β-AMINO ACIDS AS SECONDARY STRUCTURE INDUCERS IN PEPTIDES

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CONTENTS

Abbreviations

2-ClTrt	2-Chlorotrityl
Å	Ångström
ar	aromatic (NMR)
AA	Amino acid
Ac	Acetyl
ACN	Acetonitrile
AcOH	Acetic acid
Al	Allyl
Aloc	Allyloxycarbonyl
AOP	9-Azabenzotriazole-1-yl-*N*-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate
Ar	Aryl
Anh	Anhydride
Boc	tert.-Butoxycarbonyl
BOP	Benzotriazol-1-yl-*N*-oxy-tris(dimethylamino)-phosphonium hexafluoro-phosphate
Bu	Butyl
Bz	Benzoyl
Bzl	Benzyl
°C	Degrees centigrades
c-	cyclo
CD	Circular dichroism
CHL	Chloroform
COSY	Correlated spectroscopy
d	dublet (NMR)
δ	Chemical shift (NMR)
DBU	1,8-Diazobicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCHA	Dicyclohexylamine
DCM	Dichloromethane
DIC	*N*, *N'*-Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
Dmab	4-{*N*-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]--amino}benzyl
DMAP	4-Dimethylaminopyridine
Dmb	2,4-Dimethoxybenzyl
DMF	Dimethylformamide

DMSO	Dimethylsulfoxide
ee	Enantiomeric excess
Et	Ethyl
Fb	Fibrinogen
Fn	Fibronectin
FT	Fourier-Transform
Fmoc	9-Fluorenylmethoxycarbonyl
HAPyU	1-(1-Pyrrolidinyl-1*H*-1,2,3-triazolo[4,5-b]pyridinylmethylene)-pyrrolidinium-3-oxide hexafluorophosphate
HATU	1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxide hexafluorophosphate
HBTU	1-[Bis(dimethylamino)methylene]-1*H*-benzotriazolium-3-oxide hexafluorophosphate
HFIP	Hexafluoroisopropanol
HOAt	*N*-Hydroxy-9-azabenzotriazole
HOBt	*N*-Hydroxy-benzotriazole
HPLC	High performance liquid chromatography
Hz	Hertz
i-PrOH	iso-Propanol
IC_{50}	50 % Inhibitory capacity
IR	Infrared
J	Coupling constant
m	multiplet (NMR)
MALDI	Matrix-assisted laser desorption ionization
MD	Molecular dynamics
MS	Mass spectrometry
NMR	Nuclear magnetic resonance (Spectroscopy)
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect and exchange spectroscopy
Me	Methyl
MeO	Methoxy
NMP	*N*-methylpyrrolidinone
P.	Page
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
PE	Petrolether
Pg	Protective group
Ph	Phenyl
ppm	Parts per million
PyAOP	9-Azabenzotriazole-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate

PyBOP	Benzotriazole-1-yl-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate
q	quartet (NMR)
ROESY	Rotating frame Overhauser effect spectroscopy
s	singlet (NMR)
Sasrin	Super acid-sensitive resin
SPPS	Solid phase peptide synthesis
t	triplet (NMR)
TBTU	1-[Bis(dimethylamino)methylene]-1*H*-benzotriazolium-3-oxide tetrafluoroborate
TOCSY	Total correlation spectroscopy
t-Bu	tert.-Butyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
THF	Tetrahydrofuran
TIS	Triisopropylsilane
ToF	Time of flight
Trt	Trityl
UV	Ultraviolet
Vn	Vitronectin
Z	Benzlyoxycarbonyl

Amino acids:

Three letter code	One letter code	Amino acid
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
1-Nal		1-Naphtylalanine
2-Nal		2-Naphtylalanine
Phe	F	Phenylalanine
Phg		Phenylglycine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Xaa	X	Unspecified amino acid
β-hXaa	$^{\beta h}$X	Unspecified β-Homoamino acid

D-amino acids are described by the letter D in the three-letter code and the small face letter in the one-letter code.

1. INTRODUCTION

A central goal in biology is to unravel the complex molecular mechanisms by which a cell coordinates its many functions as part of a multicellular organism. The discovery and use of naturally occurring pharmacological agents to inhibit the function of intracellular signaling molecules has been of great importance in this process. Such compounds also provide starting points for development of many therapeutically important drugs. One major methodology of drug development focuses on screening of vast libraries of molecules for biological activity from which variants are produced to maximize beneficial medical characteristics. An alternative method, rational drug design, uses protein sequence and three-dimensional structure information to obtain insight in to protein functions. These informations suggest that the mechanisms by which molecules interact, and thus mediate signals, may be predictable, allowing the rational design of inhibitors of such interaction.

Many of the diverse signaling molecules found in a cell use similar mechanisms to interact, and thus mediate the propagation of a signal. Of particular importance to influence such interactions is the principle that short peptide sequences mediate both affinity and specificity for the interaction of the molecules. In many cases these short target peptides contain an amino acid sequence that allows their identification and prediction of their function by a combination of bioinformatic and biochemical methodology. This class of peptide-protein or protein-protein interaction is an ideal subject for the rational design of inhibitors. Such inhibitors have the dual benefits that they allow elucidating the roles of their target molecules in cellular function and evaluating their potential as targets of therapeutic intervention. One approach involves the identification of a possible interaction by analysis of the sequence of candidate interacting molecules, and by designing short peptides based on the target sequence. Consequently agents that can competitively and specifically inhibit the interaction may be generated. In practice, design of peptide inhibitors proved very difficult for several reasons. Firstly, peptides in general are very flexible. This flexibility makes all structural studies extremely difficult. Moreover, the relationship between the primary structure (constitution) and the so-called “bioactive conformation” is far from being obvious. The structural contribution to selectivity and activity of the peptides can be attributed to different backbone conformations or different side chain conformations. Secondly, peptides are prone to hydrolysis by different proteases present in living systems. Third, the cell membrane is a barrier for passing of large hydrophilic molecules such as peptides into cells. To overcome these problems different approaches are used. The transport problem may be overcome by the

recently developed “Trojan horse” approach. This method involves the association of the peptide (or other bioactive molecule) to one of an emerging class of cell-permeable peptide vectors, which can translocate with ease across plasma membranes and enter the cytoplasm with high efficiency without compromising normal cellular function.[1]

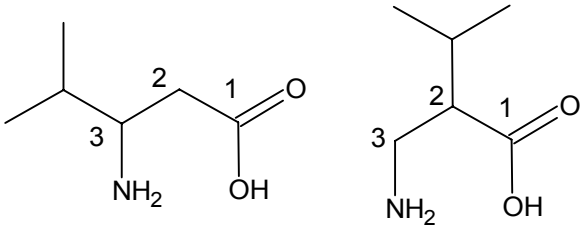
Both of the first two disadvantages can be diminished by introducing “nonstandard” amino acids, like D- or β-amino acids, in a peptide chain. These amino acids are often not recognized by different peptidases. Because of this the rate of peptide hydrolysis by a peptidase can be lower. Peptides composed of only D-amino acids exhibit resistance toward enzymatic proteolysis.[2] Recently, protease-resistant antimicrobial peptides composed of β-amino acids have also been constructed.[3] To reduce the number of possible conformations of an active sequence often cyclic constrained peptides are used with tailor made amino acids at a specific position in the peptide chain that could fix the peptide in an active conformation. As these tailor-made amino acids D-amino acids are used but β-amino acids have also served as important building blocks in natural and pharmaceutical candidates such us RGD (Arg-Gly-Asp) mimetics.[4] β-Amino acids may provide a convenient molecular tool to force a peptide segment to adopt a well determined folded conformation and incorporation of a distinct β-amino acid in cyclic peptides results in the stabilization of the overall secondary structure. The controlled employment of β-amino acids together with their structural preferences, and another structural bias, could allow the fine-tuning of biologically active cyclic peptide conformations.

In this work the influence of β-amino acids on conformations of cyclic peptides in different solutions has been examined with different techniques (CD, FTIR, NMR). The findings of these studies are used for the synthesis of cyclic constrained peptides with different recognition sequences as selective and effective inhibitors of the integrin family of heterodimeric cell surface receptors and their extracellular matrix protein ligands. Several different integrins have been implicated in disease processes and potent inhibitors of their interaction with protein ligands have aroused widespread interest as potential targets for drug discovery.

2. THEORETICAL PART

2.1. β-AMINO ACIDS

β-Amino acids are emerging as an interesting class of compounds for the medicinal chemist. They are naturally produced in humans, animals, microorganisms, marine organisms and plants either in free form or as a part of a peptide or depsipeptide. They are found as components of peptidic natural products with antibiotic, antifungal, cytotoxic, and other pharmacological properties.[5] The most well-known medicinally important class of nonpeptidic β-amino acids are found in β-lactams.



β^3-Leucine

β^3-Homovaline β^2-Homovaline

Figure 1: Nomenclature example for β-amino acids.

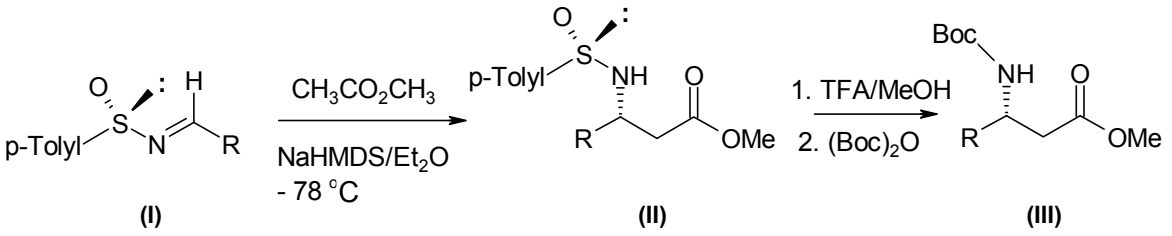
These include antibiotics, β-lactamase inhibitors, human leukocyte elastase inhibitors, and cholesterol uptake inhibitors.[5] β-Amino acids are analogs of α-amino acids in which the carboxy and amino group are separated by two carbon atoms. Trivial names for β-amino acids are used very often. The extension of the amino acid skeleton by one carbon atom is designated in nomenclature by the prefix *homo*.[6]

Consequently, β-homoamino acids are analogs of α-amino acids in which a CH_2- group has been inserted between the α-carbon and carboxy or amino group of the parent α-amino acid. Depending on the position of the side chain, β-amino acids can be β^2-amino acids (Figure 1 right), β^3-amino acids (Figure 1 left) or $\beta^{2,3}$ amino acids.

There are significant chemical differences between β-amino acids and their α-analogs. For example β-amino acids are stronger bases and weaker acids than their α-analogs. It is also known that peptidic molecules containing β-amino acids are generally more stable to enzymatic hydrolysis due to the inability of peptidases to cleave the amide bonds adjacent to the β-amino acid.

There are a variety of approaches to the synthesis of β-amino acids.[5,7,8] β^3-substituted derivatives **(III)** may be conveniently prepared using Arndt Eistert

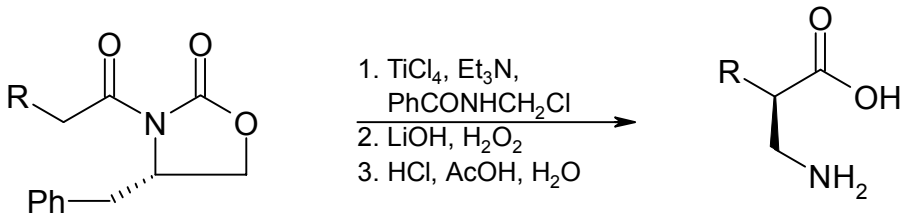
homologation of α-amino acids, which is compatible with a variety of protecting groups including Boc and Fmoc. The overall yields for the generation of the β^3-amino acid **(III)** from the corresponding α-amino acid range from 33 % to 58 % depending on amino acid and protecting groups.[9] This method is used in this work and it is in detail described in chapter 4.1, P. 25. Another important method for the synthesis of β^3-amino acids **(III)**, with broad applicability, relies on the addition of metal enolates derived from acetic acid esters to chiral sulfinimines **(I)** in an aldol type reaction.[10] The reaction (Scheme 1) proceeds with high diasteroselectivity and good overall yield. Given the variety of effective methods available for the preparation of chiral sulfinimines **(I)**, this route to β^3-amino acids **(III)** should become increasingly attractive.



(I) **(II)** **(III)**

Scheme 1: Synthesis of β^3-amino acids via chiral sulfinimines.

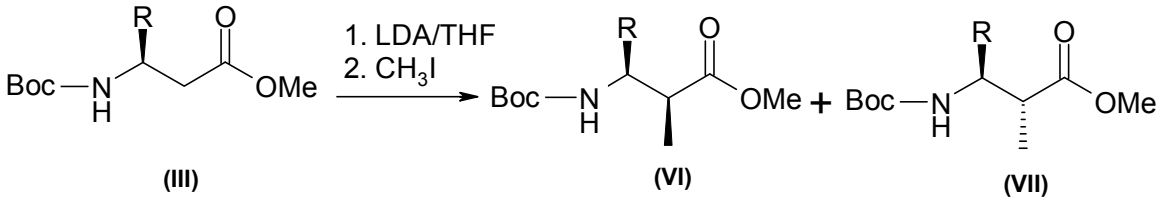
β^2-substituted amino acids **(V)** may be readily synthesized in enantiomerically pure form by aminomethylation of acyl derivatives of Evans' chiral auxiliary **(IV)** (Scheme 2).[11] Aminomethylation of the titanium-enolates proceeds with high diastereoselctivities and very good yields.[9b]



(IV) **(V)**

Scheme 2: Synthesis of β^2-amino acids by aminomethylation of Evans' chiral auxiliary (IV).

$\beta^{2,3}$-disubstituted amino acids **(VI)** or **(VII)** may be synthesized by α-alkylation of the urethane protected β^3-amino acid methyl esters **(III)** via doubly lithiated intermediates (Scheme 3). Procedures for obtaining either diastereomer with good stereochemical control are known.[12]



(III) (VI) (VII)

Scheme 3: Synthesis of $\beta^{2,3}$-amino acids via α-alkylation of β^{3}-amino acids.

2.2. PEPTIDES

Proteins play a crucial role in almost all fundamental processes in the living cell. Although they carry out an almost bewildering range of functions in living things, all proteins are composed of the same basic building blocks, being biopolymers of the 21 DNA-encoded amino acids. They are not just unstructured chains of their constituent monomers, but rather adopt characteristic, highly organized three-dimensional arrangements in solution that are intimately related to their biological function.

Peptides are simply smaller versions of proteins. While there is no clear borderline between peptides and proteins, an acceptable working distinction is that proteins are large peptides, where large is a relative term and may mean anything from perhaps 50 to several hundred amino acid residues.

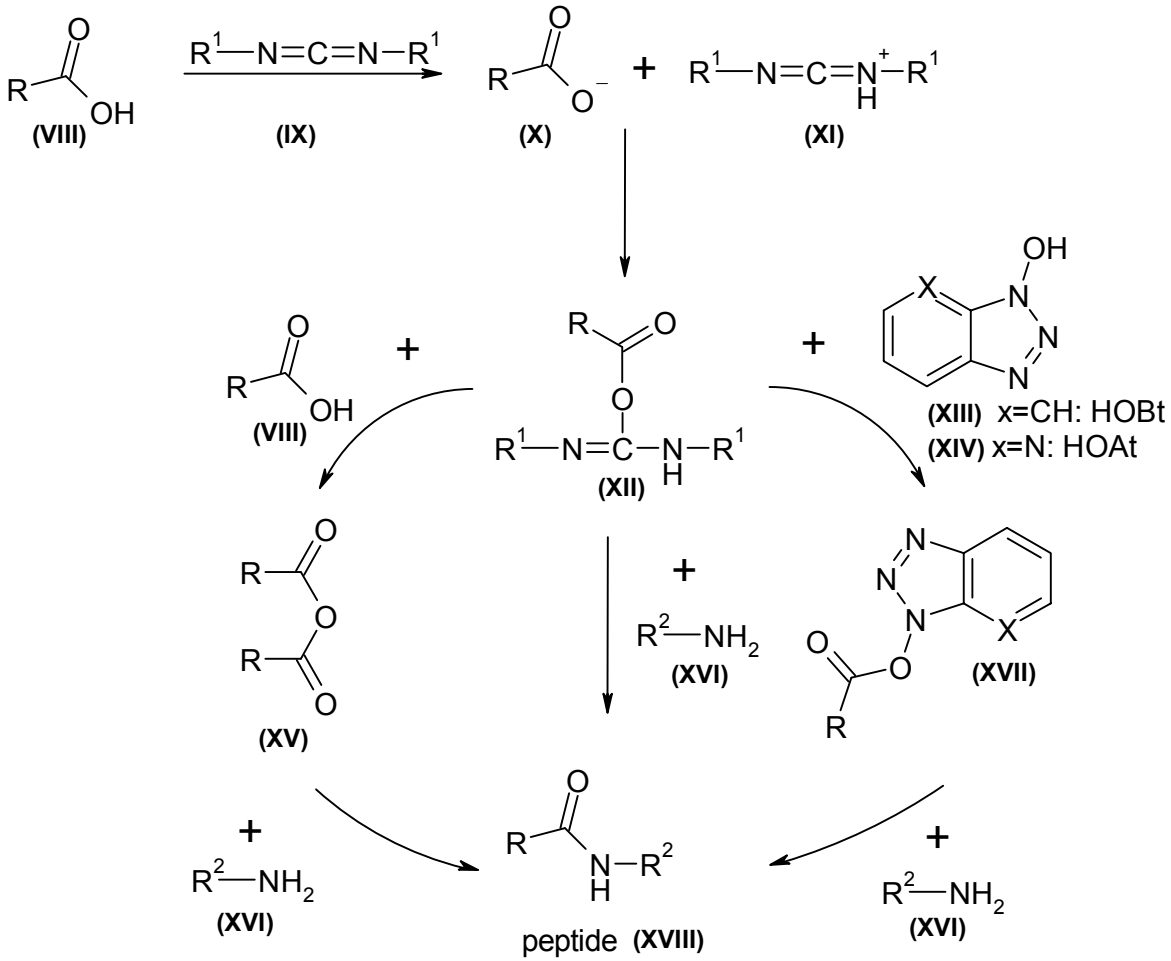
All of the 21 DNA-encoded or proteinogenic amino acid building blocks have the same basic structure that incorporates an amino and a carboxy group and differs only in the nature of the side chains. Proline is unique in having a cyclic structure with a secondary amine. With the exception of glycine, all are chiral, due to the presence of at least one stereogenic carbon atom, and belong to the L-stereochemical series. Consequently, the chiral α-amino acids all have the *S* configuration, except cysteine in which it is *R* as a consequence of the Cahn-Ingold-Prelog convention.[13] Two amino acids, threonine and isoleucine, have a second stereogenic centre at the β-carbon atom.

Since the difference between peptides and proteins is essentially one of size or length of the amide backbone, the problems involved in the chemical synthesis of proteins are basically those of the synthesis of peptides.

2.2.1.Peptide Synthesis

Generally peptides are formed by the connection of α-amino acids involving amide bonds. It is necessary to activate the carboxy group of one amino acid so that nucleophilic attack by the amino group of the second amino acid can take place forming the desired amide bond. This process of amide bond formation is called *coupling*. Using a coupling reagent is today the most common general coupling method in peptide synthesis. The coupling reagent reacts with the free carboxy group of an amino acid, generating a reactive species, which is not isolated and which is sufficiently reactive to allow amide bond formation to occur at room temperature or below.

The most common coupling reagents are the carbodiimides **(IX)** of which the most popular one is dicyclohexylcarbodiimide (DCC).[14]

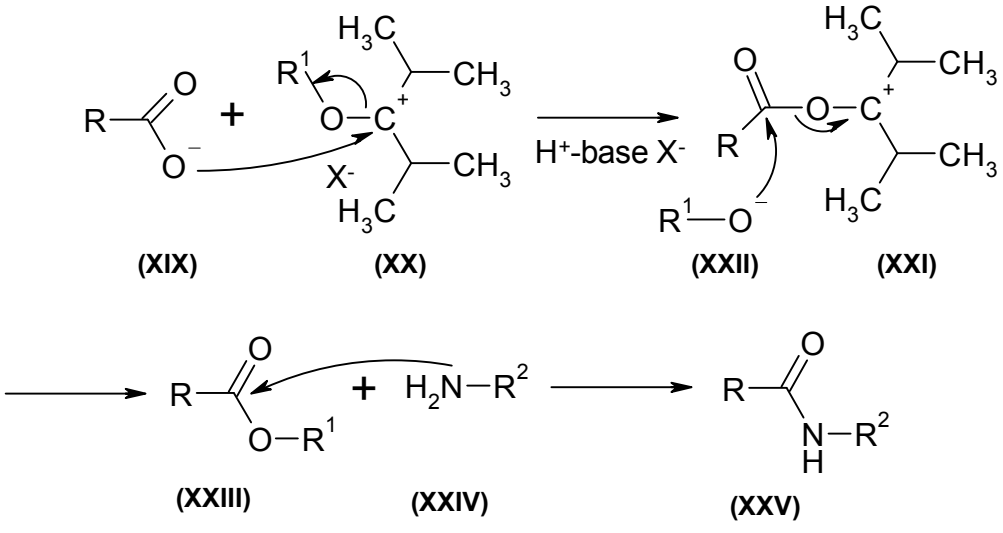


Scheme 4: Peptide bond formation via carbodiimide activation.

This activation process is often employed in the presence of a hydroxylamine derivate (HOBt **(XIII)** or HOAt **(XIV)**) that suppresses racemization and excludes dehydration of carboxamide residues like Asn and Gln. Reaction of a N-protected

amino acid **(VIII)** with a carbodiimide **(IX)** is believed to involve a labile O-acylisourea **(XII)**, which reacts with the amino component **(XVI)** to give the corresponding amide (Scheme 4).[15] If two equivalents of protected amino acids **(XII)** are employed the intermediate O-acylisourea **(XII)** reacts with the second equivalent of acid **(XII)** to give the corresponding symmetrical anhydride **(XV)**. In the presence of HOBt **(XIII)** or HOAt **(XIV)** as additives, O-acylisourea **(XII)** reacts with them to give active esters **(XVII)**.

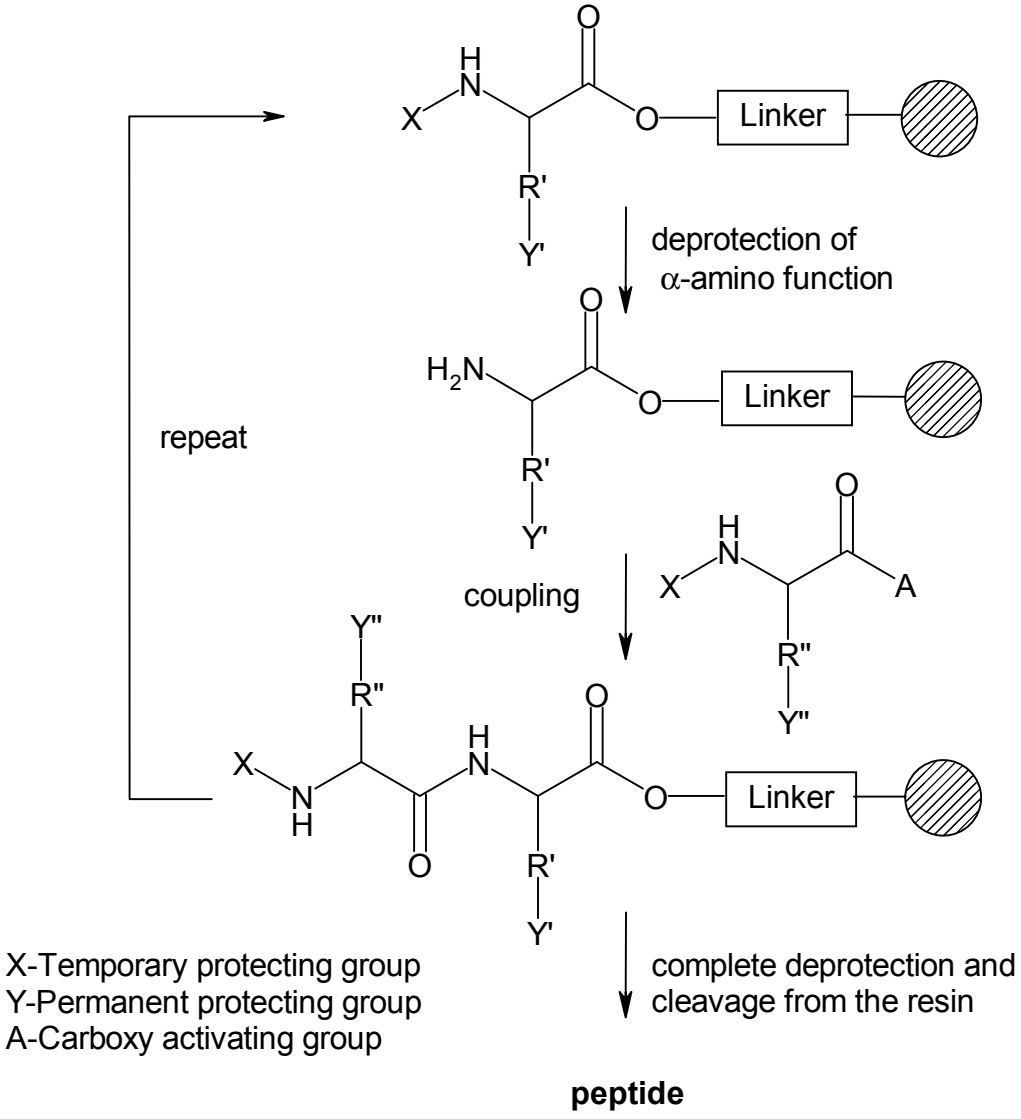
In the last few years, O-acyl uranium/guanidinium and O-acyl-phosphonium type coupling reagents (Figure 23, P. 53) enabling in situ generation of active esters in the presence of a tertiary nitrogen base have become more and more popular. Facile handling, very short coupling time and hardly any loss of configuration during coupling makes them highly suitable for automated solid phase peptide synthesis. The most commonly used coupling reagents are HBTU,[16] TBTU[17] and HATU.[18] A tertiary amine is used as a base to form the carboxylate ion **(XIX)** of the carboxy component. Initial attack of this on the uronium salt **(XX)** leads to an acyloxyuronium salt **(XXI)**. This is extremely reactive and is attacked by the oxyanion **(XXII)** of 1-hydroxy-benzotriazole, forming the benzotriazolyl ester **(XXIII)**, thought to be the predominant species suffering aminolysis (Scheme 5).[19]



Scheme 5: Peptide bond formation via uronium activation.

For the synthesis of even the smallest peptide in a controlled manner it becomes obvious that certain functional groups must be protected. In order to couple amino acids in a way that is useful for the synthesis of complete peptides, the functional groups that are not directly involved in the amide bond-forming reaction must also be protected or blocked. For peptide synthesis the N^{α}-protecting group is almost

always a urethane derivative, and there are several reasons for this. Urethane groups are easily introduced and, depending upon their structure, can be easily removed. This leads in the first instance to carbamic acids, which spontaneously decarboxylate generating the free amine of the N-terminal amino acid. In addition, the activation and coupling of amino acids with urethane N^{α}-protecting groups can be accomplished with minimal racemization of the α-stereogenic centre. The N^{α}-amino group of one of the amino acids and C-terminal carboxy group of the other are both blocked with suitable protecting groups. Formation of the desired amide bond can now occur upon activation of the free carboxy group. After coupling, peptide synthesis may continue by deprotection of the N^{α}-amino group of the dipeptide and coupling with the free C-terminus of another protected amino acid or of a suitably protected peptide.

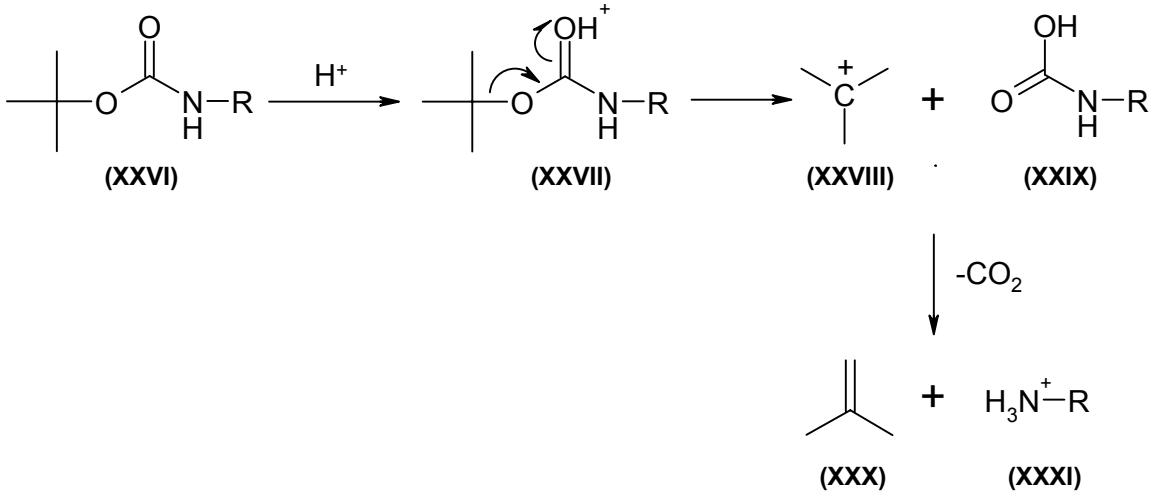


Scheme 6: Solid phase peptide synthesis.

Protecting groups are classified as either “permanent” or “temporary”. Permanent protecting groups are retained until assembly of the peptide chain is complete, while temporary protecting groups are removed at intermediate stages.

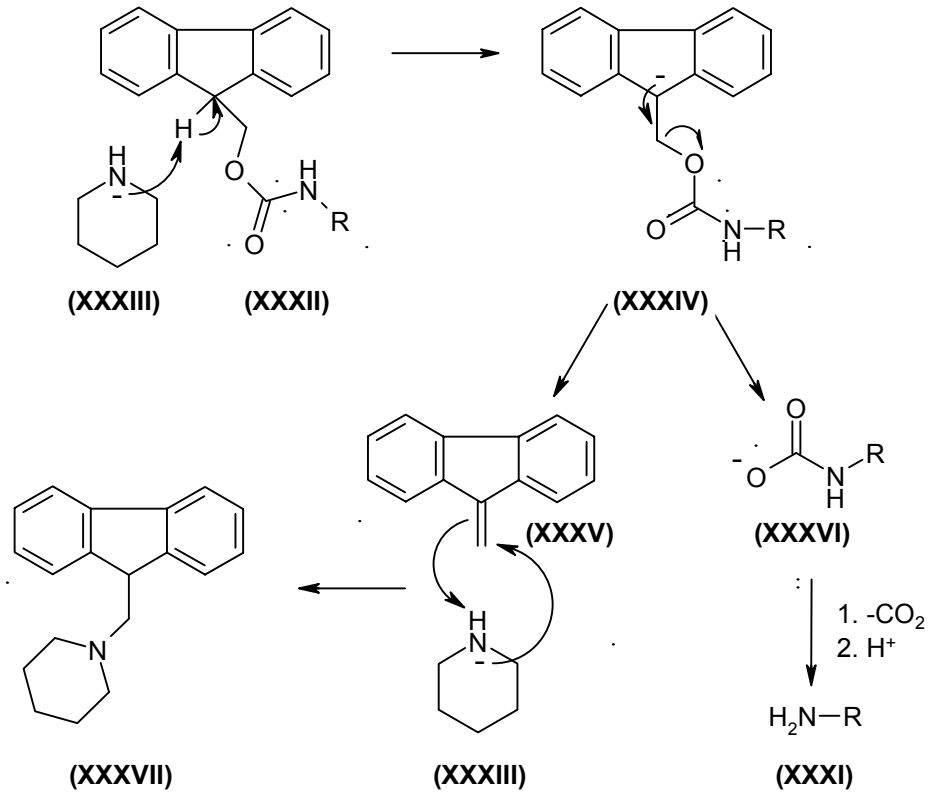
In the solid phase peptide synthesis (Scheme 6), introduced by Merrifield,[20] a C-terminal amino acid of the target peptide is anchored on the insoluble polymeric support that serves as permanent protection group, via its carboxy group. Any functional group in amino acid side chains must be masked with permanent protecting groups that are not affected by the reaction conditions employed during peptide chain assembly. The temporary protecting group masking the α-amino group during the initial resin loading is then removed. An excess of the second amino acid is added, with the carboxy group of this amino acid being activated for amide bond formation. After coupling, excess reagents are removed by washing and the protecting group is removed from the N-terminus of the dipeptide, prior to addition of the next amino acid residue. This process is repeated until the desired peptide sequence is assembled. In a final step, the peptide is released from the support and the side-chain protecting groups are removed. Generally, side-chain protecting groups and resin linkage are chosen such that protecting groups are removed and the assembled peptide released under the same conditions.

In this way, the desired peptide chain is assembled on the support in a linear fashion, almost always from the C-terminus to the N-terminus (the C→N strategy). This is the reverse of the ribosomal peptide synthesis in nature, where chain elongation takes place from the N-terminus. The main problem in the N→C solid phase peptide synthesis is racemization. Nevertheless, there are a few reports where racemization can be efficiently suppressed and the peptides are synthesized in N→C direction on solid support.[21] The two most extensively used protecting group schemes in solid phase peptide synthesis (SPPS) are Boc/Bzl, and Fmoc/t-Bu- approaches.



Scheme 7: Acidolytic removal of the Boc group.

In Boc/Bzl synthesis, all protecting groups are removed by acidolysis. The side chain-protecting groups are, however, stable to the repeated treatments with moderately strong acid solutions, such as 33% TFA in DCM, required for removing the temporary Boc group (Scheme 7). Once the peptide has been synthesized, the side chain-protecting groups are removed by treatment with strong acid, often liquid hydrogen fluoride or, less commonly, trifluoromethanesulfonic acid. This detaches the completed peptide chain from the solid support at the same time.



Scheme 8: Removal of the Fmoc protective group with piperidine.

In Fmoc/t-Bu synthesis, the temporary Fmoc protective group is labile to a solution of a secondary amine, normally 20% piperidine in dimethylformamide. The mechanism of cleavage (Scheme 8) proceeds by initial proton abstraction to give the stabilized dibenzocyclopentadienide ion **(XXXIV)**, which on elimination gives rise to dibenzofulvene **(XXXV)**. The latter is then trapped by reaction with an excess of piperidine **(XXXIII)**.[22] This leads to carbamic acid **(XXXVI)**, which spontaneously decarboxylates generating the free amine of the N-terminal amino acid **(XXXI)**. Other reagents, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[23] or fluoride ion,[24] are also effective for Fmoc group removal. The side chain-protecting groups and the peptide-resin anchorage are also labile to acid in the Fmoc/t-Bu approach. However, the use of strong acids such as liquid hydrogen fluoride is not necessary. In the majority of cases treatment with TFA is sufficient to cleave the peptide from the solid support and to remove all protecting groups.

2.2.2. Peptide Secondary Structures

The properties of peptides and proteins, which form the molecular machinery of living systems, are largely determined by their three-dimensional structure. Proteins fold into well-defined three-dimensional structures in physiological solution, while peptides often adopt unique structures only while bound to their appropriate receptors.

A polymer's secondary structure (2˚ structure) is defined as the local conformation of its backbone. For proteins and peptides, this means the specification of regular polypeptide backbone folding patterns: helices, pleated sheets and turns.

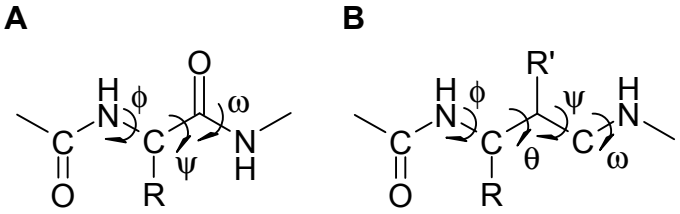


Figure 2: Torsion angles of α-amino acids (A) and β-amino acids (B) in a peptide bond.

The backbone of peptides is a linked sequence of rigid planar amide groups, and their conformation can be specified by the torsion angles about the C^{α}-N bond (ϕ) and the C^{α}-C bond (ψ) of each of its amino acid residues (Figure 2 A).

For β-amino acids an additional torsion angle θ around the central C^{α}-C^{β} bond is present (Figure 2 B). Because of the additional methylene group higher flexibility and greater structural variability is possible for β-amino acids. However, the conformational space and the flexibility might be restricted by the side chains R and R' at the C^{α}- and C^{β}-atoms. Folded helical or turn-like conformations of peptides, which contain β-amino acids, require a gauche conformation about the torsion angle θ.[25] The torsion angles ϕ and ψ of several naturally abundant periodic secondary structures are shown in Table 1.

Helices are the most striking elements of protein 2˚ structure. If a polypeptide chain is twisted by the same amount about each of its C^{α}-atoms, it assumes a helical conformation. As an alternative to specifying its ϕ and ψ angles, a helix may be characterized by the number, *n*, of peptide units per helical turn, and its pitch, *p*, the distance the helix raises along its axis per turn. A helix has also chirality, it may be either right-handed, *P*, or left handed, *M*. A right-handed helix turns in the direction that the fingers of a right hand curl when its thumb points along the helix axis in the direction that the helix raises. Additional stabilization of helix and other 2˚ structures is hydrogen bonding.

Table 1: The torsion angles ϕ and ψ of several secondary structures.

Secondary structure	ϕ/deg.	ψ/deg.
Right-handed α-helix	-57	-47
Parallel β-pleated sheet	-119	113
Antiparallel β-pleated sheet	-139	135
Right-handed 3_{10} helix	-60	-30
Right-handed π helix	-57	-70
2.2_7 ribbon	-78	59
Left-handed polyglycine II and poly-L-proline II helices	-79	150
Collagen	-51	153
Left-handed α -helix	57	47

Very often the helices are described by the notation n_m where *n* is the number of residues per helical turn, and *m* is the number of atoms, including H, in the ring that is closed by the hydrogen bond (Figure 3). The most common secondary structure element of both fibrous and globular proteins, the α-helix, is described as 3.6_{13} according to this notation. Hydrogen bonded rings are formed from the C- to the N-terminus (backward direction), between the NH group of amino acids *i* and the CO group of amino acid *i-n.* Opposite orientations of the helix dipoles, as the consequence of the structural difference, are observed for the helices formed by β-amino acids (β-peptides).[26] In these helices hydrogen bonds may also be formed in the N- to C-terminal direction (forward direction) between the NH groups of amino acids *i* and the CO groups of amino acids *i+n*.

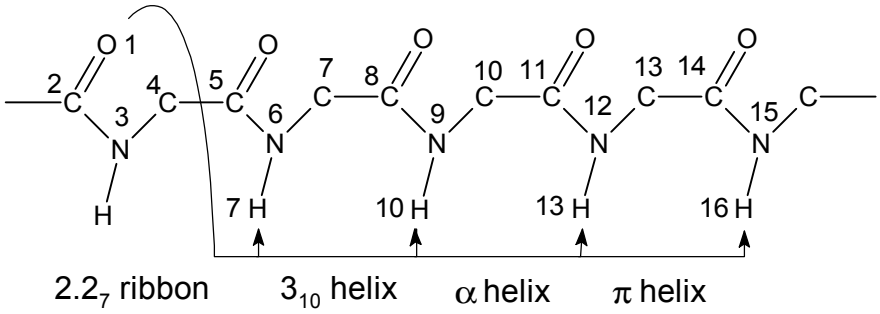


Figure 3: The hydrogen-bonding pattern of several polypeptide helices.

In β-pleated sheets hydrogen bonding occurs between neighboring polypeptide chains rather than within one as in α-helices. There are two kinds of β-pleated sheets:

a) The antiparallel β-pleated sheet, in which neighboring hydrogen bonded polypeptide chains run in opposite directions (Figure 4).

b) The parallel β pleated sheet, in which neighboring hydrogen bonded polypeptide chains extend in the same direction (Figure 4).

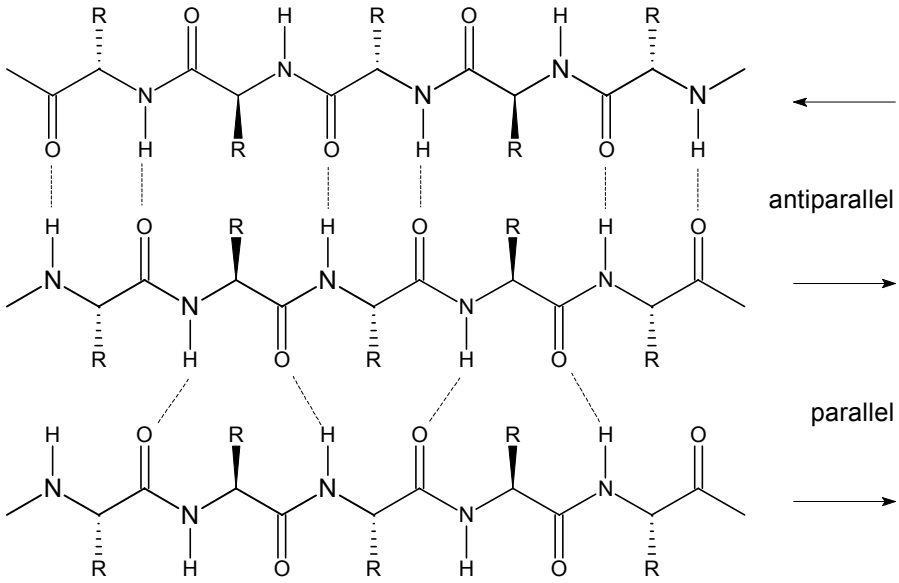


Figure 4: Antiparallel (above) and parallel (below) β pleated sheets.

The conformations in which these β structures are optimally hydrogen bonded vary somewhat from that of a fully extended polypeptide ($\phi=\psi=\pm180°$). They therefore have a rippled or pleated edge-on appearance, which accounts for the appellation "pleated sheet".

