (HRP)) to the antibody-antigen sandwich. Addition of the enzyme substrate to the reaction results in a colour change; the product of the enzyme activity is a readily detectable chromophore. A UV-Vis absorbance signal is utilised as the measurable response. The generated signal is proportional to the concentration of antigen present in the original sample.

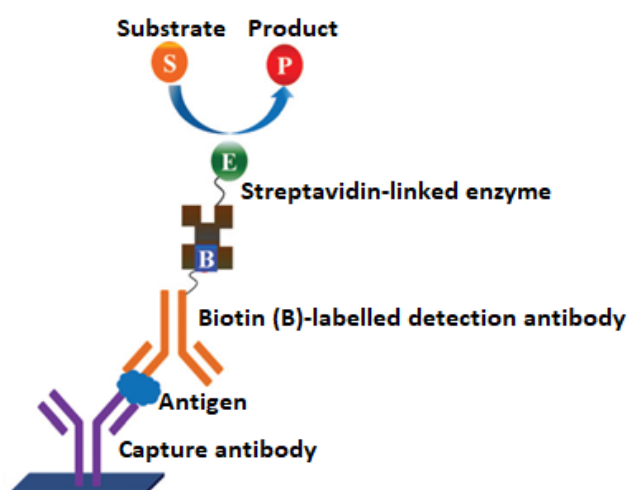


Figure 50: ELISA; use of biotin-streptavin-HRP as a reporter system. *Adapted from Kim and co-workers.*¹⁰⁰

4.3 Insulin secretion studies

4.3.1 Overview

Study 1: Assessment of insulin secretion following incubation of INS1 cells in a high-glucose condition.

Study 2: Determination of the effect of presence of TLQP-62 during incubation on insulin secretion – comparison of HA-TLQP-62 **75** and human TLQP-62 (provided by Professor Gian Luca Ferri, The Department of Biomedical Science, Università di Cagliari (Italy)).

4.3.2 Protocol

4.3.2.1 Cell culture

Rat INS1 cells (parental cell line provided by Nottingham Trent University) were cultured under 95% humidified air:5% CO₂ in complete medium composed of RPMI 1640

supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 2 mM L-Gln, 100 units/mL penicillin, 100 µg/mL streptomycin and 50 µM 2-mercaptoethanol.⁷⁰

Cells were passaged every 7 days as per standard protocol in order to maintain adequate stock levels (protocol taken, with permission, from the University of Nottingham Cell Culture Basics Handbook, unpublished). Firstly, cell culture media was discarded from the culture vessel and the cells were washed with phosphate-buffered saline (PBS) solution for a few minutes. After use, the PBS solution was removed from the culture vessel and discarded. Pre-warmed trypsin was added to the side of the flask, and to ensure complete coverage of the cell layer, the flask was gently rocked prior to incubation at 37 °C for 3-4 minutes. Detachment of cells from the flask was assessed using a light microscope and if detachment was less than 90% then incubation was repeated (dissociation was checked every 30 seconds). When ≥90% of the cells had detached, pre-warmed complete medium (as previously defined, 2 x volume of trypsin) was added to the flask. The medium was dispersed by pipetting it over the cell layer surface several times to ensure complete neutralisation of the trypsin in order to prevent cell damage. The cells were then transferred to a universal 15 mL or 50 mL conical tube and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in a minimal volume of pre-warmed complete growth medium and then a sample was removed for counting using a hemocytometer. The cells were diluted to the recommended seeding density, then transferred into new culture vessels which were returned to the incubator. For insulin secretion studies, cells were plated in 6-well plates, according to the recommended seed density per well.

4.3.2.2 Insulin secretion studies

Study 1: The cells were washed four times with modified Krebs-Ringer bicarbonate-HEPES buffer (KRBH): 134 mM NaCl, 4.7 mM KH₂PO₄, 1.4 mM MgSO₄, 1 mM CaCl₂ and 10 mM HEPES (pH 7.4) without bovine serum albumin (BSA).⁷⁰ Fresh KRBH containing 10 µM forskolin (Frsk), 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), 20 mM glucose, 100 nM tetra phorbol myristate (TPA) or 56 mM KCl was applied and the supernatant was collected after 1 hour of stimulation.

Quantification of insulin in the supernatant was determined using an ELISA kit specific for rat insulin (Merck Millipore).⁷⁰ Each assay included testing, in duplicates, of a “blank” sample (assay buffer), six insulin standards (0.2-10 ng/mL insulin), two “quality control” samples (rat insulin in quality control buffer) and the unknown(s).

Capture of insulin onto the wells of a monoclonal anti-insulin antibody-coated microplate followed by binding of a biotin-labelled polyclonal anti-insulin antibody to the immobilised insulin occurred.⁷⁰ Treatment of the immobilised insulin-antibody with streptavidin-HRP in buffer followed by addition of the 3,3',5,5'-tetramethylbenzidine substrate enabled colorimetric quantification of insulin. HRP activity was measured using a microplate spectrophotometer; increase in UV-Vis absorbance at 450 nm was directly proportional to the amount of captured insulin. Therefore, the concentration of insulin in the original sample was determined by interpolation from a plotted reference curve.

Study 2: As Study 1, but with the addition of 10 μ M TLQP-62 to the incubation medium and testing of more than one unknown per assay (HA-TLQP-62 **75** and human TLQP-62).

4.3.3 Expected results

It is widely accepted that insulin is secreted in response to high glucose, and the INS1 cell line is particularly sensitive to this nutrient.^{70,82} Therefore, it is expected that insulin secretion would have followed incubation of cells in a high-glucose condition (Study 1). Incubation of cells with TLQP-62 (as part of Study 2) should have induced a potent increase in GSIS. It is hoped that this would have also been observed for HA-TLQP-62 **75**. As previously highlighted, determination of the ability of peptide **75** to function as an insulin secretagogue would have enabled initial consideration of the effects of N-terminal modification of this peptide; significant hindrance of insulin secretion would have indicated that investigation into ¹⁸F-labelling at an internal position rather than at the N-terminus of TLQP-62 was required. Study 2 would be repeated using N-terminal ¹⁹F-fluorinated TLQP-62 (as highlighted in section 6.3), to gain further insight, prior to commencement of any animal studies.

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Chapter 5: ^{18}F -Labelling of Peptides

Abstract

Positron emission tomography imaging of mice administered with fluorine-18-labelled TLQP-62 could enable further insight into the role of this VGF-derived peptide in glucose homeostasis via determination of which tissue(s) it interacts with (ultimately leading to receptor identification). Furthermore, half-life data would infer whether longer acting analogues are required.

Key “direct” and “indirect” methods for the incorporation of fluorine-18 into peptides have been summarised in this chapter; both traditional and more recent approaches (such as use of bio-orthogonal chemistry for the attachment of a pre-fluorinated prosthetic group and radical chemistry for direct fluorination of unmodified peptides) have been discussed. It is hoped that in the future, a suitable technique will be successfully applied to TLQP-62.

5.1 Introduction

5.1.1 Fluorination of TLQP-62

Evidence suggests that the VGF-derived peptide TLQP-62 could function as an important regulator of glucose homeostasis.^{70,80,94,96,97} It has been hypothesised that this peptide functions as an insulin secretagogue via direct interaction with pancreatic islet β cells.⁷⁰ However, a receptor is yet to be identified and further understanding of the biological role of this peptide, particularly in relation to DM, is required. Further insight into which tissue(s) TLQP-62 interacts with, ultimately leading to receptor identification, would be highly impactful. Furthermore, half-life data indicating whether TLQP-62 reaches the tissue(s) in a pharmacologically active concentration would infer whether longer acting analogues are required. Synthesis of a fluorinated analogue of TLQP-62 using rapid and site-selective chemistry has the potential to yield an effective *in vivo* imaging agent.

5.1.2 Fluorination of peptides: pharmaceuticals

In comparison to other small molecules, such as proteins and antibodies, peptides represent a unique class of pharmaceutical compounds attributed to their distinct biochemical and therapeutic characteristics.³⁴ In addition to maintaining minimal immunogenicity and toxicity,

peptides generally excel in selectivity and can be chemically modified to improve their plasma half-life and pharmacokinetic profile.^{34,35,123} For example, unlike monoclonal antibodies, peptide-based drugs can typically permeate the cell membrane to reach intracellular targets.^{34,35} Furthermore, their synthesis can be carried out relatively straightforwardly due to advancements in chemical synthesis techniques. In light of this, peptide-therapeutics has become a leading industry; more than 60 peptide drugs have been approved by the US Food and Drug Administration and at least 400 are in global clinical development.¹²³

There is demand to explore and exploit fluorination of peptides with the non-radioactive fluorine-19 (¹⁹F) isotope in order to deliver safer, more effective therapeutics with enhanced properties.¹²⁴ Fluorine is a favorable atom in drug development due to its physical properties including small van der Waals radius (1.47 Å), high electronegativity, and ability to form a relatively strong bond with carbon vs the carbon-hydrogen bond (C–F bond energy is 112 kcal/mol; C–H bond energy is 98 kcal/mol).¹²⁵

5.1.3 Fluorination of peptides: *in vivo* imaging

Positron emission tomography (PET) is a sensitive, non-invasive imaging technique that produces three-dimensional images showing the *in vivo* concentration of radiolabelled molecules in tissues.¹²⁶ Positron emitting isotopes such as carbon-11, nitrogen-13, and oxygen-15 can replace their non-radioactive counterparts in target compounds to produce tracers for PET imaging that are chemically identical to the original molecules and have the same pharmacological properties.¹²⁷ Due to its favourable nuclear properties and half-life (109.8 minutes), fluorine-18 (¹⁸F) is the most frequently used PET radionuclide.^{128,129}

Magnetic resonance imaging (MRI) is a further non-invasive clinical diagnostic tool that provides high-resolution images.¹³⁰ MRI primarily involves detection of the proton NMR signal of endogenous water.¹³¹ Generation of contrast via the addition of an imaging probe (termed a “contrast agent”) is often required, and the development of those that can specifically target various biomarkers has recently been focussed on.¹³⁰ ¹⁹F-labelled contrast agents are well established, and are effective due to the fact that ¹⁹F has an alternative magnetic resonance frequency to ¹H.

Effective imaging probes have an optimal biological half-life, participate in specific, high affinity binding with the target, exhibit rapid clearance from the blood (and non-targeted

tissues) and are safe and non-toxic.^{123,132,129} Peptides are increasingly being considered for use as PET imaging probes; ^{18}F -labelling enables visualisation of these biomolecules *in vivo*.^{123,133} Peptides have also been explored for use as MRI contrast agents, with the successful targeting and labelling of over-expressed receptors on diseased cells being the ultimate aim.^{130,131}

5.1.4 ^{18}F -labelling of peptides

^{18}F -fluorine gas produced in the cyclotron via bombardment of $^{18}\text{O}_2$ can be directly used for electrophilic fluorination.¹²⁵ However, because of the extremely dangerous properties of molecular fluorine (highly toxic, strong oxidant with little or no specificity, potential runaway free-radical reactions), conversion into less reactive and more selective fluorination reagents such as *N*-[^{18}F]-fluorobenzenesulfonimide ([^{18}F]NFSI) **96** and ^{18}F -SelectfluorTM **97** is preferred (Figure 51).^{134,135} Due to the relative ease of ^{18}F -fluoride production vs ^{18}F -fluorine gas (and its derivatives), alongside its high specific activity (SA) (radioactivity/mol), use of this nucleophilic species (or $\text{K}[^{18}\text{F}]\text{F}$ /Kryptofix[®] 2.2.2 ($\text{K}_{2.2.2}$) **98**; a source of ^{18}F -fluoride (Figure 52)) to effect ^{18}F -labelling is the most favoured approach.^{125,129,136}

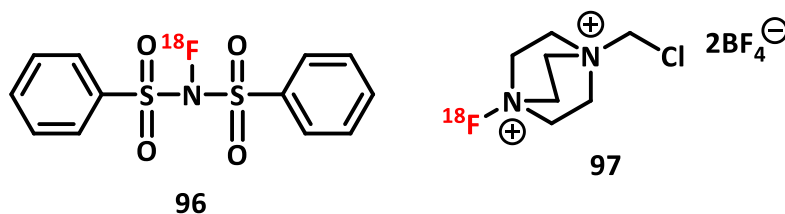


Figure 51: [^{18}F]NFSI (LHS) and SelectfluorTM (RHS).

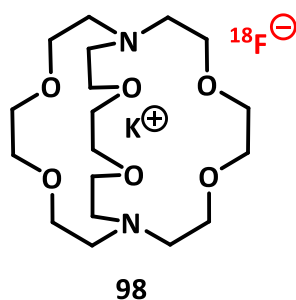


Figure 52: $\text{K}[^{18}\text{F}]\text{F}$ / $\text{K}_{2.2.2}$.

5.1.4.1 ^{18}F -fluoride production

^{18}F -fluoride is produced in a cyclotron primarily by ^1H irradiation of H_2^{18}O .¹²⁵ In aqueous solution ^{18}F -fluoride forms hydrogen bonds with the surrounding water molecules and hence

is not labile to nucleophilic reactions.¹²⁹ Therefore, the reactivity of ^{18}F -fluoride is enhanced by the addition of a cationic counter ion prior to the removal of the water; addition of base (e.g. potassium carbonate) is carried out followed by treatment with a ligand (such as $\text{K}_2.2.2$) in advance of azeotropic distillation of the fluoride salt with acetonitrile or microwave heating.¹³⁵

5.1.4.2 ^{18}F -labelling

The relatively short half-life of ^{18}F imposes limitations on the time frame to achieve fluorination; a narrower range of appropriate methods are available for use in comparison to ^{19}F -labelling.¹³⁷ All manipulations for the synthesis of ^{18}F -labelled peptides (including purification) must be completed in approximately 1–2 hours to ensure sufficient active material for use.¹³⁴ Ideally, no final HPLC purification step should be required for the formulation of the final ^{18}F -labelled peptide.¹³⁸

^{18}F -labelling is typically carried out through the “direct”, late-stage incorporation of ^{18}F into a peptide (pre-modified with a prosthetic group) via $\text{C-}^{18}\text{F}$, $\text{B-}^{18}\text{F}$ or $\text{Si-}^{18}\text{F}$ bond formation or via $\text{Al-}^{18}\text{F}$ chelation, or via “indirect” methods where the synthesis of ^{18}F -labelled prosthetic groups is carried out in advance of peptide conjugation (Figure 53).^{137,139} Additionally, recent efforts have been focussed on the use of trifluoromethylation and radical chemistry to directly label unmodified peptides.^{140–142}

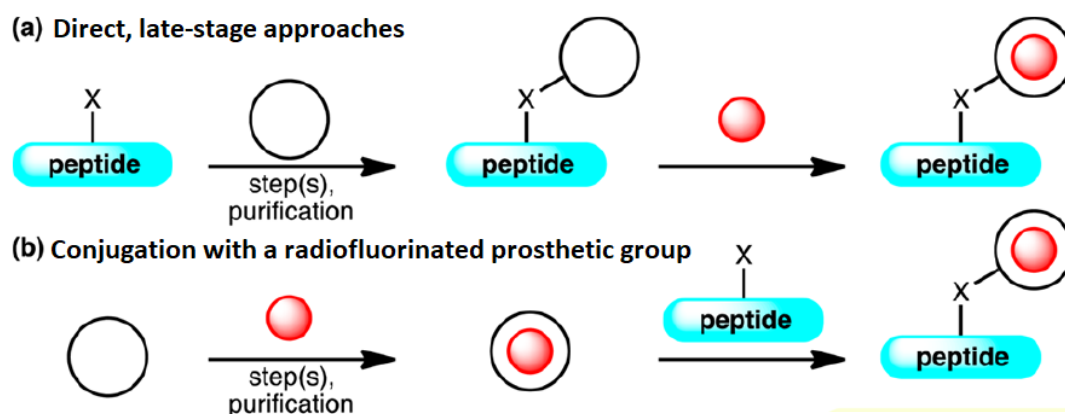


Figure 53: Traditional strategies for peptide ^{18}F -labelling: **a)** indicates direct ^{18}F -labelling of a peptide pre-modified with a prosthetic group; **b)** indicates synthesis of an ^{18}F -labelled prosthetic group in advance of peptide conjugation. *Adapted from Hooker and co-workers.*¹³⁹

Direct approaches typically require the use of harsh reaction conditions such as extremes of temperature and/or pH which are not compatible with many sensitive biomolecules.^{137,143} High reaction temperatures and activating reagents (i.e. strong bases and $K_2.2.2$) can lead to the formation of by-products which require separation from the desired ^{18}F -labelled product.¹⁴⁴ Furthermore, strict anhydrous conditions are also often necessary due to the aforementioned limited nucleophilicity of ^{18}F -fluoride in aqueous solution.¹⁴⁵ Whilst use of secondary ^{18}F -labelling agents generally enables circumvention of the challenging conditions associated with direct approaches, laborious multistep procedures are often required for prosthetic group radiosynthesis.¹²⁶ Consequently, such ^{18}F -labelling reactions are associated with an eroded radiochemical yield (RCY) and SA (typically $\sim 1 \text{ Ci}/\mu\text{mol}$).^{146,147} Whichever approach is taken, the introduction of major structural modifications to the peptide is unfavourable, due to the potential implications on biological activity.^{132,139}

5.2 Site-selective, direct, late-stage ^{18}F -labelling methodologies

Traditional direct methods are those in which ^{18}F -fluoride is reacted directly with the peptide, which has been previously modified with a suitable prosthetic group to facilitate site-selective labelling.¹²⁵ It is possible to introduce ^{18}F in the penultimate step of a radiosynthesis (in advance of purification).¹³⁷

5.2.1 Carbon- ^{18}F bond formation

^{18}F labelling of peptides is traditionally carried out via nucleophilic aromatic substitution reactions.¹²⁵ However, alternative approaches involving the introduction of a carbon- ^{18}F bond into aliphatic precursor groups also exist.

5.2.1.1 Nucleophilic aromatic substitution

^{18}F nucleophilic aromatic substitution requires sufficient activation of the phenyl ring, which can be achieved by an electron withdrawing group (EWG) (such as $-\text{CN}$ or $-\text{CF}_3$) in the ortho or para position to the leaving group.¹²⁵

In 2009, Kocsch and co-workers published methodology for the ^{18}F -labelling of peptides modified with a trimethylammonium (TMA) leaving group-containing activated aromatic ring **99** (Figure 54).¹⁴⁸ A series of model compounds containing different electron-withdrawing substituents and a TMA leaving group were synthesised. The optimal model compound, 2-

cyano-4-(methoxycarbonyl)-*N,N,N*-trimethylbenzenaminium triflate, was subsequently converted to the corresponding acid (4-carboxy-2-cyano-*N,N,N*-trimethylbenzenaminium triflate). This carboxylic acid was coupled to four model tetrapeptides (differing by one amino acid residue; inclusion of Arg, Phe, Lys or methionine (Met, M)) and two bombesin peptides (**101** and **102** in Figure 55), followed by ^{18}F -labelling in dimethyl sulfoxide (DMSO) under relatively mild conditions (50-90 °C, pH<8).

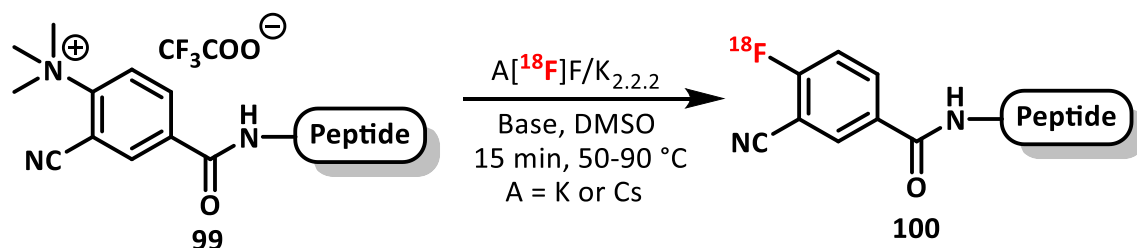


Figure 54: Nucleophilic aromatic substitution at a 4-TMA-3-CN substituted arene moiety resulting in ^{18}F -labelling of a peptide of interest.¹⁴⁸

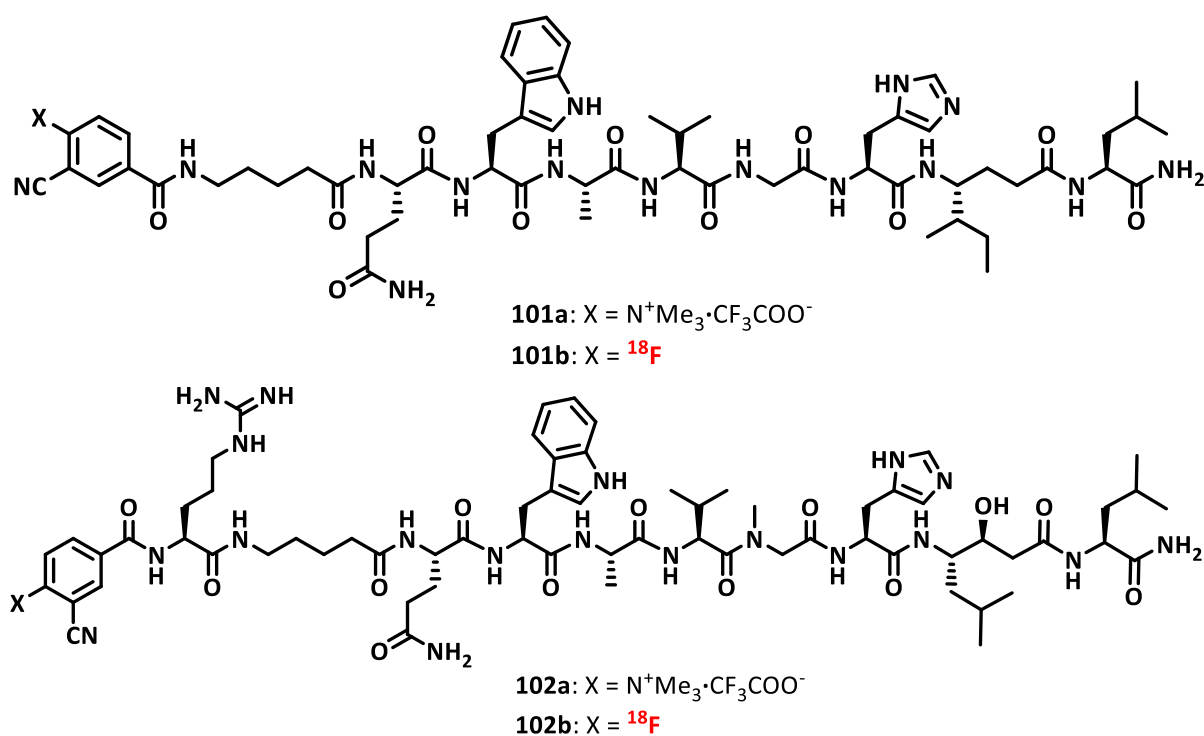


Figure 55: Bombesin peptide **101** (**101a**; 3-cyano-4-fluorobenzoyl-Ava-Gln-Trp-Ala-Val-Gly-His-FA01010-Leu-NH₂) and **102** (**102a**; 3-cyano-4-fluorobenzoyl-Arg-Ava-Gln-Trp-Ala-Val-N(CH₃)Gly-His-Sta-Leu-NH₂); the RCC for **101b** and **102b** was 70% and 51%, respectively (70 °C, caesium carbonate, 15 minutes, DMSO).¹⁴⁸

Inclusion of a 3-cyano-activating group in the aromatic ring increased the leaving potential of the TMA group, thereby resulting in faster nucleophilic aromatic substitution (15-minute reaction time) and a lower temperature requirement, whilst maintaining good conversion yields.¹⁴⁸ Optimisation of temperature and base type resulted in a high percentage of ¹⁸F-incorporation for all peptides except one; the Lys-containing tetrapeptide (>80% radiochemical conversion (RCC) for all model tetrapeptides; 70% (n = 3) and 51% (n = 2) RCC for bombesin peptides **101b** and **102b**, respectively). Furthermore, the reported RCY (decay corrected) and SA for **101b** and **102b** was ~20% and ~2 Ci/μmol respectively) after HPLC purification. For the Lys-peptide, only moderate ¹⁸F-incorporation was achieved (24%) even at elevated temperature, though this was considered as a success due to the nucleophilicity of the free Lys R group. It was also concluded that peptides containing unprotected His, Trp, and Arg (typically difficult to apply direct ¹⁸F-labelling chemistry to) are amenable to this approach.

