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PSEUDOPEPTIDES AND PEPTIDOMIMETICS MODULATING THE PROTEOLYTIC ACTIVITY OF KALLIKREIN-RELATED PEPTIDASE 3

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ACADEMIC DISSERTATION

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Cover picture: The C-4 peptide docked in its presumed binding site on KLK3. Part of the peptide has been omitted for clarity. Molecular modelling data courtesy of Henna Ylikangas.

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Every kid starts out as a natural-born scientist, and then we beat it out of them. A few trickle through the system with their wonder and enthusiasm for science intact.

Carl Sagan (1934-1996)

ABSTRACT

The highly prostate specific serine protease kallikrein-related peptidase 3 (KLK3, also known as prostate specific antigen, PSA) is widely used as a biomarker for prostate cancer and it has also been postulated that it may play a part in tumour growth. Especially interestesting is the antiangiogenic effect exerted by proteolytically active KLK3 in cell line models. In order to stimulate the proteolytic activity of KLK3, a series of peptides have been developed by phage display methodology. Even though the peptides are quite potent KLK3 stimulators, they are not directly suitable for *in vivo* studies or use as drugs.

Even though there are many natural and unnatural biologically active peptides, they suffer from rapid clearance via the liver and kidneys and proteolytic degradation of the compounds both in the gastrointestinal tract and other parts of the body. This gives peptides a poor oral bioavailability meaning that they are usually administered as intravenous or intramuscular injections. Several different strategies have been developed in order to access compounds with improved bioavailability including modifications of the peptide structure, development of pseudopeptides and development of small molecular weight peptidomimetics.

This thesis concentrates on the further development of the two most potent peptides known to stimulate KLK3, i.e. B-2 and C-4. The main part of the work was concentrated on the replacement of disulphide bridges in the peptides in order to both gain more information on which residues are necessary for obtaining the biological activity and at the same time also gain information on how changes to the geometry of the disulphide bridge affects the activity.

A series of different disulphide bridge mimicking building blocks were designed and synthesised with the intention of using them in a protocol for solid-phase synthesis of KLK3 stimulating peptides. Unfortunately, the use of these building blocks in the synthesis of pseudopeptides based on C-4 turned out to be an unsurmountable challenge and the synthesis had to be completed using a different strategy in which the key step was the use of ring-closing metathesis (RCM) for the cyclisation of the partly completed pseudopeptide. Pleasingly, the synthesis of pseudopeptide analogues of the B-2 peptide using the building blocks was more successful. In total three pseudopeptide analogues of C-4 and four of B-2 were synthesised and shown to retain the biological activity of the parent peptides.

Based on the information from the synthesised pseudopeptides and a molecular modelling study, a 4-quinolone based peptidomimetic was designed to mimic the C-4 peptide and a synthetic protocol was devised to access this compound. Even though the synthesis of the desired target compound has so far not been successful, the synthetic protocol that was designed has given access to a number of 1,2,8-trisubstituted 4-quinolone derivatives.

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LIST OF ORIGINAL PUBLICATIONS

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I. Tadd, AC; Meinander, K; Luthman, K; Wallén, EAA; Synthesis of Orthogonally Protected Disulfide Bridge Mimetics, *J. Org. Chem.* 2011, **76**, 673–675

II. Meinander, K; Weisell, J; Pakkala, M; Tadd, AC; Hekim, C; Kallionpää, R; Widell, K; Stenman, U-H; Koistinen, H; Närvänen, A; Vepsäläinen, J; Luthman, K; Wallén, EAA; Pseudopeptides with a Centrally Positioned Alkene-Based Disulphide Bridge Mimetic Stimulate Kallikrein-Related Peptidase 3 Activity, *Med. Chem. Commun.*, 2013, **4**, 549-553

III. Meinander, K; Pakkala, M; Weisell, J; Stenman, U-H; Koistinen, H; Närvänen, A; Wallén, EAA.; Replacement of the Disulfide Bridge in a KLK3 Stimulating Peptide Using Orthogonally Protected Building Blocks; *ACS Med. Chem. Lett.*, 2014, **5**, 162-165

IV. Meinander, K.; Fissers, J.; Luthman, K.; Wallén, EAA.; KLK3-Stimulating Peptide Mimetics. *Manuscript*

The publications are referred to in the text by their roman numerals. The supporting information for original publications **I-III** is not included in the thesis. This material is available from the author or via the internet at http://pubs.acs.org for original publications **I** and **III**, and http://pubs.rsc.org for publication **II**. Original publications **I-III** are included with the permission of the copyright holders. In addition, one manuscript (publication **IV**) is included.

An incorrect structure in article **II** has been corrected with an amendment published on 16th May 2013. The amendment can be found online at http://www.rsc.org/suppdata/md/c3/c3md20292e/addition.htm and after the article in the printed edition of this thesis.

ABBREVIATIONS

Aaa	Arbitrary amino acid residue
Ac	Acetyl
Acm	*S*-Acetamidomethyl
ACT	α1-Antichymotrypsin
ADME	Absorption, distribution, metabolism and excretion
All	Allyl
Alloc	Allyloxycarbonyl
Bn	Benzyl
Boc	*tert*-Butoxycarbonyl
BOP	(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BPH	Benign prostatic hyperplasia
Cbz or Z	Benzyloxycarbonyl (or carboxybenzyl)
CCK	Cholecystokinin A
CDI	*N*,*N*-Carbonyldiimidazole
CM	Cross metathesis
CMC	Comprehensive medicinal chemistry
DAS	2,7-Diaminosuberic acid
dba	Dibenzylideneacetone
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DEAD	Diethyl azodicarboxylate
DIAD	Diisopropyl azodicarboxylate
DIPEA	Diisopropylethylamine
DLR	Drug like range
DMF	*N*,*N*-Dimethylformamide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDT	1,2-Ethanedithiol
EPCA	Early prostate cancer antigen
Fmoc	9-Fluorenylmethoxycarbonyl
G2	Grubbs 2nd generation catalyst
GI	Gastrointestinal
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate
HBTU	*N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate
HG2	Hoveyda-Grubbs 2nd generation catalyst
HIV	Human immunodeficiency virus
HMPA	Hexamethylphosphoramide
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole

HPLC	High performance liquid chromatography
HRV	Human rhinovirus
HUVEC	Human umbilical-vein endothelial cells
IC_{50}	Half maximal inhibitory concentration
KLK or hK	Human kallikrein-related peptidase
Me	Methyl
MS	Mass spectrometry
α-MSH	α-Melanocyte stimulating hormone
MW	Microwave
nd	Not detected
NMR	Nuclear magnetic resonance
PCA3	Prostate cancer antigen 3
PDB	Protein data bank
PG	Protecting group
Piv	Pivaloyl
PSA	Prostate-specific antigen
PTSA	*para*-Toluenesulphonic acid
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyBroP	Bromo-tris-pyrrolidino phosphonium hexafluorophosphate
RCM	Ring-closing metathesis
RT	Room temperature
SAR	Structure-activity relationship
SIRT2	Sirtuin 2
SPMIP/SgI	Seminal plasma motility inhibitor precursor/semenogelin I
SPPS	Solid phase peptide synthesis
TBDMS	*tert*-Butyldimethylsilyl
TBTU	*O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate
^{t}Bu	Tertiary butyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol
TMSOTf	Trimethylsilyl trifluoromethanesulphonate

1 GENERAL INTRODUCTION

1.1 HUMAN KALLIKREIN-RELATED PEPTIDASE 3 AND ITS ROLE IN PROSTATE CANCER

Human kallikrein-related peptidase 3 (KLK3 or hK3), that is better known as prostate-specific antigen or PSA, is a 28 kDa, 237 residue protein belonging to the family of 15 peptidases referred to as kallikreins.[1,2] It is found almost exclusively in the epithelium of the prostate although small amounts have also been isolated from for example the ileum, testis and thyroid gland.[3,4] KLK3 exhibits chymotrypsin-like serine protease activity with a preference for cleavage of substrates after tyrosine, leucine or glutamine residues.[5-8] Its main physiological role is cleavage of the gel-forming proteins semenogelin I and II in order to dissolve seminal clots and thus improve sperm motility.[5-7] The active site of the members of the kallikrein family comprises the catalytic triad consisting of residues His57, Asp102 and Ser195.[9-12] In the so-called classical kallikreins (KLKs 1-3), the active site is located under a large, flexible insertion consisting of 11 amino acid residues following Asn95 (KLK1) or Asp95 (KLKs 2 and 3). This insertion is referred to as the kallikrein loop (coloured yellow in Figure 1) and has been suggested to play a role in the activity of KLKs 1-3.[10,12-15] The biosynthesis of KLK3 occurs mainly in the epithelium of healthy prostate and is decreased by abnormalities in the prostate although it is not completely eliminated. Both malignant (e.g. prostate cancer) and non-malignant (e.g. benign prostatic hyperplasia, BPH) conditions cause modifications to the structure of the prostatic tissue resulting in leakage of KLK3 into the plasma.[16] Once in the plasma, KLK3 is deactivated by protease inhibitors such as α1-antichymotrypsin (ACT) and α2-macroglobulin that form covalent complexes with KLK3 thus inhibiting its activity.[17,18] Various other inactive forms of KLK3 such as pro-KLK3 and nicked KLK3 have also been found in plasma.[19]

It has been shown that malign or benign changes in the prostate cause leakage of KLK3 into the plasma. This can be utilised for detecting and monitoring the risk for an individual to develop prostate cancer. This is especially helpful as prostate cancer does not cause clinical symptoms until it has spread into surrounding tissues and started affecting the urinary function or it has metastasised into other tissues, mainly the lymph nodes or bones. This is further complicated by the fact that prostate cancer can exhibit a number of different clinical behaviours, ranging from insignificant, slow-growing tumours to aggressively metastatic and fatal forms.[20-22] Taking this into account, screening individuals believed to be at higher risk of developing prostate cancer is helpful. The diagnosis can then be verified by using, in addition to the KLK3 assay, a digital rectal examination, transrectal ultrasonography and/or transrectal ultra-sound guided biopsies.[23]

Prostate cancer is the second most common form of cancer in males worldwide (the most common in the developed world) and sixth in the number of cancer deaths worldwide, although there are large variations in the incidence rates.[24] When

examining the situation in the United States, it has been shown that around one in six men will develop prostate cancer in their lifetime with the mortality rate being 3.4 %.[25]

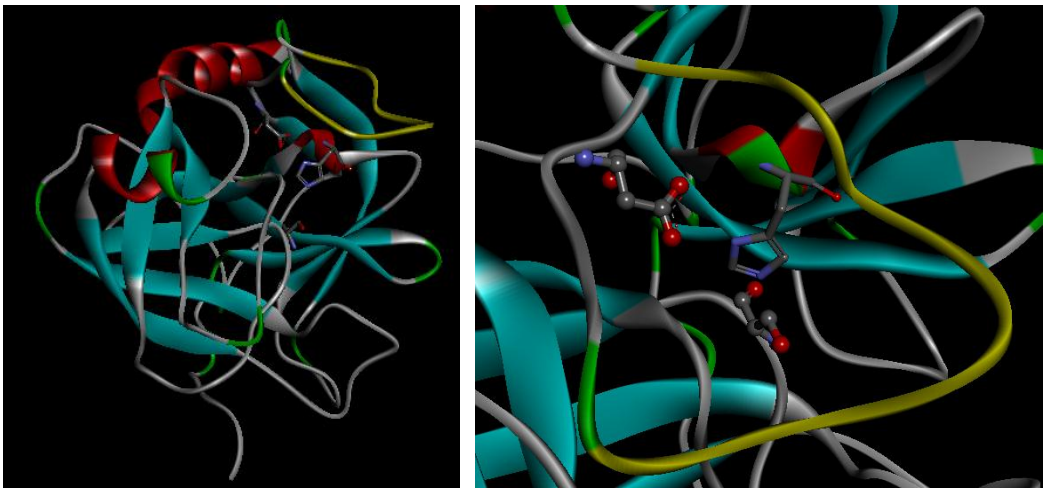


Figure 1 The structure of KLK3 with the kallikrein loop highlighted in yellow. The right-hand image shows a close-up of the position of the active site under the kallikrein loop. Images adapted from ref 11.

Even though the KLK3 assay is widely used in screening for prostate cancer and has proven highly effective, it does also have some drawbacks. One known problem is that many benign conditions (BPH, prostatitis) also cause elevations in the serum levels of KLK3 which this simple test cannot discriminate from malign conditions. This combined with the variations in the growth rates of tumours causes an overdiagnosis of prostate cancer which in turn causes many patients to have to go through unnecessary treatments such as prostatectomies or radiotherapy. Thus the patients have to suffer the side-effects of these treatments when only having a benign or latent condition.[19] In addition to the problems associated with distinguishing clinically relevant tumours from others it has also been suggested that measuring KLK3 levels will only reflect on the relative risk of cancer. Additionally, no level can be defined that would completely rule out the possibility of cancer.[26] From this, it can be concluded that other biomarkers are also required for the screening of prostate cancer. Other possible biomarkers that have been suggested include human kallikrein-related peptidase 2 (KLK2), early prostate cancer antigen (EPCA), prostate cancer antigen 3 (PCA3) and/or hepsin.[27,28] Although a combination of the serum level of KLK2 with measurements of different forms of KLK3 have been claimed to improve the diagnosis and prognosis of prostate cancer,[19,29-31] the search for new biomarkers still continues.[27,32]

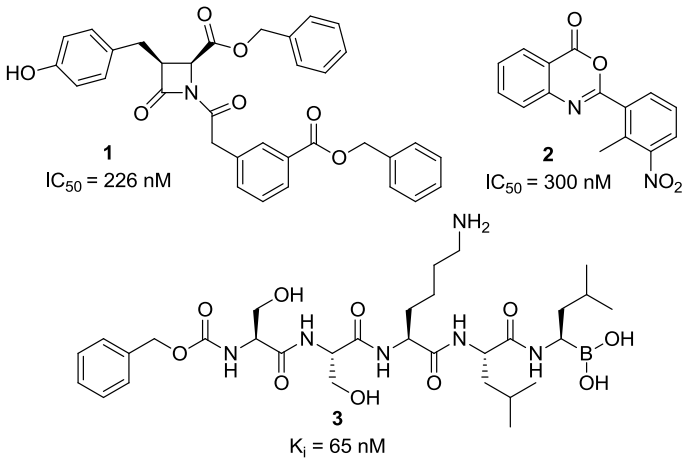
Even though the role of KLK3 in prostate cancer is still largely unknown, it has been shown that it inhibits angiogenesis. In both *in vivo* and *in vitro* models KLK3 has been proven to reduce the growth of blood vessels by inhibiting endothelial cell growth, invasion and migration.[33,34] In a HUVEC (human umbilical-vein endothelial cells) model it has been shown that KLK3 can inhibit tube formation, indicating that

the enzymatic activity of KLK3 is important for the antiangiogenic effects[35] and that peptides can be used to enhance this activity.[36] It has also been shown that poorly differentiated tumours show a lower level of KLK3 expression.[37-39]

On the other hand, several investigations have also shown that KLK3 may have a role in promoting tumour growth and the formation of metastases. For example, KLK3 has been postulated to promote osteoblast proliferation and thereby play a role in the formation of bone metastases.[40,41] A recent study has also shown that the lowering of KLK3 levels in an LNCaP human prostate cancer cell line reduced the growth rates of these cells. This would suggest that enzymatically active KLK3 is required for the progression of prostate cancer.[42]

1.2 COMPOUNDS MODULATING THE ENZYMATIC ACTIVITY OF KLK3

KLK3 and especially its enzymatic activity seems to play an important part in the development of prostate cancer. As KLK3 has been shown to both inhibit[35,39] and stimulate prostate tumour growth as well as promoting both the formation of bone metastases[43,44] in prostate cancer and stimulating tumour growth in breast cancer,[45,46] compounds both inhibiting and stimulating KLK3 have been investigated. It has been shown that metal ions such as Zn^{2+}, Hg^{2+}, and, to a lesser extent, Mn^{2+} inhibit the hydrolysis of the seminal plasma motility inhibitor precursor/semenogelin I (SPMIP/SgI) by KLK3 and chymotrypsin.[7] As KLK3 exhibits proteolytic activity similar to other serine proteases, several inhibitors of KLK3 have been found. Adlington *et al*. have used a *β*-lactam derivative known to inhibit other serine proteases to develop several compounds inhibiting KLK3, the most potent (**1**) had an IC_{50}-value of 226 nM.[47,48] The *β*-lactams have also been used to study the mechanism for KLK3 inhibition.[49] A more recent screening of a library of approximately 50 000 compounds identified two other compounds that inhibit KLK3 at micromolar concentrations and also exhibit selectivity for KLK3 over chymotrypsin, **2** was the most potent of these with an IC_{50}-value of 300 nM.[35] Based on studies of peptide substrates for KLK3,[50] several peptidyl boronic acids such as **3** that inhibit KLK3 have also been developed and utilised to further elucidate the mechanism of inhibition.[51,52]



As proteolytically active KLK3 has also been hypothesised to inhibit tumour growth, compounds stimulating this activity could aid in the treatment of prostate cancer. Wu *et al*. used phage-display methodology to identify three peptides that bind specifically to KLK3 and exhibit a significant stimulating effect. The cyclic peptides denoted *A-1*, *B-2* and *C-4* are shown in Figure 2.[53] The most potent of these was the bicyclic peptide C-4 (**4**). It has been suggested that these peptides bind in the vicinity of the active site of KLK3.[10,53,54] Additionally, the binding affinity of the peptides was increased by Zn^{2+} which is known to inhibit KLK3.[53] Some short, linear peptides that approximately double the proteolytic activity of KLK3 have also been developed by another group but this line has not been explored further.[14]

Synthetically modified analogues of peptides C-4 (**4**) and B-2 (**5**) have also been demonstrated to retain their biological activity.[54,55] Replacement studies performed on these peptides have also given some indications of which residues in the peptides are important for retaining the biological activity. In the B-2 peptide all residues appear to be important for retaining the biological activity while in the C-4 peptide, several of the residues can be replaced with alanine without a significant loss in biological activity.[54-56] It has also been shown that the stability of peptide B-2 in plasma can be increased without losing the biological activity by replacing the terminal disulphide bridge by a bridge consisting of *γ*-aminobutyric acid and aspartic acid.[55]

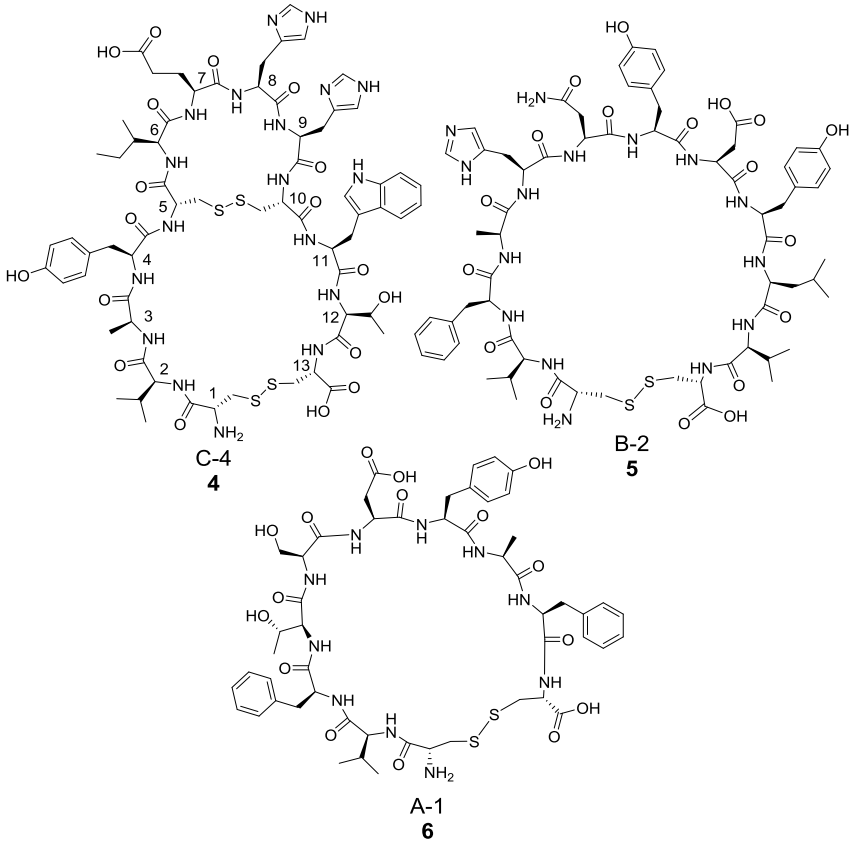
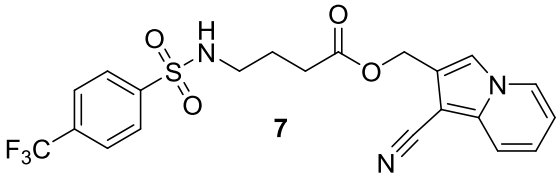


Figure 2 KLK3 stimulating peptides identified by phage display.[53]

For development of peptide mimicking compounds, it would be important to know the binding site of the peptides on the target enzyme. This information is often derived from the crystal structure of the protein in question. In the case of KLK3 this work is quite complicated as the only structural information available are two crystal structures found in the Protein Data Bank (PDB) (2ZCK and 3QUM), unfortunately both of these structures also include an antibody. The 2ZCK-structure also contains a substrate molecule which leads to some uncertainty if the crystal structure actually corresponds to the proteolytically active form of the enzyme or not.[11,12] Even though there is some conformational data available on the peptides that stimulate KLK3, the inherent flexibility of peptides adds an additional level of complexity to this work as it is difficult to determine whether the conformations observed in solution are actually the biologically active ones.[54]

Another interesting alternative to the crystal structures can be found in a recently published modelling study on KLK3.[10] In this study, a homology model of KLK3 was constructed based on the structural information available on other members of the kallikrein family, especially KLKs 1-3 that share the flexible kallikrein loop that covers the active site of the enzyme. Based on the structural information, a pharmacophore model was constructed. This model was used for virtual screening of compound libraries which identified a few compounds with a weak stimulating effect on KLK3, thereby at least in part verifying the correctness of the model.[10] Of the compounds presented in the study, **7** was the most active, increasing the proteolytic activity of KLK3 by 7 %. The small molecular weight compounds are suggested to bind on the kallikrein loop, in the same place as peptides **4**-**6** that have been discussed previously.



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1.3 PEPTIDES AND THEIR ROLE IN DRUG DISCOVERY

1.3.1 NATURAL PEPTIDES

A peptide can be seen as a polymeric chain consisting of a number of amino acid residues connected via amide bonds which in peptides are referred to as peptide bonds, the structure of a peptide bond is shown in Figure 3. A peptide containing two amino acid residues is called a *dipeptide*, three residues make a *tripeptide*, etc. A chain of up to 50 amino acid residues is denoted an *oligopeptide*, 50-100 residues a *polypeptide* and any chain longer than this would be referred to as a *protein*. These definitions are, however, somewhat arbitrary and additional definitions such as *small proteins* are also used in shorter peptide chains.