While the α-helix and β-sheet are periodically ordered conformations having on average the same ϕ, ψ angles, turns are aperiodic ordered secondary structures (their residues have different ϕ, ψ torsions angles). The X-ray structural analysis of an increasing number of proteins has revealed that turns are common in proteins, accounting for 25-30 % of the residues to the total molecule. It is also found that about one-fourth of turns does not possess hydrogen bonding. Turns have been recognized as sites where the polypeptide chain reverses its overall direction and they alone, or as a part of the larger loops, are very often located on the protein surface. As a consequence of the folded geometry of the peptide backbone, the polar side-chain groups in corner positions (i+1 and i+2) point outward and may serve as a site for molecular recognition. Indeed, turns frequently have been suggested as the bioactive conformation involved in receptor binding, immune recognition, posttranslational modifications, and other recognition processes.

Depending on the number of amino acids, three types of turns can be classified, α- (five amino acids), β- (four amino acids) and γ-turn (three amino acids). These aperiodically folded structures may or may not be stabilized by intramolecular

hydrogen bonds: NH^{i+4} to CO^{i} for α-turn, NH^{i+3} to CO^{i} for β-turn and NH^{i+2} to CO^{i} for γ-turn (Figure 5).

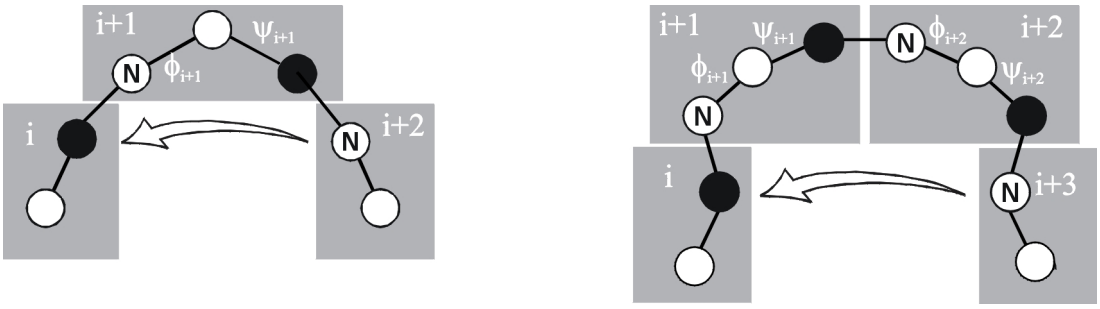


Figure 5: γ– (left) and β– (right) turn.

Like other 2° structures, turns are defined by torsion angles about the C^{α}-N (φ) and the C^{α}-C bond (ψ) of each of its amino acid residues. These torsion angles for different turns are shown in Table 2. An additional turn criterion is that the $C^{\alpha}(i)$- $C^{\alpha}(i+3)$ distance is less than 7 Å for a β-turn.[27]

Table 2: Characteristic turn torsion angles.

Turn	ϕ_{i+1}/Deg	ψ_{i+1}/Deg	ϕ_{i+2}/Deg	ψ_{i+2}/Deg	ϕ_{i+3}/Deg	ψ_{i+3}/Deg
I-α_{RS}	-60	-29	-72	-29	-96	-20
II-α_{RS}	-59	129	88	-16	-91	-32
I-α_{RU}	59	-157	-67	-29	-68	-39
I-α_{LU}	-61	158	64	37	62	39
βI	-60	-30	-90	0	-	-
βI'	60	30	90	0	-	-
βII	-60	120	80	0	-	-
βII'	60	-120	-80	0	-	-
βIII	-60	-30	-60	-30	-	-
βIII'	60	30	60	30	-	-
βVIa	-60	120	-90	0	-	-
βVIb	-120	120	-60	150	-	-
γ	75	-64	-	-	-	-
γ^{j}	-79	69	-	-	-	-

2.3. INTEGRINS

In order to understand how the extracellular matrix interacts with cells, one has to identify the cell-surface molecules (matrix receptors) that bind the matrix components as well as the extracellular matrix components themselves. Integrins are the principal receptors on animal cells responsible for binding most extracellular matrix proteins, including collagen, fibronectin and laminin. The integrins constitute a large family of homologous transmembrane linker proteins. The name of these receptors emphasizes their role in integrating the intracellular cytoskeleton with the external environment. Integrins differ from cell-surface receptors for hormones and for other soluble signaling molecules in that they bind their ligands with relatively high affinity ($K_a=10^6$-10^9 M^{-1}) and are usually present in about ten- to hundredfold higher concentration on the cell surface. This arrangement makes sense, as binding simultaneously to a large number of matrix molecules allows cells to explore their environment without losing all attachment to it. Integrins are crucially important receptor proteins because they are the main way that cells both bind to and respond to the extracellular matrix. These receptors are involved in fundamental cellular processes such as attachment, migration, proliferation, differentiation, and survival. Integrins also contribute to the initiation and/or progression of many common diseases including neoplasia, tumor metastasis, immune dysfunction, ischemia-reperfusion injury, viral infections, osteoporosis and coagulopathies.[28,100a] They are ~280 Å long heterodimeric membrane glycoproteins, composed of an α- (150 to 180 kD) and a β- (~90 kD) subunit, both of which are type I membrane proteins. Eighteen α and eight β mammalian subunits are known, which assemble noncovalently to give 24 different heterodimers. Although these subunits could in theory associate to give more than 100 integrin heterodimers, the actual diversity appears to be much more restricted. Contacts between the α and β subunits primarily involve their N-terminal halves, which together form a globular head, the remaining portions form two rod-shaped tails that also span the plasma membrane. Each integrin subunit has a large extracellular domain, a single membrane spanning domain and usually a short cytoplasmic domain (40-60 amino acids).[29] These short cytoplasmic domains of the α and β integrin subunits do not have intrinsic enzymatic activities, but can interact with a variety of cytoplasmic proteins, including cytoskeletal and signaling molecules. The α cytoplasmic domains are highly diverse, whereas the β cytoplasmic domains are somewhat conserved but they are necessary and sufficient for integrin-dependent signaling.[29] Association of α and β subunits defines distinct, although largely overlapping ligand specificity. Integrin binding to extracellular matrices can be classified as either

RGD-dependent (binding e.g. fibronectin, vitronectin and fibrinogen) or RGD-independent (binding e.g. collagen and invasin). In addition, some integrins can bind to counterreceptors (such as intercellular adhesion molecules ICAMs) on adjacent cells leadings to homotypic and heterotypic cell-cell interaction. Like other receptors, integrins transmit signals to the cell interior ("outside-in" signaling), which regulates organization of the cytoskeleton, activates kinase-signaling cascades, and modulates the cell cycle and gene expression.[30] Unlike other receptors, ligand binding to integrins is not generally constitutive but is regulated to reflect the activation state of the cell. This "inside-out" regulation of integrins protects the host from pathological integrin–mediated adhesion.[28] Inside-out and outside-in signaling are associated with distinct conformational changes in the integrin extracellular segment.

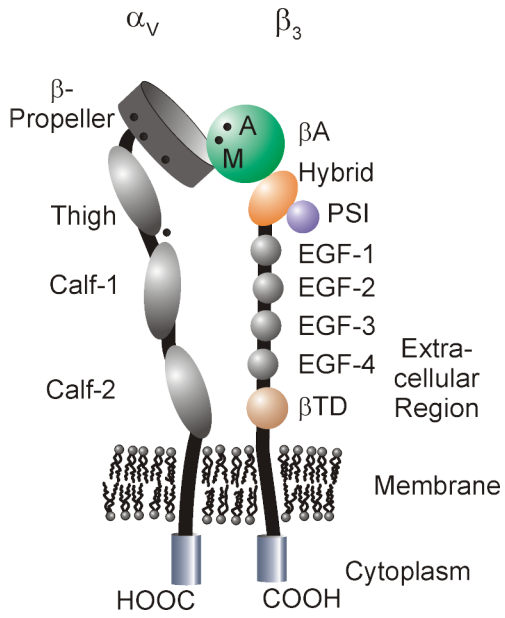


Figure 6: The domain structure of the $\alpha_v\beta_3$ integrin.

These changes vary with cell type and the state and nature of the ligand, and are modulated by divalent cations that are also required for integrin-ligand interaction.[31] Despite extensive investigation, it is not exactly clear how integrins interact with their ligands, how ligand occupancy affects integrin conformation, and how receptor activation is coupled to bi-directional signal propagation. Xiong et. al.[32] reported crystal structures of the extracellular segment of $\alpha_v\beta_3$ integrin with a cyclic RGD peptide ligand and without it and thus advanced the field an enormous stride toward achieving these goals. The extracellular segment of the αA-lacking $\alpha_v\beta_3$ integrin has been crystallized in the presence of Ca^{2+} and Mn^{2+} ions.

The overall shape of the crystallized conformer (resolved to 3.1 Å) is that of a large "head" on two "legs" (Figure 6),[33] similar to the images seen using electron microscopy.[34] The head has dimensions of ~ 90 Å by 60 Å by 45 Å and contains a seven-bladed β-propeller structure. The β-propeller (a toroidal arrangement of seven β-sheets) is found in many other proteins, including the β-subunit of heterotrimeric GTP-binding proteins (G proteins). Six Ca^{2+} binding sites (black dots), which are essential for binding of an integrin to its ligand, are seen in the structure. Four solvent-exposed Ca^{2+} binding sites are found in the A-B-β hairpin loops of blades 4-7 at the β propeller's bottom, and another site is in the knee

region of the α subunit. The top face of the βA domain contains two potential cation-binding sites. The first one is known as a metal ion-dependent adhesion site, or MIDAS,[35] although this is unoccupied in the crystal structure, the second has been named ADMIDAS by the authors.

The crystal structure of the extracellular part of the $\alpha_v\beta_3$ integrin in complex with cyclic pentapeptide ligand c-(Arg-Gly-Asp-D-Phe-Val(NMet)-) and eight Mn^{2+} ions is also resolved. In this crystal structure six of the Mn^{2+} ions occupied the same position like Ca^{2+} ions in previous crystal structure. Again is no metal ion visible at MIDAS. The cyclic pentapeptide inserts into a crevice between the propeller and βA domains on the integrin head. As predicted the RGD sequence makes the main contact area with the integrin. The Arg and Asp side chains point in opposite directions, exclusively contacting the propeller and βA domains, respectively.

The signaling pathways activated by integrins have been identified through the analysis of biochemical events that are triggered by integrin engagement, and by the identification of proteins that associate with focal adhesion complexes. These signaling pathways control activation of both protein tyrosine kinase and members of the Rho family of small GTP-binding proteins. Protein phosphorylation is one of the earliest events detected upon integrin stimulation. Increased tyrosine phosphorylation has been shown to be a common response to integrin engagement in many cell types including platelets, fibroblasts, carcinoma cells and leukocytes.

Individual cells can and do vary their adhesive properties by selective expression of integrins. Further versatility is introduced by the ability of cells to modulate the binding properties of integrins so that specificity and affinity of a given integrin receptor on a given cell are not always constant. There are numerous examples of modulation of integrin function. A particular important feature of integrins is that they undergo activation. It is commonly the case in an adhesion process that integrins provide strong adhesion only after activation by other stimuli, which can include soluble mediators (hormones, cytokine, etc.) and/or insoluble reactants (extracellular matrix or other cells). The specificity of the overall adhesion event lies in the coupling of activation of the final adhesion receptor, often an integrin that is not intrinsically highly specific, to a cascade of signals triggered by specific and /or local events. Usually an integrin is activated at the appropriate time and place by input of several specific signals. Of equal importance as activation of integrins is their inactivation. It is crucially important that cells should not attach at the wrong times and places. Platelets and leukocytes offer two prime examples in which inappropriate adhesion leads to thrombosis and inflammation, respectively. The integrins are a fascinating recognition system that has the potential of

becoming one of the best understood protein-protein interaction system with profound biological and medical significance.

2.3.1. RGD Recognition Sequence

Proteins containing an arginine-glycine-aspartic acid (RGD) attachment site, together with the integrins that serve as receptors for them, constitute a major recognition system for cell adhesion. At least eight, and possibly as many as twelve, of the currently known 24 integrins recognize the RGD sequence in their adhesion protein ligands. A partial list of adhesion proteins with RGD sites include fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, enactin, tenascin, osteopontin, bone sialoprotein, and, under some conditions, collagens.[36] Also many RGD-containing microbial proteins, such as the penton protein of adenovirus,[37] the foot and mouth virus coat protein,[38] the *Coxsackie* type virus,[39] and a surface protein of *Bordetella pertussis*[40] serve as ligands through which these microbes bind to integrins on the cell surface and gain entry into the cell. Also the Tat protein of the human immunodeficiency virus (HIV) is an RGD-containing protein with cell attachment activity. The interaction of Tat with cells is important because Tat can be internalized by cells, thus allowing Tat produced by one cell to enter another cell and turn on the production of latent HIV.[41] The role of the RGD sequence in the interactions of Tat with cell surfaces is still not obvious. Two groups[42] have found the RGD site on Tat to be active, whereas two others[43] find that the cell attachment activity of the Tat protein is linked to a highly basic sequence (RQR) in Tat. The attachment mediated by the basic sequence is found to be dependent on a $\alpha_v\beta_5$ integrin, which has both an RGD-directed binding site and a site for a basic sequence. Nevertheless, this integrin despite its ability to bind Tat does not appear to play any significant role in the internalization of Tat by cells.

Not all RGD-containing proteins mediate cell attachment. This is so because the RGD sequence may not always be available at the surface of the protein or may be presented in a context that is not compatible with integrin binding.

The presentation of the RGD sequence in the RGD proteins is important for their recognition by integrins. For example, the RGD site in fibronectin exists in a loop flanked by β-strands. A similar situation has been encountered with disintegrins, small RGD-containing snake venom proteins that bind to different integrins and inhibit their function.[44] The dependence of the integrin specificity for RGD on the conformation of the RGD site and the nature of the surrounding amino acids has been successfully utilized in the design of integrin-selective peptides. Peptides that

bind selectively to an integrin with affinities 10,000 to 50,000 fold higher than those of the original RGD peptides have been designed.[45] The peptides are cyclized to provide conformational restraint and the sequences flanking the RGD are selected to give the best possible affinity and selectivity.

Short peptides containing the RGD sequence can mimic cell adhesion proteins in two ways: When coated onto a surface, they promote cell adhesion, whereas in solution they act as decoys, preventing adhesion. Both modes of using RGD peptides have found applications: Surface-coated RGD peptides are being investigated for improvement of tissue compatibility of various implanted devices, and soluble peptides targeted towards individual integrins show promise as potential drugs for treatment of a number of diseases.

RGD peptides, or compounds that mimic the pharmacophoric guanidine and carboxy groups, inhibit the function of the $\alpha_{IIb}\beta_3$ integrin and are already in late-stage clinical trials as anti-thrombotics. Other applications being explored include the targeting of the $\alpha_v\beta_3$ integrin in osteoporosis. Osteoclasts attach to the bone through this integrin and inhibition of their attachment prevents bone degradation.[46] The RGD peptides can also prevent tumor growth *in vivo* by interfering with the angiogenic process that the growing tumor would need to maintain its blood supply.[47] Better understanding of the antitumor effects of the RGD peptides could lead to the development of promising new anticancer compounds.

Finally, protein engineering with RGD can have applications in protein targeting and gene therapy with viruses. Advances in the application of RGD and related sequences to various purposes will depend on detailed understanding of integrin-ligand recognition.

2.3.2. Vascular Cell-Adhesion Molecule

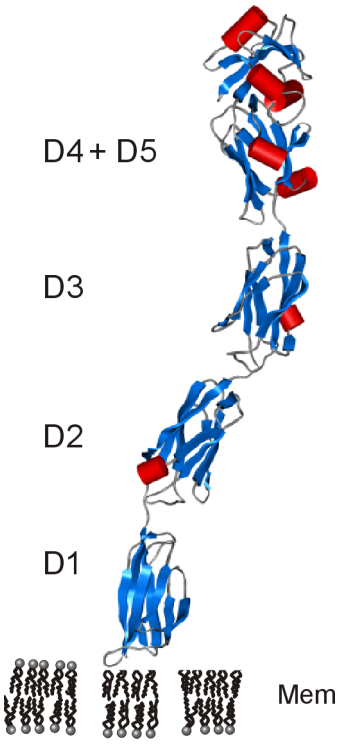
The cell-surface glycoprotein vascular cell adhesion molecule-1 (VCAM-1) is induced on the surface of vascular endothelial cells. Together with the intercellular adhesion molecules ICAM-1, ICAM-2, ICAM-3 and the mucosal vascular addressin MAdCAM-1 it forms an integrin-binding subgroup of the immunoglobulin superfamily. This protein mediates intercellular adhesion by specific binding to the $\alpha_4\beta_1$ integrin, also known as very-late antigen-4 (VLA-4).[48] This integrin is a key cell-surface receptor that is expressed on leukocytes such as lymphocytes, monocytes, mast cells, macrophages, basophils and eosinophils, but not neutrophils. It mediates cellular adhesion and activation through a variety of cell-cell and cell-matrix interactions that regulate leukocyte migration into tissues during inflammatory responses and lymphocyte trafficking.[49] Blocking of these

interactions has the potential to inhibit several processes that play important roles in inflammation and thus may be especially useful in the treatment of inflammatory diseases. The binding of the $\alpha_4\beta_1$ receptor to cytokine-induced VCAM-1 at sites of inflammation results in firm adhesion of the leukocyte to the vascular endothelium followed by extravasation into the inflamed tissue.[50] Binding of $\alpha_4\beta_1$ integrin to VCAM-1 also plays a key role in stem cell adhesion to bone marrow stroma cells[51] and may also be involved in tumor cell metastasis.[52] Monoclonal antibodies directed against $\alpha_4\beta_1$ integrin or VCAM-1 have been shown to be effective modulators in animal models of chronic inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease.[50] In addition to their clinical relevance in inflammation, these molecules act as cellular receptors for viral and parasitic agents.[53] The predominant form of VCAM-1 *in vivo* has an amino-terminal extracellular region comprising seven immunoglobulin-like domains. Structure/function studies indicate that domain 1 (or the homologous domain 4) is most important for binding, with the contiguous domain 2 (or domain 5) required at least for stabilization of structure. The X-ray crystal structure for the binding region of VCAM-1 (Figure **20**, P. 45) indicates the existence of a loop that protrudes from the surface of the molecule.[54] This region is a good target for synthetic peptides and peptide-like substances (i.e. peptidomimetics) that could inhibit the interaction of VCAM-1 with $\alpha_4\beta_1$ integrin and on this way have numerous medicinal applications.

2.3.3. Invasin

A number of bacterial pathogens is able to enter normally nonphagocytic cultured cells. There are numerous potential roles that cellular entry plays in establishing diseases. Organisms, such as the enteropathogens *Yersinia* and *Salmonella* appear to utilize cellular entry to gain access to subepithelial regions. Once microorganisms translocate across the epithelium, the routes that different organisms take to promote disease may diverge significantly from one to another. Efficient entry of enteropathogenic *Yersinia* into both cultured mammalian cells and M cells requires the bacterial protein invasin, which binds multiple members of the integrin receptor superfamiliy.[55] The protein is part of a family of adhesins encoded by enteropathogenic bacteria that includes the intimins, which are involved in promoting attachment and effacing lesions by enteropathogenic *Escherichia coli,* as well as *Citrobacter freundii*. All members of this family appear to be involved in binding to receptors present on the mammalian cell plasma

membrane that signal to the host cytoskeleton.[56] The most significant region of similarity between these family members is found in the 500 amino acids of the *Y. pseudotuberculosis* invasin, which is required for outer membrane localization and export of carboxy termini of these peptides.[57]



The integrin binding region of invasin consists of five domains in tandem (D1-D5, with D1 being at the amino terminus) of which D1-D4 display the folding pattern of the immunoglobulin superfamily. D5 has a topology similar to C-type lectin like domains. D5 + D4 is the cell adhesion module of the protein and the large interface between D4 and D5 gives the appearance of a single superdomain (Figure 7).

Invasin does not contain an RGD sequence. Three sites are involved in binding to integrin receptors. The first region is located in a disulfide loop and centered around the residue Asp911. Even a conservative change of this residue to a Glu residue results in total loss of bacterial uptake in host cells.[58,59]

Figure 7: Crystal structure of invasin.

The second region is centered around residue Asp811, although residue changes in this region have weaker effects than seen with Asp911. This region that appears to play a role in supporting integrin binding is called synergy region. The third Arg883 residue, also in the synergy region, is located about 30 Å apart from Asp911. The five integrins that bind invasin ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$) are either receptors for extracellular matrix proteins or are involved in cell-cell interactions. The best characterized representative of this group is $\alpha_5\beta_1$, which is also known as the fibronectin (Fn) receptor. Although there is no detectable similarity between invasin and fibronectin, the crystal structure suggests striking similarities in the receptor recognition properties of fibronectin and invasin. In fact, the two substrates appear to bind to the same site on the $\alpha_5\beta_1$ receptor based on competitive inhibition studies, mutational analysis and monoclonal antibody inhibition results.[60] Nevertheless, invasin binds much more efficient than fibronectin, particularly under conditions in which the amount of receptor on the target cell is relatively low. The much higher receptor binding affinity of invasin appears to be the reason for this difference.[61] There are two explanations on the structural basis for higher activity of invasin. First, the rigidity of the D4-D5 adhesion module may lock the protein in an optimal conformation for binding that

stabilizes ligand-receptor interaction. Fibronectin, in contrast appears to have great flexibility between the two domains involved binding integrins, and it may assume several conformations that are not optimal for binding. Secondly, in invasin there exists a patch of five aromatic amino acids in the region between Asp911 and Asp811 residues that forms a protrusion of the surface of D4. This region could facilitate either hydrophobic interactions or hydrogen bonding with the receptor and might contribute greatly to the binding energy. Fibronectin has a deep cleft in the corresponding region that may not contribute significantly to binding.
These invasin binding regions are good targets for design of powerful integrin inhibitors, which could have numerous medicinal and biological implementations.

2.4. TYROCIDINE A

Tyrocidine A (Figure 8) belongs to the group of peptides produced naturally by microorganisms living in different habitats, spread from aquatic to terrestrial environments. They are not genetically encoded but are synthesized non-ribosomally on large multifunctional enzymes called peptide synthetases.[62]

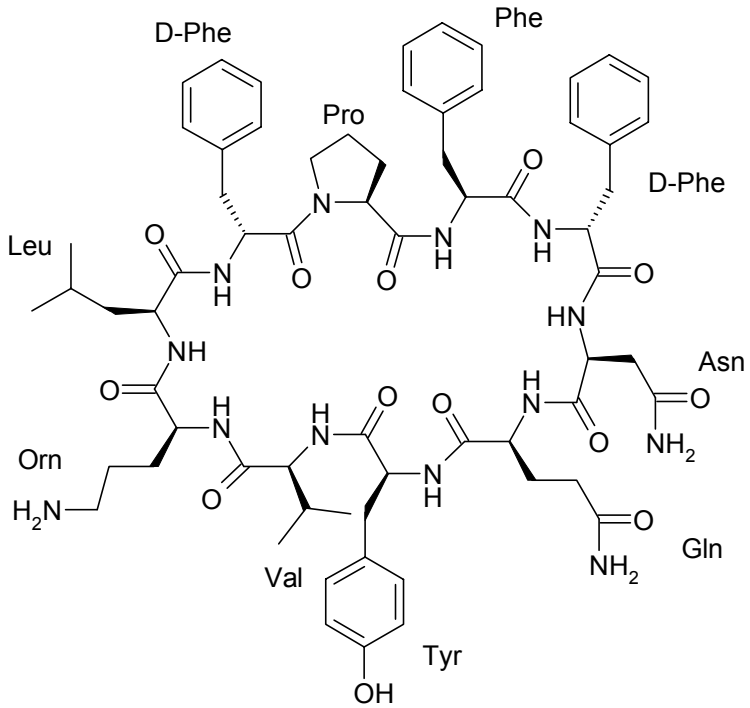


Figure 8: Tyrocidine A.

In this non-ribosomal mechanism of peptide synthesis, compounds such as lipopeptides, depsipeptides, and peptidolactones are assembled from an exceedingly diverse group of precursors (more than 300 are known[63]) including pseudo amino acids, nonproteinogenic amino acids, hydroxyl acids, N-methylated

amino acids, D- and β-amino acids. This non-ribosomal protein template directed synthesis of peptides is limited only by the length of the peptide chain formed, which has been found to range from 2 to 48 residues.[64] The peptide backbone of these short bioactive peptides can be composed of linear, cyclic, or cyclic branched structures that may be further modified by acylation, glycosylation or heterocyclic ring formation.

These structurally diverse compounds are endowed with a broad spectrum of biological properties, including antimicrobial, antiviral and antitumor activity and could be interesting targets for drug discovery.

Although diverse in structure, most of the non-ribosomally synthesized peptides share a common mode of synthesis by enzymes that employ a multiple-carrier thiotemplate mechanism. The peptide synthetases have a unique modular structure in which each module is responsible for the recognition, activation and in some cases modification (epimerization, N-methylation etc.) of a single substrate residue of the final peptide product. The modules are aligned in a sequence that is collinear with the sequence of the peptide product. With this template arrangement in peptide synthetases, the modules seem to operate independently of one another, but they act in concert to catalyze the formation of successive peptide bonds.[65]

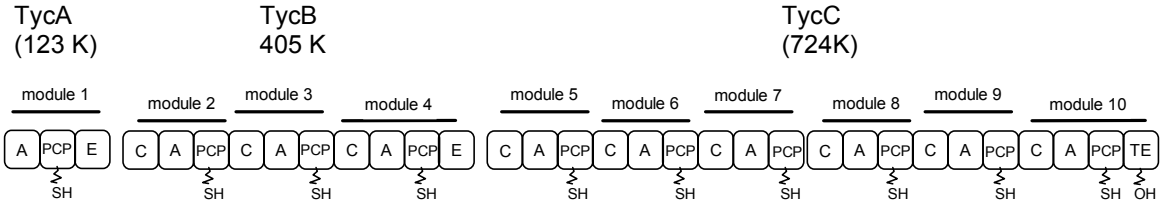


Figure 9: The tyrocidine non-ribosomal peptide synthetase.

Synthetase subunits TycA, TycB, and TycC are represented by a series of boxes. Each box represents a functional domain: A, adenylation (catalyzes amino acid activation); PCP, peptidyl carrier protein; C, condensation (catalyzes peptide bond formation); E, epimerization; TE, thioesterase.

The modular structure of peptide synthetases gives them the advantage of being suitable candidates for the combinatorial biosynthesis of non-ribosomal peptides by rearranging or substituting the modules such that a new substrate amino acid or modification is placed within the final product.[66] This technology has already been successful in the engineering of modular polyketide synthases for the rational design of altered polyketides.[67] Recently it has been shown that a carboxy terminal thioesterase domain of peptide synthetase, that is involved in cyclization and product release can also catalyze macrocyclization, and elongation in the case of symmetric cyclic peptides, independently of upstream domains.[68]

3. AIMS

Several goals were to be achieved in this work:

-Because of the growing number of evidence[69] that β-amino acids exert characteristic and predictable influence on the conformation of cyclic peptides in solution, cyclic model peptides are required. Cyclic tetra-, penta- and hexa-peptides and their analogs in which one α-amino acid is replaced with β-amino acid are ideal models for such studies. The conformation in solution of these cyclic peptides and influence of cations on conformations should be studied using different spectroscopic techniques (CD, FTIR, NMR).

-The second aim is the synthesis of cyclic penta- and hexapeptides based on the binding epitope of VCAM-1 (TQIDSPLN). In these peptides D-amino acids and β-amino acids should be incorporated to induce regular turns and to improve the stability of these peptides toward enzymatic hydrolysis. Such peptides could be strong and selective inhibitors of VCAM-1 interactions with the $\alpha_4\beta_1$ integrin and could find numerous medicinal and scientific applications. Conformational analysis in solution of these peptides together with the determination of their inhibitor activity should provide more insight in the relative orientation of the pharmacophoric groups (structure-activity relationship) and could lead to the development of even stronger nonpeptidic inhibitors.

-The third aim is the synthesis of the cyclic peptides based on the invasin binding epitope (SDMS). This sequence has to be positioned in a β-turn for optimal activity of the peptides. Also the influence of an Arg residue, which is about 30 Å distant from the SDMS loop in invasin, should be investigated and peptides that mimic these two recognition sites should be synthesized. These peptides will be tested as inhibitors of the laminin interaction with the integrin $\alpha_3\beta_1$.

-The fourth goal of this work is to synthesize linear precursors of tyrocidine A and their analogs (α-amino acids replaced by β-amino acids) using the recently developed “safety catch” linker strategy for the introduction of a thioester at the C-terminus of the linear precursor. Cyclization of these linear precursors to the Tyrocidine A analog will be performed using overexpressed and purified TycC TE domain of the large multifunctional enzymes called peptide synthetases.

-The last objective is the synthesis of different cyclic RGD peptides in which β-amino acids are incorporated. Also the influence of the hydrophobicity of the residue following the RGD sequence on the inhibitor activity toward integrins binding should be studied.

4. RESULTS AND DISCUSSION

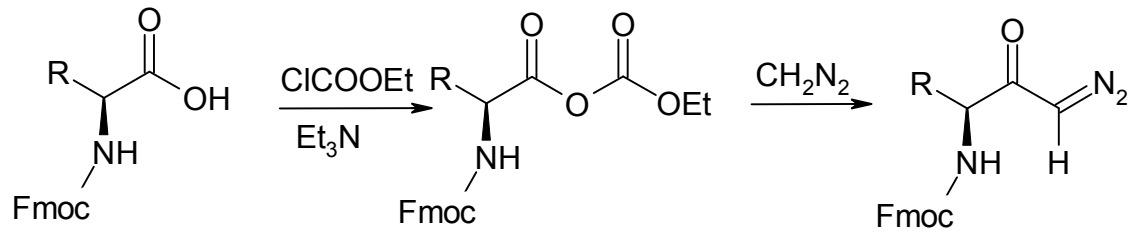
4.1. β-HOMOAMINO ACIDS

In the course of this study aimed at obtaining modified biological molecules with potential pharmacological activity, a useful approach to β-homoamino acids was needed. Among others, the homologation of α-amino acids is an important strategy for the asymmetric synthesis of β-homoamino acids.[5,7,8]

The Arndt-Eistert approach towards β-homoamino acids via Wolff rearrangement of diazo ketones derived from protected α-amino acids has been utilized since the early 1950s.[70] This protocol was reinvestigated thoroughly with respect to possible epimerization of the chiral centre.[71] The Wolff rearrangement of α-diazo ketones can be accomplished thermally, photochemically, or by metal ion (Ag^+) catalysis and has been shown to proceed in most cases with complete retention of configuration.[71,72] It has been shown that epimerization occurs only in the case of urethane protected phenyl glycine, presumably during carboxy group activation.[71,73] Recently it has been also described that base-free, Ag^+ catalyzed Wolff rearrangement of Fmoc protected α-diazo ketones proceeds smoothly within minutes at room temperature and without epimerization (except for phenylglycine) on sonication using an ultrasound-cleaning bath.[74] This method is compatible with base labile protective groups and is chosen for the synthesis of Fmoc-protected β-amino acids used later in solid phase peptide synthesis.

The instability of the acyl chloride intermediates, their pronounced tendency to undergo racemization at the α-carbon and incompatibility with acid labile protective groups prevents the application of acyl chlorides as preactivated α-amino acids. It is also possible to use acyl fluorides[75] as preactivated α-amino acids or to use DCC[76] as activating reagent. The Fmoc-protected α-amino acids **(1)** are activated as mixed anhydrides **(2)** using ethyl chloroformate.[71,77] Reaction with a sufficiently high excess of diazomethane secures complete conversion into diazo ketone **(3)**.

It has been reported that application of this protocol to the Fmoc protected substrates often gives poor yields, racemic starting materials and their methyl esters as by-products.[78]

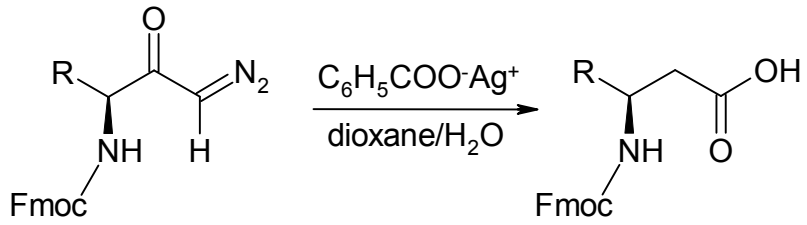


(1) **(2)** **(3)**

Scheme 9: Synthesis of diazo ketones (3).

Although methyl esters of α-amino acids **(1)** are observed in the reaction mixtures, this has no influence on the next synthetic step, and the diazo ketones **(3)** can be used without further purification. Nevertheless, some diazo ketones (Fmoc-L-isoleucyldiazomethane **(10)**, Fmoc-L-phenylalanyl-diazomethane **(13)**, Fmoc-L-prolyldiazomethane **(17)**, Fmoc-L-valyldiazomethane **(20)**) have been isolated and purified. The corresponding β-amino acids are obtained from such intermediates with good yields and purity but overall yields are almost the same as observed in reactions without isolation of intermediary diazo ketones.

Wolff rearrangement by sonication of the diazo ketone **(3)** in 1,4-dioxane in the presence of silver benzoate and a suitable hetero nucleophile (water) results in a clean formation of the β-amino acid derivative **(4)**.



(3) **(4)**

Scheme 10: Wolff rearrangement of the diazo ketones (3).

The β-homoamino acids obtained by this method can be used in peptide synthesis without further purification.

Table 3: Synthesized β-homoamino acids.

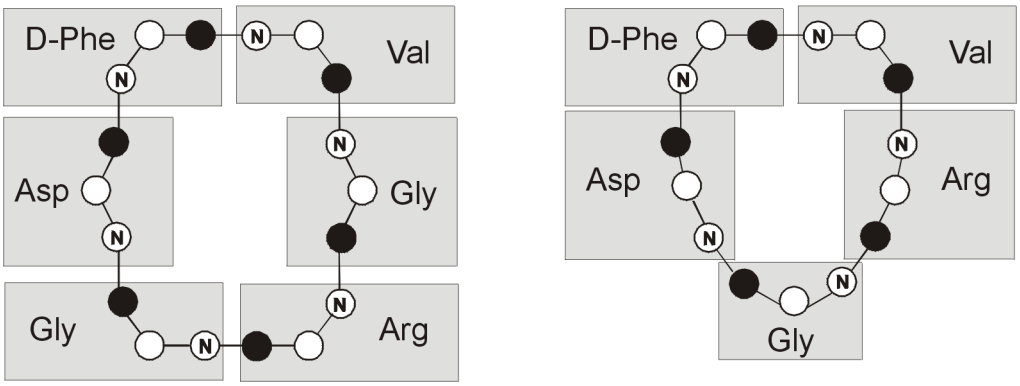
β-homoamino acids	D/L nomenclature	Yield[a] /%
Fmoc-(R)-β^3-hAla-OH **(5)**	D	50
Fmoc-(S)-β^3-hAsn(Trt)-OH **(6)**	L	75
Fmoc-(R)-β^3-hAsp(Ot-Bu)-OH **(7)**	L	39
Fmoc-(S)-β^3-hGln-OH **(8)**	L	53
Fmoc-(R)-β^3-hGln-OH **(9)**	D	42
Fmoc-(3R,4S)-β^3-hIle-OH **(11)**	L	50
Fmoc-(S)-2-β^3-hNal-OH **(12)**	L	67
Fmoc-(S)-β^3-hPhe-OH **(14)**	L	60
Fmoc-(R)-β^3-hPhg-OH **(15)**	L	52
Fmoc-(S)-β^3-hPhg-OH **(16)**	D	57
Fmoc-(S)-β^3-hPro-OH **(18)**	L	64
Fmoc-(R)-β^3-hSer(t-Bu)-OH **(19)**	L	80
Fmoc-(R)-β^3-hVal-OH **(21)**	L	62
Fmoc-(S)-β^3-hVal-OH **(22)**	D	62

[a] Yields are calculated from the starting Fmoc protected α-amino acids.

4.2. MODEL PEPTIDES

Cyclic peptides are appealing targets for combinatorial library development.[79] They are excellent tools for examination of the conformational requirements of peptide or protein recognition and serve as models for the design of bioavailable drugs. Some cyclic peptides are drugs in their own right, examples include octreotide[80] and cyclosporin A.[81] The continuously growing interest in the rational design of biologically active peptides led to the development of molecular tools that should be capable to force conformationally flexible peptides to preferentially adopt a required bioactive three-dimensional structure. One excellent example of rational drug design using molecular modeling techniques has been contributed by Kessler et al.[45] They incorporated the triad Arg-Gly-Asp (RGD), which is known to be a universal cell recognition sequence binding to cell surface-exposed integrins, in conformationally restricted penta- and hexapeptides. The hexapeptide **(23)**, a product of this rational design, efficiently inhibits binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$ involved in thrombocyte aggregation, while the cyclic pentapeptide **(24)** prevents binding of vitronectin to the integrin $\alpha_V\beta_3$ playing a role in tumor cell adhesion, angiogenesis and osteoporosis. The selectivity profile of these

cyclopeptides is rationalized by a mutually different presentation of the pharmacophoric group (RGD).



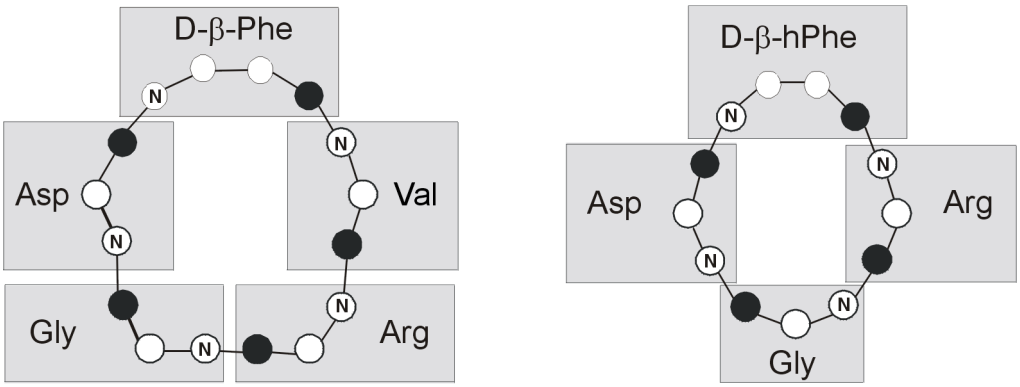
c-(-Arg-Gly-Asp-D-Phe-Val-Gly-) **(23)** c-(-Arg-Gly-Asp-D-Phe-Val-) **(24)**

Figure 10: Cyclic RGD peptides (23) and (24) as integrin antagonists.

The RGD motif is found to occupy positions i+1 to i+3 of a β-turn in the $\alpha_{IIb}\beta_3$ selective hexapeptide **(23)** while it resides in positions i to i+1 of a regular γ-turn in the $\alpha_V\beta_3$ selective pentapeptide **(24)**. One D-amino acid is used to achieve this strong conformational bias. It is known[82] that D-amino acids are inducing β-turns in which they occupy i+1 position.

Peptides containing β-amino acids are characterized by a different skeleton atom pattern. The peptide backbone is extended by a C_1 unit for each β-amino acid present. Replacement of one α- by a β-amino acid in cyclic peptides induces conformational changes in such peptides.

It has been shown that by incorporation of β-amino acids the active RGD sequence can be placed in a proper conformation and that β-amino acids may posses a higher conformational bias than D-amino acids.[69] By replacing the D-Phe residue in the cyclopentapeptide **(24)**, a strong $\alpha_V\beta_3$ integrin antagonist, with D-β-Phe, the cyclo-pentapeptide c-(-Arg-Gly-Asp-D-β-Phe-Val-) **(25)** obtained (Figure 11) has inhibitory activity toward $\alpha_{IIb}\beta_3$ integrin. This is again explained with different conformational preferences of these two amino acids. The β-amino acid in **(25)** occupies the central position of an extended γ-turn, while the D-amino acid in **(24)** occupies i+1 position of a βII'-turn. This γ-turn forces the RGD sequence to adopt a β-turn, which is responsible for selective inhibitory activity toward $\alpha_{IIb}\beta_3$ integrin of this peptide.



c-(-Arg-Gly-Asp-D-β-Phe-Val-) **(25)** c-(-Arg-Gly-Asp-D-β-hPhe-) **(26)**

Figure 11: Cyclic RGD peptides (25) and (26) as integrins antagonists.

The tetrapeptide **(26)**, which is obtained by replacing the dipeptide sequence D-Phe-Val in **(24)** by the β-homoamino acid D-β-hPhe, is a strong and selective antagonist of $\alpha_v\beta_3$ integrin. Presentation of the RGD sequence in a γ-turn seems to be a prerequisite of selective $\alpha_v\beta_3$ antagonism.

4.2.1. Synthesis of Model Peptides for CD and FTIR Studies

Cyclic model peptides in which one α-amino acid is replaced by a β-amino acid analog are required for the conformational investigation of turn structures induced by β-amino acids in cyclic peptides. The cyclic peptides should have different ring sizes. Cyclic tetra-, penta- and hexa- peptides are ideal models for such studies. The amino acids are chosen so that some conditions are fulfilled:

a) Amino acids without side-chain functionality that could interfere with spectroscopic techniques (Leu, Ala, Val, Gly, Pro) should be used.
b) Because conformational changes depending on the solvent are also part of the study, amino acids with hydrophobic side chains that can improve solubility of the corresponding cyclic peptides in organic solvents should be used.
c) A Gly residue should be present in all synthesized peptides at the C-terminus of the linear precursor to avoid epimerization during cyclization.
d) A Pro residue, which exerts strong conformational bias should also be present in all synthesized cyclic peptides to improve the cyclization rate over dimerization. Moreover, for studying the conformational preferences of β-amino acid this amino acid is chosen to be replaced by the β-homo analog (Table 4).

Table 4: Model peptides.

