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ABSTRACT

Peptides and peptide-based pharmaceuticals have been a subject of growing interest during the last decades because of their wide influence to the different biological actions. Numerous hormones, neurotransmitters, growth factors and antigens are peptides, many of them directly used as therapeutics. However, peptides are considered as poor drug candidates because they are usually rapidly metabolized by enzymatic degradation and extracted from the organism resulting in very poor bioavailability. Therefore, the administration of peptide drugs is challenging and must be repeated many times per day, often intravenously or intramuscularly. The developments in peptide synthesis, in the strategies aimed at reducing metabolism and improving bioavailability and advances in alternative delivery routes have resulted in a rapid increase in the number of peptide-based pharmaceuticals on the market. Furthermore, peptides are widely used as lead molecules in radiopharmaceuticals and imaging agents because their high selectivities and specificities.

The prostate produces several proteases including two highly specific enzymes KLK2 and KLK3 (PSA) which are used as marker for prostate malignancies. The measurement of the KLK3 concentration in the human serum has been used for detection and monitoring of prostate cancer for decades although one problem with the serum test is the high false-positive rate mainly caused by benign prostate hyperplasia. Thus, a more accurate detection method is needed. KLK2 and KLK3 have been shown to be involved prostate cancer proliferation. KLK3 has been proposed to participate in the inhibition of the angiogenesis which is necessary for tumor growth and metastasis. KLK2 participates in the proteolytic cascades which degrade extracellular matrix and thereby KLK2 may mediate tumor growth and invasion. Thus, if one could discover agents which wouyld be able to modulate the enzymatic activity of KLK2 and KLK3, it might be possible to modify the growth rate of prostate cancer and impair its ability to metastase.

In this study, peptide based modulators of KLK2 and KLK3 have been studied by the means of peptide modifications and conformational analysis with NMR and structure-activity relationship studies. The residues essential for the activity stimulation of KLK3 were identified via sequential replacement studies. The stability against degradation in plasma as well as against degradation by KLK3 could be enhanced in one selected peptide by the replacement of terminal disulphide bridge by analogous bridges without the loss of bioactivity. It was also shown that backbone cyclization of KLK2 inhibitory peptides increased stability without the loss of biological activity. A series of peptide analogs also provided valuable structure–activity relationships information for peptide mimetic studies. The improved *in vivo* stability is essential for *in vivo* applications, such as diagnostic tumor imaging and prostate cancer therapy.

TIIVISTELMÄ

Peptidit ja peptidipohjaiset lääkeaineet ovat olleet kasvavan kiinnostuksen kohde viimeisten vuosikymmenten aikana, koska peptidit osallistuvat moniin biologisiin prosesseihin. Useat hormonit, välittäjäaineet, kasvutekijät ja vasta-aineet ovat peptidejä ja useita niistä on käytetty suoraan lääkinnällisiin tarkoituksiin. Peptidejä pidetään kuitenkin heikosti soveltuvina lääkeaineaihioiksi, koska ne metaboloituvat nopeasti entsyymien vaikutuksesta ja erittyvät elimistöstä. Tällöin niiden hyötyosuus jää pieneksi ja annostelu vaikeutuu vaatien useita yleensä suonensisäisiä päiväannoksia. Menetelmäkehitys, joka on johtanut peptideihin liittyvän syntetiikan ja metaboliaa hidastavien menetelmien paranemiseen ja hyöty-osuuden kasvuun sekä vaihtoehtoisten annostelumenetelmien kehitys ovat edesauttaneet peptidipohjaisten lääkeaineiden myynnin kasvua. Lisäksi peptidien kyky sitoutua usein spesifisesti ja selektiivisesti kohteesensa edesauttaa niiden käyttöä lähtörakenteina radiolääkeaineiden ja kuvantamisaineiden kehityksessä.

Eturauhanen tuottaa useita proteaaseja mukaanlukien hyvin spesifiset KLK2 ja KLK3, joita voidaan käyttää eturauhasen poikkeuksellisen kasvun määrityksessä ja seurannassa. KLK3:n seerumipitoisuuden nousua on käytetty eturauhassyövän diagnostiikkaan jo pari vuosikymmentä. Pitoisuustestin ongelma on suuri virheellisten diagnoosien osuus johtuen pääosin eturauhasen hyvälaatuisesta liikakasvusta. Testin tarkkuutta on parannettava ja eräs mahdollisuus on seurata myös KLK2:n seerumipitoisuutta. KLK2:n ja KLK3:n on myös todettu osallistuvan eturauhassyövän kehitykseen. KLK3:n on esitetty ehkäisevän syöpäkasvaimen verisuonten kehitystä vaikuttaen myös mahdolliseen etäispesäkkeiden syntyyn. KLK2 toimii osana proteolyyttista kaskadia, joka hajoittaa soluväliainetta ja näin edesauttaa syöpäkudoksen leviämistä. Eturauhassyövän kasvua ja leviämistä voidaan täten mahdollisesti kontrolloida vaikuttamalla KLK2:n ja KLK3:n entsyymiaktiivisuuksiin.

Tämän tutkimuksen pohjana ovat peptidit, jotka vaikuttavat KLK2:n ja KLK3:n entsyymiaktiivisuuteen. Peptidien bioaktiivista rakennetta on pyritty määrittämään ja muokkaamaan käyttäen NMR-rakenneanalyysiä sekä kohdennettuja modifikaatioita ja tutkimalla niiden aiheuttamia rakenne-aktiivisuus suhteiden muutoksia. KLK3:n biologisen aktiivisuuden kannalta tärkeät aminohapot on kartoitettu. Yhden valitun peptidin vastustuskykyä sekä plasman proteaaseista että KLK3:sta aiheutuvaa entsymaattista hajoitusta kohti on pystytty kasvattamaan tuottamalla analogi, jossa rikkisilta peptidin päiden välillä on korvattu epäluonnollisella synteettisellä osalla. Myös KLK2:sta inhiboivan peptidin stabiilisuutta on kyetty lisäämään syklisaatiolla joka ei tuhoa biologista aktiivisuutta. Tutkimuksessa on lisäksi saatu arvokasta tietoa peptidin rakenteen ja rakenteen muutoksen vaikutuksesta biologiseen aktiivisuuteen. Kasvanutta *in vivo* stabiilisuutta voidaan hyödyntää uusien terapeuttisten ja diagnostisten aineiden ja niiden rakenteiden suunnittelussa.

Luokitus: QU 68, QU 136, QV 745, WJ 762
Yleinen suomalainen asiasanasto: prostatapesifinen antigeeni; kallikreiinit; eturauhassyöpä; peptidit; sykliset peptidit; kemiallinen synteesi; peptidisynteesi; aminohapposubstituutio; proteolyysi; proteiinin stabiilius; lääkesuunnittelu; rakenne-toimintasuhde

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Turku, autumn 2012

Miikka Pakkala

List of the original publications

This dissertation is based on the following original publications:

I   Pakkala, M., Jylhäsalmi, A., Wu, P., Leinonen, J., Stenman, U.-H., Santa, H., Vepsäläinen, J., Peräkylä, M. & Närvänen, A., 2004, "Conformational and biochemical analysis of the cyclic peptides which modulate serine protease activity", *Journal of Peptide Science,* vol. 10, no. 7, pp. 439-447.

II   Pakkala, M*., Hekim, C*., Soininen, P., Leinonen, J., Koistinen, H., Weisell, J., Stenman, U.-H., Vepsäläinen, J. & Närvänen A. 2007, "Activity And Stability of Human Kallikrein-2 Specific Linear And Cyclic Peptide Inhibitors", *Journal of Peptide Science,* vol. 13, no. 5, pp. 348-353.

III Pakkala. M., Weisell, J., Hekim, C., Vepsäläinen, J., Wallen, E., Stenman, U.-H., Koistinen, H. & Närvänen, A. 2010, "Mimetics of the disulfide bridge in the KLK3 stimulating peptide B-2", *Amino Acids,* vol .39, no. 1, pp. 233-242.

IV   Meinander K, Pakkala M, Weisell J, Vepsäläinen J, Hekim C, Stenman U-H, Koistinen H, Närvänen A, Wallen E, Synthesis and Evaluation of Analogs of the KLK3-activating Peptide B-2, *Manuscript*

*equal contribution

In addition, this thesis contains previously unpublished data from a multiple amino acid replacement study of the C-4 peptide (a follow up study to article I)

Contents

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Abbreviations

3D	three dimensional	Dmab	4-(*N*-[1-{4,4-dimethyl-2,6-
A2M	*α*2-macroglobulin		dioxocyclohexylidene}-3-
Acm	acetamidomethyl		methylbutyl]amino)benzyl
ACN	acetonitrile	DMF	*N,N*-dimethyl formamide
ACP	acyl carrier protein	DMSO	dimethyl sulfoxide
Aha	7-aminoheptanoic acid	DPDPE	c[D-Pen2, D-Pen5]-enkephalin
Ahx	6-aminohexanoic acid	EDT	ethanedithiol
Aib	aminoisobutyric acid	ESI	electron spray ionization
All	allyl	Fmoc	9-flourenylmethoxycarbonyl
Alloc	allyloxycarbonyl	GABA	γ-amino butyric acid
Aox	8-aminooctanoic acid	GLP-1	glucagon like peptide-1
BAL	backbone amide linker	GLP-2	glucagon like peptide-2
BBB	blood-brain barrier	GnRH	gonadotropin releasing
Bn	benzyl		hormone
Boc	tert-butyloxycarbonyl	HBTU	2-(1H-Benzotriazole-1-yl)-
BPH	benign prostatic hyperplasia		1,1,3,3-tetramethyluronium
Bt	benzotriazole		hexafluorophosphate
Bu^t	tert-butyl	HIV	human immunodeficiency
CNS	central nervous system		virus
COSY	correlation spectroscopy	Hmb	2-hydroxy-4-methoxybenzyl
DCC	dicyclohexylcarbodiimide	HOAt	hydroxy-7-azabenzotriazole
DCM	dichloromethane	HOBt	1-hydroxybenzotriazole
DCU	dicyclohexylurea	HPLC	high performance liquid
DIC	*N,N′*-diisopropylcarbodiimide		chromatography
DIEA	diisopropylethylamine	HUVEC	human umbilical-vein
DKP	diketopiperazine		endothelial cells
		IAP	inhibitors of apoptosis protein
			family

IGF	insulin-like growth factor		PCa	prostate cancer
IGFBP-3	insulin-like growth factor binding protein-3		PCI	protein C inhibitor
			PEG	polyethylene glygol
IR	infra red spectroscopy		Pen	penicillamine, thiol-D-valine
KLK2	kallikrein-related peptidase 2		PKB/Act	activated protein kinase B
KLK3	kallikrein-related peptidase 3		pNA	p-nitroaniline
LPPS	liquid-phase peptide synthesis		PS	polystyrene
mAb	monoclonal antibodies		PTHrP	parathyroid hormone-related protein
MALDI	matrix-assisted laser desorption ionization		Pyr	pyroglutamic acid
MBHA	methylbenzhydrylamine		RGD	Arg-Gly-Asp tripeptide
MMP	matrix metalloproteases		RMC	ring closing metathesis
MS	mass spectrometry		ROESY	rotating-frame Overhauser effect spectroscopy
αMSH	α-melanocyte stimulating hormone		RT	room temperature
Mtt	4-methyltrityl		SAR	structure-activity relationship
MW	microwave assisted heating		SPPS	solid-phase peptide synthesis method
Mw	molecular weight			
NCL	native chemical ligation		SST14/28	somatostatin 14/28
NET	neuroendocrine tumor		Suc	succinic acid
NMM	N-methylmorpholine		TFA	trifluoroacetic acid
NMP	1-methyl-2-pyrrolidinone		TFMSA	trifluoromethanesulphonic acid
NMR	nuclear magnetic resonance		TGF-β2	transforming growth factor-β2
NOE	nuclear Overhauser effect		TIS	triisopropylsilane
NOESY	NOE spectroscopy		TMP	trimethylpyridine (collidine)
NT	neurotensin		TOCSY	total correlation spectroscopy
PAI-1	plasminogen activator inhibitor 1		TOF	time-of-flight analysator
			Trt	trityl
Pbf	pentamethyl-2,3-dihydrobenzofuran-6-sulfonyl		Z, Cbz	benzyloxycarbonyl
PBS	phosphate buffered saline			

1 General Introduction

1.1 PEPTIDES IN DRUG DISCOVERY

The word "peptide" was first introduced by the German chemist Emil Fischer in the beginning of the 20th century (Suresh Babu 2011, Kimmerlin & Seebach 2005). The name is based on the term pepsis, meaning the digestion product of proteins. Peptides, like proteins, consist of amino acids termed as residues when they are incorporated in a peptide. The Amino acids are connected one to the next via amide bonds between the C-terminal α-carboxylic acid group of one amino acid and the N-terminal α-amino group of the following residue and therefore amide bonds are commonly called peptide bonds (*Figure 1*). Peptides are commonly depicted in a left to right direction, from the *N*-terminus to the *C*-terminus (*Figure 1*). Naturally occurring peptides and proteins consist of 20 genetically encoded amino acids with the L-configuration. The D-configuration is rare, however, it has been found for example in the bacterial cell walls and in some antimicrobial peptides (Suresh Babu 2011). Peptides are shorter than proteins, they can range from two (dipeptide) up to 50-100 (polypeptide) residues depending on the source material (Lien & Lowman 2003, Watt 2006). In fact the limit where a peptide turns into a protein is not defined. For example, the sweetener aspartame is a dipeptide (H-Asp-Phe-OMet, *Figure 1*) with molecular weight (Mw) below 300 Da and the hormone insulin containing 51 residues with Mw almost 5800 Da is also classified as a peptide.
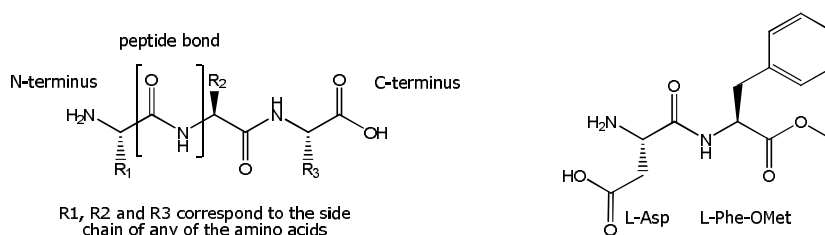
Figure 1. Structures of peptide backbone (left) and dipeptidic sweetener aspartame (right).

Peptides exert a wide range of biological actions since numerous hormones, antigens, neurotransmitters, growth factors and cytokines are peptides. Therefore, peptides have gained growing interest in pharmaceutical research. Many natural peptides such as certain hormones, insulin for treatment of diabetes, calcitonin for osteoporosis and oxytocin for labor (*Table 1*) have been used directly as drugs for many years. However, peptides have been considered as poor drug candidates mainly because they are usually rapidly metabolized and their oral bioavailability is poor, even non-exixtent. The developments in the field of synthetic chemistry, new strategies for reducing metabolism and improving productivity and advances in alternative delivery routes for example pulmonary, nasal and oral administration have led to a rapid increase in the number of peptide-based pharmaceuticals on the market (Vlieghe et al. 2010, Kimmerlin & Seebach 2005). Further, peptides are widely used as lead molecules in radiopharmaceuticals and as imaging agents (Okarvi 2004).

1.1.1 Synthetic therapeutic peptides on the market

The market for synthetic therapeutic peptides was €8 billion in 2005 and it has been estimated that it will rise to €11.5 billion in 2013 (Pichereau & Allary 2005, Vlieghe et al. 2010). Among the different therapeutic peptides in use in 2003, the largest class was the gonadotropin/luteinizing hormone releasing hormone (GnRH/LHRH) agonists (*Table 1*) used in the treatment of prostate and breast cancers, having 45% market share. Other large selling classes of peptide related drug were somatostatin and somatostatin analogs like ocreotide for the treatment of acute variceal bleeding and acromegaly (15%), immunopeptides such as glatiramer used in the therapy of relapsing multiple sclerosis (12%) and calcitonins for the treatment of osteoporosis (10%)(*Table 1*) (Pichereau & Allary 2005). The list of Pichereau and Allary excluded insulin and insulin-based products apparently because insulin is produced by several methods, not exclusively via synthesis. It has been calculated that the market of insulin products alone was around US $4.5 billion in 2002 and the need for insulin products was estimated to 4,600 kg in year 2000 in the industrial world alone (Walsh 2005). The occurrence of diabetes mellitus and the growth in the numbers of subjects with disorders requiring insulin, have made this peptide one of the most significant biopharmaceutical product categories, both in terms of medical impact and market value. Furthermore, insulin-based therapy of diabetes has been an exemplary research object not only for production of peptide-based pharmaceuticals but also for development of non-parenteral delivery systems, as well as organ-/cell-based and gene therapy-based approaches to controlling the disease (Walsh 2005).

Peptides and peptide-based pharmaceuticals have been used also as therapeutic agents for example for the treatment of oncologic (leuprolerin), gastroenterologic (GLP-2, glucagon like peptide-2, teduglutide), cardiovascular (bivalirudin, eptifibatide), immunotherapy (cyclosporine), enuresis (vasopressin analogs like desmopressin), antiviral (enfuvirtide for human immunodeficiency virus, HIV) and antimicrobial (defensin) indications (Stevenson 2009, Vlieghe et al. 2010). Examples of synthetic therapeutic peptides and peptide derivatives which have reached the market in the developed countries are listed in the *Table 1* with the sources, their indications, sequences and routes for dosing.

In the past, many of the peptide therapeutics were derived by extraction from organs, for instance insulin from porcine and bovine pancreatic glands (Johnson 1983). Nowadays therapeutic peptides are most commonly manufactured via a synthetic route due to the rapid development of synthetic chemistry and most of the compounds listed in *Table 1* are also synthetic. Some unmodified peptides with long sequences such as insulin, glucagons and calcitonins can still be manufactured using viral particles or plasmids via recombinant DNA technology (Verlander 2007, Walsh 2005). Nonetheless, the large scale synthesis of relatively short and highly structured peptides still remains a challenge. One practical example is cyclosporine which is highly hydrophobic and contains many N-methylated residues (*Table 1*). The first complete synthesis of cyclosporine was published by Wenger in 1984 (Wenger 1984) and since then numerous reports using differing synthetic method have been published. However, cyclosporine is still usually manufactured via microbial fermentation (Verlander 2007).

Table 1. Common synthetic therapeutic peptides in the market derived from different sources.

Peptide	Source	Indication(s)	Number of residues	Sequence (N→C)	Routes for dosing	Literature
insulin	hormone, pancreas	diabetes, controlling the blood glucose level	51	two polypeptide chain, A and B linked to each other via two disulphide bridge	mainly subcutaneous, nasal	Antosova et al. 2009, Nicol & Smith 1960
teduglutide	intestinal hormone GLP-2 (glucagon like peptide 2) analog, from proglucagon	enhancing intestinal growth and metabolism, Crohn's disease?	33	H-**His**-**Gly**-Asp-Gly-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Ala-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp-OH	subcutaneous injection	Stevenson 2009
liraglutide	intestinal hormone GLP-1 (glucagon like peptide 1) analog, from proglucagon	type-2 diabetes	30	H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-**Lys***-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-**NH₂**, ***Glu-C16 fatty acid**	subcutaneous injection	Stevenson 2009
somato-statin, SST14 (SST28)	hormone, pancreas, hypothalamus, CNS, gastrointestinal track	acute variceal bleeding, target of various studies because of wide variety of physiological function	14	H-Ala-Gly-cyclo[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys]-OH	intravenous infusion	Stevenson 2009, Reissmann & Imhof 2004, Patel 1999
ocreotide	synthetic analog of somatostatin	acromegaly	8	D-Phe-cyclo[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thol	subcutaneous intravenous intramuscular	Stevenson 2009
desmo-pressin	synthetic replacement for vasopressin, the hormone that reduces urine production	Central diabetes insipidus, enuresis, nocturia, and stoppage of bleeding or haemorrhage in haemophilia A Patients	9	cyclo-[**Mpa-Tyr**-Phe-Gln-Asn-Cys]-Pro-D-Arg-Gly-**NH₂**	intravenous injection, nasal spray, tablet	Stevenson 2009, Antosova et al. 2009
oxytocin	hormone, uterus, hypothalamus	labor	9	H-cyclo[Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly-**NH₂**	intravenous, nasal	Stevenson 2009, Lee et al. 2009
atosiban	synthetic, oxytocin antagonist	retard premature labor	8	cyclo-[**Mpa-D-Tyr(Et)**-Ile-Thr-Asn-Cys]-Pro-Orn-Gly-**NH₂**	intravenous	Stevenson 2009, Lee et al. 2009

Peptide	Source	Indication(s)	Residues	Sequence (N→C)	Routes for dosing	Literature
leuprorelin	GnRH (gonadotropin releasing hormone), synthetic analog, agonist	treatment of advanced prostate and breast cancers, precocious puberty, endometriosis etc.	9	**Pyr**-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-**NHEt**	depot injections and sustained release implants	Wilson et al. 2007
enfuvirtide	HIV precursor protein gp160	anti-HIV	36	**Ac**-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-**NH₂**	subcutaneous injection	Watt 2006
glatiramer	myelin basic protein	reduction of frequency of relapses in multiple sclerosis	n	random mixture consisting of H-(Ala:Lys:Glu:Tyr)-OH, 4.2:3.4:1.4:1.0	subcutaneous	Stevenson 2009
calcitonin (salmon)	ultimobranchial glands (thyroid-like glands) of fish	postmenopausal osteoporosis, hypercalcemia	32	H-cyclo[Cys-Ser-Asn-Leu-Ser-Thr-Cys]-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-**NH₂**	subcutaneous intramuscular intranasal	Antosova et al. 2009
cyclosporine	fungus	immunosuppression for propholactic treatment of organ transplants, rheumatoid arthitis	11	Cyclic **MeBmt-Abu**-Sar-MeLeu-Val-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal	injection in alcohol, oral capsule	Stevenson 2009
bivalirudin	from the saliva of the medicinal leech, synthetic	thrombin-specific anticoagulant	20	D-Phe-Pro-Arg-Pro-Gly-Gly-Gly-Gly-Asp-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu	intravenous infusion	Robson et al. 2002, Stevenson 2009
eptifibatide	synthetic derivative from protein of rattlesnake venom	inhibits platelet aggregation in blood	7	cyclo-[Mpa-homoArg-Gly-Asp-Trp-Pro-Cys]-**NH₂**	intravenous	Stevenson 2009

Abbreviation used: Ac, acetate; Thol, Threoninol; Pyr, pyroglutamic acid; MeBmt, N,4-dimethyl-4(R)-[2(E)-butenyl]-L-threonine; Abu, α-aminobutanoic acid, Sar, sarcosine (N-methylglysine); MeLeu, N-methylleucine; MeVal, N-methylvaline; Mpa, 3-mercaptopropanoic acid; homoArg, homoarginine

1.1.2 Disadvantages and advantages of using peptides as therapeutic agents

Since bioactive peptides are often derived from natural sources, they are commonly well-tolerated. However, living organisms also have mechanisms to rapidly degrade and excrete peptides out of circulation and these are considered as their main disadvantages when peptides are used as therapeutic agents. These problems and several other disadvantages and advantages are discussed in the subsequent sections and they are also listed in *Table 2*.

Bioavailability and biodistribution of a drug molecule depend on its absorption, transport and distribution through biological membranes and cellular barriers and then how it is eliminated. The most important properties affecting bioavailability are aqueous solubility, lipophilicity, H-bond formation and both chemical and metabolic stability (Witt et al. 2001, Vlieghe et al. 2010). Peptides are relatively hydrophilic, hence they cannot pass passively through physiological barriers such as hydrophobic membranes. This also means that their access is extremely limited to the central nervous system (CNS) through the blood brain barrier (BBB) (Witt et al. 2001, Vlieghe et al. 2010, Lindgren et al. 2000). Since they are bulky molecules with molecular weights usually over 1000 Da, they need active transportation if they are to penetrate through the physiological barriers. There is a certain class of peptides, called cell-penetrating peptides (CPPs) which can pass through biological membranes and they can also act as transporters for macromolecules (Lindgren et al. 2000). In addition, the membrane penetrating ability of calcitonins (*Table 1*) and the possibility to use them as carriers of macromolecules has been studied (Rennert et al. 2008). Recently, the use of various biodegradable nanoparticles in cytosolic delivery of therapeutics has attracted attention and these materials may also be used as carriers to help the peptide pass through cell membranes (Vasir & Labhasetwar 2007).

The main disadvantage of peptides is their sensitivity toward metabolic degradation by the proteolytic enzymes in the blood and gastrointestinal tract. Short peptides are rapidly cleared from the circulation by the liver and kidneys, usually within minutes. Proteases, which are responsible for the degradation of peptides, can be divided into two categories: endopeptidases which cleave peptide bonds in the middle of their peptide sequence and exopeptidases which cleave from either *N*- or *C*-terminal side and are thus called aminopeptidases or carboxypeptidases, respectively. These peptidases/proteases are mainly located in blood, pancreas, liver and kidneys (Turk 2006, Werle & Bernkop-Schnurch 2006). Endopeptidases often exhibit narrow cleavage specificity requiring the presence of one certain amino acid on the *N*-terminal side of cleavage point which is recognized by the peptidase. The most important endopeptidases are serine, cysteine and aspartic acid proteases and metalloproteases which have a wide variety of cleavage sites because they can recognize the next residue after the cleavage site. The most significant exopeptidases include dipeptidyl- of tripeptidylpeptidases cleaving *N*-terminal di- and tripeptides respectively and peptidyl-dipeptidases cleaving *C*-terminal residues. In addition, there are various amino- and carboxypeptidases cleaving specific amino acids from the *N*- and *C*-terminal end of the peptide (Vlieghe et al. 2010, Werle & Bernkop-Schnurch 2006).

It has been estimated that the oral bioavailability of peptides is a mere 1-2% due to their enzymatic degradation and low mucosal permeability (Pauletti et al. 1996). Due to the low oral bioavailability of peptide pharmaceuticals, they need to be delivered via parenteral administration e.g. intravenous infusion which can result in low patient compliance (*Table 1*). Direct injection can overcome some of the problems due to the low bioavailability and poor stability in the bloodstream. Due to their short half-life in the bloodstream, repeated

dosing is usually required causing a fluctuating concentration of the drug in the blood, painful injection or infusion and expensive therapy (Antosova et al. 2009). The production costs of the peptide pharmaceuticals are high, especially longer peptides or modified analogs. Finally, linear short peptides are highly flexible and this can result in a lack of selectivity due to unspecific binding to other receptors and thus evoking side effects and also the risk of generating an immunogenic response (Sato et al. 2006, Vlieghe et al. 2010).

The advantages of peptides as therapeutics must be compared to both small molecules and large proteins such as antibodies. Monoclonal antibodies (mAb) have been used as tumor targeting agents and in the therapy of various cancers but the large size of mAbs (160 kDa), the nonspecific uptake of antibody molecules into the reticuloendothelial system such as the liver, spleen and bone marrow have limited their use as carriers of cytotoxic drugs, toxins and radionuclides to the tumor site (Aina et al. 2007). In comparison to antibodies, the smaller size of peptides increases their potential to penetrate into the target tissues which are most commonly tumors (Reubi 2003, Sato et al. 2006, Ladner et al. 2004). Therapeutic peptides are also usually less immunogenic than antibodies mostly due to their smaller size, rapid degradation and elimination. Furthermore, the production costs are lower, storage at room temperature is possible and chemical diversity is higher when compared to antibodies and proteins (Sato et al. 2006, Vlieghe et al. 2010).

Since they often contain the reduced functional part of a protein, usually composed of 10-50 amino acids, peptides usually have higher efficacy and selectivity compared to small molecular weight drucgs (Sato et al. 2006, Ladner et al. 2004). The high specificity based on a broad interaction site is an advantage when there is a need for a specific, efficacious and extended peptide-target interaction in order to block or interfere with the biological protein-protein interaction. Thus, they can act as an antagonist, for example this is usually their mode of action in cancer therapy. Many of the peptide therapeutics are agonists or antagonists and despite the high production costs, only small amounts of peptide therapeutic are usually needed to achieve a therapeutic response if the specificity is sufficiently high (Ladner et al. 2004).

There are several well-studied technigues to routinely produce peptides of different sizes and sequences including chemical synthesis, recombinant DNA technology, transgenic plants and animals and enzymatic synthesis. In particular, synthetic production methods for peptides give the possibility to add an enormous variety of different building block into the peptides and peptide derivatives (Bray 2003, Vlieghe et al. 2010). Another advantage of synthetic peptide chemistry is that the purity of the product is usually high when the drugs are manufactured under good manufacturing practice (GMP). Synthetic chemistry makes it possible to restrict the enzymatic degradation of labile bonds, to mask recognizable side chains from proteases and to add variable bridge structures to enhance the structural rigidity of the peptides. It is also relatively easy to link chelators or radiolabeled ligands into the peptides via synthetic methods (Reubi 2003). The methods and achievements of peptide and peptide derivative synthesis will be reviewed in section 1.2.

Finally, the degradation products of peptide therapeutics are usually amino acids minimizing the risk of toxic metabolites compared to small molecule drugs. The accumulation of peptides or their metabolites to the tissues is low due to the short half-life in living organisms (Sato et al. 2006, Vlieghe et al. 2010).

Recent developments in methods for discovering peptide lead structures, the better understanding of biological and chemical nature of peptides and the improved synthetic

methods have expanded the use of peptides as pharmaceutical agents. Although intravenous administration is the main administration route for peptide pharmaceuticals, alternative delivery routes have been extensively investigated particularly for the widely used peptide hormones. Innovative examples of alternative administration routes include pulmonary (e.g. insulin), nasal (e.g. calcitonin, desmopressin), oral (e.g. insulin) and buccal (e.g. insulin) delivery (Antosova et al. 2009).

Table 2. The main advantages and disadvantages of peptides in therapeutics.

Advantages	Disadvantages
High activity	Low oral bioavailability, intravenous administration required
High affinity and high specificity for receptors	Usually low stability
Low unspecific binding to molecular structures other than desired target	Difficult delivery, challenge in transporting across membranes
Low accumulation in tissues and rapid clearance from blood and non-target tissues	Costly synthesis
Usually low toxicity	Low water solubility
Often very potent	The risk of immunogenic effects
Biological and chemical diversity	Rapid clearance from the body
Usually low immunogenicity	Structural flexibility which can cause non-specific binding
Relatively small size compared to antibodies	

1.1.3 Lead peptide sources

Therapeutic peptides have been derived mainly from three sources: nature, genetic or recombinant libraries and chemical libraries. The main natural sources, from which naturally occurring peptides, peptide hormones or fragments of larger proteins have been isolated and characterized, include plants, animals or humans (Sato et al. 2006, Vlieghe et al. 2010, Lewis & Garcia 2003). Since peptides from natural sources have undergone natural selection and therefore they usually have enhanced *in vivo* stability, most of the peptide therapeutics used today are derived from natural sources (Sato et al. 2006, Watt 2006). Salmon calcitonin, somatostatin and corticotropin-releasing factor are good examples of highly structured peptides from natural sources which also are synthesized using common peptide synthesis methods. The first commercial peptide drug based on the fragment of a protein and to be manufactured via a synthetic route was the anti-HIV drug enfuvirtide (*Table 1,* see page 3) (Watt 2006). Several peptides from natural sources act as potent agonists and antagonists targeting receptors involved in the progression of many diseases. Thus, the development of more sophisticated and powerful methods for the isolation, identification and characterization of natural peptides has led to the production of a huge number of potential new lead structures for peptide therapeutics.

Recently, developments in the field of recombinant display technologies such as phage display, and chemical library methods, together with the advanced synthetic combinatorial chemistry, and computational methods have dramatically increased the number of potential lead peptides with therapeutic applications (Marasco et al. 2008, Falciani et al. 2005). While the use of genetic and recombinant libraries is limited to only the naturally

occurring L-amino acids, both L- and D-amino acids and an enormous number of other synthetic building blocks can be incorporated via synthetic combinatorial chemistry. Furthermore, one can only create complicated bi- or multicyclic structures or branched structures by using combinatorial synthetic chemistry.

Chemical combinatorial peptide libraries are also able to yield from thousands to millions of sequences at one time. The peptides can be screened against biological targets either as cleaved free compounds or they can be assayed when still attached to their solid support (Marasco et al. 2008, Lam et al. 1991, Houghten et al. 1991). Synthetic combinatorial peptide libraries have been used successfully to identify new drugs, for example antimicrobial peptides (Blondelle et al. 1996) and an opioid receptor antagonist (Dooley et al. 1998).

However, there are some considerable drawbacks in the use of random peptide libraries. Firstly, it is possible that randomly encoded peptide libraries do not yield any sequence that will bind to target because random sequences may not fold to the structure in a stable enough way to permit a strong interaction between the target and the ligand. This may lead to poor hit rates despite the fact that a valid sequence may have been created (Watt 2006). However, although a peptide does not fold spontaneously in solution, it may be capable of adopting an induced fold within the target. Secondly, if unspecific binding does occur and there are several hits, the characterization of the sequences may be difficult. It has been noted that similar biological effects can be achieved with divergent sequences, because biological molecules usually recognize shape and charge rather than the exact primary structure (Yang & Honig 2000).

Complex peptide libraries are relatively complicated to synthesize and the individual peptide products are difficult to identify. Genetically encoded combinatorial libraries, phages, plasmids or ribosomes, offer the potential to overcome these problems. Genetically encoded libraries produce peptides with the desired binding activities. In order to optimize the specificity and affinity of the peptide, the clones can be readily amplified and re-screened (Landon et al. 2004). The phage display method was first introduced by Smith in 1985. It is based on an *in vitro* selection procedure in which the peptide is displayed on the surface of the viral particle after the peptide gene is genetically fused onto a bacteriophage coat protein (Smith 1985). In addition to linear peptides, predetermined cysteine positions in the sequence can allow for the expression of cyclic peptides. Phage display is a powerful technology to generate peptides from random peptide libraries and since its discovery, it has been used in a variety of biological and biotechnological applications (Landon et al. 2004, Laakkonen & Vuorinen 2010, Smith & Petrenko 1997). These include the search and development of enzyme inhibitors (Hyde-DeRuyscher et al. 2000), angiogenesis inhibitors (Hetian et al. 2002) protease stimulators (Wu et al. 2000) or inhibitors (Hekim et al. 2006). Phage display can also be used for specific substrate determination (Cloutier et al. 2002) or for the selection of target homing peptides such as peptides targeting cancer cells (Shadidi & Sioud 2003) and peptides which bind specifically to lymphatic vessels (Laakkonen et al. 2002).

Computer-aided drug design is a powerful tool for small molecules but in peptide research it encounters some limitations. The structure of a peptide is usually flexible and there may exist a large population of different conformations. Several factors such as solvent, pH and the surrounding temperature have major influences on the conformation. Construction of a computational model of a single peptide without any information of its

binding site on the target is pointless. For example, in enzyme research, the peptide lead structures are usually derived from substrates but some peptides also bind in close vicinity to the active site, and thus can regulate the activity of the target protein. When the lead structure has been identified, computational methods can be used to assist in the design of modifications. For example, novel protein kinase B inhibitors with enhanced pharmaceutical properties were designed by using structure-activity relationship (SAR) studies with synthetic libraries of cyclic peptides and superimposing the most active nuclear magnetic resonance (NMR) based structures computationally onto the target protein, (Tal-Gan et al. 2011a).

The computational screening methods for searching for protein binding structures usually require a knowledge of the three dimensional (3D) structure of the target molecule. The 3D structure is usually studied using NMR, using mass spectrometry (MS) for the search of sequential data and modification sites, or by solving the crystal structures of a protein using X-ray crystallography (DeLucas et al. 2005). In the case of NMR, the size of the protein is the main limitation. X-ray crystallography is the method routinely used to resolve structures of macromolecules with molecular weights over 20 kDa. Some proteins do not form crystals, for example if the surface properties of a protein do not favor the formation of crystal contact, then structural information may have to be obtained from homologous proteins from other species (Dale et al. 2003; DeLucas et al. 2003, DeLucas et al. 2005). Human kallikrein-related peptidase 3 (KLK3), is a widely studied serine protease which crystal structure has not been resolved. The crystal structures of KLK3 in various complexes have been defined (Menez et al. 2008, Stura et al. 2011). A homologue model of KLK3, based on the crystal structures of porcine kallikrein and human tissue kallikrein (HK1), was used in substrate specificity studies of KLK3 and for inhibitor design via docking experiments (Singh et al. 2009). Recently, a comparative model of KLK3 was constructed by using KLK1 as a reference structure (Härkonen et al. 2011). Using this model together with peptides, which are able to stimulate the enzymatic activity of KLK3, the small molecule stimulators of KLK3 activity were discovered (Härkonen et al. 2011).

1.1.4 Peptides in cancer research

Today cancer therapy is one of the leading areas of peptide research because there is growing evidence that several peptide receptors, such as growth factor receptors, are involved in various cancers (Landon et al. 2004). Many of these receptors are overexpressed in the cancer or become expressed in other tissues in a response to cancer growth. Thus, peptides with specific binding may be used for example as receptor antagonist for long-term use in therapy. They can also function as peptide derivatives for example with an adduct containing cytotoxic or imaging agent (Okarvi 2004, Zaccaro et al. 2009, Reubi 2003). In the following sections, some commonly known bioactive peptides will be discussed.