All peptides and proteins found in humans consist of a set of 20 amino acids collectively called the proteinogenic amino acids. In recent years selenocysteine (Sec, U) and pyrrolysine (Pyl, O) have also been added to this list as it has been found that they are also genetically encoded in the biosynthesis of peptides.[57] The common feature for all of the amino acids except glycine is the side chain attached to the stereogenic α-carbon. The amino acids found in endogenous peptides and proteins are predominantly in the L-configuration although some examples of peptides incorporating D-amino amino acids can be found in archaea and some bacteria.

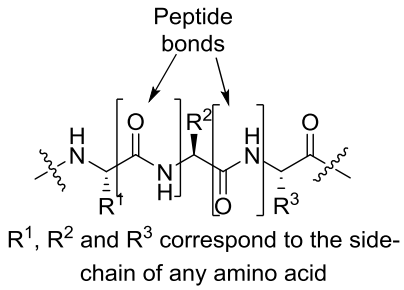


Figure 3 General structure of the backbone of a peptide chain

Another structural trait of peptides and proteins are the disulphide bridges. These bridges are formed when a covalent bond is formed between the sulphurs of two cysteine residues, forming what is often referred to as a cystine unit. The disulphide bridges are important for the correct folding and stability of proteins and peptides, either by cross-linking different parts of one peptide chain (as in ribonuclease A[58]) or by connecting separate peptide chains (as in insulin[59,60]). This stabilisation is especially important in proteins that are excreted into the extracellular medium[61] as most cell compartments are reducing environments that will degrade disulphide linkages rapidly unless a sulfhydryl oxidase is present.[62] Disulphide bridges are characterised by the *cis*-like geometry of the -S-S- bond and the dihedral angle along said bond which is close to ±90° (χ_3, Figure 4).[63,64]

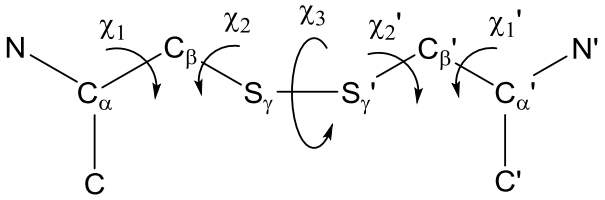


Figure 4 The structure of a disulphide bridge and its dihedral angles.[64]

As the chain length of the peptide grows the number of intramolecular interactions increases and gives rise to a number of secondary structures such as turns, helices and sheets within the peptide structure. In shorter peptides the most prevalent

secondary structure is the β-turn, whereas e.g. in proteins the different secondary structures play important roles in determining the overall 3D-structure of the protein.

Because of the large diversity of the side-chains of the amino acids present in peptides or proteins they can interact with their targets in a number of different ways. These interactions include electrostatic/polar interactions such as ionic or hydrogen bonding, π-π-interactions, and van der Waals interactions.

Peptides have many different biological activities. In humans a number of bioactive peptides function as e.g. neurotransmitters or hormones. Examples of peptide neurotransmitters are orexin,[65] somatostatin,[66] substance P[67] and the family of opioid peptides (e.g. endorphin).[68] Representative examples of peptide hormones are prolactin,[69] vasopressin[70] and oxytocin.[71]

Many toxins found in venomous snakes and other animals also contain active peptide components. Examples of these peptides include the neurotoxic conotoxins from marine cone snails,[72] melittin and apamine from bee venom that have also found use in cancer therapy[73] and the nicotinic receptor antagonist cobratoxin that can be found in the venom of some species of cobra.[74]

Peptides also have other biological effects such as flavour compounds. Examples of this are the artificial sweetener aspartame[75] and the so-called beefy meaty peptide that is responsible for the taste of meat.[76]

1.3.2 SYNTHETIC PEPTIDES

The field of peptide chemistry is generally considered to have started in the early 20th with the work of Emil Fischer beginning with the synthesis of the dipeptide glycylglycine (Gly-Gly) from 3,5-diketopiperazine in 1901.[77,78] Further developments toward modern peptide synthesis were e.g. Curtius' development of acyl azides for activating amines for the formation of peptide bonds in 1902,[79] Fischers first synthesis of the octadecapeptide Leu-Gly_3-Leu-Gly_3-Leu-Gly_9 in 1907,[80] Bergmann and Zervas' development of the concept of removable protecting groups in 1932[81] and du Vigneauds work on characterising and synthesising the biologically active peptide oxytocin in the 1950s.[82,83]

The key step in the synthesis of peptides is the formation of the amide bond; this is usually done by activation of the carboxylic acid moiety before its reaction with an amine. The first of these activating agents were the carbodiimides.[84,85] Using this methodology carries with it the limitation that it may form an aromatic, cyclic intermediate that can lead to racemisation of the amino acid,[86,87] although this problem can be minimised by using an additive such as HOBt[87,88] or HOAt.[89] † The later developments of coupling reagents are usually referred to as onium salts which

† HOBt = 1-Hydroxybenzotriazole; HOAt = 1-Hydroxy-7-azabenzotriazole; HATU = 1-[Bis(dimethylamino-)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate; HBTU = *N,N,N′,N′*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; TBTU = *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate; BOP = (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; PyBOP = (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; HMPA = hexamethylphosphoramide

are used in the presence of a base, typically diisopropylethylamine (DIPEA). Representative examples from the group of ammonium/uronium reagents are HATU,[90] HBTU[90,91] and the corresponding tetrafluoroborate salt of the latter, TBTU.[92-95] The group of phosphonium reagents can be exemplified by BOP[96] which was later developed into PyBOP[97] in order to avoid the formation of carcinogenic HMPA as a by-product.

The advent of modern peptide chemistry has also required the development of a number of different protecting groups that can be selectively manipulated. These protecting groups can be split into three different classes: acid labile, base labile and those cleaved by other methods. The amine protecting benzyloxycarbonyl group (Cbz or Z), which was the first one published, falls into the last of these classes as it is removed by catalytic hydrogenation.[81,98] Other example of this class are the allyl ester used to protect acids and allyloxycarbonyl (Alloc-) used for amines,[99] which can both be cleaved with a Pd(0)-reagent, e.g. $Pd(PPh_3)_4$[99]. Some typical examples of acid labile protecting groups are the *tert*-butyl ester for protection of acids[100] and the *tert*-butoxycarbonyl (Boc-)[101,102] and trityl groups (Tr-)[103-105] for the protection of amines. If a protecting group removable by base is required, the options include the fluorenylmethoxycarbonyl (Fmoc-)[106] and methyl/ethyl esters[107-109] for amines and acids, respectively. In addition to the examples given here there are a large number of other possibilities that can be used depending on the requirements of the specific applications.[98,110,111]

Until the early 1960s peptide synthesis was performed in solution phase which involved tedious purification and identification between every step of the synthesis. The whole field of peptide chemistry was revolutionised in 1963 when Robert Merrifield published his paper on solid phase peptide synthesis (SPPS).[112] Since this original publication numerous developments have been made to the original solid phase resin in order to improve it for specific applications. In SPPS typical resins used are the acid-labile Rink and Wang resins. One modification of these worth noting is the Rink amide resin, which upon acidic cleavage of the peptide results in a product with a *C*-terminal amide instead of a free carboxylic acid. One of the most important developments of SPPS was the advent of systems that enabled the automated synthesis of peptides which significantly reduced the number of man-hours needed for the synthesis of longer peptides.[113,114]

The strategy for SPPS can be briefly summarised as in Figure 5: the *C*-terminal residue of the target peptide is attached to the resin and its *N*-terminal protection is removed, the following *N*-protected residue is coupled and its *N*-terminal protection is removed (the resin is washed between each separate step). This process is then repeated until the desired peptide sequence is complete and finally cleaved from the resin using a suitable, often acidic, solution with additives to prevent side reactions.[115-118] The final peptide can then be isolated by precipitation, followed by purification by preparative HPLC.

In the past decades a new direction in peptide synthesis has emerged, i.e. heating by microwave irradiation to speed up the synthesis.[93,119] By heating the reaction mixture to a temperature of 60 °C during the coupling step it has been possible to

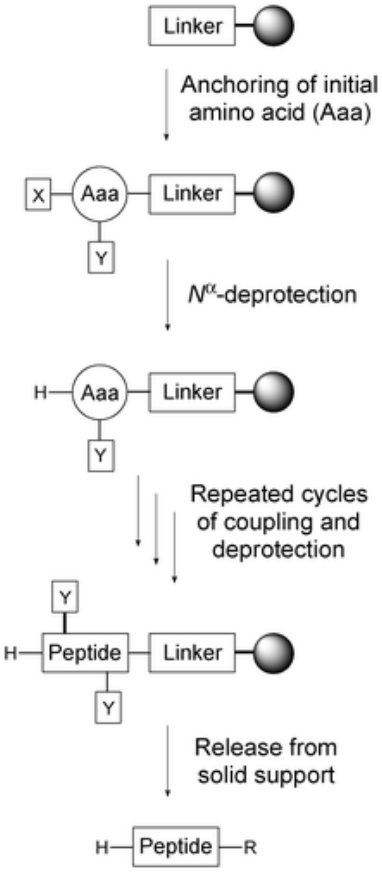


Figure 5 The general principle of SPPS. X: Temporary N^{α}-protecting group. Y: Semi-permanent side chain amino acid (Aaa) protecting groups. R: *C*-terminal functionality, typically OH or NH_2. Image reproduced from reference 93 with permission of The Royal Society of Chemistry.

shorten the reaction time required from 45 min to 5 min. The reason for this is still under debate, some sources suggest a so-called microwave effect that stretches the peptide out during the reaction thus facilitating the reaction.[120,121] Even though microwave heating can be useful in peptide synthesis care must be taken in some couplings, for example the amino acids histidine and cysteine have been shown to racemise when coupled under microwave irradiation.[93,120] Microwave heating has also later been utilised in automated peptide synthesis.[122,123]

1.3.3 PEPTIDES AS DRUGS

Even though many biologically active peptides have been isolated from natural sources, the number of peptides or derivatives thereof that are currently in use as drugs or other applications is relatively low.[124,125]

At present there are a number of natural or modified peptides in therapeutic use; e.g. insulin (treatment of diabetes), leuprorelin (treatment of hormone-responsive cancers), bivalirudin (anticoagulant, direct thrombin inhibitor), cyclosporine (immunosuppressant), calcitonin (osteoporosis and hypercalcemia) and enfuvitide (antiretroviral for the treatment of HIV-1 infections), to name a few. In addition to

these a number of synthetic peptides are also in use; for example eptifibatide (inhibits platelet aggregation), atosiban (prevents premature labour), octreotide (used in the treatment of acromegaly and gigantism) and desmopressin (treatment of diabetes insipidus, bedwetting, or nocturia).[125]

As peptides and proteins are abundant in nature and an essential part of the nutrition for humans, a number of different routes and methods for the metabolism and elimination of these types of compounds exist that significantly lower their utility as pharmaceutical compounds. When orally administered peptides and proteins reach the gastrointestinal (GI) tract, they are immediately degraded by pepsin, an endopeptidase excreted from the gastric mucosa. The oligopeptides resulting from this degradation then exit into the small intestine where they are further degraded by a number of endo- and exopeptidases resulting in single amino acids and di- or tripeptides.[117,126,127] This rapid degradation inevitably leads to a low level of oral bioavailability for peptides, it has been estimated to be only 1-2 %.[128]

Using a parenteral route for the administration of peptides can overcome part of the problems even though these peptides are also eliminated relatively quickly. Small peptides entering the circulation are typically metabolised within a few minutes by the peptidases in the plasma and larger peptides are eliminated by the kidneys or liver.[126,127,129] Once the peptide or protein has been eliminated from the blood stream it is also rapidly metabolised. It seems that smaller peptides are generally eliminated in the kidneys and larger peptides and proteins in the liver. This is, however, also affected by other factors such as hydrophobicity and overall charge of the compound.[126,129] For larger compounds other factors such as aggregation or denaturation also cause pharmaceutical compounds to lose their biological activity.[127,129,130]

In addition to the problems associated with metabolism and elimination many peptide or protein based therapeutics also exhibit physicochemical properties that are unfavourable for use as pharmaceutical compounds. The molecular weight and size of this type of compounds means that in most cases passive diffusion over cell membranes is not possible and hence a mechanism for active transport would be needed. These problems are further increased by the hydrophilicity of most peptides and proteins. As most membranes are hydrophobic, this further limits passive transport through membranes, including the blood-brain-barrier.[127]

At least some of these limitations can be overcome by administering the peptide as a subcutaneous or intravenous injection, in the case of desmopressin and oxytocin the use of a nasal spray has also been shown to be an effective way of administering the drug. In addition to the discomfort an injection can cause the patient, the rapid clearance of the peptides also means that repeated injections are required causing fluctuations in the plasma concentration of the drug.[127] Linear peptides also carry with them the additional problem that they are conformationally flexible. This flexibility can cause a lack of specificity (non-selective binding to additional targets or receptors), side effects and immunogenic responses.[131,132]

The final and possibly biggest limitation to the use of peptides for therapeutic applications is cost or complexity of production of large quantities of the active compound. Even though there are many ways for producing peptides including

chemical synthesis, recombinant DNA technology, transgenic plants and animals and enzymatic synthesis all of these methods carry with them limitations. Chemical synthesis offers virtually unlimited possibilities for the modification of peptides but only produces small amounts of the product, the large number of synthetic steps required for large peptides also severely limits the overall yield. The biotechnological methods can generally be used to produce larger amounts but offer limited possibilities for modifications of the natural product.[124] An exception to these statements is the natural peptides and peptide derivatives used as drugs, e.g. insulin can be produced cheaply in large quantities by using biological methods.[133]

With all this being said, peptides still offer many advantages that make them attractive compounds in drug-discovery projects. As the peptides used for modulating the activity of some drug targets exploit the efficacy of a natural protein while comprising only a small part of the structure they often exhibit a higher selectivity and efficacy than small molecular compounds.[131,134] As peptides are constructed from amino acids, the risk of toxic metabolites is also significantly lower than for small molecules. Given both the type and short half-life of the metabolites there is also a low risk of accumulation in the tissues.[131,132]

1.4 MODIFIED PEPTIDES, PSEUDOPEPTIDES AND PEPTIDOMIMETICS

Due to the problems with using peptides as drugs outlined in Chapter 1.3.3 considerable effort is currently being put into developing peptides into compounds with improved pharmacokinetic properties. Depending on the extent of the modification the resulting product can be referred to as a modified peptide, a pseudopeptide or a peptidomimetic. The differences between these alternatives will be discussed in this chapter using selected examples. More information can be found in any of several more comprehensive reviews that have been written on the subject.[135-137]

1.4.1 MODIFIED PEPTIDES AND PSEUDOPEPTIDES

The simplest modification of a peptide is to replace one or more of the original amino acid residues with a different natural or unnatural amino acid. One application for this is the use of an alanine scan to determine which residues are important for achieving the biological effect of the peptide or protein. When working with a small peptide this can be done by manually replacing the residues one by one,[54] when working with proteins phage display technology is commonly used in which bioactive peptides or proteins are identified from combinatorial libraries of mutant proteins.[138,139]

One limiting factor for the stability of peptides is the degradation of the peptide chain by peptidases. As there are peptidases that use either the *N*-terminal amino group or *C*-terminal carboxylic acid to identify a peptide before starting the degradation, one strategy to improve the stability can be the cyclisation of the

peptide by intramolecular amide bond formation.[140,141] This cyclisation can either be between the termini of the peptide (head-to-tail) or between one of the termini and a suitable side-chain. As peptidases work by cleaving the amide bonds in the peptide another approach for stabilisation is *N*-methylation of one or more specific residues.[140,142,143] However, this also results in a decrease in the hydrophilicity of the peptide causing problems with water solubility although it does improve the permeability.[117]

A third way of modifying the structure while still retaining the peptide structure is the synthesis of what is called a retro-inverso peptide (Figure 6). This means that the sequence of the peptide is reversed at the same time as all amino acid residues are changed to the corresponding D-configuration. This gives a peptide with the same secondary structure as the original one but with the amino acid sequence inverted, meaning it will not be degraded by the same enzymes as the natural peptide.[144,145]

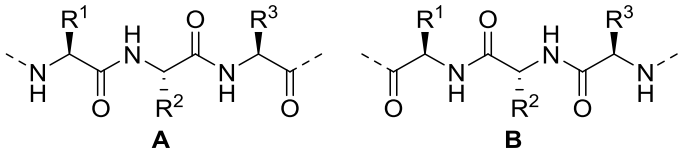


Figure 6 Comparison of the structure of a natural peptide sequence **A** and its retro-inverso counterpart **B**

1.4.1.1 Amide bond isosteres

The terminology is not well defined but most commonly a pseudopeptide refers to a compound which still retains most of its peptide structure, but contains non-peptidic features. The most common starting point for designing a pseudopeptide is the replacement of one or more of the amide bonds. A few representative examples of amide bond mimetics will be given here, a more comprehensive overview of different mimetics and their respective properties can be found e.g. in a review by Gillespie *et al*.[146] The changing of an amide bond is often denoted by the symbol Ψ followed by brackets containing the functionality replacing the amide bond. A number of amide bond replacements are presented in Figure 7.

One of the oldest amide bond mimetics is the reduced amide isostere Ψ[CH_2NH].[147] The initial interest in these compounds is often attributed to their simple synthesis both in solution and on solid phase by reductive amination of protected aminoaldehydes.[148,149] Use of the Mitsunobu reaction has increased the number of different dipeptidomimetics of this type.[150] The use of reduced amides improves both the metabolic[149,151] and chemical[152] stability of peptides. The increased basicity of the reduced amide as compared to the original amide can be a source for problems as it increases the polarity of the pseudopeptide which can potentially affect its pharmacokinetic and receptor-binding properties adversely. One possibility for avoiding this problem is to use the structurally similar ketomethylene isostere (Ψ[$COCH_2$]). Introducing a ketomethylene unit in a tripeptide does not change the affinity of the compound for the human intestinal peptide transporter,

hPEPT1, that is responsible for the active transport of peptides and peptidomimetics over the apical membrane.[153] Ketomethylene units are also successful amide bond replacements in several protease inhibitors,[154,155] at least one of which (AG7088 or rupintavir) has been clinically evaluated as an irreversible inhibitor of human rhinovirus (HRV) 3C protease[156,157] and is also a promising antiviral for combating enterovirus 71.[158] Ketomethylenes units can be easily synthesised using a tandem chain extension aldol reaction[153,159] and are also found in natural peptides such as the aminopeptidase B inhibitors arphamenine A and B that have been isolated from bacteria.[160]

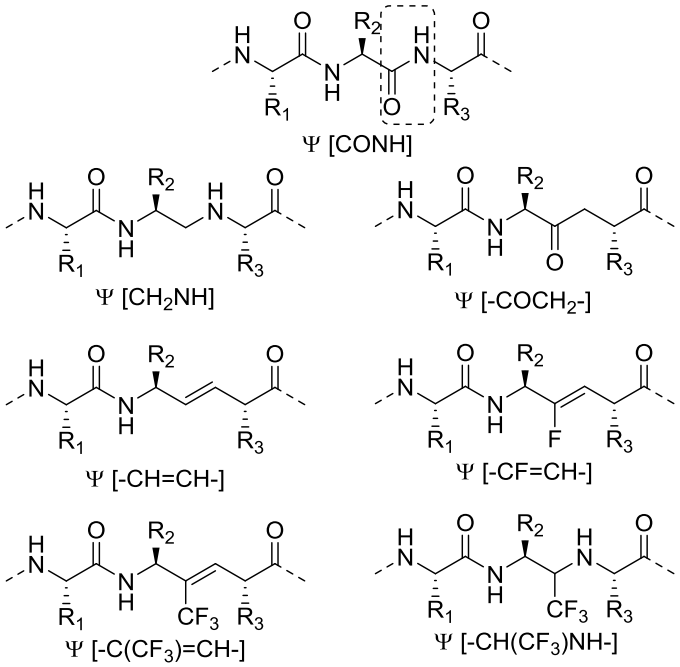


Figure 7 Selected examples of amide bond replacements.

One common amide bond isostere is the alkene Ψ[-CH=CH-], both in the *E*- and *Z*-configuration. Of these two, the *E*-isomer is the more popular one as it closely mimics the *trans*-geometry seen in most amide bonds.[161,162] The *Z*-isomer of the double bond mimics the *cis*-geometry of an amide bond equally well, although this type of amide is typically only found in –Aaa-Pro-bonds.[163] Of special interest among the alkenes are the fluoroalkenes (Ψ[-CF=CH-]) as these also retain most of the polarity of the original amide bond.[164] An interesting modification of the fluoroalkene-moiety as an amide bond isostere is the trifluoromethylated analogue (Ψ[-C(CF_3)=CH-]) which exhibits an improved mimicry of the electrostatic potential surface of the amide bond and also mimics its dipole moment.[161] A step closer to the properties of the original peptide bond is Ψ[-CH(CF_3)NH-]. In addition to the CF_3-group being electronically similar to the carbonyl, this mimetic has a backbone angle close to the 120° of an amide bond as well as having a pK_a-value closer to that of the NH of the CONH group.[165,166]

Another example of constructing pseudopeptides is to use the so-called azapeptides in which the α-carbon is replaced by a non-stereogenic nitrogen yielding a rigid urea structure (Figure 8). An interesting feature of the azapeptides is that they appear to favour the formation of turns.[167,168] A large number of bioactive azapeptides have been prepared[168] and proven to retain their biological activity.[169]

One approach for the design of pseudopeptides are the peptoids in which the amino acid side-chain has been shifted from the α-carbon to the amide nitrogen, forming an *N*-substituted polyglycine chain (Figure 8).[170] This gives a structure with a significantly more flexible backbone allowing it to adapt both the *cis*- and *trans*-conformation of the amide bond.[171] Partial replacement of the sequence of the parent peptide with peptoid moieties has yielded both a nociceptin antagonist[172] and β-sheet breakers able to inhibit amyloid formation.[173]

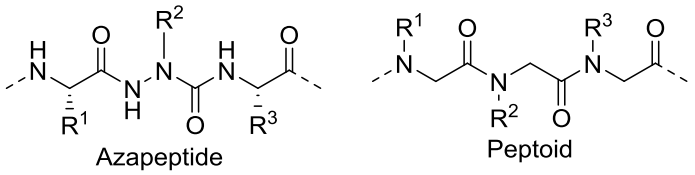


Figure 8 Structural changes to the peptide backbone in azapeptides and peptoids.