In their 2011 publication, Chen and co-workers present a new, single step method for the direct ¹⁸F radiolabelling of monomeric and dimeric cyclic Arg-Gly-Asp-D-Phe-Lys (RGDfK) peptide derivatives at 4-nitro-3-trifluoro-methyl arene sites.¹⁴⁹ One of the most recognised peptide motifs that bind to the α_vβ₃ receptor, which is found to be overexpressed in many cancer cell lines, contains the amino acid sequence Arg-Gly-Asp.¹⁵⁰ Therefore, this sequence has potential as a diagnostic agent. As indicated in Figure 56, peptide sequences **103**, having previously been modified with an arene moiety, were mixed with dried K[¹⁸F]F.K_{2.2.2} in DMSO then heated for 3.5 minutes at 130 °C to effect nucleophilic aromatic substitution. As in the aforementioned approach published by Kokschi and co-workers, incorporation of an EWG in the ortho position (in this case a trifluoromethyl substituent) results in activation of the aromatic ring.¹⁴⁸

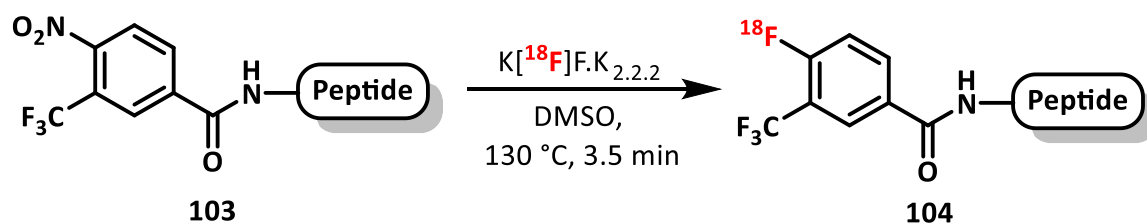


Figure 56: Nucleophilic aromatic substitution at a 4-NO₂-3-CF₃-substituted arene moiety resulting in ¹⁸F-labelling.¹⁴⁹ Peptide refers to monomeric or dimeric cyclic RGDfK.

The monomeric ^{18}F -labelled RGD peptide was achieved in a RCY (decay corrected) of 19% ($n = 6$) and the dimeric equivalent in a value of 9% ($n = 4$).¹⁴⁹ Both peptides had a radiochemical purity (RCP) of >99% and SA of 2.1 Ci/ μmol after HPLC purification (overall radiosynthesis time, including purification, was 40 minutes). These results, as well as the relatively short reaction time and ability to use a low amount of precursor, were attributed to use of the 4- NO_2 -3- CF_3 arene group. One disadvantage of this approach is the difficulty of separating the nitro-containing peptide precursor from the desired ^{18}F -labelled product. Furthermore, this method would not be expected to be generally applicable due to the requirement for a high temperature and basic conditions.¹²⁵

Kuhnast and co-workers have recently developed a generic tag that can be conjugated to a cyclic peptide of interest in advance of one-step/late-stage ^{18}F -labelling at moderate temperature (<40 °C) under basic conditions.¹⁵¹ Their approach exploits the reactivity of a pyridine-derivative ring **105** in the presence of a suitable EWG, as highlighted in Figure 57.

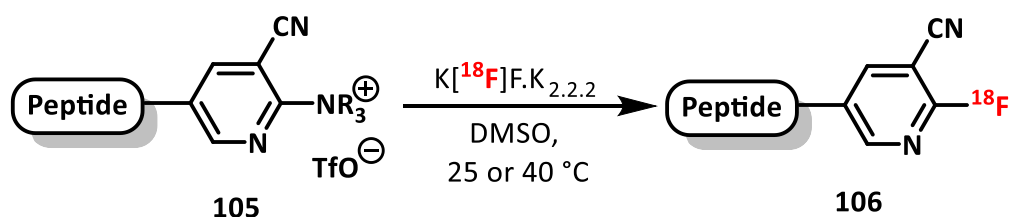


Figure 57: Nucleophilic aromatic substitution at a 4-ammonium (NR_3^+)-3-CN-substituted pyridine moiety resulting in ^{18}F -labelling.¹⁵¹ Peptide refers to cyclic RGDfK or glutathione.

Nitrile, ester (ethyl and benzyl), a combination of nitrile and ester, amide (benzyl), *N*-hydroxysuccinimide (NHS) **107** (Figure 58) and tetrafluorophenyl (TFP) activated esters were initially investigated as EWGs; the performance of each precursor toward ^{18}F -labelling was evaluated at 40 °C and room temperature.¹⁵¹ Nitriles and esters, or combination of both, gave the highest conversions reaching more than 90% even at room temperature after 15 min ($n = 3$). Activated esters NHS and TFP gave the lowest RCCs because of their relative instability under the radiofluorination conditions.¹⁵²

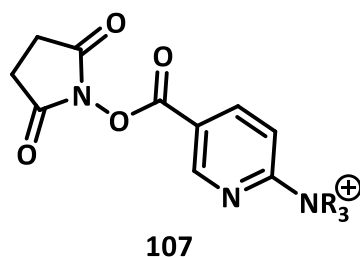


Figure 58: The NHS activated ester pyridine-derivative precursor utilised by Kuhnast and co-workers.¹⁵¹

Synthesis of a generic cyanopyridine tag **108** based on the nitrile precursor was then carried out in advance of coupling to cyclic RGDfK and glutathione (Figure 59).¹⁵¹ ¹⁸F-labelling was effected via treatment with K[¹⁸F]F.K_{2.2.2} in DMSO at 40 °C, as highlighted in Figure 59. RCYs (decay corrected) of 42% and 55% respectively were achieved for HPLC-purified cyclic RGDfK and glutathione.

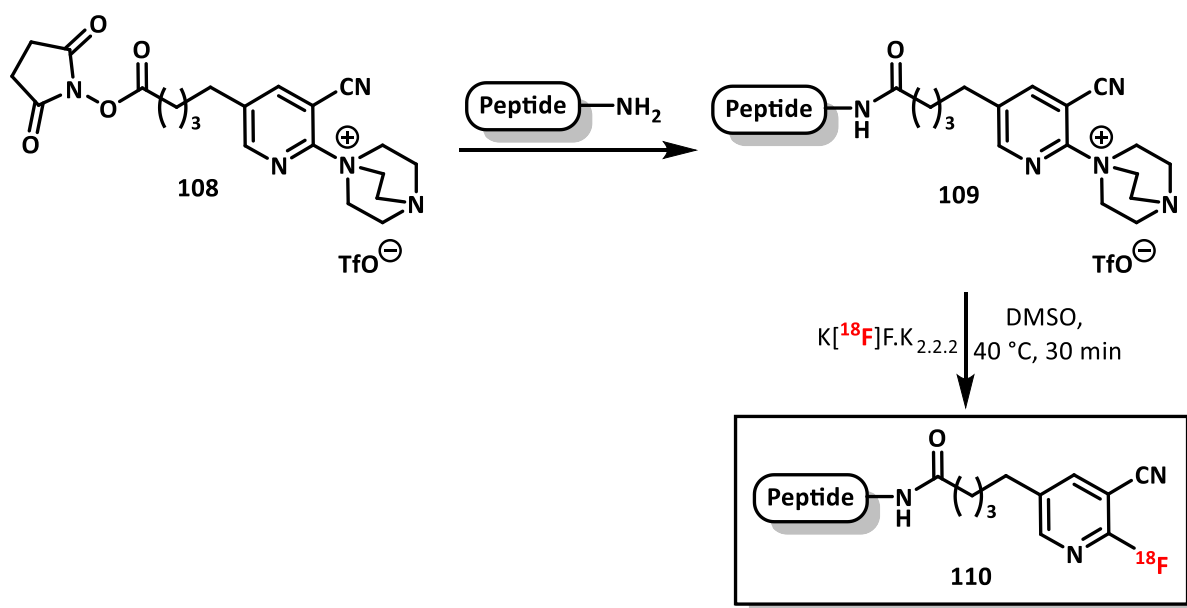


Figure 59: Schematic of late-stage ¹⁸F-labelling of a peptide of interest (cyclic RGDfK or glutathione); coupling of the peptide to a cyanopyridine tag **108** to form an activated precursor compound **109** followed by ¹⁸F-labelling at 40 °C in DMSO to form **110**.¹⁵¹

5.2.1.2 Aliphatic carbon-¹⁸F bond formation

Ametamey and co-workers successfully applied the one-step ¹⁸F-labelling via activated aziridine ring-opening to four model compounds (**111-114**) and two peptides of interest (a tetrapeptide and an octapeptide).¹²⁷ The reaction of ¹⁸F-fluoride was investigated using standard basic fluorination conditions and DMSO as solvent, as shown in Figures 60 and 61.

The influence of different activating groups (4-(CH₃)C₆H₄SO₂ **111**, 2,4,6-(iPr)₃C₆H₃SO₂ **112**, 3,4-(CH₃O)₂C₆H₃SO₂ **113** or benzoyl **114** (Figure 60)) and reaction temperature were initially considered for the model compounds. Moderate to high conversions were achieved for all *N*-sulfonated aziridines under mild labelling conditions (<70 °C, >54% RCC). The 2,4,6-triisopropylphenylsulfonyl activating moiety was identified as the optimal group to facilitate regioselective aziridine ring-opening with ¹⁸F-fluoride; the corresponding acid derivative of the 2,4,6-triisopropylphenylsulfonyl activated aziridine was selected for coupling to target peptides.¹³⁷ Temperature, base and solvent were investigated, however the conversion was rather low for both peptides (tetrapeptide: 16% RCC (90 °C, caesium carbonate; octapeptide: 7% RCC (70 °C, caesium carbonate)).¹⁴⁹

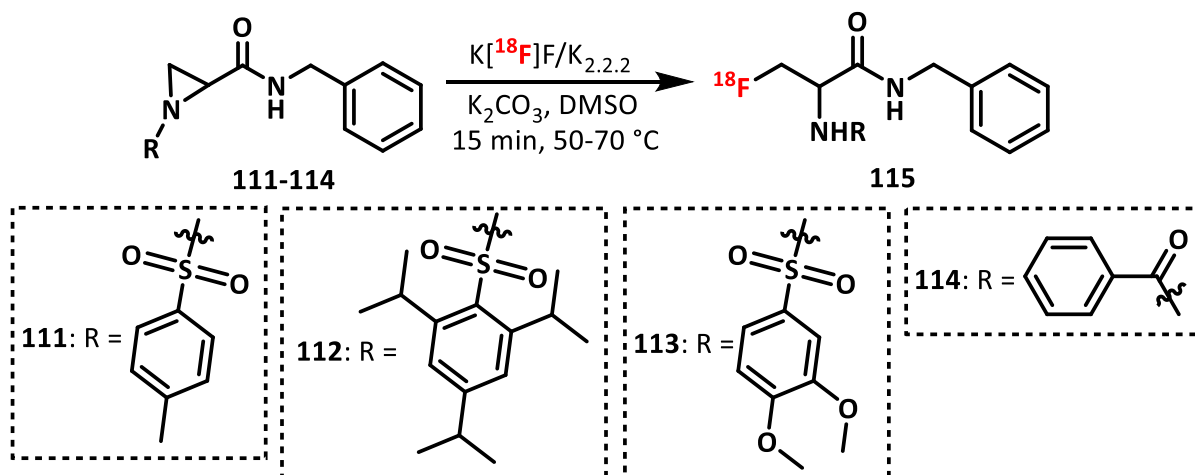


Figure 60: Regioselective nucleophilic aliphatic substitution of model compounds via activated aziridine ring-opening to form an ¹⁸F-labelled 3-fluoro-2-amino-amide.¹²⁷

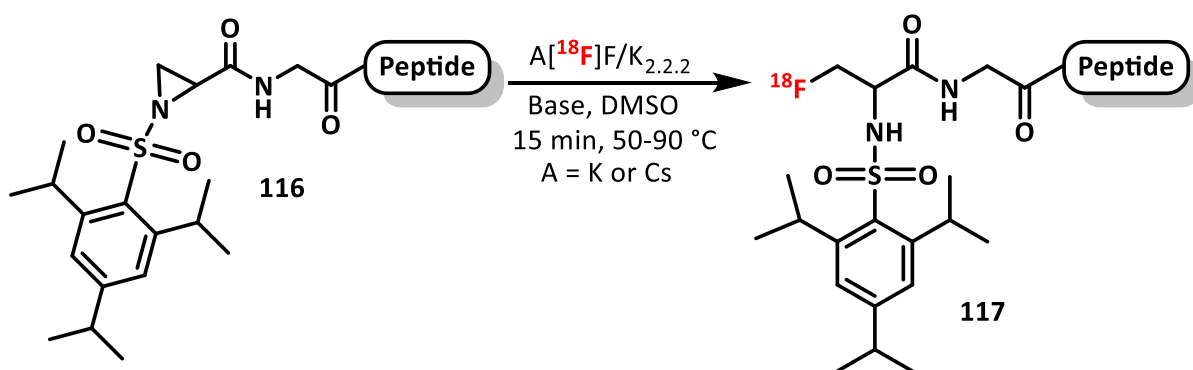


Figure 61: Regioselective nucleophilic aliphatic substitution of peptides of interest via activated aziridine ring-opening to form an ¹⁸F-labelled 3-fluoro-2-amino-amide.¹²⁷

The fluorinase enzyme, originally isolated from *Streptomyces cattleya*, catalyses the reaction between *S*-adenosyl-L-Met (AdoMet) **118** and ¹⁸F-fluoride to form 5'-fluoro-5'-

deoxyadenosine (FDA) **120** and L-Met, as highlighted in Figure 62.¹⁵³ The discovery and characterisation of four naturally occurring fluorinase enzymes offers scope for using biocatalysts to effect C-¹⁸F bond formation via nucleophilic substitution.¹⁵⁴

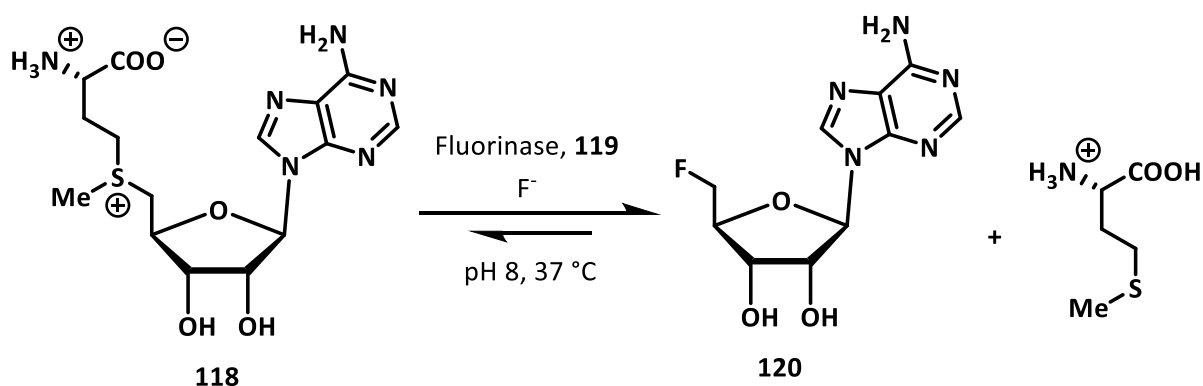


Figure 62: Transformation of AdoMet to FDA and L-Met via a fluorinase enzyme.¹⁵³

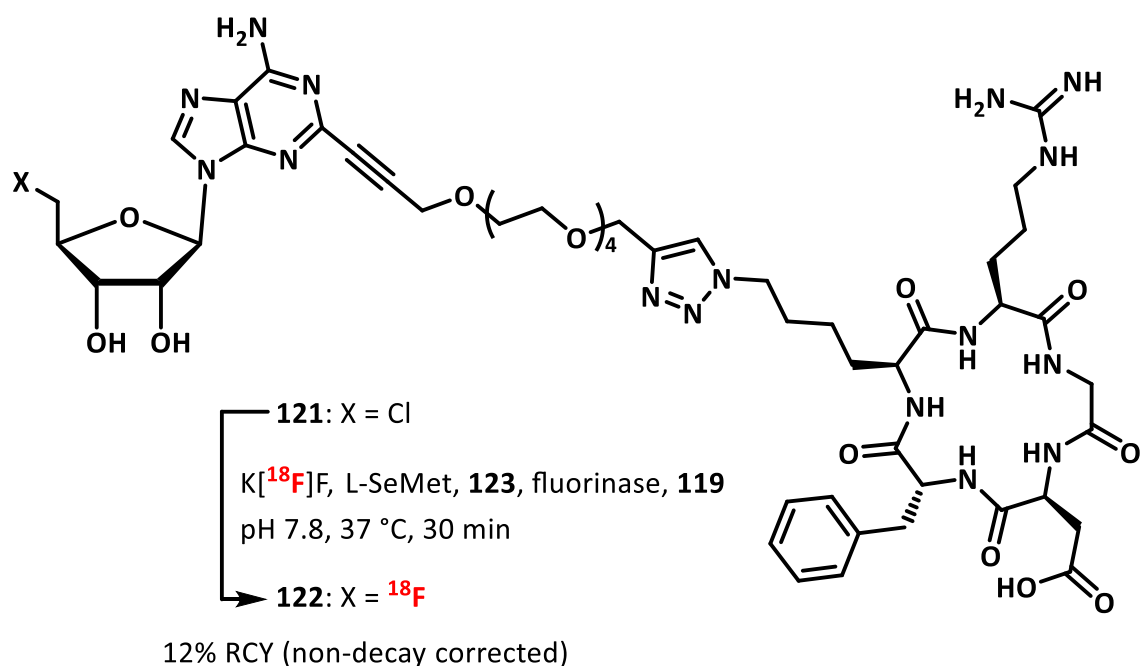


Figure 63: Fluorinase-catalysed ¹⁸F-labelling of the CIDEA-TEG-RGDfK substrate **121** in the presence of L-SeMet under mild conditions.¹³⁷

O'Hagen and co-workers recently published an approach for the fluorinase enzyme-facilitated ¹⁸F-labelling of cyclic RGDfK; use of aqueous ¹⁸F-fluoride (in the absence of base or K_{2.2.2}) was possible.¹⁵³ Following initial confirmation that modification of the carbon-2 position of the adenine ring with an acetylene moiety did not hinder the enzyme-catalysed transhalogenation reaction, 5'-chloro-5'-deoxy-2-ethynyladenosine (CIDEA)-tetraethylene glycol (TEG)-RGDfK **121** (Figure 63) emerged as a synthetic target. **121** was subsequently

incubated with fluorinase in the presence of L-selenoMet (L-SeMet) and ^{18}F -fluoride for 30 minutes under mild conditions (pH 7.8, 37 °C) to afford the ^{18}F -labelled peptide **122** (12% RCY of final formulated product (non-decay corrected)).¹³⁷

Several main group inorganic elements form a stronger bond with fluorine than carbon; in the corresponding diatomic molecule the bond dissociation energy (kJ/mol) of carbon-fluoride is 513.8 ± 10 , of boron-fluoride is 732, of silicon-fluoride is 576.4 ± 17 and of aluminium-fluoride is 675.¹³⁵ Consequently, various approaches exist for the ^{18}F -labelling of modified peptides via boron, silicon or aluminium-fluoride bond formation.¹⁵¹

5.2.2 Boron- ^{18}F bond formation

The strength of the boron-fluoride bond has led to the investigation of organotrifluoroborates as substrates for ^{18}F -labelling approaches.¹⁵⁵

As highlighted in Figure 64, Perrin and co-workers demonstrated how the rapid, direct radiolabelling of an arylborimidine-containing derivative of dimeric cyclic RGDfK (bisRGDfK) **124** can be carried out in a single step under aqueous, low temperature (30 °C) conditions using concentrated (but not dried) aqueous K^{18}F ; drying lowers the SA of ^{18}F -fluoride.^{156,157} Conjugation of bisRGDfK to a diaminonaphthalene-protected arylboronic acid followed by reaction with K^{18}F over 15 minutes results in formation of an ^{18}F -labelled aryltrifluoroborate anion **125** (Figure 64). The total radiosynthesis time was ~45 minutes. Whilst competitive RCYs (non-decay corrected) and RCPs (~10% ($n = 3$) and >98% respectively) were recorded, presence of unlabelled bisRGDfK- $\text{ArB}(\text{OH})_2$ (resulting from acidolysis of diaminonaphthalene), hindered HPLC purification of the ^{18}F -labelled product. Consequently, a relatively low minimum SA of 0.3 Ci/ μmol was determined (although based on previous studies it was suggested that this value could be improved by use of higher activity ^{18}F -fluoride).^{147,157}

Conjugation of monomeric cyclic RGDfK to a tetraphenylpinacolate boronate ester **126** (Figure 65), followed by conversion to the ^{19}F -labelled aryltrifluoroborate anion **127** (via treatment with ^{19}F - KHF_2) and then ^{19}F to ^{18}F isotope exchange (IEX) (20 minutes, pH 2, 40 °C) in the presence of concentrated (not dried) aqueous ^{18}F -fluoride (Figure 66) has been carried out by Perrin and co-workers.¹⁴⁷ This approach resulted in a much higher SA (~14 Ci/ μmol ($n = 3$)) and RCY (65% (decay corrected)), after HPLC purification to that reported by Perrin and

co-workers, and the reported RCP was 100%.¹⁵⁶ The total radiosynthesis time was 30-35 minutes.