Somatostatins are 14 (SST-14, *Figure 2*) or 28 (SST-28) residues containing cyclopeptide hormones possessing a variety of biological effects in different cells and organs. Both somatostatins have an inhibitory effect not only on the secretion of growth hormone, but also on thyroid stimulating hormone, prolactin, insulin, glucagon and cholecystokinin (Patel 1999). Somatostatins can also act as neurotransmitters and may function as modulators of motor and cognitive functions (Reissmann & Imhof 2004). Somatostatins have been also shown to inhibit both normal and tumor cell proliferation (Weckbecker et al. 2003). Therefore somatostatins are good candidates for anticancer agents and for the

treatment of neuroendocrine disorders but their specificity for certain receptors is low and their half lives in organs are very short (<3 min) (Pyronnet et al. 2008). Somatostatin has been the subject of a vast number of studies involving synthetic derivatives and in 1982 Bauer *et al*. published synthesis of ocreotide (Bauer et al. 1982, Harris 1994, Weckbecker et al. 2003). Ocreotide is cyclic octapeptide containing disulphide bridge and D-amino acids (*Figure 2*). This compound showed increased resistance against enzymatic degradation while maintaining the biological activity of the original somatostatin. In fact, its half-life of degradation was long enough to permit long-term therapy (Bauer et al. 1982, Reubi 2003). Ocreotide became the first synthetic peptide analog for the treatment of agromegaly and neuroendocrine tumors (NETs) (*table 1*, see page 3) (Zaccaro et al. 2009). Somatostatin and its analogs, especially ocreotide, have been used successfully in the diagnosis of NETs and for the radionuclide therapy of these tumors for years (Zaccaro et al. 2009, Reubi 2003).
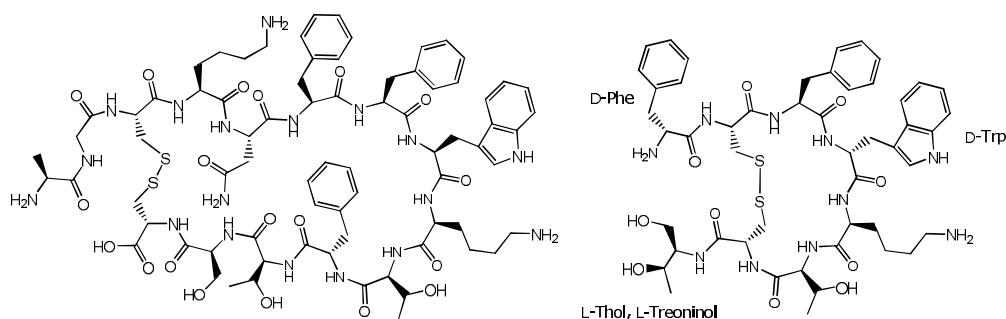
Figure 2. Somatostatin 14 (SST-14, left) and its synthetic analog ocreotide (right) with the non-naturally encoded residues indicated.

Integrin receptor binding ligands contain the tripeptide sequence RGD (Arg-Gly-Asp) and this tripeptide has been used as a lead structure in the development of various diagnostic and imaging agents. Integrins are heterodimeric transmembrane glycoproteins consisting of two subunits, α and β, and to date 18 α- and 8 β-subunits have been identified (Zaccaro et al. 2009). A group of integrins are closely connected to angiogenesis since they can promote the development of new blood vessels and they also contribute to the survival of the newly formed vasculature (Ruoslahti & Pierschbacher 1987, Ivaska & Heino 2000). The RGD-sequence is present in several extracellular matrix proteins such as fibronectin, fibrinogen, lamin, collagen and thrombin (Ruoslahti & Pierschbacher 1987). One of the integrins, $\alpha_v\beta_3$, has been found to be overexpressed in several tumor forms, including melanomas, breast cancer, ovarian and lung carcinomas, neoblastomas and glioblastomas and therefore integrin $\alpha_v\beta_3$ is interesting target for the early diagnosis and detection of metastases (Zaccaro et al. 2009). Based on the RGD-sequence, a series of studies with linear and cyclic analogs with the RGD sequence have been described including also radiolabeled derivatives (van Hagen et al. 2000, Haubner et al. 1996, Zaccaro et al. 2009, Okarvi 2004).

Neurotensin (NT, *Figure 3*) is a linear 13 amino acid long peptide which functions as both a neurotransmitter and a hormone (Vincent 1995). NT exerts its biological effects via a specific interaction with cell-surface receptors and these NT-receptors are known to be overexpressed in various human tumors including Ewing sarcoma, meningioma, astrocytoma and, medulloblastoma. This NT-receptor overexpression in these tumors has been targeted with NT analogs (Okarvi 2004). Residues 8-13 of NT are the important amino

acids determining the binding and biological activity, and the NT 8-13 residues (*Figure 3*) have been examined in various studies as a lead structure. In an attempt to enhance the stability of NT against degradation in blood several methods including the introduction of a pseudopeptide bond into the natural cleavage site between residues 8 and 9 (NT-IV) and amino acid replacements (NT-VIII) have been investigated (Okarvi 2004, Garcia-Garayoa et al. 2002). The NT analogs displayed increased stability *in vivo* and they were further used in imaging studies (Garcia-Garayoa et al. 2002, de Visser et al. 2003).

Neurotensin, NT

p-Glu——Leu—Tyr—Glu—Asn—Lys—Pro—Arg—Arg—Pro—Tyr—Ile—Leu—OH

NT 8-13

H—Arg—Arg—Pro—Tyr—Ile—Leu—OH

NT-IV

H—His—Lys—(ψCH₂-NH)—Arg—Pro—Tyr—Ile—Leu—OH

NT-VIII, (NαHis)Ac-(N-CH₃)-Arg-Lys-Pro-Tyr-Tle-Leu-OH
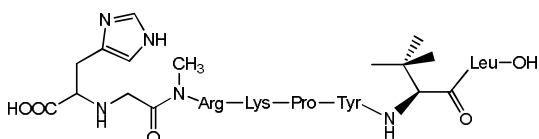
Figure 3. Neurotensin (NT), NT 8-13, NT-IV and NT-VII.

1.1.5 Proteases as therapeutic target

Proteolytic enzymes, usually known as proteases, are enzymes that catalyze the breakdown of proteins by hydrolyzing their peptide bonds. Proteases are extremely important molecules in the signaling pathways and they regulate many physiological processes like cell proliferation, cell death, DNA replication, wound healing, immune response and blood coagulation. Proteases are overexpressed in several diseases and tissue abnormalities and increased proteolytic activities are associated with tumor invasion and metastasis (Turk 2006, Koblinski et al. 2000, Overall & Kleifeld 2006). These enzymes have a broad functional diversity and also tumor suppressive effects have been proposed (Lopez-Otin & Matrisian 2007). At present, several proteases are widely used as biomarkers for many cancers such as human breast, lung, head and neck carcinomas, melanoma, colorectal carcinoma and prostate cancer (Kos & Lah 1998, Stamey et al. 1987). Cysteine proteases, serine proteases and matrix metalloproteases (MMP) have also been evaluated as targets for tumor imaging (Sloane et al. 2006, Fonovic & Bogyo 2007). Due to the potential of proteases in the diagnosis of diseases and in therapeutics, it would be important to find ligands which bind selectively and with high specificity to their target protease. Phage display and different combinatorial methods have been used to define substrate specificities of proteases (Thornberry et al. 1997, Harris et al. 2000, Matthews & Wells 1993). Specific substrates can be considered as potential lead structures for therapeutic and imagining agents.

The inhibition of proteases has been the main goal of protease targeting studies because protease inhibition, blocks the activity of downstream proteases (Turk 2006). Antibodies are well-known specific protease inhibitors, but their large size limits their specificity and uptake and therefore their use as therapeutic agents. Small molecule inhibitors often lack the requisite specificity. Thus peptides and peptide derivatives may serve as prototypes for protease activity modulators. Some natural peptidic inhibitors such as tick anticoagulant peptide (TAP), selective inhibitor of serine protease Factor Xa, and antistasin, an inhibitor of coagulation but also metastasis, have been investigated in inhibitor design tests (Dunwiddie et al. 1992, Tuszynski et al. 1987).

The stimulation of protease activity has not been a widely studied area but some activating molecules already exist. Stimulation of protease activity can be achieved using agents which block the inhibitory activities of protease inhibitors such as inhibitors of apoptosis (IAP) protein family in cancer cells (Schimmer et al. 2004). In the study of Schimmer and co-workers, small molecular-weight inhibitors for IAPs were developed and the stimulation of apoptosis was observed in several types of tumor cell lines in culture. It is not common to find agents that directly stimulate the protease activity but for example peptide-based stimulators of KLK3 protease activity have been described (Wu et al. 2000).

1.2 PEPTIDE SYNTHESIS

Peptides can be produced by various technologies, mainly depending on the source of peptide and the molecular size of the product. The naturally existing peptides can be extracted directly from the living organism. However, the most popular approaches in peptide production for pharmaceutical purposes are chemical synthesis, recombinant DNA-technology (rDNA) or enzymatic synthesis (Guzman et al. 2007, Gill et al. 1996). Chemical peptide synthesis is a flexible technology for the synthesis of small and medium size peptides, particularly due to the high purity of products, and the possibility to incorporate a wide variety of non-natural building blocks. Other technigues, i.e. rDNA and enzymatic synthesis, offer some advantages over chemical synthesis e.g. use of inexpensive and non-hazardous chemicals and mild reaction conditions. In addition, there will be no racemization present in the products. However, rDNA-methods are used mainly in the production of large peptides and proteins such as insulin and other hormones and also antibodies. Enzymatic synthesis is used for the production of very short (up to 10 amino acids) peptides including certain food additives like aspartame (*Figure 1*, see page 1) (Guzman et al. 2007, Gill et al. 1996, Kumar & Bhalla 2005, Walsh 2005). Today, the different methodologies to produce peptides and peptide derivatives are not only competitive but they can also be used in combination. For example the combination of chemical and enzymatic synthesis has been used in the large scale and low cost production of the RGD-tripeptide. Chemical synthesis of RGD (Arg-Gly-Asp) is challenging because of the hydrophilic residues and thus the low solubility in organic solvents and this lead to increases in the total costs of synthesis and purification. This problem can be bypassed when chemically synthesized Gly-Asp was coupled enzymatically with Arg (Hou et al. 2005).

Chemical peptide synthesis has advanced during the last few decades now having routine methods for the synthesis, purification and characterization of peptides. There are still problems that limit the efficacy of peptide synthesis e.g. racemization during peptide bond formation, the costs and the availability of special chemicals and commonly used protected amino acids and activators, low yield of sterically hindered couplings and the toxic nature of solvents and coupling reagents when applied to pharmaceuticals or food grade end products. Furthermore, recycling of the chemicals and solvents used in synthetic peptide chemistry is not a common practice (Guzman et al. 2007, Kumar & Bhalla 2005). However, chemical peptide synthesis is the most convenient method for small scale production of short and medium sized peptides (up to 50 residues) and it is the only method which can produce peptides with non-natural structures and modifications.

1.2.1 Development of peptide chemistry

Fischer and his group described the synthesis of first peptide, glycylglycine (Gly-Gly) in 1901[1] and a few years later they published the synthesis of 18 residues peptide (see Suresh Babu 2011, Kimmerlin & Seebach 2005). The problems with the suitable protection and deprotection of terminal amino and carboxylic groups and with racemization i.e. the conversion of the L-configuration to D-configuration (and vice versa) of the residues hindered progress in peptide chemistry for a long time. Further, there were few suitable purification and identification methods for the products. The introduction of the benzyloxycarbonyl (Z, also Cbz) –protection group (*Figure 4*) for temporary *N*-terminal amino protection in 1931 was a huge step forward, not only for protecting of the compounds but also preventing the racemization (Kimmerlin & Seebach 2005). It took more than 50 years from the first peptide synthesis when the first peptide hormone, oxytocin, could be synthesized and purified by du Vigneaud and his collaborators (du Vigneaud et al. 1953). This initiated the era of peptide pharmaceuticals. The next groundbreaking breakthrough was the introduction of a new, acid-labile protecting group, tert-butyloxycarbonyl (Boc) (*Figure 4*), which was stable toward hydrogenation and strong alkali and therefore orthogonal to the Cbz (Carpino 1957, McKay & Albertson 1957). The combination of Boc- and Cbz-protecting groups was used for the synthesis of several peptides including the synthesis of a 39-residue β-corticotropin, also known as adrenocorticotrophic hormone (ACTH) in 1963 (Schwyzer & Sieber 1963). In 1970, Carpino was once again involved in a major breakthrough in the synthetic peptide chemistry field when his group discovered the acid-labile α-amino-protecting group, 9-flourenylmethoxycarbonyl (Fmoc, *Figure 4*) (Carpino & Han 1970). This enabled the use of acid-labile protection for side-chains and selective deprotecting of the α-amino group after amide bond formation. Today, a large number of protecting groups are in common use with selective and specific coupling and deprotecting methods and these allow very specific modifications to the peptides (Isidro-Llobet et al. 2009).
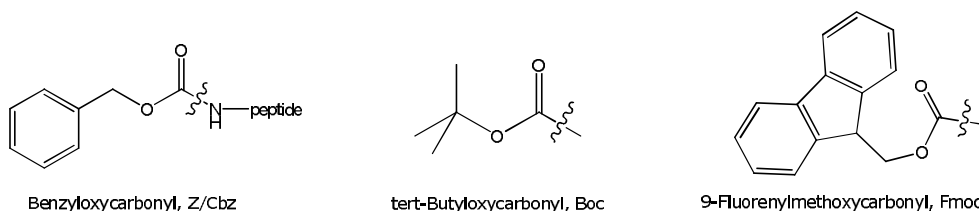
Figure 4. Selective α-N-protecting groups.

In addition to the amino acid protecting method development, new coupling methods were intensively studied. The first important innovation was the introduction of carbodiimides in 1955 which allowed the activation of carboxylic group (Sheehan & Hess 1955). However, the activation of the carboxylic group via carbodiimides such as *N,N'*-diisopropylcarbodiimide (DIC) and *N,N'*-dicyclohexylcarbodiimide (DCC) (*Figure 5*) caused the racemization of activated residue through the highly reactive intermediate of the O-acyl-isourea (*Figure 6*). The activation of the carboxylic group often causes some racemization, but the introduction of less-reactive benzotriazole based activators such as 1-

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[1] Original publication: Fischer, E. & Fourneau, E. 1901, "Ueber einige Derivate des Glykocolls", *Berichte der deutschen chemischen Gesellschaft*, vol. 34, no. 2, pp. 2868–2879.

hydroxybenzotriazole (HOBt, *Figure 5*) and 1-hydroxy-7-azabenzotriazole (HOAt, *Figure 5*) has been shown to minimize racemization (König & Geiger 1970, Kimmerlin & Seebach 2005, Carpino 1993) Subsequently, a large number of coupling reagents and methods has been described based on carbodiimides and benzotriazoles and their combinations such as uronium salt derivative of HOBt, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, *Figure 5*) (Albericio & Carpino 1997, Albericio 2004).
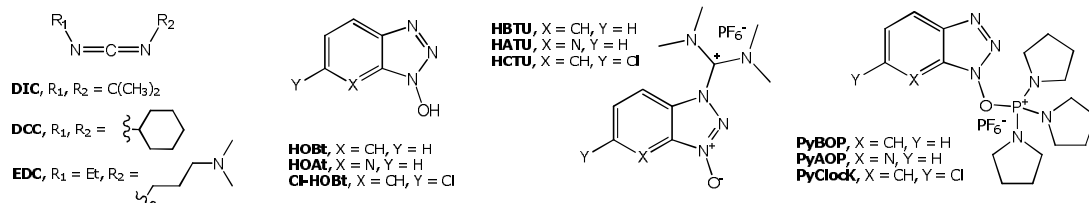
Figure 5. Commonly used activators based on carbodiimides and benzotriazoles.
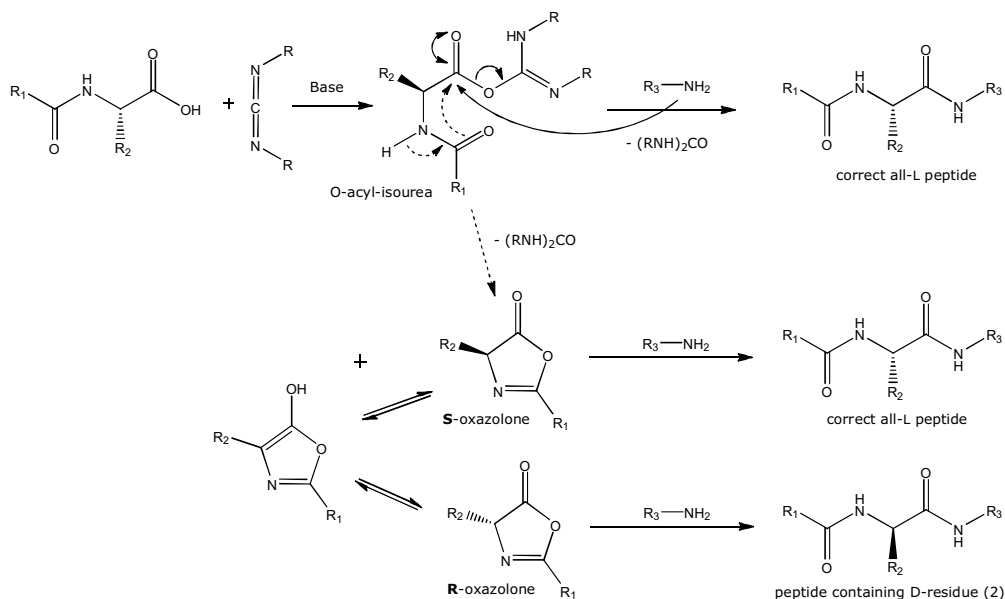
Figure 6. Activation of *C*-terminal carboxylic group by carbodiimides during coupling (above) and the mechanism for racemization via 5-(4H-)oxalozone intermediates (below).

The synthesis method used for the peptide production until 1960 was called solution- or liquid-phase peptide synthesis (LPPS, sometimes called also SPS). This consists of consecutive cycles of the synthesis, isolation, purification and characterization of intermediate products. Peptide chemistry progressed slowly until Bruce Merrifield published the revolutionary solid-phase peptide synthesis method (SPPS) in 1963 (Merrifield 1963). The main concept of SPPS method is the linking of the growing peptide to an insoluble support and after each reaction step, the byproducts are simply removed by filtration and washing. Since the peptide synthesis is a continuous cycle of washings, couplings of new residues, washings and deprotections, it can be performed in a single reaction vessel. This led to the rapid development of the first automated peptide synthesizer (Merrifield & Stewart 1965, Merrifield et al. 1966). With LPPS, it took weeks or months to produce even a short peptide, whereas with the Merrifield method syntheses

could be completed within days. The developments of SPPS together with new chromatographic purification techniques, especially high performance liquid chromatography (HPLC), and powerful product characterization via MS turned SPPS into a routine and highly automated method. There are several manufacturers of automatic peptide synthesizers e.g. AAPPtec, Biotage AB, Protein Technologies Inc and CEM Corporation. Some manufacturers sell a synthesizer which can use microwave assisted heating during synthesis.

The coupling reactions of peptide synthesis have been performed traditionally at room temperature (RT) without the need of external heating or with gentle heating. In organic chemistry, microwave (MW) assisted heating has been used widely since the middle of 1980's with the first time it was used in peptide synthesis being in 1992 (Yu et al. 1992). There was still some delay in the development of MW-assisted peptide synthesis because of the requirements of the technology including reactors which could work at specific pressures with temperature control and also chemicals stable enough to work at elevated temperatures and pressures. The MW-assisted heating also raises the number of unwanted side reactions such as the racemization of certain amino acids and possible aspartimide formation. Recently in many studies, MW-assisted peptide synthesis has been shown to have advantages in terms of speed, yields and purity. The coupling times can be even reduced to 30s instead of the commonly used ½ - 2h. More complete coupling reactions can increase the yields in terms of purity and also the synthesis of some difficult sequences can be enhanced (Sabatino & Papini 2008).

1.2.2 Solid-phase and solution-phase peptide synthesis

The main advantages of LPPS are the control of synthesis via the purification and characterization of intermediate products and the limited sterical hindrance in the coupling sites. However, when the peptide chain elongation is continued, then the purification becomes more difficult. This problem could be resolved by the condensation of short protected peptide fragments and coupling them to larger molecules (Guzman et al. 2007, Bray 2003). Several peptide-based pharmaceuticals have been manufactured via LPPS including atosiban, eptifibatide, ocreotide and, bivaluridin (*Table 1*, see page 3) (Guzman et al. 2007, Verlander 2007). LPPS could be used also in the condensation of peptide fragments produced by SPPS. This hybrid type of synthesis has been used successfully in the production of enfuvirtide, a 36 amino acid containing drug for treatment of HIV (Table 1). The annual production of enfuvirtide was believed to be 4000 kg in 2007 (Guzman et al. 2007) and it has been one of the driving forces in the development of new synthetic strategies. The yield of enfuvirtide via conventional SPPS was initially only 6-8% from the loaded resin but after several improvements, the efficiency of the hybrid synthesis via solution phase condensations of three SPPS-produced fractions reached over 30% (Bray 2003).

The main advantage of SPPS is that when the growing peptide is attached to the solid resin, the excess amino acids, coupling reagents, impurities and deprotecting products can be removed easily via simple washings. When the sequence is ready, the peptide is cleaved and isolated via conventional purification methods. There are a high number of different solid supports with a wide variety of cleaving protocols enabling the cleavage of peptide with side chains protected or deprotected and with an unmodified or modified carboxy-terminal. There are also resins which can be preloaded with a wide variety of available

chemical groups. The prepared peptides are usually cleaved from the resin using strong or mild acids. In addition, base labile resins and further photolabile resins have been developed (Guzman et al. 2007). Today almost all of the synthetic steps can be automated and the protocols have become well-established. The peptides which are manufactured by SPPS include leuprolide, calcitonins, desmopressin, somatostatin and glugacon derived peptides (Guzman et al. 2007, Verlander 2007).

The disadvantages of SPPS are more or less based on the limited space inside and around the porous resins. Protected residues in the growing sequence and the incoming bulky protected amino acid just cannot react in the limited space. The growing peptide chain can fold over onto itself or aggregate with a neighboring peptide chain. These can cause the truncation of the peptide chain and result in a high amount of incomplete sequences and impurities which are difficult to separate. The introduction of analytical and preparative HPLC-methods in the late 1960's by Horvath and coworkers and developments in the HPLC techniques have improved the purification of peptide products (Horvath & Lipsky 1969). The success of peptide synthesis is highly sequence dependent. It has been noted that peptides including <15 amino acids are generally efficiently synthesized and purified but if the number of residues increases, then the purification becomes more challenging. It is rare that peptides containing 40 or more amino acids can be successfully synthesized with the correct sequence and with clear peak when analyzed by HPLC (Bray 2003).

Long peptides can be prepared by combining fully protected peptide fragments cleaved from very weak acid labile resins. The coupling of peptide fragments in solution to the other fragments is usually the only possible method with which to produce the correct full length peptide. This usually means conducting many studies via trial and error to find the best combination as seen in the case of enfuvirtide a peptide with 36 residues (Bray 2003). The hybrid synthesis of green fluorescent protein with its 238 residues was performed using 26 fully protected fractions in solution phase coupling (Nishiuchi et al. 1998). Another approach for producing long peptide sequences is the method called native chemical ligation (NCL). This is based on the prior thiol capture strategy for the coupling of two peptide fragments and it was introduced by Kemp and collaborators (Kemp et al. 1981, Kemp & Kerkman 1981). The original NCL method was based on the acyl shift between one peptide connected to template molecule via *C*-terminal ester bond and another peptide connected to the same template via a disulphide bridge between side chain of the *N*-terminal cysteine and the template molecule. After the acyl shift has occurred forming an amide bond between peptides, the disulphide bond is cleaved yielding long peptide. One problem may be that there can be steric hindrance due to the amino acid attached to the template and this limits the efficiency of the synthesis (Kimmerlin & Seebach 2005). Improved NCL methodology was introduced in the middle of 1990's. This technique allows the selective ligation of the two purified unprotected peptide sequences (Dawson & Muir 1994, Tam et al. 1995). The ligation occurs between *C*-terminal thioester and *N*-terminal cysteine via transesterification and rapid intramolecular S to N acyl shift, forming the amide bond at the ligation site (*Figure 7*). The chemical ligation overcomes problems that usually limit the synthesis of large peptides and proteins such as poor solubility and difficulties in the purification of large protected fragments (Kimmerlin & Seebach 2005). Several specific chemical ligation methods have been developed also for the on-resin to minimize the losses during repeated purification and lyophilization steps especially in the

protein synthesis (Canne et al. 1999, Brik et al. 2000, Kimmerlin & Seebach 2005). Furthermore, it has been shown that the epimerization of the residue next to the ligation site does not occur during the ligation reaction (Lu et al. 1996).
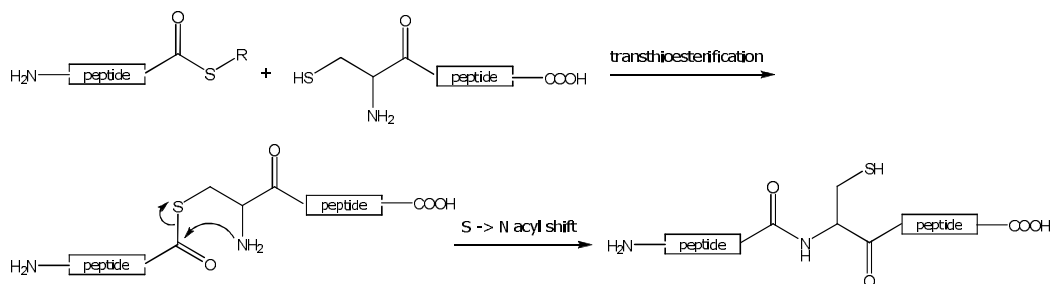
Figure 7. Native chemical ligation via *N*-terminal cysteine.

One major limitation of NCL method is that many peptides or proteins do not contain cysteine. The ligation reaction can also be performed via using for example histidine or homocysteine, which can be methylated to methionine, as the N-terminal residue (Kimmerlin & Seebach 2005). Chemical ligations have been utilized mainly in the synthesis of proteins but they also provide an interesting possibility to permit the synthesis of complex peptides.

1.2.3 Strategies in peptide synthesis

The basic principle of peptide synthesis contains repetitive steps of the couplings of protected amino acids and the deprotection of the backbone protection. This is usually performed by attaching the first residue to the solid phase via the *C*-terminal carboxylic group followed by the deprotection of *N*-terminal amino group. The next step is the attachment of the second residue via the carboxylic group, usually activated for better coupling efficiency. This synthetic direction is called the C→N direction and the basic principles are represented in *Figure 8*. *N*-terminal deprotection is usually removed under mild conditions and the reactive groups of side chains are masked with permanent protection groups which are stable towards the reaction conditions during peptide elongation. The permanent side chain protection groups can be deprotected during the cleavage of the peptide from the solid support or afterwards.

The reaction conditions such as coupling, deprotection and cleavage times, solvents and mixing method are case-dependent. The literature is full of references for peptide synthesis protocol using LPPS or both manual and automated SPPS. The main factors to be considered when a new peptide synthesis is being planned, are: **1) the choice of the synthetic strategy between Fmoc- and Boc-strategy, 2) the choice of side chain protection groups, 3) the choice of coupling reagents, 4) the solvent to be used and the concentration of reagents, 5) ratio of substrates, 6) reaction times, 7) temperature, 8) mixing method (shaking, stirring, bubbling with inert gas such as N_2 or Ar) and when SPPS is used, 9) the choice of the solid phase, the resin** (Li & Roller 2002, Amblard et al. 2006, Albericio 2004, Sabatino & Papini 2008). Several associations such as the Association of Biomolecular Resource Facilities (ABRF), reagent suppliers such as Bachem, GL Biochem Ltd, Sigma-Aldrich corporation, ChemPep Inc and Merck and also manufacturers of peptide synthesizers (earlier mentioned) have published their own protocols for basic peptide

synthesis usually covering all of the above mentioned factors. The next sections will describe the main factors to be considered.

SPG = permanent side chain protection
NPG = temporary α-N protection group

= resin

activating agent + base

deprotection of NPG

coupling step with
activating agent + base

steps are repeated n+1 times

deprotection of NPG
washings
next coupling

cleavage from resin
and side chain deprotection

peptide product

X = -OH, -NH$_2$, etc
Y = -H, -Ac, etc
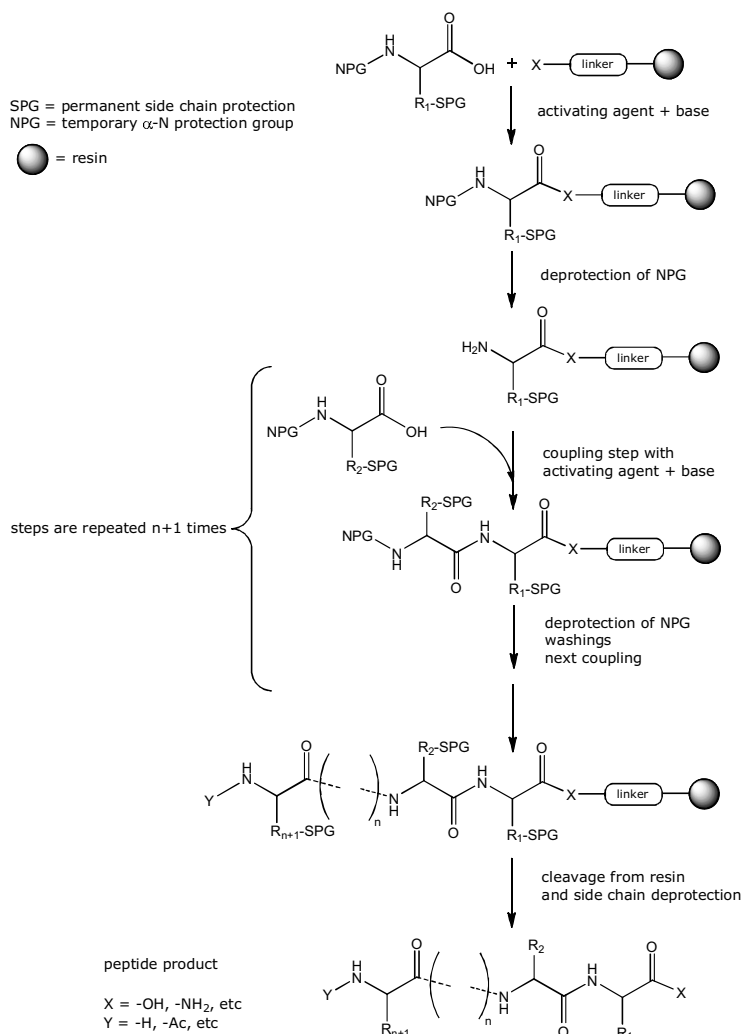
Figure 8. Basic peptide synthesis protocol.

Fmoc versus Boc. There are currently two main strategies being used with SPPS for peptide synthesis: Boc (Boc/Bn) -strategy with Boc-protected α-amino group and Fmoc (Fmoc/Bu^t) -strategy with Fmoc-protected α-amino group. The protection of the α-amino group is the most important issue in peptide chemistry in order to control the polymerization. The α-amino group should be removed rapidly and specifically without any side reactions. α-N-Protected amino acids have to be soluble in most common solvents and they should be crystalline solids with good stability to allow easy handling and storage. They also should prevent or minimize racemization during the coupling (Isidro-Llobet et al. 2009). In SPPS, Fmoc and Boc are the most widely used protecting groups. In the LPPS, also Z (*Figure 4*, see page 13) can be used for α-amino protection.

The Boc-strategy is based on the use of mild acid labile Boc-group (*Figure 4*) for the protection of α-N-group and benzyl-based protection (Bn) of the side chains of amino acids. This method has been in use since the development of SPPS by Merrifield (Merrifield 1963,

Amblard et al. 2006). The *N*-terminal Boc is usually removed by using 25-50% trifluoroacetic acid (TFA) in dichloromethane (DCM). This combination is an excellent solvent for the protected peptide chain since it reduces the amount of aggregation and usually achieves the quantitative deprotection of the N-teminal amine. The use of acidic conditions in the deprotection step results in the protonation of the amino group and this might affect the next coupling if the neutralization remains insufficient (Sabatino & Papini 2008). This can be avoided by, for example, using *in situ* neutralization during the coupling step by adding a slight excess of a weak base such as diisopropylethylamine (DIEA) (Schnölzer et al. 1992). The rapid deprotection of the N-amino group means that the Boc-strategy suitable for the synthesis of difficult and/or high molecular weight sequences (Guzman et al. 2007). Furthermore, the advantage of the Boc-strategy is the relatively low costs of Boc-protected amino acids and the other building blocks. The major disadvantage for Boc-chemistry is the need for the highly toxic chemical, hydrogen fluoride (HF), during the final cleavage step in the release of the complete sequence from resin. This means that one must utilize polytetraflouroethylene-lined equipment and this limits the use of Boc-chemistry in many research laboratories (Amblard et al. 2006). However, as an alternative for HF, trifluoromethanesulphonic acid (TFMSA) can be used and this agent can be used with normal laboratory glassware (Yajima et al. 1974). The use of the very strong acid can lead to the production of many acid catalyzed side reactions such as alkylations of aromatic moieties and deletions in sequences containing fragile bonds and residues (Kimmerlin & Seebach 2005). The extent of some of these side reactions can be reduced by the use of diluted HF in H_2S (Tam et al. 1983). However, the repeated Boc-deprotections in acidic conditions may cause the undesirable cleavage of the side chain protecting groups (Verlander 2007, Amblard et al. 2006, Sabatino & Papini 2008).

The Fmoc –strategy is based on the use of the base-labile Fmoc-group (*Figure 4*, see page 13) for the protection of N-teminal amino group and tert-butyl-based protection for the side chains (Carpino & Han 1970). The use of base-labile α-N-protection enables the use of acid-labile side chain protection and linkers in the resin. There is a wide variety of possibilities for the temporary or permanent orthogonal protection. Peptide synthesis based on the Fmoc-strategy can be performed without any special laboratory equipment and this decreases the costs of synthesis. Fmoc-based SPPS is today the method of choice for routine peptide synthesis (Amblard et al. 2006). There are however some considerable disadvantages associated in the Fmoc-strategy. Fmoc is a bulky protecting group and its presence may decrease both coupling and deprotection efficiency. Steric hindrance can be critical, especially when the Fmoc-α-N-protected amino acid does contain bulky and protected side chain such as Trp or Tyr. The deprotection reaction with Fmoc is slower than with Boc and may produce a significant amount of incomplete side products if quantitative deprotection is not achieved (Schnölzer et al. 2007). The most widely used deprotection cocktail for use with Fmoc is 20% piperidine in *N,N*-dimethyl formamide (DMF) (Atherton et al. 1978) but also many other methods has been reported including the use of 50% morpholine in DMF (Isidro-Llobet et al. 2009). The major problem in piperidine-based deprotection is aspartimide formation from aspartic acid. This may cause the conversion of α-peptide to β-peptide and also the formation of a piperidine adduct to the side chain of aspartic acid (*Figure 9*). The most widely used side chain carboxylic acid protecting group is tert-butyl (Bu^t, *Table 3*). This minimizes the aspartimide formation via sterical reasons but it does not prevent the reaction completely (Isidro-Llobet et al. 2009, Mergler et al. 2003). In

addition to Bu^t, various other side chain protection groups for Asp have been described such as more bulky 3-methylpent-3-yl (Mpe) and 9-fluorenylmethyl (Fn) (Isidro-Llobet et al. 2009). The addition of a small amount of HOBt into the deprotection step further reduces the aspartimide formation (Mergler & Dick 2005, Isidro-Llobet et al. 2009). Compared to the Boc strategy, the price of Fmoc-protected amino acids is relatively high and the solubility of some Fmoc derivatives in the commonly used solvent, DCM, can be very low (Isidro-Llobet et al. 2009).
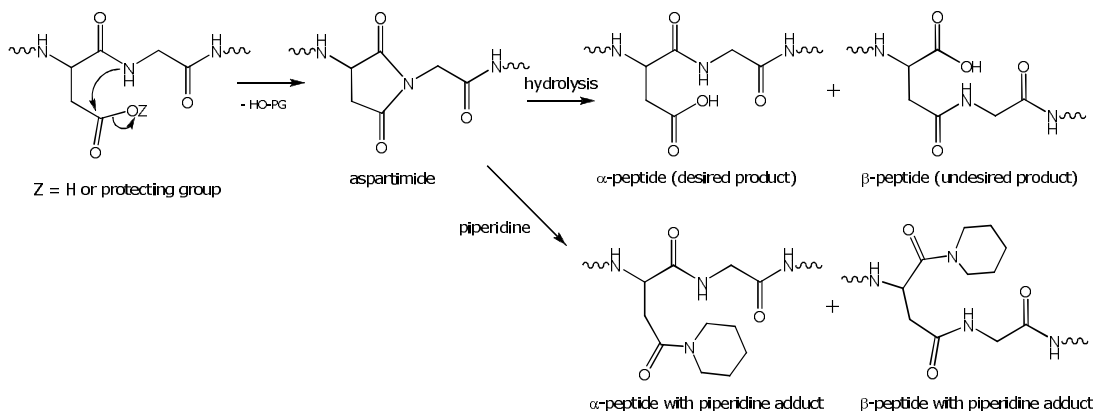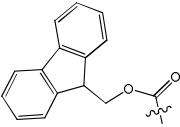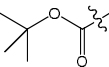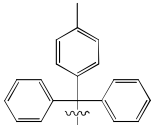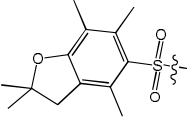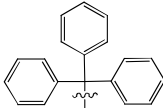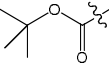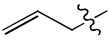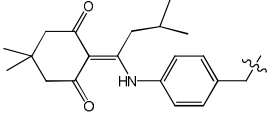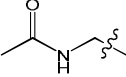
Figure 9. Aspartimide formation and piperidine adduct attachment during Fmoc synthesis.

Protection of functional groups. The protection of side chain functionalities against unwanted reactions is highly dependent on the α-N-protection method chosen. When the Boc-strategy is selected, then the side chain protection has to be stable against relatively strong acidic conditions whereas in the Fmoc-strategy, side chain protection has to be able to withstand mild bases. The focus in this section is on protection of naturally encoded amino acids but it will also cover a wide range of functionalities in common use in organic chemistry. The side chain functional groups, which should be protected are primary amino (Lys), guanidino (Arg), secondary amino groups (His, Trp), amide groups (Asn, Gln), carboxylic acids (Asp, Glu), primary alcohols (Ser, Thr), phenol (Tyr), thiol (Cys) and thioether (Met). Gly and aliphatic or aromatic hydrocarbon side chains containing amino acids (Ala, Val, Leu, Ile, Phe, Pro) do not need side chain protection. The optimal protecting group must possess a few desirable properties: a straightforfard introduction to the functional group, stability towards a broad range of reaction conditions and specific removal when needed. The most commonly used protection groups for Fmoc-based peptide synthesis will be briefly discussed below and the protecting groups with removal conditions are shown in *Table 3.*

The primary amino group can be related to the α-N-group of backbone and it can be protected in the same manner. A Free side chain amine group leads to branched peptides during Fmoc-based peptide synthesis and commonly it has to be specifically protected. The side chains of Lys and its shorter analogs such as Orn can be used as a linker in cyclization reactions but also for the conjunction of additional molecules such as chelating agents. If permanent or specifically removable protection is needed, allyloxycarbonyl (Alloc) or 4-methyltrityl (Mtt) can be used in the Fmoc-strategy (Isidro-Llobet et al. 2009).