In addition to the methods already presented for the design and synthesis of pseudopeptides, several modifications also exist that are based on an elongation of the peptide backbone. These include, but are not limited to, β-peptides,[174] vinylogous peptides,[175] ureas[176] and carbamates.[177] Such structures are shown in Figure 9.

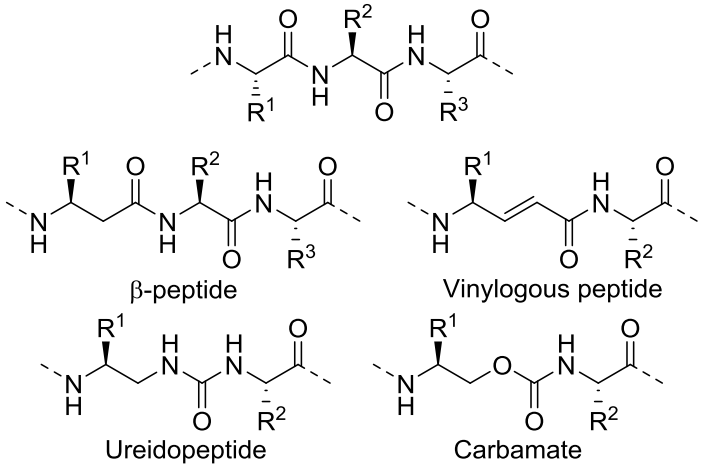


Figure 9 Various examples of backbone modifications in pseudopeptides.

1.4.1.2 Disulphide bridge mimetics

As disulphide bridges are both important for the 3D structure of peptides and proteins and are metabolically scissible, they make an interesting target for the modification of peptides into more stable analogues. The use of hydrocarbon bridges has been found to be both simple to introduce in the structure and to retain the biological activity of the parent peptide.[178-182] Other alternative modifications of the native disulphide bridge include replacing the sulphur atoms with selenium or tellurium,[183] introducing lactams for cyclisation,[184] fully reduced hydrocarbon linkers,[185] or the use of triazoles formed by click-methodology.[186]

The simplest way of introducing an unsaturated hydrocarbon linker in a peptide is to incorporate two or more allylglycine residues in the amino acid sequence, these residues can later be coupled using a ring-closing metathesis (RCM) reaction to yield the cyclic pseudopeptide containing the same number of atoms in the bridge as the native disulphide. One problem with this approach is that, given the larger size of the sulphur compared to the carbon atom (100 and 70 pm in diameter, respectively)[187] and hence the greater length of both the S-S and S-C bond than their C-C counterpart (2.0 Å, 1.8 Å and 1.5 Å, respectively),[188] the length of a four-carbon bridge will not be equal to a regular disulphide bridge containing two carbons and two sulphurs. The disulphide bond will usually adopt a *cis*-like conformation with a roughly 90° dihedral angle between the C-S bonds which further complicates a direct comparison. Comparing the energy minimised structures of peptide hormone oxytocin and an analogue containing a *cis* double bond in place of the disulphide bridge shows a high level of similarity, the only major difference is a small kink in the disulphide bridge caused by the 90° dihedral angle between the sulphur atoms

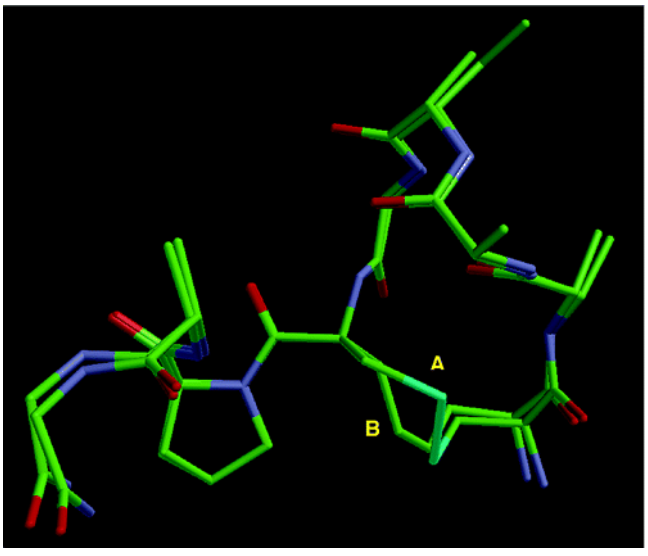


Figure 10 Overlaid energy minimised structures of oxytocin (A) and an analogue containing a hydrocarbon linker with a *cis* double bond in place of the disulphide bridge (B). Side chains on Y, I, Q, N, L are omitted for clarity. Reprinted with kind permission from ref 179. Copyright © 2005 American Chemical Society

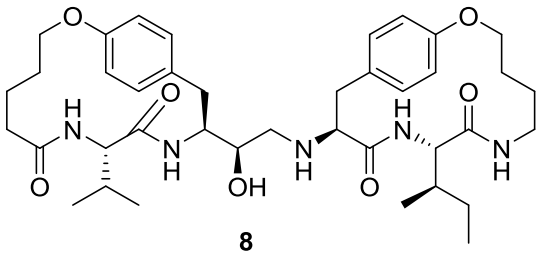
(Figure 10).[179] The analogue containing the cis double bond had a biological activity approximately 10-fold lower than oxytocin whereas the analogues containing the *trans* double bond or the fully saturated linker were 100-fold less active than the *cis* linker.[178,179]

Despite these facts a common approach to replacing disulphide bridges is to use a *trans*-double bonded four-carbon linker that is often formed *in situ* by using the RCM reaction during the peptide synthesis. Even though the length of the linker itself may not be the same as the length of a native disulphide bridge, the differences in the conformation of the bridges means that the more rigid alkene-based bridge may well give the pseudopeptide a conformation in which the α-carbons are at the same distance from each other.

1.4.1.3 Secondary structure mimetics

The properties of peptides and proteins are largely defined by their secondary structure. Common subunits found are β-strands, -turns, -sheets, and α-helices.

The simplest of these subunits is the β-strand which is a peptide chain in which the backbone is completely stretched out with the side chains of the residues protruding on alternating sides. Even though β-strands have for a long time been considered to be merely random structure elements in peptides, it has been shown that enzymes use β-strands to identify and bind substrates or inhibitors, thus making β-strand mimetics attractive as enzyme inhibitors.[136,137] Additionally, the immune system also routinely uses β-strands for recognition and destruction of infected cells.[189,190] Even though only a few amino acid residues are often responsible for the biological activity associated with a β-strand, the flexibility of short peptides will in most cases mean that a fragment containing only these residues is unlikely to adopt the correct conformation and will hence lack the biological activity of the parent peptide. If this is the case, the structure can be rigidified by e.g. the introduction of macrocyclisation (end-to-side chain and/or using unnatural amino acids) as in pseudopeptide **8** that mimics 12 different linear HIV-1 protease inhibiting peptides or pseudopeptides.[191] Another altenative is to introduce nonpeptidic elements to replace amino acid residues as in tetrapeptide mimicking p21^{ras} farnesyltransferase inhibitor **9**.[192]



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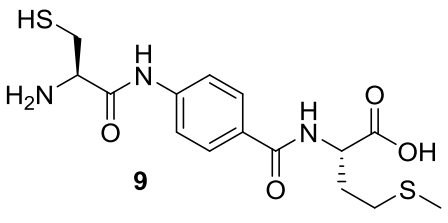

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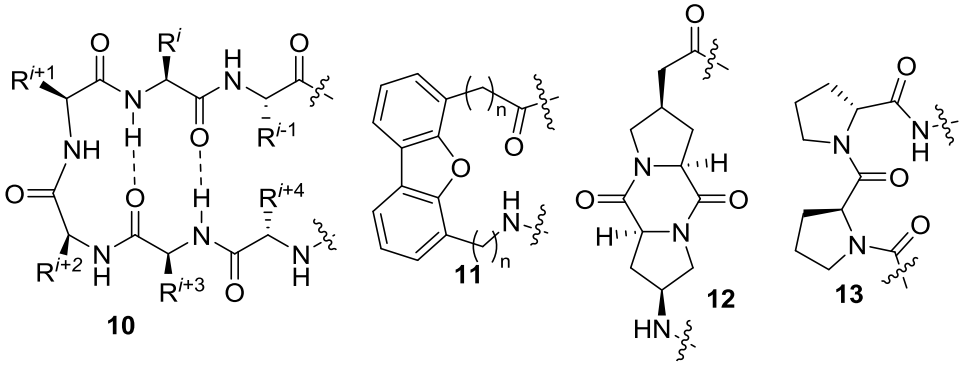
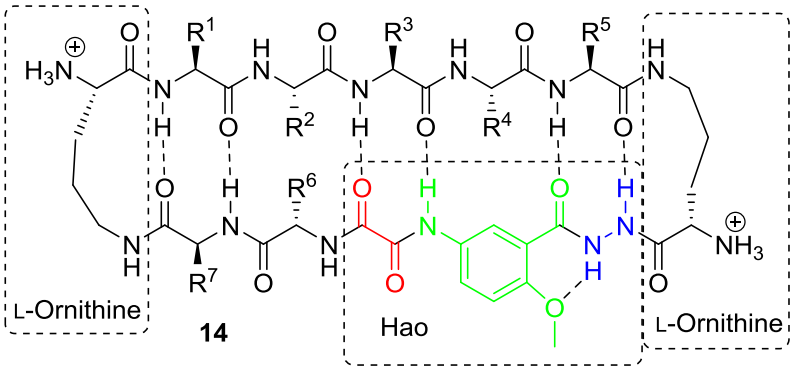
A β-sheet is usually formed by a right-hand turn in the peptide structure, resulting in so-called β-hairpin structures. The turn itself is usually referred to as a β-turn, meaning it consists of a four-residue nucleus stabilised by an *i*, *i*+3 hydrogen bond, as in **10**. As the biological significance of the β-sheet is similar to that of β-strands,

the goal when designing mimetics or pseudopeptides based on *β*-sheets is also the stabilisation or nucleation of the secondary structure. The most common mimetics for nucleating *β*-sheets are the *β*-hairpin turn mimetics, also known as (*β*-)turn mimetics. The mimetics force the peptide strand to make the turn after which the sheet itself is stabilised by hydrogen bonding between the strands just as in the natural case. Examples of sheet-inducing turn mimetics are the dibenzofuran **11**[193] and the *bis*-proline-derived diketopiperazine **12**.[194] Another interesting alternative for inducing a turn is the introduction of a D-proline residue in the amino acid sqeuence, thus forming **13**. Even though this structure is neither a peptidomimetic nor technically even a pseudopeptide but should rather be classified as a modified peptide, it shows one of the simplest way of constructing a secondary structure mimetic using only natural amino acids.[195]

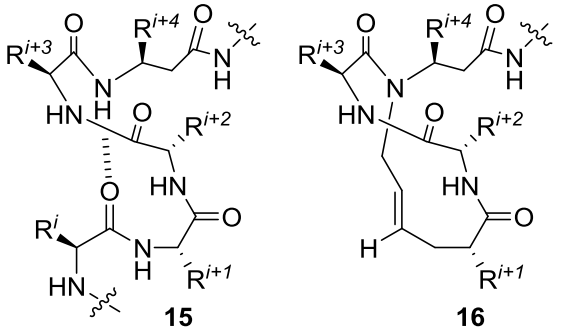


One interesting strategy for inducing *β*-sheets is the use of a synthetic amino acid called "Hao" (named after its components: hydrazine (blue), 5-amino-2-methoxybenzoic acid (green) and oxalic acid (red)) in a peptide which has been shown to allow even short pseudopeptides to adopt a *β*-strand conformation. When dissolved in chloroform these pseudopeptides readily formed *β*-sheet-like dimers (**14**). When these structures are fused together with a *δ*-linked ornithine residue to mimic a turn intramolecular *β*-sheets are formed.[196]



One of the most abundant secondary structures in peptides and proteins is the *α*-helix. The helices are important for protein-protein and protein-nucleic acid interactions, misregulations of these interactions often result in disease states.[197] Even though the biological effects of these helices mainly resides only in parts of the secondary structure, "hot spots" on the surface,[198,199] using these short peptides as

drugs suffers from the same problems as any other peptides. Hence, a number of techniques for stabilising the helical structure of short peptides have been developed: nucleation of helices, surface mimetics and, most recently, stapled peptides in which a hydrocarbon linker is used to stabilise the structure.[200-202]



15 **16**

As in the case of β-sheet nucleators, α-helices can also be initiated in several different ways. One approach is the use of non-natural residues (e.g. α,α'-dialkyl residues)[135] which stabilise the peptide in the α-helix conformation. Alternatively, using the RCM reaction to introduce a rigid structure to replace an *i*,*i*+4 hydrogen bond as in **15** has been shown to be successful e.g. in forming helices inhibiting the gp41-mediated HIV-1 fusion (**16**).[203-205]

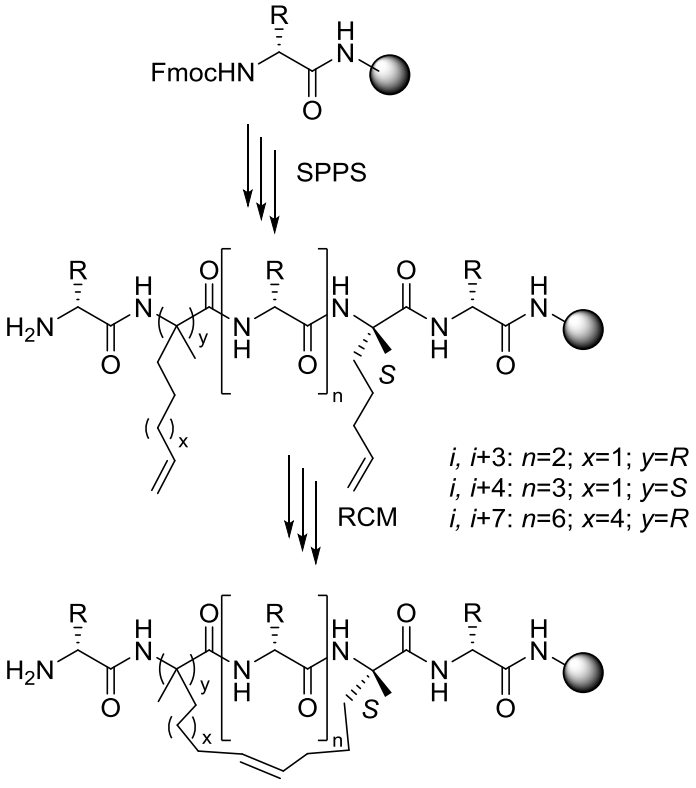


Figure 11 General strategy for the synthesis of a stapled peptide[200]

Another alternative is to use olefins attached to the side chains of selected residues to form a longer linker between the *i*,*i*+3, *i*,*i*+4 or *i*,*i*+7 residues which consists of 8-11 carbon atoms, this approach is referred to peptide stapling.[206-208] The stapled peptides are synthesised by SPPS by adding an α-disubstituted, non-natural amino acid (e.g. α-methyl-pent-4-enylglycine) and finishing the synthesis by performing the RCM reaction. The synthesis of these compounds is outlined in Figure 11.[200,201] Several stapled peptides have been shown to have a biological efficacy similar to the parent peptide,[209-211] there is even one stapled peptide that has been brought into phase I clinical testing.[212] Despite this it has been reported that the stapling can destabilise instead of stabilise the peptide structure, possibly by disrupting the network of intramolecular interactions that stabilise the natural peptide.[213]

1.4.2 PEPTIDOMIMETICS

In contrast to both the modified peptides and pseudopeptides, a peptidomimetic is typically a compound which retains or mimics the biological activity of its parent peptide while lacking any typical peptidic elements such as peptide bonds. In general, peptidomimetics have improved pharmacokinetic properties compared to the parent peptide. IUPAC has issued the following definition:*"A peptidomimetic is a compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural peptide. A peptidomimetic does no longer have classical peptide characteristics such as enzymatically scissile peptidic bonds"*.[214]

Peptidomimetics are often divided into three classes. The type I peptidomimetics are designed to mimic the structure of the backbone of the parent peptide atom-for-atom and retain the local topography such as a helix, a turn, or a sheet. Representative examples of type I mimetics are the amide bond isosteres shown in Figure 7 on page 13. The nature of the type I mímetics means that most of them can be classified as pseudopeptides. The type II mimetics are compounds that mimic the biological activity of a peptide while binding to a different subsite of the target receptor or a completely different receptor. The type III mimetics are compounds that bind to the same receptor as the parent peptide but no longer share any structural traits with it, most importantly they no longer contain any peptide bonds. Typically, a type III mimetic is a compound built around a template or scaffold that has been decorated with the side chains required for attaining the desired biological activity.[215]

One of the biggest limitations in the development of bioactive compounds partially or completely lacking peptidic elements is the large amount of work required for each new compound to be developed. Even though a number of peptidomimetics have already been published, no general methods for the development of a peptidomimetic exist. A typical workflow for the *de novo* development of a peptidomimetic is presented in Figure 12.

One approach for mimicking an α-helix using a type III peptidomimetic is the use of surface mimetics, i.e. non-peptidic compounds that encompass the important side chains from a given helix. This is based on the observation that it is often side chains

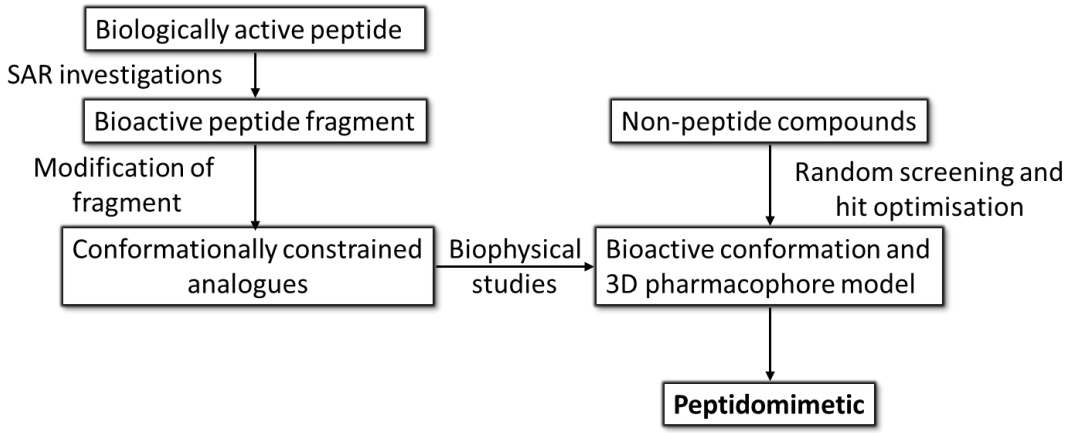
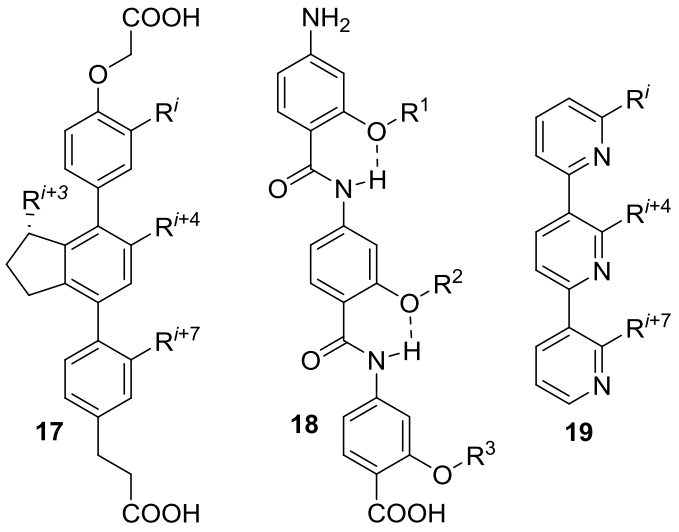


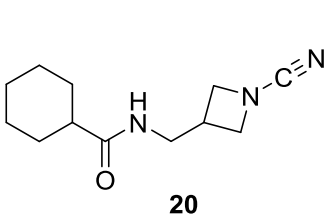
Figure 12 Typical workflow for the *de novo* approach to peptidomimetic design[216]

on only one face of the helix that interact with the target, some consensus has been reached that the important residues are often *i*, *i*+3 or *i*+4, and *i*+7.[198,217] As a consequence of this observation a number of different molecular scaffolds have been developed the main function of which is to present the required side chains in the desired orientation. In order to achieve rigidity in the backbone of the mimetic while retaining its helical nature, different oligocyclic compounds have been developed. Examples of compounds able to mimic two turns of a helix include terphenyl compound **17**[195] or the *O*-alkylated *para*-benzamide **18**[218] that also offers intramolecular hydrogen bonding to further stabilise the helical structure.[218] It has however been shown that the terphenyl structure is actually not as rigid as was originally believed; it can adopt 16 conformations with almost equal energy. Because of this it was proposed that the terpyridyl scaffold **19** is actually more suited for this particular application.[195,219]



The most complex strategy for the design of a peptidomimetic is to use a compound that is able to mimic the biological activity without any part of the peptide chain being present, a type III mimetic. In their simplest form these types of

peptidomimetics can be a molecule that covers part of e.g. a β-sheet or an α-helix as compounds **20** and **21**, respectively.[220,221]



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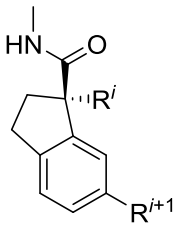

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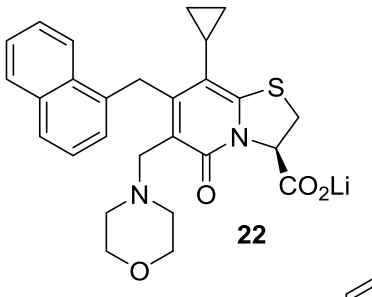
Once the structural requirements for a potential peptidomimetic have been identified, the next step in the process is usually the selection of a suitable scaffold with handles that make it possible to attach the required side chains in positions that allow interactions with the targets that are in agreement with those of the parent peptide. Several different scaffolds have been used in peptidomimetic applications; these are often based on some type of cyclic core to limit the flexibility of the final compound. Some examples of the cyclic scaffolds used are cyclohexane,[222] sugars,[223] triazoles,[224] oxadiazoles,[224] diketopiperazines,[225] chromones,[226,227] chromanones,[226,228] quinolones,[229] isoindolones[230] and tricyclic ring-fused pyrazoles.[231]

Often the peptidomimetic is designed to fit into a specific receptor or active site on e.g. an enzyme and not to specifically mimic a certain peptide, although if both of these pieces of information are available, they can be combined to create the final mimetic. The design of a peptidomimetic is often based around a cyclic, preferably heterocyclic, scaffold that is used to direct the side chains necessary for the biological activity into the position required to fit into the target. Several different compounds based around different scaffolds have been found to have biological effect with several different targets. Examples include the 2-pyridone based pilicide **22**[232] (mimicking the *C* terminus of PapG, an adhesin involved in the disease process of uropathogenic infections) and HRV 3C protease inhibitor **23** that mimics a synthetic hexapeptide substrate of the enzyme,[233] chromone **24** that mimics a Gly-