	All α-amino acid	Analogs
Tetrapeptide	c-(-Ala-Pro-Phe-Gly-) **(37)**	c-(-Ala-β-hPro-Phe-Gly-) **(38)**
Pentapeptide	c-(-Ala-Val-Pro-Phe-Gly-) **(35)**	c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)**
Hexapeptide	c-(-Leu-Ala-Val-Pro-Phe-Gly-) **(33)**	c-(-Leu-Ala-Val-β-hPro-Phe-Gly-) **(34)**

The corresponding linear peptides are synthesized on Wang resin and Sasrin, resp., in the automated peptide synthesizer. For the synthesis Fmoc chemistry is used with a solution of 2 % DBU, 2 % piperidine in DMF as the deprotection agent, TBTU as coupling reagent and DIPEA as base (Table 30, P. 114). Linear peptides are cleaved from the resin with an adequate cleavage cocktail (protocol 9, P. 118 for Sasrin and protocol 10, P. 118 for Wang resin) and purified by reverse phase preparative HPLC. Results are represented in Table 5.

Table 5: Synthesis of linear precursors of the model peptides (33) – (38).

Linear peptide	Resin	μmol	Yield		
			mg	μmol	%
H-Leu-Ala-Val-Pro-Phe-Gly-OH **(27)**.	Sasrin	100	50	80	80
H-Leu-Ala-Val-β-hPro-Phe-Gly-OH **(28)**.	Wang	150	65	89	59
H-Ala-Val-Pro-Phe-Gly-OH **(29)**.	Sasrin	200	60	123	62
H-Ala-Val-β-hPro-Phe-Gly-OH **(30)**.	Sasrin	200	68	135	68
H-Ala-Pro-Phe-Gly-OH **(31)**.	Sasrin	200	47	120	60
H-Ala-β-hPro-Phe-Gly-OH **(32)**	Wang	150	36	89	59

Two main reasons influenced the yields of linear peptides. Some quantities of peptides are lost during the purification of the peptides by reverse phase preparative HPLC. Diketopiperazine formation during deprotection of the second amino acid in the peptide, which is known[83] to be the main side reaction in peptide synthesis, also lowers the yields of the linear peptides. This reaction is kinetically and thermodynamically favored if one of the first two amino acids can easily adopt an amide bond having the cis-configuration, like proline or glycine.

These linear peptides are cyclized in solution under high dilution condition using a large amount of solvent (protocol 7, method A, P. 116) or two injection pumps (protocol 7, method B, P. 116). HATU is used as the coupling reagent with DIPEA as the base. The results are given in Table 6.

As it was expected the best yields of cyclic peptides are achieved with hexapeptides **(33)** and **(34)**. Comparing these two peptides, a higher yield is obtained for the analog with the β-homoamino acid **(34)**. This can be caused by two reasons:

a) Smaller ring strain due to an additional methylene group from the β-homoamino acid, and/or formation of a pseudo-γ-turn, which brings the peptide in a conformation that favors cyclization.

b) The method of cyclization with two injection pumps has great advantage, dimerization can be completely suppressed by fine regulation of the peptide addition rate.

Table 6: Cyclization in solution of model peptides.

Cyclic peptide	Met.	Coupling reagents	Yield		
			mg	μmol	%
c-(-Leu-Ala-Val-Pro-Phe-Gly-) **(33)**	A	1.1 equiv HATU 3.0 equiv DIPEA	20	34	41
c-(-Leu-Ala-Val-β-hPro-Phe-Gly-) **(34)**	B	1.5 equiv HATU 3.0 equiv DIPEA	45	75	76
c-(-Ala-Val-Pro-Phe-Gly-) **(35)**	A	1.1 equiv HATU 3.0 equiv DIPEA	25	53	43
c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)**	A	1.1 equiv HATU 3.0 equiv DIPEA	18	37	28
c-(-Ala-Pro-Phe-Gly-) **(37)**	A	1.1 equiv HATU 3.0 equiv DIPEA	1	2	2
c-(-Ala-β-hPro-Phe-Gly-) **(38)**	B	1.5 equiv HATU 3.0 equiv DIPEA	15	37	41

As expected pentapeptides **(35)** and **(36)** are obtained in lower yield than hexapeptides **(33)** and **(34)**. An unexpected low yield is obtained for the pentapeptide with β-homoamino acid **(36)**, caused by dimerization. The final concentration of this peptide was approximately 0.19 mmol/l, which is much higher compared to the cyclo-pentapeptide **(35)** (about 0.1 mmol/l). The latter is synthesized with relatively good yield.

Again in good agreement with theory tetrapeptide **(37)** is synthesized with very low yield. In most cases the product of the reaction was the linear dimer and the cyclic dimer of the corresponding linear peptide **(31).** An additional experiment, cyclization with two injection pumps did not give better yield. Tetrapeptides always represent a problem for cyclization because their ring is too strained and the intermolecular dimerization reaction is much faster than intramolecular cyclization despite the high dilution conditions. The additional methylene group of the β-homoamino acids in the peptide backbone proved to be beneficial for cyclization because this provides more conformational flexibility and probably arranges the peptide in a conformation that favors the intramolecular cyclization over intermolecular dimerization. The analog tetrapeptide **(38)** with β-homoamino acids is obtained in relatively high yield.

4.2.2. Spectroscopic Studies

Because the crystallization of the cyclic model peptides remained unsuccessful so far, x-ray crystallography could not be employed. Moreover, solid-state structures of flexible molecules may not reflect the conformational tendencies of isolated molecules since lattice forces are not negligible. In fact, they are sometimes the prevailing forces. The biological significance of solid-state structures is difficult to assess, not only because of the above-mentioned influence of crystal packing on conformation but also because the lattice is certainly different from any of biological environments in which the peptide can be found. Despite these limitations, solid-state structures are very valuable. They are actual low-energy conformations that represent reference structures to which conformers predicted by computational methods and found in solution can be compared.[84]

Three other techniques are frequently used. NMR is the most powerful method for conformational studies of peptides and proteins in solution. From the wealth of data provided by NMR, coupling constants (J) and nuclear Overhauser effects (NOE) may be converted into structural information. However, the interpretation of NMR data for flexible small and midsize peptides must be carried out with caution. NMR is a slow method with a time scale of second to hundreds of seconds. Hence, conformational interconversions not requiring peptide bond rotations will result in averaged NMR parameters. The interpretation of these averaged parameters is of little value for conformation analysis.[85] Despite this, multidimensional NMR experiments in combination with energy calculations and molecular modeling by means of molecular mechanics (MM) and molecular dynamics (MD) methods are prevalent in the cyclic peptide field.

The time scale of chiroptical and vibrational spectroscopic methods is much shorter than that of NMR. Circular dichroism (CD) spectroscopy, one of several chiroptical methods, has been established as a simple but sensitive tool for detecting protein and peptide secondary structure. CD reflects for instance the relative spatial orientation of consecutive amide groups. By the end of the 1980s it became evident that the information inferred from the CD spectra gave only a "low resolution" picture of the steric structure.

Fourier transform infrared (FTIR) spectroscopy is the simplest method for characterizing H-bonded folded secondary structures.[86] Amide vibrations are highly sensitive to H-bonding. Thus, vibrational techniques are of great help not only in detecting turns but also quantitating their distortions. The most important region for studying of peptides turns is the amide I spectral region (1620-1700 cm^{-1}). Using the mathematical procedure of band narrowing-Fourier self deconvolution (FSD) and Fourier derivation (FD), individual amide I components representing different secondary structures are visually separated.[87] CD and FTIR spectroscopy reflect weight-dependent spectral contribution of all components of a pool of conformers. The combined application of these simple and inexpensive methods is advantageous in preliminary conformational studies (conformational screening) because measurements performed under the same conditions provide complementary data on the three-dimensional structure.

4.2.2.1. Circular Dichroism (CD) Spectroscopy

The CD spectra of proteins are determined by the relative spatial arrangement of the amide chromophores repeated periodically along the polypeptide chain. The relative orientation of the consecutive amide groups depends on the geometry (type) of the turn. The CD spectrum reflects this relative spatial orientation of the amide groups, which in regular ordered conformations are linked together by H-bonds of different strength. CD spectroscopy is used very often for fast screening of regular ordered secondary structures in proteins. The CD curves of different helices and β-sheets are well documented. The CD curve of an α-helix has a negative band at 222 nm associated with the $n\pi^*$ transition and a $\pi\pi^*$ couplet located at 208 nm (negative band) and 192 nm (positive band).[88] The β-pleated sheet has a CD spectrum composed typically of a negative band near 215 nm, which is assigned to the $n\pi^*$ transition, and a positive band near 198 nm, which is assigned to $\pi\pi^*$ excitation components.[89] Different types of turns have characteristic chiral contributions, which permit their detection and discrimination

from other conformations. The CD spectra of different β-turns are also known in the literature. The spectrum of a type I β-turn qualitatively resembles that of the α-helix in the long-wavelength region, but the positive band at short wavelengths is weaker in the turn spectrum. Type II β-turns give rise to a spectrum that resembles the β-sheet spectrum but the bands are shifted to the red by 5-10 nm. The γ-turn represents the other main type of folded secondary structures, but contributions of γ-turns to CD-spectra are not clearly described in the literature. There are suggestions that an inverse γ-turn has a negative $n\pi^*$ CD band near 230 nm but the $\pi\pi^*$ region of their CD spectra has not been sufficiently characterized.[86, 90]

Due to geometric factors, cyclic pentapeptides often form one β- and one complementary γ-turn, while cyclic hexapeptides have a ring size, which allows the adoption of even two β-turns. Also structures with one β- and one γ-or with two or more γ-turns have been found.[91] Because of simple geometric reasons, cyclic tetrapeptides and tripeptides cannot adopt a β-turn backbone conformation and only γ-turn formation is possible. Tetrapeptides are ideal models for studying the contribution of the γ-turn in CD spectra. For fast screening of conformational changes that occur by replacing one α-amino acid by its β-analog in a cyclic peptide, the CD spectra of all model peptides (Table 4, P. 30), except for c-(-Ala-Pro-Phe-Gly-) **(37)** because of its low solubility, in different solvents have been recorded.

CD spectra of these cyclic peptides in ACN are shown in Figure 12. These spectra are results of different peptide conformations, turn contributions and the aromatic contribution by phenylalanine. In the majority of cases CD spectroscopy is applied for monitoring the conformational flexibility rather than characterizing the conformation of the cyclic molecule. Nevertheless, some similarities are to be observed. The CD curve of two peptides containing a β-homoamino acid, c-(-Leu--Ala-Val-β-hPro-Phe-Gly-) **(34)** (blue), and c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)** (brown) are very similar with a negative band near 202 nm and a shoulder near 195 nm. In the NMR and molecular modeling studies (chapter 4.2.2.3, P. 40) it has been shown that the cyclic pentapeptide **(36)** is present in two conformations in DMSO (Figure 14 and Figure 15, P. 41). The major conformation, about 70 % of the population, contains one pseudo-γ-turn with β-homoproline in the central position and one β-turn with glycine in i+1 position. The minor conformation consists of one γ-turn with alanine in the central position and one pseudo-β-turn with β-homoproline in i+1 position. From the similarity of the CD spectra of these two peptides, **(34)** (blue) and **(36)** (brown), similar conformations might be expected for cyclic hexapeptide **(34)**. The CD spectra of the third peptide with β-homoamino acid, c-(-Ala-β-hPro-Phe-Gly-) **(38)** (violet), is also characterized by a negative

minimum, but shifted near 207 nm, and with much lower intensities than in the case of two previous peptides **(34)** (blue) and **(36)** (brown). Also instead of the negative shoulder near 195 nm a positive band is present. The third band of this peptide **(38)** (violet) is very broad with low intensity between 220 and 240 nm. Obviously this curve has a completely different profile than the CD spectra of peptides **(34)** (blue) and **(36)** (brown).

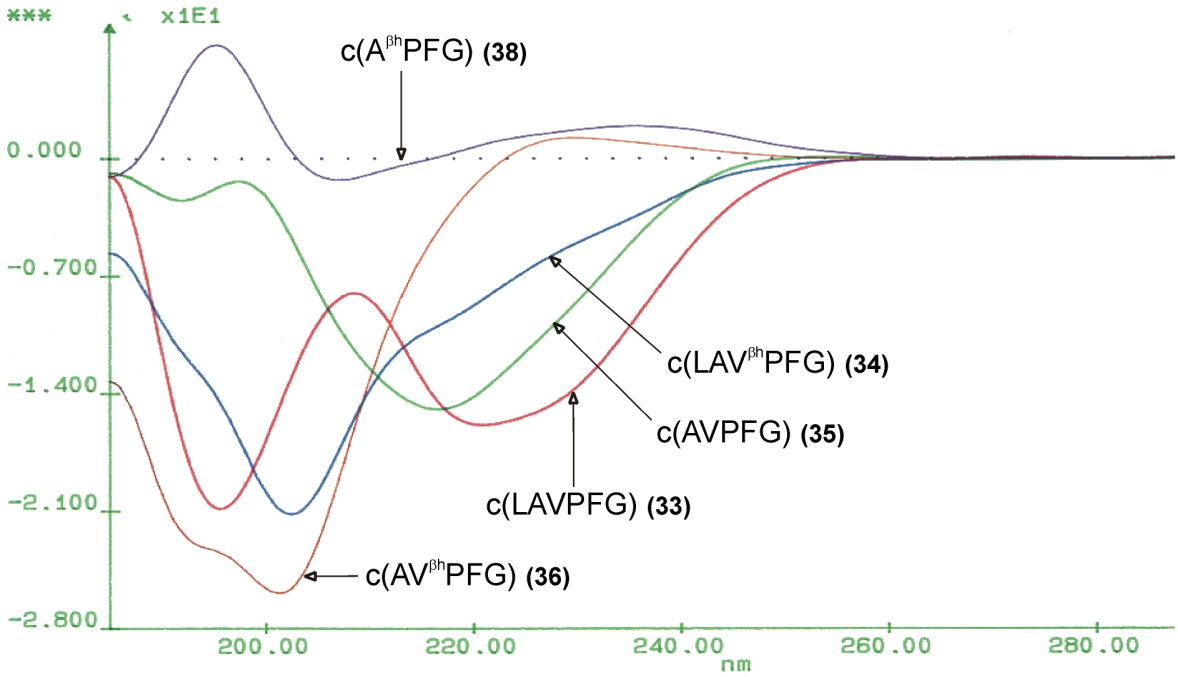


Figure 12: CD spectra of model peptides in acetonitrile.

The CD spectra of the cyclic peptide c-(-Leu-Ala-Val-Pro-Phe-Gly-) **(33)** (red), which does not contain any β-amino acids has a different curve profile, which is characterized by a negative band near 195 nm, and a negative band near 220 nm with a shoulder near 225 nm. The CD spectrum of the cyclopentapeptide without any β-amino acid c-(-Ala-Val-Pro-Phe-Gly-) **(35)** (green) shows the same profile but with much lower band intensity near 192 nm.

The influence of the solvent on the conformation of the peptides **(34)** and **(36)** has been examined for the peptides containing a β-homoamino acid by changing the solvent to more polar TFE or TFE/H_2O. A representative example is presented in Figure 13. The CD spectrum of cyclopentapeptide **(36)** in the strong proton donating solvent TFE (red or green in Figure 13) completely changes the profile of the curve. Instead of the negative shoulder near 195 nm, a positive band at the same wavelength is observed. A negative band near 202 nm is present, but with lower intensity. A third very broad band of low intensity between 220 and 230 nm is also present. This curve is now similar to the curve of the tetrapeptide **(38)** in ACN (violet, Figure 12). The NMR and molecular modeling studies (chapter 4.2.2.3, P. 40) show that the cyclic pentapeptide **(36)** is present in only one

conformation in TFE (Figure 16, P. 41) and that this conformation is similar to the major conformation of the cyclic pentapeptide **(36)** in DMSO. In this conformation the cyclic peptide **(36)** contains one pseudo-γ-turn with a β-homoproline residue in central position of the turn, but the β-turn is not present.

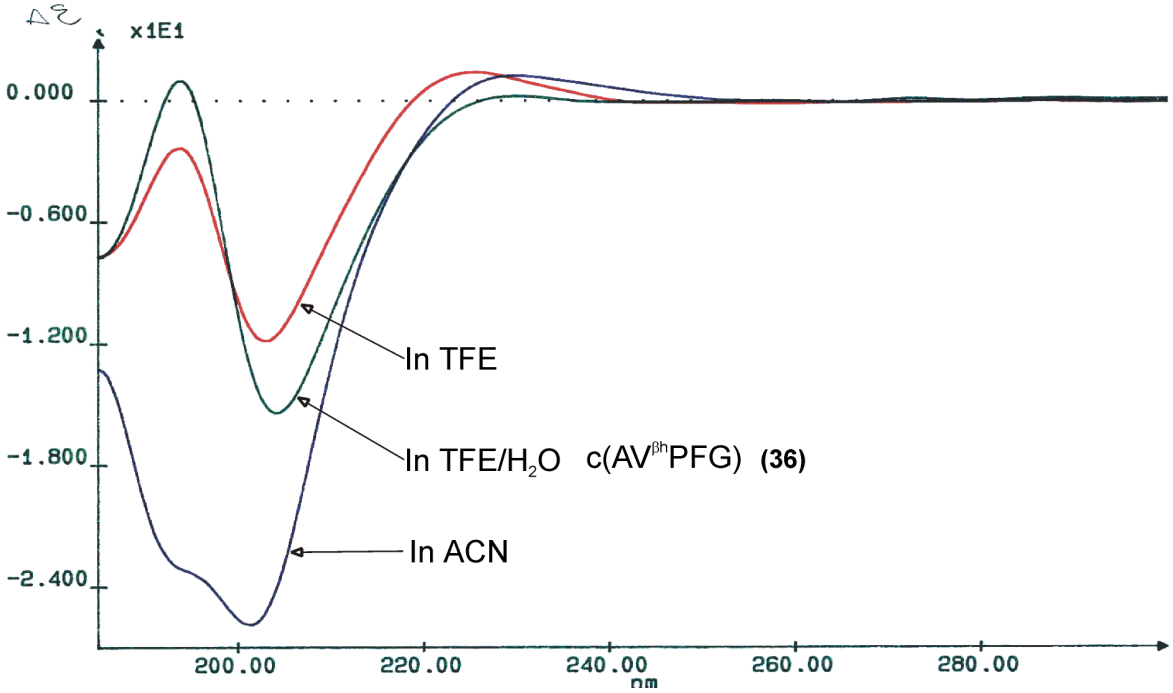


Figure 13: CD spectra of c-(Ala-Val-β-hPro-Phe-Gly-) (36) in different solvents.

Because of the similarity between the CD spectra of the cyclic tetrapeptide **(38)** (that do not change with the solvent) and cyclic pentapeptide **(36)** in TFE it is to be concluded that cyclic tetrapeptide **(38)** adopts predominantly one conformation. Also this conformation should accomplish one pseudo-γ-turn probably with β-homoproline in the central position. There are only few literature data[86, 90] about the influence of the γ-turn on the CD spectra of cyclic peptides and no data about pseudo-γ-turn but it seems that the positive band near 195 nm and the positive broad band near 230 nm could be connected with γ- and pseudo-γ-turn while the negative band near 207 nm is more likely to be influenced by phenylalanine and/or other irregular structures. A significant change of the CD spectra with the solvent is neither observed for the cyclic penta- and hexapeptides **(33)** and **(35)** that do not contain β-homoamino acids nor for tetrapeptide **(38)**.

4.2.2.2. FTIR Spectroscopy

The amide group gives rise to several strong FTIR bands, the fine structure of which depends on the various types of secondary structures and their relative amounts. However, the majority of amide bands reflecting H-bond strength and skeletal conformations are usually hidden because of the complexity of IR spectra. Among the vibrations of the amide function, it is amide I band (1620-1700 cm^{-1}) that has attracted most attention. The major factors responsible for the conformational sensitivity of the amide I band are H-bonding and the coupling of transition dipoles.[92] The band shifts caused by H-bonding and/or transition dipole coupling depend on the orientation and distance of interacting amide dipoles and thus provide information about the relative steric position of amide groups. Among the various vibrational techniques, Fourier-transform infrared (FTIR) spectroscopy, coupled with mathematical procedures for visual separation of individual component bands in the broad amide I band contour, is most widely used in the field of protein and peptide secondary structure analysis. Generally, FTIR bands appearing between 1660 and 1690 cm^{-1} have been assigned to β-turns.[93] This spectral region corresponds to weakly solvated or shielded amide carbonyls, which are not involved in H-bonds matching the strength of the H-bonds of α-helix (1650-1657 cm^{-1}), or β-sheet (1620-1640 cm^{-1}). The turns (type I or II) are fixed by 1←4 (C_{10}) intramolecular H-bonds. FTIR spectroscopic studies on many cyclic and linear peptides have given support to the proposal that characteristic acceptor amide I band of H-bonded type I and II β-turns appears near 1640 cm^{-1} in D_2O, $CHCl_3$ and ACN.[87b] In halogenated alcohols (e. g. TFE) this band may be shifted down to 1634 cm^{-1}.

There are only few literature data about FTIR of a γ-turn.[86,94] Nevertheless, bands found around 1620 cm^{-1} are assigned to the hydrogen bonding in a γ-turn.

There are no literature data about FTIR of a pseudo-γ-turn, but it should not essentially differ from the γ-turn.

The FTIR spectra of all model peptides have been recorded in acetonitrile and TFE, resp.. Data for the amide I region are summarized in Table 7. These data are in good correlation with data obtained from CD and NMR/molecular modeling studies. For all cyclopeptides different spectra are obtained in TFE and acetonitrlile.

Table 7: Assignment of Amide I component bands in the FTIR spectra of cyclic peptides.

Peptide	Solvent	Free and distorted amides/ cm^{-1}	Solvent-exposed amides/ cm^{-1}	Weak H-bonds, solvated amides/ cm^{-1}	IHB acceptor amides in β- and γ-turns**/ cm^{-1}
c(LAVPFG) **(33)**	TFE	1676vs		1658w-m	1639m-s, 1626w
	ACN	1695m, 1686s, 1671m-s		1653m-s	1630m-s
c(LAV$^{\beta h}$PFG) **(34)**	TFE	1700w, 1674s		1652m	1626s
	ACN	1695w, 1679s	1667m	1651m	1633m-s, 1619w
c(AVPFG) **(35)**	TFE	1689m, 1675s		1656m	1641m-s, 1622w-m
	ACN	1696m, 1680m		1657vs	1636w, 1619w-m
c(AV$^{\beta h}$PFG) **(36)**	TFE	1689m, 1674s		1658m	1643m, 1628s
	ACN	1698m, 1676m	1662m-s		1634s
c(A$^{\beta h}$PFG) **(38)**	TFE	1674s		1648m	1622m-s
	ACN	1692s, 1672m-s		1658m	1636

* Higher values (1690-1700 cm^{-1}) result from distorted (non-planar) amides.

** Lower values are assigned to strongly H-bonded γ-turns (C_7).

Cyclo-pentapeptide c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)** in TFE gives rise to two bands, which could be correlated with β-turn (1643 cm^{-1}) and pseudo-γ-turn (1628 cm^{-1}), which is in good correlation with data obtained with CD spectroscopy and NMR/molecular modeling experiments.

The same peptide **(36)** in acetonitrile has different spectra in the turn-region. Only one band is observed at 1634 cm^{-1} that may be the result of two conformations and overlapping of different bands. Cyclotetrapeptide c-(-Ala-β-hPro-Phe-Gly-) **(38),** because of the similarity of CD spectra of this peptide and pentapeptide **(36)**, should have only one pseudo-γ-turn probably with β-homoproline residue in the central position of the turn. The FTIR spectra of this peptide in TFE and in ACN show only one band, in TFE at 1622 and in ACN at 1636 cm^{-1}. This is in good correlation with theoretical observations that bands are shifted to lower wavenumbers in TFE than in ACN, although these shifts in acetonitrile are smaller than expected. The FTIR spectra of cyclopentapeptide c-(-Ala-Val-Pro-Phe-Gly-) **(35)**, which should have one γ- and one β-turn, are again in good correlation with CD spectra of the same peptide and with theory. Using both spectroscopic methods no changes with different solvents are observed. For both solvent systems two bands are present at 1641 cm^{-1} (1636 cm^{-1}) for one β-turn and 1622 cm^{-1} (1619 cm^{-1}) for one γ-turn. Surprisingly bands are shifted more in acetonitrile than in TFE. Hence, it might be concluded that turns probably are formed by different amino acid residues.

The situation is more complicated for the two cyclohexapeptides **(33)** and **(34)**. Cyclo-hexapeptide c-(-Leu-Ala-Val-Pro-Phe-Gly-) **(33)** that does not contain a β-amino acid residue adopts two β-turns. The FTIR spectrum of this peptide in acetonitrile has only one band at 1630 cm^{-1}, which could be connected to two β-turns although this value is shifted to smaller wavenumbers than would be expected for one β-turn. In TFE not only a band at 1636 cm^{-1}, connected to β-turns, but also an additional small band on 1626 cm^{-1} is present. In this case larger shifts are again observed in acetonitrile than in TFE.

Cyclohexapeptide c-(-Leu-Ala-Val-β-hPro-Phe-Gly-) **(34)**, because of the additional methylene group in the peptide backbone, has even more conformational flexibility than cyclohexapeptide **(33)**. Nevertheless, FTIR spectra of this peptide in acetonitrile shows one strong band on 1633 cm^{-1} and one weak on 1619 cm^{-1}. That suggest that the major conformations of peptide is accomplished of two β-turns, but conformations with γ-turns are also present. In TFE solution of this peptide **(34)** only one band is present but strongly shifted to 1626 cm^{-1}.

4.2.2.3. NMR/Molecular Modeling Studies

The results of the NMR/molecular modeling studies are in good agreement with the results obtained from FTIR and CD measurements.
Until now molecular modeling/NMR experiments have been performed only for one peptide **(36)** in two solvents by Dr. Frank Schumann and Ulf Strijowski.
The structures have been simulated using GROMACS software[95] in DMSO and TFE boxes, using parameters obtained from ROESY and NOESY NMR experiments for interproton distances as boundary conditions.

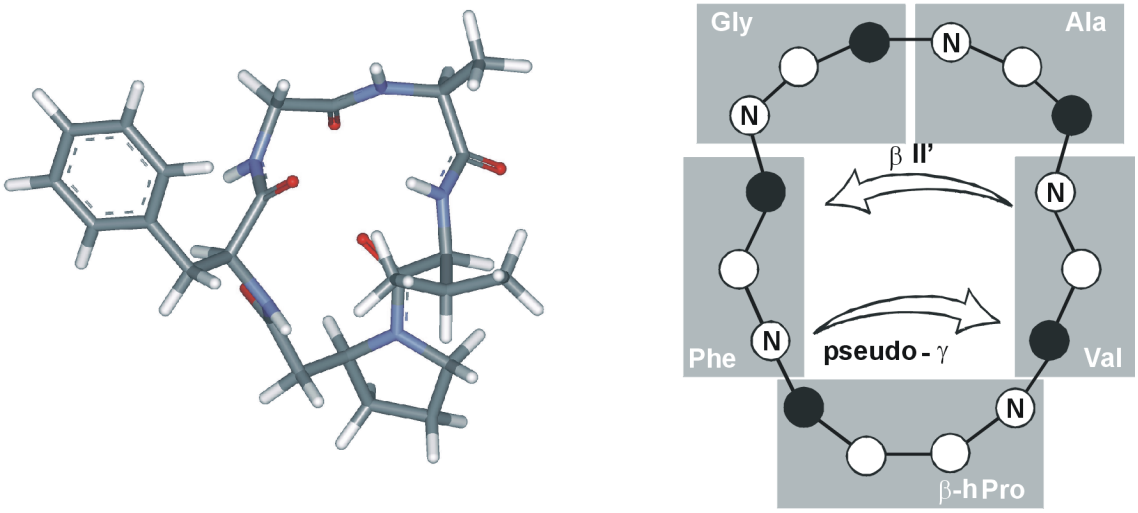


Figure 14: Conformer I c-(-Ala-Val-β-hPro-Phe-Gly-) (36) in DMSO.

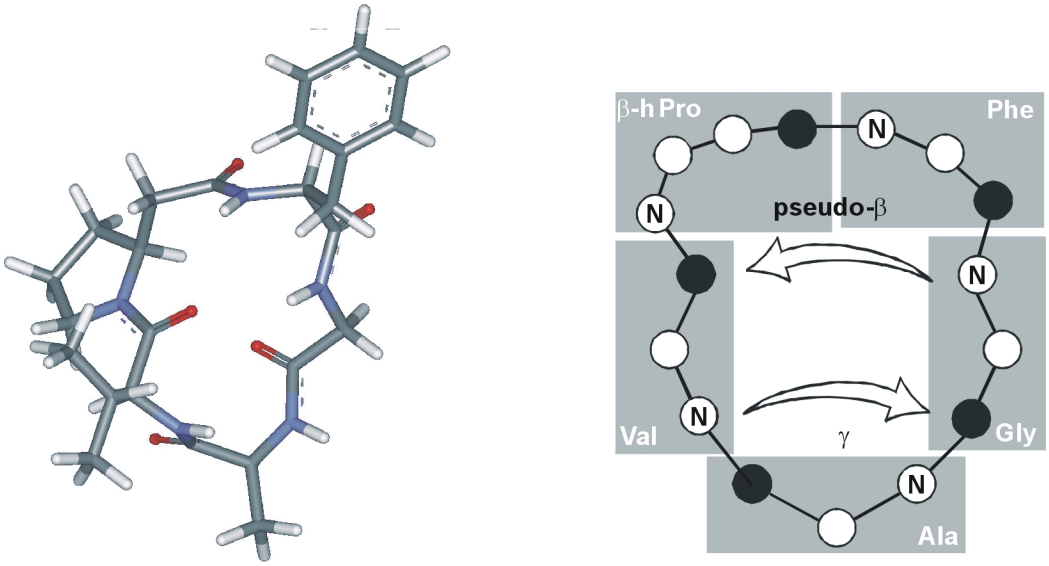


Figure 15 Conformer II c-(-Ala-Val-β-hPro-Phe-Gly-) (36) in DMSO.

Both conformations of peptide **(36)** found in the DMSO box are in agreement with the results obtained using CD and FTIR. The major conformation (~ 70 % of the population) adopts two turns, a pseudo-γ-turn with β-homoproline in the central position of the turn and a corresponding β II' turn with glycine in i+1 position

(Figure 14). The minor conformation (~ 30 % of the population) adopts a pseudo-β-turn with β-homoproline in i+1 position and a γ-turn with alanine in the central position of the turn (Figure 15).

Because the CD curve of this peptide **(36)** in TFE is significantly different from that in ACN (Figure 13, P. 36), NMR spectra of this peptide **(36)** have been recorded in CF_3CD_2OH as the solvent and these data have been used for molecular modeling in TFE box. In this solvent the cyclic pentapeptide **(36)** is found in only one conformation that is similar to the major conformation found in DMSO. This conformation has also the pseudo-γ-turn with β-homoproline in the central position of the turn and but the corresponding β- turn with glycine in i+1 position is missing (Figure 16).

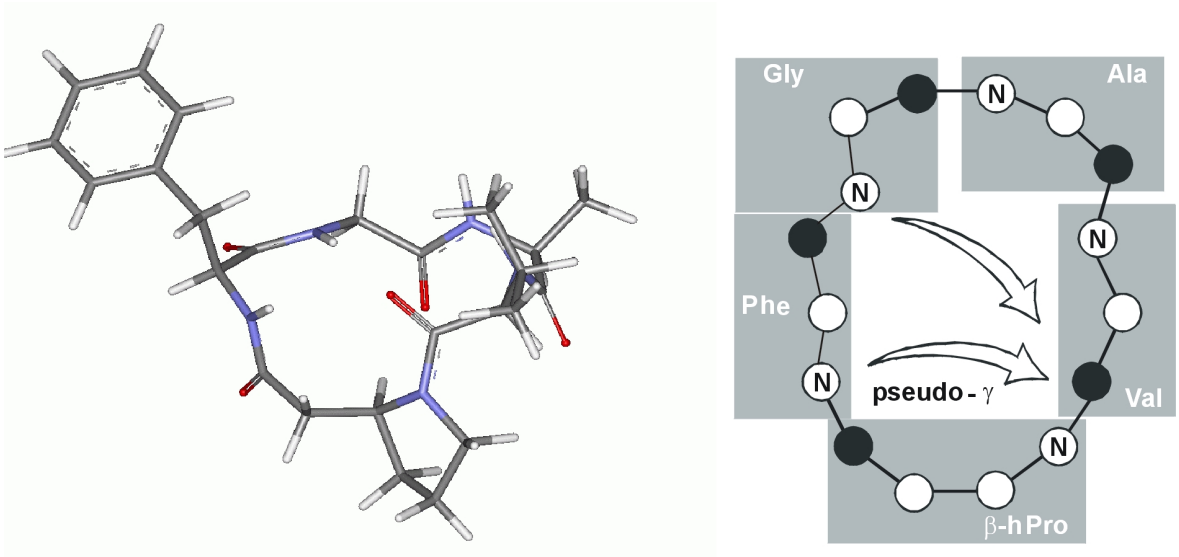


Figure 16: c-(-Ala-Val-β-hPro-Phe-Gly-) (36) in TFE.

In this case the pseudo γ-turn is stabilized by a hydrogen bonding between the valine oxygen and the phenylalanine amid bond hydrogen (distance between these two atoms is 2.5 Å). Hydrogen bonding between the same oxygen and the glycine amide bond hydrogen is also present (distance is 2.6 Å) that can stabilize a pseudo-β-turn. The ϕ, ω and ψ torsion angles of all amino acids in this conformation are given in Table 8.

Table 8: Torsion angles for c-(-Ala-Val-β-hPro-Phe-Gly-) (36) in TFE.

	ψ	ϕ	ω
Phe	-50.2	-63.3	172.2
Gly	-97.3	-130.7	176.8
Ala	-44.9	-79.8	160.2
Val	112.6	-85.8	-133.6
β-hPro	-98.5	-86.7	169.5

4.2.3. Cation Binding Studies

Cations, especially Ca^{2+}, are involved in a number of biological processes.[96] On cation binding, peptides or even proteins may undergo structural changes. These changes are often important for the biological activity of the peptides.[97] For example, cyclo^{ss}-KYGCRGDWPC is a much stronger inhibitor of fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin in presence of Mn^{2+} than in presence of Ca^{2+} ions. The IC_{50} value is 100 nM in presence of Ca^{2+} ions and 1 nM in the presence of Mn^{2+} ions.[98]

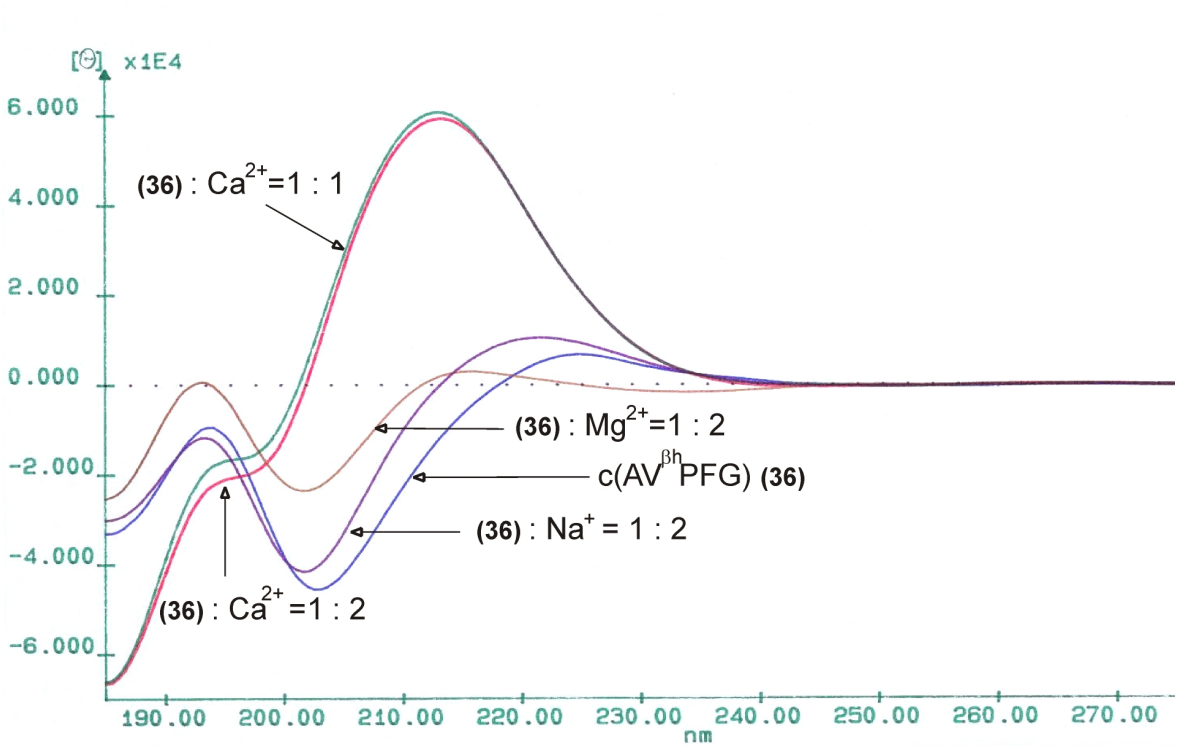


Figure 17: CD spectra of c-(-Ala-Val-β-hPro-Phe-Gly-) (36) in the presence of different cations in TFE.

CD spectroscopy has also been applied in this field of cation-binding studies. Cyclic peptides are excellent models of CD-monitored cation-binding studies.[99]
CD spectra of model cyclic peptides **(33)** - **(38)** (Table 4, P. 30) in TFE c≅1mM with different cations have been recorded. Again as a representative example, the CD spectra of pentapeptide c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)** in complex with different cations are shown in Figure 17. Calcium ions in a molar ratio peptide : Ca^{2+} = 1 : 1 or peptide : Ca^{2+} = 1 : 2 have the strongest influence on conformation of the peptide according to the CD spectra.
Complexation with Mg^{2+}(brown curve) results in small changes of the CD curve. The small and broad positive band near 220 nm is shifted to 215 nm, but there are

no changes in the positive band near 195 nm. This becomes more prominent with Ca^{2+} ions. Both spectra show a broad positive band near 213 nm (red and green curves), with much higher intensity than for pentapeptide alone **(36)** (blue curve) in the same solvent. The similarity of the spectra with peptide : Ca^{2+} ration of 1:1 and 1:2 lead to the conclusion that a 1:1 complex is formed. Sodium has not very much influence on the CD curve of this cyclic peptide.

Very similar results are obtained in the cation binding studies of cyclic hexapeptide **(34)** (Figure 18).

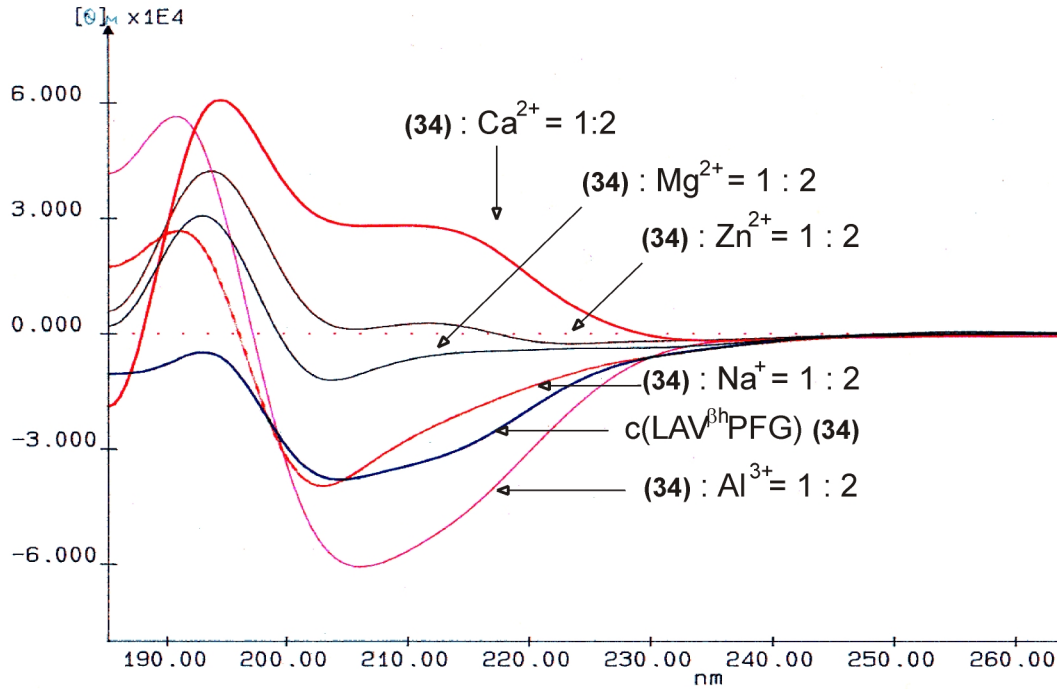


Figure 18: CD spectra of c-(-Leu-Ala-Val-β-hPro-Phe-Gly-) (34) in the presence of different cations in TFE.

Again the biggest influence on peptide conformation is observed in the experiment with Ca^{2+}, (red curve). Cation binding has no or relatively small influence on the band near 195 nm, which is shifted a little and has stronger intensity, but a new broad positive band between 210 and 220 nm is observed again. The negative band near 205 nm with shoulder near 213 nm in peptide **(34)** (blue curve) completely disappears or is covered by this new positive band. The same changes, but with smaller intensities are observed for all cations tested except Al^{3+}. CD spectra of complex of peptide **(34)** with this cation has same curve shape like hexapeptide **(34)** without cation but stronger intensity.

Analogs cation binding studies have been performed with the cyclic hexa- and pentapeptides **(33)** and **(35)**, giving very similar results that are shown in Figure 19 for c-(-Leu-Ala-Val-Pro-Phe-Gly-) **(36)**. Like before cation binding has no influence or has influence only on the band intensities for the negative band near 200 nm,

but the negative band near 220 nm completely disappears, or is covered with positive band on approximately same place. The similarity of the spectra with peptide : Ca^{2+} ration of 1:1 (red curve) and 1:2 (green curve) lead to the conclusion that a 1:1 complex is formed. The 1:1 complex is also formed with Mg^{2+} ions. Contrary with Zn^{2+} ions 1:2 complex is formed.