Table 3. Protection groups and removal conditions commonly used in Fmoc-peptide synthesis.

Protected functionality	name	structure	removal conditions	stabile to the removal of
backbone amino	**Fmoc**		20% piperidine in DMF,	Boc, Pbf, Mtt, Trt, All, ^{t}Bu, Dmab, Acm
Lys, Orn (sc amino)	**Boc**		25-50% TFA in DCM,	Fmoc, All, Trt
	Mtt		1% TFA in DCM,	Fmoc, All, Boc, Dmab, Acm
Arg (sc guanidino)	**Pbf**		90% TFA (H_2O, TIS as scavengers)	Fmoc, Trt, Alloc
His (sc imidazole) **Asn, Gln** (sc amide) **Cys** (sc thiol)	**Trt**		1% TFA-DCM,	Fmoc, Alloc, Acm
Trp (sc indole)	**Boc**		95% TFA and scavengers	Fmoc, Trt, All
backbone carboxylic acid, (sc carb. acid)	**All**		Pd(PPh$_3$)$_4$ (0,1eq), PhSiH$_3$	Fmoc, Boc, Trt, ^{t}Bu,
Asp, Glu (sc carb. acid)	**Bu^{t}**		90% TFA in DCM,	Fmoc, Trt, All
	Dmab		2% hydrazine in H_2O-DMF (1:1)	Fmoc, Boc, Trt, All, ^{t}Bu
Ser, Thr, Tyr (sc hyxroxy)	**Bu^{t}**		90% TFA in DCM,	Fmoc, Trt, All
Cys (sc thiol)	**Acm**		I$_2$, Hg (II)	Fmoc, All, Trt

Abbreviation used: sc, side chain; carb. acid, carboxylic acid

The guanidino group of Arg can undergo deguanidation under basic conditions, especially during Fmoc deprotection, converting Arg to Orn (Rink et al. 1984). The side chain of Arg has to be protected with strongly electron withdrawing and bulky groups such as pentamethyl-2,3-dihydrobenzofuran-6-sulfonyl (Pbf) in the Fmoc strategy.

The imidazole ring of His may be involved in racemization via N$^{\pi}$ of His residue during the activation step for the next amino acid coupling (Isidro-Llobet et al. 2009). The imidazole ring can also be acylated when endcapping with acetic anhydride is performed after coupling, leading to the termination of synthesis via N$^{\tau}$ to α-N-migration (Ishiguro &

Eguchi 1989). Thus, N^τ is usually protected with a bulky or an electron withdrawing group such as trityl (Trt) in the Fmoc-strategy to prevent acylation and to reduce basicity of N^π (Isidro-Llobet et al. 2009).

The nitrogen in the indole group of Trp can undergo alkylation by carbocations from deprotection or degradation products from the resin (Giraud et al. 1999). Trp can be used without protection but usually it is Boc-protected in the Fmoc strategy (Isidro-Llobet et al. 2009).

Asn and Gln can be used without deprotection but the solubility of unprotected Asn and Gln is low and there is the risk of side reactions: Asn can undergo the dehydration of its side chain yielding nitrile instead of the amide in side chain (*Figure 10*), especially when the Fmoc-strategy is being used (Mojsov et al. 1980, Gausepohl et al. 1989). The use of HF in the Boc-strategy usually reverts the dehydration (Mojsov et al. 1980). Gln can undergo the same side reaction as Asn but its most significant side reaction is the formation of cyclic pyroglutamyl leading to a truncated sequence (*Figure 10*)(Dimarchi et al. 1982). This reaction is weak acid catalyzed and is more important in the Boc-strategy. The most widely used protecting group for Asn and Gln is Trt in the Fmoc- strategy.
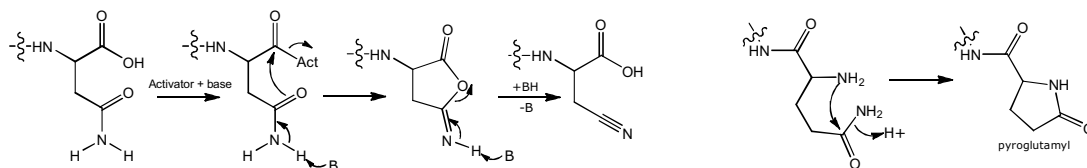
Figure 10. Dehydratation of Asn during the coupling (left) and pyroglutamyl formation (right).

In addition to the amino function, the carboxylic acid function is the most extensively used function for modification site in peptide synthesis. The *C*-terminal carboxylic acid is usually protected via resin attachment because most commonly peptide synthesis in SPPS is performed in the C→N direction. In LPPS or in the reversed peptide synthesis strategy (N→C direction), specific *C*-terminal protection will be necessary (Jensen et al. 1998, Thieriet et al. 2000, Isidro-Llobet et al. 2009). The side chains of Asp and Glu are usually protected in order to prevent their unwanted cyclization or branching of peptide. During N→C synthesis, the duplication of residues is prevented via the backbone α-carboxylic acid protection. The most important side reaction to be considered for Asp and Glu side chain protection is the prevention of the above mentioned aspartimide formation (*Figure 9*, *see page 20*) and pyroglutamic formation, respectively. However, the cyclization of Glu yielding pyroglutamic acid is much less problematic. The most commonly used side chain protection groups are Bu^t in the Fmoc-strategy despite the fact that it is only capable of minimizing aspartimide formation (Isidro-Llobet et al. 2009, Mergler et al. 2003). Allyl (All), which can be selectively removed with a Pd-catalyst, is a very useful carboxylic acid protection group when *C*-terminal modifications are needed. All has been used in the N→C synthesis strategy and for the production of *C*-terminally modified peptides using backbone amide linker (BAL) (Jensen et al. 1998, Thieriet et al. 2000) or in head-to-tail, side chain to tail and side chain to side chain cyclizations (Alcaro et al. 2004, Li & Roller 2002). When it is used in a synthesis where the target is to combine the Fmoc-protected amino group and the All-protected carboxylic acid, the latter's protection should be first removed to prevent allylation of the amino function (Kates et al. 1993). Another selectively

deprotected carboxylic function protection group which can be used in cyclization chemistry is 4-(*N*-[1-{4,4-dimethyl-2,6-dioxocyclohexylidene}-3-methylbutyl]amino)benzyl (Dmab) which is labile toward dilute hydrazine solution but nonetheless stable toward piperidine (Isidro-Llobet et al. 2009).

The amino acids which contain a hydroxyl group in the side chain include Ser, Thr and the aromatic Tyr. These molecules can undergo O-acylation followed by O-N migration after N-deprotection when excess acylating agents are used. Ser and Thr are also prone to dehydration but this is not common in the usual peptide synthetic methods. The hydroxyl group is commonly protected as an ether and Bu^t is used in the Fmoc-strategy (Isidro-Llobet et al. 2009).

Cys plays a major role in natural rigid structures and in disulphide bridges between two Cys residues are very commonly present as the stabilizing agents in 3-D structures of proteins. The nucleophilic thiol undergoes spontaneous oxidation to the disulphides in air if it is not protected. Cys can also undergo several side reactions even with protection. These include oxidation, base catalyzed β-elimination of sulphur resulting dehydroalanine, S-alkylation and racemization (Isidro-Llobet et al. 2009, Angell et al. 2002, Han et al. 1997). The racemization occurs during treatments with bases in the coupling steps and the racemization rate can be reduced either by using no base or some alternative base such as N-methylmorpholine (NMM) or 2,4,6-trimethylpyridine (TMP, collidine) in place or the commonly used DIEA in the Fmoc-strategy (Han et al. 1997). Racemization can also be controlled by the choice of activators, by protecting groups and resins (Kaiser et al. 1996, Angell et al. 2002). The choice of protecting group depends on the desired product. If a free thiol is wanted, then Trt in the Fmoc strategy is a feasible choice whereas acetamidomethyl (Acm) is the recommended protecting group if the disulphide bridge is needed (Isidro-Llobet et al. 2009). However, it should be noted that some Acm adducts may well be detected for example from Tyr after Acm removal (Engebretsen et al. 1997). The variety of different types of protection of cysteine is very important when the desired product contains more than one disulphide bridge. The disulphide bridge assembly can be controlled using different pairs of protection groups (*Table 3*).

Methionine is a challenging residue in peptide chemistry because it is prone towards oxidation of its thioether to sulfoxide and in some cases it can become S-alkylated. Met is still commonly left unprotected in the Fmoc strategy (Isidro-Llobet et al. 2009).

Backbone NH-protection is not commonly used but it can be helpful for preventing the aggregation of the growing peptide chain, especially in the Fmoc strategy. The aggregation of the protected sequence reduces coupling and deprotection yields and can result in incomplete sequences. The incorporation of dimethyloxazolidine dipeptides (*Figure 11*) which can be derived from Ser or Thr in combination with the next residue in the *N*-terminal side has been shown to enhance the synthesis of challenging sequences (White et al. 2004, Abedini & Raleigh 2005). Backbone protection can be also used for preventing aspartimide formation which is a severe problem especially when the sequence contains Asp-Gly. The rate of aspartimide formation can be affected by the choice of resin, the deprotection reagents, coupling additives and resin cleavage cocktails but the use of backbone *N*-protection such as 2-hydroxy-4-methoxybenzyl (Hmb) has been shown to prevent efficiently aspartimide formation (Packman 1995, 284 Cebrian et al. 2003).
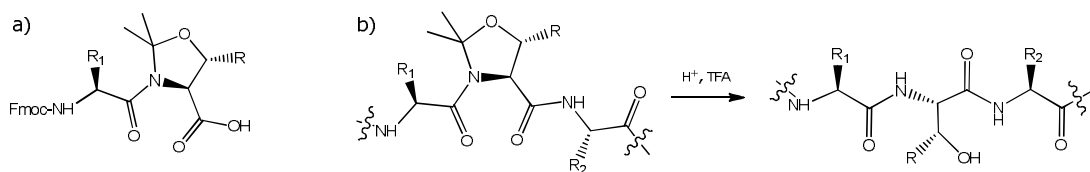
Figure 11. Fmoc-protected oxazolidine dipeptide building block (a) and Acid-catalyzed ring opening of oxazolidine to regenerate Ser or Thr (b). R = H, Ser; R = CH_3, Thr; R_1, R_2 = any amino acid side chain.

Another harmful side reaction, which can result in sequence shortening by two residues, is diketopiperazine formation (DKP, *Figure 12*) between two adjacent residues. The DKP formation has been detected mainly between the first couple of residues connected to the resin during the α-*N*-deprotection of the second and the coupling of the third residue (Albericio & Carpino 1997). The DKP formation can be prevented by using bulky and mild acid labile trityl for α-N-protection and via the choice of a resin with the use of a sterically hindered Barlos-resin (e.g. 2-Cl-trityl) (later in *Table 4*) being recommended (Albericio 2004, Amblard et al. 2006, Albericio & Carpino 1997). The prevention of DKP formation is crucial when the reverse SPPS method (N→C) or backbone amide linker (BAL) method is used for the production of *C*-terminally modified peptides. During reverse synthesis, the choices of the resin and the coupling conditions have to be considered (Thieriet et al. 2000). The use of acid labile protection group for α-*N*-group leaves the amino function protonated after cleavage, and this prevents DKP-formation (Jensen et al. 1998). DKP formation is commonly detected during the synthesis of backbone *N*-methyl rich peptides or when both D- and L-amino acids are used (Teixido et al. 2005). The DKP formation can occur also during chain elongation (Thieriet et al. 2000, Tulla-Puche et al. 2007, Isidro-Llobet et al. 2009).
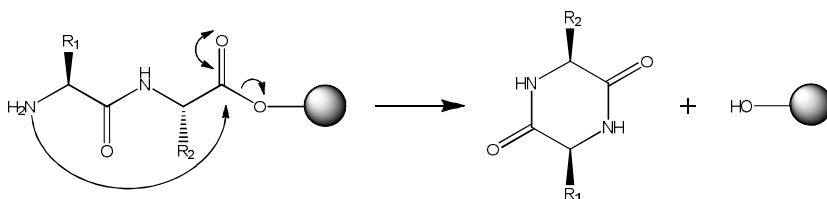
Figure 12. The diketopiperazine formation between two *C*-terminal residues.

Activation of coupling steps. The growing need for the efficient and low cost synthesis of peptides, especially peptide pharmaceuticals, has resulted in a wide variety of activation reagents which can be used in amino acid couplings. These have been widely discussed in the literature and only the most commonly used in basic peptide synthesis protocols will be discussed in this section (Albericio 2004, Amblard et al. 2006, Albericio & Carpino 1997, Li & Roller 2002). Carbodiimides (*Figure 5*, see page 14) are known to be effective coupling reagents but the racemization of the activated residue via oxazolone intermediate (*Figure 6*, see page 14) limits their use and therefore the less active uronium and phosphonium salts of HOBt are currently extensively used in peptide chemistry. However, carbodiimides are cheap, especially DIC, and they are thermally stable which makes them suitable for MW-assisted peptide synthesis (Hachmann & Lebl 2006). DIC is the recommended choice of carbodiimides for Fmoc-based SPPS, because the diisopropylurea formed after activation is more soluble in commonly used DMF than the dicyclohexylurea (DCU) formed after DCC

activation (Albericio & Carpino 1997). In the Boc strategy, DCC can be used because HF used for the α-N-deprotection is a good solvent for DCU. HOBt can be used as an additive with carbodiimides to form active HOBt-ester which is not so prone to racemization. The DIC/HOBt activation is still the recommended choice in some instances, for example in MW-assisted peptide synthesis (Bacsa et al. 2008). The most commonly used activators in conventional peptide synthesis are uronium salts of HOBt despite the fact that they can irreversibly block the N-terminus by forming tetramethylguanidium derivatives (Amblard et al. 2006). However, this can be avoided by creating the carboxylate of amino acid before adding uronium-based activators and also by using reduced equivalency for the activator compared to the amino acid (Albericio et al. 1998, Amblard et al. 2006). The most widely used uronium derivatives are HBTU, HATU and HCTU (*Figure 5*, see page 14) because of their rapid and efficient coupling and reduced racemization associated with their use. HOAt derived HATU is said to possess superior acylation kinetics and to have a better ability to prevent racemization but its use is usually limited to the challenging coupling steps such as N-methylated amino acids and cyclizations because it is expensive (Carpino 1993, Miranda & Alewood 1999). The development of Cl-HOBt derived HCTU about 10 years ago represented a relatively cheap alternative to HBTU and HATU and recently it has been shown to be very efficient in the rapid synthesis of seven biologically active peptides including oxytocin and β-amyloid 1-42 requiring only 5 min coupling times (Sabatino et al. 2002, Hood et al. 2008). The phosphonium salts of benzotriazoles such as PyBOB and PyAOP (*Figure 5*) can be used as an alternative to the uronium salts but their efficiency is not as high as the uronium-based activators (Hachmann & Lebl 2006, Sabatino & Papini 2008). However, by using the phosphonium salts of benzotriazoles, the guanidylation side reaction can be prevented (Sabatino & Papini 2008, Albericio 2004). In addition, the mixing of different types of coupling reagents can also be an option (Hachmann & Lebl 2006). Mild tertiary bases are used in conjunction with activators to convert the amino acid into an active benzotriazole-ester (OBt) with DIEA being most commonly used for this purpose although racemization can occur when Fmoc-Cys(Trt)-OH or Fmoc-Ser(Bu^t)-OH is used (Di Fenza et al. 1998, Han et al. 1997). TMP can serve as an alternative for DIEA to reduce epimerization (Carpino et al. 1996). β-Branched amino acids including Ile, Val and Thr may result in incomplete acylation due to steric hindrance. The recoupling (double couple) of the residues is recommended if incomplete coupling is suspected (Albericio & Carpino 1997). Sometimes, if there is a suspicion of incomplete coupling, a method called end-capping can be used to terminate incomplete sequences in order to prevent deletions from the sequences. α-N-functionality is terminated readily using acetic anhydride and the same method has been applied to terminate free amino groups from the resin before proceeding with the synthesis (Garcia-Martin et al. 2006, Bacsa et al. 2006, Albericio & Carpino 1997).

Solvents for peptide synthesis. The choice of the solvents is dependent on the method being used for the synthesis. In solution phase synthesis, it is important that there are good reaction rates with all reagents i.e. protected amino acids and activators are completely dissolved together with growing peptide chain. During SPPS, the complete solubility of reagents is virtually essential because insoluble particles can precipitate in an insoluble carrier and then the risk of contamination becomes significant. The most widely used solvents in peptide syntheses are DMF, DCM, and 1-methyl-2-pyrrolidinone (NMP) but also dimethyl sulfoxide (DMSO) and tetrahydrofuran (THF) can be used (Albericio & Carpino 1997). DMF is commonly used in SPPS because it is aprotic and polar and thus it

offers good solubility for the different kinds of reagents and protected amino acids (Amblard et al. 2006). The disadvantage of DMF is its tendency to form dimethylamine during long storage periods and the amine moieties can cause unwanted Fmoc-deprotection during the synthesis procedure. DMF is usually distilled after a long time storage. NMP is a feasible alternative and in fact it is usually preferred in MW assisted syntheses because of its better thermal stability (Bacsa et al. 2008). The relatively nonpolar DCM is a widely used solvent in the Boc-strategy. Another highly important factor for determining which solvent to use in SPPS is the ability to swell the resin beads because most of the coupling sites lie inside of the beads. The swelling ability of the solvent will be dependent on the resin chosen and this can affect significantly the aggregation of the growing peptide chain leading to incomplete coupling via steric hindrances. For example when a hydrophobic polystyrene-based resin is used, dichloromethane (DCM) is used for swelling. However, the solubility of the activators and protected amino acids is often limited in DCM and the synthesis may be performed in a mixture of DCM and DMF at variable concentrations. The more hydrophilic polyethylene glycol (PEG) containing resins can be swelled also in more polar solvents such as DMF or DMSO and PEG-resins are therefore useful with a variety of solvents and mixtures (Garcia-Martin et al. 2006, Kates et al. 1998).

Amount of reactants. The basic principle is that the concentration of reactants should be kept high and thus concentrations between 0.5 and 1.0 M should be utilized (Albericio & Carpino 1997). Reagent and peptide synthesizer manufacturers usually recommend suitable concentrations and methods. Usually moderate or a clear excess of amino acids and activators is used in peptide synthesis, ensuring the maximal coupling efficiency. Typically concentrations 2-10 times in excess compared to the peptide or resin load equivalency are used (Albericio & Carpino 1997, Amblard et al. 2006). If one is utilizing uronium derivatives of activators, then a slight excess of amino acid compared to the uronium derivatives is recommended to avoid guanidium adduct formation. The formation of the OBt-ester of amino acid can be achieved in the presence of a tertiary base, commonly DIEA, and an equivalent or slight excess (1.5-2 eq.) of base is commonly used (Albericio & Carpino 1997, Amblard et al. 2006).

Reaction times. There is basically no upper limit for the reaction times and usually reaction times are approximated. However, in the pharmaceutical industry, the time consumed during synthesis is very costly and a rapid synthesis is desirable. The coupling efficiency is heavily sequence dependent and thus the optimal coupling times for each step should be evaluated, if the highest yield is demanded. Difficult couplings can be predicted in certain reactions e.g. for sterically hindered amino acids with bulky side-chain protection groups (e.g. Pbf, Trt, Dmab), when N-methylated amino acids are used, for cyclization steps such as head-to-tail cyclization and when the length of the peptide sequence exceeds 10 residues. In these situations longer coupling times will be needed (Amblard et al. 2006). For example, the cyclization yield of the marine sponge cycloheptapeptide, Phakellistatin 5, never exceeded 15% although coupling times of up to 2 weeks were tested (Pettit et al. 2000). However, if the coupling time is long, the possibility for side reactions is also elevated. One possible side reaction is guanidinylation of amino functionalities when uronium-based activators are used (Hachmann & Lebl 2006, Albericio & Carpino 1997). The development of automated and MW-assisted peptide synthesis protocols have resulted in the publication ofsome studies investigating coupling efficiency with the lower limits being

defined. The optimal coupling efficiency was monitored in the study of Hachmann and Lebl and they found that with a coupling time of 2 min using HBTU as an activator, one obtained a reasonable yield for the well-known test sequence of the acyl carrier protein (ACP 65-74) (Hachmann & Lebl 2006). The use of DIC as an activator yielded no correct product and demanded 8 min for coupling times to achieve yields equivalent to those obtained with HBTU. When finally 30 min was used as coupling time, DIC gave the best yields. In most cases, the coupling times will vary from 15 to 60 minutes, and for example almost all coupling reaction examples described in the article of Albericio and Carpino used a time of 30 minutes (Albericio & Carpino 1997). In many cases the use of external heating affects both the coupling rates and efficiency. However, when MW-heating is used, the coupling times are usually still counted in minutes, for example in the study of Brandt et al the sufficient coupling efficiency was achieved for the model peptides using 3-4 min coupling times (Brandt et al. 2006).

Reaction conditions. As stated earlier, peptide synthesis is routinely performed at ambient temperature and atmosphere. However, difficult couplings can be performed at elevated temperatures up to 60-80°C and today these steps are more usually performed under MW because it enables rapid heating and short reaction times while still providing pure products (Brandt et al. 2006, Sabatino & Papini 2008). However, the higher the temperature rises, the more side reactions will occur and the degradation of reactants and products will be more likely. Most of the automated peptide synthesizers, with or without MW-heating, utilize the basic Fmoc-reaction procedures. Some reaction steps however require abnormal conditions. For example, All-deprotection has to be performed under an inert gas because of the Pd-catalyst. Oxygen poisons the Pd-catalyst and usually argon-atmosphere is preferred over N_2 (Dessolin et al. 1995, Grieco et al. 2001).

Resin for SPPS. The choice of the solid carrier for SPPS is very case dependent on the peptide or peptide derivative product needed. During the next section, the term resin refers to both a solid matrix and the linker between the matrix and the attachment site (grey dots in *Figure 8*, see page 18 and *Table 4*) and only attachment sites will be discussed in more detail. Resins for SPPS can be purchased for both Fmoc- and Boc-strategies and they have a wide variety of attachment sites for different functionalities, not only for the *C*-terminal acid formation after cleavage. The peptide chain can be attached to the resin also via the *N*-terminus, via side chains of amino acids (e.g. Asp, Glu, Lys, Ser, Thr) or via the backbone amide. By using different resins the cleavage sites can be released as free unmodified peptide acids or as ready modified moieties e.g. amides, alcohols or thioesters (Kimmerlin & Seebach 2005). Furthermore, peptides can be cleaved as unprotected product or either partially or totally side chain protected. The resin can be purchased with or without protection on the attachment site or preloaded with some residue or even with multiple residues. Loading of the first amino acid is dependent on the resin in use and therefore not discussed here. The selection of the commonly used resins with different functionalities and cleavage conditions are presented in *Table 4*.

When Merrifield invented the groundbreaking SPPS method, he ended up to utilize a polystyrene (PS) -based polymer matrix with chloromethylated attachment sites (Merrifield 1963). He already noted that the porous gel structure of resin allowed good penetration for reactants, especially in the presence of a swelling solvent. PS resins are relatively hydrophobic resulting in sequence depended coupling difficulties. The search for better swelling properties with an extended range of solvents led to the development of
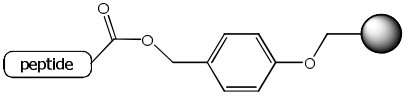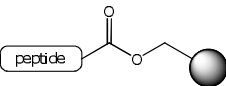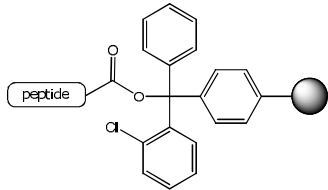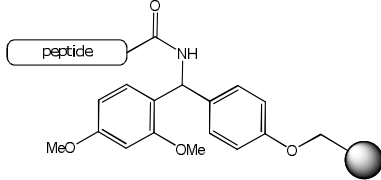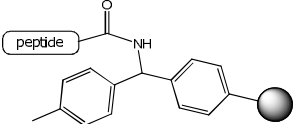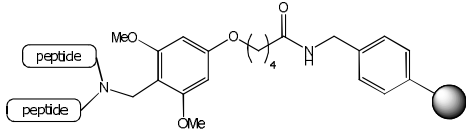
polyethyleneglycol derived PS-resins (PEG-PS) (Zalipsky et al. 1994). PEG contains both hydrophobic (ethylene) and hydrophilic moieties (oxygen). At the end of 20th century, PEG-PS resins became available in a wide variety of structures, for use with many kinds of synthetical strategies and with variable loading capacities (Kates et al. 1998). Loading capacity (load) refers to the theoretical amount of the molecules which can be produced on the resin beads and it is calculated as moles per mass unit of resin (mmol/g). Short peptides can usually be produced with higher load (0.5-1.0 mmol/g) whereas for long or difficult sequences a resin with a lower load (0.1-0.3 mmol/g) can be chosen. Recently, a highly versatile and totally PEG-based resin called chemmatrix was described (Garcia-Martin et al. 2006). It contains only the PEG-mesh with free amino groups conferring high physical stabily with excellent swelling properties and easy handling when dried. The model peptides, polyarginine containing peptide and the often aggregation prone β-amyloid (1-42) peptide were synthetized at commendable yields compared to PS- and PEG-PS resins (Garcia-Martin et al. 2006).

Peptides with an unmodified *C*-terminus can be synthetized using the Wang-resin (Wang 1973) in Fmoc-strategy or Merrifield-resin (Merrifield 1963) in the Boc-strategy (*Table 4*). In the Fmoc-strategy, the peptide is cleaved from the resin using 95% trifluoroacetic acid (TFA) which also cleaves the side chain protection exept for those with Fmoc, All, Dmab and Acm enabling possible post-cleavage modifications to the released functionalities. In the Boc-strategy, Merrifield-resin cleavage is performed using HF or trifluoromethanesulfonic acid (TFMSA) resulting in peptide acid. Trityl-based resins such as 2-Cl-trityl (Barlos et al. 1989, Barlos et al. 1991) can be used for the production of protected peptide fragments in the Fmoc strategy. The side-chain protected peptide fragment can be cleaved from the 2-Cl-trityl-resin using very mild acidic conditions, i.e. 1% TFA, with a wide variety of side chain protecting groups remaining attached. Further advantages of the trityl-resins are the loading of the first residue without activators in the Fmoc strategy creating epimerization free conditions and the bulkiness of resin attachment site prevents DKP-formation as discussed earlier (Albericio & Carpino 1997, Amblard et al. 2006). The 2-Cl-trityl-resin can be used also for the synthesis in the reverse N➔C direction (Thieriet et al. 2000). Peptides with a directly modified carboxylic group can be synthesized in different ways, for example via Rink amide resin in the Fmoc strategy (Rink 1987) or via MBHA-resin (p-methylbenzhydrylamine) (Matsueda & Stewart 1981) in the Boc-strategy. Both Rink amide and MBHA resin can be used for the backbone peptide amide synthesis. It is also possible to produce head-to-tail cyclic peptides via anchoring of Asp or Glu via the side chain to the resin producing either Asn or Gln, respectively, after cleavage. The peptide can also be attached to the resin via the backbone amide bond allowing the synthesis in both C➔N and N➔C directions and therefore also for the synthesis of cyclic peptides or *C*-terminally modified peptides (Jensen et al 1998, Bourne et al 2001).

The final cleavage step of the peptide from the resin and the cleavage procedure are highly dependent on peptide sequence, *C*-terminal amino acids and the resin being used (Jubilut et al. 2007). Thus, different cleavage methods will not be discussed in detail here. Commonly, care must be taken when the sequence includes one or several amino acids which contain electron rich side-chains. These kinds of amino acids are Cys, Met, Set, Thr, Tyr and especially Trp and they are prone to side-chain alkylation via the relatively stable deprotected carbocations such as tert-butyl or trityl cations (Amblard et al. 2006, King et al. 1990). In the Fmoc-strategy, peptides are usually cleaved with 90-95% TFA with the

remaining 5-10% consisting of scavengers. A small fraction of H_2O is used to quench tert-butyl cations (King et al. 1990). Furthermore, for example ethanedithiol (EDT) for the prevention of ^{t}Bu-triflouroacetate formation and ^{t}Bu-alkylation can be used (Lundt et al. 1978). EDT and the malodorous and less poisonous triisopropylsilane (TIS) also prevent the realkylations of Cys by trityl cations (Pearson et al. 1989, King et al. 1990). The presence of EDT and H_2O can however result in the cleavage of Acm-protection of Cys and care should be taken when there is the possibility of the production of unwanted disulphide bridges (Singh et al. 1996). Overall the cleavage conditions can affect the yields of the synthesis and the optimization of cleavage step is very important as is the optimization of the coupling steps during peptide synthesis (Sole & Barany 1992, Albericio et al. 1990).

Table 4. Selected resins for solid phase peptide synthesis.

Name	structure	attached group	leaving group	cleavage notes
Wang		RCOOH	RCOOH	95% TFA, Fmoc-strategy
Merrifield		RCOOH	RCOOH	HF, TFMSA, Boc-strategy
Barlos, CTC, 2-Cl-trityl		RCOOH	RCOOH	1% TFA in DCM, protected peptide fragments, in Fmoc-strategy
Rink amide		RCOOH	$RCONH_2$	95% TFA, Fmoc-strategy
MBHA		RCOOH	$RCONH_2$	HF, TFMSA, Boc-strategy
BAL		back-bone amide	back-bone amide	95% TFA, both C→N and N→C direction synthesis

Monitoring of coupling and deprotection. Although SPPS is today well-documented and commonly considered as a standardized method, incomplete couplings and deprotections still occur. The growing peptide chain is prone to aggregation via hydrogen bonding as well as via hydrophobic interactions and the aggregation will result in the incomplete solvation of the peptide resin and the inaccessibility of reagents to the reaction site (Amblard et al. 2006). Incomplete reactions can result in the deletion of one or several

residues and the truncation of peptide chain. The reactivity of differently protected amino acids varies and if the washing steps are insufficient between the coupling and the deprotecting steps, also the dimerization of some residues may occur. During SPPS, it is not reasonable to monitor and characterize the reaction completions via the cleavage of intermediate product because it is very time and substance consuming. In the Fmoc-strategy, the deprotection of the *N*-terminal Fmoc group results in the formation of dibenzofulvene and its piperidine adduct. Both of these compounds can be monitored by UV-spectroscopy and this can be conducted automatically during automated SPPS (Amblard et al. 2006). It is also possible to monitor couplings via infrared spectroscopy (IR) between the coupling steps (Henkel & Bayer 1998). However, there is not always equipment and time to conduct such complicated monitoring and therefore a range of colorimetric tests are commonly used. Usually it is feasible to monitor the presence of free or blocked *N*-terminal *α*-*N*-group and this can be performed via color tests which produce differing visible color changes depending on the free or blocked amino group. These tests are only qualitative, not quantitative and they do not rule out incomplete reactions. Furthermore, when the length of the peptide chain increases or aggregation occurs, the results of the color test become more difficult to interpret (Amblard et al. 2006, Albericio & Carpino 1997). The presence of the free *N*-terminal *α*-*N*-group has been monitored via a simple ninhydrin test, usually called the Kaiser-test since it was developed by Kaiser and his collaborators (Kaiser et al. 1970). Ninhydrin test results can also be monitored photometrically (Sarin et al. 1981). This test is suitable for free primary amines which give an intensive blue color but there can be problems encountered with some amino acids as *N*-terminal residues e.g. Ser, Asn and Asp. Since proline is a secondary amine, it does not give the correct positive result instead producing red/brown color (Kaiser et al. 1970). The chloranilin-test can be used for secondary amines and challenging residues (Christensen 1979, Vojkovsky 1995). Recently, an improved, rapid and sensitive method for monitoring resin-bound primary and secondary alkyl and aryl amines has been described (Blackburn 2005). The coupling end point can be monitored via the use of a test based on the acid-base indicator chromophenol blue but this test emits a positive result in the presence of any resin-bound base (Krchnak et al. 1988). Other functional groups can also be monitored during peptide synthesis and one commonly used test is the Ellman test for free sulfhydryl groups (Ellman 1959). The Ellman test can be used to follow the progress of the oxidation reactions of cysteines.

Difficult sequences. Although unsuccessful syntheses are not usually published, there are many report describing "difficult sequences" during peptide synthesis. Well-known examples are oligoalanine when it contains more than five residues and β-amyloid peptides, which are apparently difficult to synthesize with high yields using standard coupling and deprotection protocols (Abdel Rahman et al. 2007, Hachmann & Lebl 2006). The difficulties are attributable to intra- and/or intermolecular aggregation, secondary structure formation and steric hindrance of bulky residues and protecting groups. The tendency towards aggregation and folding will depend upon the nature of the peptide chain e.g. peptides with a high proportion of Asn, Gln, Ala, Val or Ile residues are associated with aggregation (Bacsa et al. 2008, Bedford et al. 1992). Hydrogen bonds, usually between the backbone amide hydrogen and the carbonyl oxygen, as well as hydrophobic interactions of hydrophobic residues are the main reason for secondary structures undergoing aggregation. Intrachain interactions result in reverse turns and

interchain interactions commonly lead to the formation of α-helixes or β-sheets (Bacsa et al. 2008, Albericio & Carpino 1997). The interchain interactions can become evident already when the fifth residue is coupled. Thus, coupling difficulties can exist even when a short peptide (5-15 residues) is being synthesized (Bacsa et al. 2008, Albericio & Carpino 1997). Incomplete acylation of the α-amino group during the coupling steps are commonly associated with β-branched amino acids such as Ile, Val and Thr (Milton et al. 1990, Albericio & Carpino 1997). The simplest way to overcome coupling difficulties is to test different coupling cocktails, variable coupling times and temperatures or to use double coupling for a specific residue but if the problems are associated with aggregation, then the disruption of hydrogen bonds may be a reasonable option. Firstly, the low-load resin (0.1-0.2 mmol/g) can be tested (Pugh et al. 1992, Tam & Lu 1995). Secondly, elevated temperatures can be used although there will be an increased probability of racemization and undesired side reactions such as asparimide formation (Bacsa et al. 2008, Albericio & Carpino 1997, Souza et al. 2004). An elevated temperature can be achieved rapidly via MW-assisted heating and for example the commonly known difficult sequence containing multiple alanines has been efficiently synthesized using MW-heating (Abdel Rahman et al. 2007). Thirdly, the coupling efficiency can be enhanced using polar solvent such as DMF, NMP, DMSO and 2,2,2-trifluoroethanol (TFE) or mixture of these solvents (Miranda & Alewood 1999, Albericio & Carpino 1997). Aggregation can also occur via hydrophobic interactions when the resin is polystyrene-based or the sequence contains the regions of apolar protection groups or residues. In that case, the addition of some nonpolar solvent such as DCM in polar solvents can be used (Albericio & Carpino 1997). Furthermore, the so called magic mixture consisting of ethylene carbonate and non-ionic detergent Triton X100 in combination with DMF and NMP can be used for breaking secondary structures (McNamara et al. 2000[2]). Fourthly, aggregation can be prevented using denaturing agents or chaotropic salts such as LiCl, LiBr, KSCN and NaClO$_4$ (Amblard et al 2006, Albericio & Carpino 1997). These agents can increase resin swelling and disrupt the β-sheet formation. Fifthly, the use of the previously mentioned backbone amide protection groups such as Hmb (Packman 1995) or the introduction of cleavage cocktail labile derivatives of amino acids such as dimethyloxazolidine dipeptides (*Figure 11*, see page 24) when sequence the contains Ser or Thr (White et al. 2004) can be used during chain elongation to help in the prevention of aggregation.

It has to be noted that every coupling and deprotection and also every cleavage step rarely results in equivalent yields. Therefore, the crude product will contain a mixture of incomplete sequences. Thus when SPPS is used, the purification and characterization steps are as important as the synthesis itself. To conclude, it can be stated that a new synthetic peptide/peptide derivative can be a very unique project. A molecule seems to be usually synthesized by the methods currently in use in the particular laboratory. Thus, the optimization of methods and the maximization of yields seem not to be the first steps undertaken when conducting peptide research and chemistry.

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[2] Original publication: Zhang, L., Goldammer, C., Henkel, B., Zühl, F., Panhaus, G., Jungs, G. & Bayer, E. 1994, '"Magic mixture', a powerful solvent system for solid-phase synthesis of 'difficult sequences'", In Epton, R. (ed.), Innovation and Perspectives in Solid Phase Synthesis, Mayflower, Birmingham, pp. 711–716.

1.2.4 Chemical optimization of peptide sequence

The chemical optimization of peptides for therapeutic agents is often intented to improve their bioavailability, reduce their biodegradation and elimination and increase their selectivity and affinity toward target (see section 1.1.2.). The chemical optimization strategy for therapeutic peptide is based on structure-activity relationship (SAR) studies between peptides or their modified analogs and the biological activity achieved. Most commonly, the chemical optimization of the peptide lead is based on experimental techniques such as residue replacement and progressive deletions. The definition of the peptide active 3D-structure, if it can be possibly determined, is additive information and may enable further the use of supplemental computational modeling (Falciani et al. 2005). Lead peptides can be investigated via biophysical studies including multidimensional NMR, X-ray crystallography, quantitative structure-activity relationship (QSAR) and molecular dynamic simulations. However, the structural information obtained may be somewhat limited because of the flexibility of peptides, especially short and linear compounds or because of inadequate information about the structure of peptide ligand target. It is very questionable whether the structure of ligand, target or target-ligand complex determined via NMR or X-ray crystallography will all be identical to those occurring in solution or involved in biological interactions (Hruby 2002, Grauer & König 2009). The flexibility of the peptide bond structure is somewhat restricted via amide-imidic acid tautomeria, but in addition every amino acid has two degrees of conformational freedom, N–C$_\alpha$ (Φ) and C$_\alpha$–CO (Ψ) bonds. In the early and pioneering work of Ramachandran and his colleagues, it was found that the conformational freedom of amino acids cover about one-third of total structural space available (Ramakrishnan & Ramachandran 1965). As a consequence, it can be observed that short polypeptides consisting of 20 naturally encoded amino acids rarely exist in stable conformations.

In addition to SAR-studies, naturally occurring bioactive peptides can be used as a guide for peptide modification. For example, the toxins of several venomous animals are peptides or peptide derivatives and in order to function as lethal peptide vemoms, they must be able to resist enzymatic degradation. The modifications present in peptide venoms include disulphide bonds formation for the stabilizing structure, amidation (*C*-terminal), sulphation (Tyr), bromination (Trp), glycosylation (Thr), γ-carboxylation (Glu), hydroxylation (Pro), pyroglutamation, head-to-tail cyclization and isomerization to D-amino acids (Lewis & Garcia 2003) .