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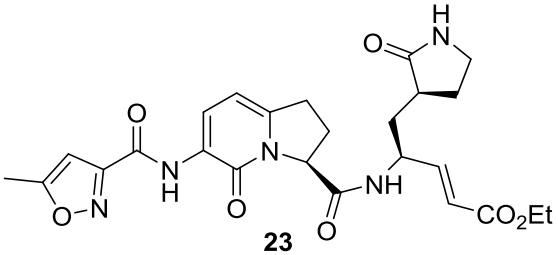

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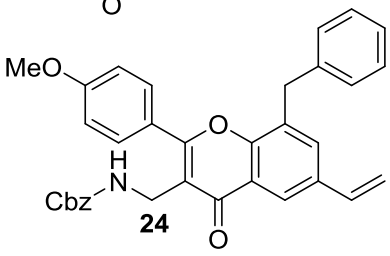

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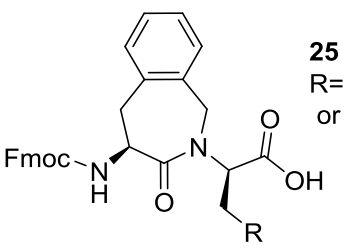


25
R=Ph, 4-ClPh
or 2'-naphthyl

Tyr-Phe-Gly β-turn,[226] and the benzazepin-3-one based human melanocortin-3 receptor ligand **25** that mimics the tetrapeptide (His-Phe-Arg-Trp) that has been found to contain the principal pharmacophore groups of α-melanocyte stimulating hormone (α-MSH).[234]

In some cases, when the starting point is a small cyclic peptide, a peptidomimetic can be designed that accurately mimics the entire structure of the peptide. An example of this is the design of mimetic **26** based on the structure of rhodopeptins C1 and B5 illustrated in Figure 13.[229,235]

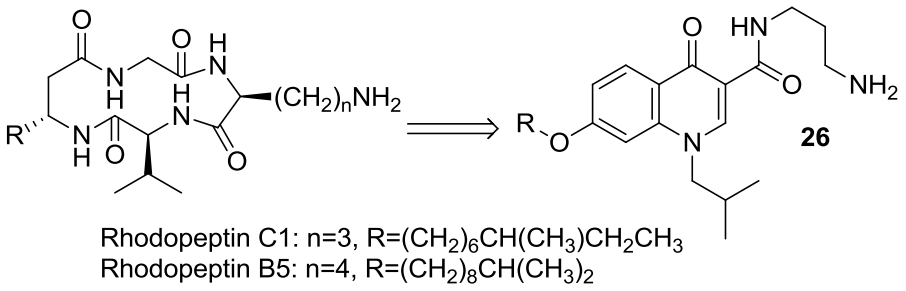
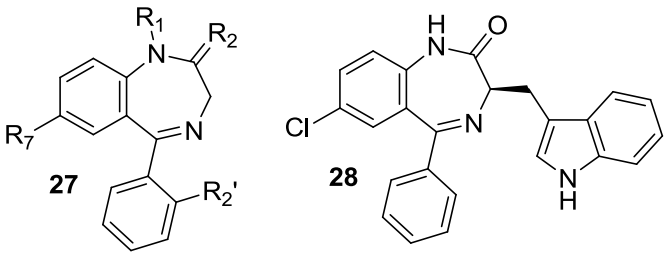


Figure 13 Design of scaffold peptidomimetics based on rhodopeptins C1 and B5.

1.4.3 PRIVILEGED STRUCTURES

One central concept in drug discovery that applies well to peptidomimetics is the idea of privileged structures. This concept was introduced by Evans *et al.* who in 1988 stated that "*certain privileged structures are capable of providing useful ligands for more than one receptor*".[236] What this phrase means in practice is that it has been observed that there are some recurring structures that can be found in drug molecules and biologically active compounds acting on different targets.

The concept of privileged structures is one that is largely concerned with molecular frameworks. The original idea presented by Evans and coworkers stems from the investigation of benzodiazepine derivatives and the discovery that these compounds have an antagonistic effect on the peptide hormone cholecystokinin A (CCK) as well as the benzodiazepine receptor. These compounds are built around the benzodiazepine core **27**; the compounds in Evans' study are further based on prototype compound **28**.[236] This work has later been expanded first by Bunin *et al.* who screened 192 compounds against CCK[237] and later by Thompson and Ellman who screened 1680 1,4-benzodiazepines and also found biological activity for additional targets.[238]



To identify privileged frameworks in drug molecules, Bemis and Murcko have proposed a systematic approach for breaking a compound down into its constituent parts (Figure 14).[239]

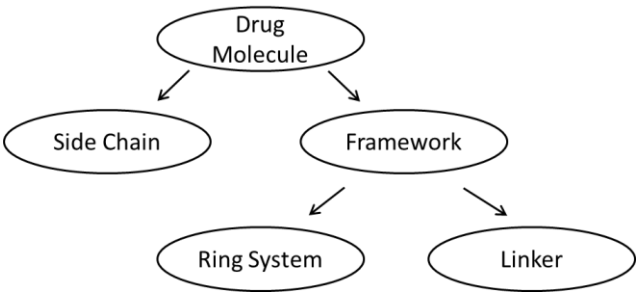
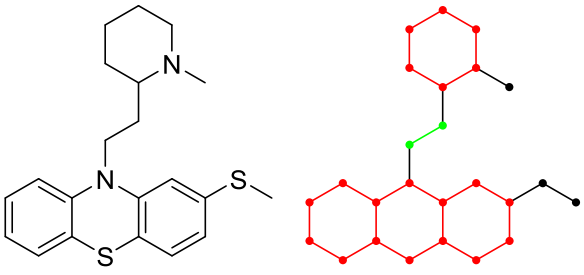


Figure 14 Strategy for hierarchical breakdown of a drug molecule[239]

The antidepressant thioridazine **29** can be used as an illustrative example of this process. In **30** the different parts of the structure have been colour-coded for clarity. The side chains are black and the graphic framework of the molecule has been divided into the red ring systems and green linker part. When illustrated in this way it is clear that the graphic framework in this particular compound consists of two cyclic units connected by a two-carbon linker (the red and green parts of **30**). Whether or not the framework can be considered to be a privileged structure is defined by how frequently it is found among bioactive or drug compounds.[239]



29 **30**

In order to investigate structures that are common in drug compounds, Bemis and Murcko screened 5120 compounds from the Comprehensive Medicinal Chemistry (CMC) database.[239] From this set of compounds it was possible to define 32 simplified or graphic frameworks that account for around 50 % of the total number of molecules. When additional properties such as atom hybridisation, type and bond order are also considered 41 atomic frameworks account for 24 % of the compounds. Although benzene is clearly the most common structure (433 compounds, 8.5 %) it is still clear that other cyclic structures are also abundant among drug molecules, including both carbo- and heterocyclic ring systems (Figure 15). In order to further refine the concept of privileged structures, Feher and Schmidt performed a statistical analysis of around 710 000 biologically active compounds from several databases

and were able to identify a number of numerical descriptors such as molecular weight and number of heteroatoms.[240]

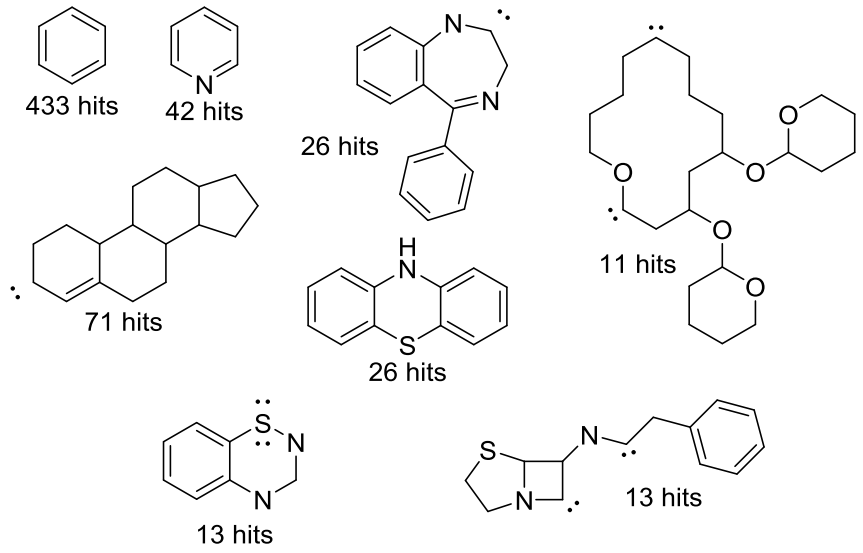


Figure 15 Examples of atomic frameworks and their respective number of hits in the CMC database[239]

Han *et al.* used the information available on privileged structures to construct a set of 29 bicyclic (both fused and linker-connected) scaffolds to identify around 160 compounds from four different databases and study their physicochemical and pharmacokinetic properties.[241] The properties of these compounds were investigated by modelling and verified by data from the literature where this was possible. In order to construct a model for predicting the properties of new compounds, the cell permeability was tested with Caco-2 cells and the metabolic stability with rat liver microsomes. Combining all the data made it possible to formulate some drug-like ranges for several descriptors of potential drug compounds. The conclusion could also be drawn that bicyclic compounds containing heteroatoms do have advantages over other types of structures when designing drug compounds.

Even though the privileged structures mostly appear in discussions on the drug-likeness of compounds they can also be utilised in the design of experimental procedures. This is exemplified in a comprehensive review on the combinatorial synthesis of compounds based on privileged structures and substructures.[242]

What conclusions can be drawn from this discussion? The consensus among researchers working in this field is, in short, that the concept of privileged structures can be useful when designing new compounds although it does have its flaws. What is clear is that there are molecular frameworks that are found in a large number of different drug compounds and natural compounds,[236,239,241] that compounds based on the same framework can have an effect on several different targets[236,242] and that some physicochemical descriptors such as molecular weight, polar surface area and number of hydrogen bond donors and acceptors have ranges where it is more

probable to find biologically active compounds.[241,243,244] Even though no rules of this type can, on their own, guarantee the success of a drug compound, consideration of these and other factors can greatly increase the probability of achieving biological activity.

2 AIMS OF THE STUDY

As the proteolytic activity of KLK3 seems to play a role in the development and progression of prostate cancer it is an attractive target for a medicinal chemistry project. Stimulating the proteolytic activity of KLK3 is an especially interesting target as it has been shown to have an antiangiogenic effect directly related to this activity. At the moment, however, the only compounds known to efficiently stimulate this activity are peptides **4**-**6** identified by phage display technology. In order to exploit this extremely interesting biologicial activity it is necessary to develop new compounds with the same activity as the peptides but with improved pharmacokinetic properties.

The aim of this work was to use the C-4 and B-2 peptides (**4** and **5**, respectively) as a starting point and develop pseudopeptides that exhibit the same biological activity as the parent peptides while being more stable *in vivo*. The ultimate goal of the project was to further refine the information from the study of the peptides in combination with molecular modelling studies to develop a small molecular peptidomimetic with the same biological activity as the peptides.

More specifically, the aims of the work were to:

- Develop orthogonally protected hydrocarbon crosslinks that can be used as building blocks to mimic a disulphide bridge.
- Investigate which amino acid residues in the C-4 peptide are necessary for retaining its biological activity.
- Develop a protocol to introduce said building blocks in pseudopeptides stimulating KLK3 using SPPS or other methodology.
- Develop a scaffold based, small molecular peptidomimetic stimulating KLK3.

3 PSEUDOPEPTIDES STIMULATING KLK3 (PAPERS I-III)

3.1 REPLACING A DISULPHIDE BRIDGE WITH A HYDROCARBON LINKER

The most potent KLK3 stimulators known so far are peptides C-4 (**4**), B-2 (**5**), and A-1 (**6**). Interestingly, previous work has shown that **4** and **5** can be modified without a significant loss in biological activity.[54,55] This makes them interesting as starting points for the development of pseudopeptides. Previous results also indicate that at least in peptides **4** and **5**, the residues closest to the disulphide bridges are important for retaining the biological activity.[54]

Even though it has long been known that disulphide bridges in biologically active peptides can be replaced without a significant loss in biological activity,[185] only the development of synthetic methodologiy has made it possible to utilise the full potential of these types of modification in the development of peptide-based drugs.

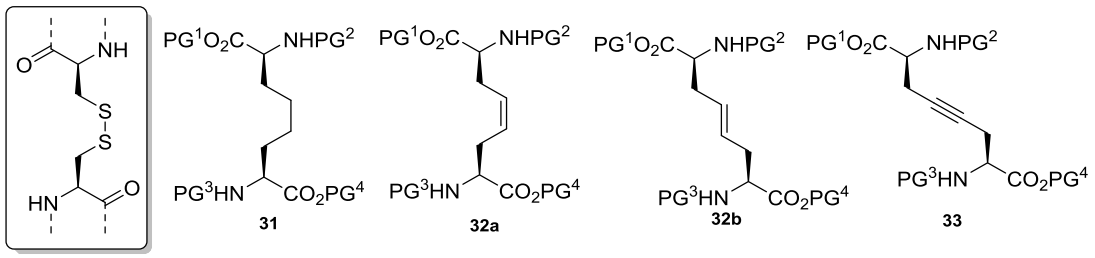
These factors indicate that an interesting starting point for modifying KLK3 stimulating peptides is the replacement of the disulphide bridge (or bridges) in **4** or **5.** There are several reasons for this:

- Some information is already available on which parts of the peptides are important for the biological activity
- The stability of **5** in plasma can be increased by removing the terminal NH_2 group without losing biological activity
- Replacing disulphide bridges with hydrocarbon linkers in peptides does not generally result in a significant lowering of the biological effect of the peptide
- The use of different hydrocarbon linkers can offer further insights into how the conformation of the disulphide bridge affects the biological activity of the peptides
- It presents a significant challenge in synthetic chemistry

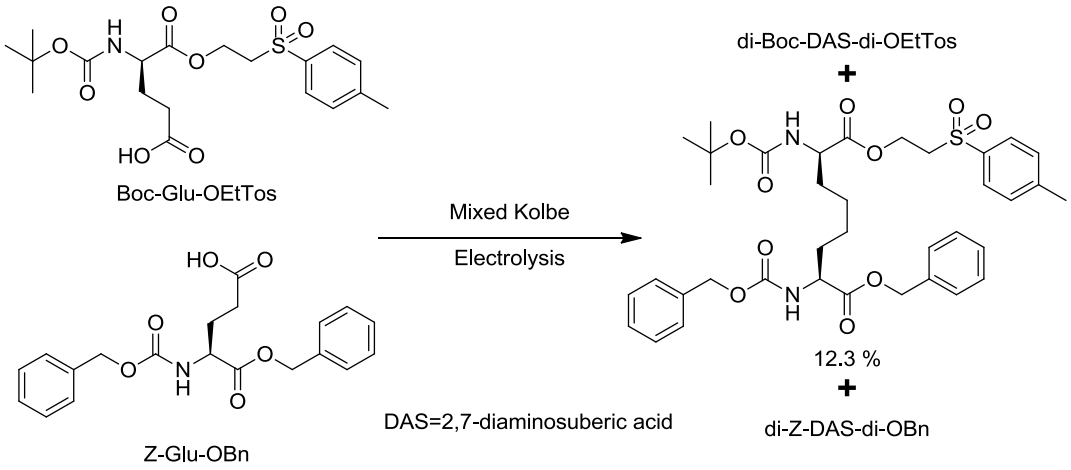
3.1.1 DISULPHIDE-BRIDGE MIMICKING BUILDING BLOCKS (PAPER I)

The C-4 peptide (**4**) contains two potentially labile disulphide bridges and as alanine replacements have shown that the residues required for biological activity are situated around the central bridge, it was of interest to construct a synthetic protocol for replacing at least this one. We also wanted to develop a general method that could be applied to other peptides as well. In order to accomplish this, we envisioned constructing four orthogonally protected building blocks based on 2,7-diaminosuberic acid **31**, the two different conformations of 2,7-diamin-oct-4-enedioic acid **32a**/**b** and 2,7-diamin-oct-4-yndioic acid **33**.

Although these core structures are known in the literature, only **31** has previously been reported to have been synthesised in an orthogonally protected form.[245] Aside



from the protecting groups used not being the ones required for the rest of our synthesis, the procedure for obtaining the product was also rather cumbersome. The key step in this procedure, outlined in Scheme 1, is a mixed Kolbe electrolysis yielding a mixture of the three possible products in a 1:2:1 ratio.[245,246] According to the literature the total yield of the desired cross-coupling product from this reaction was 12.3 %.[245]



Scheme 1 Mixed Kolbe electrolysis for obtaining an orthogonally protected building block.[245]

Other methods for the synthesis of similar compounds have also been reported using Schöllkopf bislactim ether methodology,[247] alkyne cross metathesis[248] and Katsuki-Sharpless asymmetric epoxidation[249] as the key steps for sythesising the final product. All of these methods do produce enantiopure products but the overall yields are limited both by the number of synthetic steps needed and the complex starting materials. All of the reactions are also limited to only one final product (the fully saturated linker) meaning that the other linkers needed cannot be accessed using these methods and the methods can hence not be called general.

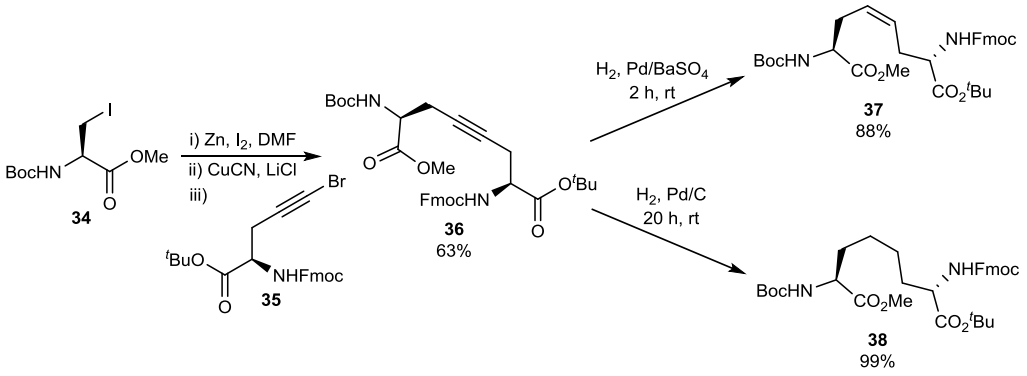
Methods can be found in the literature for the synthesis of similar building blocks containing both the alkene,[181,250,251] the alkyne[252] and the fully reduced chain[250] with non-orthogonal protecting groups. In some previously published cases the authors have not determined the orientation of the double bonds in the alkene products, possibly due to the main interest being the fully reduced analogue, meaning that the double bond was reduced in the following step in these studies.

In addition to the need for the protecting groups used to be orthogonal, they also had to be compatible with the Fmoc-protocol used in SPPS. This meant that a

method was needed that enabled the selective removal of the methyl ester and phthalimide protecting groups that are not normally used with this protocol.

The method chosen for the synthesis of the required disulphide bridge mimetics employs a copper-mediated organozinc/haloalkyne reaction originally published by Yeh and Knochel in 1989[253] to form alkyne building block **36** (Scheme 2). The same method has also previously been employed in the synthesis of the non-orhogonally protected form of linker **33** with the NH_2 groups Boc-protected and the carboxylic acids protected as methyl esters.[252] Once the reaction was found to work satisfactorily, attempts were made to modify the reaction conditions to improve the yield but neither altering the copper source to CuBr·DMS (32 % yield) nor changing the solvent to THF (<10 % yield) were successful.

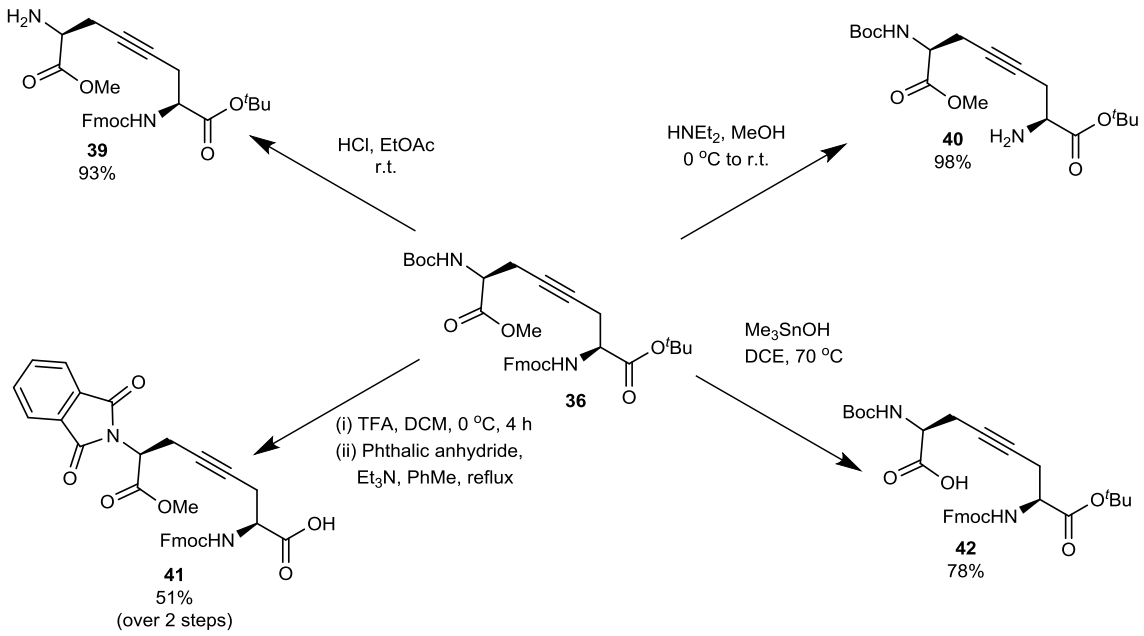
Building block **32** was found to be readily reduced by catalytic hydrogenation using either Rosenmunds catalyst (Pd on $BaSO_4$) or Pd/C to give the *Z*-double bond **37** or the fully saturated **38**, respectively (Scheme 2). Attempting the selective reduction to the *Z* double bond using Lindlar's catalyst did not yield the desired product. Both starting materials (**34** and **35**) are readily available through well-established reactions from commercially available starting materials.[254-256]



Scheme 2 Synthesis of alkyne linker **36** and its subsequent reduction to the *Z*-alkene and alkane analogues.