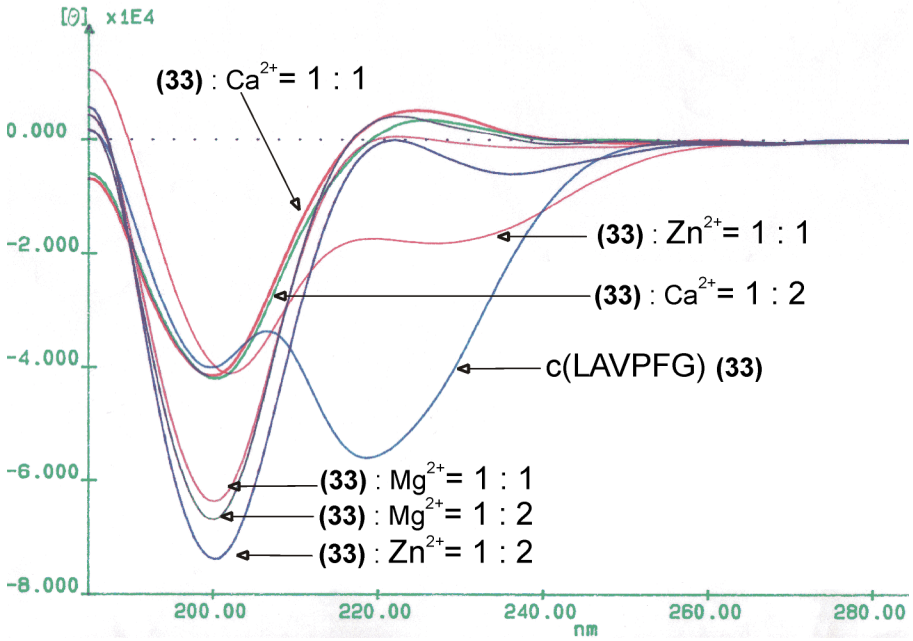


Figure 19: CD spectra of c-(-Leu-Ala-Val-Pro-Phe-Gly-) (33) in the presence of different cations in TFE.

Similar results have been obtained for all peptides examined but conclusions on the conformational properties of the cyclic peptides in complex with Ca^{2+} or other cations have to rely on NMR experiments. Nevertheless, some general observations have been also obtained with CD. Using this technique it has been shown that cation binding, especially of Ca^{2+}, has influence on conformation of the peptide in solution. Changes in conformation for all of these peptides are similar although these peptides have different conformations without cations.

4.3. CYCLIC ANALOGS OF VCAM-1 BINDING EPITOPE

The interaction between an integrin (family of heterodimeric cell surface receptors) and their protein ligands are fundamental for maintaining cell function, for example by tethering cells to a particular location, facilitating cell migration or by providing survival signals to cells from their environment.[100] Several different integrins have been implicated in disease processes and have attracted widespread interest as potential targets for drug discovery.

The integrin $\alpha_4\beta_1$ is expressed primarily on human monocytes, lymphocytes, eosinophils, basophils and macrophages.[48] The primary ligands for the $\alpha_4\beta_1$ integrin are the endothelial surface protein vascular cell adhesion molecule-1 (VCAM-1), and an alternatively spliced form of the extracellular matrix protein fibronectin containing the type III connecting segment (CS-1 fibronectin).[101] Binding of $\alpha_4\beta_1$ to VCAM-1 plays a key role in extravasations of the leukocyte into the inflamed tissue, stem cell adhesion to bone marrow stromal cells and may also be involved in tumor cell metastasis.[51,52] Monoclonal antibodies directed against $\alpha_4\beta_1$ or VCAM-1 have been shown in animal models to be effective modulators of chronic inflammatory diseases such as asthma, rheumatoid arthritis, and inflammatory bowel disease.[102] Antagonists of $\alpha_4\beta_1$ might therefore be useful for treatment of a number of inflammatory and autoimmune diseases, for mobilization of bone marrow stem cells, or even find application as antitumor agents.[103]

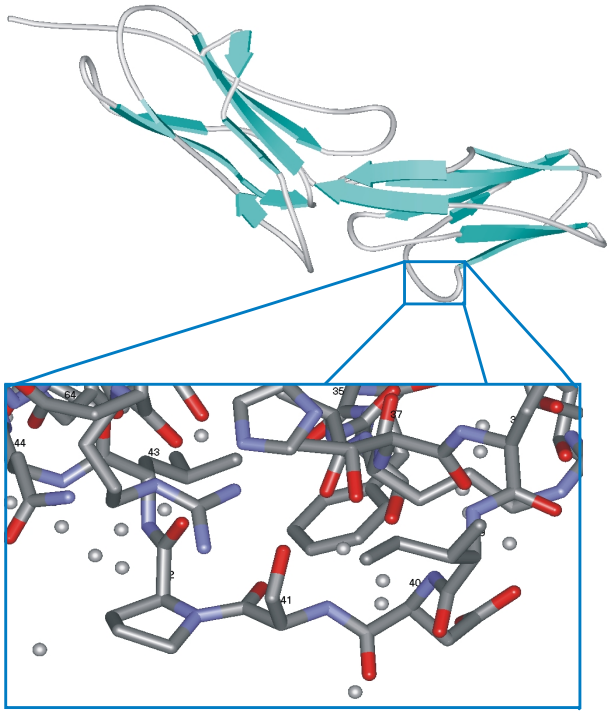


Figure 20: Recognition motif of VCAM-1.[54a]

Cyclic peptide inhibitors containing RGD or LDV sequences were shown to block binding of fibronectin to $\alpha_4\beta_1$ and $\alpha_5\beta_1$, respectively, at low micromolar concentrations.[104,105] Indeed, many integrin ligands including fibrinogen, vitronectin, and collagen display similar sequences in their binding sites.

Compounds containing such motifs will probably become useful therapeutic agents.

The integrin binding sites of VCAM-1 are thought to reside in the first and fourth domains of the seven-domain protein.[106] The crystal structure of domains 1 and 2 of VCAM, solved by two independent groups,[54] propose the sequence RTQIDSPLN in a surface-exposed loop connecting two β-strands (the CD-loop) as integrin binding-epitope of VCAM-1 (Figure **20**). By utilizing functional groups similar to those found in the binding epitope of VCAM-1, it has been found that simple replacement of arginine by tyrosine in the parent cyclic peptide c-(-Arg-Cys--Asp-Pro-Cys-) leads to nearly a 1000-fold enhancement in binding affinity for $\alpha_4\beta_1$.[107]

Head to tail cyclic peptides, important targets in peptide synthesis over decades, have attracted considerable interest in recent years.[108] Constraining highly flexible linear peptides by cyclization induces or stabilizes the conformation of the peptide and at the same time increases their resistance to enzymatic degradation.[109]

The aim of this work was to synthesize constrained cyclic peptides (Table 9) as mimics of the binding epitope of VCAM-1, which could bind to the integrin with higher affinity and selectivity and at the same time be resistant to enzymatic degradation. For that reasons D-and β-homoamino acids as a strong conformational inducers have also been incorporated in the cyclic peptides.[69,82]

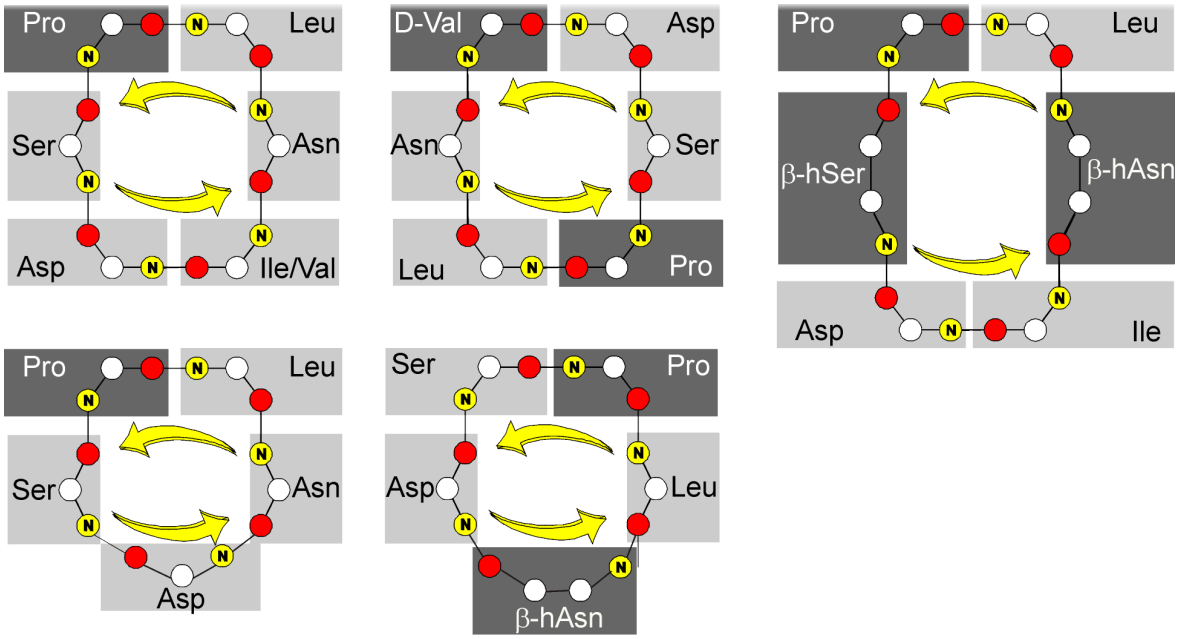


Figure 21: Design principle for the C-terminal analogs of VCAM-1 binding epitope.

Both proline and D-amino acids induce a β-turn in cyclic peptides and have the strong conformational preference to i+1 position in the β-turn. Contrary β-amino acids induce a pseudo-γ-turn and occupy the central position of the turn (see

chapter 4.2.2, P. 32). Using different combinations of these amino acids various spatial orientation of the pharmacophoric group could be achieved. An example of such spatial screening is schematic presented in Figure 21 for C-terminal analogs of the VCAM-1 binding epitope.

Table 9: Cyclic analogs of VCAM-1 binding epitope.

Analogs of the C-terminal region	Analogs of the N-terminal region	Analogs of the central part
c-(-Ile-Asp-Ser-Pro-Leu-Asn-) **(39)**	c-(-Thr-Gln-Ile-Asp-Ser-Pro-) **(49)**	c-(-Gln-Ile-Asp-Ser-Pro-) **(55)**
c-(-Ile-Asp-Ser-β-hPro-Leu-Asn-) **(40)**	c-(-Thr-Gln-Ile-Asp-Ser-β-hPro-) **(50)**	c-(-Gln-Ile-Asp-Ser-β-hPro-) **(56)**
c-(-Ile-Asp-β-hSer-Pro-Leu-β-hAsn-) **(41)**	c-(-Thr-β-hGln-Ile-Asp-β-hSer-Pro-) **(51)**	c-(-Gln-Leu-Asp-Ser-Pro-) **(57)**
c-(-Val-Asp-Ser-Pro-Leu-Asn-) **(42)**	c-(-Thr-Gln-Val-Asp-Ser-Pro-) **(52)**	c-(-Ile-Asp-Ser-β-hPro-Leu-) **(58)**
c-(-D-Val-Asp-Ser-Pro-Leu-Asn-) **(43)**	c-(-Thr-Gln-Val-Asp-Ser-β-hPro-) **(53)**	
c-(-Val-Asp-Ser-β-hPro-Leu-Asn-) **(44)**	c-(-Thr-Gln-D-Val-Asp-Ser-Pro-) **(54)**	
c-(-D-Val-Asp-Ser-β-hPro-Leu-Asn-) **(45)**		
c(-Asp-Ser-Pro-Leu-Asn-) **(46)**		
c-(-Asp-Ser-Pro-Leu-β-hAsn-) **(47)**		
c-(-β-hAsp-Pro-Leu-Asn-) **(48)**		

Among many other methods,[108] on-resin cyclization is a promising method for cyclization of linear peptides. As compared with solution phase procedures, on-resin cyclization has the following advantages:

a) Coupling reagents can be separated from the resin-bound peptide by simple filtration.

b) Excess of coupling reagents can be used to ensure faster ring closure, which minimizes epimerization.

c) Pseudo–dilution minimizes dimerization[110].

Among the three strategies for on-resin cyclization, side chain-to side-chain, side-chain to backbone, and head-to tail, the latter has been investigated extensively. Two distinct protocols are employed in the solid phase synthesis. One extensive studied approach is the on-resin cyclization of a peptide anchored via a side chain functional group such as imidazole,[111] carboxylic acid,[112] amine[113] or alcohol[114]

which commonly is distinguished from the cyclization-cleavage approach, in which the peptide is cyclized with simultaneous cleavage from the resin.[115] One major advantage of the latter method is that the side chain does not need to be anchored, making the approach more general than the first one. The published methods used in the latter approach employ either the Kaiser oxime resin[115a-c] with a Boc/benzyl-based protocol or thioester resin[115d, 116] employing both Fmoc- and Boc/benzyl-based protocols for the synthesis of the linear peptides. The first approach for on-resin synthesis of head to tail cyclic peptides comprises (I) attachment of orthogonally protected amino acid on resin, (II) stepwise solid phase synthesis of the linear peptide, (III) orthogonal deprotection to liberate selectively the C-terminal carboxy function, (IV) activation of the C-terminal carboxy group and its condensation with the free amino group at the N-terminus and finally (V) deprotection and cleavage to release the required free cyclic peptide into solution.[117]

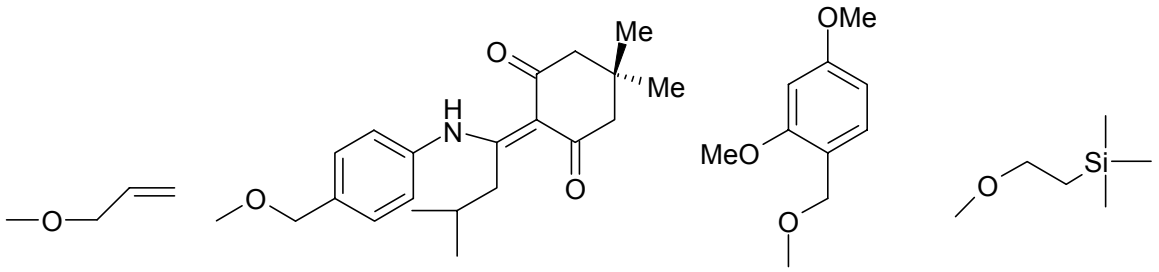
Depending on the synthetic goal, the anchoring bond should be compatible to most common peptide synthesis tactics, (e.g. Fmoc) and orthogonal to the C-terminal protecting group. The latter is to be cleaved selectively under mild conditions at the end of the peptide chain assembly prior to the cyclization step. The anchoring bond, e.g. for trifunctional amino acids present in binding epitope of VCAM-1 can be an ester (Asp, Glu, Ser, Thr), amide (Asn, Gln) or mixed carbonate (Ser, Thr).

These requirements in turn suggest that a trifunctional amino acid can be attached directly to a polystyrene resin (hydroxymethyl resin, aminomethyl resin) or to a modified or activated polystyrene resin.

4.3.1. Orthogonally Protected Aspartic and Glutamic Acid

It is obvious that orthogonally protected aspartic or glutamic acids are essential for this kind of head to tail cyclization on resin. There are several possibilities for orthogonal protection of the C^{α} carboxy function of aspartic or glutamic acid. The most widely used protective groups for the α-carboxy function of these amino acids are presented in Figure 22.

a) Allyl ester (Figure 22a), cleaved with $[Pd(PPh_3)_4]$ in different mixtures of solvents and in the presence of a nucleophile (morpholine, dimedone, N-methyl aniline), the role of which is to scavenge the allyl group released during deprotection.[118]
b) Dmab ester (Figure 22b), cleaved with 2 % hydrazine in DMF.[119]
c) Dmb ester (Figure 22c), cleaved with 1 % TFA in DCM.[112]
d) Trimethylsilylethyl ester, (Figure 22d) cleaved with fluoride ion.[120]

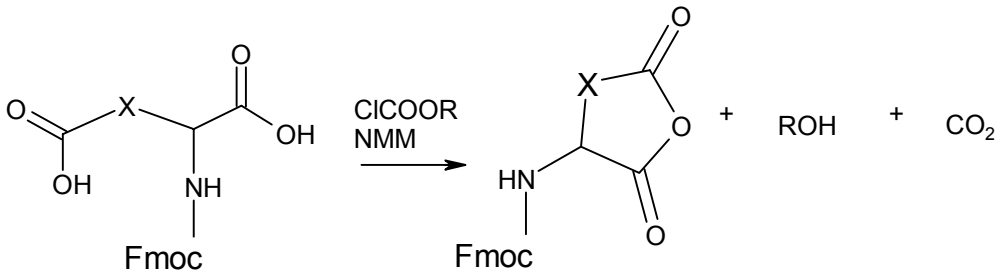


(a) **(b)** **(c)** **(d)**

Figure 22: Orthogonal protective groups for α-carboxy function.

There are also other examples of orthogonal protection for these and other amino acids.[121] Allyl protection is an excellent approach but often yields significant amounts of by-products and/or low yields. Side reactions and low yield is also reported for the Dmab protection strategy.[122] For these reasons Dmb esters of aspartic and glutamic acid have been chosen as protection tactics for cyclization on resin.

In the first step of the synthesis Fmoc protected aspartic **(59)** or glutamic **(60)** acid is converted to the corresponding acid anhydride **(61)** and **(62)**.[123]



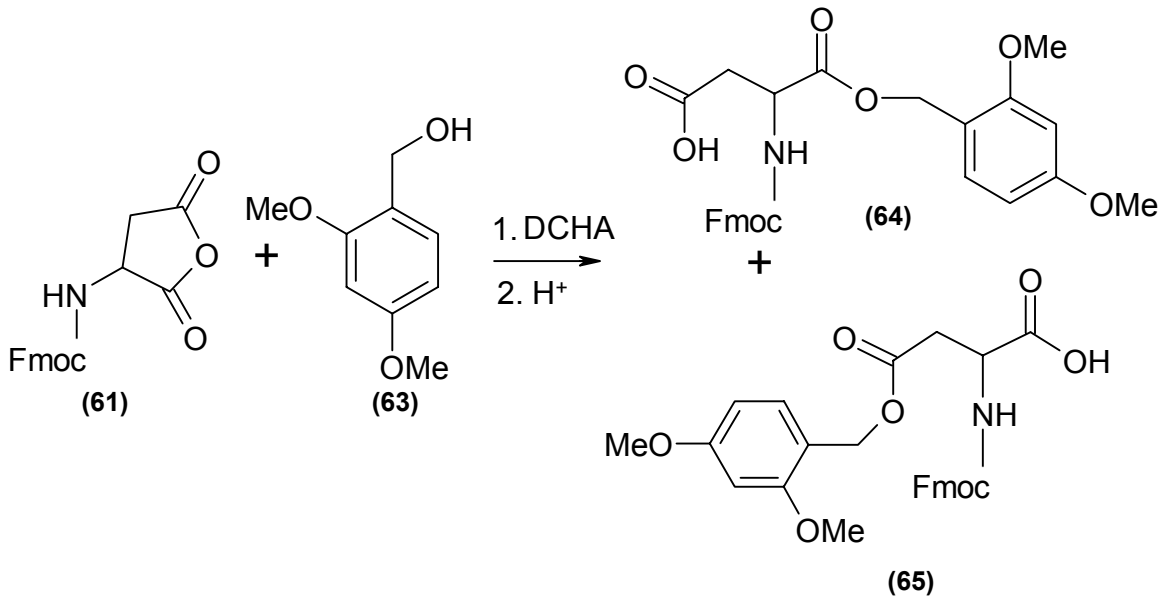
(59) X = CH_2 **(61) X = CH_2**

(60) X = $(CH_2)_2$ **(62) X = $(CH_2)_2$**

Scheme 11: Synthesis of anhydrides.

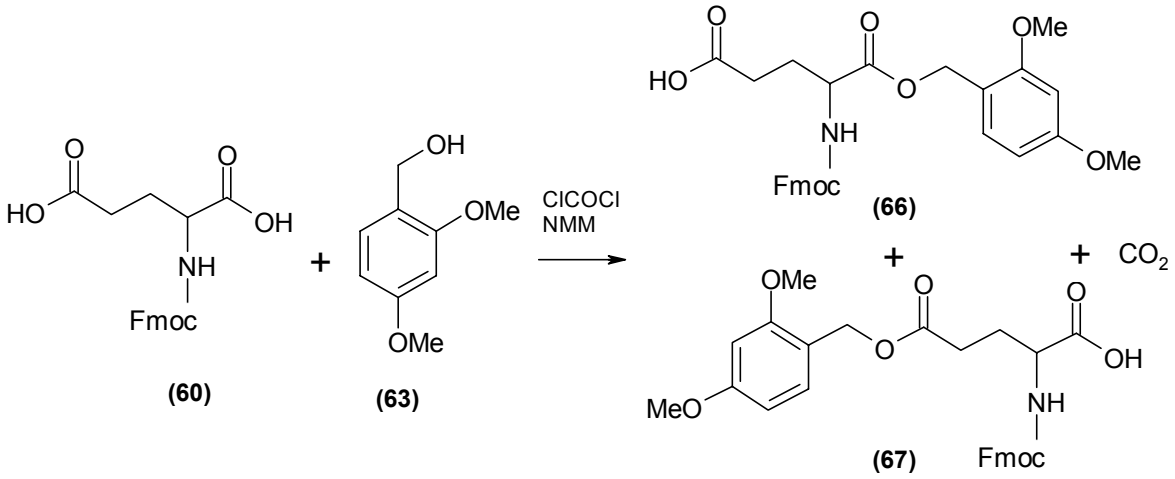
By this method both anhydrides **(61)** and **(62)** are synthesized with good yields (87 % starting from Fmoc-Asp-OH **(61)** and 94 % starting from Fmoc-Glu-OH **(62)**). In this process the main by-product is an ester formed by nuclephilic attack of the alcohol, which is the chloroformate hydrolysis product, on cyclic anhydride. This can be suppressed by using isopropenyl chloroformate as activating reagent, which upon hydrolysis produces acetone.[124]

Fmoc-Asp-ODmb **(64)** is prepared by addition of an etheral solution of DCHA to a solution of Fmoc-aspartic acid anhydride **(61)** and 2,4-dimethoxybenzyl alcohol **(63)** in DCM.[125]



Scheme 12: Synthesis of Fmoc-Asp-ODmb.

The α-carboxy group is rendered more electrophilic by the electron-withdrawing amino group, allowing its selective esterification. A mixture of α- **(64)** and β- **(65)** isomers is obtained, which can be separated by flash chromatography. The α/β ratio depends on the solvent and the substrate used. The best results (α/β ratio 25 according to HPLC) for Fmoc protected aspartic acid **(59)** are obtained with a mixture of THF and diethyl ether. Because of the acid sensitivity of the Dmb group, the conditions for liberating the carboxylic acid from the dicyclohexyl ammonium salt may be the reason for the lower yield (33 %).

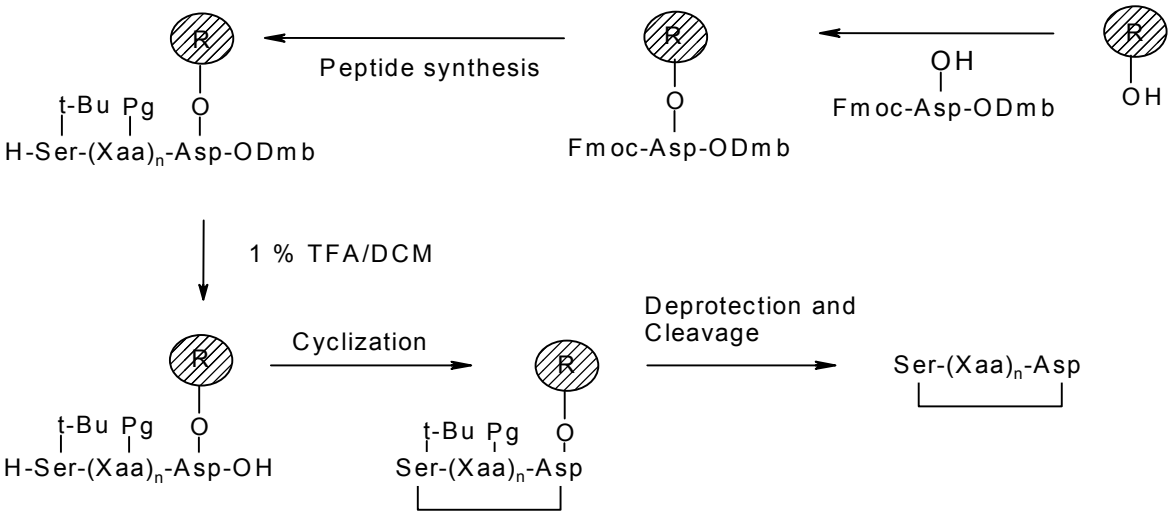


Scheme 13: Synthesis of Fmoc-Glu-ODmb.

The synthesis of Fmoc-Glu-ODmb **(66)** by this protocol proceeds with low yield and bad regioselectivity. Fmoc-Glu-ODmb **(66)** is synthesized *in situ* mixing Fmoc protected glutamic acid **(60)** with one equivavelnt of phosgene, one equvivalent of

2,4-dimethoxybenzyl alcohol **(63)** and two equivalents of a base (N-methylmorpholine). Two isomers, α **(66)** and γ **(67)**, can be separated with flash chromatography. The best α/γ isomeric ratio (α/γ ratio is 14) for this reaction is obtained in DCM as solvent. The structure of the isomers has been elucidated by ^{1}H-NMR spectroscopy. According to the literature, benzyl protons of α isomers should be shifted downfield compared to the corresponding γ isomer.[123] The benzyl protons of the α isomer **(66)** are found as two doublets at 5.09 ppm and 5.19 ppm with coupling constants of 11.9 Hz while the benzyl protons of the γ isomer **(67)** are found as a singlet at 4.99 ppm.

4.3.2. On Resin Cyclization



Scheme 14: On resin cyclization according to McMurray.

Asp and Ser residues are essential for the binding affinity of the peptides and are both present in all planned peptides (Table 9, P. 47). It is also known that bonds between Asp and Ser residues are very often prone to base or acid catalyzed hydrolysis and other side reactions. For these reasons it was decided to use the McMurray approach for the synthesis of cyclic constrained analogs of the binding epitope of VCAM-1, peptides **(39)** - **(58)**. In this approach aspartic acid, with the α-carboxy function being orthogonally protected by the Dmb group, is attached to the resin using the β-carboxy group. The linear peptides are assembled using Fmoc chemistry. The last amino acid residue to be connected is serine in the TQIDSP series. The α-carboxy function of aspartic acid is deblocked with 1 % TFA in DCM and peptides are cyclized on the resin. Although the original procedure

suggests that yields and purity of the product are better if the amino function is deprotected after α-carboxy function, no difference has been observed in this work. Finally the cyclic peptides are completely deprotected and simultaneously cleaved from the resin.

Anchoring of the first amino acid on Wang resin:

Orthogonally protected Asp is attached with its β-carboxy group on Wang resin using DMAP-catalyzed coupling with carbodiimide. To achieve optimal conditions for cyclization the loading of the resin should range between 0.5 and 0.7 mmol/g resin. Alternatively this amino acid could be attached to the Rink amide resin, which, after cleavage with concentrated TFA, gives an amide functionality (Asn, Gln).[126]

Assembly of linear peptides:

Linear peptides are assembled in an automated peptide synthesizer by a standard protocol (Table 30, P. 114).[127] Fmoc is used as temporary protective group for the amino function. Permanent protective groups are used as follows:
t-butyl ethers for the hydroxy function of serine and threonine, trityl for the amide function of asparagine and glutamine. These protective groups are stable to basic conditions used for Fmoc-deprotection and to acidic conditions (1 % TFA/DCM) used for Dmb deprotection, and they are easily cleaved under strongly acidic conditions used for cleavage of the peptides from the resin. All attempts to synthesize the target peptides without protection on the amide functional group of asparagine and glutamine failed.
Onium salts based on 1-hydroxybenzotriazole (HOBt) and 1-hydroxy-7--azabenzotriazole (HOAt), (Figure 23, P. 53) are widely used as coupling reagents in peptide synthesis because of their high efficiency and low racemization rate. Their aza derivates are even more reactive than the parent benzotriazole derivatives in both activation and coupling. [128]
TBTU in the presence of base (DIPEA) in DMF was used as a relatively cheap, commercially available and efficient coupling reagent. The solvent can enhance coupling efficiency and shorten coupling time.[129] DMSO, NMP and DMF display best characteristics. Although DMSO very often is superior regarding acylation yield and reaction rate, DMF has been chosen because of the much lower price. Coupling and/or deprotection have been repeated twice or the coupling time was

prolonged if necessary. Fmoc deprotection is achieved with a mixture of 2 % DBU, 2 % piperidine in DMF. Additional quantities (0.1 mmol/l) of HOBt are added to the deprotection mixture because this can suppress base or acid catalyzed side reactions like aspartimide formation for Asn and Asp residues,[130] pyroglutamyl and glutarimide formation for Glu and Gln residues[131] and asparagine or glutamine deamidation.[132] Peptides are especially prone to base catalyzed aspartimide formation as a side reaction when an Asp residue follows Asn or Gly residues. The partial sequence -Asp-Ser- is susceptible to acid catalyzed aspartimide formation.[130c]

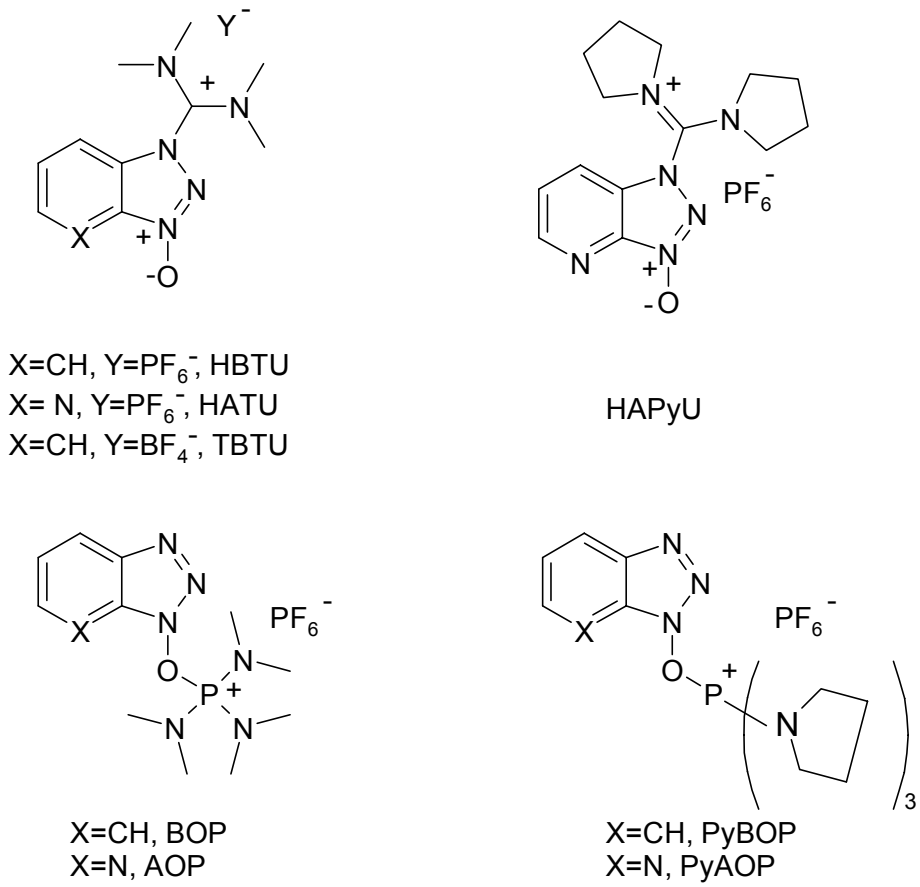


Figure 23: Highly efficient coupling reagents.

Small samples of the peptides are cleaved from the resin, completely deprotected and analyzed with analytical HPLC and MALDI-ToF MS to monitor the reaction progress. Although, in MALDI-ToF MS the desired linear peptides are detected, HPLC chromatograms showed a mixture of compounds, which consists of desired peptides, by-products of synthesis, cleavage products of protective groups and scavengers. From these chromatograms it is not possible with certainty to determine the yield and purities of the linear peptides. For the cyclization in solution linear protected peptides with the same amino acid residues, but different

order of amino acids, are synthesized using the same protocol on the 2-ClTrt resin. After cleavage and purification linear peptides are isolated with varying yields of 50-90 % depending on amino acid sequence.

Cyclization:

The first series of on-resin cyclizations was done with 3.0 equiv of HATU (relative to resin loading) as a coupling reagent, and 6.0 equiv of DIPEA (relative to resin loading) as a base in DMF during 45 min at room temperature. After the reaction, small samples have been cleaved from resin and analyzed with MALDI-ToF MS and analytical HPLC. The cyclization was repeated until no more starting linear peptide is present in the mixture. The peptide was completely deprotected and cleaved from the resin with cleavage reagents TFA/TIS/H_2O = 95 : 2.5 : 2.5.
Analytical HPLC chromatograms of the crude products showed a mixture of compounds from which the isolation of the desired cyclic peptides by reverse phase preparative HPLC was a laborious task. Results are given in Table 10.
Overall yields are calculated for isolated pure cyclic peptides referring to the first amino acid anchored on the resin. Because the complete peptide synthesis, cyclization and cleavage consist of 14 chemical synthesis steps (for hexapeptides) or 12 steps (for pentapeptides) high overall yields are not to be expected (if every step for a cyclic hexapeptide proceeds with a yield of 90 % the calculated overall yield is 0.9^{14}x100 = 23 %). The critical step in cyclic peptide synthesis is the cyclization of the linear precursor. Although ring closure of hexa- and pentapeptides sometimes is hampered, the ease of cyclization is often enhanced by the presence of turn structure-inducing amino acids such as glycine, proline, or D-amino acids.[133] Obviously, the success in cyclization depends on the propensity of the linear precursor to adopt a conformation similar to the transition state required for cyclization.[134] For the linear peptides, which do not contain amino acid residues that stabilize turn structures, the cyclization reaction may be an inherently improbable or slow process, and side reactions, such as dimerization, may dominate even at high dilutions. For such slow cyclizations the increased lifetime of the intermediate activated linear peptide provides an opportunity for increased epimerization at the C-terminal residue. Some amino acids are prone to side reactions (Asp, Asn, Gln) and coupling and deprotection of sterically hindered (Thr, Ile, Val) proceeds with lower efficiency. Also amide bond hydrolysis of some combinations of amino acids (-Asp-Ser-) represents an additional problem.

Table 10: Overall yields for the on-resin cyclization.

Resin			Peptide	Yield[a]		
	mg	mmol		mg	μmol	%
Wang	400	0.20	c-(-Ile-Asp-Ser-Pro-Leu-Asn-) **(39)**	6	9	4
Rink amide	100	0.06		-	-	-
Wang	300	0.20	c-(-Ile-Asp-Ser-β-hPro-Leu-Asn-) **(40)**	1	2	1
Rink amide	170	0.10		-	-	-
Wang	170	0.09	c-(-Ile-Asp-β-hSer-Pro-Leu-β-hAsn-) **(41)**	3	4	4
Wang	400	0.20	c-(-D-Val-Asp-Ser-Pro-Leu-Asn-) **(43)**	9	14	7
Wang	330	0.20	c-(-D-Val-Asp-Ser-β-hPro-Leu-Asn-) **(45)**	13	30	15
Wang	400	0.20	c-(-Val-Asp-Ser-β-hPro-Leu-Asn-) **(44)**	10	16	8
Wang	500	0.20	c-(-Thr-Gln-Ile-Asp-Ser-Pro-) **(49)**	10	15	8
Wang	400	0.20	c-(-Thr-Gln-Ile-Asp-Ser-β-hPro-) **(50)**	6	8	4
Wang	200	0.09	c-(-Thr-β-hGln-Ile-Asp-β-hSer-Pro-) **(51)**	3	5	5
Wang	330	0.20	c-(-Thr-Gln-Val-Asp-Ser-β-hPro-) **(53)**	10	16	8
Wang	330	0.20	c-(-Thr-Gln-Val-Asp-Ser-Pro-) **(52)**	6	10	5
Wang	400	0.20	c-(-Thr-Gln-D-Val-Asp-Ser-Pro-) **(54)**	9	14	7
Wang	130	0.10	c-(-Gln-Ile-Asp-Ser-Pro-) **(55)**	3	6	6
Wang	125	0.05	c-(-Gln-Ile-Asp-Ser-β-hPro-) **(56)**	4	7	14
Wang	130	0.10	c-(-Asp-Ser-Pro-Leu-Asn-) **(46)**	12	23	23

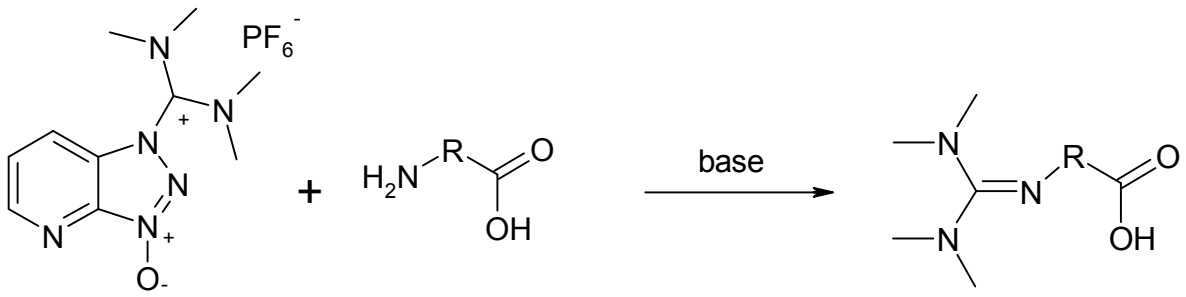
[a] Overall yields are calculated on isolated pure products for all synthesis from loading of the first amino acid (Fmoc-Asp-ODmb) on the resin.

The largest problem is the profile of by-products, mismatch sequences, and core sequences accumulated during synthesis. These by-products have very similar

retention times in reverse phase HPLC like the desired cyclic peptides, which represents an additional problem and lowers the overall yields.

Efforts are made to identify the by-products and to improve of the cyclization efficiency. Analysis of mass spectra obtained from MALDI-ToF MS of reaction mixtures gave some insight into by-products formed during the synthesis. In almost all cases dimeric and cyclic dimeric products were found in the mixture despite the high dilution effects of the low resin loading. This can be explained by a too high local concentration of the amino acid on the resin because of unequal distribution of the linker on the resin, different size of the resin pores and bad mixing conditions during anchoring of the first amino acid on the resin. Using such an inappropriate resin, formation of dimers and cyclodimers cannot be avoided during peptide synthesis.

One more by-product very often identified in mass spectra of the reaction mixtures is characterized by a mass difference of +116 compared to the cyclic peptide (and + 98 compared to the linear peptide). This by-product **(68)** occurs because of guanylation of the free N-terminus of the linear peptide (Scheme 15).[135] Such side reactions are not prevalent during the solid-phase coupling of single amino acids, since the activation step is fast and the coupling reagent is rapidly consumed by reaction with the carboxy group of the amino acid.

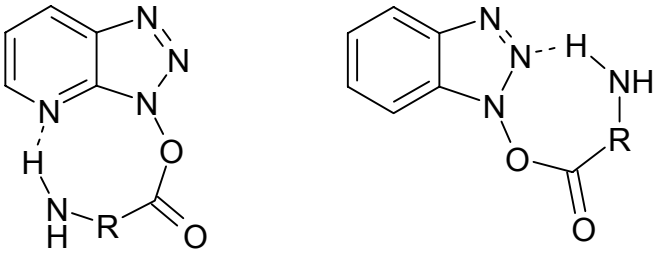


HATU Linear peptide **(68)**

Scheme 15: N-terminal guanylation.

However, because of the much slower activation of hindered amino acids and the slower cyclization, protected peptide segments or carboxylic acids involved in cyclization steps, the coupling reagent may undergo reaction with the amino component. Coupling reagents used in cyclization are in large excess to the linear peptide (3.0 equiv relative to the resin loading, and 4.0 equiv assuming that the linear peptide is synthesized with an approximate yield of 70 %). Therefore, it is not completely consumed by activation of the carboxy function of the linear peptide. Additives such as HOAt and HOBt can suppress side reactions, improve

cyclization yields and suppress racemization forming ring transition states **(69)** and **(70)** (Figure 24).[136] The better effects of HOAt over HOBt are explained with the electron withdrawing properties of the pyridine nitrogen atom, than provide for a better leaving group and thus increased reactivity for the derived O-acyl ester.



(69) **(70)**

R = peptide chain

Figure 24: Proposed transition states of HOAt and HOBt esters with linear peptide.

Indeed, cyclization of the linear peptide H-Ser(t-Bu)-Pro-Gln(Trt)-Ile-Asp(Wang)-OH **(116)** with a smaller excess of HATU (1.1 equiv relative to resin loading), and additional HOAt (2.0 equiv relative to resin loading) gives a better overall yield (Table 37, P. 157). However, the guanidino by-product **(68)** is again detected in the reaction mixture.

Newly developed coupling reagents like PyAOP[137] (Figure 23, P. 53) have also been employed in cyclizations (Table 31, P. 127 and Table 36, P. 144), although side reactions with these coupling reagents have also been reported.[138] Nevertheless, in the cyclization with this reagent the guanidino by-product is not observed, but overall yields are not significantly improved.