Whilst use of $^{18}\text{F}\text{-ArBF}_3^-$ for the ^{18}F -labelling of cyclic RGDfK has yielded promising results, it is likely that this method will not be suitable in the case of acid-sensitive peptides. Approaches for the direct ^{18}F -labelling of aluminium chelates, as highlighted in section 5.2.3, also are dependent on acidic conditions.

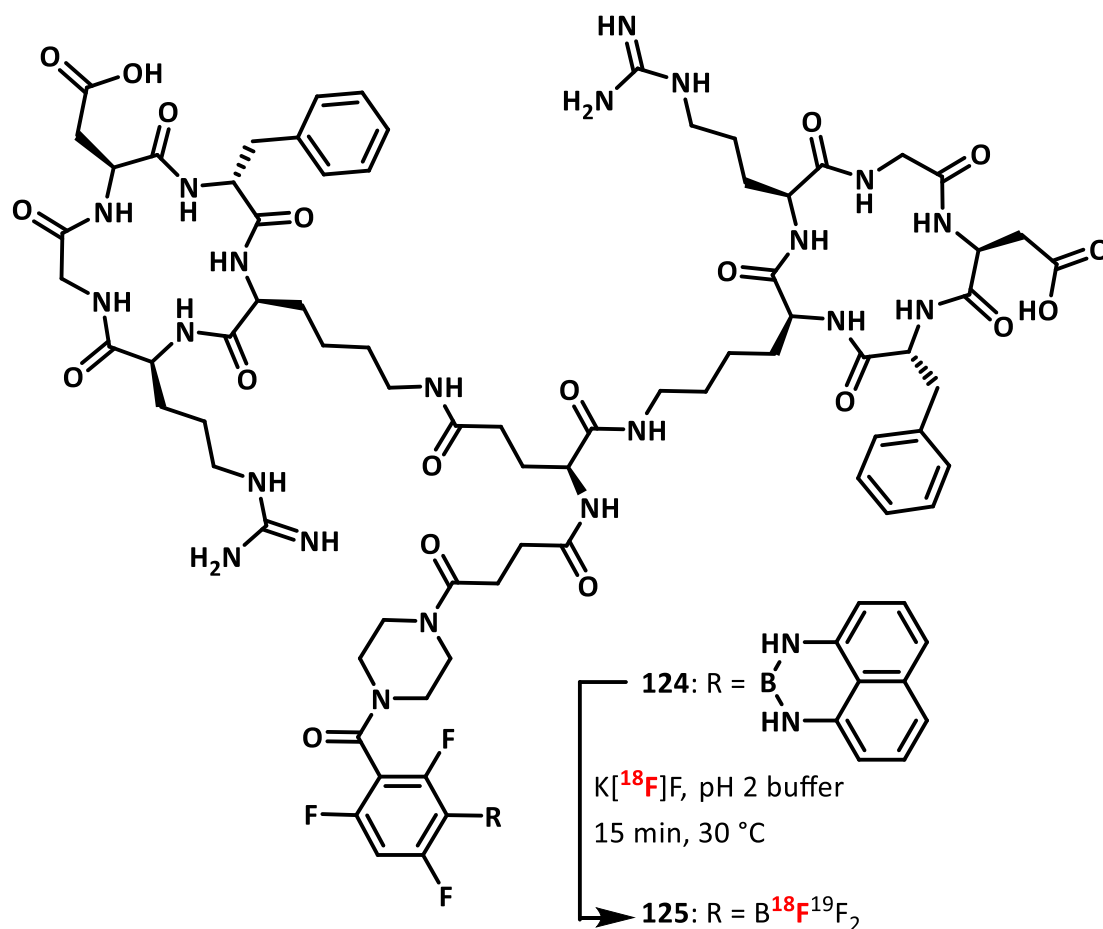
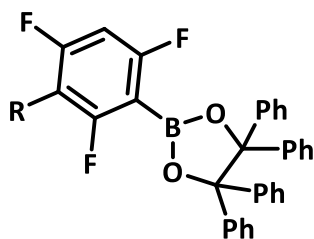


Figure 64: One-step aqueous ^{18}F -labelling of diaminonaphthalene-protected arylboronimide conjugated to dimeric cyclic RGDfK **124** resulting in formation of $^{18}\text{F}\text{-ArBF}_3^-$ **125**.¹⁵⁶



126

Figure 65: The tetraphenylpinacolate boronate ester utilised by Perrin and co-workers; subsequent conversion to the corresponding ^{19}F -labelled aryltrifluoroborate anion then ^{19}F to ^{18}F IEX in the presence of ^{18}F -fluoride results in ^{18}F -labelling.¹⁴⁷ R represents conjugated monomeric cyclic RGDfK.

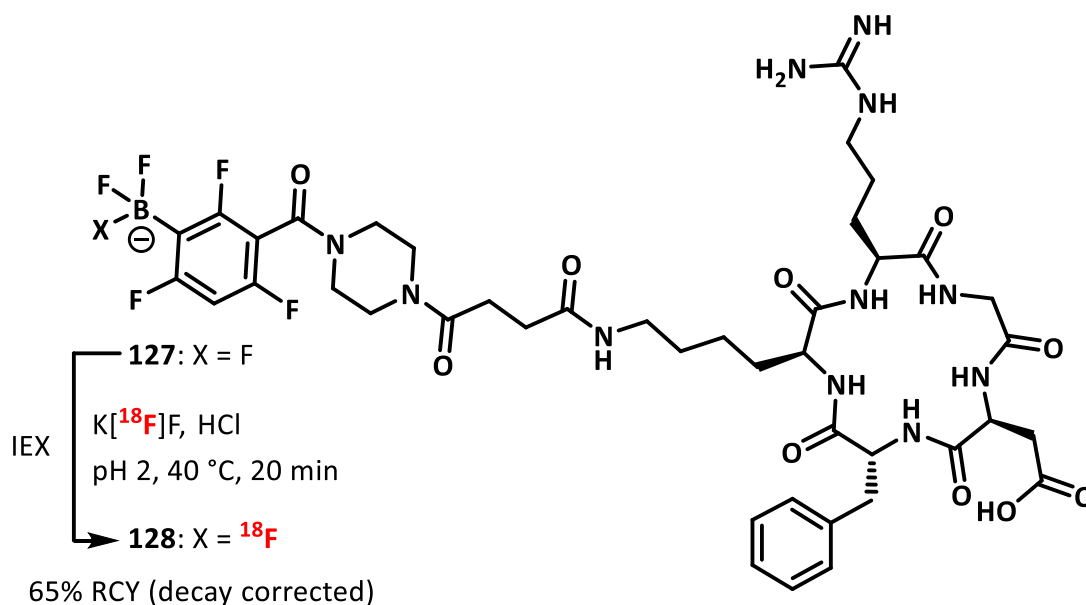


Figure 66: ^{19}F to ^{18}F IEX, resulting in ^{18}F -labelling of monomeric cyclic RGDfK under acidic conditions.¹³⁷

5.2.3 Aluminium complex- ^{18}F bond formation

Formation of stable ^{18}F -aluminium complexes with polyaminocarboxylates enables the ^{18}F -labelling of peptides that are conjugated to a chelating agent.¹³⁵ Introduction of an aluminium fluoride species in this manner exploits the strong binding of fluorine to aluminium and allows ^{18}F -labelling via numerous chelating systems.¹⁴³

In 2009, Goldenberg and co-workers published the first study related to use of a macrocyclic hexadentate chelator for rapid ^{18}F -labelling of peptides under aqueous conditions.¹⁵⁸ The group employed the use of a derivatised 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) chelator; an elevated reaction temperature of 100 °C was required to enable efficient

complexation of aluminium monofluoride at pH 4. ^{18}F -labelling of a NOTA-di-(histamine-succinyl-glycine (HSG)) hapten-peptide (termed “IMP 449”; NOTA-*p*-Bn-CS-D-Ala-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH₂) **129** (Figure 67) was carried out using this approach over 15 minutes; RCYs (non-decay corrected) of 5-20% and SAs of 0.5-1.3 Ci/ μmol after HPLC purification were recorded. The purified ^{18}F -labelled peptide **130** was produced in the absence of an azeotropic drying step within 60 minutes and was stable in serum at 37 °C for 4 hours, thereby suggesting scope for use as an imaging agent *in vivo*.

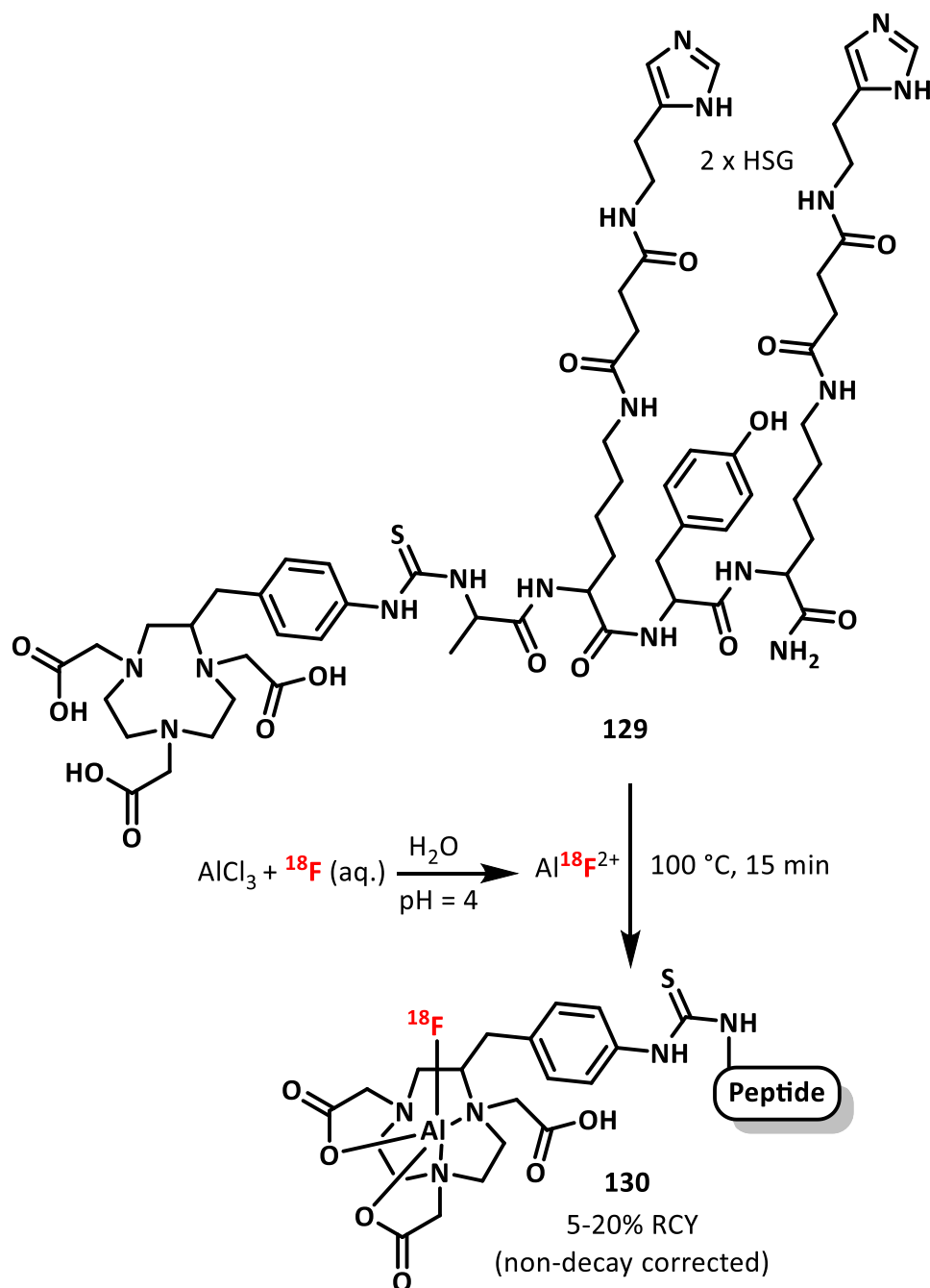


Figure 67: ^{18}F -labelling of IMP 449 via complexation of $(\text{Al}^{18}\text{F})^{2+}$.¹³⁷

Use of NOTA as a pentadentate chelator was subsequently investigated by Boerman and co-workers.¹⁵⁹ In their 2010 publication, they present a method for the ^{18}F -labelling of the somatostatin analogue octreotide based on the chelation of aqueous aluminium fluoride by NOTA. Various buffers and peptide concentrations were tested to optimise the ^{18}F -labelling efficiency; a RCY (non-decay corrected) and SA of 50% and 1.2 Ci/ μmol respectively were achieved in sodium acetate buffer (100 °C, pH 4.1, 15 minutes). The labelled product was purified by HPLC to remove unbound aluminium monofluoride and unlabelled peptide. The total radiosynthesis time was 45 minutes. Adding a solvent, such as ethanol or acetonitrile, increased the labelling yield to >97%.¹⁶⁰ Furthermore, cyclic dimeric Arg-Gly-Asp-D-Tyr-Lys (bisRGDyK) with mini-PEG-ylation (termed “PRGD2”)-NOTA **131** (Figure 68) was directly ^{18}F -labelled over 10 minutes in RCYs (non-decay corrected) of 5-25% at 100 °C at pH 3-4 by chelation of aqueous aluminum- ^{18}F -fluoride generated *in situ*.¹⁶¹ However, in these studies, carried out by Chen and co-workers, ^{18}F -Al-NOTA-PRGD2 could not be separated from unlabelled PRGD2-NOTA via HPLC, thereby reducing the SA.¹⁵⁶

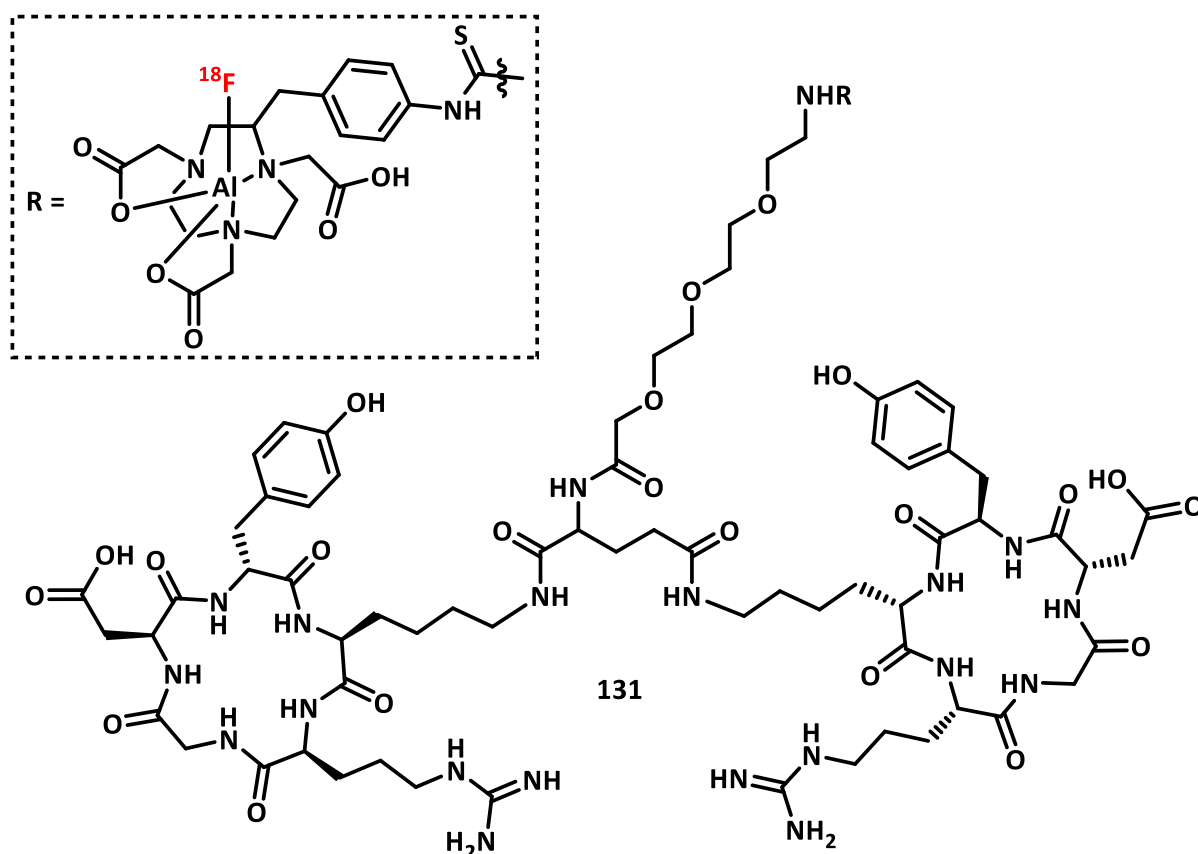


Figure 68: PRGD2-NOTA that has been ^{18}F -labelled via complexation with aqueous $(\text{Al}^{18\text{F}})^{2+}$.¹⁶¹

A short analogue of the VGF-derived peptide TLQP-21, modified at the N-terminus by 1,4,7 triazacyclononane-1,4-diacetic acid (NODA)-methyl phenylacetic acid (NODA-MPAA) **132** (Figure 69) (NODA- $\beta\alpha\beta$ ARRRHFFHALPPAR), has successfully been ^{18}F -labelled using aqueous $(\text{Al}^{18}\text{F})^{2+}$ by Todde and co-workers for biodistribution studies.¹⁴⁶ The aforementioned Goldenberg and co-workers methodology was adopted; a RCY (non-decay corrected) and RCP of 34% and ~98% respectively were reported for the HPLC-purified product.¹⁵⁸ The total radiosynthesis time was 30 minutes. Furthermore, a prototype kit containing a NODA-MPAA-di-HSG hapten-peptide (termed “IMP 485; NODA-MPAA-D-Lys(HSG)-D-Tyr-D-Lys(HSG)-NH₂) was generated by Goldenberg and co-workers.¹⁶² For optimal ^{18}F -labelling, the kit was reconstituted with an aqueous solution of aluminium monofluoride and ethanol (1:1), heated at 100–110 °C for 15 minutes (optimal pH was 4) and then rapidly purified via solid-phase extraction (SPE) (purification via HPLC was not necessary).¹³⁷ Al^{18}F -IMP 485 was isolated within 20 minutes; RCYs (non-decay corrected) and SAs of 45–97% and 6 Ci/ μmol respectively were reported, dependent on buffer, solvent and pH.

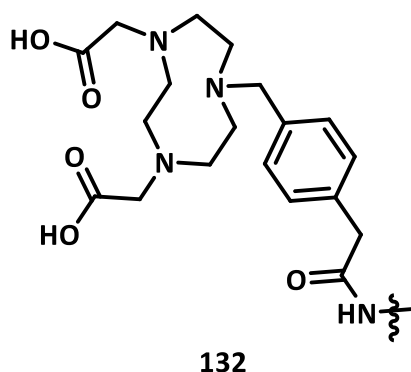


Figure 69: The N-terminus of the truncated VGF-derived peptide TLQP-21; modification of the peptide via conjugation to NODA-MPAA in advance of ^{18}F -labelling via complexation with $(\text{Al}^{18}\text{F})^{2+}$.¹⁴⁶

The aforementioned methodologies for the ^{18}F -labelling of chelator-conjugated molecules can be carried out rapidly and afford relatively good RCYs; ^{18}F -AlF complexation to numerous peptides and proteins that have high affinity for receptors which are overexpressed on tumours has been carried out over recent years.¹³⁵ However, the requirement for high temperatures may limit the scope of application to potential *in vivo* imaging agents.¹²⁵

In 2016, Bormans and co-workers developed a new acyclic polydentate ligand (termed “H₃L3”) for complexation of $(\text{Al}^{18}\text{F})^{2+}$ at 40 °C, as highlighted in Figure 70.¹²⁶ The aim was to

develop a flexible chelator in order to reduce the activation energy (and hence temperature) required for complexation. It was assumed that a more flexible, acyclic structure would be less stable once complexed. Therefore, optimisation of the coordinative set was carried out. An acidic pH (4–4.5) was required for complexation in order to prevent hydrolysis of aluminium fluoride in solution. At this pH, carboxyl groups were more likely to be deprotonated than phenol moieties (pK_a of 3–4 ($R-COOH$) and 9–10 ($Ar-OH$)); use of carboxylate as a donor was associated with increased complexation efficiency. Furthermore, pyridine was prone to exist in a positively charged state (pK_{aH} of 5.2 (tertiary amine)), which hindered complexation. H_3L3 was used for the ^{18}F -labelling of a urea-based inhibitor of the prostate-specific membrane antigen (PSMA) Glu-NH-CO-NH-Lys(Ahx); this ligand was conjugated to the peptide to form **133** in advance of complexation of aqueous $(Al^{18}F)^{2+}$ over 12 minutes (RCY of HPLC-purified product **134** was 25% (non-decay corrected); RCP was 98% and SA was 0.7 Ci/ μ mol).