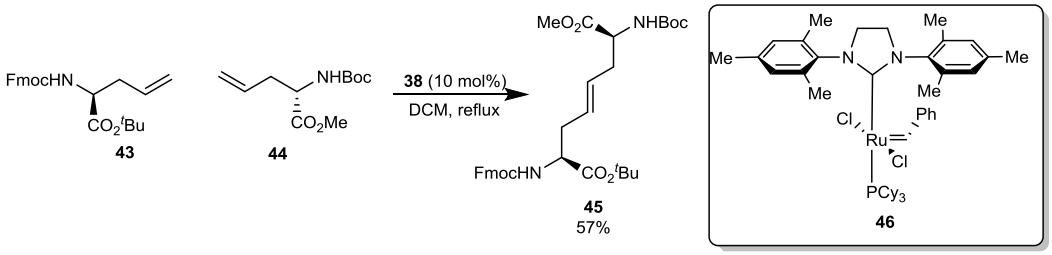
Once building block **36** had been synthesised, the orthogonality of the protecting groups was proven by the selective removal of each one to access the four different mono-deprotected linkers. The Fmoc moiety was removed using diethylamine and the Boc group by hydrolysis using hydrochloric acid in ethyl acetate, both using standard methods. The selective hydrolysis of the methyl ester turned out to be more complicated; using lithium hydroxide resulted in an unsatisfactory yield as well as partial cleavage of the Fmoc group. Fortunately, it was possible to selectively hydrolyse the ester in good yield by using trimethyltin hydroxide in 1,2-dichloroethane at 60 °C, conditions reported by the Nicolau group.[108] The removal of the *tert*-butyl ester to give carboxylic acid **41** required a two step strategy including

the removal of both acid-labile protecting groups followed by reprotection of the amine as a phthalimide.[‡]



Scheme 3 Synthetic strategies for the selective mono-deprotection of alkyne linker **36**.

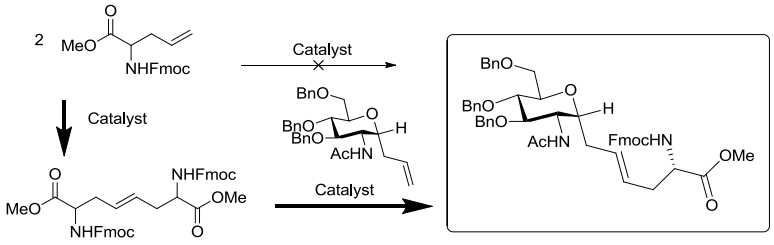
In order to have a complete set of the different linkers, a method was also needed for synthesising the linker containing the *E*-alkene (**45**). As there are no methods available in the literature for selective reduction of an alkyne to an *E*-alkene, a different method for this synthesis was needed. For this purpose, the cross metathesis (CM) reaction was employed using conditions found in the literature,[257] which afforded the desired product in good yield (Scheme 4). Using microwave instead of thermal heating proved disadvantageous as the yield decreased from 57 % to 36 %. Lowering the loading of catalyst **46** from 10 % to 5 % decreased the yield to 46 %.



Scheme 4 The CM reaction used for synthesis of the *E* alkene-containing linker **45**.

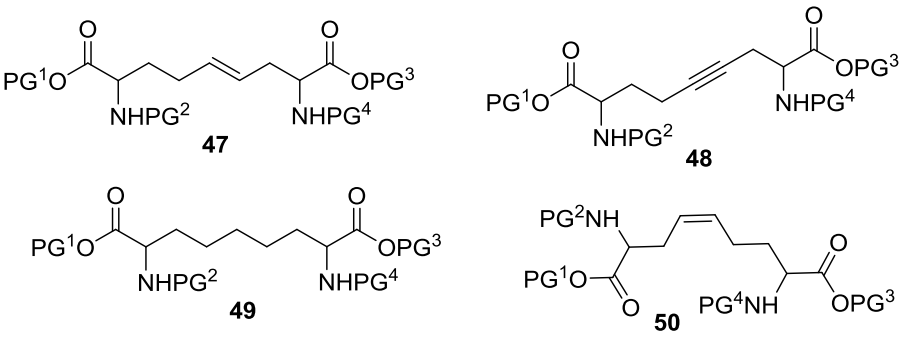
[‡] The phthalic anhydride used for the reprotection step was old and partly decomposed which may have lowered the yield of this step. This was discovered after the completion of the syntheses and was not investigated further.

Interestingly, the only product isolated after the CM reaction was the cross-coupling product **45**. This is in stark contrast to previous investigations where the CM reaction has been shown to exclusively give homodimers when two different allylglycine derivatives are present in the reaction.[181] By following a similar reaction by NMR spectroscopy, Schmidtmann and coworkers have shown that the reaction initially forms a homodimer of one of the starting materials which then undergoes a second CM reaction to form the final product; as illustrated in Scheme 5.[251] One exception to this is the use of the CM reaction to either couple two allylglycine residues that are part of a pseudopeptide or the coupling of an allylglycine to another, resin-bound, allylglycine residue.[180] In these cases, formation of heterodimers has been reported. Such reactions will be further discussed in the following chapter.



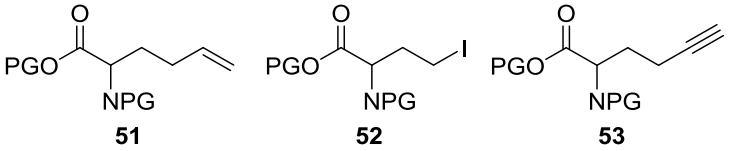
Scheme 5 Proposed route for the formation of a heterodimer via a CM reaction.[251] Bold reaction arrows indicate the presumed reaction pathway.

In order to investigate how the length of the hydrocarbon chain in the linker impacts the biological activity of the pseudopeptides, we also investigated the possibilities for synthesising linkers containing more that four carbon atoms. (Unpublished results) Of special interest were linkers **47** - **50** that are five carbon atoms long as these should be roughly equal in length to their sulphur-based parent structures. Bridges shorter than four carbons are most probably too short to be able to accurately mimic the structure of the disulphide bridge and were excluded from these considerations.

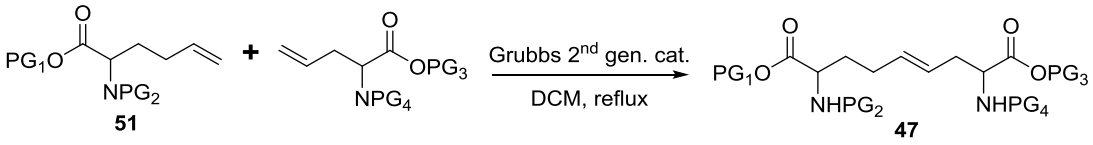


In order to access these compounds, we wanted to use similar chemistry as the methods that had been used to synthesise the orthogonally protected building blocks discussed earlier in this chapter. In addition to investigating the viability of the reactions in question with different starting materials, this also required starting materials containing an additional carbon atom. In the case of the CM reaction this

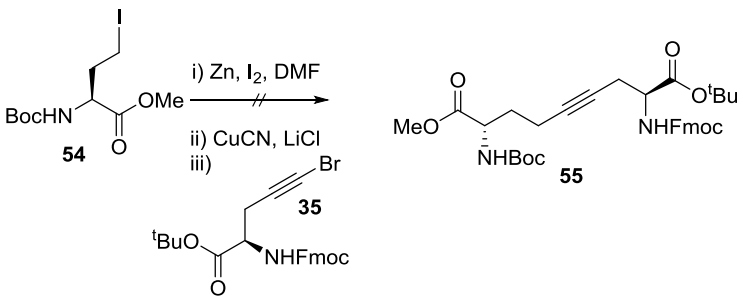
means replacing one of the allylglycines in the reaction with 2-amino-5-hexenoic acid **51**. Even though this compound is available from commercial sources, the price makes it more attractive to synthesise, especially as a simple procedure for this is well known in the literature.[258,259] For building block **48** containing the triple-bonded linker, we opted to extend the chain using iodo-homoserine (2-amino-4-iodobutanoic acid) **52** instead of 2-amino-5-hexynoic acid **53**. Even though both of them are known in the literature, **52** only requires the protection and iodination of commercial homoserine whereas **53** requires more complex chemistry.[260]



With the starting materials in hand, both of the coupling reactions were attempted. Even though our previous syntheses of orthogonally protected building blocks using the CM reaction had run smoothly, the coupling outlined in Scheme 6 was more complicated than anticipated. NMR-spectroscopic analysis of the product mixture from the CM reaction did show the desired product in decent yield, unfortunately all attempts at purifying the product resulted in inseparable mixtures of two compounds. The original theory that these two compounds would simply be isomers containing the two different double-bond orientations was easily disproven by a catalytic hydrogenation of the mixture that also gave a mixture of two compounds. What is worth noting in this context is that even though the CM reaction using α-3-butyleneglycine **51** as one component seems to be unsuccessful, several reports exist detailing using this when cyclising pseudopeptides via the RCM reaction.[179]



Scheme 6 Proposed CM reaction to form a building block containing a five-carbon linker



Scheme 7 Attempted Cu-mediated coupling to access longer alkyne-based linkers.

A similar attempt at synthesising a longer building block (**55**) by using copper chemistry was even less successful (Scheme 7). Using the same conditions for the reaction that were used previously failed to yield any product. This lack of success in synthesising building block **55** also meant that the building block corresponding to **50** was out of reach as it would have been synthesised by reduction of **55**.

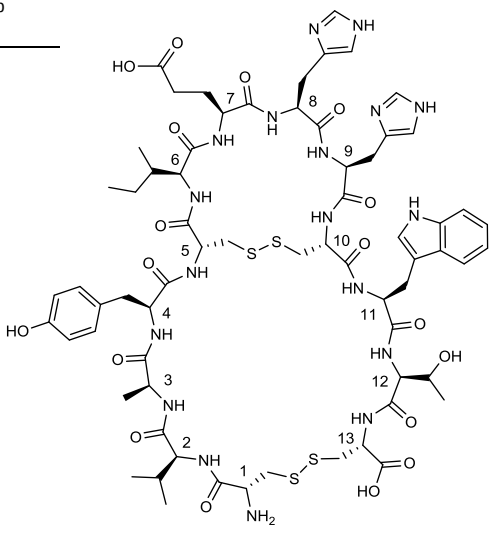
3.1.2 INTRODUCTION OF A HYDROCARBON LINKER IN THE C-4 PEPTIDE USING RING-CLOSING METATHESIS (PAPER II)

One of the objectives of the thesis was to complement the information gained from a previous single-alanine replacement study[54] to build a more detailed picture of which amino-acid residues are important or necessary for the KLK3-stimulating activity of peptide **4**.

In order to achieve this goal, a number of peptides were synthesised and their biological activity evaluated. In the first study, it was shown that residues Glu7, His8 and His9 could be replaced with alanine without a significant loss in biological activity.[54] These results were further expanded in our second study (Paper II). Ile6 was also replaced with other non-polar residues which led to the interesting observation that this residue could be replaced with amino acids with lipophilic side chains, whereas residues with smaller side chains such as alanine and glycine were not tolerated. The results from the multiple-alanine scan are listed in Table 1.

Table 1. *KLK3 stimulating effect of analogues of peptide **4** in the multi Ala-replacement study.Concentration of all peptides in the assay was 20 µg/ml.*

Peptide	Replacements[a]	KLK3 proteolytic activity [%][b]
56	CVAYCIE**AA**CWTC-NH_2	226
57	CVAYCI**AA**HCWTC-NH_2	476
58	CVAYCI**A**H**A**CWTC-NH_2	239
59	CVAYCI**AAA**CWTC-NH_2	165
60	CVAY**AC**EH**CA**WTC-NH_2	84
61	C**A**AYCIEHHCW**A**C-NH_2	127
62	CVAYC**F**EHHCWTC-NH_2	583
63	CVAYC**L**EHHCWTC-NH_2	436
64	CVAYC**A**EHHCWTC-NH_2	299
65	CVAYC**G**EHHCWTC-NH_2	152



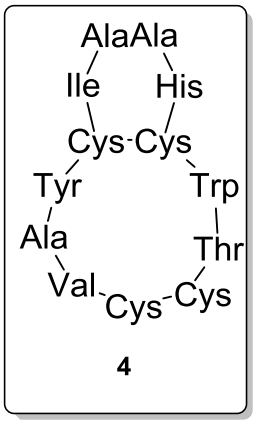
4 (*C-4*)

[a]amino acid replacements are marked in bold face; [b]the activity of KLK3 alone is 100 %

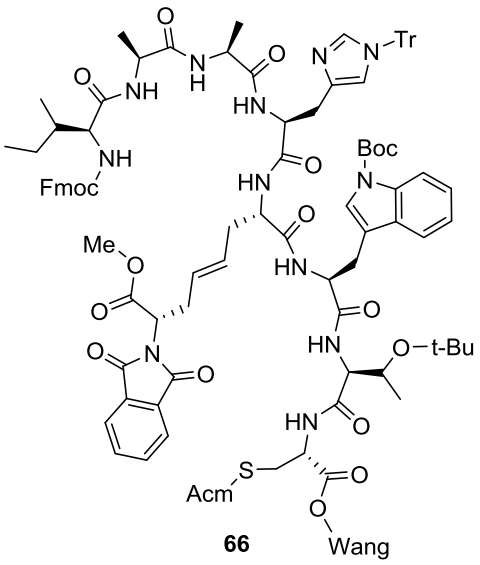
From these results it was possible to conclude that the residues next to both disulphide bridges seem to be important for the biological activity of the peptide.

Based on this, the internal disulphide bridge was considered to be most important for the development of pseudopeptides.

Our first thought was to use the previously synthesised building blocks directly in solid-phase peptide synthesis to replace the internal disulphide bridge of **4**. The simplest strategy for the synthesis of the target peptide is to use mono-deprotected linker **41** and couple this to the resin-bound tripeptide Cys13-Thr12-Trp11 using standard peptide coupling conditions (but using a longer coupling time). The peptide synthesis is then continued normally to the Ile6-residue, the methyl ester and Fmoc groups in intermediate **66** are removed and the ring is closed using a head-to-tail peptide coupling. The phthalimido protecting group is removed and the peptide synthesis is completed by the addition of the three remaining residues. Finally, the pseudopeptide is cleaved from the resin and the terminal disulphide bridge is closed by oxidation using I_2 in acetic acid with aqueous HCl. Unfortunately, the hydrolysis of the methyl ester and/or the cleavage of the phthalimido group in **66** was unsuccessful despite several different attempts and methods.



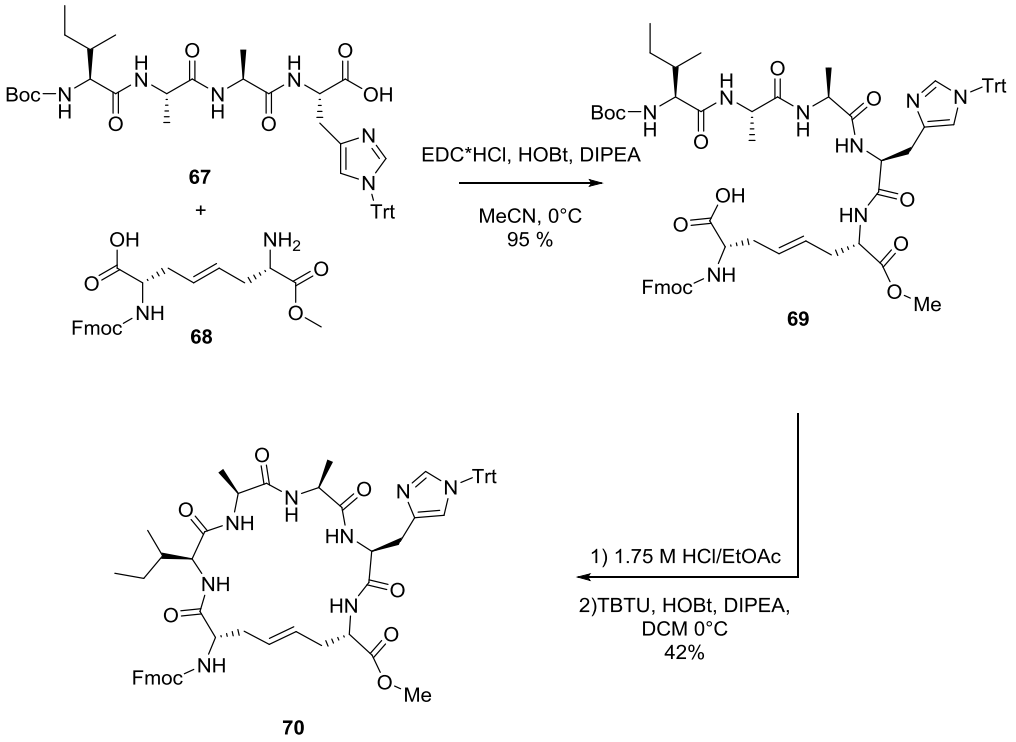
4



66

This initial failure required the development of another method for synthesising pseudopeptide analogues of peptide **4**. In our second attempt, tetrapeptide **67** was synthesised in solution before coupling to building block **68**. As racemisation of the histidine residue after coupling the tetrapeptide to the building block was observed, all of the isolated products were closely examined by NMR spectroscopy after purification. With the help of these analyses it was concluded that the addition of EDC·HCl was required to suppress the racemisation and it was possible to isolate cyclic pseudopeptide **70** in pure form.

Disappointingly, the hydrolysis of the methyl ester, which is required for the continued peptide synthesis failed even when using trimethyltin hydroxide in 1,2-dichloroethane, a procedure previously found to selectively hydrolyse the methyl esters of orthogonally protected building blocks **36** and **45** without any cleavage of the Fmoc group. The reason for this is believed to be the steric hindrance caused by the Fmoc- and trityl groups close to the methyl ester in the non-polar solvent.



Scheme 8 Solution phase synthesis of the smaller ring of the C-4 peptide

Several different explanations for this observation were initially considered and molecular modelling of the compound was used to attempt to further elucidate the problem. This showed that the pseudopeptide will likely adopt a conformation wherethe Fmoc- and/or trityl groups twist close to the methyl ester creating significant steric hindrance around the carbonyl group of the ester. As trimethyltin hydroxide is also a bulky reagent[261] it appears unlikely that it would have the access to the carbonyl group required for the reaction to take place. A smaller reagent such as lithium hydroxide might be more successful at hydrolysing the ester, but as this could also lead to cleavage of the much more accessible Fmoc group, it is not an approach that is useful in this specific application. Thus the conclusion from this discussion was that this is not a track worth pursuing any further and a completely different approach was needed.

Inspired by the recent success of using ring-closing metathesis (RCM) to cyclise a number of different pseudopeptides,[262] it was also decided to investigate the applicability of this approach to the problem at hand. In the initial attempt, the entire sequence of the target peptide was synthesised on Wang resin using standard Fmoc-chemistry. Peptide **71** was cleaved from the resin and subjected to RCM conditions (Hoveyda-Grubbs 2nd generation catalyst (HG2), trifluoroethanol:DCM 4:1, RT, 24h) which unfortunately did not give even trace amounts of the desired product. The same procedure was also tried for resin-bound pseudopeptides **72a** and **72b** but the results were equally disappointing. Altering reaction times (24 or 48 h) or temperature (room temperature or 60 °C) or changing the catalyst to the Grubbs 2nd generation catalyst **46** did not prove beneficial either. Performing the RCM reaction

on a resin-bound pseudopeptide fragment **73** where the synthesis was halted after addition of the second allylglycine residue also failed to produce any product, the different conditions used are listed in Table 2.

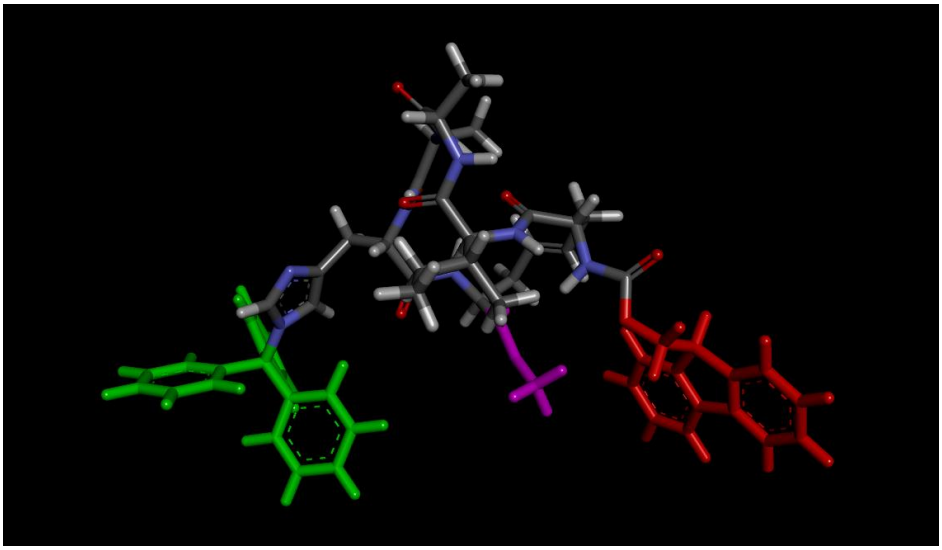
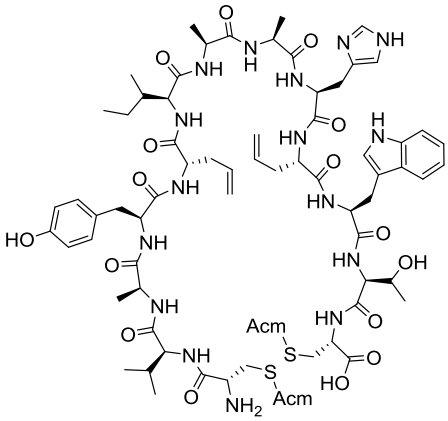
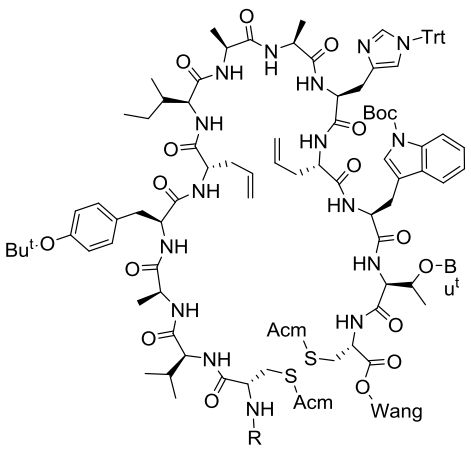


Figure 16 One possible conformation of peptide intermediate **70**. For clarity, the methyl ester is coloured purple, the Fmoc group red and the trityl green.