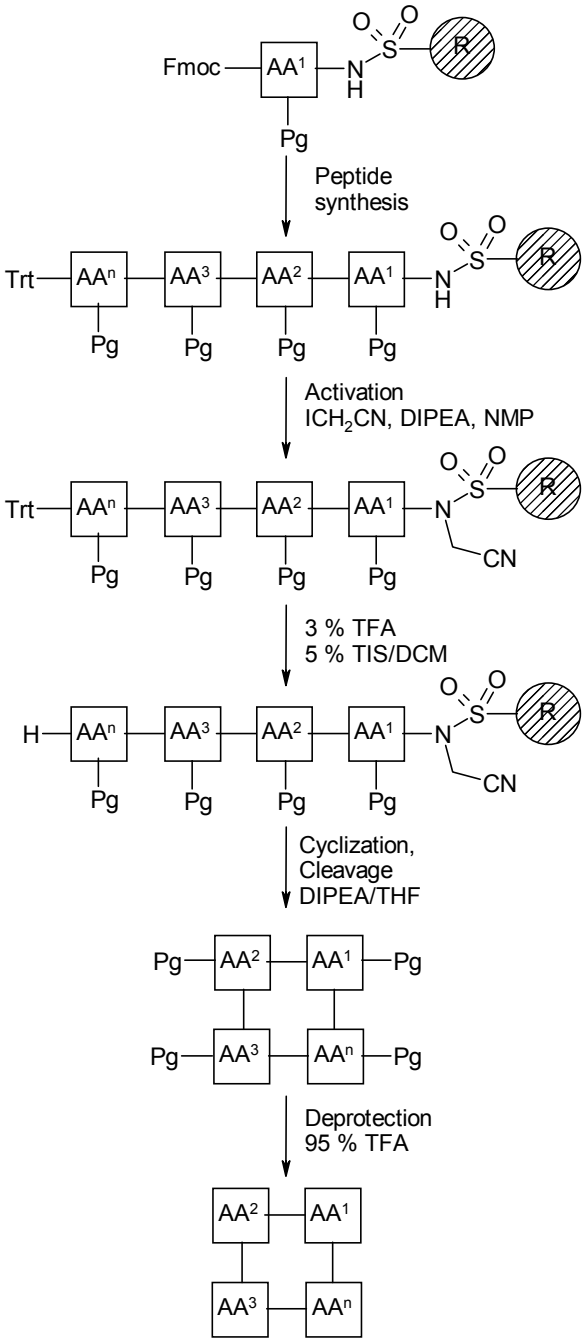
Another possible reason for this side reaction could be incomplete deprotection of the α-carboxy function of the linear peptide. This is also the most important disadvantage of this approach: there are no analytical methods to monitor the efficiency of Dmb-protective group hydrolysis. Nevertheless, experiments with prolonged reaction times or repeated deprotection of the α-carboxy function with 1 % TFA/DCM did not give better results.

The mechanism of the amino acid acylation with the phosphonium and uronium compounds (Scheme 5, P. 7) suggests that the role of the base is only an abstraction of a proton from the carboxy group of the amino acid. However, the nature of the base has an important influence on the reaction rate, yield and level of racemization. A lot of work have been done to find the best base,[139] but it seems to depend on the case and should be optimized for every peptide individually. However, the best results are obtained with DIPEA and/or collidine.

Finally cleavage of the cyclic peptides from the resin and deprotection presents also a source of different side reactions and by-products especially for peptides that contain the -Asp-Ser- sequence which undergoes acid catalyzed side reactions as has been mentioned before.
Several cleavage mixtures have been employed:
95 % TFA, 2.5 % H_2O, 2.5 % TIS,
(47-49) % TFA, (47-49) % DCM, (3-6)% TIS,
82.5 TFA, 5 % Phenol, 5 % H_2O, 5 % Thioanisol, 2.5 % EDT (reagent K),
88 % TFA, 5 % Phenol, 2 % TIS, 5 % H_2O (reagent B)
The appropriate cleavage reagent should also be chosen individually for every peptide.[140] Anyway, these cleavage mixtures are a good starting point for the optimization of cleavage and deprotection conditions.
Several cases have been reported in the literature in which cyclization yields depend strongly on the sequence of the linear peptide precursor.[137,141] Moreover, several linear precursors, all leading to the same cyclic product, often showed highly variable yields in the cyclization.[141b-e]
For taking advantages from this conformational effect two more strategies have been employed: on-resin cyclization-cleavage approach and cyclization in solution.

On-resin cyclization-cleavage approach:

In this approach (Scheme 16) the first amino acid is anchored on Kenner's "safety catch" sulfonamide linker, which is stable to nucleophilic attack, is compatible with Fmoc chemistry, but can be activated when required.[142]
The peptide sequence is synthesized following Fmoc chemistry. The last amino acid should not be Fmoc protected at the N-terminus before activation of the sulfonamide linker by cyanomethylation because the Fmoc protective group is unstable under this condition. In this work the trityl group is used for the protection of the last amino acid. This can be achieved by using a trityl-protected amino acid in the last step of the peptide synthesis or with two additional steps in the synthesis: Fmoc deprotection and introduction of trityl protection on the peptide N-terminus. After activation by cyanomethylation, the resin is treated with a solution of 3 % TFA and 5 % TIS in DCM to remove the trityl protecting group. Cyclization and cleavage by nucleophilic attack of the N-terminal amino group are accomplished by treatment with DIPEA in THF at room temperature. If necessary the cyclic fully protected peptide can be purified by reverse phase preparative HPLC. After that, the cyclic peptide is completely deprotected by treatment with an appropriate cleavage cocktail.



Scheme 16: On-resin cyclization-cleavage approach.

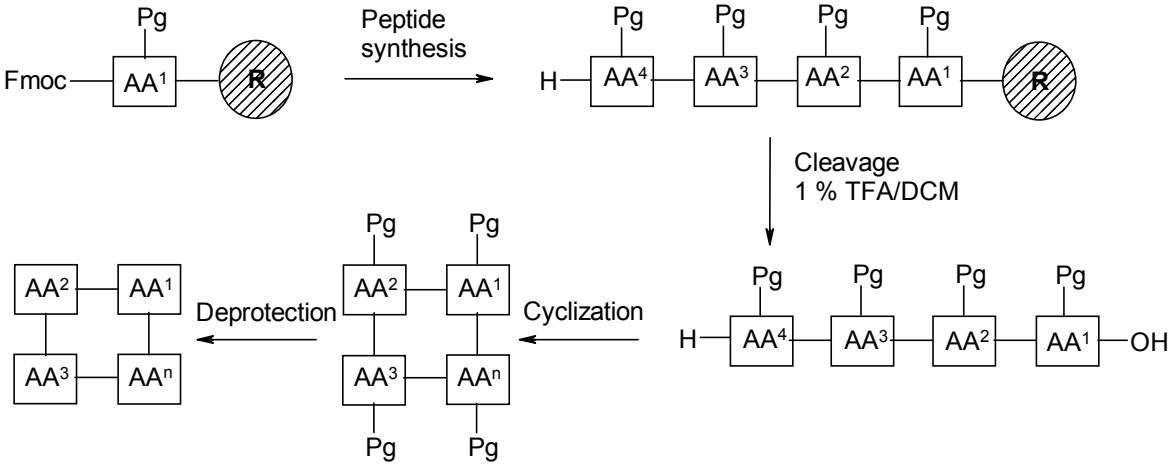
The anchoring of the first amino acid on the alkanesulfonamide “safety catch” linker proceeds smoothly and without racemization According to an optimized method[143] using PyBOP and DIPEA as coupling reagents in $CHCl_3$ at -20 ºC during 8 h. Nevertheless, the maximum loading with this method was 0.7 mmol/g and a repeated procedure did not give better loading. The rate of loading and racemization are strongly dependent on the temperature and time of the reaction. Only few syntheses have been tried with this strategy. Nevertheless, in all cases almost only dimeric and cyclodimeric products have been obtained, the wanted cyclic product was formed only in traces.

The disadvantage of this strategy is the relatively long procedure for cleavage of the product from the resin, so that analytical monitoring during the synthesis is time-consuming.

For these reasons it is decided to try the synthesis with more traditional cyclization in solution.

4.3.3. Cyclization in Solution

In this approach, the linear peptide is synthesized on 2-ClTrt resin or Sasrin and cleaved from the resin with 1 % TFA/DCM so that all side chain functional groups are still protected. The linear peptide is then cyclized in solution under high dilution conditions to minimize dimerization and finally completely deprotected (Scheme 17).



Scheme 17: Cyclization in solution.

For this strategy it is essential that the peptide is cleaved from the resin without concomitant cleavage of the protective groups from the amino acid side chain functional groups. 2-Cl trityl resin or Sasrin is chosen, because it is possible to cleave the peptide from these resins with 1 % TFA/DCM while under this condition all protective groups used (t-butyl for serine and threonine[144], t-butoxy for aspartic acid[145], trityl for asparagine and glutamine[146]) are stable.[147] The chloro trityl resin has the advantage over Sasrin that it, because of its bulkiness, can suppress diketopiperazine formation during assembly of the first three amino acid residues. Moreover, relatively fast esterification of the first Fmoc-protected amino acid residue on this resin proceeds without enantiomerization and dipeptide formation.[148]

4.3.3.1. Cyclic Analogs of the C-terminal VCAM-1 Binding Epitope

c-(-Ile-Asp-Ser-Pro-Leu-Asn-) **(39)** was used as a test peptide for the synthesis of this group of cyclic peptides. The linear protected precursor H-Asn(Trt)-Ile-Asp(Ot--Bu)-Ser(t-Bu)-Pro-Leu-OH **(72)** of this peptide is synthesized on Sasrin so that Leu is the first amino acid anchored on the resin and the C-terminal residue of the linear peptide for the following reasons:

a) Isoleucine should be avoided at the C- or N- terminus of the peptide chain, because this amino acid is sterically hindered and represents a problem for cyclization.
b) Proline is in a position in the peptide chain where, by cis-trans isomerization, it can introduce a proper conformation for cyclization.[149]
c) The number of basic Fmoc deprotection steps for the peptide, once aspartic acid and asparagine residues have been incorporated should be minimal, because that undergo different base catalyzed side reactions.

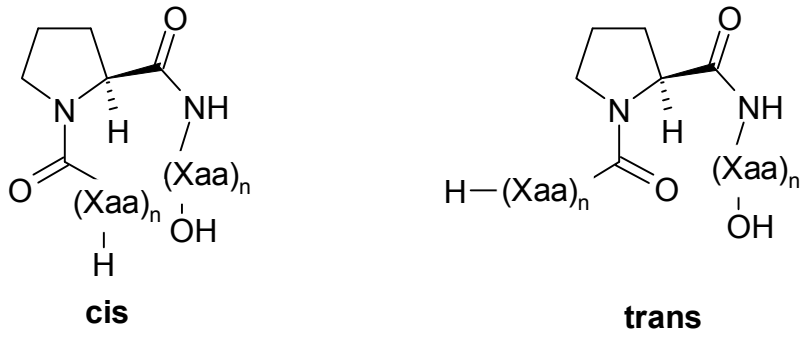


Figure 25: The influence of cis-trans isomerization of the -Xaa-Pro- amide bond on the cyclization.

After the synthesis of the linear protected peptide H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser-(t-Bu)-Pro-Leu-OH **(72)** it is cleaved from the resin with 1 % TFA in DCM, and the mixture is immediately neutralized with pyridine. For removal of the pyridinium salt of trifluoracetic acid from the linear protected peptide **(72)** a recently developed precipitation-extraction process is used.[150] The technique uses normal approaches of chromatography and solid-phase extraction sorbents with a precipitation or drying procedure so that the sorbent becomes a support matrix for thin-film deposition of the compounds of interest. Reverse phase C_{18} material is used. The purity of the linear peptide thus obtained was approximately 70 %, and the linear peptide is cyclized without further purification. Alternatively, the linear peptide could be purified by reverse phase preparative HPLC, but this is a time and solvent consuming process, that is not necessary at this stage of the synthesis.

The linear protected peptide **(72)** is cyclized under high dilution conditions using three different coupling reagents (Table 32, P. 128).

The best results are obtained with PyAOP and DIPEA as a coupling reagent, but the reaction was relatively slow. The reaction is monitored by MALDI-ToF MS every ½ h. Because the reaction was not completed after ½ h additional quantities of PyAOP are added to the reaction (PyAOP is stable in basic solution for approx. 30 min[128]). After evaporation of DMF, the cyclic fully protected peptide is purified by reverse phase preparative HPLC. The cyclic dimer and the unreacted linear peptide are isolated as by-products in this reaction. With DIC and HOAt as coupling reagents[151] only traces of product are detected after 24 h. HATU and DIPEA lead to lower yield than with PyAOP, but the reaction is faster. After ½ h no more starting compound is detected by MALDI-ToF MS (Table 32, P. 128). The cyclic dimer was present as a by-product but no epimerization or guanylation by-product is detected with analytical HPLC and MALDI-ToF MS. The full amount of the protected cyclic peptide **(73)** is completely deprotected using the cleavage mixture TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 3 h and finally purified by reverse phase preparative HPLC. The yield of this final deprotection was only 74 %. Sufficient quantities of high purity peptide needed for biological tests and conformational analysis are obtained with this methodology. The overall yield was again low (18 %), but much better compared to on-resin cyclization. Moreover, this methodology allows analysis and additional purification after every step. The linear protected peptide **(72)** was synthesized with a yield of 70 % and purity (HPLC) of 70 %. It is possible to purify the linear protected peptides before cyclization. The cyclization proceeds with moderate yield as expected, accompanied by dimerization despite the low peptide concentration. However, no epimerization has been noted. Surprisingly low yield (~70 %) is obtained in the final cleavage of all protective groups, which can be due to the low stability of these peptide under acidic conditions, and especially of the -Asp-Ser- amide bond.

The synthesis of the C-terminal analogs of the binding epitope of VCAM-1 (Table 9, P. 46) was performed an Sasrin or 2-ClTrt resin. In most cases Fmoc-protected leucine was used as the first amino acid anchored on the resin, but proline have also been used. Eight new linear peptides have been synthesized via this route. The results are presented in Table 11. From these results it is obvious that Sasrin has better characteristics for the synthesis of these linear peptides compared to 2-ClTrt resin. Peptides are obtained from Sasrin with yield of 60-75 % and good purity. With 2-ClTrt resin yields are much lower, but the purity of the peptides was also satisfactory. Only in one case (H-Asn(Trt)-Val-Asp(Ot-Bu)-Ser-(t-Bu)-Pro-Leu--OH **(80)**) the linear peptide had to be purified by reverse phase preparative HPLC before cyclization. Also by-products (mismatch sequences) have not been detected upon purification of the peptides (MALDI-ToF MS).

The problem is probably the instability of the 2-ClTrt resin after anchoring the first amino acid on the resin. Normally, resin is stored on 4 °C until peptide synthesis, after the loading the resin with the first amino acid, estimation of the level of loading and removing the Fmoc protective group.

Table 11: Synthesis of linear C-terminus analogs of the binding epitope of VCAM-1.

Linear peptide	Resin/ μmol	Yield		
		mg	μmol	%
H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(72)**	Sasrin 100	95	65	65
	2-ClTrt 200	213	173	86
H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Leu-OH **(75)**	Sasrin 200	134	120	60
H-β-hAsn(Trt)-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-Leu-OH **(78)**	Sasrin 200	180	140	70
H-Asn(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(80)**	2-ClTrt 200	90	72	36
H-Leu-Asn(Trt)-D-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(83)**	2-ClTrt 200	133	99	50
H-Leu-Asn(Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(88)**	2-ClTrt 200	101	90	45
H-β-hAsn Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(90)**	Sasrin 200	167	150	75
H-Asn(Trt)-β-hAsp(Ot-Bu)-Pro-Leu-OH **(92)**	2-ClTrt 200	129	137	69

[a] After purification by reverse phase preparative HPLC

In this series also peptides with proline at the C-terminus have been synthesized. Proline at the C-terminus is very important because cyclization of such peptides can be achieved without epimerization[152].

For the cyclization of these linear peptides an apparatus with two injection pumps was used. The linear peptide and the coupling reagent are separately slowly added to the solution of the base in DMF. The low rate of addition of the peptide and coupling reagent in solution is necessary to minimize intermolecular condensation that results in dimer and cyclic dimmer formation. The rate of addition of peptide and HATU in solution is calculated from Equation 1.

Equation 1.

$$v_m = \frac{v_a * 60 * n}{V}$$

v_m = rate of addition of peptide in solution in μmol/h

v_a = rate of addition of peptide solution to reaction mixture in ml/min.

n = molarity of the peptide in μmol

V = volume of peptide solution in ml

Dimerization can thus be completely suppressed and quantities of the solvent may be drastically reduced, but the reaction time is relatively long. Because of the previous results obtained in the cyclization of the test peptide c-(-Ile-Asp-Ser-Pro--Leu-Asn-) **(39)**, HATU was chosen as a coupling reagent and DIPEA as base. With HATU the reaction was faster than with PyAOP what is essential for using pumps for cyclization. HATU is also not stable in basic solution and must be separately added to the reaction mixture. In some cases, HOAt has been added to the reaction mixture because this substance makes the reaction faster and suppresses on this way epimerization and dimerization. This method for cyclization is new and should be optimized with regard to quantities of HATU, base, rate of addition and additives.

The results of cyclizations are presented in Table 12.

After the cyclization, the peptides have been purified by reverse phase preparative HPLC or by filtration through a C_{18} plug and all yields are calculated on pure isolated peptide. The rate of dimerization is inverse proportional to the addition rate of peptide to the reaction mixture, but for some peptides, like tetrapeptide c-(-β-hAsp-Pro-Leu-Asn-) **(92)**, it is not possible to avoid this side reaction. DMSO and NMP have not been used before as solvents for cyclization in solution because of the high boiling points of these solvents and the impossibility to isolate the cyclic peptide from large quantities of solvent. Using the advantageous method of cyclization with a dual pump (smaller solvent volumes) and purification by precipitation extraction method it is possible to use any solvent for cyclization. After the reaction, water is added to the mixture to precipitate the product. The mixture is then filtered through a small plug filled with C_{18} reverse phase material. The product on the plug is additionally washed with water and diethyl ether. After that, the product is eluted from the plug with acetonitrile, DCM or any other adequate solvent (powerful solvent mixtures TFE/DCM or HFIP/CHL, which proved to be useful for dissolving of sparingly-soluble protected peptides,[153] can also be used), which is subsequently evaporated and the product is additionally purified by reverse phase preparative HPLC if necessary.

The best yield (85 %) for cyclization of the linear peptide H-Asn(Trt)-Val-Asp-(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(80)** is obtained with this method, but epimerization has been observed.

Table 12: Cyclization of C-terminus analogs of the binding epitope of VCAM-1.

Linear peptide	n/ μmol	Coupl. reagent	Solvent	v/ μmol/h	Cyclic peptide	Yield		
						mg	μmol	%
(75)	120	HATU 3.0 equiv DIPEA 6.0 equiv	DMF	29	**(76)**	72	72	60
(78)	140	HATU 1.6 equiv DIPEA 3.0 equiv	DMF	11	**(79)**	78	76	54
(80)	72	HATU 1.0 equiv DIPEA 3.0 equiv HOAt 0.1 equiv	DMSO	9	**(81)**[a]	75	61	85
(83)	99	HATU 2.0 equiv DIPEA 6.0 equiv HOAt 3.0 equiv	DMF	4	**(84)**	59	60	61
(88)	90	HATU 1.5 equiv DIPEA 3.0 equiv HOAt 1.0 equiv	DMF	5	**(89)**	90	60	68
(90)	140	HATU 1.5 equiv DIPEA 3.0 equiv HOAt 1.0 equiv	DMF	19	**(91)**[a]	94	110	70
(92)	137	HATU 4.0 equiv DIPEA 8.5 equiv	DMF	8	**(93)**	51	48	35
(72)	173	HATU 1.2 equiv DIPEA 3.4 equiv HOAT 1.2 equiv	DMF	5	**(73)**	205	126	73

[a] Epimerization

An influence of the proline on the C-terminus of the peptide and conformation of the linear peptide on racemization is observed with next two peptides. Val residue is replaced with D-Val in peptide H-Leu-Asn(Trt)-D-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro--OH **(83)** and pentapeptide H-Leu-Asn(Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(88)** contains one amino acid less than analog **(80)**. In both cases cyclization proceeds without epimerization.

In the pentapeptide H-β-hAsn(Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(90)**, in which Asn is replaced by β-hAsn (β-hAsn is at the N-terminus of the peptide chain and

can not influence the conformation of the linear peptide), and Leu is on the C-terminus, epimerization is again observed.

Surprisingly the tetrapeptide H-Asn(Trt)-β-hAsp(Ot-Bu)-Pro-Leu-OH **(92)** can be cyclized without epimerization, probably due to location of the β-homoamino acid in central position of the peptide chain, where it can induce a pseudo-γ-turn and bring the peptide in a proper conformation for cyclization. In summary, quite good yields are obtained using this cyclization method for all synthesized peptides. After the optimization had been done, the synthesis of the c-(-Ile-Asp-Ser-Pro-Leu-Asn-) **(39)** is repeated using new cyclization method. The linear precursor **(72)** is cyclized without epimerization or dimerization with very good yield (73 %).

For the final deprotection of all protective groups, two different cocktails have been used. Results are presented in Table 13.

Table 13: Deprotection of cyclic analogs of the binding epitope (C-terminal part) of VCAM-1.

Cyclic protected peptide	n/ μmol	Cleavage cocktail	Time/ h	Cyclic deprotected peptide	Yield		
					mg	μmol	%
(73)	23	TFA 95 % H_2O 2.5 % TIS 2.5%	3	**(39)**	10	17	70
(73)	126	Reagent K	5	**(39)**	30	46	37
(76)	72	Reagent K	4	**(40)**	47	71	99
(79)	76	TFA 95 % H_2O 2.5 % TIS 2.5 %	1	**(41)**	33	49	65
(81)	61	Reagent K	5	**(42)**	29	47	77
(84)	60	Reagent K	5	**(43)**	32	51	85
(89)	60	Reagent K	5	**(46)**	79	60	100
(91)	110	TFA 95 % H_2O 2.5 % TIS 2.5%	2	**(47)**	25	46	44
(93)	48	Reagent K	5	**(48)**	16	28	58

Obviously some of these peptides are not stable in acidic medium, and the cleavage cocktail should be carefully chosen for every peptide individually.

All epimeric mixtures are successfully separated using reverse phase preparative HPLC (method 3) under high flow (25 ml/min) with a small gradient.

4.3.3.2. Cyclic Analogs of the N-terminal VCAM-1 Binding Epitope

Within this series of cyclic peptides, c-(-Thr-Gln-Ile-Asp-Ser-Pro-) **(49)** was taken as a model peptide for cyclization studies. This peptide contains two sterically hindered amino acids (Thr, Ile) that should be avoided as N- or C-terminal residues of the precursor linear peptide. This limits cyclization to the position between Ser and Pro or Asp and Ser. The latter combination is avoided because bad results have been obtained for on-resin cyclization of the same sequences. Nevertheless, some linear fully protected peptides are synthesized (Table 14).

Table 14: Synthesis of linear precursors of c-(-Thr-Gln-Ile-Asp-Ser-Pro-) (49).

Linear peptide	Resin/ μmol	Yield		
		mg	μmol	%
H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-OH **(95)**	200	209	119	58
H-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(97)**	200	-	-	-
H-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-OH **(98)**	200	91	68	34
H-Pro-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-OH **(99)**	200	-	-	-

All peptides were synthesized on 2-ClTrt resin and cleaved with 1 % TFA/DCM with immediate neutralization of the mixture with pyridine. Two peptides, H-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(97)** and H-Pro-Thr(t-Bu)--Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-OH **(99)** have not been separated from pyridinium trifluroacetate. Instead, cyclization was attempted with this mixture (Table 15). These cyclizations completely failed for peptide **(99)**, or proceeded with low yield for peptide **(97)**. Two others (linear protected peptides **(95)** and **(98)**) cyclized with improved but still relatively low yields (29 and 30 % respectively). Epimerization is also observed in both cases probably because the sterically hindered amino acids (Thr and Ile) are present on the N- or C-terminus of the linear precursors. Nevertheless, in both cases epimers can be separated by reverse phase preparative HPLC.

Table 15: Synthesis of c-(-Thr-Gln-Ile-Asp-Ser-Pro-) (49)

Linear peptide	n/ μmol	Coupling reagent	v/ μmol/h	Yield		
				mg	μmol	%
(95)	120	4.5 equiv HATU 10 equiv DIPEA	7	37	35	30
(97)	-[c]	2.0 equiv[a] HATU 3.0 equiv[a] DIPEA	-[c]	16 [b]	15	8
(98)	70	1.5 equiv HATU 3.0 equiv DIPEA	10	21	20	29
(99)	-[c]	2.0 equiv[a] HATU 3.0 equiv[a] DIPEA	-[c]	0	0	0

[a] Relative to resin loading. [b] Calculated from resin loading. [c] Precursor was not purified

Despite of these bad results, all peptides from this series are synthesized following the same methodology.

The linear precursor peptides were synthesized on 2-ClTrt resin (Table 16). This time good yields were obtained for all peptides, probably because the resin was used immediately after loading with the first amino acid. Two linear protected peptides, **(104)** and **(109)**, were not purified before cyclization.

Table 16: Synthesis of linear analogs of the N-terminal binding epitope of VCAM-1.

Linear peptide	μmol	Yield		
		mg	μmol	%
H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Thr(t-Bu)-Gln(Trt)-OH **(101)**	200	190	147	74
H-β-hGln-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-Thr(t-Bu)-OH **(104)**	100	-	-	-
H-Thr(t-Bu)-β-hGln-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-OH **(106)**	200	180	174	87
H-Gln(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-OH **(108)**	200	-	-	-
H-Gln(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Thr(t-Bu)-OH **(111)**	200	160	134	67
H-D-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-OH **(114)**	200	160	130	65

Cyclizations without isolation of the linear peptide from TFA/pyridinium salts have been tried because in a solid phase peptide synthesis using Boc chemistry it was found that in situ neutralization of peptide trifluoroacetates with bases such as DIPEA or collidine gives better results than separate neutralization, washing and than coupling.[129a,154] In the cases tried, cyclization without removing the TFA salt gave bad results. For example, the linear protected peptide **(104)** was cyclized with only 18 % yield (calculated from resin loading). The same cyclic peptide **(105)** was obtained from a different precursor **(106)** that was previously purified by filtration through a C_{18} plug with much better, but still low yield (38 %) and without epimerization (Table 17).

This peptide **(106)** is only one example in this series that cyclizes without epimerization. This is obviously due to the proline residue at the C-terminus of the peptide. One reason for the relatively low yields is that all of these peptides contain a β-hGln residue, where the side chain amide groups are not protected, which can lead to numerous by-products in every step of the synthesis.

Table 17: Cyclization of the linear N-terminus analogs of the binding epitope of VCAM-1.

Linear peptide	n/ μmol	Coupl. reagent	v/ μmol/h	Cyclic peptide	Yield		
					mg	μmol	%
(101)	147	1.8 equiv HATU 6.0 equiv DIPEA 1.0 equiv HOAt	6	**(102)**[a]	114	107	73
(104)	-[d]	1.1 equiv HATU[c] 3.0 equiv DIPEA[c]	-[d]	**(105)**[a]	15	18	18[b]
(106)	174	2.3 equiv HATU 4.5 equiv DIPEA 2.3 equiv HOAt	5	**(105)**	92	66	38
(108)	-[d]	1.6 equiv HATU[c] 3.0 equiv DIPEA[c]	-[d]	**(109)**[a]	16	15	8[b]
(108)	155	3.0 equiv HATU 6.0 equiv DIPEA	26	**(109)**[a]	35	34	22
(111)	134	3.0 equiv HATU 6.0 equiv DIPEA	32	**(112)**[a]	84	50	37
(114)	130	1.1 equiv HATU 3.0 equiv DIPEA 1.0 equiv HOAt	5	**(115)**[a]	52	47	36

[a] Epimerization is observed. [b] Yield is calculated on first amino acid loading on the resin. [c] Calculated on resin loading. [d] Precursor was not purified.

The linear protected peptide **(108)** is the first example in this methodology, where a large quantity (155 μmol from starting max 200 μmol) of starting linear peptide **(108)** was isolated after cyclization. This material was used for a second cyclization. In this reaction 22 % of cyclic peptide **(109)** and 16 % of linear peptide **(108)** was again isolated. Surprisingly not only cyclization but also dimerization is suppressed in this example. This can be due to strong sterical hindrance of the bulky t-butyl protective group in beta position of the threonine residue at the C-terminus of the linear peptide **(108)**, although other peptides (**(98), (104), (111)**) have been synthesized with the same residue at the C-terminus without this effect but also with moderate yields. The linear protected peptide H-Ile-Asp(Ot-Bu)-Ser(t--Bu)-β-hPro-Thr(t-Bu)-Gln(Trt)-OH **(101)**, with a β-homoamino acid in the central position of the peptide chain is cyclized again with high yield (73 %) but epimerization is not suppressed. Obviously epimerization is the main problem for cyclization of these peptides and can be suppressed only by using a proline residue at the C-terminus of the linear peptides.
All these cyclic peptides are completely deprotected (Table 18) with two different cleavage cocktails and purified by reverse phase preparative HPLC.

Table 18: Deprotection of the linear N-terminus analogs of the binding epitope of VCAM-1.

Cyclic protected peptide	n/ μmol	Cleavage cocktail	Time/ h	Cyclic deprotected peptide	Yield		
					mg	μmol	%
(96)	35	Reagent K	2	**(49)**	11	17	49
(102)	107	Reagent K	5	**(50)**	36	55	51
(105)	18	TFA 95 H_2O 2.5 TIS 2.5	2	**(51)**	4	5.5	31
(105)	66	Reagent K	5	**(51)**	14	21	32
(109)	49	TFA 95 H_2O 2.5 TIS 2.5	2	**(52)**	19	30	61
(112)	50	Reagent K	2	**(53)**	27	42	84
(115)	47	Reagent K	5	**(54)**	27	43	92

Once again these peptides show instability during deprotection, and yields for the deprotection are lower as it was expected. Reagent K is better for deprotection of these peptides but it is also not always a good choice. For example, only a slightly better yield is obtained with reagent K than with another mixture for peptide **(105)**.

Once again all epimeric mixtures are successfully separated using reverse phase preparative HPLC (method 3) under high flow (25 ml/min) with a small gradient. Following this methodology, some analogs of the binding epitope of VCAM-1 were synthesized, which mimics the central position of the epitope (Table 9, P. 47.)

4.3.3.3. Cyclic Analogs of the Central Part of VCAM-1 Binding Epitope

The linear peptides were synthesized on 2-ClTrt resin using Fmoc chemistry using an automated peptide synthesizer. Results are shown in Table 19.

Table 19: Linear analogs of the central part of VCAM-1 binding epitope.

Linear peptide	Resin/ μmol	Yield		
		mg	μmol	%
H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Gln(Trt)-OH **(117)**	200	150	89	45
H-β-hPro-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-OH **(120)**	200	74	72	36
H-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Gln(Trt)-Leu-OH[a] **(122)**	200	130	142	71
H-Ile-Asp(Ot-Bu)-Ser(t-Bu)- β-hPro-Leu-OH **(124)**	200	98	130	65

[a] Purified by reverse phase preparative HPLC

These four peptides are a good example of sensitivity to hydrolysis and other side reactions (already mentioned in chapter 4.3.2, P. 51) of -Asp-Ser- amide bond under basic conditions during Fmoc deprotection. The yield of the linear peptide is inverse proportional to the numbers of treatment with a base (Fmoc deprotection) for the -Asp-Ser- amide bond. For example, in the peptide H-β-hPro-Gln(Trt)-Ile--Asp(Ot-Bu)-Ser(t-Bu)-OH **(120)**, where the sequence -Asp-Ser- is attached to the resin, the yield of the linear protected peptide is very low (36 %). In the opposite case, when the sequence -Asp-Ser- is at the N-terminus of the linear peptide **(122)**, the yield is much better (71 %).
Results of cyclization of these linear protected peptides are shown in Table 20.

Table 20: Cyclization of linear analogs of the central part of VCAM-1 binding epitope.

Linear peptide	n/ μmol	Coupling reagent	v/ μmol/h	Cyclic peptide	Yield		
					mg	μmol	%
(117)	89	HATU 6.0 equiv DIPEA 13.0 equiv	5	**(118)**[a]	29	26	29
(120)	72	HATU 3.0 equiv DIPEA 6.0 equiv	22	**(121)**[a]	40	26	36
(122)	142	HATU 1.1 equiv DIPEA 2.0 equiv HOAt 1.0 equiv	4	**(123)**	120	101	71
(124)	130	HATU 3.0 equiv DIPEA 6.0 equiv	31	**(125)**	8	10	8

[a] Epimerization is observed

In this series of cyclic peptides epimerization was also observed but fully protected epimers are separated by reverse phase preparative HPLC. The rate of cyclization can be improved enormously with addition of HOAt and with slower addition rate of the peptide to the reaction solution. Using these conditions, peptide **(122)** is cyclized with a yield of 71 % and racemization is not observed. Other peptides (**(117)**, **(120)**, and **(121)**), are cyclized without addition of HOAt with moderate yields. These cyclic peptides are completely deprotected with reagent K. The results are shown in Table 21.

Table 21: Deprotection of cyclic analogs of the central part of VCAM-1 binding epitope.

Cyclic protected peptide	n/ μmol	Cleavage cocktail	Time/ h	Cyclic deprotected peptide	Yield		
					mg	μmol	%
(118)	26	Reagent K	5	**(55)**	15	24	92
(121)	26	Reagent K	2	**(56)**	13	23	88
(123)	101	Reagent K	5	**(57)**	32	44	44
(125)	10	Reagent K	2	**(58)**	4	6	62

The above mentioned problems with final deprotection could be avoided only by using a different protection scheme. A synthesis of the linear peptides with a benzyl type protective group instead of t-butyl type and without any protective groups on Gln and Asn residues, which could be deprotected by simple catalytic hydrogenation, failed. Analytical HPLC of the linear peptide after cleavage from the resin shows a mixture of compounds and in the MALDI-ToF MS only a product

with a mass of 108 less than expected is identified. The analytical HPLC in this case shows a mixture of compounds.

All epimeric mixtures obtained during the cyclizations have been separated by preparative HPLC. After separation, some of these peptides are found by NMR to adopt at least two distinct conformations probably due to a cis-trans isomerization of a peptide bond. These species are characterized by the presence of two different sets of signals in the ^{1}H NMR spectrum. The ratio of conformers differs from 10 : 90 to 50 : 50. Conformers with a ratio less than 20 % have not been further examined. For other cases the signals of both conformations were elucidated using two-dimensional ^{1}H-^{1}H COSY spectra.

Although only one symmetric peak was observed in the analytical HPLC for all synthesized peptides, additional experiments were needed to prove that the two sets of signal are a result of two peptide conformations and not of two diastereomers:

Peptide c-(-Ile-Asp-Ser-Pro-Leu-Asn-) **(39)** was chosen for additional NMR experiments. The changes of the ratio major/minor intensities of the several protons with temperature are given in the Table 22. The ratio between:

a) Ile amide proton in the major conformation (8.73 ppm at 300 K) and Asn amide proton in the minor conformation (7.07 ppm at 300 K),
b) Asx H^{β} proton in the major conformation (2.88 ppm at 300 K) and Asx H^{β} proton in the minor conformation (2.37 ppm at 300 K),
c) Asx H^{β} proton in the major conformation (2.88 ppm at 300 K) and Asx H^{α} proton in the minor conformation (4.41 ppm at 300 K) are shown.

Table 22: The ratio major/minor intensities of the several protons with temperature in ^{1}H NMR spectra of the peptide c-(-Ile-Asp-Ser-Pro-Leu-Asn-) (39).

Temp./K	300	305	310	315	320	330
Major Ile NH/minorAsn NH	2.4	2.5	2.7	2.8	2.9	3.2
Major Asx H^{β}/minor Asx H^{β}	3.0	3.1	3.2	3.8	3.7	4.4
Major Asx H^{β}/minor Asx H^{α}	2.4	2.6	2.8	3.0	3.0[a]	-[a]

[a] The signal of the minor proton is partially covered with another signal.

Because of the noise and overlapping of the signals the more accurate integration of the peaks was not possible. Nevertheless for all three cases the ratio major/minor signal intensity increase with increasing the temperature. This effect is not possible for the diastereomers.

4.4. CYCLIC ANALOGS OF INVASIN BINDING EPITOPE

The protein invasin encoded by enteropathogenic *Yersinia Sp.* allows entry of bacteria into intestinal M cells by binding to multiple members of the integrin receptor superfamily. The five integrins that bind invasin ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$) are either receptors for extracellular matrix proteins or are involved in cell-cell interactions.[55,100a] The best characterized member of this group is $\alpha_5\beta_1$, which is also known as the fibronectin (Fn) receptor. Integrins are primarily localized on the basal surfaces of cells in the epithelium, and are normally not available for contact with bacteria in the lumen of the intestine. The exception to this rule is the M cell, which has at least two integrin invasin receptors uniformly distributed on its surface.[59]

Because binding of the invasin to the integrins is very strong especially by low concentrations of the integrines on the cell surface it represents a good target for developing new integrine antagonists. Such antagonists could not only have medical application than also can be useful for studying ligand binding and signal transduction mediated by different integrins.

The integrin binding motif consists of one loop region with conservative Asp911 residue and two synergistic regions (see chapter 2.3.3, P. 20).

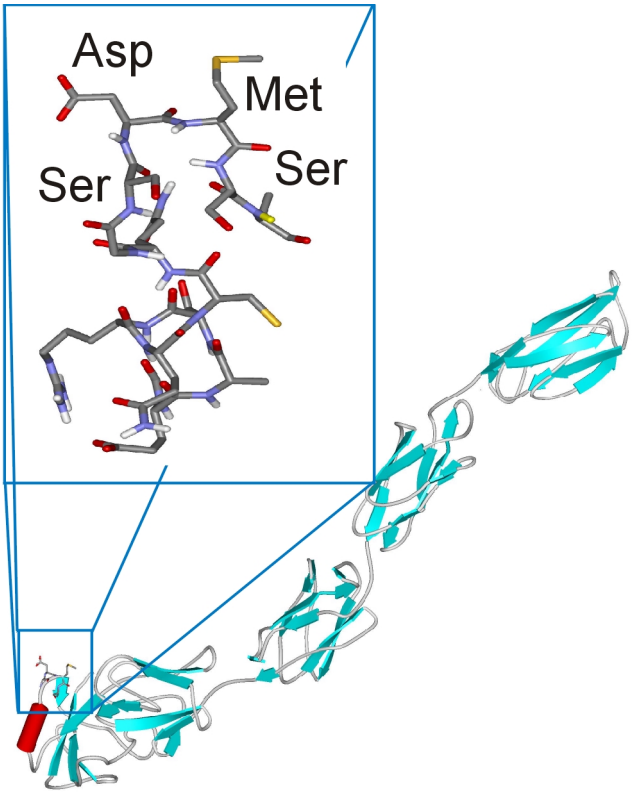
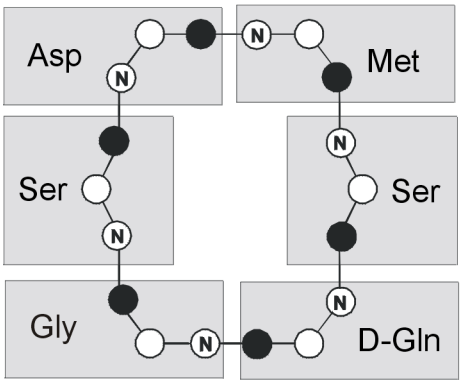


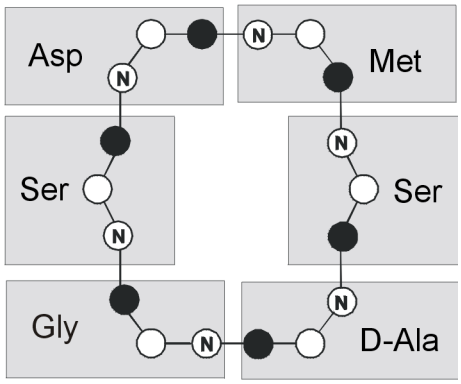
Figure 26: Binding epitope of Invasin with the critical Asp residue in the β-turn.

The critical Asp911 residue is found in the i+1 position of the β-turn in the sequence -Ser-Asp-Met-Ser- (Figure 26).

Two cyclic constrained peptides c-(-Ser-Asp-Met-Ser-D-Gln-Gly-) **(126)** and c-(-Ser-Asp-Met-Ser-D-Ala-Gly-) **(127)** were synthesized on Wang resin using the on-resin cyclization strategy already used for the synthesis of the cyclic analogs of VCAM-1 binding epitope (chapter 4.3.2, P. 51) Peptides present in the recognition sequence (-Ser-Asp-Met-Ser-) is locked in a suitable conformation for binding to the integrin by incorporation of a single D-amino acid (Figure 27). The D-amino acid induces usually a βII' turn in a cyclic hexapeptide and leads to the formation of an additional complementary β-turn.



c-(-Ser-Asp-Met-Ser-D-Gln-Gly-) **(126)**



c-(-Ser-Asp-Met-Ser-D-Ala-Gly-) **(127)**

Figure 27: Proposed conformations for two cyclic SDMS peptides.

These two peptides have been tested as inhibitors of the laminin-5 interaction with the integrin $\alpha_3\beta_1$ by Dr. Johannes Eble, Universität Münster.

The integrin $\alpha_3\beta_1$/laminin-5 interaction is a target for modulation of tumor cell invasion and tissue remodeling.[155] There are indications that elucidation of the mechanisms of the $\alpha_3\beta_1$ /laminin-5 interaction is critical for understanding the roles of $\alpha_3\beta_1$ in biological and pathological processes and for designing potential therapeutics.[156]

The inhibitor activity of both peptides **(126)** and **(127)** is similar, which confirms that the sequence -Ser-Asp-Met-Ser- in the β-turn is responsible for binding activity (Figure 28) and that the two other amino acid residues are not important for binding activity.