The process in which the peptide is converted into a drug or drug-like molecule can be crudely divided into three groups (Adessi & Soto 2002, Grauer & König 2009). At first, the focus is to modify the peptide without altering the peptide bonds. This can be done via peptide length optimization, amino acid sequence optimization, via chirality changes (L to D), via non-genetically encoded amino acids, via *N*- and *C*-terminal end modification and via backbone *N*-amide alkylations (*Figure 13*). Cyclization can also be used to stabilize peptide conformational flexibility without dramatically altering the peptide pharmacophore. Cyclization will be discussed in its own section subsequently. The second step is to alter the peptide bonds by replacing chemical groups and produce amide bond surrogates, peptoids and azapeptides (*Figure 13*). The last step is to produce peptide mimetics, organic compounds whose essential elements (pharmacophore) mimic a natural peptide or protein in 3D space. The Peptide mimetic has to retain the ability to interact with the biological target and to produce the same biological effect as the parent peptide (Vagner

et al. 2008). Since the peptide mimetic may be a totally non-peptide molecule, peptide mimetics will be only briefly discussed in this thesis referring only to the short structural motifs of peptides.

The first step after the lead peptide structure has been obtained is to determine the key amino-acid(s) responsible for biological activity. Usually the biologically active peptide sequence covers a relatively large binding site on the surface of the target such as the protein or a membrane component and traditionally the length of the sequence responsible for the biological effect can be derived via truncation or deletion of its lead sequence (Hruby 2002, Vlieghe et al. 2010). During truncation, the peptide is shortened from the *N*- or *C*-terminus at one residue at a time to determine the shortest biologically active sequence. One or more consecutive residues can also be omitted from the middle of the sequence. One common example of successful truncation and key residue determination was work done with somatostatin (SST-14, *Figure 2*, see page 10) a peptide hormone containing 14 residues. Veber and his collaborators discovered that residues 7-10 were mainly responsible for the biological activity of SST:s. They developed cyclic hexapeptide analogs with one non-naturally encoded amino acid, ω-aminoheptanoic acid (Aha), to stabilize the cyclic conformation and to decrease the *in vivo* reduction of the disulphide bridge (Veber et al. 1979, Veber et al. 1981). Finally, the most widely used analog of SST, ocreotide, was developed by replacing Aha with D-Phe in the *N*-terminus and Thr- derivative L-treoninol in the *C*-terminus resulting in analog with a total of 8 residues (Bauer et al. 1982, Harris 1994). Ocreotide was 3-times more potent in inhibiting the secretion of the growth hormone than the parent hormone SST-14 and its elimination half-life was elevated from 3 minutes up to 90 minutes (Harris 1994).
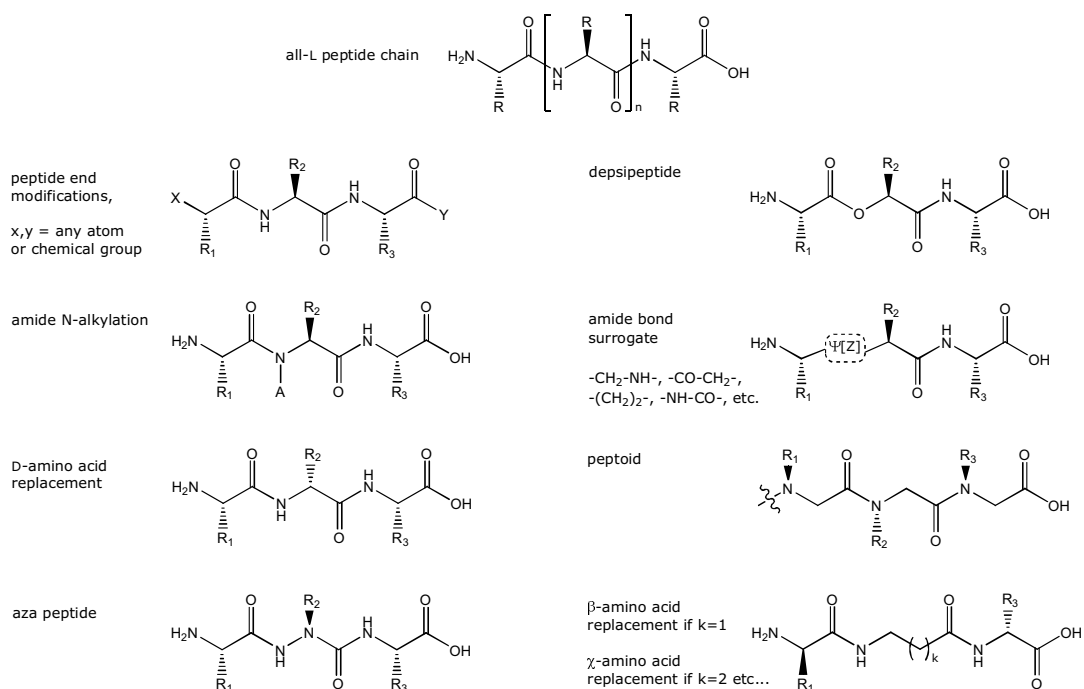
Figure 13. Schematic presentation of some commonly used peptide modifications, which are reported to increase proteolytic stability.

The importance of the side chains can be investigated via the so-called scanning procedure meaning the replacement studies of certain/all residues in the sequence by some other amino acids. The most used replacements are L-alanine scan which has been used for both peptides and protein and D-amino acid scan (Hruby 2002, Vlieghe et al. 2010, Falciani et al. 2005, Morrison & Weiss 2001). During an alanine scan, each amino acid in the sequence is replaced by an alanine, one at a time to examine the importance of the side chain for binding affinity and biological activity. The binding affinity and biological activity of Ala-replaced mutations can be determined using *in vitro,* even *in vivo* assays and the results can be related to the known or presumed function of the parent peptide. Substitution with alanine removes all side chain atoms beyond β-carbon but still maintains structural rigidity with the usual dihedral angles in the backbone. However, it should be noted that the conformational space covered by the methyl side chain of alanine is not the same as the space of bulky side chains present in groups such as Trp, Tyr and Phe. Thus, the conformational restrictions are much more limited compared to the bulky residues. Furthermore, the charge of the side chain of alanine is neutral and it will not form strong hydrophobic or hydrophilic interactions with other residues. However, during alanine scans, it is usually assumed that the overall conformation of peptide is not significantly changed (Hruby 2002). The most problematic amino acids during Ala-scans are Gly (no side chains, flexible), Pro (cyclic, restricted dihedral angles) and Cys (usually forms a disulphide bridge with another Cys). The Ala-scan technique was used successfully when the important residues of neurotensin were being monitored (*Figure 3* see page 11) and as a result Leu13 was found to be the most important amino acid (Henry et al. 1993). In the same study, also truncation was used to determine the shortest active sequence. In particular, the Ala-scan of somatostatin-14 (SST-14, *Figure 2*, see page 10) revealed the importance of residue Trp8 for biological activity and Trp has been incorporated into almost all somatostatin analogs during few decades when there has been intensive studies of this interesting peptide (Vale et al. 1975, Reissmann & Imhof 2004).

The positional or combinatorial D-amino acid scan has been used to eliminate the potential cleavage sites of peptidases, most notably endopeptidases. D-amino acids have also been found to be less immunogenic than L-amino acids (Adessi & Soto 2002) and D-amino acids have been shown to be able to stabilize certain reverse turns and destabilize α-helices (Hruby 2002). For example, substitution of Phe7 by D-Phe in α-melanocyte stimulating hormone (αMSH) led to the synthesis of a potent analog with prolonged activity since it avoided enzymatic degradation by serum enzymes perhaps also the β-turn stabilized the molecule (Sawyer et al. 1980, Sawyer et al 1982). D-Amino acid replacement studies have revealed the potential of the D-Trp substitution for Trp8 in somatostatin. This led to the synthesis of an analog with around 8 times higher activity (Rivier et al. 1975, Voyles et al. 1979). It was later concluded that Trp8 is essential for generating activity. Therefore, the D-Trp8-modification can be found in most of the SST-analogs including ocreotide (*Figure 2*, *Table 1*, see page 10 and 3, respectively) which is the much studied octapeptide analog of somatostatin (Bauer et al. 1982, Reissmann & Imhof 2004). D-amino acid substitutions can also be found in several other pharmaceutical peptides including desmopressin, atosiban and leuprorelin and even in cyclosporin A which is derived directly from a natural source (*Table 1*). One major drawback during D-amino acids replacement work is the possible loss of activity because of conformational changes. Therefore often only the residue near to the cleavage site is replaced. In some studies, resistance against

enzymatic degradation has been shown to be reduced when D-amino acids have been incorporated into the peptide. For example, the D-amino acid replacement in the scissile bond of Dermorphin, an opioid heptapeptide, was not as resistant against *in vitro* enzymatic degradation as the native peptide (Darlak et al. 1988). Chirality changes have been used also in the design of so called retro-inverso peptides (Adessi & Soto 2002, Chorev & Goodman 1995). The parent peptide sequence is synthesized in an inversed direction using D-amino acids. Structurally, the retro-inverso peptide maintains the side chain topology compared to the original sequence, only the peptide ends contain opposite charges and the peptide bonds are reversed. However, only a limited number of retro-inverso derivatives have displayed comparable activity to their native sequences, evidence of the clear importance of the backbone for the structure and binding properties of peptides (Grauer & König 2009). The retro-inverso synthesis can be used simply to partially cover the enzymatically unstable region of the sequence. The partial retro-inverso analog of dermorphin has been shown to maintain the biological opioid activity of the original peptide (Salvadori et al. 1985).

The importance of side chain groups for biological activity can also be examined by modifying the steric, acid-base and both hydrophilic and hydrophobic properties of certain key residues (Hruby 2002). Current knowledge in synthetical chemistry enables the design and synthesis of a wide variety of derivatives of genetically encoded amino acids and organic molecules containing amino and carboxylic functions. The structural knowledge of the ligand and binding site is extremely helpful when the modifications and replacements are being engineered but modifications can also be based on SAR-studies. It should be noted that the binding pocket can modify the ligand structure, at least the orientation of the side-chain can be altered (Hruby 2002). In brief, the selected residues in the enzyme recognition site can be modified or replaced by the structures which are close to the original amino acids. The simplest substitutions might be Ile for Leu, Tyr for Phe, Asn for Asp, Lys for Arg, norleucine (Nle) for Met or naphthylalanine for Phe and vice versa (Adessi & Soto 2002, Hruby 2002). However, the results of SAR-studies must be assessed carefully, when substitutions are considered, because sometimes one or two substitutions can result in very different biological activity. For example, the peptide hormone oxytocin, which causes uterine contraction and vasopressin, which is an antidiuretic hormone are identical in all but two residues i.e. their biological responses are very different (Suresh Babu 2011).

Oxytocin: H-cyclo[Cys-Tyr-**Ile**-Gln-Asn-Cys]-Pro-**Leu**-Gly-NH$_2$
Vasopressin: H-cyclo[Cys-Tyr-**Phe**-Gln-Asn-Cys]-Pro-**Arg**-Gly-NH$_2$

The functional groups containing side-chains can be modified relatively easily and it is not possible to cover all of the reactions here, just a few examples will be provided. Aromatic side chains can be modified via methylation or halogenations (Adessi & Soto 2002). For example, the chlorination of Phe resulting para-Cl-Phe in the *N*-terminus of enkephalin analogs has enhanced their analgesic effects, evidence that these analogs possess better penetration properties through the blood-brain barrier (BBB) (Weber et al. 1991, Weber et al 1993). The methylation of the Tyr (2,6-dimethyl-tyrosine, DMT) on the synthetic Met-enkephalin analog enhanced significantly the analgesic effect due to better BBB penetration and thus increased the bioavailability (Hansen et al. 1992). Hydroxyl groups of Thr, Ser and Tyr can be blocked reversibly via esterification or irreversibly via

methylation (Witt et al. 2001). Alkylation, including methylation of a hydroxyl group also reduces possibilities for hydrogen bonding and increases lipophilicity (Witt et al. 2001). The alkylation of side chain hydroxyl group can be found in the Tyr2 of the oxytocin antagonist atosiban (*Table 1*, see page 3).

Conformationally constrained derivatives of amino acids can be used to increase backbone rigidity. Proline is known to play a special role in the structural and conformational properties of peptides and proteins because of its cyclic and conformationally restricted structure (*Figure 14*). Proline plays a very important role in β-hairpin structures such as β-turns and it can be used along with its analogs to disrupt helical conformations and to form turned structures (Hayashi et al. 1997, Cowell et al. 2004). Modifications of Pro can be used to improve the stability and the activity of a peptide drug. Pseudoprolines, such as serine and threonine-derived oxazolidines, are also a helpful tool in the synthesis of difficult sequences as discussed before (Cowell et al. 2004, Adessi & Soto 2002). Another commonly used conformationally restricted, although non-naturally encoded residue is α-aminoisobutyric acid (Aib, also known as 2-methylalanine, *Figure 14*) (Cowell et al. 2004, Adessi & Soto 2002). Compared to alanine, the α-hydrogen is replaced by a methyl group resulting in an achiral analog with more steric hindrance creating a derivative which is known to promote the helix formation (Cowell et al. 2004). α-Hydrogen substitution with other bulkier substituents than methyl can be used to further increase steric restrictions (Cowell et al. 2004, Adessi & Soto 2002). Conformational constraints can also be included in β-carbon. A couple of examples of β-carbon modified residues are tert-Leu (Tle) in the neurotensin analog NT-VIII (*Figure 3*, see page 11) as well as penicillamine (Pen, thiol-D-valine, *Figure 14*) in the cyclic opioid peptide enkephalin analog c[D-Pen2, D-Pen5]-enkephalin (DPDPE) (Mosberg et al. 1983).

L-Proline; R_1, R_2, R_3 = H
Proline derivative if
R_1, R_2 or R_3 = any chemical group

Pyroglutamic acid, Pyr

α-aminoisobutyric acid, Aib; R_1, R_2 = CH_3
α,α-dialkylated glysine; R_1, R_2 = any alkyl group

Penicillamine, Pen
(thiol-D-valine)
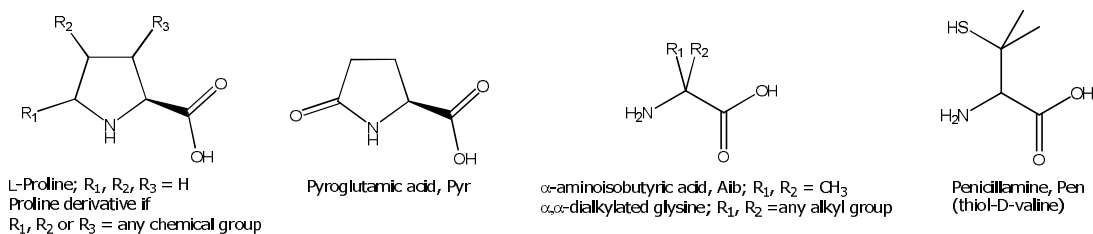
Figure 14. Proline and selected conformationally constrained non-natural amino acids.

Most L-amino acid peptides with free *N-* and *C-*terminus are degraded rapidly within minutes by exopeptidases (Adessi & Soto 2002). Since it is desirable to slow down or to block biodegradation via exopeptidases, the *N-*and *C-*termini can be modified using covalently bound groups such as an acetyl in the *N-*terminus or an amide in the *C-*terminus (*Figure 13* see page 33) or for example via head-to-tail cyclization. These peptide end modifications will affect the lipophilicity of peptides, for example *N-*terminal acetylation increases the lipophilicity of the resulting peptide (Witt et al. 2001, Adessi & Soto 2002). Many natural peptides hormones and neuropeptides such as the previously mentioned oxytocin and vasopressin are naturally protected using amino-acetylation or carboxy-amidation. Both protections are also found in α-melanocyte-stimulating hormone (αMSH). Therefore, αMSH is degraded practically by endopeptidases, not exopeptidases (Adessi & Soto 2002). Peptide end modifications are also common in peptide pharmaceuticals, for example a *C-*terminal amide can be found in liraglutide, desmopressin, oxytosin,

enfuvirtide and calcitonin (*Table 1*, see page 3). An example *of N*-terminal acetylation can be found in enfuvirtide (*Table 1*). The intra residual cyclization of *N*-terminal Glu resulting in pyroglutamic acid (Pyr, *Figure 14*) can also be used for *N*-terminal protection against exopeptidases, for example this can be found in the natural peptide hormone neurotensin (*Figure 3*, see page 11) and the peptide drug leuprolerin (*Table 1*). Both *N*-terminal acetyl and additional *N*-terminal pyroglutamyl has been shown to completely resist the *N*-terminal degradation of glucagon like peptide 1 (GLP-1) by dipeptidyl peptidase. In addition *N*-terminal modifications can improve plasma half-life significantly (Green et al. 2004). Peptide end modifications are commonly used as standard procedures in the synthetic chemistry of peptides, for example a wide variety of *C*-terminal end modifications can be directly added via the choice of the resin and the cleavage method (Alsina & Albericio 2003). However, it has to be noted that end modifications rarely prevent enzymatic degradation completely, it is only the activity of exopeptidases that is impaired or prevented, not the endopeptidases. In fact, the modifications intended to stabilize peptide or protein drugs towards endopeptidases are commonly more challenging than protecting the *N*- or *C*-terminal sites from exopeptidase cleavage (Werle & Bernkop-Schnürch 2006).

The alkylation of the amide nitrogen has been discussed before in the consideration of its use in peptide synthesis, but it is also one of the most commonly used methods to stabilize the peptide bond against enzymatic degradation by endopeptidases. The *N*-alkylation also produces a conformational constraint on the peptide bond, promoting the cis-conformation and eliminating the ability of the hydrogen bond to undergo amide hydrogen (Cowell et al. 2004, Adessi & Soto 2002, Teixido et al. 2005). Systematic *N*-alkylation can be used to improve biostability and it represents a powerful tool in SAR-studies (Vagner et al. 2008, Hruby 2002, Grauer & König 2009). *N*-Methyl scanning has been conducted on somatostatin octapeptide agonists and antagonists and different *N*-methylations have been shown to affect receptor subtype selectivity (Rajeswaran et al. 2001a, Rajeswaran et al. 2001b). Mother nature, especially many marine species, produces several *N*-methylated peptides which have been the topics of various conformational studies. These molecules have also been subjected to synthetic studies because of the much reduced reactivity of *N*-methylated residues (Adessi & Soto 2002, Teixido et al. 2005). One of the most widely studied peptide, which contains *N*-methylated residues, seems to be cyclosporin A (*Table 1*, see page 3). This cyclic undecapeptide contains seven *N*-methylated residues and although it is a short peptide, it is exceptionally conformationally rigid. Proline can be considered also as an *N*-alkylated amino acid. Thus proline analogs with differing ring substituens as well as other cyclic amino acid derivatives with differing ring sizes are commonly used to achieve conformational restrictions (Cowell et al. 2004).

In addition to the above mentioned modifications, several other additional chemical groups can be attached to the *N*- or *C*-terminus, to side chains, to the backbone amide nitrogen or to α-carbons. Glycosylation (glucose, xylose, hexose etc.) can be used to increase plasma stability as a peptide end modification. Glycosylation can also be used for enhancing hydrophilicity or improving biodistribution to the brain via BBB nutrient carriers (Vlieghe et al. 2010, Witt et al. 2001, Egleton et al. 2000). Polymer conjugation, most commonly the addition of a variable PEG chain, can be used to increase stability against enzymatic degradation, to decrease elimination by increasing peptide size making the molecule too large for renal clearance, to decrease toxicity and to decrease protein binding.

PEGylation can also be used in the desing of potential control-release drugs (Veronese & Pasut 2005, Witt et al. 2001). Lipidization, i.e. conjugation with a hydrophobic lipid molecule, is another way to increase the membrane permeability of peptide. Lipidization can also result in increased plasma protein binding of the hydrophobic conjugates (Witt et al. 2001). This can be used if one wishes a controlled administration rate as in the case of the peptide-based drug liraglutide (*Table 1*). The addition of the C16 fatty acid moiety to Lys20 resulted in binding of the drug to serum albumin, which extended its biological lifetime to permit once daily injection (Stevenson 2009).

Peptide backbone modifications via the alterations or replacements of some atoms in the peptide backbone result in the formation of a molecule which is not peptide but an analog called a pseudopeptide or a peptidemimetic, depending on the source material. According to one definition, pseudopeptide is any peptide analog which has a peptide backbone modification such as amide bond surrogates or isosteres, peptoids or azapeptides (*Figure 13*, see page 33) (Adessi & Soto 2002). Nevertheless, the introduction of the peptide backbone modification is targeted against enzymatic degradation but can be also used to control structural flexibility/rigidity and therefore for example in the selectivity of peptide-based pharmaceutical. Furthermore, there may be different types of backbone modifications e.g. those intended to modify hydrophobicity, hydrogen bonding ability and the length of the backbone (Vagner et al. 2008, Adessi & Soto 2002). The peptide bond (-CO-NH-) replacements can refer to the alterations of carbonyl- or amino-moieties or both of them (*Figure 13*). *N*-alkylations have been already discussed above and they are not discussed here. The carbonyl group can be reduced (-CH$_2$-NH-); this change can be found for example in the neurotensin analog NT-IV (*Figure 3*, see page 11). Furthermore, the carbonyl group can be modified to endothiopeptide (-CS-NH-) or phosphoamide [-PO(-OH)-NH-]. The amide-NH can be replaced by O (-CO-O-, depsipeptide), by S (-CO-S-, thioester) or by –CH$_2$- (-CO-CH$_2$-, ketomethylene). The whole peptide bond can also be modified to make it a retro-inverso peptide (-NH-CO- bonds) or some of the amide bonds can be replaced by thiomethylene bonds (-CH$_2$-S-), dicarba bonds (-CH$_2$-CH$_2$-) or hydroxyethylene bonds (-CHOH-CH$_2$-) (Vlieghe et al. 2010, Adessi & Soto 2002). Recently, the incorporation of ester and urea bonds and the ester-urea motif instead of peptide bonds has been shown to reduce the aggregation ability of peptide sequences due to the loss of the backbone's hydrogen bonding ability (Hartwig et al. 2010).

Peptides with the α-carbon replaced by nitrogen are called azapeptides (*Figure 13*). The azapeptide bond lacks the α-hydrogen resulting in the loss of chirality and a reduction of flexibility; those alterations to the specific secondary structure will tend to influence the recognition and cleavage of the modified bond by proteases (Zega 2005). Therefore azapeptides have been used extensively as protease inhibitors, for example targeting cysteine and serine proteases (Magrath & Abeles 1992). Recently, a method for conducting an azapeptide scan using MW-assisted heating has been published (Freeman et al. 2011). A selective linear heptapeptide inhibitor for activated protein kinase B (PKB/Act), an enzyme which is associated with many human cancers, was aza-scanned and its stability against proteases trypsin and chymotrypsin was examined (Tal-Gan et al. 2011b). The inhibition potency declined while the stability increased and the authors emphasized that this synthetical method enabled a rapid azapeptide scan in SAR-studies.

Another type of modification, which yields the loss of chirality in the α-carbon is the use of peptoids (*Figure 13*, see page 33). The side chain of amino acid is "switched" by one

position to the amide nitrogen yielding *N*-substituted glycines. This results in the creation of a more flexible structure and the loss of recognition by proteases (Adessi & Soto 2002, Patch & Barron 2002). Peptoids are based on the proteinlike backbone with both the hydrophobicity and charge being readily modified. Furthermore, these molecules can adopt helical structures (Patch & Barron 2002). A peptoid has been used in the synthesis of the antibacterial magainin mimetics (Patch & Barron 2003) and in the development of potent and selective somatostatin analogs (Tran et al. 1998).

The last possibility for converting a bioactive peptide into a drug is to create a peptidomimetic. One definition for a peptidomimetic is that it is a molecule which no longer contains peptide bonds (Adessi & Soto 2002). However, a peptide, which contains non-natural structures mimicking the local topography of peptide such as peptide bond isosteres and retro-inverso peptides, can also be considered to be a kind of peptidomimetic. These mimetics often match the peptide backbone length atom-for-atom and retain functionality (pharmacophore) allowing them to make important contacts with binding sites. According to Ripka and Rich, this type of peptidomimetics can be classified as Type-I peptidomimetics (Ripka & Rich 1998). A peptide mimetic can also be a small non-peptide molecule that binds to the peptide receptor, being as a functional mimetic and these molecules can be classified as Type-II peptidomimetics (Ripka & Rich 1998). Functional mimetics do not necessarily resemble the structure of the parent peptide. Morphine was one of the first structures identified as a peptide mimetic because it is functional mimetic to Leu5- and Met5-enkephalins cabable of binding to the morphine receptor (Adessi & Soto 2002). Type-III mimetics have novel templates which contain the necessary groups positioned on a novel non-peptide scaffold to serve as topographical mimetics. These molecules can appear to be unrelated to the original peptide and thus represent the ideal in peptide mimetics (Ripka & Rich 1998). Peptide mimetics can be rationally designed or can be identified from randomly obtained structures. However, the rational design of the peptide mimetic requires knowledge of the electronic, conformational and topochemical properties of the native peptide in its target (Grauer & König 2009). Traditionally, random screening has been the most successful method for producing peptide mimetics but developments in synthetic and combinatorial chemistry combined with computational improvements in conformational analysis (NMR, X-ray), molecular modelling and virtual screening-based methods are today making an important contribution to peptide mimetic research.

The flexibility of peptides and the dependence of their structure on their environment is still a major problem in the peptide mimetic research. Therefore, the most widely used peptide mimetics are unnatural amino acids and dipeptide surrogates for short structurally constrained regions such as β-turn mimetics (Vagner et al. 2008). β-Turns are the reversed turns commonly present in protein structures and they are stabilized via hydrogen bonding between the adjacent residues in the turns. The β-turns consist of four adjacent (i – i+3) residues and typically the residues in the corner of turns (i+2, i+3) are replaced by non-natural dipeptide mimetics (Kieber-Emmons et al. 1997) with a variety of typically bicyclic structures being utilized (Cowell et al. 2004, Vagner et al. 2008). Another group of commonly used molecules in peptide mimetic research are β-amino acids. The backbone of β-amino acid between the amino and carboxylic group includes an additional carbon atom compared to the α-amino acids. The structurally simplest β-amino acid is called β-alanine i.e. 3-amino propanoic acid. The advantages of β-peptides include their ability to form

predictable helical structures and their increased stability against peptidases (Gademann et al. 2001). β-Amino acids have been used in many experiments, for example in the synthesis of a β-tetrapeptide derivative which mimics somatostatin in its binding properties (Gademann et al. 2001). β-Amino acids and β-peptides have also been used in the design and synthesis of peptide mimetics e.g. antimicrobial peptides such as magainin (Patch & Barron 2002, Patch & Barron 2003). The helical conformation can be affected by the substituents in the α- and β-carbon and a knowledge of the structural behavior enabled the de novo design of synthetic mimetics of antimicrobial peptides (Patch & Barron 2002, Scott et al. 2008). α-Helical structures can be mimicked also by using both α-helix inducing linkers such as side chain to side chain bridges or by using totally non-peptidic molecules. In addition to β-turn mimetics and helical mimetics, also various β-sheet mimetics have been developed and studied. β-Sheets are common structural motifs in proteins. Peptides can be stabilized into artificial β-sheets resembling the conformation using non-natural building blocks (Grauern & König 2009).

The peptide hormones somatostatin and bradykinin are two molecules which are served as model compounds to examine and explore what can be done when the lead peptide is transformed and/or modified to a peptide-based pharmaceutical (Stewart 2007, Janecka et al. 2001, Harris 1994, Reissmann & Imhof 2004). Somatostatin (*Figure 2* see page 10) is almost ubiquitous in the brain and the gastrointestinal tract possessing a wide variety of properties including the modulation of motor and cognitive functions, acting as neurotransmitter and the causing the inhibition of nutrient absorption (Janecka et al. 2001, Harris 1994, Reissmann & Imhof 2004). Bradykinin also possesses many physiological and pathological roles e.g. its involvement in the inflammatory response (Stewart 2007, Reissmann & Imhof 2004).

1.2.5 Peptide cyclization

The cyclization of peptide is a major tool for the introduction of global conformational restriction into the peptide sequence. The cyclization reduces conformational flexibility and may thus enhance potency, selectivity and specificity for a particular receptor. Cyclization may also reduce hydrogen bonding and increase lipophilicity and in that way improve membrane barrier permeability and bioavailability (Li & Roller 2002, Adessi & Soto 2002, Witt et al. 2001). In particular, head-to-tail cyclization is a very powerful tool against exopeptidases because the peptide no longer contains ionizable *N*- and *C*-terminal residues. The proportion of cis-amide bonds is also higher in cyclic peptides than their linear counterparts preventing enzymatic degradation (Li et al. 2002). The more constrained geometry can be utilized in conformational studies, SAR-studies and molecular modeling because of the more predictable structure, especially when the ring size is small (Li et al. 2002, Landon et al. 2004). Many naturally occurring bioactive peptides are cyclic. Cyclosporin A (*Table 1*, see page 3) is head-to-tail cyclic peptide whereas oxytocin, vasopressin and somastostatins all contain a disulphide bridge (*Figure 2*). Several peptide-based pharmaceuticals also contain the cyclic structural motif (*Table 1*, see page 3).

Methods for cyclization are traditionally divided into two classes, the backbone to backbone cyclization and the cyclization between side chains (Li & Roller 2002, Davies 2003). Head-to-tail backbone cyclization and disulphide bridge formation between side chains are the most notable examples although the development of sophisticated chemical, biophysical and computational methods has resulted in an enormous number of

possibilities with which to derive cyclic structures. However, in most cases one has to be start from the basics, especially when structural data is limited, and some aspects of heat-to-tail macrolactamization and disulphide bridge formation and the properties of disulphide bridge mimetics will be discussed in the next sections.

   The cyclization of a peptide can be performed homodetically (all amide bonds) via the amide bond between *N*- and *C*-terminus resulting in the production of a head-to-tail cyclic peptide. The amide bond can be also formed between the side chain amino group and the *C*-terminal carboxyl group, between the side chain carboxyl group and the *N*-terminal amine or between the side chain amine and the side chain carboxyl group (*Figure 15*) (Adessi & Soto 2002). Cyclization via the amide bond is also called lactam bridge formation. A cyclic peptide is called heterodetic if the bridge is formed in some way other than amide bonding. The most common heterodetic cyclization is attained via the disulphide bridge between two cysteine residues (*Figure 15*). A disulphide bridge can be found universally as a protein 3D-structure stabilizing component. Peptides can be cyclized heterodetically also via the ester bond (lactone), ether, thioether (lanthionine) or hydrocarbon bridges (Adessi & Soto 2002). Cyclization can be performed also using different specific linkers or spacers with variable lengths or chemical structures. The use of the parent peptide side chains for cyclization can achieve to the prevention of the interaction between the peptide and its receptor. The possible involvement of the side chains of peptide in the interactions with receptors can be circumvented in some cases by backbone cyclization (Reissmann & Imhof 2004). In backbone cyclization, two N^{α} and/or C^{α} can be substituted and linked to each other to a yield cyclic molecule. Backbone cyclization chemistry can be more challenging than the cyclization chemistry via side chains or between N- and C- terminis mainly due to sterical reasons (Reissmann & Imhof 2004).

   The cyclization site and procedure including the length of the bridge, structural motif and chemistry being used etc. are highly dependent on the available structural data because the biological activity of the parent peptide can be lost when conformational restrictions are used in the wrong position or/and with the wrong composition. Without any or with only limited knowledge of 3D-conformation, the systematic cyclization between different positions and SAR-studies based on this cyclic scan products might be the only way to discover the cyclic bioactive peptide (Reissmann & Imhof 2004). A knowledge about particular conformational regions including the α-helix and β-turn is usually beneficial and the cyclization can be targeted to stabilize those local conformations. Cyclization can be performed using residues already present in the parent sequence. Residues to be utilized in cyclization can be also added via replacing residues which are not important for biological activity (Hruby. 2002). This type of cyclization scan has been used in the systematic positional-cyclization of glucacon to clarify the structural motifs of glucagon (Ahn et al. 2001). In the study of Ahn and his collaborators, the selected residues, which were not essential for binding affinity, were replaced by cysteines for disulphide bridge formation or by lysine and glutamine for lactam bridge formation. Cyclization via disulfide bridges between Cys_i and Cys_{i+5} was used to induce and stabilize turn conformations, while cyclization via the lactam bridges between Lys_i and Glu_{i+4} was intended to induce the helical conformation. As a result, the helical structures were achieved in the *N*- and *C*-terminal regions of glucagon, a peptide containing 29 residues (Ahn et al. 2001). Recently, backbone cyclic peptide libraries with varying ring sizes, bridge chemistry and modes of cyclization were synthesized to enhance the pharmacological properties of a linear heptapeptide

inhibitor of activated protein kinase B (PKB/Act), an enzyme associated with many types of human cancers (Tal-Gan et al. 2011a). The synthesis and SAR-studies were performed by creating over 60 linear and cyclic analogs and these molecules were then evaluated for in vitro PKB/Akt inhibition. As a result, more potent analogs of selective PKB/Akt inhibitors were identified and the importance of addressing all conformational parameters such as ring position, ring size, ring chemistry and the mode of cyclization were clarified (Tal-Gan et al. 2011a).
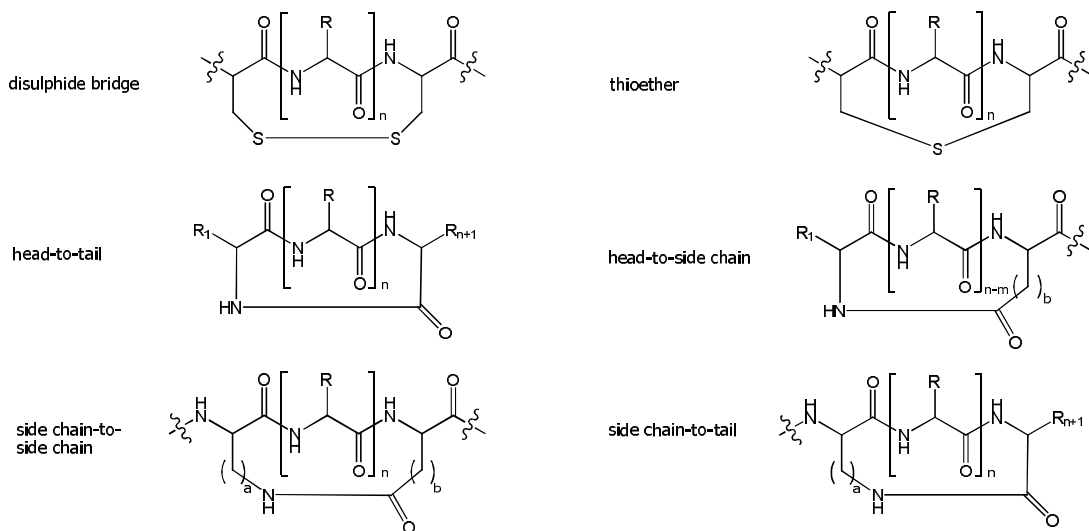
Figure 15. Some representative cyclic peptide structures, a,b ≥ 1.

During head-to-tail synthesis macrolactamization is the rate-limiting step. Thus it was formerly performed in solution using a highly diluted concentration of partially protected peptide in order to avoid the dimerization or polymerization of peptide (Davies 2003). The slow cyclization step resulted in tetramethylguanidium mediated end capped amino group when uronium-based activators were used for cyclization step and the racemization of *C*-terminal residues commonly occurred (Davies 2003). From the beginning of 1990, cyclization on-resin gained popularity and the growing peptide chain was anchored to the resin via the side chain of *C*-terminal residue or *C*-terminal carboxylic acid (Li & Roller 2002, Davies 2003). One of the most useful methods is the use of allyl-protection for α-carboxylic group of Asp or Glu which, depending on the resin being used, yield head-to-tail cyclic peptide containing Asp or Asn and Glu or Gln at the resin cleavage site (Kates et al. 1993). The peptide can also be cyclized via the so-called cleavage-by-cyclization method (Li & Roller 2002, Davies 2003). In this method, the peptide is anchored to the resin via a specific eletrophilic linker which is prone to nucleophilic attack by a free basic amino group and the cleavage occurs during this attack (Davies 2003). For example, a cyclic human calcitonin fragment analog has been synthesized using the cleavage-by-cyclization method (Kapurniotu & Taylor 1993). The third method for backbone cyclization is the use of backbone amide linker (BAL) which permits standard peptide chemistry and cleavage steps (Davies 2003, Jensen et al. 1998, Li et al. 2002). For example, a cyclic peptide library based on the somatostatin sequence has been synthesized using the backbone amide linker and

the usefulness of the BAL-method for the synthesis of library was highlighted (Bourne et al. 2001).

The history of peptide backbone cyclization contains numerous publications with techniques reported using a wide variety of chemistries but still the cyclization step and sequence dependence on the cyclization potency are the major yield-limiting factors (Li & Roller 2002, Davies 2003). The most important factor is the length of sequence to be cyclized and the influence of residues to be included in the sequence. Cyclodipeptides, in other words diketopiperazines (*Figure 12*, see page 24), are usually not wanted as earlier mentioned, but if desired, they can usually be easily prepared (Fischer 2003). Cyclotripeptides are almost impossible to construct with all-L amino acids because all of the residues should adopt cis-conformation. If one uses *N*-alkylated residues or β-amino acids, then cyclotripeptides can be produced (Davies 2003). Cyclotetrapeptides also are unfavourable if one tries only to incorporate L-amino acids because the ring should contain a trans-amide bond in all residues (Davies 2003). The universal cell-recognition sequence RGD, found in ligands binding to integrins, has attracted considerable attention for example in cancer research. The RGD sequence has also been intensively studied in the peptide chemistry field, for example in a study of the cyclization of tetrapeptides including the RGD-sequence (Alcaro et al. 2004). The cyclization of penta- and hexapeptides is a challenging task if the sequence does not contain any turn-inducing residue such as a D-residue or Gly/Pro (Davies 2003). Certain factors such as the choice of the terminal residues, the turn inducing properties and activators for cyclization have been studied to see how they influence the cyclization of pentapeptides (Tang et al. 2002). The same factors have been evaluated in heptapeptides. When the number of residues in the cyclic peptide is seven or more, the ring system becomes more and more flexible and there is no universal method available for cyclization (Davies 2003). There are a couple of backbone cyclic peptides worth considering from the synthetic point of view, the cyclodecapeptide gramicidin S with Gram-positive activity and the previously mentioned cycloundecapeptide cyclosporine A, which are subjects of tens of thousands of publications including numerous synthetical studies (Davies 2003).

