Synthesis of Cyclodextrin Composites Incorporating Targeting and Drug Carrying Capabilities

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ABSTRACT

Synthesis of Cyclodextrin Composites Incorporating Targeting and Drug Carrying Capabilities

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A selective, versatile, and robust methodology for the bi-functionalisation of β-cyclodextrin has been developed which allows for the attachment of peptides and/or sulfonamides in varying *C*- and *N*-terminal combinations on resin using *N*-Fluorenylmethoxycarbonyl (Fmoc) Solid Phase Peptide Synthesis (SPPS). Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin, an amino acid based bi-functionalised derivative of β-cyclodextrin, has been functionalised with the bioactive peptide, bradykinin, and/or sulfonamides using Fmoc SPPS on Rink resin. The all-in-one molecule contains a carrier (cyclodextrin), targeting agent (bradykinin) and/or drug (sulfonamide). Varying combinations of these bradykinin-focused molecules have been synthesised in an attempt to determine the structure-function relationship against cancer cell lines using cell-based screening *in vitro*.

This study commenced with the synthesis of two linkers on to cyclodextrin. This enabled selective binding directly on to the resin, or a peptide attached to the resin. Peptide growth and/or cleavage from the resin followed allowing for the synthesis of peptide-cyclodextrin species in various combinations. Fmoc SPPS techniques have been employed to allow for the addition and synthetic extension of peptides on to cyclodextrin. Peptide purification was achieved by reverse phase high pressure liquid chromatography, and nuclear magnetic resonance spectroscopy and mass spectrometry were used to determine the success of the coupling reactions and identification of cyclodextrin regio isomers. Sulfonamide additions to the cyclodextrin and/or peptide compounds were obtained after numerous studies investigating the optimal reaction conditions. 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl chloride was found to give the highest yields for the synthesis of *C*-terminal peptide sulfonamides with 4-carboxybenzenesulfonamide giving the highest optimal yields for *N*-terminal peptide sulfonamides. Peptide coupling efficiency of cyclodextrin and sulfonamides were investigated and optimised by comparing different SPPS resins and solvents. The incorporation of spacers between the peptide/cyclodextrin and/or resin have also been investigated in an attempt to improve overall reaction yields.

Preliminary bioassay testing against tumour cell lines HT-29 Human Duodenum, Hs700T Human pancreatic adenocarcinoma, and MA-104 Human pancreatic adenocarcinoma were performed. The MTT assay and the flow cytometry assay were used to show the effect of varying combinations of these cyclodextrin-peptide-sulfonamide molecules against the three cell lines and compared to a known anticancer drug, 5-Fluorouracil. Despite employment of simple entities in the construction of these compounds, an increase in cell proliferation (*ca* 10-20%) was seen for some cyclodextrin-bradykinin complexes. In addition, an exposed *C*-terminus on the bradykinin-sulfonamide moiety and an exposed *N*-terminus on the cyclodextrin-bradykinin sulfonamide moiety both gave positive results. Mixed results were obtained with the addition of a linker between the cyclodextrin and the bradykinin molecules (less then 5% increase or decrease) compared to their non-linker counterparts.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Amino acid
Ala	Alanine (amino acid)
aq	Aqueous
Arg	Arginine (amino acid)
β-CD	Beta-cyclodextrin
B_1, B_2	Bradykinin receptors
Bi-	Two units added
BK	Bradykinin
Boc	*Tert*-butyloxycarbonyl
BOP	l-Benzotriazolyloxy-tris-dimethylamino-phosphonium-hexafluorophosphate
br	Broad (spectral)
Bn-	Benzyl ester (protecting group)
^{13}C NMR	Carbon. In reference to NMR spectroscopy utilising the ^{13}C isotope of carbon
2ClZ	2-Chloro-Z, *N*-(2-Chlorobenzyloxycarbonyloxy)
C-18	HPLC column coated with a carbon 18 reverse phase
CA	Carbonic anhydrase
Cbz	Carbobenzyloxy (protecting group)
Cbz-OSu	*N*-α-(Benzyloxycarbonyloxy) succinimide
CD	Cyclodextrin
CD OH2, OH3	Cyclodextrin secondary hydroxy groups labelling
CD C1-C6	Carbon labelling for an amylose ring
cm	Centimetres
COSY	Correlation spectroscopy
***C*-terminus**	Carboxy terminal (of a peptide)
Cys	Cysteine (amino acid)
4-DMAP	4-Dimethylaminopyridine
d_6-DMSO	Dimethyl-d_6 sulfoxide
d	Doublet (spectral)

D-	D-optical isomer (of amino acids)
Da	Daltons
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-Dicyclohexylcarbodiimide
De-Boc	Removal of the *tert*-butyloxycarbonyl protecting group
DEPT	Distortionless enhancement by polarization transfer
Des	One less of something
Dhbt	3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DHPS	Dihydropteroate synthase
DIEA	*N,N*-Diisopropylethylamine (Hunig's base)
DIPCD	*N,N'*-Diisopropylcarbodiimide
DMEM	Delbecco's modified eagle's medium
E7070	Sulfonamide
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDT	Ethanedithiol
EDTA	Ethylenediaminetetraacetic acid
Endo-	Internal functionalisation
EPR	Enhanced Permeability and Retention factor
equiv	Equivalent(s)
ESI-MS, ESI	Electrospray Ionisation Mass Spectrometry
Exo-	External functionalisation
5-FU	5-Fluorouracil
FCS	Foetal calf serum
Fmoc	*N*-Fluorenylmethoxycarbonyl
Fmoc-OSu	*N*-(9-Fluorenylmethoxycarbonyloxy) Succinimide
G0/G1	Cell cycle phase(s)
G1	Cell cycle phase
G2	Cell cycle phase
G2/M	Cell cycle phase(s)
g	Gram(s)
Gly	Glycine (amino acid)
g/mol	Grams per mole

^{1}H NMR	Proton (hydrogen). In reference to NMR spectroscopy utilising the ^{1}H isotope of hydrogen
H^{+}	Proton
h	Hour(s)
HATU	2-(6-Aza-1-H- Benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HBTU	O-Benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HCTU	2-(6-Chloro-1-H-Benzotriazole-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
Hexa-	Six units added
His	Histidine (amino acid)
HMP resin	4-Hydroxymethylphenoxyacetyl-4'-methylbenzyhydryl-amine resin (Wang resin)
HOBT	Hydroxybenzotriazole
HPLC	High pressure liquid chromatography
HR-MS	High resolution mass spectrometry
Hs700T	Cancer cell line
HT-29	Cancer cell line
Hz	Hertz
IFNHH	Institute of Food Nutrition and Human Health
Igl	α-Amino-2-indanacetic acid
Ile	Isoleucine (amino acid)
In situ	In the same place
in vacuo	In vacuum
IR	Infrared spectroscopy
J	Coupling constant (in NMR spectrometry)
K	Kilo
K-562	Cancer cell line
K^{+}	Potassium ions
L-	L-optical isomer (of amino acids)
Leu	Leucine (amino acid)
Linker	Joins the resin to the functional group in solid phase peptide synthesis

l/h	Litres per hour
LR-MS	Low Resolution Mass Spectrometry
m	Multiplet (spectral)
M	Cell cycle phase
M^+	Parent molecular ion
MA-104	Cancer cell line
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight
MBHA	*p*-Methyl-benzylhydrylamine
MCF-7	Cancer cell line
Met	Methionine, amino acid
mg	Milligram
MHz	Megahertz
min	Minute(s)
mL	Millilitre(s)
mL/min	Millilitre per minute
mmol	Millimole
MMP	Matrix metalloproteinases
mol	Mole(s)
mol/L	Moles per litre
Mono-	Single, one unit added
MS	Mass spectrometry
Mtr	4-Methoxy-2,3,6-trimethylbenzensulfonyl group
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
m/z	Mass-to-charge ratio
Na^+	Sodium ions
nm	Nanometre(s)
NMM	*N*-Methylmorpholine
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung carcinoma
***N*-terminus**	Amide terminal (of a peptide)
Nu^-	Nucleophile
OBt	Oxy-benzotriazole (active ester)
OC	Oxalyl chloride

OD	Optical density
PABA	Para-aminobenzoic acid
PAM resin	4-Hydroxymethylphenylacetamidomethyl resin
Pbf	2,2,7,7,8-Pentamethylchromane-6-sulfonyl
Pd/C	Palladium on carbon (catalyst)
PEG	Poly(ethylene glycol)
PEGA-based support	PEG copolymers, e.g. bis 2-acrylaminoprop-1-yl-poly-ethylene glycol
Pfp	Pentafluorophenylesters
Phe	Phenylalanine (amino acid)
PI	Propidium iodide
Pip	Piperidine
ppm	Parts per million
Pro	Proline (amino acid)
PS	Polystyrene
PSA	Preformed symmetrical anhydrides
PSG	Penicillin-streptomycin-L-glutamine
Psi	Pounds per square inch
PyBOP	1-Benzotriazolyloxy-tris-pyrrolidino-phosphonium hexafluorophosphate
PyBroP	Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate
q	Quartet (spectral)
rbf	Round bottom flask(s)
R_f	Retention factor (in chromatography)
RP-	Reverse phase
rpm	Revolutions per minute
t_R	Retention time (in chromatography)
rt	Room temperature
s	Singlet (spectral)
S	Cell cycle phase
S/G2/M	Cell phases of a cell cycle
SCLC	Small cell lung carcinoma
Sec	Second(s)

SEM	Standard error of the mean
Ser	Serine (amino acid)
Spacer	Moiety used to separate various components in cyclodextrin/sulfonamide/peptide complexes
SPPS	Solid phase peptide synthesis
t	Triplet (spectral)
***t*-butyl, *t*-Bu**	Tertiary-butyl group
TBTU	O-Benzotriazol-1-yl-*N,N,N'*-tetra-methyluronium tetrafluoroborate
TCFC	Tetramethyl chloro formamidinium hexachlorophosphate
TCFH	Tetramethyl chloro formamidinium hexafluorophosphate
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFFH	Tetramethyl fluoro formamidinium hexafluorophosphate
TFMSA	Trifluoromethanesulfonic acid
THC	Tetrahydrocannabinol
Thr	Threonine, amino acid
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
TMU	Tetramethyl urea
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TOF	Time-of-flight
Tri-	Three units added
Trp	Tryptophan (amino acid)
Ts, Tos	*N*-Tosyl
Tyr	Tyrosine (amino acid)
UV-Vis	Ultraviolet-visible spectroscopy
V	Voltage
Val	Valine (amino acid)
WSCD	Water soluble carbodiimide
XXX_1	Refers to the position of the amino acid in a peptide chain
6^A-	Number location for the attachment of functional groups onto cyclodextrins primary hydroxy groups

6^X-	Unknown positional attachment to cyclodextrins primary hydroxy groups
°C	Degrees centigrade
µL	Microlitre(s)
µg	Microgram(s)
µmol	Micromolar(s)
µg/mL	Microgram per millilitre
µL/min	Microlitre per minute
γ-	Gamma
δ	Chemical Shift in parts per million
🟣	Spacer (ε-aminocaproic acid)
🔴	Rink amide resin
🟣	TentaGel resin
🟢	PAM resin
🔴–🟣	Rink-spacer resin
○	Peptide(s)
🔵	Sieber resin
⚪	Sulfonamide replacement for PABA
◉	Polymer support
🟠	Side-chain protecting group
●	N-α-protecting group
🔵	Activating group(s)
⚫	Para-amino-benzoic acid (PABA)
🟦	Enzyme(s)
🟪	Glutamic acid substrate
🟦🟪	Folic acid
⬜	*N*- and/or *C*-terminal peptide
⬛	Protecting group(s)
🟪	*C*-terminal sulfonamide, 4-aminobenzenesulfonyl chloride
🟦	*N*-terminal sulfonamide, 4-carboxyenzenesulfonamide
🟥	Bradykinin peptide
🟩	Val-Gly-Ala (tri-peptide)

Chapter One

Introduction

1.0 Cancer

The second most common health affliction affecting humans from unborn foetuses to elderly people is cancer. Plants and animals are also affected by cancer. Although knowledge of this disease dates back to the late 18th century, its causes and prevention still elude us. Cancer can affect all body parts, from skin to the vital organs and blood, with the chance of getting cancer increasing as people become older. In New Zealand, women are more affected than men with Maori people more highly affected than any other race.[1] About 80% of cancer is thought to be lifestyle or environmentally related with 25% of all cancer deaths associated with smoking.[1] With early detection and screening for cancer, rates have clearly decreased for certain types, including cervical and breast cancer, but overall cancer remains a big problem.[1]

Cancer is a group of aggressive, invasive, and often mobile cells that divide without the control that usually limits or inhibits normal bodily processes and can result in death. There are two types of cancerous cells. Malignant cancer cells are able to spread invasively throughout the body destroying healthy tissues, whilst benign tumour cells are self-limited in their growth and do not invade other cells. It is possible however, that benign tumour cells can change into malignant cells over time.[1]

Abnormalities within the genetic material of the cells can be passed on when the cells are transformed. These abnormalities may be due to carcinogens or cancer-forming agents such as tobacco smoke, sprays, or radiation. Cancer abnormalities may also be formed by randomly acquired genetic errors in the DNA replication cycle, or are inherited from birth.[1]

There are two genes associated with cancer and these have been the targets of much cancer research. Oncogenes are cancer-promoting genes that are activated in cancer cells determining the phenotype of the cancer. These genes give cancer cells the ability to increase their growth rate and division, as well as protecting them against programmed cell death. Tumour-suppressor genes are inactivated in cancer cells thereby resulting in the loss of normal cellular functions such as accurate cell replication, control of the cell cycle, cell orientation and adhesion within tissues, and interaction with protective cells of the immune system. Therefore, tumours are a mass of uncontrollable cells, aligned randomly, with the ability to spread throughout the body.[1]

1.0.1 *Current Treatment Methods*

Cancer classification is based upon the tissues the cancerous cells originate from, as well as their histology. For example, carcinoma can be a type of malignant tumour derived from epithelial cells. This group represents the most common forms of cancer including breast, lung, prostate, and colon cancer. Most cancers can be treated by an array of treatments depending upon the location, type, and stage the cancer is at. Common treatment methods include a combination of surgery, radiation, and chemotherapy. However, each treatment has potential side effects arising from toxicity (chemotherapy) or damage (radiation) to normal cells within the body. Surgical removal of tumours is only applicable where the tumour cell is easily accessed without damage to any other tissues or organs. Complete removal of the tumour is hard to determine as tumour cells easily spread throughout other parts of the body.[1]

Metals have also been important for the control of cancer. A cis-platinum complex (Figure 1-1) can be injected into tumour cells successfully killing them. However, it does cause side effects of nausea, vomiting, and damage to healthy cells. In most cases, decisions regarding treatment depends upon the quality of life that the cancer patient will have during and after the treatment.[1]

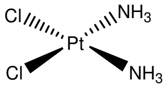


Figure 1-1 Structure of cis-platinum, a drug used in the treatment of cancer

1.0.1.1 Radiation Therapy

Radiation therapy uses ionising radiation to kill cancer cells and shrink tumours. It is a localised treatment but it can also destroy or injure other healthy cells in the area being treated. It can be used on almost all types of solid tumours, such as in the brain or in the breast, as well as for the treatment of leukaemia and lymphoma.[1]

1.0.1.2 Chemotherapy

Chemotherapy is the treatment of cancer with cytotoxic drugs that destroy the cancer cells in an untargeted manner.[1] Cytotoxic drugs are a heterogeneous class of chemical compounds that treat cancer primarily by toxicity to cells that are rapidly growing and dividing. Because cancer cells often undergo rapid growth and proliferation, these agents preferentially kill them. These substances are usually administered intravenously in the form of a free drug solution and despite the long history of their use and development, treatment failures are still frequently encountered. For example, breast cancer treatment using these drugs has low positive clinical outcomes.[2] Some pitfalls for cytotoxic drugs include:[2]

- The hydrophobic nature of these drugs promotes their precipitation in aqueous media
- They suffer from poor selectivity for target tissues
- They undergo bio-distribution in the body volume with severe side-effects in sensitive non-target areas
- The rapid leakage of fluid/macromolecules from cells can produce damage to healthy cells
- Rapid clearance from the body
- Metabolic degradation *in vivo* means larger dose volumes need to be administered
- They are susceptible to resistance

5-Fluorouracil (5-FU, Figure 1-2) is an example of a cytotoxic drug and is an antimetabolite of the pyrimidine analogue type, which shows a broad spectrum of activity against solid tumours, alone or in combined chemotherapy regimes. 5-FU interacts with the nucleosides metabolism due to its structure and is able to be incorporated into RNA and DNA, therefore leading to cell death.[2]

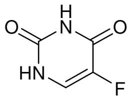


Figure 1-2 Structure of 5-Fluorouracil (5-FU), a drug used in the treatment of cancer

1.0.2 *Cancer Research*

Current cancer research is focused on the causes of cancer, as well as methods for prevention, better diagnosis, treatments, and cures. This research ranges from molecular bioscience bench to clinical trials, to epidemiology and biotechnology. During the mid-1990's, immuno- and gene-therapy as potential treatments for cancer were developed. This came after the better understanding of the genes that control cancer cells, as well as the progression of cancerous cells throughout tumour growth.[1] However, tumour resistance to drugs still poses a large problem in prolonged cancer treatments. New drug design has focused on the specific targeting of cancer drugs in order to reduce the side effects of current treatments and increase the drug's potential in the fight against cancer.[1]

1.1 Peptides in Drug Design

1.1.1 *Drug Discovery*

Past drug design was based upon serendipity or by the identification of active ingredients from traditional medicines. However, due to new technology and a wider knowledge of the chemical and biomedical world, a new approach in the understanding of how disease and infection are controlled at the molecular and physiological level is now being used for the design of new drugs.[4]

Effective drug design is based upon identifying suitable biological targets and tailor making a drug to suit. Ideally, the target molecule is involved in the specific diseased signalling or metabolic pathway affecting its survival. However, such drugs run into problems of minimal drug specificity where they have the potential to target other similar pathways. This is where specific drug targeting peptides/proteins come into play. These have potential in the delivery of a specific drug to a particular target maximising the effect of the treatment and minimising side effects.[4]

1.1.2 *Peptides*

Peptides are natural or synthetic compounds containing two or more amino acids linked by the carboxyl group of one amino acid, to the amino group of another amino acid. Peptides are structurally identical to a protein, but are smaller in length. Peptide classes include many hormones, antibiotics, and other compounds that participate in the metabolic functioning of living organisms. The number of amino acids in a peptide is indicated by the prefix where a dipeptide contains two amino acids, a nonapeptide contains nine amino acids, and a polypeptide contains many amino acids. In some literature, a peptide is classified as a protein when its molecular weight is 10,000 Da or 100 amino acids in length.[5]

1.1.3 *Methods of Peptide Preparation*

Various routes depending upon the structure, length, and application can be employed to prepare peptides. These methods include enzymolysis, partial hydrolysis (for example; gelatine is prepared from the partial hydrolysis of collagen)[6] and chemical synthesis as described below.[5, 7]

1.1.3.1 Solution Synthesis

Peptide solution or liquid phase peptide synthesis is a classical method for large-scale syntheses. It comes with a considerable labour component in that each segment of the growing peptide should be purified. This method involves high labour input, considerable chemical input, and potential problems often arise from solubility. Due to this, most laboratories employ solid phase peptide synthesis (SPPS).[5,7]

1.1.3.2 Solid Phase Peptide Synthesis (SPPS)

Solid phase peptide synthesis (SPPS) was first proposed in 1962 by Bruce Merrifield[5,7-8] and is based upon the sequential addition of suitably protected α-amino and orthogonal protected amino acids to an insoluble polymeric support. This support contains reactive sites, which chemically attach to the *C*-terminal amino acid of the developing peptide chain. This enables higher yields and faster peptide production followed by an easier and more successful separation and purification using HPLC than peptide solution synthesis. This insoluble support system also enables the removal of excess reagents and amino acids without loss of the growing peptide. Thus individual peptide intermediates do not have to be isolated, purified, and coupled further. Preparation of peptides using this method involves three distinct steps as follows: [6-9]

- Stepwise chain assembly on a resin. This procedure offers various chemical strategies that enable a high synthesis success rate including the use of various activating agents to aid amino acid coupling.

- Sequential cleavage and deprotection of the fully protected resin-bound chain leading to a cleaved, deprotected crude peptide. Alternatively, a partially protected peptide can be obtained.

- Purification and characterisation of the target peptide. This step is more or less invariant to the methods used to generate the crude peptide product. Purification is usually carried out on a reversed-phase (RP) HPLC column using C-18, C-8, or C-4 columns with characterisation by MALDI or ESI-MS methods. However, ion exchange and silica columns can be used for purification of 'greasy' hydrophobic peptides.

There are two major chemistries for SPPS. The base-labile Fmoc-group (*N*-fluorenylmethoxycarbonyl) or the acid-labile Boc-group (*tert*-butyloxycarbonyl) is used for *N*-α-amino-protection (Figure 1-3).[5,7-8] After removal of this protecting group, the next protected amino acid is added either using a coupling reagent *in situ* or a pre-activated protected amino acid derivative.[10]

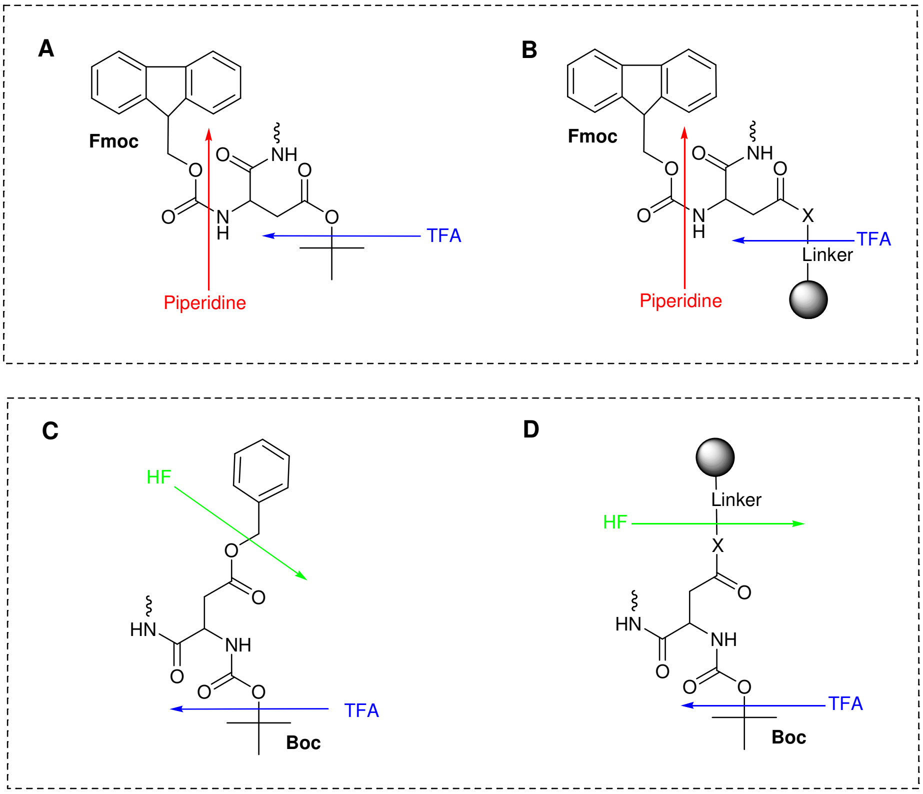


Figure 1-3 Protecting group strategies in SPPS. *A)* General protecting and side chain group cleavage strategy for Fmoc SPPS. *B)* General protecting group and resin cleavage strategy for Fmoc SPPS. *C)* General protecting and side chain group cleavage strategy for Boc SPPS. *D)* General protecting group and resin cleavage strategy for Boc SPPS. Where X is oxygen or NH functionality depending upon the resin used.[10]

Cleavage of the Boc protecting group is achieved by trifluoroacetic acid (TFA) and the Fmoc group by piperidine (pip). Final peptide cleavage from the resin and orthogonal deprotection requires strong acid, such as hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA) in the case of Boc chemistry, and TFA in the case of Fmoc chemistry (see Figure 1-3).[5,10] Figure 1-4 describes the general scheme for both Fmoc- and Boc-SPPS. The resulting peptide is attached to the resin via a linker, through its *C*-terminus and may be cleaved to yield a peptide acid or amide, depending upon the linker used. Orthogonal protecting groups are often chosen so as to be cleaved simultaneously with peptide detachment from the resin.[10]

Peptide synthesis can be carried out in a batch-wise or continuous manner. In batch-wise synthesis, the peptide-resin is contained in a fritted reaction vessel and reagents are added and removed under manual or computer control. In the continuous flow method, the resin is contained in a column through which reagents and solvents are pumped continuously, again under manual or automatic control. Only the Fmoc synthesis is fully compatible with the continuous flow method which also enables spectroscopic monitoring of the deprotection/coupling steps.[5,10]

1.1.4 *Fmoc- versus Boc-SPPS*

Concerns about the repetitive TFA acidolysis employed for the Boc-deprotection, which can lead to a rearrangement of sensitive peptide bonds, and acid catalysed side reactions lead to the development of a milder synthesis technique, Fmoc synthesis. In Fmoc synthesis, the growing peptide is subjected to milder base treatment and TFA is only used in the cleavage of the peptide from the resin (last step). By contrast, Boc cleavage requires the use of dangerous anhydrous HF and expensive all-Teflon laboratory apparatus. This is not available to many researchers.[5,8,10] Fmoc SPPS chemistry generally generates higher yields and better quality peptides with the use of less harsh reagents.[8] Impurities in Boc SPPS are attributed to the strong acid cleavage reagents, dehydration, and *t*-butylation.[5,10] However, for some peptides, Boc SPPS may be better than Fmoc SPPS and vice versa. Surveys have shown that with skilled and experienced hands, either Fmoc or Boc SPPS can give good results, but Fmoc SPPS in the hands of an ‘average user’ are more likely to provide the best avenue for routine synthesis of peptides.[5,8,10]

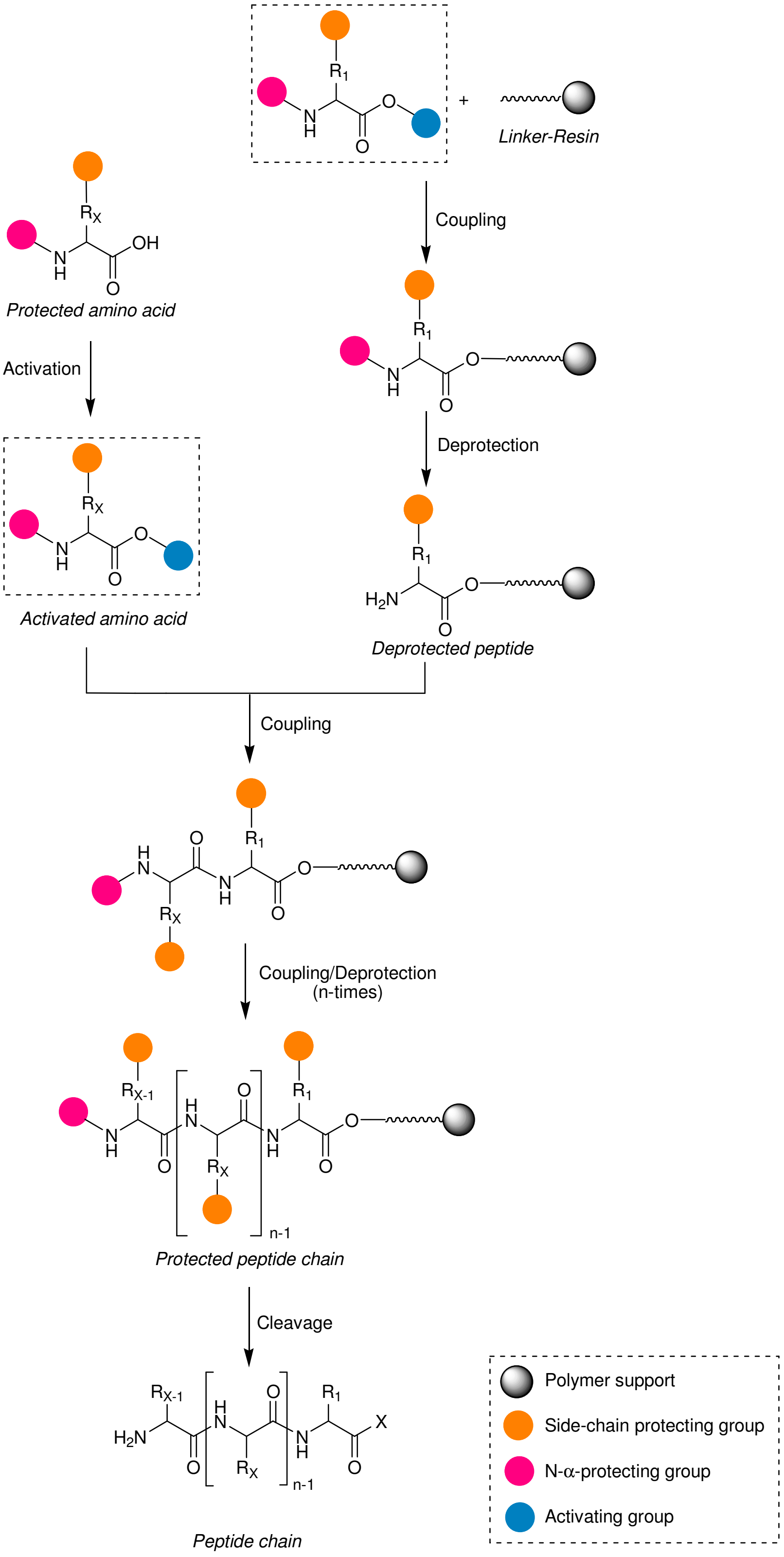


Figure 1-4 General scheme of SPPS.[5,8,10]

1.1.5 *Resins*

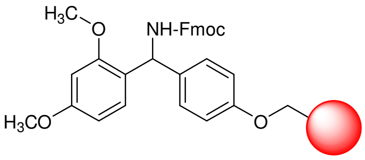
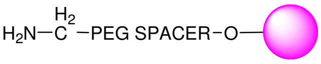
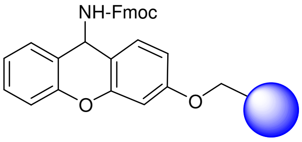
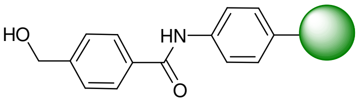
Advantages of SPPS over solution synthesis include:[5,8,10]

- Excess reagents allows the reactions to be driven to completion
- Excess reagents and soluble by-products can be eliminated by washing the resin
- Physical losses are minimal since the product remains attached to the resin throughout the synthesis
- The process is readily automated
- Single resin beads can function as “micro-reactors enabling large numbers of compounds to be prepared simultaneously by parallel or serial combinational methods

For peptide synthesis, the use of resins with low cross-linking and small particle size is favoured. These resins allow for the rapid diffusion of reagents into and around the beads giving good swelling capability that enables them to better accommodate the bulk of the growing peptide chain. Polystyrene-based supports are normally employed for batch-wise synthesis, but can also be used in continuous synthesis, provided that the flow rate is low, or the resin is co-packed with glass beads. However, for continuous flow synthesis, polyethylene glycol (PEG) based resins are preferred. This is due to the solvation of the PEG which confers stability on the polymer, making them suitable for use in pumped-flow systems.[10] Ideally the peptide and the polymer support should be equally solvated during peptide synthesis to allow for an enhanced synthesis rate and yield. This is not the case with polystyrene-based resins. Polyamide resins based upon polyacrylamide polymers were therefore developed for peptide synthesis to closely mimic the properties of a peptide chain.[7] These resins are ideal for continuous flow SPPS methodology but not suitable for automated synthesis if the vessels are small due the large swelling capabilities.[7]

Solvation and swelling potential of the resin are also important in obtaining maximum couplings and yields. Dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) are the primary solvents used for resin deprotection, coupling, washing, and solvation.[5,10] Resins used in this study include Rink amide, Sieber amide, TentaGel, HMP, and PAM resins (see Table 1-1 for more details):[10]

Table 1-1 Resins used in this study

Resin	*C-terminal functionality*	*Cleavage conditions*	*Fmoc- or Boc-SPPS*	*Structure*[5,10]
Rink amide	Amide	90-95% TFA	Fmoc-SPPS	
Tentagel	Amide	20-95% TFA	Fmoc-SPPS	
Sieber amide	Amide	1-5% TFA	Fmoc-SPPS	
PAM	Carboxy	HF, TFMSA	Boc-SPPS	

1.1.5.1 Rink Amide Resin

The Rink amide producing functionality can be added to a variety of polystyrene (PS), polystyrene-polyethylene glycol graft copolymers, and polyethylene glycol copolymers (PEGA) generating the Rink amide resin. Rink resin is also more chemically stable towards strong reducing agents however, breakdown of the amide linker can occur in high concentrations of TFA leading to the formation of highly coloured by-products.[5,10]

1.1.5.2 TentaGel Resin

TentaGel resin is a composite of low cross-linked polystyrene and 3000-4000 molecular weight PEG, which has been terminally amino functionalised. As a result, high diffusion rates, excellent swelling properties in a range of solvents, and small bead size makes it ideally suited for the synthesis of serial and parallel libraries by SPPS. Cleavage conditions are similar to that of Rink amide resin.[5,10]

1.1.5.3 Sieber Amide Resin

The Sieber amide resin is the preferred support for the synthesis of protected peptide amide fragments and acid-labile carboxamides as the product can be cleaved by mild acidolysis (1-5% TFA in DCM). This is due to the amino group being less hindered than that of Rink amide resin making it better suited to applications where there are sensitive amino acids or steric factors involved. Thus, Sieber amide resin was found to give better results than Rink amide resin in synthesis studies conducted by Chan *et al.*[5,10]

1.1.5.4 PAM Resin

PAM resin has become the most widely used resin in Boc chemistry as it has greater acid stability than the Merrifield resin (a polystyrene based resin), making it more suited for synthesising medium to large peptides. Attaching the initial amino acid to the resin requires special conditions to activate the amino acid, including the use of HOBt, which is added to reduce epimerisation.[5,10]

1.1.5.5 Potential Problems

Peptides containing cysteine (Cys), histidine (His), proline (Pro), methionine (Met), and tryptophan (Trp) residues at the *C*-terminus require special coupling and/or cleavage conditions. For example, Cys and his are prone to racemisation when attached to hydroxymethyl-based resins such as Wang. Trityl-based resins are also recommended for the preparation of peptides containing *C*-terminal Pro, Met, and Trp residues due to the formation of example, diketopiperazine (proline cyclisation, Figure 1-5) during Fmoc deprotection.[8] Alkylation of Met and Trp by cations generated during the cleavage reaction can result in the reattachment of the peptide to the support. In the latter two instances, by the use of the suitable resins, these reactions are reduced or eliminated due to the bulkiness of the linker employed.[5,8,10]

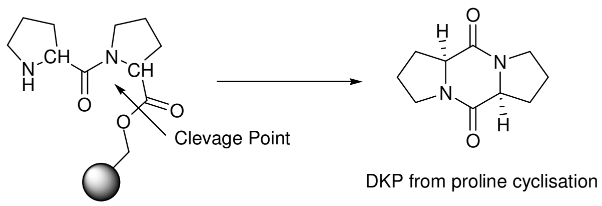


Figure 1-5 Diketopiperazine formation from the cleavage of di-proline peptides attached to resin.[8]

1.1.5.6 Basic Resin Handling

To ensure all possible reaction sites are exposed, it is important to ensure that the resin is fully swollen before use and the best solvent is employed. For example, polystyrene resins swelling ability varies depending upon the solvent used. Therefore, the simplest way to swell such resins would be to initially swell in DCM and then exchange this for the reaction solvent, such as DMF.[5,10]

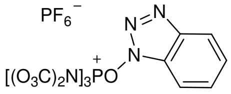
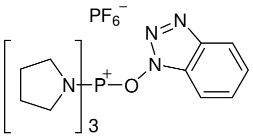
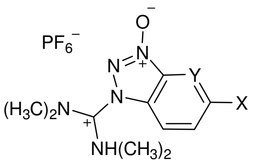
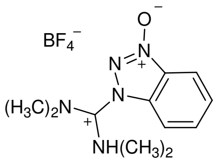
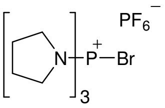
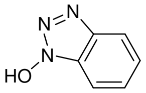
1.1.6 *Coupling Methods*

Efficient peptide-bond formation requires chemical activation of the carboxyl component of the N-α-protected amino acid. The activating group or reaction must be chosen carefully to achieve very high coupling efficiency, and to avoid potential side reactions. There are four basic types of coupling techniques employed for stepwise introduction of the N-α-protected amino acids in SPPS.

- Carbodiimides and preformed symmetrical anhydrides (PSA)
- Phosphonium and uronium/aminium salts
- Fmoc amino acid active esters
- Fmoc amino acid chlorides and fluorides

The most frequently used coupling agents are given in Table 1-2.[8,10]

Table 1-2 Common coupling agents used in SPPS[8,10]

Reagent	*Full Name*	*Structure*
BOP	1-Benzotriazolyloxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate	
PyBOP	1-Benzotriazolyloxy-tris-pyrrolidino-phosphonium hexafluorophosphate	
HBTU	O-Benzotriazole-*N,N,N',N'*-tetramethyluronium hexafluorophosphate	 HBTU; X = H, Y = CH HATU; X = H, Y = N HCTU, X = Cl, Y = CH
HATU	2-(6-Aza-1-H-Benzotriazole-1-yl)-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate	
HCTU	2-(6-Chloro-1-H- Benzotriazole-1-yl)-*N,N,N',N'*-tetramethyl-uronium hexafluorophosphate	
TBTU	O-Benzotriazol-1-yl-*N,N,N'*-tetra-methyluroniumtetra-fluoroborate	
PyBroP	Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate	
HOBt	Hydroxybenzotriazole	

1.1.6.1 Carbodiimides and Preformed Symmetrical Anhydrides

Carbodiimides are *in situ* activating agents for peptide synthesis. For example, *N,N'*-dicyclohexylcarbodiimide (DCC) was first described in the early 1950's and remains popular for use in apolar solvents (i.e. DCM) for polystyrene resins. Reactions are carried out at room temperature (rt) in DCM or DMF. The drawback of using this method is the formation of insoluble dicyclohexylurea during activation and acetylation.[10] Water soluble carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) are also used for amino acid couplings. EDC and its urea have the added advantage of being water soluble.[5,8]

Preformed symmetrical anhydrides (PSA) have also been used due to their higher reactivity in Boc chemistry. These are generated *in situ* using two equivalents (equiv) of protected amino acid and one equivalent of DCC in a suitable solvent. The urea formed is removed by filtration and the couplings then proceed. The method has the drawback of being wasteful as more equivalents of amino acid are used and wasted to remove the urea formed in the reaction. Symmetrical anhydrides are best used fresh.[5,8,10]

1.1.6.2 Phosphonium and Uronium/Aminium Salts

In situ activating agents are widely accepted due to their ease of use, fast reactions, applicability for sterically hindered amino acids, and are generally free from side reactions. Most are based upon the phosphonium (BOP/PyBOP) or uronium/aminium (HBTU/TBTU) salts, which in the presence of a base (e.g. DIEA), can smoothly convert protected amino acids to a variety of activated species (Oxy-Benzotriazole (OBt) active esters).[5,8,10]

The use of HATU and HCTU generates more reactive active esters than HBTU in the presence of base. This higher reactivity compared to the OBt ester is due to the lower pKa of HOAt and HOCt compared to HOBt. Furthermore, HOAt has the added benefit of the pyridine nitrogen which provides assistance in the coupling reaction by the neighbouring group (in this case, the carboxylic group on the coupling amino acid) reducing the loss of chiral integrity.[5,8,10-11]

1.1.6.3 Fmoc Amino Acid Active Esters

Several classes of active esters are stable for isolation and prolonged storage, but still react rapidly in peptide coupling. These include the Fmoc-AA-OPfp (pentafluorophenyl) and Fmoc-AA-ODhbt (3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine) esters which are simply dissolved and added to the resin. The addition of HOBt to these reactions significantly increases the rate of reaction making them very useful for SPPS.[5,8,11]

1.1.6.4 Role of HOBt in Coupling Reactions

It is known that the addition of HOBt to preformed active ester reaction mixtures promotes the formation of the OBt ester and thereby leads to more efficient couplings.[10] This approach has been used in many peptide syntheses, especially difficult ones. However, due to HOBt's recent classification as a desensitised explosive (UN3380), transport of this product by air or sea is no longer possible. It can however be made with extreme care, if required. There is currently no alternative purchasable replacement for HOBt in the peptide industry although, for some reactions, it has been found that there is no loss in yield with its omission.[5,10] Nevertheless it would seem a suitable replacement for HOBt is desirable in the case of a synthesis of a large number of peptides or long peptide fragments.[5,10-11]

1.1.7 ***Deprotection***

Deprotection of the *N*-terminal protecting group is normally achieved by TFA (neat or in DCM) in Boc-SPPS and by pip in DMF in Fmoc-SPPS. These methods are suffice for most sequences, however, it has been shown that for long peptides, incomplete Fmoc-deprotection can occur, even after considerable use of piperidine. Therefore, an increase in deprotection time or a stronger base (such as DBU) is advised.[5,10]

1.1.8 *Coupling Tests*

The ninhydrin test is widely used for the presence or absence of free amino groups (deprotection/coupling) and was devised by Kaiser.[5,10] The test is simple and quick but some amino acids fail to give the expected dark blue colour typical of a free primary amino group. Namely, serine, asparagine, and aspartic acid. In addition, proline, being a secondary amino acid, does not yield a positive reaction. Furthermore, in aggregated sequences, occasionally false negatives are observed. Therefore, other methods for monitoring should also be employed where necessary. These include mass spectrometry (MS) whereby a small amount of the peptide is cleaved from the resin and analysed for presence of the des-amino acid peptide fragment, another colour test performed, for example using 2,4,6-trinitrobenzene sulfonic acid (TNBS) which gives red resin beads for a positive result (free amine), or the chloranil test to detect for amino acids such as proline by indication of blue resin beads for secondary amines.[5,10]

1.1.9 *Orthogonal Protecting Groups for Amino Acids*

Compromises to yields, purity, or both must be considered when choosing the appropriate amino acid protection for use in peptide synthesis. Things that must be considered include:[8,10]

- Orthogonal protection of the amino acid
- When and how to remove this protection
- Side reactions associated with its presence and/or removal
- Coupling when using a particular protecting orthogonal protecting group

Ideally, all three arginine nitrogen atoms should be blocked because there is evidence a single blocking unit does not fully protect the guanidino group from activity, however, in practise; the majority of guanidino protecting groups only block the ω-nitrogen (Figure 1-6). These groups can be divided into four classes (nitro, urethane, arenylsulfonyl, and trityl, Figure 1-6) and all exhibit differing requirements for coupling and removal.[8,10]

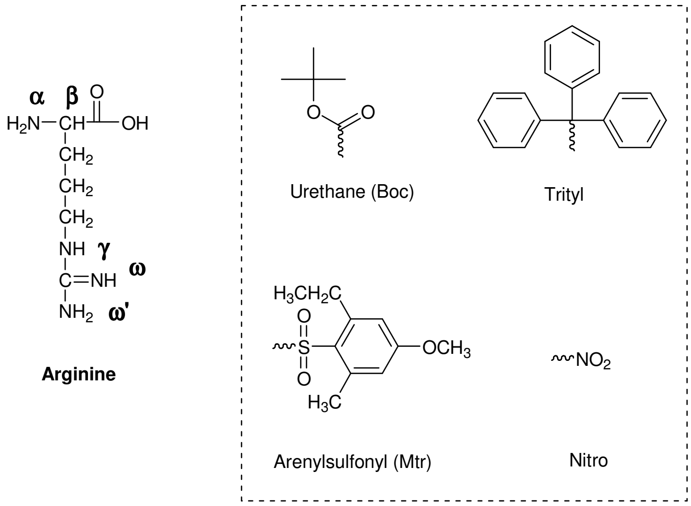


Figure 1-6 Greek labelling of the nitrogens in arginine (left) and examples of four differing types of protecting groups available for arginine protection (right).[10]

For example, the Mtr protecting group (Figure 1-6) belongs to the arenylsulfonyl class. It contains a single arenesulfonyl-based arginine protecting group and appears to offer complete blocking of the guanidino side chain. Other protecting groups used in this class include the Pmc (2,2,7,7,8-pentamethylchromane-6-sulfonyl, see **Chapter 3** for structural details) and tosyl (Tos). This group is extremely stable and may only be removed using high HF/anisole, Na/NH_3 cleavage, or strong TFA cleavage conditions. Peptides containing this group generally require extended cleavage times, which can affect sensitive residues in the sequence. The Pmc protecting group within this set is more labile than that of the Mtr protecting group making it ideally suited for the preparation of multiple-arginine containing peptides, but like the Mtr group, can also cause sulfonation of Trp residues during TFA mediated cleavage.[5,8,10]

Other amino acids that contain various protecting groups with associated problems are asparagine and glutamine. These amino acids can undergo several side chain reactions such as nitrile formation via dehydration of the carboxamide side chain during acylation when in the presence of *in situ* coupling agents. This can be minimised by using HOBt or by using orthogonal protecting groups to protect the free amide side chain. Aspartic and glutamic acids, cysteine, histidine, methionine, and tryptophan are also known to have side reactions whereby protection of the orthogonal group is essential.[5,10]

1.1.9.1 Cleavage Conditions

Cleavage of peptides from the resin in Fmoc SPPS is performed with varying strengths of acid (TFA). The strength of acid required to give an effective cleavage depends both on the nature of the peptide, the orthogonal protecting groups on the amino acids, and the nature of the linker attached to the resin. In general, the more electron-rich the linker, the easier it is to cleave the product. Conversely, electron-poor linkers (i.e. CO_2H) require more harsh acidic cleavage conditions such as HF.[8,10]

Scavengers are added to the cleavage reaction mixtures to prevent side reactions. The use of scavengers is based upon the peptide chain to be cleaved and includes the use of water, thioanisole, *m*- or *p*-cresol, and ethanedithiol (EDT). Water is an effective scavenger for any peptide not containing Met, Trp, and Cys. Water has the ability to scavenge *t*-butyl cations and the products from the cleavage of arylsulfonyl-based protection groups (such as Mtr). Thioanisole is added when the peptide contains Met, Trp, and Arg (although care must be taken to avoid premature cleavage of side-chain protecting groups such as *t*-butyl), as it has the ability to accelerate the removal of protecting groups from arginine as well as suppressing methionine oxidation. EDT is used if the peptide contains Ser, Thr, Tyr as it is good for the removal of *t*-butyl cations and provides some protection for Trp when it has no orthogonal protecting groups.[5,8,10] It also assists in the removal of the trityl group from Cys. Ethanedithiol (or thioanisole) suppresses methionine oxidation and phenol aids in the protection of Tyr and Trp.[5,8,10]

1.1.10 ***Purification***

Semi-preparative or preparative HPLC, usually reverse phase, is employed to purify the peptide from the crude cleavage mixture, which contains by-products as well as salts and scavengers. Depending upon the size and hydrophobicity of the peptide, reversed phase packings from C-18 to C-4 are used with solvent systems such as water/acetonitrile, water/methanol, or water/isopropanol with acidic ion pairing using reagents such as TFA or phosphoric acid buffers. This ion pairing gives better resolution. Column effluent monitoring is usually performed using a wavelength in the range of 210-220 nm however, peptides which contain aromatic residues can be monitored in the range of 240-280 nm. Solubility problems can be associated with hydrophobic residues, aggregation, disulfide bond formation, secondary structure formation, or a high amount of acidic residues. To overcome this, dissolving the peptide in basic (up to pH 8 otherwise damage will occur to the RP-column), acidic, or neutral solvents can be trialled. Storage of peptides is ideal at -20ºC under a nitrogen atmosphere.[5,9-10]

1.1.11 *Improving Synthetic Efficiency in SPPS*

The following issues must be taken into account when trying to improve the efficiency of peptide yields of difficult sequences when using SPPS.[10,12]

- Synthesis method
- Resins
- Amino acid and side chain protecting groups
- Solvents
- Coupling, deprotection, and cleavage times
- Coupling reagents
- Cleavage mixtures
- Purification

1.1.12 *Chemical Ligation*

Proteins of up to 200 to 300 amino acids can be synthesised with several fragment-coupling steps referred to as chemical ligation. The most common native chemical ligation method involves an *N*-terminal cysteine reacting with a *C*-terminal thioester.[5,7] Each segment can be synthesised on resin (using SPPS) and then joined together forming a larger chain. This enables better yields and easier purification of the final molecule.[5,7,12]

1.1.13 *Peptides in Drug Design*

The two main problems with proteins as drug candidates are their poor bioavailability and lability to proteolysis/acidolysis (i.e. in the stomach).[13] Truncated peptides and peptidomimetics are alternatives and can be more effective targeting agents against cancer. These molecules are often more chemically stable, small, easy to synthesise and can be readily conjugated to cytotoxic drugs, or toxins. Peptides that are *N*- or *C*-terminally blocked, cyclised, and/or contain D- or unnatural amino acids are generally more stable to proteolysis (but not necessarily hydrolysis). These molecules can be developed into cancer-targeting agents through the modification of known native ligands against cancer associated receptors (e.g. BK analogues against the BK receptors) or molecular modelling if the structure of the receptor is known and/or screening combinational peptide (or chemical) libraries.[13]

1.2 Sulfonamides

In medicine, there exist several groups of sulfonamides known as ‘sulfa drugs’. Sulfonamides are derived from sulfanilamide (4-aminobenzenesulfonamide, Figure 1-7 where R and R’’ is hydrogen). These sulfa drugs were found to display good antibacterial properties as far back as the early 19th century. Many studies since then have gone on to exchange the carbonyl group in amino acids and DNA for a sulfonyl group.[14] This exchange has been taking place for many years in drug design. Therapeutically active derivatives are usually substituted on the N^1 nitrogen and the N^4 position is generally unsubstituted (e.g. Figure 1-7, where R’ is any organic compound and R’’ can be a hydrogen or an aliphatic/aromatic group).[14]

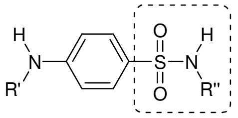


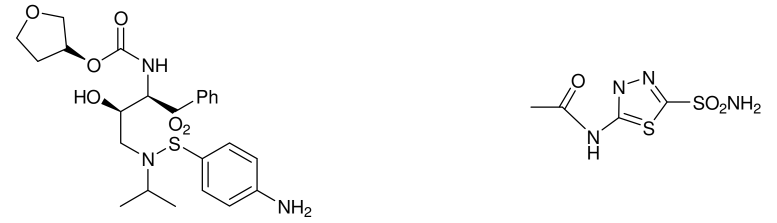
Figure 1-7 General structure of a bioactive sulfanilamide moiety where R’ is any organic compound and R” can be a hydrogen or an aliphatic/aromatic group. The sulfonamide moiety is highlighted.

Although most sulfonamide containing drugs have been replaced with natural and semisynthetic antibiotics, they still play an important part in the third world countries whereby storage and lack of medical personnel make them appropriate to use. They are especially useful for urinary tract infections.[14]

The clinical usefulness of sulfonamides relies not only on their antimicrobial properties, but also on their ability to have, as required, hydro- and lipo-solubility, protein binding capability, variable half-lives, and good metabolic characteristics. Currently used bioactive sulfonamides vary widely in their absorption, distribution, and excretion patterns. This is applicable to the targeting of the sulfa drugs as some are absorbed and excreted very quickly, therefore are able to be used for urinary tract infections whilst others are absorbed very slowly, ideal for gastrointestinal infections.[14b,15]

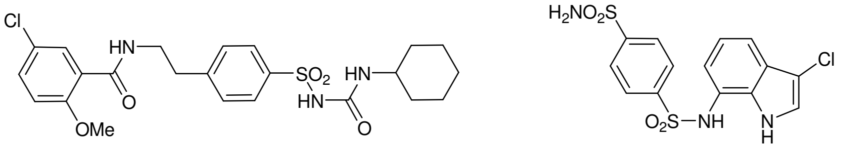
Sulfonamides with biological half-lives of up to 10h are considered short acting while half-lives longer than 24h are long acting. These wide pharmacokinetic properties along with their ease of administration, broad-spectrum antimicrobial activity, and non-interference with host-defence mechanisms are responsible for their wide-spread use six decades after their discovery.[14b,15a-c]

Apart from bacterial infections, sulfonamides have also been used in other medicinal areas with some success. These include anti-AIDS agents (such as the HIV protease inhibitor amprenair), carbonic anhydrase inhibitors (such as acetazolamide), and hypoglycaemic agents (such as glibenclamide).[14b,15a-c] They have also branched out into potential anti-cancer agents including E7070 which is in advanced clinical trials (see Figure 1-8).[14b,15a-c]



Anti-AIDS agents: HIV protease inhibitor amprenair

Carbonic anhydrase inhibitor: acetazolamide



Hypoglycemic agent: gilbenclamide

Possible anticancer drug: E7070

Figure 1-8 Structures of non-antibacterial sulfonamide drugs[15c]

1.2.1 *Sulfonamides as Anti-cancer Agents*

Despite improvements in prevention, early detection, surgical management, and advances in treatments such as chemo- and radio-therapy, the ability to cure cancer patients remains an elusive goal, with a strong need to develop alternative, more effective treatments.[15c]

As noted previously, considering the disadvantages with current cancer treatment methods, doctors are also faced with the ever-evolving emergence of multi-drug resistant tumours which escape treatment by a variety of new biochemical mechanisms.[15c] Thus, a massive search for new anti-cancer therapies and drugs has primarily been fuelled by unveiling new molecular targets on which to intervene. This is followed by the discovery of a novel class of compounds that interact with such targets where some protease inhibitors are among the most promising.[15c]

1.2.1.1 Sulfonamide Anti-cancer Targets

Although sulfonamide drugs work by interacting with a variety of targets, they are commonly known to act as a competitive inhibitor of the enzyme dihydropteroate synthase (DHPS) essential for the production of folate and alternatively nucleotide synthesis. Without nucleotide synthesis, replication of DNA and RNA is halted, stopping cell replication and division (Figure 1-9). Humans require folate in the daily diet due to it not being synthesised within mammalian cells, thereby allowing selectivity between bacterial and human cell lines by these sulfonamide drugs.[15c,16]

Other targets for biologically active sulfonamides include matrix metalloproteinases (MMPs) which are a family of zinc-containing endopeptidases that are shown to play a critical role in the extracellular matrix (ECM) for integrity and structure of various tissues in higher order invertebrates. The main interest here, with respect to cancer, is tumour invasion by the inhibition of selective MMPs.[15]

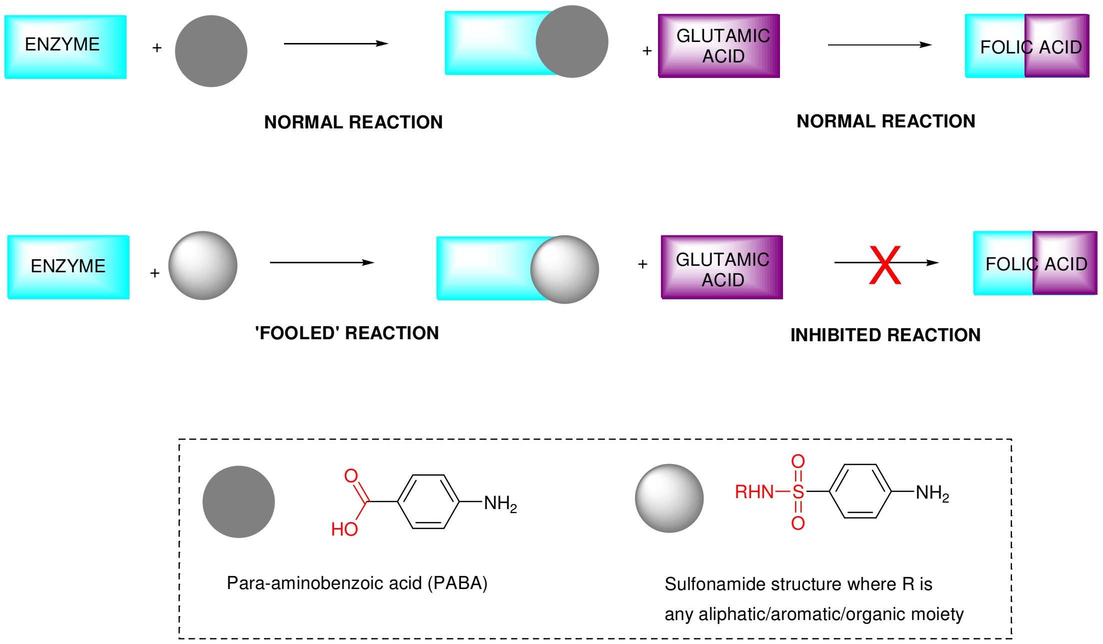


Figure 1-9 Schematic representation of the inhibition of enzymes by sulfonamide based molecules[16]

Sulfonamide-type inhibitors that interact with amino acids associated with enzymes which contribute synergistically to the pathophysiology of many diseases has emerged as an interesting target for the drug design. The dual inhibition of these enzymes is applicable for tumour invasion.[15c]

1.2.2 *Physiochemical Properties*

Sulfa drugs are weak acids with a pKa in the range of 5.8 units. This acidity, due to the sulfonamide moiety, improves the solubility in basic aqueous solutions. The pKa can be modified with the functionalisation of the N^1-substitutent, but clinically useful sulfonamides generally have pKa values which give them solubility at physiological pH. For example, the addition of a carboxylic group at the N^4-terminus enables them to be soluble in this range.[15c] It has also been noted that the presence of a primary aromatic amino group has been found essential for activity, and the presence of N^1-substituents has a significant influence in the degree of acid dissociation of the sulfonamide group.[15c]

Sulfonamides are hydrolysed at a slower rate than carboxamides due to a number of reasons outlined in Figure 1-10 and Table 1-3 including both steric and electronic factors.[17]

Figure 1-10 Nucleophilic attack (Nu^-) to form the alkoxide (**I**, 4 groups) or sulfonyl (**II**, 5 groups) intermediates[17]

Hence, sulfonamides are more sterically impaired with respect to nucleophilic attack leading to an electronically unfavoured intermediate. Both these factors will tend to produce longer life times for bioactive sulfonamides with respect to, for example, hydrolysis.[17]

Table 1-3 Steric and electronic factors of carboxamides and sulfonamides[17]

Variable	*Carboxamide*	*Sulfonamide*
Hydrolysis	-	Slower
Nucleophilic attack	-	Slower
Nucleophilic attack at	Trigonal acyl carbon, unhindered	Tetrahedral sulfur, hindered
Intermediate structure	Tetrahedral carbon, stable octet	Pentavalent sulfur, unstable decet
-NH_2 acidity	Acidic, negative charge dispersed over one oxygen and one nitrogen atom	Much greater acidity, negative charge dispersed over two oxygen and one nitrogen atom

1.2.3 *Structure-activity Relationships*

The following generalisations have arisen from a review of more than 5000 sulfonamides used and tested as drugs.[15d]

- The amino and sulfonyl groups on the benzene ring should be in the 1,4-positions
- The amino group should be unsubstituted or converted to a free amino *in vitro*
- In most cases, the replacement of the benzene ring by other ring systems, or the introduction of additional substituents, decreases or abolishes activity
- Exchange of the -SO_2NHR with -SOC_6H_4-*p*-NH_2, -$CONH_2$, -CONNOR, or -COC_6H_4R generally reduces activity
- N^1-monosubstitution may result in greater activity but N^1-disubstitution, in general, leads to inactive compounds

From these studies, the best activity for sulfonamide drugs has been found where the R' on the sulfanilamide (Figure 1-7) is a heterocycle and R'' is a hydrogen, isocyclic, or an acyl group.[15d]

1.2.4 *Toxicity*

A small percentage of patients treated with sulfonamides have suffered side effects, such as fever, rashes, and mental disturbance. These effects are due to poor excretion or over-dosing levels, and are more often associated with high inherent patient blood pressure. Long lasting sulfonamides appear to be the cause of most of these problems, as they tend to crystallise out in the kidneys and build up in the blood system. However, in general, sulfa-based drugs are considered relatively safe.[15c]

1.3 Vasodilators

Blood vessels contain smooth muscles allowing them to dilate or compress to control blood flow and pressure. Drugs or chemicals that allow the relaxation of these muscles are known as vasodilators. Vasodilators are especially important in warmblooded animals when temperature control is essential. Here, vasodilators divert the flow of warm blood to the skin allowing it to cool. Vasoconstriction is the opposing process to vasodilation, which involves different chemicals for the increase of blood pressure. For example, vasoconstriction is associated with pupil dilation.[18]

The bioactive molecules that naturally modulate vasodilation within the body include paracrine agents from endothelial cells, (including bradykinin and adenosine), the adrenal glands, and the automatic nervous system. These latter two secrete catecholamines such as norepinephrine and epinephrine respectively, and are important in the body’s reaction to stress by the fight or flight response.[16]

Vasodilation works by one of two mechanisms and is dependent upon the endogenous nature of different vasodilators. There is either a decrease in the intracellular calcium ion concentration within the surrounding smooth muscles or by phosphorylation of myosin. Exogenous vasodilator signals depend upon environmental factors such as the absence of noise, illumination, or chemical signals such as the change in ethanol or nitric oxide concentration. An example of this is the chemical tetrahydrocannabinol (THC) that is the major active ingredient in marijuana. THC has mild vasodilating effects on cannabis smokers seen by the reddening of their eyes. Medicinal uses of vasodilators include the regulation (decrease) of blood pressure as well as treatment of angina and congestive heart failure.[16]

1.3.1 *Bradykinin*

Bradykinin (BK, Figure 1-11) is a physiologically and pharmacologically active vasodilating nonapeptide in the kinin group of proteins consisting of the amino acid sequence, NH_2-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-C(O)-NH_2. BK is released from the venules and is involved in the response to pain similar to that of histamine. It is also known that BK raises the calcium levels within a cell causing them to release glutamate.[15d,18]

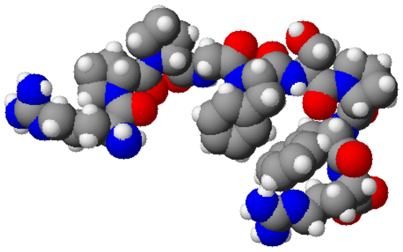


Figure 1-11 Spacefilling model of bradykinin. Red = oxygen, blue = nitrogen, gray = carbon, and white = hydrogen

BK is synthesised from the kinin-kallikrein system of proteins by proteolytic cleavage and is rapidly broken down by kinases in the human body at three places, Arg_1, Phe_6, and Arg_9. Phe_6 is the first place of attack giving the BK peptide a life time of only a few seconds.

In mammals, there are two types of BK receptors known. The first one is the B_1 receptor, which is only expressed as a result of injury to the tissue. In this receptor, BK plays a positive role in inflammation recruiting white blood cells in the response to pain. The second receptor is the B_2 receptor, whose only active role is in BK's vasodilating response and is only expressed when the cell is under stress.[16] Both these kinin receptors belong to the G-protein coupled receptor family. This large family of eukaryotic transmembrane receptor proteins is able to initiate a cellular response by sensing molecules outside the cell and activating signaling pathways within the cell. The ligands that bind and activate these receptors include hormones (such as BK), neurotransmitters, odours, and vary in size from small peptides to large proteins. They therefore make a good target for many modern day drug studies.[18b-i]

1.3.2 *Bradykinins Importance in Tumour Cells*

More recently BK has been found to be a crucial and potent factor in the growth of tumour cells. Here, BK has been linked to the stimulation of growth in tumour cells causing them to migrate and invade normal cells by stimulating the release of the vascular endothelial growth factor. Since tumour cells grow and divide at a rate faster than normal cells, angiogenesis is employed to help cater for the ever-demanding oxygen and nutritional needs of tumour cells.[19]

Tumour cell growth differs greatly from that of normal cell growth as the blood vessels are irregular in shape, dilated, leaky, and the cells themselves are poorly aligned. This results in the leakage of blood plasma components such as macromolecules, lipid particles, and nanoparticles into the tumour tissue. There is also an accumulation of macromolecules within tumour cells due to a slow venous return and poor clearance of unwanted particles. This is known as the Enhanced Permeability and Retention factor (EPR) effect and has become the target for tumour specific anticancer drugs.

The EPR effect does not accumulate low molecular weight molecules as they diffuse more readily into the circulating blood flow. Factors that affect the EPR effect include BK and prostaglandins. BK is produced by tumour tissues sustaining tumour growth by increasing angiogenesis within these cells. Due to this, antagonists of BK and BK analogues that block all these aspects offer great potential as anti-tumour agents.[18b-i]

1.3.3 *Bradykinin Agonists and Antagonists*

Inhibitors of BK include aloe and polyphenols which are substances found in red wine and green tea. However, research into other BK inhibitors has focused on molecules that can interact with hormone receptors blocking (antagonist) or amplifying (agonist) the biological response of BK. These may help to define the biological or pathological processes of BK leading to drug design. It has been found that the best anti-cancer BK derivatives need bulky hydrophobic amino acids at the *C*- and *N*-terminus and that slight changes in the sequence of BK can alter its role dramatically, including the change from D- to L-amino acids.[18b-e,18g-i,20] See Table 1-4 for more details into how the structure of BK can be altered to produce agonist or antagonist behavior in the cell.

Table 1-4 A se\lection of studies for the synthesis of bradykinin (BK) analogues

Brief Description	*References*
Antagonist B9430 acts an anti-inflammatory drug, no cancer cell inhibition. When bound by *N*-terminal cross-linking (B9870) it becomes a potent growth inhibitor of SCLC. *N*-terminal acylation of the agonist B9430 (B10238) with a bulky hydrophobic fluorine-rich group increases the growth inhibition. Small BK mimics (e.g. BKM-638) inhibited the growth of SCLC when injected into nude mice. A change of D- to L-amino acids have significant changes in its anticancer activity.*	21
Dimeric BK antagonist (CP-127, bradycor) and related dimers produced modest growth inhibition of SCLC cell lines at high concentrations. E.g. CU201 acts as a biased agonist, inhibits the growth of both SCLC and NSCLC cell lines by a unique mechanism. CU201 and related compounds may be useful in the treatment of lung cancers and other cancers with neuroendocrine features.[†]	18g
Exchange of the hydrophobic Phe$_8$ amino acid in des-Arg$_9$ BK to an even more hydrophobic aliphatic residue (such as Leu or Ile) produces an antagonist. Replacement of the three prolines in BK for α-aminoisobutric acid (singly and in varying combinations) gives high agonist activity. Replacement of Pro$_7$ with several D- and L-aromatic amino acids results in a competitive BK antagonist. Most potent BK position 7 analogues contained the hydrophobic residues-Val, Phe, and Trp.	18h
Exchange of Pro$_7$ with a D-aromatic amino acid such as Phe produces a BK B$_2$ receptor antagonist. Exchange of BK residues 5 and 8 for thienylalanine gave an enhancement of receptor affinity. A D-Arg at the *N*-terminus locks aminopeptidase activity and hydroxyproline at position 3 lowers the affinity for receptors. Octahydroindole-2-carboxylic acid into position 8 blocked degradation and increased life time of action. Dimerisation of a Cys antagonist with a cross linker gave an antagonist. Removal of Arg$_9$ along with Igl gave potent long lasting antagonist	22

* B9430 = D-Arg-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-Arg where Hyp = *trans*-4-hydroxyproline, Igl is α-(2-indanyl)-glycine, and Oic is octahydroindole-2-carboxylic acid, B9870 = SUIM-(B9430)$_2$ where SUIM is suberimidyl , B10238 = 5F$_c$-B9430 where 5F$_c$ is 2,3,4,5,6-pentafluorocinnamol, BKM-638 = DDD-($_d$-Arg-Igl-Arg-Matp)$_2$ where Matp is 4-(methylamino)-2,2,6,6-tetramethylpiperidine

[†] CP-127 = (D-Arg-Arg-Pro-Hyp-Gly-Phe-Cys-D-Phe-Leu-Arg-COOH)$_2$ linkage at Cys via di-sulfide bond

1.4 Cyclodextrin

Cyclodextrin (CD) makes up a family of non-reducing cyclic oligosaccharides comprised of five or more R-α-1,4-linked D-(+)-glucopyranose units resulting from the enzymatic breakdown of starch. The three most common CDs are comprised of 6 (alpha, α), 7 (beta, β, most commonly studied for drug release due to it being readily available), and 8 (gamma, γ) cyclic glucose monomers creating a truncated cone shape. This cone shape is stiffened by the hydrogen bonding between the secondary hydroxy situated at the 2- and 3-position on each glucopyranose unit groups around the outer rims of the CD cavity (Figure 1-12).[23] CD also has primary hydroxy groups situated at the 6-position on the glucopyranose unit (Figure 1-12) and these are able to stabilize the inclusion of molecules (e.g. drugs), inside the CD bucket.[23]

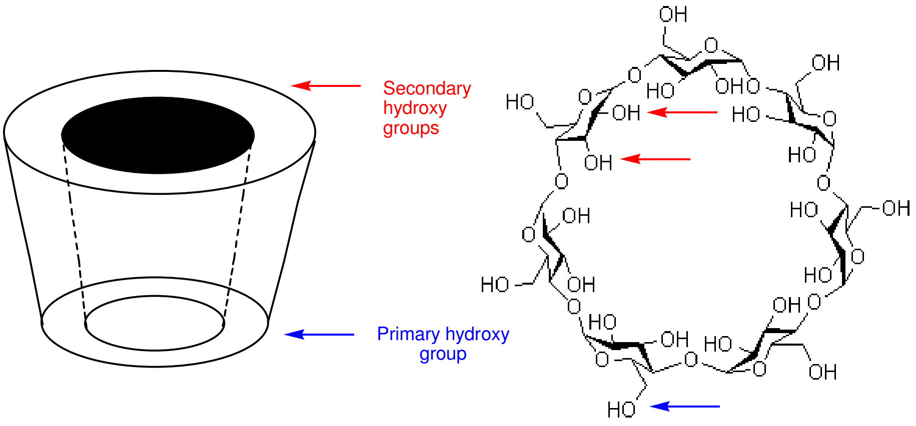


Figure 1-12 This CD figure shows the hydroxy groups exposed to the solvent resulting in a hydrophobic cavity enabling CD to act as a host for hydrophobic molecules and metal complexes[23]

The more glucose units present, the stronger the cavity due to an increase in hydrogen bonding. CD contains a hydrophilic outer surface enabling moderate water solubility and a hydrophobic cavity which is able to contain non-polar guest compounds. Hence, for many years CD has been important in the study of drug release within biological systems. Encapsulation of a molecule within CD depends upon the size of the cavity and the drug. α-CD is generally too small, γ-CD is not stable enough, and hence β-CD has been extensively used in this area (Table 1-5).[23-24]

Table 1-5 Some important characteristics of α-, β-, and γ-CD[23-24]

Type of Cyclodextrin (CD)	*Cavity Diameter (Å)*		*Molecular Weight (g/mol)*	*Solubility (g/100 mL)*
	Inner Rim	*Outer Rim*		
α-CD	4.7	5.3	972	14.5
β-CD	6.0	6.5	1135	1.85
γ-CD	7.5	8.3	1297	23.2

1.4.1 *Cyclodextrin in Drug Release*

β-CD has been most commonly used in the food, agricultural, and environmental engineering industries indicating its large range of applications. Excipients have also been used to aid with the delivery and dispersion of the active ingredients. More recently though, β-CD has become of considerable interest in the pharmaceutical industry for drug delivery and release studies. This is due to CD being easy to handle, its ability to increase the bioavailability and solubility of drugs, decrease drug irritation, stabilise active ingredients, and to reduce drug-drug interactions. CD is also able to decrease the integrity of cell membranes through the inclusion of some cell components leading to the increase in the permeability of some impermeable drugs. CD is stable in acid down to a pH of approximately 3 and thermally stable below 200°C and these properties are readily exploited for drug release. External functionalisation of CDs primary or secondary hydroxy groups can increase its water solubility therefore allowing easier transportation of molecules around the body.[23-24]

In aqueous solution, the CD cavity is able to weakly hold between three (α-CD) and nine (γ-CD) water molecules, which can easily be displaced. This cavity is also able to accommodate aliphatic and aromatic compounds, including drugs. They can bind in differing ratios from 1:1, 2:1, and 1:2 depending upon the CD involved and the size of the guest molecule. This binding is driven by enthalpic and entropic forces controlling the release of water from the cavity increasing the solubility of CD in aqueous medium.[23-24]

CDs can recognise the size, shape, and chirality of amino acids, however since peptides and proteins are generally quite large, they are not able to fit entirely inside the CD cavity. Therefore, there are only local interactions between the CD and the accessible hydrophobic parts of the peptides/proteins. Such interactions affect the overall three-dimensional structure of the peptide/proteins and hence also affect their biological and chemical properties.[23-24]

It has been found that non-covalent interactions between peptides and CD derivatives occur in solution forming host-guest complexes. An example of this can be seen in the interaction of the BK peptide with permethylated β-CD which has been studied by mass spectrometry (MS).[23-24] The guest is driven to the CD cavity by its hydrophobic nature, such as the phenyl group of phenylalanine. Studies indicate that BK forms a non-covalently bound complex with the CD in the region where the basic-amino acid residues, such as arginine and phenylalanine are located. This non-covalent interaction is likely to be from an ion-dipole interaction, with no evidence of an inclusion complex formed.[23-24]

Encapsulation of metals and other small molecules by CD is also possible. These are held in the cavity by the functionalisation of the primary and secondary faces, as well as by hydrophobic and hydrophilic interactions of the CD cavity. Due to this, CD is also able to act as a molecular receptor important for the separation of chiral and/or impure compounds.[23-24]

1.4.1.1 Drug Bioavailability

CD enhances the bioavailability of drugs by increasing their solubility, dissolution, and/or membrane permeability. For example, CD can aid the drug permeability by direct action on mucosal membranes by interacting with cholesterol increasing the membranes fluidity and induce membrane invagination through a loss of bending resistance and cause cell lysis. CD can also cause the removal of phospholipids, especially phosphatidylcholine and sphingomyelin from the outer half of the membrane bilayer causing an imbalance.[23c,25]

1.4.1.2 Drug Safety

The ability of CD to increase drug solubility ideally means that less drug is required therefore reducing potential side effects or toxicity by making the drug more effective at lower doses.[23c,25]

1.4.1.3 Drug Stability

CDs have the ability to increase drug stability against, e.g., dehydration, hydrolysis, and oxidation therefore increasing the drug's shelf life. This stability has been proposed to be due to CD shielding the drug and particularly the drug's active site thus insulating the drug from degradation.[25] The stabilisation effect is dependent upon the nature of the groups attached to CD, and the nature of the drug.[25]

1.4.2 ***Cyclodextrins Application in Drug Delivery***

Various types of delivery methods using CD-based drugs have been studied. These include oral, parenteral, ocular, nasal, rectal, and more controlled delivery methods including, e.g., colon-specific delivery.[23,25-26] Each method demands specific requirements that focus the selection from such a broad range of drug delivery systems.

For example, oral drug delivery requires improvement to the drug's bioavailability with increased solubility, a better rate and extent of dissolution, and the stability of the drug at the absorption site, such as the stomach or the intestine. Elimination of taste and odour of the drug must also be taken into consideration. For oral delivery to be successful, the time of drug release is important in order to get the maximum effect of the drug, so release in transit is not ideal. CDs enhance the absorption of drugs by oral delivery by increasing the free drug availability at the absorptive surface and their complexation provides better and uniform absorption of desired low-soluble drugs with poor and erratic absorption. However, when considering the type of delivery method, safety, efficacy in terms of complexation, cost, and acceptance in pharmacopeia should be considered.[25] Ocular delivery, such as CD in eye drops, has been used to aid solubilisation and chemical stabilisation of drugs, reduction of eye irritation, and enhancement of drug permeability. CD is ideal for this instance as it is non-irritating to the eyes surface maximising the absorption of the drug.[25] Nasal delivery of drugs has optimised the use of CDs due to increasing the aqueous solubility of the drug and/or by enhancing the drug's permeability. This is also the case with suppository and parenteral drug delivery with the addition of minimising toxicity and increasing drug stability.[25]

Controlled drug release has been extensively studied with CD based drugs due to their ability to carry or complex drugs. The hydrophilic and hydrophobic nature of CD enables exploitation and transport to differing areas within the body, and therefore differing release times. For example, hydrophilic derivatives improve the aqueous solubility and dissolution rate of poorly soluble drugs, while hydrophobic derivatives retard the dissolution rate of water-soluble drugs from vehicles. Therefore, hydrophilic and hydrophobic combinations are used in immediate and prolonged release type formulations, respectively. pH can also be applied to induce a delayed and specific release time of a drug.[25]

1.4.3 *Cyclodextrin-peptidyl Conjugates*

There is a continuing challenge for using cyclodextrin for solubilisation and drug targeting, and this has lead to the preparation of a wide variety of chemically modified derivatives in order to improve the properties of the host molecule.[16] Peptide and proteins in drug delivery have various problems due to their chemical and enzymatic instability, poor absorption through biological membranes, rapid plasma clearance, peculiar dose response curves, and immunogenicity. Therefore, the use of CD aids in the bioadaptability in pharmaceutical use. Its ability to interact with cellular membranes means CD can act as a potential carrier for the delivery of proteins, peptides, and oligonucleotide drugs.[23c,25]

Little work has been devoted to the covalent attachment of large bioactive molecules including peptides to CD, although smaller amino acids and peptides have been attached and carried. Studies show that there is a tendency for self-inclusion of these small peptides/amino acids when in the presence of CD in aqueous solution.[23a-b,27]

A study on the grafting of a *C*-terminal tetrapeptide onto β-CD using both per- and mono-CD substitution has been performed.[23a-b,27c] A spacer was used to enhance the peptides response. It was shown that by separation of the CD and peptides molecules, per-substitution of CD led to a strong interference of the biological properties of the peptide, with mono-CD substitution better suited for activity. Overall, it has been shown that for bioactive peptide-CD units to be successful, these following properties are essential.[23a-b,27c]

- The free hydroxy groups of the cyclodextrin cores must be retained since they play a key role in the stabilisation and solubility of the complex.

- The direction of the peptide's recognition signal must be accessible to the receptor, unless detachment of the peptide is employed once the target is reached. This involves attachment of the peptide to either the *C*- or *N*-terminal depending upon the active end of the peptide. In the case of this study, the active end of the BK peptide is Arg_1 at the *N*-terminus which is essential for binding to the B_2 receptor. Agr_9 at the *C*-terminus is essential for binding to the B_1 receptor.[28]

- A spacer between the CD and the peptide moieties could be used to reduce possible aggregation and steric hindrance effects induced by the grafting of the peptide onto CD.