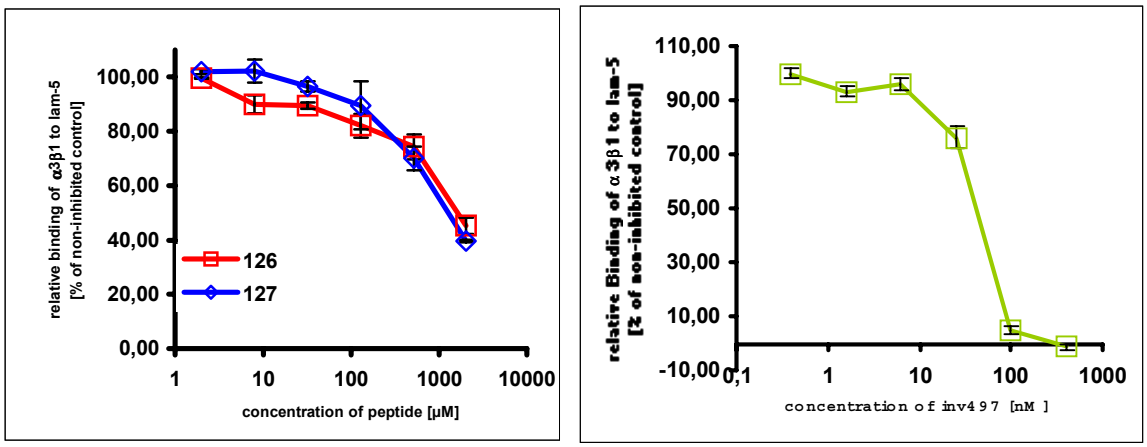


Figure 28: Binding affinity of c-(-Ser-Asp-Met-Ser-D-Gln-Gly-) (126) and c-(-Ser-Asp-Met--Ser-D-Ala-Gly-) (127) and invasin on $\alpha_3\beta_1$ integrin.

Both peptides show same inhibitory activity with IC_{50} value in the mM range). The binding of the laminin-5 to $\alpha_3\beta_1$ integrin is not completely inhibited. The inhibitory activity is much weaker than for invasin (IC_{50} in nM range). Obviously there are other contact points present in invasin that are also involved in binding of this protein to the integrin. Although there is no striking similarity between invasin and fibronectin, sequence determinants involved in receptor recognition appear to be similar. In fact, the two substrates appear to bind to the same site on the $\alpha_5\beta_1$ receptor.[60] Nevertheless, invasin is much more efficient than fibronectin.[61] Comparing the invasin and fibronectin[157] binding domains, two synergy regions in invasin are found (Figure 29). Invasin Asp811 is also involved in binding, but mutations on this place have much weaker effects than observed for Asp911.[158] Both invasin and fibronectin synergy regions contain Arg residues that are located at identical distances (about 30 Å) from the critical Asp residues found in their respective carboxy terminal cell adhesion domains.[159]

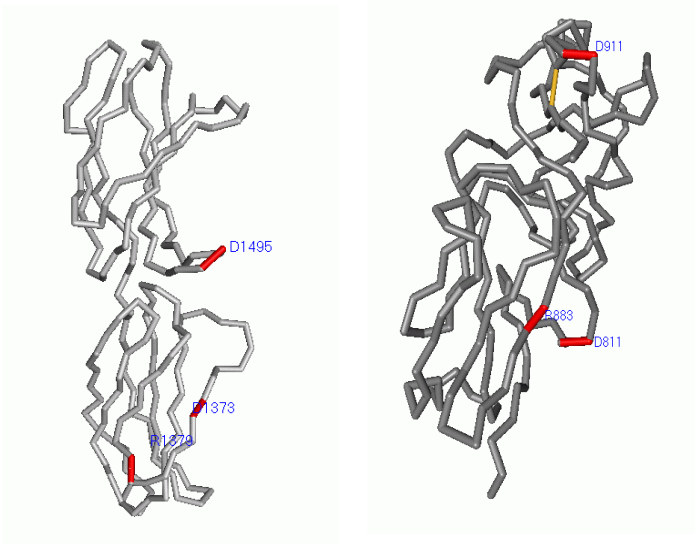


Figure 29: Cell adhesion domains of invasin (right) and fibronectin (left).[59]

As a “second generation” $\alpha_3\beta_1$ antagonist, a peptide c-(-Ser-Asp-Met-Ser-Lys(H--Arg-(β-Ala)$_5$-)-Gly-) **(130)** which contains both the cyclic constrained SDMS binding epitope present in a β-turn and an Arg residue in approximately 30 Å distance is synthesized by the following methodology.

Two peptide segments, the fully protected cyclic peptide c-(-Ser(t-Bu)-Asp(O-tBu)--Met-Ser(t-Bu)-D-Lys(Aloc)-Gly-) **(134)** and the linear peptide Fmoc-Arg(Pbf)-(β-Ala)$_5$-OH **(131)** are synthesized separately.

The linear peptide Fmoc-Arg(Pbf)-(β-Ala)$_5$-OH **(131)** is synthesized on 2-ClTrt resin, cleaved from the resin with 1 % TFA in DCM (protocol 9) and purified by reverse phase preparative HPLC. Protective groups are chosen so that additional purification of the partially protected peptide after segment condensation reaction and cleavage of the Fmoc protective group is possible if necessary.

The linear peptide H-Ser(t-Bu)-Asp(Ot-Bu)-Met-Ser(t-Bu)-D-Lys(Aloc)-Gly-OH **(133)** is synthesized on 2-ClTrt resin and purified by reverse phase HPLC. The t-butyl group for serine, t-butoxy group for aspartic acid and Aloc for lysine are chosen for orthogonal protection of the linear peptide. These protective groups are stable under the basic conditions of Fmoc chemistry and Aloc can be selectively removed with Pd^0 The fully protected cyclic peptide c-(-Ser(t-Bu)-Asp(O-tBu)-Met-Ser(t-Bu)--D-Lys(Aloc)-Gly-) **(134)** is obtained by cyclization of this linear precursor **(133)** in DMF using a previously developed method (chapter 4.3.3, P. 60) with 1.5 equiv HATU as coupling reagent, 4.7 equiv DIPEA as base and 1.5 equiv HOAt as additive, which should improve the reaction rate and suppress dimerization and epimerization.

All attempts made for selective deprotection of the Aloc protective group using [Pd(PPh$_3$)$_4$] and N-methylmorpholine, morpholine or N-methyl aniline as scavengers[118] failed. The main product in the MALDI-ToF MS has a mass 16 larger than the desired peptide, probably due to oxidation of methionine residue. Completely (100 % by analytical HPLC) and fast deprotection is achieved using phenylsilane[160] as scavenger. The selectively deprotected peptide c-(-Ser(t-Bu)--Asp(Ot-Bu)-Met-Ser(t-Bu)-D-Lys-Gly-) **(132)** is used in the next step without further purification.

An excess of segment **(131)** is coupled to **(132)** in DMF using HATU as the coupling reagent and DIPEA as the base. The fully protected peptide **(135)** is deprotected first with a solution of 2 % DBU, 2 % piperidine in DMF to remove the Fmoc group and then with reagent K to cleave the t-Bu type protective groups. The fully deprotected peptide **(130)** is purified by reverse phase preparative HPLC.

Peptide c-(-Ser-Asp-Met-Ser-Lys(H-Arg-(β-Ala)$_5$-)-Gly-) **(130)** shows increased inhibitory activity (IC_{50} ~200 μmol). Although binding not as strongly as invasin,

this peptide **(130)** completely inhibits the binding of laminin-5 protein to the $\alpha_3\beta_1$ (Figure 30), which was not the case with previously tested peptides.

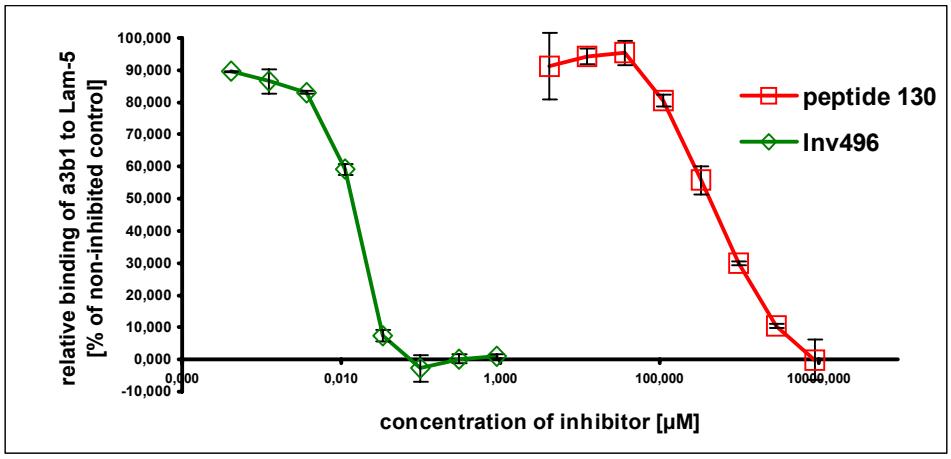


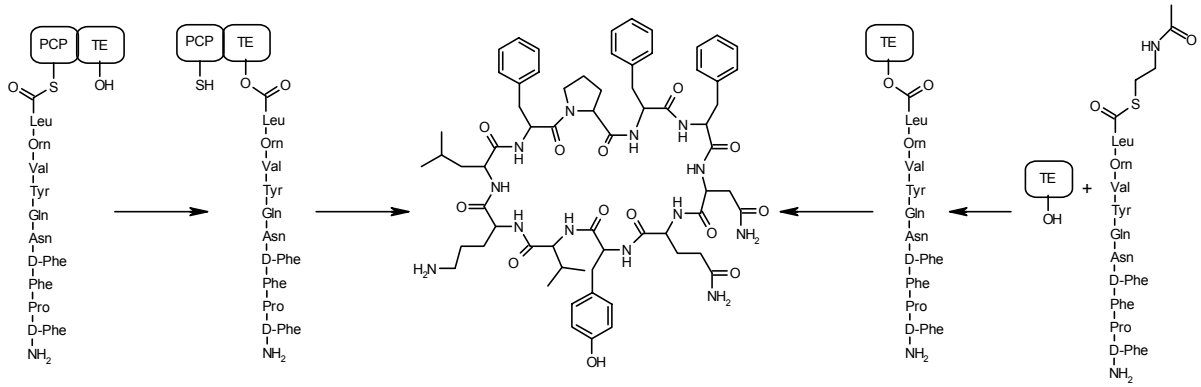
Figure 30: Binding affinity of (130) and invasin on $\alpha_3\beta_1$ integrin.

Still better inhibitory activity could be achieved by optimization of the length and the flexibility of the peptide chain between the Arg residue and the cyclic part of the peptide. Also incorporation of the second synergistic part of invasin (Asp811 residue) should also improve the inhibitory activity of this compound.

4.5. LINEAR PRECURSORS OF THE ANTIBIOTIC TYROCIDINE A AND THEIR ANALOGS

Antibiotic tyrocidine A (Figure 8, P. 22) belongs to the group of the natural peptides synthesized non-ribosomally by large enzymes able to form peptide bonds from thiol-linked amino acids called peptide synthetases.[66] Peptides synthesized in this manner are small in size and may acylated, glycosylated, methylated, heterocyclized or cyclized. This, non-ribosomal protein template directed synthesis of peptides is limited only by the length of the peptide chain formed, which has been found to range from 2 to 48 residues.[64] The modular mechanism of action of these enzymes is shown on Figure 9, P. 23. Each module is responsible for the addition of a single amino acid. Modules activate and modify a specific amino acid and form the peptide bond between activated amino acids. Each module is made up of distinct domains. The adenylation domain (A-domain) is responsible for the recognition and activation as acyaladenylates of their substrate. In the next step activated amino acids are thioesterifed to the thiol group present in the thiolation domain, also called the peptidyl carrier protein (PCP). Peptide bond formation is catalyzed by condensation domain. During elongation, the intermediate peptides remain covalently attached to the peptide synthetase.

Release of the thioester-bound peptide is achieved via the action of a thioesterase. Recently it has been shown that a carboxy terminal thioesterase domain of peptide synthetase, that is involved in cyclization and product release can also catalyze macrocyclization, and elongation in the case of symmetric cyclic peptides, independent of upstream domains.[161] The full length TycC (M_r 724K) is replaced with overexpressed and purified TycC TE domain (M_r 28K). This domain successfully catalyzes cyclization of synthetic peptide N-acetylcysteamine (NAC) thioesther. N-acetylcysteamine is structurally identical to the terminal portion of phosphopantetheine and thus a good mimic of the natural substrate decapeptide-S-PCP.[162]



Peptide-S-PCP Peptide-O-TE Tyrocidine A Peptide-O-TE **(136)**

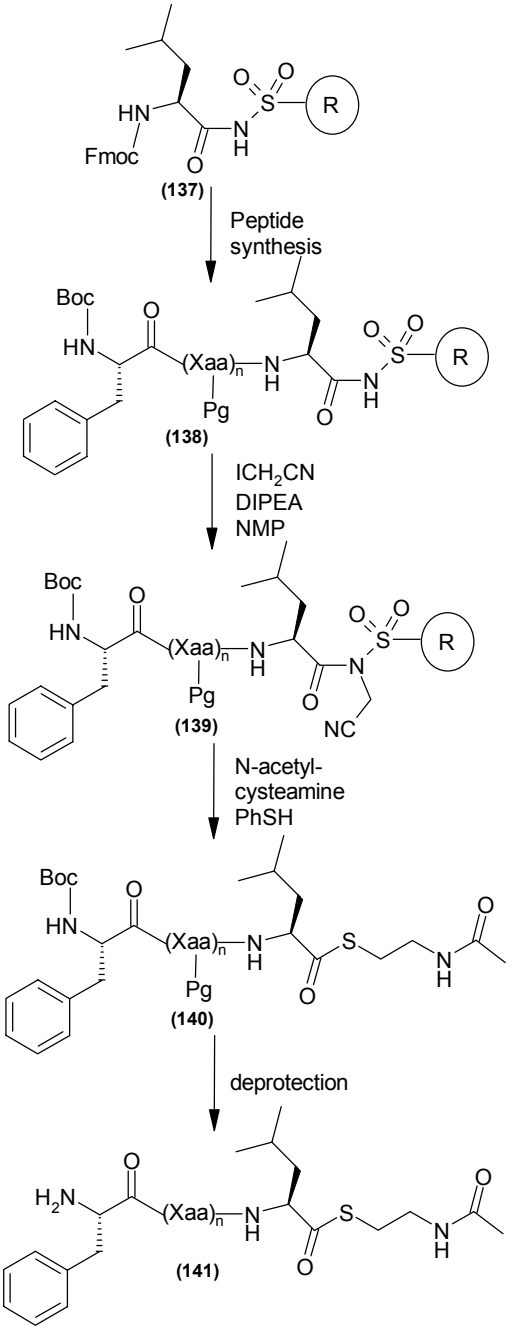
Scheme 18: Mechanism of TE-domain catalyzed macrocyclization and product release.[161]

On the left side of Scheme 18 the proposed mechanism of thioesterase (TE)-domain catalyzed macrocyclization and product release is shown, in which the peptide is transferred from peptidyl carrier protein (PCP) to peptide O-TE where it is cyclized and released from the enzyme. On the right side the mechanism is shown where the natural enzyme-substrate complex peptidylthio-PCP is replaced with a synthetic peptidyl N-acetylcysteamine thioester (peptidyl-SNAC) **(136)** and cyclized with overexpressed and purified TycC TE domain. Cyclization with synthetic peptide **(136)** proceeds smoothly and with negligible rate of hydrolysis. Mutation experiments with replacement of each residue in the linear precursor **(136)** by alanine show that the N-terminal residue D-Phe and C-terminal residue Orn are essential for cyclization. Recognition of both stereochemistry and the side chain of these residues is essential for cyclization. These results[161] suggest that Tyr7, Val8, and Leu10 also contribute to substrate recognition, although their contribution is smaller than that of D-Phe1 and Orn9.

The C-terminal peptidyl thioester is the key intermediate for this enzymatic macrocyclization. The C-terminal peptidyl thioester can be prepared by standard

solid-phase synthesis using t-butoxycarbonyl methodology but this requires final cleavage of the side chain protecting groups with liquid hydrogen fluoride in the last step of the synthesis.[163] Some problems must be solved for using the Fmoc-chemistry for synthesis of C-terminal thioesters. First of all, resin bound thioesters are unstable to repeated exposure to piperidine, which is used to remove the Fmoc protective group in Fmoc-based chemistry.

Moreover, the susceptibility of thio esters to epimerization under basic conditions imposes an additional difficulty. Nevertheless, several strategies for solving these problems have been reported:



Scheme 19: Synthesis of peptides with thioester on the C-terminus.

In one approach[164] a thioester-compatible Fmoc-cleavage cocktail (25 % 1-methyl--pyrrolidine, 2 % hexamethylenimine, 2 % HOBt) has been used.

Good results are obtained using a mixture of Me_2AlCl and EtSH for cleaving the peptide from Wang and Pam resin to give the corresponding thioester.[165]

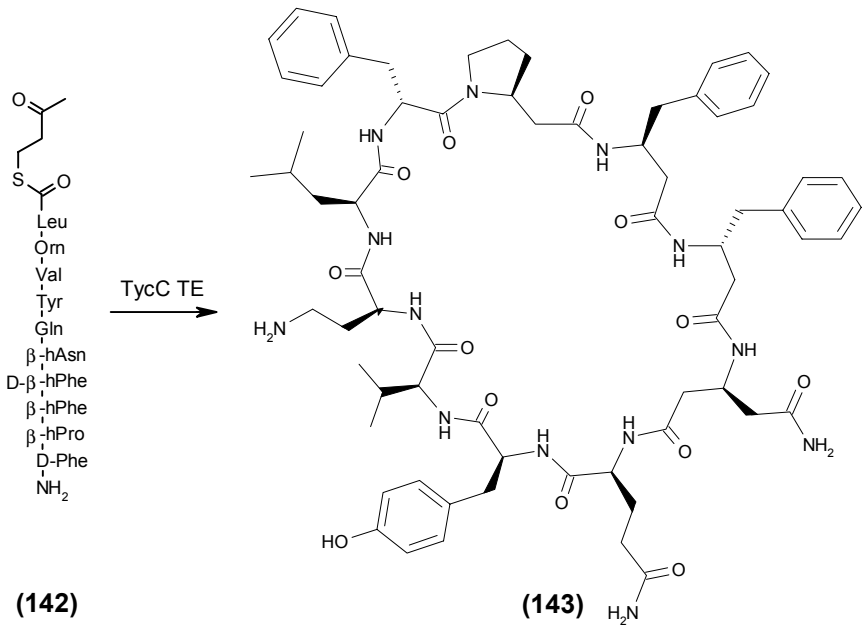
Alternatively, the labile thioester can be introduced at the end of the synthesis. The backbone amide linker (BAL) strategy may be employed to achieve this.[166] The C-terminal residue of the peptide, anchored to a solid support through its backbone nitrogen, is being activated and coupled to an amino acid thioester prior to final cleavage and deprotection.

In this work the more general method using Kenner's sulfonamide "safety-catch" linker,[143] used in a "native ligation" approach, a recently developed method for synthesis of larger peptides, was applied.[167]

The peptide was synthesized on the “safety catch” resin using Fmoc-chemistry. Then the linker was activated using iodoacetonitrile, and the peptide was cleaved with a nucleophiles, in this case N-acetyl cysteamine. Finally the peptide is deprotected and purified by reverse phase preparative HPLC (Scheme 19).

Fmoc protected leucine is anchored on the “safety catch” resin using Ellman´s method.[143] The maximal loading of amino acid on resin achieved by this method was about 0.7 mmol/g, and repeated reaction does not give better results. Racemization is not observed. The linear peptide **(138)** is synthesized using Fmoc chemistry only the N-terminal residue (D-Phe) is Boc protected.

Activation of the resin can be achieved using iodoacetonitrile or diazomethane. Iodoacetonitrile was preferred in most experiments, although it is published[168] that better results could be obtained with diazomethane if catalytic amounts of thiophenol are present in the reaction mixture during nucleophilic displacement in the next step of the synthesis. This is in good agreement with previous observations showing that diazomethane provides more efficient alkylation, while the product of alkylation with haloacetonitrile displays enhanced reactivity toward nucleophilic displacement.[142c]



(142) **(143)**

TycC TE - overexpressed and purified thioesterase domain of the C subunit of the Tyrocidine synthetase.

Scheme 20: Synthesis of Tyrocidine A analog.

The C-terminus of the peptide on the activated resin **(139)** is then reacted with N-acetylcysteamine as a nucleophile that is accompanied by cleavage of the peptide **(140)** from the resin. Better yields in this step are obtained if catalytic amounts of thiophenol or sodiumthiophenolate are added to the reaction mixture.[168,169] The reason for this catalytic effect is that for both an aliphatic

sulfonamide and an aliphatic mercaptan the pK_a in DMSO is 17, while for thiophenol the pK_a in DMSO is 10.[170] The strongly nucleophilic thiophenolate anion initially cleaves the peptide to give a phenyl-thioester, which exchanges in situ with the large excess of aliphatic thiol to produce a more stable aliphatic α-thioester. The rationale for this procedure is analogous to the use of thiol additives to improve the kinetics of native chemical ligation.[167]

Composition of the cleavage mixture and reaction time should be fine-tuned on the specific peptide sequence. However, for these peptides reagent B is used and a cleavage time of 1-2 h is found to be optimal.

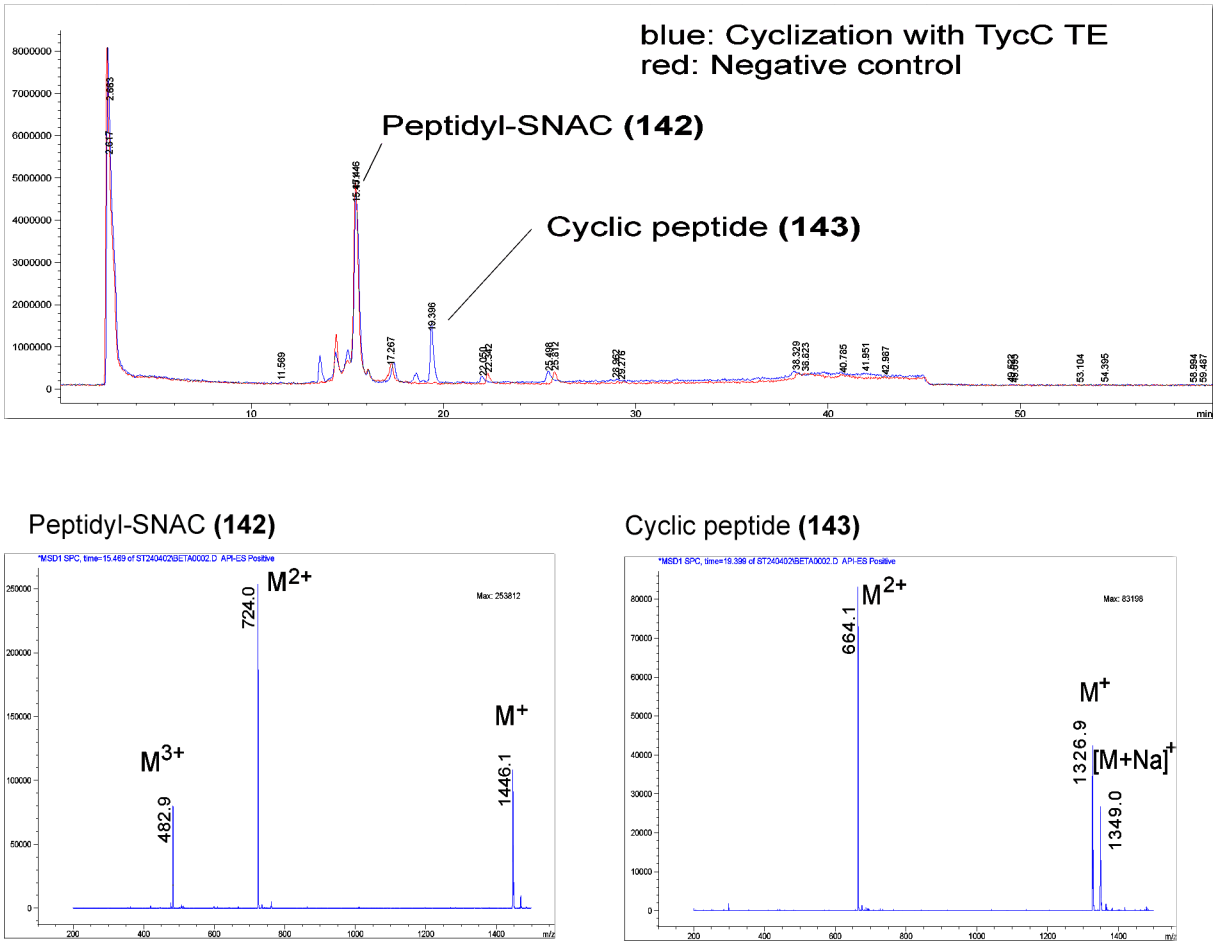


Figure 31: HPLC chromatogram and mass spectra of peptidyl-SNAC (142) and cyclic peptide (143)

Using this methodology the peptide H-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val--Orn-Leu-S-CH_2-CH_2-$NHCOCH_3$ **(136)** can be synthesized with an overall yield of 63 % and in very high purity. An analogous peptide in which four amino acids are replaced by their corresponding β-homoamino acids, H-D-Phe-β-hPro-β-hPhe-D-β--hPhe-β-hAsn-Gln-Tyr-Val-Orn-Leu-S-CH_2-CH_2-$NHCOCH_3$ **(142)** is obtained with an overall yield of only 11 %. The major disadvantage of this methodology is that

there are no analytical methods for analyzing activation and nucleophilic displacement steps. Hence, there are no possibilities for selection and optimization of the problematic step.Cyclization of H-D-Phe-β-hPro-β-hPhe-D-β-hPhe-β-hAsn-Gln-Tyr-Val-Orn-Leu-S-CH_2-CH_2-$NHCOCH_3$ **(142)** with the isolated TycC TE domain was performed in the group of prof. A. Marahiel, Philipps-Universität Marburg.

Preliminary results shows smooth cyclization to give a cyclic analog of Tyrocidine A **(143)**, with a minor rate of hydrolysis (Figure 31). This experiment is performed under following conditions:

2.5 μL peptidyl-SNAC (2.5 mM, final concentration 100 μM)

0.5 μL TycC TE final concentration 1 μM

47 μL puffer (50 mM Hepes, 0.1 M NaCl, pH 7.0)

Temperature : 25 °C

Reaction time: 10 min.

4.6. RGD PEPTIDES

The Arg-Gly-Asp (RGD) sequence serves as the primary integrin recognition site in extracellular matrix proteins, and peptides containing this sequence can mimic the activities of matrix proteins. They bind to integrins, the main cell surface receptors mediating cell adhesion to extracellular matrices.[171] Peptides having little secondary structure bind to all integrins usually with low affinity, whereas conformational restriction of the RGD sequence in a peptide, combined with an appropriate choice of the amino acids that flank the RGD sequence, can yield more selective peptides. These conformationally restricted peptides can bind specifically to one integrin only or to a small group of closely related integrins.[172] Peptides that are specific for individual integrins are of considerable interest and medical significance. RGD peptides and peptidomimetics that block activity of the platelet integrin $\alpha_{IIb}\beta_3$ almost without interfering with other integrins are already in clinical use. The integrin $\alpha_v\beta_3$ receptor is present in a number of tumor cells, like osteosarcoma, neuroblastoma, prostate, breast, and lung cancer, and melanoma cells.[173] RGD peptides that are selective antagonists of this integrin have numerous medical applications. They can be used as inhibitors of angiogenesis,[174] retinal angiogenesis,[175] and osteoporosis.[176]

RGD peptides selectively binding to $\alpha_v\beta_3$ are used for targeting anti-cancer drugs into tumor vasculature.[177] Anticancer drugs coupled to the RGD peptide are less toxic and more active against human breast cancer tumors than unmodified drugs. RGD peptides are also used for selective targeting tumor cells ($\alpha_v\beta_3$ integrin) with

radiolabeled ligands, what may provide information about the receptor status and enable early discovery of cancer cells and specific therapeutic planning.[178] Moreover, RGD peptides grafted to surface proteins of adenoviruses change the infectivity pattern of the virus from all human cells to those expressing RGD-directed integrins.[179] Such viral vectors, if specific for $\alpha_v\beta_3$, could be particularly useful in gene therapy.

For all of these applications, two properties of RGD peptides, affinity and selectivity are important. Both are achieved by bringing these three amino acid residues in an appropiate conformation. In conformational analysis of model peptides (chapter 4.2.2, P. 32) it is shown that β-homoproline, when incorporated in one cyclic pentapeptide, most likely will occupy the central position of a pseudo-γ-turn. Consequently, β-amino acids can be used to force the rest of the cyclic peptide, which contains the active RGD sequence, to adopt a predefined conformation.

The cyclic pentapeptide c-(Arg-Gly-Asp-D-Phe-β-hPro-) **(146)** was synthesized and cyclized on Wang resin, using previously described method (chapter 4.3.2, P. 51), with good yield and purity.

A cyclopropyl (Cpr) analog of aspartic acid **(148)** (Figure 32) was incorporated in two other RGD peptides. Highly constrained β-aminocyclopropane carboxylic acids, recently recognized as conformationally rigid amino acids for the synthesis of peptides,[180] exert strong conformational bias,[181] and could be used to induce a pseudo-γ-turn in cyclic peptides. This cyclopropane rest **(148)** is introduced in the peptide chain as dipeptide because it is known that donor-acceptor-1,2-disubstituted cyclopropanes rapidly undergo ring opening.[182]

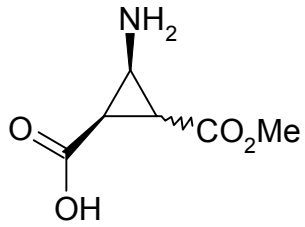


Figure 32: Cyclopropyl analog of aspartic acid γ-methyl ester (148).

The cyclopentapeptide c-(Arg-Gly-Asp-Phe-Cpr(OMe)-) **(149)** was synthesized and cyclized on Wang resin using the same method as described in chapter 4.3.2, P. 51. The cyclopropyl ring is incorporated to the peptide as a dipeptide with Fmoc protected Phe residue at the N-terminus.[183] After complete deprotection and cleavage from the resin, cyclopentapeptide **(149)** is obtained as a mixture of epimers, which could not be separated by reverse phase preparative HPLC, because the incorporated cyclpropyl rest was a diastereomeric mixture.

For the synthesis of the cyclopentapeptide c-(Arg-Gly-Asp-Cpr(OMe)-Val-) **(151)** another method was used. First the linear peptide H-Asp(Ot-Bu)-Cpr(OMe)-Val--Arg(Pbf)-Gly-OH **(152)** is synthesized on 2-ClTrt resin. As a C-terminal residue glycine is used to avoid racemization during the cyclization in the next step. The

cyclopropyl rest is incorporated, as a diastereomeric mixture, in to the peptide as a dipeptide with an Fmoc protected Asp residue at the N-terminus. After cleavage from the resin and purification, the linear peptide **(152)** is obtained in only 27 % yield. Several reasons for this low yield are possible:

-Instability of the cyclopropane ring under the acidic cleavage conditions.

-Instability of the cyclopropane rings under the basic conditions of the deprotection of the last Fmoc protective group.

-Bad coupling efficiency of the dipeptide Fmoc-Phe-Cpr(OMe)-OH residue because of steric hindrance of the cyclopropyl ring or its instability.

Nevertheless, this linear peptide **(152)** is cyclized, using method A (chapter 6.4.3.2, P. 116) for the cyclization in solution, and completely deprotected with good overall yield (55 %).

A hydrophobic residue following the RGD sequence is very important for the inhibitory activity of the RGD peptides.[184] For example, antithrombotic activity of the RGDX peptides increases in the series X= Phe>Val>Cys>Gln>Ser.[185] The Arg-Gly-Asp-Xaa sequence is the essential pharmacophore for effective inhibition of the $\alpha_{IIb}\beta_3$-integrin, with Xaa being a hydrophobic residue.[186] Similar results are obtained for the binding activity of the RGD peptides to the $\alpha_v\beta_3$ integrin.[187]

For the further investigation of this hydrophobic effect on the binding activity of the RGD peptides, two cyclic tetrapeptides, c-(-Arg-Gly-Asp-D-1-Nal-) **(154)** and c-(-Arg-Gly-Asp-2-β-hNal-) **(155)** with D-1-Naphthylalanine and 2-β-homo--naphthylalanine residues are synthesized.

The linear precursors of these peptides, H-Asp(Ot-Bu)-D-1-Nal-Arg(Pbf)-Gly-OH **(156)** and H-Asp(Ot-Bu)-2-β-hNal-Arg(Pbf)-Gly-OH **(158)** are synthesized on 2-ClTrt resin. Gly is used as the C-terminal amino acid attached to the resin to avoid epimerization during cyclization, despite lower yields of linear peptides because of diketopiperazine formation on the resin-bound dipeptide stage.

Peptides **(156)** and **(158)** are cyclized in solution using a method developed for the synthesis of cyclic analogs of the binding epitope of VCAM-1 (chapter 4.3.3, P. 60). An excellent yield for the cyclization (94 %) of the linear tetrapeptide **(156)** is obtained. This is unusually high for cyclic tetrapeptides. Both of these cyclic peptides, **(157)** and **(159)**, are not stable and hydrolyze very fast during purification by reverse phase preparative HPLC, what is the probable cause for the low yield (19 %) obtained on the cyclic tetrapeptide **(159)**. The instability of peptides in acidic medium was also observed in the final deprotection and purification of these peptides. During the final purification of the cyclic tetrapeptide **(154)**, by reverse phase preparative HPLC, two new, linear peptides with a mass difference of + 18 compared to the cyclic peptide are observed.

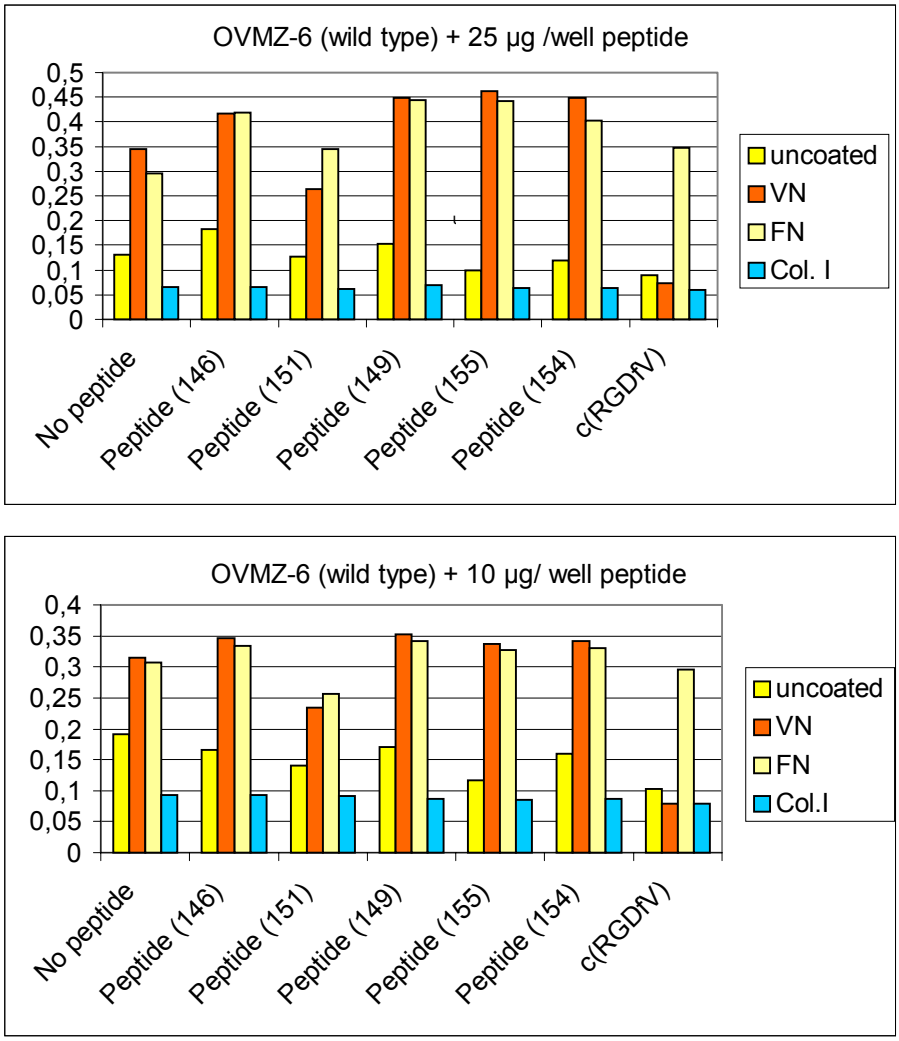


Figure 33: Adhesion tests with wild type human ovarian carcinoma cells.

Several adhesion tests with these peptides were performed by Dr. Ute Reuning, TU München. In the first assay the inhibitor affinity of these peptides toward the interaction between the wild type human OV-MZ-6 ovarial carcinoma cells and different extracellular matrix proteins (fibronectin, vitronectin and collagen type I) is tested. The cells were incubated with two different concentrations of a peptide. The number of the adhering cells on the plate was determined photometrically using the hexoaminidase activity. As a reference value c-(-Arg-Gly-Asp-D-Phe--Val-) **(24)**, which is a selective $\alpha_V\beta_3$ antagonist (see chapter 4.2, P. 27), is used. Results are presented in Figure 33.

From these diagrams it is obvious that collagen type I is not a ligand for these type of cells. In both concentrations peptides **(146)**, **(149)**, **(154)** and **(155)** display no inhibition of the interaction of the cells with vitronectin and fibronectin.

The cyclopeptide c-(Arg-Gly-Asp-Cpr(OMe)-Val-) **(151)** inhibits cell adhesion to vitronectin stronger than to fibronectin. When the peptide is present in higher concentration the interaction of the cells with fibronectin is surprisingly not

inhibited. Inhibition of cell adhesion to vitronectin by this peptide is not as strong as for the reference peptide.

Because of solubility problems of two cyclotetrapeptides **(154)** and **(155)** the adhesion test for these peptides was repeated. Small quantities of DMSO are added to the peptide solutions. Peptide **(155)** has higher inhibitor capacity in vitronectin binding (Figure 34) than peptide **(154)**, but slightly lower activity than the reference peptide.

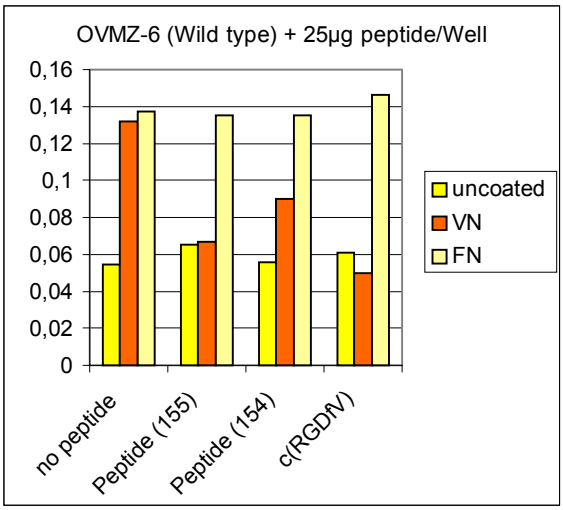


Figure 34: Adhesion test for peptides (154) and (155).

In the next assay cells overexpressing $a_v ß_3$ integrin (the concentration of the $a_v ß_3$ integrin is 8-10 times higher than in wild type cells) were used with the same ligands and peptides. Different results compared to the wild type cells were obtained (Figure 35). This type of cells interacts also with collagen type I and this interaction is inhibited by all tested peptides except **(146)**. This peptide has no inhibitor affinity on any of the ligands tested. Surprisingly all tested peptides have higher inhibitor activity in lower concentration. Interaction of fibronectin with this type of cells is inhibited slightly with cyclopeptide the c-(Arg-Gly-Asp-Cpr(OMe)--Val-) **(151)**. However, these experiments have to be repeated, because the cells display high adhesion to uncoated wells, too. Peptide **(151)** shows also the strongest inhibition of the cell interaction with vitronectin (in the same range like reference peptide c(RGDfV) **(24)**, which IC_{50} is in nm scale[188]). This peptide is tested as a diastereomeric mixture and probably one of the diasteromers is a strong $a_v ß_3$ antagonist. The second peptide tested as a diasteromeric mixture, c-(Arg-Gly-Asp-Phe-Cpr(OMe)-) **(149)**, also suppresses the interaction between the cells and vitronectin but this effect decreases with the concentration of the peptide. Very strong inhibition of the same interaction is also achieved with cyclotetrapeptides c-(-Arg-Gly-Asp-2-β-hNal-) **(155)** and c-(-Arg-Gly-Asp-D-1-Nal-) **(154)** in lower concentration.