The high frequency of disulphide bridges between two Cys-residues in the natural peptides and protein fueled the research and developments in the structural characteristics and synthetic chemistry of disulphide bridges. For example, disulphide bridges can be found in many natural hormones somatostatin, oxytocin, calcitonin and insulin (*Table 1*, see page 3). The intramolecular disulphide bonds play a fundamental role in the stabilization of bioactive conformations such as loops and turns in peptides and proteins, and these are usually crucial for biological activity. The disulphide bridges are important in protein folding and if the protein is denatured, the disulphide formation is responsible for destabilizing the denatured state (Li et al. 2002).

During the past few decades, the chemistry concerning disulphide bridge formation has been intensively studied (Annis et al. 1997, Li & Roller 2002, Davies 2003), and the practical procedures are not discussed here in detail. The chemistry around disulphide bridge formation is focusing on the maximum amount of correct and the desired product and also targets the avoidance of unwanted bridge formation (mispairing) including dimerization and further polymerization in the presence of multiple cysteines. Unwanted disulphide bridges can be reduced back to free thiols but this recycling is time and reagent consuming. A disulphide bridge between two sulphur atoms can be formed in solution using high

dilution to avoid unwanted polymerization. Both free thiols and S-protected precursors can be used (Annis et al. 1997). A disulphide bridge can be created by spontaneous oxidation under air by using slightly alkaline reaction conditions. In that case, Trt as thiol-protecting group (*Table 3*, see page 21) is commonly used in the Fmoc-strategy, because Trt is already cleaved during the final cleavage of the peptide from resin. Often a disulphide bridge is formed by using some oxidative reagents, such as iodine or thallium trifluoroacetate. Both of these reagents also remove Acm-protecting group enabling the selective deprotection for cysteines (Annis et al 1997). Disulphide can also be formed on solid phase and the cyclization yields can be enhanced via using low-load resin (Albericio et al. 1991). On-resin cyclization is enhanced via so called pseudo-dilution phenomena, a kinetic phenomenon which favors intramolecular prosecces (Albericio et al. 1991). The Acm is a usable protection group for thiol also during on-resin cyclization process (Annis et al. 1997).

The main advantages of the chemistry of cysteines and disulphide bonds are the regioselective removal of protective groups permitting the formation of multiple specific disulfide bridges within the same peptide (*Figure 16*), the construction of oligomeric peptides in multi Cys-containing precursors and the nucleophilic conjugation of thiol group with variable agents such as labeling agents (Li & Roller 2002). Furthermore, if the lead peptide is derived from a phage display library, directly disulphide bridge constrained peptides can be screened (Landon et al. 2004). However, the disulphide bridge is known to be metabolically labile under reductive conditions as well as in the presence of nucleophilic and basic agents (Williams & Liu 1998) and thus it should be replaced in peptide based pharmaceutical by other structural units or by mimetics.
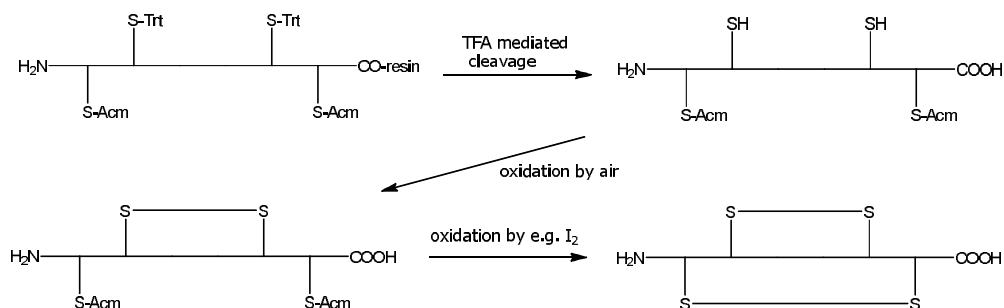
Figure 16. Regioselective formation of two specific disulphide bridge by using spontaneous air oxidation and iodine oxidation.

The structural importance of the disulphide bridge in somatostatin is commonly known and the disulphide bridge exists usually in most SST-analogs such as ocreotide. It has been demonstrated that the shortest active sequence is the cyclic hexapeptide including the active sequence and the disulphide bridge as a stabilizer for an active conformation (Reissmann & Imhof 2004). Another one well-known successful example of disulphide bridge incorporation is the development of the δ-opioid receptor ligand [D-Pen2, D-Pen5]-enkephalin (DPDPE, *Figure 17*), an analog of the opioid pentapeptide Met5-enkephalin (Mosberg et al. 1983). The replacement of Gly2 and Met5 by two Pen-residues and the oxidation of thiols to the disulphide bridge resulted in the creation of a highly potent and selective δ-opioid receptor ligand (Mosberg et al. 1983, Cowell et al. 2004). The disulphide bridge was proposed to constrain the biologically active conformation. Another example is the cyclic analog of the previously mentioned α-melanocyte stimulating hormone (αMSH)

which was found to be superpotent due to its stabilized β-turn structure via disulphide bridge although it did not exhibit as prolonged biological activity as the analog with the D-amino acid in the possible cleavage site for endopeptidases (Sawyer et al. 1982).
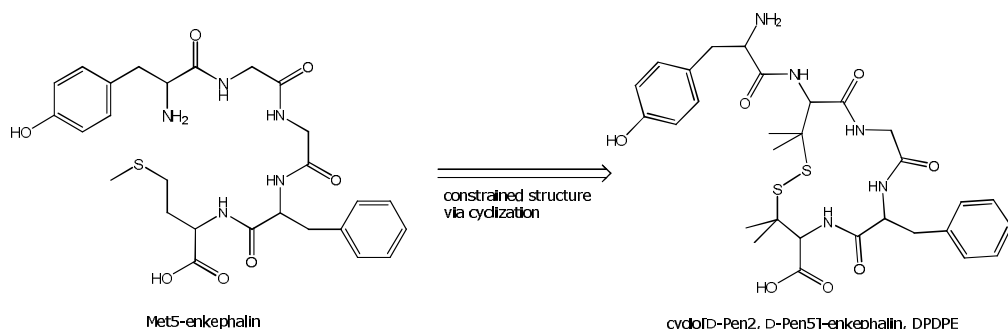
Figure 17. The structures of Met5-enkephalin and its analog DPDPE.

Disulphide bonds are known to be very important in proteins and one very interesting group is small, around 30 residues containing head-to-tail cyclic, and three disulphide bridges containing miniproteins, called cyclotides. Cyclotides are present in some plant materials and their main property is to defend the host. They also possess a diverse range of biological activities including anti-microbial, cytotoxic and anti-HIV activities (Cemazar & Craik 2006). Cyclotides have been shown to possess exceptional stability against enzymatic, thermal and chemical degradation mostly due to the cyclic backbone, the cystine knot formed by disulphide bridges and their extensive hydrogen bonding network. Several biological activities will be lost if disulphide bridges are removed and the cyclotides become linearized (Cemazar & Craik 2006).

The importance of the disulphide bridge in biological structures has fueled the research into disulphide bridge mimetics. The challenges of creating structures mimicking disulphide bridge is that the atomic radius of sulphur differs from other atoms present in peptides (e.g. C, O, N). The dihedral angle between S-C and S-S is almost 90° and thus it differs from the bond angles in peptide bonds or all-hydrocarbon linker bonds. Furthermore, cystine contains a total seven rotable bonds whereas all-peptide bridge contains only four rotable bonds in the area covering approximately the same size (*Figure 18*) (Stymiest et al. 2005, Cowell et al. 2004). By using all-hydrocarbon linkers it is possible to maintain the number of rotable bonds compared to the disulphide bridge, between residues which are connected to a bridge structure (*Figure 18*).
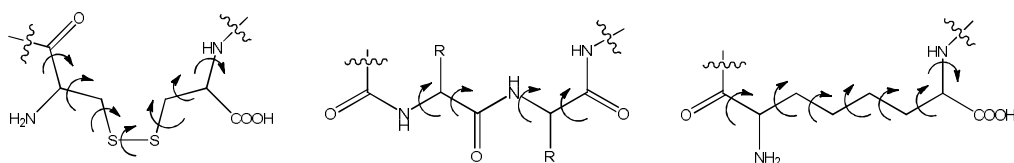
Figure 18. The structure and rotating bonds in the disulphide bridge (on the left), all-peptide bridge (in the middle) and olefin linker (on the right).

Bacterial microorganisms produce antimicrobial peptides such as nisin and epidermin derivatives, which are called lantibiotics because they contain a building block called lanthionine (Li & Roller 2002). Lanthionine consists of two alanines joined together via a

thioether bond between the β-carbons, in other words lanthionines are monosulfide analogs of cystine. The main advantage of thioether-cyclized analogs compared to the disulfide bridge containing peptide is its stability against redox conditions. Lanthionines have been successfully used in the formation of ocreotide analogs. When the disulphide bridge of the ocreotide (*Figure 2* see page 10) was replaced with a lanthionine bridge, the serum stability of lanthionine-ocreotide was increased by 2.4 fold (Osapay et al. 1997). In addition, highly potent and selective lanthionine analogs of above mentioned enkephalin derivative DPDPE has been developed (Rew et al. 2002).

Another widely used disulphide bridge mimetic procedure is the use of dicarba analogs to form olefin-linked cyclic structures. An olefin-bridge can be formed via ring closing metathesis (RMC) i.e. olefin metathesis and this has been shown to increase the metabolic stability and helicity of certain peptides. The olefin bridge can be used also for the stabilization of the peptide's secondary structures like β-turns and β-sheet (Miller et al. 1996, Grubbs 2004, Li & Roller 2002). Furthermore, it has been described in the study of Miller and his collaborators that dicarba analog of β-turn tetrapeptide Cys-Pro-Aib-Cys exhibit structural similarity with the parent peptide, based on NMR- and IR-studies (Miller et al. 1996). RCM can be performed using ruthenium-based catalysts which are air and water tolerant and they can be used with a wide variety of functional groups and protecting groups present in peptide synthesis (Li et al. 2002, Grubbs 2004). In general, in the synthesis of the dicarba analogs for disulphide bridge, the L-cysteines are replaced by (S)-allylglycines in the linear precursor of the peptide (*Figure 19*). After RCM-reaction, the olefinic double bond instead of the disulphide is formed (Li et al. 2002). This double bond can be further reduced to olefin (*Figure 19*). Recently, the dicarba analogs of both somatostatin (D'Addona et al. 2008) and oxytocin and its antagonist atosiban (*Table 1*, see page 3) (Stymiest et al. 2005) have been synthesized and their importance in structure-affinity studies and in a pharmacophore model was highlighted.
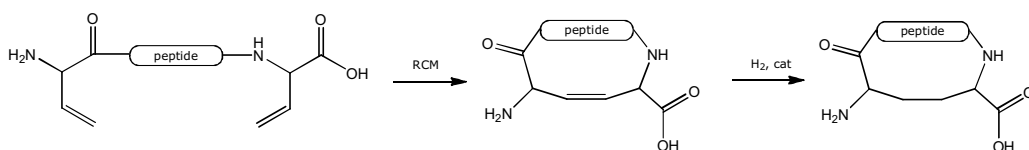
Figure 19. Cyclization of a peptide by using RCM reaction between terminal allylglycines and the reduction of a double bond to olefin

1.3 PROSTATE CANCER AND KALLIKREINS

1.3.1 Prostate Cancer

Prostate cancer (PCa) is the most common cancer in males and the third leading cause of cancer deaths after lung and colorectal cancers in the developed countries (Jemal et al. 2011). For example, in the United States, one in six men have a lifetime risk of receiving a PCa diagnosis and the chance of death due to PCa is 3,4% (Jemal et al. 2010). Most commonly, PCa only causes clinical symptoms when the cancer has spread to the surrounding tissues affecting directly on urinary function or after metastasis to the lymph nodes or other organs, mostly bones. Unfortunately, PCa displays a wide range of clinical behaviour, from clinically insignificant slow-growing tumors to aggressively metastatic and

fatal disease posing remarkable challenges in the diagnosis and treatment of prostate cancer. The risk of PCa has been monitored and detected via screening based on the determination of KLK3 in serum (Stenman et al. 2005, Catalona et al. 1991, Stamey et al. 1987). Eventually PCa diagnosis is based on the combined use of KLK3 assay, a digital rectal examination (DRE), transrectal ultrasonography (TRUS) and transrectal ultrasound-guided biopsies (Cooner et al. 1990).

Human kallikrein 3 (KLK3), formerly known as prostate-specific antigen (PSA) has been commonly used as a serum PCa marker for detecting early-stage PCa, for monitoring disease progress and for evaluating the therapeutic response. However, the disadvantages of the currently used KLK3 serum test are that it detects indiscriminately many types of prostate abnormalities. The level of KLK3 increases in the serum of a patient with nonmalignant prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatitis and thus the test is not cancer specific. These facts together with the reality that most PCa tumors grow slowly has lead to a remarkable rate of overdiagnosis with many patients having to undergo unpleasant examinations and unnecessary treatments such as radical prostatectomy or radiotherapy with its attendant side effects for benign or latent tumors (Lilja et al. 2008). The use of opportunistic screening with artificial limits of KLK3 in serum has caused dramatic increase in the PCa incidence although many prostate cancers patients would not have surfaced clinically without screening and the cancer would have posed no threat to life or health (Stenman et al. 2005). Thus, KLK3 is currently used as a marker for diagnosis but it has been stated that KLK3 values indicate only the relative degree of risk and there is no KLK3 concentration which could rule out cancer (Thompson et al. 2005). It is important to devise methods to distinguish clinically relevant tumors with an ability to metastasize using more specific markers and methods for more accurate prognosis are needed. KLK3 remains the most useful marker but a wide variety of candidates for new biomarkers of PCa have been described including KLK2, early prostate cancer antigen (EPCA), prostate cancer antigen 3 (PCA3) and hepsin and these have been widely discussed in the literature (Sardana et al. 2008, Steuber et al. 2008). The serum levels of KLK2 and the combination of KLK2 with different forms of KLK3 have been claimed to improve diagnosis and the prognosis of PCa (Recker et al. 2000, Steuber et al. 2007, Lilja et al. 2008, Becker et al. 2000). However, the search for markers or their combinations still continues (Sardana et al. 2008, Pavlou & Diamandis 2009).

1.3.2 KLK3

KLK3 is a serine protease which belongs to the kallikrein related peptidase family (Lundwall et al. 2006, Yousef & Diamandis 2001). It is a 28-kDa glycoprotein consisting of 237 amino acids and it is expressed as a proenzyme mainly in the epithelial cells of the prostate but it has been shown that minuscule amounts can be detected in some other organs such as mammary gland and ileum (Olsson et al. 2005, Shaw & Diamandis 2007). KLK3 has a chymotrypsin-like activity and its physiological role is to degrade gel-forming proteins semenogelin-I and –II, cleaving preferentially after tyrosine, leucine and glutamine residues, causing liquefaction and thereby promoting sperm motility (Lilja 1985, Malm et al. 2000, Robert et al. 1997). KLK3 is synthetized in healthy prostate but it is also expressed in prostate abnormalities. In both malignant and some non-malignant conditions like BPH, KLK3 is leaked to the circulation due to the altered tissue architecture (Stenman 1997). When it leaks into the circulation, most of the catalytically active KLK3 is inactivated by

protease inhibitors like α1-antichymotrypsin (ACT) and α2-macroglobulin (A2M) which inhibit any catalytic activity of KLK3 by forming stable covalent complexes (Christensson et al. 1990, Stenman et al. 1991). KLK3 also exists in blood in various noncomplexed forms such as pro-KLK3, intact and nicked KLK3 (Lilja et al. 2008).

The role of KLK3 in cancer is still largely unknown but it has been shown that KLK3 has an anti-angiogenic potential. KLK3 inhibits endothelial cell growth, invasion and migration both *in vivo* and *in vitro* indicating the reduction of activities needed for blood vessel formation (Fortier et al. 2003, Fortier et al. 1999). The mechanism for the anti-angiogenic activity is unclear. It has been shown that only enzymatically active KLK3 can inhibit tube formation in a cell culture model using human umbilical-vein endothelial cells (HUVEC) (Mattsson et al. 2008). This indicates that the enzymatic activity of KLK3 is needed for its anti-angiogenic properties. Recently it has been shown that by using peptides which stimulate the enzymatic activity of KLK3, the antiangiogenic activity of KLK3 can be enhanced (Mattsson et al. 2012). Furthermore, it has been shown that KLK3 can cleave plasminogen into angiostatin-like fragments achieving an anti-angiogenic effect (Heidtmann et al. 1999). High KLK3 expression is associated with low microvessel density and in poorly differentiated tumors, the expression decreases suggesting a tumor suppressing role for KLK3 (Abrahamsson et al. 1988, Paju et al. 2007, Papadopoulos et al. 2001).

Conversely it has also been reported that KLK3 can promote tumor growth and the formation of metastases by degrading extracellular matrix proteins and activating matrix – metalloproteinase-2 (Webber et al. 1995, Ishii et al. 2004). KLK3 has been shown to cleave insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) (Cohen et al. 1992). Increased IGF-1 activity is associated with the stimulation of cell proliferation and thus it influences the cancer growth in many cancers including prostate cancer (Samani et al. 2007). KLK3 also activates the latent form of transforming growth factor-β2 (TGF-β2) which can promote the growth of the malignant cells (Killian et al. 1993, Dallas et al. 2005). Furthermore, KLK3 has been postulated to play a role in the development of bone metastases via the promotion of osteoblast proliferation (Goya et al. 2006, Romanov et al. 2004). This includes the hydrolyzation and inactivation of parathyroid hormone-related protein (PTHrP) which has been shown to inhibit osteoblastic processes (Cramer et al. 1996, Iwamura et al. 1996). In a recent study, lowering the KLK3 levels in LNCaP human prostate cancer cell line reduced LNCaP growth rates suggesting that enzymatically active KLK3 is needed for PCa progression (Williams et al. 2011).

In summary, the levels of KLK3 in tissue are easily measured and they have been used for a long time to predict the incidence of PCa. Furthermore, KLK3's activity as a serine protease has been extensively studied. However, its role in both the normal prostate and the PCa remains unclear. The fact that mice or rats do not possess the KLK3 gene or express a functional KLK3 homolog in the prostate has hampered PCa research. KLK2 and KLK3 transgenic mice have been created but the levels of KLK3 produced in this model species were estimated to be 1000-fold lower than in human prostate (Williams et al. 2010). In addition, KLK3 expression level in human PCa cell lines varies and the tumor growth rate *in vivo* is slow in immune-compromised animals (Williams et al. 2007a). Furthermore, there have been difficulties in producing structural data such as obtaining a crystal structure for KLK3.

1.3.3 KLK2

The KLK2 is 78% homologous in its amino acid sequence to KLK3 and is its the closest relative (Schedlich et al. 1987). Like KLK3, KLK2 expression is mainly restricted to the prostate and it has been considered as a prostate cancer marker in addition to KLK3 (Shaw & Diamandis 2007, Darson et al. 1997, Sardana et al. 2008). KLK2 has trypsin-like activity differing from KLK3 (Frenette et al. 1997a) and it cleaves substrates mainly after lysine and arginine. Like KLK3, KLK2 is secreted into the seminal fluid and its main physiological function is believed to be the activation of KLK3 via cleaving proKLK3 (Lövgren et al. 1997, Williams et al. 2010). It has been shown to cleave also semenogelins but from different sites than KLK3 (Deperthes et al. 1996). It is rapidly covalently bound to protein C inhibitor (PCI) when released into the seminal plasma (Deperthes et al. 1996) and in the circulation it is mainly complexed with protease inhibitors such as A2M, PCI, α_2-antiplasmin and plasminogen activator inhibitor 1 (PAI-1) (Mikolajczyk et al. 1998, Mikolajczyk et al. 1999, Heeb & Espana 1998). KLK2 is also involved in proteolytic cascades which facilitate prostate cancer growth and metastasis. KLK2 cleaves IGFBP-3 which may promote both tumor growth and a more aggressive cancer phenotype (Rehault et al. 2001, Hekim et al. 2010). It also activates the zymogen form of urokinase-type plasminogen activator (pro-uPA) (Frenette et al. 1997b) and inactivates PAI-1 (Mikolajczyk et al. 1999). Moreover, several other cancer-related proteins are potential substrates for KLK2 based on its substrate specificities (Cloutier et al. 2002). The expression of KLK2 is increased in prostate tumors but it differs from KLK3 and increased serum levels of KLK2 are associated with poor prognosis (Steuber et al. 2007, Darson et al. 1997).

Like KLK3, the role of KLK2 in prostate cancer is not clear and studies are complicated also by the facts that KLK2 is not expressed in mouse and its concentrations in the seminal plasma are much lower than those of KLK3 (Deperthes et al. 1996). Furthermore, even though KLK2 is enzymatically active after ejaculation, it becomes quickly bound to PCI as an inactive complex.

1.3.4 Peptides binding KLK3 and KLK2

The KLK2 and KLK3 are potential targets for the treatment of PCa because of their possible influence on cancer development. In addition, they are normally highly prostate-specific and thus represent an optimal target for specific imaging and therapy of PCa without side effects. Usually small molecule protease inhibitors lack specificity and to overcome this problem peptides or peptide derivatives that specifically bind to KLK2 and KLK3 have been developed. Wu and his collaborators used phage-display technology to develop peptides, which would specifically bind to KLK3 (Wu et al. 2000). As a result, three differing 10-13 residue-long peptides[3], which are cyclic via disulphide bridges, were identified. These peptides bound to the same region as mAbs specific free KLK3, which indicates that they were binding near to the active site of KLK3. Peptide C-4 with two disulphide bridges exhibited the highest binding affinity. Zn^{2+} which is known to act as a KLK3 inhibitor increased the binding affinity toward KLK3 for all three peptides studied (Wu et al. 2000). In addition, the synthetic analogs of the peptides have been shown to promote the proteolytic activity of KLK3 (Wu et al. 2004, Pakkala et al. 2004). KLK3 modulating peptides have also been used in peptide affinity chromatography to separate

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[3] Sequences are: cyclo[Cys-Val-Phe-Thr-Ser-Asn-Tyr-Ala-Phe-Cys] (which was nominated as A-1), cyclo[Cys-Val-Phe-Ala-His-Asn-Tyr-Asp-Tyr-Leu-Val-Cys] (B-2) and bicyclo[Cys-Val-Ala-Tyr-(Cys-Ile-Glu-His-His-Cys)-Trp-Thr-Cys] (C-4)

the enzymatically active and inactive forms of KLK3 (Wu et al. 2004). Recently also small molecule stimulators for KLK3 protease activity have been investigated (Härkönen et al. 2011). The phage display was also used to develop linear KLK2 binding peptides which inhibit enzyme activity of KLK2 (Hekim et al. 2006)[4]. Peptides based activity inhibitors of KLK3 have also been developed by other groups (Ferrieu-Weisbuch et al. 2006, Hassan et al. 2007). Recently, LeBeau and co-workers reviewed peptide-derived inhibitors which may have valuable therapeutic applications and advantages in imaging (LeBeau et al. 2010).

The role of KLK3 in PCa is still unknown as mentioned above, but for the modulation of the anti-angiogenic activity of KLK3, the activity stimulation may be important. KLK3 has a narrow chymotrypsin like substrate-specificity and for most substrates, its activity is low when compared to chymotrypsin (Coombs et al. 1998). Recently it has been found that the difference between substrate specificity of chymotrypsin and KLK3 is dependent on the kallikrein loop of KLK3 which seems to regulate the entry of substrates into the active site (Menez et al. 2008). The same study revealed that an antibody which stimulates the activity of KLK3 could stabilize the kallikrein loop and it has been postulated that KLK3-binding peptides possess a similar effect (Koistinen et al. 2008). KLK2 is highly active trypsin-like protease and although it has also kallikrein loop, this might allow easier substrate entry causing less sensitivity to modulation (Frenette et al. 1997a, Koistinen et al. 2008). Compared to KLK3, the concentration of KLK2 in seminal plasma is very low but due to its potential role in PCa cancer growth, it remains an interesting target for therapy. Thus, peptides which inhibit KLK2 activity have been developed, and their influence on KLK2 activity studied (Hekim et al. 2006).

Peptides which bind specifically to prostate cancer serum markers KLK3 and KLK2 have also been studied for therapeutic and imaging purposes. The specific expression of KLK3 and KLK2 in prostate and in PCa metastases has made it possible to develop peptide based prodrugs activated by the KLK3 or KLK2 in PCa tumors. These prodrugs have been shown to be able to kill prostate cancer cells *in vitro* and to reduce the size of the tumor in mouse xenocraft models (DeFeo-Jones et al. 2000, Janssen et al. 2004, Janssen et al. 2006, Christensen et al. 2009, Williams et al. 2007b, Denmeade et al. 2003). Peptide sequences for prodrugs were modulated by the sequence modifications of the cleavage site of semenogelin 1, the natural substrate of KLK3 (DeFeo-Jones et al. 2000, Denmeade et al. 2003) or via a combinatorial chemistry library (Janssen et al. 2004). LeBeau and his collaborators have synthesized a selective KLK3-imaging agent using peptide-based KLK3-inhibitor but the results from imaging studies have still not been published (LeBeau et al. 2010).

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[4] Sequences contain either motif Arg-Phe-Lys-Xxx-Trp-Trp or Ala-Arg-Arg-Pro-Xxx-Pro

2 Aims of the study

Human kallikrein-related peptidases KLK2 and KLK3 (PSA) are produced nearly exclusively by the healthy prostate but also by the prostate based tumors. Because of the high specificity of these proteases and their possible roles in prostate cancer progress and proliferation, they are potential targets for prostate cancer therapy and have diagnostic possibilities. Previously it has been shown that peptides, which bind specifically to these proteases, are able either to inhibit or stimulate the proteolytic activity. These peptides are therefore potential lead molecules not only for therapeutic but also for diagnostic purposes. However, peptides tend to suffer from certain limitations such as low bioavailability due to rapid metabolic degradation and rapid elimination. Their pharmacokinetic properties need to be enhanced before they can be used in the clinical applications. One powerful method to enhance the usefulness of peptides for therapeutic applications is to convert bioactive peptide into peptide mimetics. Therefore it is important to identify the enzymatically labile regions of bioactive peptides and evaluate their replacement with more stable compositions resulting in analogs which still maintain their biological activity.

The main objectives in this study were:

I. To identify the structural motifs essential for the biological activity of KLK3 binding peptides.

II. To identify the parts of peptides which can be replaced without losing biological activity.

III. To enhance stability against enzymatic degradation by developing alternative cyclization strategy for an enzymatically labile disulphide bridge

IV. To enhance the stability of linear KLK2 binding peptides against enzymatic degradation by synthesizing cyclic analogs

V. To identify the structural motifs essential for the biological activity of KLK2 binding peptides by comparing the structural data obtained with linear and cyclized active peptides

3 General experimental procedures

3.1 PEPTIDE SYNTHESIS

The peptides were mainly synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY, USA). In the earliest syntheses also a PerSeptive 9050 Plus automated peptide synthesizer was used. Difficult couplings such as non-naturally encoded molecules and cyclizations were performed manually. All amino acids and other reagents (GLS Biochem, Aldrich, Fluka, Bachem, Novabiochem) were purchased in standard qualities and used without further purification.

3.1.1 Synthesis of linear peptides

The peptides were synthesized with the Fmoc strategy using HBTU/DIEA or TBTU/DIPEA as the coupling reagents. NovaSyn TGA with 4-hydroxymethylphenoxyacetic acid linker, Gly-preloaded Wang resin and Rink Amide AM resin were used as the solid phase depending on peptide needed and also on the desired peptide end modification and cyclization. The side-chain protecting groups used in parallel synthesis were Trt for Asn, Gln and His, Boc for Trp and Bu^t for Glu, Asp Ser, Thr and Tyr. For Cys, both Acm and Trt protection groups were used. The additional side chain protecting groups used in bridge forming residues OAll for C-terminus and both Bu^t and ODmab for side chain protection of aspartic acid and glutamic acid, Mtt for ornithine and lysine side chain protection.

For peptide C-terminal amides, Rink Amide Am resin was used for direct amide formation during the cleavage step with 94-96% TFA. For N-terminal acetylation, the free amino group was treated with 20% acetic anhydride in DMF twice for 10 min before cleavage.

The final cleavage steps were performed using a high concentration of TFA 94-96% and as scavengers; small aliquots of H_2O, TIS and EDT were used. After cleavage, the peptides were precipitated in diethyl ether and centrifuged. The precipitates were collected and dissolved in 0-50% AcOH in H_2O and lyophilized. The lyophilized peptide was purified by HPLC as described later on chapter 3.2.

3.1.2 Peptide cyclization via disulphide bridge

For Cys, both Acm and Trt protection groups were used. During cleavage from the resin with 94-96% TFA, the Acm protection group remained in the Cys side chain and Trt was cleaved resulting in direct air oxidation and the formation of a disulphide bridge between two cysteines.

Peptides with cysteines (Acm) were cyclized using the iodination method. Lyophilized peptide was purified by HPLC and fractions the containing crude acyclic peptide were pooled, lyophilized and then dissolved in 50% acetic acid (AcOH) in H_2O at a concentration of 2 mg/ml., 1 M HCl (0.1 ml/mg of peptide) was added followed immediately by 0.1 M iodine solution in 50% AcOH (5 eq./Acm). The solution was stirred vigorously at RT for 40-120 min. The reaction was terminated with 0.1 M sodium thiosulphate. After filtration (0.45 μm,) the peptides were purified with HPLC and the formation of the disulphide bonds was verified in a mass spectrometer as described later on chapter 3.2.

3.1.3 Peptide cyclization via amide bonds

The efficient head-to-tail cyclization method on-resin with side-chain anchoring of Asp or Glu to the resin and with the use of All-protection for *C*-terminal carboxylic function was first described by Kates and his collaborators (Kates et al. 1993). They achieved a good yield for their selected cyclodecapeptide with all four differing anchoring and cyclization sites. Over the next decade, an enormous number of studies were published concerning different activators and reaction conditions for cyclization yield maximization and many useful properties have been described in literature reviews (Li & Roller 2002, Davies 2003). During the studies concerning this thesis, no reaction optimization was performed and therefore basic peptide synthesis procedures were mainly used and if cyclic product was not detected, the cyclization step was not repeated with an alternative procedure.

During this study, allyl-protected aspartic acid (Fmoc–Asp(OH)–OAll) was used for head-to-tail cyclization. Carboxy terminal-protected aspartic acid was coupled to the Rink Amide AM resin via the free side chain. *C*-terminal allyl deprotection was performed using a method which has been described in the publication of Grieco and his collaborators (Grieco et al. 2001). A catalytic amount of Pd(PPh$_3$)$_4$ with PhSiH$_3$ as a scavenger was added to the resin in anhydrous DCM. The mixture was purged with an argon flow and stirred under argon for 1.5 h. The resin was washed with DMF, 0.5% DIEA in DMF), 0.5% sodium diethyldithiocarbamate trihydrate in DMF to remove the palladium catalyst, DMF and finally with DCM. When a significant level of All-protection attached molecules were detected after this procedure, also the method described in the publication of Alcaro and her collaborators (Alcaro et al. 2004) was tested. C-terminal allyl deprotection was performed using Pd(PPh$_3$)$_4$ in the mixture of CHCl$_3$:AcOH:NMM (37:2:1). The mixture was stirred under argon for 2 h at RT and washing steps were performed as described. No traces of All-protection were detected. After the All-deprotection, the N-terminal Fmoc protecting group was removed with 20 % piperidine in DMF.

Head-to-tail, sidechain to tail and side chain to side chain cyclizations were performed on resin using HBTU or HATU/HOAt as the coupling agent and DIEA as the base. The Kaiser test was used for monitoring of the cyclization success (Kaiser et al. 1970). When the Kaiser test was negative, the peptides were cleaved from the resin, lyophilized, purified and characterized as described in chapter 3.2.

3.2 PEPTIDE PURIFICATION AND CHARACTERIZATION

Typically, the peptides were cleaved from the resin using TFA:TIS:H$_2$O:EDT (94:1:2.5:2.5) and lyophilized. The disulphide bridge containing peptides was purified if needed or cyclized as described in the previous chapter and purified immediately after oxidation. The peptides were purified by HPLC (Shimadzu, Kioto, Japan) with a C18 reverse phase column and acetonitrile (ACN) as eluent (0.1% TFA in H$_2$O/0–60% ACN gradient for 60 min) and verified by mass spectrometry with a MALDI-TOF mass spectrometer (Bruker Analytic GMBH, Karlsruhe, Germany) or an ABI QSTAR XL hybrid mass spectrometer using the MALDI interface (Applied Biosystems, Foster City, CA). The purity was determined by analytical HPLC with 240 × 1.4 mm C18 column eluted with 0–60% ACN for 30 min or with 0–90% ACN for 42 min. The degradation rates were determined by analytical HPLC on a 240 x 1.4 mm C18 column eluted with 0–90% ACN for 42 min.

3.3 STRUCTURAL ANALYSIS BY NMR SPECTROSCOPY

3.3.1 Background

Different spectroscopic and spectrometric methods have been used to obtain information about the 3D-structure of peptides and the flexibility of their structures, but also the influences of pH and polarity of the solvent on the structure have been investigated (Reissmann & Imhof 2004). Structural information can be obtained using UV-VIS-, Raman- or IR-spectroscopy or circular dichroism but most commonly information about the 3D structure in solution is obtained by NMR spectroscopy (Reissmann & Imhof 2004, Dyson & Wright 1991). The most important data derived from NMR measurements were sequential and long-range nuclear Overhauser effects (NOEs), $C^\alpha H$ conformational shifts, temperature coefficients of amide protons and appropriate coupling constants, $^3J_{NHC\alpha H}$, for conformational determinations (Wüthrich 1986, Wishart et al. 1991). Long-range NOEs are important for distance geometry determinations through space and can be used in structural analysis performed with the appropriate software. Sequential NOEs, $C^\alpha H$ conformational shifts and coupling constants can be used to determine residual conformations. The conformations can be roughly divided into α-conformations common in tight turns like β-turns and in helical conformations or the β-conformations common in extended and β-sheet structures (Wüthrich 1986, Dyson & Wright 1991). The solvent exposures of NH groups can be detected by determining the temperature coefficients of the NH groups (Wüthrich 1986, Dyson & Wright 1991). Short peptides are known to have flexible conformations, especially linear peptides and in NMR-based structural analysis conducted in this thesis, the main focus was to determine if there was some local conformational restriction such as hydrogen bonding and reversed turn structures. The main procedure will be described in the next section. It has to be noted however, that because the timescale of NMR-experiment is slow related to conformational fluctuation, all of the information gained is a population-weighted average over all existing structures of peptides in solution (Dyson & Wright 1991). Therefore, considerable caution is required when the information of NMR-experiments is interpreted but after careful consideration the information of the dominant conformations of the peptides in solution can be obtained.

3.3.2 Procedure

At first, the temperature coefficients of the amide protons were determined. The chemical shift of the amide proton ^1H-resonance depends upon the temperature and it is upfield shifted when the temperature of peptide sample is raised. The temperature coefficient, $\Delta\delta/\Delta T$ (ppb/K) provides information about the interaction of the amide proton with hydrogen-bond-accepting solvent molecules. A lowered temperature coefficient *(< 3 ppb/K)* is evidence of the protection of the amide proton from solvent and is most commonly associated with intramolecular hydrogen bonding (Wüthrich 1986, Dyson & Wright 1991). Another chemical shift heavily affected by local backbone conformations is the ^1H chemical shift of $C^\alpha H$. By analyzing, a large library of protein chemical shifts, it has been noted that in the α-conformation which is present in turns and helices, the ^1H chemical shift of $C^\alpha H$ is upfield shifted by around 0.39 ppm compared to a so called random coil value. Random coil values are the approximated averages of chemical shifts from the differing 3D conformations of peptides and proteins (Wüthrich 1986, Wishart et al. 1991). In contrast, in β-conformations which exist in extended structures, the ^1H chemical shift of $C^\alpha H$ is

downfield shifted around 0.37 ppm compared to random coil values (Wishart et al. 1991). Other possible explanations for chemical shift differences from random coil values are dependent on pH, solvent used and ring-current effect existing nearby aromatic rings but their influence is more or less local and does not cover all residues (Wishart et al. 1991). If a low temperature coefficient was detected indicating solvent shielding for amide proton, attempts to find the reason for low value were sought by further using NOESY-correlations and structural analysis. The direct NOE-signal between a given pair of protons can be observed in the presence of at least some population of conformers in which the distance between these protons is relatively short. For flexible peptides the limit for observation is defined as being less than 3.5 Å in solution (Dyson et al. 1988). Sequentially the most important NOEs connectivities are between the $C^{\alpha}H$ of a given residue i and the NH of the following residue (i+1) which is termed the $d_{\alpha N}(i, i+1)$ NOE connectivity and between the amide protons of adjacent amino acid residues which is termed $d_{NN}(i, i+1)$ connectivity (*Figure 20*). For example, the observation of a strong $d_{\alpha N}(i, i+1)$ NOE connectivity in the absence of any observable $d_{NN}(i, i+1)$ NOEs indicates extended chain conformation as the backbone dihedral angles (*Figure 21)* are predominantly in the β region of ϕ,ψ space in the Ramachandran plot (Ramakrishnan & Ramachandran 1965, Dyson & Wright 1991). The observation of strong $d_{NN}(i, i+1)$ NOE connectivities is indicative of local structures with dihedral angles in the α region including turned and/or helical structures (*Figure 20*). The observation of both $d_{\alpha N}(i, i+1)$ and $d_{NN}(i, i+1)$ types of sequential NOE connectivity at a single site in a peptide can be an indication of a highly preferred local structure such as a type II β-turn (*Figure 21*) (Dyson & Wright 1991). If both $d_{\alpha N}(i, i+1)$ and $d_{NN}(i, i+1)$ are detected in extended regions, this is a clear indication of a conformational fluctuation and averaging (Dyson & Wright 1991). Thus, if indications of local structures were found, the medium (i, i+2) and long range (i, i+n) NOE connectivities were used to confirm observations since especially tight loop containing structures NOEs between opposite chains could be present.
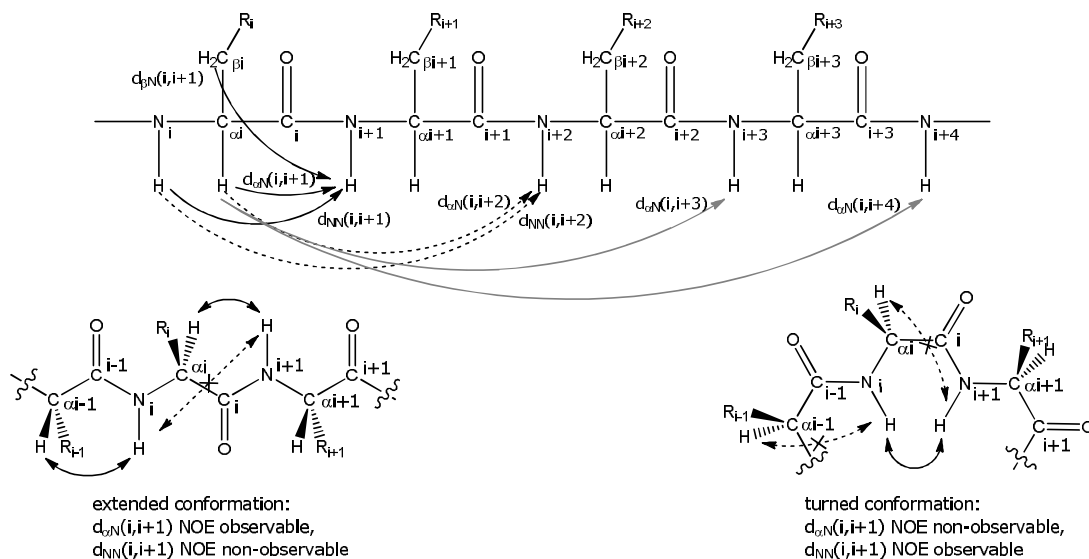

Figure 20. Main observable NOEs indicated as sequential (solid arrows) and medium-range (dashed and grey arrows) ^{1}H-^{1}H connectivities in the polypeptide chain (above). The main differences in NOE-observations between extended and turned structures (below).