1.4.3.1 Cyclodextrin-peptidyl Conjugates in the Literature

Synthesis of CD-peptidyl conjugates has become more prominent over the last few decades, perhaps due to CDs increased availability, the need to develop better, more effective drugs, or the increasing knowledge into its exploitable drug delivery properties.[23a-c,25,27b-c] Many groups have been involved in the functionalisation of CDs for a number of reasons including supramolecular chemistry and drug delivery and release. A selection of these groups is detailed in Table 1-6.

Table 1-6 A selection of studies involved with the functionalisation of cyclodextrin (and its derivatives) for use in supramolecular chemistry, and drug design.

Group leaders	*Description*	*References*
Fujita *et al.*‡	Unsymmetrically di-substituted CD in isomeric mixtures for enzyme mimics (or receptors) offers a promising approach to access hetero-bi-functional CDs.	29
Bols *et al.*	Bi-functionalisation of α-CD for the use as artificial glycosidases. Only got one isomer. Use in supramolecular chemistry.	30
Tabushi *et al.*	Bi-functionalisation of CD using capping.	31
Jankowski and Hanessian *et al.*‡	Bi-functionalisation of CD with amino and carboxylic acids using solution synthesis. Total yield of 9% (8 steps) with one isomeric form. Potential in peptide synthesis and drug delivery.	32
Sollogoub *et al.*	CD capping for the selective synthesis of CD bearing three different functionalities. Uses include asymmetrical caps, catalysis materials and peptide addition.	33

‡ Selective bi-functionalisation of the primary hydroxy groups with two different functional groups (see text, **Section 1.4.3.1** for more details)

Table 1-6 (continued) A selection of studies involved with the functionalisation of cyclodextrin (and its derivatives) for use in supramolecular chemistry, and drug design.

Group leaders	*Work Outline*	*References*
Moroder *et al.*	Perfacial functionalisation of a peptide onto CDs primary hydroxy groups using a condensation reaction. A spacer is employed between the peptide and the CD moiety. Looked at the inhibition of the peptide towards a substrate.	27c, 34
Stoddart *et al.*	Neoglycoconjugate synthesis based on CD and calixranes which includes the mono- or hepta-functionalisation of CD. Metal inclusion and amino acid addition (off resin) onto CD also studied.	24, 35
Armspach *et al.*[‡]	Regioselective multi-functionalisation of CD obtaining three isomers.	36
Sinay *et al.*	Head-to-tail homo-dimers of CD joined by alky chains synthesised by selective functionalisation of the outer hydroxy groups. Uses include looking at their water solubility compared to unfunctionalised versions.	33b, c, 37
Mauclaire *et al.*	Lipid and amino acid functionalisation of CD off resin by condensation reactions for the mono-functionalisation of CD to check the aggregation behaviour of the lipid-CD species.	27b

[‡] Selective bi-functionalisation of the primary hydroxy groups with two <u>different</u> functional groups (see text, **Section 1.4.3.1** for more details)

From Table 1-6 it can be seen that the mono-functionalisation of CD has been successful with either selective functionalisation of a single primary or secondary hydroxy group, or the complete functionalisation of all primary (or all secondary) hydroxy groups. However, the selective bi-functionalisation of the primary hydroxy groups with two different functional groups has only recently been achieved (see ‡ in Table 1-6). However, all these methods employ global-protection of the unutilised hydroxy groups and have a large number of steps involved with limited examples of uses explained and demonstrated. The use of linkers, that may be essential for the retention of the bioactivity of the attached group (e.g. peptides), have not been examined.

The rotation of the CDs secondary hydroxy groups is restricted due to hydrogen bond formation between them, whereas the primary hydroxy groups are free to rotate reducing the effective diameter of the cavity on the primary side of the molecule.[23a] The cavity of CD is rendered hydrophobic due to the presence of the glycosidic oxygens and the C-H units. The presence of the hydroxy groups on the outer cavity makes it hydrophilic. When functionalising the CD molecule, two primary faces must be taken into account, the nucleophilicity of the hydroxy groups and the ability of CD to form complexes with the reagents used. All modifications of CD take place at the hydroxy groups, and since hydroxy groups are nucleophilic in nature, the initial reaction, which directs regioselectivity, and the extent of modification (mono-, di- to hepta-) of all subsequent reactions, is an electrophilic attack on these positions.[23a] Until now, general modifications of CD can be achieved as summarised in Table 1-7.[23a]

Table 1-7 Overview and examples for the modification of CDs[23a]

Hydroxy position on CD	*Modification Method*
6-	Normal reactivity; weak base (e.g. pyridine) and a non-complexing electrophile. Any base and electrophile but need to protect 2-OH position.
2	Strong base (deprotonated) and a non-reducing electrophile.
2- or 3-	Weak base and any electrophile, need to protect 6-OH position.
2- or 3- or 6-	Reaction by complex formation using a complexing nucleophile.

The hydroxy groups at position 2 are the most acidic so will be the first to be deprotonated, whereas those at position 6 are most nucleophilic (basic) and are most easily functionalised. The hydroxy groups at position 3 are the least accessible. It is also important to note that the more reactive the reagent, the less selectivity is obtained due to exchange at one or more hydroxy positions. Isomers (such as AB, AC, AD for the addition of two substituents, Figure 1-13) are obtained when there is the addition of two functional groups to CD.[23a]

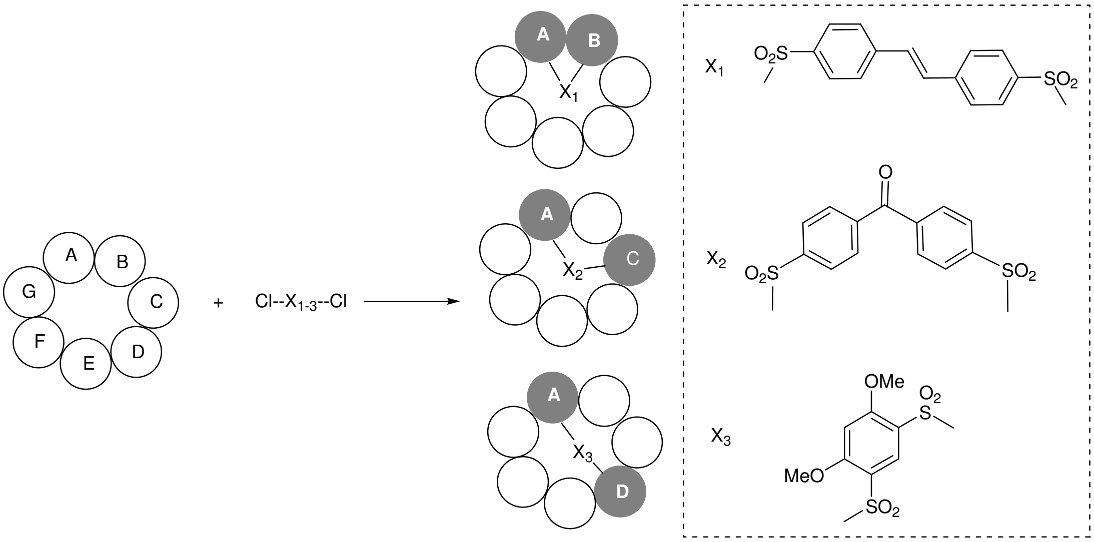


Figure 1-13 Use of geometry of reagents to direct the regiospecificity in di-substitution of CDs[23a]

1.4.3.2 Di-substitution of Cyclodextrin

Di-substitution of CD is obtained from the careful addition of more than one equivalent of reagent to CD under suitable reaction conditions to give a mixture of products. Statistical analysis suggests that di-substitution can produce 33 regioisomers in the case of β-CD in which purification and identification of each is difficult.[23a] Using oversized reagents is not effective in the synthesis of fewer numbers of substituents, but bridging reagents (a reagent that joins to two differing glucose units on one CD molecule) such as 1,3-benzenedisulfonyl chloride in pyridine gives the AB isomer in a moderate yield after purification by HPLC (Figure 1-13).[23a]

1.4.4 *Cyclodextrin as a Drug Carrier*

CD, being hydrophilic, remains outside the membrane to which the drug is delivered to and released. Therefore, it is possible to regulate the speed of release for certain drugs that use these macromolecules as delivery systems. There are two proposed methods for the use of CDs as drug delivery vehicles:[25,26b]

- The synthesis of CD derivatives covalently bound to pharmaceutically active molecules so as to guarantee where the absorption takes place

- The functionalisation of the CD with messenger molecules such as biologically active peptides, which act as specific ligands for certain membrane receptors. The cavity of these derivates can transport the drug guaranteeing a specific release site. The direct functionalising with drugs seems the most promising strategy to increase the stability, the solubility, and the site-specific release.

In 1998, Moroder and co-workers[27c] reported an extensive study of β-CD as a carrier of peptide hormones by mono- or multi-valent display. The *C*-terminal tetrapeptide amide sequence of the gastrointestinal hormone, gastrin, is the shortest sequence capable of exhibiting all of the required biological properties, although with reduced potency.[23b] Functionalisation of CD with many bioactive peptides, as per Schaschke and co-workers, showed that the peptides interfered with the binding capabilities of CD to the receptor, decreasing its selectivity.[27c] The reduced selectivity was ascribed to steric influences of the peptide moieties as well as the collapse of the peptide chains onto the CD core such that all potential activity disappears. However, they found indications for beneficial interactions of the CD with the receptor surface. Further studies have shown CD to act as a scaffold to control the distance-geometry in the display of ligands with multimeric protein complexes.[27c]

1.4.5 *Cyclodextrin-sulfonamide Conjugates*

Non-covalent interactions between sulfonamide drugs have been studied for the protection of the drug against the environment (e.g. photo-stability) and for utilisation of the carrier properties of the CD molecule. Other sulfonamide studies have also investigated the use of CD for an increase in solubility and stability against illumination. For example, the CD encapsulation of Furosemide (a sulfonamide-type drug) by non-covalent bonds has been achieved using co-precipitation methods and has been shown to impart greater aqueous stability and potentially better therapeutic properties to the drug.[38] However, little work has been done on the covalent binding of sulfonamides to CD (in or outside the CD core).

1.5 PhD Project

The studies reported in this thesis were directed towards the development of a methodology which would allow the attachment of one or more peptides, with the same or different biological properties, to be attached to a carrier system using SPPS.

CD was chosen as the carrier molecule for this study because it fulfilled many of the conditions, as outlined above, required for a useful support for drug delivery. In addition, CDs structure enabled the attachment of a differing number of peptides, as well as having the potential for inclusion compounds bound within its cavity, if required. See **Chapter 2** for the synthesis of CD to enable mono- and bi-functionalisation.

BK was chosen as the bioactive peptide to be attached to the CD molecule due to its known tumour-related properties and hence targeting potential. See **Chapters 3** and **4** for the synthesis of BK and CD-peptides respectively. Model sulfonamides were also bound and modelled as the ‘drug’ in this potential drug delivery system (**Chapters 5** and **6**). Therefore, an investigation was carried out to determine whether CD-peptide-sulfonamides could be assembled and hence be used as a drug delivery vehicle. This study was further supported using preliminary bioassay testing (**Chapter 6**).

A small number of studies have been published (see Table 1-6) in which CD has been functionalised with one or more linkers. However, global protection was employed in these studies and a large number of steps used. It appears that no studies have been reported where the bi-functionalisation of CD has been performed without global protection. This thesis study reports the achievement of this goal with fewer steps than previously reported (**Chapter 2**). For CD-peptide complexes, a linker has also been employed to separate the CD-peptide molecule with a view to retain the bioactivity of the peptide/drug being carried.

Although studies into the synthesis of *C*- and *N*-terminal peptide sulfonamides have been reported (see **Chapter 5**), investigation into acceptable coupling conditions was still required for this study. Once these methodologies were established, various combinations of CD-peptide-sulfonamides were synthesised and characterised. See **Appendix 1-I** for the study proposal at the start of the PhD project and how everything fits in together.

Goal: A range of studies were envisaged as necessary to obtain satisfactory reaction conditions for the coupling of bi-functionalised CD moieties onto resin/peptides for peptide synthesis using Fmoc SPPS.

Experimental rationale: Proof-of-concept was the prime objective for this study. As will be revealed later, yields were low and much work to improve them certainly needs to be carried out in furthering this study.

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Chapter Two:

Mono- and Bi-functionalisation of β-Cyclodextrin

2.0 Introduction

The main goal of this PhD project was to synthesise a multi-functional drug delivery vehicle for the transportation of sulfonamide drugs into cells based upon the carrier molecule, β-cyclodextrin (β-CD). This chapter describes the functionalisation of β-CD with one or two linkers to enable attachment to a resin or other compounds, and subsequent peptide growth. The functionalisation of β-CD with one functional group was synthesised for comparison against the bi-functionalised β-CD molecule.

β-CD has been exploited in host-guest complexation with the non-covalent binding of hydrophobic compounds within its cavity.[1] This property has been extensively studied in the past to change the physicopharmaceutical properties of lipophilic drugs such as water-solubility, bioavailability, improved stability, and bio-effectiveness. CDs have also been important in the food industry for the protection of active ingredients against oxidation, elimination (or reduction) of undesired tastes or odours, and extension of shelf life for some encapsulated compounds.[2] Covalent linkage of bioactive peptides to CD has also been proposed[1a,1e,3] to possibly take advantage of this complexation in terms of solubility and reduced catabolism, although such conjugates with the relatively large CD carrier, were expected to impair recognition processes at the molecular level.[1a]

2.0.1 *Chapter Outline*

This chapter will describe a selective, versatile and robust methodology for the bi-functionalisation of β-CD which enables the attachment of peptides in varying *C*- and/or *N*-terminal combinations on resin using Fmoc SPPS. Two linkers are attached to β-CD enabling selective binding to the resin or a peptide attached to the resin. Then continuation of peptide growth and/or cleavage from the resin follows, thus various combinations of peptide-CD species are achieved. Model peptides (Gly-Ala, Val-Gly-

Ala, and tri-glycine) and a bioactive peptide (BK) were used (**Chapters 4** and **6**) in this study to illustrate the potential of this system for attaching one or more bioactive peptides for drug transport. Preliminary bioactive testing of these compounds has also been achieved (**Chapter 6**).

Figure 2-1 illustrates the plan for this chapter. *Part a* describes the functionalisation of β-CD with one functional group which can lead to mono-peptide addition on- (**Chapter 4**) or off-resin (solution synthesis). The functionalisation of β-CD with an azide (*Part b*) was also done to enable peptide addition via click chemistry (**Appendix 2-I**). Subsequently, leading on from this, the synthesis is described for the addition of a second linker (*Part c*) enabling the attachment of one or more peptides in varying *C*- and/or *N*-terminal combinations (see **Chapters 4** and **6**). There are also options for peptide/non-peptide combinations synthesised on/off-resins which were not explored in this study but are discussed in **Chapter 7**.

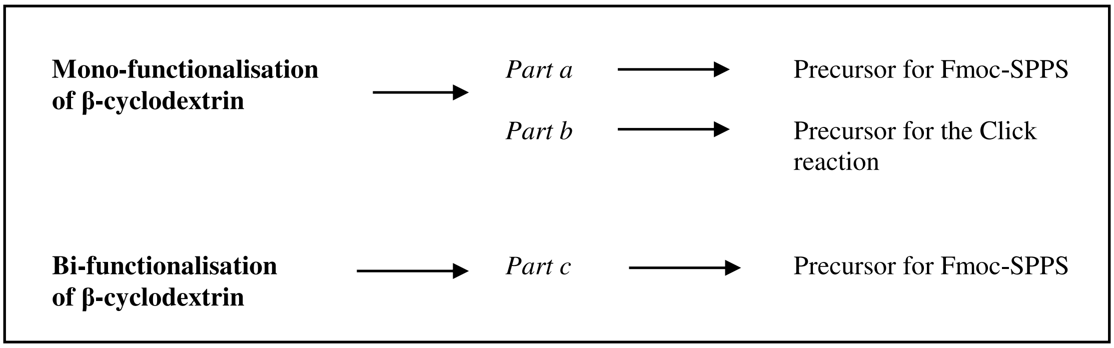


Figure 2-1 A schematic for the layout of **Chapter 2**

Within this section (*Part c*), the coupling properties of the bi-functional molecule under Fmoc-SPPS reaction conditions are examined. This includes studying couplings to different resins using different solvents, coupling reagents, and times. Standard resins (Figure 2-2) were tried as well as Rink resin with a spacer attached (Rink-spacer resin, Figure 2-2). The results indicated that TentaGel resin (which has a long PEG spacer between the resin core and the resin's reactive site) gave more successful couplings than Rink or Sieber resin. It also showed that the Rink-spacer resin gave a better coupling compared to Rink resin, indicating that this bulky bi-functional β-CD molecule needs a long, unhindered, 'floppy' chain with a terminal reactive group to enable successful attachment to resin. Differing coupling conditions also showed a significant change in the coupling efficiency of β-CD to each resin indicating that investigation into the best system is important.

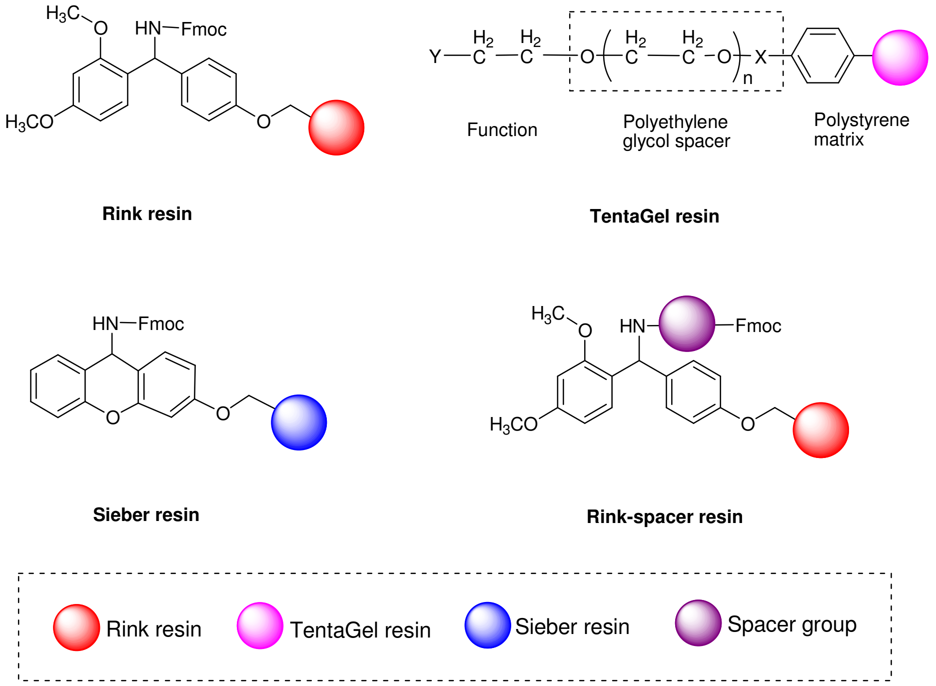
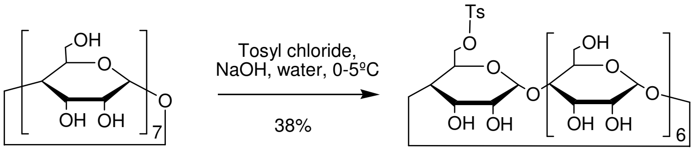


Figure 2-2 Structures of Rink, TentaGel (where X and Y are functional groups dependent on the type of functional groups desired at the peptides *C*-terminal and type of SPPS undertaken), Sieber, and Rink-spacer (prepared in **Chapter 4, Section 4.7.7**) resins. In the following chapters, each resin will be represented by a coloured ball (red = Rink, pink = TentaGel, blue = Sieber, and red/purple = Rink-spacer resin)

2.1 Results and Discussion for Part a

2.1.1 *Synthesis of Mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin (**2**)*

Mono-derivatisation of β-CD (**1**) was successful in a high yielding synthesis obtaining an easily purified product. The first step (parallel to procedures by Bryn *et al.*) [4] was the key with the tosylation reaction enabling only one functional group to be attached at this point (Figure 2-3) to give mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin (**2**). The tosyl group occupies the cavity of the CD for the reaction to take place;[5] hence the mono-tosyl species is the most prominent in this reaction. However, if left long enough, bi- and tri-tosylation is possible and especially prominent with an increase in reaction temperature and tosyl chloride concentration. The product was purified by recrystallisation and analysed to be of a sufficient purity for continuation to the next step.

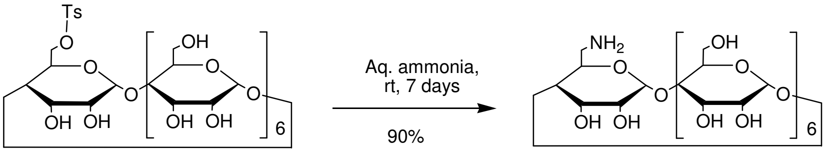


1 2

Figure 2-3 Synthesis of mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin (**2**)

2.1.2 *Synthesis of Mono-6^A-amino-β-cyclodextrin (3)*

Synthesis of mono-6^A-amino-β-cyclodextrin (**3**) was then achieved by stirring **2** in aqueous ammonia (Figure 2-4) over a seven-day period, refreshing the ammonia solution daily. Conversion of **2** to the azide and then to the amine was also an option however, this ammonia reaction was very clean and went with a near quantitative yield (90%).



2 3

Figure 2-4 Synthesis of mono-6^A-amino-β-cyclodextrin (**3**)

2.1.3 *Synthesis of Mono-6^A-succinylamino-β-cyclodextrin (4)*

Coupling of a spacer to the amino group of **3** now gives a free carboxy terminus enabling attachment to resin (and/or peptide on resin) and subsequent peptide synthesis. Mono-6^A-succinylamino-β-cyclodextrin (**4**, Figure 2-5) was synthesised using modified reaction conditions from Lincoln *et al.*[6] and went in a good yield of 89%. Precipitation of the product from acetone gave the pure compound with no need for any further purification. From here, peptide synthesis using compound **4** is detailed in **Chapter 4**.

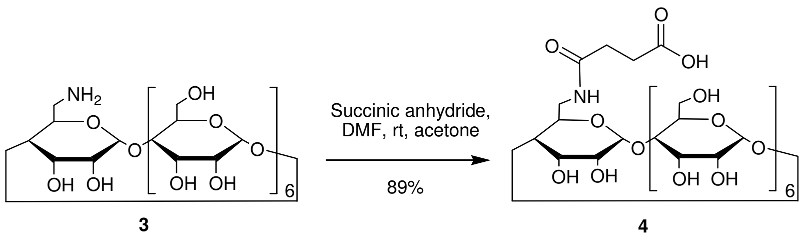


Figure 2-5 Synthesis of mono-6^{A}-succinylamino-β-cyclodextrin (**4**)

2.2 Results and Discussion for Part b

2.2.1 *Synthesis of Mono-6^{A}-(α-azidoacetamido)-β-cyclodextrin (**5**)*

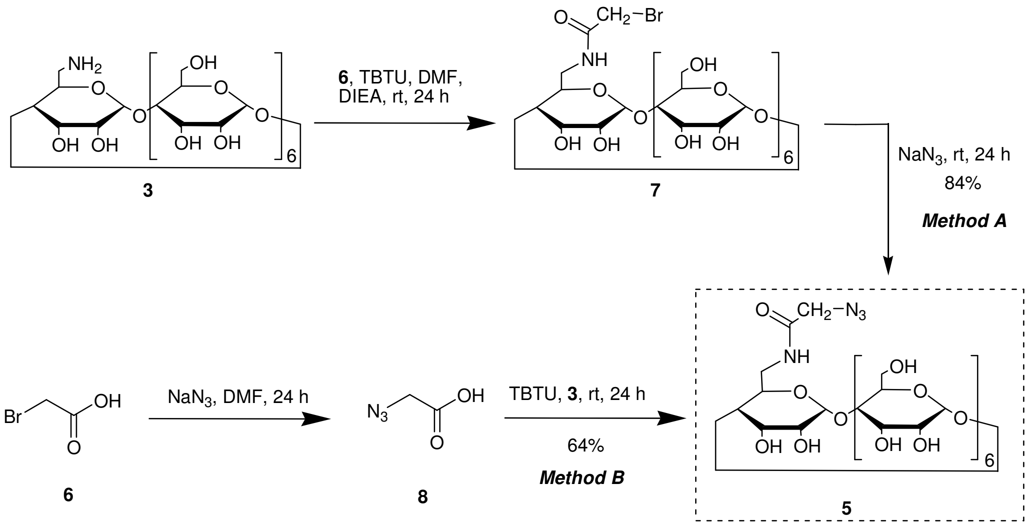


Figure 2-6 Potential synthesis routes for the functionalisation of **3** with an azido functionality for peptide attachment on resin using click chemistry (**Appendix 2-I**)

Peptide addition onto the mono-functionalised β-CD moiety via click chemistry was also tried. Click chemistry is a reasonably new chemistry method which uses a catalyst (copper in this instance) to join an azide with an alkyne (called the azide alkyne Huisgen cycloaddition reaction) forming a heterocyclic linkage (1,4- or 1,5-substituted 1,2,3-triazole depending upon the catalyst used) and has been used for the attachment of molecules to CD.[6] The following conversion of β-CD (**3**) to an azide for use in the click reaction follows procedures by Emrick *et al.*[6a] and Eichler *et al.*[6b] Refer to Figure 2-6 for details.

It was found that *Method A* (Figure 2-6) worked best giving an 84% yield (two synthetic steps) due to the formation and isolation of the mono-bromo-β-CD species (**7**) before continuation with the reaction as opposed to the *in situ* reaction (*Method B,* 64%, Figure 2-6). IR (right, Figure 2-7) shows the azide peak at 2100 (weak) which is not present for compound **3** (left, Figure 2-7). NMR was also used to confirm the product (see experimental section).

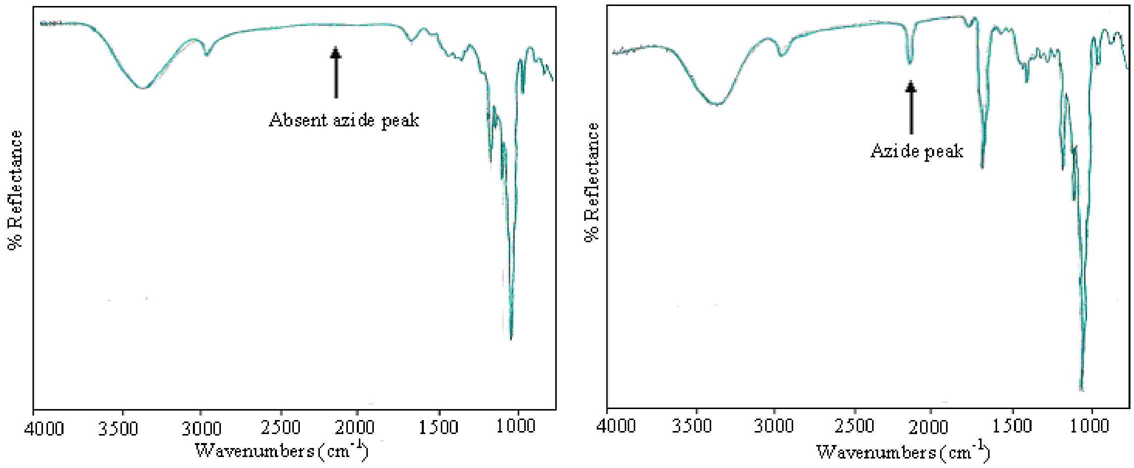


Figure 2-7 IR spectra for compound **3** (left) and compound **5** (right)

This synthesis later led to the mono-functionalisation of β-CD with peptides by click chemistry on resin (**Appendix 2-I**). Only a small number of preliminary experiments were tried for the addition of the peptide to β-CD using click chemistry and overall some success was achieved (**Appendix 2-I**). However, more in depth studies are required to obtain better workable yields for this part of this study.

2.3 Results and Discussion for Part c

Bi-functionalised β-CD exists in the literature, however, the authors employed global protection of all unutilised hydroxy groups (see **Chapter 1**, **Section 1.4.3.1** for details) and therefore the aim of this study was to functionalise β-CD without the use of mass protection, in a precise and controlled manner in as few steps as possible.

2.3.1 *Synthesis of Mono-6^A-carbobenzyloxyamino-β-cyclodextrin (**9**)*

The bi-functionalisation of β-CD was achieved by protection of **3** using the carbobenzyloxy (Cbz) protecting group as per Liskamp *et al.* (**9**, Figure 2-8).[7] This group was chosen as it has both acid and base stability under moderate conditions, hence enabling more chemical freedom for sequential reactions. It is also easy to remove by a hydrogenation at rt thus enabling conversion to another, more user-friendly SPPS protecting group if desired. In our case, the Fmoc group was chosen for addition in the final synthesis step (see **Section 2.3.4**) as Fmoc-SPPS was to be undertaken for peptide addition (**Chapters 4** and **6**). It should be noted that the conversion from the Cbz-protected molecule to the Fmoc-protected molecule was undertaken in one step (**Appendix 2-VII**).[8] However, this one step approach was not very successful and hence, three separate reactions isolating each intermediate species were used (see the following three sections).

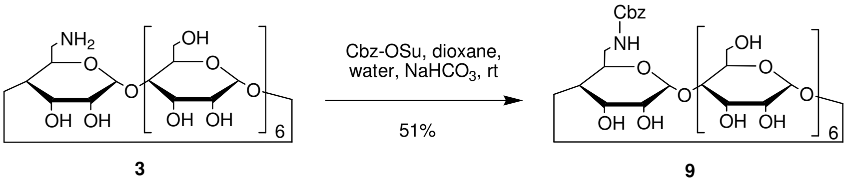


Figure 2-8 Synthesis of mono-6^A-carbobenzyloxyamino-β-cyclodextrin (**9**)

2.3.2 *Synthesis of Mono-6^A-carbobenzyloxyamino-mono-6^X-succinyl-β-cyclodextrin (**10**)*

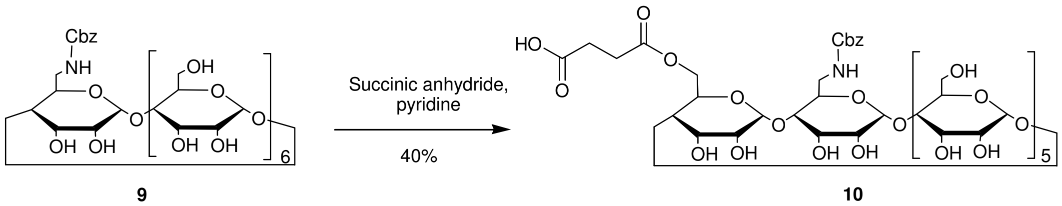


Figure 2-9 Synthesis of mono-6^A-carbobenzyloxyamino-mono-6^X-succinyl-β-cyclodextrin (**10**)

The addition of a second linker onto **3** (a succinyl group, **10**, Figure 2-9) results from a large number of attempts (see **Appendix 2-III**) by means of changes to reaction conditions including solvents, temperature, and molecular ratios, where the most successful reaction conditions (based upon final yield of the desired product) are presented here. In this study, yields and final placement of functional groups (primary or secondary hydroxy attachment) were compared.

It was found that with very mild, controlled conditions (succinic anhydride in pyridine under anhydrous conditions, see **Appendix 2-III**) that the succinyl group could be attached to the primary hydroxy groups of compound **9** (Figure 2-9). Bi- and tri-succinyl substitution was also noted, but in trace amounts. Purification of the reactants can be achieved by recrystallisation, but for full characterisation purposes, RP-HPLC purification was carried out.

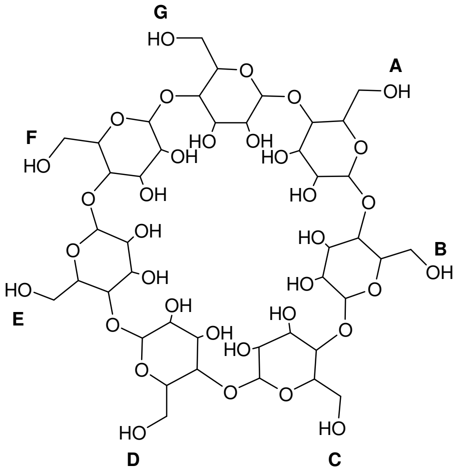


Figure 2-10 β-cyclodextrin with the identification of all the different α-D-glucopyranoside units. These groups make up three pairs of isomers (AB/AG, AC/AE, AD/AF) when two different moieties are bound

Isomer purification was the limitation of the method, resulting in a mixture of six isomers of compound **10** (AB, AC, AD, AE, AF, and AG, Figure 2-10). Although the regio-isomeric mixture was used in the rest of the study, the purification of the regio-isomers into 3 pairs (AB/AG, AC/AF, and AD/AE) was achieved using RP-HPLC (Figure 2-11).

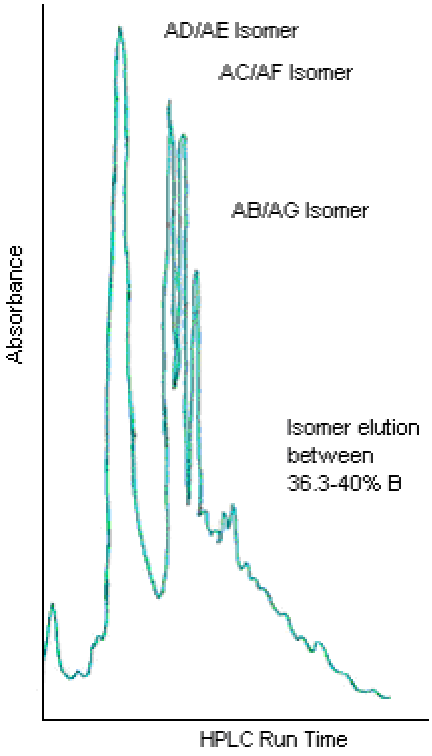


Figure 2-11 Compound **10** RP-HPLC trace

Isomeric purification of compound **10** was achieved by RP-HPLC using a MeOH:H_2O:TFA gradient followed by a MeCN:H_2O:TFA gradient in consecutive runs. This afforded a 1:2:2 ratio of the (AB/AG):(AC/AF):(AD/AE) regioisomers (Figure 2-10) with the elution pattern of (AD/AE):(AC/AF):(AB/AG), confirmed by ESI-MS studies as per Sforza *et al.*[14] This ratio pattern is due to the steric hindrance of the complex at positions AB and AG and has been seen before.[9] Differentiation between the (AB/AG), (AC/AF), and (AD/AE) pairs using this method is not possible. Further RP-HPLC purification was attempted but separation of the isomers was not accomplished (see **Appendix 2-II**).

- Instrumental Setup

All solutions for the MS analysis reported here were prepared at a 50 µmol concentration in the presence of NaCl (100 µmol). MS were obtained on a Micromass ZMD electrospray instrument by perfusing the solutions into the mass spectrometer at a 10 µL/min flow rate. MS conditions: ESI interface, positive ions, single quadrupole analyser. Capillary voltage 3500 V, cone voltage 190 V, source temperature 80ºC, desolvation temperature 150ºC, cone gas ($N_{2(g)}$) at 60 l/h, desolvation gas ($N_{2(g)}$) 450 l/h. Spectra were acquired in the positive ion mode (290-1700 Da), scan time 2.82 sec, inter-scan delay 0.1 sec.[14]

- Sample analysis:

β-Cyclodextrin fragmentation:

To understand the fragmentation pattern of the substituted (β-CDs), the ESI pattern for compound **1** was determined. The mass spectrum collected showed a simple fragmentation pattern generating a series of peaks always with a difference of 162 Da (995/ 833/ 671/ 509/ 347 *m/z*) as shown in Figure 2-12. The fragmentation events occur at every acetal junction of the opened β-CD molecule relating to 2, 3, 4, 5, and 6 monomeric units respectively (A, Figure 2-13).[14]

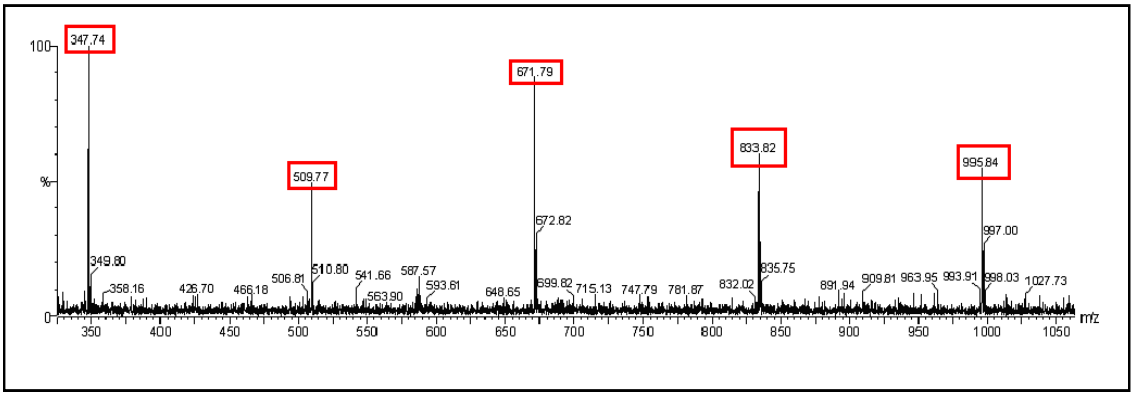


Figure 2-12 β-CD (**1**) fragmentation pattern

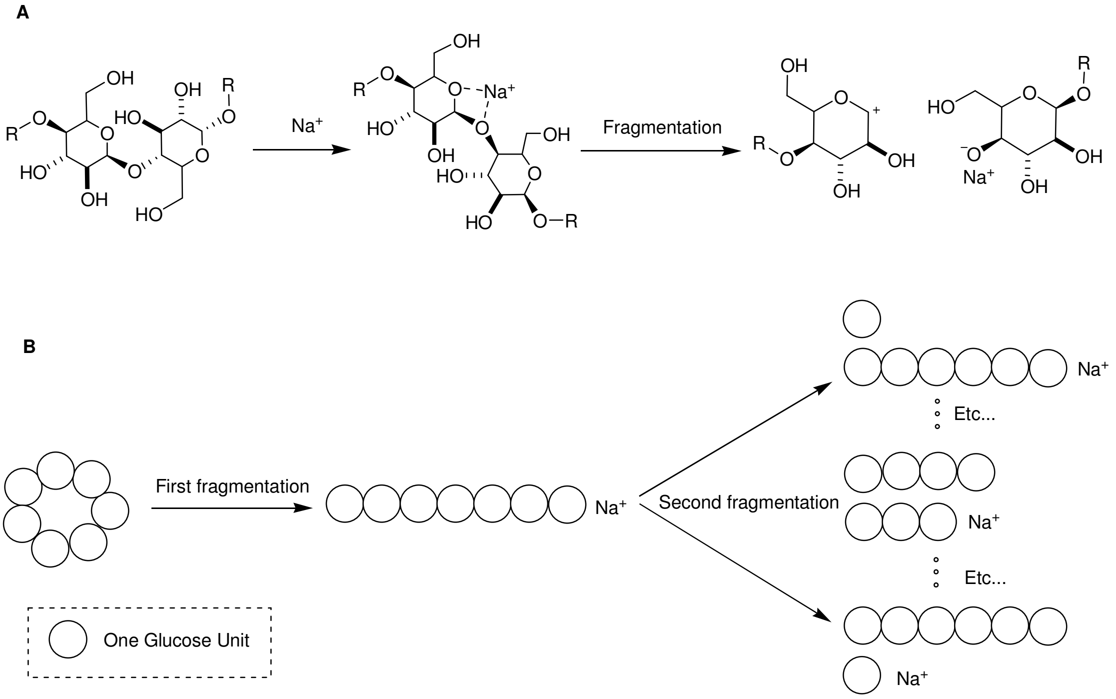


Figure 2-13 *A)* Hypothetical fragmentation at the acetal junction of cyclodextrin. *B)* Second fragmentation generation of β-cyclodextrin through a two-fragmentation random process. Every circle represents a glucose moiety.[14]

*Mono-6^{A}-carbobenzyloxyamino-β-cyclodextrin (**9**) fragmentation:*

The mass spectrum collected for compound **9** (Figure 2-14) shows the fragmentation pattern consistent with the expected values given in Table 2-1. As per compound **1**, a difference of 162 Da is observed due to breakages at each acetal junction of **9**.

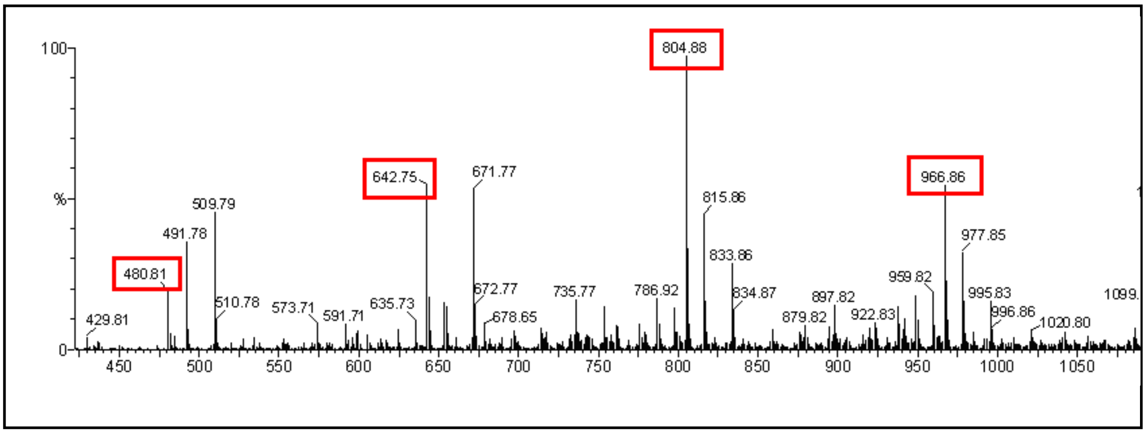


Figure 2-14 ESI-MS fragmentation pattern for compound **9**. Peak 1128 (Table 2-1) is not shown due to complete fragmentation of the product. A softer MS method would be required in order to show this peak.

Table 2-1 Expected fragmentation peaks for the ESI-MS ionisation of compound **9** in the presence of excess sodium

Fragments Glucose Units	*Glucose Unit Fragments*				
	2	3	4	5	6
Mono-substituted	480	642	804	966	1128

*Mono-6^A-carbobenzyloxyamino-mono-6^X-succinyl-β-cyclodextrin (**10**) fragmentation:* Isomer identification for compound **10** was achieved from ESI-MS analysis by first determining, by statistical analysis, the relative percentages of the un-, mono-, and di-substituted peaks. These were then correlated to the expected number of fragments (out of seven) for every possible isomeric pair for compound **10** (Table 2-2).[14]

From here, a quick identification for the three pairs of regioisomers was achieved as per reference 14 by checking the ESI-MS spectra (Figures 2-15 and 2-16) for the peaks corresponding to those who have three glucose units and one or no dehydrated groups (highlighted in Table 2-3). The ratios between these peaks were found to be in agreement with the literature values[14] and consistent with the expected pattern of fragmentation (insert, Figure 2-16) for every regioisomer. The 642 peak is higher for the AD/AE isomer pair, the 742 peak is higher for the AB/AG isomer pair, and the two peaks are of similar intensity for the AC/AF isomer pair (left, Figure 2-16).

Therefore, the first eluted RP-HPLC isomer is the AD/AE isomeric pair, the second eluted RP-HPLC isomer is the AC/AF isomeric pair, and the third eluted RP-HPLC isomer is the AB/AG isomeric pair.

Table 2-2 Expected distribution of the un-, mono-, and di-substituted peaks for every fragment of compound **10** characterised by a defined number of monomeric units in the case of AB/AG, AD/AE, and AC/AF regioisomers, given a random fragmentation at the acetal junctions.[14]

Fragments glucose units	*AB/AG ISOMER*					*AD/AE ISOMER*					*AC/AF ISOMER*				
	2	3	4	5	6	2	3	4	5	6	2	3	4	5	6
Di-substituted	1/7	2/7	3/7	4/7	5/7	0/7	1/7	2/7	3/7	5/7	0/7	0/7	1/7	3/7	5/7
Mono-substituted	2/7	2/7	2/7	2/7	2/7	4/7	4/7	4/7	4/7	2/7	4/7	6/7	6/7	4/7	2/7
Un-substituted	4/7	3/7	2/7	1/7	0/7	3/7	2/7	1/7	0/7	0/7	3/7	1/7	0/7	0/7	0/7

Table 2-3 Expected fragmentation pattern for the isomers of compound **10** in the presence of excess sodium with ESI-MS ionisation. Bolded numbers enable quick identification of the three regioisomers, AB/AG, AD/AE, and AC/AF by comparing the ratios between the highlighted peaks. Molecular weight of **10** = 1367.44 g/mol (H^+) or 1390.44 g/mol (Na^+)

Fragments glucose units	*Glucose unit fragments*				
	2	3	4	5	6
Di-substituted	378	540	702	864	1026
Mono-substituted	480	***642***	804	966	1127
Un-substituted	580	***742***	904	1066	1228

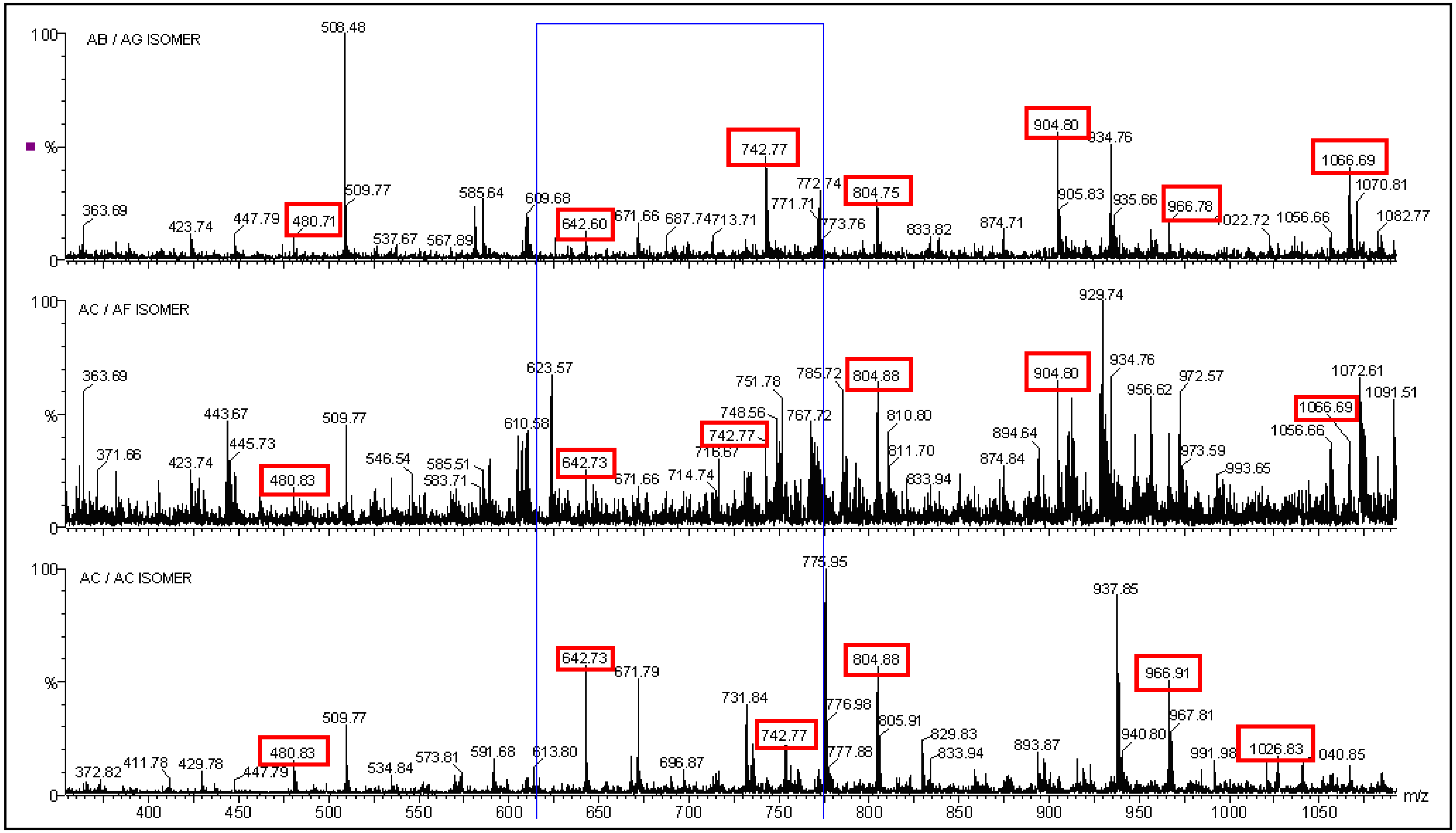


Figure 2-15 Full spectrum for the ESI fragmentation pattern of compound **10** with the enhancement area of Figure 2-16 highlighted. AB/AG isomer (top), AC/AF isomer (middle), AD/AE isomer (bottom)

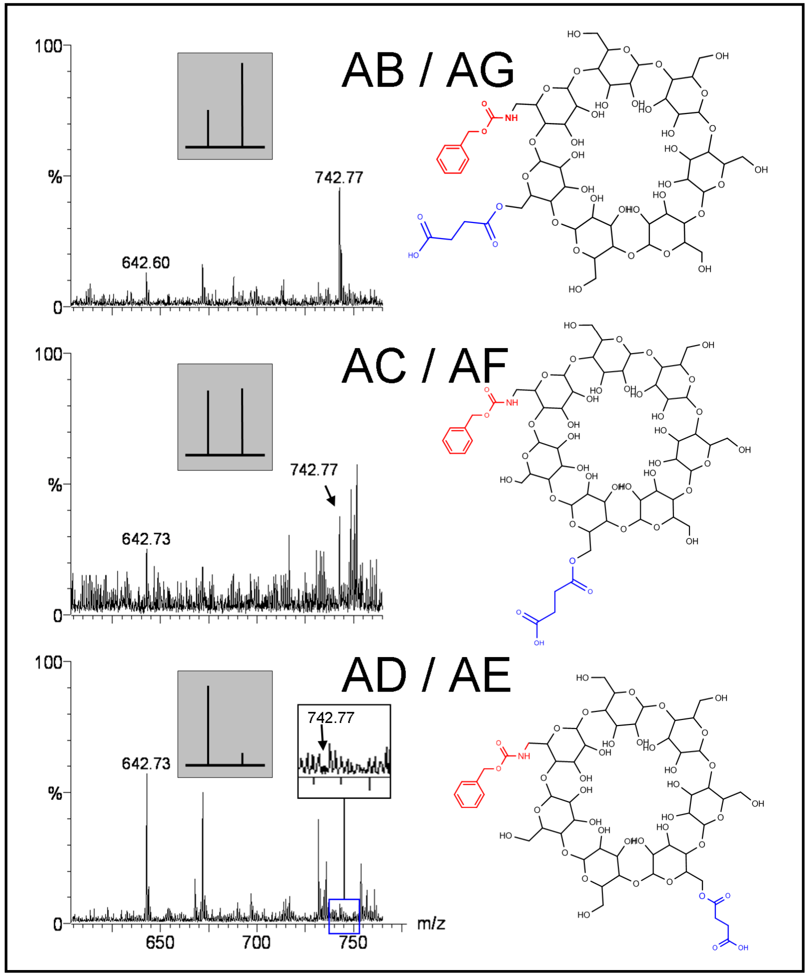


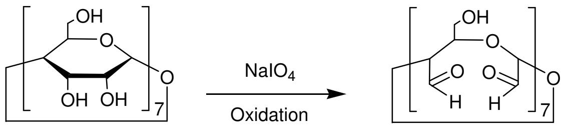
Figure 2-16 Enhancement of the highlighted section in Figure 2-15. Experimental ESI-MS isomer spectra (left) with the expected pattern of fragmentation (insert); structures of mono-6^{A}-carbobenzyloxyamino-mono-6^{X}-succinyl-β-cyclodextrin, **10** isomers (right). AB/AG isomer (top), AC/AF isomer (middle), AD/AE isomer (bottom)

The stability of compound **10** under standard Fmoc-SPPS coupling conditions was determined by exposing a small amount of **10** to each of the reaction conditions stated in Table 2-4. MALDI analysis was used to look for any breakdown products. It was found that compound **10** was stable to all standard Fmoc SPPS reaction conditions (Table 2-4)

Table 2-4 Stability test conditions and results (determined by MALDI analysis) for compound **10** when exposed to standard Fmoc SPPS reaction conditions

Reaction Conditions	*Experimental Time (Hours)*	*Results*
100% DMF	24	No change
100% DCM	24	
20% Pip in DMF	0.5	No change
20% Pip in DMF	1.0	
0.5 mmol TBTU in DMF	24	No change
0.5 mmol HOBt in DMF	24	
0.5 mmol DIEA in DMF	24	
0.5 TBTU/HOBt in DMF	24	
0.5 TBTU/HOBt/DIEA in DMF	24	
20% TFA in DCM	0.5	No change
95% TFA in DCM	0.5	
95% TFA in DCM	24	

A periodate reaction[9] was used to determine the placement of the succinyl- and Cbz-functional groups onto the β-CD ring (the primary or secondary hydroxy positions). This reaction works by oxidising the bonds between the two adjacent secondary hydroxy groups on the glucose molecule forming two aldehydes (Figure 2-17) and the consumption of the oxidant in the reaction is monitored by UV spectroscopy.[9]



1

Figure 2-17 Oxidisation of β-cyclodextrin using sodium metaperiodate ($NaIO_4$) forming two aldehydes at the secondary hydroxy position[9]

Sodium metaperiodate ($NaIO_4$) was used as the oxidant for these reactions in 10% excess (in relation to the number of possible secondary hydroxy groups) to ensure the reaction goes to completion. Four reaction cells were used throughout the reaction, cell 1 was the blank containing water, cell 2 and 3 were sample blanks to monitor the reaction without either the compound or the oxidant (periodate) present, respectively. The absorbance of cell 3 was followed in order to correct for the slow change in absorption that takes place in solutions of metaperiodate. This change was presumably due to temperature, light, and pH effects over time.[8] The reactant to be oxidised and the oxidant (sodium metaperiodate) was in cell 4.

The reaction was monitored over a period of days until the absorbance had reached equilibrium. By using Equation 2-1, the amount of free hydroxy groups could be determined, and hence the position of the Cbz and succinyl-groups placements onto compound **10**. The absorbance of cell 4 subtracted from the sum of absorbances of cells 2 and 3 represents the decreases in absorbance due to the consumption of periodate by the sample. When this absorbance difference becomes constant, the reaction is considered complete. This difference divided by the absorbance of cell 3 at time zero is equal to the fraction of the known amount of added periodate that is consumed on oxidizing the vicinal hydroxy groups.[8]

A = [reaction cell (cell 4)] – [sample blank cells (cell 3 + cell 2)]

B = [A/cell 3] / [initial absorbance reading]

Where:

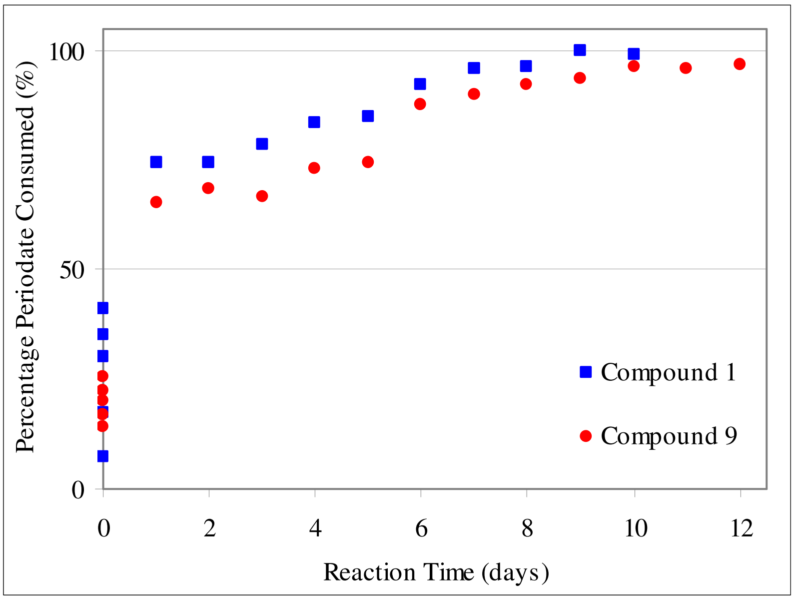
***A** = Absorbance due to consumption of sodium periodate*

***B** = Fraction of the known amount of added periodate consumed in oxidising vicinal hydroxy groups*

Equation 2-1 Periodate equation used to determine the placement of substituents around the cyclodextrin cone (primary or secondary hydroxy groups).[14]

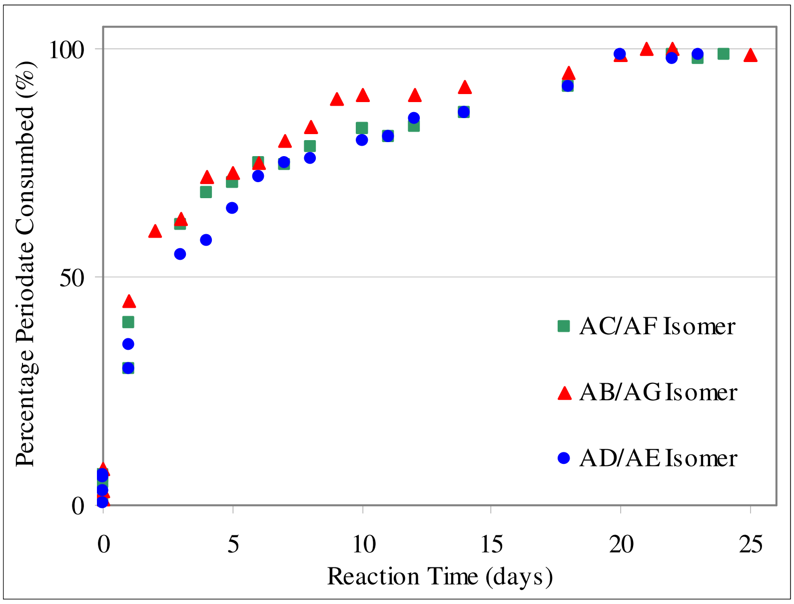
It was found that the reaction of compound **1** with an excess of periodate (Figure 2-17 and Graph 2-1) leads to a rapid consumption of one mole of periodate per glucose on each glucose residue which levels off after a number of days. This indicates that nothing was attached to the secondary hydroxy groups. It also indicates that a 10% excess of periodate is suitable for the reactions and has therefore been used in the oxidation of the functionalised compounds, **9** and **10**. Compound **9** (Graph 2-1) also led to the complete consumption of periodate indicating that the Cbz group had attached to the primary hydroxy group of β-CD.

Graph 2-1 Percentage of periodate consumed for the reaction with compounds **1** and **9**



Graph 2-2 indicates the attachment of the succinyl group was also at the primary hydroxy groups of β-CD as indicated by the 100% consumption of periodate. Again, the reaction took much longer to complete than that of compounds **1** and **9** (Graph 2-1) indicative of the lower reaction concentration. All three isomeric pairs (of compound **10**) completed the reaction within a few days of each other, following a very similar reaction path. The consumption of periodate (100%) for all tested compounds (**1**, **9**, and the three isomeric pairs of **10**) suggests that the functionalisation of β-CD has been successful at the primary hydroxy position.

Graph 2-2 Percentage of periodate consumed for the reaction with the AB/AG, AC/AF, and AD/AE isomers of compound **10**



2.3.3 *Synthesis of Mono-6^A-amino-mono-6^X-succinyl-β-cyclodextrin (**11**)*

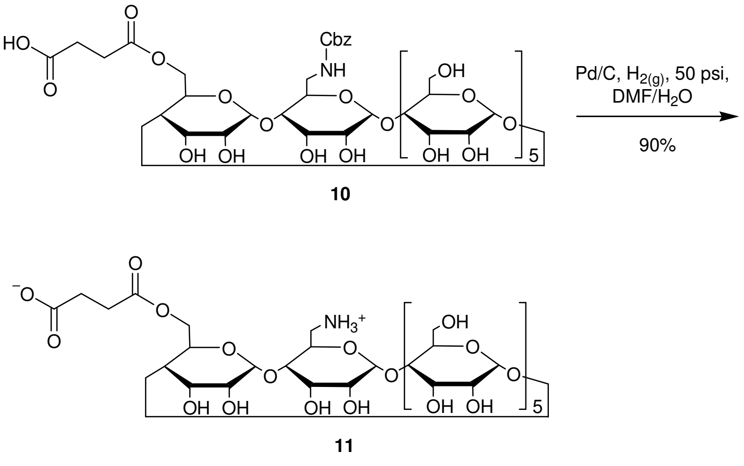


Figure 2-18 Synthesis of mono-6^A-amino-mono-6^X-succinyl-β-cyclodextrin (**11**)

Removal of the Cbz protecting group was achieved using hydrogenation using a heterogeneous catalyst (Pd/C, $H_{2\,(g)}$) as per Liskamp *et al.* (Figure 2-18).[7]

2.3.4 *Synthesis of Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**)*

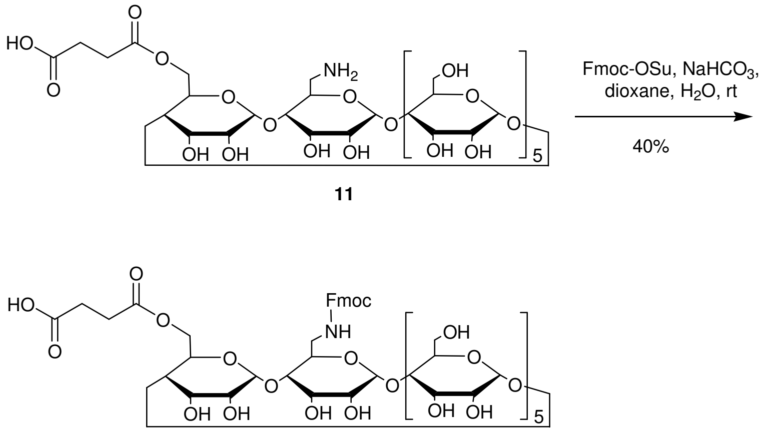


Figure 2-19 Synthesis of mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**)

Fmoc protection of **11** as per Liskamp *et al.*[7] gave the desired bi-functional product (**12**, Figure 2-19) ready for Fmoc SPPS (**Chapters 4** and **6**). RP-HPLC purification enabled determination of the isomer elution pattern as AC/AF: AD/AE: AB/AG in a ratio of 2:2:1 with retention times (t_R) of 34.9%B, 38.2%B, and 39.1%B respectively (Figure 2-20).

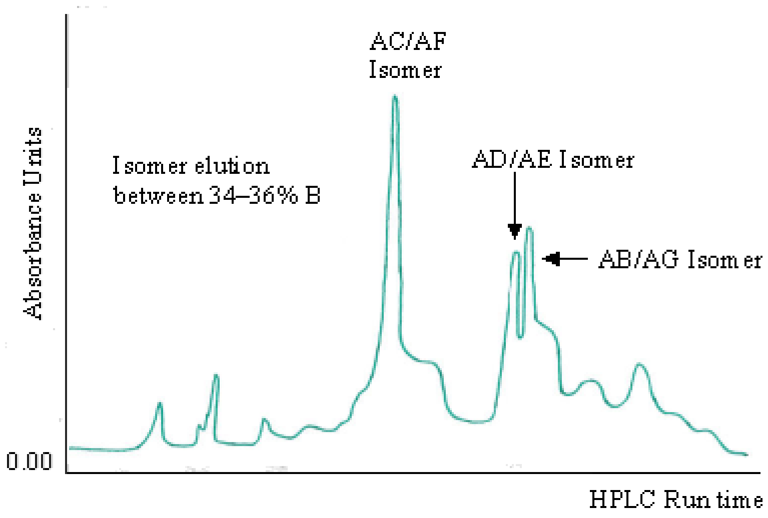


Figure 2-20 Compound **12** RP-HPLC trace

The stability of compound **12** to standard Fmoc SPPS coupling conditions was determined by exposing a small amount of pure sample to each of the reaction conditions stated in Table 2-5. Product breakdown was monitored by MALDI and the results show a loss of the Fmoc group (as expected) when exposed to a 20% pip in DMF solution, but no further product breakdown is seen (Table 2-5). This meant that compound **12** is stable and suitable for Fmoc SPPS reactions under these conditions.

Table 2-5 Stability test conditions and results for compound **12** when exposed to standard Fmoc SPPS reaction conditions

Reaction Conditions	*Experimental Time (Hours)*	*Results*
100% DMF	24	No change
100% DCM	24	
20% Pip in DMF	0.5	Fmoc protection lost
20% Pip in DMF	1.0	
0.5 mmol TBTU in DMF	24	No change
0.5 mmol DIEA in DMF	24	
0.5 TBTU/DIEA in DMF	24	
20% TFA in DCM	0.5	No change
95% TFA in DCM	24	

From here, coupling studies (as per White *et al.*)[10] were performed with differing coupling reagents, resins, coupling times, and solvents to find the best Fmoc-SPPS coupling conditions (Tables 2-6, 2-7, and 2-8) to test the coupling efficiency of each reaction. A small amount of resin was removed, dried, weighed and allowed to react with a fresh 20% pip in DMF solution for 2h with shaking. Its absorbance (detection of the free Fmoc group) was then measured at a 290 nm wavelength using a Varian Cary 50 UV-Vis spectrometer. Each experiment was carried out in duplicate. The coupling efficiency was then calculated based upon the theoretical substitution (mmol/g), the substitution of the starting resin (mmol/g), and the molecular weight of the target peptide (g/mol) taking into account the loss of the Fmoc group (Equation 2-2).[10]

Table 2-6 Experimental details to determine the coupling efficiency of compound **12** to Rink resin with a coupling time of overnight with one re-coupling

Experiment Number	*Coupling Conditions*
A-r	TBTU (4 equiv)/DIEA (8 equiv) in DMF
B-r	HBTU (4 equiv)/DIEA (8 equiv) in DMF
C-r	EDC (4 equiv)/DIEA (8 equiv) in H_2O
D-r	EDC (4 equiv, no base) in H_2O
E-r	EDC **(4 eq)/DIEA (8 eq) in Pyridine/ DMF (1:1)**
F-r	EDC (4 equiv)/TEA (8 equiv) in H_2O
G-r	EDC (4 equiv)/DIEA (8 equiv) in DMF
H-r	EDC (4 equiv)/DIEA (8 equiv) in DCM
I-r	EDC (4 equiv)/DIEA (8 equiv) in EtOH
J-r	EDC (4 equiv)/DIEA (8 equiv) in THF
K-r	EDC (4 equiv) in pyridine

Table 2-7 Experimental details to determine the coupling efficiency of compound **12** to Sieber resin with a coupling time of overnight with one recoupling

Experiment Number	*Coupling Conditions*
A-s	TBTU (4 equiv) /DIEA (8 equiv) in DMF
B-s	EDC (4 equiv) /DIEA (8 equiv) in 1:1 DMF/H_2O

Table 2-8 Experimental details to determine the coupling efficiency of compound **12** to TentaGel resin with a coupling time of overnight with one recoupling

Experiment Number	*Coupling Reagents*
1-t	TBTU (4 equiv)/DIEA (8 equiv) in DMF
2-t	TBTU (4 equiv)/HOBt (4 equiv)/ DIEA (8 equiv) in DMF
3-t	HBTU (4 equiv)/DIEA (8 equiv) in DMF

A = B x 1000 / [1000 + (B x (M – X))]

Where:

A *= theoretical substitution (mmol/g)*

B *= substitution of starting resin (mmol/g)*

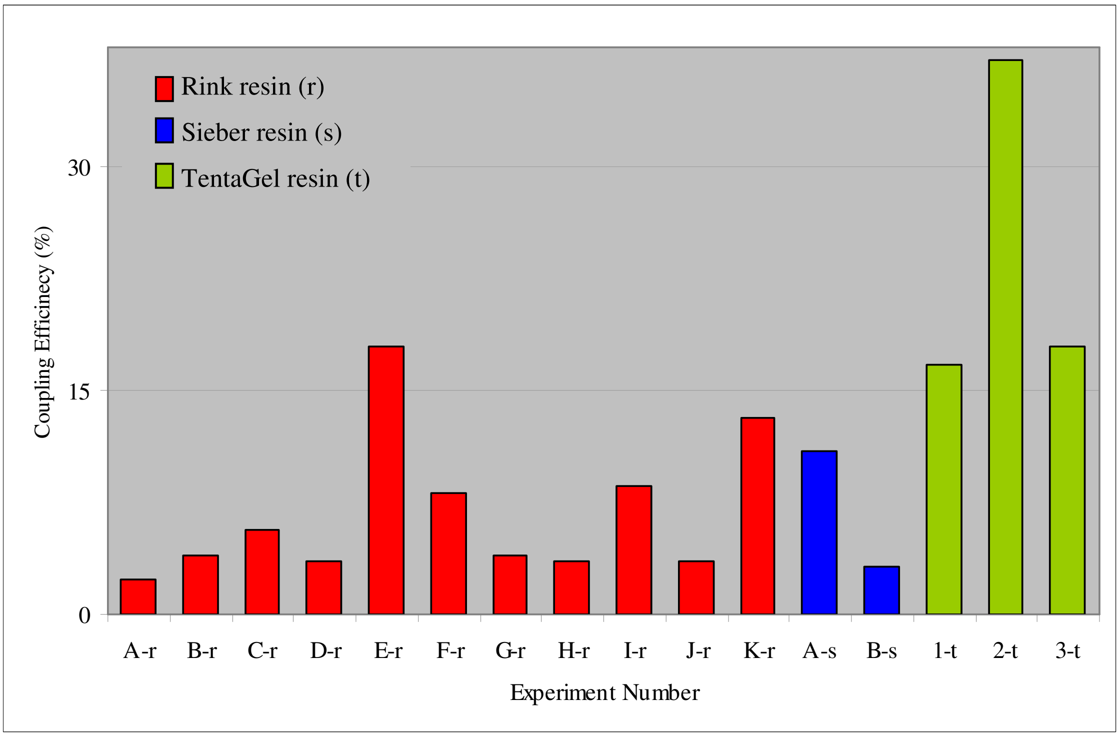
M *= molecular weight of target peptide, plus all protecting groups*

X *= addition of 239 for the Fmoc-protecting group of the resin*

Equation 2-2 Equation to calculate the coupling efficiency of Fmoc-compounds to Resin[10]

It was found that TentaGel resin gave the most efficient coupling compared to Rink and Sieber resin (Graph 2-3). This could be due to the lower loading of the resin which could reduce the amount of chain aggregation allowing for better coupling of the peptide to and cleavage from the resin.[11]

Graph 2-3 Coupling efficiencies for the coupling for compound **12** to Rink (r), Sieber (s), or TentaGel (t) resin under varying reaction conditions (Tables 2-6, 2-7, and 2-8 respectively)



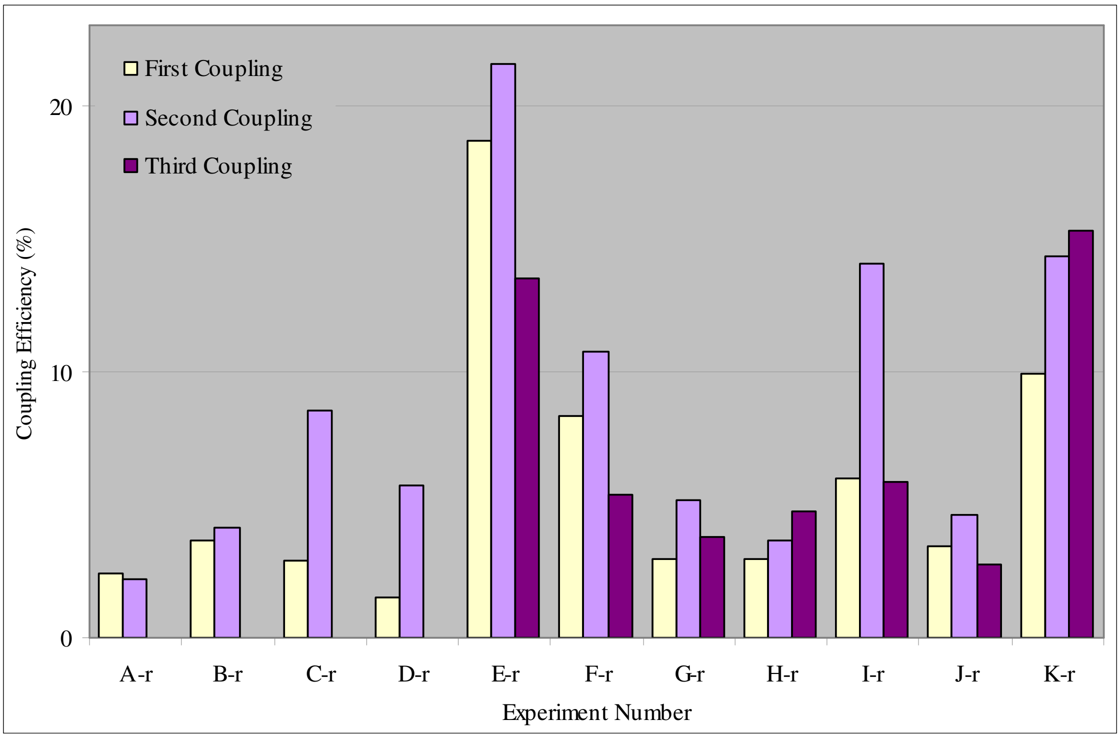
From Graph 2-3, the best coupling conditions for mono-6^{A}-fluorenylmethyloxycarbonylamino-mono-6^{X}-succinyl-β-cyclodextrin (**12**) to Rink resin was using WSCD (4 equiv, EDC used in this study), DIEA (8 equiv) in a 1:1 mixture of pyridine in DMF, followed by one recouple. The most common coupling conditions, namely variations of TBTU, HBTU, and HOBt with DIEA in DMF, were surprisingly low yielding in this experiment (<5%). This could possibly be due to the large size of compound **12**, the nature of the compound in relation to its hydrophobic bucket and/or solubility problems.

The coupling of compound **12** to Sieber resin gave only slightly better yields compared to that of Rink resin (3% and 11% in the two experiments tried, Graph 2-3). This could also be attributed to the same reasoning as for that of Rink resin. The coupling of **12** to Tentagel resin [TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight, *Experiment 2-t*, Graph 2-3] gave the highest coupling efficiency of 37%. It can be seen from Graph 2-3 that both Rink and Sieber resin give very low couplings under the same conditions tried compared to Tentagel resin (*Experiments A-r*, *1-t*, and *A-s*, Graph 2-3. One of the advantages of SPPS is its potential for high yields. Overall yields of amino acid addition in this study were much less than 100%. Of course peptide yields are variable and can be highly dependent upon their structure. Ideally, amino acid addition should be well above 90% with a high cleavage rate (greater than 99%) to give a reasonable yield for a long peptide. Hence, the coupling of **12** in these results is very inefficient.

These results suggest that perhaps steric problems were a major contributing factor in this study that have led to low coupling efficiencies of **12** to both Rink and Sieber resin. TentaGel resin provides a long PEG spacer from the core resin, and the reactive site needed for amino acid addition is potentially well out into the solvent from the support. Knowing that these resins are acceptable for the addition of standard amino acids, the large difference between compound **12** and amino acids is size. Compound **12** is a large, bulky molecule and the short linkers employed in this study could tend to produce problems for **12** accessing the resin reactive sites, hence low couplings and the low reaction yield. Different solvents were investigated to encourage an increase in accessibility through, for example, better resin swelling. It was found that the solvents all gave very similar coupling efficiencies. *Experiments G-r*, *H-r*, *I-r*, and *J-r* (Graph 2-3), all had efficiencies around 4% when using solvents DMF, DCM, EtOH, and THF. However, when using just water alone the efficiency doubled to *c.a.* 8% (*Experiments C-r* and *F-r*). This could be attributed to better solvation (and swelling) of the resin and solubility of **12** allowing for better activation with the coupling agent (EDC), and therefore a better overall coupling.

It can be seen from comparison of *Experiments D-r* to *Experiments E*-r, *F-r*, *G-r*, *H-r*, *I-r*, *J-r*, and *K-r* (Graph 2-4) that coupling using EDC with no base present (*Experiment D-r*, 4%) is less efficient than when a base is present (6-13%). This change is associated with the need for the CD compound to be deprotonated before attack of the EDC can commence. The large increase in coupling efficiency seen in *Experiment E-r* indicates that CD requires a stronger base presence in order to be fully deprotonated and for the experiment to proceed at a better rate. A better solubility of the resin/**12** is also thought to be important. The optimum coupling was found to be using EDC and DIEA in excess in a pyridine:DMF solvent system (*Experiment E-r*) giving 22% efficiency after two successive couplings.

Graph 2-4 Coupling efficiencies for one-, two-, or three-recouplings of compound **12** to Rink resin under varying reaction conditions (found in Table 2-6)



It was interesting to note that there was little change from one to two-recouplings on Rink resin in some cases (Graph 2-4). Namely, *Experiments A-r*, *B-r*, *G-r*, *H-r*, and *J-r*.

It was also interesting to note the small decrease in coupling efficiency from the second to a third coupling (E*xperiments E-r*, *F-r*, *G-r*, *I-r*, and *J-r*).

Further experiments were done to test the stability of the Fmoc group attached to compound **12** under each of these solvent conditions (namely, E*xperiments E-r*, *F-r*, *G-r*, *I-r*, and *J-r*) over a 3 day time period (the length of the experiment graphed in 2-4). Results showed no loss of the Fmoc under any conditions tested indicating that the lower coupling efficiency could be due to aggregation of the resin bound β-CD or trapping of the Fmoc group in the β-CD-resin once cleaved.

2.3.5 *Spacer Extension Studies of Compound **12***

From here, it is proposed that addition of a spacer to **12** would aid in its addition to Rink resin by making it more accessible for compound **12** to react with the active part of the resin, similar to that of TentaGel resin, which contains a long, unhindered PEG spacer. This would, in-turn, improve the final β-CD-peptidyl yields (in **Chapter 4**). This was attempted in two ways (see Figure 2-21).

The first was to couple a spacer (Fmoc-protected ε-aminocaproic acid used in this study) onto compound **12** and then add this to resin (A in Figure 2-21). The second was to add the spacer to the resin, and then couple compound **12** (B in Figure 2-21, see **Chapter 4** for synthesis details). From here, coupling efficiencies of the spacer-CD molecules were obtained and compared to the coupling of **12** to the unfunctionalised resin.