71



72a: R = H
72b: R = acetyl

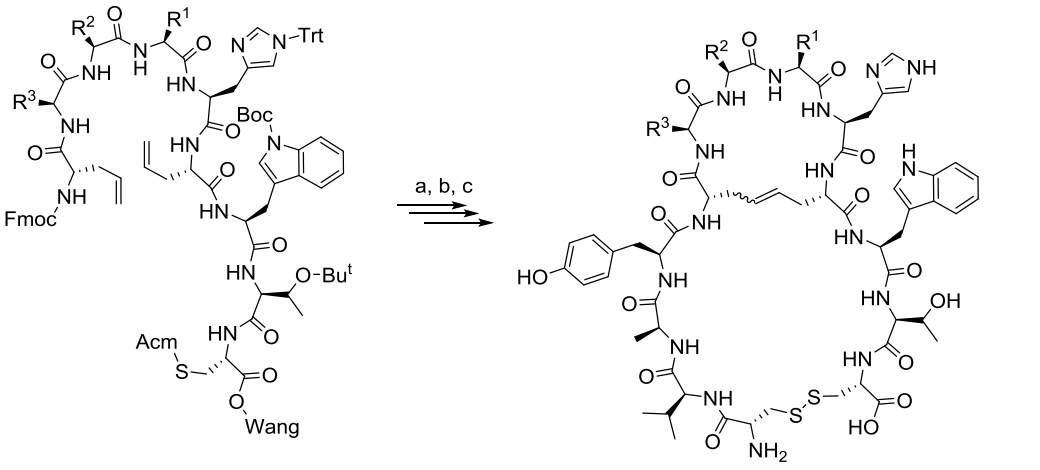
A search in the literature revealed that other groups facing similar problems had been able to overcome them by the addition of LiCl in DMF to the RCM reaction. The published procedures were modified slightly and the reaction was performed on intermediate **73** at 100 °C in 1,2-dichloroethane for 1 h with microwave heating. After cleaving the peptide from the resin we were pleased to find that the mass spectrum (MS) of the crude product showed mainly cyclised peptide, the only by-products were minute quantities of unreacted starting material. The reaction was repeated for intermediates **74** and **75**; the peptide synthesis was finished, the peptides

were cleaved from the resin, cyclised at the terminal disulphide bridge and purified by HPLC.

Table 2. *Reaction conditions tested during optimisation of the RCM reaction.*

Starting material	Catalyst[a]	Additive[b]	Solvent[c]	Temperature[d] [°C]	Time [h]	Product[e]
71	HG2		TFE:DCM 4:1	RT	24	nd
72a	HG2		TFE:DCM 4:1	RT	48	nd
72b	HG2		DCM	RT	24	nd
72b	HG2		TFE:DCM 4:1	60	1	nd
72b	HG2		DCM	60	1	nd
72b	HG2		DCE	60	20	nd
72b	G2		DCE	60	20	nd
72b	HG2	LiCl/DMF	DCE	100 (MW)	1	nd
72b	G2	LiCl/DMF	DCE	100 (MW)	1	nd
73	HG2		DCE	RT	48	nd
73	HG2	LiCl/DMF	DCE	100 (MW)	1	> 50% conv.
74	HG2	LiCl/DMF	DCE	100 (MW)	1	> 50% conv.
75	HG2	LiCl/DMF	DCE	100 (MW)	1	> 50% conv.

[a]HG2, Hoveyda-Grubbs 2nd gen. catalyst; G2, Grubbs 2nd gen. catalyst; [b]DMF, *N*,*N*-dimethylformamide; [c]TFE, trifluoroethanol; DCM, dichloromethane; DCE, 1,2-dichloroethane; [d]RT, room temperature; MW, microwave heating; [e]nd, not detected by MS; conv., conversion in comparison to unreacted starting material by MS.



73: side chains; R^1 = Ala, R^2 = Ala, R^3 = Ile
74: side chains; R^1 = His(*N*-Trt), R^2 = Glu(*O*-Bu^t), R^3 = Ile
75: side chains; R^1 = His(*N*-Trt), R^2 = Glu(*O*-Bu^t), R^3 = Phe

76: side chains; R^1 = Ala, R^2 = Ala, R^3 = Ile
77: side chains; R^1 = His, R^2 = Glu, R^3 = Ile
78: side chains; R^1 = His, R^2 = Glu, R^3 = Phe

Scheme 9 RCM reaction and final SPPS to give pseudopeptide analogues of **4**. Materials and methods: a) HG2, 0.4 M LiCl in DMF (10% (v/v)) / DCE, 100°C, MW; b) i. SPPS (coupling of Tyr(^{t}Bu), Ala, Val and Cys(Acm)), ii. Reagent K (TFA:H_2O:phenol:thioanisol:EDT 82.5:5:5:5:2.5); c) I_2, HCl / AcOH, H_2O.

The main drawback of using this strategy is that the reaction forms a mixture of the *E*- and *Z*-isomers of the double bond and that there is no way of controlling this ratio. It is also unlikely that it will be possible to separate the peptides containing the two different isomers. It is, however, possible to determine the ratio of the two isomers using NMR-spectroscopy.[263,264]

A close examination of the NMR spectra of both the peptide cleaved after the RCM reaction and the purified final product showed that the ratio of the *E* and *Z*

isomers in the products is roughly 1:1. The NMR spectrum of peptide intermediate **74** after the RCM reaction is shown in Figure 17.

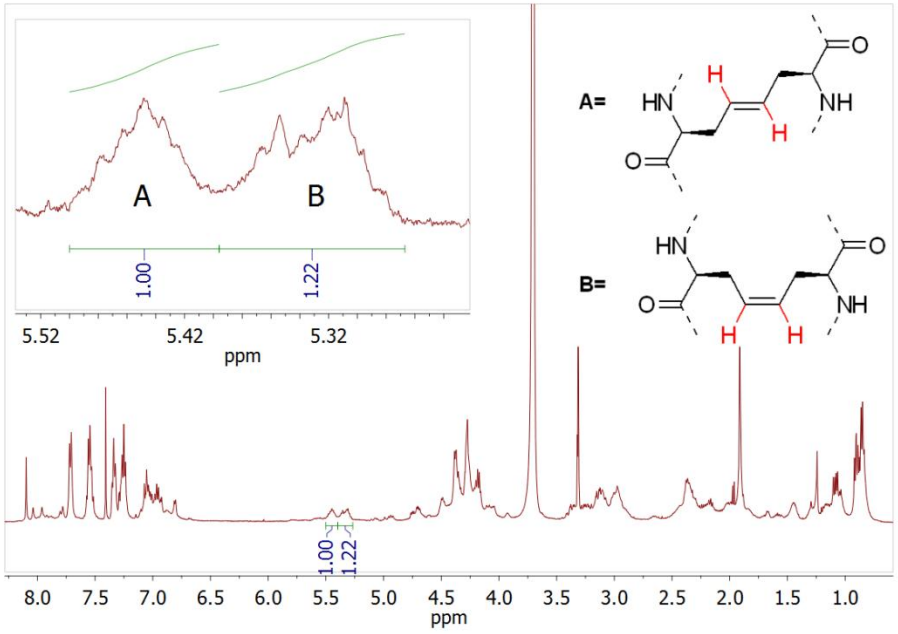


Figure 17 NMR spectroscopic analysis of the double bond in peptide intermediate **74** after the RCM reaction.

Even though papers can be found where alkene-containing disulphide mimetics in terminal position have been reduced using standard H_2, Pd/C-methodology,[178,179] none seem to exist when the disulphide bridge is internal. Inspired by this, several attempts were made at reducing the double bonds in the pseudopeptides using hydrogen gas and a suitable catalyst. The reducuction of the double bonds in the pseudopeptides is also motivated by the observation that the peptides we synthesised contain a mixture of the two isomers of the double bond. Under the assumption that the peptides with the different double bonds will not be equally active it is also difficult to assess the actual potency of the pseudopeptides when they can only be tested as a mixture.

The reduction of the pseudopeptides was attempted both using standard solution-phase methodology (a balloon of H_2) and using a continuous flow hydrogenation reactor that makes it possible to run reactions both at elevated temperatures and under a hydrogen pressure of up to 100 bar. The reactions were attempted both with regular and elevated pressure of hydrogen and the material was cycled through the catalyst several times. The catalysts used were Pd/C and Raney nickel. Unfortunately, MS analysis did not show any trace of the desired product. Reduction of only the smaller ring **70** gave essentially full conversion of starting material to product (as determined by MS), thus proving the general viability of the method itself.

Finally, the biological activity of the pseudopeptides was evaluated compared to the parent peptide. The results, presented in graphical form in Figure 18, were

encouraging for the continuation of the project. Our new pseudopeptides **76**-**78** increased the activity of KLK3 to 225 %, 189 % and 223 %, respectively, of that of KLK3 alone. This can be compared to an increase in activity to 625 % by peptide **4**. All of the previous results were obtained at a peptide concentration of 20 µg ml^{-1} (corresponding to 13 µM for **4**, **76** and **77**, and 14 µM for **78**). When increasing the concentration of the pseudopeptides to 200 µg ml^{-1}, they reached a level of stimulation approximately fourfold to that of KLK3 alone. It is, however, unclear at present if this is an absolute maximum or if higher concentrations would give an even higher level of stimulation. It is also possible that the simulation of KLK3 is only caused by the peptide containing one of the two isomers of the double bond, this could account for the seemingly lower potency of pseudopeptides **76**-**78** compared to **4**.

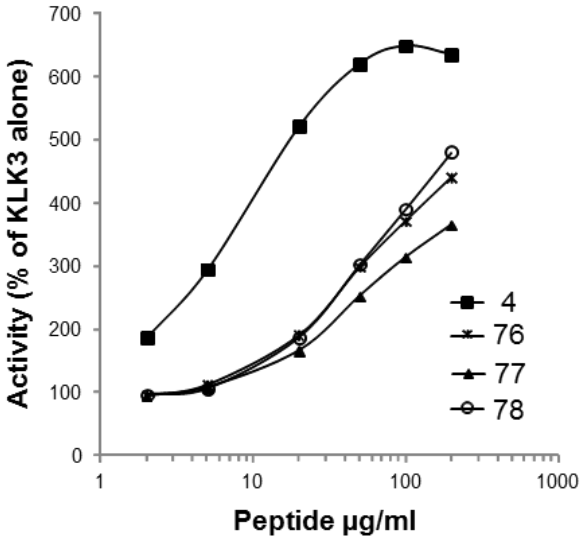
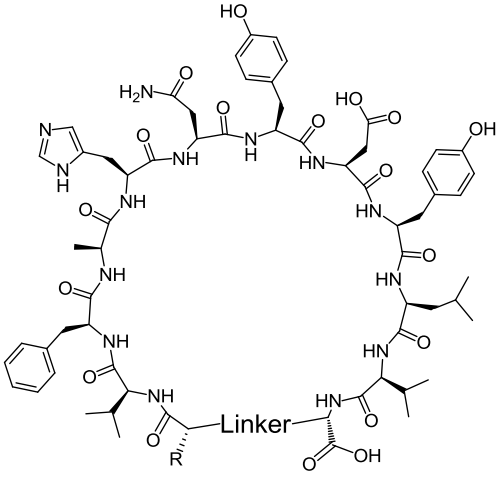


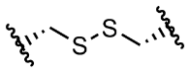
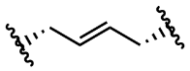
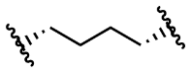
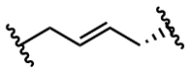
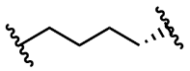
Figure 18 Dose response curves of pseudopeptides **76**-**78** compared to parent peptide **4**

3.1.3 REPLACING THE DISULPHIDE BRIDGE IN PEPTIDE B-2 WITH A HYDROCARBON LINKER (PAPER III)

Based on the success of pseudopeptides 7**6**-**78** bearing a hydrocarbon linker in place of a disulphide bridge, we wanted to further elaborate this finding by producing pseudopeptides containing only one isomer of the double bond and also the fully saturated linker in place of the disulphide bridge. As our original attempt at using our previously synthesised building blocks failed due to problems with the removal of protecting groups, we instead decided to direct our attention towards the monocyclic, KLK3 stimulating, peptide B-2 (**5**). As a previous investigation showed that a peptide lacking the *N*-terminal amino group retains its biological activity and displays a higher stability both in the presence of KLK3 and in plasma,[55] we also wanted to expand our set of building blocks to cover this type of structure. The set of target compounds was thus pseudopeptides **79a**-**79d**.

The building blocks for **79a** and **79b** were synthesised following the CM-based procedure already described. The only difference was that the fully reduced building block was synthesised by a reduction of the *E*-alkene before hydrolysis of the methyl ester.

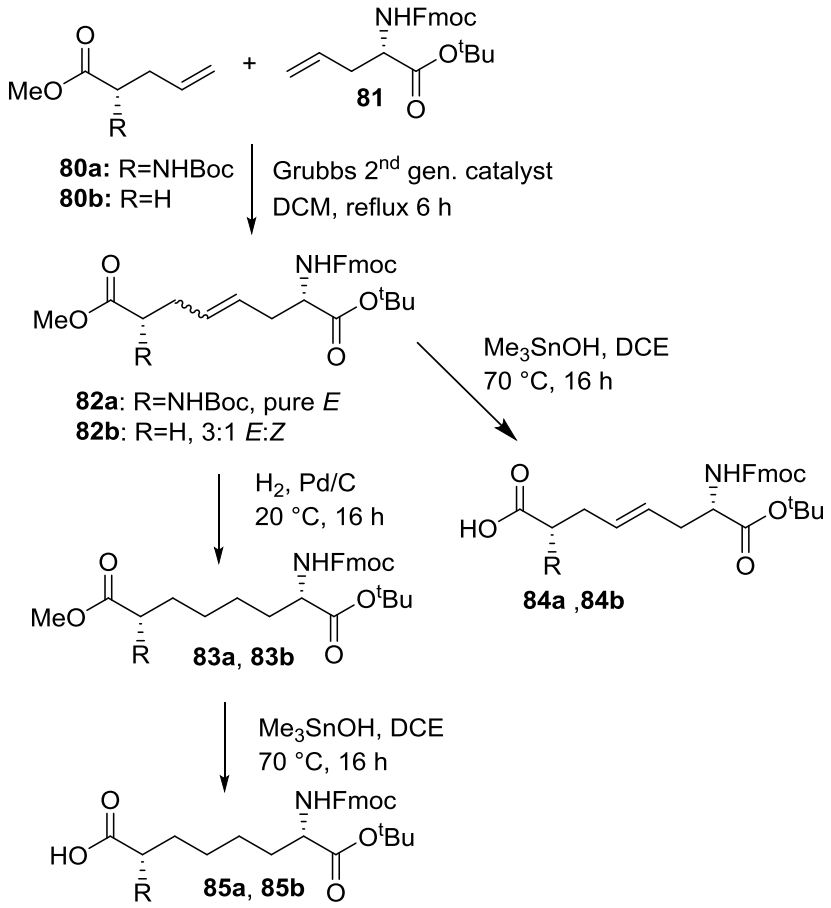


	R	Linker
5	NH_2	
79a	NH_2	
79b	NH_2	
79c	H	
79d	H	

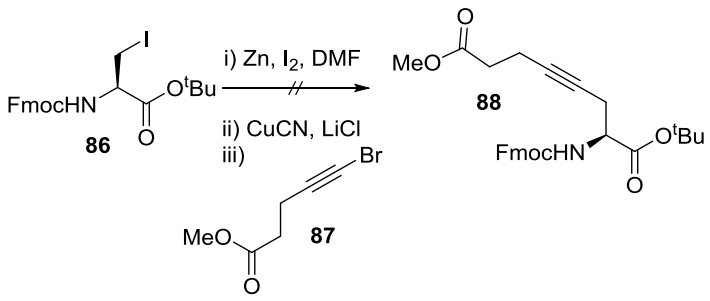
The building block for the pseudopeptide lacking the terminal amino group was also synthesised in a similar way, the only modification to the procedure was the replacement of one of the allylglycines in the CM reaction with methyl 4-pentenoate **80b** (Scheme 10). These methods gave us easy access to all four building blocks required. Unfortunately, using the CM reaction for this procedure resulted in building blocks with an inseparable, roughly 3:1 mixture of the *E* and *Z* double bond isomers. Interestingly, however, hydrolysis of the methyl ester yielded only the product containing the *E* double bond in pure form. The cause for this difference in reactivity of the two starting materials is unknown, but it was confirmed by NMR spectroscopy of both the isolated product and the recovered starting material.

In order to diversify the set of pseudopeptides synthesised, we also wanted to use building blocks containing the triple- and *Z* double bond in the linker but lacking the terminal NH_2 group. (Unpublished results) The copper-mediated coupling of methyl 5-bromo-4-pentynoate **87** and Fmoc-protected iodoserine-O^{t}Bu **86** seemed to run smoothly giving a crude product from which the desired compound could be easily separated. Disappointingly, NMR spectroscopic analysis of both crude and purified products showed that this reaction yields only traces of the desired product, thus making the reaction unfeasible as a tool for accessing the desired compound(s).

With the required building blocks **84a**, **84b**, **85a**, and **85b** in hand we employed standard Fmoc chemistry to synthesise the pseudopeptides on solid phase using Rink amide AM resin resulting in products carrying a *C*-terminal amide instead of a carboxylic acid after cleavage from the resin. In this particular case, the peptide was attached to the resin from the side chain carboxylic group of Fmoc-Asp-OAll, corresponding to the Asn6-residue in the original peptide (**5**). This means that the synthesis of the peptide needs to be finished by the hydrolysis of the allyl ester of the Asp6/Asn6-residue, removal of the Fmoc-group and finally a head-to-tail cyclisation of the pseudopeptide (Scheme 12). This approach has been successful in previous syntheses of modified analogues of peptide B-2.[55]



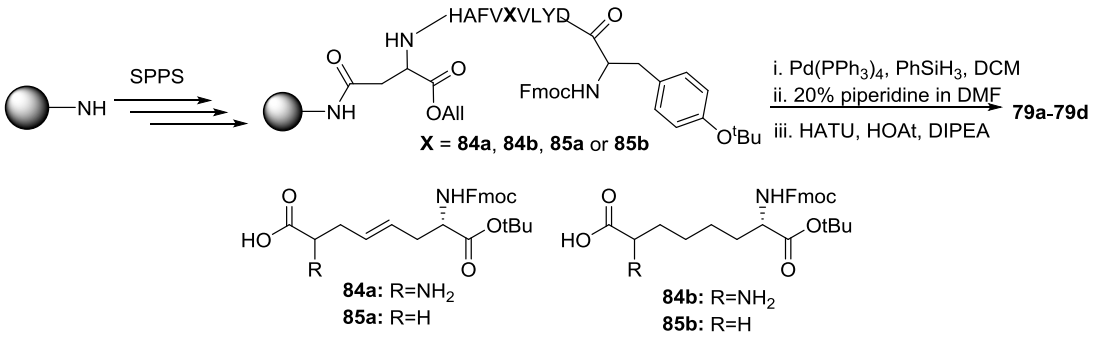
Scheme 10 Synthesis of building blocks used for pseudopeptide analogues of the B-2 peptide.



Scheme 11 Attempted synthesis of an alkyne linker lacking the terminal NH_2 group

As previously, the resulting pseudopeptides were purified by HPLC and their identity was verified by mass spectrometry.

In order to further verify the exact structure of the pseudopeptides and more specifically the orientation of the double bond, an NMR study was undertaken. By using the same method as previously mentioned,[178,179,263] we investigated the chemical shifts of the methine protons in the hydrocarbon linkers. This showed that the final pseudopeptide contained the *E*-isomer exclusively.



Scheme 12 Strategy used fot the solid-phase synthesis of pseudopeptides **79a**-**79d**

All four pseudopeptides stimulated the activity of KLK3 but the effects were weaker compared to original peptide **5** and slightly weaker compared to the earlier analogues with a linker consisting of aspartic acid and γ-amino butyric acid.[55] The increase in enzymatic activity of KLK3 in the presence of pseudopeptides **79a** and **79b** as compared to KLK3 alone were 161 and 247 %, respectively. Similarly, activities for pseudopeptides **79c** and **79d** were 150 and 167 %, respectively.

4 QUINOLONE-BASED SCAFFOLD PEPTIDOMIMETICS (PAPER IV)

4.1 DESIGN OF A MIMETIC OF THE C-4 PEPTIDE

When using a peptide as the starting point one alternative for improving the bioavailability is the development of a scaffold-based peptidomimetic, i.e. a small-molecular weight compound which retains the biological effect by interacting with the same target as the parent peptide. Even though this approach to the development of peptidomimetics is quite difficult, it was chosen for taking the step from peptides to peptidomimetics in this project.

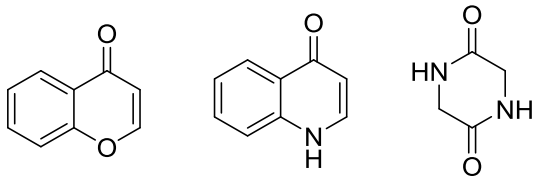
One of the most important tasks of this approach is to identify the part or parts of the peptide that are required for the biological activity. This in turn requires knowledge of the bioactive conformation of the peptide. In an ideal case the bioactive conformation and binding site of the peptide would be determined experimentally by e.g. either NMR spectroscopy or X-ray diffraction experiments performed on the crystallised peptide-protein complex. If experimental data are not available, molecular modelling studies can instead be used to form an educated guess.

Unfortunately, the amount of structural information available on KLK3 is limited and even the available information has some degree of uncertainty as the available crystal structures also contain antibodies and substrate molecules.[11,12] What is however known is that KLK3 has an active site consisting of three residues (His57, Asp102 and Ser159) situated under a flexible loop-structure (the kallikrein loop).[9,10,54,56] It has also been hypothesised that peptides and antibodies can bind to this loop "locking" it in an open conformation in order to make it easier for a substrate to reach the active site, thus stimulating the activity of KLK3.[54,56] Based on this observation and NMR-studies of the C-4-peptide (**4**) a docking study was performed to determine the binding site of the peptide on the kallikrein loop and consequently also the interactions needed for binding the peptide to the enzyme.[10]

As there is only a limited amount of data available on the binding interactions of the peptides stimulating KLK3 it is difficult to reliably design a peptidomimetic with a specific activity on KLK3. Instead, we chose a scaffold structure that contains enough possibilities for varying the substitution pattern to be considered a general scaffold for peptidomimetics. The simpler the protocol for synthesis of the scaffold and further substitution of it, the easier it will be to adapt the mimetic to different targets.