Figure 21. Standard nomenclature for the atoms and dihedral angles in peptide chain (on the left). The structure demonstrating a β-turn structure stabilized via the hydrogen bond between O_i and NH_{i+3} (on the right). The main rotable bonds differing between the different types of β-turns displayed with arrows.

The main problem, when measuring the NOESY-spectra of short peptides, is the misleading NOE-information due to aggregation (Dyson & Wright 1991). It is possible that in the samples with a relatively high concentration of significantly large molecules like peptides, the correlation time of the oligomer is greater than that of the monomer. Therefore the intensities of the NOEs observed in the aggregate can be remarkably high compared to the NOEs of the monomer (Dyson & Wright 1991). Additional information about restricted conformations can be derived through the coupling constant between the amide proton and H^α of the same residue; this is termed as $^3J_{HN\alpha}$ coupling constant. In α-helical structure this value can be ~4 Hz, whereas in a regular β-strand it is ~9 Hz. However, for the detection of folded conformations in flexible peptides, the determination of $^3J_{HN\alpha}$ is usually one of the least sensitive NMR parameters and values will be ~6-7 Hz despite there may be rather large population of defined conformations (Dyson & Wright 1991).

The structural characterization based on distance geometry and restrained molecular dynamics and the quantitative calculations of structure are meaningful only for peptides that adopt some defined unique conformations in solution. For most linear peptides in aqueous solution, the NOEs and $^3J_{HN\alpha}$ coupling constants will be a population weighted average over all conformations but if conformational constraits such as cyclization exist, then the population averaging is usually decreased. Thus, distance constraints and torsion angle constraints collected by NMR experiments can be used to help in the estimation of 3D peptide and protein structures using computer software which can evaluate molecular dynamics in torsion angle space.

During the studies included in this thesis all spectra were recorded on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Karlsruhe, Germany) operating at 500.13 MHz for ^1H. All one dimensional experiments were recorded at four or five different temperatures and all two dimensional experiments were recorded at two different temperatures depending on the quality and clarity of the spectra. All chemical shifts are reported with respect to the 5 mM trimethylsilyl propanoic acid (TSP) peak at 0.00 ppm when 10% D_2O in H_2O were used or to the DMSO peak at 2.50 ppm when used. For all 2D experiments, standard pulse programs from the Bruker software library were used. For 2D-spectra (COSY, TOCSY, NOESY, ROESY) various mixing times were tested with the optimal time being determined based on the quality and clarity of the spectra. The temperature coefficients (dδ/dT) of the amide protons were calculated by analysing the chemical shifts at four temperatures.

3.4 BIOSTABILITY STUDIES

The stability of peptides towards systemic metabolism is commonly evaluated by incubating the peptide at +37°C and pH 7.4 in serum or plasma (Werle & Bernkop-Schnurch 2006). If more detailed knowledge about cleavage proteases is needed, the peptide can be incubated in a medium containing one or more isolated enzymes. Trypsin and chymotrypsin are two of the most widely used proteases in metabolic stability studies because they have major roles in protein and peptide digestion (Tal-Gan et al. 2011b). Usually a purified cocktail of peptidases does not completely mimic the *in vivo* metabolic behavior of peptides and it is reasonable to determine the metabolic stability also *in vitro* with plasma and serum (Adessi & Soto 2002). Further, incubation with different tissue homogenates or membrane preparations can also be attempted (Adessi & Soto 2002).

3.4.1 Stability of peptides against proteases
During this study, the stabilities against selected proteases were determined by incubating selected peptides in a buffered solution at +37°C with proteases. Small samples were collected at several time points. Proteases were removed from reaction mixture with Microcon Centrifugal Filter Device (Microcon YM-10, cut off 10 kDa, Millipore, Bedford, MA, USA) by centrifugation at 14000 – 15000 rpm in an Eppendorf 5415 D centrifuge (Eppendorf, Hamburg, Germany) for 10 min. The filtrates were analyzed by HPLC and peptide fragments were characterized by MSMS as described in chapter 3.2. Fragment ions were calculated by using Prowl (http://prowl.rockefeller.edu/) if needed.

3.4.2 Stability of peptides in plasma
The stability of the peptides in human plasma was studied by mixing fresh human plasma which was collected into EDTA-coated tubes with each peptide in phosphate buffered saline (PBS) solution. The reaction mixtures were incubated for up to 24h at +37°C. The peptides and peptide fragments were separated from plasma, analyzed and characterized as described above.

3.5 EFFECT OF THE PEPTIDES ON THE ENZYME ACTIVITY OF KLK3 AND KLK2

The effect of different peptides in the activity of KLK3 was measured using the chromogenic chymotrypsin substrate S-2586 (MeO-Suc-Arg-Pro-Tyr-pNA) purchased from Chromogenix Instrumentation Laboratory (Milano, Italy). During activity measurements of KLK2, chromogenic substrate S-2302 (H-D-Pro-Phe-Arg-pNA, Chromogenix, Milano, Italy) were used. KLK3 or KLK2 was incubated for 30 min at RT with synthetic peptides in Tris-buffer solution. After the addition of the substrate, the absorbance at 405 nm was measured at 5 min intervals for 30 min with a Victor 1420 Multilabel fluorometer (Perkin-Elmer-Wallac, Turku, Finland) if not otherwise stated.

4 Optimization of Amino Acid Composition and Conformational Analysis of the Cyclic Peptides which Stimulate KLK3 Protease Activity[5]

4.1 INTRODUCTION

By using phage display libraries, specific KLK3 binding peptides A-1 (cyclo-[CVFTSNYAFC]), B-2 (cyclo-[CVFAHNYDYLVC]) and C-4 (bicyclo-[CVAYCIEHHCWTC]) have been identified. They bind only to the enzymatically active form of KLK3 (Wu et al. 2004). The structures of three KLK3 activating synthetic peptides having one or two disulfide bridges were studied by NMR spectroscopy to determine possible conformational information of these bioactive peptides. Furthermore, the amino acid sequences of the three peptides were studied using alanine replacement-based structure-activity studies in order to determine the amino acid residues important for the activity of the peptides. Based on NMR studies and peptide modifications, a common structure and the amino acid residues that are important for the KLK3 promoting activity of the peptides, were proposed.

In an additional study*, the structure-activity relationships of C-4 peptide, which displays the highest activity stimulation toward KLK3, were examined using selective amino acid and multi-alanine replacements (unpublished data). The aim of the additional study was to determine residues or sections which can be simplified without decreasing biological activity and possibly replaced by mimicking structures.

4.2 RESULTS AND DISCUSSION

4.2.1 Conformation of peptides

Every peptide investigated (A-1, B-2, C-4) has NH group or groups showing very low $\Delta\delta/\Delta T$ values *(<3 ppb/K)* characteristic of strong solvent shielding. Low temperature coefficients of amide protons were found for Tyr7 and especially for Ala8 in peptide A-1, for Tyr7 in peptide B-2 and for His9 in peptide C-4 (*Table 5*). The upfield chemical shifts of C^αHs suggest that there exist α-conformations between residues Ser5-Tyr7 in peptide A-1, between His5-Asp8 in peptide B-2 and between Glu7-His8 in peptide C-4 (*Figure 22*) (Wishart et al. 1991, Maynard et al. 1998). Very strong sequential NH(i)-NH(i+1) NOEs were found for Asn6-Tyr7 in peptide A-1, for His5-Asn6, Tyr7-Asp8 and Asp8-Tyr9 in peptide B-2 and for Val2-Ala3 and His8-His9 in peptide C-4 indicating α-conformations and possible turn structure. Strong sequential C^αH(i)-NH(i + 1) NOEs were found between residues Val2-Asn5 in peptide A-1, between Val2-Ala4 and Tyr9-Cys12 in peptide B-2 and between Cys1-Glu7 and His9-Cys13 in peptide C-4 indicating the existence of β-

conformations. Long-range inter-residual NOEs were found for peptides B-2 and C-4. No significant inter-residual NOEs were observed for peptide A-1 (*Figure 23*).

Table 5. ^{1}H chemical shifts measured in DMSO-d$_6$ and amide proton temperature coefficients of KLK3-binding peptides. The temperatures of the NMR-samples, when the ^{1}H chemical shifts presented in this table were detected, were 305K for peptides A-1 and C-4 and 310K for peptide B-2.

Peptide Residue	NH	C$^\alpha$H	C$^\beta$H	C$^\chi$H	C$^\delta$H	Other	-Δδ/ΔT (ppb/K)
A-1							
Cys1		4.20	2.98				
Val2	8.52	4.23	1.88	0.81			6.1
Phe3	8.36	4.83	3.08, 2.88		7.18	7.13-7.23 (C$^\varepsilon$H, C$^\zeta$H)	6.0
Thr4	8.02	4.38	4.18	1.04			7.4
Ser5	8.06	4.14	3.72, 3.65				4.7
Asn6	7.99	4.46	2.46, 2.39			6.93, 7.38 (N$^\delta$H)	7.2
Tyr7	7.81	4.36	2.86, 2.67		6.94	6.62 (C$^\varepsilon$H)	3.7
Ala8	7.89	4.37	1.09				1.8
Phe9	8.00	4.60	3.06, 2.82			7.13-7.23 (C$^\varepsilon$H, C$^\zeta$H)	7.0
Cys10	8.48	4.58	3.17, 2.96				7.5
B-2							
Cys1	-	4.39	3.03, 2.82				
Val2	8.62	4.42	2.07	0.87, 0.81			4.7
Phe3	8.40	4.77	3.07, 2.73		7.25	7.21-7.24 (C$^\varepsilon$H, C$^\zeta$H)	9.3
Ala4	8.02	4.25	1.16				7.6
His5	8.42	4.27	3.13, 3.02		7.27 (4H)	8.81 (C$^\varepsilon$H, 2H)	8.7
Asn6	7.99	4.26	2.59, 2.59			6.87, 7.44 (N$^\delta$H)	5.0
Tyr7	7.85	4.13	2.86, 2.75		6.84	6.58 (C$^\varepsilon$H)	0.7
Asp8	8.16	4.53	2.68, 2.50				7.3
Tyr9	7.53	4.58	2.94, 2.72		6.94	6.57 (C$^\varepsilon$H)	4.0
Leu10	8.30	4.50	1.35, 1.35	1.33	0.55, 0.53		13.1
Val11	8.21	4.29	1.92	0.88, 0.87			10.8
Cys12	8.75	4.67	3.21, 2.89				10.3
C-4							
Cys1	-	4.22	3.23				
Val2	8.61	4.28	1.98	0.89, 0.88			6.7
Ala3	8.15	4.65	1.20				6.4
Tyr4	7.96	4.71	2.87, 2.66		6.89	6.53 (C$^\varepsilon$H)	4.5
Cys5	8.67	5.38	2.83, 2.72				9.9
Ile6	8.55	4.20	1.81	1.14, 1.55 0.92 (C$^\chi$H$_3$)	0.86		6.5
Glu7	8.98	3.57	2.17, 1.95	2.28			8.5
His8	8.41	3.93	3.27		7.24 (4H)	8.74 (C$^\varepsilon$H, 2H)	5.9
His9	7.86	4.72	3.04, 2.87		7.18 (4H)	8.63 (C$^\varepsilon$H, 2H)	1.0
Cys10	8.60	5.27	2.74, 2.65				9.1
Trp11	8.58	4.76	3.09, 2.89		7.14 (2H)	7.65 (4H), 6.95 (5H), 6.98 (6H), 7.26 (7H), 10.62 (NH)	4.3
Thr12	8.20	4.40	3.94	1.10			10.3
Cys13	7.97	4.50	3.17				4.0
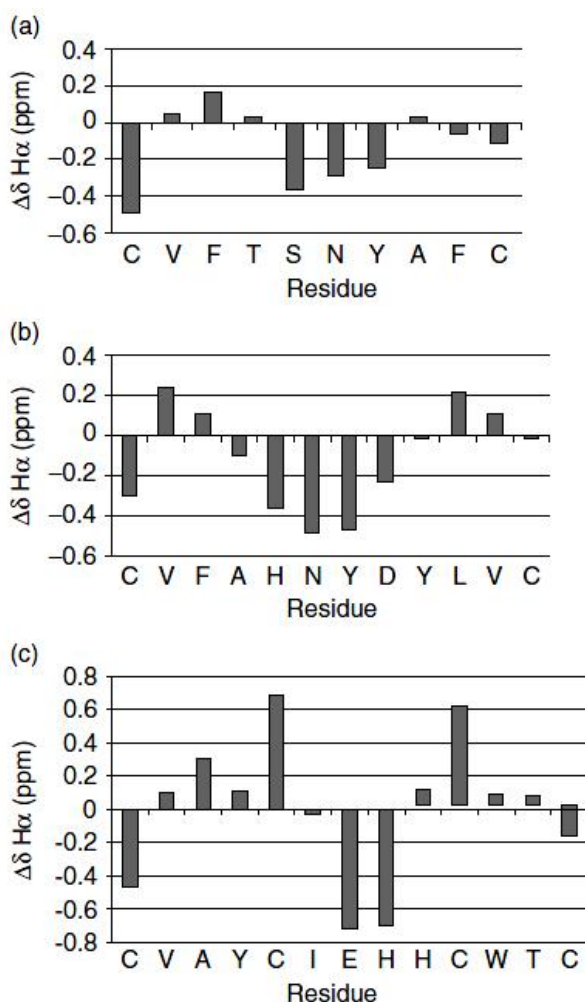
Figure 22. Differences between the C$^{\alpha}$H chemical shift values in the random coil and values determined experimentally in DMSO-d$_6$ for A-1 (above), B-2 (middle) and C-4 (bottom).

A part of the NH peaks was broadened indicating solvent-exposed NH groups or conformational fluctuation and dynamic behavior (Wüthrich 1986, Raghothama et al. 1996). Because of broadening all $^3J_{NHC\alpha H}$ could not be detected and the detected coupling constants were usually between 6.0 and 8.0 Hz.

The common turn structures in the peptides investigated were identified as a type I β-turn between residues Ser5-Ala8 for peptide A-1, a type I β-turn between residues Ala4-Tyr7 for peptide B-2 and a type II β-turn between residues Ile6-His9 for peptide C-4[6]. However, peptide C-4 has a disulphide bond between residues 5 and 10, which causes a tight turn in the sequence between those residues. Whenever a loop structure was formed in the peptides studied the aromatic side chains of phenylalanine, tyrosine or tryptophan were found to locate on the same side of the peptide loop and near each other.

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[6] β-turn is formed by four consecutive residues which are not part of α-helix and the distance between C$^{\alpha}$(i) and C$^{\alpha}$(i+3) is less than 7Å. The nomenclature is depending on backbone torsion angles of two center residues, i+1 and i+2. The most commonly existing β-turn types are classified as type I, I', II, II' VIII, Via, VIb and IV (Wilmot and Thornton 1998, Hutchinson and Thornton 1994).

In peptide A-1, a relatively strong NH(i)-NH(i+1) correlation between Asn6 and Tyr7 was detected (*Figure 23*) supporting the existence of an α-conformation for the backbone of Asn6 and thereby proposing the existence of a type I β-turn between residues Ser5-Ala8 (Wüthrich 1986). A moderately low temperature coefficient for the amide proton of Tyr7 (3.7 ppb) was detected (*Table 5*), which is likely due to an interaction between the NH-proton of Tyr7 and the side chain oxygen of Ser5. This is in line with serine's potential to be at position i of the type I turn (Hutchinson & Thornton 1994, Wilmot & Thornton 1988). However, strong CαH(i)-NH(i + 1) correlation for Asn6 and Tyr7 provides evidence for the existence of a significant population of type II turns in the same area (*Figure 23*). Thus, these mutually exclusive NOE-correlations indicate high flexibility or conformational interconversion in the turn (Gunasekaran et al. 1998).
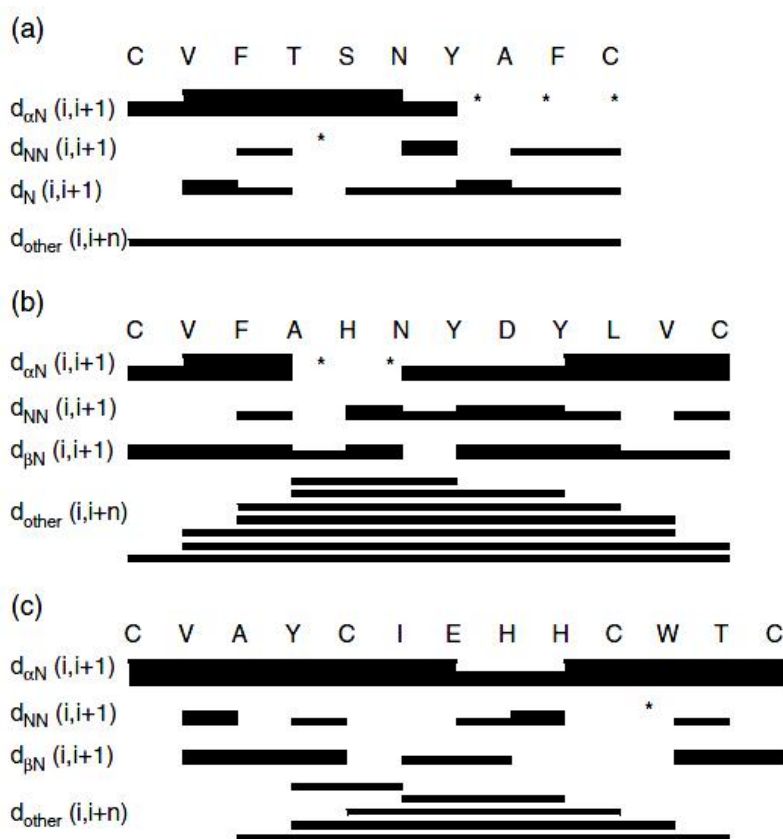
Figure 23. Summary of significant NOE-correlations for peptides A-1 (a), B-2 (b) and C-4 (c) in DMSO-d₆. Relative cross-peak intensities were estimated from volume integration and depicted as strong, medium and weak. Mark * means disturbed NOE-correlation by some other NOE. NOEs indicated as "other" interaction include long range main chain-main chain, side chain-main chain or side chain-side chain interactions.

In peptide B-2, a strong NH(i)-NH(i+1) correlation between residues His5 and Asn6, and a weak correlation between Asn6 and Tyr7 were found (*Figure 23*), indicating that there is a type I β-turn at residues 4–7 (Wüthrich 1986). Furthermore, weak NOE-correlations between Ala4 CᵝH₃-protons and both Tyr7 and Tyr9 amide protons indicate that a tight turn exists.

In peptide C-4, both strong NH(i)-NH*(i+1)* correlation between His8 and His9 (*Figure 23*) and strong intraresidual C$^{\alpha}$H(i)-NH(i) correlation for His8 indicate a type II turn. A very strong downfield shift for Glu7 NH evidences its solvent-exposed conformation, which is most probably intrinsic for a type II turn. A NOE was observed between Ile6 NH and Tyr9 NH indicating the existence of a tight turn.

The cyclization of the peptides is suggested to force the peptides in turned conformations essential for the KLK3-promoting activity. Long-range NOE analyses indicate loop structures and common β- turn structures for each peptide. In A-1 Tyr7 is located at position i+2 (turn 5–8) and in B-2 at position i+3 (turn 4–7) of a β-turn. Due to the fluctuation of the A-1 backbone, Tyr7 may also locate at position i+3. It is suggested that this fluctuation is more favoured in water solutions, because DMSO-d$_6$ may act only as a hydrogen bond donor, whereas water has hydrogen bond donor and acceptor properties. Therefore, distances between the aromatic residues and the conformations of A-1 and B-2 are likely to be similar. In peptide C-4, a disulphide bond close to Tyr and Trp is the main factor determining the overall structure of the peptide. In addition, hydrophobic interactions may exist between Tyr, Ile and Trp forming a hydrophobic cluster on one side of the peptide. NOE-correlation peaks were found between the side chains of Tyr4 and Ile6.

The distance between a pair of protons has to be less than 3.5 Å in flexible structures for a NOE to be observed (Dyson & Wright 1991). The sequential NH(i)-NH*(i+1)* NOE cross peaks are evidences of folded or turned conformations (Dyson & Wright 1991). In the case of short peptides, the NH(i)-NH*(i+1)* NOE is seen if the population of a turn conformation is at least 10% (Wüthrich 1986, Dyson et al. 1988). Both α- and β-conformations were found for Phe3, Asn6, Ala8 and Phe9 in peptide A-1, for Phe3, Ans6, Tyr7, Asp8, Tyr9 and Val11 in peptide B-2 and for Val2, Tyr4, Glu7, His8 and Trp11 in peptide C-4. Thus, in spite of the fact that these peptides are relatively short cyclic structures, several conformations were observed.

4.2.2 The KLK3-promoting activity of A-1, B-2, C-4 and their modifications

The activities of the three KLK3-promoting peptides and corresponding alanine replacement sets were compared with the activity of KLK3 alone (*Figure 24*). Most of the changes inactivated or reduced the activity of peptides A-1 and B-2. The exception was the replacement of His5 by Ala in B-2, which improved the activity. In addition, two replacements (Phe3→Ala and Asn6→Ala) in the A-1 sequence slightly decreased the KLK3 reactivity. In the B-2 sequence the carboxy terminal Leu and Val, which are not present in the A-1 sequence, could not be replaced by alanine without losing the activity. The C-4 peptide contains 13 amino acids and two disulphide bridges. None of the alanine replacements totally inhibit the activity. Reduced activities were observed in Tyr→Ala, Ile→Ala and Trp→Ala modifications. In contrast to this, slightly increased activities were seen when Val2, Glu7, His8, His9 and Thr13 were mutated to Ala.
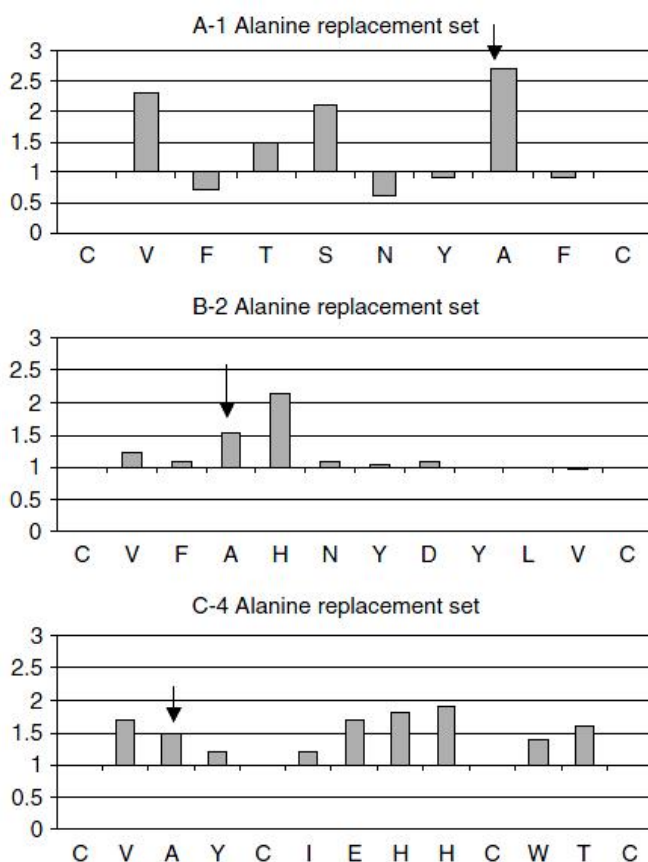
Figure 24. Alanine replacement sets of A-1, B-2 and C-4 peptides. Every column represents amino acid, which is changed with alanine and the height is calculated from the equation: absorbance of peptide KLK3/KLK3 alone. Value 1 illustrates KLK3 activity alone. In peptide A-1 some of the modifications have values below 1 suggesting KLK3 inhibitory activity. The parent sequences are shown by the arrow.

On the basis of the results from alanine replacements, three sequences of 10 amino acid residues were synthesized and their activities were compared with the A-1 sequence. Reduced activities resulted in the peptides having alanines at positions 2, 4 and 5 (54%) or 2, 4, 5 and 6 and Asp at position 8 (37%). When Tyr7 was replaced by alanine the activity was completely lost (*Table 6*).

Table 6. Sequences with more than one residue replaced with alanine. The activities of the modified peptides reported are relative to the activity of the parent sequence. S/C means singnal to control ratio, where control is the activity of KLK3 alone (value = 1.00).

peptide	S/C	%
CVFTSDYAFC	2.52	100
CAFAADYAFC	1.83	54
CAFASAADYC	1.08	5
CAFAAAYDYC	1.57	37

Alanine replacement studies showed that the biological activities of all three peptides arise mainly from the aromatic and hydrophobic interactions of Tyr and Phe with the

KLK3. However, also small hydrophobic residues, Ile, Leu and Val, have a role in the activities of the peptides. This was further studied with consensus peptides, in which the replacement of Tyr totally inactivated the peptide, whereas alanines in other positions reduced the activity. It can be assumed that the aromatic rings or hydrophobic side chains are the contact residues, and their spatial arrangement is important for activity while the rest of the peptide acts as a backbone for the active side chains, influencing the conformation of the peptides. In B-2 peptide, the replacement of Leu10 or Val11 with alanine inactivates the peptide. On the other hand, A1-peptide, which contains sequential similarity to B-2 but lacks carboxyterminal Leu and Val, is active.

Aspartic acid stabilizes the turn structures by hydrogen bonding via its side chain. The replacement of Asp with Ala both in A-1 and B-2 sequences inactivated the peptides. Histidine is not as favoured in position i+1 in type I β-turns (Hutchinson & Thornton 1994). It can be assumed that removing this relatively large and basic side chain near the contact residues increases the biological activity. The same effect can be seen in peptide C-4, where replacement of His8 or His9 increases the activity. It is suggested that the biological activity of the three peptides is based on the interaction of the aromatic and hydrophobic side chains with KLK3. The turn structures of the peptides place the important contact residues in an arrangement crucial for the KLK3 recognition. Since it has been shown that B-2 and C-4 peptides bind only to the intact and active form of KLK3, the interaction of the peptides is dependent on the native structure of KLK3 (Wu et al. 2004).

4.2.3 Cross-inhibition of the peptides

The binding of GST-peptide C-4 to KLK3 was blocked in a dose dependent fashion by each of the synthetic peptides A-1, B-2 and C-4. The inhibition was up to 60%–70% when using 100-fold molar excess of the peptides showing that these three peptides bind to the same site or to sites which are very close to each other on KLK3 (*Figure 25*).

Although the three peptides contain tyrosine, which has the aromatic side chain of the substrates recognized by KLK3, the peptides are not supposed to bind to the active site of the protease (Wu et al. 2000). The cross-inhibition studies indicate that, despite significant differences in the sequences of A-1 and B-2 compared with C-4, all three peptides bind to the same area of the KLK3. Furthermore, in the presence of Zn^{2+}, the affinities of the three peptides increase three- to sevenfold, suggesting the involvement of Zn^{2+} in the binding of the peptides to KLK3 (Wu et al. 2000). It is reasonable to suggest that the KLK3-promoting peptides do not directly interact with Zn^{2+} but upon binding of Zn^{2+} the KLK3 structure is altered so that the affinities of peptides are increased.

The KLK3-promoting activities of the three cyclic peptides are comparable in spite of the differences in the amino acid sequences and the number of disulphide bonds. Originally these sequences were determined by using phage display technology (Wu et al. 2000). This technology allows the synthesis of millions of different combinations of short peptides, which can be screened with a binding assay to the target molecule. During the selection, several sequences with similar binding properties can be characterized (Scott & Smith 1990, Devlin et al. 1990, Cwirla et al. 1990). These sequences serve as valuable lead structures in order to study the mechanism of the biological interactions.
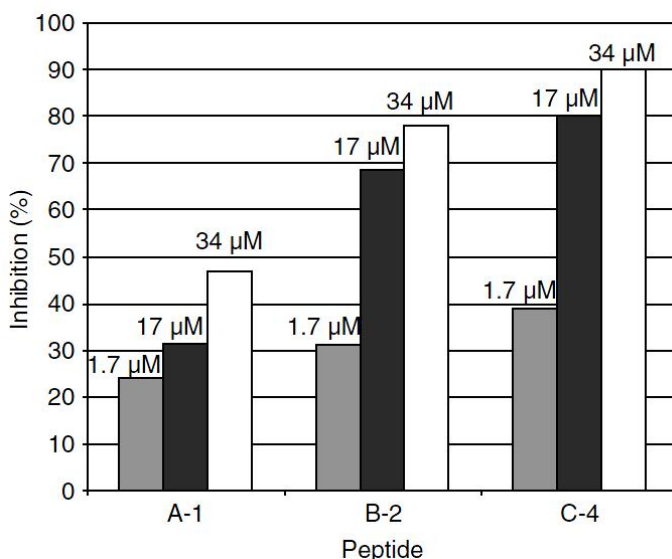
Figure 25. Effect of synthetic peptides on the binding of GST-peptide fusion protein C-4 to immobilized KLK3. 1.7, 17 and 34 µM concentrations of synthetic peptides together with 0.17 µM GST-peptide C-4 were added to wells containing KLK3 bound by MAb 5E4 resulting concentration dependent inhibition of GST-peptide binding.

The crystal structure of human KLK3 is not known. However, the 3D-structure of the corresponding horse prostate kallikrein (HPK) has been determined. The common prostate origin and significant sequence homology between the human and horse PSA suggest that HPK may serve as a model for the structure of human KLK3. The catalytic site of HPK is similar to the other chymotrypsin-like proteases containing a catalytic triad of His57, Asp102 and Ser195. A common feature for all kallikreins is the kallikrein loop near the active site of the protease but there are major differences in the kallikrein loop sequences between HPK and other kallikreins. In HPK, the kallikrein loop and the loop comprising Trp215-Cys220 form lid-like structures covering the catalytic site. This lid-like structure is suggested to act as a regulator of the protease activity and specificity (Carvalho et al. 2002).

In human KLK3, the regulatory mechanism may be similar. Zn^{2+} is a non-competitive inhibitor of KLK3 and it is coordinated by Asp91, His101 and His234 close to the residues of the catalytic triad. The exact binding site and mechanism of the peptides are not known but it is proposed that the peptides bind, like Zn^{2+}, near the active site of KLK3 and they interfere with the possible regulatory function of the kallikrein loop. The recognition of KLK3 takes probably place via hydrophobic interactions and aromatic rings of phenylalanine or tyrosine. The specific binding of the peptides may provide a diagnostic tool for selective recognition of an active form of KLK3.

4.2.4 Additional SAR-study using single and multiple replacements

Because the single alanine replacement sets were tested earlier for C-4 (chapter 4.2.2), this additional study focused on point mutations with other amino acids and further alanine replacements in two or three positions at the same time (unpublished data). The main focus was the CIEHHC-loop because of its structural role nearby essential amino acids Tyr4 and Trp11 determined in single alanine replacement studies. The loop could be an object for a peptide mimetic structure with more stable molecule in sights. Ile6 is supposed to have a possible conformational stabilizing effect with Tyr4 and Trp11 because their close location

in space (*Figure 23*). Therefore it was one focus of the mutations. The inner disulphide bridge between cysteines 5 and 10 with alanines was replaced in order to monitor essentiality of bridge. Further, the analog, in which both Val2 and Thr12 were replaced with alanines, or the analogs, in which either Val2 or Thr 12 were replaced with asparagines, were synthetized for further modifications, in order to define if there was a possibility to link peptide to the resin in another way than via C-terminal cysteine. Stimulation of KLK3 activity for all synthesized peptides was measured. Both high and totally reduced stimulation of KLK3 activity were detected, which gives valuable information about the structure activity relationships between KLK3 and activity stimulating peptides. The results are also helpful for peptide mimetic design.

Sythesized sequences shown in *Table 7* and the stimulation of the KLK3 activity of successfully cyclized peptides are represented in *Figure 26*. The activity studies of some of the intended sequences were declined due to the low cyclization rates of peptides. The low cyclization rates caused significantly low yields of peptides with satisfactory purity. However, in the method used in the activity stimulation studies includes high excess of peptide versus KLK3. Thus, despite of the low purity of some peptides, the activity stimulation results are still indicative. All peptides **2-21** were synthesized with *C*-terminal amide because in our previous studies, we had noticed that in the case of C-4, the charge in *N*-terminus was not essential for the activity.

Table 7. Sequences of peptide C-4 analogs in replacement study.

peptide	mutation	sequence	peptide	mutation	sequence
1	parent	CVAYCIEHHCWTC	11	Ala7,8	CVAYCIAAHCWTC
2	Gly8	CVAYCIEGHCWTC	12	Ala7,9	CVAYCIAHACWTC
3	Phe6	CVAYCFEHHCWTC	13	Ala8,9	CVAYCIEAACWTC
4	Gly6	CVAYCGEHHCWTC	14	Ala6,9	CVAYCAEHACWTC
5	Leu6	CVAYCLEHHCWTC	15	Ala6,8	CVAYCAEAHCWTC
6	Val6	CVAYCVEHHCWTC	16	Ala7,8,9	CVAYCIAAACWTC
7	Ala6	CVAYCAEHHCWTC	17	Ala6,7,9	CVAYCAAHACWTC
8	Asn12	CVAYCIEHHCWNC	18	Ala6,7,8	CVAYCAAAHCWTC
9	Asn2	CNAYCIEHHCWTC	19	Ala4,6	CVAACAEHHCWTC
10	Cys6-Cys9	CVAYACEHCAWTC	20	Ala6,10	CVAYCAEHHCATC
			21	Ala2,12	CAAYCIEHHCWAC

Single replacements. The replacement of His8 with glysine (**2**) maintained the equal activity indicating possibilities to modify some residues heavily (*Figure 26*). Glysine is also highly favored in the place i+2 in type II β-turn (Hutchinson & Thornton 1994). The activity stimulation results show the essentiality of hydrophobic side chain in position 6. Replacing

Ile6 with especially bulky phenylalanine (**3**) increased the activity but the replacement by glysine (**4**) reduced the activity stimulation almost totally. Replacement by leusine (**5**) reduced the activity stimulation slightly. Via decreasing the size of the hydrophobic side chain (**6**, **7**) the activity stimulation was reduced. Involvement of asparagines (**8**, **9**) to either side of the disulphide bridge resulted remarkable loss of activity stimulation excluding the possibility for alternative resin attachment site for possible disulphide bridge replacement synthesis.
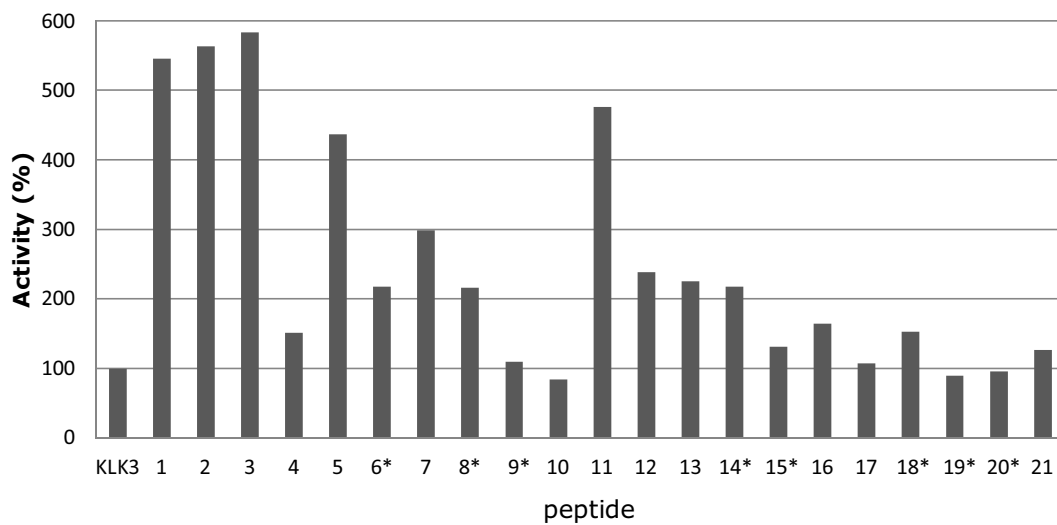
Figure 26. Results of KLK3 activity stimulation by C4 analogs. Purity of sequences marked with asterisk (*) was moderate 50-80%.