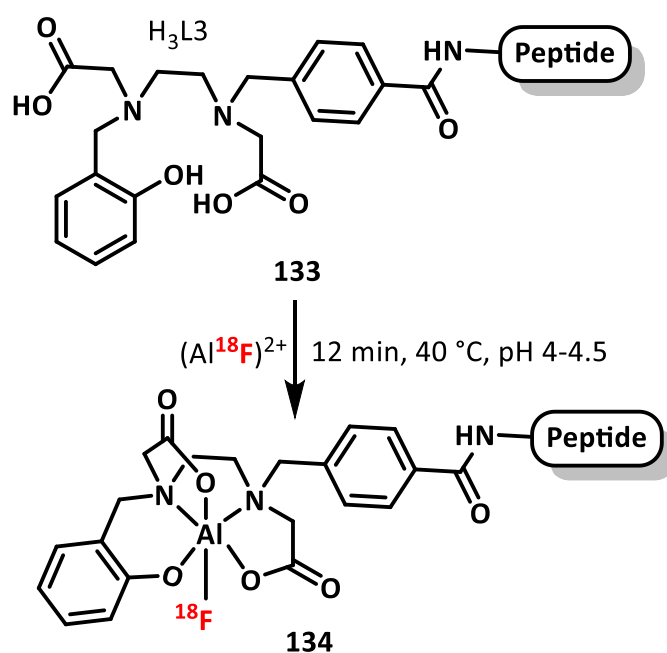


Figure 70: Complexation of aqueous $(Al^{18}F)^{2+}$ to a peptide of interest at 40 °C under acidic conditions using the H_3L3 chelator developed by Bormans and co-workers.¹²⁶

5.2.4 Silicon- ^{18}F bond formation

Silicon- ^{18}F labelling methods have been increasingly exploited in recent years due to their inherent simplicity and efficiency compared to conventional strategies.¹⁴⁴

^{18}F -fluorotrimethylsilane was initially proposed as an ^{18}F -labelling reagent by Rosenthal and co-workers (synthesised in a RCY (decay corrected) of 80%).¹³⁸ However, a preliminary *in vivo* evaluation revealed fast hydrolysis of the compound (due to the highly polar nature of the Si-F bond) followed by bone uptake of free ^{18}F , suggesting an unsuitable labelling reagent.^{135,144} It has since been determined that hydrolysis of ^{18}F -labelled silanes can be significantly reduced by the introduction of bulky substituents (such as *t*Bu groups) to the silicon moiety.¹³⁵

The greater covalent radius of silicon versus carbon contributes to the enhanced propensity of organosilanes to undergo nucleophilic substitutions at the silicon atom compared to their carbon-centered counterparts.¹⁴⁴ Srinivasan and co-workers reported on the direct fluorination of bombesin analogues by nucleophilic displacement of fluoride on alkoxy, hydroxy or hydride leaving groups.¹⁶³ ^{18}F -labelling of a hydrolytically stable, modified di-*t*Bu-silyl-bombesin peptide **135** was effected under acidic conditions via treatment with $\text{K}[^{18}\text{F}]\text{F} \cdot \text{K}_{2.2.2}$ in DMSO at 110 °C, as highlighted in Figure 71. Following HPLC purification, after 20 minutes, a RCY (decay corrected, $n = 4$) of 13% and RCP of >95% was reported. The total radiosynthesis time was 115-155 minutes. Whilst this approach yielded the ^{18}F -labelled bombesin analogue **136** with a high SA (1.7 Ci/ μmol), heating was required for ^{18}F -displacement.¹²⁹

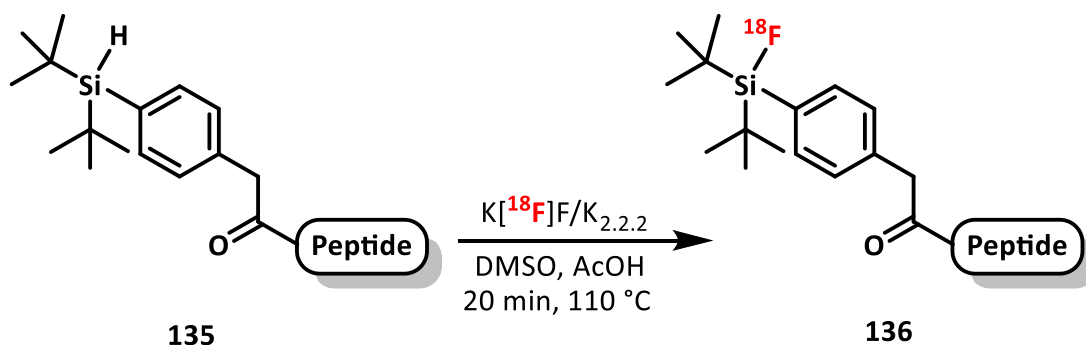


Figure 71: Nucleophilic displacement of ^{18}F -fluoride on a hydride leaving group; ^{18}F -labelling of a hydrolytically stable, modified *t*Bu-silyl-bombesin peptide under acidic conditions.¹⁶³

The organosilicon-based fluoride acceptor (SiFA) ^{18}F -labelling approach was initially presented by Jurkschat and co-workers; the ^{19}F to ^{18}F IEX of an organosilicon-functionalised aldehyde coupled to an aminoxy-derivatised Tyr³-octreotate **137** (via an oxime bond) was carried out, as highlighted in Figure 72.¹³⁸ Reaction with $\text{K}[^{18}\text{F}]\text{F} \cdot \text{K}_{2.2.2}$ over 10 minutes (acetonitrile, room temperature) yielded the ^{18}F -labelled peptide **138** in RCCs and RCPs of 95-

97% and 98-100%, respectively. The calculated SA of the desired product was 0.08–0.1 Ci/ μ mol, and it was hypothesised that this could be increased by increasing the amount of ^{18}F -fluoride used in the IEX. The radiosynthesis time was 25 minutes; a final HPLC purification step was not necessary. It is evident that this method is also compatible with aqueous ^{18}F -fluoride; high RCCs of 70-90% were achieved within 30 minutes at 95 °C, under non-basic conditions.

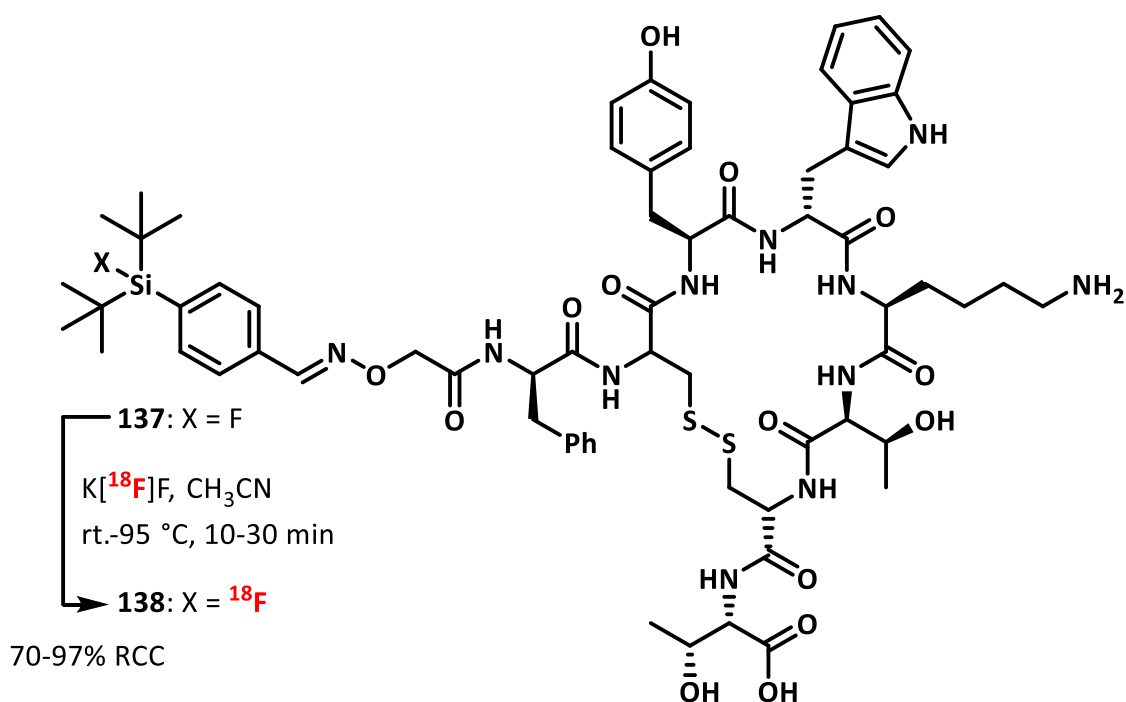


Figure 72: SiFA ^{18}F -labelling; ^{19}F to ^{18}F IEX of an organosilicon-functionalised aldehyde coupled to an aminoxy-derivatised Tyr³-octreotate (via an oxime bond).¹³⁷

Substitution of [^{18}F]fluorosilanes with *t*Bu is associated with an increase in lipophilicity leading to disrupted specific uptake of the PET imaging agent.¹⁴⁴ The development of lipophilicity-reducing auxiliaries was carried out by Wngler and co-workers; hydrophilic ^{18}F -labelled SiFA-modified somatostatin analogues **140** were synthesised (Figure 73) and investigated *in vitro* and *in vivo*.¹⁶⁴ Reaction with K[^{18}F]F.K_{2.2.2} over 5 minutes (acetonitrile, room temperature) via IEX afforded a RCY (non-decay corrected) and RCP of 53% and >98%, respectively. The calculated SAs were between 1.3 and 1.7 Ci/ μ mol. The radiosynthesis time was 25 minutes; a final HPLC purification step was not necessary. Synthesised peptides exhibited high binding affinities to somatostatin receptor-positive tumour cells and specific uptake/tumour accumulation.

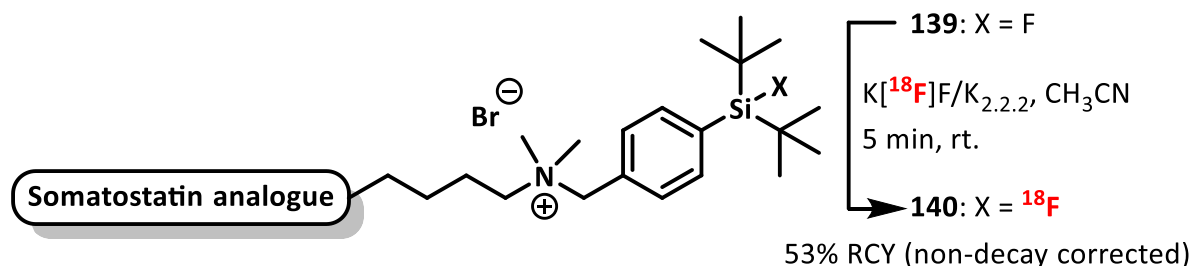


Figure 73: SiFA ^{18}F -labelling; ^{19}F to ^{18}F IEX of a hydrophilic SiFA-modified somatostatin analogue.¹³⁷

5.3 Indirect ^{18}F -labelling methodologies

Indirect methods require the prior radiosynthesis of a prosthetic group and subsequent bioconjugation to a peptide (traditionally via acylation, alkylation, hydrazone or oxime bond formation, to name a few).¹²⁵

^{18}F -labelling by $\text{C}_{\text{sp}}^2\text{-C}_{\text{sp}}^2$ bond formation with prosthetic groups has recently been explored.¹³⁷ For example, Davis and co-workers successfully demonstrated how an aqueous Suzuki-Miyaura reaction between a non-proteinogenic *para*-iodo-L-phenylalanine (pIPhe) residue **141** (installed via genetic code expansion) and $[^{18}\text{F}]4$ -fluorophenylboronic acid **142** can effect site-selective ^{18}F -labelling of peptides (Figure 74).¹⁶⁵ Their work built upon concepts introduced by Wood and co-workers regarding the use of an optimised palladium/ligand catalyst system plus a boronic acid derivative for the modification of peptides.¹⁶⁶ Whilst the bioconjugation reaction conditions were mild (pH 8, 37 °C, 30 minutes), radiosynthesis of the prosthetic group occurred over 3 steps (^{18}F -fluoride nucleophilic substitution on a diaryliodonium precursor followed by conversion to 4-fluorophenylboronic acid), thereby resulting in relatively low RCYs (decay corrected) and SAs of 5-10% and 0.08-0.40 Ci/ μmol ($n = 3$), respectively. Furthermore, whilst RCYs (decay corrected) of a functionalised peptide were on the order of 30-50% (95% RCP), unfortunately RCYs of a larger protein were quite low (2-5%, decay corrected), following column purification.¹²⁵

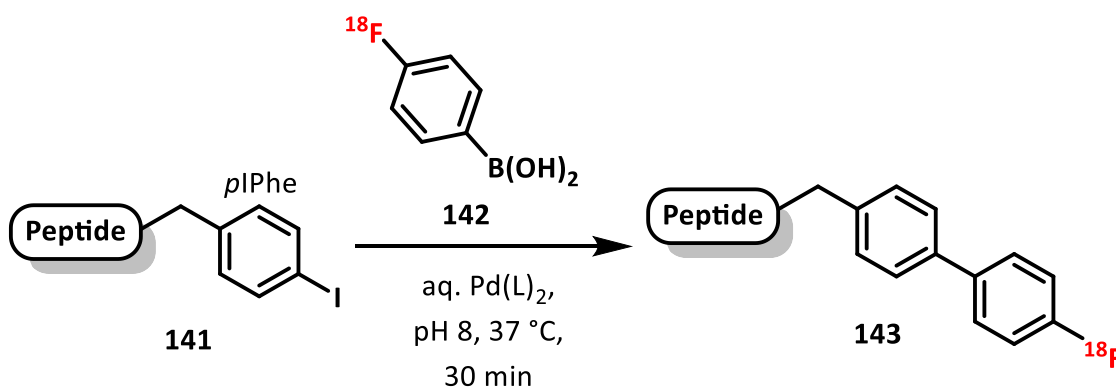


Figure 74: Site-selective Suzuki-Miyaura cross coupling of a peptide *pI*Phe residue R group to $[^{18}\text{F}]$ 4-fluorophenylboronic acid under mild, aqueous reaction conditions.¹⁶⁵ $\text{Pd}(\text{L})_2$ refers to a palladium-ligand catalyst.

A great deal of work has been published using amino-selective (N-terminal or primary amine of Lys R group) acylating NHS ester prosthetic groups, e.g. *N*-succinimidyl-4- $[^{18}\text{F}]$ fluorobenzoate ($[^{18}\text{F}]$ SFB) **144** (Figure 75), in relation to the ^{18}F -labelling of peptides.^{125,137,143} Furthermore, various ^{18}F -labelled maleimides, e.g. *N*-(2-(4- $[^{18}\text{F}]$ fluorobenzamido)ethyl) maleimide ($[^{18}\text{F}]$ FBEM) **145** (Figure 75), exist for reaction with free thiols.¹⁶⁷ Whilst use of these prosthetic groups typically enables the use of mild reaction conditions, they are also associated with complex and multi-step radiosyntheses which are incompatible with the relatively short half-life of ^{18}F .^{135,143} For example, the radiosynthesis of **144** traditionally requires two to three steps followed by HPLC purification (due to interference of the activated ester with the ^{18}F -labelling reaction).¹⁶⁸ Consequently, in recent years, development of time-efficient approaches for the synthesis of and subsequent bioconjugation of novel amino and thiol-selective ^{18}F -labelled prosthetic groups has been carried out; preservation of RCY and SA has been a major focus.

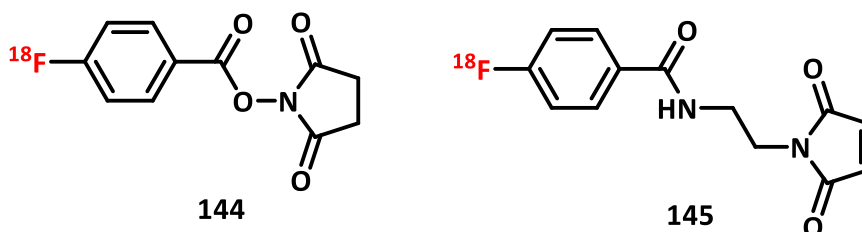


Figure 75: An amino-selective (**144**; $[^{18}\text{F}]$ SFB) and a thiol-selective (**145**; $[^{18}\text{F}]$ FBEM) prosthetic group.

5.3.1 Amino-selective prosthetic groups

5.3.1.1 [^{18}F]Nicotinic acid *N*-hydroxysuccinimide ester (6- ^{18}F SFPy)

Active esters of nicotinic acid functionalised with a TMA group at the 2-position are highly activated for nucleophilic aromatic substitution with ^{18}F -fluoride and allow direct labelling under mild conditions where the active ester moiety is not prone to degradation.¹⁶⁸ In their 2020 publication, Swenson and co-workers present an efficient approach for the radiosynthesis of [^{18}F]nicotinic acid NHS ester (6- ^{18}F SFPy) **151** via use of “Sep-Pak” ^{18}F -labelling, as highlighted in Figure 76.¹⁵²

The activated TMA-aromatic precursor compound **150** (Figure 76) was prepared over three steps starting with commercially available 6-chloronicotinic acid.¹⁵² Conversion of **150** to the ^{18}F -labelled prosthetic group 6- ^{18}F SFPy **151** was achieved in one step; purification via HPLC was not required in advance of peptide conjugation.

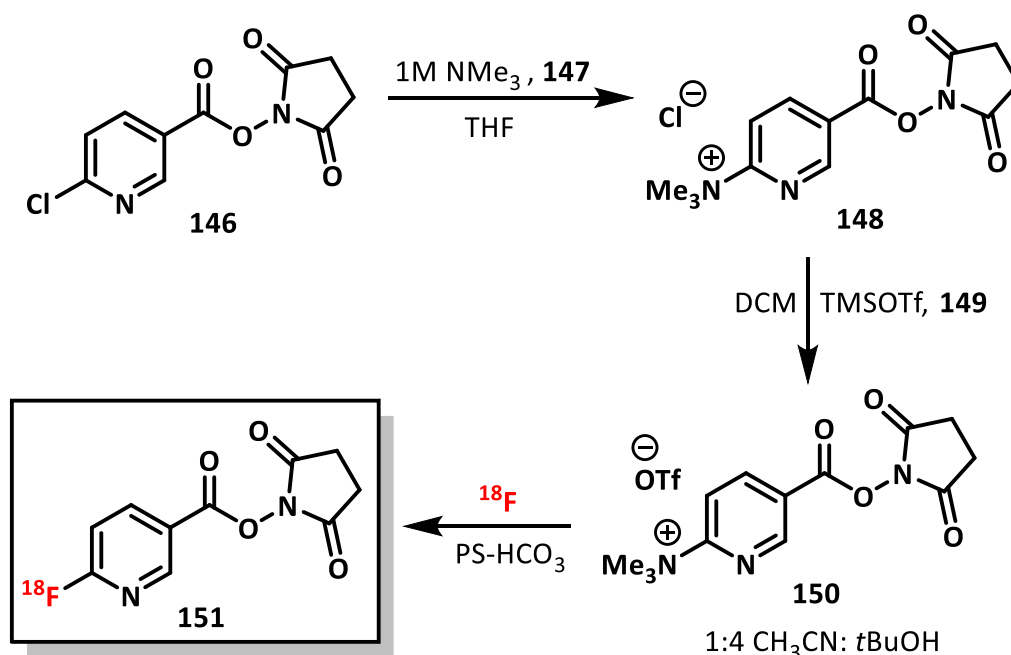


Figure 76: Schematic of radiosynthesis of 6- ^{18}F SFPy from an activated TMA-aromatic precursor compound **150** via Sep-Pak ^{18}F -labelling. **150** was synthesised over three steps: 1) A 6-chloronicotinic acid-derivative **146** was obtained using a literature-based method; 2) chloride substitution via reaction of **146** with trimethylamine in tetrahydrofuran (THF) and 3) anion exchange with trimethylsilyl triflate (TMSOTf).¹⁵²

The C-X-C motif chemokine receptor 4 (CXCR4) is a seven-transmembrane G protein-coupled receptor that is overexpressed in numerous diseases, particularly in various cancers (hence is

an attractive target for imaging and therapeutic purposes).¹⁵² After optimisation of ^{18}F -labelling, Swenson and co-workers applied their methodology to a CXCR4 ligand (termed “T140”; **152** Figure 77).¹⁵² Amide bond formation between *N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) (Dde)-protected **152** in DMSO and dried 6- ^{18}F SFPy **151** was carried out at 60 °C over 20 minutes followed by Dde removal via treatment with hydrazine solution, as highlighted in Figure 77. Radiochemically pure 6- ^{18}F FPy-T140 **155** (Figure 77) was obtained in RCYs and SAs (decay corrected) of 6-17% and 0.8-2.7 Ci/ μmol ($n = 15$) respectively, after HPLC purification. The total radiosynthesis time was 90 minutes.

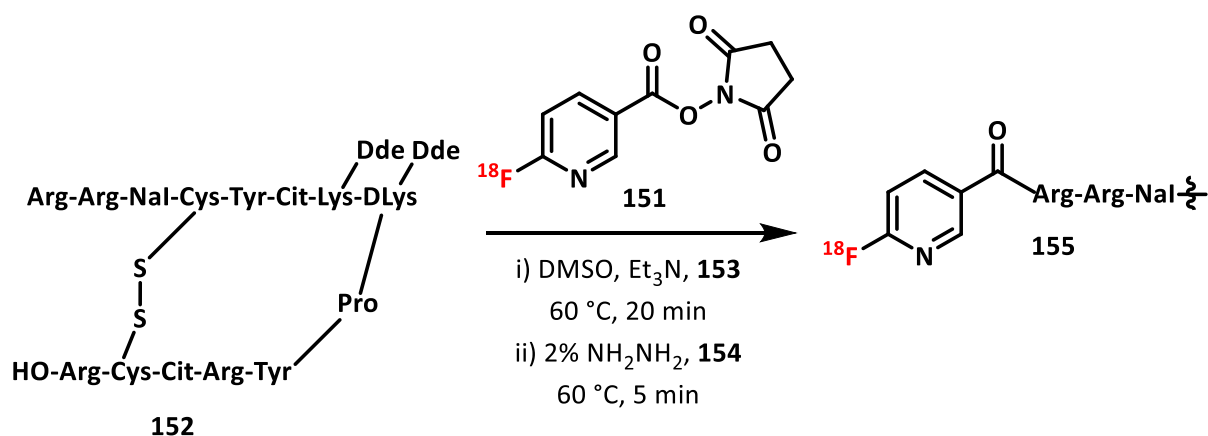


Figure 77: ^{18}F -labelling of T140 via conjugation with dried, Dde-protected 6- ^{18}F SFPy under mild reaction conditions (i) followed by Dde group removal (ii).¹⁵²

5.3.1.2 3- ^{18}F Fluoropropanesulfonyl chloride

Another prosthetic group with specificity for reactive amine functionalities, 3- ^{18}F fluoropropanesulfonyl chloride **157** (Figure 78), was first reported by Kisewetter and co-workers for the ^{18}F -labelling of molecules by forming sulfonamide derivatives.¹⁶⁹

An improved, two-step synthesis of this prosthetic group via the intermediate 3- ^{18}F fluoropropyl thiocyanate was subsequently reported by Steinbach and co-workers, as highlighted in Figure 78.¹⁷⁰ The ^{18}F -labelled prosthetic group was reacted with different model peptides under mild reaction conditions (room temperature, 3 minutes) (Figure 79).¹²⁵ High RCYs were achieved for most of the reactions without the addition of an auxiliary base. For example, cyclohexylamine was obtained in a RCY (non-decay corrected) of 35-38% after HPLC purification (total radiosynthesis time was 85-90 minutes).