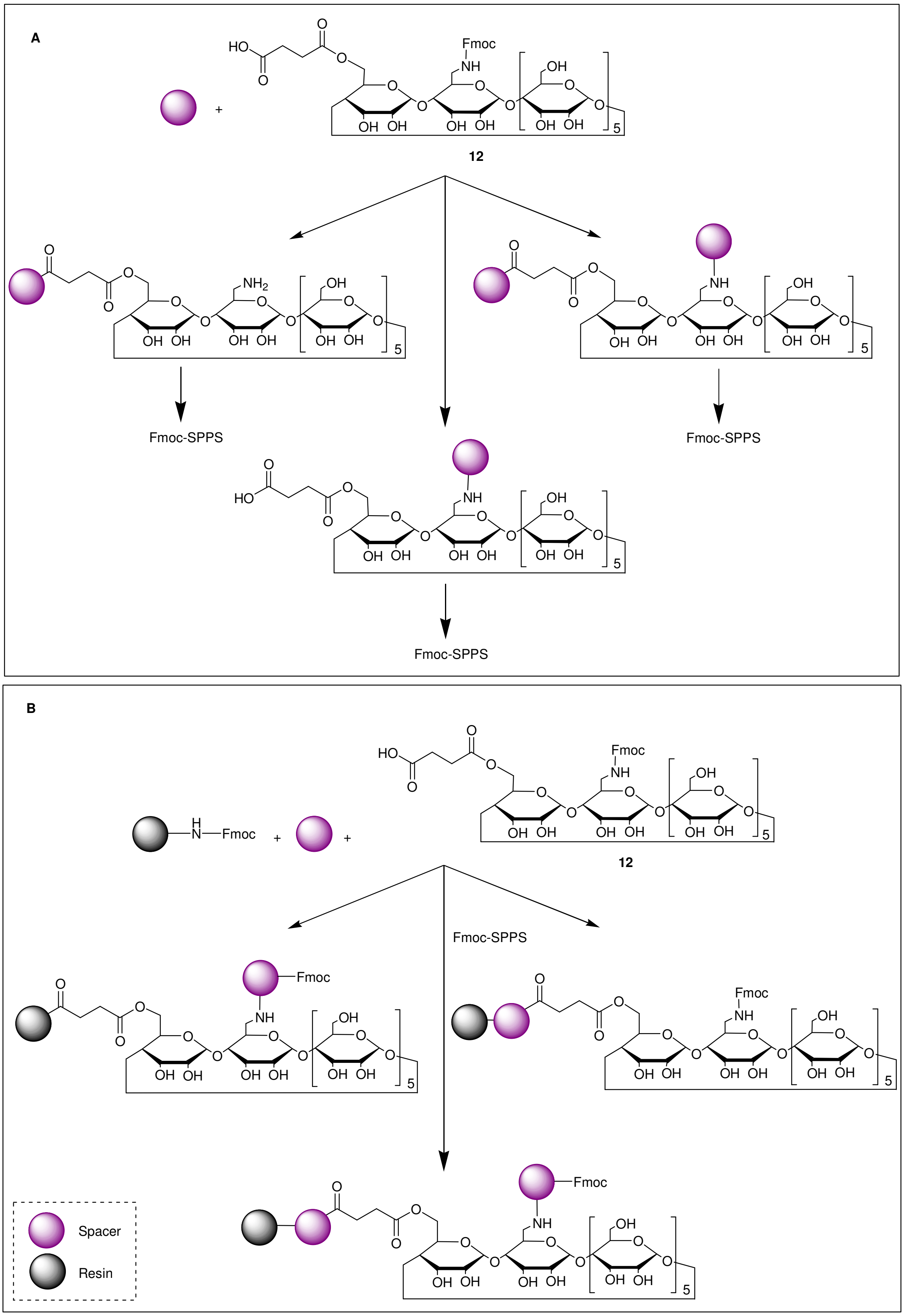


Figure 2-21 Spacer addition for better yield in peptide synthesis of compound **12**. *A*) attachment of the spacer directly to **12** followed by SPPS *B*) attachment of the spacer and **12** to the Resin using SPPS

Compounds **12i** (Figure 2-22), **12ii** (Figure 2-23), and **12iii** (Figure 2-24) were synthesised (in relation to A, Figure 2-21), but very low yields made this synthesis route unattractive (<1%, see **Sections 2.4.4.5** to **2.4.4.7**). It is presumed that the reactions were low yielding due to the complexity of the molecules. Therefore, an alternative synthesis route was used (B, Figure 2-21). This involved the treatment of the spacer as an amino acid, and involved coupling the spacer (ε-aminocaproic acid used in this study) to the resin followed by the addition of compound **12**, in various combinations (for synthesis details see **Chapter 4, Sections 4.6** and **4.7.7**).

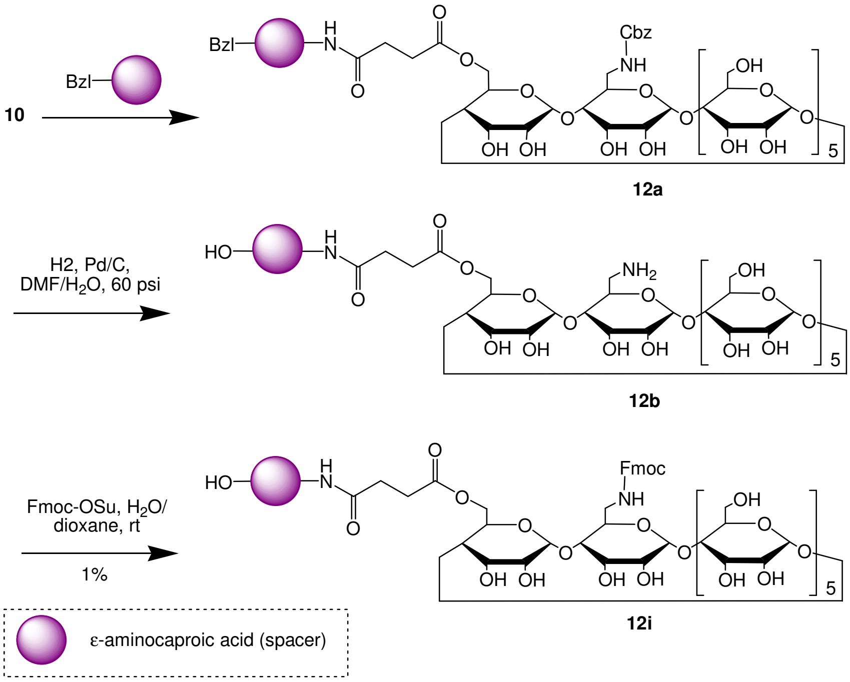


Figure 2-22 Synthesis of mono-6^{A}-fluorenylmethyloxy-carbonylamino-mono-6^{X}-succinyl-6^{X}-ε-amino-caproic acid-β-cyclodextrin (**12i**)

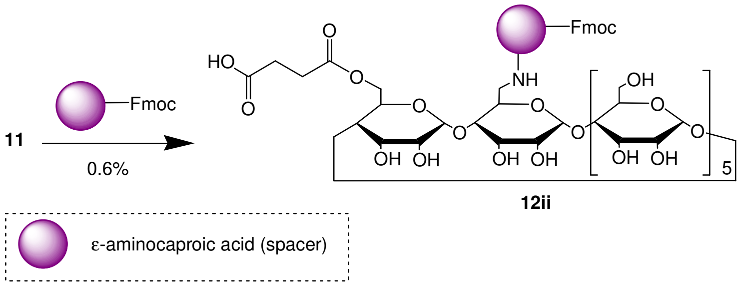


Figure 2-23 Synthesis of mono-6^{A}-ε-aminocaproic acid-6^{A}-fluorenylmethyloxycarbonyl-amino-mono-6^{X}-succinyl-β-cyclodextrin (**12ii**)

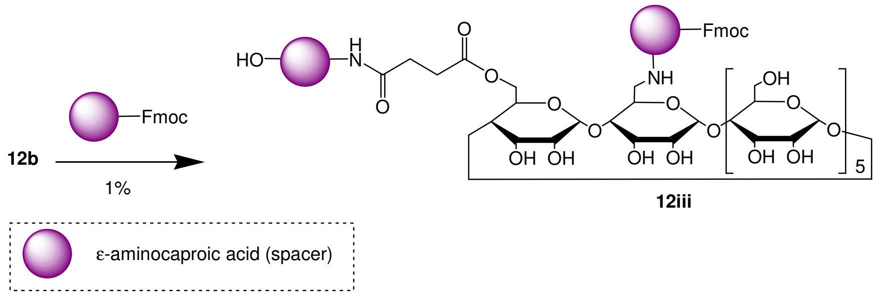
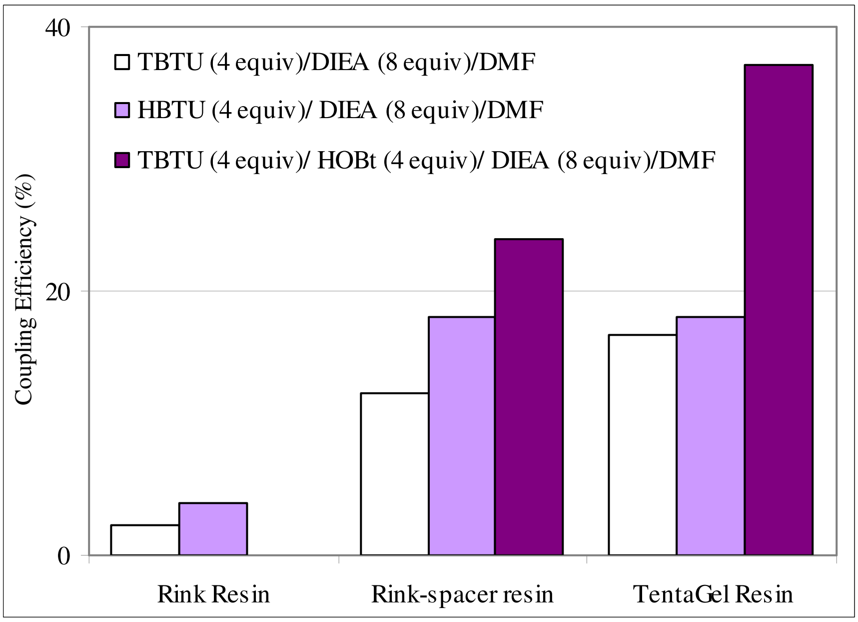


Figure 2-24 Mono-6^A-ε-aminocaproic acid-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-6^X-ε-aminocaproic acid β-cyclodextrin (**12iii**)

Coupling studies for compound **12** to resin-spacer were also completed and are able to be compared to the non-spacer experiments. The experiment was conducted by the addition of the spacer to Rink resin and then the coupling of compound **12** to this spacer (for more details refer to **Chapter 4**, **Sections 4.6** and **4.7.7**). *Fmoc-UV results (Graph 2-5) are for the addition of compound **12** to the resin-spacer, not for the spacer to the resin.*

Graph 2-5 Comparison of coupling efficiencies for compound **12** to Rink-spacer resin* compared to Rink and TentaGel resin, under the same reaction conditions



* For synthesis details refer to **Chapter 4**, **Section 4.7.7**

The results indicate that the addition of the spacer does indeed increase the coupling efficiency (Graph 2-5) of **12** dramatically, when coupling to Rink resin. This supports the contention that the low coupling efficiency is due to steric hindrance. Future studies should investigate this further, using longer spacers to see if the coupling can be improved further.

However, when compared to TentaGel resin, which has a long PEG spacer between the reactive site of the resin and the core, the coupling efficiencies for the Rink-spacer resin are still low (Graph 2-5). It can also be noted that the coupling mixture of TBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in DMF (dark purple bars, Graph 2-5) is the most efficient from these results.

Graph 2-5 also shows that coupling with TBTU and HOBt (4 equiv of each) with DIEA (8 equiv) is better than that of either TBTU or HBTU (4 equiv each respectively with 8 equiv DIEA). In relation to coupling with TBTU or HBTU, HBTU was slightly more efficient in this circumstance. It is known that the addition of HOBt to active ester reaction mixtures promotes the formation of the OBt ester faster and thereby leads to a more efficient coupling.[12] This approach has been used in many peptide syntheses, especially difficult ones. However, due to HOBt's recent classification as a desensitized explosive (UN3380), transport of this product by air or sea is no longer possible. It can however be made with extreme care, if required. There is currently no alternative purchasable replacement for HOBt in the peptide industry although, for some reactions, it has been found that there is no loss in yield with its omittance.[12]

2.4 Materials and Methods

2.4.1 *Reagents and Instruments*

Unless specified, all reagents were analytical reagent grade. For chromatography, all reagents were of High Pressure Liquid Chromatography (HPLC) grade. Water was of Milli-Q quality (Millipore, Biocel Synthesis A10). A Varian Cary 50 UV-Vis spectrometer was used to measure the absorbance for all compounds unless stated otherwise. β-cyclodextrin (hydrate) was obtained from Aldrich (USA), tosyl chloride and succinic anhydride were obtained from Merck (Schuhardt, Germany), *N*-α-

(benzyloxycarbonyloxy) succinimide (Cbz-OSu) and *N*-(9-flurenylmethoxy-carbonyloxy) succinimide (Fmoc-OSu) were obtained from Bachem (California, USA), EDT and Pd/C was purchased from Alfa Aesar (Heysham, Lancaster). Fmoc amino acids (i.e. Fmoc-Gly-OH, Fmoc-Ser-OH, Fmoc-Arg-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Pro-OH), Rink resin (0.69 or 0.72 mmol/g, 150 mesh), TBTU, HOBt, and HBTU were obtained from Auspep (Sydney, Australia). TentaGel resin (0.32 mmol/g) and ε-aminocaproic acid are from Fluka Analytical (Switzerland) and Sieber amide resin (0.72 mmol/g) was from NovaBiochem (Germany). The manufacturer's reported resin loading was used in the calculation of the yield of the final peptide. DIEA and thioanisole were purchased from Acros Organics (Belgium) and sodium azide was from BDH limited (Poole, England). Phenol (detached crystals) was purchased from May & Baker Ltd (Dagenham, England).

Dry DMF was obtained by stirring over CaH, distilled under vacuum, and stored over oven dried KOH pellets in the dark. Dry pyridine was obtained by drying over KOH, distilled, and stored over KOH. Bromoacetic acid was purified by recrystallisation from petroleum ether (60°C) and dried *in vacuo*.[13]

Unless stated elsewhere, compounds were purified by recrystallisation. For full characterisation purposes, compounds were further purified using RP-HPLC. All RP-HPLC experiments were performed with a Shimadzu LC-6 system equipped with a 300-Å Phenomenex guard column and a C-18 Jupiter, 250 by 21.20 mm reverse phase 15-µm separation column. The fractions were analysed with either a Waters Alliance HT 2790 HPLC instrument coupled to a Micromass ZMD electrospray mass spectrometer with infusion injection or a Waters Alliance Micromass LR-MS (TOF) mass spectrometer using α-cyanohydroxycinnamic acid as the matrix (2 mg/mL in 1:1 MeCN:MeOH). HR-MS data was obtained from the University of Auckland (Auckland) on a micro-time-of-flight single quadrupole electrospray with infusion injection using a 50% proponal:H_2O gradient. Water was removed from samples by freeze-drying using a FTS system (New York, USA).

All NMR spectroscopy were recorded on Bruker 500 MHz or 700 MHz spectrometers as stated. Chemical shift (δ) values are stated in parts per million (ppm). Samples were dissolved in deuterated solvents as stated.

2.4.2 *Experimental for Part a*

2.4.2.1 Mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin (**2**)

The synthetic route is illustrated in Figure 2-3. Mono-6^A-(4-methylbenzenesulfonyl)-β-cyclodextrin (**2**) was synthesised according to known procedures.[4] Briefly, **1** (5 g, 4.4 mmol, 1 equiv) and NaOH (2.5 g, 62.5 mmol, 14 equiv) was dissolved in H_2O (150 mL, 0-5ºC) with stirring. Tosyl chloride (3 g, 15.7 mmol, 3.5 equiv) was added to the reaction mixture which was then stirred vigorously for 2h on ice. A second portion of tosyl chloride (3 g, 15.7 mmol, 3.5 equiv) was added and stirred for 3h with TLC monitoring.[†] The reaction mixture was filtered through a bed of celite, cooled to 0-5ºC and 1M 10% HCl solution added (35 mL). The reaction was stored in the fridge overnight, filtered, and the solid was recrystallised (twice) from hot H_2O yielding a white solid (2.1g, 38%). For characterisation purposes, RP-HPLC analysis on a C-18 preparative column at a wavelength of 254 nm using an 8-mL/min MeOH stepwise gradient (Table 2-9). Buffer A: 100% H_2O + 0.1% TFA, buffer B: 100% MeOH was carried out. Samples were dissolved up in buffer A with sonication and filtered. The pure product eluted at 37% B.

Table 2-9 Methanol stepwise gradient time programme for the separation of **2** using a C-18 preparative RP-HPLC column

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	65	30
10	0	70	40
15	10	80	40
30	10	85	100
35	20	90	100
50	20	95	0
55	30	100	STOP

[†] A small amount of **2** was dissolved in DMF and spotted on Merck silica gel 60 plates with methylethylketone:H_2O:MeOH (4:1:1) as the elutant. It was developed by dipping in a ninhydrin stain (1.5 g ninhydrin in 100 mL n-butanol with 0.3 mL acetic acid) and heated. The mono-tosyl product (**2**) gave a purple spot (R_f of 0.75) and the starting material (**1**) gave a yellow spot (R_f of 0.5).

HR-MS: *m/z* $C_{49}H_{76}O_{37}SNa$ $(M+Na)^+$ 1311.3678, actual 1311.3642, LR-MS: *m/z* (%, assignment) 1311.668 (100, $M+Na)^+$, 1327.616 (45, $M+K)^+$; ^1H NMR (500 MHz, d_6-DMSO): δ = 2.42 (s, 3H, tosyl CH_3), 3.16-3.74 (m, 40H, CD H2-H6), 4.15-4.23 (m, 1H, CD H1), 4.33 (d, 1H, J =9.94 Hz), 4.74-4.88 (m, 13H), 5.44-6.12 (br s, 14H, CD OH2, OH3), 7.41 (d, J =7.6 Hz, 2H, tosyl aromatic), 7.75 (d, J =7.6 Hz, 2H, tosyl aromatic); ^{13}C NMR (500 MHz, d_6-DMSO): δ 21.6 (CH_3), 59.7, 59.9, 60.2, 60.4 (non-tosyl-C6), 69.3, 70.1 (tosyl-C6), 72.3, 72.4, 72.5, 72.6, 72.8, 72.8, 72.9, 73.1, 73.1, 73.4, 73.5 (non/tosyl-CD C3-5), 81.2, 81.6, 81.8, 81.9, 81.9, 82.1 (non/tosyl-CD C2), 101.7, 102.3, 102.3, 102.4, 102.4, 102.7 (non/tosyl-CD C1), 128.1, 130.4 (tosyl aromatics).

2.4.2.2 Mono-6^A-amino-β-cyclodextrin (**3**)

The synthetic route is illustrated in Figure 2-4. A solution of **2** (3.96 g, 3.02 mmol, 1 equiv) in 35% aq ammonia (refreshed daily) was stirred at rt for 7 days. The reaction was monitored by TLC[‡] where the product (**3**) gave a purple spot (baseline R_f), naked CD (**1**) gave a yellow spot (R_f of 0.5), and the starting material (**2**) gave a brown spot (R_f of 0.7). Solvents were removed *in vacuo* and freeze-dried overnight. Recrystallisation (three times) from hot H_2O yielded a pure white solid (3.56 g, 90%).

HR-MS: *m/z* $C_{42}H_{72}N_1O_{34}$ $(M+H)^+$ 1134.3930, actual 1134.3917, LR-MS: *m/z* (%, assignment) 1157.431 (100, $M+Na)^+$, 1173.447 (65, $M+K)^+$; ^1H NMR (500 MHz, d_6-DMSO): δ = 3.26-3.74 (m, 52H, CD C2-C6, including HOD peak), 4.46-4.69 (m, 6H, CD OH6), 4.96-4.79 (m, 7H, CD H1), 5.52-5.95 (br m, 14H, CD OH2, OH3), 7.12 (d, J= 7.6 Hz, 2H, NH_2); ^{13}C NMR (500 MHz, d_6-DMSO)**:** δ 60.4 (CD C6), 72.4, 72.5, 72.7, 72.8, 73.2, 73.4, 73.5 (CD C3-5), 81.9, 82.1, 83.3 (CD C2), 101.6, 102.1, 102.4 (CD C1).

[‡] A small amount of the solid (**3**) dissolved in DMF was spotted on Merck silica gel 60 plates with methylethylketone:H_2O:MeOH (4:1:1) as elutant, developed by dipping in an acid stain (10% sulfuric acid in EtOH) and heated.

2.4.2.3 Mono-6^A-succinylamino-β-cyclodextrin (**4**)

The synthetic route is illustrated in Figure 2-5. Mono-6^A-succinylamino-β-cyclodextrin (**4**) was synthesised using modified reaction conditions from Lincoln *et al.*[5] Briefly, **3** (1.5 g, 1.32 mmol, 1 equiv) was dissolved in dry DMF (20 mL). Succinic anhydride (145.6 mg, 1.46 mmol, 1.1 equiv) was added and the reaction was stirred at rt overnight. The reaction was monitored by TLC.[§] The solution was then added dropwise to acetone (600 mL) forming a very fine white precipitate. The solution was centrifuged at 2000 rpm for 15 minutes, the supernatant decanted off; the white solid washed with acetone twice, and freeze-dried overnight yielding a pure white solid (1.46 g, 89%).

LR-MS: *m/z* (%, assignment) 1256.348 (100, $M+Na)^+$, 1272.320 (65, $M+K)^+$; ^{1}H NMR (500 MHz, d_6-DMSO): δ 2.32-2.46 (m, 4H, succinyl CH_2 groups), 3.12-3.90 (m, 42H, CD C2-C6, including HOD peak), 4.39-4.56 (m, 10H, CD OH6), 4.79-4.88 (m, 11H, CD H1, 5.66-5.88 (m, 23H, CD OH2, OH3), 7.66-7.72 (t, J= 4.6 Hz, 1H, NH), 11.89-12.18 (br s, 1H, succinyl OH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 29.6 (succinyl CH_2), 30.3 (succinyl CH_2), 60.3, 60.4 (CD C6), 70.3, 72.4, 72.6, 72.8, 73.4 (CD C3-C5), 81.8, 82.0, 82.1 (CD C2), 102.2, 102.4 (CD C1)

2.4.3 *Experimental for Part b*

2.4.3.1 Mono-6^A-(α-azidoacetamido)-β-cyclodextrin (**5**)

The synthetic route is illustrated in Figure 2-6.

Method A:

Bromoacetic acid (**6**, 245 mg, 0.88 mmol, 2 equiv) dissolved in a solution of TBTU (850 mg, 1.32 mmol, 3 equiv) in DMF was added to a solution of **3** (500 mg, 0.44 mmol

---

[§] A small amount of the solid (**4**) dissolved in DMF was spotted on Merck silica gel 60 plates with methylethylketone:H_2O:MeOH (4:1:1) as elutant, developed by dipping in an acid stain (10% sulfuric acid in ethanol) and heated. The product (**4**) gave a brown spot with an R_f of 0.27, and the starting material (**3**) gave a brown spot with a baseline R_f.

1 equiv) dissolved in DMF. DIEA (464 μL, 1.32 mmol, 3 equiv) was added and the reaction mixture allowed to stir at rt overnight forming an amber coloured solution (**7**). NaN_3 (287 mg, 4.4 mmol, 10 equiv) was added to the crude reaction mixture of **7** and the solution was allowed to stir at rt overnight. The aq layer was washed with EtOAc (2 x 10 mL) and ether (2 x 10 mL), then the product was precipitated with acetone, and the pure product isolated by centrifugation (**5**, 450 mg, 84%).

Method B:

Compound **6** (1.21 g, 8.76 mmol, 10 equiv) dissolved in DMF and NaN_3 (564.3 mg, 0.88 mmol, 10 equiv) was shaken overnight at rt forming a milky white suspension (**8**). TBTU (1.40 g, 4.4 mmol, 5 equiv) was added to the reaction mixture of **8** for preactivation in the dark for 25 minutes, followed by addition of **3** (990 mg, 0.088 mmol, 1 equiv). The reaction was allowed to stir at rt in the dark overnight. The solvent was concentrated *in vacuo*, then the product was precipitated with acetone, and the pure product isolated by centrifugation as a white solid (**5**, 674 mg, 64%).

^{1}H NMR (500 MHz, d_6-DMSO): δ 3.20-3.44 (m, 31H, CD H6-5, including HOD peak), 3.49-3.77 (m, 29H, CD H4-2, including HOD peak), 3.78-3.88 (m, 2H, CH_2), 4.35-4.55 (m, 6H, CD OH6), 4.80-4.91 (m, 7H, CD H1), 5.64-5.85 (m, 14H, CD OH2, OH3); ^{13}C NMR (500 MHz, d_6-DMSO): δ 50.9 (CH_2), 60.2 (CH_2), 60.3 (CH_2), 70.1, 72.1, 72.5, 72.6, 72.8, 72.8, 72.9, 73.4, 73.5, 81.7, 81.8, 81.9, 84.3, 102.4, 102.4, 102.5, 102.7

See **Appendix 2-I** for peptide synthesis using compound **5**

2.4.4 *Experimental for Part c*

2.4.4.1 Mono-6^A-carbobenzyloxyamino-β-cyclodextrin (**9**)

The synthetic route is illustrated in Figure 2-8. *N*-α-(benzyloxycarbonyloxy) succinimide (Cbz-OSu, 220.0 mg, 0.88 mmol, 1 equiv) was dissolved in dioxane (20 mL) and added dropwise to a solution of $NaHCO_3$ (92.6 mg, 0.88 mmol, 1 equiv) and **3** (1.0 g, 0.88 mmol, 1 equiv) dissolved in H_2O (20 mL). The reaction was stirred at rt for 4h. Solvents were removed *in vacuo* and freeze-dried overnight yielding an off-white coloured solid. Recrystallisation (twice) from hot H_2O with overnight precipitation at 4°C yielded a white coloured solid (1.14 g, 51%). For characterisation purposes, RP-HPLC analysis was performed at 254 nm on a C-18 preparative column using an 8-mL/min MeOH stepwise gradient (Table 2-10). Buffer A: 100% H_2O + 0.1% TFA, buffer B: 100% MeOH. The sample was dissolved in buffer A with sonication and filtered. The pure product eluted between 33-35% B.

Table 2-10 Methanol stepwise gradient time programme for the separation of **9** using a C-18 preparative RP-HPLC column

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	80	100
10	0	90	0
60	50	100	STOP
70	100	-	-

HR-MS: *m/z* $C_{50}H_{77}NO_{36}Na$ $(M+Na)^+$ 1290.4117, actual 1290.4118, LR-MS: *m/z* (%, assignment) 1291.058 (100, $M+Na)^+$; ^1H NMR (500 MHz, d_6-DMSO): δ 3.21-3.76 (m, 42H, CD H2-H6), 4.78-4.86 (m, 7H, CD H1), 4.99 (q, J= 13.1 Hz, 2H, Cbz CH_2), 7.03-7.09 (m, 1H, NH), 7.28-7.38 (m, 5H, Cbz aromatic); ^{13}C NMR (500 MHz, d_6-DMSO): δ 60.3, 60.3 (C6), 65.8 (Cbz CH_2), 70.4, 72.3, 72.4, 72.5, 72.6, 72.9, 73.4 (CD C3-C5), 81.8, 82.0, 84.1 (CD C2), 102.1, 102.4 (CD C1), 128.2, 128.3, 128.7 (Cbz aromatics)

2.4.4.2 Mono-6^A-carbobenzyloxyamino-mono-6^X-succinyl-β-cyclodextrin (**10**)

The synthetic route is illustrated in Figure 2-9. Succinic anhydride (1.40 g, 14.05 mmol, 1 equiv) dissolved in dry pyridine was added dropwise to a solution of **9** (17.8 g, 14.05 mmol, 1 equiv, dried at 120°C overnight) dissolved in dry pyridine. The reaction mixture was stirred at rt overnight under a $N_{2(g)}$ atmosphere. The reaction was quenched with H_2O and the solvents were removed *in vacuo* yielding a fluffy white solid.[¶] The solid was recrystallised (twice) from hot H_2O yielding 7.6 g of white product (40%)

[¶] Products in the reaction mixture include mono-, bi-, and tri-succinyl species of compound **10** and starting material (**9**).

with trace amounts of the bi- and tri-succinyl species still present. Two RP-HPLC purifications were performed at a wavelength of 254 nm on a C-18 preparative column using first an 8 mL/min MeOH stepwise gradient and second an 8 mL/min MeCN stepwise gradient. Buffer A: 100% H_2O + 0.1% TFA, buffer B: 100% MeOH (or 100% MeCN). The sample was dissolved up in buffer A. The time programme used for both purifications is shown in Table 2-11. The pure product eluted between 36.3-40% B (Figure 2-11) after the second (MeCN) purification. Three isomeric pairs (AD/AE: AC/AF: AB/AG), the location of which are shown on β-CD in Figure 2-10, eluted with a 2:2:1 ratio as determined by ESI-MS analysis.[\\] Stability studies for compound **10** under standard Fmoc SPPS coupling conditions were also investigated.[**] Studies into the identification and confirmation of the placement of the Cbz and succinyl groups around the CD cone,[††] were also carried out. For further purification attempts and other reaction conditions attempted see **Appendix 2-II** and **Appendix 2-III** respectively.

Table 2-11 Methanol and acetonitrile stepwise-gradient time programme for the separation of **10** using a C-18 preparative RP-HPLC column

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	80	100
10	0	90	0
70	60	100	STOP

HR-MS: *m/z* $C_{54}H_{82}NO_{39}$ $(M+H)^+$ 1368.4458, actual 1368.4401 and $C_{54}H_{81}NO_{39}Na$ $(M+Na)^+$ 1390.4278, actual 1390.4259, LR-MS: *m/z* (%, assignment) 1389.720 (100, $M+Na)^+$, 1405.650 (30, $M+K)^+$; ^{1}H NMR (500 MHz, d$_6$-DMSO, regio isomeric mixture): δ 2.41-2.48 (m, 4H, succinyl CH_2), 3.21-3.78 (m, 63H, CD H2-6, including HOD peak), 4.11-4.53 (m, 6H, CD OH6, succinyl OH), 4.78-7.93 (m, 6H, CD H1), 4.93-5.06 (m, 2H, Cbz CH_2), 5.38-6.23 (br s, 14H, CD OH2, OH3), 7.25-7.39 (m, 5H, Cbz aromatic group); ^{13}C NMR (500 MHz, d$_6$-DMSO): δ 28.9 (CH_2), 29.0 (CH_2), 60.3

[\\] ESI-MS isomeric identification method as per Sforza *et al.*[14]

[**] Using a periodate reaction as per reference 9

[††] The stability of compound **10** under standard Fmoc-SPPS coupling conditions was determined by exposing a small amount of **10** to each of the reaction conditions stated in Table 2-4. MALDI analysis was used to look for any breakdown products.

(CH_2), 65.8 (CH_2), 72.3, 72.4, 72.5, 72.6, 72.7, 72.8, 72.9, 73.3, 73.4, 81.5, 81.8, 81.9, 82.6, 84.1, 102.2, 102.4, 102.4, 102.5, 102.6, 102.9, 103.0, 128.1, 128.2, 128.3, 128.4, 128.7

- Periodate reaction[9]

Briefly, four plastic cuvette cells (1.00 cm path length) were filled with water and absorbance readings, at 300 nm, were taken using one of the cells (cell 1) as a blank. Any absorbance differences for these cells were later applied as corrections. Cells 2, 3, and 4 were emptied, dried, and filled in the following manner:

Equal and known volumes of a solution containing the sample to be oxidised (**1**, **9**, or **10**) were added to cells 2 and 4 and the same volume of water is added to cell 3. Similarly, equal and known volumes of periodate solution (in approximately 10% excess of the total number of free vicinal hydroxy groups present) were added to cells 3 and 4. An equal volume of water was added to cell 2. The solutions in the cells are mixed and their absorbances were measured at regular intervals, appropriate for the speed of each individual reaction.[8]

Specific Experimental Details:

- Periodate reaction for CD (compound **1**):

- Cell 2: 1 mL of a stock solution of **1** (2.218 mmol, 2.515 g in 250 mL distilled water, 0.0088 mol/L) and 1.7 mL water

- Cell 3: 1.7 mL periodate stock solution (2.00 mmol, 427.8 mg in 100 mL distilled water, 0.02 mol/L) and 1 mL water

- Cell 4: 1.7 mL periodate stock solution and 1 mL of **1** stock solution.

*Moles of periodate stock solution required in 10% excess accounting for the 14 free hydroxy groups on compound **1** is 34.0 mmol.*

- Periodate reaction for compound **9:**

- Cell 2: 1 mL of a stock solution of **9** (0.0789 mmol, 100 mg in 50 mL distilled water, 1.578 mmol/L) and 260 μL water

- Cell 3: 260 μL periodate stock solution (2.00 mmol, 427.8 mg in 100 mL distilled water, 0.02 mol/L) and 1 mL water

- Cell 4: 260 μL periodate stock solution and 1 mL of **9** stock solution

*Moles of periodate stock solution required in 10% excess accounting for the 14 free hydroxy groups on compound **9** is 1.04 mmol.*

- Periodate reaction for compound **10**:

- Cell 2: 1 mL **10** (isomers AB/AG, AC/AF, or AD/AE, 0.0107 mmol, 14.7 mg in 10 mL distilled water, 1.075 mmol/L) and 1 mL water

- Cell 3: 1 mL periodate stock solution (3.277 mmol, 70.01 mg in 100 mL distilled water, 3.277 mmol/L) and 1 mL water

- Cell 4: 1 mL **10** and 1 mL sodium metaperiodate

*Moles of periodate stock solution required in 10% excess accounting for compound 14 free hydroxy groups compound **10** is 0.013 mmol.*

2.4.4.3 Mono-6^A-amino-mono-6^X-succinyl-β-cyclodextrin (**11**)

The synthetic route is illustrated in Figure 2-18. Compound **10** (500 mg, 0.365 mmol, 1 equiv) was dissolved in a DMF:H_2O mixture (2:1, 2 mL) in a 10 mL test tube. Pd/C (1 g) was added and the reaction mixture was hydrogenated at 50 psi for 7h at rt, then filtered through filter paper, and then a bed of celite. Solvent was removed *in vacuo* and the reaction mixture was freeze-dried overnight yielding a pale brown coloured solid. Recrystallisation (twice) from hot H_2O yielded a white product (405 mg, 90%). RP-

HPLC purification was achieved using a preparative C-18 RP-column (at a wavelength of 254 nm) with an 8 mL/min MeOH stepwise gradient (Table 2-11). Buffer A: 100% H_2O + 0.1% TFA, buffer B: 100% MeOH. Sample was dissolved up in buffer A and filtered. The pure product eluted at 23-25% B.

HR-MS: *m/z* $C_{46}H_{76}NO_{37}$ $(M+H)^+$ 1234.4091, actual 1234.4052, LR-MS: *m/z* (%, assignment) 1255.543 (95, M+Na)$^+$; ^{1}H NMR (500 MHz, d_6-DMSO): δ 2.45-2.50 (m, 2H, succinyl CH_2), 2.53-2.85 (m, 2H, succinyl CH_2), 3.20-3.77 (m, 53H, CD H2-6, including HOD peak), 4.08-4.70 (m, 6H, CD OH6, succinyl OH), 4.82-4.97 (m, 6H, CD H1), 5.59-5.91 (m, 14H, CD OH2, OH3), 7.72-7.87 (m, 2H, NH_2); ^{13}C NMR (500 MHz, d_6-DMSO): δ 29.1 (succinyl CH_2), 30.0 (succinyl CH_2), 60.0 (CH_2), 60.2 (CH_2), 60.41 (CH_2), 60.44 (CH_2), 60.48 (CH_2), 60.5 (CH_2), 68.2, 69.3, 72.4, 72.5, 72.6, 72.7, 72.8, 72.9, 72.8, 73.0, 73.1, 73.21, 73.25, 73.39, 73.47, 73.5, 73.6, 102.413, 102.4, 102.5

2.4.4.4 Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**)

The synthetic route is illustrated in Figure 2-19. Adapted from Liskamp *et al.*[7] Fmoc-OSu (4.51 g, 13.35 mmol, 1 equiv) dissolved in dioxane was added dropwise to a solution of **11** (16.77 g, 13.35 mmol, 1 equiv) and Na_2CO_3 (83 mg, 13.35 mmol, 1 equiv) dissolved in H_2O. The pH was kept below 8. The reaction mixture was allowed to stir at rt overnight. 1M HCl (40 mL) was added to the reaction mixture to form a milky white coloured solution. Upon addition of EtOAc (40 mL) a clear solution formed. The aq layer was isolated, solvent was removed by freeze-drying overnight, and then dissolution (twice) from hot H_2O, filtration, and freeze-drying yielded a pale brown coloured solid (18.80 g, 40%). RP-HPLC analysis was carried out at a wavelength of 254 nm using an 8 mL/min MeCN stepwise gradient (Table 2-12). Buffer A: 100% H_2O + 0.1% TFA, buffer B: 100% MeCN. The sample was dissolved up in H_2O and filtered. The pure product eluted at 34-36% B (Figure 2-20). The stability of compound **12** to standard Fmoc coupling conditions was explored.[‡‡] Fmoc UV-vis coupling efficiency experiments were performed as per reference 10 to determine the best method of

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[‡‡] A small amount of sample was exposed to each of the reaction conditions stated in Table 2-5. Product breakdown was monitored by MALDI

coupling **12** to resin, under Fmoc-SPPS conditions. The method for determining the isomer elution pattern of AC/AF: AD/AE: AB/AG was examined by ESI-MS analysis.[§§]

HR-MS: *m/z* $C_{61}H_{85}NO_{39}Na$ $(M+Na)^+$ 1478.4591, actual 1478.4496, LR-MS: *m/z* (%, assignment) 1478.867 (100, $M+Na)^+$; ^{1}H NMR (500 MHz, d_6-DMSO, regio isomeric mixture): δ 2.35-2.49 (m, 2H, succinyl CH_2), 2.52-2.68 (m, 2H, succinyl CH_2), 2.99-3.99 (m, 74H, CD H2-6, OH6, including HOD peak), 4.18-4.29 (m, 4H, succinyl OH, Fmoc CH and CH_2), 4.78-4.95 (m, 7H, CD H1), 5.30-6.15 (m, 14H, CD OH2, OH3), 7.08 (s, 1H, NH), 7.29-7.44 (m, 4H, Fmoc aromatics), 7.68-7.78 (m, 2H, Fmoc aromatics), 7.85-7.91 (m, 2H, Fmoc aromatic); ^{13}C NMR (500 MHz, d_6-DMSO): δ 29.02 (CH_2 succinyl), 29.08 (CH_2 succinyl), 47.2 (Fmoc CH), 60.07 (CH_2), 60.08 (CH_2), 60.1 (CH_2), 60.2 (CH_2), 60.3 (CH_2), 60.4 (CH_2), 66.0 (Fmoc CH_2), 69.2, 69.3, 70.4, 70.5, 72.4, 72.5, 72.5, 72.6, 72.7, 72.8, 72.9, 73.3, 73.46, 73.42, 73.9, 81.5, 81.7, 81.8, 82.5, 84.1, 102.2, 102.31, 102.34, 102.41, 102.45, 102.5, 102.92, 102.96, 120.5, 125.7, 127.5, 128.0

Table 2-12 Acetonitrile stepwise gradient time programme for the separation of **12** using a C-18 preparative RP-HPLC column

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	90	100
10	0	100	100
50	40	110	0
80	60	120	STOP

2.4.4.5 Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-6^X-ε-aminocaproic acid-β-cyclodextrin (**12i**)

The synthetic route is illustrated in Figure 2-22. In this synthesis, the benzyl (Bn) ester

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[§§] Each isomeric pair (AB/AG, AC/AF, and AD/AE) of compound **10** (10 mg, 0.00811 mmol, 1 eq) was reacted under parallel reaction conditions converting each into compound **12**. RP-HPLC purification was performed on each isomer separately as per the same conditions for compound **12**, to establish the individual retention times for each isomeric pair. The elution pattern is AC/AF: AD/AE: AB/AG in a ratio of 2:2:1 with retention times of 34.9%B, 38.2%B, and 39.1%B respectively.

protected spacer (synthesis details is **Appendix IX**) is used as the Bn group can be removed with hydrogenation in the same step as the loss of the Cbz group (also removed with hydrogenation). Fmoc protection will lead to the final target molecule (**12i**) in a 1% yield, which can be added to resin. For experimental details see **Appendix 2-IV.**

2.4.4.6 Mono-6^A-ε-aminocaproic acid-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12ii**)

The synthetic route is illustrated in Figure 2-23. In this synthesis, the Fmoc protected spacer would be added in the final step of the synthesis to make compound **12ii**, in a 0.6% yield, giving an extension of the amine terminus. For experimental details see **Appendix 2-V**.

2.4.4.7 Mono-6^A-ε-aminocaproic acid-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-6^X-ε-aminocaproic acid-β-cyclodextrin (**12iii**)

The synthetic route is illustrated in Figure 2-24. Fmoc-spacer protection of the free amine on **12b** gives **12iii** in a 1% yield. For experimental details see **Appendix 2-VI.** See **Chapters 4** and **6** for peptide synthesis using compound **12**

2.5 Conclusions

Overall, this methodology avoids the use of bulk protection of the unutilised hydroxy groups, a first for β-CD bi-functionalisation. It should allow for the potential control of peptide synthesis onto β-CD without the need for the extra involvement of hydroxy protection and subsequent deprotection. This method also shows a more succinct methodology, resulting in a lower number of synthetic steps from basic β-CD in current literature.[13-14]

This leads us into **Chapters 4** and **6** with the attachment of bioactive peptides and bioactive peptide sulfonamides respectively. However, before such peptide addition can take place, the investigation into the synthesis of the bioactive peptide bradykinin (BK) was investigated (**Chapter 3**) as well as the synthesis of bioactive peptide sulfonamides (**Chapter 5**).

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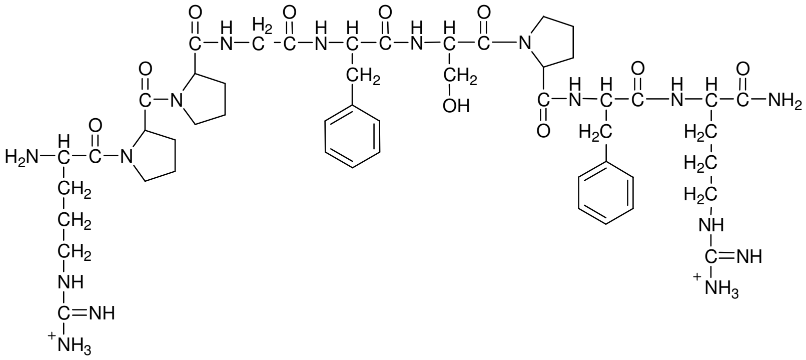
Chapter Three:

Synthesis and Purification of Bradykinin

3.0 Introduction

The synthesis of bradykinin (BK, Figure 3-1) dates back to the early days of Merrifield's career in 1964 where it was first used to demonstrate the efficiency of his newly proposed Solid Phase Peptide Synthesis (SPPS) method. BK was chosen for its complexity as it contains the trifunctional amino acids arginine and serine, as well as proline, the latter adding rigidity to the peptide secondary structure. It was also a peptide of physiological importance with specific biological activities, as well as offering a comparison against the conventional solution synthesis method, which had already been used to synthesise BK. Since then many analogues of BK have been investigated for potential anticancer activity, as well as other therapeutic activities.[1]



Bradykinin, **13**

Figure 3-1 Amino acid sequence for the *C*-terminal amide bradykinin (BK, **13**) peptide

The synthesis of BK has been primarily performed using Boc chemistry with few changes since Merrifield's synthesis in 1964.[1] Yields are in the range of 39% to 83% (Table 3-1).[1d-f,2] There have been slight improvements to Merrifield's method including the changing of coupling reagents, side chain and amino acid protecting groups, and an alteration in the cleavage procedures as more knowledge was gained about peptide chemistry. To my knowledge, there has been little mention of BK synthesis using Fmoc methods throughout the literature[2] with the exception of BK analogues (inclusion/loss of amino acids) which have more recently been synthesised using Fmoc chemistries.[3]

Alterations to the BK sequence have been very common for purposes of structure-function studies. These have been mainly associated with the use of BK analogues as antagonists towards cancer in cells.[1a-c,4] An alteration to the sequence of the BK peptide or to the BK receptor (in the cell) has been shown to display anticancer properties. This is attributed to the loss of function of the BK peptide for the importation of molecules required for the sustainment of tumour cell growth. These studies into BK analogue activities have focused on molecules that can interact with hormone receptors (antagonists) or ones that can amplify (agonists) the biological response of BK, leading to drug design. Research has found that the best anti-cancer derivatives need bulky hydrophobic amino acids at the *C*- and *N*-terminus. It has also been shown that slight changes to the sequence of BK can alter its role dramatically within the cell. This includes the change from D- to L-amino acids.[1a-c,4]

Dimers of BK (e.g. BK joined with disulfide bridges) and their analogues have also been shown to produce anticancer activity in certain cell lines (i.e. SCLC cells).[1a-c,5] The retention of the proline residues within BK analogues has been shown to be important for the interaction of BK with its receptor. This is due to proline offering a high degree of rigidity and structural control within the BK peptide because it is a cyclic amino acid. It has also been shown that exchange of the cleavage point amino acids, Arg_1, Phe_5, and Arg_9 with other amino acids can lead to an increase in BK's half-life. For ease of tracking peptides/drugs when used in various studies, fluorescent tags, and/or radiolabelling have been used.[1a-c,4]

So far there have been few major *N*- and *C*-terminal <u>extensions</u> of the BK nonapeptide sequence. Oral peptide analogues based upon BK have shown a low bioavailability. Conversion to non-peptidic molecules has been achieved without a loss of activity. Modifications to the BK sequence have also seen BK agonists and antagonists designed so that there is a decrease in their peptidyl nature allowing better functioning and longer half-lives (e.g. in cells) paving the way for a new group of drugs.[1a-c,5]

Table 3-1 A selection of literature search results for the synthesis of bradykinin (BK) with a carboxy C-terminus

Brief Synthesis Details	*Yield*	*References*
Boc SPPS on chloromethylated copolystyrene-2% divinylbenzene as the support. De-Boc with HCl in acetic acid. Amino acid (4 equiv, except proline, 8 equiv) coupling with DCC for 2h. Get urea formation. No acylation. HBr cleavage. 8 days to synthesise.	83%	1e
Boc SPPS on a chloromethylated copolystyrene-2% divinylbenzene is the support. Boc removal with HCl in acetic acid for 30 minutes, amino acid (4 equiv, except proline, 8 equiv) coupling with DCC for 2h with urea formation. No acylation. HBr cleavage of peptide from resin. 8 days to synthesise.*	65%	5
Boc SPPS onto *t*-Boc-L-phenylalanyl-nitro-L-arginyl resin with acetic anhydride (Ac_2O) capping. DCC couplings. Peptide cleavage using HBr in TFA. Ion exchange with acetic acid gradient to purify.*	51%	1d
Boc SPPS on poly-4-hydroxy-3-nitrostyrene support using amino acid active esters. DCC couplings in DMF. Removal of Boc with HBr in glacial acetic acid followed by neutralisation with TEA. Peptide cleavage and orthogonal protecting groups removal by HF.	39%	1f
Fmoc SPPS on Wang resin (with hydroxmethyl groups as the solid support). Amino acid coupling DIPCD/HOBt in DMF pre activation. Deprotection with 20% pip in DMF. HPLC purification.	69%	2

* Arginines' nitro deprotection using catalytic hydrogenation with palladium black

3.0.1 *Chapter Outline*

This chapter describes the synthesis of the BK peptide, having both *C*-terminal amide and carboxy ends. Boc-SPPS (**Appendix 3-I** to **3-II**) was initially tried for the synthesis of the BK peptide, but was less successful as compared to that of Fmoc-SPPS. Fmoc-SPPS is also easier to conduct, with less harsh cleavage conditions (TFA instead of HF reaction mixtures). Hence, Fmoc-SPPS has been used throughout the rest of this study. L-amino acids have been used in this study due to availability.

3.1 Results and Discussion

Throughout the literature, the synthesis of BK has predominately been achieved using Boc-chemistry, with a few Fmoc-syntheses (see Table 3-1). In this Chapter, synthesis of BK using Fmoc-SPPS was investigated to achieve a method giving the best yield, ease of purification, and suitability for continuation of this study by addition to cyclodextrin (**Chapters 4**-**6**). The investigation into differing coupling reagents, resins, sequence capping, and arginine protecting groups was performed. Six syntheses were carried out (*A*-*F*, Table 3-2), whereby the specific synthesis details can be found under the Materials and Methods section (**Section 3.2**) in this chapter.

Table 3-2 Overview of the yields associated with the synthesis of bradykinin using Fmoc-SPPS

Synthesis	*Purified Yield (%)*
A	26
B	12
C	2
D	27
E	30
F	52

Standard Fmoc SPPS was used to couple each of the Fmoc-amino acids overnight using TBTU (2-4 equiv) and DIEA (4-8 equiv) in excess as activating reagents. A ninhydrin test was used to check the coupling success of each amino acid and re-coupling was performed as required. The peptide was cleaved from the resin using a standard TFA cleavage mixture, inclusive of scavengers, followed by liquid-liquid extraction of the aq layer with EtOAc and ether, and freeze-drying. RP-HPLC purification on a C-18 preparative column using a MeCN/H_2O/TFA stepwise gradient was used to purify the peptide at a wavelength of 214 nm (See **Section 3.2.2.2** for details).

Synthesis A (Figure 3-2) employed 4 equiv of TBTU and 8 equiv of DIEA as coupling reagents for the Fmoc-amino acids and both arginines were protected using the Mtr protecting group (Figure 1-6). This synthesis generated a product yield of 26%. A RP-HPLC trace of *Synthesis A* is given in Figure 3-3.

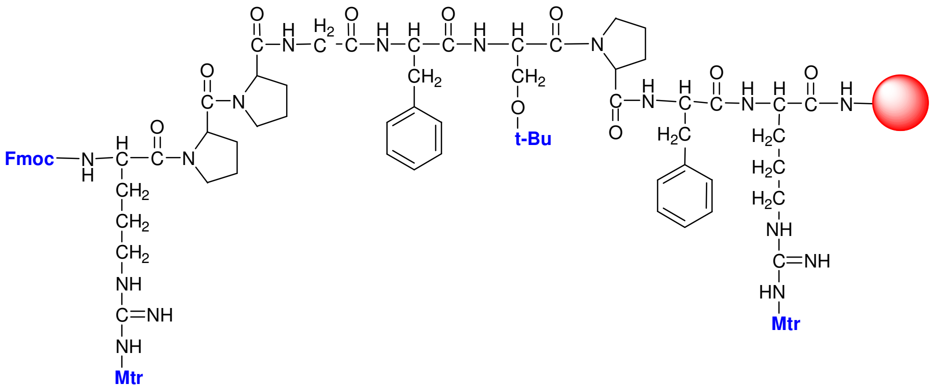


Figure 3-2 Amino acid sequence for the fully protected bradykinin peptide synthesised by Fmoc-SPPS on Rink resin

Synthesis B was a parallel to that of *Synthesis A* with the use of only 2 equiv of TBTU and 4 equiv of base giving a yield of only 12%. A comparison of *Synthesis A* and *B* (yields 26% and 12% respectively) shows that a higher ratio of coupling reagents and amino acids gave a better synthesis. Overall, this is quite a significant increase in the final product yield (*Synthesis A*). The presence of the des-arginine species (a prominent by-product in the synthesis of BK) suggested an incomplete coupling of the last arginine to the sequence. This could be attributed to the nature of the peptide chain such as the formation of aggregates or secondary structures.[8] Synthesis of BK was then tried using the Pmc protected arginine (*Synthesis C*, **Section 3.2.2.4**, Figure 3-4) under the same reaction conditions as per *Synthesis A*.

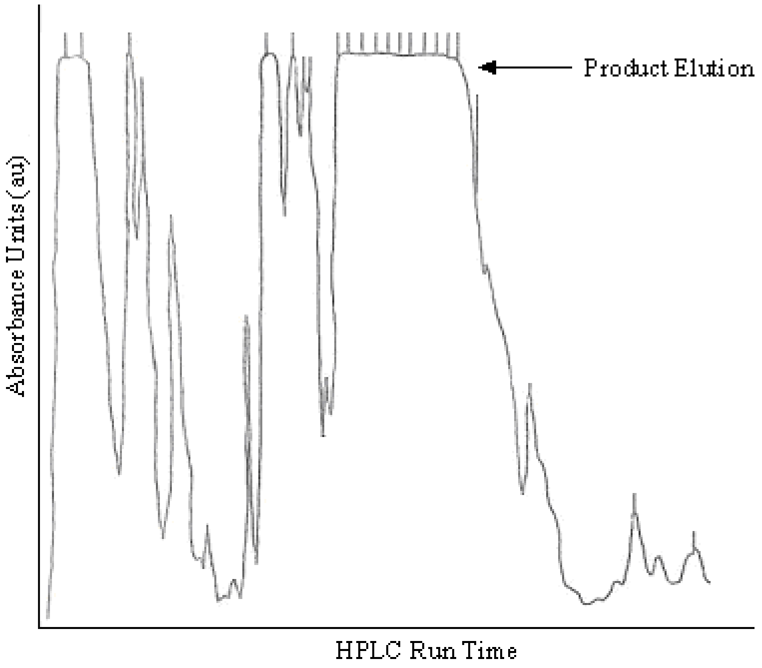


Figure 3-3 RP-HPLC trace for the purification of bradykinin prepared using *Synthesis A*. Vertical lines above each peak represent the fractions analysed by MS.

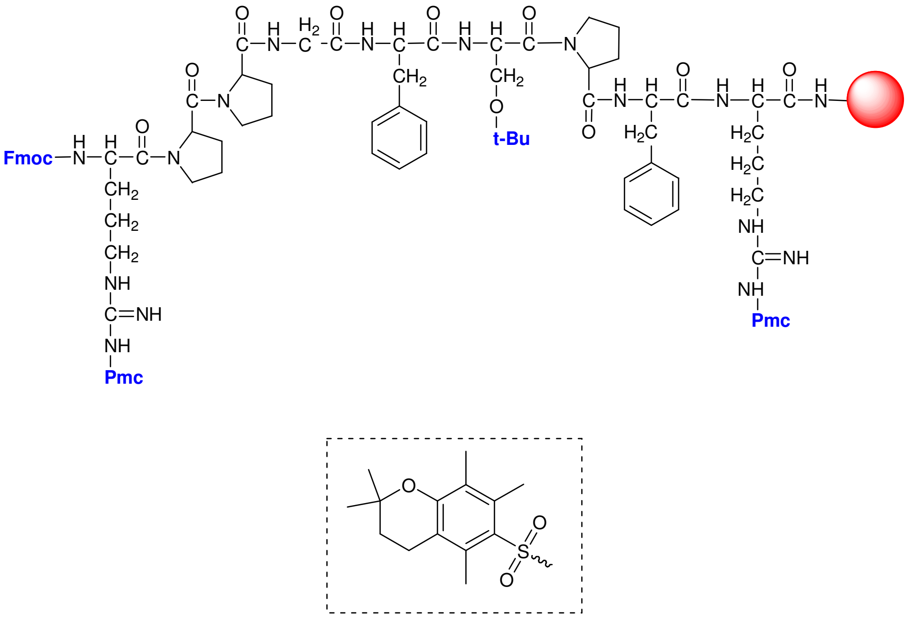


Figure 3-4 Amino acid sequence for the fully protected bradykinin peptide synthesised by Fmoc-SPPS on Rink resin using *Synthesis C* (top). Pmc (2,2,7,7,8-pentamethylchromane-6-sulfonyl) protecting group used for arginine protection in Fmoc-SPPS (bottom).

The Pmc protecting group, compared to the Mtr protecting group, is potentially easier to couple to the resin and requires less harsh cleavage conditions for its removal at the end of the synthesis (50% TFA cleavage mixture instead of the 95% TFA cleavage mixture required for removal of the Mtr group). It is therefore recommended for routine syntheses of peptides.[9] However, the overall yield for *Synthesis C* (2%), using Pmc protection, was very low compared to that of the other Fmoc syntheses (Table 3-2). This indicates that the coupling of the Arginine(Pmc) has not been successfully added to the resin and therefore the Mtr protected species is recommended from this limited study for a higher yielding synthesis of BK.

A synthesis was therefore performed whereby addition of arginine(Mtr) was used for the first arginine, and arginine(Pmc) was used for the last arginine (*Synthesis E,* Figure 3-5) in sequence. It can be seen from comparison of *Syntheses C* and *E* (yields of 2% and 30% respectively) that by using the Mtr species first, the yield is overall better indicating a better coupling to the resin.

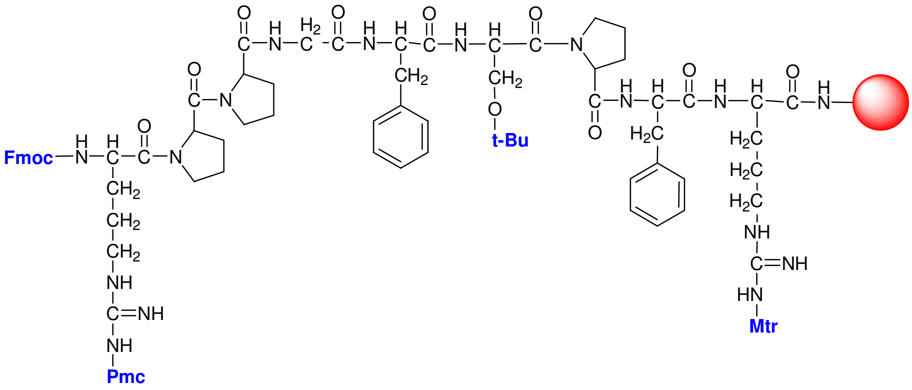


Figure 3-5 Amino acid sequence for the fully protected bradykinin peptide synthesised by Fmoc-SPPS on Rink resin using *Synthesis E and F.*

In the purification of *Synthesis A*, the BK species eluted in one broad band (Figure 3-3). Fractionation of this band, into small fractions, enabled a good separation of the product from any by-products in the crude reaction mixture. These included the BK(Mtr) species and any des-amino acid BK species. *Synthesis D* is a parallel synthesis of *A*, except that capping with acetic anhydride (Ac_2O) was used to aid purification. Acetic anhydride capping is used to react with unreacted polypeptide chains, after addition of the amino acid but before the deprotection step, thus ensuring that incomplete peptide chains can not be extended throughout the rest of the synthesis. Ac_2O capping also aids in purification as the acetylated fragments stick to the column longer, due to less positive charges than that of the parent peptide.[9] By comparison of each RP-HPLC trace (Figures 3-3 and 3-6), elution of the product from *Synthesis D* was in one peak, free of other shoulder peaks seen in *Synthesis A*. The yield of *Synthesis D* was 27% and *Synthesis A* 26%.

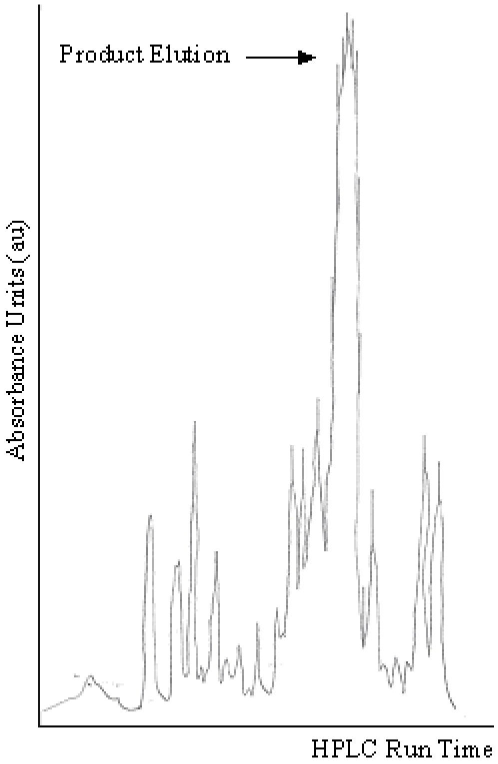


Figure 3-6 RP-HPLC trace for the synthesis of bradykinin using *Synthesis D*. Vertical lines above some of the peaks represent fractions analysed by MS.

Using the information gained from *Syntheses A-E*, a good yield has been obtained for the Fmoc-SPPS of BK (*Synthesis F*, 52%, Figure 3-5). This synthesis uses 4 equiv of the Fmoc-L-amino acids and coupling reagents together with 8 equiv of DIEA to assemble the amino acids onto Rink resin. Mtr protection has been employed for the first arginine in the sequence and Pmc protection for the last arginine in sequence. Capping with Ac_2O was used to aid in the purification of the peptide and routine double coupling was also implemented to ensure a maximum possible yield. Therefore, this Fmoc-SPPS method was implemented for the synthesis of BK throughout the rest of this project.

The yields obtained could be attributed to the nature of the peptide. There are many bulky amino acids in the sequence which will affect the coupling efficiency, seen in the production of BK des-amino acid species.[9] As the peptide is elongated, it is able to form secondary structures during synthesis. Conceivably it could self-aggregate or aggregate with the polymeric support. This then contributes to lower reaction yields and rates. The effects can range from just subtle slowing down of the coupling rates to reduced solubility of the growing peptide thus hindering the reaction. This can often be seen by a shrinking of the resin matrix in manual syntheses.[8] A change in solvent, resin, or coupling reagent can decrease the possibility of these occurring. The use of secondary amino acid surrogates, such as analogues of proline or *N*-alkylamino acids are suitable for inserting into the backbone of difficult sequences for disrupting secondary structure formation.[8] However such modifications were beyond the scope of this study.

The coupling success was assessed by the qualitative ninhydrin test.[9] However, although the results show negative tests (yellow) indicating that minimal free amines are present, the MS indicates the presence of small amounts of des-amino acid BK species. To monitor the overall yield of this peptide, MS tests should be used after each amino acid addition to gauge the coupling completion of each amino acid in conjunction with the ninhydrin test. Other alternative methods to attempt for yield improvement include the introduction of a spacer between the resin and the BK peptide. This may aid the coupling of the arginine to the resin since both the resin and the arginine(Mtr) are very bulky. Or, alternatively, a change in resin could be tried such as Tentagel which has a long, unhindered PEG spacer from the core of the resin.[9] Studies on this resin were not tested as this part of the study was undertaken first, before the synthesis and knowledge gained from the studies in **Chapter 2**.

To remove all of the Mtr groups, a longer TFA cleavage could be used (currently 3h) or the TMSBr cleavage could be employed.[9] It should also be noted that other coupling reagents were also tried, namely mixtures of HBTU and/or HOBt instead of TBTU with minimal difference in yields. It is also likely that the RP-HPLC purification method does not give a very good recovery when using the C-18 column. To improve this, other columns such as the C-8 or C-4 column may give better yields for such a polar peptide.[9] Resins of lower-loading have been shown to reduce the occurrence of inter-chain aggregation in order to facilitate peptide synthesis, as does the removal of a hydrophobic linker which renders the resin more hydrophilic and allows chemistry, such as the cleavage step to be performed in a more greener environment (aq).[10]

3.2 Materials and Methods

3.2.1 *Reagents and Instruments*

With the exception of the following details, the same reagents and instruments as per **Chapter 2**, **Section 2.4.1** was used throughout this chapter.

Unless stated elsewhere, compounds were purified by RP-HPLC. HPLC grade Dimethylformamide (DMF) was used straight from the bottle without further purification or degassing. Trifluoroacetic acid (TFA) was distilled at atmospheric pressure over a short column. Boc protected amino acids (i.e. Boc-Arg-OH, Boc-Gly-OH, Boc-Phe-OH, Boc-Pro-OH, Boc-Ser-OH) were purchased from Auspep (Australia), Bachem (Torrance, Calif) or Peptides International (Louisville, Kentucky, USA)**.** *t*-Boc Arg(Tos)OCH_2 PAM resin (133.32 mmol/g) was from Peptides International (Louisville, Kentucky, USA). Solvents for peptide synthesis (DCM and DMF) were from APS Finechem (Asia) and Votre Partenair Chemie (France). *p*-Cresol was from Acros Organics (New Jersey, USA).

3.2.2 *Fmoc-Solid Phase Peptide Synthesis of L-Bradykinin*

3.2.2.1 *General Fmoc-Solid Phase Peptide Synthesis Method*

See **Appendix 3-III** for these details

3.2.2.2 Synthesis A

BK was assembled manually on Rink resin (685 mg, 0.50 mmol, 1 equiv) in the order stated in Figure 3-2. For more detailed information on any of the following steps refer to **Appendix 3-III**. The resin was swollen overnight in DMF (100%). A 20% pip in DMF solution (2 x 10 minutes) was used for Fmoc deprotection of all the amino acids.

Fmoc-L-amino acids (2.00 mmol, 4 equiv) were coupled overnight (followed by a second re-coupling overnight) using TBTU (2.00 mmol, 4 equiv) and DIEA (4.00 mmol, 8 equiv) in excess as activating reagents. The Kaiser test[9] was used to determine whether the amino acids had coupled successfully. For the final deprotection, the resin was washed with a 20% pip in DMF solution (four times) and then agitated (2 x 5 minutes) in this solution. The resin was first washed with DMF and then a 50% solution of DCM/MeOH before being well suction dried. The resin bound peptide was then freeze-dried prior to cleavage (2.10 g). The resin bound peptide was deprotected and cleaved from the support using a freshly made solution of TFA/H_2O/thioanisole/EDT as per **Section 3.2.2.1** to yield 1.40 g of crude product. RP-HPLC was used to purify the parent peptide. The initial time programme used to purify the product is given in Table 3-3 however, with a faster elution at the start of the gradient and a slower gradient in the middle of the time programme (changes highlighted in Table 3-3), a better separation between the parent and other BK(Mtr) species was obtained. The parent peptide eluted at 36% B (140 mg, 26%). See Figure 3-7 for MS data. Figure 3-3 shows a RP-HPLC trace for the purification of BK using *Synthesis A*.

LR-MS: *m/z* (%, assignment) 1059.58 (100, M+H)$^+$, 1081.56 (50, M+Na)$^+$, 1097.54 (25, M+K)$^+$; ^{1}HR-MS: *m/z* $C_{50}H_{74}N_{16}O_{10}$ (M+2H)$^{+2}$ 530.2960, actual 530.2971 Elemental Analysis. Calculated for $C_{50}H_{74}N_{16}O_{10}$ expected C: 56.70, H: 7.04, N: 21.16, actual C: 56.68, H: 6.99, N 21.00; NMR assignment in d_6-DMSO conclusive with reference.[11]

Table 3-3 Acetonitrile stepwise gradient time programmes for the separation of the bradykinin peptide using a preparative RP-HPLC column

Time	*% Mobile phase 'B'*	
(min)	*Initial Time Programme*	*Altered Time Programme*
0	5	5
5	5	5
15	12	12
20	12	**20**
100	55	**50**
105	100	100
110	5	5
120	STOP	STOP

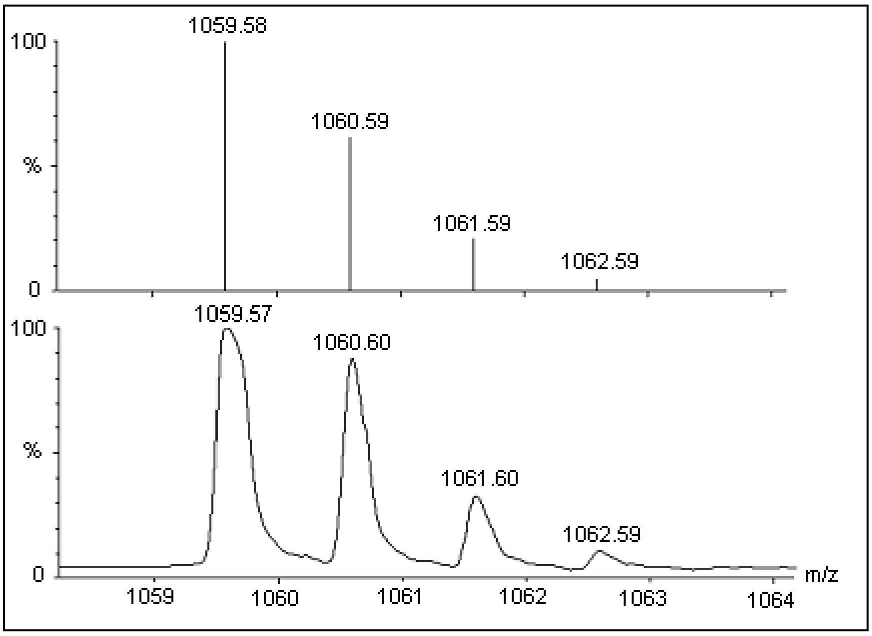


Figure 3-7 Predicted (M+H)$^+$ fragmentation pattern for the bradykinin (top) and actual bradykinin obtained from *Synthesis A* (bottom). A similar pattern for the sodium and potassium isotope was also seen (not shown)

3.2.2.3 Synthesis B

The same procedure as per **Section 3.2.2.2**, was used for *Synthesis B* with the exception of using resin (1 equiv), Fmoc-L-amino acids (2 equiv), coupling agents (2 equiv), and base (4 equiv). Peptide cleavage, purification, and characterisation as per **Section 3.2.2.2**. The yield for *Synthesis B* was 65 mg (12%).

3.2.2.4 Synthesis C

BK was assembled manually on Rink resin (342 mg, 0.25 mmol, 1 equiv) as per **Section 3.2.2.2** with the exception of the Arg protecting group. In this synthesis, the Pmc protecting group (Figure 3-4) was used (instead of the Mtr group seen in *Syntheses A*, *B*, and *D*). Peptide cleavage, purification, and characterisation as per **Section 3.2.2.2**. Crude results indicate no presence of the Pmc protected BK peptide. The pure product eluted at 36% B (5.2 mg, 2%).

3.2.2.5 Synthesis D

BK was assembled manually on Rink resin (1 equiv) as per **Section 3.2.2.2**. After each successful amino acid coupling, the resin was washed well with DMF. The peptide was capped with a solution of Ac_2O (4 equiv) in DMF and allowed to agitate for 10 minutes. The resin was then washed well with DMF, deprotected, and then the next amino acid was coupled as per **Section 3.2.2.2**. Cleavage, purification, and characterisation as per **Section 3.2.2.2**. The pure product eluted at 36% B (72 mg, 27%) with elution of the BK des-amino acid products after the elution of the parent species (*c.a.* 43% B). See Figure 3-6 for the RP-HPLC trace of *Synthesis D.*

3.2.2.6 Synthesis E

BK was assembled manually on Rink resin as per **Section 3.2.2.2**. The Mtr protecting group was used to protect the first Arg in sequence, and the Pmc protecting group was used to protect the last Arg in the sequence (Figure 3-5). Peptide cleavage, purification, and characterisation as per **Section 3.2.2.2**. Crude results indicate no presence of the Pmc protected BK peptide with trace amounts of the Mtr species. The pure product eluted at 35.4% B (79.5 mg, 30%).

3.2.2.7 Synthesis F

BK was assembled manually on Rink resin as per **Section 3.2.2.2**. The Mtr and Pmc protecting groups were used to protect the first and last arginines respectively (Figure 3-5). The amino acids were capped at the end of each successful coupling with Ac_2O (see **Section 3.2.2.5**). Peptide cleavage, purification, and characterisation as per **Section 3.2.2.2**. Crude results indicate no presence of the Pmc protected BK peptide with trace amounts of the Mtr product. The pure product eluted at 34.3% B (138 mg, 52%).

3.3 Conclusion

The synthesis of the BK peptide using Fmoc-SPPS gave a useful yield of 52% (*Synthesis F*) using TBTU (4 equiv) and DIEA (8 equiv) as coupling reagents on Rink resin. Capping with Ac_2O allowed easier purification of the parent peptide from BK des-amino acid products, whilst exchange of the Arg_9 Mtr protecting group for Pmc allowed for a more successful synthesis with only traces amount of the BK-Mtr present in the cleaved mixture. Therefore, the reaction conditions for *Synthesis F* were used throughout the rest of the study for the synthesis of the BK peptide. This now leads into **Chapters 4** and **5** with the addition of peptides, both BK and other model peptides, onto cyclodextrin and the synthesis of peptide sulfonamides, respectively.

3.4 References

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Chapter Four:

Synthesis and Purification of Cyclodextrin-Peptides

4.0 Introduction

This chapter follows on from **Chapter 2** where one or two linkers were attached to β-CD and although **Chapter 2** does not display any new chemistries,[1] the synthetic approach and versatility achieved allows for the addition and growth of *C*- and/or *N*-terminal peptides with the same and/or different functional properties on a solid phase support. Peptide attachment off resin in solution using stepwise or ligation synthetic protocols should also be possible. Direct coupling to unfunctionalised β-CD as desired, was unsuccessful under all Fmoc procedures used in this study (**Appendix 4-I**), hence functionalisation was essential for peptidyl attachment using SPPS. This system has great developmental potential for the transportation of drugs and/or other molecules. Owing to the large variety of cellular receptors, peptides appear to be amongst the most versatile compounds for such targeting purposes. The grafting of peptides onto CD also adds potential therapeutic dimensions.[2]

4.0.1 *Chapter Outline*

This chapter describes, for the first time, the bi-functionalisation of β-CD with a series of model and bioactive peptides using Fmoc-SPPS. The bioactive peptide used in this study is bradykinin (BK). For synthesis and other details of the BK peptide, refer to **Chapters 1** and **3**. This synthetic approach allows the functionalisation of β-CD with one or two peptides in various combinations; e.g., the attachment of β-CD to the *C*- and/or *N*-terminus of peptides as well as the functionalisation of β-CD with differing peptidyl chains (Figure 4-1).

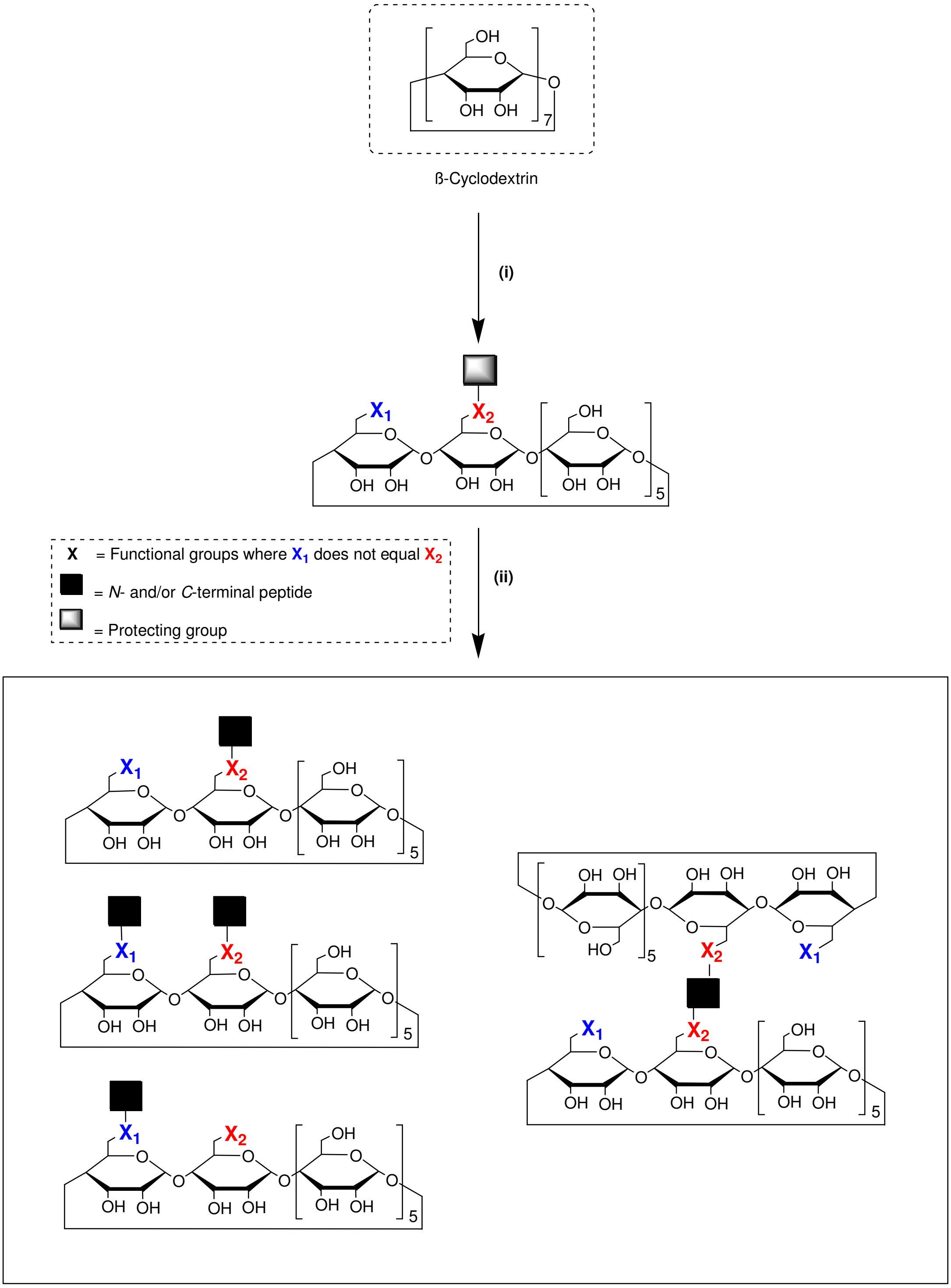


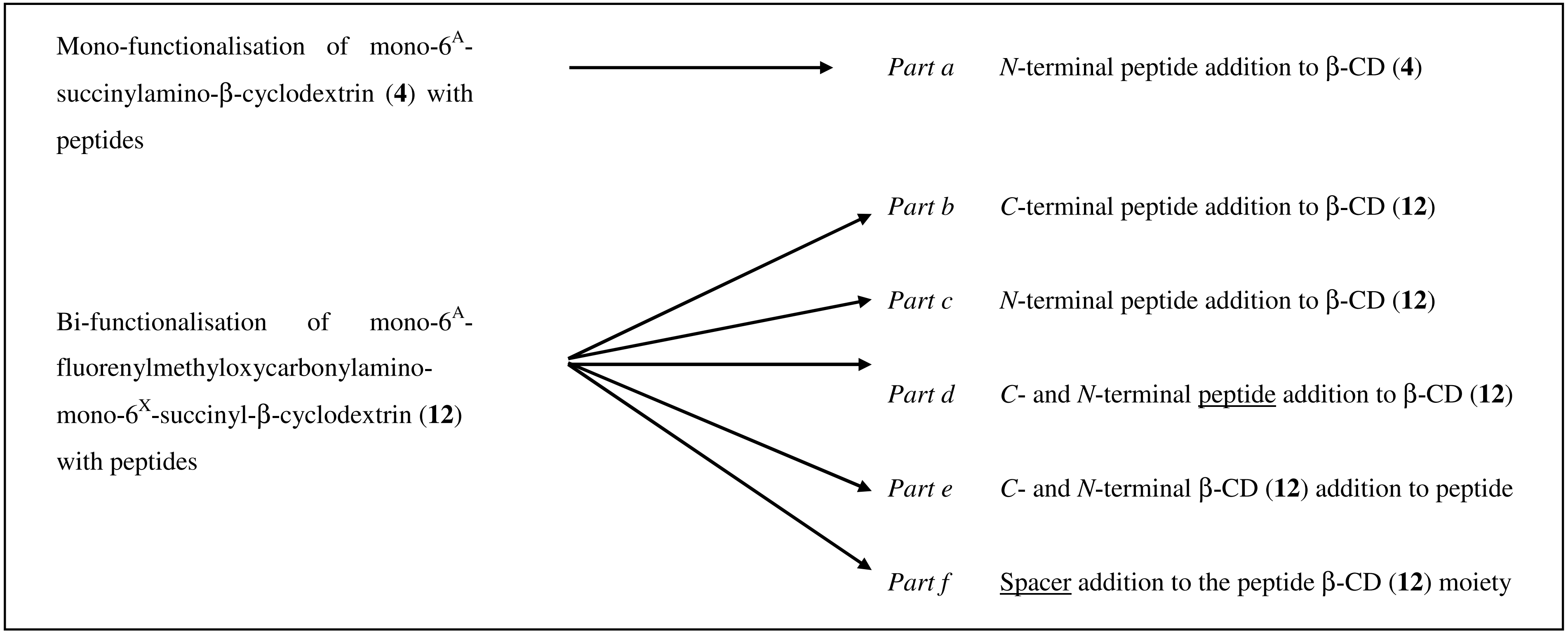
Figure 4-1 Derivatisation of β-CD using solid phase peptide synthesis. (i) Synthesis of bi-functional β-CD to enable selective peptidyl attachment in SPPS. (ii) Attachment of peptides to β-CD using Fmoc-SPPS to synthesise a number of *C*- and/or *N*-terminal derivatised products.