Multiple replacements. The replacement of the inner disulphide bridge between cysteines 5 and 10 with alanines (data not shown) or changing the site of bridge (**10**) eliminated the biological activity, indicating that correct conformation of inner bridge is essential for biological activity. The residues in the loop-area between Cys5 and Cys 10 could be replaced with two or three alanines without losing the activity. Peptide **11** with residues Glu7 and His8 replaced with alanines at the same time maintained stimulation of activity almost equally compared to peptide **1**. It seems however that replacement of any other two residues in the loop between cysteines 5 and 10 resulted reduced activity (**12**, **13**, **14**, **15**) and especially, if Ile6 is among the replaced residues, the activity stimulation was reduced heavily (**14**, **15**). Replacing three residues at the same time reduced the activity stimulation no matter which residues were replaced (**16**, **17**, **18**). Both combinations in which one of the essential residues, Tyr4 or Trp11, in combination with Ile6 (**19**, **20**) were replaced were totally inactive. The replacement of valines nearby the terminal disulphide bridge resulted almost total elimination of activity ensuring the essentiality of residues, which are directly connected to the cysteine residues in the terminal bridge (**21**).

4.3 CONCLUSIONS

The structures of three KLK3 activating synthetic peptides having one or two disulfide bridges were studied by NMR spectroscopy, and a population of reversed turn structures

was found for each peptide. Turn types were identified as a type I β-turn between residues Ser5-Ala8 for peptide A-1, a type I β-turn between residues Ala4-Tyr7 for peptide B-2 and a type II *β*-turn between residues Ile6-His9 for peptide C-4. Furthermore, the amino acid sequences of the three peptides were studied using alanine replacement-based structure-activity studies. The alanine replacement studies showed that the biological activities of all three peptides arise mainly from the aromatic and hydrophobic interactions of Tyr and Phe with the KLK3. The cross-inhibiton test showed that despite different structures, the peptides bind to the same area on the KLK3. Further, it was shown that commonly know KLK3 inhibitor Zn^{2+} increases the binding affinity of KLK3 stimulating peptides suggesting that peptides bind near to catalytic triad of KLK3.

In the additional study of KLK3 activity stimulating peptides, the presence of essential residues Tyr4 and Trp11 in combination with hydrophobic residues, especially Ile, Leu or Phe in position 6, was found to be highly favored (*Figure 26*). The loop between Cys5 and Cys10 enables minor modifications, especially at positions 7 and 8. This site is important for peptide mimetic design toward more biologically stable conformation as it eases the design and synthesis of mimetics.

4.4 EXPERIMENTAL

4.4.1 Synthesis of linear peptides

The single peptides for NMR were synthesized using a PerSeptive 9050 Plus automated peptide synthesizer, with Fmoc strategy, TBTU/DIPEA as the coupling reagent and NovaSyn TGA with 4-hydroxymethylphenoxyacetic acid linker as the solid phase. Alanine-replacement sets and other modified peptides were synthesized with an Apex 396 DC multiple peptide synthesizer. In original alanine replacement sets, all amino acids except cysteines were replaced one by one. In the additional study, peptide **1** was obtained from AnaSpec (San Jose, CA). Peptides 2-21 were synthesized using Rink Amide AM resin yielding peptides with *C*-terminal amide.

4.4.2 Cyclization of the peptides

For peptides A-1, B-2 and their modifications Cys(Acm) was used in the Cys position 1: C(Acm)VFTSNYAFC(Trt) and C(Acm)VFAHNYDYLVC(Trt), for peptide C-4 and its modifications Cys(Acm) in positions 6 and 9: C(Trt)VAYC(Acm)IEHHC(Acm)WTC(Trt) was used. The solution was stirred vigorously at RT for 40 min. In the additional study, the only side-chain protecting group used for cysteine was Acm.

4.4.3 KLK3 activity measurements

77 pmoles *(2 μg)* of KLK3 substrate and 20 nmoles of B-2 and C-4 series and 9 nmoles with A-1 were incubated in Tris-buffer (10 mM Tris and 150 mM NaCl at pH 8.0) with 1.6 mM concentration of the substrate at RT. KLK3 reaction without any peptide was used as the basic level control. The reaction was measured at 405 mm at 10 min intervals for 60 min using a Multiscan RC photometer (ThermoLabsystems, Helsinki, Finland). The effects of the peptides were calculated as a ratio of the optical density (OD) value of the KLK3-peptide complex to the KLK3 alone after 60 min incubation.

In the additional study, different concentrations were used as KLK3 (0.3 μM, 30 pmol) was incubated for 30 min at RT with a multifold molar excess of synthetic peptide (13 μM,

1,3 nmol) in 0.05 M Tris buffer, pH 7.7, containing 0.154 M NaCl, 8 mM NaN_3 and 0.1% bovine serum albumin. After the addition of the substrate to a final concentration of 0.2 mM, the absorbance at 405 nm was measured as described in the chapter 3.4.

4.4.4 Cross-inhibition test

The relative binding sites of the peptides on KLK3 were mapped by competition experiments with chemically synthesized peptide and GST-peptide fusion protein (Wu et al. 2000). Briefly, KLK3 specific monoclonal antibody (mAb5E4) was coated in microtitration plate wells (ThermoLabsystems, Helsinki, Finland) and KLK3 was bound to the antibody. The GST fusion peptide C-4 (0.17 µM) and each of the synthetic peptides (A-1, B-2 or C-4) at 10–100 fold molar excess were incubated with KLK3. After 1 h, the wells were washed and Eu^{3+}-labelled anti-GST antibody was added. After a further incubation for 1 h and washing, the bound fluorescence was measured with a 1234 Delfia Research fluorometer (Perkin Elmer Wallac, Turku, Finland).

4.4.5 NMR spectroscopy

Because of the low solubility in water (especially C-4) all spectra were recorded in DMSO-d_6. NMR samples were prepared by dissolving purified and lyophilized peptides in 600 µl DMSO-d_6 to 5–10 mM. The pHs of the samples were not tested. All one-dimensional experiments were recorded at five different temperatures over the range 300 K to 320 K. All two-dimensional experiments were recorded at either 305 K or 310 K depending on the quality and clarity of the spectra. All the chemical shifts are reported with respect to the DMSO peak at 2.50 ppm (*Table 5*).

For all 2D experiments, standard pulse programs from the Bruker software library were used. TOCSY spectra were recorded with mixing times of 80 ms by means of a MLEVTP mixing sequence with TPPI phase cycling (Bax & Davis 1985). NOESY spectra were mainly recorded with mixing times of 400 ms for A-1, 420 ms for B-2 and 300 ms for C-4 using TPPI phase cycling (Jeener et al. 1979). Various mixing times were tested and the best were determined based on the quality and clarity of the spectra. NOE-build up curves were not determined. Some COSY spectra were recorded with mixing times of 30 ms. Data sets were processed with phase-shifted sine bell functions. Typically the data were recorded with a resolution of 1024 points for both $t1$ and $t2$.

4.4.5 Structural analysis

Starting structures for structural analysis were constructed by DYANA software (Guntert et al. 1997). NOE-intensities were calibrated approximately relative to the β-protons of Tyr7 and Cys10 and the aromatic ring protons of Tyr7 in A-1, the aromatic ring protons of Tyr7 in B-2 and the aromatic ring protons of Trp11 in C-4. NOE correlations were classified either as strong (1.8–2.5Å), medium (1.8–3.5Å) or weak (1.8–5.0Å). Pseudo-atom corrections of 1.5Å for methyl, 1.0Å for methylene protons and 2.0Å for tyrosine ring protons were added when needed. Dihedral angles were not restricted mainly because of fluctuation of 3-D structures (Wüthrich 1986). The final DYANA structures were modelled and visualized with the software package QUANTA with help of the CHARMM program on a Silicon Graphics workstation (Brooks et al. 1983).

5 Mimetics of the disulfide bridge between the N- and C-terminal cysteines of the KLK3-stimulating peptide B-2[7]

5.1 INTRODUCTION

This chapter is based on the findings of two separate studies[7] in which the strategies to improve the *in vivo* stability and bioavailability of KLK3 binding peptides were investigated. In the study presented in the chapter 4, it has been shown that most of the amino acid side chains are essential for the promoting activity of the KLK3 binding peptide B-2, making the side chain modifications difficult. The KLK3-stimulating peptides, A-1, B-2 and C-4 are cyclic, containing disulfide bridge between the *N*- and *C*-terminal cysteines. Peptide C-4 contains also a second internal disulfide bridge. The first step in stabilizing the peptides was to use alternative cyclization methods. It has been shown that the plasma stability of linear KLK2 inhibiting peptide is increased by head-to-tail cyclization. This study is presented later in the chapter 6.

The earlier attempts to perform head-to-tail cyclization of the two most potent peptides B-2 and C-4 have failed without detectable yields of cyclic peptides (data not shown). Typically, the cyclization of peptides is highly dependent on the sequence, amino acid composition and the length of the peptide. Several research groups have published successful cyclization of penta- and hexapeptides but the head-to-tail cyclization of longer sequences is more challenging, as recently discussed by Davies (Davies 2003). Of the two most active peptides, B-2 (**1**) (*Figure 27*) was chosen as the lead peptide, since its conformation is less restricted than that of C-4 (chapter 4).

In the first study presented in this chapter, the strategy was to use various types of linking units, which are connected by amide bonds and also suitable for automated SPPS, for the replacement of conventional cysteines in *N*- and *C*-terminus. In some cases, also one of the valines on either side of the cysteines was replaced. A set of differently cyclized peptides with lysine, ornithine, β-alanine or γ-amino butyric acid (GABA) at the *N*-terminal site and aspartic acid at the *C*-terminal site was generated. The *C*-terminal aspartic acid was linked to the resin via either the backbone or side chain carboxylic group depending on the synthesized bridge structure. In addition, longer linking units, such as 6-aminohexanoic acid (Ahx) and 8-aminooctanoic acid (Aox), were also investigated. Successfully cyclized structures are shown in *Figure 28*.

Figure 27. Structure of peptide B-2. Area for replacement studies is shadowed with gray.

The stimulation of the KLK3 activity was evaluated for the successfully cyclized peptides. The stability in plasma was evaluated for the peptides which significantly stimulated KLK3 activity. As KLK3 cleaves substrates mainly at the *C*-terminal side of tyrosine and glutamine (Coombs et al. 1998, Malm et al. 2000) it is feasible that the B-2 peptide also acts as a substrate for KLK3. Therefore, the stability against enzymatic degradation by KLK3 was also studied.
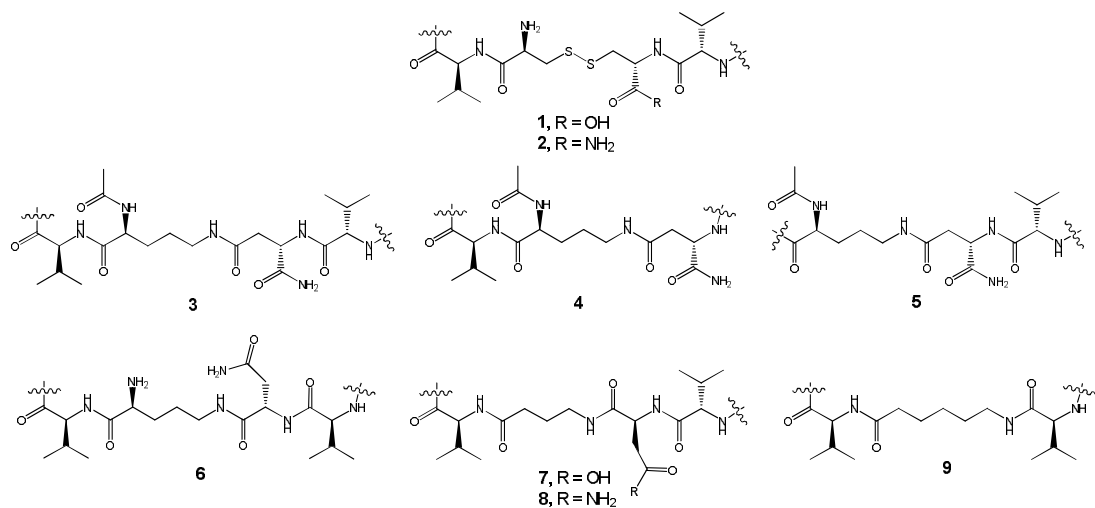
Figure 28. The structures of the successfully synthesized disulfide bridge replacements.

In the second study presented in this chapter, the disulfide bridge was replaced by other types of linkers. Based on the first study, the use of the *E*-alkene and the alkane linkers for mimetics of the disulfide bridge in peptide **1** were also studied. The strategy of replacing

disulphide bridges with carbon isosteres has already been proven to yield peptides that are both structurally (Ghalit et al. 2007a) and biologically (Ghalit et al. 2007b) equivalent to the original compound. Tadd and his collaborators have recently presented the synthesis of a series of disulfide bridge mimetics with different hydrocarbon cross links (Tadd et al. 2011). The same type of hydrocarbon cross linkers have recently been successfully used to cross link α-helices (stapled peptides) while retaining biological activity (Kim & Verdine 2009).

5.2 RESULTS AND DISCUSSION

5.2.1 Synthesis of B-2 variants

Initially, the original B-2 peptide (**1**) was synthesized with *C*-terminal amide using Rink Amide AM resin, yielding peptide **2**. The synthesis strategies for alternatively cyclized peptides are represented in *Figure 29*. In the first set of alternatively cyclized peptides, the disulfide bridge was replaced with a side chain to side chain bridge, using side chain ODmab-protected aspartic acid at the *C*-terminus and side chain Mtt-protected lysine or ornithine at the *N*-terminus. The aspartic acid residue was coupled to Rink Amide AM resin via the carboxyl terminus, leaving the side chain free for cyclization after Dmab-deprotection (**10**, **11**, **12**). The length of the modified bridge area (marked with gray background in *Figure 27*) was also changed by removing either Val11 or Val2 (**11**, **12**). The carboxylic acid group attached to the resin was converted to the corresponding amide using Rink Amide AM resin.
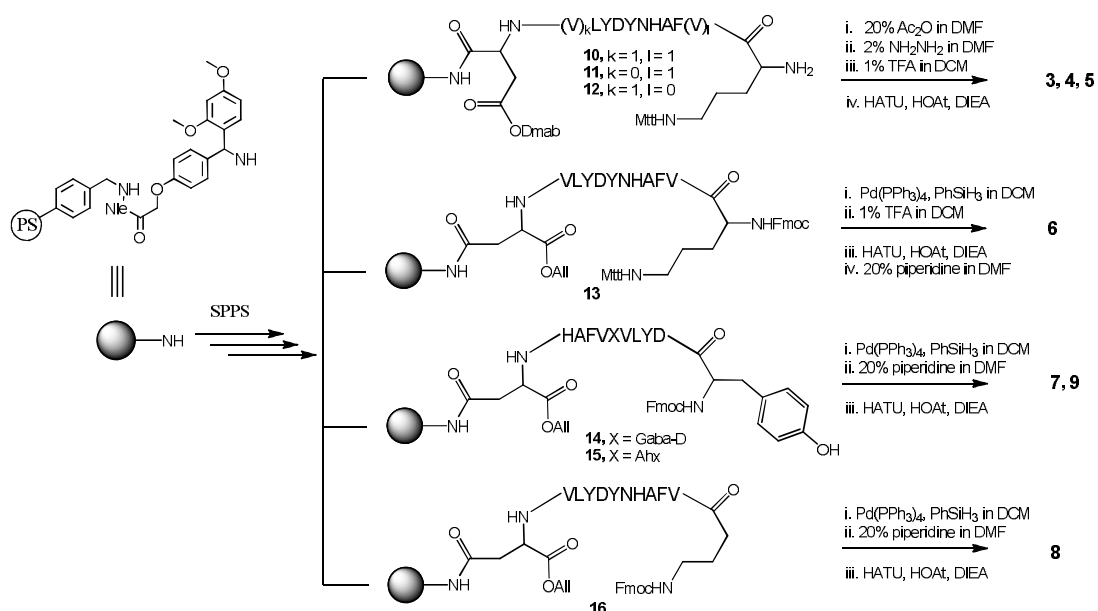
Figure 29. Representative solid-phase synthesis of peptides **3**–**9**.

The cyclization of the peptides having an *N*-terminal ornithine was successful, yielding peptides **3**–**5** but only when the amino group of the *N*-terminus of the peptides was acetylated. The cyclization of Fmoc-protected peptides was not successful. The cyclization of the peptides having an *N*-terminal lysine failed, although the α-amino group was acetylated. Earlier studies with peptide **1** have shown that modification of the *N*-terminus

does not reduce the activity of the peptides (Wu et al. 2004). The peptide with an *N*-terminal ornithine and *C*-terminal aspartic acid was also coupled to the resin via the side chain of aspartic acid (**13**), leaving the *C*-terminal carboxyl group free for cyclization. The cyclization of this peptide, yielding peptide **6**, was successful although the amino group of the *N*-terminus was not acetylated. In peptide **6,** the length of the bridge replacement was reduced by one –CH₂– group as compared to peptide **3**.

Peptides **7** and **8** have a bridge where the amino group of the GABA at the *N*-terminus is coupled to the main chain carboxylic acid group of aspartic acid at the *C*-terminus. Peptide **1** contains asparagine at position **6**, which provides an additional resin binding site. Peptide **7** was synthesized using *C*-terminal All-protected aspartic acid, conjugated to Rink Amide resin via the side chain producing Asp6 in free peptide (**14**). After completing the synthesis, the peptide was cyclized via the free amino terminus of tyrosine (original position 7) to the deprotected resin-bound aspartic acid. In peptide **8**, aspartic acid at position 12 was conjugated to Rink Amide resin via the side chain and the bridge was formed via GABA (**16**). Peptide **9** was synthesized with the same method as peptide **7** by attaching Asp6 to the resin (**15**). The bridge in peptide **9** was constructed by using Ahx as a linker.

Also other bridge structures were tried to be synthesized, but the cyclization of them failed or gave only extremely low yields and purities (*Figure 30*). These included a cyclization of an *N*-terminal β-alanine with the side chain of a *C*-terminal aspartic acid (**17**), where the peptide was attached to the resin from the *C*-terminal carboxylic acid of the aspartic acid. Another unsuccessful trial (**18**) was the analog of peptide **9** with an Aox linker instead of Ahx. Furthermore, a peptide with a tri-alanine bridge between valines (**19**), using resin-bound Asp6 as a cyclization site, was also unsuccessful.
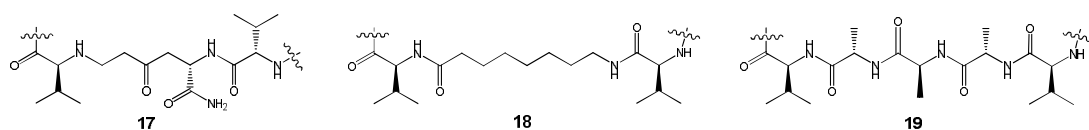
Figure 30. The structures of the unsuccessfully synthesized disulfide bridge replacements.

5.2.2 Synthesis of hydrocarbon disulphide bridge mimetics and peptides containing these mimetics

The replacement of disulphide bridge with *E*-alkene and the alkane linkers resulted pseudopeptides **20a** and **20b** (*Figure 31*). The orthogonally protected bridge mimetics were synthesized using the Grubbs metathesis reaction (*Figure 32*) and the conformation of the double bond determined as previously published (Tadd et al. 2011). The methyl ester group of the building blocks was hydrolyzed and the building blocks were incorporated in the peptide synthesis as described in the experimental section.
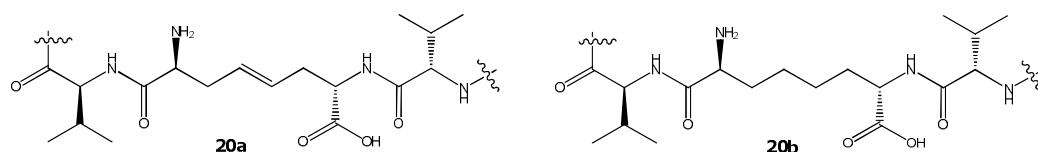
Figure 31. The structures of *E*-alkene **20a** and alkane **20b** linkers.
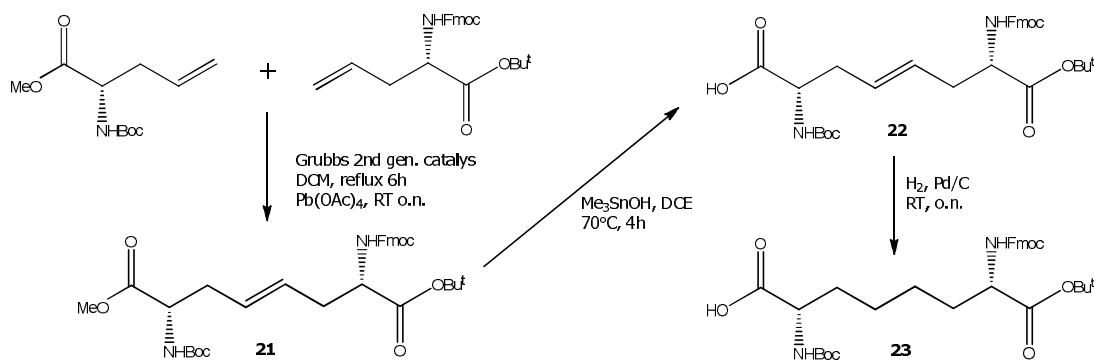
Figure 32. Synthesis of building blocks **22** and **23**.

The syntheses of the peptides were performed on-resin attaching an aspartic acid to the Rink Amide AM resin (*Figure 33*). That made it possible to maintain the sequence of the original B-2-peptide unmodified with just the disulphide bridge replaced. Longer reaction times were used to improve the coupling efficiency of the bridge mimetic building blocks. Syntheses of the linear sequences were straightforward but the cyclization step on-resin was obviously the yield limiting step. The length of the peptide and the bulky tyrosine in the N-terminal site of the cyclization point were affecting the cyclization rates and the low yields were expected. The head-to-tail cyclization with different building blocks has resulted in low yields also in the first study. Peptide **20a** with a double bond gave the highest yields with the yields decreasing when the double bond was removed. It seems that the more flexible the bridge mimetic is, the lower the yield of the cyclization. It was common for all of the sequences synthesized that there were a lot of impurities, and the purifications with HPLC were repeated two or three times yielding decreasing amounts of the products. One common impurity according to MS studies was incomplete deprotection of OAll-group of the aspartic acid preventing the head-to-tail cyclization.
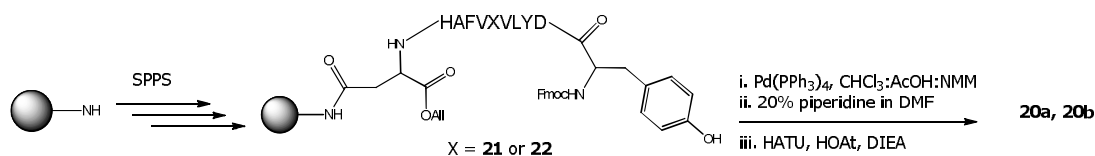
Figure 33. Representative solid-phase synthesis of peptides **20a** and **20b**.

5.2.3 Determination of activity

The ability of the synthesized peptides **1–9** to stimulate KLK3-activity in the first study is shown in *Figure 34*. Peptides **3–5** did not show any activity against KLK3. These peptides had side chain to side chain cyclizations between an ornithine at the *N*-terminus and aspartic acid at the *C*-terminus, and peptides **4** and **5** were shortened with one valine from either side of the bridge. These results suggest that the structure of the bridge is important for activity, especially the length of the bridge. The B-2 peptide (**1**) has 14 atoms counting the atoms in the bridge fragment. In peptide **3** the number of atoms is 16. Peptides **4** and **5**, on the other hand, have only 13 atoms in the bridge and they lack one of the adjacent valine residues. Peptide **6** stimulated KLK3 activity but less than peptide **1**. Peptide **6** has 15 atoms in the bridge, suggesting again that the length of the bridge is important and it is getting closer to the optimal length. In addition, in peptide **6** the configuration of the stereocenter

derived from L-aspartic acid is reversed with respect to the main chain in the ring, and the carboxylic acid group side chain is one –CH₂– group further apart as compared to the peptide **1**. Peptide **7** was almost as potent as the peptide **1** and peptide **2**. Peptide **7** has the same number of atoms in bridge (14) as the peptide **1**, again emphasizing the importance of the length of the bridge. Furthermore, peptide **7** clearly shows that the amino group at the *N*-terminus is not needed for the KLK3-stimulating activity. Peptide **8** differs from peptide **7** only in that the carboxylic acid in the side chain in peptide **7** is an amide in peptide **8**. This reduces the activity, which is in agreement with the earlier observation that the free carboxylic acid is preferred over an amide, i.e., peptide **2** is slightly less active than peptide **1** (*Figure 34*). Interestingly, the relative configuration of the stereocenter next to the carboxylic acid group is not important. Peptide **9** showed no activity, indicating again the importance of a *C*-terminal carboxyl group. However, peptide **9** is one atom shorter than peptides **7** and **8**, having only 13 atoms in the bridge, which might also have an effect on the activity.
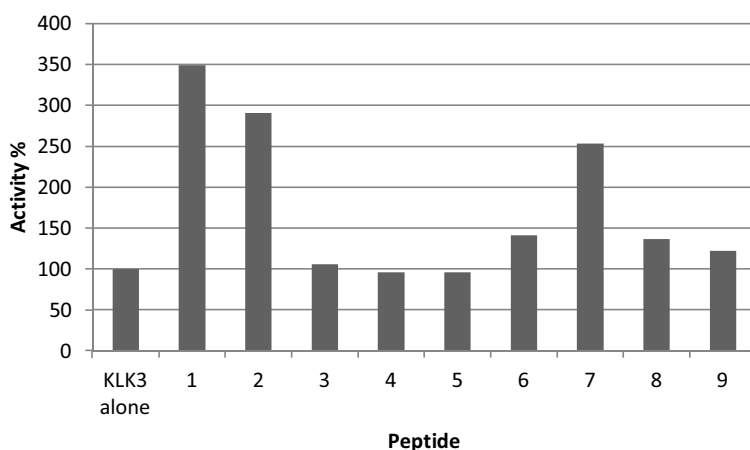
Figure 34. Effect of the synthetic peptides on KLK3 activity. Stimulation of activity of synthetic peptides as compared to KLK3 without peptides (KLK3 alone is indicated as 100%). Peptide concentration used was 13 µM.

The ability of the synthesized peptides **20a** and **20b** to stimulate the activity of KLK3 in the second study is shown in *Figure 35*. Both peptides stimulated the activity of KLK3 but the stimulations were lower compared to the original peptide **1** and slightly lower compared to the earlier analogs with a linker consisting of aspartic acid and γ-amino butyric acid as the bridge mimetic. The pseudopeptides containing the *E*-alkene (**20a**) and the alkane (**20b**) replacements for the disulphide bridge increased the enzymatic activity of KLK3 as compared to the original peptide **1** by 76 % and 22 %, respectively.
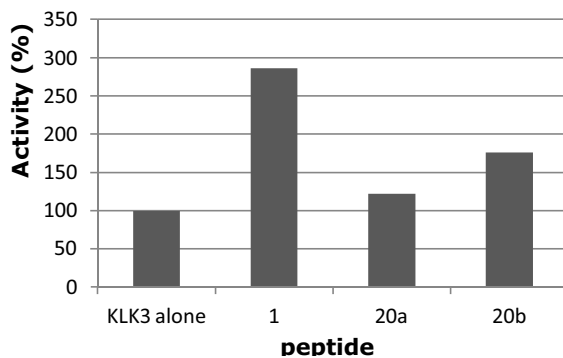
Figure 35. Effect of the synthetic peptides on KLK3 activity. Stimulation of activity of synthetic peptides as compared to KLK3 without peptides (KLK3 alone is indicated as 100%).

5.2.4 Concentration dependency of KLK3-stimulation by the peptides

The concentration dependency was studied using the most active peptides, i.e., **1**, **2** and **7** in the first study. While these peptides showed a dose-dependent stimulation of KLK3-activity at low concentrations, the original B-2 (**1**) started to show reduced stimulation at higher concentrations (*Figure 36*). The results suggest that peptide **1** could also act as a substrate for KLK3 (see below), especially at high concentrations at which the activation by peptide **2** reaches its maximum. Preliminary molecular modeling has suggested that while the major binding site of peptide **1**, where it is likely to exert its stimulatory effect, is outside of the active center of KLK3, there might be a second binding site at the active site where it could compete with substrate (Henna Härkönen, personal communication). Unlike the peptides **1** and **2**, peptide **7** stimulated KLK3 activity dose dependently at all concentrations studied, exceeding the effect of other peptides. This finding together with a lower degradation rate of peptide **7** by KLK3 or plasma proteases (see below) suggests that the binding of peptide **7** to KLK3 is different from that of peptide **1**.
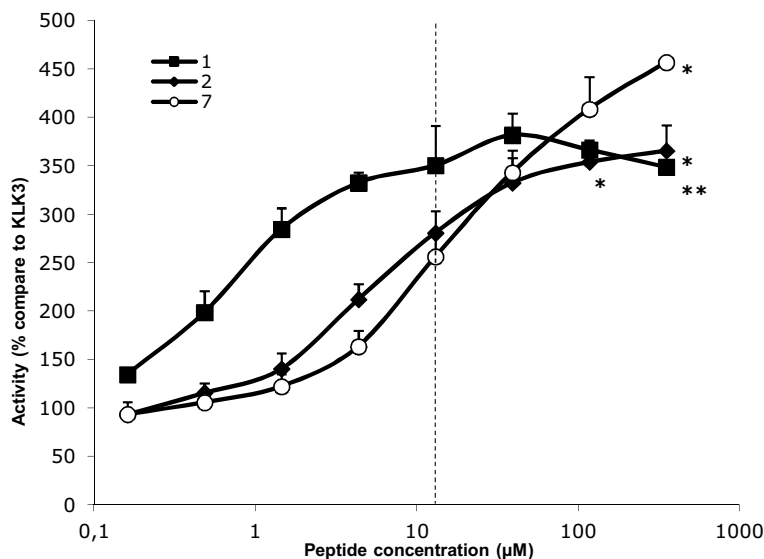
Figure 36. Concentration dependency of the KLK3 stimulation by peptides **1**, **2** and **7**. Data represent average +SD of three independent experiments, except for *n = 2 and **n = 1. Solid line shows the concentration (13 µM) used for the data shown in *Figure 34*.

5.2.5 Stability studies

The original peptide **1** and peptide **7** were cleaved by KLK3 at high concentrations in the first study. The cleavage rate of peptide **7** was slower than that of peptide **1**. Using a ninefold molar excess of peptides and after 2 h incubation, 59% of peptide **1** and 72% of peptide **7** were intact. After 24 h 5.7% (**1**) and 34% (**7**) were found intact, respectively (*Figure 37A*). Mass spectrometry analysis indicates that the ring opens between Tyr7 and Asp8 (data not shown). Although the amino acid sequence of peptide **7** is identical to the original peptide, excluding the bridge, it is more stable against KLK3, suggesting different binding behavior.

The original peptide **1** and peptide **7** were tested for plasma stability. During the first 2 h, 26% of peptide **1** and 13% of peptide **7** disappeared, either by degradation or binding to plasma proteins. After 2 h, the disappearance rate became slower, and finally after 24 h, 58% of peptide **1** and 73% of peptide **7** were found intact (*Figure 37B*). These results suggest that the replacement enhances the stability of the KLK3-stimulating peptides against plasma proteases or decreases binding to plasma proteins.
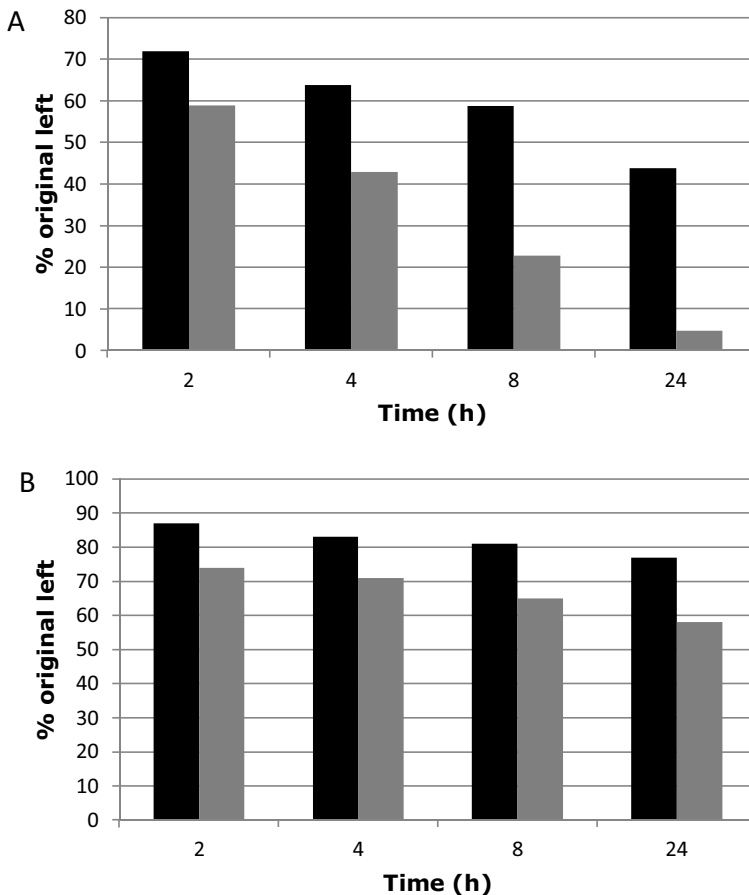
Figure 37. Stability of peptides **7** (black columns) and **1** (gray columns) over the time (A) with purified KLK3 and (B) in plasma.

5.3 CONCLUSIONS

Since the earlier studies have shown that Ty7 and Asp8 are essential for the promoting activity of B- 2 peptide (chapter 4), the alternative cyclization methods were used in order to improve the stability of B-2 peptide instead of side chain modifications. Based on the new analogs of peptide B-2 (**1**), it can be concluded that the bridge between the N- and C-termini is highly important for the KLK3-stimulating activity. The number of the atoms in the alternative bridge structure was found to be important. A free carboxylic acid group at the *C*-terminus was also important for the KLK3-stimulating activity but there was no difference whether the free carboxylic acid was in side chain or in the main chain of L-aspartic acid. The free carboxylic acid could be replaced by an amide group but this replacement reduced the KLK3-stimulating activity. On the other hand, the amino group at the *N*-terminus could be removed without affecting the KLK3-simulating activity. Peptide **7** with a cysteine based disulfide bridge mimetic consisting of GABA and aspartic acid, where the amino group from the former was linked to the main chain carboxyl group of the latter, is more stable than peptide **1** and, at high concentrations, the activity exceeds that of peptide **1**.

Further in the second study, it was shown that no heteroatoms are necessary for biological activity, a linker consisting of a simple hydrocarbon chain is sufficient. Peptide **20a** displayed the lowest activity, possibly due to incorrectly restricted conformation of the peptide compared to the conformation of the parent peptide. Peptide **20b** displayed moderate activity stimulation. This is likely due to the more flexible linker which eases the peptide to adopt the biologically active conformation.

5.4 EXPERIMENTAL

5.4.1 Peptide synthesis
Rink Amide AM resin (50 mg, loading 0.575 mmol/g) was used as solid-phase with Fmoc strategy and HBTU/DIEA as coupling reagent. The side chain protecting groups used in bridge forming residues were Acm for cysteine, OAll for *C*-terminus and both Bu^{t}O and ODmab for side chain protection of aspartic acid and glutamic acid, Mtt for ornithine and lysine side chain protection. Additional amino acids used for bridge formation were Fmoc-protected β-alanine, GABA, Ahx and Aox. Fmoc group was not removed from peptidyl resins **13–16** after final amino acid coupling. Peptide **1** was obtained from AnaSpec (San Jose, CA, USA) and was >95% pure as detected by HPLC.

5.4.2 Purification and mass spectrometry of peptides
The purity and degradation were verified by mass spectrometry on an ABI QSTAR XL hybrid mass spectrometer using MALDI or ESI interface (Applied Biosystems Inc., Foster City, CA, USA) for the disulfide bridge containing sequences. Negative mode was used for the detection of head-to-tail, side chain to tail and side chain to side chain cyclic peptides.

5.4.3 Cyclization of the disulfide bridged peptide
Peptide **2**, containing cysteines protected with Acm, was cyclized by the standard iodination method in solution. Peptide was purified by HPLC and lyophilized yielding peptide **2**. ESI–MS: (m/z) 1.443.6 [M + H]$^+$. HPLC: t$_R$ = 15.6 (purity >95%).

5.4.4 Cyclization method for alternatively cyclized peptides

Head-to-tail, side chain to tail and side chain to side chain cyclizations were performed on resin using HATU and HOAt as the coupling agent and DIEA as the base (*Figure 29*, see page 72). The yields were not expected to be higher than a few percent due to the long sequences. Furthermore, bulky Tyr7 adjacent to the cyclization sites in case of the B-2 analogs connected to resin via Asp6 (peptides **7** and **9**) expected to cause problems in cyclization. Therefore, the coupling step was performed repeatedly and with different solvent concentrations between DMF and DCM because those solvents swell the resin and peptides differently due to their different polarities. Yields of crude peptides after lyophilization varied between 25 and 35 mg and after purification by HPLC between 1 and 3 mg (2–8%) depending on peptide synthesized. Unsuccessfully cyclized peptides yielded no analytical HPLC detectable amounts of pure peptide.

The Kaiser test was performed for all alternatively cyclized peptides before the cyclization step and all tests were found positive indicating a free amino group (Kaiser et al. 1970). Usually the peptidyl resin was swelled for 30 min with the solvent used in the coupling step, the solvent was removed and the coupling agents, HATU (3 equiv), HOAt (3 equiv) and DIEA (4.5 equiv), were added with a small amount of anhydrous solvent achieving a final volume of 3 ml. The mixture was stirred for 2.5 h at RT under argon, the solvent was then removed, and the resin was washed with DMF (3 ml, 3 x 5 min) and with anhydrous DCM (3 ml, 3 x 5 min). The Kaiser test was performed to monitor the success in cyclization. If the Kaiser test results turned out to be positive, the cyclization was repeated. Usually the first cycle was performed in DMF, the second in 25% DCM in DMF and the third in 50% DCM in DMF. Higher concentrations of DCM were discarded due to the low solubility of HATU in DCM. When the Kaiser test was negative, the peptides were cleaved from resin and then lyophilized.