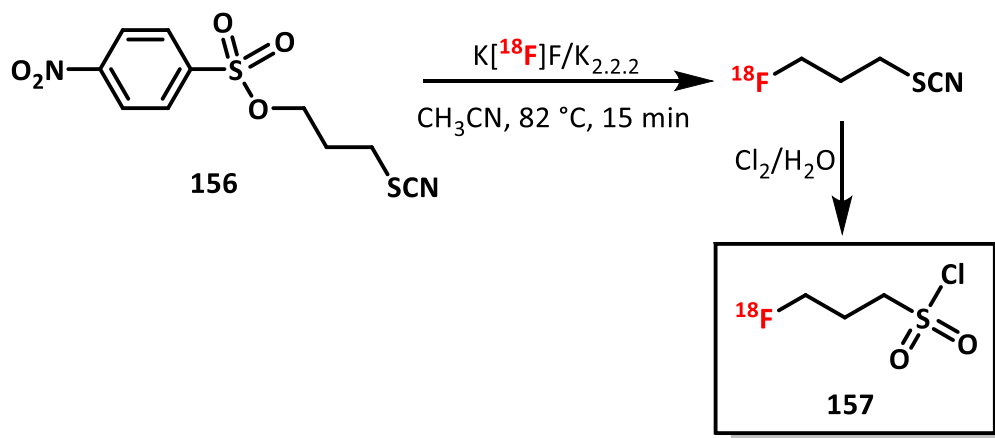


Figure 78: Schematic of radiosynthesis of 3-[^{18}F]fluoropropanesulfonyl chloride via the intermediate 3-[^{18}F]fluoropropyl thiocyanate.¹⁷⁰

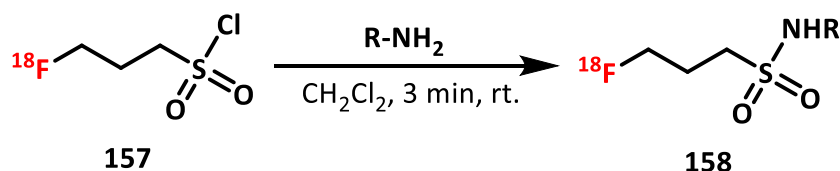


Figure 79: Rapid, ^{18}F -labelling of model peptides via sulfonamide bond formation; conjugation with 3-[^{18}F]-fluoropropanesulfonyl chloride under mild reaction conditions.^{125,170}

5.3.1.3 ^{18}F -labelled organosilicon-based fluoride acceptors (SiFAs)

As previously highlighted, use of SiFA-facilitated ^{19}F to ^{18}F IEX chemistry for ^{18}F -labelling can be carried out relatively rapidly and does not require HPLC purification.

In their 2009 publication, Schirmacher and co-workers report the radiosynthesis of [^{18}F]SiFA-isothiocyanate ([^{18}F]SiFA-ITC) **160**; treatment of an SiFA-ITC precursor **159** with $\text{K}[^{18}\text{F}]\text{F}.\text{K}_{2.2.2}$ over 10 minutes was carried out (Figure 80).¹⁷¹ Despite the high reactivity of the isothiocyanate, the ^{19}F to ^{18}F IEX proceeded nearly in quantitative yields (95% RCY (non-decay corrected)).¹⁴⁴ Conjugation of **160** via a thiourea linkage afforded ^{18}F -labelled unprotected model proteins under relatively mild reactions conditions (pH 9, room temperature, 10-20 minute reaction time). ^{18}F -labelled rat serum albumin (RSA), apotransferrin and bovine immunoglobulin (IgG) were obtained in RCYs (non-decay corrected) and RCPs of 20-40% and >99% respectively, after HPLC purification. The total radiosynthesis time was 40-50 minutes.

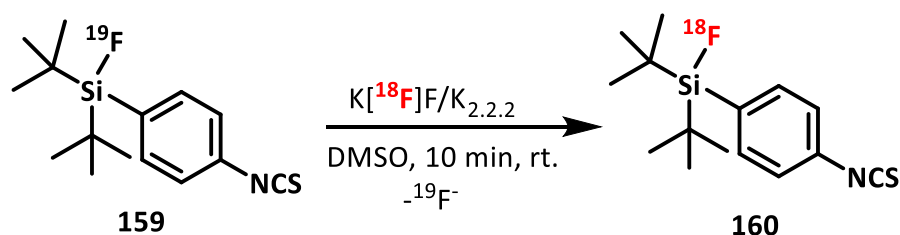


Figure 80: ^{19}F to ^{18}F IEX of an SiFA-ITC precursor in DMSO to form (^{18}F)SiFA-ITC within 10 minutes at room temperature.¹⁷¹

A procedure for the rapid radiosynthesis of the prosthetic group *N*-succinimidyl 3-(di-*tert*-butyl(^{18}F)fluorosilyl) benzoate (^{18}F)SiFB **162** via ^{19}F to ^{18}F IEX (treatment of an SiFB precursor **161** with $\text{K}[^{18}\text{F}]\text{F}.\text{K}_{2.2.2}$ over 30 minutes) was carried out by Schirmacher and co-workers (Figure 81).^{172,173} Anhydrous ^{18}F -fluoride was prepared in the absence of azeotropic drying (via the “Munich” method”) and purification of **162** was carried out via SPE, thereby time-costly steps were avoided. It was hypothesised that this methodology could be applied to unprotected peptides/proteins of interest; incubation with **162** in an aqueous solution (relatively mild reaction conditions; pH 9, room temperature) for 30 minutes, followed by HPLC purification would afford high SAs. RSA and erythropoietin were subsequently labelled with **162** in RCYs (non-decay corrected) of 35% ($n = 3$) and 19% ($n = 2$), respectively.¹⁷³ The total radiosynthesis time was ~90 minutes.

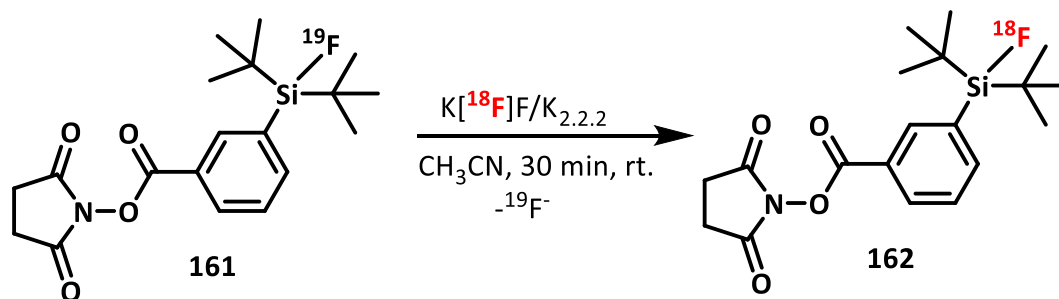


Figure 81: Synthesis of (^{18}F)SiFB from an SiFB precursor via ^{19}F to ^{18}F IEX (treatment with $\text{K}[^{18}\text{F}]\text{F}.\text{K}_{2.2.2}$ over 30 minutes in acetonitrile).^{172,173}

Despite the advantages associated with ^{19}F to ^{18}F IEX for ^{18}F -labelling, the lipophilic nature of the SiFA moiety on the prosthetic group has the potential to have a negative influence on the pharmacokinetics of small peptides.¹⁴³

5.3.1.4 Oxime and hydrazone bond formation via ^{18}F -labelled prosthetic groups

Significant developments have been made to bioconjugation reactions featuring oxime and hydrazone formation; these are now widely used for the site-selective ^{18}F -labelling of aminooxy and hydrazine-functionalised peptides, respectively.^{137,174}

As highlighted in section 5.2.4, anchorage of a prosthetic group can be achieved via oxime bond formation; such an approach is based on the highly chemoselective reaction between an aminooxy group and an aldehyde or ketone.^{137,175} Kung and co-workers developed a novel ^{18}F -labelled prosthetic group termed [^{18}F]PEG-benzaldehyde ([^{18}F]FPBA) **163** (Figure 82); radiosynthesis of **163** was achieved in one step from a tosylate precursor using $\text{K}[^{18}\text{F}]\text{F.K}_{2.2.2}$, followed by HPLC purification (which was costly in terms of the overall radiosynthesis time).¹⁷⁶ During ^{18}F -labelling, the aldehyde group of **163** was protected (as the corresponding acetal) to avoid side product formation; deprotection of the aldehyde was effected via treatment with TFA solution over two minutes in advance of bioconjugation, as shown in Figure 83. Three cyclic peptides bearing an aminooxy functional group at the N-terminus position underwent oxime bond formation with **163** under aqueous reaction conditions (ethanol/water, 70 °C, 30 minutes). The peptides **164** were obtained in RCYs (non-decay corrected) and RCPs of 21-24% and >99% respectively, after purification by SPE. The total radiosynthesis time was ~130 minutes.

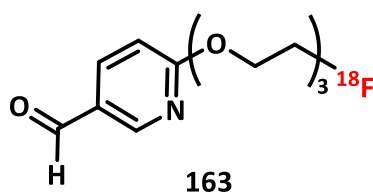


Figure 82: [^{18}F]FPBA.¹⁷⁶

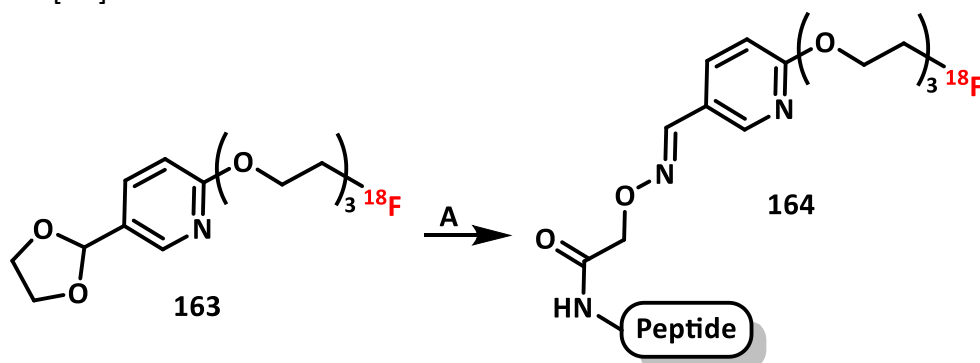


Figure 83: Oxime bond formation between [^{18}F]FPBA and a peptide of interest containing an N-terminal aminooxy group. Reagents and conditions (A): i) acetal deprotection; TFA, EtOH/H₂O, 70 °C, 2 minutes, ii) peptide, EtOH/H₂O, 70 °C, 30 minutes.¹⁷⁶

Bioconjugation of an aldehyde-based prosthetic group to a pre-modified peptide was also utilised by Suh and co-workers for the site-selective ^{18}F -labelling of cyclic RGDyK peptide; [^{18}F]fluorobenzaldehyde ([^{18}F]FBA) **166** (Figure 84) was used to form a conjugate via hydrazone formation with the amino group in the peptide.¹⁷⁷ Cyclic RGDyK-hydrazinonicotinic acid **165** (Figure 84) was initially synthesised via a straightforward procedure, followed by reaction with **166** (^{18}F -labelling occurred via nucleophilic substitution of 4-*N,N,N*-trimethylaminebenzaldehyde triflate in presence of ^{18}F -fluoride) at 50 °C for 30 minutes, as per Figure 84. The RCY (decay-corrected) and RCP of the ^{18}F -labelled peptide **167** was ~15% and >99% respectively ($n = 4$), after HPLC purification. The recorded SA was 0.6 Ci/ μmol and the total radiosynthesis time was 90 minutes.

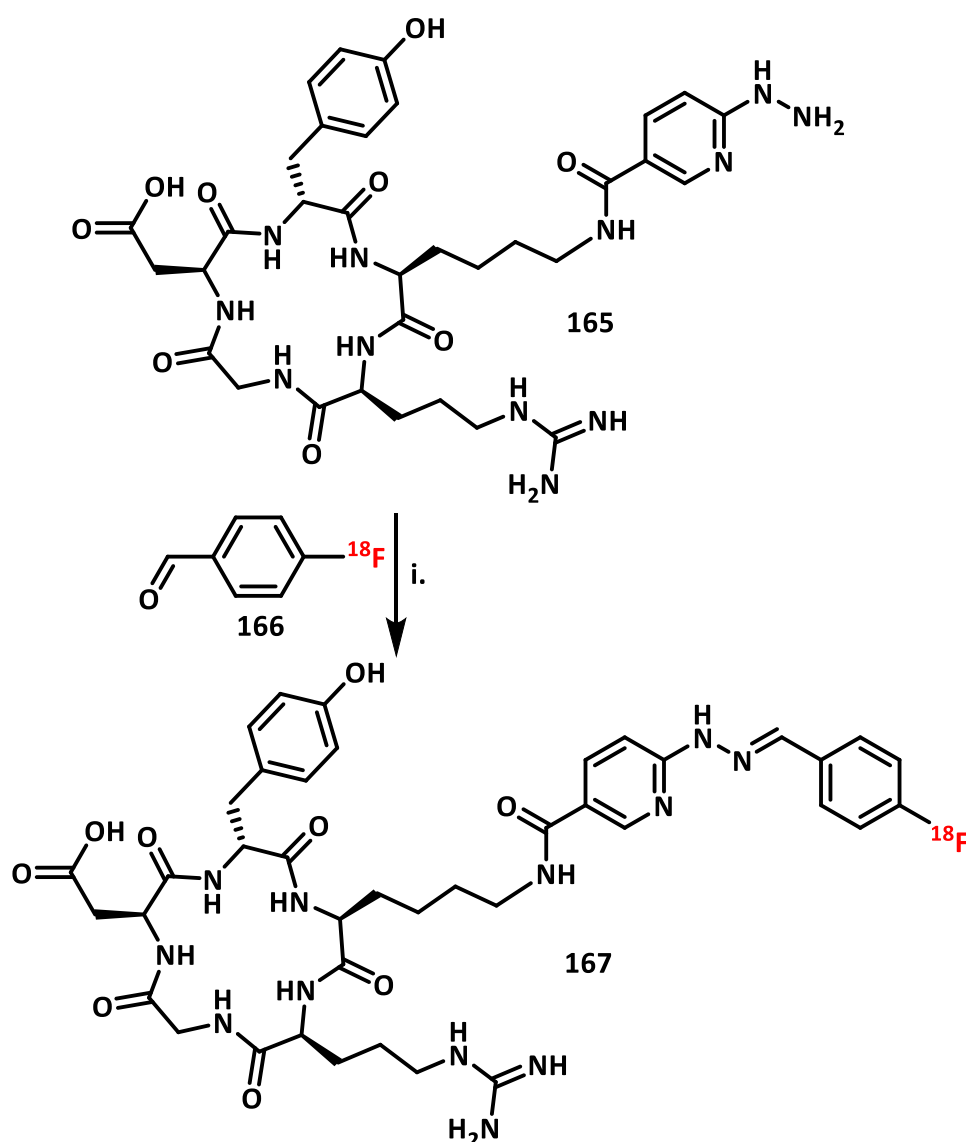


Figure 84: ^{18}F -labelling of cyclic RGDyK peptide: bioconjugation of cyclic RGDyK-hydrazinonicotinic acid with [^{18}F]FBA to form a hydrazone linkage; i. = EtOH/NaOAc buffer solution, pH 4.5, 50 °C, 30 minutes.¹⁷⁷

5.3.2 Thiol-selective prosthetic groups

The most common indirect peptide ^{18}F -labelling methods utilise the reactivity of the amino group.¹⁷⁸ However, alternative Cys residue-dependent (native or pre-installed) strategies affording superior chemoselectivity also exist.

5.3.2.1 [^{18}F]*N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-ethyl)-6-fluoronicotinamide ([^{18}F]FNEM)

^{18}F -labelling is possible via the participation of maleimide-derivative prosthetic groups in Michael addition at Cys.¹⁷⁴ Interestingly, thiol-maleimide chemistry has also successfully been applied to peptide maleimide-derivatives; it is possible to ^{18}F -label, in a site-selective manner, via bioconjugation of an [^{18}F]SiFA-SH prosthetic group to a suitably pre-modified peptide of interest.¹⁷⁹

Unfortunately, the radiosynthesis of maleimide-derivative prosthetic groups is typically complex and multi-step in nature.¹⁴³ A more straightforward strategy was recently published by Chen and co-workers for [^{18}F]*N*-(2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-ethyl)-6-fluoronicotinamide ([^{18}F]FNEM) **171** (Figure 85).¹⁷⁸ A one-pot, two-step, 75 minute strategy was utilised for the radiosynthesis of **171**; ^{18}F incorporation by nucleophilic displacement of a TMA substrate **168**, followed by amidation of the resultant 6-[^{18}F]fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester **169** with *N*-(2-aminoethyl)maleimide trifluoroacetate salt **170** (Figure 85). Subsequent chemoselective bioconjugation of **171** with [Cys⁴⁰]-exendin-4 under mild reaction conditions (room temperature, 50 minutes) resulted in the ^{18}F -labelled peptide in RCYs (non-decay corrected) of 30-40% and SAs of between 0.2 and 0.6 Ci/ μmol .

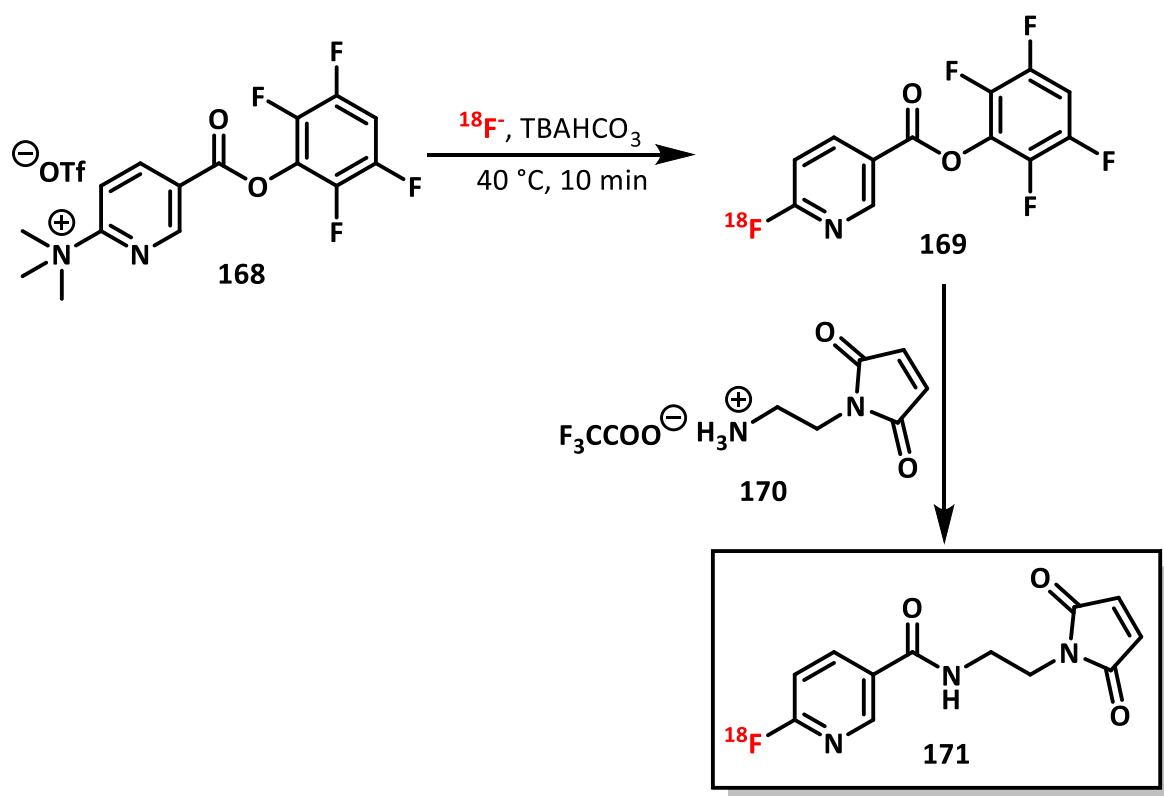


Figure 85: Schematic of the one-pot, two step-radiosynthesis of $[^{18}\text{F}]$ FNEM: ^{18}F incorporation by nucleophilic displacement of a trimethylammonium substrate, followed by amidation of the resultant 6- $[^{18}\text{F}]$ fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester with N -(2-aminoethyl)maleimide trifluoroacetate salt.¹⁷⁸

The most evident limitation of using thiol-maleimide chemistry for the ^{18}F -labelling of peptides is the susceptibility of the formed succinimidyl thioether linkage to undergo degradation via a retro-Michael reaction.¹⁸⁰

5.3.2.2 $[^{18}\text{F}]$ 2-Cyanobenzothiazole ($[^{18}\text{F}]$ CBT)

Recently, prosthetic groups based on heteroaromatic derivatives have provided an alternative class of thiol-reactive synthons with improved stability over maleimides.¹⁸⁰ Chin and co-workers devised an approach for the site-selective ^{18}F -labelling of peptides based on a rapid condensation of $[^{18}\text{F}]$ 2-cyanobenzothiazole ($[^{18}\text{F}]$ CBT) **172** and an N-terminal Cys, as highlighted in Figure 86.¹⁸¹

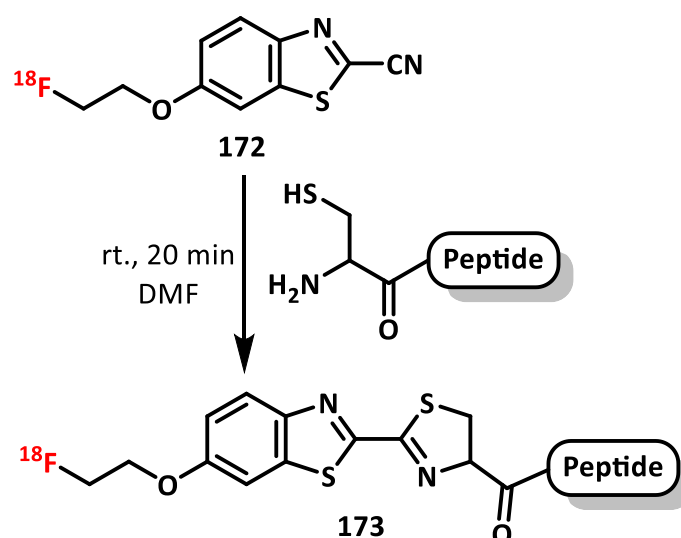


Figure 86: A rapid condensation reaction between [^{18}F]CBT and an N-terminal Cys to afford site-selective ^{18}F -labelling of a peptide of interest under mild reaction conditions.^{180,181}

Radiosynthesis of [^{18}F]CBT **172** was achieved using tosylated CBT; reaction with an anhydrous 18-Crown-6/ K^+ /[^{18}F] F^- complex (90 °C, 10 minutes), followed by HPLC purification afforded **172** in a good RCY (decay corrected) and RCP (20% and >99%, respectively).¹⁸¹ Bioconjugation of **172** to a derivative of dimeric cyclic RGDyK (pre-modified with N-Boc-Cys(Trt) succinimidyl ester) was subsequently carried out under mild reaction conditions; a RCY (non-decay corrected) and RCP of 8% and >99% ($n = 3$) were recorded respectively, following HPLC purification. The SA of the final product was 1.3 Ci/ μmol (total radiosynthesis time was 100 minutes).