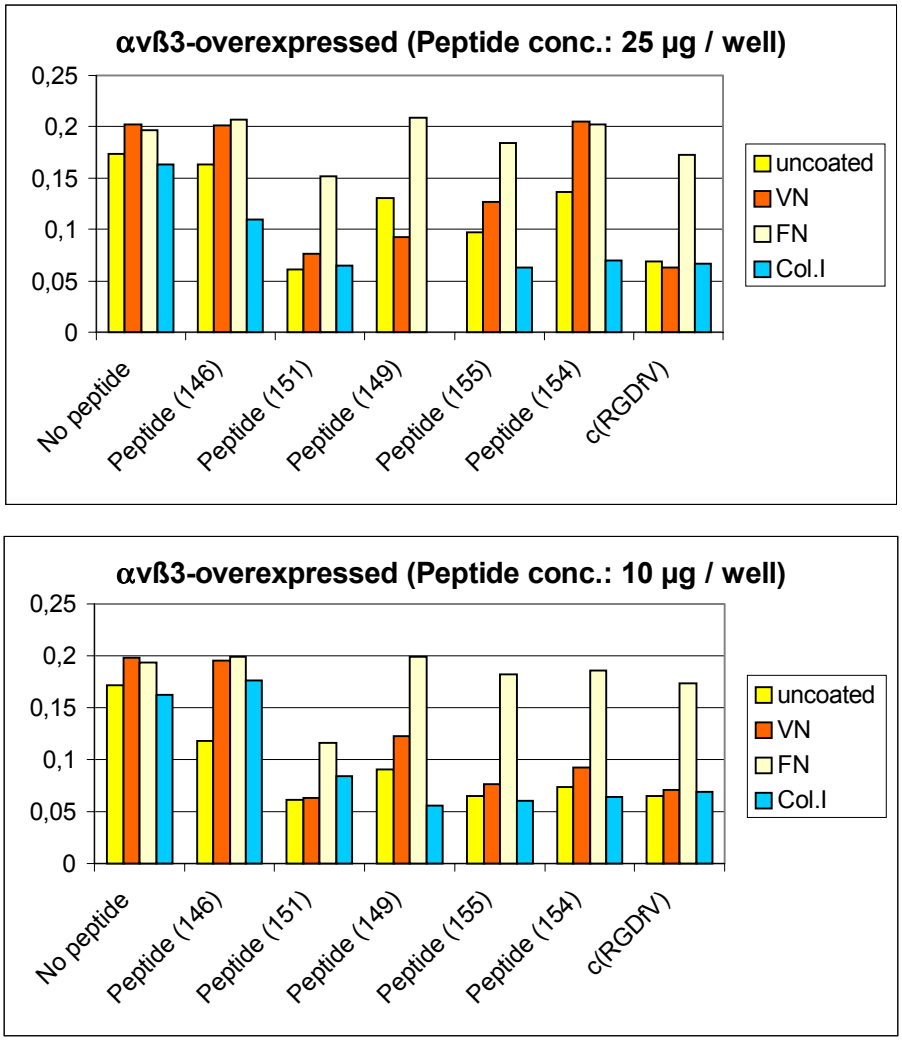


Figure 35: Adhesion tests with $\alpha_v\beta_3$-overexpressing human ovarian carcinoma cells

For the more insight in the selectivity and strength of the binding of these peptides to different integrins, more sophisticated tests are needed. Nevertheless, from these adhesion tests it is obvious that peptides c-(Arg-Gly-Asp-Cpr(OMe)-Val-) **(151)** and c-(-Arg-Gly-Asp-D-1-Nal-) **(154)** are good candidates for the development of strong and selective $a_v ß_3$ antagonists, but peptides c-(-Arg-Gly-Asp-2-β-hNal-) **(155)** and c-(Arg-Gly-Asp-Phe-Cpr(OMe)-) **(149)** should also be tested in different assays.

5. CONCLUSIONS

Starting from protected α-amino acids the Arndt-Eistert approach via sonochemical Wolff rearrangement in the presence of Ag^+ ions is a fast and efficient method for the synthesis of β-homoamino acids without racemization. Fourteen different β-homoamino acids were obtained using this method with good yields and purity. These β-homoamino acids have been incorporated into cyclic hexa-, penta-, and tetrapeptides, where they exert influence on the secondary structure.

Different spectroscopic methods (FTIR, CD, NMR) have been used to study the conformational properties of cyclic hexa-, penta-, and tetrapeptides, with or without β-homoproline, and to examine the influence of this β-amino acid on the peptide conformation in different solvents. Combination of these three analytical techniques revealed that β-homoproline is an excellent inducer of secondary structure when inserted into a cyclic peptide. This residue stabilizes a pseudo-γ-turn, or less frequently a pseudo-β-turn. Nevertheless, different conformations of peptides are found in different environments. Two-dimensional NMR experiments combined with molecular modeling techniques confirm observations obtained with CD and FTIR spectroscopy for the cyclic pentapeptide c-(-Ala-Val-β-hPro-Phe-Gly-) **(36)**. In dipolar aprotic solvents, like DMSO or ACN, this peptide is found to adopt two conformations. About 70 % of the population is found in one conformation consisting of one pseudo-γ-turn with the β-homoproline residue in central position of the turn and one βII-turn with glycine in i+1 position. Another conformation, adopted by 30 % of the population, consists of one pseudo-β-turn with β-homoproline in i+1 position and a γ-turn with alanine in the central position of the turn. In polar solvents, like H_2O or TFE, this peptide is found in only one conformation very similar to the major conformation of this peptide described for DMSO solution, with the β-homoproline residue in the central position of a pseudo-γ-turn. There are only few literature data about γ-turns, and no literature data about pseudo-γ-turn properties in CD and FTIR spectroscopy. These studies suggest that CD spectra of a pseudo-γ-turn are characterized by a positive band near 195 nm and a broad and positive band between 230 and 240 nm. Hydrogen bonding within a pseudo-γ-turn shifts the amide I band to 1620-1630 cm^{-1} in FTIR spectra. CD and FTIR techniques allow a fast and simple screening of the peptides under different conditions. However, because of the complexity of these spectra more accurate conformational analysis currently is not possible. Low-resolution results

obtained by this way can be interpreted using much slower but accurate two-dimensional NMR and molecular modeling techniques. These findings can generally be utilized in the de novo design of biologically active cyclopeptides, since the spatial orientation of side chains exposing potential pharmacophoric groups can be pre-defined by incorporation of tailor-made β-amino acids in appropriate sequential positions.

CD spectroscopy was used in cation binding studies to investigate the influence of different cations (Al^{3+}, Ca^{2+}, Mg^{2+}, Na^{+}, Zn^{2+}) in a different molar ratio on the conformation of all model peptides. Calcium ions have in the greatest influence on peptide conformation. The same change, a new positive broad band between 220 and 230 nm, is observed in the CD spectra for all peptides regardless of the previous conformation upon titration with calcium ions.

Twenty new cyclic peptides that mimic the VCAM-1 binding epitope ($R^{36}TQID^{40}SPLN^{44}$) have been synthesized using Fmoc chemistry and cyclization in solution. D-amino acids, β-amino acids and proline are used as secondary structure inducers for the spatial screening of the pharmacophoric groups. These peptides, designed as $\alpha_4\beta_1$ integrin antagonists, could have numerous medical applications and might be very useful in future studies of cell-cell and cell-extracellular matrix interactions.
A new method for cyclization in solution using two injection pumps for separate addition of peptide and coupling reagent solutions to the reaction mixture and a purification by precipitation extraction method was developed. Combination of these two methods allows any solvent to be used for peptide cyclization. HOAt as additive, a rational design of the linear peptide precursor and slow addition rate of the peptide solution to the reaction mixture suppresses dimerization and epimerization, the main side reactions of the cyclization. The best results have been obtained with proline at the C-terminus of the linear peptide. A β-homoamino acid in a central position of the linear precursor enhances the cyclization rate, and suppresses dimerization by bringing the peptide in a proper conformation.

Invasin, a bacterial adhesion protein, is one of the most potent natural ligands of β_1 integrins. Small compounds that mimic the invasin binding epitope might find pharmacological application. The crystal structure of the *Y. Pseudotuberculosis* invasin suggested the epitope -Ser^{910}-Asp^{911}-Met^{912}-Ser^{913}- to be critical for binding of invasin to integrins. Two additional residues important for binding were identified, Arg^{883} and Asp^{811}. Mutations of these two residues affect the binding affinity to integrins but not so drastically as mutations of the Asp^{911} residue.

Two cyclic constrained peptide mimics of the invasin binding epitope c-(-Ser-Asp-Met-Ser-D-Gln-Gly-) **(126)** and c-(-Ser-Asp-Met-Ser-D-Ala-Gly-) **(127)** in which D-amino acids were designed to lock the peptide conformation in such a way that the critical Asp residue occurs in i+1 position of the β-turn were synthesized. The adhesion test shows that these peptides bind to $\alpha_3\beta_1$ integrin with low affinity (mM range) and inhibit binding of laminin-5 to the integrin. Peptide **(130)** is a combination of a cyclic hexapeptide with the SDMS sequence with an arginine residue connected via a linker to mimic the synergistic activity of Arg^{883} located 30 Å apart from the binding epitope. This peptide **(130)** completely suppresses the binding of laminin-5 to the $\alpha_3\beta_1$ integrin and the IC_{50} value is in the μM range. Still better inhibitory activity could be achieved by optimization of the length and the flexibility of the peptide chain between Arg residue and the cyclic part of the peptide. Also incorporation of the third synergistic residue of invasin, Asp^{811} should improve the inhibitory affinity of this molecule.

The Arg-Gly-Asp (RGD) sequence serves as the primary integrin recognition site in extracellular matrix proteins. Peptides containing this sequence can prevent binding of matrix proteins to integrins. RGD peptides that are specific for individual integrins are of considerable interest and medical significance. Small cyclic peptides containing this sequence are already in clinical trial. Affinity and selectivity are important for medical applications. Both could be achieved by locking the RGD amino acid sequence in an appropriate conformation. Three peptides, c-(Arg-Gly-Asp-D-Phe-β-hPro-) **(146)**, c-(Arg-Gly-Asp-Phe-Cpr(OMe)-) **(149)** and c-(Arg-Gly-Asp-Cpr(OMe)-Val-) **(151)**, all with one unnatural β-amino acid residue that exerts a strong conformational bias, were synthesized using both solid phase cyclization and cyclization in solution strategies. Hydrophobicity of the residue following the RGD triad is also very important for the binding affinity of the RGD peptides. For investigation of this influence, two cyclic tetrapeptides, c-(Arg-Gly-Asp-D-1-Nal-) **(154)** and c-(Arg-Gly-Asp-2-β-hNal-) **(155)** with D-1-naphtylalanine and β-homo-2-naphtylalanine, respectively, were synthesized by cyclization in solution. These peptides were tested as inhibitors of the interaction between OV-MZ-6 carcinoma cells with three different extracellular matrix proteins (vitronectin, fibronectin and collagen typ I). The cyclopentapeptide c-(Arg-Gly-Asp-Cpr(OMe)-Val-) **(151)**, although present as a diastereomeric mixture, is a very strong inhibitor of the interaction of $a_v ß_3$ with vitronectin. Cyclotetrapeptide c-(Arg-Gly-Asp-D-1-Nal-) **(154)** shows also good inhibitor affinity. Both are very good starting points for developing even stronger and more selective $a_v ß_3$ antagonists.

Peptide synthetases are very large and complex multienzymes needed for nonribosomal peptide synthesis in bacteria and fungi. The mechanism of action of these enzymes is modular. Each module activates and modifies specific amino acid and forms the peptide bond. Modules are made up of distinct domains that catalyze different reactions. It was shown that a carboxy terminal thioesterase domain of peptide synthetase, that is involved in cyclization and product release can also catalyze macrocyclization, and elongation in the case of symmetric cyclic peptides, independently of upstream domains. Linear peptides with a C-terminal thioester are key intermediates for this enzymatic macrocyclization. These precursors were synthesized on Kenner's sulfonamide "safety-catch" linker. N-acetylcysteamine is used for nucleophilic displacement and cleavage of the peptides from the activated linker. Cyclizations of linear precursors **(136)** and **(142)** of the antibiotic tyrocidine A and its analogs proceed smoothly and with negligible rate of hydrolysis using isolated and purified TycC TE domain.

6. EXPERIMENTAL PART

6.1. INSTRUMENTS AND MATERIALS

6.1.1. Instruments

Centrifuge:
Eppendorf centrifuge 5810 R

Circular Dichroism:
Jobin-Yvon mark VI dichrograph

Elemental Analysis:
CHN-O-Rapid (Heraeus)
Vario EL (Heraeus)
CHNS-932 (Leco)

Flash Chromatography:
Silica Gel 60, 40-63 μm (Merck)
Silica Gel 60, 15-40 μm (Merck)

FTIR Spectroscopy:
FT-IR-Spectrometer Genesis (Mattson)
Bruker IFS-55 FTIR
Jasco FT/IR-410 spectrometer

HPLC (Preparative):

Software:	Chromquest
Pump:	Thermo Separation Products P 4000
Controller:	Thermo Separation Products SN 4000
Detector:	Thermo Separation Products UV 1000
Columns:	Vydac^{TM} 218 TP 1022 Efficiency, C_{18} 250x220 mm,
	Vydac^{TM} 300, C_{18}, 10 μm, 250x220 mm
Eluent A:	95 % H_2O, 5 % ACN, 0.1 % TFA
Eluent B:	95 % ACN, 5 % H_2O, 0.1 % TFA

Methods used for preparative HPLC:

Method 1 (used for protected peptides):

Table 23: Preparative method for fully protected peptides.

Time (min)	Eluent A (%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	92	8	10	220 or 245
50	0	100		
60	0	100		
65	92	8		

Method 2 (used for deprotected peptides):

Table 24: Preparative method for cyclic deprotected peptides.

Time (min)	Eluent A (%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	92	8	25	220
10	92	8		
25	50	50		
30	92	8		

Method 3 (used for deprotected peptides):

Table 25: Preparative method used for separation of epimeric mixtures.

Time (min)	Eluent A (%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	92	8	25	220
20	68	32		
23	92	8		

HPLC (Analytical):

Software: Chromquest
Autosampler: Thermo Separation Products AS 100
Pump: Thermo Separation Products P 4000
Controller: Thermo Separation Products SN 4000
Detector: Thermo Separation Products UV 6000
Columns: Vydac^{TM} 300 C_{18} (218 TPB), 5 μm, 250x4 mm,
Phenomenex, Jupiter C_{18}, 5 μm, 250x 4.6 mm
Vydac^{TM} 218TP54 efficiency C_{18}, 5 μm, 250x4.6 mm
Eluent A: H_2O/ACN/TFA = 95 : 5 : 0.1
Eluent B: ACN/H_2O/TFA. = 95 : 5 : 0.1

Methods used for analytical HPLC:

Method 1:

Table 26: The gradient for analytical method 1.

Time (min)	Eluent A (%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	100	0	1	220 and 245
3	100	0		
40	0	100		
50	100	0		

Method 2:

Table 27: The gradient for analytical method 2.

Time (min)	Eluent A(%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	100	0	1	220 and 245
5	100	0		
25	50	50		
40	0	100		
45	100	0		

Method 3:

Table 28: The gradient for analytical method 3.

Time (min)	Eluent A(%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	100	0	1	220 and 245
5	100	0		
25	70	30		
30	100	0		

Method 4:

Table 29: The gradient for analytical method 4.

Time (min)	Eluent A (%)	Eluent B (%)	Flow (ml/min)	UV (nm)
0	100	0	1	220 and 245
15	100	0		
35	0	100		
40	100	0		

MALDI-ToF Mass Spectrometer:

Voyager-DE™ BioSpectrometry™ Workstation (PerSeptive Biosystems)

Laser:	Nitrogen Laser, α = 337 nm
Delay-Time:	100 ns
Accelerating Voltage:	20000 V
Grid Voltage:	93.8 %
Guide Wire Voltage:	0.05 %
Scans:	up to 50
Matrix:	2,5-dihydroxy benzoic acid or
	α-cyano-4-hydroxycinnamic acid

Melting Point Apparatus (uncorrected values):

Tottoli Melting Point Apparatus (Büchi)

Melting Point B-540 (Büchi)

Electrothermal® Melting Point Apparatus IA 6304 (Electrothermal)

NMR-Spectroscopy:

Gemini 200 (Varian): ^{1}H: 199.975 MHz, ^{13}C: 50.289 MHz

Gemini 2000 (Varian): ^{1}H: 200.041 MHz, ^{13}C: 50.305 MHz
DRX 500 (Bruker): ^{1}H: 500.130 MHz, ^{13}C: 125.758 MHz
AC 250-P (Bruker): ^{1}H: 250.133 MHz, ^{13}C: 62.896 MHz

Optical Rotations:
Digital Polarimeter DIP-360 (Jasco)
Polatronic-D (Schmidt & Haensch), Sodium D-Line

Sonification Baths:
Sonorex RK 510 H (Bandelin)
Sonorex TK 52 (Bandelin)
T 480/H Digital (Elma)

Peptide synthesizer:
Advanced ChemTech 496 MOS
Advanced ChemTech ACT 90.

Shaker:
IKA® KS 130 basic

TLC:
Silica Gel 60 F_{254} on aluminum sheets (Merck)

Two-injection pump:
Kd Scientific model 200

6.1.2. Materials

Commercially available amino acids, coupling reagents and resins were purchased from: Advanced ChemTech, Bachem, Fluka, and Novabiochem.
Trifluoroethanol (TFE, n.m.r. grade, ≥99.5 %), $NaClO_4$, $Al(ClO_4)_3 \times 9H_2O$, $Ca(ClO_4)_2 \times 4H_2O$ and $Mg(ClO_4)_2$ were purchased from Aldrich.
Fmoc-Phe-Cpr(OMe)-OH, and Fmoc-Asp(Ot-Bu)-Cpr(OMe)-OH were obtained from the group of Prof. Dr. O. Reiser, Institut für Organische Chemie, Universität Regensburg.

DMF:	Refluxed for 2 h over ninhydrine (100 mg/l) and then distilled under reduced pressure
Chloroform:	Distilled over calcium chloride and phosphorus pentoxide

Ethylacetate: Distilled from calcium chloride
Diethylether: Distilled from calcium chloride and finally from sodium
Petrolether: Distilled from calcium chloride and finally from sodium
Methylenchloride: Distilled from calcium chloride and finally from calcium hydride
THF: Distilled from calcium chloride and finally from sodium
All mixtures of the solvents are given in the volume percents (v/v).

HPLC solvents:

Acetonitrile**:** Merck (gradient grade)
Water: Purified Millipore water purification system
TFA: Solvay Fluor and Derivate GMBH

6.1.3. Biological Experiments

Binding activity of laminin-5 on $\alpha_3\beta_1$ integrin in the presence of cyclic inhibitors peptide inhibitors were performed by Dr. Johannes Eble, Institut für Physiologische Chemie und Pathobiochemie, Universität Münster, Germany.

Cyclization of the linear precursors of Tyrocidine A analogs by the carboxy terminal thioesterase domain of peptide synthetase were performed in the group of Prof. Dr. A. Marahiel, Biochemie/Fachbereich Chemie, Philipps-Universität Marburg, Germany.

Adhesions tests of human ovarian carcinoma cells on the extracellular matrix proteins in the presence of RGD cyclic peptide inhibitors were performed by Dr. Ute Reuning at the Frauenklinik der Technische Universität München, Germany.

6.2. SYNTHESIS OF β-HOMOAMINO ACIDS

6.2.1. General Procedure

The Fmoc protected α-amino acid derivative (5-15 mmol) is dissolved in dry THF (5 ml/mmol), triethylamine (1.0 equiv) and ethylchloroformate (1.0 equiv) are added subsequently at -15 °C. Stirring is continued for 15 min at the same temperature, then the solution is warmed up to 0 °C. A solution of diazomethane (3.0 equiv) in Et_2O is added slowly at 0 °C. The solution is allowed to reach room temperature and stirred for further 3 h. Excess diazomethane is decomposed by dropwise addition of AcOH. The mixture is washed with satd. $NaHCO_3$, satd. NH_4Cl and brine. The organic layer is dried over Na_2SO_4 and evaporated in vacuo. The resulting diazo ketone is dissolved in dioxane/water (5 : 1; 50 ml/mmol). After addition of silver benzoate (0.1 equiv) the mixture is sonicated using an ultrasound-cleaning bath for ca. 30 min. The reaction progress is monitored by TLC (EtOAc/petroleum ether 1:1 or/and DCM/MeOH/AcOH 10:1:0.1). When the reaction has reached completion, the solution is acidified to pH 2 with 1M HCl and extracted with Et_2O (1 x 100ml, 3 x 30 ml). The organic layers are pooled, dried over Na_2SO_4 and evaporated in vacuo. The resulting residue is crystallized from Et_2O and/or PE. The resulting Fmoc protected β-amino acids are used in the peptide synthesis without further purification.
For analysis small quantities of substances are purified by flash chromatography on silica gel with DCM/MeOH/AcOH = 10:0.5:0.1 as eluent.
It is possible to purify the intermediate diazo ketone by chromatography on neutral aluminum oxide with PE/EtOAc = 4:1 as eluent.

6.2.2.Synthesized β-Homoamino Acids

(3*R*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}butanoic acid (5)
Fmoc-D-β^3-hAla-OH

$C_{19}H_{19}NO_4$ (325.37 g/mol).
Synthesized from 1.56 g (5.0 mmol) Fmoc-D-Ala-OH.
Yield: 0.81 g (2.5 mmol), 50 %.
Mp.: 164 °C (Lit. 165-166 °C).[9a]
$[\alpha]_D^{24}$: -7.0 (c=1 in MeOH) (Lit. $[\alpha]_D^{20}$: -7.4, c=1. in MeOH).[9a]
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 326.14 $[M+H]^+$, 348.12 $[M+Na]^+$, 364.10 $[M+K]^+$
Found: 348.14 $[M+Na]^+$, 364.02 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3438m, 1712s, 1603s, 1510m, 1257m, 1008w.
^{1}H NMR (250 MHz, DMSO-d$_6$), δ (ppm): 1.09 (d, ^{3}J=6.5 Hz, 3H, H$^{\gamma}$), 2.27 (dd, ^{2}J=15.3 Hz, ^{3}J=7.3 Hz, 1H, H$^{\alpha}$), 2.45 (dd, ^{2}J=15.3 Hz, ^{3}J=6.5 Hz, 1H, H$^{\alpha}$), 3.86 (m, 1H, H$^{\beta}$), 4.18-4.30 (m, 3H, Fmoc CH_2, CH), 7.23 (d, ^{3}J=7.7 Hz, 1H, NH), 7.33-7.88 (m, 8H, H^{ar}), 12.10 (br, 1H, COOH).
^{13}C NMR (63 MHz, DMSO-d$_6$), δ (ppm): 20.37 (C$^{\gamma}$), 40.77 (C$^{\alpha}$), 43.71, 46.69 (CH), 65.10 (Fmoc CH_2), 119.99, 125.04, 126.95, 127.49 (CH_{ar}), 140.68, 143.90 (C_{ar}), 155.17 (N-C=O), 172.28 (C=O).
Element. Anal. (%): Calcd.: C 70.13, H 5.88, N 4.30
Found: C 70.26, H 5.95, N 4.29.

(3*S*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-5-oxo-5-(tritylamino)-pentanoic acid (6)
Fmoc-L-β^3-hAsn(Trt)-OH

$C_{39}H_{34}N_2O_5$ (610.72 g/mol).
Synthesized from 5 g (8.4 mmol) Fmoc-L-Asn(Trt)-OH.
Yield: 3.85 g (6.3 mmol), 75 %.
Mp.: 187 °C.
$[\alpha]_D^{24}$: -14.0 (c=0.25 in MeOH).
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 611.25 $[M+H]^+$, 633.24 $[M+Na]^+$, 649.21 $[M+K]^+$
Found: 633.39 $[M+Na]^+$, 649.42 $[M+K]^+$

IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3316m, 2969w, 1713s, 1517s, 1256m, 1047m, 740m.
^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 2.61 (dd, ^{2}J=15.4 Hz, ^{3}J=7.2 Hz, 1H, H^{α} or H^{γ}), 2.55-2.68 (m, 2H, H^{α}, H^{γ}), 2.78 (dd, ^{2}J=14.1 Hz, ^{3}J=4.1 Hz, 1H, H^{α} or H^{γ}), 4.09-4.31 (m, 4H, H^{β}, Fmoc CH_2, CH), 6.08 (d, ^{3}J=7.5 Hz, NH), 7.08 (s, 1H, NH), 7.16-7.76 (m, 23H, H^{ar}).
^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 37.83, 39.96 (CH_2), 45.78, 47.03 (CH), 67.00 (Fmoc CH_2), 70.92 (Trt C), 119.94, 125.12, 127.15, 127.69, 127.70 128.00, 128.57 (CH_{ar}), 141.21, 143.73, 144.13 (C_{ar}), 155.82 (N-C=O), 170.54, 174.63 (C=O).
Element. Anal. (%): Calcd.: C 76.69, H 5.61, N 4.58
Found: C 76.65, H 5.81, N 4.33.

(3*R*)-5-*tert*-butoxy-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-5-oxopentanoic acid (7)
Fmoc-L-β^{3}-hAsp(Ot-Bu)-OH

$C_{24}H_{27}NO_6$ (425.49 g/mol).
Synthesized from 2.05 g (5.0 mmol) Fmoc-L-Asp(Ot-Bu)-OH.
Yield: 0.82 g (1.9 mmol), 39 %.
Mp.: 90 °C (Lit. 88 °C).[74]
$[\alpha]_D^{24}$: 0.4 (c=2 in MeOH) (Lit. $[\alpha]_D^{28}$: 0.3, c=1.9 MeOH).[74]
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 426.19 $[M+H]^+$, 448.17 $[M+Na]^+$, 464.15 $[M+K]^+$
Found: 448.19 $[M+Na]^+$, 464.27 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3347w, 2977w, 2927w, 1720s, 1531m, 1261m, 1153m, 740m.
^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 1.43 (s, 9H, t-Bu), 2.54-3.00 (m, 4H, H^{α}, H^{γ}), 4.18-4.39 (m, 4H, H^{β}, Fmoc CH_2, CH), 5.65 (d, ^{3}J=8.8 Hz, 1H, NH), 7.28-7.73 (m, 8H, H^{ar}).
^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 28.04 (t-Bu, CH_3), 37.82, 39.19 (CH_2), 44.90, 47.15 (CH), 66.90 (Fmoc CH_2), 81.64 (t-Bu, C), 119.97, 125.08, 127.05, 127.70 (CH_{ar}), 141.29, 143.82 (C_{ar}), 155.63 (N-C=O), 170.51, 175.66 (C=O).

(3*S*)-6-amino-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-6-oxohexanoic acid (8)
Fmoc-L-β^3-hGln-OH

$C_{21}H_{22}N_2O_5$ (382.42 g/mol).
Synthesized from 3.68 g (10.0 mmol) Fmoc-L-Gln-OH.
Yield: 2.02 g (5.3 mmol), 53 %.
Mp.: 206 °C.
$[\alpha]_D^{24}$: 3.7 (c=1 in DMF).
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 383.16 $[M+H]^+$, 405.14 $[M+Na]^+$, 421.12 $[M+K]^+$
Found: 383.21 $[M+H]^+$, 405.20 $[M+Na]^+$, 421.15 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3397s, 3100m, 1695s, 1648s, 1535m, 1448m, 1267m.
^{1}H NMR (250 MHz, DMSO-d$_6$), δ (ppm): 1.58-1.68 (m, 2H, H^{γ}), 2.01-2.06 (m, 2H, H^{α}), 2.34-2.37 (m, 2H, H^{δ}), 3.75 (m, 1H, H^{β}), 4.19-4.26 (m, 3H, Fmoc CH_2, CH), 6.72 (br, 1H, NH), 7.24 (s, 1H, NH_2), 7.26 (s, 1H, NH_2), 7.32-7.87 (m, 8H, H^{ar}).
^{13}C NMR (63 MHz, DMSO-d$_6$), δ (ppm): 29.69, 31.48, 39.75 (CH_2), 46.79, 47.89 (CH), 65.18 (Fmoc CH_2), 120.83, 125.28, 127.15, 127.69 (CH_{ar}), 140.76, 143.94 (C_{ar}), 155.65 (N-C=O), 172.48, 174.02 (C=O).
Element. Anal. (%): Calcd.: C 65.95, H 5.80, N 7.33
Found: C 65.81, H 6.16, N 7.22.

(3*R*)-6-amino-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-6-oxohexanoic acid (9)
Fmoc-D-β^3-hGln-OH

Synthesized from 1.84 g (5.0 mmol) Fmoc-D-Gln-OH.
Yield: 0.80 g (2.1 mmol), 42 %.
$[\alpha]_D^{24}$: -3.6 (c=1 in DMF).
Element. Anal. (%): Calcd.: C 65.95, H 5.80, N 7.33
Found: C 65.81, H 6.20, N 7.24.

*Fmoc-L-Isoleucyldiazomethane **(10)***

$C_{22}H_{23}N_3O_3$ (337.45 g/mol).
Synthesized from 1.77g (5.0 mmol) Fmoc-L-Ile-OH.
Yield: 1.28 g (3.8 mmol), 76 %.
Mp.: 137-140 °C (Lit. 136-137 °C).[77]
^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 0.91-0.97 (m, 6H, 2xCH_3), 1.10-1.46 (m, 2H, H^{γ}), 1.85 (m, 1H, H^{β}), 4.18-4.25 (m, 2H, H^{α}, Fmoc CH), 4.37-4.45 (m, 2H, 2 Fmoc CH), 5.31 (s, 1H, CHN_2), 5.41 (d, ^{3}J=8.4 Hz, 1H, NH), 7.26-7.78 (m, 8H, H^{ar}).
^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 11.90, 16.04 (CH_3), 25.00 (CH_2), 38.05, 47.72, 55.32, 62.73 (CH), 67.11 (Fmoc CH_2), 120.50, 125.62, 127.62, 128.24 (CH_{ar}); 141.90, 144.34, (C_{ar}), 156.78 (N-C=O), 193.92 (C=O).

(3*R*,4*S*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-4-methylhexanoic acid (11)
Fmoc-L-β^3-hIle-OH

$C_{22}H_{25}NO_4$ (367.45 g/mol).
Synthesized from 1.28 g (3.8 mmol) Fmoc-L-Isoleucyldiazomethane **(10)**.
Yield: 0.92 g (2.5 mmol), 66 %.
Mp.: 133-135 °C (Lit. 138 °C).[74]
$[\alpha]_D^{24}$: 3.6 (c=1 in MeOH) (Lit. $[\alpha]_D^{28}$: 3.7, c=4.0. MeOH).[74]
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 368.19 $[M+H]^+$, 390.17 $[M+Na]^+$, 406.14 $[M+K]^+$
Found: 390.18 $[M+Na]^+$, 406.22 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3427br, 3327m, 2963m, 1700m, 1659m, 1544m, 1449m, 738m.
^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 0.77-0.91 (m, 6H, 2xCH_3), 1.05-1.22 (m, 1H, H^{δ}), 1.46-1.50 (m, 2H, H^{γ}, H^{δ}), 2.54-2.57 (m, 2H, H^{α}), 3.88 (m, 1H, H^{β}), 4.21 (t, ^{3}J=6.6 Hz, 1H, Fmoc CH), 4.39-4.23 (m, 2H, Fmoc CH_2), 5.17 (d, ^{3}J=10.0 Hz, 1H, NH), 7.26-7.75 (m, 8H, H^{ar}).
^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 11.82, 15.82 (CH_3), 25.95, 36.77 (CH_2), 38.52, 47.79, 52.80 (CH), 67.11 (Fmoc CH_2), 120.43, 125.52, 127.53, 128.14 (CH_{ar}); 141.81, 144.34, (C_{ar}), 156.56 (N-C=O), 177.54 (C=O).
Element. Anal. (%): Calcd.: C 71.91, H 6.85, N 3.81
Found: C 72.04, H 6.67, N 3.60.

(3*S*)-4-(5,8-dihydro-2-naphthalenyl)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]-amino}butanoic acid (12)
Fmoc-L-2-β^3-hNal-OH

$C_{29}H_{25}NO_4$ (451.53 g/mol).
Synthesized from 1.00 g (2.3 mmol) Fmoc-L-2-Nal-OH.
Yield: 0.69 g (1.5 mmol), 67 %.
Mp.: 144 °C.
$[\alpha]_D^{24}$: -15.1 (c=0.21 in MeOH).
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 452.19 $[M+H]^+$, 474.17 $[M+Na]^+$, 490.14 $[M+K]^+$
Found: 452.08 $[M+H]^+$, 474.14 $[M+Na]^+$, 490.12 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3329m, 3049w,2922w, 1695s, 1536s, 1448m, 1271s, 738s.
^{1}H NMR (250 MHz, DMSO-d$_6$), δ (ppm): 2.32-2.51 (m, 2H, H^α), 2.91-2.93 (m, 2H, H^γ), 3.31 (m, 1H, H^β), 4.13-4.20 (m, 3H, Fmoc CH_2, CH), 6.91 (br, 1H, NH), 7.21-7.97 (m, 15H, H^{ar}), 12.26 (br, 1H, COOH).
^{13}C NMR (63 MHz, CDCl$_3$), δ (ppm): 37.51, 40.40, (CH_2), 47.24, 49.32 (CH), 66.82 (Fmoc CH_2), 119.97, 125.00, 125.68, 126.18, 127.44, 127.60, 127.66, 127.94, 128.36, 128.49, 130.20, (CH_{ar}), 132.40, 133.51, 134.84, 141.33, 143.86 (C_{ar}), 171.45 (N-C=O), 176.70 (C=O).
Element. Anal. (%): Calcd.: C 77.14, H 5.58, N 3.10
Found: C 76.80, H 5.89, N 2.82.

Fmoc-L-phenylalanyldiazomethane (13)

$C_{25}H_{21}N_3O_3$ (411.47 g/mol).
Synthesized from 1.94 g (5.0 mmol) Fmoc-L-Phe-OH.
Yield: 1.40 g (3.4 mmol), 68 %.
Mp.: 128-130 °C (Lit. 136-137 °C).[78]
^{1}H NMR (200 MHz, acetone-d$_6$), δ (ppm): 3.23 (dd, ^{2}J =14.0 Hz, ^{3}J=5.0 Hz, 1H, H^β), 2.88 (dd, ^{2}J=13.8 Hz, ^{3}J=9.4 Hz, 1H, H^β), 4.17 (m, 1H, Fmoc CH), 4.30-4.47 (m, 3H, Fmoc CH_2, H^α), 5.87 (s, 1H, CHN_2), 6.86 (d, ^{3}J=8.6 Hz, 1H, NH), 7.28-7.86 (m, 13H, H^{ar}).

(3*S*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-4-phenylbutanoic acid (14)
Fmoc-L-β^3-hPhe-OH

$C_{25}H_{23}NO_4$ (401.47 g/mol).
Synthesized from 1.40 g (3.4 mmol) Fmoc-L-phenylalanyldiazomethane **(13)**.
Yield: 1.20 g (3.0 mmol), 88 %.
Mp.: 155 °C (Lit. 157 °C).[74]
$[\alpha]_D^{26}$: -23.6 (c=1 in MeOH) (Lit. $[\alpha]_D^{28}$: -25.0, c=0.6 in MeOH).[74]
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 402.17 $[M+H]^+$, 424.15 $[M+Na]^+$, 440.13 $[M+K]^+$
Found: 424.40 $[M+Na]^+$, 440.32 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3330m, 3062w, 2921w, 1697s, 1532m, 1447m, 1261m, 738m.
^{1}H NMR (200 MHz, acetone-d$_6$), δ (ppm): 2.55-2.59 (m, 2H, H^{α}), 2.90-2.94 (m, 2H, H^{γ}), 4.20-4.31 (m, 4H, H^{β}, Fmoc CH_2, CH), 6.52 (br. 1H, NH), 7.26-7.86 (m, 13H, H^{ar}).
^{13}C NMR (50 MHz, acetone-d$_6$), δ (ppm): 38.46, 40.50 (CH_2), 47.62, 50.52 (CH), 66.27 (Fmoc CH_2), 120.38, 125.74, 126.76, 127.53, 128.09, 128.77, 129.89 (CH_{ar}), 139.17, 141.74, 144.78 (C_{ar}), 156.20 (N-C=O), 172.35 (C=O).

(3*R*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-3-phenylpropanoic acid (15)
Fmoc-L-β^3-hPhg-OH

$C_{24}H_{21}NO_4$ (387.44 g/mol).
Synthesized from 2.80 g (7.5 mmol) Fmoc-L-Phg-OH.
Yield: 1.50 g (3.9 mmol), 52 %.
Mp.: 178-184 °C (Lit. 184 °C).[74]
$[\alpha]_D^{24}$: 19.6 (c=1 in DMF) (Lit. $[\alpha]_D^{25}$: -22.0, c=1 in $CHCl_3$).[75]
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 388.15 $[M+H]^+$, 410.14 $[M+Na]^+$, 426.11 $[M+K]^+$
Found: 388.30 $[M+H]^+$, 410.19 $[M+Na]^+$, 426.13 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3363m, 1704s, 1531m, 1284m, 740m.
^{1}H NMR (250 MHz, acetone-d$_6$), δ (ppm): 2.80 (m, 2H, H^{α}), 4.16-4.32 (m, 3H, Fmoc CH_2, CH), 5.17 (dd, ^{3}J=7.8 Hz, ^{3}J=6.6 Hz, 1H, H^{β}), 7.01 (br, 1H, NH), 7.20-7.42 (m, 13H, H^{ar}).

^{13}C NMR (63 MHz, acetone-d_6), δ (ppm): 41.45 (CH_2), 48.22, 53.08 (CH), 67.08 (Fmoc CH_2), 120.89, 126.19, 126.22, 127.51, 128.03, 128.04, 128.18, 128.59, 129.40 (CH_{ar}), 142.03, 143.47, 144.98 (CH_{ar}), 156.31 (N-C=O), 172.00 (C=O).

(3*S*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-3-phenylpropanoic acid (16)
Fmoc-D-β^3-hPhg-OH

Synthesized from 1.90 g (5.0 mmol) Fmoc-D-Phg-OH.
Yield: 1.10 g (3.9 mmol), 57 %.
$[\alpha]_D^{24}$: -21.5 (c=1 in DMF) (Lit. $[\alpha]_D^{24}$: -22.2, c=1 in DMF).[74]

Fmoc-L-prolyldiazomethane (17)

$C_{21}H_{19}N_3O_3$ (361.41 g/mol).
Synthesized from 1.67 g (5.0 mmol) Fmoc-L-Pro-OH.
Yield: 1.37 g (3.8 mmol), 76 %.
Mp.: yellow oil (Lit. 136-138 °C).[77]
^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 1.80-2.10 (m, 4H, 2H$^\beta$, 2H$^\gamma$), 3.45-3.48 (m, 2H, 2H$^\delta$), 4.10-4.53 (m, 4H, H$^\alpha$, Fmoc CH_2, CH), 5.30 (s, 1H, CHN_2), 7.26-7.77 (m, 8H, H^{ar}).

{(2*S*)-1-[(9*H*-fluoren-9-ylmethoxy)carbonyl]pyrrolidinyl}acetic acid (18)
Fmoc-L-β^3-hPro-OH

$C_{21}H_{21}NO_4$ (351.41 g/mol).
Synthesized from 1.37 g (3.8 mmol) Fmoc-L-prolyldiazomethane **(17)**.
Yield: 1.11 g (3.2 mmol), 84 %.
Mp.: 171 °C (Lit. 191-192 °C).[77]
$[\alpha]_D^{24}$: -37.6 (c=0.5 in MeOH) (Lit. $[\alpha]_D^{20}$: -33.6, c=0.9, in DMF).[77]
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 352.15 $[M+H]^+$, 374.14 $[M+Na]^+$, 390.11 $[M+K]^+$
Found: 352.28 $[M+H]^+$, 374.35 $[M+Na]^+$, 390.31 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3453s, 3065m, 2926w, 1689vs, 1623s, 1436m, 1336m, 739m.

^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 1.67-2.14 (m, 4H, H^{γ}, H^{δ}), 2.39 (m, 1H, H^{α}), 3.04 (m, 1H, H^{α}), 3.39-3.42 (m, 2H, H^{ε}), 4.20-4.56 (m, 4H, H^{β}, Fmoc CH_2, CH), 7.27-7.75 (m, 8H, H^{ar}), 9.40 (br, 1H, COOH).
^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 23.98, 31.39, 38.85, 47.02 (CH_2), 47.75 (CH), 54.79 (Fmoc CH), 67.61 (Fmoc CH_2), 120.50, 125.59, 127.57, 128.21, (CH_{ar}), 141.92, 144.54 (C_{ar}), 155.49 (N-C=O), 177.06 (C=O).
Element. Anal. (%): Calcd.: C 71.77, H 6.02, N 3.99
Found: C 71.63, H 6.29, N 3.85.

(3*S*)-4-*tert*-butoxy-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}butanoic acid (19)
Fmoc-L-β^3-hSer(t-Bu)-OH

$C_{23}H_{27}NO_5$ (397.48 g/mol).
Synthesized from 3.83 g (10.0 mmol) Fmoc-L-Ser(t-Bu)-OH.
Yield: 3.16 g (8.0 mmol), 80 %.
Mp.: 96-98 °C (Lit. 96-98°C).[9a]
$[\alpha]_D^{24}$: 20.8 (c=1, MeOH) (Lit. $[\alpha]_D^{20}$: 15.7, c=1, $CHCl_3$)[9a].
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 397.19 $[M+H]^+$, 420.18 $[M+Na]^+$, 436.15 $[M+K]^+$
Found: 420.24 $[M+Na]^+$, 436.21 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3353m, 2974m, 1697s, 1528m, 1449m, 1193m, 1061m, 739m.
^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 1.17 (s, 9H, t-Bu), 2.68-2.69 (m, 2H, H^{α}), 3.42-3.52 (m, 2H, H^{γ}), 4.17 (m, 1H, H^{β}), 4.23 (t, ^{3}J=7.2 Hz, 1H, Fmoc CH), 4.34-4.42 (m, 2H, Fmoc CH_2), 5.43 (d, ^{3}J=8.8 Hz, 1H, NH), 7.31-7.76 (m, 8H, H^{ar}).
^{13}C NMR (125 MHz, $CDCl_3$), δ (ppm): 27.34 (t-Bu, CH_3), 36.02 (CH_2), 47.18, 47.89 (CH), 62.52, 66.83 (CH_2), 73.42 (t-Bu, C), 119.97, 125.06, 127.04, 127.69 (CH_{ar}), 14.29, 143.85 (C_{ar}), 155.89 (N-C=O), 176.48 (C=O).
Element. Anal. (%): Calcd.: C 69.50, H 6.84, N 3.52
Found: C 69.48, H 6.64, N 3.41.