5.4.5 Deprotection and characterization of alternatively cyclized peptides

Peptidyl resins **10**, **11** and **12** with Orn–Asp bridge as well as corresponding lysine based peptidyl resins (schemes not shown) from automated SPPS were placed in reaction columns, and the free *N*-terminal amino groups were acetylated manually adding 20% Ac$_2$O in DMF (3 ml) and stirring resulting solution 10 min at RT. Treatments were repeated and resins washed with DMF (3 x 3 ml). Side chain Dmab-protection was removed using 2% hydrazine in DMF (3 ml) agitating 10 min at RT. The treatment was repeated twice, followed by washing with 20% DIEA in DMF:H$_2$O; 9:1 (3 ml) to make sure that all traces of Dmab were removed. Resins were washed with DMF (3 x 3 ml) and DCM (3 x 3 ml). *N*-terminal Mtt was deprotected using 1% TFA in DCM (3 ml) agitating 10 min at RT. Treatment was repeated three times and the deprotection was monitored by disappearance of the orange color characteristic for traces of Mtt. Resins were washed with DMF (3 x 3 ml) and DCM (3 x 3 ml) and dried. Peptides were cyclized on resin using HATU/HOAt/DIEA-method as described above. After cleavage peptides were lyophilized yielding peptides **3**, **4** and **5**. Peptide **3**, ESI–MS: (m/z) 1,493.0 [M - H]⁻, HPLC: t$_R$ = 16.0 (purity >90%). Peptide **4**, ESI–MS: (m/z) 1,393.95 [M - H]⁻, HPLC: t$_R$ = 16.8 (purity >95%). Peptide **5**, ESI–MS: (m/z) 1,393.90 [M - H]⁻, HPLC: t$_R$ = 16.0 (purity >85%).

Peptidyl resin **13** from automated SPPS was placed in reaction column and washed with DCM (3 x 3 ml). *C*-terminal allyl deprotection was performed using a catalytic amount of Pd(PPh$_3$)$_4$ (0.1 equiv) with PhSiH$_3$ (10 equiv) as a scavenger in anhydrous DCM (3 ml). The

mixture was purged with an argon flow and stirred under argon for 1.5 h. The resin was washed with DMF (3 x 3 ml), 0.5% DIEA in DMF (3 x 3 ml), 0.5% sodium diethyldithiocarbamate trihydrate in DMF (3 x 3 ml), DMF (3 x 5 ml) and finally with DCM (3 x 5 ml). The treatment was repeated to drive the deprotection to completion. Deprotection of side chain Mtt was performed as described in previous chapter. Peptidyl resin **13** was additionally deprotected using 2% TFA in DCM (3 ml) to increase the deprotection rates, although this might also lead to cleavage of the peptides from the Rink-resin (Bourel et al. 2000). Peptide was cyclized on resin using HATU/HOAt/DIEA-method as described above. *N*-terminal Fmoc deprotection was performed after cyclization using 20% piperidine in DMF (3 ml, 2 x 15 min), followed by washing with DMF (3 ml, 3 x 5 min) and DCM (3 ml, 3 x 5 min). After cleavage peptide was lyophilized yielding peptide **6**. ESI–MS: (m/z) 1,451.0 [M - H]$^-$, HPLC: tR = 15.5 (purity >95%).

Peptidyl resins **14**, **15** and **16** from automated SPPS were placed in reaction columns and washed with DCM (3 x 3 ml). *C*-terminal allyl deprotection and *N*-terminal Fmoc deprotection were performed as described in previous chapter. After deprotection peptides were cyclized on resin using HATU/HOAt/DIEA-method as described above. After cleavage peptides were lyophilized yielding peptides **7**, **8** and **9**. Peptide **7**, ESI–MS: (m/z) 1,422.5 [M - H]$^-$, HPLC: tR = 16.8 (purity >95%). Peptide **8**, ESI–MS: (m/z) 1,421.5 [M - H]$^-$, HPLC: tR = 16.4 (purity >85%). Peptide **9**, ESI–MS: (m/z) 1,335.8 [M - H]$^-$, HPLC: tR = 18.3 (purity >90%).

5.4.6 Synthesis of building blocks

Compound **21** was synthesized according to a previously published procedure in similar yields (Tadd et al. 2011).

(7S,E)-7-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-8-(tert-butoxy)-2-((tert-butoxycarbonyl)amino)-8-oxooct-4-enoic acid (22). Trimethyltinhydroxide (76.3 mg, 0.42 mmol) was added to a stirred solution of compound **21** (*Figure 32*, see page 74, 50 mg, 0.08 mmol) in 1,2-dichloroethane (2.0 mL) under argon. The reaction mixture was then heated at 70 °C for 4 hours. After this time, the reaction mixture was concentrated *in vacuo*, and the residue dissolved in ethyl acetate (ca. 15 mL). The organic phase was washed with 0.5 M HCl(aq) (3 × 15 mL) and brine (ca. 15 mL), dried over anhydrous sodium sulphate, filtered and concentrated *in vacuo* before purification by flash column chromatography.

(7S)-7-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-8-(tert-butoxy)-2-((tert-butoxycarbonyl)amino)-8-oxooctanoic acid (23). Compound **22** was dissolved in EtOAc, Pd/C added and the mixture stirred 24h at RT under an atmosphere of H$_2$. The Pd/C was filtered off using a small pad of silica at the mixture concentrated *in vacuo*. This gave **23** which was used without further purification.

5.4.7 Synthesis of peptides with all-hydrocarbon disulphide bridge mimetics

The peptides were synthesized using an Apex 396 DC multiple peptide synthesizer (Advanced ChemTech, Louisville, KY, USA). Manual couplings were performed using a PD-10 column with a magnetic stirring. A Rink Amide AM resin (50 mg, loading 0.575 mmol/g) was used as a solid-phase.

General method for the manual SPPS: The resin was swelled for 30 min in DMF and DMF was removed. All natural residues (D, H, A, F, V, L, Y, N, 4.9 eq.) were coupled using HBTU (5 eq.) as the activating agent and DIEA (10 eq.) as the base. Fmoc-protected amino

acids were dissolved in DMF. HBTU and DIEA were added to the mixture and the mixture was added to the resin. The coupling times used were 40 min. The resin was then washed with DMF (3 x 3 ml). A N-terminal Fmoc deprotection was performed using 20% piperidine in DMF (2 ml, 3 x 5 min). The resins were carefully washed with DMF (3 x 5 ml) after the Fmoc-deprotection. Coupling and deprotection steps were monitored usually after couple of cycles with Kaiser-tests.

The couplings and deprotection of orthogonally protected disulphide bridge mimetics (*Figure 33*, see page 74) was performed as above using 4 h as a coupling time to ensure higher coupling efficiency. The Fmoc-deprotections were performed as mentioned above.

5.4.8 Deprotection of terminal protecting groups of peptides with an all-hydrocarbon bridge

C-terminal allyl deprotection was performed using Pd(PPh$_3$)$_4$ (1 eq.) in 2 ml mixture of CHCl$_3$:AcOH:NMM (37:2:1) and stirred under argon for 2 h at RT. Washing, catalyst removal and *N*-terminal Fmoc-deprotection was performed as described in chapter 5.4.4.

5.4.9 Cyclization of peptides with an all-hydrocarbon bridge

Head-to-tail cyclizations were performed on resin using HATU and HOAt as the coupling agent and DIEA as the base (*Figure 33*). Reactions were performed using HATU (4.8 equiv), HOAt (4.8 equiv) and DIEA (7.2 equiv) as described in chapter 5.4.4. After cleavage peptides were lyophilized and purified as described previously. After the first purification cycle, the purity varied between 30 -70% and second purification was needed. After the second purification, peptides were lyophilized yielding ca. 1.0 mg of peptide **20a** (~ 5%), and around 0.1 mg **20b** (<1%). Peptide **20a**, ESI–MS: (m/z) 1406.01 [M - H]-, HPLC: tR = 15.3 (purity > 90%). Peptide **20b**, ESI–MS: (m/z) 1408.03 [M - H]-, HPLC: tR = 15.0 (purity 90%).

5.4.10 Effect of the peptides on KLK3 activity

KLK3 (0.3 µM) was incubated for 30 min at RT with synthetic peptides (13-14 µM) in 0.05 M Tris buffer, pH 7.7, containing 0.154 M NaCl, 8 mM NaN$_3$ and 0.1% bovine serum albumin. After the addition of the substrate to a final concentration of 0.2 mM, the absorbance at 405 nm was measured at 5 min intervals for 30 min. The concentration dependency of the activity was determined by measuring the activity of KLK3 in the presence of the synthetic peptides in a series of concentration from 0.16 to 351 µM.

5.4.11 Stability tests

Proteolytic cleavage of selected peptides with KLK3 was followed for 24 h. Peptide (0.18 mM) was incubated with KLK3 (0.02 mM in PBS final concentration) in 150 µl volume at +37°C or with PBS as the control. Peptide samples of 35 µl were collected at 2, 4, 8 and 24 h time points.

Plasma stability tests for the selected peptides were performed by incubating the peptides with 250 µl fresh human EDTA plasma, 125 µl PBS and 125 µl peptide solution (0.5 mg/ml in H$_2$O) for 24 h at +37°C. For controls, the peptides were added to PBS. Peptide samples of 100 µl were collected at 2, 4, 8 and 24 h time points.

6 Activity and stability of human kallikrein-2-specific linear and cyclic peptide inhibitors[8]

6.1 INTRODUCTION

Previously, Hekim and his collaborators have identified KLK2-specific peptides by using the phage display methodology. These peptides are enzyme-specific and do not inhibit other proteases (Hekim et al. 2006). The motifs required for biological activity of KLK2-specific peptide inhibitors were characterized by alanine substitution analysis, and also sequences with improved inhibitory capacity were found (Hekim et al. 2006).

The proteolytic stability is an important factor for the use of peptides *in vivo*. Most peptides have to be modified to prevent enzymatic degradation. Several approaches including the use of D-amino acids, unusual amino acids, peptidomimetics or cyclization have been used to improve the peptide stability (Adessi & Soto 2002, Okarvi 2004). In this study, cyclic variants of KLK2 inhibitory peptides were prepared and their inhibitory activities were compared to the original peptides. Furthermore, the effect of cyclization on the peptide stability against digestion by trypsin and human plasma were studied. The conformational constraints of the peptides were studied by proton NMR spectroscopy.

6.2 RESULTS AND DISCUSSION

Phage display is a powerful method to produce peptides with novel biological activities, which may be useful for forming lead structures for the design of new diagnostic and therapeutic molecules. Hekim and his collaborators have identified novel peptide inhibitors that are specific to the serine protease KLK2 by phage display (Hekim et al. 2006). Two linear synthetic analogs, KLK2a (ARRPAPAPG) and KLK2b (GAARFKVWWAAG), display specific and efficient inhibition of KLK2. Although the amino acid sequences of the two peptides are dissimilar, their inhibition of KLK2 is competitive, suggesting that they bind to the same site. Both peptides contain arginine, which cannot be replaced with alanine, suggesting its important role for binding (Hekim et al. 2006). Substrate-specificity studies indicate that KLK2 has strict preference for arginine at position P1 in cleavage site followed by a serine at position P'1 (Cloutier et al. 2002). In spite of this, peptides KLK2a and KLK2b are not cleaved by KLK2 but its protease activity *in vitro* is inhibited. The replacement of arginine with lysine in the peptide KLK2b did not abolish its inhibitory effect, suggesting that inhibition is based on the positive charge that guides binding of the peptides to KLK2.

6.2.1 Inhibitory activity of the peptides

In order to study the correlation between structure and function, a set of cyclic variants of both peptides KLK2a and KLK2b were synthesized by adding cysteines to the amino and carboxyl ends. The inhibitory effect of the linear and cyclic forms of the KLK2a and KLK2b peptides was measured by using 0.17 μM concentration of the KLK2 enzyme and a 100-fold molar excess of each peptide. There was a clear difference in the effect of cyclization between the two KLK2-specific peptides. Most of the cyclic forms of KLK2a were totally inactive and only one KLK2a(c2) displayed slight (23%) inhibition, while all cyclic KLK2b peptides were active and some of them were even more active than the original linear peptide. The most active peptide (KLK2b(c1)) inhibited 97% of the activity, while the linear peptide displayed 74% inhibition. A variant with the point mutation R4K in the linear form of KLK2b displayed equal activity compared to the original peptide (*Table 8*). Inhibition assays showed that essentially all the cyclic KLK2a peptides were inactive, while only one showed a slight inhibitory activity. The corresponding cyclic forms of the KLK2b peptides were active. Since the disulfide-bridged cyclic forms of KLK2b were biologically active, a set of cyclic peptides were further synthesized using the head-to-tail cyclization strategy. After cyclization, the inhibitory activity of the KLK2b peptides with various sequence lengths was similar and slightly higher than that of the corresponding linear peptide.

Table 8. Activities of linear KLK2 peptides and the corresponding cyclic forms (c = cyclic form with cysteine bridge, ht = head-to-tail cyclic peptide). Activities indicate the degree of inhibition of KLK2 activity.

Peptide	Sequence	MW (g/mol)	KLK2 inhibition (%)	±SD (%)
KLK2a linear	A R R P A P A P G	891.30	72	1.6
KLK2a(c1)	C A R R P A P A P C G	1095.35	−4	0.0
KLK2a(c2)	C A A R R P A P A P C G	1166.56	23	1.2
KLK2a(c3)	C A A A R R P A P A P C G	1237.36	3	0.1
KLK2a(c4)	C R R P A P A C G	927.49	−9	0.1
KLK2a(c5)	C A R R P A P A C G	1098.34	1	0.2
KLK2a(c6)	C A R R P A P A A C G	1069.13	−4	0.0
KLK2a(c7)	C A A R R P A P A A C G	1140.28	7	0.2
KLK2a(c8)	C A A A R R P A P A A C G	1211.42	0	0.0
KLK2b linear	G A A R F K V W W A A G	1318.53	74	0.3
KLK2b(c1)	G C A A R F K V W W A A C G	1522.60	97	0.0
KLK2b(c2)	C A A R F K V W W A A C	1408.60	95	0.2
KLK2b(c3)	G C A R F K V W W A C G	1380.59	94	1.7
KLK2b(c4)	G C A A R F K V W W A A C G	1522.80	90	1.8
KLK2b(c5)	C R F K V W W C	1124.40	72	1.4
KLK2b(c6)	G C R F K V W W C G	1238.50	70	1.2
KLK2b linear	G A A R F K V W W A A G	1318.53	72	1.2
KLK2b linear R4K mutation	G A K F K V W W A A G	1219.60	82	1.0
KLK2b ht1	A R F K V W W N	1087.69	93	0.8
KLK2b ht2	A R F K V W W A N	1158.62	94	0.6
KLK2b ht3	A A R F K V W W A N	1229.66	94	0.8
KLK2b ht4	A A R F K V W W A A N	1300.69	96	0.3
KLK2b ht5	A A A R F K V W W A A N	1371.73	95	2.1
KLK2b ht6	A R F K V W N	901.48	68	0.7

6.2.2 Biological stability

The stability of linear acetylated and two cyclic forms of KLK2b were determined by using modified trypsin, which cuts peptide bonds after lysine and arginine. Complete degradation of the linear forms occurred in 30 min, producing fragments cleaved after the positively charged amino acids (*Table 9*). Proteolytic cleavage of the peptide with cysteine bridges, produced a peptide with an additional 18 Da because of the degradation of one amide bond while the cysteine bridge remained intact. The same addition was seen with the head-to-tail form of the KLK2 peptide (KLK2b ht1). The cleavage rate of this peptide was much slower than that of the three other forms. HPLC analysis showed that only 57% of the KLK2b ht1 peptide was cleaved by trypsin in 4 h (*Figure 38A*). According to MSMS analyses, the major cleavage site was after arginine.

Table 9. Four different forms of KLK2b peptides digested with trypsin for 30–240 min and analyzed by mass spectrometry. Sequences of the fragments were deduced from the masses of the fragments (MW) by mass spectrometry. A dash (−) in the sequence indicates a possible cleavage site but the resultant peptides are still attached by cysteine bridges.

	Sequence	Mw
linear		
uncleaved	ARFKVWWG	1048,55
fragment 1	ARFK	520,30
fragment 2	VWWG	546,25
fragment 3	AR	245,14
fragment 4	FKVWWG	821,41
acetylated linear		
uncleaved	AcARFKVWWGG	1147,57
fragment 1	AcARFK	562,30
fragment 2	VWWGG	603,27
fragment 3	AcAR	287,14
fragment 4	FKVWWG	821,41
cyclic with Cys-bridge		
uncleaved	GCAARFKVWWACG	1451,470
estimated fragments +18 D	GCAARFK - VWWACG	1469,480
estimated fragments +18 D	GCAAR - FKVWWACG	1469,480
head-to-tail cyclic		
uncleaved KLK2b ht1	ARFKVWWN	1088,53
linear fragment 1	VWWNARFK	1105,57
linear fragment 2	FKVWWNAR	1105,57

To simulate the proteolytic environment *in vivo*, all four peptides were incubated in human plasma. The two linear forms as well as the cyclic form with a cysteine bridge were degraded within 30 min, whereas the head-to-tail peptide remained intact for 24 h (*Figure 38B*).
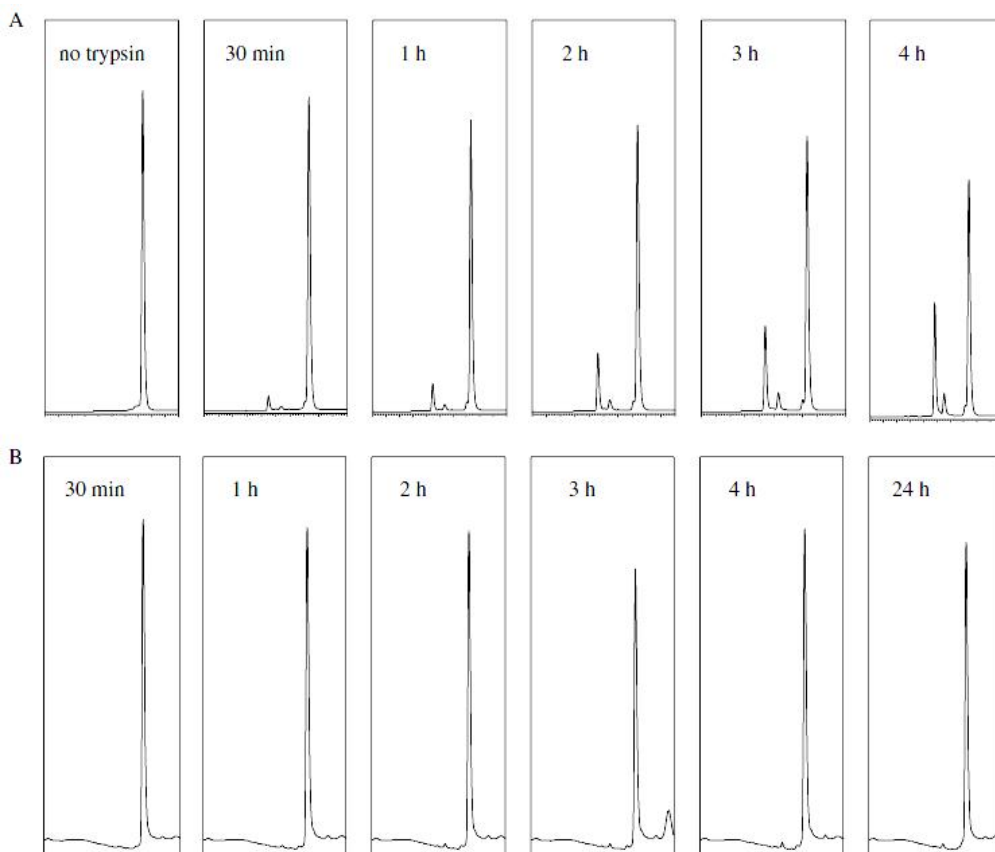
Figure 38. (A) HPLC analyses of the degradation of the head-to-tail form KLK2 ht1 with trypsin. About 57% of the peptide was cleaved after 4 h. (B) HPLC analyses of KLK2 ht1 peptide incubated with plasma. No significant degradation occurred during 24 h. Peptides and peptide fragments corresponding to the masses are shown in *Table 9*.

Although the linear forms of the peptides were not degraded by KLK2, they were sensitive to trypsin digestion and they were also degraded in plasma. The fragments detected after trypsin digestion indicate that the linear forms were cleaved after arginine and lysine. An adduct of 18 Da was detected after protease treatment of the cyclic form with a cysteine bridge, indicating that the peptide was cleaved after arginine or lysine, leaving the cysteine bridge intact. The most stable form was the cyclic peptide without terminal amino acids, i.e. head-to-tail cyclic form. After 4 h incubation with trypsin, 57% of the KLK2b ht1 peptide was still intact, and even after 24 h no fragmentation was observed in plasma. It is noteworthy that the used trypsin concentration was very high, i.e. fivefold higher than generally used for protein digestion for mass spectrometry. Head-to-tail cyclization has also been found to improve the stability of peptides against proteases of α-conotoxin, while maintaining the biological activity (Clark et al. 2005). The cyclic form of the conotoxin was sensitive to the size of the linker between the amino and carboxy termini. As opposed to peptides in the present study, the 3D structure of the cyclic conotoxin could be determined, which indicated a rigid conformation and influence of the sequence length on the correct conformation of the peptide. Absence of a rigid conformation in both the linear and cyclic forms of the KLK2b peptide could explain why both cyclic and linear forms with variable sequence lengths displayed similar activity towards KLK2. Usually

cyclization of peptides has been used to restrict conformational mobility and to enhance the affinity to the target molecule (Osapay et al. 1997, Weiner & Thakur 2005). In the case of KLK2 inhibitory peptides, a rigid structure does not seem to be needed for biological activity or stability. The results indicate that backbone cyclization is an efficient and simple approach to improve the resistance to proteolytic digestion also of flexible structures, and this approach, without any sequence modifications, could be useful for modification of other peptides that are used for *in vivo* studies. Although there were no defined rigid structural elements in cyclic forms, the slightly higher activity compared to the linear ones may be due to the restricted conformational space during binding. Lower internal rotational entropy may translate into higher binding affinity for the cyclic analogs.

6.2.3 NMR analyses

Solvent exposures of the NH groups were detected by determining their temperature coefficients. None of the investigated peptides had an amide proton showing very low Δδ/ΔT values (<3 ppb/K) characteristic of strong solvent shielding (Raghothama et al. 1996). No reasonable differences were found between the chemical shifts of CαH's and random coil values (Wishart et al. 1991). Medium or strong sequential CαH(i)–NH(i+1) NOEs were found between almost all residues in both peptides, indicating predominant β-conformations. Furthermore, no long-range interresidual NOEs were detected. Some broadening of the NH peaks was detected, indicating solvent-exposed NH groups or conformational fluctuation and dynamic behavior (Raghothama et al. 1996, Wüthrich 1986).

Structural studies of linear and cyclic peptides by NMR spectroscopy indicated the absence of major rigid conformations. Neither sequential NH(i)–NH(i+1) NOEs nor low-temperature coefficients were found for acyclic forms of KLK2a. Cyclic forms of KLK2a were not investigated with NMR because of their lack of biological activity. In the case of cyclic forms of KLK2b, only weak sequential NH(i)–NH(i+1) NOEs were found between some of the residues, and no low temperature coefficients for amide protons were found in the cyclic and linear forms of KLK2b. A majority of the recognized NOE correlation peaks were intraresidual or sequential (i − i+1). This suggests that there is no major conformational constrains in the KLK2b sequences. Furthermore, the chemical shifts between cyclized and acyclic forms of KLK2b were almost equal in all residues enabling structural similarity and therefore similar biological activity.

6.3 CONCLUSIONS

In conclusion, the head-to-tail cyclization of peptide inhibitors of KLK2 dramatically improves their resistance to proteolytic digestion and enhances their inhibitory capacity. Due to the long term stability in plasma, these peptides are potential candidates for the *in vivo* studies of prostate cancer in animal models.

6.4 EXPERIMENTAL

6.4.1 Peptide synthesis

The peptides (Table 1) were synthesized with Fmoc strategy and TBTU/DIPEA as the coupling reagent. Fmoc-Gly-Wang and Rink resins were used as the solid phase. For Cys,

both Acm and Trt protection groups were used. Allyl-protected aspartic acid (Fmoc–Asp(OH)–OAll) was used for head-to-tail cyclization. For stability tests, one of the peptides, KLK2b, was acetylated on the resin. The free amino group was treated with 20% acetic anhydride in DMF twice for 10 min before cleavage.

6.4.2 Cyclization of the peptides

Peptides containing cysteines with Acm were cyclicized by the iodination method. Head-to-tail cyclization was performed on the resin. Deprotection of the allyl ester was performed manually with Pd(0) under argon. The catalyst dissolved in a small amount of dry DCM was transferred to a sealed vial containing the Fmoc–peptidyl(resin)–OAll and $PhSiH_3$. The reaction mixture was stirred for 2.5 h at RT under argon. The catalyst was removed and the resin was washed and dried. The *N*-terminal Fmoc group was removed and head-to-tail cyclization of the peptides was performed twice by the addition of HBTU/DIEA (8 equiv., 1 : 1, mol/mol) in DMF for 2 × 3 h at RT. Peptides were cleaved from the Rink resin with 95% TFA, which introduced an asparagine into the final cyclic peptide. The yields of the both cyclic forms varied between 10 and 15%.

6.4.3 Effect of the peptides on the enzyme activity of KLK2

KLK2 (0.17 µM) was incubated with a 100-fold molar excess of synthetic peptides in 20 mM Tris-buffer, pH 8.0, containing 0.1% BSA for 30 min at RT. After the addition of the substrate to a final concentration of 0.2 mM, the absorbance was monitored at 405 nm at 5–10 min intervals for 1 h.

6.4.4 Stability studies

Initially, stability was studied by using modified trypsin (sequencing-grade modified trypsin V5113, Promega, Madison, USA). Four hundred microliters of each peptide (1 mg/ml) dissolved in 200 mM NH_4HCO_3 buffer (pH 8.0) was mixed with 1 µl of trypsin solution (1 mg/ml in NH_4HCO_3, pH 8.0). The peptides were incubated at +37 °C and 30 µl samples were taken every 30 min. Thirty microliters of 2% TFA and 5% ACN in water were added, and the samples were analyzed by HPLC and by mass spectrometry. The open rings of the KLK2 ht1 peptide were analyzed by MSMS.

The stability of the peptides in human plasma was studied by mixing 400 µl of human male plasma and 400 µl of each peptide in PBS (1 mg/ml). The peptides were incubated at +37 °C and 100 µl aliquots were taken after 30 min and then every hour up to 4 h. In the case of the head-to-tail form, the peptide was incubated for 24 h. The remaining plasma proteins on the filter were washed after centrifugation with 50 µl of 0.1% TFA and 0.1% Tween 20 in water and centrifuged again for 10 min.

6.4.5 NMR spectroscopy

Samples were prepared for NMR by dissolving the purified and lyophilized peptides in 500 µl 10% D_2O in H_2O to a peptide concentration of 20–25 mM. The pH of the samples varied between 2 and 3. One-dimensional experiments were recorded at different temperatures over the range 285–340 K. Two-dimensional (2D) experiments were recorded at 300 or 330 K depending on the quality and clarity of the spectra. All chemical shifts are reported with respect to the 5 mM TSP peak at 0.00 ppm. Two-dimensional experiments included COSY, TOCSY and ROESY with mixing times of 80 ms for KLK2a and 225 ms for KLK2b.

7 General discussion and Conclusions

7.1 GENERAL DISCUSSION

Prostate cancer is one of the most common cancers in the developed countries and also the one of the leading causes of cancer deaths. PCa is most commonly a clinically insignificant slow-growing tumor but it can also be expressed as an aggressively metastatic and lethal disease. Clinically PCa is very challenging because of its non-accurate, complicated diagnosis and unpleasant therapy. The decision to initiate therapy is difficult because in the most cases slow-growing tumors do not affect to the quality of the life of patients. The increased serum concentrations of KLK3 have been used as a marker for the detection of early-stage PCa but the drawback of serum tests is the high rate of false-positives, mostly due to benign prostate hyperplasia. KLK3 and its relative KLK2 are mainly expressed in prostate, in both healthy and abnormal conditions and are both very prostate specific. Both enzymes have been also shown to participate the prostate cancer development, KLK3 may have anti-angiogenic properties due its ability to inhibit endothelial cell growth whereas KLK2 is associated with tumor growth via proteolytic cascades. Therefore, agents with specific binding and interference with KLK2 and KLK3 activities may hold diagnostic and therapeutic potential.

Previously several peptides with specific binding and activity modulation toward KLK2 (Hekim et al. 2006) and KLK3 (Wu et al. 2000) have been identified. The therapeutic potential of peptides is limited due to their low *in vivo* stability. In this study, these peptides were further characterized by conducting structure-activity relationship studies and NMR-based structural characterization. The aim was to develop anologues with increased potential for *in vivo* applications.

Due to the limited information of the structures of the target proteins, the binding structures of these peptide ligands were unknown. Short peptides rarely maintain rigid conformations in solution and their binding is dependent on the target conformation and the surrounding chemical and physical environment. KLK3 binding peptides were developed using the phage display method with one or two pre-determined disulphide bridges as a conformational restriction. The sequential differences between KLK3 binding peptides in combination with the similarities of their biological response were striking. The cross-inhibition test revealed that these peptides bind to the same region of KLK3. Therefore, at the beginning of this study, the existence of common structural motifs in KLK3 binding peptides were studied to determine if it would be possible to modulate the structure by incorporating non-natural synthetic building blocks. For all three KLK3 binding peptides, A-1, B-2 and C-4 (*Figure 39*), the populations of reversed turn structures were found. All peptides contained one amide proton protected from solvent indicating that there were hydrogen bonds between the backbone carbonyl group and the amide proton. Intra- and inter-residual NOE-connectivities were used in the detection of turn types and the presence of NOE-connectivities suitable for the certain types of β-turns were observed. However, strong indications about conformational fluctuation were also detected reflecting the reality that the bioactive conformation cannot be derived solely from NMR-

based structure determinations. In peptide C-4, close connectivity between isoleucine and aromatic residues was detected.
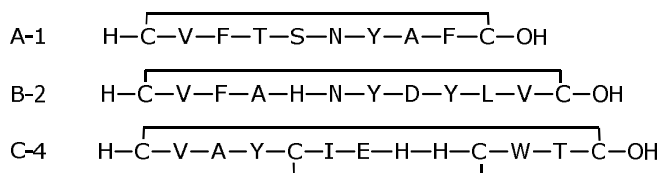
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A-1     H—C—V—F—T—S—N—Y—A—F—C—OH

B-2     H—C—V—F—A—H—N—Y—D—Y—L—V—C—OH

C-4     H—C—V—A—Y—C—I—E—H—H—C—W—T—C—OH
```

Figure 39. The original sequences of KLK3 binding peptides.

   The essential residues for biological activity and residues whose side chains can be removed without losing biological activity in terms of peptide mimetic design were mapped via alanine replacement studies. The replacement of a certain residue by alanine removes all atoms beyond C^β but it still maintains the conformational limitations of the backbone indicating the essentiality of the side chain for the biological response. In shorter peptides and those with only one disulphide bridge containing peptides A-1 and B-2, most of the replacements reduced the activity emphasizing the importance of correct sequence in the structure-activity relationship. The most dramatic effects were noted when aromatic and hydrophobic residues were replaced highlighting their importance. In the case of C-4, some activity stimulation remained in the most of cases, evidence that several residues are responsible for the maintenance of biological activity. The most important residues were once again aromatic and hydrophobic side chains. The importance of several residues for biological activity was studied by using peptide A-1 and by replacing two or three out of its ten residues at the same time. Moderate activity stimulations were detected except for one analog when tyrosine was replaced in position 7. In an additional study, peptide C-4, which had the highest stimulatory activity, was studied using multiple alanine replacements. Further, a targeted positional scan examined the possibility to replace valines nearby to the terminal disulphide bridge with residues suitable for use in a head-to-tail cyclization strategy. The multi-alanine replacement studies showed the importance of aromatic residues with hydrophobic isoleucine in position 6. The replacement of those residues eliminated the activity stimulation, although the replacement of one of them alone only reduced the stimulation of activity. It was possible to replace Ile6 with a hydrophobic residue. The importance of the correct location for disulphide bridge between Cys5 and Cys10 was demonstrated and no modifications near to the terminal disulphide bridge between Cys1 and Cys13 were permitted. Both Glu7 and His8 could be replacede by alanines at the same time. This is important for peptide mimetic design since it eases both the design and the synthesis.

   Peptides are rapidly degraded in living organism by peptidases, both endo- and exopeptidases. Endopeptidases are usually selective for certain residues whereas exopeptidases degrade peptides from their terminal ends. Head-to-tail cyclization is a widely used method to increase the *in vivo* stability of peptides. KLK3 binding peptides already contained disulphide bridges and those bridges were subjected to alternative cyclization strategies. A series of the cyclic analogs of peptide B-2 with head-to-tail, side chain to tail and side chain to side chain cyclic structures were synthesized and their activity stimulation examined. Based on the results obtained with the peptide B-2 analogs, it was clear that the bridge between the *N-* and *C*-termini was very important for the KLK3-

stimulating activity. The number of the atoms in the alternative bridge structure was found to be important although the conformational fluctuation of these analogs was restricted as compared to the original disulphide bridge. The presence of a free carboxylic acid moiety around the *C*-terminus was also important for the KLK3-stimulating activity. The carboxylic acid could be replaced by an amide group, but this replacement reduced the KLK3-stimulating activity. One analog included a disulfide bridge mimetic consisted of GABA and aspartic acid, where the amino group from the former was linked to the main chain carboxyl group of the latter. This derivative was found to be more stable than the original peptide B-2 against enzymatic degradation in plasma and with the presence of KLK3.

The possibility to replace disulphide bridge by all-hydrocarbon linker was studied. The results revealed that no heteroatoms were necessary to maintain biological activity, a linker consisting of a simple hydrocarbon chain was sufficient. A peptide with a flexible linker containing all-sp^3-hybridized carbon atoms displayed moderate activity stimulation whereas structurally more restricted *E*-double bond containing peptide exhibited low activity stimulation.

The original KLK2 binding peptides characterized by phage display were linear. In order to increase their stability, several cyclic analogs using additional disulphide bridge and head-to-tail cyclization were synthesized (*Figure 40*). After cyclization, the structural motifs common to both biologically active linear and cyclic peptides were studied by NMR. However, the NMR studies emphasized the conformational flexibility of the peptides studied and no remarkable conformational motif could be found, not even hydrogen bonding. The cyclization of peptides increased the inhibitory activity of KLK2 binding peptides and their stability against enzymatic degradation by trypsin or plasma proteases. Both the linear and cyclic forms with a disulphide bridge were degraded in plasma within 30 minutes but the head-to-tail cyclized peptide was stable against degradation for at least 24 h. The head-to-tail cyclic form was relatively stable against degradation by purified trypsin, which is known to cleave the peptide bond after Lys or Arg. In all, 57% of head-to-tail cyclic peptide remained uncleaved after 4 h whereas the linear forms were completely broken down within 30 minutes suggesting that cyclic peptides could be suitable for *in vivo* applications.

KLK2b linear   H—A—R—F—K—V—W—W—G—OH

KLK2b(c1)   H—G—C—A—A—R—F—K—V—W—W—A—C—G—OH

KLK2b ht1   A—R—F—K—V—W—W—N
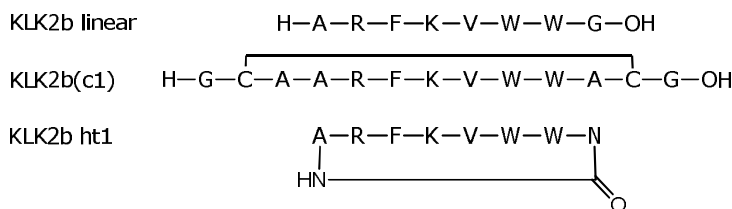
Figure 40. The sequences of linear, disulphide bridged and head-to-tail cyclized versions of KLK2 binding peptides.

7.2 CONCLUSIONS

The present study describes the structural characterization, sequential optimization and the enhancement of the plasma stability of specific KLK2 and KLK3 binding peptides. The modified peptides could modulate the enzymatic activities of KLK2 and KLK3. The results lead to the following conclusions.

I. Populations of reversed turn structures were found for KLK3 binding peptides but also there were clear indications of conformational fluctuation. Further studies will be needed to clarify the interactions between KLK3 and ligand.

II. The important residues of KLK3 binding peptides for biological activity response were defined. The residues which were replaceable in the peptide mimetic design were also determined.

III. The stability of KLK3 binding peptide B-2 against enzymatic degradation was improved via an alternative cyclization strategy. Both amide bond and all-hydrocarbon containing disulphide bridge mimetics were synthesized without losing biological activity.

IV. The stability of KLK2 binding peptides against enzymatic degradation in plasma was improved by head-to-tail cyclization. In addition, head-to-tail cyclization improved the stability of the molecules against degradation by KLK2. Thus increased stability of the cyclic peptide means that this agent has therapeutic potential for use in prostate cancer.

V. Several KLK2 binding peptide analogs had preserved inhibitory activity but no common structural motifs were found highlighting the essentiality of a flexible structure for binding and biological activity of KLK2 binding peptides.

VI. Valuable information about the structure-activity relationships of KLK2 and KLK3 binding peptides was gained.

7.3 FUTURE PERSPECTIVES

Although no rigid conformational motifs were detected for KLK2 and KLK3 modulating peptides and the structures responsible for their biological activity have not been defined, the mapping of residues important for biological activity would help in identifying new modified peptides with *in vivo* applications. C-4 peptide, which has been shown to decrease angiogenesis in cell cultures and has the highest activation capacity for KLK3, contains two disulphide bridges. The new cyclization methods and the knowledge of replaceable residues in C-4 peptide sequence may lead to the discovery of a more active and stable KLK3 modulating analog. Analog with increased *in vivo* stability would be essential if one wishes to undertake future studies of the KLK3 and KLK2 producing tumors in animal models.

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Peptide Based Modulators of Prostate Specific Kallikrein-Related Peptidases 2 and 3

Prostate cancer (PCa) is common cancer in males with challenging diagnosis. Prostate specific proteases KLK3 (PSA) and KLK2 are used in monitoring of PCa but they are also involved in prostate cancer progression. Synthetic peptides, which modulate the protease activity of KLK2 and KLK3, have low bioavailability limiting the use of the peptides in therapeutic applications. This study focuses on the improvements in the activity and stability of peptide-based activity modulators of KLK2 and KLK3.

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