This Chapter details the synthesis, purification, and characterisation of peptide addition to mono-6^A-succinylamino-β-cyclodextrin (**4**, synthesis details outlined in **Chapter 2**, **Sections 2.4.2.3**) and mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**, synthesis details outlined in **Chapter 2**, **Section 2.4.4.4**) using Fmoc SPPS.

Model peptides were first used in this study to show proof-of-concept and to obtain reaction conditions that gave acceptable yields. The model peptides used include the di-peptide, Gly-Ala, and the tri peptides, tri-glycine (Gly-Gly-Gly) and Val-Gly-Ala. These peptides were chosen due to availability and cost enabling a number of small preliminary studies to be undertaken, to ensure successful syntheses. This was important when it came to the addition of the bioactive peptide which was much longer, and much more complicated in structure to make (see **Chapter 3** for specific details).

Figure 4-2 outlines the layout of this chapter. *Part a* details the synthesis of mono-peptidyl-CD using mono-6^A-succinylamino-β-cyclodextrin (**4**) where the peptide is a model or BK. *Part b* and *Part c* describe the addition of the model and BK peptides to mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**) in either *N*- or *C*-terminal combinations, respectively. *Part d* details the addition of a peptide to both the *N*- and *C*-terminus of **12**, whilst *Part e* shows the addition of **12** to both ends of the peptide (model peptide or BK) creating a CD-peptide-CD moiety.

In *Part f*, spacers are added between the resin and/or β-CD and/or peptides in varying combinations. The spacer employed is ε-aminocaproic acid. This was done for bioassay purposes (**Chapter 6**) to see what effect of the spacer had on the activity of the bioactive peptide in relation to the non-spacer molecules (*Parts a* and *b*). Yield studies were also looked into for these and compared against non-spacer experiments. It was thought that the longer spacer between the resin and β-CD or peptide may improve its coupling by decreasing steric factors. See **Chapter 2**, **Section 2.4.4.4** for more details on the coupling of **12** to resin.



Mono-functionalisation of mono-6^A-succinylamino-β-cyclodextrin (**4**) with peptides

Part a *N*-terminal peptide addition to β-CD (**4**)

Bi-functionalisation of mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**) with peptides

Part b *C*-terminal peptide addition to β-CD (**12**)

Part c *N*-terminal peptide addition to β-CD (**12**)

Part d *C*- and *N*-terminal peptide addition to β-CD (**12**)

Part e *C*- and *N*-terminal β-CD (**12**) addition to peptide

Part f Spacer addition to the peptide β-CD (**12**) moiety

Figure 4-2 Outline for the layout of **Chapter 4**

4.1 Results and Discussion for *Part a*

Mono-derivatisation of β-CD with one peptide has been achieved in the literature before (see **Chapter 1**, **Section 1.4.3.1** and Table 4-1 for more details) and was used in this study to obtain experience for reaction conditions required for the use of the bi-functional molecule (**12**) in SPPS.

In *Part a*, mono-peptidyl-derivatisation of β-CD (Figure 4-3) where the peptide is either a model (tri-glycine, **14a**) or BK (**14b**) was successful. The peptide was coupled to the modified β-CD (**4,** synthesised in **Chapter 2**), by means of standard amino acid couplings.[*] The yields for both compounds (**14a** and **14b**) were very low (4% and 5% respectively). This is probably due to the size, sterics, polarity, and coupling chemistries and hence, more studies were obviously warranted to formulate higher yielding syntheses. Other species present in the crude mixture of **14b** were BK(Mtr)-CD (1.6 mg, 5%) and the BK peptide (1.1 mg, 4.5%). Also collected was the unreacted functionalised β-CD moiety (**4**). This was isolated, further purified, and reused for subsequent syntheses.

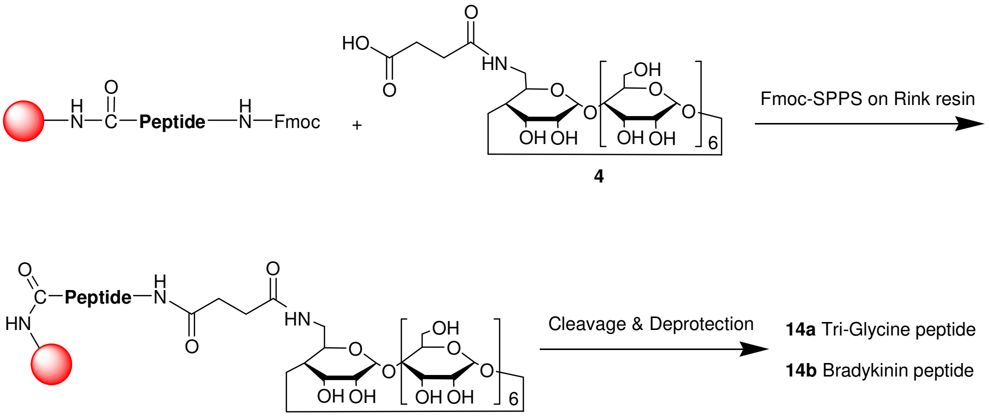


Figure 4-3 Placement of the peptide onto compound **4** for *Part a*

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[*] Fmoc amino acids (4 equiv) were assembled on resin and/or β-CD using HBTU or TBTU (4 equiv) and DIEA (8 equiv) overnight monitoring with ninhydrin and re-coupling where necessary. Compound **4** (4 equiv) was coupled to resin and/or peptides using this same method. See Experimental section for specific details.

Table 4-1 A selection of studies for cyclodextrin-amino acid/peptidyl conjugates

Brief Synthesis Details	*Yield*	*References*
Monconjugates of gastrin peptides starting with 6-amino-6-deoxy-β-cyclodextrin with 5-7 amino acids attached using a succinyl spacer. Synthesised the peptide first, then attached to the CD unit off resin in solution.	18-39%	3
Stepwise Fmoc-SPPS on Rink resin with addition of 6-amino-6-deoxy-β-cyclodextrin attached orthogonally to glutamate (*t*-butyl protected). Synthesis of cyclodextrin-glutamine moiety in solution before addition onto resin. Peptide is 17 amino acids long.	2-21%	4
Two peptides were grafted (in solution, off resin) onto 6-amino-6-deoxy-β-cyclodextrin at different positions. Peptides were 8 and 11 amino acids long.	66-67%	2
Peptide/amino acid dendrimers based on a β-CD core synthesised by first constructing the amino acid or peptide dendrons, followed by their coupling to heptakis-6-deoxy-6-iodo-β-cyclodextrin using grafting techniques onto resin. 1 or 2 amino acids added to each primary hydroxy group.	76-82%	5
Stepwise synthesis of the tri-peptide and then grafting onto a β-CD hepta-conjugate with a succinyl spacer (off resin)	70%	6
Stepwise synthesis of a hexa-peptide and then grafting (off resin) onto a β-CD hepta-conjugate with a *ε*-aminocaproic acid spacer.	74%	7
Single amino acids addition to β-CD off-resin. CD is functionalised with iodo- or amino-groups.	35-94%	1

4.2 Results and Discussion for *Part b*

Bi-derivatisation of β-CD has also been achieved with the coupling of one and/or two peptides to a modified β-CD compound (**12**, synthesised in **Chapter 2**), by means of standard amino acid couplings. Literature searches for mono-peptidyl CD conjugates reveal the synthesis of a large number with varying yields (Table 4-1). All these synthesises used grafting addition of the peptides onto the functionalised β-CD moiety with the exception of the synthesis by Toyoda *et al.*[4a] In this synthesis, β-CD was added orthogonally to the peptide sequence, not as a linear addition as per the synthesises described in this thesis.

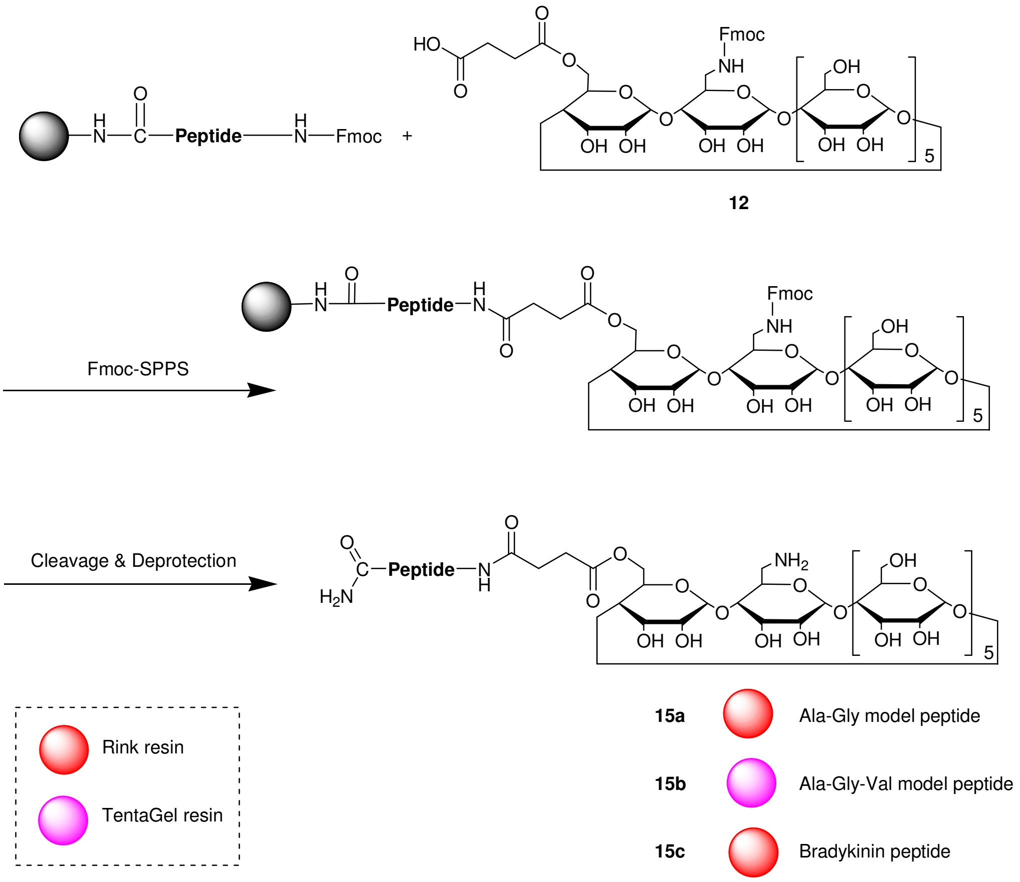


Figure 4-4 Peptidyl placement onto compound **12** for *Part b*

Part b peptides were attached to the succinyl terminus of compound **12** (Figure 4-4). First the peptide was synthesised on the resin and following Fmoc-deprotection of the peptide, compound **12** was coupled to it.[†] Deprotection of the β-CD compound (**12**)

Fmoc group and subsequent cleavage from the resin yielded a mono-peptidyl-β-CD product. The peptides used in this section (*Part b*) were Gly-Ala (**15a**), Val-Gly-Ala (**15b**), and BK (**15c**). As the complexity of the peptide increased (**15a**, **15b**, and **15c**), the yields decreased from 33% to 14% and 2%, respectively. This is presumably due to the nature of the peptides being coupled, i.e. the shape of the peptide which it undertakes depending upon its amino acid sequence. For example, proline gives the peptide chain a bent structure compared to glycine, see Figure 1-11, **Chapter 1**. See **Chapter 6** for preliminary bioassay testing of compounds **15b** and **15c**.

4.3 Results and Discussion for *Part c*

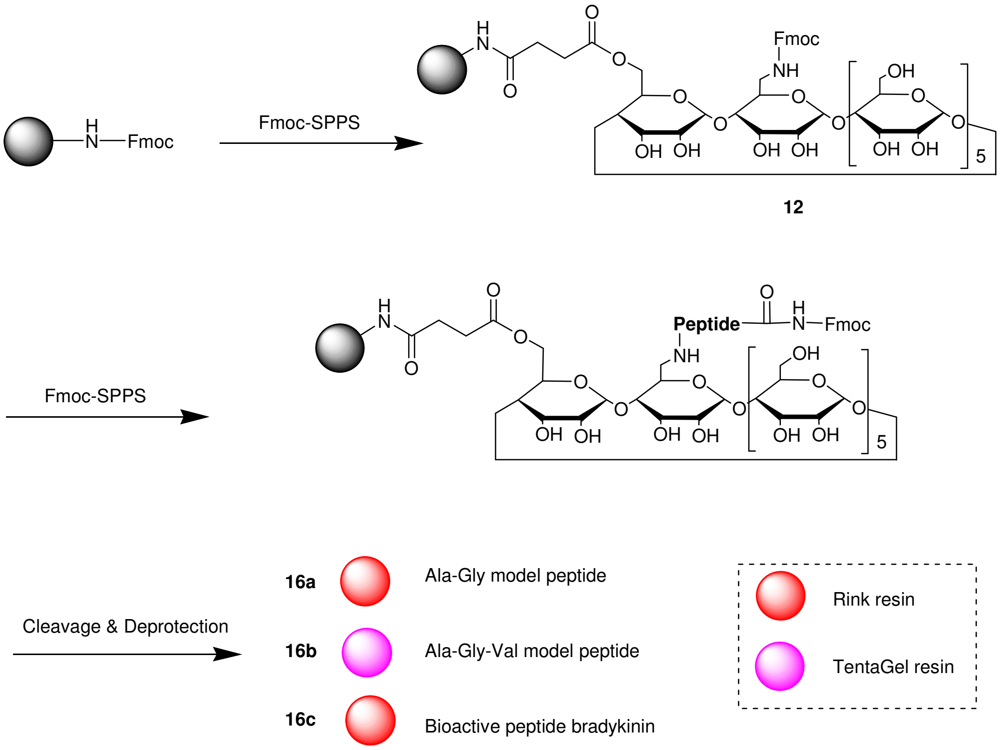


Figure 4-5 Peptidyl placement onto compound **12** for *Part c*

Part c, attached the peptides to the amino terminus of compound **12**[†] on resin (Figure 4-5) giving low yields. Namely, **16a** (Gly-Ala, 15%) and **16b** (Val-Gly-Ala, 7%).

[†] Compound **12** was assembled on the resin and/or peptide using the optimum reaction conditions found in **Chapter 2** (**Section 2.4.4.4**). More specifically, **12** (4 equiv), EDC (4 equiv), and DIEA (4 equiv) in pyridine/DMF overnight.

However, for the addition of the BK peptide, a 49% yield was obtained. In this limited study (*Part b* and *Part c*), there appears to be little correlation between the attachment of peptides to either the carboxy or the amino terminus of **12**. Both give the desired products in workable yields. See **Chapter 6** for preliminary bioassay testing of compounds **16b** and **16c**.

It must be noted for all synthesises that following the use of compound **12** in SPPS, the resin was washed with DMF and the β-CD washings collected. These were then purified using RP-HPLC, re-functionalised with the Fmoc protecting group and used in further couplings. Amongst the reaction mixture from the addition of **12** to resin/peptides were also β-CD-des-peptide products (notably for the BK peptide; des-serine, des-proline, and des-arginine, collectively the second yielding by-product eluted from the resin), unfunctionalised peptide products (peptide only, this made up the main percentage of the by-products cleaved from the resin), and β-CD-peptide-protecting group products (namely β-CD-BK(Mtr) species). This latter species was then re-cleaved using the same TFA cleavage mixture as per removal from the resin and combined with the product in some instances. In other instances, the protecting group was unable to be removed. The des-amino acid β-CD products were not retained or used in this study.

4.4 Results and Discussion for *Part d*

Part d attached two peptides to compound **12** (β-CD) and the results showed the capabilities of this bi-functional system (Figure 4-6). The peptide can be the same (Val-Gly-Ala, **17a**) or different (Val-Gly-Ala and Tri-Gly, **17b**). However, as the complexity of the peptides increase (length and amino acid composition), the yields decreased. This is seen by comparison of **17a** (*c.a.* 15%) with **17c** (BK peptides, trace).

The synthesis of **17a** was completed on both Rink (14%) and TentaGel (16%) resins (see **Chapter 1** and **Chapter 2**, **Section 2.0.1** for resin details) to see if there was any change in yield. Rink resin has a short, hindered spacer between the reactive site of the resin and the resin core whereas TentaGel resin has a long, less hindered PEG spacer between the core of the resin and its reactive site. It is presumed that the bulkier and larger the molecule, the harder it is to bind to the resin, especially the Rink resin.

However, if there is a long spacer between the resin core and reactive site, the easier it will couple. It is interesting to note that there is little difference in yields when using either Rink or TentaGel resin. This could perhaps be due to the peptide chain being added to the resin first, essentially extending the spacer (on both resins). It could also be due to the peptide used (3 amino acids long) and the result may be more noticeable when a larger peptide is tested which could incur more aggregation on the Rink resin due to this short spacer. See **Chapter 6** for preliminary bioassay testing of compound **17a**.

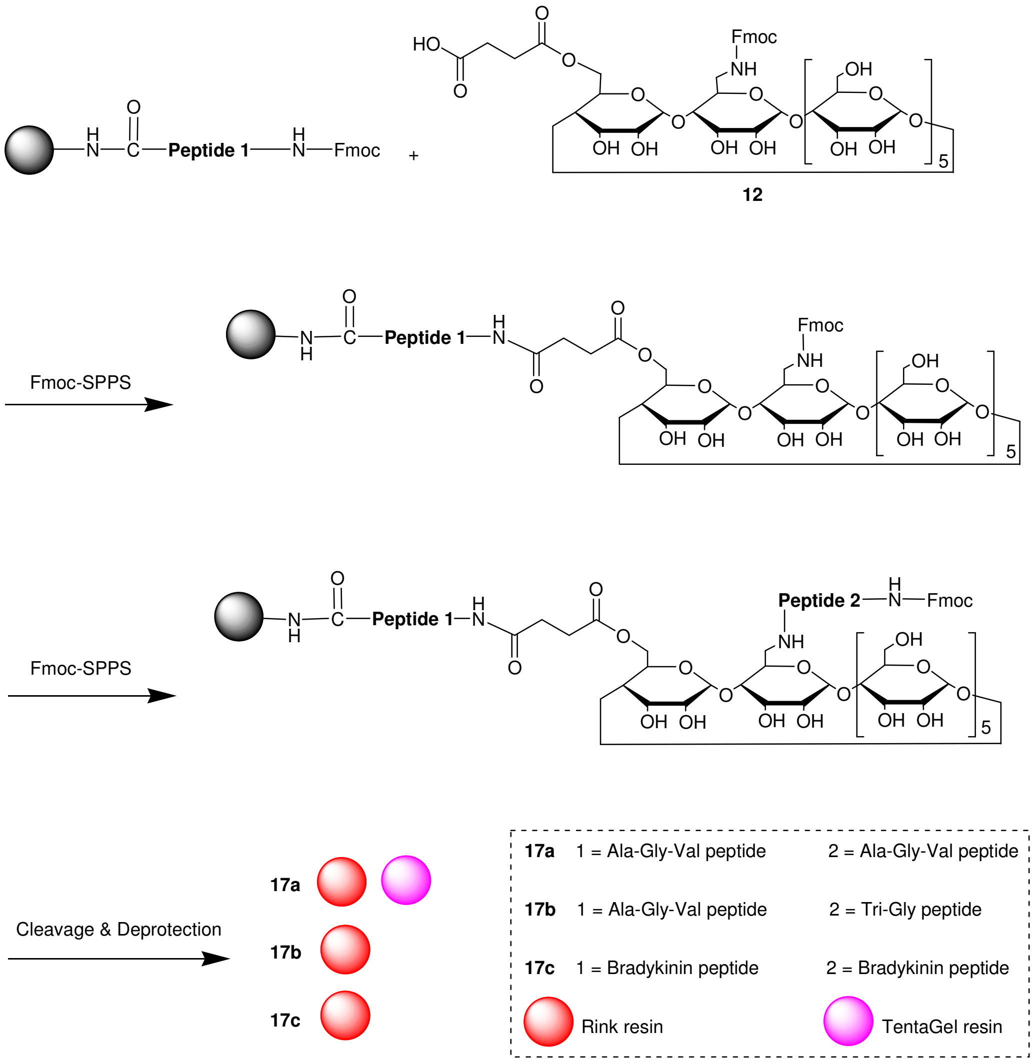


Figure 4-6 Placement of peptide(s) onto compound **12** for *Part d*

4.5 Results and Discussion for *Part e*

Part e attached β-CD (**12**) to both ends of a peptide using Fmoc-SPPS (Figure 4-7). Firstly, **12** is attached to resin,[‡] followed by the addition of a peptide,[§] deprotection, and addition of **12**[‡] again. This shows the capability of the system with the multiple attachment of peptides and/or β-CD molecules, all using SPPS. The di-peptide Gly-Ala (**18a**, 15%) and the tri-peptide (**18b**, Val-Gly-Ala, 1%) β-CD complexes were all synthesised and purified and the results imply the larger the peptide, the less successful each coupling is. This could be attributed to the complexity of the peptide in relation to peptide-resin aggregation, secondary peptide structure formation, or a decrease in peptide solubility on the resin. See **Chapter 6** for preliminary bioassay testing of compound **18b**. The synthesis of the BK conjugate was unsuccessful likely due to its large size.

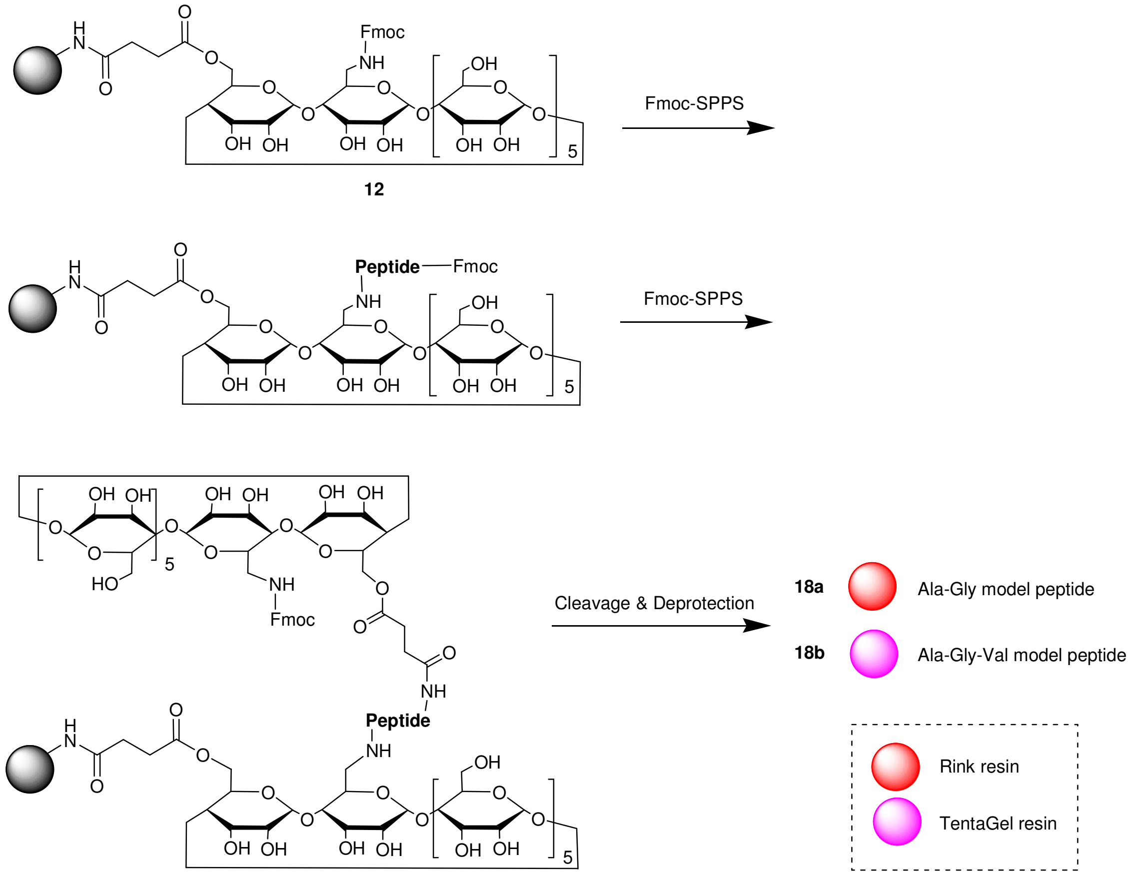


Figure 4-7 Placement of the peptide onto compound **12** for *Part e*

[‡] Using the optimum reaction conditions found in **Chapter 2** (**Section 2.4.4.4**). Namely, **12** (4 equiv), EDC (4 equiv), and DIEA (4 equiv) in pyridine/DMF overnight.

[§] Fmoc amino acids (4 equiv), HBTU (or TBTU, 4 equiv), DIEA (8 equiv) overnight monitoring with ninhydrin and re-coupling where necessary.

4.6 Results and Discussion for *Part f*

Part f was performed to investigate the use of spacers (Fmoc protected ε-aminocaproic acid in this study) for the attachment of peptides to β-CD. As discussed in **Chapter 2**, **Section 2.3.4**, the Fmoc-coupling efficiencies of compound **12** to resin were low (3-40%) compared to efficiencies expected for normal amino acid couplings (98-99%).[8] This low coupling of **12** (as well as the nature of the peptides being coupled to it) could be used to explain the low yields obtained for the β-CD-peptides obtained in *Parts b-e*. It was therefore proposed that a spacer between the resin, and/or peptides and/or **12** would aid its coupling and achieve better yields. It further allows for investigation using preliminary bioassays to see if the peptides are less/more reactive when bound close or further away from the β-CD moiety.

In this section, the five β-CD compounds (**19a–19e**) were synthesised to study the effect of a spacer molecule on a peptide coupling to β-CD (model or BK, Figure 4-8). Compound **19a** (no peptide) was synthesised to ensure that a spacer-β-CD complex can be achieved in a reasonable yield using Fmoc SPPS. A 6% yield was obtained (**19a**).

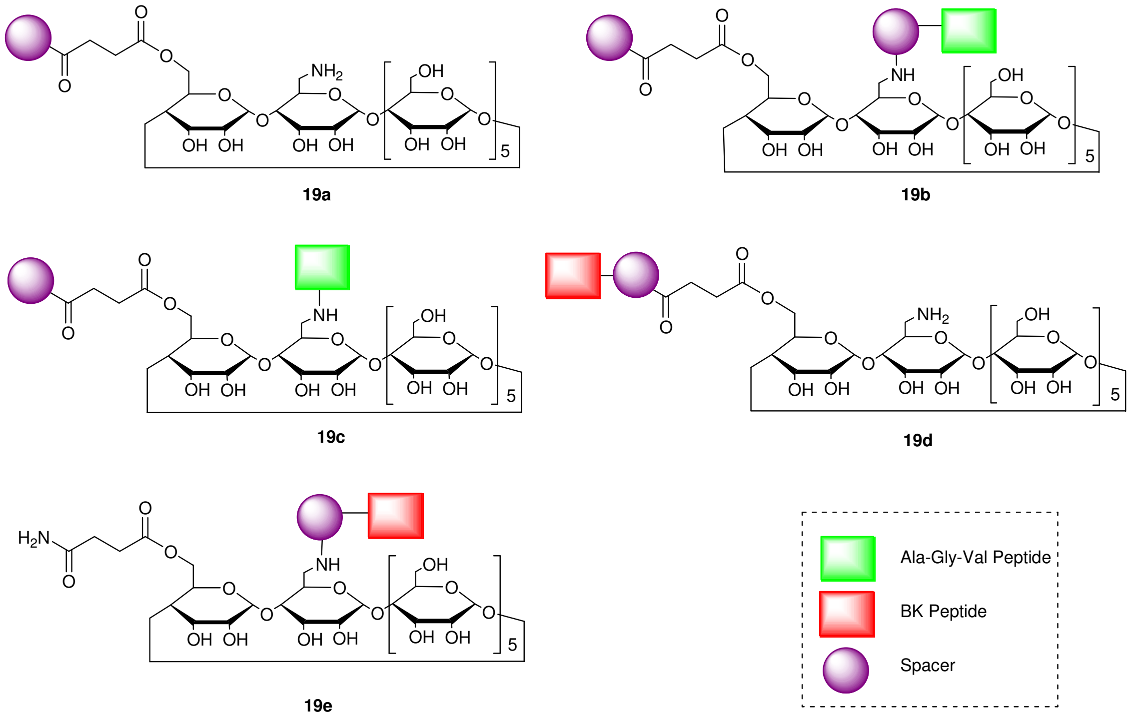
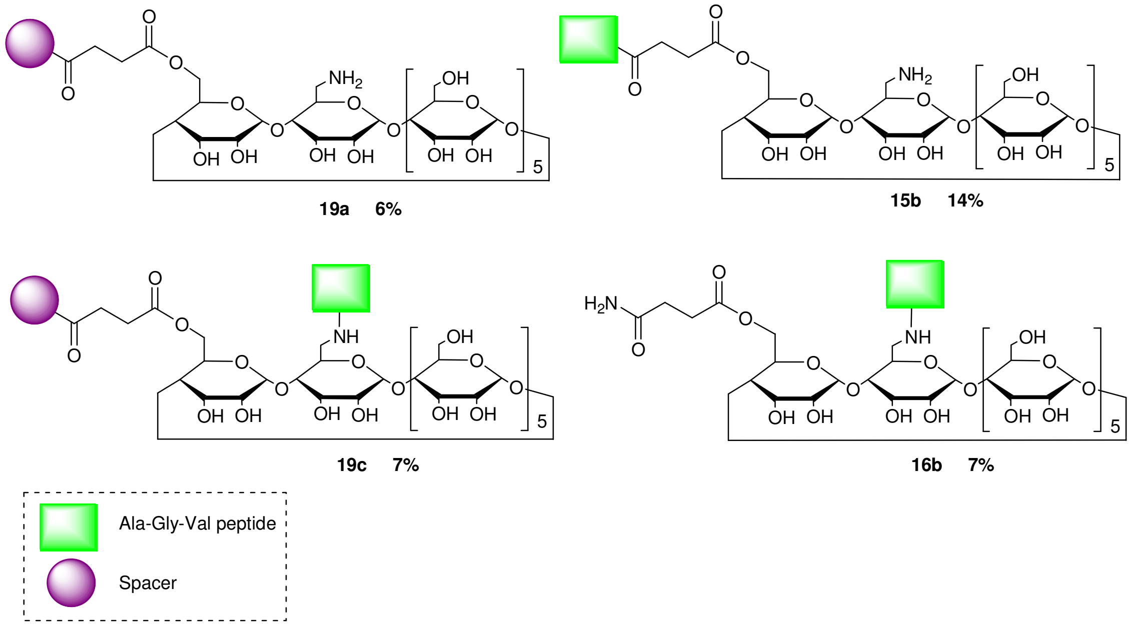

19a **19b** **19c** **19d** **19e**

Figure 4-8 Compounds synthesised in *Part f*

A comparison of the yields for compounds **19b** with **19c** (where the peptide is Val-Gly-Ala) showed **19c** (7%) having the higher yield than **19b** (3%). This could be attributed to the complexity of the molecule and its ability to form higher ordered structures, making it harder for the amino acids to couple. Minimal differences in yield were observed between **19d** and **19e** (BK peptide) perhaps due to addition of only one spacer possibly meaning that they can undertake similar conformational changes.

It was shown (**Chapter 2**, Graph 2-5) that the coupling efficiency of **12** to Rink resin with a spacer compared to Rink resin with no spacer increased almost three-fold for the standard Fmoc coupling reagents (10-25% compared to *c.a.* 4%). Therefore, inclusion of a spacer to the resin and/or peptide is thought to increase the coupling efficiency generating better yielding peptides onto β-CD.

It can be seen by comparison of yields from experiments **19a** (no peptide, 6%) with **15b** (Val-Gly-Ala, 14%) and **19c** (Val-Gly-Ala, 7%) with **16b** (Val-Gly-Ala, 7%) that there is little increase in the yields of these molecules when a spacer is employed (Figure 4-9).



19a 6%

15b 14%

19c 7%

16b 7%

Figure 4-9 Structural comparison of compounds **19a** to **15b** and **19c** to **16b**

However, there is also little difference when comparing larger peptide compounds **19d** (BK, 2%) with **15c** (BK, 2%, Figure 4-10). This could be due to a number of reasons, including the size and complexity of the molecule that is now being attached to the resin. The only exception to this is found when comparing compounds **19e** (BK, 2%) with **16c** (BK, 49%). This large increase in yield when no spacer is employed could be attributed to less aggregation around β-CD (less bulk without the presence of the spacer). Further studies are needed to investigate this. See **Chapter 6** for preliminary bioassay testing of compounds **19d** and **19e**.

It should be noted that the succinyl linker is not removed at the end of any of these syntheses. Although the succinyl group is converted into an amide functionality, it can be retained as a free carboxyl with the appropriate resin.

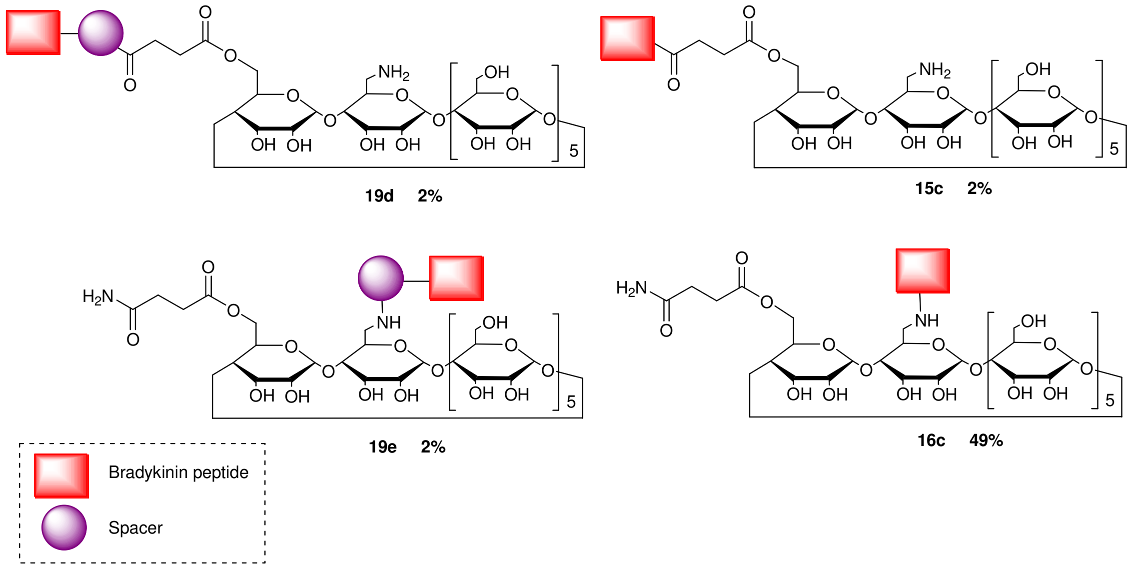


Figure 4-10 Structural comparison of compounds **19d** to **15c** and **19e** to **16c**

4.7 Materials and Methods

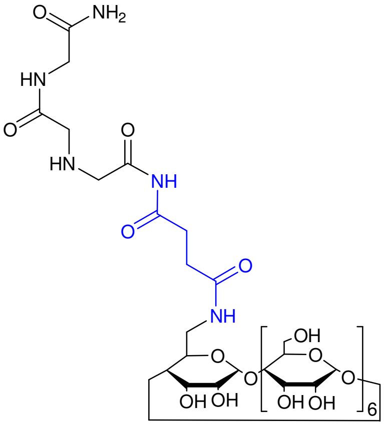
4.7.1 *Reagents and Instruments*

The same reagents and instruments as per **Chapter 2**, **Section 2.4.1** and **Chapter 3**, **Section 3.2.1** were used throughout this chapter. Unless stated otherwise, compounds were purified by RP-HPLC.

4.7.2 *Experimental for Part a*

The synthetic route is illustrated in Figure 4-3.

4.7.2.1 Mono-6^A-succinylamino-6^A-Gly-Gly-Gly-β-cyclodextrin (**14a**)

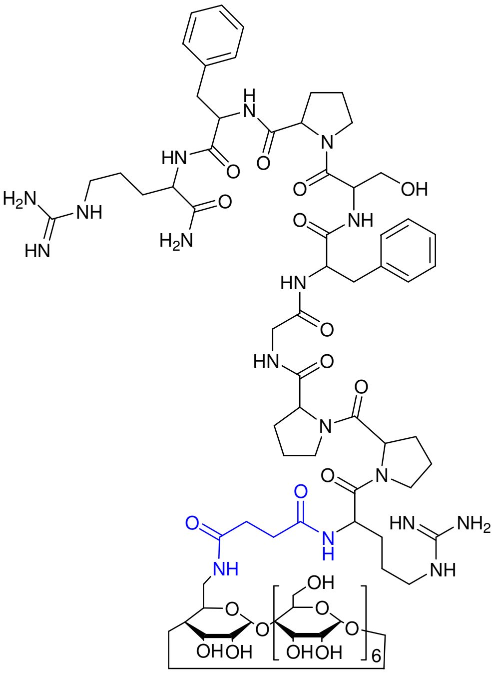


14a

The tri-glycine peptide was assembled manually on Rink resin (342.0 mg, 0.25 mmol, 1 equiv) using Fmoc-SPPS as per **Chapter 3, Section 3.2.2.1**. Fmoc-tri-glycine (197.5 mg, 0.50 mmol, 2 equiv), and mono-6^A-succinylamino-β-cyclodextrin (**4**, 727 mg, 0.50 mmol, 2 equiv) were coupled overnight using HBTU (321 mg, 1.0 mmol, 4 equiv) and DIEA (175 µL, 1.0 mmol, 4 equiv). Cleavage of the peptide from the resin as per **Chapter 3**, **Section 3.2.2.1**. RP-HPLC purification as per **Chapter 2**, **Section 2.4.4.4** using a MeOH stepwise gradient. The product came off at approximately 19% B on a shouldered peak (12.6 mg, 4%).

LR-MS: *m/z* (%, assignment) 1427.76 (50, M+Na)$^+$ HR-MS: *m/z* $C_{52}H_{85}N_5O_{39}$ $(M+H)^+$ 1404.4894, actual 1404.4876 and $C_{52}H_{85}N_5O_{39}Na$ $(M+Na)^+$ 1426.4714, actual 1426.4658; ^{1}H NMR (500 MHz, d$_6$-DMSO): δ 2.40-2.35 (m, 4H, succinyl CH$_2$), 3.19-3.75 (m, 69H, CD H2-H6, glycine CH$_2$, including HOD peak), 3.41-4.56 (m, 6H, CD OH6), 4.77-4.89 (m, 6H, CD H1), 5.58-5.88 (m, 14H, CD OH2, OH3), 7.65-7.72 (m, 3H, glycine NH); ^{13}C NMR (500 MHz, d$_6$-DMSO): δ 28.9 (succinyl CH$_2$), 31.9 (succinyl CH$_2$), 42.3 (glycine CH$_2$), 42.7 (glycine CH$_2$), 42.8 (glycine CH$_2$), 60.3 (CD C6), 60.3 (CD C6), 60.45 (CD C6), 60.47 (CD C6), 72.43 (CD), 72.45 (CD), 72.5 (CD), 72.6 (CD), 72.7 (CD), 72.85 (CD), 72.89 (CD), 73.3 (CD), 73.4 (CD), 73.51 (CD), 73.54 (CD), 81.9 (CD), 82.0 (CD), 82.11 (CD), 82.17 (CD), 83.9 (CD), 102.2 (CD C1), 102.34 (CD C1), 102.38 (CD C1), 102.4 (CD C1), 102.6 (CD C1)

4.7.2.2 Mono-6^A-succinylamino-6^A-bradykinin-β-cyclodextrin (**14b**)



14b

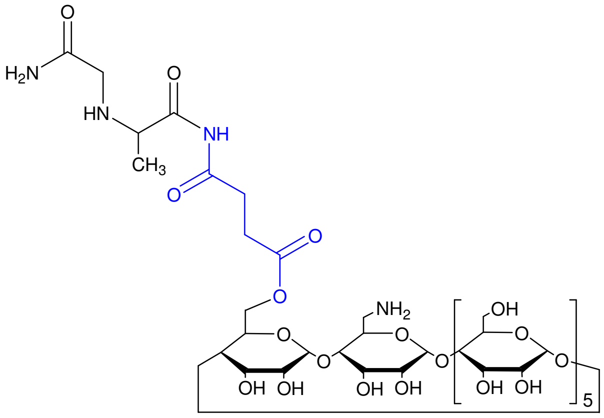
Compound **14b** was synthesised as per compound **14a** (**Section 4.7.2.1**) except the tri-glycine peptide is replaced with BK (see **Chapter 3** for specific synthesis details). Cleavage and full deprotection of the resin bound peptide as per compound **14a**. RP-HPLC purification as per **Chapter 3**, **Section 3.2.2.2** gave a good separation (1.8 mg, 3%). Other species present and separated were BK(Mtr)-CD (1.6 mg, 5%) and the BK peptide (1.1 mg, 4.5%).

LR-MS: *m/z* (%, assignment) 2275.48 (80, M+H)$^+$, 2297.49 (30, M+Na)$^+$; HR-MS: *m/z* $C_{96}H_{149}N_{17}O_{46}$ (M+2H)$^{+2}$ 1137.9916, actual 1137.9919; *NMR: too weak signal to obtain a good spectrum*

4.7.3 *Experimental for Part b*

The synthetic route is illustrated in Figure 4-4.

4.7.3.1 Mono-6^A^-fluorenylmethyloxycarbonylamino-mono-6^X^-succinyl-6^X^-Ala-Gly-β-cyclodextrin (**15a**)



15a

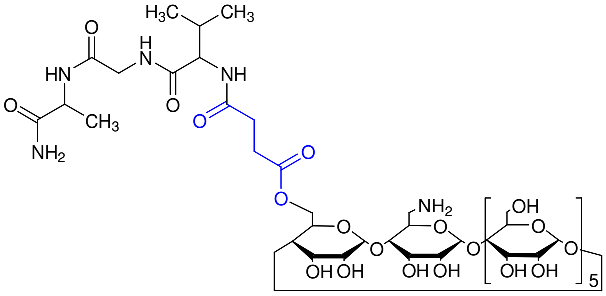
The Gly-Ala peptide was assembled manually on Rink resin (50 mg, 0.036 mmol, 1 equiv) using Fmoc-SPPS as per **Chapter 3**, **Section 3.2.2.1**. Fmoc-L-amino acids (0.144 mmol, 4 equiv) were coupled using TBTU (46 mg, 0.144 mmol, 4 equiv), HOBt (19 mg, 0.144 mmol, 4 equiv), and DIEA (20μL, 0.288 mmol, 8 equiv) and mono-6^{A}-fluorenylmethyloxycarbonylamino-mono-6^{X}-succinyl-β-cyclodextrin (**12**, **Chapter 2**, **Section 2.4.4.4**, 209 mg, 0.144 mol, 4 equiv) was coupled using EDC (4 equiv, 0.144 mmol), DIEA (4 equiv, 0.144 mmol) in pyridine/DMF respectively. TFA (**Chapter 3**, **Section 3.2.2.1**) was used to cleave the resin bound peptide yielding 135 mg of crude product. RP-HPLC purification as per **Chapter 3**, **Section 3.2.2.2** using a MeCN stepwise gradient (Table 4-2). The product eluted between 9-33.7% B (67.1 mg, 135%). A repeat purification was performed eluting the pure product between 9-11% B (16.2 mg, 33%).

LR-MS: *m/z* (%, assignment) 1383.88 (100, M+Na)$^+$ HR-MS: *m/z* $C_{51}H_{85}N_4O_{38}$ (M+H)$^+$ 1361.4855, actual 1361.4836; ^{1}H NMR (500 MHz, d_6-DMSO): δ1.23 (d, J = 6.9 Hz, 4H, alanine CH_3), 2.28-2.39 (m, 4H, succinyl CH_2), 3.19-3.92 (m, 90H, CD H2-H6, glycine CH_2, including HOD peak), 4.21-4.34 (m, 2H, alanine CH), 4.35-4.62 (m, 6H, CD OH6), 4.79-4.96 (m, 6H, CD H1), 5.65-5.98 (m, 14H, CD OH2, OH3), 7.74 (s, 1H, CD NH), 7.92-80.20 (s, 1H, glycine NH), 8.47-8.58 (s, 1H, alanine NH); ^{13}C DEPT NMR (500 MHz, d_6-DMSO): δ 22.1 (succinyl CH_2), 22.4 (succinyl CH_2), 22.6 (succinyl CH_2), 22.9 (succinyl CH_2), 34.7 (alanine CH), 44.1 (glycine CH_2), 48.4 (alanine CH_3), 48.6 (alanine CH_3), 48.7 (alanine CH_3), 60.3 (CD C6), 60.42 (CD C6), 60.45 (CD C6), 60.50 (CD C6), 60.52 (CD C6), 72.3-73.7 (CD), 81.9-82.3 (CD), 102.2-102.6 (CD C1)

Table 4-2 Stepwise time programme for the separation of the **15a**

Time (min)	*% Mobile phase 'B'*
0	0
10	0
20	15
25	15
50	50
70	100
75	0
80	STOP

4.7.3.2 Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-6^X-Ala-Gly-Val-β-cyclodextrin (**15b**)



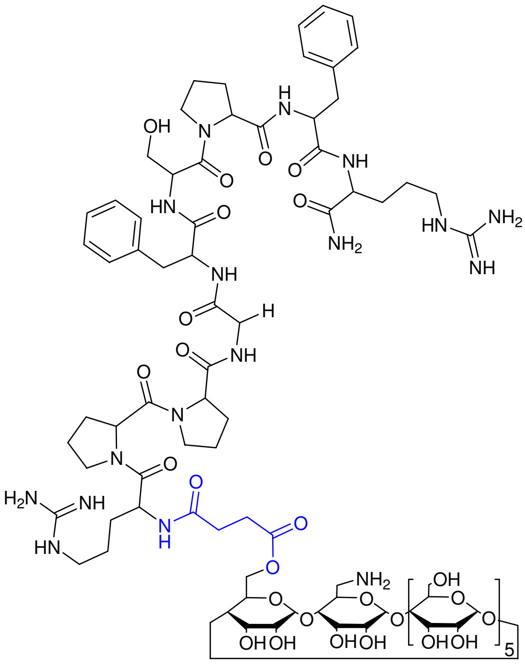
15b

The Val-Gly-Ala peptide was assembled manually on TentaGel resin (0.24 mmol/g, 0.096 mmol, 400 mg) coupling Fmoc-L-amino acids (0.384 mmol, 4 equiv) and **12** (0.384 mmol, 4 equiv) as per compound **15a** (**Section 4.7.3.1**). The crude product was purified and cleaved as per compound **15a** eluting between 0.1% and 12% B with a yield of 22.4 mg (14%).

LR-MS: *m/z* (%, assignment) 1482.42 (100, M+Na)$^+$; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.86 (d, J = 6.1 Hz, 6H, Val CH_3), 1.21 (d, J = 7.3 Hz, 3H, Ala CH_3), 1.94-2.03 (m, 1H, Val CH), 3.17-3.87 (m, 12H, CD H2-H6, Gly CH_2 including HOD peak), 4.07-4.22 (m, 2H, Ala CH, Val CH), 4.44-4.57 (m, 4H, succinyl CH_2), 4.81-5.00 (m, 7H, CH H1), 5.59-5.94 (m, 14H, CH OH2, OH3), 7.84-7.88 (m, 1H, Ala NH), 7.98-8.02 (m, 1H, Val

NH), 8.15-8.20 (m, 1H, Gly NH); ^{13}C NMR (500 MHz, d_6-DMSO): *very weak signal after 12h of recording*

4.7.3.3 Mono-6^{A}-fluorenylmethyloxycarbonylamino-mono-6^{X}-succinyl-6^{X}-bradykinin-β-cyclodextrin (**15c**)



15c

BK was assembled manually on Rink resin (0.69 mmol/g, 0.138 mmol, 200 mg, 1 equiv) coupling Fmoc-L-amino acids (0.552 mmol, 4 equiv) and **12** (803 mg, 0.552 mmol, 4 equiv) using the same reaction conditions as per compound **15a** (**Section 4.7.3.1**). Peptide cleavage and purification as per compound **15a** eluted the pure product at 42% B as a white fluffy solid (2.0 mg, 2%).

LR-MS: *m/z* (%, assignment) 2388.52 (80, M+H)$^+$ HR-MS: *m/z* $C_{102}H_{158}N_{18}O_{47}$ (M+H)$^+$ 2387.0532, actual 2387.0601; ^1H NMR (d6-DMSO, 500 MHz): δ 1.14-2.10 (m, 19H, proline$_{2\beta}$, proline$_{2\gamma}$, proline$_{3\beta}$, arginine$_{1\beta}$, arginine$_{1\alpha}$, proline$_{3\beta}$,[¶]), 2.14-2.24 (m, 1H, phenylalanine$_{2\beta}$), 2.59-3.89 (m, 51H, phenylalanine$_{2\beta,}$[¶] phenylalanine$_{5\beta}$, arginine$_{9\alpha}$,

---

[¶] All unassigned peaks associated with arginine$_{1\alpha}$, phenylalalaine$_{8\alpha}$, proline$_{3\beta}$, proline$_{3\gamma}$, proline$_{7\beta}$, proline$_{7\gamma}$, arginine$_{9\beta}$, arginine$_{1\gamma}$, arginine$_{9\beta}$, arginine$_{9\gamma}$, proline$_{7}$, or proline$_{4}$

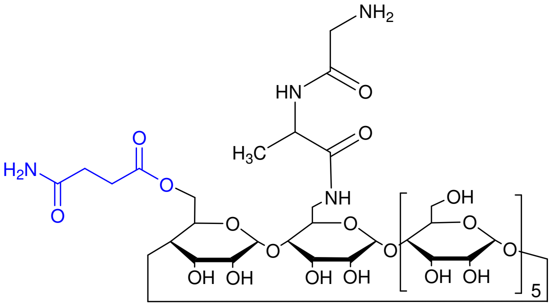
arginine$_{9\alpha}$, proline$_{7\gamma}$, proline$_{3\gamma}$, arginine$_{1\gamma}$, serine$_{6\beta}$, proline$_{7\gamma}$, proline$_{3\gamma}$, proline$_{2\gamma}$, glycine$_{\alpha}$, CD H2-H6, CD OH6), 4.07-4.41 (m, 4H,[¶] arginine$_{9}$, proline$_{3}$, proline$_{7}$), 4.45-4.74 (m, 4H, phenylalanine$_{8}$, phenylalanine $_{5}$, serine$_{8}$, proline$_{2}$), 4.80-4.99 (m, 7H, CH H1), 5.56-5.94 (m, 14H, CH OH2, OH3), 6.94-7.03 (br s, 1H, NH[¶]), 7.09-7.32 (m, 17H, phenylalanine aromatics), 7.47-7.53 (m, 3H, phenylalanine aromatics), 7.60-7.62 (m, 1H, phenylalanine aromatics), 7.62-7.65 (m, 2H, phenylalanine aromatics), 7.75-7.80 (m, 1H, glycine NH), 7.84-7.87 (m, 1H, phenylalanine aromatics), 7.87-7.97 (m, 4H, sulfonamide aromatics), 8.01-8.07 (m, 2H, sulfonamide aromatics, arginine$_{9}$ NH), 8.37-8.42 (m, 1H, phenylalanine$_{5}$ NH), 8.68-8.72 (m, 1H, serine$_{6}$ NH); ^{13}C-DEPT NMR (d$_{6}$-DMSO, 700 MHz): δ 25.20 (succinyl CH$_{2}$), 25.28 (succinyl CH$_{2}$), 25.3 (BK), 27.6 (BK), 28.2 (BK), 28.3 (BK), 29.21 (BK), 29.22 (BK), 29.3 (BK), 29.4 (BK), 29.5 (BK), 29.80 (BK), 29.81 (BK), 29.8 (BK), 29.9 (BK), 30.1 (BK), 37.5 (BK), 38.2 (BK), 40.7 (BK), 40.9 (BK), 42.2 (BK), 46.4 (BK), 47.3 (BK), 47.4 (BK), 52.5 (BK), 53.16 (BK), 53.19 (BK), 53.8 (BK), 53.9 (BK), 54.2 (BK), 58.3 (BK), 58.8 (BK), 59.5 (BK), 59.6 (BK), 59.7 (BK), 59.9 (BK), 60.0 (CD C6), 60.02 (CD C6), 60.07 (CD C6), 60.27 (CD C6), 60.3 (CD C6), 60.4 (CD C6), 60.6 (CD C6), 60.8 (CD C6), 60.9 (CD C6), 62.2 (CD), 63.73 (CD), 63.78 (CD), 69.3 (CD), 69.45 (CD), 69.46 (CD), 72.55 (CD), 72.59 (CD), 72.8 (CD), 72.9 (CD), 73.3 (CD), 73.4 (CD), 73.5 (CD), 81.3 (CD), 81.4 (CD), 81.6 (CD), 81.7 (CD), 81.9 (CD), 82.14 (CD), 82.15 (CD), 82.30 (CD), 82.31 (CD), 82.4 (CD), 82.5 (CD), 82.6 (CD), 82.9 (CD), 84.0 (CD), 102.2 (CD C1), 102.34 (CD C1), 102.38 (CD C1), 102.5 (CD C1), 102.6 (CD C1), 102.7 (CD C1), 102.8 (CD C1), 102.9 (CD C1), 126.6 (phenylalanine aromatics), 126.7 (phenylalanine aromatics), 128.4 (phenylalanine aromatics), 128.5 (phenylalanine aromatics), 129.51 (phenylalanine aromatics), 129.59 (phenylalanine aromatics)

[¶] All unassigned peaks associated with arginine$_{1\alpha}$, phenylalaine$_{8\alpha}$, proline$_{3\beta}$, proline$_{3\gamma}$, proline$_{7\beta}$, proline$_{7\gamma}$, arginine$_{9\beta}$, arginine$_{1\gamma}$, arginine$_{9\beta}$, arginine$_{9\gamma}$, proline$_{7}$, or proline$_{4}$

4.7.4 *Experimental for Part c*

The synthetic route is illustrated in Figure 4-5.

4.7.4.1 Mono-6^A-amino-6^A-Gly-Alal-6^X-succinyl-β-cyclodextrin (**16a**)

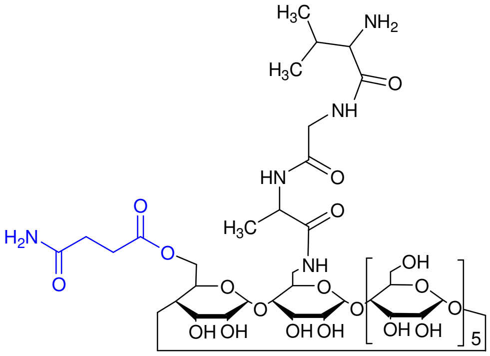


16a

Compound **16a** was synthesised on Rink resin (50 mg, 0.036 mmol, 1 equiv) coupling Fmoc-L-amino acids (0.144 mmol, 4 equiv) and compound **12** (209 mg, 0.144 mmol, 4 equiv) as per **15a** (**Section 4.7.3.1**). TFA cleavage and purification as per compound **15a** eluted the pure product between 37-40% B (7.4 mg, 15%).

LR-MS: *m/z* (%, assignment) 1383.82 (100, M+Na)$^+$ HR-MS: *m/z* $C_{51}H_{85}N_4O_{38}$ (M+H)$^+$ 1361.4838, actual 1361.4836; ^{1}H NMR (500 MHz, d_6-DMSO): δ 1.23 (d, J = 6.9 Hz, 4H, alanine CH_3), 2.27-2.39 (m, 4H, succinyl CH_2), 3.22-3.97 (m, 90H, CD H2-H6, glycine CH_2, including HOD peak), 4.21-4.35 (m, 2H, alanine CH), 4.35-4.61 (m, 6H, CD OH6), 4.79-4.94 (m, 6H, CD H1), 5.62-5.93 (m, 14H, CD OH2, OH3), 7.75 (s, 1H, CD NH), 7.90-80.11 (s, 1H, glycine NH), 8.47-8.57 (s, 1H, alanine NH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 29.2 (glycine CH_2), 29.9 (glycine CH_2), 60.3 (succinyl CH_2), 60.5 (succinyl CH_2), 67.6 (CH_2 C6), 68.3 (CD C6), 68.9 (CD HC), 72.44 (CD), 72.46 (CD), 72.49 (CD), 72.5 (CD), 72.65 (CD), 72.68 (CD), 72.7 (CD), 72.8 (CD), 72.92 (CD), 72.94 (CD), 73.2 (CD), 73.31 (CD), 73.39 (CD), 73.45 (CD), 73.47 (CD), 73.6 (CD), 81.6 (CD), 82.2 (CD), 82.3 (CD), 102.3 (CD C1), 102.4 (CD C1), 102.8 (CD C1)

4.7.4.2 Mono-6^A-amino-6^A-Val-Gly-Ala-mono-6^X-succinyl-β-cyclodextrin (**16b**)

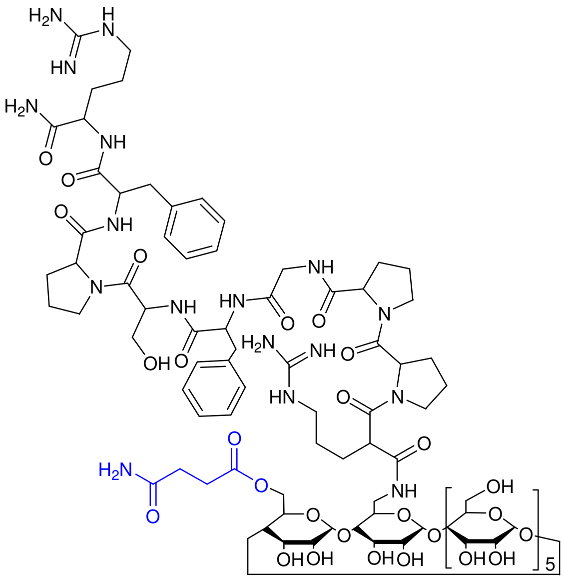


16b

The Val-Gly-Ala peptide was assembled manually on TentaGel resin (0.24 mmol/g, 0.096 mmol, 400 mg) using Fmoc-SPPS coupling Fmoc-L-amino acids and **12** as per compound **15a** (**Section 4.7.3.1**). Peptide cleavage and purification as per compound **15a** eluting at 43% B with a yield of 12.8 mg (7%).

LR-MS: *m/z* (%, assignment) 1483.19 (100, M+Na)$^+$ HR-MS: *m/z* $C_{56}H_{93}N_5O_{39}$ (M+H)$^+$ 1459.5423, actual 1459.5430; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.81-0.92 (d, J = 6.1 Hz, 6H, Val CH_3), 1.20-1.25 (d, J = 7.3 Hz, 3H, Ala CH_3), 1.95 -2.20 (m, 1H, Val CH), 3.21 -3.90 (m, 12H, CD H2-H6, Gly CH_2 including HOD peak), 4.09 -4.34 (m, 2H, Ala CH, Val CH), 4.48 -4.61 (m, 4H, succinyl CH_2), 4.68 -4.98 (m, 7H, CH H1), 5.82-6.02 (m, 14H, CH OH2, OH3), 7.85-7.93 (m, 1H, Ala NH), 7.99-8.10 (m, 1H, Val NH), 8.17-8.31 (m, 1H, Gly NH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 18.1 (valine CH_3), 18.2 (valine CH_3) 18.5 (valine CH_3), 19.1 (alanine CH_3), 19.2 (alanine CH_3), 21.6 (alanine CH_3), 30.1 (succinyl CH_2), 34.6 (valine CH), 35.8 (alanine CH), 36.8 (valine CH), 59.7 (glycine CH_2), 59.9 (glycine CH_2), 60.3 (CD C6), 60.4 (CD C6), 70.0 (CD C6), 72.3-73.5 (CD), 81.2 (CD), 81.3 (CD), 81.62 (CD), 81.65 (CD), 81.94 (CD), 81.95 (CD), 82.00 (CD), 82.06 (CD), 101.7 (CD C1), 101.8 (CD C1), 102.40 (CD C1), 102.42 (CD C1), 102.43 (CD C1), 102.46 (CD C1), 102.47 (CD C1), 102.7 (CD C1)

4.7.4.3 Mono-6^A-amino-6^A-bradykinin-mono-6^X-succinyl-β-cyclodextrin (**16c**)



16c

The BK was assembled manually on Rink resin (0.73 mmol/g, 0.0365 mmol, 50 mg) using Fmoc-SPPS as per experiment **15c** (**Section 4.7.3.3**). Cleavage and purification as per experiment **15a** (**Section 4.7.3.1**) eluted the pure product at 31.5% B as a white fluffy solid (40.5 mg, 49%).

LR-MS: *m/z* (%, assignment) 2275.32 (100, M+H)$^+$, 1137.99 (90, M+2H)$^{+2}$ HR-MS: *m/z* $C_{96}H_{149}N_{17}O_{46}$ (M+2H)$^{+2}$ 1137.9912, actual 1137.9916; ^{1}H NMR (d_6-DMSO, 700 MHz): δ 1.21-1.28 (m, 1H, proline), 1.43-2.24 (m 17H, arginine, arginine[\\]), 2.31-2.41 (m, 4H, phenylalanine), 2.58-2.63 (m, 1H, phenylalanine), 2.69-2.80 (m, 3H, arginine, proline[\\]), 2.94-3.14 (m, 9H, serine, glycine), 3.43-3.92 (m, 33H, CD H2-H6, CD OH6), 4.25-4.68 (m, 11H, arginine, proline$_3$, proline$_7$, phenylalanine$_8$, phenylalanine$_5$, serine$_8$, proline$_2$[\\]), 4.79-4.91 (m, 7H, CH H1), 5.59-5.88 (m, 14H, CH OH2, OH3), 6.73-6.85 (br s, 2H, terminal NH_2), 7.13-7.28 (m, 10H, phenylalanine aromatics), 7.30-7.43 (m, 2H, terminal NH_2), 7.88-8.02 (s, 1H, NH), 8.08-8.17 (m, 2H, NH), 8.28-8.33 (m, 1H, NH); ^{13}C NMR (d_6-DMSO, 700 MHz): δ 25.1 (succinyl CH_2), 25.2 (succinyl CH_2), 25.3 (BK), 27.6 (BK), 28.2 (BK), 28.3 (BK), 29.1 (BK), 29.2 (BK), 29.3 (BK), 29.4 (BK), 29.5 (BK), 29.6 (BK), 29.7 (BK), 29.8 (BK), 30.00 (BK), 30.08 (BK), 37.5 (BK), 38.2 (BK), 40.7 (BK), 40.9 (BK), 42.2 (BK), 46.4 (BK), 47.2 (BK), 47.4 (BK), 52.4 (BK),

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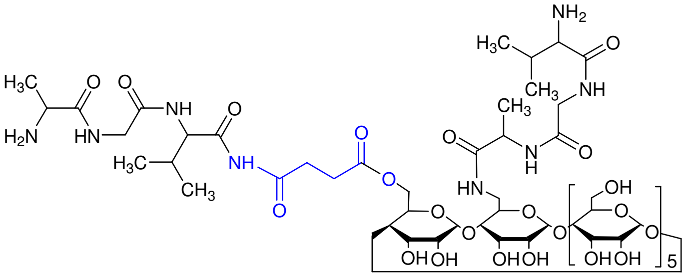
[\\] All unassigned peaks associated with the bradykinin peptide – not very good resolution for assignment

53.1 (BK), 53.1 (BK), 53.8 (BK), 53.9 (BK), 54.2 (BK), 58.3 (BK), 58.8 (BK), 59.5 (BK), 59.7 (BK), 59.92 (BK), 59.95 (BK), 60.00 (CD C6), 60.02 (CD C6), 60.07 (CD C6), 60.2 (CD C6), 60.3 (CD C6), 60.4 (CD C6), 60.6 (CD C6), 60.7 (CD C6), 60.9 (CD C6), 62.1 (CD), 63.73 (CD), 63.78 (CD), 69.3 (CD), 69.4 (CD), 69.50 (CD), 72.51 (CD), 72.6 (CD), 72.83 (CD), 72.88 (CD), 73.3 (CD), 73.4 (CD), 73.5 (CD), 81.3 (CD), 81.4 (CD), 81.5 (CD), 81.7 (CD), 81.9 (CD), 82.13 (CD), 82.17 (CD), 82.3 (CD), 82.40 (CD), 82.41 (CD), 82.5 (CD), 82.6 (CD), 82.9 (CD), 83.0 (CD), 102.2 (CD C1), 102.31 (CD C1), 102.37 (CD C1), 102.4 (CD C1), 102.62 (CD C1), 102.65 (CD C1), 102.8 (CD C1), 102.9 (CD C1), 126.7 (phenylalanine aromatics), 126.8 (phenylalanine aromatics), 128.4 (phenylalanine aromatics), 128.5 (phenylalanine aromatics), 129.5 (phenylalanine aromatics), 129.6 (phenylalanine aromatics), 138.02, 138.07, 157.0, 157.2, 158.0, 158.2, 158.4, 158.5, 168.8, 169.0, 170.10, 170.12, 170.31, 171.32, 171.41, 171.45, 171.6, 171.74, 171.79, 172.1, 172.2, 172.80, 172.82, 172.86, 173.43, 173.48, 173.6

4.7.5 *Experimental for Part d*

The synthetic route is illustrated in Figure 4-6.

4.7.5.1 Mono-6^A-amino-6^A-Val-Gly-Ala-mono-6^X-succinyl-6^X-Val-Gly-Ala-β-cyclodextrin (**17a**)



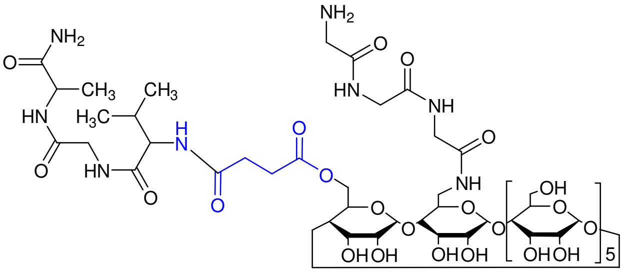
17a

The Val-Gly-Ala di-peptide was assembled manually on Rink resin (0.72 mmol/g, 0.3321 mmol, 460 mg) using the same reaction conditions as per compound **15a** (**Section 4.7.3.1**). RP-HPLC purification as per compound **15a** eluted the pure product at 22-23% B (91.2 mg, 16%).

- A parallel synthesis of **17a** was also performed using TentaGel resin (0.24 mmol/g, 0.048 mmol, 200 mg, 1 equiv) coupling Fmoc-L-amino acids (0.096 mmol, 4 equiv) and **12** (140 mg, 0.096 mmol, 4 equiv) as per **15a** (**Section 4.7.3.1**). Cleavage and RP-HPLC purification as per **15a** with the pure product eluting between 22.5-24% B (1.1 mg, 14%).

LR-MS: *m/z* (%, assignment) 1710.39 (100, M+Na)$^+$ HR-MS: *m/z* $C_{66}H_{111}N_8O_{42}$ (M+H)$^+$ 1687.6781, actual 1687.6790; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.82-0.91 (m, 10H, valine $(CH_3)_2$, succinyl $(CH_2)_2$), 0.92-1.02 (m, 6H, valine $(CH_3)_2$), 1.16-1.25 (m, 6H, alanine CH_3), 1.97 (q, J = 6.7 Hz, 1H, valine CH), 2.07 (q, J = 6.5 Hz, 1H, valine CH), 3.16-3.95 (m, 138H, CD H2-H6, 2 x glycine CH_2, including H_2O peak), 4.03-4.73 (m, 9H, 2 x alanine CH, 2 x valine CH, CD OH6), 4.78-4.94 (m, 7H, CD H1), 5.58-5.96 (br s, 14H, CD OH2, OH3), 7.76-8.24 (m, 8H, alanine NH, alanine NH_2, valine NH, valine NH_2, glycine NH, CD NH), 8.55-8.64 (m, 1H, glycine NH); ^{13}C DEPT NMR (500 MHz, d_6-DMSO): δ 18.1 (valine CH_3), 18.3 (valine CH_3), 18.4 (valine CH_3), 18.5 (valine CH_3), 18.6 (valine CH_3), 18.7 (valine CH_3), 18.81 (valine CH_3), 18.82 (valine CH_3), 19.00 (alanine CH_3), 19.05 (alanine CH_3), 19.6 (alanine CH_3), 19.7 (alanine CH_3), 21.2 (alanine CH_3), 25.6 (alanine CH_3), 29.2 (succinyl CH_2), 29.3 (succinyl CH_2), 30.2 (valine CH), 30.4 (valine CH), 30.5 (alanine CH), 30.7 (alanine CH), 30.9 (valine CH), 31.1 (valine CH), 42.1 (glycine CH_2), 42.2 (glycine CH_2), 42.51 (glycine CH_2), 42.54 (glycine CH_2), 48.5, 48.6, 48.8, 57.91, 57.95, 58.7, 58.8, 59.1, 60.1-60.4 (CD C6), 72.4-73.0 (CD), 73.2-73.6 (CD), 81.5-82.6 (CD), 102.1-103.0 (CD C1)

4.7.5.2 Mono-6^{A}-amino-6^{A}-Gly-Gly-Gly-mono-6^{X}-succinyl-6^{X}-Val-Gly-Ala-β-cyclodextrin (**17b**)

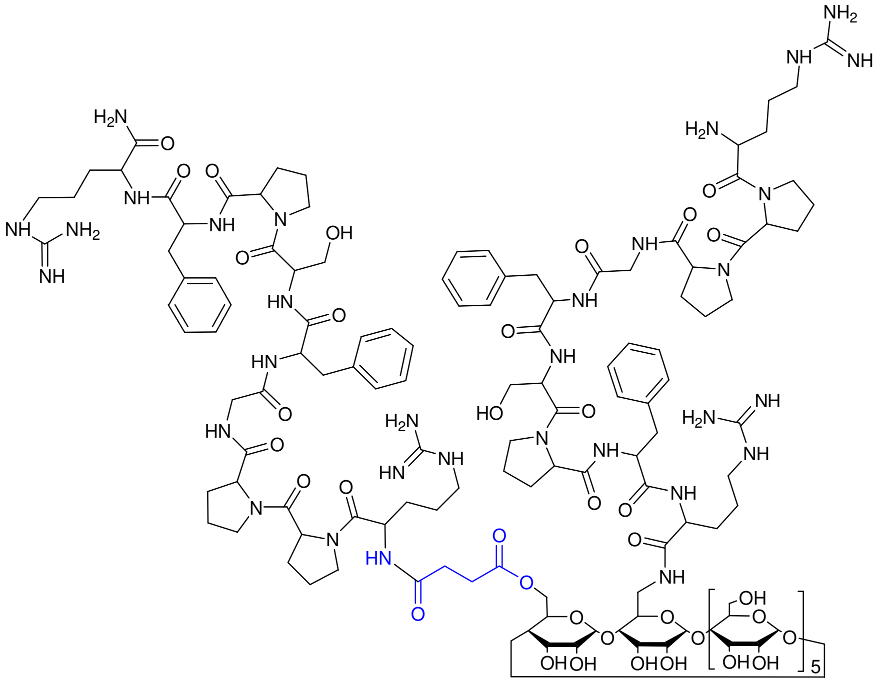


17b

The di-peptide was assembled manually on Rink resin (0.72 mmol/g, 0.144 mmol, 200 mg, 1 equiv) using Fmoc-SPPS as per **Chapter 3, Section 3.2.2.1**. Fmoc-L-amino acids (0.576 mmol, 4 equiv) and **12** (840 mg, 0.576 mmol, 4 equiv) were coupled as per **15a** (**Section 4.7.3.1**). Cleavage and purification as per **15a** with the pure product eluting between 24-25% B (20.1 mg, 8.5%).

LR-MS: *m/z* (%, assignment) 1654.25 (100, M+Na)$^+$; ^{1}H NMR (500 MHz, d$_6$-DMSO): δ 0.86 (d, J = 6.7 Hz , 6H, valine CH$_3$), 1.21 (d, J = 7.2 Hz , 3H, alanine CH$_3$), 1.9 (q, J = 6.3 Hz , 1H, valine CH), 2.36-2.45 (m, 4H, succinyl CH$_2$), 3.50-3.96 (m, 48H, CD H2-H2, glycine CH$_2$), 4.02-4.13 (m, 1H, valine CH), 4.13-4.25 (m, 1H, alanine CH), 4.38-4.63 (m, 12H, CD OH6), 4.80-5.02 (m, 7H, CD H1), 5.64-6.00 (m, 12H, CD OH2, OH3), 7.01 (s, 1H, NH), 7.25 (s, 1H, NH), 7.80-7.90 (m, 1H, alanine NH), 7.91-8.03 (m, 1H, valine NH), 8.14-8.29 (m, 2H, glycine NH), 8.57-8.69 (m, 1H, glycine NH); ^{13}C DEPT NMR (500 MHz, d$_6$-DMSO): δ 18.5 (valine CH$_3$), 18.6 (valine CH$_3$), 19.0 (alanine CH$_3$), 29.6 (succinyl CH$_2$), 30.3 (succinyl CH$_2$), 30.4 (valine CH), 42.0 (glycine CH$_2$), 42.2 (glycine CH$_2$), 42.3 (glycine CH$_2$), 42.5 (glycine CH$_2$), 47.9 (alanine CH), 48.4 (valine CH), 58.7 (CD), 58.8 (CD), 60.3 (CD C6), 72.40 (CD), 72.4 (CD), 73.8 (CD), 102.4 (CD C1)

4.7.5.3 Mono-6^{A}-amino-6^{A}-bradykinin-mono-6^{X}-succinyl-6^{X}-bradykinin-β-cyclodextrin (**17c**)



17c

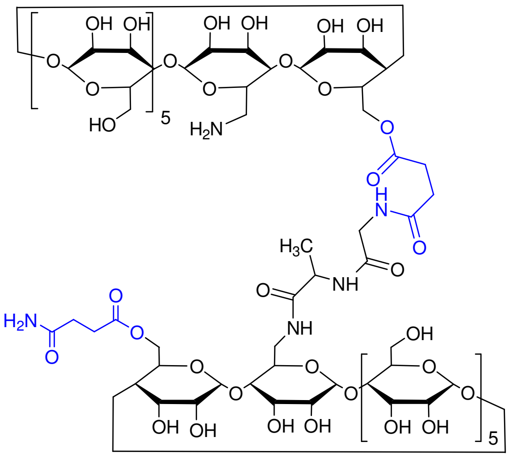
The BK di-peptide was assembled manually on Rink resin (0.69 mmol/g, 200 mg, 0.138 mmol) using the same reaction conditions as per **17b** (**Section 4.7.5.2**). The coupling order was the BK peptide, compound **12**, and then the BK peptide again. RP-HPLC purification as per **15a** (**Section 4.7.3.1**) using a semi-analytical C-18 column eluting the pure product between 43-45% B (0.75 mg, trace %).

LR-MS: *m/z* (%, assignment) 3319.13 (75, M+H)$^{+}$

4.7.6 *Experimental for Part e*

The synthetic route is illustrated in Figure 4-7.