5.3.2.3 [^{18}F]Hexafluorobenzene ([^{18}F]HFB)

In their 2015 publication, Chen and co-workers report a novel approach for the chemoselective ^{18}F -labelling of cyclic and dimeric peptides using mildly basic conditions at room temperature.¹⁸² Their methodology was applied to cyclic RGDfK; dimerisation of two thiolated monomers was effected using [^{18}F]hexafluorobenzene ([^{18}F]HFB) **175** (Figure 87).

The strongly electronegative fluorine atoms in HFB deactivate the aromatic ring, making it highly susceptible to nucleophilic substitution.¹⁸² Consequently, *para*-substitution of HFB via ^{19}F to ^{18}F IEX (treatment with $\text{K}[^{18}\text{F}]\text{F} \cdot \text{K}_{2.2.2}$) was possible, which was subsequently reacted with the two monomer peptides to simultaneously ^{18}F -label and dimerise, resulting in **176**. For the bioconjugation reaction, **175** was reacted with 2 equivalents of thiolated cyclic RGDfK **174**, an excess amount of 2-amino-2-(hydroxymethyl)propane-1,3-diol (TRIS) base and TCEP **59** in

DMF (room temperature, 20-25 minutes). **176** was isolated in a RCY (non-decay corrected) of 40% (n = 4) and SA of 15 mCi/μmol, after HPLC purification.

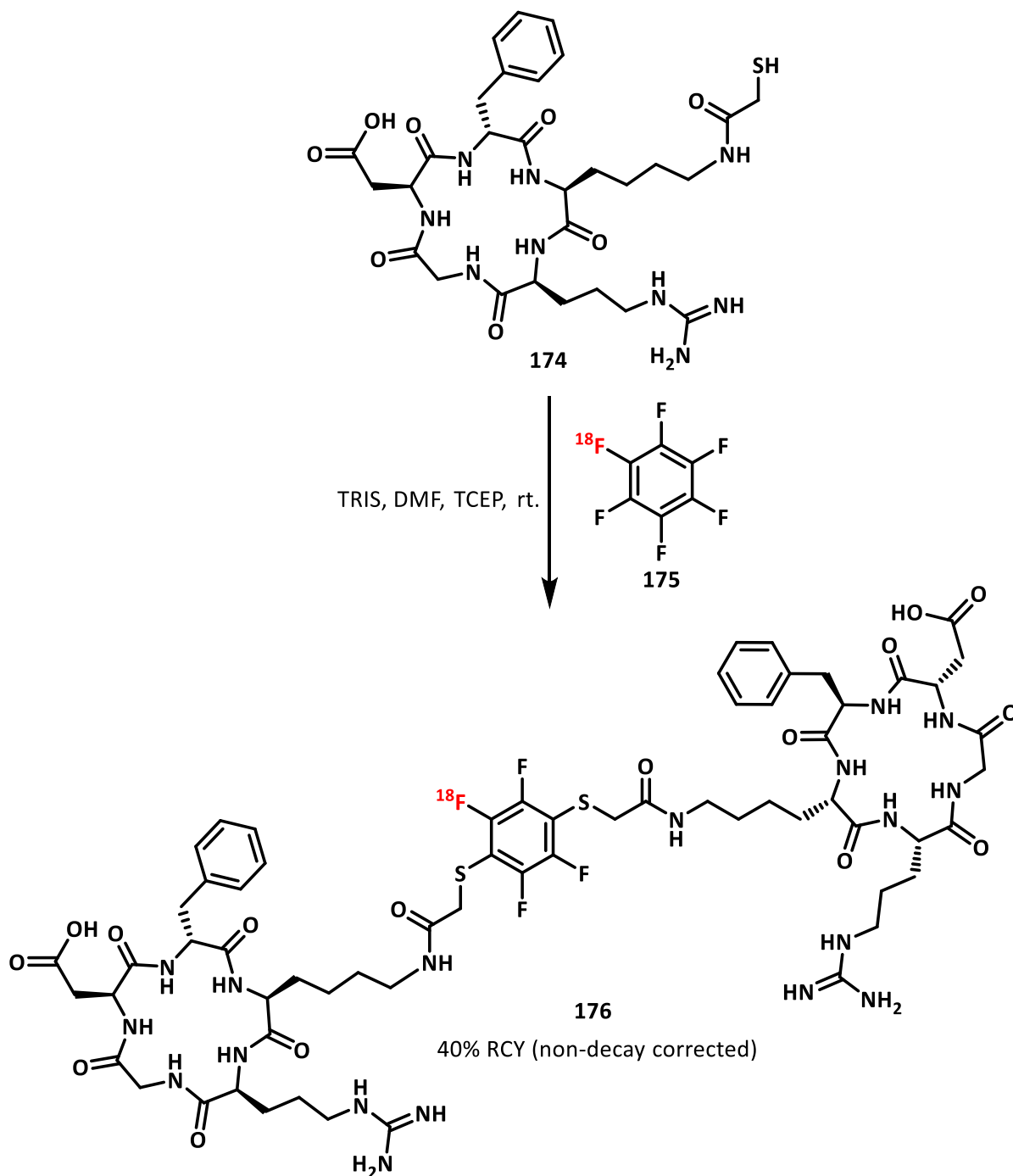


Figure 87: Dimerisation and ^{18}F -radiolabelling of thiolated cyclic RGDfK using ^{18}F HFB under mild reaction conditions.¹³⁷

5.3.2.4 [^{18}F]Fluoro-4-(vinylsulfonyl)benzene ([^{18}F]FVSB)

In their 2021 publication, Murphy and co-workers report an approach for the chemoselective ^{18}F -fluorination of peptides via a novel prosthetic group, [^{18}F]fluoro-4-(vinylsulfonyl)benzene ([^{18}F]FVSB) **177**.¹⁸⁰ Using their methodology, as highlighted in Figure 88, prosthetic group-peptide bioconjugation can be carried out under mild reaction conditions within 30 minutes.

Radiosynthesis of [^{18}F]FVSB **177** was achieved within a single step, in the absence of HPLC purification and azeotropic drying of ^{18}F -fluoride (isolation within 45 minutes); a RCY (decay corrected) and RCP of 46% and 85% ($n = 10$) respectively were reported.¹⁸⁰ Bioconjugation of **177** to cyclic Arg-Gly-Asp-D-Phe-Cys (RGDfC) was subsequently carried out under mild reaction conditions (1:1; sodium borate buffer:methanol, 35 °C); a RCY (decay corrected) of 84% ($n = 3$) was attained.

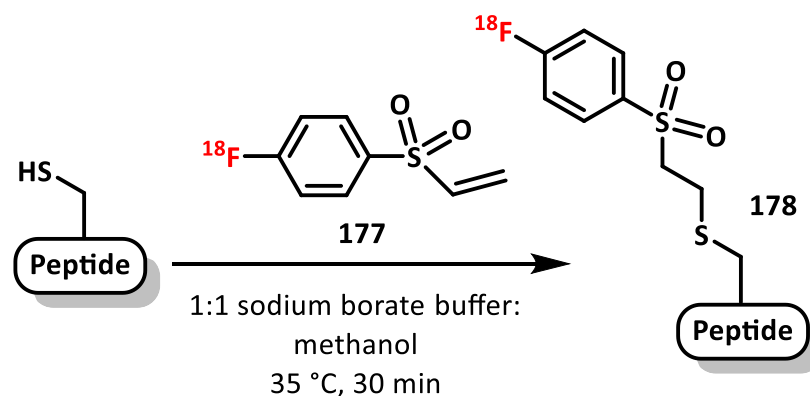


Figure 88: ^{18}F -labelling of a peptide of interest via thiol-[^{18}F]FVSB conjugation.¹⁸⁰

5.3.3 Site-selective click chemistry

Click chemistry is rapid, bio-orthogonal, high-yielding, site- and stereoselective, and reactions can be carried out under mild conditions (typically aqueous media).^{125,174} Recently, click chemistry has emerged as a powerful and versatile synthesis approach for yielding ^{18}F -labelled peptides; a class of prosthetic groups containing functional groups that participate in click-reactions have been developed for reaction with suitably functionalised peptides.¹⁸²

5.3.3.1 Copper(I)-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC)

Copper(I)-catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is characterised by the regioselective formation of a 1,4-disubstituted [1,2,3]-triazole via the reaction of an alkyne with an azide.^{174,183} The reaction can be catalysed with copper(I) sources such as copper

bromide/iodide, but is typically achieved with copper sulphate, which affords copper(I) in the presence of a reducing agent such as sodium ascorbate.¹²⁵ Recently, CuAAC has become a popular technique for the ¹⁸F-labelling of peptides under mild reaction conditions (pre-modified with either an alkyne or azide moiety), due to the ease of formation and stability of the triazole.^{137,184,185} For example, as highlighted in section 5.2.2, an arylborimidine can capture ¹⁸F-fluoride in aqueous, acidic media resulting in formation of ¹⁸F-ArBF₃⁻.¹⁵⁶ Application of this chemistry to an alkyne-arylborimidine **179** followed by rapid, copper-mediated coupling to cyclic RGDfK-azide was carried out by Perrin and co-workers (Figure 89).¹⁵⁰ RGD-trAz-¹⁸F-ArBF₃⁻ **183**, as highlighted in Figure 90, was obtained in a RCY (non-decay corrected) and SA of ~7% and 0.06 Ci/μmol respectively, following HPLC purification.

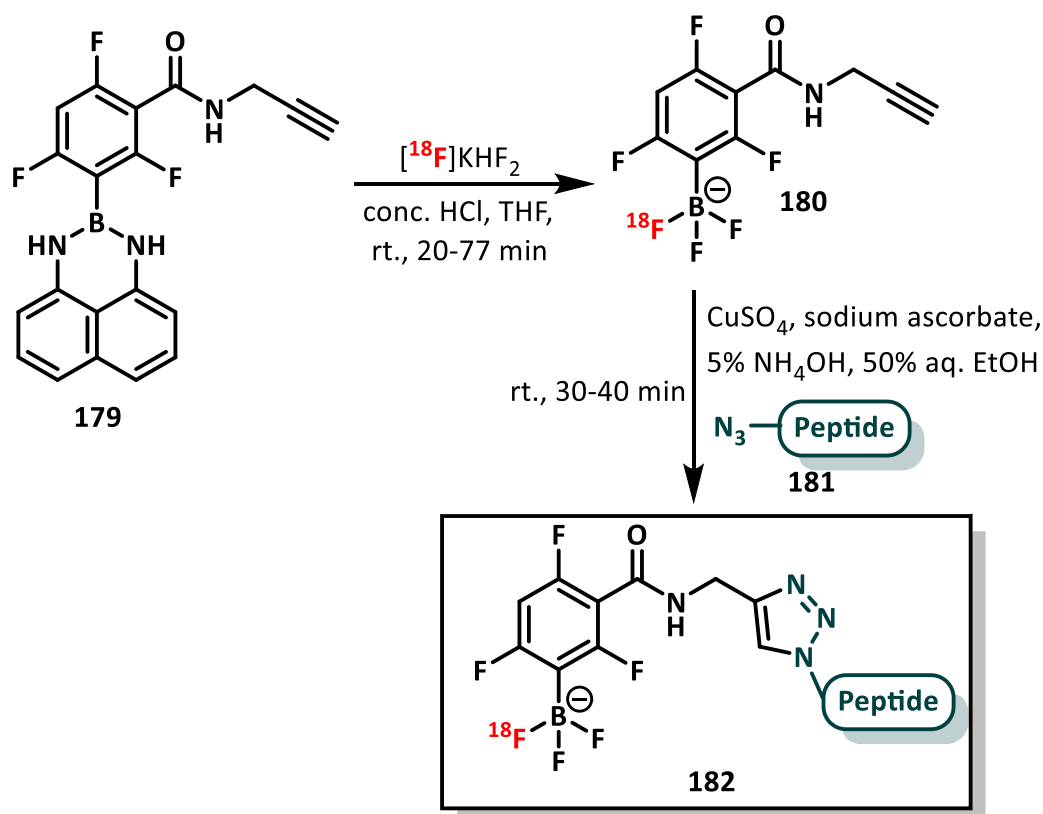


Figure 89: Schematic of CuAAC-based ¹⁸F-labelling of an azide-modified peptide of interest: conversion of an alkyne-arylborimidine to the corresponding ¹⁸F-ArBF₃⁻ followed by CuAAC.¹⁵⁰ N₃-Peptide refers to a peptide-azide.

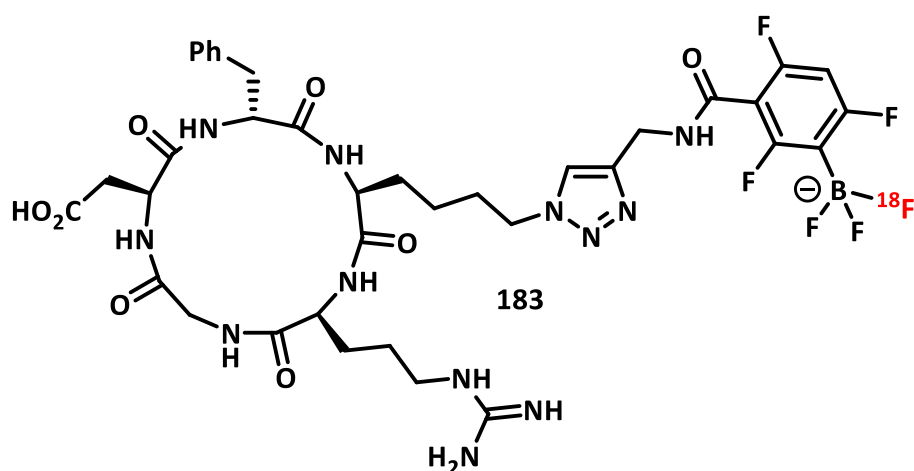


Figure 90: RGD-trAz-¹⁸F-ArBF₃⁻.¹⁵⁰

Recent efforts have been focussed on the chemoselective modification of the relatively rare Met amino acid (accounts for ~2.3% of all residues in human proteins) in advance of site-selective CuAAC to afford ¹⁸F-labelling.^{186,187} For example, in their 2020 publication Bonacorsi and co-workers report the formation of a Met-bioconjugate via reaction with an oxaziridine bearing a terminal alkyne **184**; subsequent CuAAC of the derivatised peptide **185** with an ¹⁸F-labelled PEG-ylated azide **186** afforded the corresponding triazole **187** under mild, aqueous conditions, as highlighted in Figure 91.¹⁸⁷ This approach was applied to two model peptides (each containing a single Met); RCCs of 76% and 35% were recorded, following HPLC purification.

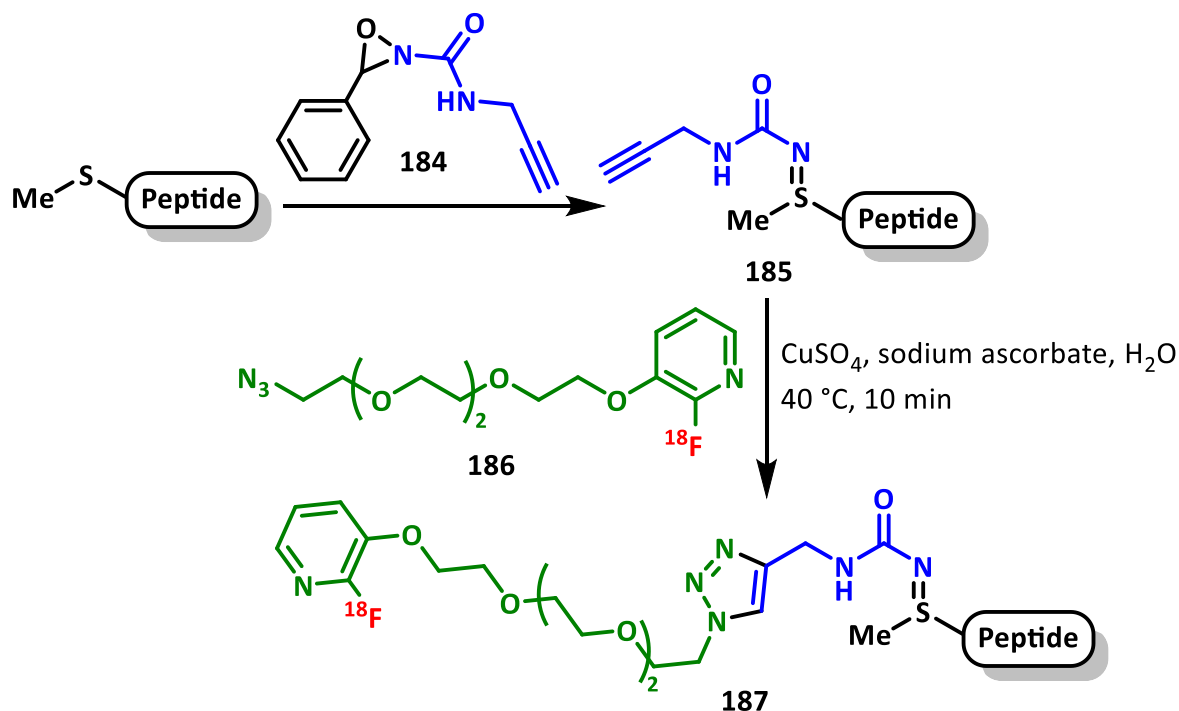


Figure 91: Schematic of CuAAC-based ^{18}F -labelling of a peptide of interest: Met-bioconjugation via reaction with an oxaziridine bearing a terminal alkyne followed by CuAAC with an ^{18}F -labelled PEG-ylated azide.¹⁸⁷

Despite the advantages of CuAAC-based chemistry for the ^{18}F -labelling of peptides, the ability of copper to bind to peptides can lead to a reduction in biological activity and rate of the CuAAC reaction.¹⁸⁸ Furthermore, the potential for residual copper (toxic in nature) to exist in ^{18}F -labelled products typically results in a requirement for complex separation protocols.¹³⁷ Consequently, alternative, copper-free click approaches are becoming more widely favoured.^{143,184} Such methodologies are associated with an increased lipophilic character of the ^{18}F -labelled peptide, which, as previously highlighted, has the potential to negatively affect the pharmacokinetics.¹⁴³ Therefore, recent efforts have been focussed on development of novel ^{18}F -labelled prosthetic groups to participate in transition-metal free click reactions.^{188,189}

5.3.3.2 Strain-promoted alkyne-azide cycloaddition (SPAAC)

Strain-promoted alkyne-azide cycloaddition (SPAAC) involves the reaction of an azide with the bent triple bond of a cyclooctyne ring to afford a triazole, in the absence of any catalyst.¹³⁷ The activation barrier of a SPAAC reaction is significantly reduced by the bent geometry of the triple bond of the eight-membered ring.¹²⁵ Modifications of the cyclooctyne-moiety, such as introduction of EWGs or conformation-restricting aromatic rings adjacent to the triple bond, have significantly increased the reactivity towards terminal azides, making SPAAC an attractive option for ^{18}F -labelling of peptides under physiological conditions.¹⁸⁵

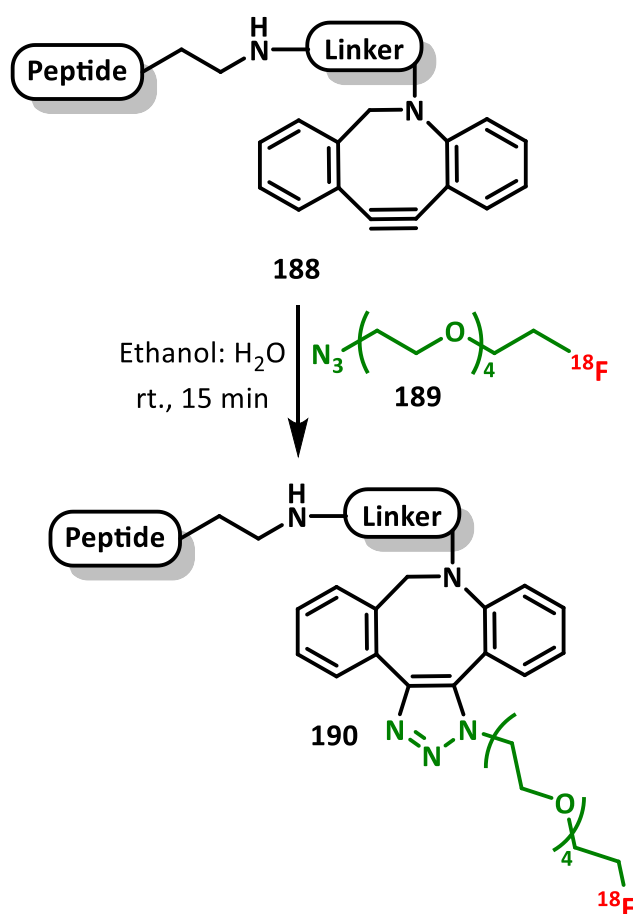


Figure 92: Schematic of a SPAAC-based ^{18}F -labelling approach: SPAAC between an ADIBO-modified peptide and an ^{18}F -azide prosthetic group under nontoxic, physiological conditions.¹²⁵

Kim and co-workers developed an ^{18}F -labelling approach based on SPAAC between an azadibenzocyclooctyne (ADIBO)-modified peptide of interest **188** and an ^{18}F -azide prosthetic group **189** (Figure 92).¹⁸⁸ Radiosynthesis of **189** was achieved within 45 minutes (including HPLC purification) via ^{18}F -fluoride displacement of a mesylated precursor compound with *n*-

Bu₄N[¹⁸F]F. Rapid, site-selective bioconjugation was effected over 15 minutes under mild and aqueous reaction conditions (room temperature, pH 7) in the absence of a final HPLC purification step (Figure 92); a unique azide-containing scavenger resin was utilised to effectively remove unreacted **188** from the desired ¹⁸F-labelled triazole product **190**.¹²⁵

This approach was applied to cyclic RGDyK peptide; an ADIBO-modified derivative was synthesised and subsequently participated in the aforementioned site-selective SPAAC reaction with the ¹⁸F-azide prosthetic group **189**, followed by ADIBO-precursor-scavenger-resin purification (total time was 35 minutes).¹⁸⁸ RGDyK-ADIBOT-¹⁸F was obtained in a RCY (decay corrected) and RCP of 92% and 98%, respectively. The total radiosynthesis time was ~80 minutes.