Three scaffolds were considered for the mimetics in this project; chromone **89**, 4-quinolone **90** and 3,5-diketopiperazine **91**. Even though these structures seem quite different they do share some of the critical properties, namely they are all fairly planar and rigid structures with possibilities for substitution in several positions. The main reason for selecting these scaffolds was the flexibility of allowed substitution

patterns combined with the previous use of the chromone and diketopiperazine scaffolds within the Luthman group at the University of Gothenburg.[226,265,266]



89 **90** **91**

Using a previously published homology model of KLK3,[10] an energy minimised conformation of peptide **4** (C-4) was docked in the presumed binding site and we proceeded to study the interactions between the peptide and KLK3 to identify the amino acid side chains that are necessary for the biological activity of the peptide. An image of part of the peptide docked on KLK3 can be seen on the cover of this thesis. This study showed that His9, Trp11 and Thr12 in **4** seem to be responsible for the peptide's interaction with KLK3. This is in agreement with the alanine-replacement studies that showed that His9 and Trp11 are required for biological activity, the study also showed that replacing Thr12 together with Val2 causes a significant drop in the biological activity of the peptide.[267] In order to design a peptidomimetic of **4**, we were looking for a scaffold that could allow the important side chains to be placed in correct relative positions. Additionally, the scaffold should enable alternative substitution patterns if fine tuning of the peptidomimetic is needed.

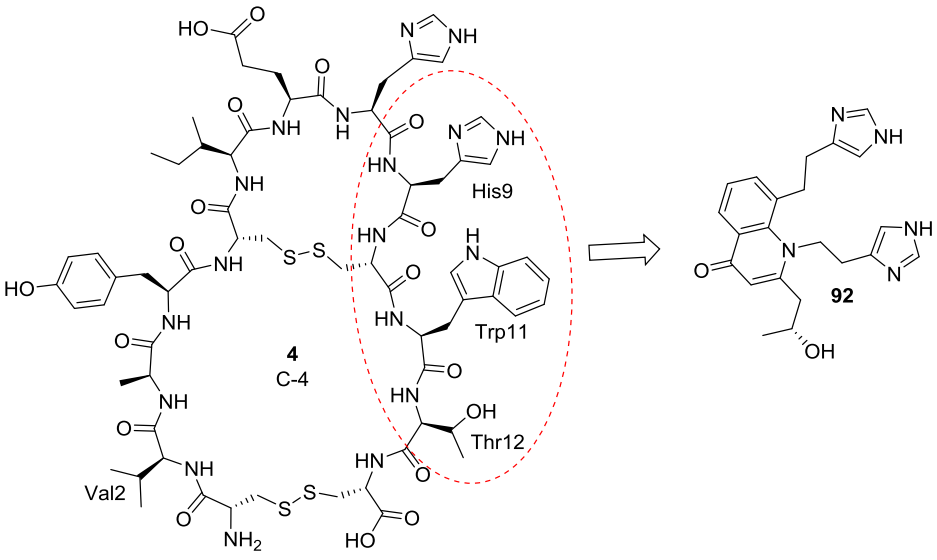
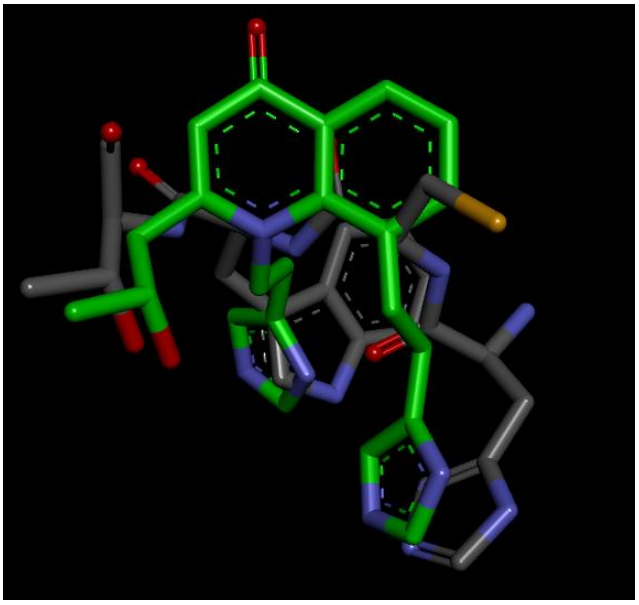
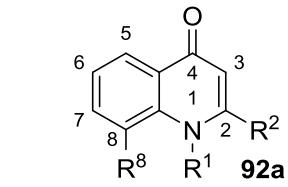


Figure 19 The structure of peptide **4** and the corresponding peptidomimetic **92**

A 1,2,8-trisubstituted 4-quinolone scaffold (**92a**) was found to fulfil both of these criteria. Based on the information gained from the docking study of peptide **4** and the preliminary evaluation of different scaffolds **92** was chosen as the structure for the

peptidomimetic. The generalised structure of the 4-quinolone based mimetic together with a superposition of the energy minimised structure of **92** and the peptide fragment in question (His9-Cys10-Trp11-Thr12 in C-4) are shown in Figure 20. In this structure the groups R^1, R^2, and R^8 correspond to the side chains of the amino acid residues the compound it is intended to mimic.





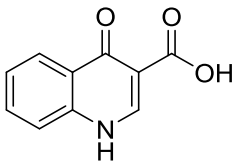
92a

R^2: Linker + oxygen containing functional group

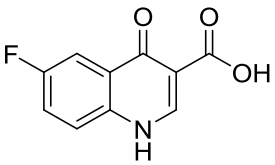
R^1 and R^8: Linker + hetero-aromatic ring corresponding to e.g. His and Trp side chains

Figure 20 General structure of quinolone-based peptidomimetic **92a** (right), an overlay of the energy minimised structure of **92** on the corresponding peptide fragment in C-4, i.e. His9-Cys10-Trp11-Thr12 (left). The hydrogen atoms are omitted for clarity.

Even though compounds based on 4-quinolones have been used as drugs since the late 1960s, these compounds are mainly based on the 4-quinolone-3-carboxylic acid core **93** or the analogous 6-fluorinated core **94**. These compounds have been extensively used as antimicrobials and broad-spectrum antibiotics,[268,269] although concerns have been raised regarding their side effects and their susceptibility to antibiotic resistance.[270-272] When considering the classification of the quinolone scaffold as a privileged structure[240,269,273] and it also having been shown to have biological activity for several different targets[269] it seems like an attractive scaffold for a peptidomimetic.



93



94

4.2 SYNTHESIS OF A PEPTIDOMIMETIC BASED ON THE 4-QUINOLONE SCAFFOLD

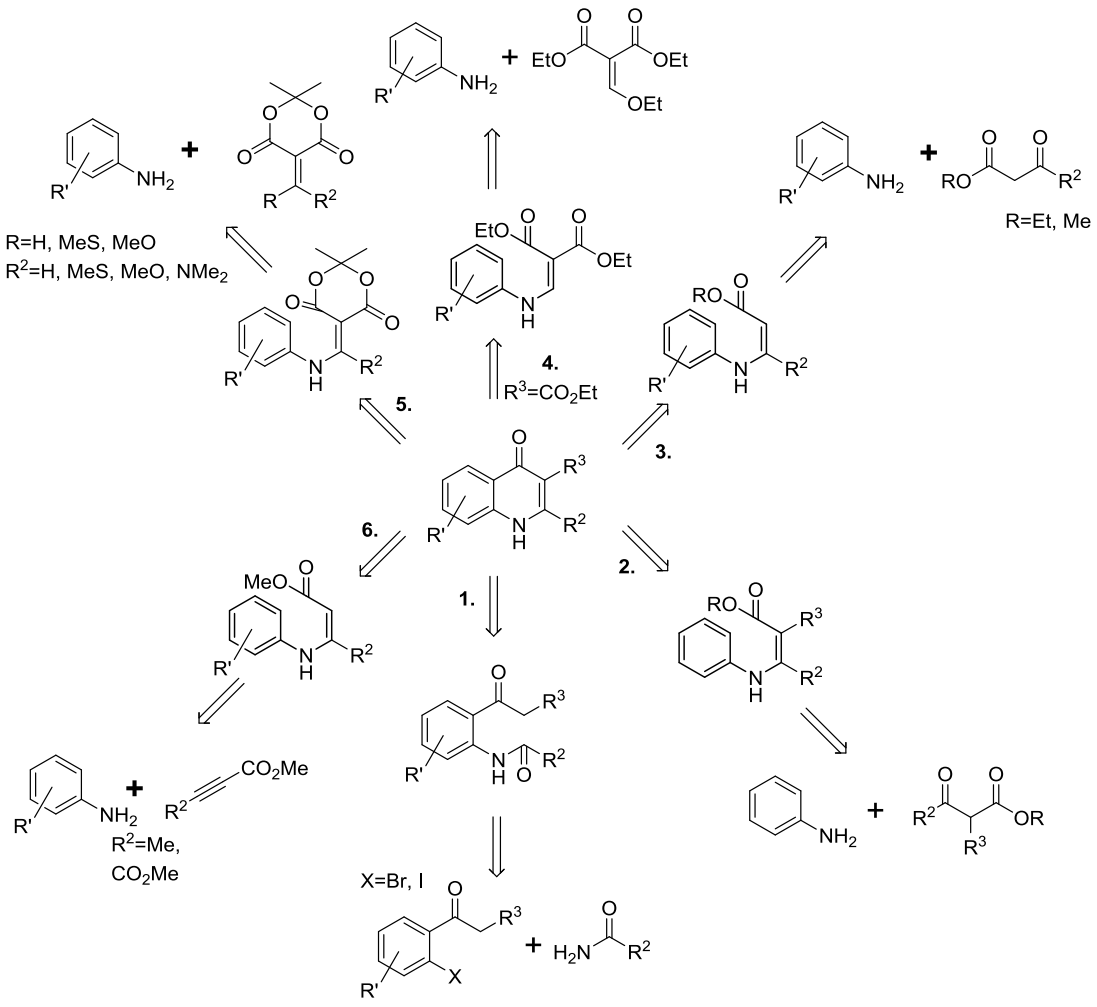
One of the consequences of the success of the fluoroquinolones is that most synthetic protocols published in this field are aimed at 4-quinolone-3-carboxylic acids.[269] It should also be noted that even though isolated examples of decarboxylation of quinolone carboxylic acids exist in the literature, even the decarboxylation of the simpler quinolones requires heating to 240-270 °C[274,275] or alternatively refluxing in a high-boiling solvent such as diphenylether,[276] making this approach less attractive for more complex compounds.

Despite the interest in the 4-quinolone-3-carboxylic acids, several methods for the synthesis of other quinolones have also been published. Six of the more common routes are outlined in Scheme 13. These strategies have in common the use of an aniline as the starting material in a condensation reaction followed by a cyclisation to yield the final product. The exception to this is Camps quinoline synthesis presented as number 1 in the scheme. In this method an *ortho*-acylaminoacetophenone is transformed into a 4-quinolone in the presence of hydroxide ions.[277] Routes 2 and 3 utilise *β*-ketoesters to form the intermediate enaminoester through an acid-catalysed substitution on the aniline nitrogen followed by cyclisation in a high-boiling solvent.[278-283] Routes 4 and 5 on the other hand use methoxy (MeO-) or methylthio (MeS-) substituted alkenes for the formation of the enaminoester by removal of the corresponding alcohol or thiol.[284-287] A modification of this method uses trimethyl orthoformate to generate the methoxymethylene moiety *in situ* (not shown in Scheme 13).[287] A modification of the two previous methods is outlined in route 6 in Scheme 13. Here, an aniline is reacted with acetylene dicarboxylate to afford the intermediate.[288-290] This reaction has also been shown to be successful when using butynoic acid or esters to give 4-quinolones.[291,292]

Even though much of the older literature concerning these syntheses report the product as being a 4-hydroxyquinoline it has later been shown that the keto-isomer (4-quinolone) dominates over the enol in the product. This is clear e.g. in NMR-spectroscopy where only a minute portion of the enol tautomer is visible at room temperature. If the 4-hydroxyquinoline or derivatives thereof are needed, one possible strategy for synthesis is to use phosphorous oxychloride ($POCl_3$) to get the 4-chloroquinoline which can further be reacted with benzyl alcohol to get the corresponding benzyl ether.[281]

Other methods for the synthesis of 4-quinolones have been published, but as these are even more limited in the scope of substitution on the final product they will not be covered here.[293-299] These limitations are caused both by the requirement of synthesising complex starting materials[293,294,299] and the limitations to the diversity of the product caused either by the mechanism of the reaction or the starting materials used.[295-297]

When reviewing the literature on the synthesis of 4-quinolones it becomes obvious that even though most types of substituents can be found, the different methods all have limitations to their compatibility with different substitution patterns. For example route 5, using Meldrums acid, gives the possibility to use anilines substi-



Scheme 13 Retrosynthetic analysis of different routes found in the literature for the synthesis of 4-quinolones

tuted in many different ways but the variations possible in the R^2-position are extremely limited.[285-287,294,300,301] This can partially be worked around by modifying the R^2 side-chain after the synthesis of the ring system but this is also limited to substituents linked to the ring by a heteroatom (O, N or S).[286,294,300] Route 4 in the scheme also suffers from this limitation as it is not possible to introduce an R^2-substituent using this method and it also results in an ester group in the 3-position.[284]

The synthetic routes starting from a *β*-ketoester and an aniline offer excellent possibilities for the synthesis of a variety of 4-quinolones as a large number of different *β*-ketoesters can be synthesised from their corresponding acids using simple procedures.[302,303] The problem in this context is the limited compatibility towards substitution especially in the 8-position of the final product (the *ortho*-position relative to the aniline nitrogen). Even though examples have been published of 5-, 6- or 7-halo substituted quinolones synthesised using this method,[275,282,283] compounds bearing a halogen substituent in the 8-position are rare. In fact, only one example can be found reporting the synthesis of an 8-bromo substituted 4-quinolone starting from

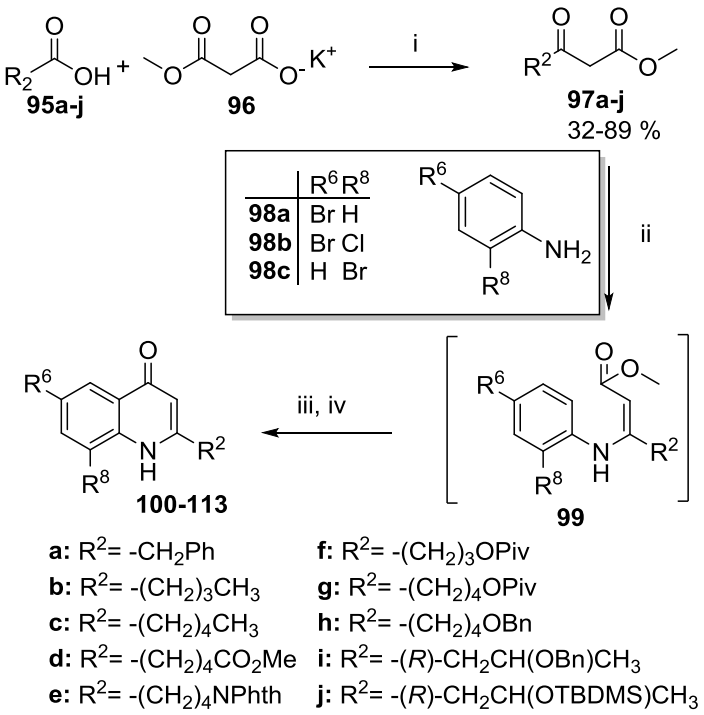
a *β*-ketoester.[304] Additionally, there has also been only limited work done on the synthesis of e.g. 6,8-dihalosubstituted quinolones bearing two different halogen substituents which would offer the possibility of attaching two different side chains to the quinolone core regioselectively. Examples do exist of 5,7-dichloro-4-quinolone[283], 6,8-dibromo-4-quinolone[305] and 6,8-difluoro-4-quinolone.[306] It should be possible to further derivatise these compounds on both halogen positions but as it is unlikely that it will be possible to selectively substitute only one of the halogens the usefulness of these compounds is somewhat limited. It is also possible to synthesise a 6-amino-8-methoxy-4-quinolone[307] in which both of the substituents on the aromatic ring can be reacted further, although there are significantly more possibilities for the substitution of halogens than of substituents of other heteroatoms, such as O, N and/or S.

In order to make the series of compounds as diverse as possible, 4-quinolones with several different R^2-side chains were synthesised. The side chains were chosen to represent the different types of side chains found in the natural amino acids. The chosen alternatives were alkyl (butyl and pentyl), aromatic (benzyl), amino (4-aminobutyl), carboxylic acid (methyl 4-pentenoate) and hydroxyl ((*R*)-2-hydroxypropyl, 3-hydroxypropyl and 4-hydroxybutyl). The different chain lengths in the substituents also show if this affects the reactivity.

The first step in the synthesis of the quinolones was the preparation of *β*-ketoesters **97a-j** containing the side chain which will end up in the 2-position of the final 4-quinolone. They were obtained in good yields by reacting the corresponding carboxylic acids **95a-j** and monomethyl potassium malonate **96** with *N*,*N*'-carbonyldiimidazole (CDI) in anhydrous THF at room temperature.[302] Protected (*R*)-3-hydroxybutanoic acids were prepared from methyl (*R*)-3-hydroxybutyrate via benzyl[308] or TBDMS[309] protection of the alcohol and subsequent hydrolysis of the ester.[310] *N*-Phthalimide-protected 5-aminovaleric acid **95e** was prepared by modifying a known procedure.[311] Pivalic esters **95f** and **95g** were prepared from pivaloyl chloride and methyl 4-hydroxybutyrate, and methyl 5-hydroxypentanoate, respectively, the methyl esters were subsequently hydrolysed using lithium hydroxide in aqueous methanol. Methyl 4-hydroxybutyrate and methyl 5-hydroxypentanoate were prepared from *γ*-butyrolactone[312] and *δ*-valerolactone,[313] respectively, according to known procedures.

4-Quinolones **100-113** were synthesised employing the Conrad-Limpach reaction. The *β*-ketoesters were stirred together with the corresponding anilines **98a-c** under argon at 50 °C for 48 h to afford the intermediate enaminoesters **99a-e**. After removal of excess aniline and *p*-toluenesulphonic acid (PTSA) the cyclisation was achieved by heating the enaminoesters to 250 °C in diphenyl ether for 45 min using microwave heating. The quinolone products **100-113** were finally isolated by crystallisation from hexane.

The yields for successful reactions run using the developed conditions are reported in Table 3. The results indicate that several substituents with varying chain lengths could be introduced in the 2-position. A major exception was the compounds with substituents with a protected hydroxyl group in the 2-position. Synthesis of these using the same method as the other 4-quinolones was unsuccessful and

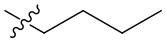
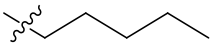
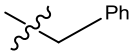
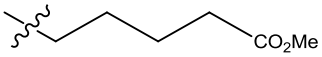
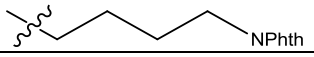


Scheme 14 Synthesis of 4-quinolone scaffolds. Reagents and conditions: i) CDI, $MgCl_2$, THF, 20 °C, 16 h. ii) **98a**-**c**, PTSA, neat, 50 °C, 48 h. iii) Cyclohexane, reflux, 30 min. iv) Diphenyl ether, MW, 250 °C, 45 min. Piv= pivaloyl (-$OC(CH_3)_3$)

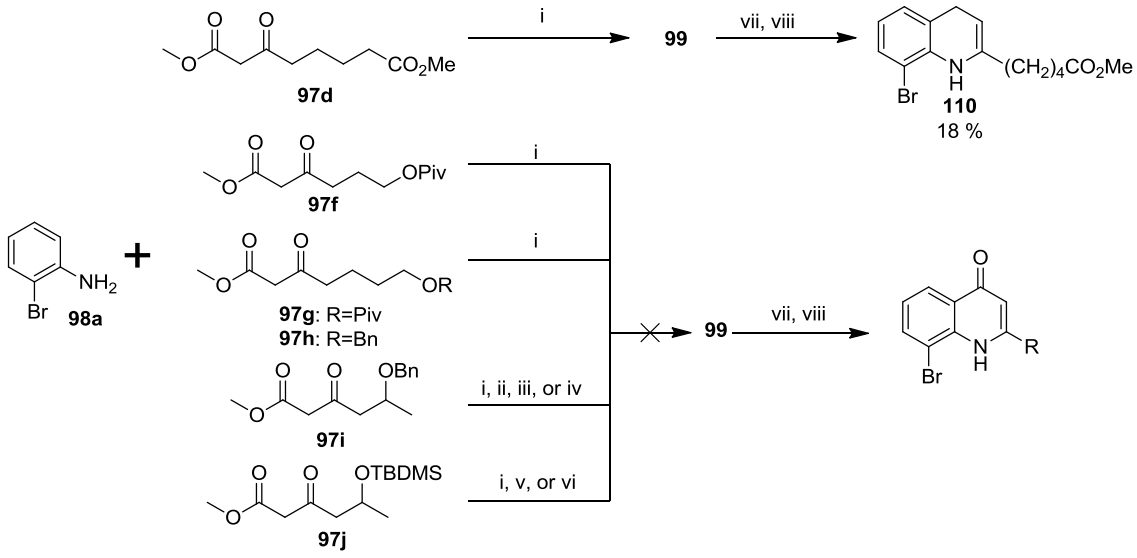
attempts to use different conditions did not improve the situation (Scheme 15). A number of different protecting groups (pivaloyl ester, benzyl ether and TBDMS), chain lengths and synthetic methods were attempted. However, the hydroxyl group can also be introduced via an ester-bearing side chain in the 2-position (as in **110**-**112**) which it should be possible to subsequently reduce to the corresponding alcohol using a previously reported method.[314]

When examining the yields of the successful reactions, it is obvious that reactions resulting in a product with bromine in the 8-position give low yields (14-31%), whereas reactions resulting in a product with hydrogen in the 8-position give better yields (34-55%). This is a clear trend in the successful reactions. Interestingly, whether or not the product has a halogen atom (chlorine or bromine) in the 6-position does not seem to affect the yield of the reaction.