4.7.6.1 Mono-6^{A}-amino-6^{A}-Gly-Ala-6^{A}-[mono-6^{A}-amino-amono-6^{X}-succinyl-β-cyclodextrin]-mono-6^{X}-succinyl-β-cyclodextrin (**18a**)

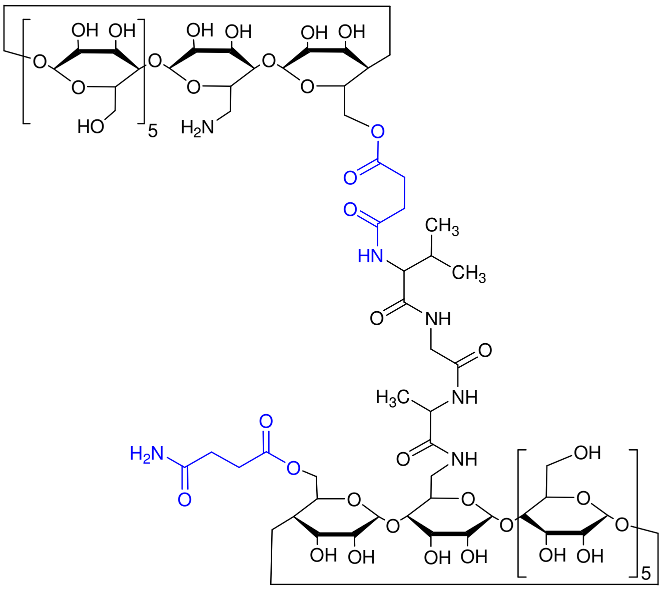


18a

Compound **18a** was synthesised on Rink resin (50 mg, 0.036 mmol, 1 equiv) coupling Fmoc-L-amino acids (0.144 mmol, 4 equiv) and **12** (209 mg, 0.144 mmol, 4 equiv) as per **15a** (**Section 4.7.3.1**). Compound **12** was initially coupled to resin, followed by the addition of the Gly-Ala peptide, and then followed by the addition of compound **12**. Cleavage and purification as per compound **15a**. The product eluted at 30% B (7.4 mg, 15%).

LR-MS: *m/z* (%, assignment) 2601.48 (100, M+Na)$^{+}$ HR-MS: *m/z* $C_{97}H_{158}N_5O_{74}$ (M+Na)$^{+}$ 2577.2723, actual 2277.2730

4.7.6.2 Mono-6^A-amino-6^A-Val-Gly-Ala-6^A-[mono-6^A-amino-mono-6^X-succinyl-β-cyclodextrin]-mono-6^X-succinyl-β-cyclodextrin (**18b**)



18b

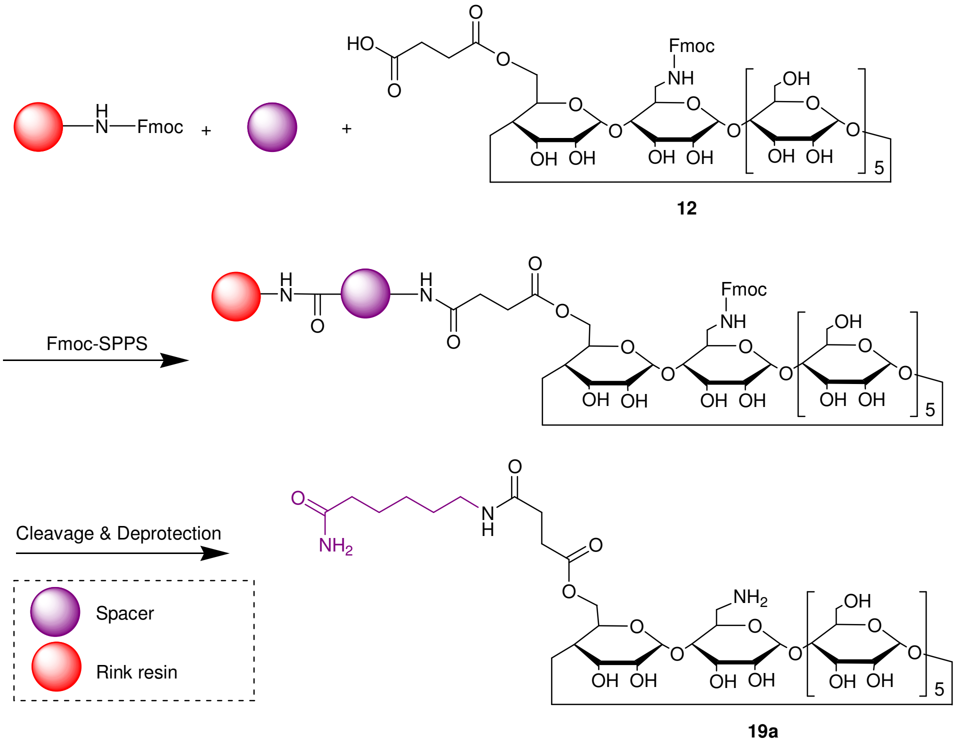
The Val-Gly-Ala peptide was assembled manually on TentaGel resin as per compound **15a**, **Section 4.7.3.1**. Cleavage and RP-HPLC purification as per **15a** eluting the product between 23% B (1.6 mg, 1%).

LR-MS: *m/z* (%, assignment) 2699.06 (100, M+Na)$^+$ HR-MS: *m/z* $C_{102}H_{167}N_6O_{75}K$ (M+H+K)$^{+2}$ 1357.4536, actual 1357.4532

4.7.7 *Experimental for Part f*

For an overview of compounds synthesised in this section see Figure 4-8.

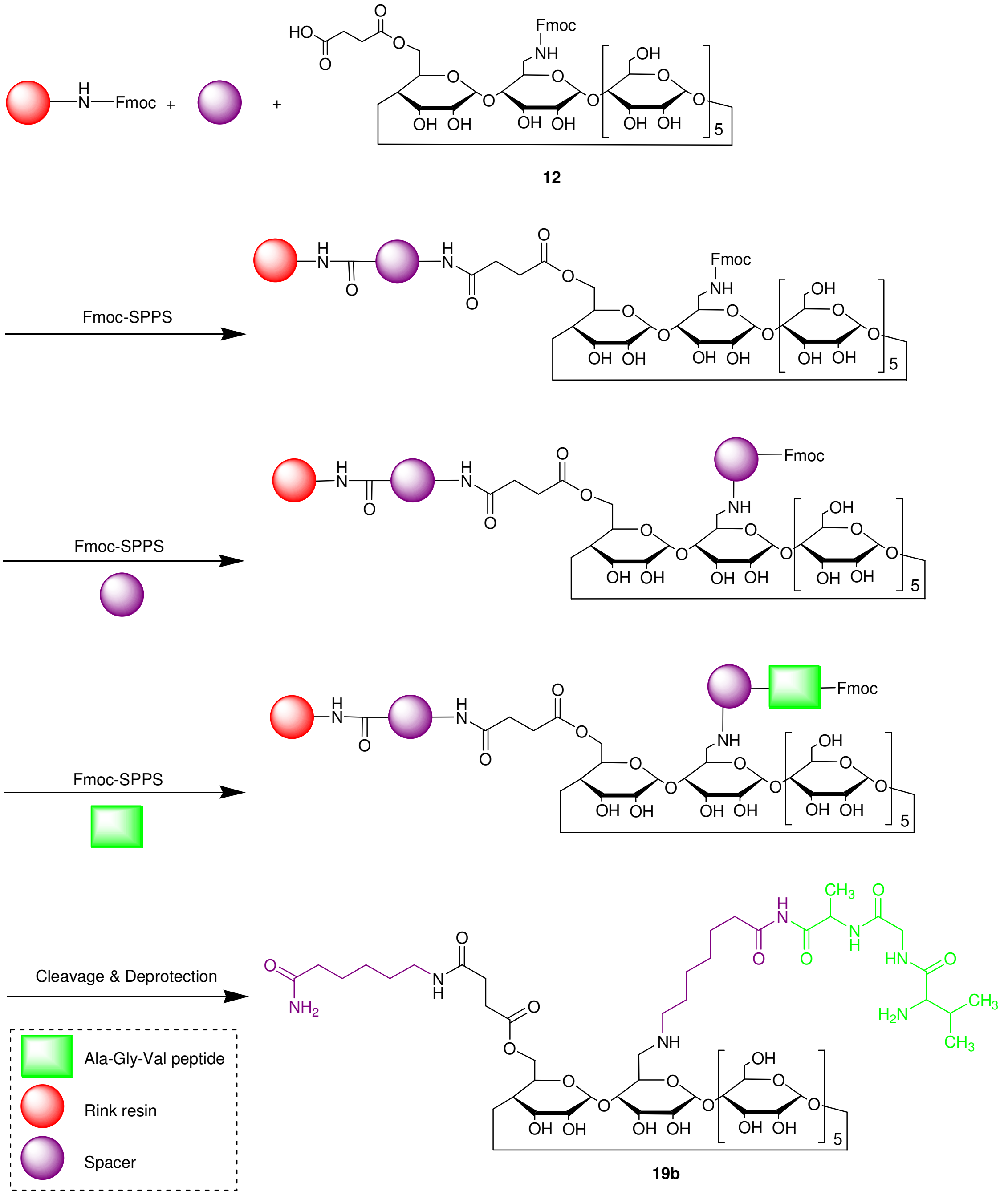
4.7.7.1 Mono-6^{A}-amino-6^{A}-mono-6^{X}-succinyl-6^{X}-ε-aminocaproic acid-β-cyclodextrin (**19a**)



Compound **19a** was manually assembled on Rink resin (0.73 mmol/g, 0.108 mmol, 150 mg, 1 equiv) coupling compound **12** (628 mg, 0.432 mmol, 4 equiv) as per **15a** (**Section 4.7.3.1**). Fmoc-ε-aminocaproic acid (for synthesis details see **Appendix 2-VIII**, 153 mg, 0.432 mmol, 4 equiv) was coupled using TBTU (139 mg, 0.432 mmol, 4 equiv), HOBt, (58 mg, 0.432 mmol, 4 equiv) and DIEA (151 µL, 0.864 mmol, 8 equiv) in excess. A RP-HPLC MeCN stepwise gradient as per **15a** was used to purify the product from the crude material at a wavelength of 254 nm. The partially-purified product eluted between 7.2-15% B. A repeat RP-HPLC was performed eluting the pure product at 8.3% B with a yield of 2.8 mg (6%) as a white fluffy solid.

LR-MS: *m/z* (%, assignment) 1368.81 (100, M+Na)$^+$ HR-MS: *m/z* $C_{52}H_{88}N_3O_{37}$ (M+H)$^+$ 1346.5012, actual 1346.5013; ^{1}H NMR (500 MHz, d$_6$-DMSO): δ 1.19-1.28 (m, 1H, ε-aminocaproic acid CH_2), 1.34-1.43 (m, 1H, ε-aminocaproic acid CH_2), 1.44-1.51 (m, 1H, ε-aminocaproic acid CH_2), 1.52-1.69 (m, 5H, ε-aminocaproic acid CH_2), 2.00-2.06 (m, 2H, ε-aminocaproic acid CH_2), 2.24-2.45 (m, 4H, succinyl CH_2), 3.88-2.97 (m, 101H, ε-aminocaproic acid CH_2, CD H2-H6, including HOD peak), 4.80-4.89 (m, 5H, CH OH6), 4.89-4.93 (m, 5H, CD H1), 4.94-4.98 (s, 15H, CD H1), 4.99-4.93 (s, 15H, CD H1), 5.52-5.91 (m, 13H, CD OH2, OH3), 7.72-7.98 (br s, 1H, NH_2), 8.25-8.53 (br s, 21H, terminal NH_2); ^{13}C DEPT NMR (500 MHz, d_6-DMSO): δ 22.0 (succinyl CH_2), 22.6 (succinyl CH_2), 25.3 (ε-aminocaproic acid CH_2), 25.8 (ε-aminocaproic acid CH_2), 34.7 (ε-aminocaproic acid CH_2), 44.1 (ε-aminocaproic acid CH_2), 60.3 (CD), 60.4 (CD), 60.5 (CD), 72.5 (CD), 72.7 (CD), 72.8 (CD), 72.9 (CD), 73.4 (CD), 81.9 (CD), 82.0 (CD), 82.1 (CD), 102.40 (CD C1), 102.46 (CD C1), 102.5 (CD C1)

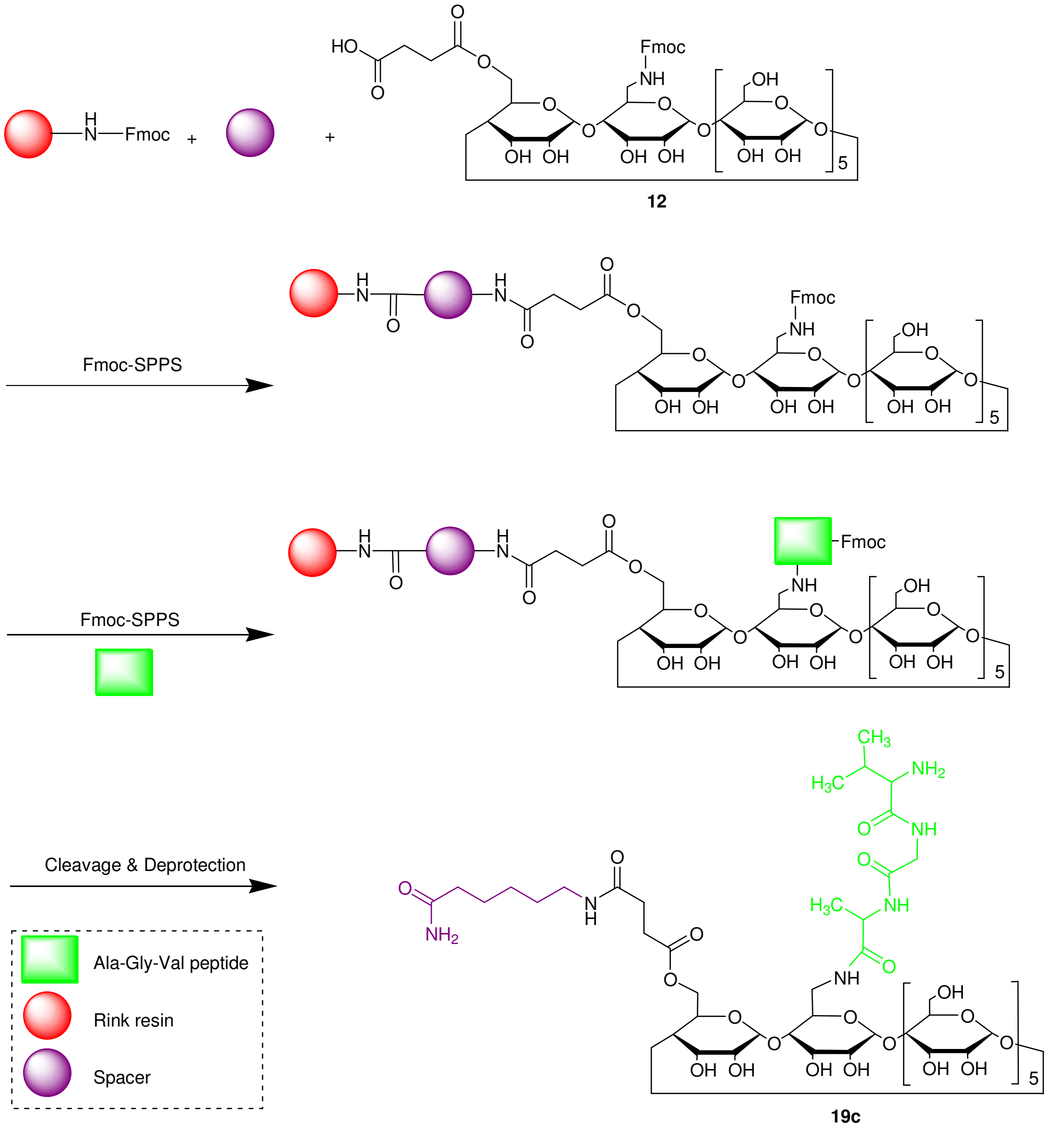
4.7.7.2 Mono-6^A-amino-6^A-mono-6^A-ε-aminocaproic acid-6^A-Val-Gly-Ala-6^X-succinyl-6^X-ε-aminocaproic acid-β-cyclodextrin (**19b**)



Compound **19b** was manually assembled on Rink resin as per **19a**, **Section 4.7.7.1**. RP-HPLC purification as per **15a** (**Section 4.7.3.1**) eluting the pure product at 23.5% B with a yield of 1.8 mg (3%).

LR-MS: *m/z* (%, assignment) 1708.80 (100, M+Na)$^+$ HR-MS: *m/z* $C_{68}H_{116}N_7O_{41}$ (M+H)$^+$ 1686.7231, actual 1686.7202; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.83-1.00 (m, 10H, valine CH_3, succinyl CH_2), 1.15-1.28 (m, 7H, alanine CH_3, 2 x ε-aminocaproic acid CH_2), 1.33-1.52 (m, 8H, 4 x ε-aminocaproic acid CH_2), 1.95-2.06 (m, 5H, 2 x ε-aminocaproic acid CH_2, valine CH), 2.99-3.07 (m, 17H, 2 x ε-aminocaproic acid CH_2, CD H1-H6), 2.99-3.07 (m, 33H, valine CH, glycine CH_2, CD H1-H6, including H_2O peak), 4.24 (t, J = 7.2 Hz, 1H, alanine CH), 4.42-4.57 (m, 5H, CD OH6), 4.78-4.95 (m, 7H, CD H1), 5.60-5.95 (m, 14H, CD OH2, OH3), 6.62-6.72 (br s, 2H, terminal NH_2), 7.15-7.27 (br s, 2H, terminal NH_2),7.69-7.92 (m, 3H, ε-aminocaproic acid NH, CD NH), 8.10 (d, J = 7.4 Hz, 1H, alanine NH), 8.55-8.62 (m, 1H, glycine NH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 18.0 (valine CH_3), 18.11 (valine CH_3), 18.15 (valine CH_3), 18.7 (alanine CH_3), 18.83 (alanine CH_3), 18.86 (alanine CH_3), 25.2 (ε-aminocaproic acid CH_2), 26.5 (ε-aminocaproic acid CH_2), 26.60 (ε-aminocaproic acid CH_2), 26.62 (ε-aminocaproic acid CH_2), 27.3 (ε-aminocaproic acid CH_2), 27.4 (ε-aminocaproic acid CH_2), 28.41 (ε-aminocaproic acid CH_2), 28.46 (ε-aminocaproic acid CH_2), 29.3 (ε-aminocaproic acid CH_2), 29.50 (ε-aminocaproic acid CH_2), 35.51 (ε-aminocaproic acid CH_2), 35.57 (ε-aminocaproic acid CH_2), 38.7-39.0 (succinyl CH_2), 42.2 (glycine CH_2), 48.6 (alanine CH), 57.93 (valine CH), 57.97 (valine CH), 60.2-60.5 (CD C6), 72.3-72.9 (CD), 73.3-73.6 (CD), 81.5 (CD), 81.8 (CD), 82.0 (CD), 82.1 (CD), 83.90 (CD), 83.94 (CD), 84.0 (CD), 102.1-102.5 (CD C1)

4.7.7.3 Mono-6^A-amino-6^A-mono-6^A-ε-aminocaproic acid-6^X-succinyl-6^X-ε-aminocaproic acid-6^X-Val-Gly-Ala-β-cyclodextrin (**19c**)

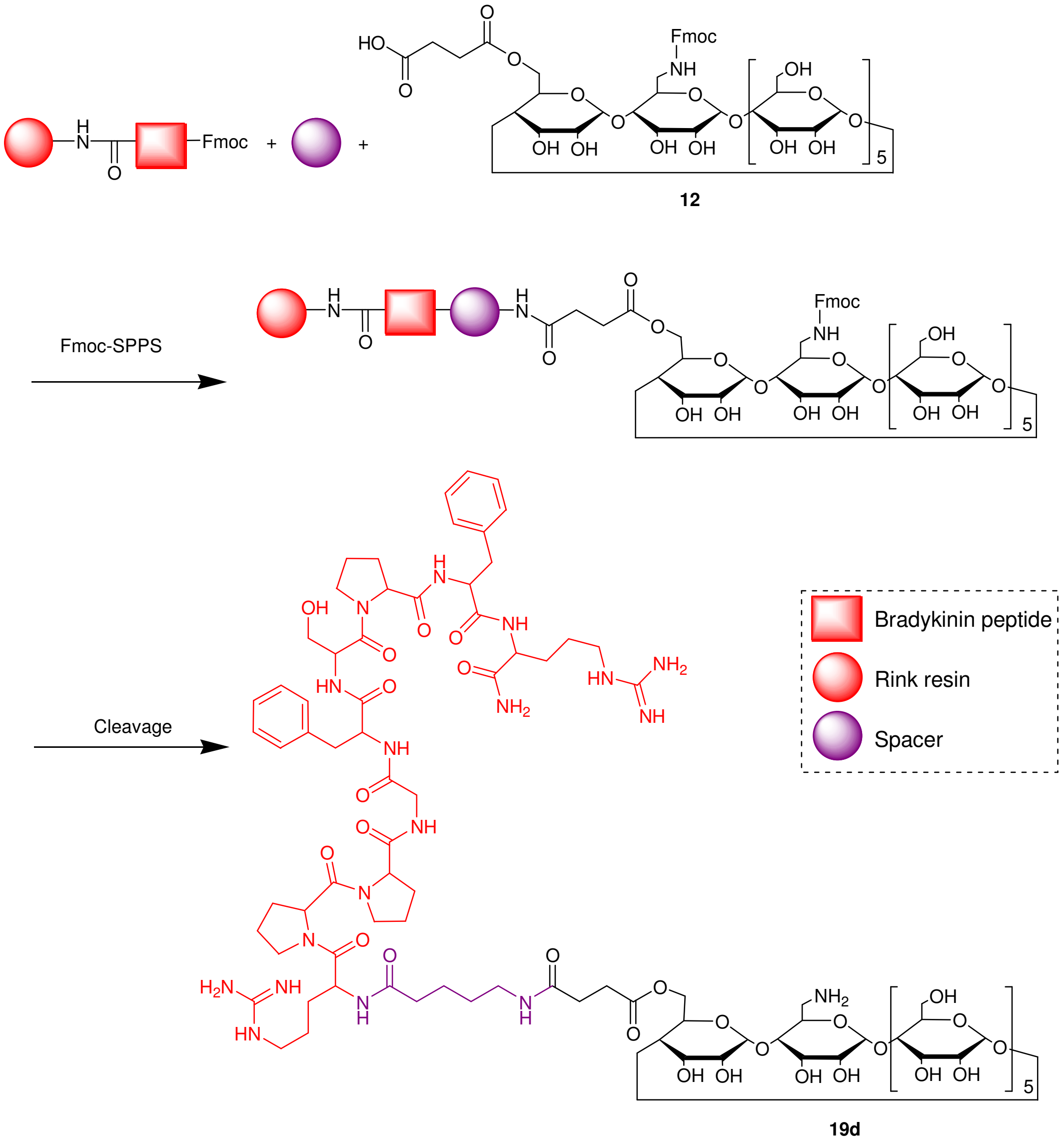


Compound **19c** was manually assembled on Rink resin as per **19a**, **Section 4.7.7.1**. Purification as per compound **19a**. The clean product eluted at 17.2% B after two RP-HPLC purifications using the same method as per **19a** with a yield of 10.8 mg (7%).

LR-MS: *m/z* (%, assignment) 1595.81 (100, M+Na)$^+$ HR-MS: *m/z* $C_{62}H_{105}N_6O_{40}$ (M+H)$^+$ 1573.6389, actual 1573.6361; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.89-0.98 (m, 6H, valine CH_3), 1.14-1.27 (m, 5H, ε-aminocaproic acid CH_2, alanine CH_3), 1.29-1.40 (m, 2H, ε-aminocaproic acid CH_2), 1.41-1.53 (d, J = 7.37 Hz, 2H, ε-aminocaproic acid

CH_2), 1.98-2.07 (m, 3H, ε-aminocaproic acid CH_2, valine CH), 2.30-2.37 (m, 4H, succinyl CH_2), 2.96-3.06 (m, 2H, ε-aminocaproic acid CH_2), 3.14-3.93 (m, 268H, CD, valine CH, glycine CH_2, including DMSO and H_2O peaks), 4.36-4.34 (m, 1H, alanine CH), 4.49-4.71 (m, 5H, CD OH6), 4.79-4.95 (m, 7H, CD H1), 5.68-5.94 (m, 14H, OH2, OH3), 6.61 (s, 2H, terminal NH_2), 7.57-7.62 (m, 1H, valine NH), 7.79-7.91 (m, 2H, CD NH, ε-aminocaproic acid NH), 8.13-8.21 (m, 1H, alanine NH), 8.53-8.59 (M, 1H, glycine NH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 25.0 (valine CH_3), 25.2 (valine CH_3), 25.3 (valine CH_3), 26.4 (alanine CH_3), 26.5 (alanine CH_3), 26.6 (alanine CH_3), 27.4 (alanine CH), 28.4 (valine CH), 29.31 (succinyl CH), 29.39 (succinyl CH), 30.2 (alanine CH), 30.3 (ε-aminocaproic acid CH_2), 31.4 (ε-aminocaproic acid CH_2), 35.3 (ε-aminocaproic acid CH_2), 35.51 (ε-aminocaproic acid CH_2), 35.57 (ε-aminocaproic acid CH_2), 38.2 (ε-aminocaproic acid CH_2), 38.86 (ε-aminocaproic acid CH_2), 38.89 (ε-aminocaproic acid CH_2), 39.6 (ε-aminocaproic acid CH_2), 51.7 (glycine CH_2), 59.4-60.8 (CD C6), 72.3-72.9 (CD), 73.1-73.5 (CD), 81.5-82.5 (CD), 101.9-102.4 (CD C1)

4.7.7.4 Mono-6^{A}-amino-6^{A}-mono-6^{X}-succinyl-6^{X}-ε-aminocaproic acid-6^{X}-bradykinin-β-cyclodextrin (**19d**)



Compound **19d** was manually assembled on Rink resin as per **19a**, **Section 4.7.7.1**. Purification as per **19a**. The clean product eluted between 38-42% B after two RP-HPLC purifications using the same method as per **19a** with a yield of 4.5 mg (2%).

LR-MS: *m/z* (%, assignment) 2388.41 (80, M+H)$^{+}$ HR-MS: *m/z* $C_{102}H_{160}N_{18}O_{47}$ (M+2H)$^{+2}$ 1194.5315, actual 1194.5336; ^{1}H NMR (500 MHz, d$_6$-DMSO): δ 1.13-2.21 (m, 33H, ε-aminocaproic acid 3 x CH$_2$**), 2.30-2.40 (m, 2H,**), 2.59-3.18 (m, 21H,

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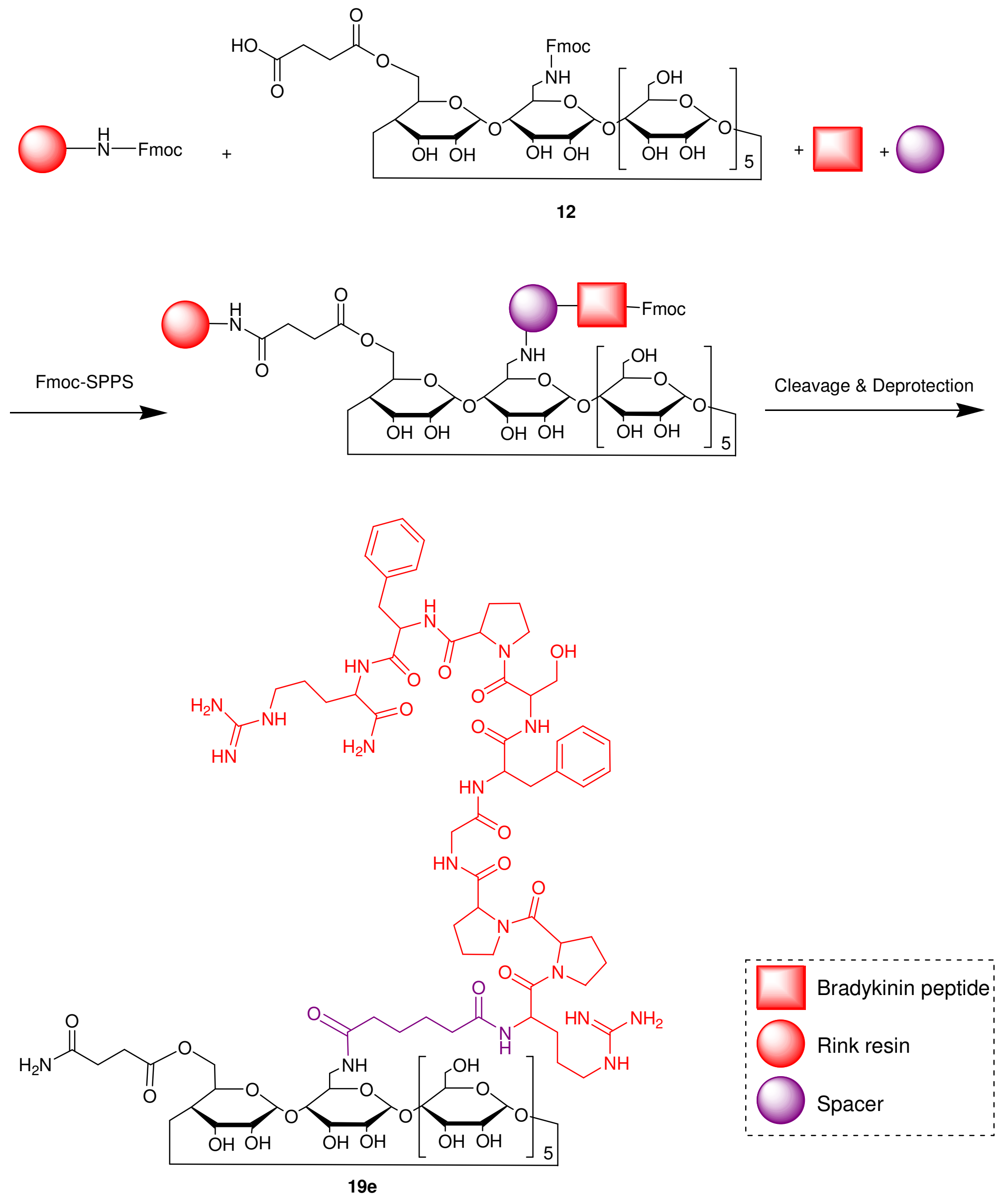
[**] Represents unassigned bradykinin peaks

ε-aminocaproic acid 1 x CH_2, glycine CH_2,[**]), 3.41-3.91 (m, 44H, CD H2-H6,[**]), 4.11-4.32 (m, 6H, glycine CH_2,[**]), 14.39-4.60 (m, 9H, CD OH1,[**]), 4.79-4.97 (m, 7H, CD H1), 5.60-5.87 (m, 14H, CD H2, OH3), 7.06-7.29 (m, 18H, Phe aromatics,[**]), 7.46-7.53 (m, 1H,[**]), 7.83-8.04 (m, 4H, glycine x CH_2,[**]); DEPT ^{13}C NMR (500 MHz, d_6-DMSO): δ 24.1 (ε-aminocaproic acid CH_2), 24.3 (ε-aminocaproic acid CH_2), 24.9 (ε-aminocaproic acid CH_2), 25.0 (BK), 25.2 (BK), 25.6 (ε-aminocaproic acid CH_2), 27.9 (BK), 29.0, (ε-aminocaproic acid CH_2), 29.1 (BK), 29.36 (ε-aminocaproic acid CH_2), 29.42 (BK), 29.6 (ε-aminocaproic acid CH_2), 38.0 (BK), 30.2 (ε-aminocaproic acid CH_2), 34.2 (ε-aminocaproic acid CH_2), 34.4 (ε-aminocaproic acid CH_2), 34.5 (ε-aminocaproic acid CH_2), 35.4 (BK), 36.4 (BK), 39.9 (BK), 40.2 (ε-aminocaproic acid CH_2), 40.9 (BK), 41.0 (BK), 41.1 (BK), 42.3 (BK), 46.9 (BK), 47.2 (BK), 51.9 (BK), 53.1 (BK), 54.5 (BK), 54.8 (BK), 58.0 (BK), 60.1 (BK), 60.3 (BK), 60.4 (CD C6), 60.50 (CD C6), 60.51 (CD C6), 60.54 (CD C6), 62.4 (BK), 72.4-72.9 (CD), 73.3-73.6 (CD), 81.9-82.4 (CD), 102.3-102.6 (CD C1), 126.82 (BK), 126.84 (BK), 128.4 (BK), 128.5 (BK), 129.53 (BK), 129.55 (BK), 129.6 (BK)

[**] Represents unassigned bradykinin peaks

4.7.7.5 Mono-6^A-amino-6^A-ε-aminocaproic acid-6^A-bradykinin-mono-6^X-succinyl-β-cyclodextrin (**19e**)



Compound **19e** was manually assembled on Rink resin using as per **15a**, **Section 4.7.3.1**. RP-HPLC purification as per **15a** with the clean product eluting at 42% B (2.0 mg, 2%) as a white fluffy solid.

LR-MS: *m/z* (%, assignment) 2388.52 (80, M+H)$^+$ HR-MS: *m/z* $C_{102}H_{160}N_{18}O_{47}$ (M+2H)$^{+2}$ 1194.5315, actual 1194.5336

4.8 Conclusion

Overall, this chapter details the synthesis of mono- and di-peptidyl β-CD moieties using Fmoc-SPPS which is a literature first. The peptides added can be the same or different allowing great flexibility with this bi-functional system. This system has great developmental potential for the transportation of drugs and/or other molecules. Overall, more studies are required to look into the coupling chemistries of these compounds, as well as the coupling of other peptides to improve existing (or develop new) strategies. The attachment of sulfonamides to some of these cyclodextrin-peptide complexes and preliminary bioassay analysis are reported in **Chapter 6**.

4.9 References

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Chapter Five:

Synthesis and Purification of Peptide Sulfonamides

5.0 Introduction

Sulfonamide drugs (also known as ‘sulfa drugs’, A in Figure 5-1) and closely related analogues have been extensively used in medicine since the discovery of sulfanilamide (B, Figure 5-1 where R’ and R’’ are hydrogen). Many bioactive sulfanilamide’s (B, Figure 5-1 where R’ is any organic moiety and R’’ is an aliphatic/aromatic group) have evolved since the early 1930’s.

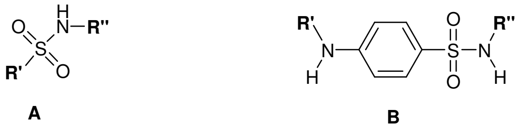


Figure 5-1 *A)* General structure of a bioactive sulfonamide whereby R’ is any organic compound and R’’ can be a hydrogen or an aliphatic/aromatic group *B)* Sulfanilamide where R’ is any organic moiety and R’’ is a hydrogen or an aliphatic/aromatic group

They showed strong antimicrobial activity paving the way for many antibacterial and antibiotic drugs and have since led onto the development of drugs for antitumour, anticarbonic anhydrase, diuretic, hypoglycemia, antithyroid, and protease activity to mention a few (Figure 5-2).[1] Bioactive sulfonamides generally have the structure shown in B (Figure 5-1) whereby R’ is any organic grouping and R’’ can be a hydrogen or an aliphatic/aromatic group.[1-2]

Within the last decade, heterocyclic and aromatic sulfonamides have been discovered that show a wide range of interesting and useful properties (Figure 5-2)[2] including the sulfonamide E7070 which is in advanced clinical trials against cancer growth.[2b-c] E7070 inhibits the cell cycle progression at multiple checkpoints blocking mitosis (see **Appendix 6-V** for details on the cell cycle) and inhibiting tubulin polymerisation. This sulfonamide molecule contains a SO_2NHR moiety giving it a high anti-tumour activity as shown by the carbonic anhydrase (CA) assay (see **Appendix 5-I** for details on the CA assay). It has also been shown to have tumour type selectivity.[2b-c] Sulfonamides can be positioned externally (**A**, exo, Figure 5-2) or internally (**B**, endo, Figure 5-2) providing differing drug bioactivities. Side effects are common with most medicines and sulfa drugs are no exception, and continuing research and studies are ongoing in order to decrease the drugs toxicity and improve their effectiveness.[2c,3]

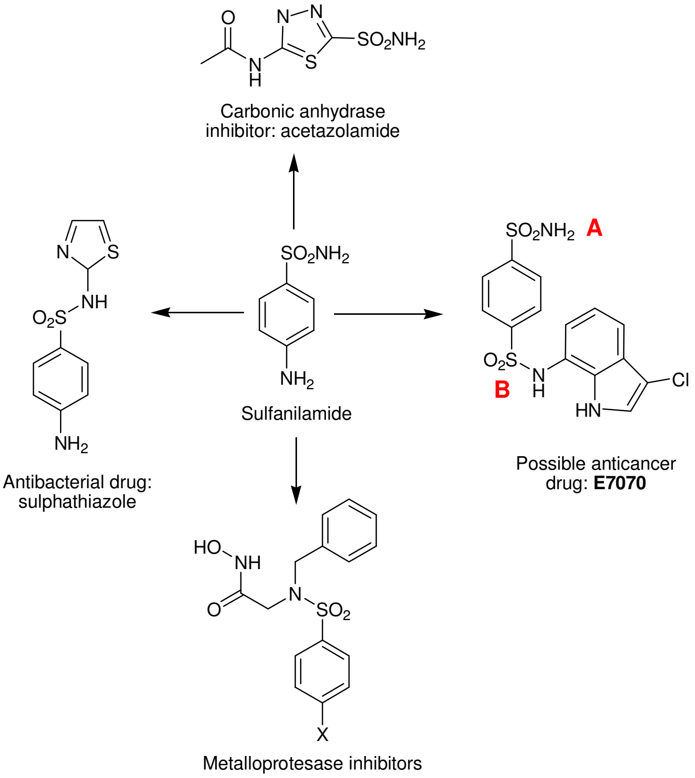


Figure 5-2 Structure of the sulfonamide drugs. E7070 is currently in clinical trials stage II as a potential anti-cancer drug[1,2b-c]

5.0.1 *Chapter Outline*

In this study, two basic sulfonamide moieties were targeted. 4-Carboxybenzenesulfonamide and 4-aminobenzenesulfonamide (Figure 5-3, compounds **30** and **34**) were chosen as model sulfonamides to be attached to the *C*- or *N*-terminus of bioactive peptides (exo-substitution) using SPPS conditions enabling the formation of external *C*- and/or *N*-terminal peptide sulfonamides respectively.

30 **34**

Figure 5-3 Structure of 4-carboxybenzenesulfonamide (**30**) and 4-aminobenzenesulfonamide (**34**)

There has been little reported on the formation of exo peptide sulfonamides using SPPS (see Table 5-1).[2a,4] Preliminary bioassay testing of these compounds should determine if the presence of the peptide would affect the activity of the sulfonamide and vice-versa (see **Chapter 6**).

Table 5-1 A selection of studies for the synthesis of (peptide) sulfonamides (exo-substituted)* synthesised on solid supports

Brief Synthesis Details	*Reference*
Addition of a non-aromatic sulfonyl bromide chain to Rink resin using 4-DMAP and DBU as coupling reagents followed by addition of a heterocyclic group to the resin bound sulfonamide. Resin cleavage using TFA/DCM (66-92%). Exo-sulfonamide generated.	5
Addition of sulfonyl chloride to Rink resin using 1:1 pyridine:DCM followed by addition of organic molecules. A 20% TFA in DCM cleavage generated exo-sulfonamides (73-95% purified yields).	6
A suspension of sulfonyl chlorides in DCM were coupled to modified resins using NMM for 14 h at rt. Resin cleavage using 0.5-5% TFA in DCM (75-83% unpurified yields). No further extension off the resin bound sulfonamide.	7

* Endo-sulfonamide examples can be found in references[2a,8]

By the attachment of a sulfonamide to either end of the BK peptide, it can be anticipated that the peptide might take the masked drug to the tumour cell where it could promote cell death. It is also possible that the attachment of the sulfa drug to the BK peptide will increase the life time of the BK peptide by blocking (or at least hindering) the enzyme-attack site at either end of the molecule.[2c,9] However, it must be acknowledged that BK's activity could also be reduced or lost with this sulfonamide addition. See **Chapter 6** for preliminary bioassay testing results. This part of the study was broken down into five sub-sections, each of which uses different sulfonyl compounds in an attempt to create *C*- and/or *N*-terminal peptide-sulfonamides using Fmoc SPPS chemistries. The five divisions are shown in Figure 5-4. Once a method was established for the synthesis of *C*-and/or *N*-terminal peptide sulfonamides, this method could then be applied and used for the synthesis of CD-peptide-sulfonamides (see **Chapter 6**). The layout of this chapter is detailed in Figure 5-5.

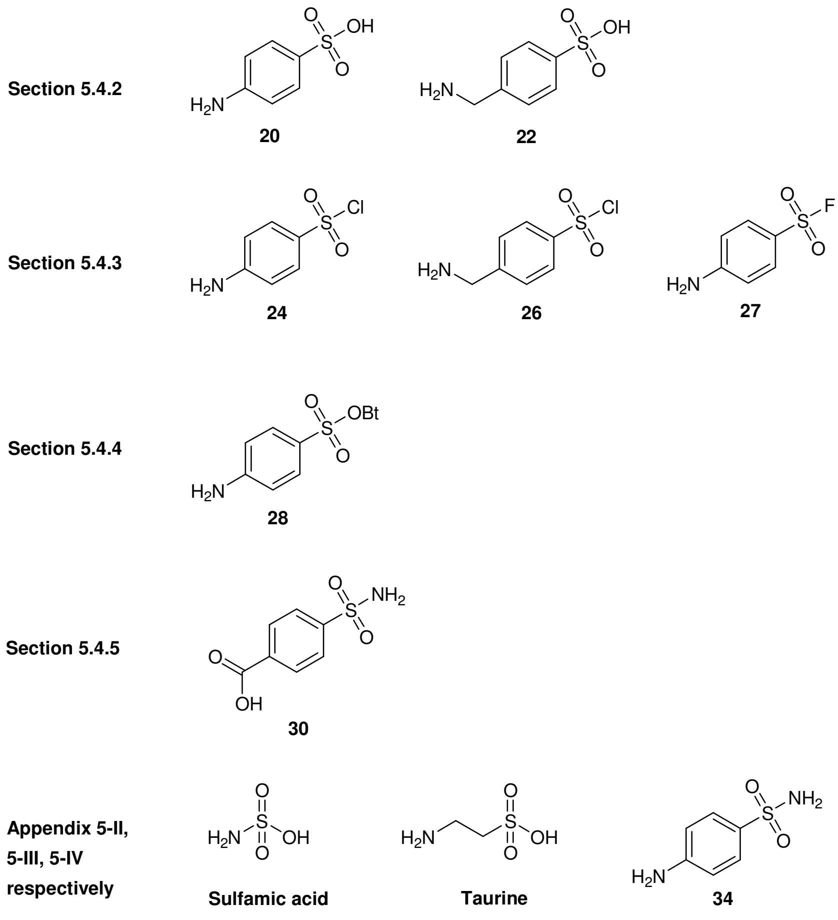


Figure 5-4 Sulfonyl precursors used in this study for the synthesis of *C*- and/or *N*-terminal peptide-sulfonamides. **20** (4-aminobenzene sulfonic acid), **22** (4-aminomethylbenzene sulfonic acid), **24** (4-aminobenzenesulfonyl chloride), **26** (4-aminomethylbenzenesulfonyl chloride), **27** (4-aminobenzenesulfonyl fluoride), **28** (4-aminobenzenesulfonyl benzotriazole), **30** (4-carboxybenzenesulfonamide), sulfamic acid, taurine, and **34** (sulfanilamide)

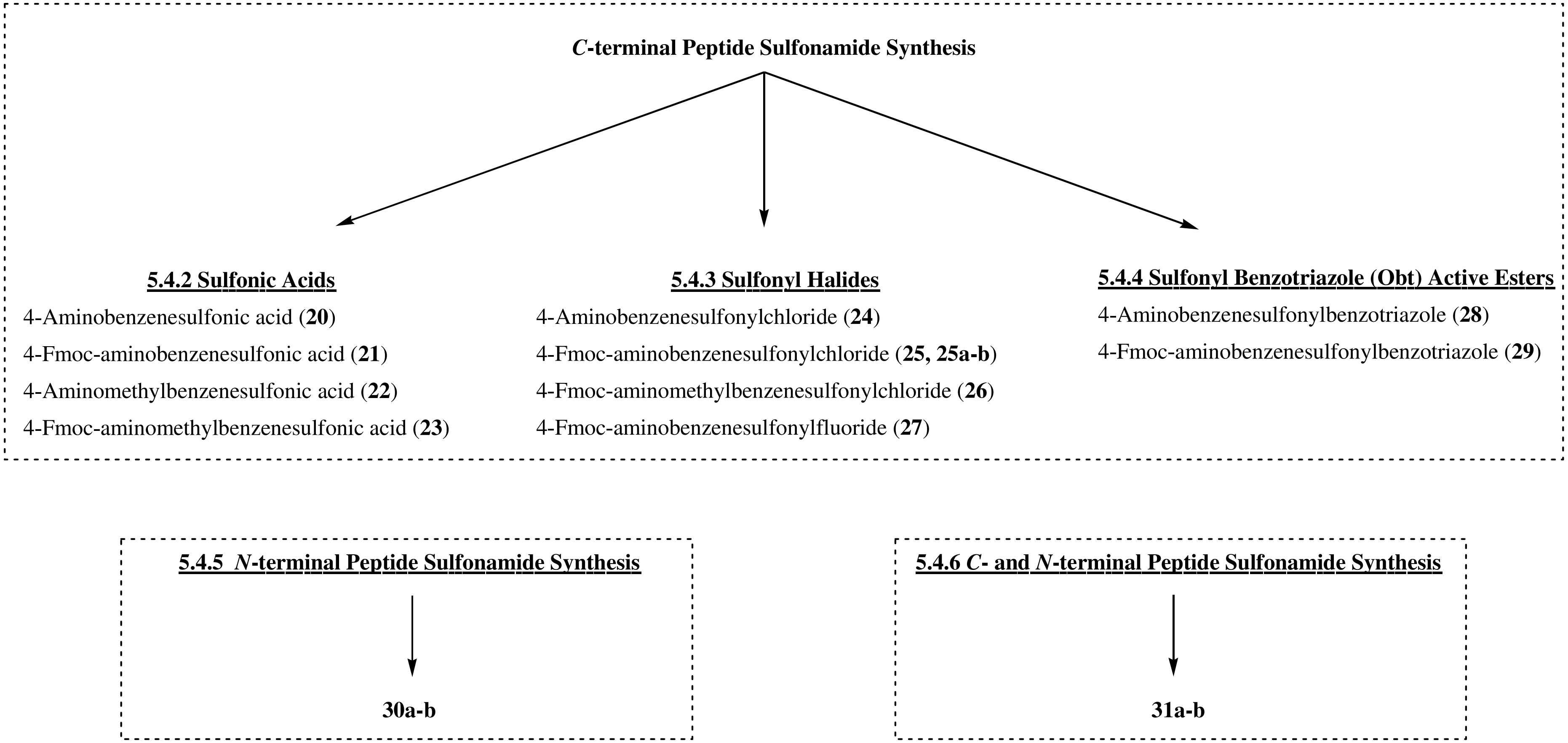


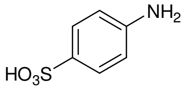
Figure 5-5 Detailed outline for **Chapter 5**. Preliminary studies were investigated for the unprotected sulfonyl compounds **20**, **22**, and **24**

5.1 Results and Discussion for the Synthesis of *C*-terminal Peptide Sulfonamides

5.1.1 *Peptide Synthesis using Sulfonic Acids*

5.1.1.1 Synthesis using 4-Aminobenzene Sulfonic Acid (**20**)

Addition of unprotected **20** (Figure 5-6, standard Fmoc SPPS coupling conditions) to resin for the synthesis of *C*-terminal peptide sulfonamides was first investigated.† Peptide yields of these *C*-terminal peptide sulfonamides were low and resulted, presumably due to polymer formation with this approach confirming that it was necessary to protect the amino group. From here, **20** was Fmoc protected to achieve better yields in peptide synthesis by eliminating the formation of sulfonamide polymers. See **Section 5.1.1.2** for details. This basic anilino sulfonyl compound can later be expanded to more complex molecules.



20

Figure 5-6 4-Aminobenzene sulfonic acid (**20**)

5.1.1.2 Synthesis of 4-Fluorenylmethyloxycarbonylaminobenzene Sulfonic Acid (**21**)

Fmoc protection of **20** (Figure 5-7) as per Liskamp *et al.*[10] was achieved and followed by stability tests. It was shown that there was the loss (as expected) of the Fmoc group upon treatment with a 20% pip in DMF solution. No further breakdown was observed, even with exposure to the TFA cleavage mixture used to cleave the peptide from the resin. See Table 5-2 for more details.

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† For specific details into stability tests, peptide synthesis, and results see **Appendix 5-V**

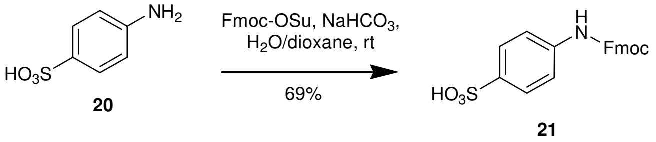

Figure 5-7 4-Aminobenzene sulfonic acid (**21**)

Table 5-2 Stability test[‡] conditions and results for compound **21** when exposed to standard Fmoc SPPS reaction conditions as determined by MALDI analysis

Reaction Conditions	*Reaction Time (Hours)*	*Results*
100% DMF	24	No change
100% DCM	24	
20% Pip in DMF	0.5	Fmoc protection lost
20% Pip in DMF	1.0	
95% TFA cleavage mixture	3.0	No change
100% TFA	24	

Peptide synthesis was then undertaken by addition of the protected sulfonic acid (**21**) to resin using a range of standard coupling conditions. Following deprotection of the Fmoc group, addition of Fmoc-tri-glycine (as per **Chapter 3**, **Section 3.2.2.1**) was attempted. TFA was used to cleave the sulfonyl-peptide from the resin. The yield of the crude product obtained was very low (<1%, Table 5-3).

[‡] Testing the compound against standard Fmoc SPPS reaction conditions to see if any breakdown occurs which would make it unsuitable for use in peptide synthesis

Table 5-3 Coupling conditions for the coupling of **21** to Rink resin for *C*-terminal peptide (tri-glycine) sulfonamide synthesis

Experiment Number	*Coupling Conditions*	*Coupling Time (Hours)*	*Product Yield (%)*
A21r	TBTU (4 equiv)/ DIEA (8 equiv) in DMF/H_2O	24, 24[§]	< 1
B21r	HBTU (4 equiv)/ DIEA (4 equiv) in H_2O	24	< 1
C21r	EDC (4 equiv)/ DIEA (4 equiv) in DMF/H_2O	24	Nothing
D21r	TBTU (4 equiv)/ TEA (8 equiv) in DMF/H_2O	24	< 1
E21r	TBTU (4 equiv)/ TEA (8 equiv) in DMF	24	< 1
F21r	HBTU (4 equiv)/ DIEA (8 equiv) in DMSO	24	< 1

[§] Recoupled for 24h

Fmoc-UV coupling tests[¶] determined that the reason for the low yields of the sulfonic acid was due to a low coupling to the resin (between 4-6% efficiency depending upon the reaction conditions used, see Table 5-4).

Table 5-4 Coupling efficiency and peptide yields for compound **21** where the peptide is Fmoc-tri-glycine (see Table 5-3 for reaction conditions).

Experiment Number	*Coupling Efficiency (%)*	*Product Yield (%)*
A21r	6.38	< 1
B21r	4.65	< 1
C21r	4.95	Nothing

These results show that the addition of the sulfonic acids, **20** and **21** (unprotected and protected) to Rink resin under the conditions attempted showed little promise for the synthesis of *C*-terminal peptide sulfonamides. The pKa for the α-amino groups on

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[¶] See **Chapter 2**, **Section 2.4.4.4** for more details amino acids ranges from 8.80 to 10.60, i.e. basic with reasonable expectations in many cases for being good nucleophiles. However, the pKa for the amino group of 4-aminobenzene sulfonic acid is 4.65 which is far less nucleophilic. These couplings were expected to be poor but were attempted for completeness of this study. It was then proposed that the formation of an aliphatic amino sulfonyl compound with the addition of a small -CH_2- spacer between the amino and the aromatic group of **20** may aid in the coupling of amino acids. Aromatic amines are generally much weaker bases than aliphatic amines. This can be seen when comparing the pKa values for benzylamine (aliphatic amine, 9.33) and aniline (aromatic amine, 4.63). These values indicate that benzylamino functionality should be the better nucleophile and lead to better couplings with amino acids. The next sections (**5.1.1.3** and **5.1.1.4**) detail the synthesis of compounds **22** and **23** respectively, containing this -CH_2- spacer group. Peptide synthesis using compound **23** is also investigated.

5.1.1.3 Synthesis of 4-Aminomethylbenzene Sulfonic Acid (**22**)

4-Aminomethylbenzene sulfonic acid (**22**, Figure 5-8) was synthesised according to known procedures[11] yielding an off white coloured solid (40%).

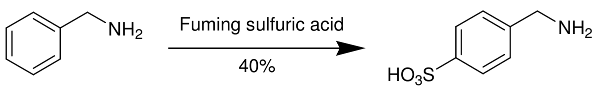

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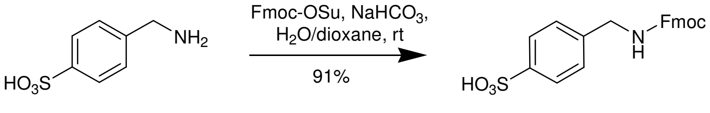
Figure 5-8 Synthesis of 4-aminomethylbenzene sulfonic acid (**22**)

5.1.1.4 Synthesis of 4-Fluorenylmethyloxycarbonylaminomethylbenzene sulfonic acid (**23**)

4-Fluorenylmethyloxycarbonylaminomethylbenzene sulfonic acid (**23**) was synthesised as per **Section 5.1.1.2**, starting from compound **22** (3.21 mmol, 1 equiv) yielding a white coloured solid (91%, Figure 5-9).



22 **23**

Figure 5-9 4-Fluorenylmethyloxycarbonylaminomethylbenzene sulfonic acid (**23**)

Coupling tests were performed on compound **23** to assess its success rate compared to the aromatic amine **21** (**Section 5.1.1.2**). The reaction conditions and results are shown in Tables 5-5 and 5-6 respectively.

Table 5-5 Fmoc-coupling data for the coupling of **23** to Rink resin overnight

Experiment Number	*Coupling Conditions*
A23r	TBTU (4 equiv)/DIEA (8 equiv) in DMF
B23r	HOBt (4 equiv)/DIEA (8 equiv) in DMF
C23r	TBTU (3 equiv)/ HOBt (3 equiv)/ DIEA (3 equiv) in DMF

Table 5-6 Coupling efficiency and peptide (Fmoc-tri-glycine) yield results for the coupling of **23** to Rink resin

Experiment Number	*Coupling Efficiency (%)*	*Product Yield (%)*
A23r	6.1	< 1
B23r	0.4	< 1
C23r	58	< 2

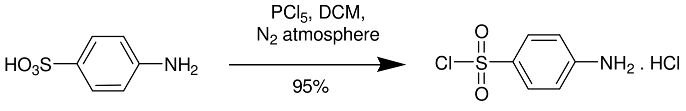
It was found that the coupling efficiency was greatly improved when coupling the benzylamino sulfonyl compound **23** (*Experiment C23r*, Tables 5-5 and 5-6, 58%) compared to the aromatic sulfonyl amino compound **21** (*Experiment A21r*, Table 5-4, 6%) with TBTU (3 equiv), HOBt (3 equiv) and DIEA (3 equiv) in DMF overnight (*Experiment C23r*, Table 5-6). The yields of the peptide-sulfonamides produced (when coupling with varying reaction conditions stated in Table 5-6) using **23** as a starting material were still very low with little difference in peptide yields between compounds **23** and **21** (Table 5-3). This suggests perhaps cleavage or purification problems with retention of the product on the resin or column respectively. Purification problems with the product sticking to the RP-HPLC column can be ruled out as the crude yield obtained from the cleaved product was low, before purification was attempted. A TMSBr cleavage was then trialled to see if any product remained attached to the resin (see **Appendix 5-VI** for details). Nothing further was cleaved indicating that the first cleavage had been complete. A milder set of cleavages was also performed (1-5% TFA in DCM for 3h) to ensure the product was not breaking down during the cleavage with similar yield results (trace-1%). Therefore, any yield problems are associated with the coupling of the components to the resin. Next, the sulfonyl chloride for compounds **20-21** and **23** were synthesised and their coupling ability to resin tested. See **Section 5.1.2** for details.

5.1.2 Peptide Synthesis using Sulfonyl Halides

Amino acid chlorides have been known since the early 1900s but have rarely been used in peptide synthesis until recently when they have been applied to Fmoc-couplings.[12] However, because of their high reactivity and sensitivity to hydrolysis, acid chlorides should be prepared from the parent amino acids immediately before use and a large excess of reactant is typically required for their synthesis.[12] Acid fluorides on the other hand are known to be more stable to hydrolysis than acid chlorides but they have been rarely used in organic synthesis because of their presumed low reactivity towards common nucleophiles (benzenesulfonyl fluoride is *c.a.* 5-times less reactive than the corresponding chloride towards acidic water and hydrolytic solvents). These results can be explained in terms of the strong S-F bond and the strong electron withdrawal capabilities of the fluorine.[12c] Even so, it was shown that fluorides such as indole 2-carbonyl fluoride have been shown to react readily with aliphatic and aromatic amines.[12b] Amino acid fluoride couplings have been successful in solution and solid phase reactions, both methods without appreciable racemisation and normally rapid reactions. Therefore, both sulfonyl chlorides (**Sections 5.1.2.1-3**) and sulfonyl fluorides (**Section 5.1.2.4**) have been synthesised and their resin coupling efficiency studied. It was found that the sulfonyl chlorides achieved better resin coupling yields than the sulfonyl fluorides.

5.1.2.1 Synthesis of 4-Aminobenzenesulfonyl Chloride Hydrochloride (**24**)

Compound **24** (Figure 5-10) was synthesised from adapted procedures by Liskamp *et al.*[10] yielding a fluffy white coloured solid (95%).



20

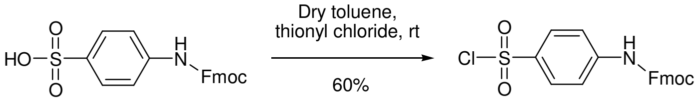
24

Figure 5-10 Synthesis of 4-aminobenzenesulfonyl chloride hydrochloride (**24**)

Peptide synthesis using the **24** (unprotected, coupling with a 1:1 DCM:pyridine slurry) generated a 4% yield of the expected sulfonamide-peptide (refer to **Section 5.4.3.1**) whereby the peptide coupled is Fmoc-hexaglycine. It was interesting to note the small product increase from the sulfonic acid **20** (trace, **Appendix 5-V**) to **24** (4%) perhaps indicating that Fmoc-protection is not necessary for such a reactive species, but to better the yields, it is important. Polymer formation may still dominate in this reaction. Next, Fmoc protection of **24** was carried out to see if yields could be improved. See **Section 5.1.2.2** for details.

5.1.2.2 Synthesis of 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl Chloride (**25**)

The synthesis of **25** (Figure 5-11) was achieved by converting **21** (Fmoc protected sulfonic acid) into the Fmoc-protected sulfonyl chloride under dry conditions using excess thionyl chloride as per reference 10. Product identification by NMR was achieved (see experimental section). Recrystallisation from benzene/hexane gave a white coloured solid (60%).



21

25

Figure 5-11 Synthesis of compound **25**

To further confirm the validity of compound **25**, the sample was dissolved up in DMSO and allowed to react with benzylamine in the presence of base (TEA) overnight. MALDI results showed the presence of the $(M+2H)^{2+}$ peak for compound **25i**, Figure 5-12). A small loss of the Fmoc group was also observed (as expected). It is also noted that the solubility of the chloride species (**25**, soluble in DMF) was notably different from the acid starting material (**21**, only sparingly soluble in DMSO) indicating the reaction had occurred (see NMR data, **Section 5.4.3.2**).

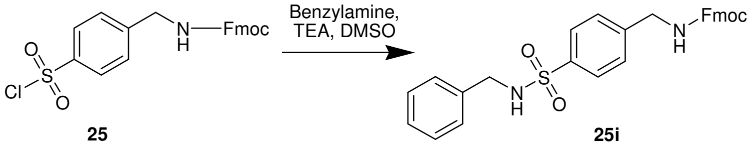


Figure 5-12 Scheme for the reaction of compound **25** with benzylamine

Stability tests performed on **25** showed it to be stable under standard Fmoc reaction conditions (see Table 5-7) with the loss (as expected) of the Fmoc group under piperidine treatment.

Table 5-7 Stability test conditions and results (as determined by MALDI analysis) for compound **25** when exposed to standard Fmoc SPPS reaction conditions

Reaction Conditions	*Coupling Time (Hours)*	*Results*
100% DMF	24	No change
100% DCM	24	
20% Pip in DMF	0.5	Fmoc protection lost
20% Pip in DMF	1.0	
95% TFA cleavage mixture\\	3.0	No change
100% TFA	24	

\\ See **Appendix 3-III** for details

Fmoc-UV coupling tests (Table 5-8) were also performed and showed that compound **25** had a maximum coupling efficiency of 40% when coupling to Rink resin with a 1:1 pyridine:DCM slurry (*Experiment C25r*). These reaction conditions (*Experiment C25r*, Table 5-8) were used for the synthesis of a number of *C*-terminal peptide sulfonamides (Table 5-9).

The model peptides (Ala-Phe-Gly and Pro-Phe-Arg) and the bioactive BK peptide were used to test the reaction conditions. It was found that all of these peptides gave useable amounts when utilised in the synthesis of *C*-terminal peptide sulfonamides, greater than for any other sulfonyl group tested with a 5% pure peptide yield for the BK peptide and 2-9% pure yields for the model peptides (see Table 5-9 for more details).

These reaction conditions were implemented throughout the rest of this study where *C*-terminal peptide sulfonamides are required and include the synthesis of compounds **25a** (Figure 5-13) and **25b** (Figure 5-13) which have been used in bioactive testing (**Chapter 6**).

Table 5-8 Experimental details and results for the coupling of **25** (4 equiv, 24h coupling time) to Rink resin

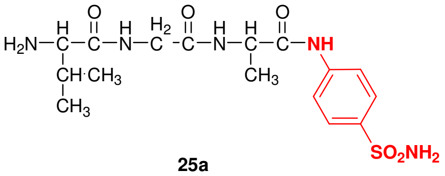
Experiment Number	*Coupling Reagents*	*Coupling Efficiency (%)*
A25r	TBTU (4 equiv)/DIEA (8 equiv)	18
B25r	HOBt (4 equiv)/DIEA (8 equiv)	19
C25r	**1:1 Pyridine:DCM slurry**	**40**
D25r	1:1 Pyridine:DMF	18
E25r	1:1 Pyridine:THF	36
F25r	1:1 Pyridine:H_2O	7
G25r	1:1 Pyridine:DMSO	21
H25r	100% Pyridine	30

Table 5-9 Peptide synthesis details and results for the coupling of **25** to Rink resin

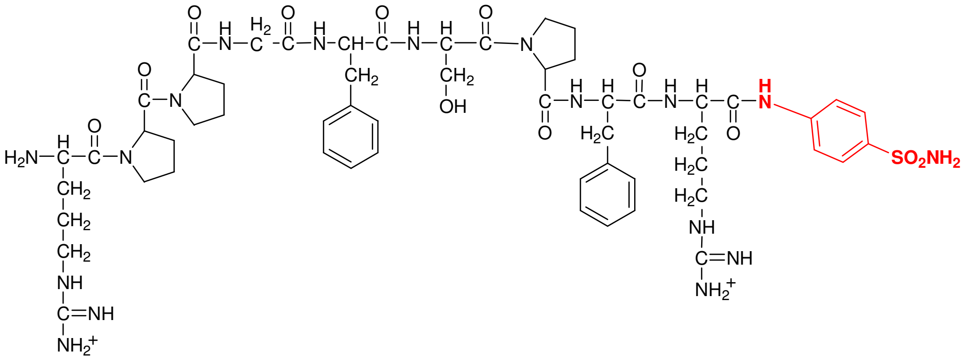
Experiment Number	*Peptide*[**]	*Coupling Details for 25*[§§]	*Product Yield*	
			mg	*%*
A-25	Pro-Phe-Arg[††]	4 equiv, 24h	4.0,	7
B-25	Pro-Phe-Arg[††]	4 equiv, 24h plus 1 recouple	4.2	8
C-25	Pro-Phe-Arg[††]	8 equiv, 24h	4.1	7
25a	Val-Gly-Ala[‡‡]	4 equiv, 24h	1.3	2
D-25	Val-Gly-Ala[‡‡]	4 equiv, 24h	8.3	9
E-25	Val-Gly-Ala[‡‡]	4 equiv, 24h plus 1 recouple	9.1	9
25b	Bradykinin	4 equiv, 24h	1.4	5

[**] Coupled with TBTU (4 equiv) and DIEA (8 equiv) in DMF overnight [††] BK model peptide

[‡‡] Basic model peptide [§§] Coupled with a 1:1 slurry of DCM:pyridine



25a

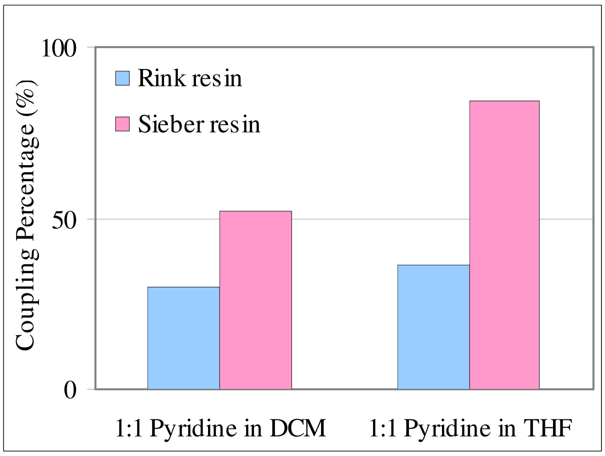


25b

Figure 5-13 Compounds **25a** and **25b**. Experimental details found in **Section 5.4.3.3**

Also investigated was the use of Sieber resin for the synthesis of *C*-terminal peptide sulfonamides using compound **25**. It was found that by coupling this sulfonyl chloride to Sieber resin with a 1:1 pyridine:THF slurry the efficiency almost doubled (Graph 5-1). This could be associated with the milder nature of cleavage used to remove the peptide-sulfonamide from Sieber resin (less harsh reaction conditions).[18] It could also be associated with a better solvation/solubility of Sieber resin and compound **25** compared to Rink resin under the same reaction conditions. Unfortunately these results with the Sieber resin were discovered very late in this study. However, they point to one way of extending this study beyond proof-of-concept to higher yields.

Graph 5-1 Comparison of the coupling efficiencies for compound **25** for its coupling to Rink and Sieber resin under variable reaction conditions



5.1.2.3 Synthesis of 4-Fluorenylmethyloxycarbonylaminomethylbenzene Sulfonyl Chloride (**26**)

As per **Sections 5.1.1.3** and **5.1.1.4**, a methylene group was introduced into compound **25** producing amino **26** (4-fluorenylmethyloxycarbonylaminomethylbenzenesulfonyl chloride). The Fmoc free compound was expected to have reasonable nucleophilicity for SPPS coupling. The synthesis of **26** (Figure 5-14) was as per the synthesis of compound **25** (**Section 5.1.2.2**) creating a product which is sparingly soluble in DMSO. To confirm the existence of the expected product, the same method as per compound **25** was performed using benzylamine (see **Section 5.1.2.2**). MALDI results showed presence of the $(M+2H)^{2+}$ peak for **26i** with a small loss (as expected) of the Fmoc group. It can be concluded from these results and NMR data that the product was synthesised successfully (60%).

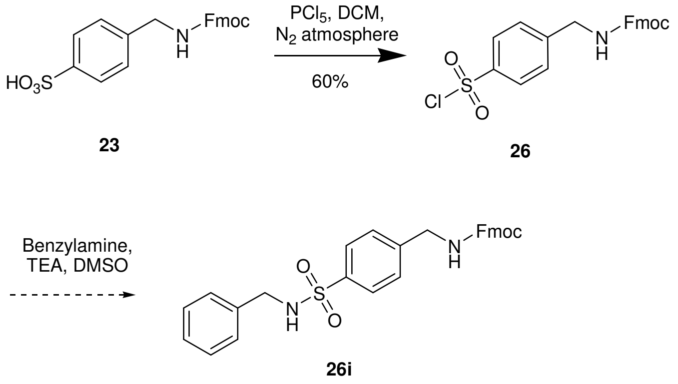


Figure 5-14 Synthesis of 4-fluorenylmethyloxycarbonylaminomethylbenzenesulfonyl chloride (**26**)

Addition of the single methylene group between the amino group and the benzene ring generating an aliphatic sulfonyl compound (**23** and **26**) ensured increased coupling of the amino acids to the sulfonyl group (now attached to resin). These results can then be compared to their anilino aromatic counterparts (**21** and **25**). Coupling studies and peptide synthesis using **26** was undertaken using the coupling conditions stated in Table 5-10 with results shown in Table 5-11.

Table 5-10 Fmoc-coupling experimental data for the coupling of **26** (4 equiv, 24h coupling) to Rink resin

Experiment Number	*Coupling Conditions*
A26r	TBTU (4 equiv)/DIEA (8 equiv) in DMF
B26r	HOBt (4 equiv)/DIEA (8 equiv) in DMF
C26r	1:1 Pyridine:DCM slurry
D26r	1:1 Pyridine:DMF
E26r	1:1 Pyridine:THF
F26r	1:1 Pyridine:H_2O
G26r	1:1 Pyridine:DMSO
H26r	100% Pyridine
I26r	TBTU (3 equiv)/HOBt (3 equiv)/DIEA (3 equiv) in DMF

Table 5-11 Coupling efficiency and peptide cleavage results for the coupling of **26** to Rink resin where the peptide is Fmoc-Val -Gly-Ala

Experiment Number	*Coupling Efficiency (%)*	*Peptide Yield (mg, %)*
A26r	5	0.8, < 1
B26r	4.2	Trace crude product
C26r	< 1	Nothing
D26r	< 1	Nothing
E26r	< 1	Nothing
F26r	< 1	Trace crude product
G26r	< 1	Nothing
H26r	< 1	Nothing
I26r	52	1.5, 3

Coupling efficiencies for compounds **25** (*Experiment* C25r, Table 5-8, 40%, coupling with 1:1 pyridine:DCM) and **26** [*Experiment I26r*, Table 5-11, 52%, coupling with TBTU (3 equiv)/HOBt (3 equiv)/DIEA (3 equiv) in DMF] indicated that compound **26** was more successful at coupling to Rink resin under these reaction conditions. However, in general, resin coupling followed by peptide synthesis generated lower yielding products (best purified peptide yield for **26** is 3% where the peptide is Val-Gly-Ala, *Experiment I26r*, Table 5-11) compared to compound **25** (2-9%, Table 5-9 where the peptide is either a model or BK). Cleavage and purification problems were ruled out as clevage using either TFA or TMSBr gave comparable yields (with variable strengths and reaction times). Next, sulfonyl fluorides were investigated to see if yields could be improved. See **Section 5.1.2.4** for details.

5.1.2.4 Synthesis of Tetramethyl Chloro Formamidinium Hexafluorophosphate (**TCFH**)

Amino acid fluorides are better at coupling in peptide synthesis due to the slower rates of racemisation, fast couplings, being less prone to hydrolysis, and therefore should provide better peptide yields than that of the amino acid chlorides (investigated in the previous sections).[14-16] In this study, tetramethyl fluoro formamidinium hexafluorophosphate (**TFFH**) was used to convert the protected sulfonic acid to the fluoride to see if peptide synthesis was more successful than that of previous precursors (sulfonyl acids and chlorides, see previous sections). The synthesis of **TFFH** is performed in two steps converting tetramethyl urea (**TMU**) and oxalyl chloride (**OC**) into tetramethyl chloro formamidinium hexafluorophosphate (**TCFH**) according to procedures by Fürstner *et al.*,[13] affording a light green foamy solid (**TCFH**, 40 %, Figure 5-15).

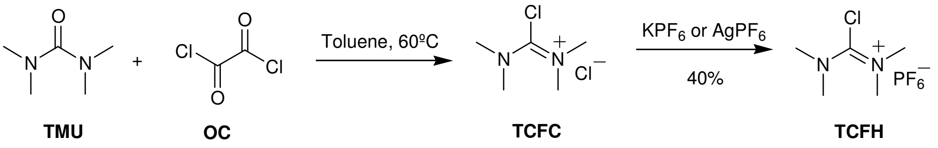


Figure 5-15 Synthesis of tetramethyl chloro formamidinium hexafluorophosphate (**TCFH**)

Following a salt exchange (Figure 5-15), **TFFH** was synthesised from **TCFH** according to adapted procedures by Carpino *et al.*[14] and Christensen *et al.*[15] to afford a white crystalline solid (**TFFH**, 40%, Figure 5-16). The samples are stored under a nitrogen atmosphere at 4ºC ready for use.

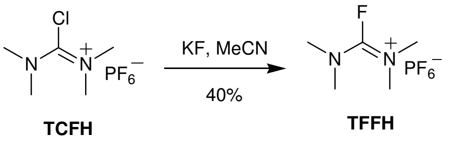


Figure 5-16 Synthesis of tetramethyl fluoro formamidinium hexafluorophosphate (**TFFH**)

From here, **TFFH** was used for the synthesis of sulfonyl amino acid fluorides (**27**, Figure 5-17).

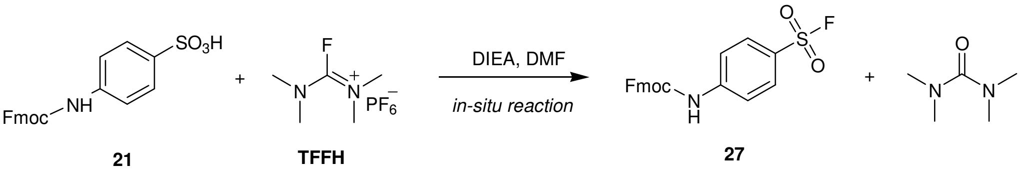


Figure 5-17 Synthesis of 4-fluorenylmethyloxycarbonylaminobenzenesulfonyl fluoride (**27**)

Compound **27** (Figure 5-17) was synthesised by an *in situ* reaction using **TFFH** (see **Section 5.4.3.7** for experimental details) followed by peptide synthesis forming a low yielding *C*-terminal peptide sulfonamide product. The ninhydrin test showed that the coupling of the sulfonyl fluoride to the resin was successful, as did an Fmoc-UV coupling test (13% coupling efficiency, Table 5-12).

Table 5-12 Experimental details and results for the coupling of **27** to Rink resin

Experiment Number	*Coupling Conditions*	*Coupling Time (hours)*	*Coupling Efficiency (%)*
A27r	*In situ* formation and coupling	24	13

However, synthesis of a *C*-terminal peptide using the Fmoc protected amino acid fluoride (**27**) was less successful than the Fmoc protected sulfonyl chloride (**25**, 7-40% coupling efficiencies, Table 5-8) despite the fact that it is known that amino acid fluorides are generally easier and faster to couple and have less chance of racemisation.[16] Next, the sulfonyl benzotriazole (OBt) active ester of **24** was synthesised, Fmoc protected, and used for peptide synthesis. The results are detailed in **Section 5.1.3**.

5.1.3 *Peptide Synthesis using Sulfonyl Active Esters (OBt)*

5.1.3.1 Synthesis of 4-Aminobenzenesulfonyl Benzotriazole (**28**)

Sulfonyl active ester (benzotriazole, OBt) coupling to the resin was attempted to see if any greater success could be obtained. However, these produced lower yielding peptide sulfonamides than the sulfonyl chlorides (**24-25**), and hence were not studied any further. Details into their synthesis are given below.

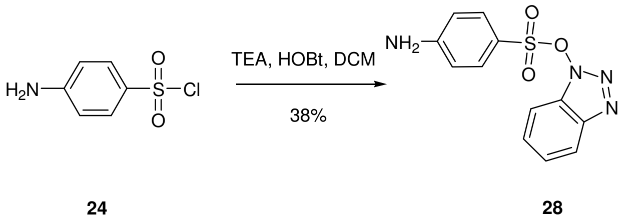


Figure 5-18 Synthesis of 4-aminobenzenesulfonyl benzotriazole (**28**)

Compound **29** (Figure 5-19) was successfully synthesised using Fmoc-OSu (1 equiv, 0.34 mmol) $NaHCO_3$ (1 equiv, 0.34 mmol) and **28** (0.34 mmol, 1 equiv) yielding a white solid (57%).

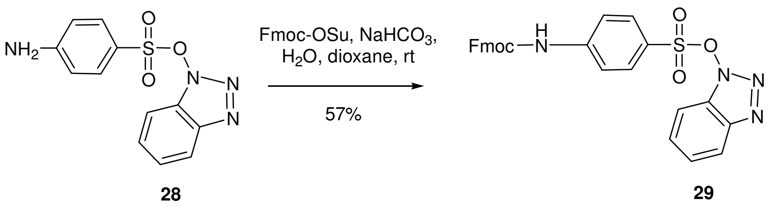


Figure 5-19 Synthesis of 4-fluorenylmethyloxycarbonylaminobenzenesulfonyl benzotriazole (**29**)

The coupling efficiency of **29** to Rink resin was reflected in the low product yields with *c.a.* 1% pure peptide sulfonamide obtained (Table 5-13). Addition of the preformed sulfonyl activated ester (compound **29**) to resin generated a slightly better yielding peptide-sulfonamide (1%) than formation of the active ester *in situ* (of compound **21**, <1%, see Table 5-3). This could be due to the different reaction conditions used for the synthesis of the isolated active ester, and for future synthesises, trying these conditions *in situ* should be investigated. The peptide used in this part of the study was Val-Gly-Ala.

Table 5-13 Peptide synthesis details and results for the coupling of **29** to Rink resin where the peptide is Val-Gly-Ala

Experiment Number	*Peptide*	*Coupling Details for **29***	*Product Yield*	
			mg	*%*
A29r	Ala-Gly-Phe	4 equiv, 24h	0.7	1
B29r	Ala-Gly-Phe	4 equiv, 24h plus recouple	0.8	1
C29r	Pro-Phe-Arg	4 equiv, 24h	0.5	< 1

5.1.4 *Section summary*

An overview of the study is presented in Table 5-14.

From these results, *C*-terminal peptide synthesis for the rest of this study was undertaken using the following reaction conditions:

i. Resin: Rink resin was used for all *C*-terminal peptide sulfonamide synthesis

ii. Compound **25** was used as the sulfonyl species added to resin for the synthesis of *C*-terminal peptide sulfonamides in the remainder of this study

iii. Coupling conditions for compound **25**: A 1:1 pyridine in THF mixture with overnight coupling were used. The ninhydrin test was used to determine if the coupling was successful.