5.3.3.3 Tetrazine ligation

Tetrazine ligation is a cycloaddition based on an inverse electron demand Diels–Alder reaction between 1,2,4,5-tetrazine and a dienophile (a strained alkene or alkyne such as *trans*-cyclooctene (TCO)), followed by a retro-Diels-Alder reaction (resulting in nitrogen release).^{125,137} This approach for the ¹⁸F-labelling of peptides is characterised by fast, efficient and site-selective prosthetic group bioconjugation in the absence of a transition metal catalyst and in aqueous solvent. Furthermore, the rapidity of the tetrazine ligation enables peptide ¹⁸F-labelling at a low concentration.¹⁹⁰

Akin to the aforementioned work carried out by Bonacorsi and co-workers, recent efforts have been focussed on the chemoselective modification of a native residue in advance of site-selective tetrazine ligation to afford ¹⁸F-labelling.^{187,189} For example, in their publication, Conti and co-workers report the chemoselective modification of Cys via reaction of a tetrazinyl-maleimide **191** (Figure 93) with a peptide of interest.¹⁸⁹

Radiosynthesis of [¹⁸F]TCO **193** from the corresponding nosylate **192** via treatment with ¹⁸F-fluoride/tetrabutylammonium bicarbonate (TBAB) in acetonitrile was achieved by Conti and co-workers, as shown in Figure 94.¹⁸⁹ ¹⁸F-labelling of a cyclic RGDyC-tetrazinyl-maleimide conjugate (RGD-TM) **194** via treatment with **193** under ambient reaction conditions was subsequently achieved over 5 minutes (Figure 95). The site-selective bioconjugation afforded the ¹⁸F-labelled peptide **195** in a quantitative RCY. Furthermore, excellent RCPs and SAs of

>95% and 3-6 Ci/ μmol were recorded respectively, following HPLC purification. The total radiosynthesis time was ~ 90 minutes.

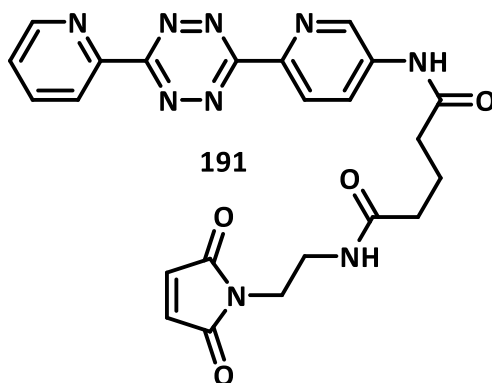


Figure 93: The tetrazinyl-maleimide utilised by Conti and co-workers.¹⁸⁹

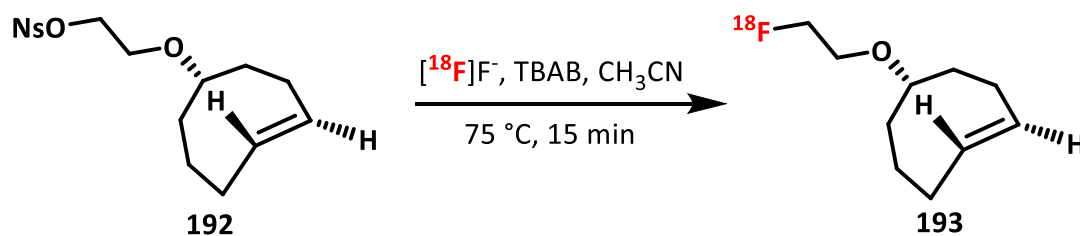


Figure 94: Radiosynthesis of [^{18}F]TCO.¹³⁷

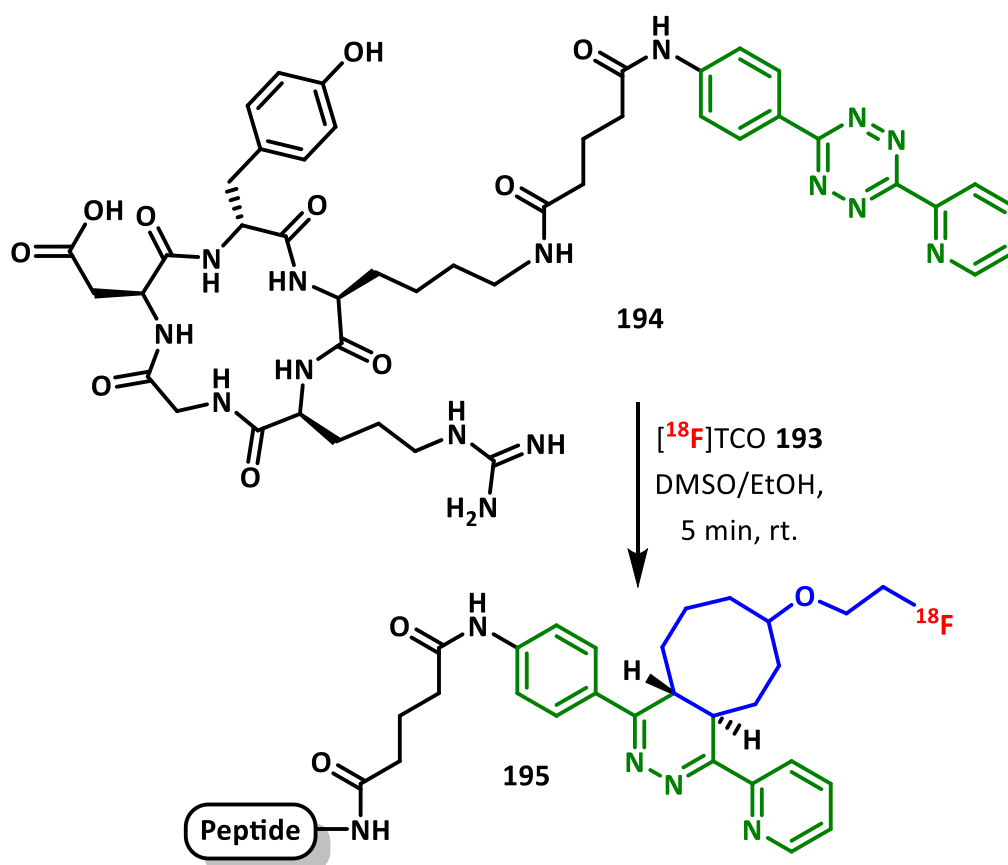


Figure 95: A tetrazine ligation-based ^{18}F -labelling approach: rapid, site-selective tetrazine ligation between RGD-TM and [^{18}F]TCO under mild reaction conditions.¹³⁷

5.4 Direct, late-stage methodologies for the ^{18}F -labelling of unmodified peptides

Direct ^{18}F -labelling of peptides devoid of a pre-installed prosthetic group, as represented in Figure 96, enables circumvention of structural modification.

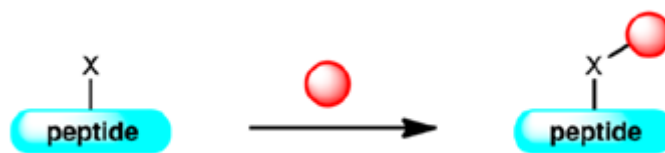


Figure 96: Direct ^{18}F -labelling of a prosthetic-group free peptide. Adapted from Hooker and co-workers.¹³⁹

5.4.1 ^{18}F -trifluoromethylation

In their 2018 publication, Gouverneur and co-workers report use of a novel agent for the ^{18}F -labelling of unmodified peptides at Cys under basic reaction conditions, as shown in Figure 97.¹⁴⁰ ^{18}F -labelled 5-(trifluoromethyl) dibenzothiophenium trifluoromethanesulfonate ([^{18}F]-Umemoto reagent) **196** (Figure 97) was initially synthesised, then used as an electrophilic source of fluorine; R group-unprotected model di- and tetrapeptides underwent ^{18}F -trifluoromethylation at room temperature in the presence of DMAP in a 4:1 mixture of DMSO:water. RCCs of >55% were obtained, even in the presence of nucleophilic groups, such as the R group of Lys (100% RCP). Optimisation of base type, solvent mixture, temperature and reaction time was carried out for each model peptide.

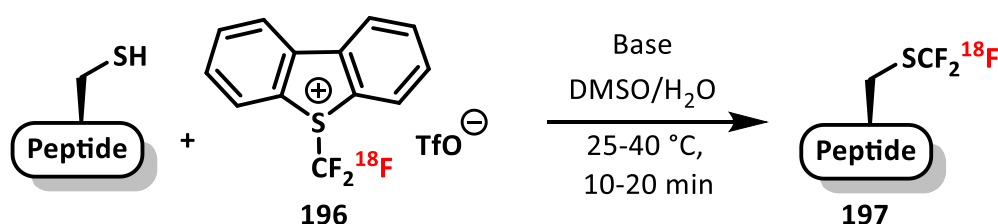


Figure 97: Direct ^{18}F -trifluoromethylation of a prosthetic-group free peptide using [^{18}F]-Umemoto reagent.¹⁴⁰

The aforementioned methodology was subsequently applied to unprotected cyclic RGDfC (Et_4NHCO_3 , 40 °C, 1:1 ratio; DMSO:water).¹⁴⁰ After 20 minutes, cyclic RGDfC-([^{18}F]CF₃) **198** (Figure 98) was purified via HPLC; a RCY (non-decay corrected) and RCP of 19% and >99% (n = 2) were reported. The SA of the ^{18}F -labelled product was ~4.1 mCi/ μmol .

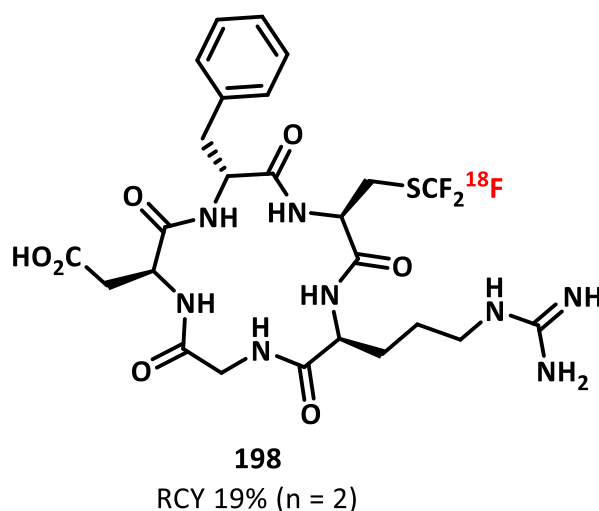


Figure 98: cRGDfC-([¹⁸F]CF₃).¹⁴⁰

Dipeptides featuring His and Trp resulted in the formation of more than one ¹⁸F-radiolabelled product (RCP of 68% and 64% respectively); ¹⁸F-trifluoromethylation at the imidazole ring was evident (i.e. lack of chemoselectivity).¹⁴⁰ Maximisation of thiol ¹⁸F-trifluoromethylation was attempted using 1 equivalent of KHCO₃ as a base instead of DMAP, but the side reaction could not be entirely suppressed (RCC of desired product was 41% (vs 21%) (His) and 59% (vs 28%) (Trp) (n = 4)).

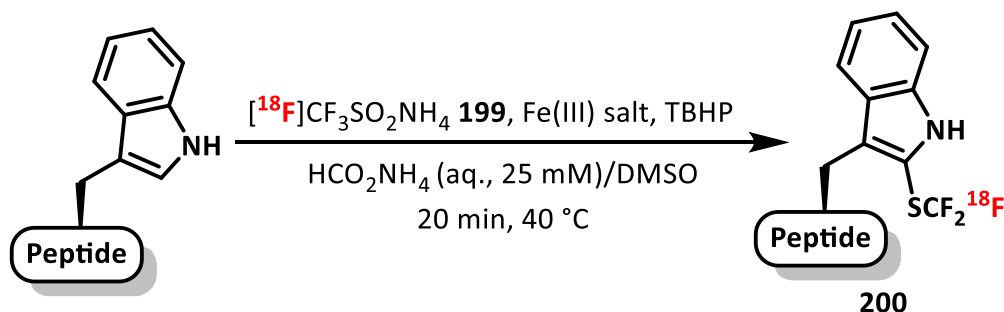


Figure 99: Chemoselective radical ¹⁸F-trifluoromethylation at Trp.¹⁴¹

Chemoselective radical ¹⁸F-trifluoromethylation was subsequently achieved by Gouverneur and co-workers; [¹⁸F]CF₃SO₂NH₄ (a source of ·CF₃) was used to direct ¹⁸F-labelling at unmodified Trp under oxidative conditions (Figure 99).¹⁴¹ Unprotected endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂), a tetrapeptide associated with AD, underwent Trp-selective ¹⁸F labelling in a RCC and RCY (non-decay corrected) of 11% (n = 4) and 23% (n = 1) after HPLC purification; the combined RCC for endomorphin-1[Trp(4-CF₂¹⁸F)] and endomorphin-1[Trp(7-CF₂¹⁸F)] was 5% (n = 4). The total radiosynthesis time was 90 minutes. Furthermore, octreotide[Trp(2-CF₂¹⁸F)] was obtained in a RCC of 29% (n = 3); C-H ¹⁸F-trifluoromethylation in the presence of

$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and *t*Bu hydroperoxide (TBHP) afforded a SA of 7.6 mCi/ μmol after purification by HPLC. The total synthesis time was 133 minutes. Interestingly, in the absence of Trp, Tyr-selective ^{18}F -trifluoromethylation was also demonstrated; a RCY (non-decay corrected) of 33% ($n = 3$) was reported for cyclic RGDyK.

5.4.2 C-H ^{18}F -labelling via radical chemistry at branched leucine

Radical chemistry is a relatively underexplored approach with regards to ^{18}F -labelling of peptides. In 2018, Britton and co-workers published a protocol for the use of photoactivated sodium decatungstate (NaDT) and ^{18}F -NFSI **96** for ^{18}F -labelling of unprotected peptides at the branched R group of leucine (Leu, L), as highlighted in Figure 100.¹⁴² Light of an optimum wavelength was used to photoactivate the decatungstate polyoxometalate (POM) catalyst which then catalysed hydrogen atom abstraction from Leu. ^{18}F transfer from **96** to the resultant carbon radical then occurred. The reaction could be completed rapidly under mild, aqueous conditions (acetonitrile-water, room temperature).

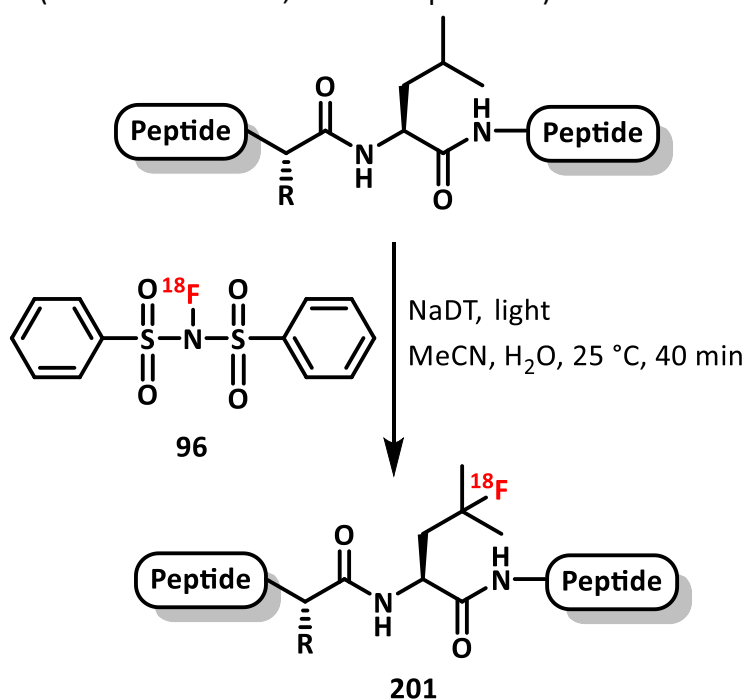


Figure 100: Direct ^{18}F -labelling at the branched R group of Leu.¹⁴²

^{18}F -labelling of various di- and tetrapeptides (all containing one Leu) was carried out.¹⁴² Generally, good RCYs (decay corrected) and RCCs were recorded (ranging from 16-40% and 26-55% respectively), and all peptides were obtained in a RCP of >95% (after HPLC purification). SAs ranged from 0.04-0.14 mCi/ μmol ; it was suggested that significant improvements in SA could be achieved via use of higher SA ^{18}F -fluorine gas to produce **96**.¹²⁵

The most significant shortcoming associated with this approach is lack of chemoselectivity; as well as Leu, the tertiary R group site of Ile and Val are also prone to fluorination; difluorinated dipeptides had an abundance of ~5% after 30 minutes.¹⁴²

5.5 Conclusion

Traditional methodologies for the ¹⁸F-labelling of peptides are associated with various disadvantages; direct, late-stage approaches typically require the use of harsh, non-physiological reaction conditions which are not compatible with sensitive biomolecules and ¹⁸F-labelled prosthetic group radiosyntheses are often multi-step in nature which is generally incompatible with the relatively short half-life of ¹⁸F.^{126,137,143} Significant efforts have recently been focussed on developing alternative strategies to circumvent these limitations; ¹⁸F-labelling of peptides using 'click chemistry' prosthetic groups has proved to be particularly valuable.^{150,182,187–189}

Unfortunately, attachment of a prosthetic group has the potential to influence the biological activity of a peptide, particularly in the case of bulky chelators.^{132,139} Trifluoromethylation of unmodified Cys/Trp has recently been presented as a way to directly ¹⁸F-label unprotected peptides without the prior introduction of a prosthetic group.^{140,141} Furthermore, the installation of a single fluorine atom using [¹⁸F]NFSI **96** and photoactivated NaDT to unmodified and unprotected Leu-containing peptides has been demonstrated by Britton and co-workers via the use of radical chemistry.¹⁴² However, lack of site-selectivity is associated with both of these approaches.

Precise ¹⁸F-introduction onto peptides provides significant advantages over non-selective methods including formation of predictable, homogeneous, fully characterisable PET imaging agents, whilst minimising the potential for disruption of their ability to engage a binding site.¹⁸⁷ Consequently, novel approaches for the direct, rapid and site-selective ¹⁸F-labelling of unmodified and unprotected biomolecules under mild reaction conditions are required. Despite the relative complexity of production of electrophilic sources of fluorine such as [¹⁸F]NFSI **96** and ¹⁸F-Selectfluor™ **97** vs ¹⁸F-fluoride, these compounds offer great potential as versatile reagents for the ¹⁸F-labelling of peptides.

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6.1 Synthesis of prosthetic group-modified TLQP-62 peptide

As highlighted in section 5.2.1.1, rapid, direct and site-selective ^{18}F -labelling of peptides can be achieved under relatively mild reaction conditions via nucleophilic aromatic substitution at a TMA-leaving group-containing activated aromatic ring.¹⁴⁸ This prosthetic group handle is small and planar and thus unlikely to interfere with the biological activity of TLQP-62.

6.1.1 Aromatic precursor compound synthesis

2-cyano-4-(methoxycarbonyl)-*N,N,N*-trimethylbenzenaminium triflate will be synthesised from 2-fluoro-5-formylbenzonitrile **202** (Figure 101).¹⁴⁸ Oxidation of **202** will result in a carboxylic acid **203**, which will be converted to an ester **204** via reaction with methanol in the presence of an acid. Crude **204** will be reacted with dimethylamine hydrochloride under basic conditions to result in formation of an aromatic dimethylamino group **205**. Methylation of this group will yield the positively charged aminium ion **206**.