Table 3. *Successfully synthesised quinolone scaffolds*

Compound	R^2	R^6	R^8	Yield %[a]
100		Cl	Br	31
101		Cl	H	42
102		H	Br	14
103		Br	H	39
104		H	Br	29
105		Cl	Br	25
106		Cl	H	34
107		H	Br	25
108		Cl	Br	24
109		Cl	H	55
110		H	Br	18
111		Cl	Br	26
112		Cl	H	39
113		H	Br	26

[a]Yields calculated over two steps from **97a-e**



Scheme 15 Syntheses of 4-quinolones with an oxygen-bearing subsituent in the 2-position. i) 0.1 eq. PTSA, neat, 50 °C 2-16 days. ii) catalytic amount of silica gel, neat, 50°C, 2 days. iii) cat. conc. HCl, neat, 50 °C, 2 days. iv) PTSA, AcOH, 50 °C, 2 says. v) EtOH, catalytic AcOH, reflux 2 days. vi) EtOH, catalytic AcOH, $CaSO_4$, 50 °C, 2 days. vii) Cyclohexane, reflux, 30 min. viii) Diphenyl ether, MW, 250 °C, 45 min. Piv=pivaloyl

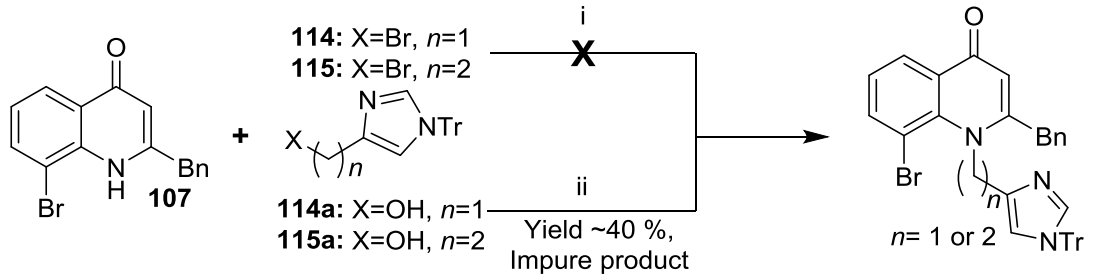
Alkylation of the nitrogen in the pyridinone-ring (the 1-position) of the 4-quinolone is one of the key features that makes it an attractive alternative for a peptidomimetic scaffold. The most common way of doing this substitution is to use a suitable alkyl

halide in the presence of a base. This has been shown to be successful using even mild conditions; K_2CO_3 is often used for the reaction.[315] If an *N*-methylation is to be done the substitution is usually performed as described above, using methyl iodide and a suitable base.[316] The use of diazomethane has also been reported although the *N*-methylated compound was only the product of a side reaction in this case.[317] If the required halide is not easily available another alternative for the substitution is to use an alcohol in a Mitsunobu reaction. One example of a Mitsunobu reaction on a 4-quinolone has been published[318] but this is clearly an approach that has not been thoroughly investigated. When looking at the patent literature, a few examples of similar Mitsunobu reactions can be found where the alcohol has been activated to a mesylate.[319]

The final step in the decoration of the quinolone scaffold is the introduction of substituents on the aromatic ring. If the aromatic ring bears e.g. a bromo substituent, this can be done by a palladium-mediated Heck-coupling as has been shown for 6-bromo-4-quinolone.[320] When using the chromone scaffold, which is similar to the 4-quinolone, the Heck-reaction has been shown to be selective for an 8-Br-substituent also when a 6-Cl-substituent is present on the same ring.[265]

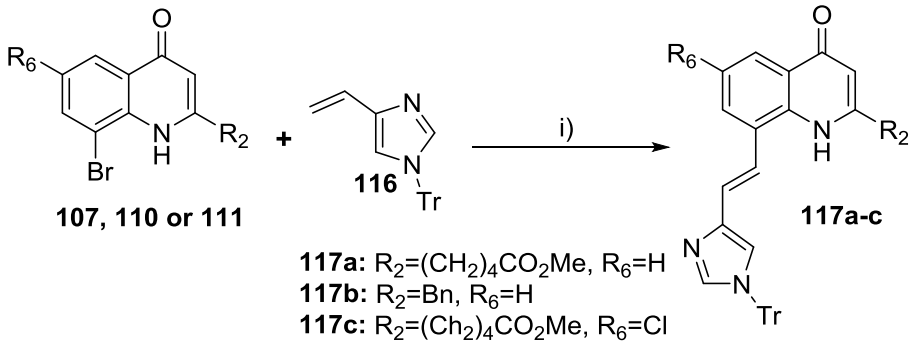
Our initial strategy for introduction of substituents in the 4-quinolones was to first substitute the 1-position followed by a Pd-mediated Heck reaction on the brominated 8-position to obtain the final compound. Initially, attempts were also made to alkylate the nitrogen atom in the 1-position using an alkyl bromide (**114** or **115**) and a base, but unfortunately none of the conditions tried for this reaction yielded the desired product.

The DIAD or DEAD-mediated Mitsunobu reaction was chosen as a second alternative for this substitution and did produce the desired product although unfortunately as an inseparable mixture of the product and amine byproducts produced from DIAD or DEAD in the reaction. Based on NMR spectroscopic analysis of the product mixture the yield of the reaction was estimated to approx. 40 %. Performing the Heck reaction on the impure product from the Mitsunobu reaction did not give any of the desired product. Whether this is caused by the impurities or some other reason was not investigated.



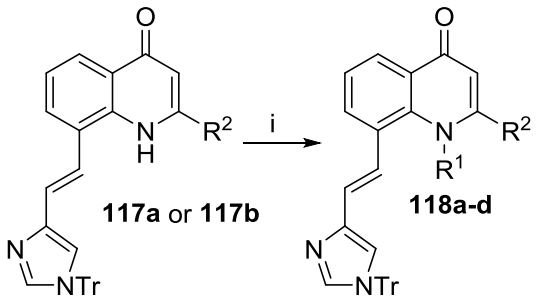
Scheme 16 Reactions attempted for substitution of 4-quinolone **107**. Reagents and conditions: i) NaH or Cs_2CO_3, DMF or THF, 25 or 100 °C, 4 – 16 h. ii) DIAD or DEAD, PPh_3, chloroform, reflux, 16 h.

These problems required a change in strategy. The substituent in the 8-position of the quinolone was successfully introduced by a Pd(0)-mediated Heck-coupling that substitutes the bromide present on the aromatic ring. The reaction was performed in benzene using tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$) and tri-*tert*-butylphosphonium tetrafluoroborate ($[(t\text{-}Bu)_3PH]BF_4$) with microwave heating. Performing the reaction on the 6-chloro-8-bromo-substituted quinolone **111**, we were also able to show selectivity of the bromide over the chloride in the Heck-reaction.



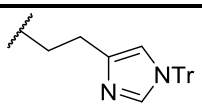
Scheme 17 Heck reactions used for the substitution of the 2-position of quinolone scaffolds **107**, **110**, and **111**. Reagents and conditions: i) $Pd_2(dba)_3$, $[(t\text{-}Bu)_3PH]BF_4$, benzene, 160 °C, 30 min, MW.

Substitution of the nitrogen atom in the 1-position of the final 4-quinolone was accomplished with an alkyl bromide under basic conditions (Scheme 18, Table 4). This reaction also simultaneously hydrolysed the methyl ester in compound **117a** to yield **118a**. Attempts to perform the same substitution by a DIAD-mediated Mitsunobu reaction failed to produce any product, probably due to the sterically crowded reaction site in combination with the bulky nature of the intermediate formed in this type of reaction.



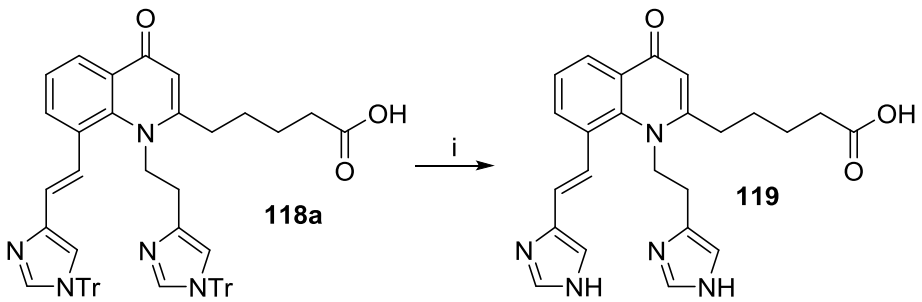
Scheme 18 Alkylation of intermediates **117a** and **117b**. The structures of the compounds are presented in Table 4. Reagents and conditions: i) R^1Br, Cs_2CO_3, DMF, 100 °C, 16 h.

Table 4. *1-Alkylated 4-quinolones*

Starting Material	Product	R_1	R_2	Yield
117a	**118a**		$-(CH_2)_4CO_2H$[a]	64
117b	**118b**		$-CH_2Ph$	50
117b	**118c**	$-CH_2Ph$	$-CH_2Ph$	38
117b	**118d**	$-(CH_2)_2Ph$	$-CH_2Ph$	54

[a]R^2 refers only to **118a**.

The final product was achieved by removal of the trityl groups from the imidazoles. This was done using 20 % TFA in DCM at room temperature in a yield of 92 %.



Scheme 19 Deprotection of **118a** to yield final product **119**. Reagents and conditions: i) 20 % TFA in DCM, 90 min, room temperature.

The ability of compound **119** to stimulate the proteolytic activity of KLK3 was determined in an assay using a chromogenic substrate.[54-56] Unfortunately, the compound showed no KLK3-stimulating effect.

5 CONCLUSIONS AND FUTURE PROSPECTS

The work presented in this thesis covers several years of work involving a large number of people. Even though there have been many successes, many things have also been unsuccessful and/or still remain to be done. This chapter aims to summarise what has been learned from the work and also add some considerations as to how the work could be continued in the future.

5.1 REPLACING DISULPHIDE BRIDGES IN PEPTIDES

Much of the work discussed has been aimed at the replacement of disulphide bridges in biologically active peptides in such a way that the pseudopeptides retain the biological activity of the parent peptide. The different building blocks presented in Chapter 3.1 represent interesting possibilities for replacing disulphide bridges in peptides. The four different general structures (**31**, **32a**, **32b** and **33**) allow for the investigation of whether the sulphur atoms in the bridge are essential for the biological activity, they also represent different conformations of the bridge.

The tolerance for different types of substrates for both the CM and copper-mediated coupling reactions was further investigated in connection to the work on pseudopeptides based on the B-2 peptide (**5**) as it has been determined that the terminal NH_2-group of said peptide is not necessary for biological activity. In order to further investigate this, it was decided to synthesise a set of pseudopeptides using two of the previously synthesised building blocks and analogues lacking the terminal amine. The synthesis of the building blocks required for these peptides once again brought with it some surprises concerning the reactivities of different substrates in these reactions.

The conclusion from this is that the reactions discussed are apparently quite sensitive to both the structure of the starting materials involved and possibly also the reaction conditions. The exact cause of this observation remains unclear based on the number of experiments performed within this project. It is fully possible that the problems with low yields and mixtures of products could be solved just by optimising or changing the reaction conditions. Due to time constraints this was, however, deemed to be outside the scope of this project and not investigated further.

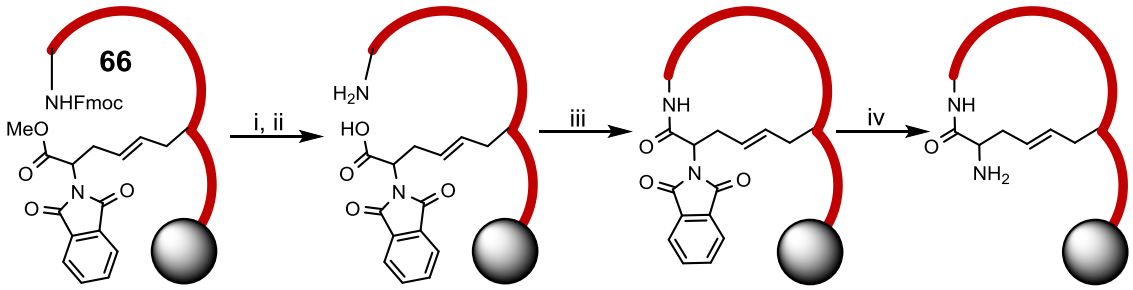
As was discussed in some detail in Paper II (Chapter 3.1.2 of this thesis), the synthesised building blocks were originally intended to be used in in the synthesis of a pseudopeptide analogue of the C4-peptide (**4**), unfortunately with no success so far. As our original method for this synthesis using the building blocks directly in SPPS was unsuccessful due to problems with the removal of protecting groups from intermediate **66**, we decided to first synthesise the smaller ring **70** of peptide **4** and attach this to the peptide chain as a building block in SPPS. Even though the synthesis of the ring itself was completed successfully, the selective hydrolysis of the

methyl ester proved to be a significant obstacle to this approach. The use of trimethyltin hydroxide has been useful for selective ester hydrolysis in many other applications, but in this case it seemed to mainly cleave the Fmoc from the peptide. The methods used for this reaction included both thermal and microwave heating for various lengths of time as well as changing the solvent.

Despite the initial failures in the synthesis of the pseudopeptide analogues of **4** it was possible to synthesise these compounds using the RCM reaction for closing the smaller ring of the partially synthesised peptide. In this case the conditions found in the literature were not initially successful, but systematic modification of the conditions did eventually yield an efficient method for the synthesis of the pseudopeptides in Paper II.

Even though the results from these syntheses and the biological evaluation of the pseudopeptides were extremely pleasing, the peptides were still not as potent as the peptides containing the native disulphide bridge. As has already been noted, the structure most likely to be able to mimic the conformation of the parent peptide is the one containing the linker with a *Z* double bond or the fully saturated chain. As the attempts to use the orthogonally protected building blocks in the synthesis of pseudopeptides in the place of an internal disulphide bridge had so far proved unsuccessful, the obvious method for obtaining these compounds would be the reduction of the double bond in the finished pseudopeptide.

The lack of success in reducing the internal double bonds in pseudopeptides **76-78** inevitably meant that a method for using the orthogonally protected building blocks is required to access pseudopeptides containing the various bridge-mimetics in pure form. This, of course, meant that the work on deprotection of intermediate **66** had to be restarted, the strategy for this is outlined in Scheme 20.



Scheme 20 Proposed route for the synthesis of C-4 analogues using an orthogonally protected building block. Reactions: i) Hydrolysis of methyl ester. Ii) Removal of Fmoc group. Iii) Peptide coupling. Iv) Removal of phthalimide group.

Assuming that the problems concerning the deprotection of intermediate **66** can be solved and the synthesis of the pseudopeptide finished, this would lead to two significant advances in the investigation of the pseudopeptides of the C-4 peptide (**4**). Firstly, it would make it possible to investigate what effect the different linkers replacing the disulphide bridge have on the biological activity of the peptide. Secondly, it would also enable the use of the RCM-method for replacing the terminal

disulphide bridge in peptide **4** simultaneously with the internal one. In addition to giving some more information about how modifications of the peptide affect its biological activity, replacing the terminal disulphide could also have an effect on the stability of the peptide in plasma as the terminal disulphide bridge is likely to be more susceptible to reduction than the internal one. As has been proven with pseudopeptides based on **5**, removing the terminal NH_2-group from a peptide can also increase the stability.[55] Using a method analogous to that used to synthesise building block **84b** it should be possible to introduce this type of structure in these pseudopeptides as well. Even if the RCM-reaction turns out to produce a mixture of the *E* and *Z* double bonds in this case as well, they should be separable by chromatographic methods and reducible using Pd/C in a hydrogen atmosphere according to what has been seen with other peptides containing hydrocarbon replacements for terminal disulphides.

5.2 QUINOLONE-BASED PEPTIDOMIMETICS THAT MODULATE THE ACTIVITY OF KLK3

The motivation for the study of the chemistry of the 4-quinolones lies in the original goal of the project to create a small molecular weight compound with a biological activity like that of the peptides and pseudopeptides discussed in this work. The starting point for the work in designing a compound with this activity was to study the (partly unpublished) work that has been done on modelling the interactions between KLK3 and the C-4 peptide (**4**). In this work, the C-4 peptide has been docked onto the kallikrein loop of KLK3 in order to identify both a possible binding site for the peptide and also what interactions are required for binding.

By closely studying the structure of the peptide docked on a homology model of the protein it is possible to identify His9, Trp11 and Thr12 as the residues that interact with KLK3 via hydrogen bonding. Especially interesting is the observation that the imidazole ring of His9 fits neatly into a cavity in the kallikrein loop directly above the active site of the enzyme. Figure 21 shows the peptide docked on the protein, for clarity the parts of that are not directly participating in the interaction have been omitted.

Based on the information from this study, a few different scaffolds were investigated for their ability to mimic the structure of the peptide backbone. When the side chains corresponding to the residues mentioned previously had been attached to the 4-quinolone scaffold using spacers of suitable length, the compound energy minimised and subjected to a conformational search, it showed good agreement with the His9-Thr12 fragment of the peptide as illustrated in Figure 20 on page 45.

One surprising and unfortunate observation that was made in the course of the synthesis of the 4-quinolones was the difference in reactivities of the β-ketoesters depending on the type of oxygen-bearing substituent (Scheme 15). The exact cause for this problem has not been investigated more closely, although NMR analyses and MS analysis of intermediate **99** from these reactions did indicate that for some

reason the product formed in the first reaction is not the desired enaminoester. From the analyses that have been performed it is not immediately apparent what has actually happened in the reaction.

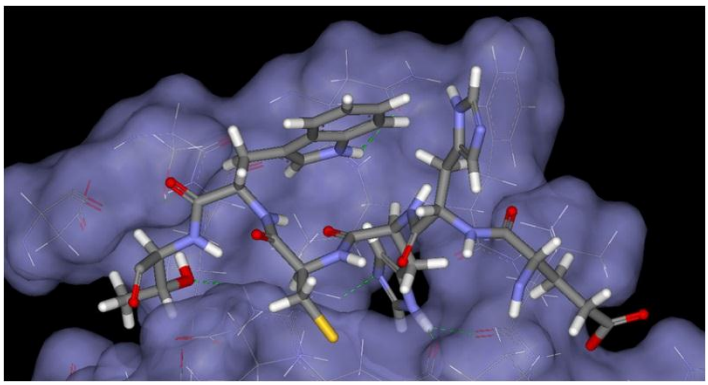
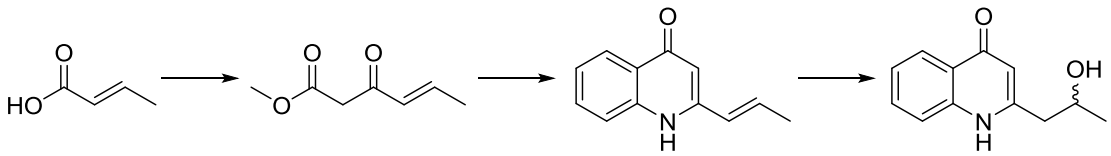


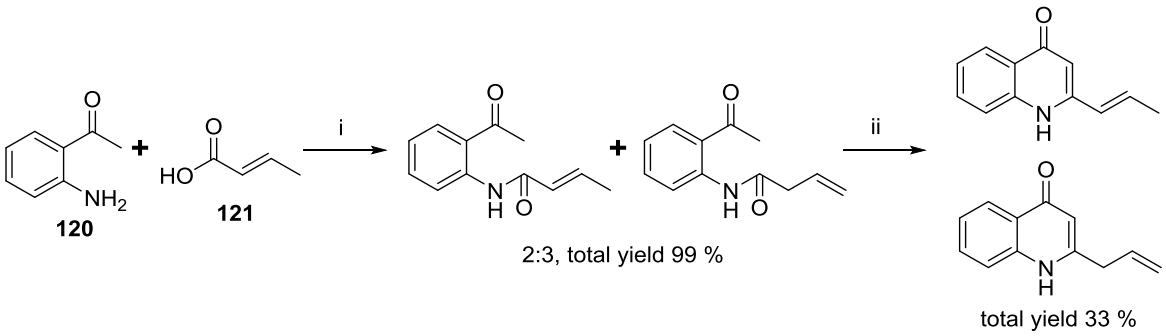
Figure 21 The C-4 peptide docked on the kallikrein loop of KLK3. Amino acid residues in the peptide not directly participating in the interaction with KLK3 have been removed for clarity.

As a consequence of these problems, it is clearly necessary to find a different strategy for the synthesis of the quinolones with a hydroxyl-bearing substituent in the 2-position. One possibility is to first synthesise the 4-quinolone with an unsaturated R^2 side chain, then add the hydroxyl at a later stage through a suitable reaction to complete the synthesis as outlined in Scheme 21.



Scheme 21 Proposed synthesis of a hydroxyl-bearing 4-quinolone using the Conrad-Limpach reaction.

There are also two interesting alternative routes found in the literature that utilise modified Camps' cyclisations to access a quinolone with an unsaturated side chain in the 2-position.[277,321] Of these, the procedure outline in Scheme 22 uses commercially available *o*-aminoacetophenone **120** and crotonic acid **121** to give a 2:3 mixture of the two quinolones in two steps.[321] Whether or not it is possible to separate the two compounds is not discussed in the paper.



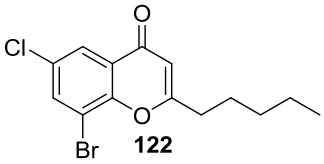
Scheme 22 Use of a modified Camps' quinoline synthesis to access 4-quinolones with a substituent in the 2-position containing an alkene moiety.[321] Materials and methods: i) PyBroP (1.1 eq.), TEA (1.1 eq.), DCM (0.1 M), r.t., 16 h. ii) TEA (3 eq.), TMSOTf (6 eq.), DCE (0.05 M), reflux.

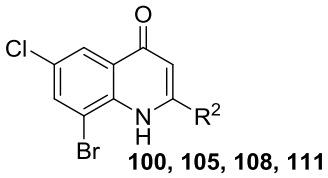
5.3 SIRT2 INHIBITORY ACTIVITY OF 4-QUINOLONE BASED COMPOUNDS

Even though none of the quinolones intended to be tested for stimulation of KLK3 have so far been successfully synthesised and tested, some of the compounds have instead been tested as inhibitors of sirtuin 2 (SIRT2), a family of NAD^- dependent histone deacetylases. The sirtuins have become highly interesting targets for drug discovery as they are involved in a number of important cellular processes and have been implicated in certain disease states related to aging and consequently to neurodegenerative diseases such as Alzheimer's, Parkinson's, or Huntington's.[322-325] One highly potent and selective inhibitor of SIRT2, 8-bromo-6-chloro-2-pentylchromone **122** (82 % inhibition at 200 µM, IC_{50} = 5.5 µM) and SAR-studies indicate that large, electron withdrawing groups in the 6- and 8-positions are favoured.[326] As some of the synthesised 4-quinolones are also 8-bromo-6-chloro-substituted and structurally quite close to chromone **122**, they were also tested for SIRT2-inhibition, the results are shown in Table 5.

Table 5. *SIRT2 inhibition of 4-quinolone compounds*

Compound	R^2	Inh. %, 200 µM (± SD)
122		82 (0.4)
100	$-(CH_2)_3CH_3$	47 (0.9)
105	$-(CH_2)_4CH_3$	26 (1.4)
108	$-CH_2Ph$	58 (2.1)
111	$-(CH_2)_4CO_2Me$	53 (1.1)





Even though none of the tested 4-quinolones are exceptionally potent, they are quite good for being a first, un-optimised set of compounds. One of the obvious differences between the quinolone and chromone is the NH-group which makes the

quinolone more hydrophilic than the chromone which can be problematic as the inhibitors have been proposed to bind in a hydrophobic pocket on SIRT2.

With some additional compounds it should be possible to improve the activity at least to some degree making this an interesting application for the 4-quinolone scaffold. Even as such these compounds serve as proof that 4-quinolone is indeed a privileged structure and also proves it is a good bioisostere of the chromone ring system. This clearly underlines one of the major advantages with working with privileged structures, the simple transition from one target to another one.

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