Further unsuccessful attempts (**Appendix 5-II** to **5-IV**) included the use of taurine and sulfamic acid for the preparation of external non-aromatic *C*-terminal peptide sulfonamides.

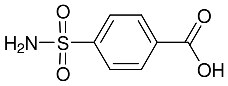
Table 5-14 Overview for the C-terminal sulfonyl compounds synthesised in the first part of **Chapter 5**

Sulfonyl Molecule	*Discussion Section*	*Best Coupling Efficiency (%)*	*Optimum Coupling Conditions*	*Peptide Yields (%)*
20	5.1.1.1	N/A	HBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight	< 1
21	5.1.1.2	6.4[¶¶]	See Table 5-4 for details	< 1
22	5.1.1.3	-	-	-
23	5.1.1.4	58[¶¶]	TBTU (3 equiv), HOBt (3 equiv), DIEA (3 equiv) in DMF overnight	< 2
24	5.1.2.1	N/A	1:1 pyridine:DCM slurry coupled overnight	4
25	5.1.2.2	40[¶¶]	1:1 pyridine:DCM slurry coupled overnight	1-9 See Table 5-9 for details
		84[\\\\]	1:1 pyridine:THF slurry coupled overnight	N/A
26	5.1.2.3	52[¶¶]	TBTU (3 equiv), HOBt (3 equiv), DIEA (3 equiv) in DMF overnight	3
27	5.1.2.4	13[¶¶]	*In situ* formation and coupling overnight	Trace
28	5.1.3.1	-	-	-
29	5.1.3.1	N/A	HBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight	1

[¶¶] Rink resin

[\\\] Sieber resin

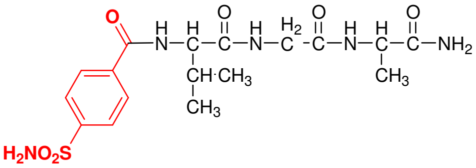
5.2 Results and Discussion for the Synthesis of *N*-terminal Peptide Sulfonamides



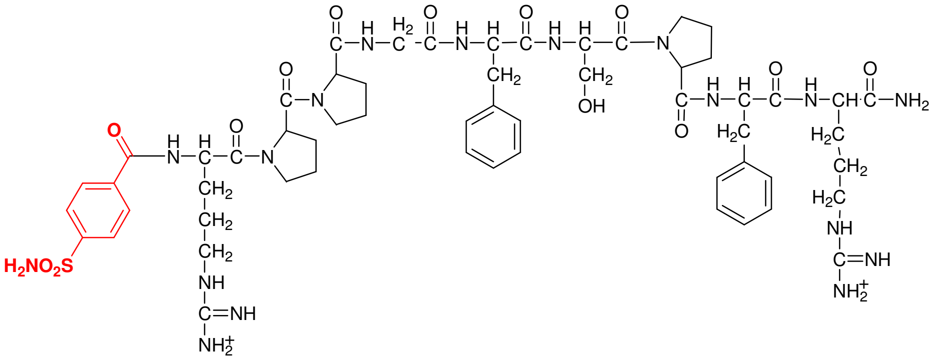
30

Figure 5-20 4-carboxybenzenesulfonamide (**30**)

4-Carboxybenzenesulfonamide (Figure 5-20) has been successfully employed for the synthesis of *N*-terminal peptide sulfonamides which, in **Chapter 6**, leads onto the formation and biological testing of β-CD-based compounds. Peptide synthesis on Rink resin followed by the addition of 4-carboxybenzenesulfonamide coupling under normal Fmoc SPPS reaction conditions[***] readily generates *N*-terminal peptide sulfonamides.



30a



30b

Figure 5-21 *N*-terminal peptides **30a** and **30b**

[***] See experimental (**Section 5.4.5**) for more details

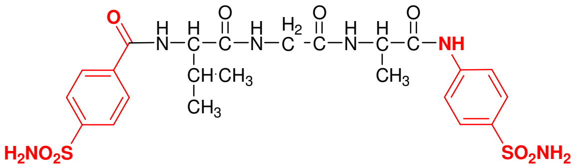
In this section, **30** was coupled to the model peptide (**30a**, Val-Gly-Ala, Figure 5-21) and BK (**30b**, Figure 5-21). The yields for compounds **30a** and **30b** were 2% and 4% respectively (Table 5-17, **Section 5.4.5.1**). The unfunctionalised BK peptide (synthesised in **Chapter 3**, **Section 3.2.2.7**) had a yield of 52%.

This significant decrease in yield could possibly be attributed to an incomplete coupling of the sulfonamide moiety to the resin leaving unfunctionalised reactive sites. It could also be attributed to the incomplete addition of the Fmoc-amino acids that make up the BK peptide chain.

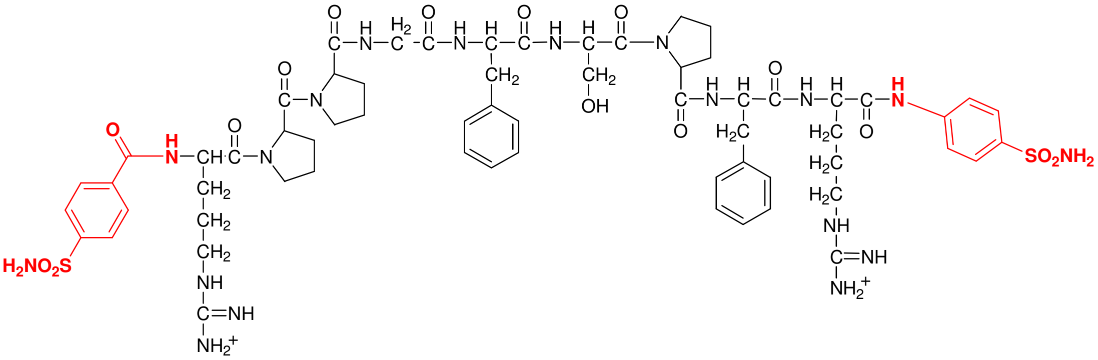
5.3 Results and Discussion for the Synthesis of *C*- and *N*-terminal Peptide Sulfonamides

The synthesis of *C*- and *N*-terminal peptide sulfonamides combines the methods from both **Sections 5.1** and **5.2**. 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl chloride (**25**) is coupled to resin using a 1:1 slurry of pyridine in DCM overnight (as per **Section 5.1.2.2**). Following Fmoc deprotection of resin-bound **25**, Fmoc-amino acids are then coupled using standard Fmoc SPPS coupling conditions of TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight. The *N*-terminal sulfonamide 4-carboxybenzenesulfonamide (**30**) is then coupled using these same reaction conditions. All Fmoc deprotection has been achieved using a 20% pip in DMF solution (2 x 5 minutes). The yields for this section (Table 5-18, **Section 5.4.6.1**) indicate that the longer and more complicated the peptide, the lower the reaction success (a comparison of compounds **31a** and **31b**, Figure 5-22) whereby the model peptide has a yield of 14% and the BK peptide only 0.7% respectively.

Overall, although very low yielding this study produced useable amounts (mg) of peptidic material for chemical and bioassay analysis. Obviously, future studies must involve generating better yields starting with coupling methodologies and testing a wider range of solid phases. Cleavage conditions and reaction times should also be optimised. These include investigations into the stability of the cleaved products versus varying cleavage conditions, the inclusion of suitable scavengers in the cleavage mixture, and the optimal time for the removal of protecting groups employed in the reaction.



31a



31b

Figure 5-22 *N*-terminal peptides **31a** and **31b**

5.4 Materials and Methods

5.4.1 *Reagents and Instruments*

With the exception of the following details, the same reagents and instruments as per **Chapter 2**, **Section 2.4.1** and **Chapter 3**, **Section 3.2.2.1** were used throughout this chapter.

4-Aminobenzene sulfonic acid was from Baker Chemical Company (Phillipsburg, New Jersey), 4-carboxybenzene sulfonic acid was from Sigma-Aldrich (Germany). Taurine and sulfamic acid was purchased from Sigma Chemical Company (USA). Tetramethyl urea was obtained from Sigma-Aldrich (Germany), potassium hexafluorophosphate and oxalyl chloride were from Acros Organics (Belgium).

5.4.2 *Experimental for Sulfonic Acid Precursors*

5.4.2.1 Peptide Synthesis using 4-Aminobenzene sulfonic Acid (**20**)

For the structure of **20** refer to Figure 5-6. See **Appendix 5-V** for details on stability tests, peptide synthesis, and their results.

5.4.2.2 4-Fluorenylmethyloxycarbonylaminobenzene sulfonic acid (**21**)

The synthetic route is illustrated in Figure 5-7. Adapted from Liskamp *et al.*[10] Fmoc-OSu (1.94 g, 5.77 mmol) dissolved in dioxane (20 mL) was added dropwise to a solution of **20** (1.00 g, 5.77 mmol) and $NaCO_3$ (0.612 g, 5.77 mmol) dissolved in H_2O (20 mL). The reaction was stirred at rt overnight. Solvents were removed *in vacuo* followed by addition of HCl (50 mL, 1 mol/L). After extraction with EtOAc (1 x 50 mL) and ether (1 x 50 mL), the organic layer was washed with H_2O (2 x 50 mL) and then dried over $MgSO_4$, filtered, then dried *in vacuo.* The solid was then recrystallised from hot H_2O, filtered, and the liquid freeze-dried to yield a cream coloured powder (1.58 g, 69%).[†††]

LR-MS: *m/z* (%, assignment) 394.16 (100, M-1)$^-$ HR-MS: *m/z* $C_{21}H_{16}NO_5S$ (M-H)$^-$ 394.0755, actual 394.0740; ^{1}H NMR (500 MHz, d_6-DMSO): δ 4.32 (t, J = 6.6 Hz, 1H, Fmoc CH), 4.48-4.80 (m, 2H, Fmoc CH_2), 6.43 (d, J = 6.6 Hz, 2H, sulfonamide aromatics), 7.24 (d, J = 6.4 Hz, 2H, sulfonamide aromatics), 7.36 (t, J = 7.6 Hz, 2H, Fmoc aromatics), 7.43 (t, J = 7.3 Hz, 2H, Fmoc aromatics), 7.76 (d, J = 7.5 Hz, 2H, Fmoc aromatics), 7.91 (d, J = 7.5 Hz, 2H, Fmoc aromatics); ^{13}C NMR (500 MHz, d_6-DMSO): δ 40.0 (Fmoc CH_2), 73.4 (Fmoc CH), 120.6, 125.7, 126.6 (Fmoc aromatics),127.0 (Fmoc aromatics), 127.6 (Fmoc aromatics), 128.1 (Fmoc aromatics)

[†††] Stability tested as per **Chapter 2**, **Section 2.4.4.4** using the conditions stated in Table 5-4. Fmoc-UV coupling tests as per **Chapter 2**, **Section 2.4.4.4**. Compound **21** was coupled to Rink resin (50 mg, 0.0365 mmol) overnight using the conditions stated in Table 5-3.

5.2.2.3 4-Aminomethylbenzene Sulfonic Acid (**22**)

The synthetic route is illustrated in Figure 5-8. 4-Aminomethylbenzene sulfonic acid (**22**) was synthesised according to known procedures[11] yielding an off white coloured solid (7 g, 40% when starting with 12.2 mL, 93 mmol of the benzylamine).

^{1}H NMR (500 MHz, d_6-DMSO): δ 3.57 (s, 2H, CH_2), 3.97 (s, 2H, NH_2), 7.50 (s, 4H, aromatics); ^{13}C NMR (500 MHz, d_6-DMSO): δ 42.4 (CH_2), 125.9 (aromatics), 129.1 (aromatics)

5.2.2.4 4-Fluorenylmethyloxycarbonylaminomethylbenzene sulfonic acid (**23**)

The synthetic route is illustrated in Figure 5-9. 4-Fluorenylmethyloxycarbonyl-aminomethyl benzene sulfonic acid (**23**) was synthesised as per **Section 5.4.2.2**, starting from compound **22** (6 g, 3.21 mmol, 1 equiv) yielding (11.98 g, 91%) of white solid.[‡‡‡]

LR-MS: *m/z* (%, assignment) 408.84 (10, M-H); ^{1}H NMR (500 MHz, d_6-DMSO): δ 3.77 (d, J = 7.3 Hz, 2H), 4.0 (t, J = 6.8 Hz, 1H), 4.28 (d, J = 7.3 Hz, 4H), 7.28-7.44 (m, 6H), 7.67-7.74 (m, 2H), 7.83-7.89 (m, 2H); ^{13}C NMR (500 MHz, d_6-DMSO): δ 50.6, 64.3 (CH_2), 65.5 (CH_2), 120.3, 120.5, 125.6, 125.7, 127.2, 127.5, 127.6, 128.1

5.4.3 *Experimental for Sulfonyl Halide Precursors*

5.4.3.1 4-Aminobenzenesulfonyl Chloride (**24**)

The synthetic route is illustrated in Figure 5-10. Synthesised from adapted procedures by Liskamp *et al.*[10] 4-Aminobenzene sulfonic acid (**20**, 3.0 g, 17.3 mmol) was suspended in a minimum amount of DCM under an $N_{2(g)}$ atmosphere. Phosphorus pentachloride (9.00 g, 43.25 mmol, 2.5 equiv) was added and the reaction was stirred at rt overnight. The organic layer was washed with H_2O, a saturated $NaHCO_3$ solution, then isolated, dried over anhydrous Na_2SO_4, and filtered. The solvent was removed *in vacuo* and freeze dried overnight yielding a fluffy white solid (**24**, 3.174 g, 95%).

[‡‡‡] Coupling tests were performed as per **Chapter 2**, **Section 2.4.4.4** using the conditions stated in Table 5-5. Peptide synthesis using **23** as per **Section 5.4.2.1** using the conditions stated in Table 5-5.

LR-MS: (%, assignment) 190.6091 (40, M+H); ^{1}H NMR (500 MHz, d_6-DMSO): δ 8.08 (d, J = 9.1 Hz, 2H), 7.45 (d, J = 8.8 Hz, 2H)

See **Appendix 5-VII** for details on peptide synthesis using compound **24**.

5.4.3.2 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl Chloride (**25**)

The synthetic route is illustrated in Figure 5-11.

Adapted from Liskamp *et al.*[10] Compound **21** (1.0 g, 2.5 mmol, 1 equiv), dry toluene (50 mL), dry DMF (5 mL), and excess thionyl chloride (2 mL) were allowed to stir at rt overnight followed by quenching with ice-H_2O, neutralisation with $NaHCO_3$ and extraction of the aq phase with EtOAc (2 x 10 mL). The organic phase was then concentrated *in vacuo* and recrystallised (twice) with a benzene/hexane mixture forming a white solid (620 mg, 60%). MS and IR analysis shows no sign of the product (**25**).

To further assess the nature of (**25**), 50 mg of the sample was dissolved in DMSO. TEA (1.1 equiv) and Bn amine (1.1 equiv) were added, and the reaction allowed to stir at rt overnight. The synthetic route is illustrated in Figure 5-12.[§§§]

^{1}H NMR (500 MHz, d_6-DMSO): δ 4.31 (t, J = 6.5 Hz, 1H, Fmoc CH), 4.49 (d, J = 6.2 Hz, 2H, Fmoc CH_2), 7.32-7.52 (m, 8H, aromatics), 7.76 (d, J = 6.9 Hz, 2H, aromatics), 7.91 (d, J = 7.7 Hz, 2H, aromatics); ^{13}C NMR (500 MHz, d_6-DMSO): δ 47.0, 66.1, 117.6, 120.6, 125.6, 126.6, 127.6, 128.2

[§§§] Stability tests using the reaction conditions stated in Table 5-7. Coupling tests as per **Chapter 2**, **Section 2.4.4.4** using the conditions stated in Table 5-8. Peptide synthesis using **25** was undertaken using the coupling conditions stated in Table 5-9

5.4.3.3 Synthesis of **25a** and **25b**

For structures of **25a** and **25b** refer to Figure 5-13. Val-Gly-Ala or BK was assembled manually on Rink resin (see Table 5-15 for details) using Fmoc SPPS as per **Chapter 3**, **Section 3.2.2.1**. Fmoc-L-amino acids (4 equiv) and mono-6^A-fluorenylmethyloxy-carbonylaminomono-6^X-succinyl-β-cyclodextrin (**12**, **Chapter 2**, **Section 2.4.4.4**, 4 equiv) were coupled as per **15a**, **Chapter 4**, **Section 4.7.3.1**. 4-Fluorenylmethyloxy-carbonylaminobenzenesulfonyl chloride, (**25**, **Section 5.1.2.2**, 4 equiv) was coupled using a 1:1 pyridine:DCM slurry (3 mL) overnight. A 20% TFA in DCM mixture was used to cleave the peptide from the resin. Purification of **25a** and **25b** as per below.

Table 5-15 *C*-terminal peptide sulfonamides synthesised using compound **25** on Rink resin

Experiment Number	*Peptide*	*Synthesis Details*	*Yield*	
			mg	*%*
25a	Val-Gly-Ala	Rink resin , 0.073 mmol, 100 mg	1.3	2
25b	Bradykinin		1.4	5

*Purification and Results for Compound **25a**:*

RP-HPLC (twice) using a MeCN:H_2O:TFA stepwise gradient on a C-18 preparative column was used to purify the crude peptide (for details see **Chapter 3**, **Section 3.2.2.1**). The product eluted between 42-45%B after RP-HPLC purification with a yield of 1.3 mg (2%) when using the time programme given in Table 5-16.

LR-MS: *m/z* (%, assignment) 400.56 (100, M+H)$^+$, 212.23 (100, M+2H)$^{+2}$; ^{1}H NMR (d_6-DMSO, 500 MHz): δ 0.95 (t, J = 6.8 Hz, 6H, valine CH_3), 1.20-1.25 (m, 3H, alanine CH_3), 2.05-2.13 (m, 1H, valine CH), 3.63-3.67 (m, valine CH), 3.72-3.97 (m, glycine CH_2), 4.15-4.25 (m, 1H, alanine CH), 7.02 (s, 1H, NH), 7.29 (s, 1H, NH), 7.49 (s, 2H, terminal NH_2), 7.85-7.95 (m, 3H aromatic, valine NH), 8.04 (d, J = 3.9, 2H, aromatic), 8.10-8.18 (m, 1H, alanine NH), 8.70-8.78 (m, 1H, glycine NH); ^{13}C DEPT-135 NMR (d_6-DMSO, 500 MHz): δ 18.2 (valine CH_3), 18.4 (valine CH_3), 18.7 (alanine CH_3), 18.9 (valineCH_3), 30.2 (valine CH), 40.0 (glycine CH_2), 40.1 (glycine CH_2), 48.0 (alanine

CH), 48.6 (alanine CH), 57.8 (valine CH), 126.0 (sulfonamide aromatic), 127.0 (sulfonamide aromatic)

Table 5-16 Acetonitrile stepwise gradient time programme for the separation of the **25a** peptide

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	40	100
10	0	45	0
30	30	50	STOP

*Purification and Results for Compound **25b**:*

Compound **25b** was purified as per **Chapter 3**, **Section 3.2.2.2**. The pure product eluted between 39-46% B with a yield of 1.4 mg (5%).

LR-MS: *m/z* (%, assignment) 1214.79 (100, M+H)$^+$ ^{1}HR-MS: *m/z* $C_{56}H_{81}N_{17}O_{12}S$ (M+2H)$^{+2}$ 607.7967, actual 607.7980; *Partial NMR analysis* ^{1}H NMR (d_6-DMSO, 500 MHz): δ 7.10-7.31 (m, 10H, phenylalanine aromatics); COSY ^{1}H NMR (d_6-DMSO, 500 MHz): δ 7.4, 7.5, 7.71, 7.75, 7.8, 7.9 (m, 1H, glycine NH), 8.1, 8.2, 8.34, 8.38, 8.4

5.4.3.4 4-Fluorenylmethyloxycarbonylaminomethylbenzenesulfonyl Chloride (**26**)

The synthetic route is illustrated in Figure 5-14. The synthesis of **26** was as per the synthesis of compound **25** (**Section 5.4.3.2**) creating a product which is sparingly soluble in DMSO. To see if the chloride species was made (the synthetic route is illustrated in Figure 5-14), the same method as per compound **25** was performed. MS results showed presence of the (M+2H)$^{+2}$ peak for **26i** (Figure 5-14) with an expected loss of the Fmoc group also observed. It can be concluded from these results and NMR data that the product has been synthesised successfully (640 mg, 60% when starting with 1 g, 2.5 mmol of **23**).[¶¶¶]

[¶¶¶] Coupling test as per **Chapter 2**, **Section 2.4.4.4** using the experimental conditions found in Table 5-11. Peptide synthesis using the conditions in Table 5-12.

^{1}H NMR (500 MHz, d_6-DMSO): δ 1.23 (br s, 1H CH_2), 2.92 (d, J = 5.9 Hz, 2H, CH_2), 4.27 (t, J = 5.9 Hz, 1H, CH_2), 7.30 (dt, J = 7.9 Hz, 2H, aromatics), 7.35 (t, J = 7.3 Hz, 2H, aromatics), 7.82 (d, J = 7.6 Hz, 2H, aromatics), 7.90 (d, J = 7.6 Hz, 2H, aromatics), 8.51 (s, 2H, NH); ^{13}C NMR (500 MHz, d_6-DMSO): δ 47.2, 65.1, 121.6, 125.6, 126.6, 128.72, 128.74

5.4.3.5 Tetramethyl Chloro Formamidinium Hexafluorophosphate (**TCFH**)

The synthetic route is illustrated in Figure 5-15. Tetramethyl chloro formamidinium hexafluorophosphate (**TCFH**) was synthesised according to procedures by Furstner *et al.*[13] A 250 mL round bottom flask (rbf) was charged with dry toluene (50 mL) and a flea, stoppered with a rubber septum and tetramethyl urea (**TMU**, 4.64 g, 40 mmol, 4.70 mL, 1 equiv) added via a syringe. Oxalyl chloride (**OC**, 7.61 g, 60 mmol, 5.15 mL, 1.5 equiv) was added dropwise via syringe and the clear reaction mixture heated at 60ºC under a $N_{2(g)}$ atmosphere overnight. A white/cream coloured solid precipitated. The solvent was carefully drawn off via pipette and the solid was washed with diethyl ether (3 x 20 mL), each time pipetting off the filtrate/washes. The solid was dried under vacuum obtaining a white solid (**TCFC**, 7.32 g, 42 mmol). **TCFC** was dissolved in DCM (20 mL) before addition of vacuum dried potassium hexafluoro phosphate (7.54 g, 1.02 mmol, 41 equiv) and the suspension was stirred at rt overnight (colour change of the suspension from light yellow to light green). The light green suspension was filtered, and the solid KCl washed with DCM (50 mL), the filtrate was then concentrated *in vacuo*, affording a light green coloured foamy solid (**TCFH**, 11.6 g, 40 %).

^{1}H NMR (500 MHz, CD_3CN): δ 3.25 (s, 12H); ^{13}C NMR (500 MHz, CD_3CN): δ 44.7, 118.3

5.4.3.6 Tetramethyl Fluoro Formamidinium Hexafluorophosphate (**TFFH**)

The synthetic route is illustrated in Figure 5-16. Tetramethyl fluoro formamidinium hexafluorophosphate (**TFFH**) was synthesised according to adapted procedures by Carpino *et al.*[14] and Christensen *et al.*[15] **TCFH** (11.64 g, 9.73 mol, 1 equiv) was dissolved in MeCN (30 mL) before the addition of vacuum dried KF (2.65 g, 10.7

mmol, 1.1 equiv). The white coloured suspension was stirred at rt overnight. The solid was filtered off using suction filtration and the organic filtrate was concentrated to oil *in vacuo*. Addition of ether (40 mL) resulted in recrystallisation of the product and the sample as left at 4°C overnight. The crystalline solid was isolated, washed with ether and dried under vacuum to afford a white coloured crystalline solid (**TFFH**, 10.53 g, 40%). The samples are stored under a $N_{2(g)}$ atmosphere at 4°C ready for use.

LR-MS (%, assignment): 120.14 (100, $M+H)^+$; ^1H NMR (500 MHz, $CDCl_3$): δ 3.17 (d, J = 5.2 Hz, 12H, CH_3), 7.45 (d, J = 8.7 Hz, 2H)

5.4.3.7 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl Fluoride (**27**)

The synthetic route is illustrated in Figure 5-17. Adapted from procedures by Carpino *et al.*[14] Compound **21** (100 mg, 0.25 mmol. 1 equiv) and **TFFH** (66.9 mg, 0.25 mmol, 1 equiv) were reacted with DIEA (88.6 μL, 0.50 mmol, 2 equiv) in DMF (20 mL) and stirred slowly for 2h at rt. MS and IR showed no sign of the product. Compound **27** was also not able to be synthesised using this method.

From here, an *in situ* preparation and coupling of **27** to Rink resin was attempted. In this reaction, **TFFH** (66.9 mg, 0.25 mmol, 1 equiv) dissolved in dry DMF was added dropwise to a solution of **21** (100 mg, 0.25 mmol, 3.5 equiv) dissolved in dry DMF. This mixture was stirred under an $N_{2(g)}$ atmosphere. DIEA (306 μL, 3.5 mmol, 7 equiv) was added to the reaction mixture and this was stirred at rt until a colour change from clear to yellow colour was observed. This solution was then added to deprotected Rink resin (for details see **Chapter 3**, **Section 3.2.2.1**, 100 mg, 0.073 mmol, 1 equiv) and allowed to couple at rt overnight. The resin was then washed with DMF. A ninhydrin test gave a yellow result indicating that the coupling was successful.[\\\\\\]

[\\\\\\] Coupling tests was used to determine the % of successful couplings to the resin (Table 5-13, experimental details see **Chapter 2**, **Section 2.4.4.4**). From here, Fmoc-Val, -Gly, -Ala were added stepwise to the resin using TBTU (93 mg, 0.29 mmol. 4 equiv) and DIEA (98 μL, 0.56 mmol, 8 equiv) in DMF as per standard Fmoc-SPPS procedure detailed in **Chapter 3**, **Section 3.2.2.1**. TFA (95%) was used to cleave the peptide from the resin (**Chapter 3**, **Section 3.2.2.1**). MS analysis of the crude product showed a weak signal of the product which was not purified due to only trace amounts of crude material present after the cleavage.

5.4.4 *Experimental for Sulfonyl Benzotriazole (OBt) Active Ester Precursors*

5.4.4.1 4-Aminobenzenesulfonyl Benzotriazole (**28**)

The synthetic route is illustrated in Figure 5-18. Compound **28** was synthesised according to procedures by Carpino *et al.*[17] TEA (365.5 µL, 2.6 mmol, 1 equiv) was added to a suspension of HOBt (353.5 mg, 2.6 mmol, 1 equiv) in anhydrous DCM. The reaction was stirred on ice under an argon atmosphere forming a clear coloured solution. Compound **24** (500 mg, 2.6 mmol, 1 equiv) was added to the reaction mixture and it was stirred for 30 minutes at 0ºC, then at rt for 2h forming a clear coloured solution. After dilution with DCM, the organic phase was washed with H_2O, then saturated HCl solution, and then dried over $MgSO_4$. The solvent was removed *in vacuo* and then the white solid dissolved up in a small amount of DCM. The solid was precipitated out using hexane, isolated by filtration then freeze dried overnight yielding a white coloured solid (**28**, 291.7 mg, 38%).

^{1}H NMR (500 MHz, d_6-DMSO): δ 7.41 (t, J = 7.6 Hz, 2H, aromatic), 7.54 (t, J = 7.6 Hz, 2H, aromatic), 7.72 (d, J = 4.1 Hz, 2H, aromatic), 7.98 (d, J = 4.1 Hz, 2H, aromatic); ^{13}C NMR (500 MHz, d_6-DMSO): δ 110.2, 119.6, 124.9, 127.7

5.4.4.2 4-Fluorenylmethyloxycarbonylaminobenzenesulfonyl Benzotriazole (**29**)

The synthetic route is illustrated in Figure 5-19. Compound **29** was synthesised as per **Section 5.4.2.2** using Fmoc-OSu (88.3 mg, 1 equiv, 0.34 mmol) $NaHCO_3$ (22.00 mg, 1 equiv, 0.34 mmol) and **28** (76 mg, 0.34 mmol, 1 equiv) producing 100 mg (57%) of a white coloured solid.[****]

LR-MS: (%, assignment): 511.3395 (85, M+H)$^+$; ^{1}H NMR (500 MHz, d_6-DMSO): δ 4.31 (d, J = 6.7 Hz, 1H, Fmoc CH), 4.49 (d, J = 6.4 Hz, 2H, Fmoc CH_2), 7.28-7.45 (m, 6H), 7.55-7.59 (dd, 2H), 7.33-7.65 (m, 4H), 7.84-7.92 (m, 4H); ^{13}C NMR (500 MHz, d_6-DMSO): δ 50.5 (Fmoc CH), 64.2 (Fmoc CH_2), 120.3, 120.5, 120.6, 124.8, 125.3, 125.63, 125.69, 127.2, 127.5, 128.0, 128.2

[****] Peptide synthesis undertaken onto Rink resin using the conditions stated in Table 5-14.

5.4.5 *Experimental for N-terminal Peptide Synthesis*

5.4.5.1 Synthesis of **30a** and **30b**

For structures of **30a** and **30b** refer to Figure 5-21. Peptide synthesis (Table 5-17) as per **Chapter 3**, **Section 3.2.2.1** was used to synthesise *N*-terminal peptide sulfonamides on Rink resin. It was found that coupling 4-carboxybenzenesulfonamide (**30**, Figure 5-21, 4 equiv) worked best using TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight gave the best reaction yields. Rink resin was swollen in DMF overnight followed by deprotection using a 20% pip in DMF solution (2 x 5 minutes). Fmoc-L-amino acids (4 equiv) were coupled to the resin using TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF with the coupling time depending upon the amino acid. Compound **30** (4 equiv) was coupled to the resin using TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight. A ninhydrin test was used to determine if the coupling was successful for the Fmoc-protected amino acids. No test was carried out for compound **30** due to a free amino group present before and after coupling to the resin. TFA cleavage and purification as per **Chapter 3**, **Section 3.2.2.1**. For specific details, refer to each individual compound (**30a-b**) below.

Table 5-17 *N*-terminal peptide sulfonamides synthesised using compound **30** on Rink resin

Experiment Number	*Peptide Sequence*	*Synthesis Details*	*Purified Yield*	
			mg	*%*
30a	Val-Gly-Ala	Rink resin, 0.073 mmol, 100 mg	2.4	2
30b	Bradykinin	Rink resin , 0.25 mmol, 342 mg	11.9	4

*Purification and Results for Compound **30a**:*

RP-HPLC using a MeCN:H_2O:TFA stepwise gradient on a C-18 preparative column was used to purify the crude peptide (for details see **Chapter 3**, **Section 3.2.2.1**). The product eluted between 22 and 27%B after two successive RP-HPLC purifications with a yield of 2.4 mg (2%) when using the time programme given in Table 5-16.

LR-MS: *m/z* (%, assignment) 450.25 (100, M+Na)$^+$ HR-MS: *m/z* $C_{17}H_{26}N_5O_6S$ (M+H)$^+$ 428.1598, actual 428.1601; $C_{17}H_{25}N_5O_6SNa$ (M+Na)$^+$ 450.1418, actual 450.1419; $C_{17}H_{25}N_5O_6SK$ (M+K)$^+$ 466.1157, actual 466.1157; ^{1}H NMR (d$_6$-DMSO, 500 MHz): δ 0.958 (br s, 6H, valine CH$_3$), 1.20 (d, J = 6.9 Hz, 3H, alanine CH$_3$), 2.14 (q, J = 6.7 Hz, 1H, alanine CH), 3.75 (q, J = 5.7, 2H, glycine CH$_2$), 4.19 (t, J = 6.6, 1H, valine CH), 4.27 (t, J = 7.4, 1H, alanine CH), 7.02 (s, 1H, NH), 7.29 (s, 1H, NH), 7.49 (s, 2H, terminal NH$_2$), 7.85-7.95 (m, 3H aromatic, valine NH), 8.04 (d, J = 3.9, 2H, aromatic), 8.36 (s, 1H, glycine NH), 8.53 (d, J = 3.7, 1H, alanine NH); ^{13}C DEPT-135 NMR (d$_6$-DMSO, 500 MHz): δ 18.6 (valine CH$_3$), 19.4 (alanine CH$_3$), 19.7 (valine CH$_3$), 30.2 (valine CH), 42.5 (glycine CH$_2$), 48.4 (alanine CH), 60.6 (valine CH), 125.9 (sulfonamide aromatic), 128.7 (sulfonamide aromatic)

Purification and Results for Compound ***30b****:*

Compound **30b** was purified as per **Chapter 3**, **Section 3.2.2.2**. The pure product eluted at 41% B (12 mg, 4%).

LR-MS (MALDI): *m/z* (%, assignment) 1243.027 (100, M+H)$^+$ LR-MS (ESI-MS): *m/z* (%, assignment) 623.04 (100, M+2H)$^{+2}$ HR-MS: *m/z* $C_{57}H_{79}N_{17}O_{13}S$ (M+H)$^+$ 1242.5837, actual 1242.5788; ^{1}H NMR (d$_6$-DMSO, 500 MHz): δ 1.14-2.10 (m, 19H, proline$_{2\beta}$, proline$_{2\gamma}$, proline$_{3\beta}$, arginine$_{1\beta}$, arginine$_{1\alpha}$, proline$_{3\beta}$,[††††]), 2.14-2.24 (m, 1H, phenylalanine$_{2\beta}$), 2.72-2.90 (m, 2H, phenylalanine$_{2\beta}$,[††††]), 2.99-3.17 (m, 6H, phenylalanine$_{5\beta}$, arginine$_{9\alpha}$, arginine$_{9\alpha}$), 3.41-3.87 (m, 11H, proline$_{7\gamma}$, proline$_{3\gamma}$, arginine$_{1\gamma}$, serine$_{6\beta}$, proline$_{7\gamma}$, proline$_{3\gamma}$, proline$_{2\gamma}$, glycine$_{\alpha}$), 4.07-4.41 (m, 4H, arginine$_9$, proline$_3$, proline$_7$[††††]), 4.45-4.74 (m, 4H, phenylalanine$_8$, phenylalanine $_5$, serine$_8$, proline$_2$), 6.94-7.03 (br s, 1H, NH[††††]), 7.09-7.32 (m, 17H, phenylalanine aromatics), 7.47-7.53 (dd, 3H, phenylalanine aromatics), 7.60-7.62 (s, 1H, phenylalanine aromatics), 7.62-7.65 (dt, 2H, phenylalanine aromatics), 7.75-7.80 (m, 1H, glycine NH), 7.84-7.87 (dd, 1H, phenylalanine aromatics), 7.87-7.97 (m, 4H, sulfonamide aromatics), 8.01-8.07 (m, 2H, sulfonamide aromatics, arginine$_9$ NH), 8.37-8.42 (m, 1H, phenylalanine$_5$ NH), 8.68-8.72 (d, 1H, serine$_6$ NH); ^{13}C DEPT-135 NMR (d$_6$-DMSO, 500 MHz): δ 24.9 (CH$_2$), 25.0 (CH$_2$), 25.1 (CH$_2$), 29.3 (CH$_2$), 51.4, 58.1, 60.1, 122.9,

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[††††] Unassigned peaks associated with arginine$_{1\alpha}$, phenylalaine$_{8\alpha}$, proline$_{3\beta}$, proline$_{3\gamma}$, proline$_{7\beta}$, proline$_{7\gamma}$, arginine$_{9\beta}$, arginine$_{1\gamma}$, arginine$_{9\beta}$, arginine$_{9\gamma}$, proline$_7$, or proline$_4$

126.0, 126.2, 126.6, 126.7, 127.5, 128.4, 128.5, 128.6, 129.1, 129.4, 129.50, 129.57, 130.5, 130.6, 185.3

5.4.6 *Experimental for C- and N-terminal Peptide Synthesis*

5.4.6.1 Synthesis of **31a** and **31b**

For structures of **31a** and **31b** refer to Figure 5-22. Peptide synthesis (Table 5-18) as per **Chapter 3**, **Section 3.2.2.1** was employed to synthesise *N*- and *C*-terminal peptide sulfonamides on Rink and TentaGel resins using the sulfonamides **25** and **30**. It was found that coupling **30** (4 equiv) worked best using TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF overnight gave the best reaction yields (see **Section 5.4.5**). It was found that **25** couplings worked best using a 1:1 pyridine:DCM slurry overnight (**Section 5.1.2.2**). Rink resin was swollen in DMF overnight followed by deprotection using a 20% pip in DMF solution (2 x 5 minutes). Amino acids (4 equiv) were coupled to the resin using TBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv) in DMF with the coupling time depending upon the amino acid. A ninhydrin test was used to determine if the coupling was successful for the Fmoc-protected amino acids. TFA cleavage and purification as per **Chapter 3**, **Section 3.2.2.1**. For specific details, refer to each individual compound (**31a-b**) reported below.

Table 5-18 *N*- and *C*-terminal peptide sulfonamides synthesised using compounds **25** and **30** on Rink and TentaGel resin

Experiment Number	*Peptide Sequence*	*Synthesis Details*	*Purified Yield*	
			mg	*%*
31a	Val-Gly-Ala	Tentagel resin, 0.24 mmol/g, 0.096 mmol, 400 mg	22.4	14
31b	Bradykinin	Rink resin, 0.73 mmol/g, 0.073 mmol, 100 mg	1.0	0.7

*Purification and Results for Compound **31a**:*

Compound **31a** was purified as per **Chapter 3**, **Section 3.2.2.2**. The pure product eluted 10-12%B.

LR-MS: *m/z* (%, assignment) 606.58 (100, M+Na)$^+$; ^{1}H NMR (d_6-DMSO, 500 MHz): δ 0.95 (dd, J = 6.9 Hz, 6H, valine CH_3), 1.20 (d, J = 7.2 Hz, 3H, alanine CH_3), 2.14 (q, J = 6.8 Hz, 1H, valine CH), 3.75 (dd, J = 5.5 Hz, 2H, glycine CH_2), 4.19 (t, J = 7.7 Hz, 1H, alanine CH), 4.27 (t, J = 8.0 Hz, 1H, valine CH), 7.01 (s, 1H, aromatic NH), 7.29 (s, 1H, aromatic NH), 7.49 (s, 2H, terminal NH_2), 7.87 (br s, 1H, alanine NH), 7.88-7.92 (m, 2H, aromatic), 8.02-8.05 (m, 2H, aromatic), 8.36 (t, J = 6.0 Hz, 1H, glycine NH), 8.58 (d, J = 8.1 Hz, 1H, valine NH); ^{13}C DEPT-135 NMR (d_6-DMSO, 500 MHz): δ 18.6 (alanine CH_3), 19.4 (valine CH_3), 30.3 (valine CH), 42.7 (glycine CH_2), 48.4 (alanine CH), 60.0 (valine CH), 125.9 (sulfonamide aromatics), 128.7 (sulfonamide aromatics)

*Purification and Results for Compound **31b**:*

Compound **31b** was purified as per **Chapter 3**, **Section 3.2.2.2**. The pure product eluted at 45-47%B.

LR-MS: *m/z* (%, assignment) 1397.68 (95, M+H)$^+$; ^{1}H NMR (d_6-DMSO, 500 MHz): δ 1.37-2.22 (m, 13H, proline$_{2\beta}$, proline$_{2\gamma}$, proline$_{3\beta}$, arginine$_{1\beta}$, arginine$_{1\alpha}$, proline$_{3\beta}$, phenylalanine$_{2\beta}$[‡‡‡‡]), 2.63-2.82 (m, 3H,[‡‡‡‡]), 2.87-3.86 (m, 54H,[‡‡‡‡] including H_2O peak), 4.14-4.37 (m, 4H,[‡‡‡‡]), 4.42-4.76 (m, 6H, phenylalanine$_8$, phenylalanine$_5$, serine$_8$, proline$_2$), 5.33-5.45 (m, 1H,[‡‡‡‡] , 7.09-7.30 (m, 18H, phenylalanine and sulfonamide aromatics), 7.46-7.60 (m, 5H,[‡‡‡‡]), 7.71-7.80 (m, 1H, glycine NH), 7.83-8.05 (m, 9H, [‡‡‡‡]), 8.26-8.33 (d, J = 7.2 Hz, 1H, phenylalanine$_5$ NH), 8.71-8.76 (d, J = 7.5 Hz, 1H, serine$_6$ NH); ^{13}C DEPT-135 NMR (d_6-DMSO, 500 MHz): δ 24.9 (CH_2), 25.0 (CH_2), 25.2 (CH_2), 25.4 (CH_2), 28.30 (CH_2), 28.35 (CH_2), 29.3 (CH_2), 29.5 (CH_2), 37.5 (CH_2), 40.8 (CH_2), 41.0 (CH_2), 47.2 (CH_2), 47.40 (CH_2), 47.42 (CH_2), 47.8 (CH_2), 51.4, 52.42, 52.45, 53.1, 53.9, 54.4, 58.1, 59.3 (CH_2), 59.94, 59.97, 60.2, 62.2 (CH_2), 112.9, 126.0, 126.70, 126.75, 126.8, 128.4, 128.5, 128.6, 129.5, 129.6

[‡‡‡‡] Unassigned peaks associated with the bradykinin peptide

5.5 Conclusions

C- and/or *N*-terminal peptide sulfonamides have been synthesised on Rink resin under varying Fmoc SPPS reaction conditions in low but useable yields. The synthesis of cyclodextrin peptide sulfonamides can be found in **Chapter 6**. Bioassay analysis of compounds **25a-b, 30a-b**, and **31a-b** are also reported in **Chapter 6**.

5.6 References

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Chapter Six

Cyclodextrin-Peptide Sulfonamide Synthesis & Preliminary Bioassay Testing Results

6.0 Introduction

This chapter follows on from the Fmoc-SPPS (**Chapters 2-5**) and leads to preliminary biological testing of the cyclodextrin-peptide-sulfonamide complexes using two assays for screening (MTT assay and flow cytometry assay).

6.0.1 *MTT assay for Biological Testing*

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay allows determination on the effectiveness that a potential drug may have on cell proliferation (cell death or cell growth). Traditionally, the determination of cell growth was performed by counting viable cells after staining with a dye.[1] Several approaches have been used in the past, including trypan blue staining (a simple way to evaluate a cell membrane integrity, and therefore cell proliferation or death), but this method is not very sensitive and can not be adapted for high-throughout put screening.[1a-c] Radioactive labelling and uptake of substances is more accurate but this method is time consuming and carries toxicity risks to the operator. Therefore, the most common method currently in use is a MTT stain.

The MTT stain changes colour from yellow to purple upon reduction of the dye by mitochondrial reductase.[1] This colour change indicates the conversion of the dye into formazin (see Figure 6-1). This reduction can only take place in living cells and the absorbance of this purple formazin can be measured spectrophotometrically (between 500 and 600 nm) where the absorbance max is determined by the solvent system used.[1] Therefore, the conversion from yellow to purple is directly related to the number of viable (living) cells. Quantification of the amount of formazin produces a dose-dependent curve that indicates the effectiveness of the agent in causing cell death.[1]

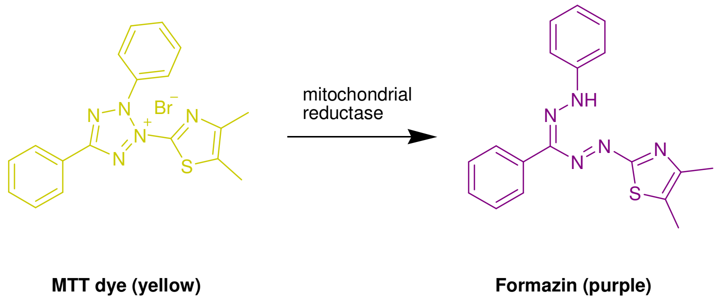


Figure 6-1 MTT dye (yellow) conversion into formazin (purple) by mitochondrial reductase in living cells

6.0.2 *Flow Cytometry Assay for Biological Testing*

The flow cytometry assay is a test used to measure the amount of DNA in cells, thereby determining the proportions of cells at different parts of the cell growth cycle.[2] It can also be used to detect populations of cells that have abnormal amounts of DNA. Previously, flow cytometry was primarily used as a research tool in the study of cancer and other conditions in humans.[2] However, this test is now widely used as a routine screening tool for the determination and characterisation of different types of human cancers as well as a lab screening tool for potential cancer treatments. It has also been used for decades to assess various physical properties (size, granularity, presence of surface proteins) of particles including mammalian cells, bacteria, and microspheres.[2]

This method involves rupturing the cell membrane (using ice-cold ethanol which keeps the cell intact allowing the movement of small molecules in/out of the cell). RNase is then added to chop up the RNA, leaving intact DNA which is able to be isolated.[2] A dye is then bound to the DNA (by inter-chelating with it) and then the dyes absorbance measured using a flow cytometer. The flow cytometer contains a laser light source which excites the fluorescent dye bound to DNA. The intensity measured is proportional to the amount of DNA at different stages in the cells cycle.[2]

Bioactive sulfonamides, CD, and bioactive peptides (e.g. BK) have been tested in the literature using these above tests (MTT and flow cytometry assays) as a method to identify potential anti-cancer activity of new molecules.[3] These assays are commonly used to screen potential anti-cancer targets and this is perhaps attributed to the ease of carrying out these assays, their low toxicity, and high accuracy. Another common anti-cancer screening assay is the carbonic anhydrase assay (specifically for sulfonamide based drugs, see **Chapter 5** for more details).

6.0.3 *Other uses for Sulfonamides, Cyclodextrins, and Bioactive Peptides*

Sulfonamide and CD based compounds have shown medicinal activity other than anti-cancer. A selection of these is listed in Table 6-1. Numerous bioactive peptides have also been studied for treatments including cancer, fungal and bacterial infections, and span other subfields including chemistry, biology, and physics.

Table 6-1 A selection of alternative uses for sulfonamides and cyclodextrins

Sulfonamides[4]	
Antibiotics Anti-inflammatory Antiviral agents	see **Chapter 5** for more details
Cyclodextrins[5]	
Environmental Protection - metal removal	
Food Industry – masking of bad tastes/smell, stabilising active ingredients	
Agriculture Industry – germination delay of seeds,	
Chemical Industry – isomeric separation and purification, catalysis reactions	

6.0.4 *Chapter Outline*

This chapter continues the study from **Chapter 4** and describes the bi-functionalisation of β-CD with a series of model and bioactive peptide sulfonamides using Fmoc-SPPS. The bioactive peptide used in this study was BK. For synthesis and other details of the BK peptide, refer to **Chapter 3**. For synthesis and other details of the bi-functionalised β-CD (**12**) refer to **Chapter 2**. This synthetic approach allows the functionalisation of β-CD with one or two peptides as well as the addition of model sulfonamide drugs (see

Chapters 1 and **5** for more details on sulfonamides) in various combinations. For example, the attachment of β-CD to the *C*- and/or *N*-terminus of peptides functionalised with exo-sulfonamides (Figure 6-2) has been achieved.

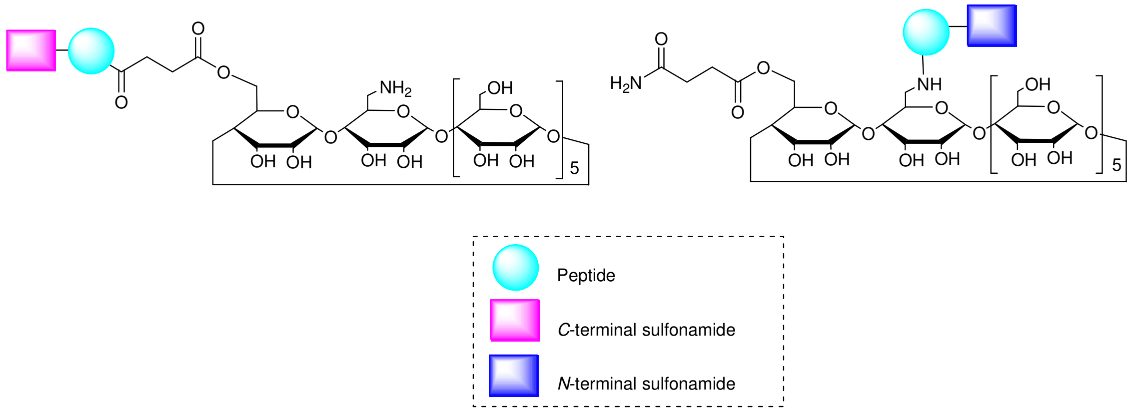


Figure 6-2 Placement of the peptide-sulfonamide onto compound **12**

Following on from the synthesis of these β-CD-peptide-sulfonamides, preliminary bioassay testing has been performed on these compounds, as well as other variations from previous chapters (see Table 6-2 and Figure 6-3).

Table 6-2 Summary of the compounds that have undergone preliminary bioassay analysis

Chapter	*Synthesis Details*	*Compounds*
4	4.7.3.2-3	**15b, 15c**
	4.7.4.2-3	**16b, 16c**
	4.7.5.1	**17a**
	4.7.6.1	**18b**
	4.7.7.4-5	**19d, 19e**
5	5.4.3.3	**25b**
	5.4.5.1	**30a, 30b**
	5.4.6.1	**31a, 31b**
6	6.2.2	**32a, 32b**
	6.2.3	**33a, 33b**

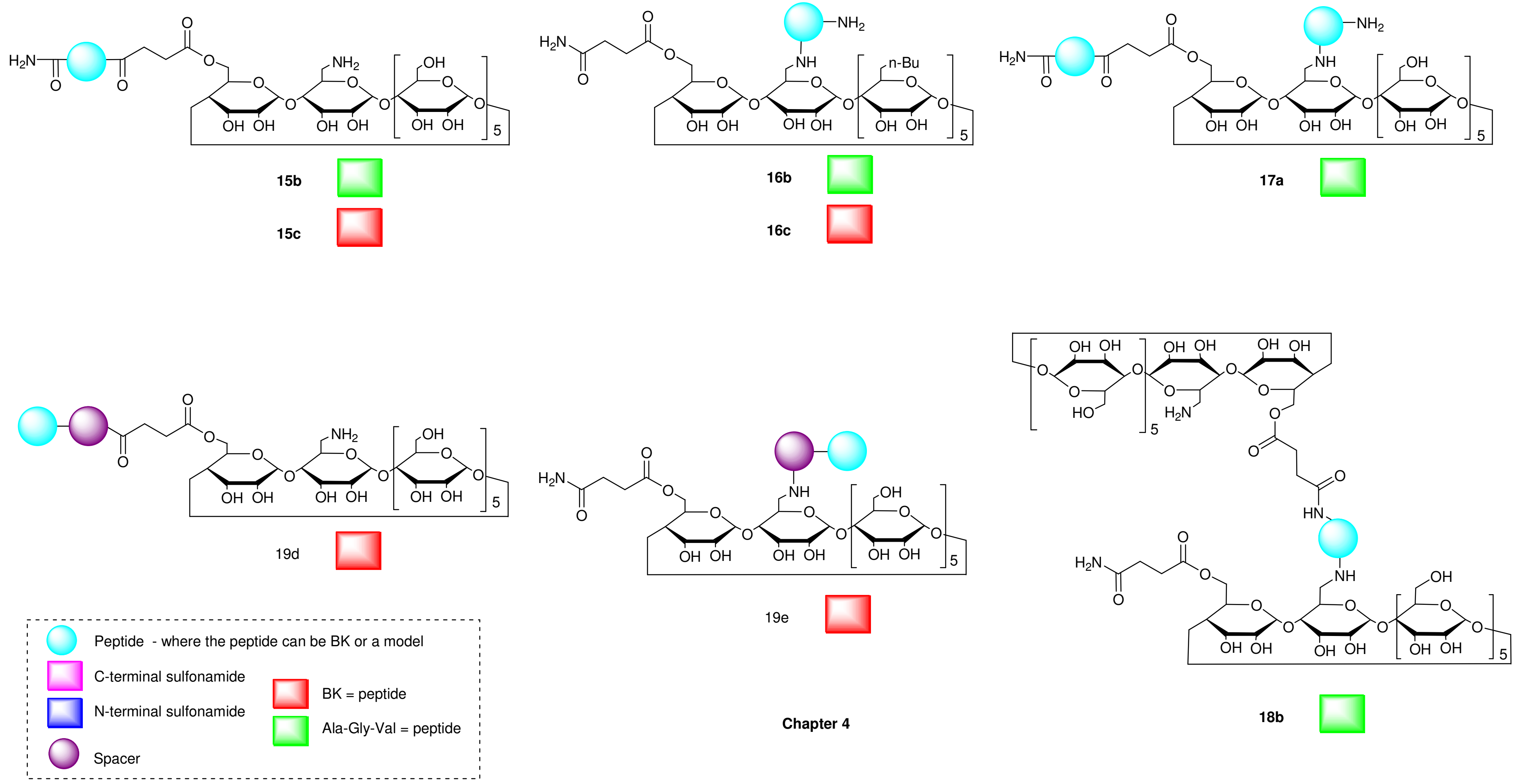


Figure 6-3 Overview of the bioassay tested molecules

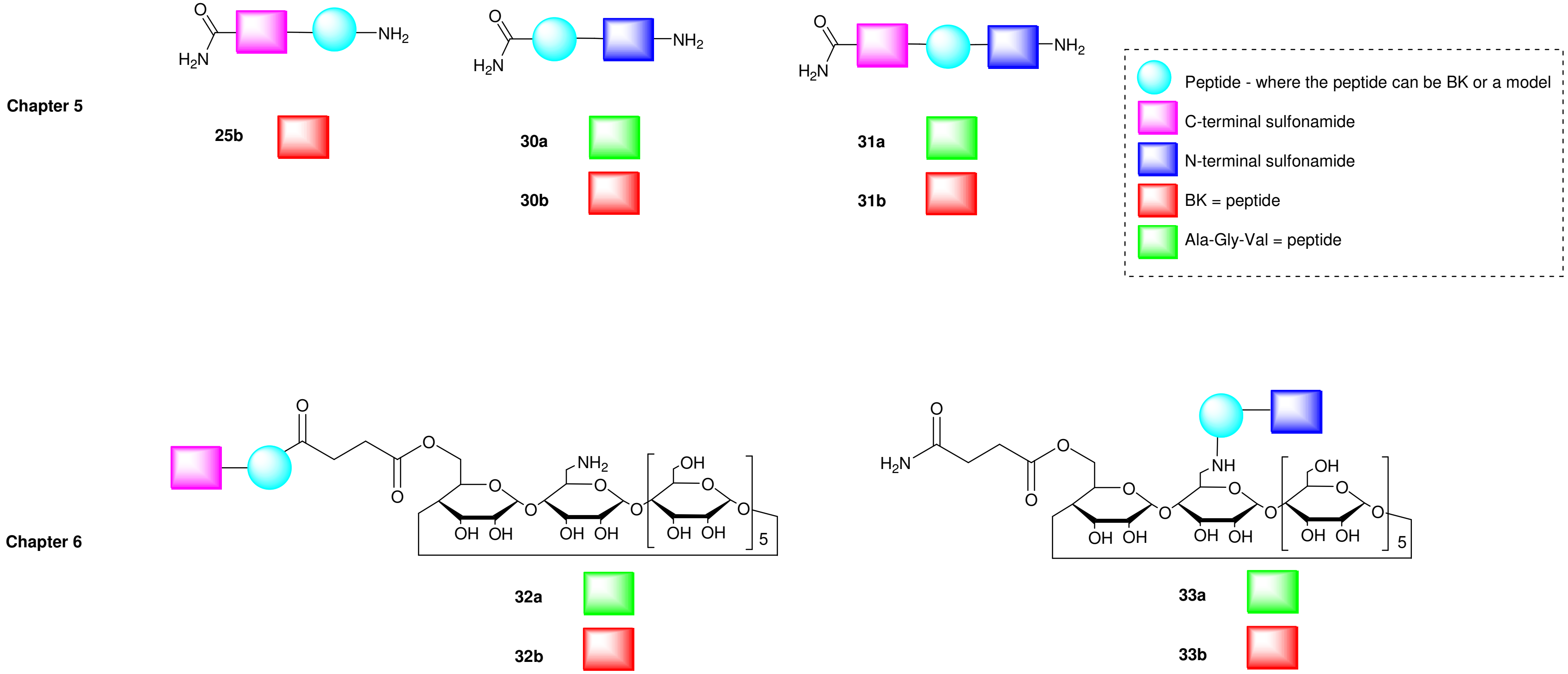


Figure 6-3 (Continued) Overview of the bioassay tested molecules

Control compounds (Table 6-3) were included in the bioassays. For example, unfunctionalised BK and β-CD were assayed.

Table 6-3 Control compounds analysed in the bioassays

Synthesis Details	*Compounds*	*Abbreviations*
Chapter 3, **Section 3.2.2**	Bradykinin	**13**
Appendix 6-IV	Model peptide (Val-Gly-Ala)	**35**
Appendix 5-IV	Sulfanilamide	**34**
Sigma-Aldrich (Germany)	4-Carboxybenzenesulfonamide	**30**
Auspep (Sydney, Australia)	Bradykinin	BK
Aldrich (USA)	β-Cyclodextrin	β-CD
Aldrich (USA)	5-Fluorouracil	5-FU

Two assays (MTT and flow cytometry) were performed on these compounds by Dr Fran Wolber's research group (IFNHH, Turitea Campus, Massey University, Palmerston North, New Zealand). Preliminary results from these assays are reported below.

5-Fluorouracil (5-FU) was also used as a control in these bioassays. This drug is known to be an effective treatment against cancer (especially stomach and rectal cancer). See **Chapter 1** for more information on 5-FU and its application to cancer treatment.

Initially studies were performed on two simple sulfonamides, 4-carboxybenzenesulfonamide (**30**) and sulfanilamide (**34**, see Figure 6-4) to determine the concentration range required for both assays.

30

34

Figure 6-4 Simple compounds used to find potential concentration ranges for the MTT assay. 4-carboxybenzenesulfonamide (**30**) and sulfanilamide (**34**)

Five cell lines were initially screened (see Table 6-4) and the ones that showed considerable cell death from the sulfonamides (**30** and **34**) were used throughout the rest of the study. These are indicated by * in Table 6-4.

Table 6-4 Cell lines used in the study for bioassay testing

Clone	*Species*	*Organ*	*Cell*	*Type*
HT-29*	Human	Duodenum	Adenocarcinoma	-
Hs700T*	Human	Pancreas	-	Carcinoma
MCF-7	Human	Breast	Adenocarcinoma	Carcinoma
MA-104*	Rhesus Monkey	Kidney	Epithelial	Transformed
K-562	Human	Haematopoietic	Erythromyeloblastomia	Leukaemia

*Cell lines used for the test compounds in this study

The MTT cell proliferation assay was used to assess the bioactivity of the more complex β-CD-peptide-sulfonamide compounds (and variations) where the peptide was the model (Val-Gly-Ala) or the bioactive peptide BK.

6.0.5 *Aims of the Bioassays*

The bioassays were carried out to answer the following questions:

1. Will the effect of the addition of the β-CD to BK result in minor or major bioactivity changes
2. Will the effect of the addition of the sulfonamide to BK result in minor or major bioactivity changes
3. Will the effect of the peptide and/or β-CD on the activity of the sulfonamide result in minor or major bioactivity changes
4. Will the effect of the spacer between the peptide and the β-CD result in minor or major bioactivity changes
5. Will the model peptides confirm the effects of the peptide, β-CD, and the sulfonamide compared to BK as the peptide

6.1 Results and Discussion

6.1.1 *C-terminal Peptide Sulfonamide Addition onto Cyclodextrin*

For the synthesis of *C*-terminal peptide sulfonamides onto β-CD, first the sulfonamide was added to the resin (as per **Chapter 5**), followed by peptide assembly (either a model or BK), and then following Fmoc-deprotection of the peptide, compound **12** was then coupled to it (as per **Chapter 4**). Deprotection of the β-CD complex Fmoc group and subsequent cleavage from the resin yielded a mono-succinimide-peptidyl-β-CD product (Figure 6-5). The peptides used in this section were Val-Gly-Ala (**32a**) and BK (**32b**). The yields for these products are low due to reasons explained in **Chapters 4** and **5**. Bioassay analyses of compounds **32a-b** are reported later in this Chapter.

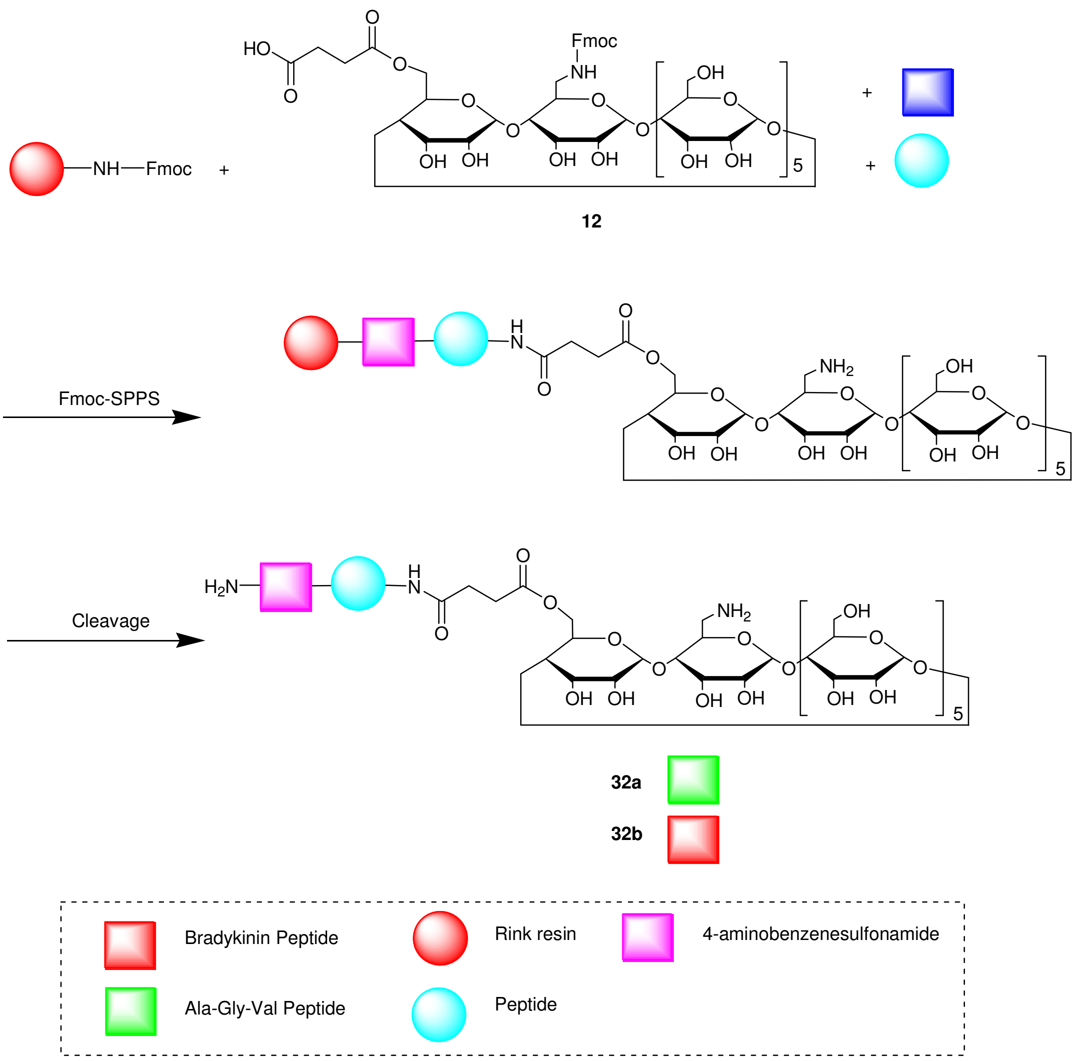


Figure 6-5 Attachment of the sulfonamide-peptide onto compound **12**

6.1.2 *N-terminal Peptide Sulfonamide Addition onto Cyclodextrin*

For synthesis of *N*-terminal peptide sulfonamides onto β-CD, first compound **12** was added to deprotected Rink resin (as per **Chapter 4**) and following Fmoc deprotection, assembly of the peptide and the sulfonamide (4-carboxybenzene sulfonamide coupled as per **Chapter 5**, **Section 5.4.5**). This can be seen in Figure 6-6. The peptides used in this section were Val-Gly-Ala (**33a**) and BK (**33b**) and although the yields were overall low, useful amounts of the complexes were obtained for bioassay analysis. Bioassay analyses of compounds **33a-b** are reported later in this Chapter.

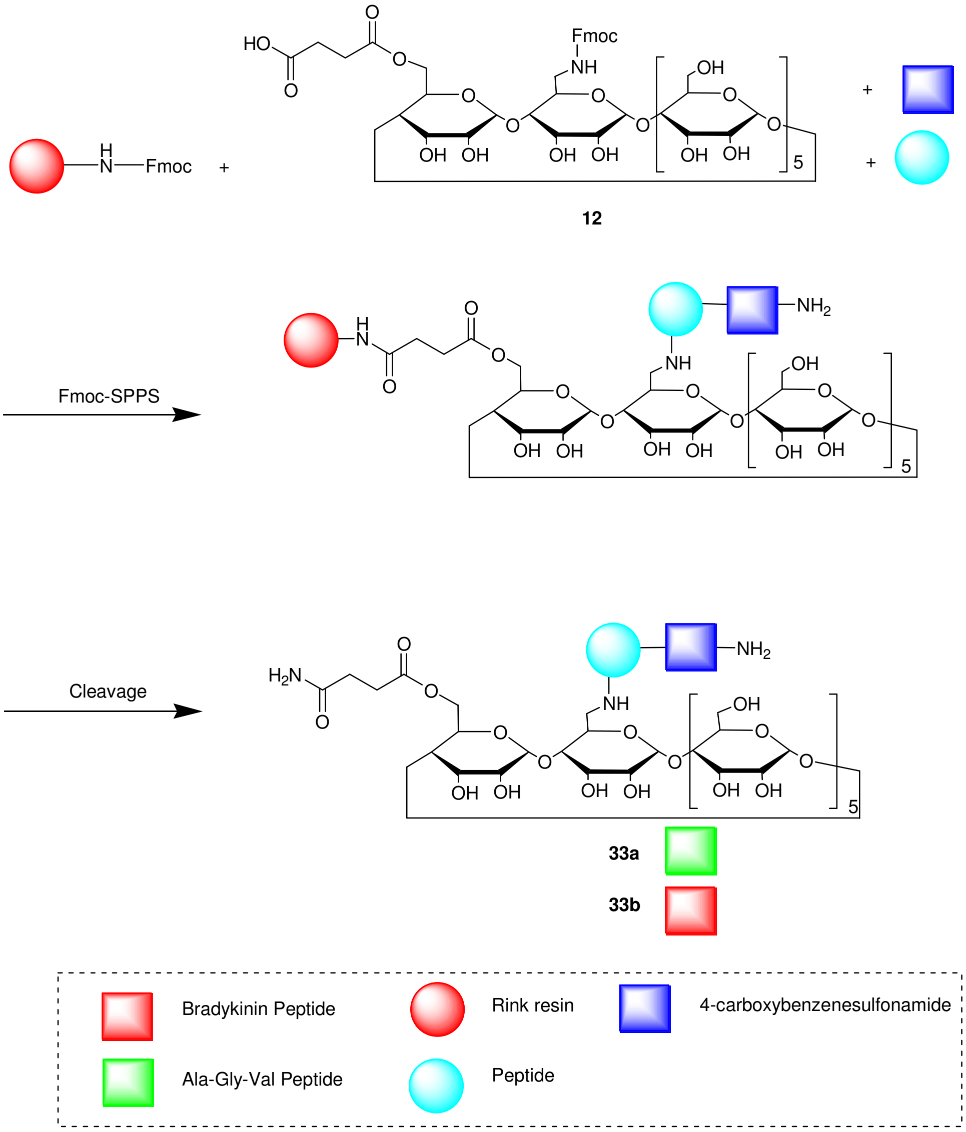


Figure 6-6 Attachment of the sulfonamide-peptide onto compound **12**

By-products present in the crude cleavage mixture of syntheses **32b** and **33b** include β-CD-BK(Mtr), BK peptide, BK(Mtr) peptide, BK-sulfonamide, BK(Mtr)-sulfonamide, and des-BK species. All indicate incomplete growth and attachment of the amino acids and β-CD/sulfonamide species and this should be investigated further in future studies. Also collected at the end of coupling was the unfunctionalised β-CD moiety (**12**, as per **Chapter 4**). This was isolated, purified, and reused for subsequent synthesises. By-products for syntheses **32a** and **33a** were similar to those of **32b** and **33b** with the exception of the Mtr species presence.

6.1.3 *Bioassay Testing of Compounds **30** and **34***

Refer to Figure 6-4 for structural details of compounds **30** and **34**. The MTT assay and the flow cytometry assay were used to screen the BK and model peptide based compounds and gave information on their structure-function relationships. 5-FU was used as a control for this part of the study as this molecule is a known anti-cancer agent (see **Chapter 1**, **Section 1.0.1.2** for more details). For information on stages of a cell cycle refer to **Appendix 6-V**.

6.1.3.1 HT-29 (Human Duodenum) Cell Line

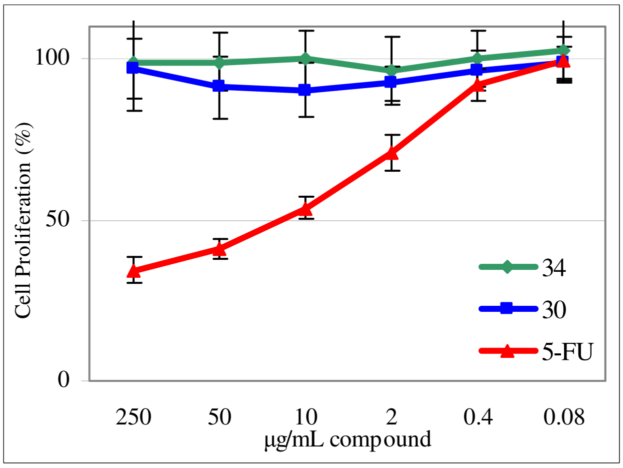
5-FU significantly diminished proliferation in a linear dose-dependent fashion after 72h of treatment compared to cells only for the following cell lines:

- Human duodenal adenocarcinoma line HT-29 (Graph 6-1). 5-FU was ineffective on the HT-29 cells at concentrations less than 0.4 μg/mL, and the effect of 5-FU was maximal at doses greater than 50 μg/mL
- Human pancreatic adenocarcinoma line Hs700T (**Section 6.1.3.2**)
- Human breast carcinoma cell line MCF-7 (**Section 6.1.3.3**)
- Monkey kidney carcinoma line MA-104 (**Section 6.1.3.4**)
- Human leukaemia K-562 (**Section 6.1.3.5**)

The model sulfonamides, **30** and **34** had little effect on the cell proliferation in the following cell lines:

- HT-29 cell line (Graph 6-1) had no effect
- MA-104 cell line (**Section 6.1.3.4**) had a slight effect at low concentrations but paradoxically appeared again to have little effect at 50–250 µg/mL

Graph 6-1 Cell proliferation effect (MTT assay) of **30**, **34**, and 5-FU on HT-29 (human duodenal adenocarcinoma) cells after 72h

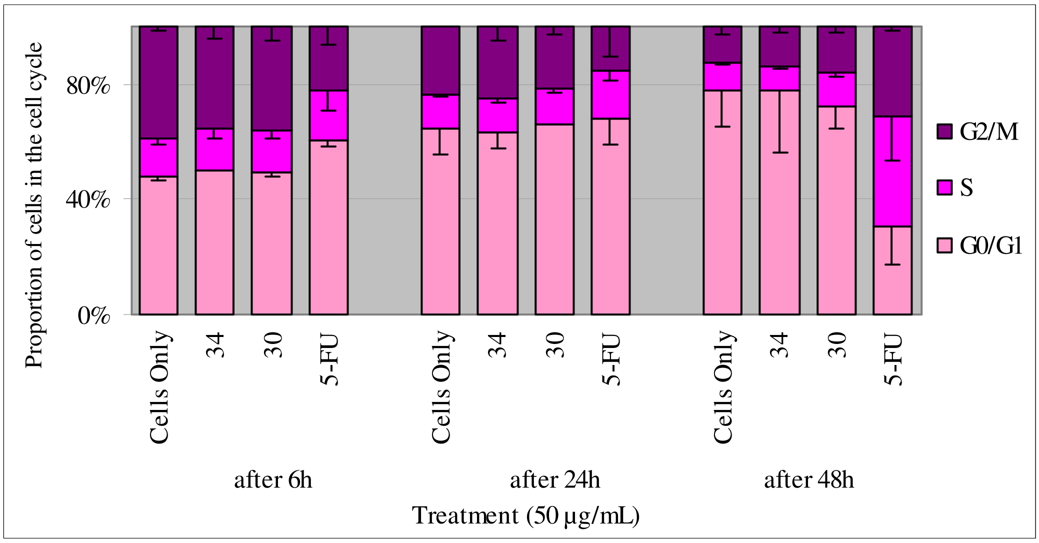


In the flow cytometry assay (duplicate experiments), 5-FU treatment after 48h resulted in the arrest of HT-29 and K-562 cells in the S/G2/M phase of cell cycle (Graph 6-2 and **Section 6.1.3.5** respectively) with treatment with 5-FU at 50 µg/mL for 24h or 48h causing MA-104 cells to partially arrest in the S/G2/M phase of the cell cycle (**Section 6.1.3.4**).

Compounds **30** and **34** had no marked effect at any time point in the following cell cycles:

- Human duodenal adenocarcinoma line HT-29 (Graph 6-2)
- Human pancreatic adenocarcinoma line Hs700T (**Section 6.1.3.2**) – only compound **30** showed no discernible effect on cell cycle
- Human breast carcinoma cell line MCF-7 (**Section 6.1.3.3**)
- Monkey kidney carcinoma line MA-104 (**Section 6.1.3.4**)

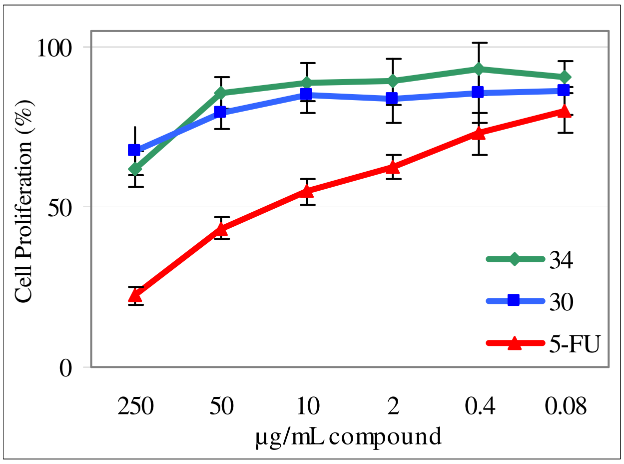
Graph 6-2 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on HT-29 (human duodenal adenocarcinoma) cells after 72h



6.1.3.2 HS700T (Human Pancreas) Cell Line

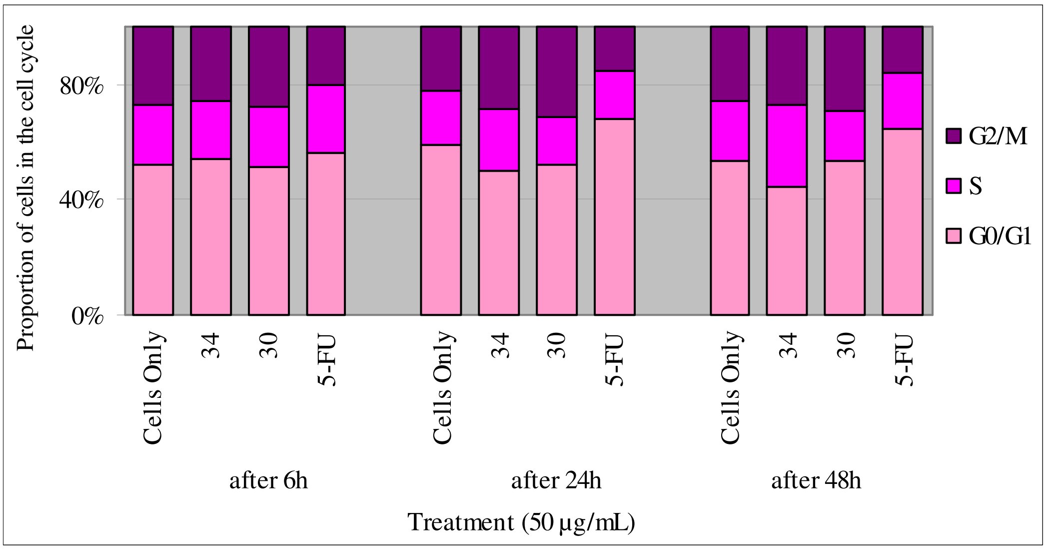
Compounds **30** and **34** slightly decreased Hs700T cell proliferation when present from 0.08–50 µg/mL, with the effect not appearing to be dose-dependent (Graph 6-3). At 250 µg/mL, **30** and **34** caused a more pronounced decrease in Hs700T proliferation. Neither **30** nor **34** were as effective as 5-FU, but at 250 µg/mL showed *c.a.* 25% reduction in cell proliferation compared to the control sample (cells only). Every concentration of 5-FU from 250-0.08 µg/mL was significantly lower ($p \leq 0.015$ by two-tailed Student's t-test).

Graph 6-3 Cell proliferation effect (MTT assay) of **30**, **34**, and 5-FU on Hs700T (human pancreatic adenocarcinoma) cells after 72h



In the flow cytometry assay, the proportion of Hs700T cells in G0/G1 was higher after 48h of treatment with 5-FU compared to the control (Graph 6-4).

Graph 6-4 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on Hs700T (human pancreatic adenocarcinoma) cells after 72h

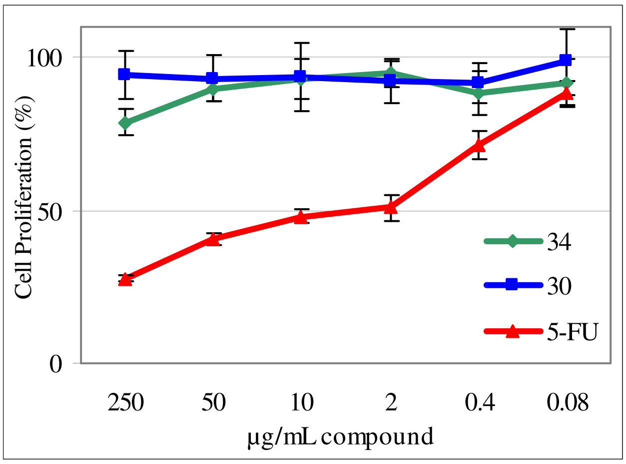


This was also observed, though to a lesser extent, after 24h. Compound **34** treatment increased the proportion of cells in the S/G2/M stages at both 24h and 48h. In future, it may be worthwhile assessing 3, 4, and 5 day time points and/or assessing the combined effect of the test compounds with 5-FU to look for possible synergistic effects.