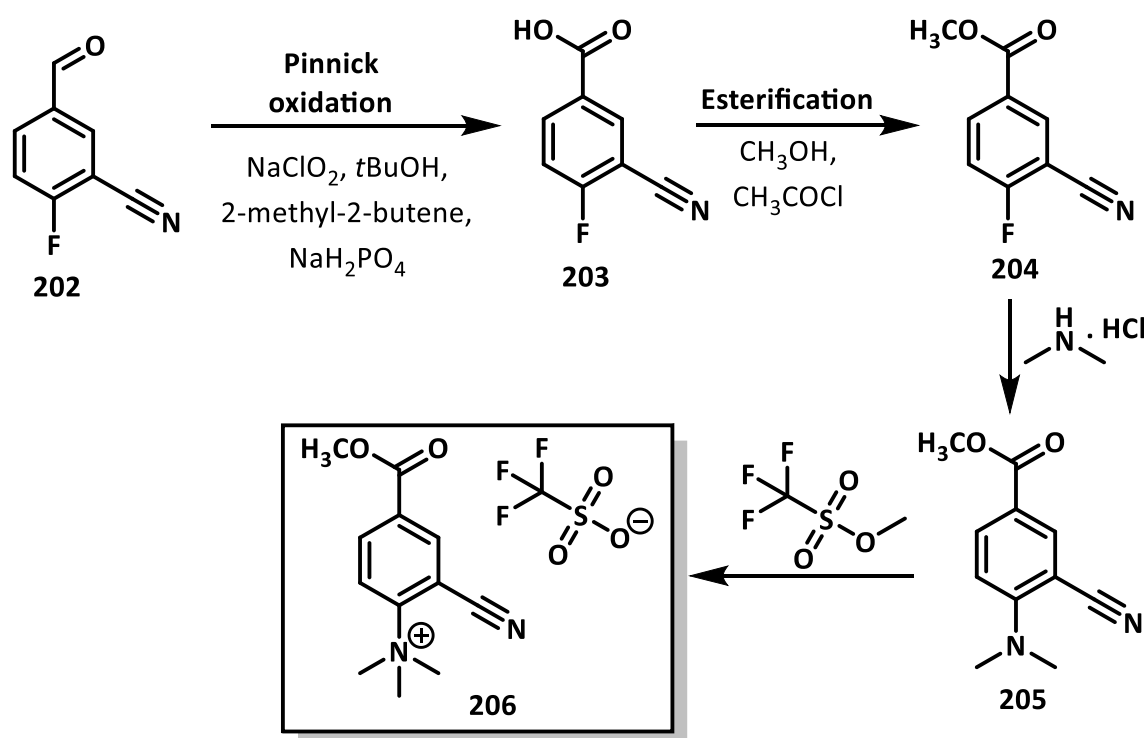


Figure 101: Schematic of synthesis of 2-cyano-4-(methoxycarbonyl)-*N,N,N*-trimethylbenzenaminium triflate from 2-fluoro-5-formylbenzonitrile.¹⁴⁸

6.1.2 Aromatic precursor acid synthesis

2-cyano-4-(methoxycarbonyl)-*N,N,N*-trimethylbenzenaminium triflate **206** will be converted to the corresponding acid (4-carboxy-2-cyano-*N,N,N*-trimethylbenzenaminium TFA salt) **207**, as shown in Figure 102.¹⁴⁸

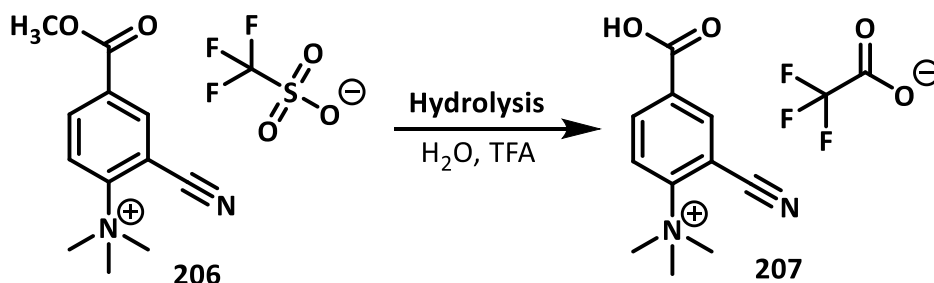


Figure 102: Hydrolysis of 2-cyano-4-(methoxycarbonyl)-*N,N,N*-trimethylbenzenaminium triflate via treatment with H₂O and TFA.¹⁴⁸

6.1.3 Synthesis of aromatic precursor acid-modified TLQP-62

As highlighted in Figure 103, 4-carboxy-2-cyano-*N,N,N*-trimethylbenzenaminium TFA salt **207** will be coupled to the N-terminus of a suitable resin bound, R group-protected, Fmoc-deprotected peptide of TLQP-62 [TLQP-62(N)] **208**.¹⁴⁸ **207** (2 eq.) and OxymaPure® **11** (2 eq.) will be dissolved in DMF.¹⁸ To this solution, DIC (2 eq.) **4** will be added and left to activate for 5 minutes. The solution will then be added to the resin (original amino acid loading = 1 eq.) and left to shake at room temperature for at least 4 hours. Coupling will be followed by peptide cleavage from resin using 30 vol% HFIP in DCM followed by treatment with DPDS and Bu₃P followed by acidolytic R group deprotection (TFA, TIS and water), precipitation and centrifugation. Purification of the resultant pellet by preparative RP-HPLC and lyophilisation will then be carried out. The precursor acid-modified selenoester **210** will be ligated to a purified TLQP-62 N-terminal (Sec)₂ dimer peptide [TLQP-62(C)] **211** (synthesised via Fmoc-SPPS); rapid additive-free DSL will be carried out followed by *in situ* deselenisation of the resultant peptide (Figure 104) to form **212**.

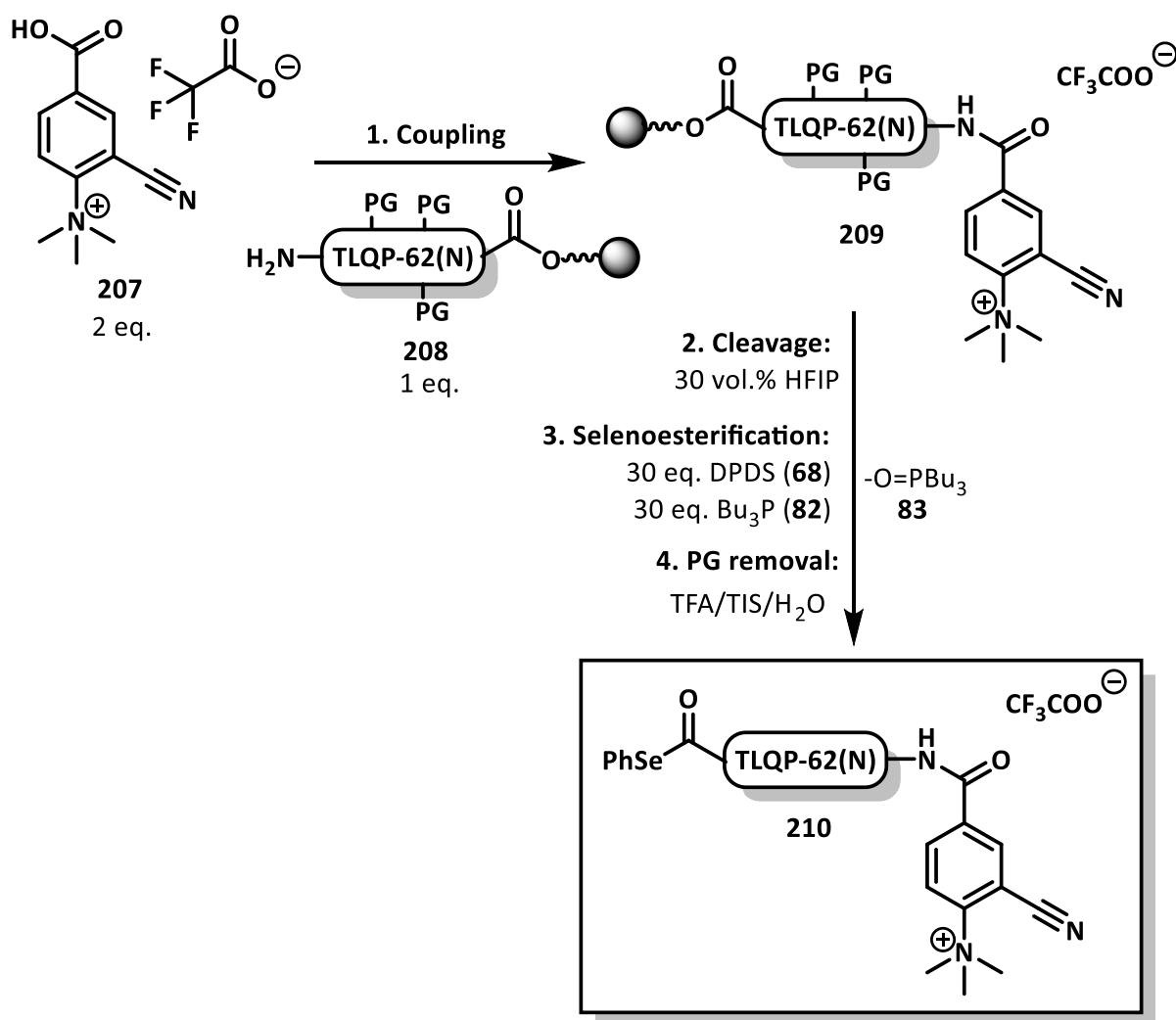


Figure 103: Schematic of synthesis of aromatic precursor acid-modified selenoester peptide of TLQP-62: coupling of 4-carboxy-2-cyano-*N,N,N*-trimethylbenzenaminium TFA salt **207** to TLQP-62(N) **208** (on resin), followed by peptide cleavage from resin using 30 vol% HFIP in DCM followed by treatment with DPDS and Bu₃P followed by acidolytic R group deprotection (TFA, TIS and water).^{18,148}

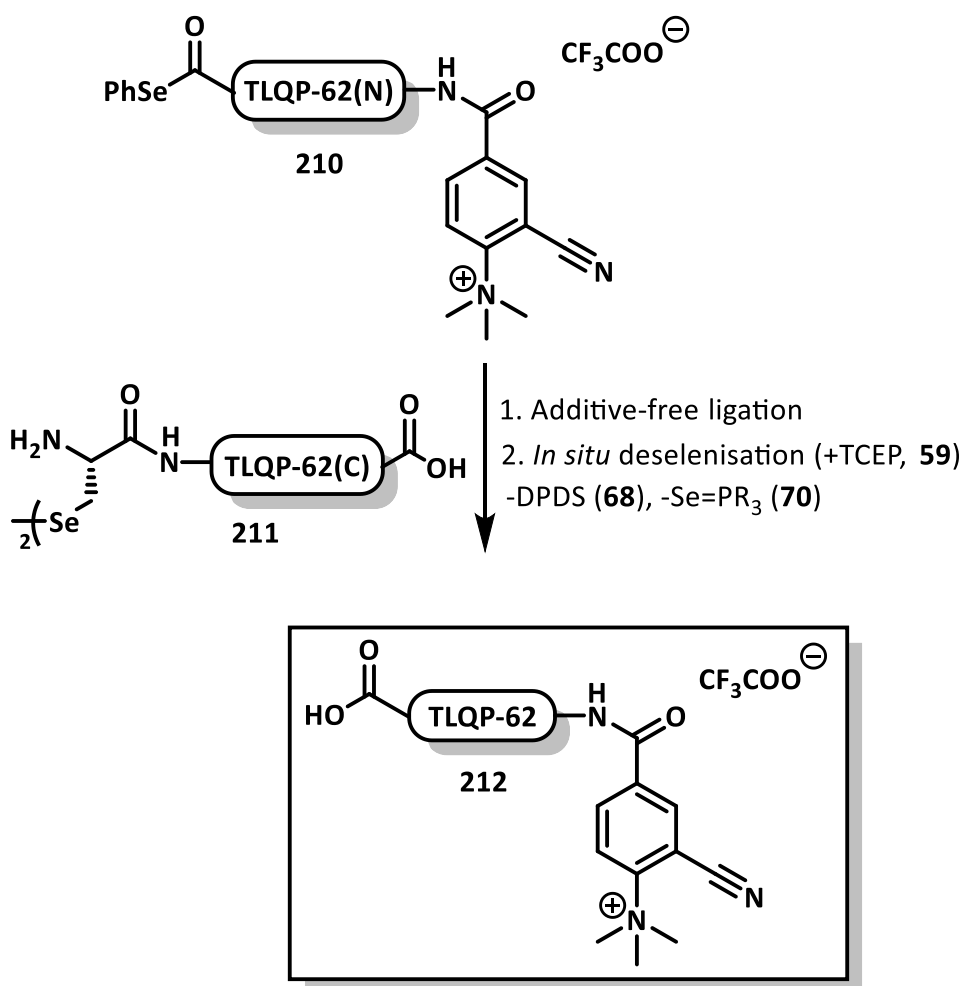


Figure 104: Schematic of synthesis of aromatic precursor acid-modified TLQP-62 **212**: DSL between the purified precursor acid-modified selenoester peptide **210** and purified TLQP-62(C) **211**, followed by deselenisation. R=C₂H₄CO₂H. Conditions: additive-free ligation: 6 M Gdn·HCl, 0.1 M Na₂HPO₄, pH 5.0-7.7; *in situ* deselenisation: (i) hexane extraction, (ii) TCEP (50 eq.), DTT (5 eq.), 16 h.^{18,148}

6.2 Fluorination of TLQP-62 peptide

N-terminal fluorination of purified aromatic precursor acid-modified TLQP-62 **212** at the pre-installed prosthetic group will be carried out in Dr Nicholas Mitchell's laboratory, University of Nottingham using K[¹⁹F]F/K_{2.2.2}, as per the established procedure (Figure 105).¹⁴⁸ Fluorination with the non-radioactive isotope ([¹⁹F]) will be used to infer ¹⁸F placement.

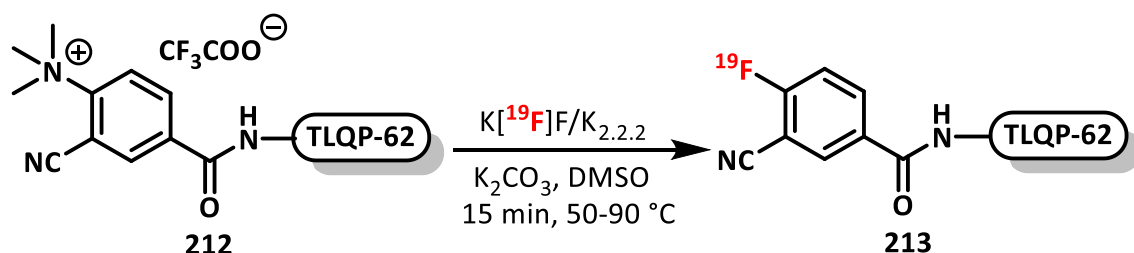


Figure 105: Nucleophilic aromatic substitution at a substituted arene moiety using K[¹⁹F]F/K_{2.2.2} resulting in ¹⁹F-labelling of TLQP-62.¹⁴⁸

6.3 Insulin secretion: fluorinated TLQP-62 peptide

The ability of [¹⁹F]TLQP-62 **213** to function as an insulin secretagogue will be assessed in relation to HA-TLQP-62 **75** and human TLQP-62 using INS1 cells, in advance of ¹⁸F-labelling.⁷⁰ Use of the non-radioactive isotope of fluorine will enable use of standard laboratory equipment. INS1 cells will be incubated with **213** (10 μM) in the presence of glucose (20 mM). The supernatant will be collected 1-hour post-treatment and insulin release will be measured using an ELISA according to manufacturer's instructions (Merk Millipore). The aromatic precursor group is small and planar; it is hoped that it will not interfere with the biological activity of the peptide.

6.4 [¹⁸F]TLQP-62 peptide studies

N-terminal fluorination of purified aromatic precursor acid-modified TLQP-62 **212** at the pre-installed prosthetic group will be carried out at the Medical School, University of Nottingham using K[¹⁸F]F/K_{2.2.2}, as per the established procedure (Figure 106).¹⁴⁸

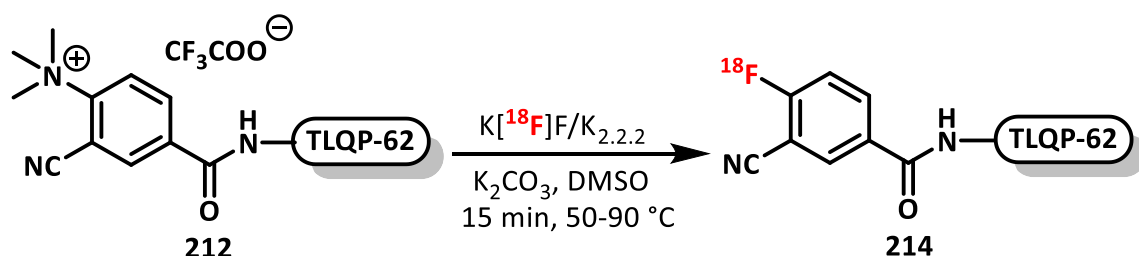


Figure 106: Nucleophilic aromatic substitution at a substituted arene moiety using K[¹⁸F]F/K_{2.2.2} resulting in ¹⁸F-labelling of TLQP-62.¹⁴⁸

Biodistribution data for [¹⁸F]TLQP-62 **214** will enable determination of which tissue(s) the peptide interacts with. Mice will be divided into two groups; they will receive a single IV infusion of either saline (control) or **214**. They will be placed on a PET-computed tomography

(CT) scanner bed at the Queen's Medical Centre whilst under anaesthesia (2% isoflurane). PET data acquisition will be made for 30 minutes. Additionally, a single IV infusion of ^{18}F -deoxyglucose (^{18}F FDG) will be made and PET data will be acquired for 120 minutes (time points will be based on a study published by Todde and co-workers).¹⁴⁶ The magnitude of tissue-specific uptake of ^{18}F FDG will be expressed as the average radioactivity in each region of interest (in kBq/cm^3) divided by the injected dose (kBq) and multiplied by the body weight of each animal (kg).

A second study will involve catheterisation of mice in a tail artery under local anaesthesia. Following recovery, they will receive a single IV infusion of ^{18}F TLQP-62 **214**; blood will be collected at 5-minute intervals over 120 minutes.¹⁹¹ The radioactivity of blood plasma will be determined using a gamma-ray spectrometer. Acquisition of **214** half-life data will enable determination of whether TLQP-62 reaches the tissue(s) in a pharmacologically active concentration, inferring whether longer acting analogues are required.

6.5 Cleavage site-modified ^{18}F TLQP-62 peptide studies

Proposed work, as indicated in sections 6.1 and 6.4 will be carried out using a modified version of human TLQP-62; removal of the internal cleavage motif will occur via substitution of residues 573-576 (SRHY). It is predicted that non-polar, hydrophobic amino acids will be included in the peptide as alternatives. Such an alteration of TLQP-62 will ensure that acquired data will relate to TLQP-62 and not the truncated version of this peptide (TLQP-21).

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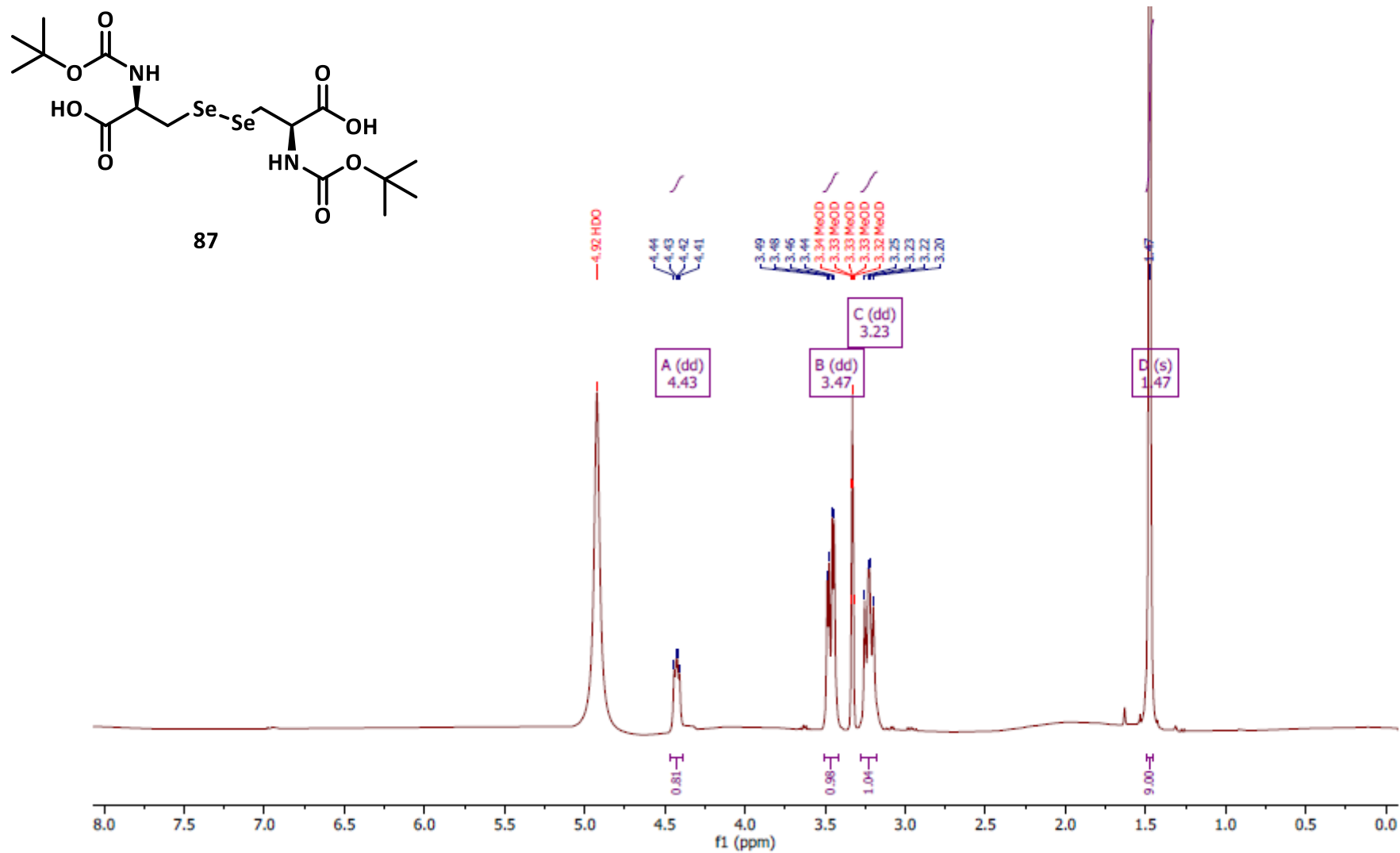
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^1H NMR spectrum for (Boc-Sec-OH) $_2$ 

^{13}C NMR spectrum for (Boc-Sec-OH) $_2$ 