6.1.3.3 MCF-7 (Human Breast) Cell Line

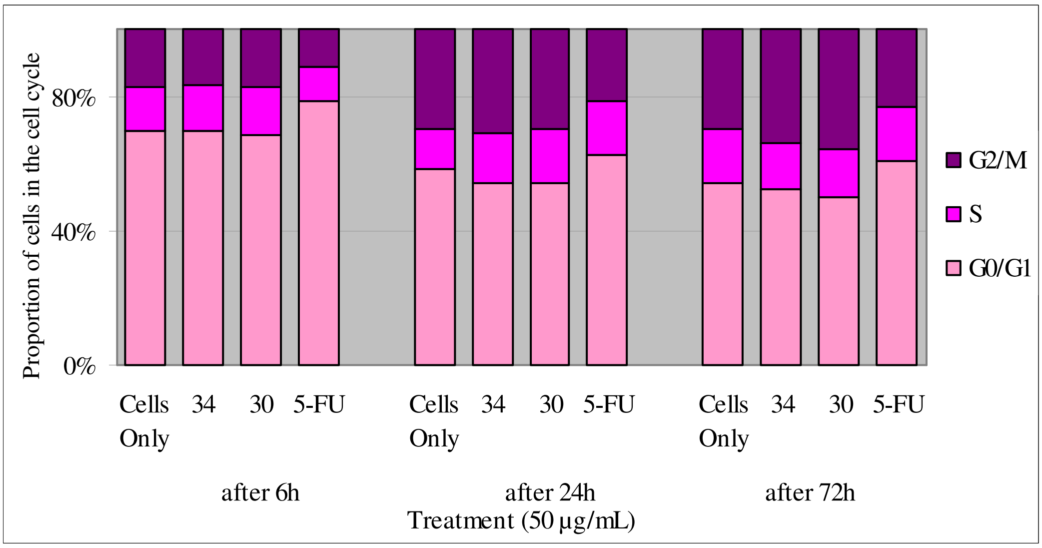
The sulfonamides, **30** and **34** (structures given in Figure 6-4) had very minor effects on these cells when present from 0.08–50 µg/mL, with the decrease in cell proliferation not appearing to be dose-dependent (Graph 6-5). At 250 µg/mL, **34** caused a more pronounced decrease in MCF-7 cell proliferation. Neither **30** nor **34** were as effective as 5-FU with only a 15-20% reduction compared to the control.

Graph 6-5 Cell proliferation effect (MTT assay) of **30**, **34**, and 5-FU on MCF-7 (human breast carcinoma) cells after 72h



5-FU partially blocked the progression of MCF-7 cells into the G2/M phase of cell cycle after 48h (Graph-6-6). This effect was evident as early as 6h.

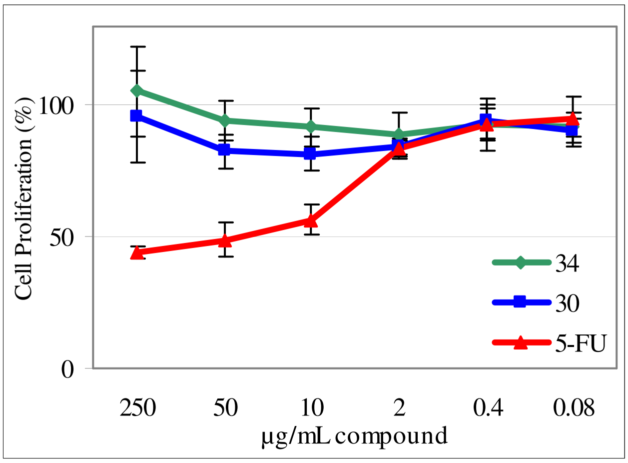
Graph 6-6 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on MCF-7 (human breast carcinoma) cells after 72h



6.1.3.4 MA-104 (Monkey Kidney) Cell Line

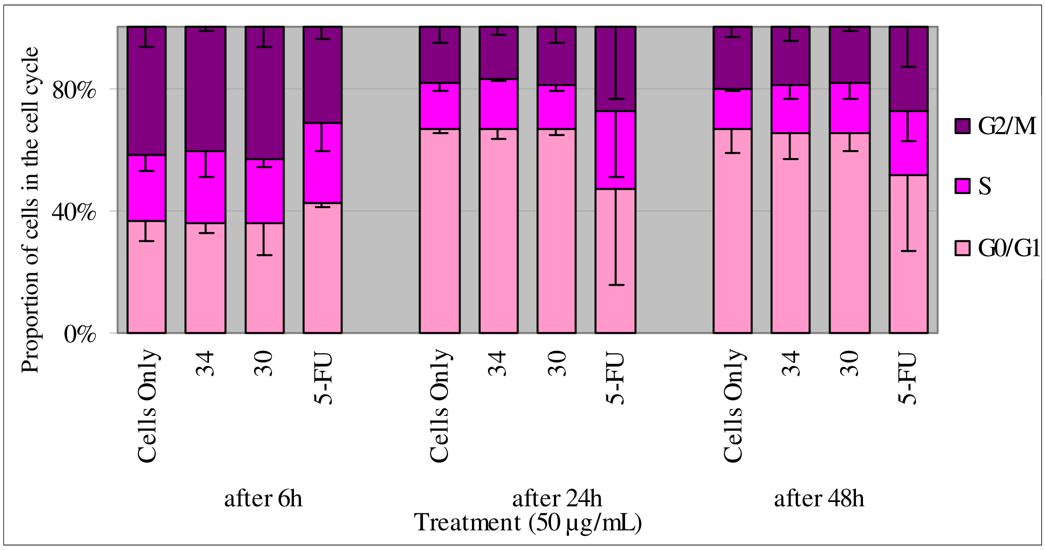
The cancer control, 5-FU at 2–0.08 µg/mL had a minor effect on proliferation of the monkey kidney carcinoma line MA-104. When present from 10–250 µg/mL, 5-FU diminished MA-104 proliferation in a dose-dependent fashion (Graph 6-7). Every concentration of 5-FU from 250-0.8 µg/mL was significantly lower ($p \leq 0.015$ by two-tailed Student's t-test). However, the effect was weak at 0.4-0.08 µg/mL and remained maximal at greater than 50 µg/mL.

Graph 6-7 Cell proliferation effect (MTT assay) of **30**, **34**, and 5-FU on MA-104 (monkey kidney carcinoma) cells after 72h



As per **Section 6.1.3.1** with no observable effect of compounds **30** and **34** on this cell line (graph 6-8).

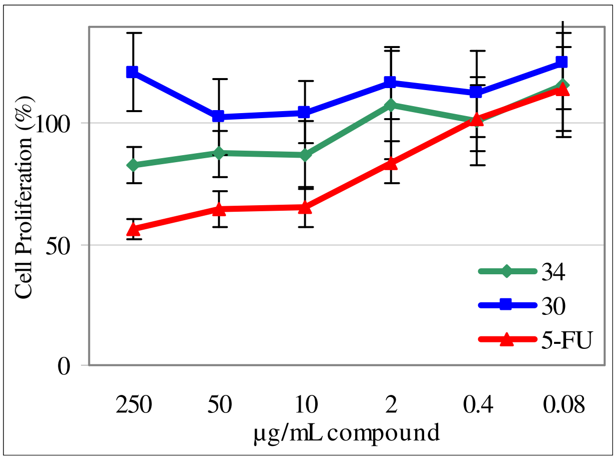
Graph 6-8 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on MA-104 (monkey kidney carcinoma) cells after 72h



6.1.3.5 K-562 (Human Leukaemia) Cell Line

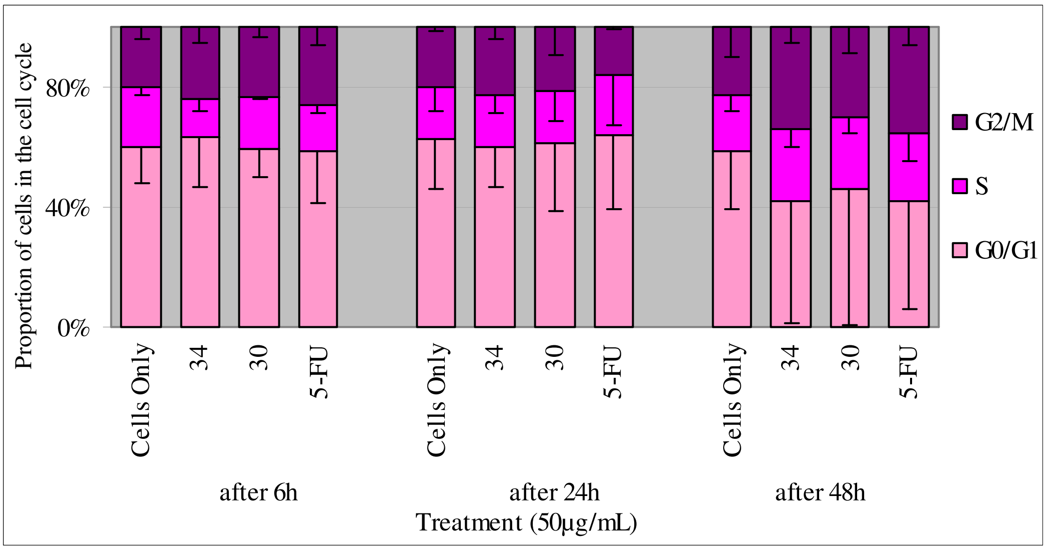
As per **Section 6.1.3.1** with no dramatic effect of compounds **30** and **34** on this cell line (graph 6-9).

Graph 6-9 Cell proliferation effect (MTT assay) of **30**, **34**, and 5-FU on K-562 (human leukaemia) cells after 72h



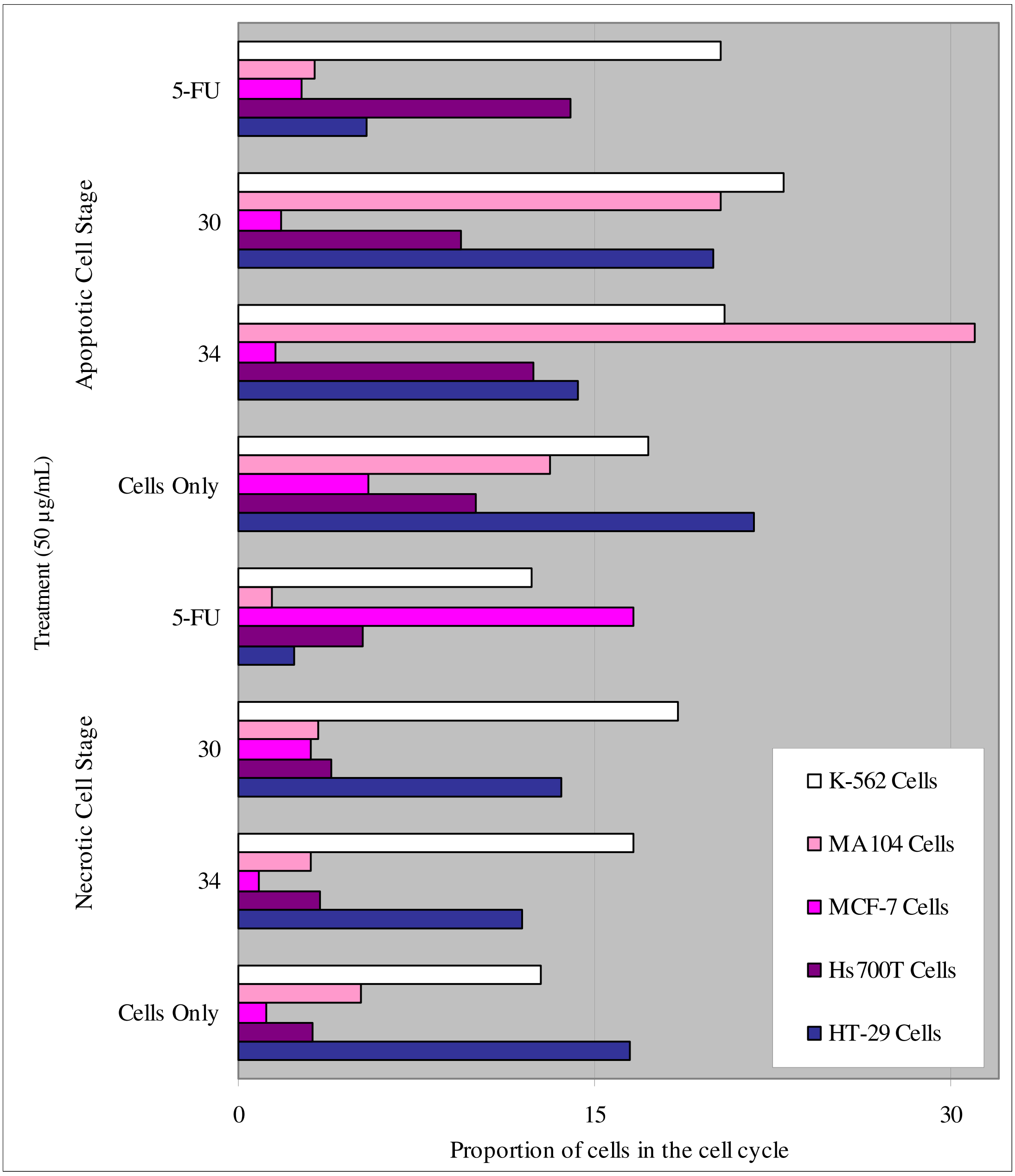
Interestingly, **30** and **34** caused K-562 cells to arrest in the S/G2/M phase of cell cycle after 48h (Graph 6-10). This was not evident at 6h or 24h.

Graph 6-10 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on K-562 (human leukaemia) cells after 72h



Treatments with 5-FU, **30**, and **34** also appeared to increase the proportion of apoptotic/necrotic cells (Graph 6-11). For future studies, it would be worth repeating the cell cycle experiment, and perhaps also to assess these compounds in other leukaemia cell lines.

Graph 6-11 Cell cycle analysis (flow cytometry assay) for **30** and **34** and 5-FU on K-562 (human leukaemia) cells after 72h looking at the necrotic and apoptotic cell stages



6.1.3.6 Section Summary

It was found that 5-FU diminished cell proliferation in all the cell lines tested at a linear dose dependent rate. Model sulfonamides, **30** and **34** were used to determine the scope for each assay (concentration ranges, solvent conditions and reaction conditions), and possible results.

From these tests, it was found that compounds **30** and **34** had little effect in either the MTT or cell cycle assay on all 5 cell lines tested (see Table 6-5 for an overview of this data). It was however, interesting to note that in the HS700T, HT-29, and the MA-104 cell lines, there was slight cell proliferation (both increased and decreased) although it was not dose dependent. Another interesting point is the cell growth caused by **34** at stages S/G2/M in the HS700T cell line, not seen in the other 4 cell lines.

From these results, three cells lines were chosen to be used throughout the rest of the study. These were the HT-29, Hs700T, and the MA-104 cell lines. These were chosen as the most cooperative adherent cell lines. The next section details bioassay testing on the cyclodextrin and/or peptide sulfonamide compounds. Cell cycle arrest was also caused by both **30** and **34** in the K-562 cell line, however more studies are required to further investigate this data.

Table 6-5 Overview of the results for compounds **30** and **34** when assayed using the MTT and flow cytometry assays on a number of different cell lines

Cell Line	*Cell proliferation (MTT assay)*	*Cell stage (flow cytometry assay)*	*Comments*
HT-29	No effect	No effect	-
HS700T	Slight decrease from 0.08-50 µg/mL but not dose dependent. More pronounced at 250 µg/mL but not as effective as 5-FU.	Compound **34** increases the growth in cell stages S/G2/M at the 24h and 48h marks. Compound **30** has no effect.	Look at 3, 4, and 5 day marks for any patterns.
MCF-7	Minor decrease in cell proliferation from 0.08-50 µg/mL. Not dose dependent. **34** caused pronounced proliferation at 250 µg/mL. Neither as effective as 5-FU	Little effect on cell cycle at any point	-
MA-104	Had slight effect at low concentrations but appeared ineffective at 50-250 µg/mL.	No effect	-
K-562	No effect of either **30** or **34**	Both caused cell arrest to the S/G2/M phase of the cell cycle after 48h, not evident at 6h or 24h. Both also seemed to increase the proportion of apoptotic/necrotic cells.	Repeat the cell cycle experiment and asses other leukaemia cell lines

6.1.4 *Bioassay Testing of Cyclodextrin-peptide-sulfonamide Complexes and Components Thereof*

The MTT assay was used to investigate the bioactive activity of BK and model peptide-based compounds synthesised in previous chapters. Refer to Tables 6-2 and 6-3 and Figure 6-3 for synthesis and structural details of the compounds used in this section.

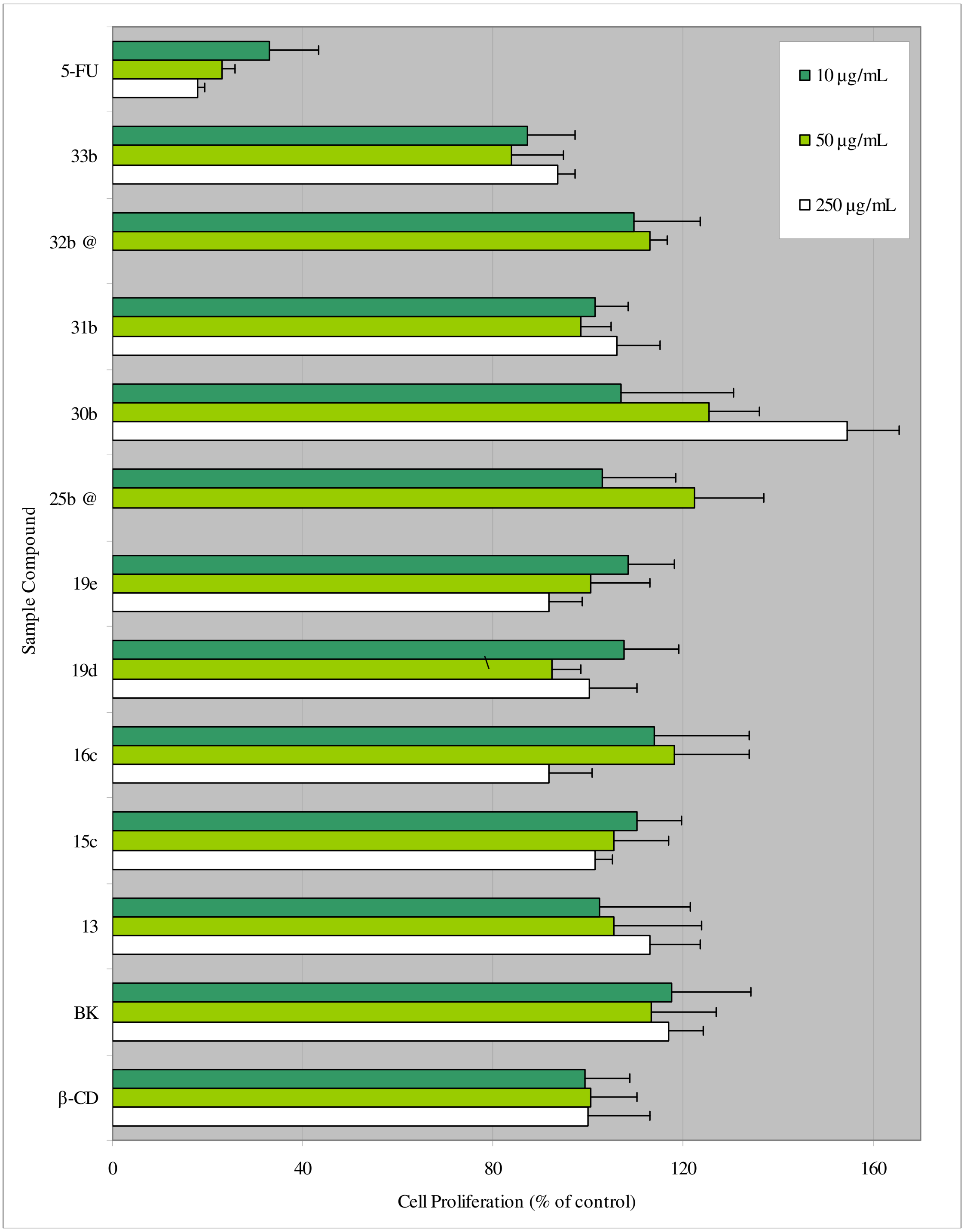
6.1.4.1 Bradykinin Peptide Effect on HT-29 (Human Duodenum), Hs700T (Human Pancreas), and MA-104 (Monkey Kidney) Cell Lines

5-FU significantly diminished proliferation of the human duodenal adenocarcinoma line HT-29 (Graph 6-12), the human pancreatic adenocarcinoma line Hs700T (Graph 6-13), and the monkey kidney carcinoma line MA-104 (Graph 6-14), in a linear dose-dependent fashion. Commercial bradykinin (BK) and bradykinin (**13**, synthesised in our laboratory) slightly increased proliferation of HT-29 and Hs700T cells when present at 250 µg/mL, while having no effect on the MA-104 cells. When BK was present at approximately 10 µg/mL, it was seen to increase cell proliferation by 10–20% in some epithelial cells as shown for commercial and self-made BK.[6] β-CD itself had little effect on any of the cell lines.

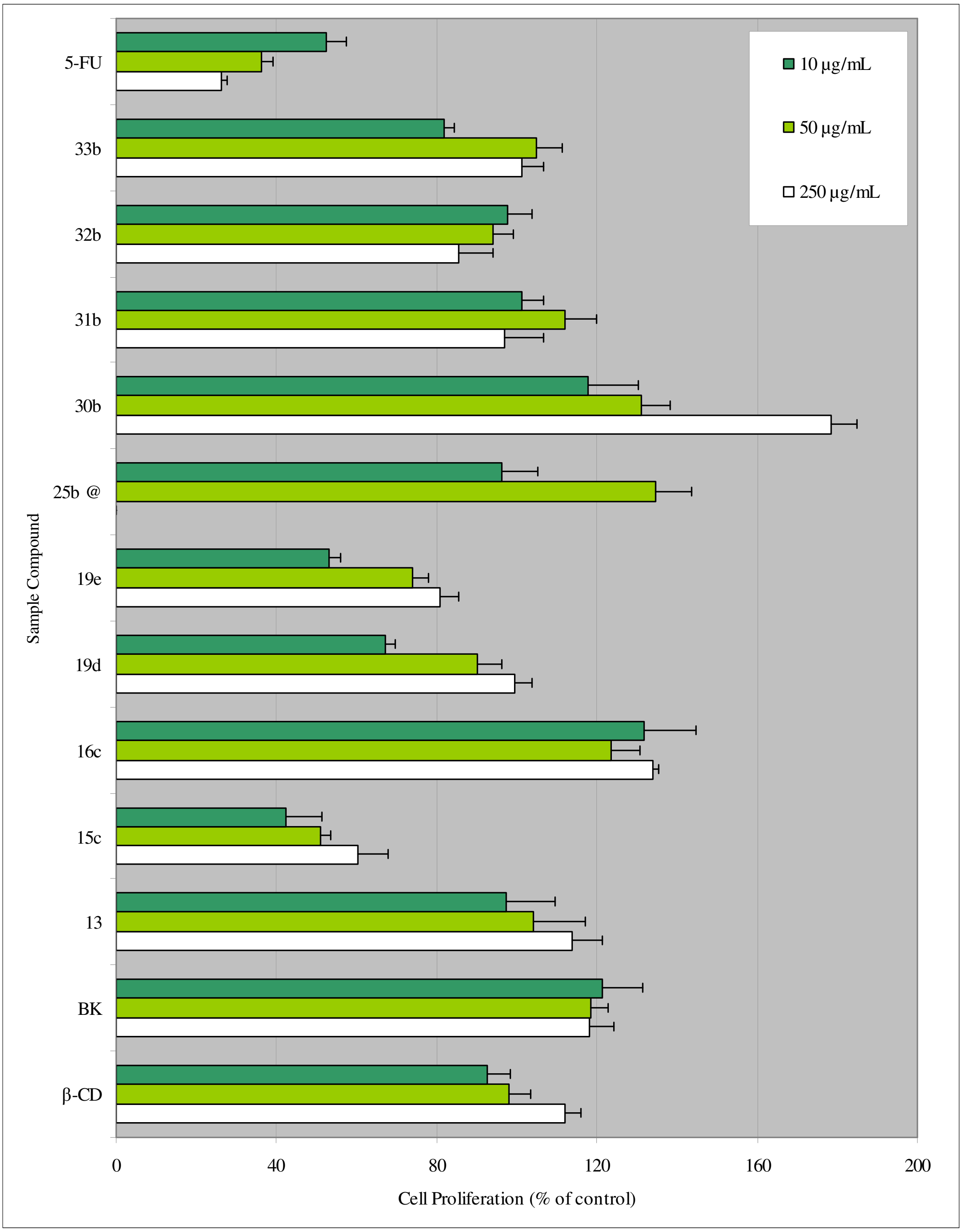
Test compound **31b** (*C*- and *N*-terminal BK sulfonamide) did not diminish cell proliferation in any cell line tested. **13** (lab synthesised BK) nor did any of the other test compounds significantly decrease cell proliferation in the three cell lines (compared to naked β-CD or untreated cells). Testing of compounds **25b** (*C*-terminal BK peptide) and **32b** (*C*-terminal BK sulfonamide-β-CD) were incomplete due to an insufficiency of test sample (no 250 µg/mL test completed). These are marked with @ in Graphs 6-12, 6-13, and 6-14.

Most of the β-CD-bradykinin-sulfonamide combinations had little effect or showed very slight (*c.a.* 10-20% in a few cases) decreased proliferation relative to β-CD or BK alone. Sample **30b**, an *N*-terminal BK-sulfonamide in all three cell lines and **16c** (spacer-β-CD-BK) in the Hs700T and HT-29 cell lines did increase the percentage of cell proliferation when present at 250 µg/mL. This suggests that adding the sulfonamide and the β-CD to the BK peptide in these configurations did not detract from the slight proliferation-enhancing quality observed with bradykinin alone, and may have slightly augmented it or given an additive β-CD or sulfonamide plus BK effect. It is interesting to note that cell proliferation was decreased for the MA-104 cell line when treated with **16c**.

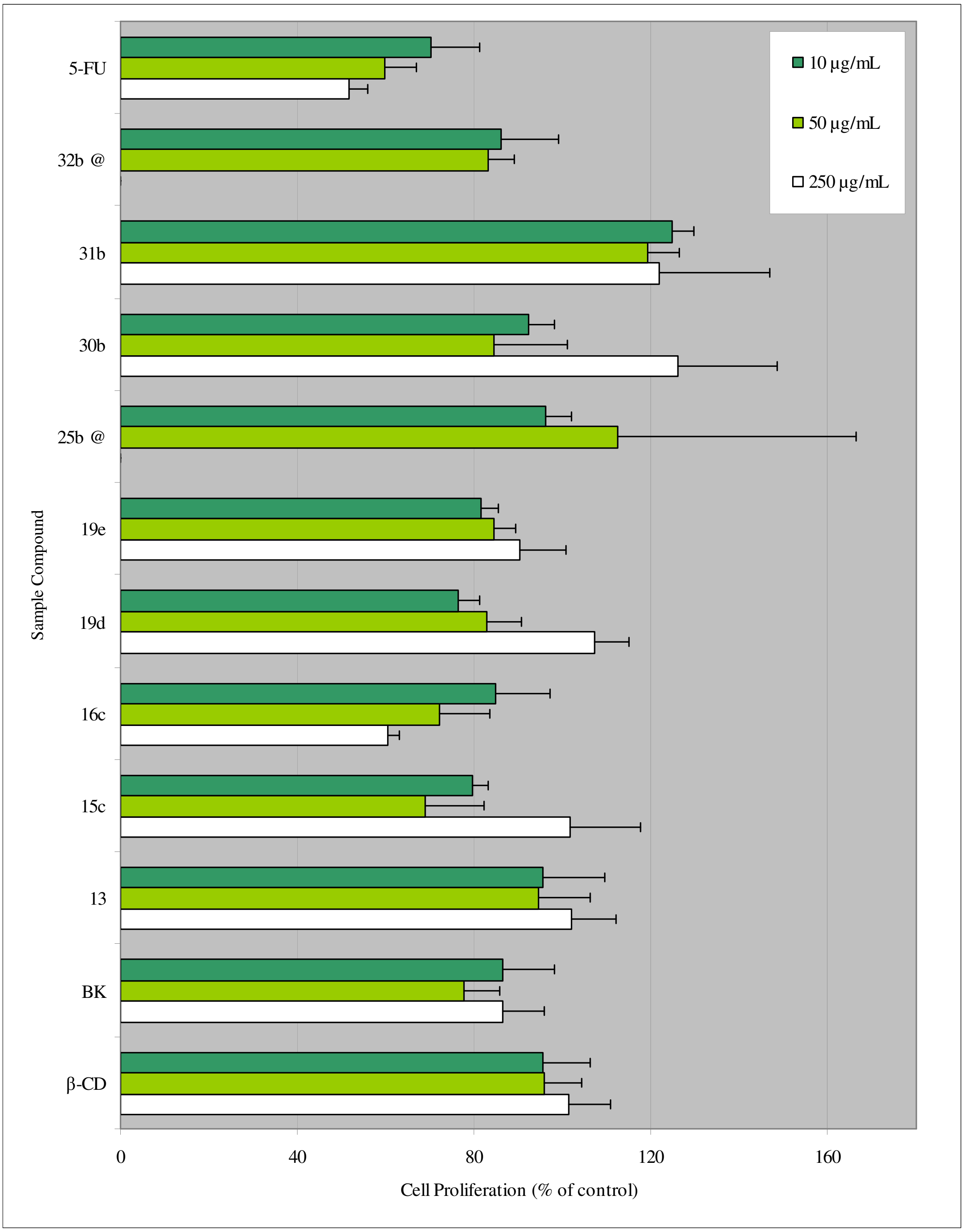
Graph 6-12 Cell proliferation effect (MTT assay) of cyclodextrin-bradykinin-sulfonamide complexes on HT-29 (human duodenal adenocarcinoma) cells after 72h



Graph 6-13 Cell proliferation effect (MTT assay) of cyclodextrin-bradykinin-sulfonamide complexes on Hs700T (human pancreatic adenocarcinoma) cells after 72h



Graph 6-14 Cell proliferation effect (MTT assay) of cyclodextrin-bradykinin-sulfonamide complexes on MA-104 (monkey kidney carcinoma) cells after 72h



Test compound **15c** (BK-spacer-β-CD) had the opposite effect to **16c** (spacer-β-CD-BK) in the Hs700T cell line with high cell proliferation at all three tested concentrations (not present in the other two cell lines). This suggests that the free carboximide terminus of BK is important for an enhanced activity compared to the commercial and lab-made BK (**13**). This change in cell growth could be associated with an increase in BK's activity by the addition of a bulky substituent at one terminus. It has been shown that introduction of a large bulky amino acids at the *C*-terminus is crucial for antagonist activity of BK (see **Chapter 3**, **Section 3.0** for more details).[7]

It has also been shown that exchange of the cleavage point amino acids, Arg_1, Phe_5, and Arg_9 with other amino acids can lead to an increase in BK's half-life.[10,16] Therefore addition of the BK peptide onto β-CD alters its activity. Further tests need to be done to determine the half life of these compounds in the cell and what can be attributed to this high cell proliferation compared to other test compounds.

Overall, the Hs700T cell line responded best to the BK test compounds giving an increase (or decrease) in cell proliferation for some samples. However, apart from **15c** which showed moderate anti-proliferation activity, none of the test compounds compared well with 5-FU as an anti-cancer agent. It may be worthwhile putting 5-FU in the bucket of β-CD (with or without BK) to see if this affects the efficacy of this known anti-cancer agent. Alternatively, if β-CD (or BK) can be tagged with a label such as FITC or biotin, it may be possible to measure the binding of the individual and combined components to cells and/or to track their movement *in vivo*. Using known powerful bioactive sulfonamide drugs would also aid in the analysis of these results.

6.1.4.2 Model Peptide Effect on HT-29 (Human Duodenum), Hs700T (Human Pancreas), and MA-104 (Monkey Kidney) Cell Lines

For the bioactivity of compounds based upon model peptides (**15b**, **16b**, **17a**, **18b**, **30a**, **31a**, **32a**, **33a**), refer to **Appendix 6-VI**. Model peptides in this study were used to show:

- Proof-of-concept that the syntheses of β-CD-peptide-sulfonamide complexes (and components thereof) can be achieved using Fmoc SPPS on resin
- Expand the scope of this limited β-CD-peptide-sulfonamide bioassay study

The data showed that **17a** (model peptide-spacer-β-CD-model peptide) had very interesting results with a high amount of cell proliferation and this would be interesting to test with the BK peptide (not done). The model peptides did not diminish cell growth and little else was observed with the tests performed on the model test compounds.

6.2 Materials and Methods

6.2.1 *Reagents and Instruments*

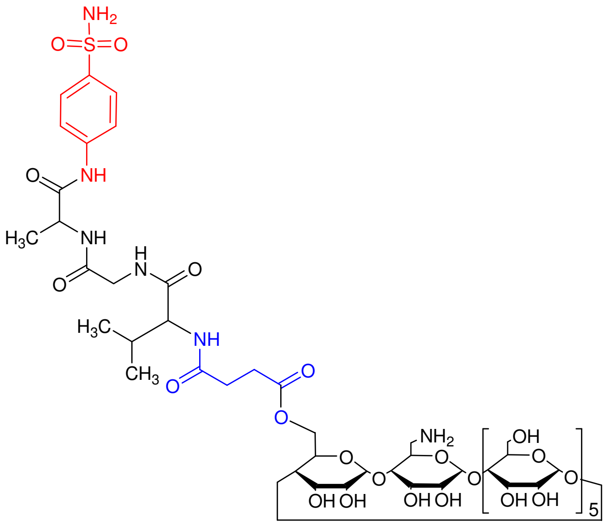
The same reagents and instruments were used as per **Chapter 2**, **Section 2.4.1** and **Chapter 3**, **Section 3.2.1** throughout this chapter, except where noted otherwise.

Delbecco's Modified Eagle's Medium (DMEM), (RPMI), penicillin-streptomycin-L-glutamine (PSG), heat-inactivated foetal calf serum (FCS), sodium bicarbonate solution, and trypsin-EDTA were obtained from Invitrogen. Culture flasks and 96-well flat-bottom plates were purchased from RayLabs. Dimethyl sulfoxide (DMSO), propidium iodide (PI), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich.

6.2.2 *Experimental for C-terminal cyclodextrin-peptide-sulfonamides*

The synthetic route is illustrated in Figure 6-5

6.2.2.1 Mono-6^{A}-amino-mono-6^{X}-succinyl-6^{X}-(4-aminobenzenesulfonamide-Val-Gly-Ala)-β-cyclodextrin (**32a**)

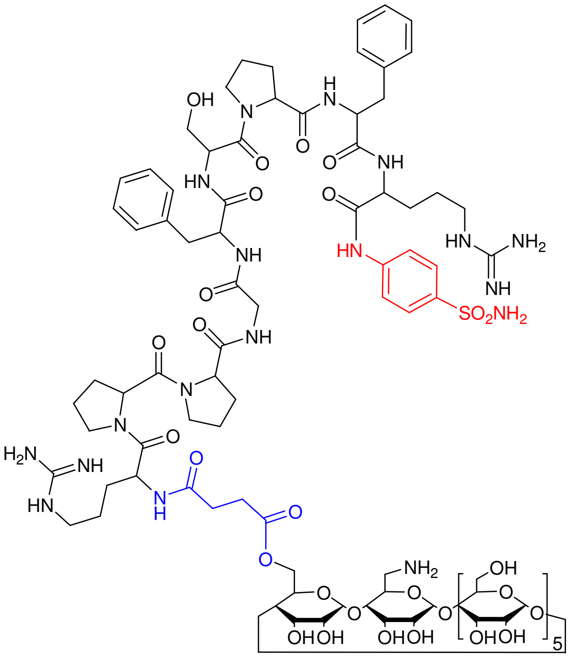


32a

Val-Gly-Ala was assembled manually on Rink resin (400 mg, 0.292 mmol, 1 equiv) using Fmoc-SPPS as per **Appendix 6-IV**. Fmoc-L-amino acids (1.168, 4 equiv) and mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12**, **Chapter 2**, **Section 2.4.4.4**, 1.699 g, 1.168 mmol, 4 equiv) were coupled as per **15a**, **Chapter 4**, **Section 4.7.3.1**. 4-Fmoc-aminobenzenesulfonyl chloride (483 mg, 1.168 mmol, 4equiv) was coupled using a 1:1 pyridine:DCM slurry (3 mL) overnight (see **Chapter 5**, **Sections 5.4.3.2** and **5.4.3.3** for more details). A 20% TFA in DCM mixture was used to cleave the peptide from the resin. RP-HPLC purification using a MeOH stepwise gradient as per **Chapter 2**, **Section 2.4.4.4** eluting the product at 46% B (13.2 mg, trace %).

LR-MS: *m/z* (%, assignment) 1616.51 (80, $M+H)^+$, 1229.46 (80, $M+2)^{2+}$ HR-MS: *m/z* $C_{62}H_{98}N_5O_{42}S_1$ $(M+H)^+$ 1616.3321, actual 1615.3395; ^{1}H NMR (500 MHz, d_6-DMSO): δ1.00-1.02 (m, 10H, succinyl CH_2, valine CH_3), 1.19-1.24 (m, 3H, alanine CH_3), 2.10-2.18 (m, 1H, valine CH), 3.48-3.81 (m, 43H, CD H2-H6), 3.81-3.94 (m, 2H, glycine CH_2), 4.19 (t, J = 7.6 Hz, 1H, alanine CH), 4.26 (t, J = 7.7 Hz, 1H, valine CH), 4.44-4.56 (m, 5H, CD OH6), 4.82-4.89 (m, 7H, CD H1), 5.64-5.86 (m, 12H, CD OH2, OH3), 7.42 (t, J = 7.9 Hz, 2H, aromatic), 7.55 (d, J =7.9, 2H, aromatic), 7.71-7.75 (m, 1H, alanine NH), 7.96-8.26 (m, 1H, valine NH), 8.15-8.22 (m, 1H, glycine NH); partial ^{13}C DEPT NMR assignment (500 MHz, d_6-DMSO): δ 17.6 (valine CH_3), 18.3 (valine CH_3), 18.4 (valine CH_3), 18.5 (valine CH_3), 18.60 (valine CH_3), 18.62 (valine CH_3), 18.67 (alanine CH_3), 18.69 (alanine CH_3), 18.8 (alanine CH_3), 19.6 (valine CH), 28.6 (succinyl CH_2), 29.6 (succinyl CH_2), 30.3 (succinyl CH_2), 30.6 (succinyl CH_2), 42.5 (glycine CH_2), 42.7 (glycine CH_2), 47.6 (alanine CH), 48.4 (valine CH)

6.2.2.2 Mono-6^{A}-amino-mono-6^{X}-succinyl-6^{X}-(4-aminobenzenesulfonamide-bradykinin)-β-cyclodextrin (**32b**)



32b

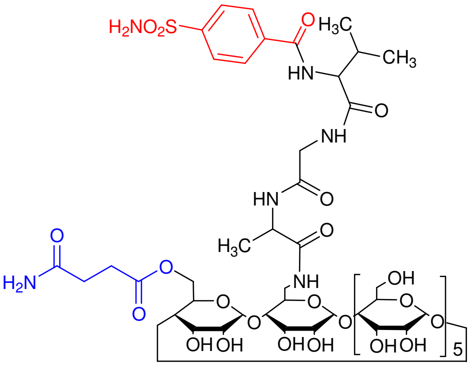
BK was assembled manually on Rink resin (100 mg, 0.072 mmol, 1 equiv) using Fmoc-SPPS as per **Section 6.2.2.1**. TFA cleavage as per **Chapter 3**, **Section 3.2.2.1** was used to cleave the peptide from the resin. RP-HPLC purification as per **Section 6.2.2.1** with the pure product eluting at 47% B (1.2 mg, 0.7%).

LR-MS: *m/z* (%, assignment) 2429.81 (50, M+Na)$^{+}$ HR-MS: *m/z* $C_{102}H_{154}N_{18}O_{48}S_1$ (M+2H)$^{+2}$ 1215.4851, actual 1215.4860

6.2.3 *Experimental for N-terminal Cyclodextrin-peptide Sulfonamides*

The synthetic route is illustrated in Figure 6-6

6.2.3.1 Mono-6^A-amino-6^A-(4-aminobenzenesulfonamide-Val-Gly-Ala)-mono-6^X-succinyl-6^X-β-cyclodextrin (**33a**)



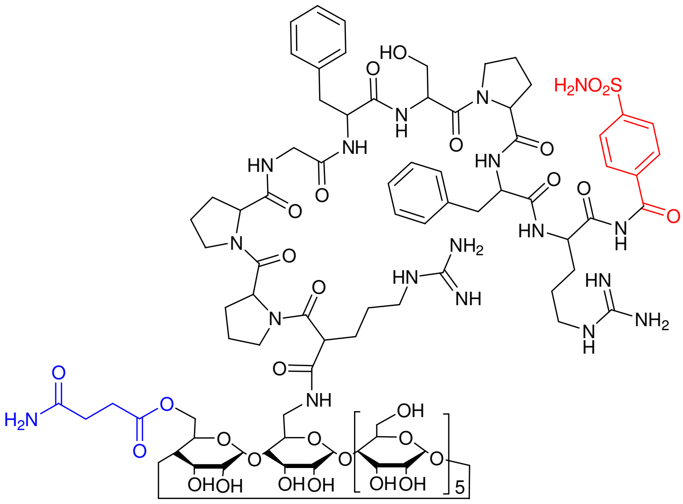
33a

Val-Gly-Ala was assembled manually on Rink resin (550 mg, 0.401 mmol, 1 equiv) using Fmoc-SPPS as per **Chapter 3**, **Section 3.2.2.1**. Fmoc-L-amino acids (1.606, 4 equiv), **12** (**Chapter 2**, **Section 2.4.4.4**, 2.336 g, 1.606 mmol, 4 equiv) were coupled as per **32a** (**Section 6.2.2.1**). 4-carboxybenzene sulfonic acid (**30**, 235 mg, 1.168 mmol, 4 equiv) was coupled using the same reaction conditions as per **30** (**Chapter 5**, **Section 5.4.5**). RP-HPLC purification using a MeOH stepwise gradient as per **Chapter 2**, **Section 2.4.4.4**. The product came off at 46% B (3.4 mg, trace %).

LR-MS: *m/z* (%, assignment) 1666.07 (100, M+H)$^+$ HR-MS: *m/z* $C_{63}H_{98}N_6O_{42}S$ (M+H)$^+$ 1665.5330, actual 1665.5337; ^{1}H NMR (500 MHz, d$_6$-DMSO): δ 0.92-0.98 (m, 10H, succinyl CH$_2$, valine CH$_3$), 1.18-1.22 (m, 3H, alanine CH$_3$), 2.10-2.17 (m, 1H, valine CH), 3.47-3.80 (m, 43H, CD H2-H6), 3.80-3.93 (m, 2H, glycine CH$_2$), 4.18 (t, J = 7.7 Hz, 1H, alanine CH), 4.27 (t, J = 7.7 Hz, 1H, valine CH), 4.43-4.54 (m, 5H, CD OH6), 4.80-4.90 (m, 7H, CD H1), 5.63-5.87 (m, 14H, CD OH2, OH3), 7.80-7.88 (m, 1H, alanine NH), 7.90 (d, J = 4.2 Hz, 2H, aromatic), 8.04 (d, J = 4.7, 2H, aromatic), 8.31-8.40 (m, 1H, valine NH), 8.53-8.60 (m, 1H, glycine NH); ^{13}C NMR (500 MHz, d$_6$-DMSO): δ 18.6 (valine CH$_3$), 19.3 (valine CH$_3$), 19.4 (alanine CH$_3$), 19.7 (alanine CH$_3$), 30.2 (succinyl CH$_2$), 30.32 (succinyl CH$_2$), 30.35 (succinyl CH$_2$), 42.4 (glycine

CH_2), 42.5 (glycine CH_2), 48.4 (valine CH), 60.0 60.2-60.5 (CD C6), 72.3-73.0 (CD), 73.1-73.5 (CD), 81.3-82.8 (CD), 102.0-102.5 (CD C1), 124.9 (aromatic), 128.7 (aromatic)

6.2.3.2 Mono-6^A-amino-6^A-(4-aminobenzenesulfonamide-bradykinin)-mono-6^X-succinyl-6^X-β-cyclodextrin (**33b**)



33b

BK was assembled manually on Rink resin (50 mg, 0.0365 mmol, 1 equiv) using Fmoc-SPPS as per **Section 6.2.2.1**. TFA cleavage as per **Chapter 3**, **Section 3.2.2.1** was used to cleave the peptide from the resin. RP-HPLC purification as per **Section 6.2.2.1** eluting the pure product at 42% B (0.4 mg, 0.4%).

LR-MS: *m/z* (%, assignment) 2458.31 (80, $M+H)^+$, 1229.46 (80, $M+2H)^{2+}$ HR-MS: *m/z* $C_{103}H_{154}N_{18}O_{49}S$ $(M+2H)^{+2}$ 1229.4911, actual 1229.4896; *Partial NMR analysis* ^1H NMR (500 MHz, d_6-DMSO): δ 4.80-4.92 (m, 7H, CD H1), 5.65-5.88 (m, 14H, CD OH2, OH3), 7.16-7.31 (m, 10H, phenylalanine aromatics)

6.2.4 *Experimental for the Bioassays*

See **Appendix 6-I** for cell preparation and **Appendix 6-V** for information on the stages of a cell cycle.

6.2.4.1 MTT Assay[1]

Test compounds were compared to 5-FU for their effect on cancer cell proliferation. Cells were plated in 96 well plates for 24h, and the test or control substances added in serial 1:5 dilutions from 250 µg/mL downwards (250, 50, or 10 µg/mL) in 2-4 replicate wells per treatment. After 3 days, cell proliferation was assessed as a factor of MTT incorporation by measuring the optical density (OD) of each well. The OD reading of each test well was normalised to the mean OD of the control (medium-only) test wells on the same plate and the mean determined for each experiment. Data are shown as mean plus SEM (standard error of the mean) of 2-4 replicate experiments. See **Appendix 6-II** for more details.

6.2.4.2 Flow Cytometry Assay[2]

The test compounds were compared to 5-FU for their effects on cell cycle arrest. Cells were plated in 24 well plates for 24h, and the test or control substances then added at a final concentration of 50 µg/mL. After 6h, 24h, or 48h, cells were collected, fixed in ethanol, and stained with propidium iodide (PI) in the presence of RNase. Cell cycle was determined by flow cytometric analysis on a minimum of 5000 cells per sample. See **Appendix 6-III** for more details.

6.3 Conclusions

Preliminary bioassay testing was performed on three cell lines (HS700T, HT-29, and MA-104) ranging from human pancreatic adenocarcinoma, human duodenal adenocarcinoma, and monkey kidney carcinoma respectively. Results for the bioactive BK samples indicated that varying the positions of the β-CD, peptide, and/or sulfonamide had only small affects on cell proliferation (either a slight increase or decrease). Further studies are required to pin-point the reasons for this change, as well as further bioassay analysis using bioactive sulfonamides. Overall, this screening provides an approach for the next research step in this area.

6.4 References

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Chapter Seven

Cyclodextrin-Bradykinin-Sulfonamide Complexes as a Drug Delivery System: A Summary and Assessment of Possible Future Directions

7.0 Summary of Results

The bi-functionalisation of β-cyclodextrin has been achieved without the use of global protection of unutilised hydroxy groups. This study has established proof-of-concept for the controlled assembly of β-cyclodextrin-peptide-sulfonamide complexes. In addition, this study offers potential for utilizing the drug carrying capabilities of the β-cyclodextrin "bucket". β-cyclodextrin has also been employed directly in the controlled addition of peptides via SPPS to the extent that attachment of one or more peptides, exhibiting the same or differing (bioactive) properties, can be achieved. This method is more succinct resulting in a lower number of synthetic steps from basic β-cyclodextrin than previous literature examples of multi-functionalisation.

Fmoc SPPS was chosen as the method of peptide assembly on β-cyclodextrin due to its ease, lower risk compared to Boc SPPS, and often greater reaction successes. Methods were developed for the assembly of the bioactive peptide in this system. Coupling studies were performed in an attempt to ensure maximum couplings for these reactions. Bradykinin was chosen as a model bioactive peptide due to its known vasodilator activity. Cancer agonist or antagonist activities have been reported when slight changes to its structure are achieved (**Chapter 1**, **Section 1.3.3**). Initial thoughts centred on the possibilities of using it as a targeting peptide as part of a drug laden complex for controlled drug release at the target site. Proof-of concept would then present a complex that could be adapted to a wide range of peptides and drugs.

The assembly of peptides onto the bi-functionalised β-cyclodextrin core was designed to develop suitable conditions for the assembly of the bioactive peptide, bradykinin, all using Fmoc SPPS. The trial peptides used were: tri-glycine, Val-Gly-Ala, Gly-Ala, and Pro-Phe-Arg. Once reaction conditions were obtained for assembly of these to cyclodextrin, the assembly of the bradykinin peptide was then employed in varying *C*- and/or *N*-terminal combinations onto the cyclodextrin functionality.

Addition of sulfonamides (only simple model compounds were used in this study to establish viability) were then coupled to the bradykinin peptide and/or β-cyclodextrin-peptide moieties using Fmoc SPPS. Coupling studies were also performed to ensure the most successful couplings were obtained. Varying combinations of β-cyclodextrin-peptide-sulfonamides and peptide-sulfonamides whereby the sulfonamide was attached at differing *C*- and *N*-terminal positions was achieved.

Spacers were also used to separate the peptide from the β-cyclodextrin core, to study the effect these would have on the relationship of the various elements of the synthesis and possible bioactivity of the β-cyclodextrin complex. The spacer used in this part of the study was ε-aminocaproic acid and was attached to the β-cyclodextrin-peptide-sulfonamide system using Fmoc SPPS methodology. It was also found that the addition of a spacer had little effect on the overall yields of the final products compared to products without a spacer group. This is presumably due, at least in part, to an added steric hindrance for the peptide or β-cyclodextrin couplings and perhaps a slight decrease in product solubility, seen with an increase in peptide growth onto resin.

Preliminary bioassay testing was performed by Dr Fran Wolber's group (IFNHH, Massey University) on three cell lines (HS700T, HT-29, and the MA-104) using the MTT assay to investigate the possibility of some bioactivity of the bradykinin based compounds. The tests indicated that varying the order of the β-cyclodextrin, and/or peptide, and/or sulfonamide resulted in a small increased/decreased change in cell proliferation. However, further bioassay studies are required to pin-point the exact reasons for this change and enhance either effect. Overall this screening has provided a basis for the next step in this research programme. Continuation of this project would ideally see the injection of these compounds directly into tumours as the best method of administration.

7.1 Future Prospects

Advancement of this study for the use of bi-functionalised β-cyclodextrin complexes as potential drug carriers requires of course a number of considerations.

Of high importance is the issue of yields which needs to be addressed to enable a more successful synthesis of the β-cyclodextrin-peptides and β-cyclodextrin-peptide-sulfonamides. Therefore yields for all stages of the bi-functionalised β-cyclodextrin molecules must be improved. If realistic yields were to be obtained, a move to push this study beyond proof-of-concept would be the ideal goal. New chemistry strategies may be required, if Fmoc SPPS chemistries cannot be significantly improved, for this to happen.

Isomer purification or method adoption to enable a single isomer to be synthesised for the bi-functional β-cyclodextrin is also very important for use in the pharmaceutical world. It is well known that isomers can all exhibit differing chemical properties, some more desired than others.

Peptide assembly onto β-cyclodextrin should also be investigated further. This includes the use of differing resins, such as Sieber resin which was shown to give better yields than that of Rink resin in the small study tried. The addition of whole peptides onto β-cyclodextrin may also better the yields in this study. The addition of peptides to β-cyclodextrin off resin is also possible, but directing the β-cyclodextrin to the correct location on the peptide requires the correct protection to be employed to ensure predicated terminal or orthogonal placement. The option of non-peptidyl bioactive molecules added to cyclodextrin is also viable preferably with suitable linkers enabling attachment.

Further spacer studies should also prove helpful. For example, the addition of differing spacers to study their effect in the bioactivity of the β-cyclodextrin complexes. Thus spacer addition could also be used to study the steric effects the cyclodextrin molecule has when coupling to resin. Spacer studies should be expanded to use for example short polyethylene glycols (PEG 2, PEG 3, or PEG 4) which are available as pure compounds.

A much more extensive range of known powerful bioactive sulfonamides should be used in this system and further testing and broader screening should also be initiated to study the effects on a much broader range of cell lines.

In this study cancer cell lines were chosen due to bradykinin's reported[1] vasodilating activity in tumour cells.[*] However, bradykinin also shows anti-fungal activity and hence screening beyond anti-cancer activity is recommended and may have some interesting results. Other assays, including the mentioned carbonic anhydrase assay, should also be used to screen the current and future sulfonamide-peptide-β-cyclodextrin complexes.

[*] *"We previously have shown that BK contributed to tumor-induced angiogenesis and tumor growth via the B_2 receptor……BK also is reported to be involved in the enhancement of tumor growth via increased permeability of the tumor vasculature. The expressions of (a) a kinin-generating enzyme, kallikrein; (b) KGNs, which are substrates for kallikrein; and (c) BK receptors (B_1 or B_2) in various types of tumor cells were reported (23–27). This accumulating evidence suggests that BK may be one of the primary mediators responsible for tumor angiogenesis, and thereby for tumor growth, but the precise process of BK production associated with tumor growth and the mechanisms underlying BK-induced angiogenesis have not been fully demonstrated."*[1]

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[1] Ikeda, Y., Hayashi, I., Kamoshita, E., Yamazaki, A., Endo, H., Ishihara, K., Yamashina, S., Tsutsumi, Y., Matsubara, H., and Majima, M., Host stromal bradykinin B_2 receptor signaling facilitates tumor-associated angiogenesis and tumor growth. *Cancer Research* **2004**, *64*, 5178-5185.

Appendix for Chapter One

Project Plan

Figure A1-1 indicates all the parts of the project at the initial planning stages and how they all fit together to create a potential peptide drug delivery system.

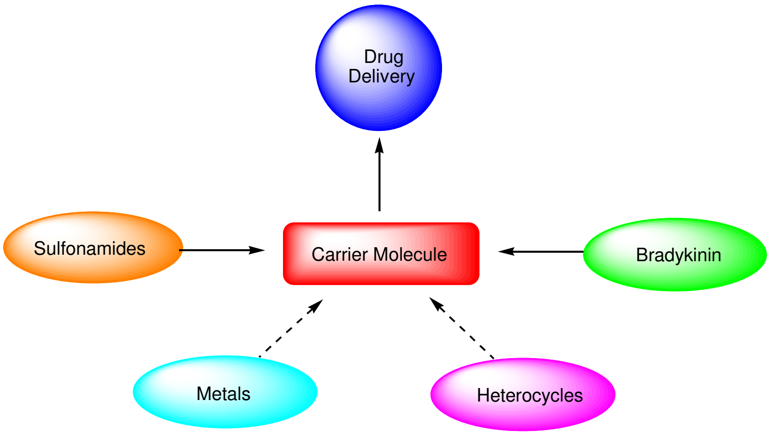

Figure A1-3 Proposed project plan

From here, focus of this study was on the development of skills in SPPS, to establish whether sulfonamides (with and/or without linkers) will be able to be attached to the *C*- and/or *N*-terminus of a bioactive peptide. Functionalisation of β-CD was performed to enable variable-peptidyl addition. The bioactive peptide chosen for this study is BK. Cyclic carbohydrates and/or heterocycles were to be investigated as carriers of potential therapeutic agents with BK as a possible targeting device. Alternatively, BK could also act as an added therapeutic agent. In addition, SPPS methodology was used to attach the peptide-sulfonamides to CD and/or heterocycles. The aim of this study was to evaluate the potential of the modified targeting agent (e.g. BK) for directing the drug to the required (tumour) site as well as the potential for the composite to increase drug concentration and rate of action. β-CD has the capability of altering the overall solubility of the transporter and/or the transported molecules.

Outline

- In this study, BK was to be synthesised using Fmoc and/or Boc SPPS. In later studies, analogues of BK were also to be considered. At the completion of this study, this had not been investigated.

- Sulfonamides ($R\text{-}SO_2\text{-}NH_2$) were to be attached to the BK peptide using SPPS chemistries. In this study, sulfonamides with and/or without linkers were to be attached to the *C*- and/or *N*-terminus of the target peptide/s. Exo-functionalisation was to be the main focus.

- Cyclic carbohydrates, namely β-CD were to be functionalised with bioactive peptide-sulfonamides. The plan was to include attachment of the peptide and or peptides to one or more locations around the β-CD outer core via the *C*- and/or *N*-terminus of the peptide. A spacer might also be employed between β-CD and the peptides to enable efficient coupling and possibly after any bioactivity achieved.

- Biological screening of the samples was also to be investigated if possible.

- Heterocycles were also to be investigated as potential passengers with bioactivity[*]

- Metals have been important in the development of drugs for many years. These include the development of anti-microbial drugs using silver, cardiovascular drugs using iron, and anticancer drugs using cis-platinum. In this study, encapsulation of metals/metal complexes inside the β-CD cavity may increase the solubility and transport capability of these molecules and hence aid in the fight against the tumour.[*]

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[*] Preliminary studies performed but not reported in this thesis

Appendix for Chapter Two

Page

APPENDIX 2-I *Click chemistry*

*Synthesis of mono-6^A-(acetamido-[1,2,3]-triazole-3-carboxylic acid)-β-cyclodextrin (**5i**)*

A 'blank' azide alkyne cycloaddition reaction (Figure A2-1) was performed to see if the addition of compound **5** (mono-6^A-(α-azidoacetamido)-β-cyclodextrin) to an alkyne (propiolic acid, Figure A2-1) would be successful.

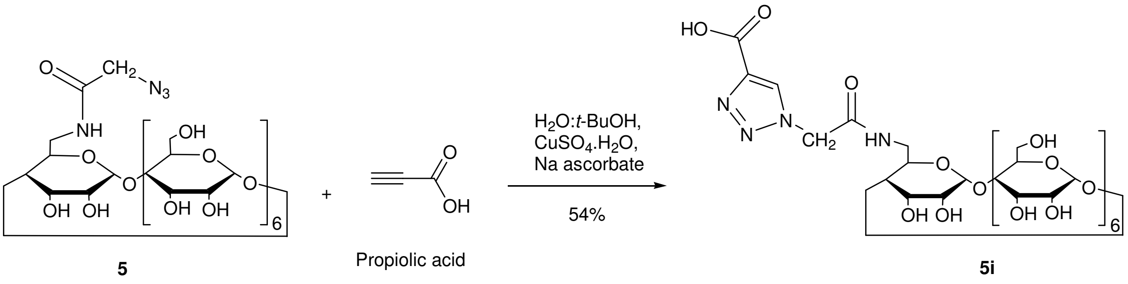

Figure A2-1 Synthesis of mono-6^A-(acetamido-[1,2,3]-triazole-3-carboxylic acid)-β-cyclodextrin (**5i**)

Adapted from references.[1] Propiolic acid (1 μL, 0.0162 mmol, 1 equiv) and **5** (see **Chapter 2**, **Section 2.4.3** for synthesis details, 39.5 mg, 2 equiv, 0.032 mol) were dissolved in a 1 mL 1:1 solution of H_2O: *t*-butanol. $CuSO_4.5H_2O$ (100 μL of a stock solution consisting of 24.3 mg in 1 mL H_2O, total $CuSO_4.5H_2O$ added to the CD reaction is 2.43 mg, 0.6 equiv) and sodium ascorbate (9.656 mg in 100 μL H_2O, 3 equiv) freshly prepared was added to the reaction mixture (clear to orange colour change) and the reaction was stirred at rt overnight (*c.a.* 17h). Precipitation with acetone, isolation by centrifugation and MS analysis showed the product in the solid layer. RP-HPLC purification as per **Chapter 2**, **Section 2.4.4.2** yielded 22 mg (54%) of pure product.

LR-MS: *m/z* (%, assignment) 1284.60 (80, M+H)$^+$

*Synthesis of **5a** (a resin-bound peptide-alkyne)*

Peptide-alkyne synthesis (Figure A2-2) as per standard Fmoc-SPPS using TBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) in excess as coupling reagents on Rink resin (0.73 mmol/g, 0.25 mmol, 395 mg, 1 equiv). The peptide (Val-Gly-Ala) was first assembled on the resin and following deprotection of the last amino acid, propiolic acid (309 μL, 5 mmol, 20 equiv) was coupled* overnight. A ninhydrin test was used to determine if the coupling was successful (as per **Appendix 3**). A small amount of this resin-bound peptide-alkyne (**5a**) was cleaved using a 20% TFA in DCM solution (3h) to ensure a successful synthesis. MS results indicated the successful formation of the peptide-alkyne.

LR-MS: *m/z* (%, assignment) 296.3 (90, M+H)$^+$, 319.2 (90, M+Na)$^+$, 335.3 (90, M+K)$^+$

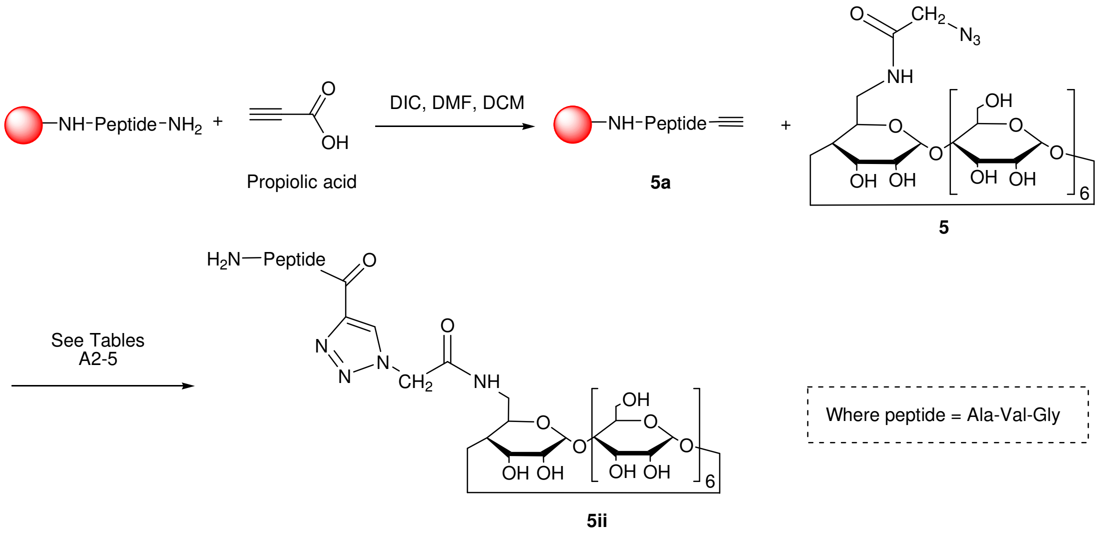

Figure A2-2 Synthesis of mono-6^A-(L-Val-Gly-Ala-acetamido-[1,2,3]-triazole-3-carboxylic acid)-β-cyclodextrin (**5ii**) on Rink resin

*Synthesis of Mono-6^A-(L-Val-Gly-Ala-acetamido-[1,2,3]-triazole-3-carboxylic acid)-β-cyclodextrin (**5ii**)*

The synthetic route is illustrated in Figure A2-2. Coupling of **5** to **5a** (Figure A2-2) was investigated using varying reaction conditions and results shown in Table A2-1.

LR-MS: *m/z* (%, assignment) 1536.4 (100, M+Na)$^+$, 1552.4 (90, M+K)$^+$

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* Pre-activation by mixing DIC (228.6 μL, 2.5 mmol, 10 equiv) in DMF (500 μL) and allowing crystal formation. After *c.a.* 2 minutes, addition of DCM (500 μL) dissolved the solid before addition to deprotected resin-bound peptide.

Table A2-5 Click chemistry coupling conditions for **5** to a resin bound alkyne (**5a**). All reactions were cleaved using a 20% TFA in DCM solution (3h) at rt

Experiment Number	***5a*** *(μmol, 1equiv)*	***5***	*Copper Catalyst*	*Base*	*Solvent*	*Reaction Conditions*	*Purification Results*[∕∕]
C1	0.0365	5.6 equiv	CuI, 3.0 equiv	DIEA, 120 equiv	DMF	[§]	t_R of 38-40% B (2.2 mg, 4%)[¶]
C2	**0.0365**	**5.6 equiv**	**CuI, 3.0 equiv**	**DIEA, 120 equiv**	**DMF**	[‡]	**t_R of 44-45% (12.7 mg, 23%)**[**]
C3	0.0187	6 equiv	CuI, 2.8 equiv	DIEA, 122 equiv	DMF:H_2O	[§]	No product
C4	0.0187	6 equiv	CuI, 2.8 equiv	DIEA, 122 equiv	DMF:H_2O	[‡]	t_R of 38-40% B (trace)[¶]
C5	0.0146	2 equiv	$CuSO_4.5H_2O$, 2 equiv	Sodium ascorbate, 3 equiv	DMF:H_2O	[§]	No product
C6	0.0187	6 equiv	$CuSO_4.5H_2O$, 1.0 equiv	Sodium ascorbate, 5 equiv	THF:H_2O	[†]	No product
C7	0.0365	5.6 equiv	$CuSO_4.5H_2O$, 1.0 equiv	Sodium ascorbate, 6 equiv	THF:H_2O	[†]	No product
C8	0.0365	5.6 equiv	$CuSO_4.5H_2O$, 0.6 equiv	Sodium ascorbate, 3 equiv	1:1:*t*-BuOH:H_2O	[§]	No product
C9	0.0365	5.6 equiv	$CuSO_4.5H_2O$, 0.6 equiv	Sodium ascorbate, 3 equiv	DMF	[§]	t_R of 43% B (1.1 mg, trace %)[**]

[†] Agitated overnight at 60ºC

[‡] Agitated at rt for 3 days

[§] Agitated overnight at rt

[¶] RP-HPLC as per **Chapter 2**, **Section 2.4.4.1**. Buffer A: 100% H_2O, 0.1% TFA; Buffer B: 100% MeOH

[**] RP-HPLC as per **Chapter 2**, **Section 2.4.4.1**. Buffer A: 98% H_2O, 2% MeCN, 0.1% TFA; Buffer B: 90% MeCN, 10% H_2O, 0.1% TFA

[∕∕] Also see cyclodextrin-$Cu(OH)_2$ complex formation [2]

Summary

The reaction conditions that gave the best reaction yields for the synthesis of **5ii** were mono-6^A-(α-azidoacetamido)-β-cyclodextrin (**5**, 5.6 equiv), CuI (3 equiv) and DIEA (120 equiv) in DMF agitated for 3 days at rt producing a 23% pure yield (*Experiment C2*,
Table A2-5).

Due to these experiments being conducted late in this study, no further studies could be carried out. However, is would be interesting to investigate the use of these reactions for the attachment of peptides to the bi-functional β-CD molecule (**12**) and may be a way to potentially better the synthesis yields reported in **Chapter 2**. Future studies should also include the elimination of oxygen from these reactions in an attempt to better the yields. This can be achieved by using degassed solvents and performing these reactions under a nitrogen or argon atmosphere. This would eliminate the side reaction of copper II with oxygen known as the Glaser Reaction (Figure A2-3) which is common in CuI reactions.

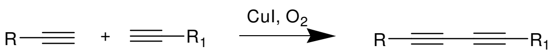
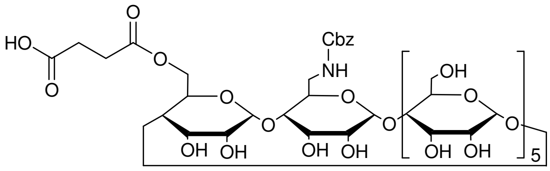


Figure A2-3 Glaser Reaction

APPENDIX 2-II *Extended Purification of Compound **10***



10

RP-HPLC purification of each isomeric pair for compound **10** (**Chapter 2**, **Section 2.4.4.2**, AB/AG, AC/AF, and AD/AD) using variable columns, gradients, and solvent systems was attempted to gain better NMR characterisation. Table A2-6 details all methods tried and the ensuing results.

It can be seen from Table A2-6 that the best purification was achieved using the conditions labelled *C*. This purification gave 6 peaks (shouldered) along with a small amount of starting material eluting just prior to the peaks t_R (as shown in Figure A2-4).

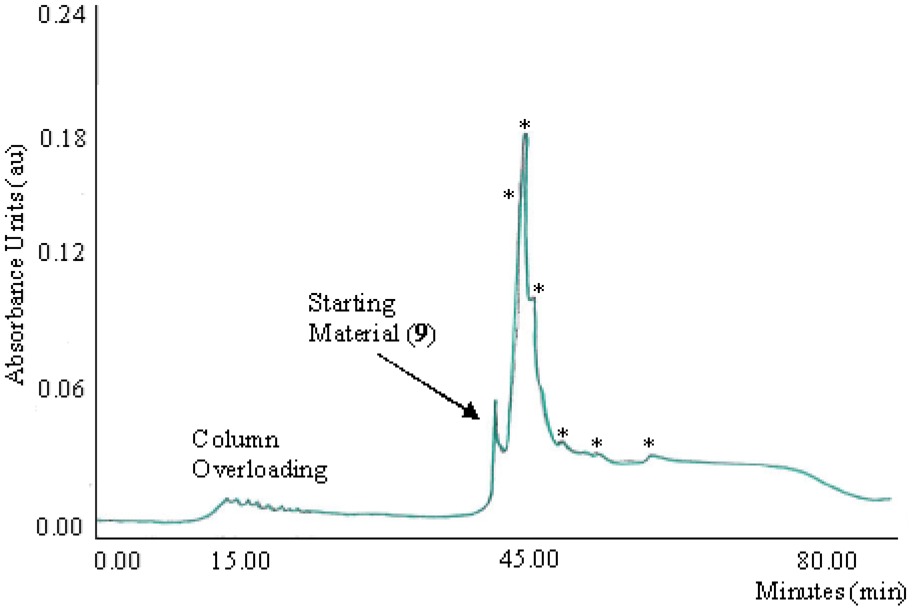


Figure A2-4 RP-HPLC elution spectra for the separation of compound **10** isomers using purification *C* (Table A2-2) where * represents each isomer

Table A2-6 RP-HPLC purification methods for the attempted purification of the isomeric pairs of compound **10** using a flow rate of 0.3 mL/min and measuring at a wavelength of 254 nm.

Purification Number	*Column*[††]	*Solvent system*	*Time Program*	*Sample*	*Results*
A	Jupiter C-18	[‡‡]	18.3%/h gradient from 25% B until 60% B	AD/AE isomer	No separation
B	Jupiter C-18	[§§]	12%/h gradient from 25% B until 75% B	All isomers	No separation
C	**Jupiter C-18**	[§§]	**Hold at 0% for 10 minutes, 12%/h gradient from 0 to 100%**	**All isomers**	**6 shouldered peaks, varying t_R**
D	Jupiter C-18	[§§]	Hold at 0% for 10 minutes, 86%/h gradient from 0 to 100%	All isomers	5 shouldered peaks
E	Econsil C-18	[§§]	Hold at 0% for 10 minutes, 120%/h gradient from 0 to 100%	All isomers	No separation
F	Jupiter C-4	[§§]	66.6%/h gradient from 25% B until 75% B	All isomers	3 shoulder peaks
G	Jupiter C-4	[§§]	66.6%/h gradient from 25% B until 75% B	AC/AF isomer	No separation
H	Jupiter C-4	[§§]	66.6%/h gradient from 25% B until 75% B	AB/AG isomer	No separation

[††] Analytical (250 x 4.60 mm) column

[‡‡] Buffer A: 100% H_2O, 0.1% TFA; Buffer B: 100% MeCN

[§§] Buffer A: 98% H_2O, 2% MeCN, 0.1% TFA; Buffer B: 90% MeCN, 10% H_2O, 0.1% TFA

Hence, each isomeric pair was RP-HPLC purified as per *Purification C* (Table A2-2) in order to locate the t_R of each peak and see if separation was achievable. It was found that the AD/AE isomer pair eluted at 71.60 minutes (Figure A2-5) as one peak.

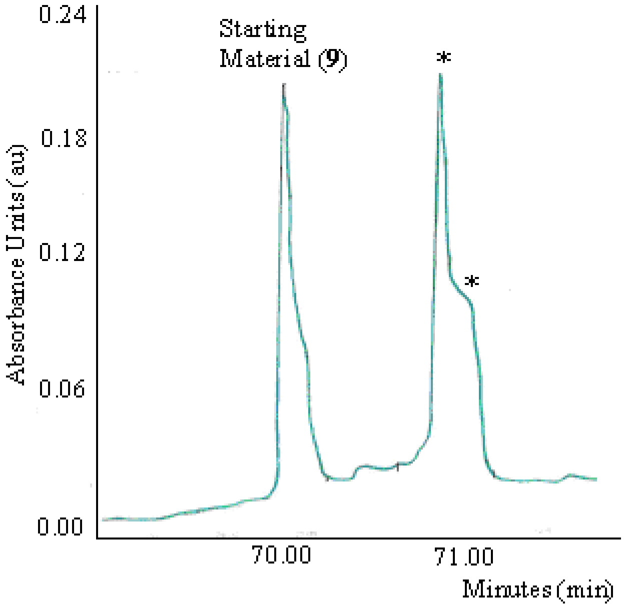


Figure A2-5 RP-HPLC elution spectra for the AD/AE isomers where * represents each isomer. Flow rate of 0.2 mL/min

Figure A2-6 shows the AC/AF isomer pair eluting between 30.30-30.90 minutes as one shouldered peak.

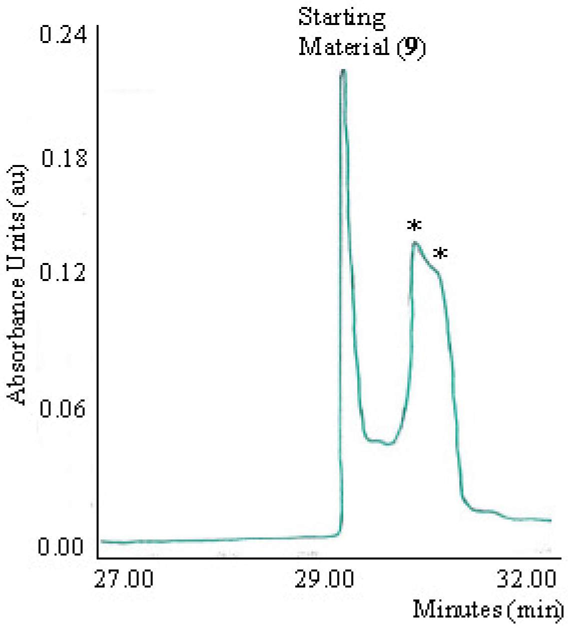


Figure A2-6 RP-HPLC elution spectra for the AC/AF isomers where * represents each isomer. Flow rate of 0.3 mL/min

The AB/AG isomer pair eluted at 77.40 and 78.27 (Figure A2-7) as separate peaks. Therefore, separation of the AB/AG isomer is possible using this method but further characterisation has not been attempted.

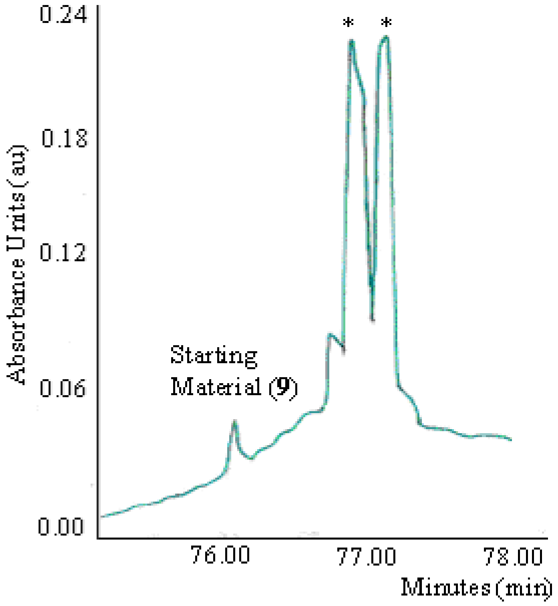


Figure A2-7 RP-HPLC elution spectra for the AB/AG isomers where * represents each isomer. Flow rate of 0.2 mL/min

The flow rate for the AD/AE and AB/AG isomer pairs was 0.2 mL/min and this results in an extended t_R compared to the AC/AF isomer pair (0.3 mL/min).

APPENDIX 2-III *Alternative Synthesis of Compound **10***

The succinylation of compound **10** (**Chapter 2**, **Section 2.4.4.2**) was obtained from a number of studies to optimise the yield by formation of only the mono-succinylated product. This included changing the reaction temperature, ration of succinic anhydride, and solvent. These studies and results can be seen in Tables A2-3 and A2-4. From these experiments, the method used in **Chapter 2**, **Section 2.4.4.2** was found to be the best and hence was used throughout the rest of this study.

Table A2-7 Alternative synthesis methods for compound **10**

Experiment Number	*Succinic Anhydride Ratio*	*Reaction Conditions*
A1	104 equiv[////]	A solution of succinic anhydride in dry pyridine (5 mL) was added to a solution of **9** (dried at 120ºC overnight, 52.7 mg, 42 mmol, 1 equiv) dissolved in dry pyridine (5 mL) and stirred at 80ºC overnight under $N_{2(g)}$. Reaction quenched with H_2O, and freeze-dry. MS analysed.
A2	25 equiv[////]	Reaction as per *Experiment A1* with the exception of concentration and temperature (rt)
A3	1 equiv	Reaction as per *Experiment A1* with the exception of concentration and temperature (rt)
A4	1 equiv	Reaction as per *Experiment A1* with the exception of time (allowed to stir for 7 days)
A5	1 equiv 20 equiv	A solution of succinic anhydride and **9** (10 mg, 7.75 $x10^{-3}$ mmol, 1 equiv) in dry DMF (1 mL)/pyridine (3.67 mL) was added and the solution, heated at 70ºC for 2 days under $N_{2(g)}$. The reaction mixture was diluted with ether forming a yellow solution, MeOH was added forming a white precipitate which was H_2O soluble. MS analysis.
A6	1 equiv	Reaction as per *Experiment A1* with the exception of concentration, temperature (rt), and pyridine volume (25 mL) [¶¶]

[////] To determine the efficiency of the experiment by enforcing full (primary and/or secondary) succinyl functionalisation

[¶¶] To enforce the mono-succinyl species via the altering of dilution

Table A2-8 Results for Table A2-3; alternative synthesis for compound 10

Experiment Number	*Results*	*Purification*
A1	Succinyl functionalisation of 5-13, no sign of the starting material	RP-HPLC purification as per **Chapter 2**, **Section 2.4.4.2**. No separation of **10** from the higher-succinyl functionalised species (combined yield of 13.6 mg, 24%)
A2	Succinyl functionalisation of 1-7, starting material present	RP-HPLC purification as per Experiment *A1* generating trace amount of **10**
A3	Succinyl functionalisation of 1-2, starting material present	RP-HPLC purification as per Experiment *A1* generating 3.4 mg (6%) amount of **10**
A4	Mono-succinyl functionalisation, starting material present	RP-HPLC purification as per Experiment *A1* generating 0.4 mg (0.7%) amount of **10**
A5	Succinyl functionalisation of 1-4, starting material present	RP-HPLC purification as per Experiment *A1* generating trace amount of **10**
	Succinyl functionalisation of 2-8, no starting material present	RP-HPLC purification as per Experiment *A1* generating trace amount of **10**
A6	Mono-succinyl functionalisation, starting material present	RP-HPLC purification as per Experiment *A1* generating 13.5 mg (12.5%) amount of **10**

Bi-functionalisation of β-CD was also attempted using:

(a) Double-tosylation

(b) Bi-functionalisation on resin

(a) *Alternative Synthesis of Mono-6^{A}-carbobenzyloxyamino-mono-6^{X}-succinyl-β-cyclodextrin (**10**) using Double-Tosylation*

This method involved the tosylation of compound **10** as per (**Chapter 2**, **Section 2.4.2.1**) to make **9i** (Figure A2-8). The specific details can be found in Tables A2-5 and A2-6 where *Experiment B9* gave the best reaction yields and was therefore used for subsequent reactions.

LR-MS (**9i**, water): *m/z* (%, assignment): 1444.770 (35, M+Na)$^+$, 1483.838 (35100 M+K)$^+$ HR-MS: *m/z* $C_{57}H_{84}NO_{38}S$ (M+H)$^+$ 1422.4387, actual 1422.4299 and $C_{57}H_{83}NO_{38}SNa$ (M+Na)$^+$ 1444.4206, actual 1444.4156

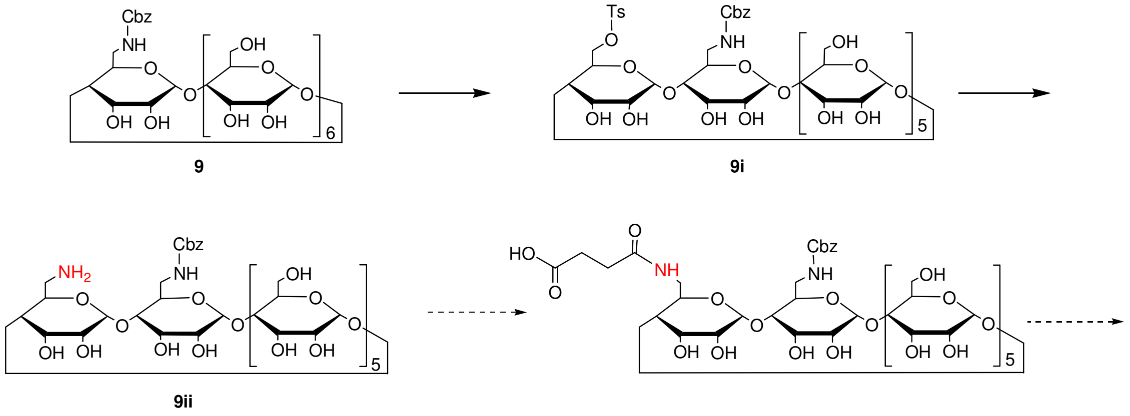


Figure A2-8 Alternative synthesis route of mono-6^{A}-carbobenzyloxyamino-mono-6^{X}-succinyl-β-cyclodextrin (**10**) using (a) the double-tosylation method and leading to Mono-6^{A}-fluorenylmethyloxycarbonylamino-mono-6^{X}-succinyl-β-cyclodextrin (**12**). This method has the succinyl group attached through a nitrogen atom (highlighted). Previously (compound **10**, **Chapter 2**) it was attached through an oxygen atom.

Table A2-9 Experimental reaction conditions for the synthesis of **9i**

Experiment Number	*Tosyl Chloride Ratio*	*Reaction Conditions*	*Experiment Number*	*Tosyl Chloride Ratio*	*Reaction conditions*
B1	5 equiv	Y = 3h[***]	*B5*	25 equiv	Y = 4h at rt[***]
B2	5 equiv	Y = 4h[***]	*B6*	25 equiv	Y = 8h at rt[***]
B3	5 equiv	Y = 4h at rt[***]	*B7*	7.1 equiv	**Chapter 2**, **Section 2.1.1**.
B4	2 equiv	Y = 12h at rt[***]	*B8*	1.5 equiv, 1.5 equiv, 1.0 equiv	[†††]
B5	2 equiv	Y = 24h at rt[***]	*B9*	5 equiv	Experiment *B1* except 9 was dried at 120ºC overnight[†††]

[***] Tosyl chloride dissolved in dry pyridine was added dropwise to a solution of **9** (1 equiv) dissolved in dry pyridine stirring on ice. The reaction was stirred for 3h on ice with MS monitoring each hour. The reaction was quenched with H_2O at Y (see Table); liquid was removed *in vacuo* and freeze-dried overnight yielding a crude yellow oily solid.

[†††] Tosyl chloride was added to a cooled mixture (-10ºC) of **9** (100 mg, 0.078 mmol) dissolved in dry pyridine and allowed to stir at 0-$^{-}$5ºC for 25h. A second portion of tosyl chloride was added and the reaction stirred at 5ºC overnight. A third portion of tosyl chloride was added and the reaction stirred for 5h. Methanol is added to the solution and cooled to 0ºC for 1.5h, followed by the addition of chloroform forming a white precipitate. The reaction is allowed to warm to rt (the precipitate was not isolatable by centrifuge) so the mixture was taken to dryness and freeze-dried overnight.

Table A2-10 Results for the synthesis of **9i**

Experiment Number	*Results*	*Purification*
B1	Tosyl functionalisation of 1-3, starting material	RP-HPLC purification as per **Chapter 2**, **Section 2.4.2.1** gave an inseparable mixture
B2	Tosyl functionalisation of 1-2, starting material	Recrystallisation from H_2O, then HPLC purification as per *B1* giving a better separation of **9i** (trace yield)
B3	Same as *Experiment B1*	RP-HPLC gave only trace yields of **9i**.
B4	Mono-tosyl functionalisation, starting material	Recrystallisation from H_2O, then HPLC purification as per *B1* giving a better separation of **9i** (trace yield)
B5	Same as *B1*	RP-HPLC gave only trace yields of **9i**.
	Tosyl functionalisation of 5-, 6-, and 7	RP-HPLC gave only trace yields of **9i**.
B7	Synthesis failed, only see starting material	N/A
B8	Same as *Experiment B1*	Recrystallisation gave the product in the supernatant. TLC analysis as per compound **2**, **Chapter 2**, **Section 2.4.2.1** shows the product having an R_f of 0.625. HPLC as per *B1* gave 0.7 mg (0.7%) of the clean product.
B9	Same as *Experiment B2*	RP-HPLC as per *B1* gave 7% (0.8 mg) of the clean product with 48 mg (48%) starting material.

Synthesis of **9ii** (Figure A2-8) was performed using parallel reaction conditions as per **Chapter 2**, **Section 2.1.2**. Purification of this material as per **Chapter 2**, **Section 2.4.4.1** with the product eluting at *c.a.* 44% B in low yields (2%).

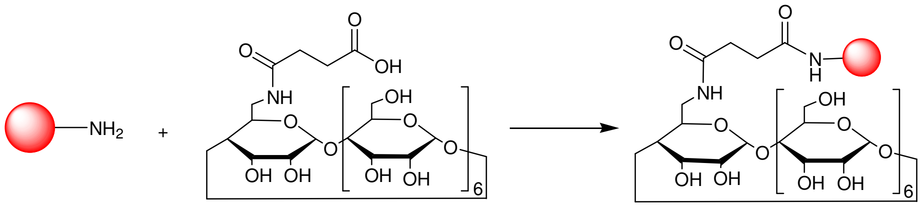
LR-MS (**9ii**, water): *m/z* (%, assignment): 1288.701 (70, M+Na)$^+$

Summary

Low yields were obtained for the product **9ii** and potential problems arose associated with purification and identification (only 1 mass unit difference between compound **9,** *m/z* = 1267, and compound **9ii** m/z = 1268). Also, RP-HPLC is the only method available to give a suitable purification so the scale of the reaction is limited. Hence, this reaction pathway was not used for the bi-functionalisation of β-CD.

(b) *Alternative Synthesis of Mono-6^A-carbobenzyloxyamino-mono-6^X-succinyl-β-cyclodextrin (**10**) using Bi-functionalisation on Resin*

Bi-functionalisation of β-CD on resin (Figure A2-9) allows for an easy purification of the non-bound species via subsequent washings as per SPPS chemistries. In this method, the same synthesis conditions as per **Chapter 2**, for compounds **2**, **3**, **9**, **10**, **11**, and **12** were attempted on Rink resin as per the following procedures.



4

4i

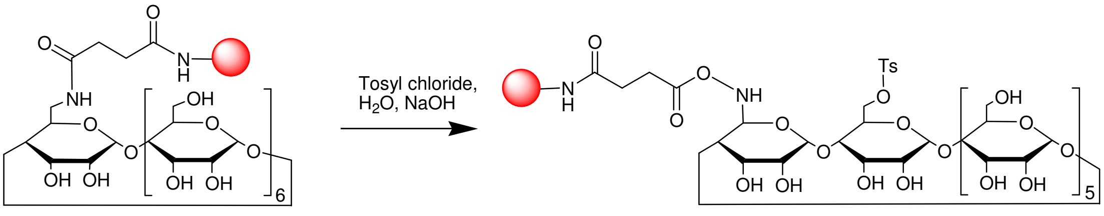
Figure A2-9 Alternative synthesis of compound **10** using bi-functionalisation on Rink resin

Synthesis conditions for **4i** (Figure A2-9) were as per **Chapter 4**, **Section 4.1** on Rink resin (600 mg, 0.438 mmol, 1 equiv). Compound **4** (2.158 g, 1.752 mol, 4 equiv) was coupled using TBTU (562 mg, 1.752 mmol, 4 equiv), HOBt (236 mg, 1.752 mmol, 4 equiv) and DIEA (613 μL, 3.504 mmol, 8 equiv) overnight. The ninhydrin test showed a successful coupling (yellow, see **Appendix 3-III**). A small amount of the resin was cleaved to confirm a successful coupling using MS.

LR-MS (**4i**, water): *m/z* (%, assignment): 1232.508 (100, M+H)$^+$

Synthesis conditions for **4ii** (Figure A2-10) were as per **Chapter 2**, **Section 2.4.2.1** on Rink resin. Tosyl chloride (3 equiv) was added to ice-cooled resin (**4i**) and allowed to agitate for 2h followed by the addition of a second portion of tosyl chloride (5 equiv). This reaction was then allowed to stir overnight at rt followed by removal of the solvent via suction, washing with H_2O and then DMF. A ninhydrin test gave a yellow colour indicating a successful coupling. MS analysis of a small portion of the cleaved resin showed presence of the product as well as presence of the bi- and tri-tosyl species.

LR-MS (**4ii**, water): *m/z* (%, assignment): 1386.70 (55, M+H)$^+$



4i **4ii**

Figure A2-10 Alternative synthesis of compound **10** using bi-functionalisation on Rink resin

The resin was then dried overnight by freeze-drying and divided into two. Half the resin was allowed to react with 35% aq ammonia (*Method A*, Figure A2-11) and agitate overnight for 7 days (refreshing the ammonia solution daily) for the synthesis of **4iii** performed as per **Chapter 2**, **Section 2.4.2.2**). MS analysis of the cleaved product gave only trace amounts of **4iii** using this method. The other half of the resin was suspended in dry DMF and warmed to 63℃ followed by the addition of crystalline KI (0.5 equiv) and NaN_3 (1 equiv).[3] The reaction was allowed to stir at this temperature for 24h, followed by removal of the solvents by suction filtration, washing with H_2O, DMF, and DCM (Method B, **4iia**, Figure A2-11). IR analysis of a small portion of the cleaved resin showed a weak presence of the azide peak. Hydrogenation of this resin-azide (**4iia**, same as per **Chapter 2**, **Section 2.3.3**)[3] and MS analysis of the cleaved product gave only trace amounts of **4iii** using Method B.

LR-MS (**4iii**, water): *m/z* (%, assignment): 1256. 01 (85, M+Na)$^+$, 1272.05 (30, M+K)$^+$

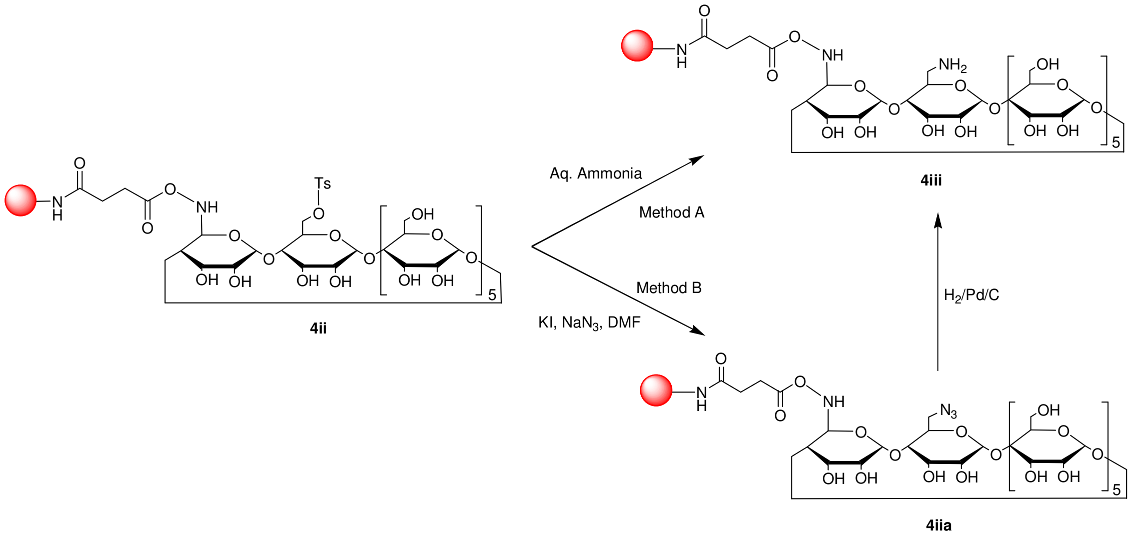
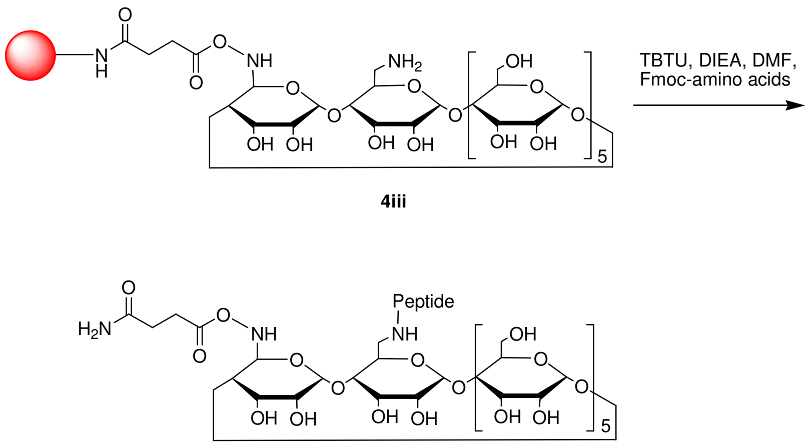


Figure A2-11 Alternative synthesis of compound **10** using bi-functionalisation on Rink resin

Peptide addition to **4iii** (Figure A2-12, samples of Methods A and B were reacted in separate reactions) was performed using parallel reaction conditions as per **Chapter 4, Section 4.1** where the peptide is Gly-Ala (4 equiv) coupled with HBTU (4 equiv), HOBt (4 equiv), and DIEA (8 equiv) overnight followed by a TFA cleavage **Chapter 4**, **Section 4.1**. *Method A*'s sample of **4iii** gave only trace amounts of the product (**4iv**) after RP-HPLC purification (as per **Chapter 4**, **Section 4.7.2.1**).

Method B's sample of **4iii** gave no product after purification.

LR-MS (**4iv**, water): *m/z* (%, assignment): 1384.10 (20, M+Na)$^+$



4iii

4iv

Figure A2-12 Peptide (Gly-Ala) addition onto Rink resin bound **4iii**

Summary

The bi-functional synthesis of β-CD on Rink resin is much faster than that of the synthesis in **Chapter 2** due to the faster removal of all impurities. However, only trace amounts of peptide-β-CD product (**4iv**) is generated. This low yield could be attributed to the mixture of products generated in the tosylation reaction (mono- through to tri-tosyl species of compound **4ii**) which are not purified at this stage, but are instead carried through to the end. Further studies are required to investigate if this method would be ultimately more successful than that used in **Chapter 2** for the synthesis of bi-functional β-CDs.

APPENDIX 2-IV *Synthesis of Mono-6^A-fluorenylmethyloxycarbonylamino-mono-6^X-succinyl-6^X-ε-aminocaproic acid-β-cyclodextrin (**12i**)*

The synthetic route is illustrated in Figure 2-22, **Chapter 2**. ε-Aminocaproic acid Bn ester (see **Appendix 2-VIII** for synthesis details, 2.1 equiv) and **10** (1 equiv) dissolved in DMF was added to a solution of TBTU (4 equiv) and DIEA (8 equiv) in DMF forming a yellow solution and stirred overnight at rt. Addition of H_2O formed a white precipitate. The solution was filtered and the aq layer taken to dryness on the freeze drier. No further purification was done (**12a**).

LR-MS (**12a**, water): *m/z* (%, assignment): 1378.89 (100, M+Na)$^+$

Removal of the Bn- and the Cbz-protecting groups (**12b**) was achieved using the same method as per the synthesis of compound **11** (**Chapter 2**, **Section 2.4.4.3**).

LR-MS (**12b**, water): *m/z* (%, assignment): 1370.31 (100, M+Na)$^+$

Fmoc protection using the same method as per the synthesis of compound **12** (**Chapter 2**, **Section 2.4.4.4**) generated **12i** in a low yield (1%).

LR-MS (**12i**, water): *m/z* (%, assignment): 1592.015 (80, M+Na)$^+$

APPENDIX 2-V *Synthesis of Mono-6^A-ε-aminocaproic acid-6^A-fluorenylmethyloxy-carbonylamino-mono-6^X-succinyl-β-cyclodextrin (**12ii**)*

The synthetic route is illustrated in Figure 2-23, **Chapter 2**. Compound **12ii** was coupled to Rink resin as per **Chapter 3**, **Section 3.2.2.1**. Fmoc-ε-aminocaproic acid (synthesised in **Appendix 2-VII**, 30 mg, 2.1 equiv) and **11** (50 mg, 1 equiv) dissolved in DMF was added to a reaction mixture of TBTU (5 equiv) and DIEA (5 equiv) in DMF forming a yellow solution and stirred overnight at rt. Solvent was removed *in vacuo* forming a yellow oil, addition of H_2O forming a white precipitate which was isolated by centrifugation. RP-HPLC purification of the solid (dissolved in a small amount of DMF/H_2O) using an acetonitrile gradient (buffer A: 100% H_2O, 0.1% TFA, buffer B: 100% acetonitrile) as per **Chapter 2**, **Section 2.4.4.4** at a wavelength of 254 nm. The product eluted at 42% B (3.9 mg, 0.6%).

LR-MS (**12ii**, water): *m/z* (%, assignment): 1591.714 (50, M+Na)$^+$

APPENDIX 2-VI *Synthesis of Mono-6^A-ε-aminocaproic acid-6^A-fluorenylmethyloxy-carbonylamino-mono-6^X-succinyl-6^X-ε-aminocaproic acid-β-cyclodextrin (**12iii**)*

The synthetic route is illustrated in Figure 2-24, **Chapter 2**. See **Appendix 2-IV** for synthesis details of **12a** and **12b**. Addition of Fmoc-ε-aminocaproic acid (synthesis in **Appendix 2-VIII**) as per the synthesis of **12ii** (**Appendix 2-IV**) generating 1% of the pure product (**12iii**) after RP-HPLC purification using the same method as per **Appendix 2-IV** with the product eluting at 33% B.

LR-MS (**12iii**, water): *m/z* (%, assignment): 1682.019 (50, M+H)$^+$, 1705.062 (100, M+Na)$^+$

APPENDIX 2-VII *One-pot Conversion of Cbz (**10**) to Fmoc Protected Compound (**12**)*

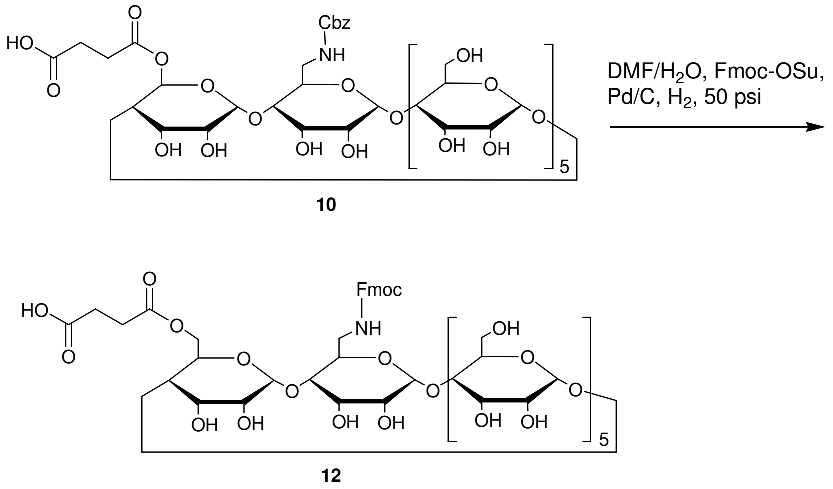


Figure A2-13 Alternative synthesis of **12**

Adapted from Schneider *et al.* (Figure A2-13).[4] Compound **10** (500 mg, 0.365 mmol, 1 equiv) was dissolved in a DMF:H_2O mixture (2:1, 2 mL) in a 10 mL test tube. Pd/C (10%), Fmoc-OSu (0.365 mmol, 1 equiv) was added and the reaction mixture was hydrogenated at 50 psi for 7h at rt, then filtered through filter paper, and then a bed of celite. Solvent was removed *in vacuo* yielding a pale yellow oil. The product was purified using the same conditions as per compound **12** (**Chapter 2**, **Section 2.4.4.4**) generating trace amounts of the Fmoc product.

See **Chapter 2**, **Section 2.4.4.4** for characterisation details.

APPENDIX 2-VIII *Fmoc-ε-Aminocaproic Acid Synthesis*

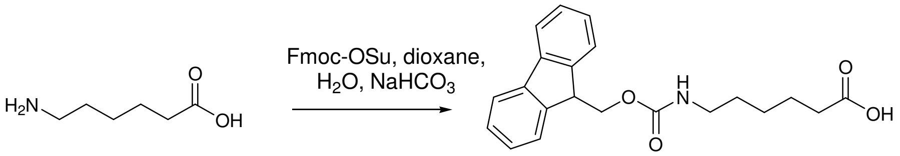


Figure A2-14 Synthesis of Fmoc-ε-aminocaproic acid

Adapted from Liskamp *et al.* (Figure A2-14).[4b] Fmoc-OSu (2.55 g, 7.5 mmol, 1 equiv) dissolved in dioxane (5 mL) was added dropwise to a stirred solution of ε-aminocaproic acid (1.00 g, 7.5 mmol, 1 equiv) and $NaHCO_3$ (0.804 g, 7.5 mmol, 1 equiv) dissolved in H_2O (5 mL). This solution was stirred overnight at rt. Solvent was removed *in vacuo* followed by addition of EtOAc (40 mL) and HCl (50 mL, 1 mol/L). Extraction with ether (1 x 50 mL), washed with H_2O (2x 50 mL) and the organic extracts dried over $MgSO_4$, filtered, and then taken to dryness *in vacuo* to yield a cream coloured powder (1.65 g, 61.5%). No further purification was carried out.

LR-MS: *m/z* (%, assignment) 354.28 (100, M+H)$^+$; ^{1}H NMR (500 MHz, d_6-DMSO): δ 1.24 (m, 2H, CH_2), 1.38 (dd, J = 7.0 Hz, 2H, CH_2), 1.48 (p, J = 7.4 Hz, 2H, CH_2), 2.17 (dt, J = 7.4 Hz, 2H, CH_2), 2.96 (q, J = 6.7 Hz, 2H, CH_2), 4.21 (t, J = 7.3 Hz, 2H, Fmoc CH), 4.29 (d, J = 6.9 Hz, 2H, Fmoc CH_2), 7.26 (t, J = 5.3 Hz, 2H, NH), 7.32 (q, J = 8.0 Hz, 2H, aromatics), 7.41 (t, J = 6.3 Hz, 2H, aromatics), 7.69 (d, J = 7.1 Hz, 2H, aromatics), 7.89 (d, J = 7.4 Hz, 2H, aromatics); ^{13}C NMR (500 MHz, d_6-DMSO): δ 24.8, 26.3, 29.6, 34.4, 40.5, 47.2, 65.1, 120.4, 120.5, 121.8, 125.6, 127.7, 128.0, 129.3

APPENDIX 2-IX *ε-Aminocaproic Acid Benzyl Ester Synthesis*

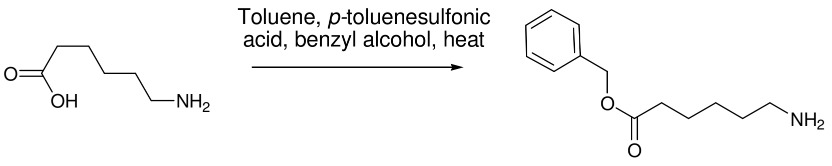


Figure A2-15 Synthesis of ε-Aminocaproic acid benzyl ester

Adapted from procedures by Stolle *et al.* (Figure A2-15).[5] To a suspension of ε-aminocaproic acid (20 g, 152.5 mmol, 1 equiv) in toluene (100 mL) was added *p*-toluenesulfonic acid (29.15 g, 152.5 mmol, 1 equiv) and Bn alcohol (63 mL, 609.8 mmol, 4 equiv). The reaction was heated and the H_2O removed by a Deans-Stark apparatus. Upon heating, all the solids dissolved and the solution became pale brown in colour. After 4h, the reaction was allowed to cool overnight forming a pale brown solid. Extra toluene was added to the reaction mixture (100 mL) along with ether (500 mL) decolourising the brown solid upon addition. The solid was then isolated by filtration, washed twice with ether and freeze-dried overnight (25.6 g, 76%). NMR and MS analysis was then acquired.

LR-MS: *m/z* (%, assignment) 222.33 (100, $M+H)^+$; ^1H NMR (500 MHz, d_6-DMSO): δ 1.29 (m, 2H, CH_2), 1.50 (m, 4H, CH_2), 2.09 (t, J = 7.1 Hz, 2H, CH_2), 2.29 (s, 2H, CH_2), 2.76 (t, J = 7.1 Hz, 2H, CH_2), 7.13 (d, J = 4.1 Hz, 2H, aromatic), 7.32 (d, J = 2.26 Hz, 2H, aromatic), 7.50 (d, J = 4.1 Hz, 2H, aromatic); ^{13}C NMR (500 MHz, d_6-DMSO): δ = 24.4, 25.8, 27.1, 33.9, 39.2, 125.9, 126.8, 128.6

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Appendix for Chapter Three

Page

APPENDIX 3-I *General Boc-Solid Phase Peptide Synthesis Method*[1]

Rink Resin Swelling

Resin (1 equiv) was placed into a reaction vessel, washed three times with DMF, and then allowed to swell in DMF overnight at rt. This swelling creates solvated reaction cavities in the resin enabling the reaction to take place internally as well as at the resin's surface.

Stepwise Resin Deprotection of Boc Groups

Suction filtration was used to remove the DMF. The reaction vessel was then filled *c.a.* three-quarters full with a 50% TFA in DCM solution and allowed to agitate for 1 minute. This was repeated twice, decanting the solvent between each repeat. DMF was used to wash the resin.

Batch-wise Addition of L-amino Acids

Excess amino acid (2 equiv) was added to the resin in the appropriate order and coupled using TBTU (8.0 equiv) and DIEA (23 equiv) in excess as activating reagents. This solution was allowed to agitate with the coupling times dependent upon the nature of the amino acid to be coupled. The Kaiser test[1] (ninhydrin) was used to determine if the amino acid had coupled with yellow signifying no free amines present. The Boc group was then deprotected with TFA and this cycle of operation was sequentially repeated for the stepwise incorporation of the remaining amino acids.

- *Kaiser Test*[1]

A few milligrams of the resin was placed into a test tube and 2 drops of 40% phenol in ethanol, 4 drops of 5% ninhydrin in EtOH, and 2 drops of 0.014 mol/L KCN in pyridine were added. This was then allowed to heat at 110°C for 5 min. A resulting yellow solution indicates that there are no free amines so the coupling reaction has gone to completion. A resulting blue/black resin shows that some free amines are still present and the amino acid needs to be recoupled.

Final N-terminal Deprotection of the Boc Protecting Group

The resin was washed with a DMF solution and then agitated twice with a 50% TFA in DCM solution for 1 min. The resin was first washed with DMF and then the resin bound peptide was then freeze-dried overnight.

Cleavage from Resin and Amino Acid Orthogonal Protecting Group Removal

The fully protected dry resin bound peptide (*c.a.* 340 mg) was transferred to an all Teflon cleavage vessel for the reaction with anhydrous HF (22 mL) containing 5% (1.5 mL) scavenger (*p*-cresol) stirring at 0°C (ice bath) for one hour. After HF evaporation, the residues were then triturated with ether (100 mL) for five minutes at 0°C, the suspension was gravity filtered and washed with ether (2 x 50 mL). The peptide was dissolved away from the resin in HPLC mobile phase B (*c.a.* 100 mL), transferred to a rbf and freeze-dried to a white solid prior to analysis. Figure A3-4 shows the cleavage point producing carboxy *C*-terminal peptides.

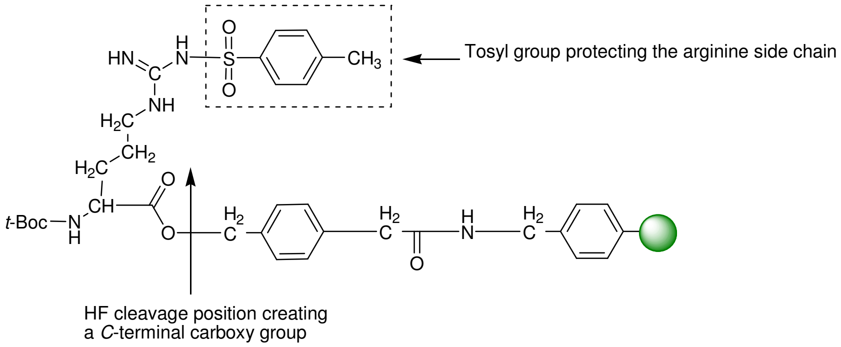

Figure A3-4 t-Boc Arg(Tos)OCH$_2$ PAM resin used for Boc synthesis indicating the cleavage point of the peptide from the resin yielding a COOH terminus upon cleavage.

Purification of the Peptide

See general Fmoc-solid phase peptide synthesis method (**Appendix 3-III**) for details.

APPENDIX 3-II *Synthesis of BK using Boc SPPS on t-Boc Arg(Tos)OCH$_2$ PAM Resin*

See **Appendix 3-I** for more specific details on the method

Method

BK was assembled manually on PAM resin (333.3 mg, 0.25 mmol, 1 equiv) preloaded with Arg(Tos) (Figure A3-1). Boc amino acids (0.5 mmol, 2 equiv) was coupled to the resin using TBTU (4 mL, 2.0 mmol, 8.0 equiv) and DIEA (1 mL, 5.74 mmol, 23 equiv) in excess as activating reagents. An HF mixture was used to cleavage the peptide from the resin.

Purification and Results

See **Chapter 3**, **Section 3.2.2.2** for MS and NMR data. The pure product eluted at 35% B (20 mg, 8%).

Conclusions from Boc SPPS of the BK peptide

The synthesis of BK using PAM resin was successful generating an 8% yield. Although not as good as the Fmoc-SPPS, it allows the generation of the carboxy *C*-terminus as opposed to the amide terminus generated using Fmoc chemistry in **Chapter 3**.

APPENDIX 3-III *General Fmoc-Solid Phase Peptide Synthesis Method*

Adapted from White *et al.*[1] This method was used for peptide synthesis throughout this study.

Rink Resin Swelling

See **Appendix 3-I** for details

Stepwise Resin Deprotection of the Fmoc Group

Suction filtration was used to remove the DMF and a 20% solution of pip in DMF was used to rinse the resin four times. The reaction vessel was then filled *c.a.* three-quarters full with this 20% pipe in DMF solution and allowed to agitate for 5 minutes. This was repeated twice, decanting the solvent between each repeat. DMF was used to wash the resin.

Batch-wise Addition of L-amino Acids

See **Appendix 3-I** for details. See Table A3-1 for Fmoc amino acid coupling times. The Kaiser test[1] (ninhydrin, see **Appendix 3-I**) or cleavage/MS test was used to determine if the amino acid had coupled with yellow signifying no free amines present.

Table A3-1 Average coupling times for Bradykinin amino acids used in this study

Fmoc-L-amino acid	*Average Coupling Time (hours)*
Arginine(Mtr)	12-24
Arginine(Pmc)	12-24
Proline	2-6
Serine(*t*-Bu)	2-6
Glycine	1-2
Phenylalanine	2-4

- *Cleavage-MS Coupling Test*

This test is used for hard-to-couple amino acids (e.g. arginine). A few milligrams of the resin was cleaved using the TFA cleavage mixture (see below) and the crude sample was analysed using MS for the presence of the des-amino acid fragment. A further coupling was performed if presence of the des-product was detected in an attempt to ensure coupling completion and high yields.

Final N-terminal Deprotection of Fmoc Protecting Group

The resin was washed with a 20% pip in DMF solution four times and then agitated twice for 5 minutes in this solution. The resin was first washed with DMF and then a 50% solution of DCM/MeOH before being suction dried well. The resin bound peptide was then freeze-dried and weighed.

General Cleavage from Resin and Removal of Amino Acid Side Chain Protecting Groups

An ice-cooled mixture of phenol (0.7g), thioanisole (0.5mL), H_2O (0.5mL), ethandithiol (0.25mL), and TFA (8.75mL) was prepared in the order stated. The dried crude product (*c.a.* 340 mg) was added to this cleavage mixture and allowed to stir for 3h at rt. The solution was filtered, washed with TFA (20mL) then H_2O (50mL), and the filtrate collected. A liquid-liquid extraction with ether (2 x 10 mL) and EtOAc (2 x 10 mL) was performed, collecting the aq layer in each case. The final aq solution was then freeze-dried overnight to yield a crude product. Figure A3-4 shows the cleavage site creating a *C*-terminal amide ($CONH_2$) peptide.

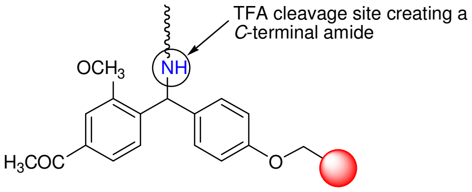

Figure A3-4 Rink amide resin used in Fmoc solid phase peptide synthesis showing the TFA cleavage site creating a *C*-terminal amide

Purification of the Peptide

RP-HPLC with a Shimadzu LC-6 system equipped with a 15-µm, 300-Å Phenomenex guard column and a separation column (250 by 21.20 mm) was used to purify the peptides. Wavelength of analysis was 214nm, unless stated elsewhere. The solvent system and time programme are specific for each peptide (see individual compounds for more details). The peptide fractions (50µL) were analysed with either a Waters Alliance

HT 2790 HPLC instrument coupled to a Micromass ZMD ESI-MS using infusion injection or a Waters Alliance Micromass LR-MS (MALDI-TOF) MS using α-cyano-hydroxycinnamic acid as the matrix (2 mg/mL in 1:1 acetonitrile:methanol). Collation of the pure fractions and freeze-drying yielded a pure product which was stored under a $N_{2(g)}$ atmosphere in the dark.

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Appendix for Chapter Four

To asses the reactivity of the un-derivatised β-CD hydroxy groups to Fmoc SPPS reaction conditions, direct coupling of β-CD to Rink resin was performed.

Direct Coupling of Peptides onto β-CD using Fmoc SPPS

The Val-Gly-Ala peptide was synthesised onto Rink resin (100 mg, 0.073 mmol, 1 equiv) as per **Appendix 6-IV** and following deprotection of the Fmoc group, β-CD (331 mg, 0.292 mmol, 4 equiv) was coupled using standard reaction conditions (**Chapter 3**, **Section 3.2.2.2**) of TBTU (94 mg, 0.292 mmol, 4 equiv) and DIEA (102 µL, 0.584 mmol, 8 equiv). A TFA cleavage (**Appendix 3-III**) gave no peptide-CD product. Unsuccessful coupling was due to no activation of the primary (or secondary) hydroxy groups on CD. This is also beneficial as no unpredicted coupling will occur during the reactions in **Chapters 4** and **6**.

Appendix for Chapter Five

APPENDIX 5-I *Carbonic Anhydrase (CA) Assay*

The carbonic anhydrase (CA) assay is based upon the α-carbonic anhydrases which are widespread metalloenzymes found in humans. These metalloenzymes have crucial physiological roles for the conversion of carbon dioxide to bicarbonate, but have more recently been found to be prominently associated with and over expressed in many tumour cells. Here they are connected with cancer progression and hence provide a measure of the response to therapy, making CA assays a good indicator for anticancer drugs.[1]

CA is a zinc metalloenzyme that catalyses the reversible hydration of carbon dioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and a proton (H^+). This metalloenzyme has become the target for drugs including acetazolamide as well as being found to have an importance in the over expression of tumour cells.[1-2]

CA is found in all animals and photosynthesising organisms, as well as some non-photosynthesising bacteria. In animals, it plays an important role in respiration by facilitating transport of CO_2 and is involved in the transfer and accumulation of H^+ and HCO_3^-. In chloroplasts of plant cells, their roles relate to the photosynthetic fixation of CO_2.[2b]

There are three CA families, α-, β-, and γ-CAs, all of which contain the zinc enzyme. However, α-CA is the one associated with the animal kingdom. α-CA has seven genetically similar sub-CA enzymes, all having differing tissue and intracellular locations. Some also exhibit differences in their catalytic efficiency and inhibitor properties. The one most studied is CA II due to its higher CO_2 hydration turnover rate. CA II is found in a wide range of cells and organs including bone, kidney, and the brain.[2b]

Sulfonamide drugs as well as inorganic and organic anions, including cyanide and fluoride, can achieve inhibition of the CA II enzyme. Divalent anions such as SO_4^{2-} do not inhibit the CA II enzyme due to the presence of negatively charged amino acids (glutamate) around the active site, which prevent binding of the divalent anions.

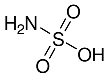
Inhibitors work by either displacing the zinc-bound water molecule or by distorting the overall active site of the enzyme, disallowing the conversion of CO_2 into HCO_3^-.[2b]

It has been found that certain molecules inhibit the functioning of these CAs. These include inorganic and organic anions as well as sulfonamides. Here, aromatic and certain heterocyclic sulfonamides are powerful and specific inhibitors of most CAs. These include compounds with unsubstituted $–SO_2NH_2$ or $–SO_2NH(OH)$ groups that bind to the metal ions associated with CA by the NH_2 or SO_2 of the sulfonamide group hence inhibiting their function. In other words, CA assays can provide a good starting point for the testing of samples as potential anti-cancer drugs.[1] It has been shown that by attaching different 'tails' to the aromatic and heterocyclic sulfonamides, it is possible to obtain different CA results indicating an increase in their potency, solubility changes, and membrane impermeability. For example, Supuran *et al.*[1] has shown that by attaching water-soluble tails such as 8-quinoline-sulfonyl- and nicotinoyl- groups to aromatic/heterocyclic sulfonamides it is possible to obtain efficient water-soluble inhibitors of the zinc enzyme CA.[1]

It has been shown that aliphatic sulfonamides such as methyl-sulfonamide are very weak inhibitors (KI is 10^{-4} molar) and extremely weak acids, whereas stronger acids such as trifluoromethyl-sulfonamide act as potent inhibitors of CA (KI is 2×10^{-9} molar).[2b]

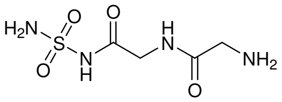
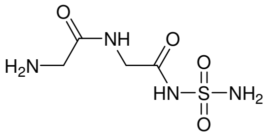
Unfortunately, the CA assay was not set up at the time of completion of this study. However, it points to one way of expanding this study beyond proof-of-concept to better understand the activity of these CD-peptide-sulfonamide moieties.

APPENDIX 5-II *Sulfamic Acid Experiments*



Sulfamic acid was trialled in these studies for the synthesis of non-aromatic peptide sulfonamides to compare what influence the aromatic ring has on their synthesis. In these experiments, attempts to add sulfamic acid directly to the *N*-terminus of a peptide chain or to the resin followed by the addition of a peptide for the synthesis of non-aromatic *N*- and *C*-terminal peptide sulfonamides respectively, were attempted all using standard Fmoc SPPS procedures. The experiments and results are given in Table A5-1. The peptide used for these studies was bi-glycine.

Table A5-11 Sulfamic acid experiments for the synthesis of *C*- or *N*-terminal non-aromatic peptide sulfonamides where the peptide is bi-glycine

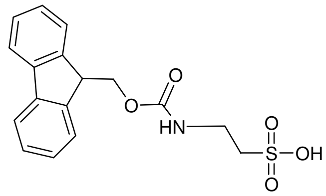
Experiment Number	*Reaction Details*	*Result*
S1	*C*-terminal peptide sulfonamide	Trace amounts in the crude product detectable in ESI-MS, nothing present after RP-HPLC. 
S2	*N*-terminal peptide sulfonamide	Trace amounts in the crude product detectable in ESI-MS, nothing present after RP-HPLC. 

These syntheses were unsuccessful due to the nature of the sulfonyl coupling groups and the low activation achieved. Fmoc protection of sulfamic acid was also attempted to aid the yields by preventing polymer formation, but this also was unsuccessful and hence no further studies were attempted using this compound.

APPENDIX 5-III *Taurine Experiments*

Taurine, an amino sulfonic acid was briefly studied for addition to resin for the formation of external non-aromatic peptide sulfonamides. Taurine is soluble only in water and therefore the addition of an Fmoc protecting group was carried out in a H_2O/dioxane mixture. Even with the addition of the Fmoc group, it did not improve its organic solvent solubility to any useful extent.

Synthesis of 2-Fmoc-aminoethanesulfonic acid

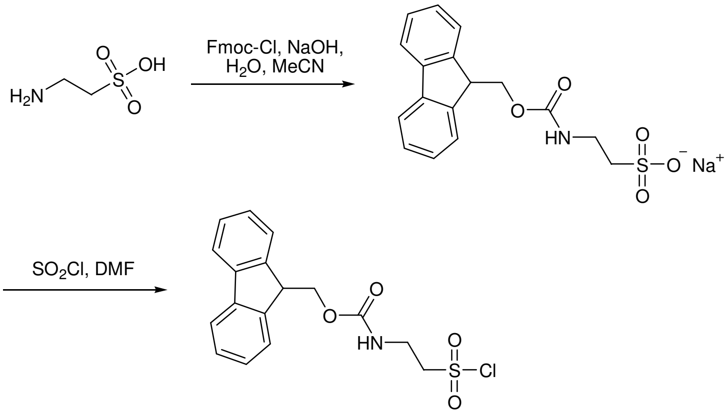


As per Liskamp *el al*[3] and Gobec *et al.*[4] 2-Fmoc-aminoethanesulfonic acid was investigated for its use to synthesise a *C*- and/or *N*-terminal peptide sulfonamide using standard Fmoc SPPS reaction conditions. It was found that 2-Fmoc-aminoethanesulfonic acid had a limited solubility in DMF so H_2O was required as the solvent. Rink resin (100 mg, 0.073 mmol, 1 equiv), 2-Fmoc-aminoethanesulfonic acid (101 mg, 0.292 mmol, 4 equiv), HBTU (94 mg, 0.292 mmol, 4 equiv), and DIEA (102 μL, 0.584 mmol, 8 equiv) were coupled in a 1:1 DMF:H_2O mixture overnight, and following deprotection the addition of a short peptide (Fmoc-tri-glycine). TFA cleavage (**Appendix 3-VI**) and RP-HPLC purification showed trace amounts of the desired product present.

EDC was also trialled as a coupling reagent for the synthesis of peptide sulfonamides. However, again only trace amounts of the product were detected after RP-HPLC purification.

It is thought that the low yields are due to the limited solubility of 2-Fmoc-aminoethanesulfonic acid in the solvent system used, in combination with the lack of reactivity of the taurine in this reaction system. From here, 2-Fmoc-aminoethanesulfonyl chloride was synthesised. It was thought that by making the chloride form, the molecule would be more easily added to resin in the presence of base.

Synthesis of 2-Fmoc-aminoethanesulfonyl Chloride



2-Fmoc-aminoethanesulfonic acid sodium salt and 2-Fmoc-aminoethanesulfonyl chloride was synthesised according to procedures by Byun *et al.,*[5] Humlian *et al.,*[4] and Bobec *et al.*[4]

Coupling of 2-Fmoc-aminoethanesulfonyl chloride (106 mg, 0.292 mmol, 4 equiv) to Rink resin (100 mg, 0.073 mmol, 1 equiv) was attempted using various coupling reagents but resulted in little or no product. Again solubility problems hampered the reactions. The coupling conditions and results used are stated below:

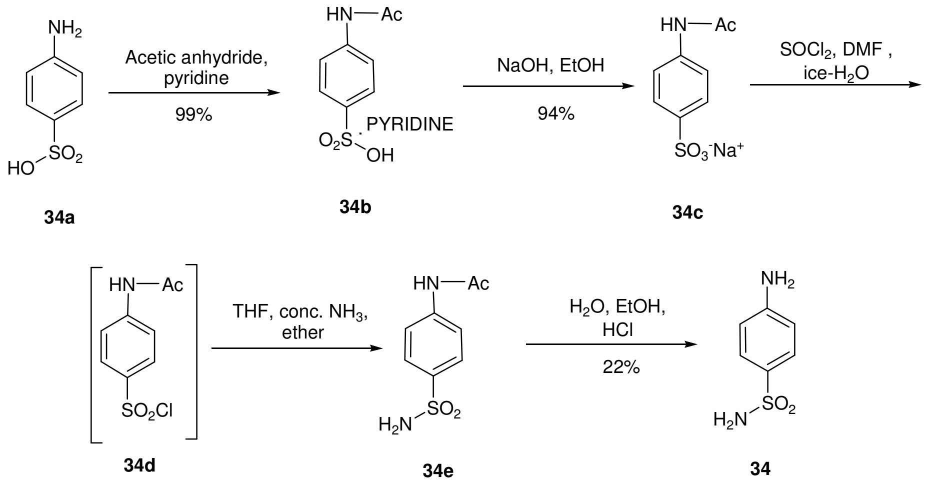
- HBTU (94 mg, 0.292 mmol, 4 equiv), and DIEA (102 μL, 0.584 mmol, 8 equiv) in DMF or 1:1 DMF: H_2O did not work well – trace amounts of the product – low solubility of the 2-Fmoc-aminoethanesulfonyl chloride species.

- 1:1 pyridine in DCM solution also resulted in a trace amount of the product

- H_2O with a 1.1 equiv TEA resulted in limited amounts of the product

Only small yields were obtained for the synthesis of *C*-terminal peptide sulfonamides and hence this method was not investigated further.

APPENDIX 5-IV *Sulfanilamide Synthesis (**34**)*



As per a paper by Boone *et al.*[6] 4-Aminobenzenesulfonamide (**34**) was synthesised by an adaptation of known procedures.[6] Ac_2O (8.2 mL, 53.6 mmol) was added dropwise by an addition funnel to a solution of 4-aminobenzene sulfonic acid (**34a**, 10 g, 57.8 mmol) and pyridine (68 mL). The resulting mixture was allowed to stir at rt for 2h. The solvent were removed *in vacuo*. EtOH (3 x 10 mL) was re-added and the resulting suspension was cooled in an ice bath and filtered. The solid obtained was washed with cold EtOH and ether, dried *in vacuo*, affording **34b** as a pale pink solid (16.83 g, 99%).

NaOH (1 M, 29 mL) at 0ºC and **34b** (8.35 g, 28.6 mmol) were added to a rbf and the resulting mixture was allowed to warm to rt. EtOH was added and the mixture was concentrated under reduced pressure by 75%. An additional portion of EtOH was added, and the mixture concentrated to dryness *in vacuo*. The solid was triturated with EtOH, filtered, washed with ether, and dried *in vacuo* (**34c**, 6.36 g, 94%).

Thionyl chloride (7.5 mL, 101.2 mmol, 3.7 equiv) was added dropwise to a solution of **34c** (6.36 g, 26.9 mmol) and DMF (73.5 mL) and stirred under a $N_{2(g)}$ atmosphere at -4ºC in an ice-brine bath. The mixture was kept below 0ºC for an additional 2h, poured into a beaker of crushed ice-H_2O (107 mL). The resulting yellow solid was collected by filtration, washed with several portions of H_2O, and dried under a stream of $N_{2(g)}$. The solid was then added portion wise to a stirred solution of THF (57.5 mL, 709 mmol, 26 equiv) and concentrated NH_3(aq) (30.5 mL) for 20 minutes, concentrated *in vacuo*, and triturated with cold ether. The solid obtained was washed with ether and dried *in vacuo* (**34e**, 3.07 g).

Compound **34e** (3.07 g, 14.4 mmol), H_2O (1.0 mL), and EtOH (16.95 mL) are stirred in a rbf under a $N_{2(g)}$ atmosphere. Concentrated HCl (7.06 mL) was added and the mixture was brought to reflux for 1h. The reaction mixture was cooled to rt, quenched with saturated $NaHCO_3$, and extracted with EtOAc (3 x 10 mL). The organic layer was washed with H_2O (2 x 10 mL) and brine (2 x 10 mL) and dried over $MgSO_4$, filtered, and concentrated *in vacuo* (**34**, 550 mg, 22%).

^{1}H NMR compound **34** (500 MHz, d_6-DMSO): δ 5.79 (s, 2H, SO_2NH_2), 6.58 (d, J = 7.0 Hz, 2H, NH_2) 6.88 (s, 2H, aromatic), 7.44 (d, J = 7.0 Hz, 2H, aromatic); ^{13}C NMR compound **34** (500 MHz, d_6-DMSO): δ 112.8 (s, 2C), 127.8 (s, 2C), 130.4 (s, 1C), 152.3 (s, 1C)

APPENDIX 5-V *Peptide Synthesis using 4-Aminobenzene Sulfonic Acid (**20**)*

Stability Tests

Before addition to resin, compound **20** was tested for its stability under standard Fmoc-SPPS reaction conditions as per compound **12** (**Chapter 2**, **Section 2.4.4.4**). These conditions and results are given in Table A5-12.

Table A5-12 Stability test conditions and results for compound **20** when exposed to standard Fmoc SPPS reaction conditions.

Reaction Conditions	*Experimental Time (Hours)*	*Results (Determined by MALDI-TOF)*
100% DMF	24	No change
100% DCM	24	
20% Pip in DMF	0.5	No change
20% Pip in DMF	1.0	
95% TFA cleavage mixture	3.0	No change
100% TFA	24	

Peptide Synthesis

Peptide synthesis using compound **20** gave only trace amounts of the *C*-terminal peptide sulfonamide when coupling with varying reaction conditions (Table A5-13). Peptide synthesis was carried out as per **Appendix 3-III** on Rink resin (292 mg, 0.25 mmol, 1 equiv) using a 20% pip in DMF solution for deprotection. The sulfonyl species (**20**) was coupled to the resin first (using the reaction conditions stated in Table A5-13) followed by the coupling of Fmoc-tri-glycine (395 mg, 1 mmol, 4 equiv, twice). The Kaiser test[7] was used to determine if the coupling of Fmoc-tri-glycine was successful. Cleavage as per **Appendix 3-III**. MS detection was used to determine the presence of the crude product. A MeOH stepwise RP-HPLC purification (**Chapter 2**, **Section 2.4.4.4**) was only applied if the crude yield was suitable. Samples were dissolved up in buffer A with sonication and filtered.

Table A5-13 Coupling conditions for the coupling of **20** to Rink resin for *C*-terminal peptide sulfonamide synthesis where the peptide was Fmoc-tri-glycine

Experiment Number	*Coupling conditions*	*Coupling Time*	*Result*
A-19	HBTU (2 equiv)/ DIEA (4 equiv) in DMF	overnight	Nothing
B-19	HBTU (4 equiv)/ DIEA (8 equiv) in DMF	Overnight plus one recoupling	Trace, < 1%
C-19	TBTU (4 equiv)/ HOBt (4 equiv)/DIEA (8 equiv) in DMF	overnight	Trace, < 1%

Overall, this unprotected sulfonic acid was unsuitable for synthesis of peptide sulfonamides. See **Chapter 5** for alternative methods.

APPENDIX 5-VI *TMSBr Cleavage*

As per White *et al.*[7] Place the dry resin in a flask and add *m*-cresol (0.25 mL/g resin), EDT (1.25 mL/g resin), thioanisole (9.35mL) and TFA (18mL/g resin). Add peptide resin and cool to 0ºC. Flush flask with $N_{2(g)}$ and quickly add TMSBr (3.3 mL/g resin) and flush the flask again before sealing. Leave to stand for 15 minutes to 3h (depending upon the sequence) with occasional agitation. Remove the resin by suction filtration though a sintered glass funnel. Wash resin twice with clean TFA then H_2O. Combine filtrates and wash twice with ether then again with ethyl acetate. Combine aq layers, and freeze dry overnight.

Table A5-14 Approximate volumes to be used for a TMSBr cleavage on 450 mg of dry resin

For 450mg of resin:	
TFA	6.9mL
Thioanisole	1.1mL
EDT	0.55mL
P-cresol	0.2mL
TMSBr	1.25mL

APPENDIX 5-VII *Peptide Synthesis using 4-Aminobenzenesulfonyl Chloride (**24**)*

Peptide synthesis using **24** (70 mg, 0.365 mmol, 5 equiv) was undertaken as per **Chapter 5** (**Section 5.4.3.1**) using the coupling conditions stated in Table A5-15. The results are given in Table A5-5. The peptides (Table A5-15) were cleaved from the resin using a 20% TFA in DCM cleavage mixture and stirred for 3h at rt, followed by filtration, removal of the solvent *in vacuo*, and freeze-drying the solid. Fmoc amino acids coupled with TBTU (2 equiv) and DIEA (4 equiv) in DMF overnight

Table A5-15 Coupling conditions and results for the coupling of **24** to Rink resin coupling overnight

Experiment Number	*Peptide Chain*	*Coupling Reagents for 24*	*MS results*
A-23	Ala-Phe-Gly	1:1 Pyridine in DCM slurry	Unsuccessful
B-23	Gly-Gly-Gly-Ala-Phe	10 equiv TBTU, 10equiv DIEA in DMF	Unsuccessful
C-23	Ala-Phe-Gly-Gly-Gly	1:1 Pyridine in DCM slurry	Unsuccessful
D-23	Hexaglycine	1:1 Pyridine in DCM slurry	1.5 mg Crude product

The peptide sulfonamide (*Experiment D-23*) was purified by RP-HPLC at a wavelength of 214 nm using a MeCN stepwise gradient (Table A5-12). An 8 mL/min flow rate was used along with the following buffers: buffer A, 2% MeCN and 0.1% TFA; buffer B, 90% MeCN and 0.1% TFA (30%). The product eluted at 37% B (1.5 mg, 4%) with tri-glycine eluting at 26%B. The product peak was very small compared to the non-sulfonated tri-glycine peptide peak suggesting that more work was needed to achieve a better yield in this reaction.

Table A5-16 Acetonitrile step-gradient time program for the separation of the peptide sulfonamide (*Experiment D-23*) using a preparative RP-HPLC column

Time (min)	*% Mobile phase 'B'*	*Time (min)*	*% Mobile phase 'B'*
0	0	60	60
10	0	80	100
15	5	90	0
60	60	100	STOP

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Appendix for Chapter Six

APPENDIX 6-I *Cell Preparation for Assays*

The cell lines K562 (human haematopoietic leukaemia), MA-104 (rhesus monkey kidney epithelial carcinoma), HT-29 (human duodenal carcinoma), MCF-7 (human mammary carcinoma), and Hs700T (human pancreatic carcinoma) were obtained from the American Type Culture Collection (ATCC, Australia) and grown as recommended by the supplier. Cells were cultured in DMEM or RPMI as appropriate containing 10% FCS, 1% PSG, and 1% $NaHCO_3$ (complete medium). Cell lines were routinely passaged (adherent lines using trypsin/EDTA) once or twice weekly at *c.a.* 1:10 split.

APPENDIX 6-II *MTT (Cell Proliferation) Assay*

A single-cell suspension of cells in complete medium was placed into 96-well plates at *c.a.* 6×10^3 cells per well in a volume of 100 µL. Plates were incubated overnight to allow the cells to attach. The medium was then removed and replaced with matching medium containing no additive (negative control), test additive, or 5-FU (positive control) at various concentrations. For each assay condition, 3-4 replicate wells were set up.

Plates were incubated with stimuli for an additional 3 days. Then an aliquot of 10 µL of MTT (5 mg/mL in phosphate-buffered saline) was added to each well. 3–4h later when purple crystals had formed, the medium in each well was removed. The reaction was stopped and the crystals solubilised by the addition of DMSO. The plates were read on a microtiter plate reader at a wavelength of 550 nm and the optical density (Equation A6-1) of each well recorded.

Equation A6-1 Equation to calculate the optical density for the MTT assay

$$\textbf{Optical Density (OD)} = A_\lambda = -\log (I/I_o)$$

Where:

I = *Intensity of light at a specific wavelength (λ) passed through the sample*

I_o =*Light before passing through the sample*

APPENDIX 6-III *Flow Cytometric (Cell Cycle) Assay*

Cells were plated in replicate wells of a 12 well plate at a concentration of *c.a.* 0.2 x 10^6 per well in a volume of 1 mL and allowed to adhere overnight in a 37ºC incubator. Medium was removed and replaced with fresh medium containing no additive, 5-FU at 50 µg/mL, or test substances at 50 µg/mL, and the plates returned to the incubator. After 6, 24, or 48h the contents of the well were collected by washing the monolayer with cold PBS and incubating with trypsin-EDTA. Medium, PBS washes, and trypsinised cells from a well were pooled and centrifuged at 1500 rpm for 5 minutes, and the cell pellet resuspended in ice-cold 70% ethanol. After refrigeration for 1–2h, the permeabilised cells were washed in cold PBS, resuspended in cold PBS containing RNase, and incubated in a 37ºC waterbath for 20 minutes. PI was then added to a final concentration of 10 µg/mL and samples were scanned on a flow cytometer (FACSCalibur, Becton Dickinson) using CellQuest software to assess fluorescence (FL-3). Histogram gates for cells in G0/G1, S, and G2/M were selected manually based on each cell line's characteristics, as shown below.

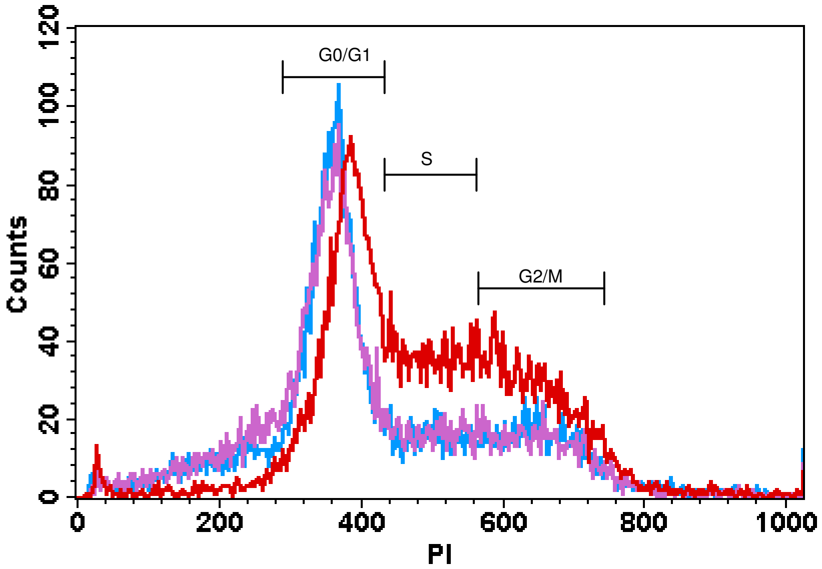
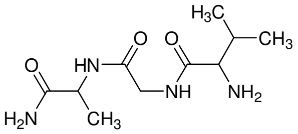


Figure A6-5 Histogram gates for cells in G0/G1, S, and G2/M. Representative experiment of cell cycle analysis for the MA-104 cell line. Cells were harvested after 24h treatment with no additive ▬, C1 ▬, or 5-FU ▬, with the latter treatment resulting in a build-up of cells arrested in S/G2/M.

APPENDIX 6-IV *Synthesis of Val-Gly-Ala Model Peptide (**35**)*

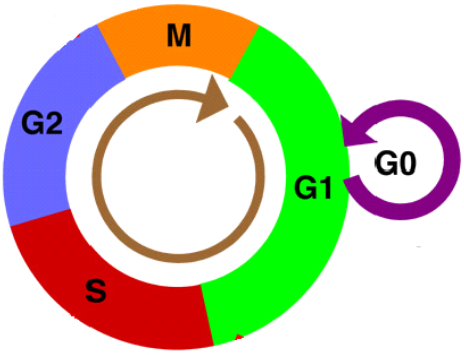


35

The Val-Gly-Ala peptide (**35**) was assembled manually on Rink resin (4.5 g, 3.24 mmol, 1 equiv) as per **Appendix 3-III**. Fmoc amino acids (13.7 mmol, 4 equiv) were coupled overnight followed by a second re-coupling with TBTU (4.2 g, 13.7 mmol, 4 equiv) and DIEA (4.8 mL, 28 mmol, 8 equiv) in excess as activating reagents. TFA (95%, **Appendix 3-III**) was used to cleave the peptide from the resin. An MeCN RP-HPLC stepwise gradient as per **Chapter 5**, **Section 5.4.5** was used to purify the product eluting the pure peptide at 28% B (560 mg, 71%).

LR-MS: *m/z* (%, assignment) $C_{10}H_{20}N_4O_3$ $(M+H)^+$ 244.15, actual 244.20 (100, M^+) HR-MS: *m/z* $C_{10}H_{21}N_4O_3$ $(M+H)^+$ 145.1608, actual 245.1613; ^{1}H NMR (500 MHz, d_6-DMSO): δ 0.94 (d, J = 7.0 Hz, 6H, CH_3, CH_3), 1.21 (d, J = 7.3, 3H, CH), 2.07 (q, J= 6.2 Hz, 1H, CH), 3.66 (m, 1H, CH), 3.83 (dd-dd, J = 5.4 Hz, 2H, CH_2), 41.19 (q, J= 6.6 Hz, 1H, CH), 7.39 (s, 1H, NH), 8.49 (d, J = 7.2 Hz, 1H, NH), 8.69 (t, J = 6.60 Hz, 3H, NH, NH_2); DEPT-135 NMR (500 MHz, d_6-DMSO): δ 18.1, 18.5, 30.2, 34.6, 39.9, 48.6, 57.9

APPENDIX 6-V *Stages of the Cell Cycle*[1]



Where:

M	Cell division (mitosis)	
G1	Cell growth	*Interphase*
S	DNA replication	
G2	Cell prepares to divide	
G0	Resting cell	

Mitosis

Production of daughter cells through nuclear division. Consists of 4 stages and makes up only a small part of the cell cycle. These stages are:

- Prophase: Replicated coil condenses and the joined chromosomes divide. Spindle also appears
- Metaphase: Spindle fibres attach to the centromeres of the chromosomes and they gradually migrate to the middle of the cell
- Anaphase: Separation of the chromosomes into two cells
- Telophase: Chromosomes uncoil and two cells formed.

Interphase:

The cell spends most of its time in this phase and it consists of 3 stages (G1, S, G2) where:

- G1 is the first gap stage following mitosis and is a period of growth and metabolic activity.
- S phase follows G1 and is a period of DNA replication and synthesis
- G2 follows S phase and precedes the next mitotic division. This stage contains a duplicate set of chromosomes in the cell

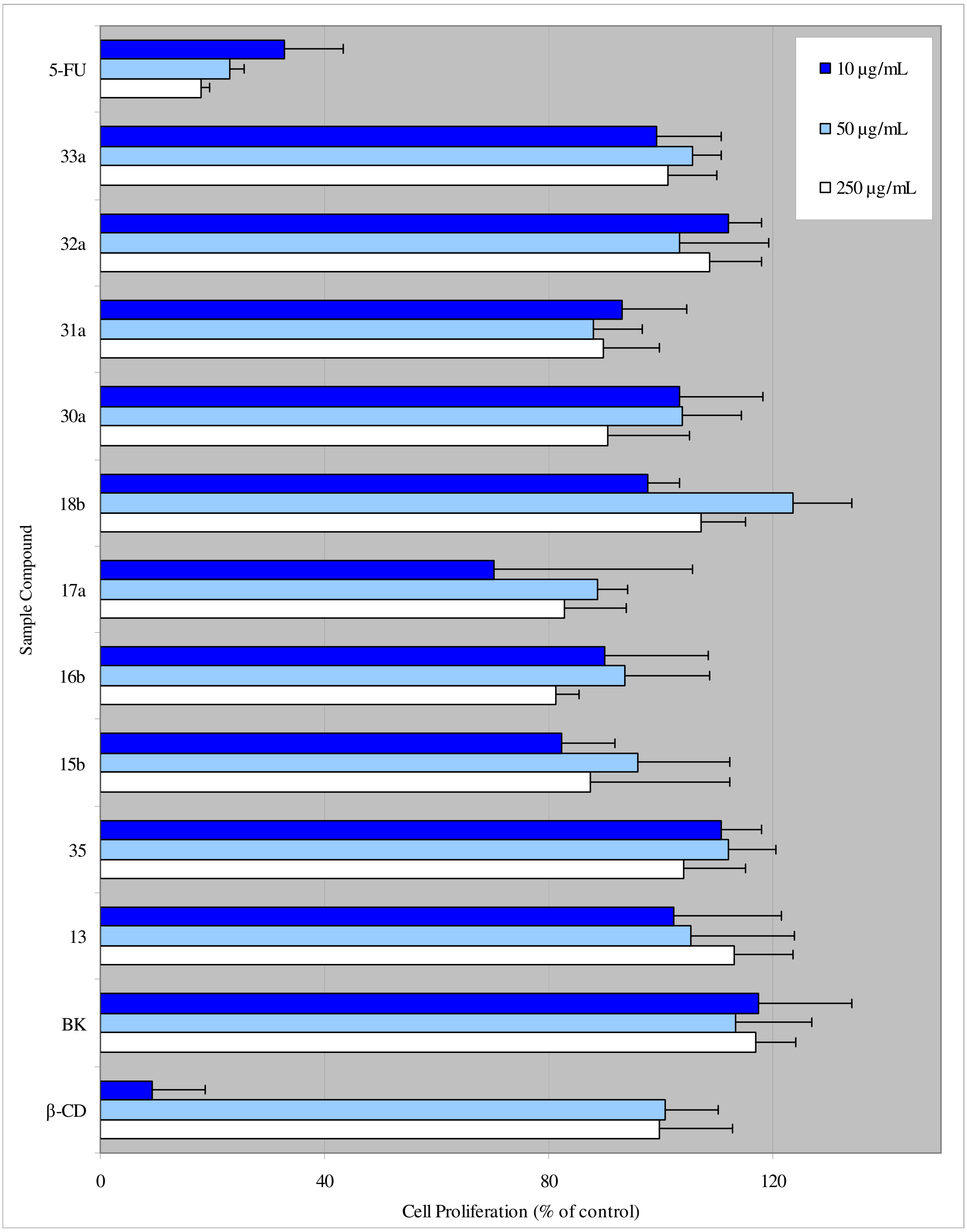
APPENDIX 6-VI *Model Peptide Effect on HT-29 (Human Duodenum), Hs700T (Human Pancreas), and MA-104 (Monkey Kidney) Cell Lines Stages of the Cell Cycle*

5-FU significantly diminished proliferation in all three cell lines (Graph A6-6, Graph A6-7, and Graph A6-3) in a linear dose-dependent fashion. Commercial bradykinin (BK) and lab synthesised bradykinin (**13**) slightly increased proliferation of HT-29 and Hs700T cells when present at 250 μg/mL, while having no effect on the MA-104 cells. BK was shown to increase cell proliferation by 10–20% at 10 μg/mL in some epithelial cells as shown for commercial and self-made BK.[2] β-CD itself had little effect on any of the three cell lines. Test compound **35** did not diminish cell proliferation in the three cell lines compared to β-CD or untreated cells.

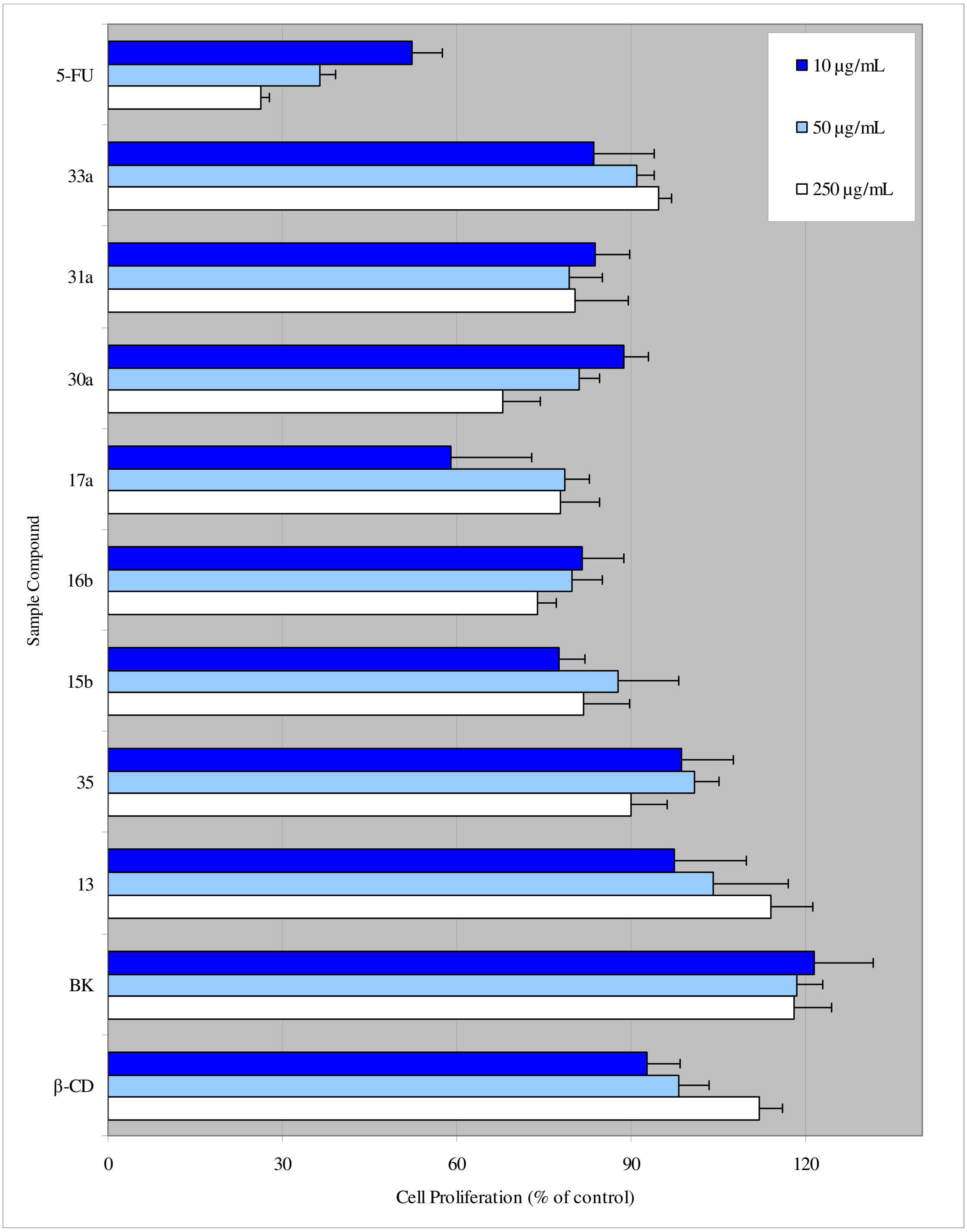
In contrast, compound **17a** (mono-6^A-amino-6^A-Val-Gly-Ala-mono-6^X-succinyl-6^X-L-Val-Gly-Ala-β-cyclodextrin) configuration appeared to decrease cell proliferation in all three cell lines when present at a low (10 μg/mL) concentration. It would be worth repeating the assay of **17a** at even lower concentrations to see where the maximal effect is observed. It is presumed that the affect seen here is due to the two β-CD moieties as the peptide alone has no activity. Unfortunately this effect can not be compared to the BK form of this compound (mono-6^A-amino-6^A-BK-mono-6^X-succinyl-6^X-BK-β-cyclodextrin) as it was not bioassay tested. It would be worth testing in future studies.

Compound **18b** also had a slight increase in cell proliferation for the MA-104 cell line when compared to β-CD and the model peptide (**35**). As for all other tested samples, no significant positive or negative activity was found.

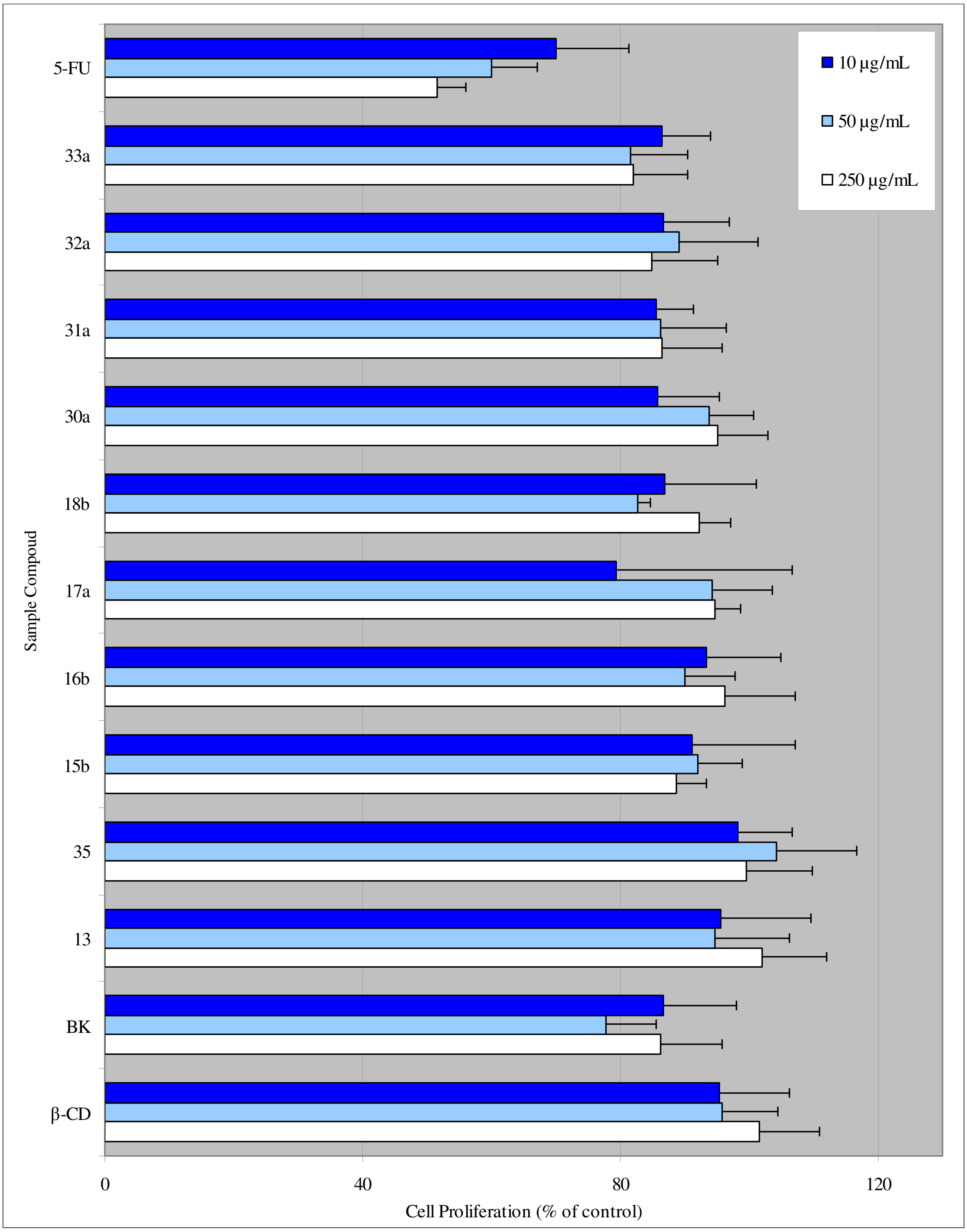
Graph A6-6 Cell proliferation effect (MTT assay) of cyclodextrin-model-sulfonamide complexes on HT-29 (human duodenal adenocarcinoma) cells after 72h



Graph A6-7 Cell proliferation effect (MTT assay) of cyclodextrin-model-sulfonamide complexes on Hs700T (human pancreatic adenocarcinoma) cells after 72h



Graph A6-8 Cell proliferation effect (MTT assay) of cyclodextrin-model-sulfonamide complexes on MA-104 (monkey kidney carcinoma) cells after 72h



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