Fmoc-L-valyldiazomethane (20)

$C_{21}H_{21}N_3O_3$ (315.41 g/mol).
Synthesized from 1.70 g (5.0 mmol) Fmoc-L-Val-OH.
Yield: 1.50 g (4.8 mmol), 95 %.
Mp.: 129-130 °C (Lit. 123-125 °C).[78]

^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 0.90 (d, ^{3}J= 6.8 Hz, 3H, H^{γ}), 1.15 (d, ^{3}J= 6.8 Hz, 3H, H^{γ}), 2.08 (m, 1H, H^{β}), 4.12 (m, 1H, H^{α}), 4.22 (t, ^{3}J=6.8 Hz, 1H, Fmoc CH), 4.41-4.46 (m, 2H, Fmoc CH_2), 5.31 (s, 1H, CHN_2), 5.41 (d, ^{3}J=8.8 Hz, 1H, NH), 7.26-7.77 (m, 8H, H^{ar}).
^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 17.79, 19.79 (CH_3), 31.45, 47.70, 54.50 (CH), 67.30 (Fmoc CH_2), 120.49, 125.53, 127.61, 128.23 (CH_{ar}), 141.89, 144.33 (C_{ar}), 156.70 (N-C=O), 193.77 (C=O).

(3*S*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-4-methylpentanoic acid (21)
Fmoc-L-β^3-hVal-OH

$C_{21}H_{23}NO_4$ (353.42 g/mol).
Synthesized from 1.50 g (4.8 mmol) Fmoc-L-valyldiazomethane **(20)**.
Yield: 1.10 g (3.1 mmol), 65 %.
Mp.: 155-156 °C (Lit. 157 °C).[74]
$[\alpha]_D^{24}$: -19.0 (c=1.0 in $CHCl_3$) (Lit. $[\alpha]_D^{27}$: -17.9, c=0.6 in $CHCl_3$).[74]
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 354.17 $[M+H]^+$, 376.15 $[M+Na]^+$, 392.13 $[M+K]^+$
Found: 354.30 $[M+H]^+$, 376.31 $[M+Na]^+$, 392.31 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3460br, 2924s, 1658s, 1512m, 1413m, 1306m, 1150m, 740m.
^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 0.83 (d, ^{3}J= 6.2 Hz, 3H, H^{δ}), 0.93 (d, ^{3}J=6.2 Hz, 3H, H^{δ}), 1.85 (m, 1H, H^{γ}), 2.38-2.59 (m, 2H, H^{α}), 3.82 (m, 1H, H^{β}), 4.21 (t, ^{3}J=6.6 Hz, 1H, Fmoc CH), 4.38-4.43 (m, 2H, Fmoc CH_2), 5.15 (d, ^{3}J=9.6 Hz, 1H, NH), 7.26-7.75 (m, 8H, H^{ar}), 8.80 (br, 1H, COOH).
^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 19.01, 21.12 (CH_3), 32.03 (CH), 37.22 (CH_2), 47.73 (CH), 53.97 (Fmoc CH), 67.10 (Fmoc CH_2), 120.46, 125.55, 127.56, 128.18 (CH_{ar}), 141.86, 144.41 (C_{ar}), 156.70 (N-C=O), 177.67 (C=O).
Element. Anal. (%): Calcd.: C 71.36, H 6.55, N 3.96
Found: C 71.29, H 6.56, N 3.94.

(3*R*)-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}-4-methylpentanoic acid (22)
Fmoc-D-β^3-hVal-OH

$C_{21}H_{23}NO_4$ (353.42 g/mol).
Synthesized from 1.70 g (5.0 mmol) Fmoc-D-Val-OH.

Yield: 1.10 g (3.1 mmol), 62 %.
$[\alpha]_D^{24}$: 19.9 (c=1.0 in $CHCl_3$).
Element. Anal. (%): Calcd.: C 71.36, H 6.55, N 3.96
Found: C 71.27, H 6.42, N 3.86.

6.3. SYNTHESIS OF α-DMB PROTECTED AMINO ACIDS

General Procedure for the Synthesis of Anhydrides

1.0 equiv NMM is added at room temperature to the suspension of Fmoc protected amino acid **(59)** or **(60)** in 10 ml DCM. The mixture is cooled to –20 °C and 1.0 equiv of ethylchloroformate is added dropwise. The mixture is allowed to reach room temperature and stirred at the same temperature for 20 min. Charcoal is added and the solution is filtered. DCM is evaporated under reduced pressure and the residue is crystallized from PE/DCM (anhydrides are used in the next step without further purification).

9*H*-fluoren-9-ylmethyl (3*S*)-2,5-dioxotetrahydro-3-furanylcarbamate **(61)**
Fmoc-L-Asp-Oanh
Synthesized from 6.00 g (17.0 mmol) Fmoc-L-Asp-OH **(59)**
Yield: 5.00 g (14.8 mmol), 87 % white crystals, mp 220 °C (Lit. 224-225 °C).[123]

9*H*-fluoren-9-ylmethyl (3*S*)-2,6-dioxotetrahydro-2*H*-pyran-3-ylcarbamate **(62)**
Fmoc-L-Glu-Oanh
Synthesized from 6.30 g (18.0 mmol) Fmoc-L-Glu-OH **(60)**
Yield: 5.80 g (17.0 mmol), 94 %, mp. 132-135 °C (Lit. 134-136 °C).[123]

(3*S*)-4-[(2,4-dimethoxybenzyl)oxy]-3-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]-amino}-4-oxobutanoic acid (64)
Fmoc-L-Asp-ODmb

$C_{28}H_{27}NO_8$ (505.53 g/mol).
Procedure:
A solution of 1.0 equiv of 2,4-dimethoxybenzylalcohol **(63)** (2.47 g, 14.7 mmol) and 1.3 equiv dicyclohexylamine (3.50 g, 19.1 mmol) in 10 ml dry DCM is slowly added to a suspension of Fmoc protected anhydride **(61)** (5.20 g, 14.7 mmol) in dry THF (10 ml). 300 ml Dry Et_2O is added and the suspension is stirred at room

temperature overnight. Crystals are filtered, washed with Et_2O and recrystallized from 450 ml 96 % EtOH.

Yield: Fmoc-Asp-ODmb dicyclohexylamine salt 5.50 g (8.2 mmol), 55 % white crystals, mp 156 °C.

Crystals are suspended in 20 ml EtOAc. 20 ml 10 % citric acid is added, and mixture is stirred at room temperature for 30 min. The layers are separated and the organic layer is washed 3 times with water, dried over sodium sulfate and evaporated. The product is purified by flash chromatography on silica gel with DCM/i-PrOH = 10:1.

Yield: 3.03 g (4.00 mmol), 33.3 %.

Mp.: 79 °C.

$[\alpha]_D^{25}$: 15.4 (c=1 in $CHCl_3$).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 506.18 $[M+H]^+$, 528.16 $[M+Na]^+$, 544.14 $[M+K]^+$

Found: 528.39 $[M+Na]^+$, 544.37 $[M+K]^+$

IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3347m, 2967w, 1725s, 1700s, 1617m, 1268m, 1209m, 1157m.

^1H NMR (500 MHz, $CDCl_3$), δ (ppm): 2.93 (dd, ^2J=17.6 Hz, ^3J=4.3 Hz, 1H, H^β), 3.06 (dd, ^2J=17.3 Hz, ^3J=4.6 Hz, 1H, H^β), 3.75 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 4.19 (dd, ^3J=7.2 Hz, ^3J=7.2 Hz, 1H, Fmoc CH), 4.32 (dd, ^2J=10.4 Hz, ^3J=7.2 Hz, 1H, Fmoc CH_2), 4.42 (dd, ^2J=10.7 Hz, ^3J=6.9 Hz, 1H, Fmoc CH_2), 4.65 (m, 1H, H^α), 5.18 (s, 2H, Dmb CH_2), 5.84 (d, ^3J=8.8 Hz, 1H, NH), 6.36-6.43 (m, 2H, $DmbH^{ar}$), 7.20-7.75 (m, 9H, H^{ar}).

^{13}C NMR (50 MHz, $CDCl_3$), δ (ppm): 36.37 (CH_2), 47.05, 50.30 (CH), 55.34 (CH_3), 63.46, 67.31 (CH_2O), 98.48, 103.87, 119.95, 125.15, 127.08, 127.70, 131.58 (CH_{ar}), 115.78, 141.25, 143.66, 156.05, 159.03 (C_{ar}), 161.45 (N-C=O), 170.57, 175.52 (C=O).

(4*S*)-5-[(2,4-dimethoxybenzyl)oxy]-4-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]-amino}-5-oxopentanoic acid (66)

Fmoc-L-Glu-ODmb

$C_{29}H_{29}NO_8$ (519.53 g/mol).

Procedure:

1.6 ml (14.0 mmol) N-methylmorpholine are added to a suspension of 2.55 g (6.9 mmol) Fmoc-L-Glu-OH **(24)** and 1.20 g (7.0 mmol) of 2,4-dimethoxybenzyl alcohol **(31)** in 100 ml dry DCM. The mixture is stirred at room temperature until the Fmoc-Glu-OH **(24)** is dissolved. The solution is cooled to -20 °C and 3.55 ml (7.1

mmol) of a 20 % solution of phosgene in toluene is added dropwise. The mixture is allowed to reach room temperature and stirred overnight.
DCM is evaporated under reduced pressure, and the product is purified by flash chromatography on silica gel with DCM/i-PrOH = 10:1 as eluent. Two isomers are isolated with ratio α/γ=35.
Yield: 2.80 g (5.4 mmol), 78 %.
Mp.: 98 °C.
$[\alpha]_D^{24}$: -7.4 (c=0.5 in MeOH).
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 520.20 $[M+H]^+$, 542.18 $[M+Na]^+$, 558.15 $[M+K]^+$
Found: 542.45 $[M+Na]^+$, 558.48 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3332m, 2961m, 1748s, 1692s, 1614m, 1535m, 1211m, 1158m.
^{1}H NMR (250 MHz, DMSO-d$_6$), δ (ppm): 1.84-2.18 (m, 2H, H^{β}), 2.34-2.44 (m, 2H, H^{γ}), 3.79 (s, 6H, OCH_3), 4.18 (m, 1H, Fmoc CH), 4.36-4-46 (m, 3H, Fmoc CH_2, H^{α}), 5.09 (d, ^{2}J=11.8 Hz, 1H, Dmb CH_2), 5.19 (d, ^{2}J =11.8 Hz, 1H, Dmb CH_2), 5.52 (d, ^{3}J=7.9 Hz, 1H, NH), 6.41-6.43 (m, 2H, DmbH^{ar}), 7.19-7.73 (m, 9H, H^{ar}).
^{13}C NMR (50 MHz, DMSO-d$_6$), δ (ppm): 27.68, 29.71 (CH_2), 47.20, 53.36 (CH), 55.39, 55.41 (CH_3O), 63.28, 67.12 (CH_2O), 98.62, 104.10, 119.99, 125.08, 127.11, 127.73, 131.72 (CH_{ar}), 98.62, 141.32, 143.75, 155.98, 159.14 (C_{ar}), 161.67 (N-C=O), 171.77, 177.40 (C=O).

2-(9H-Fluoren-9-ylmehtoxycarbonylamino)-pentanedioic acid 5-(2,4-dimethoxy-benzyl) ester (67)
Fmoc-L-Glu(ODmb)-OH

$C_{29}H_{29}NO_8$ (519.53 g/mol).
Yield: For the analysis small quantity (~80 mg, 0.15 mmol, 2 %) in above reaction is isolated as by-product.
Mp.: 90 °C.
MALDI-ToF MS$_{monoisotopic}$, m/z:
Calcd.: 520.20 $[M+H]^+$, 542.18 $[M+Na]^+$, 558.15 $[M+K]^+$
Found: 542.34 $[M+Na]^+$, 558.40 $[M+K]^+$
IR (KBr), $\tilde{\nu}$ (cm^{-1}): 3383m, 3319m, 2923m, 1722s, 1656m, 1591m, 1511m, 1293m.
^{1}H NMR (200 MHz, DMSO-d$_6$), δ (ppm): 1.79-2.04 (m, 2H, H^{β}), 2.35-2.41(m, 2H, H^{γ}), 3.74 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 3.97 (m, 1H, Fmoc CH), 4.06-4.41 (m, 3H, Fmoc CH_2, H^{α}), 4.99 (s, 2H, CH_2O), 6.48-6.56 (m, 2H, DmbH^{ar}), 7.20-7.87 (m, 9H, H^{ar}).

^{13}C NMR (50 MHz, DMSO-d_6), δ (ppm): 26.46, 30.16 (CH_2), 46.65, 53.29 (CH), 55.16, 55.46 (OCH_3), 60.91, 65.51 (CH_2O), 99.35, 104.52, 120.02, 125.16, 126.99, 127.55, 130.90 (CH_{ar}), 115.99, 140.63, 143.74, 155.93, 158.46 (C_{ar}), 160.87 (N-C=O), 172.25, 173.62 (C=O).

6.4. PEPTIDE SYNTHESIS

6.4.1. Attachment of the First Amino Acid on the Resin

6.4.1.1. PROTOCOL 1: Attachment of the First Amino Acid on Wang Resin

In a round-bottom flask Wang resin is covered with DMF and allowed to swell for 30 min.
In another round-bottom flask 1.0 – 10.0 equiv (1.5 equiv for cyclization on resin) of amino acid (relative to resin capacity) is dissolved in dry DCM under argon atmosphere. A small amount of DMF may be needed to achieve complete dissolution. This solution is cooled to 0 °C and 5.0 equiv of DIC (relative to resin capacity) are slowly added. The mixture is stirred for 20 min at the same temperature and DCM is evaporated. The residue is dissolved in a minimum of DMF and added to the resin suspension followed by 0.1 equiv of DMAP (relative to resin capacity). The suspension is shaken at room temperature for 1 h. In order to determine the resin loading, a small quantity of resin is removed and washed with DMF, DCM, MeOH. Loading should be 0.4 – 0.8 mmol/g resin for synthesis of the cyclic peptides with cyclization on resin. For capping the rest of the free hydroxyl groups, 2.0 equiv of acetic anhydride (relative to resin capacity) and 2.0 equiv of pyridine (relative to resin capacity) are added to the reaction mixture. The mixture is shaken at room temperature for additional 30 min. Resin is filtered, washed with DMF (3x), DCM (3x), MeOH (3x) and dried.

6.4.1.2. PROTOCOL 2: Attachment of the First Amino Acid on "Safety Catch" Resin

In a round-bottom flask the resin is covered with $CHCl_3$ (1 ml/100 mg resin) that was previously filtered through a plug of basic alumina. 5.0 Equiv of DIPEA (relative to resin capacity) and 3.0 equiv of an amino acid (relative to resin capacity) are added to this mixture. The reaction mixture is stirred for 10 min at

room temperature followed by cooling to –20 °C. After mixing for 20 min at -20 °C, 3.0 equiv of PyBOP (relative to resin capacity) are added to the reaction mixture, as a solid, and the reaction mixture is stirred for 8 h at the same temperature, filtered, washed with $CHCl_3$ (3x), DMF (3x), DCM (3x), MeOH (3x) and dried under vacuum.

6.4.1.3. PROTOCOL 3: Attachment of the First Amino Acid on 2-Chlorotrityl chloride Resin

2.0 Equiv of an amino acid (relative to resin capacity) are dissolved in DCM (approximately 10 ml per gram of resin). A small amount of DMF may be needed to achieve complete dissolution. This solution is added to the resin followed by 2.0 equiv (relative to resin capacity) of DIPEA. The mixture is agitated at room temperature for 5 min. 3.0 Equiv (relative to resin capacity) of DIPEA are added and mixture is agitated at room temperature for additional 1 h.
Methanol (1 ml/g resin) is added to the mixture and shaking is continued for 15 min at room temperature. The resin is drained, washed with DCM (3x), DMF (3x), DCM (3x), MeOH (3x), and dried in vacuum. After the determination of the resin loading, the Fmoc protective group is removed for long term storage.

Removal of the Fmoc protective group:
The resin is swollen in DMF for 20 min and drained. A solution of 2 % DBU and 2 % piperidine in DMF (5 ml/g resin) is added. The mixture is shaken for 5 min at room temperature and drained. An additional portion of the solution of 2 % DBU and 2 % piperidine in DMF (5 ml/g resin) is added. The mixture is shaken at room temperature for 20 min, drained, washed with DMF (3x), DCM (3x), MeOH (3x), and dried in vacuum.

6.4.1.4. PROTOCOL 4: Attachment of the First Amino Acid on Rink Amide Resin

The resin is swollen in DMF and a solution of 20 % piperidine in DMF is added to cleave the resin-bound Fmoc groups. The reaction mixture is shaken for 20 min at room temperature. The resin is drained and washed with DMF (5x). Alternatively, the previously described protocol for Fmoc cleavage can be used.
A solution of 3.0 equiv of amino acid (relative to resin capacity) in DMF is added to the resin. The reaction mixture is shaken at room temperature for 5 min. A solution

of 3.0 equiv TBTU (relative to resin capacity) in DMF (total volume of DMF is about 15 ml/g resin) is added to the reaction mixture, followed by 6.0 equiv of DIPEA (relative to resin capacity). The reaction mixture is shaken for 45 min at room temperature.
The resin is drained and washed with DMF (3x), DCM (3x), MeOH (3x). Small quantities of resin are tested for unreacted amine with Kaiser test.[189] If the test is positive, the coupling reaction (with 3.0 equiv amino acid, 3.0 equiv TBTU, 3.0 equiv DIPEA) is repeated.

6.4.1.5. PROTOCOL 5: Estimation of Level of First Residue Attachment

The cuvetee of the UV spectrophotometer is filled with 3 ml of a 20 % solution of piperidine in DMF and placed in a spectrophotometer. Absorbance at 290 nm is adjusted to zero. 1 - 2 mg of resin are added. The resin is shaken for 5 min and allowed to settle on the bottom of the cell. The cell is placed in the spectrophotometer and absorbance at 290 nm is read.

Fmoc loading: mmol amino acid/g resin = Abs_{sample}/(1.65 x mg of resin)

6.4.2. Solid Phase Peptide Synthesis

Table 30: Protocol for peptide automated solid phase synthesis

Step	Operation	Reagent	Volume/ ml	Repeats Time/min
1[a]	Swelling	DMF	5	2 x 10
2[b]	Deprotection	2 % DBU, 2 % piperidine in DMF[c]	5	1 x 5 1 x 20
3[b]	Washing	DMF	5	7 x 7
4	Coupling	3.0 equiv Xaa 3.0 equiv TBTU 6.0 equiv DIPEA in DMF	3	1 x 45
5	Washing	DMF	5	5 x 7

[a] Only for the first amino acid

[b] On the end of the synthesis both steps are repeated to obtain the deprotected peptides

[c] To the solution for deprotection, HOBt (0.1 M) can be added to suppress side reactions like aspartimide formation.
The protocol is used for the manual and automated peptide synthesis on 200–300 mg of different resins (Wang, 2-ClTrt, Safety Catch, Rink amide resin). All steps are carried out at room temperature. The peptides synthesized by this protocol were fully protected (Fmoc on the N- terminus, resin at the C-terminus). For the cyclization peptides should be deprotected at the N-terminus, for that purpose on the end of the syntheses step 2 and 3 can be employed.

6.4.3. Cyclization of the Peptides

6.4.3.1. PROTOCOL 6: Cyclization on Wang and Rink Amide Resin

The N-terminus of the fully protected peptide is deprotected by the protocol previously described (chapter 6.4.1.3, P. 113).
The peptidyl resin is washed with DMF (7 x) and DCM (5 x).

Deprotection of the C-terminal carboxy group:
A solution of 1 % TFA in DCM (2 ml/100 mg resin) is added to the resin. The reaction mixture is shaken for 5 min at room temperature and filtered. This is repeated 20 times. The resin-bound peptide is washed with DCM (5 x) and DMF (5 x).

Cyclization:
A solution of 1.0-3.0 equiv (relative to resin loading) of coupling reagent (HATU, PyAOP, DIC/HOAt) in 3 ml DMF followed by 6.0 equiv of DIPEA (relative to resin loading) is added to the resin. The reaction mixture is shaken for 45 min at room temperature and filtered. A small quantity of resin is washed with DMF (7 x) and DCM (5 x), and the peptide is cleaved from the resin, using protocol 10 (chapter 6.4.4.2, P. 118). The progress of the cyclization is checked by MALDI-ToF MS or/and analytical HPLC. If the linear peptide is still detected, the coupling step is repeated. After the cyclization is complete, the resin is washed with DMF (7 x) and DCM (5 x). The cyclic peptide is cleaved from the resin and completely deprotected following protocol 10 (chapter 6.4.4.2, P. 118).

6.4.3.2. PROTOCOL 7: Cyclization in Solution

By this method fully protected cyclic peptides are synthesized.

6.4.3.2.1. Method A

The protected linear peptide is dissolved in DMF, so that the final concentration is 0.2 mmol/l for penta- and hexapeptides or 0.1 mmol/l for tetrapeptides. 1.1 Equiv of a coupling reagent (HATU, PyAOP, DIC/HOAt) and 3.0 equiv of DIPEA are added to this solution. The solution is stirred for 30 min at room temperature and cyclization is monitored by MALDI-ToF mass spectroscopy. If the linear peptide is still present in solution, an additional quantity of coupling reagent is added to the solution. After the cyclization is complete, DMF is evaporated under high vacuum (temperature of the bath must not exceed 30 °C) and peptide is purified by reverse phase preparative HPLC or by filtration through a C_{18} plug.

6.4.3.2.2. Method B

A solution of the linear peptide in DMF is added to a solution of 3.0-6.0 equiv of DIPEA and 1.0 equiv of HOAt in DMF using a syringe pump. Simultaneously, with a second syringe pump a solution of 1.0-3.0 equiv (relative to the peptide) of the coupling reagent HATU in DMF is added at the same rate. Then the solution is stirred for 10 min after the addition of the reagents is finished. DMF is evaporated under high vacuum. The temperature of the bath must not exceed 30 °C and the peptide is purified by reverse phase preparative HPLC. If DMSO or NMP are used instead of DMF as a solvent, solution is diluted with water after cyclization, filtered through a small reverse phase C_{18} plug and washed with water (3x) and Et_2O (3x). The cyclic peptide is eluted from the plug with ACN or any other adequate solvent. The solvent is evaporated under reduced pressure and the cyclic peptide is purified by reverse phase preparative HPLC.

6.4.3.3. PROTOCOL 8: Cyclization on "Safety Catch" Resin

By this method fully protected cyclic peptides are synthesized. Peptide chain elongation follows the usual Fmoc protocol.

Protection of N-terminus with trityl protective group:
After completion of the linear peptide synthesis and Fmoc deprotection of the N-terminus of the peptide the resin is washed with DCM (3x). Then, a solution of 6.0 equiv of DIPEA (relative to resin loading) and 3.5 equiv of trityl chloride (relative to resin loading) in DCM (2 ml/100 mg resin) is added to the resin. The reaction mixture is shaken overnight at room temperature, drained and washed with DCM (3x), and NMP (3x).
Alternatively, the N-terminal amino acid could be introduced as N^{α}-trityl protected amino acid during the synthesis of linear peptide.

Activation of "Safety catch" resin:
After the N-terminus of the peptide has been protected with a trityl group and washed with DCM and NMP, NMP (1 ml/100 mg resin), 10.0 equiv DIPEA (relative to resin loading) and 25.0 equiv (relative to resin loading) of iodoacetonitrile (previously filtered through a plug of basic alumina) are added to the resin. The reaction mixture is protected from light and shaken for 24 h at room temperature. The resin is drained and washed with NMP (5x) and DCM (5x).

N-terminal deprotection:
To the activated and washed resin a solution of 3 % TFA and 5 % TIS in DCM is added and shaken at the room temperature for 2 h. The resin is drained and washed 3 times with DCM and 3 times with dry THF.

Cyclization and cleavage:
Dry THF (2 ml/100 mg resin) and 3.0 equiv of DIPEA (relative to resin loading) are added to the activated, deprotected and washed resin. The reaction mixture is shaken overnight at room temperature. The solution is collected and the resin is washed with THF (3x) and DCM (3x). The combined washings are evaporated under reduced pressure and the peptide is purified by reverse phase preparative HPLC.

6.4.4. Cleavage from the Resin and Deprotection of the Peptides

6.4.4.1. PROTOCOL 9: Cleavage of the Peptides from the 2-ClTrt Resin and Sasrin

Before peptide cleavage the resin is washed with DCM (3x). 1 % TFA in DCM (2ml/100 mg resin) is added to the washed resin and the reaction mixture is shaken for 5 min at room temperature. The solution is filtered into a round bottom flask, and immediately neutralized with pyridine. This procedure is repeated until no more peptide is present in the filtered solution (test with MALDI-ToF MS and/or analytical HPLC); usually 6 – 10 times. All filtrates are collected and evaporated under reduced pressure. 10 ml water is added and the suspension is filtered through a small column filled with reverse phase C_{18} material (the material should be washed with methanol and water before use). Column and flask are washed with water (3x) and Et_2O (3x). The cyclic peptide is eluted from the plug with ACN or any other adequate solvent. The solvent is evaporated under reduced pressure and the peptide is lyophilized.

6.4.4.2. PROTOCOL 10: Cleavage of the Peptides from Wang and Rink amide Resins

Before peptide cleavage the resin is washed with DCM (3x). One of the following cleavage cocktails (3 ml/100 mg of resin) is added to the washed resin:
95 % TFA, 2.5 % H_2O, 2.5 % TIS;
82.5 TFA, 5 % Phenol, 5 % H_2O, 5 % Thioanisol, 2.5 % EDT (Reagent K);
88 % TFA, 5 % Phenol, 2 % TIS, 5 % H_2O (reagent B).
The resin is shaken at room temperature for 2-20 h, the solvent is evaporated and cold diethyl ether is added to the residue. Diethyl ether is decanted, centrifuged for 1 h at 0 ºC with 4000 rpm, and again decanted. The residue (after both decantations) is dissolved in a minimum of solvent and purified by reverse phase preparative HPLC. Alternatively for preliminary purification before preparative HPLC and instead of precipitation with diethyl ether, the filtration method over a small C_{18} column described in protocol 9 could be used.

6.4.4.3. PROTOCOL 11: Cleavage of the Peptides from the "Safety Catch" Resin with Formation of a Thioester Bond at the C-terminus

Activation of "Safety catch" resin:

After the synthesis of the linear peptide (the N-terminus should be trityl or Boc protected) the resin is washed with DCM (3x) and NMP (3x). The NMP (1 ml/100 mg resin), 10.0 equiv (relative to resin loading) of DIPEA and 25.0 equiv (relative to resin loading) of iodoacetonitrile (previously filtered through a plug of basic alumina) are then added to the resin. The reaction mixture is protected from light and shaken for 24 h at room temperature. The resin is drained and washed with NMP (5x) and DCM (5x).

Cleavage of the peptide:

A solution of 50.0 equiv (relative to resin loading) ethyl-3-mercaptopropionate in DCM (1 ml/100 g resin), and 0.5 equiv (relative to resin loading) Na-thiophenolate dissolved in a minimum of DMF is added to the activated and washed resin. The mixture is shaken for 24 h at room temperature. The solvent is filtered and the resin washed with DCM. The combined filtrates are evaporated under reduced pressure and the peptide is purified by reverse phase preparative HPLC.

6.4.4.4. PROTOCOL 12: Cleavage of the Aloc Protective Group in Solution

24.0 Equiv of $PhSiH_3$ and a solution of 0.1 equiv $[Pd(PPh_3)_4]$ in DCM are added to the solution of the peptide in a minimum quantity of dry DCM (if the peptide is not soluble small amounts of DMF can be added) under argon. The mixture is stirred at room temperature for 10 min and the progress of the reaction is monitored by MALDI-ToF MS and/or analytical HPLC. After completion of reaction, DCM is evaporated and the product is purified by reverse phase preparative HPLC.

6.4.4.5. PROTOCOL 13: Complete Deprotection of the Cyclic Peptides

One of the following cleavage cocktails is added to the cyclic protected peptide:
95 % TFA, 2.5 % H_2O, 2.5 % TIS;
82.5 TFA, 5 % Phenol, 5 % H_2O, 5 % Thioanisol, 2.5 % EDT (Reagent K);
88 % TFA, 5 % Phenol, 2 % TIS, 5 % H_2O (reagent B).
The solution is shaken at room temperature for 2-20 h (depending on the peptide and protective groups), the solvent is evaporated and cold diethyl ether is added to the residue. Diethyl ether is decanted, centrifuged for 1 h on 0 ºC with 4000 rpm, and again decanted. The residue (after both decantations) is dissolved in a minimum of solvent and purified by reverse phase preparative HPLC. Alternatively for preliminary purification before preparative HPLC and instead of precipitation with diethyl ether, the filtration method over a small C_{18} column described in protocol 9 could be used.

6.4.5. Synthesis of Model Peptides

c-(-Leu-Ala-Val-Pro-Phe-Gly-) (33)

$C_{30}H_{44}N_6O_6$ (584.72 g/mol).
The linear peptide H-Leu-Ala-Val-Pro-Phe-Gly-OH **(27)** is synthesized on 150 mg (104 μmol) Fmoc-Gly-Sasrin (loading 0.69 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9), and purified by reverse phase preparative HPLC.
Yield (Linear peptide **(27)**): 50 mg, 83 μmol, 80 %.
HPLC (anal. method 3): t_R=16.5 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 603.35 $[M+H]^+$, 625.33 $[M+Na]^+$, 641.31 $[M+K]^+$
Found: 603.69 $[M+H]^+$, 625.66 $[M+Na]^+$, 641.59 $[M+K]^+$

The linear peptide **(27)** (50 mg, 83 μmol) is dissolved in 500 ml DMF and cyclized following protocol 7, method A:
1.1 equiv HATU (91 μmol, 34 mg).
3.0 equiv DIPEA (250 μmol, 32 mg, 42 μl).
After evaporation of DMF, the cyclic peptide **(33)** is purified by reverse phase preparative HPLC.
Yield (cyclic peptide **(33)**): 20 mg, 34 μmol, 41 %.

HPLC (anal. method 3): t_R=19.2 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 585.34 $[M+H]^+$, 607.32 $[M+Na]^+$, 623.30 $[M+K]^+$

Found: 585.52 $[M+H]^+$, 607.49 $[M+Na]^+$, 623.48 $[M+K]^+$

^{1}H NMR (500 MHz, $CDCl_3$), δ (ppm): 0.90 (m, 1H, H^{γ} (Pro)), 0.94 (d, ^{3}J= 6.3 Hz, 3H, H^{δ} (Leu)), 0.95 (d, ^{3}J= 6.3 Hz, 3H, H^{δ} (Leu)), 0.99 (d, ^{3}J= 6.3 Hz, 3H, H^{γ} (Val)), 1.12 (d, ^{3}J= 6.3 Hz, 3H, H^{γ} (Val)), 1.38 (d, ^{3}J=6.9 Hz, 3H, H^{β} (Ala)), 1.50-1.77 (m, 4H, $2H^{\beta}$ (Leu), H^{γ} (Leu), H^{γ} (Pro)), 1.89-2.06 (m, 2H, H^{β} (Val), H^{β} (Pro)), 2.22 (m, 1H, H^{β} (Pro)), 3.13 (dd, ^{2}J =14.1 Hz, ^{3}J=6.0 Hz, 1H, H^{β} (Phe)), 3.26 (dd, ^{2}J=14.1 Hz, ^{3}J=10.4 Hz, 1H, H^{β} (Phe)), 3.40 (dd, ^{2}J=14.1 Hz, ^{3}J=6.6 Hz, 1H, H^{α} (Gly)), 3.42-3.53 (m, 2H, $2H^{\delta}$ (Pro)), 3.58 (d, ^{3}J=8.8 Hz, 1H, H^{α} (Val)), 3.77 (dd, ^{2}J=14.1 Hz, ^{3}J =5.3 Hz, 1H, H^{α} (Gly)), 4.07 (ddd, ^{3}J =10.4 Hz, ^{3}J =5.0 Hz, ^{3}J =5.0 Hz, 1H, H^{α} (Leu)), 4.44 (d, ^{3}J =8.8, 1H, H^{α} (Pro)), 4.59 (ddd, ^{3}J =10.5 Hz, ^{3}J=8.6 Hz, ^{3}J=5.8 Hz, 1H, H^{α} (Phe)), 4.69 (qd, ^{3}J =9.1, ^{3}J =7.2, 1H, H^{α} (Ala)), 6.20 (d, ^{3}J =5.0 Hz, 1H, H^N (Leu)), 6.82 (s, 1H, H^N (Val)), 6.92 (d, ^{3}J=9.4 Hz, 1H, H^N (Ala)), 7.20-7.32 (m, 5H, H^{ar}), 7.66 (dd, ^{3}J=6.3 Hz, ^{3}J=5.7 Hz, 1H, H^N (Gly)), 9.03 (d, ^{3}J=8.2 Hz, 1H, H^N (Phe)).

c-(-Leu-Ala-Val-β-hPro-Phe-Gly-) (34)

$C_{31}H_{46}N_6O_6$ (598.75 g/mol).

The linear peptide H-Leu-Ala-Val-β-hPro-Phe-Gly-OH **(28)** is synthesized on 200 mg (152 μmol) Wang resin previously loaded with Fmoc-Gly-OH (loading 0.76 mmol/g), cleaved with reagent B during 2 h (protocol 10), and purified by preparative HPLC.

Yield (linear peptide **(28)**): 65 mg, 89 μmol, 59 %.

HPLC (anal. method 3): t_R=17.2 min, 84 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 617.37 $[M+H]^+$, 639.35 $[M+Na]^+$, 655.32 $[M+K]^+$

Found: 617.65 $[M+H]^+$, 639.59 $[M+Na]^+$, 655.54 $[M+K]^+$

The linear peptide **(28)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(28)** (65 mg, 89 μmol) in 10 ml DMF.

Second syringe: Solution of HATU (1.5 equiv, 133 μmol, 51mg) in 10 ml DMF.

Addition rate for both syringes: 0.05 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 267 μmol, 34 mg, 46 μl) in 20 ml DMF.

The cyclicpeptide **(34)** is purified by reverse phase preparative HPLC after evaporation of DMF.

Yield (cyclic peptide **(34)**): 45mg, 75 μmol, 76 %.
HPLC (anal. method 3): t_R=19.6 min, 90 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 599.36 $[M+H]^+$, 621.34 $[M+Na]^+$, 637.31 $[M+K]^+$
Found: 599.77 $[M+H]^+$, 621.73 $[M+Na]^+$, 635.75 $[M+K]^+$

^{1}H NMR (500 MHz, $CDCl_3$), δ *(ppm):* 0.83 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.86 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.91 (d, ^{3}J=6.3 Hz, 3H, H^γ (Val)), 0.96 (d, ^{3}J=6.3 Hz, 3H, H^γ (Val)), 1.47 (d, ^{3}J=7.5 Hz, 3H, H^β (Ala)), 1.53-1.69 (m, 2H, H^γ (β-hPro)), 1.75-1.85 (m, 2H, H^γ (Leu), H^β (Val)), 1.86-2.00 (m, 3H, 2H^β (Leu), H^δ (β-hPro)), 2.10-2.27 (m, 3H, 2H^α (β-hPro), H^δ (β-hPro)), 3.10 (dd, ^{2}J=14.4 Hz, ^{3}J =5.0 Hz, 1H, H^β (Phe)), 3.28 (dd, ^{2}J=14.4 Hz, ^{3}J =5.0 Hz, 1H, H^β (Phe)), 3.42 (dd, ^{2}J=17.6 Hz, ^{3}J=8.2 Hz, 1H, H^ε (β-hPro)), 3.69-3.86 (m, 2H, H^α (Gly), H^ε (β-hPro)), 3.96 (m, 1H, H^α (Leu)), 4.05 (m, 1H, H^α (Ala)), 4.24 (m, 1H, H^β (β-hPro)), 4.41 (m, 1H, H^α (Val)), 4.48 (dd, ^{2}J=17.3 Hz, ^{3}J=7.9 Hz, H^α (Gly)), 4.65 (m, 1H, H^α (Phe)), 5.60 (br, 1H, H^N (Phe)), 7.07-7.40 (m, 8H, 5H^{ar}, H^N (Ala), H^N (Leu), H^N (Val)), 8.23 (br, 1H, H^N (Gly)).

c-(-Ala-Val-Pro-Phe-Gly-) (35)

$C_{24}H_{33}N_5O_5$ (471.56 g/mol).
The linear peptide H-Ala-Val-Pro-Phe-Gly-OH **(29)** is synthesized on 300 mg (0.21 mmol) Fmoc-Gly-Sasrin (loading 0.69 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9), and purified by reverse phase preparative HPLC.
Yield (linear peptide **(29)**): 60 mg, 0.12 mmol, 59 %.
HPLC (anal. method 3): t_R=15.0 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 490.27 $[M+H]^+$, 512.25 $[M+Na]^+$, 528.22 $[M+K]^+$
Found: 512.27 $[M+Na]^+$, 528.29 $[M+K]^+$

The linear peptide **(29)** (60 mg, 0.12 mmol) is dissolved in 1000 ml DMF and cyclized following protocol 7, method A:
1.1 equiv HATU (0.14 mmol, 51 mg).
3.0 equiv DIPEA (0.37 mmol, 48 mg, 63 μl).
After evaporation of the solvent the cyclic peptide **(35)** is purified by reverse phase preparative HPLC.
Yield (cyclic peptide **(35)**): 25 mg, 53 μmol, 43 %.
HPLC (anal. method 3): t_R=18.1 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 472.26 $[M+H]^+$, 494.24 $[M+Na]^+$, 510.21 $[M+K]^+$

Found: 472.51 $[M+H]^+$, 494.46 $[M+Na]^+$, 510.46 $[M+K]^+$

^1H NMR (500 MHz, $CDCl_3$), δ (ppm): 0.87 (d, ^3J=6.9 Hz, 3H, H^γ (Val)), 0.90 (d, ^3J=6.9 Hz, 3H, H^γ (Val)), 1.38 (m, 1H, H^β (Pro)), 1.48 (d, ^3J=6.9 Hz, 3H, H^β (Ala)), 1.58 (m, 1H, H^γ (Pro)), 1.72 (m, 1H, H^γ (Pro)), 1.99 (m, 1H, H^β (Val)), 2.21 (m, 1H, H^β (Pro)), 2.93 (dd, ^2J=14.1 Hz, ^3J=9.7 Hz, 1H, H^β (Phe)), 3.00 (d, ^2J=14.4 Hz, 1H, H^α (Gly)), 3.18 (dd, ^2J=13.8 Hz, ^3J=5.0 Hz, 1H, H^β (Phe)), 3.48 (ddd, ^2J=11.6 Hz, ^3J=6.9 Hz, ^3J=6.9 Hz, 1H, H^δ (Pro)), 3.72 (ddd, ^2J=11.3 Hz, ^3J=6.9 Hz, ^3J=6.9 Hz, 1H, H^δ (Pro)), 4.00 (qd, ^3J=6.9 Hz, ^3J=11.0 Hz, 1H, H^α (Ala)), 4.55-4.62 (m, 2H, H^α (Val), H^α (Pro)), 4.92 (dd, ^2J=14.4 Hz, ^3J=10.6 Hz, 1H, H^α (Gly)), 5.23 (ddd, ^3J=9.9 Hz, ^3J=9.9 Hz, ^3J=5.2 Hz, 1H, H^α (Phe)), 7.04 (d, ^3J=10.5 Hz, 1H, H^N (Phe)), 7.12 (d, ^3J=6.9 Hz, 1H, H^N (Val)), 7.15-7.30 (m, 5H, H^{ar}), 7.9 (d, ^3J=10.1 Hz, 1H, H^N (Gly)), 8.56 (d, ^3J=10.7 Hz, 1H, H^N (Ala)).

c-(-Ala-Val-β-hPro-Phe-Gly-) (36)

$C_{25}H_{35}N_5O_5$ (485.59 g/mol).

The linear peptide H-Ala-Val-β-hPro-Phe-Gly-OH **(30)** is synthesized on 300 mg (0.21 mmol) Fmoc-Gly-Sasrin (loading 0.69 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9), and purified by reverse phase preparative HPLC.

Yield (linear peptide **(30)**): 68 mg, 0.14 mmol, 68 %.

HPLC (anal. method 3): t_R=16.0 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 504.28 $[M+H]^+$, 526.26 $[M+Na]^+$, 542.24 $[M+K]^+$

Found: 504.28 $[M+H]^+$, 526.11 $[M+Na]^+$, 542.06 $[M+K]^+$

The linear peptide **(30)** (68 mg, 0.14 mmol) is dissolved in 700 ml DMF and cyclized following protocol 7, method A:

1.1 equiv HATU (0.15 mmol, 56 mg).

3 equiv DIPEA (0.41 mmol, 52 mg, 69 µl).

After evaporation of DMF the cyclic peptide **(36)** is purified by reverse phase preparative HPLC.

Yield (cyclic peptide): 18 mg, 37 µmol, 28 %.

HPLC (anal. method 3): t_R=18.2 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 486.27 $[M+H]^+$, 508.25 $[M+Na]^+$, 524.23 $[M+K]^+$

Found: 486.48 $[M+H]^+$, 508.47 $[M+Na]^+$, 524.40 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 0.82 (d, ^{3}J=6.9, 3H, H$^\gamma$ (Val)), 0.87 (d, ^{3}J=6.8, 3H, H$^\gamma$ (Val)), 1.33 (d, ^{3}J=7.2 Hz, H, 3H$^\beta$ (Ala)), 1.46 (dddd, ^{2}J=12.1 Hz, ^{3}J=11.9 Hz, ^{3}J=11.9 Hz, ^{3}J=6.4 Hz, 1H, H$^\gamma$ (β-hPro)), 1.69 (m, 1H, H$^\delta$ (β-hPro)), 1.82 (m, 1H, H$^\delta$ (β-hPro)), 1.94-2.04 (m, 2H, H$^\alpha$ (β-hPro), H$^\gamma$ (β-hPro)), 2.16 (m, 1H, H$^\beta$ (Val)), 2.29 (dd, ^{2}J=15.1 Hz, ^{3}J=7.5 Hz, 1H, H$^\alpha$ (β-hPro)), 2.68 (dd, ^{2}J=14.3 Hz, ^{3}J= 11.7 Hz, 1H, H$^\beta$ (Phe)), 3.32 (dd, ^{2}J=14.3 Hz, ^{3}J= 2.7 Hz, 1H, H$^\beta$ (Phe)), 3.39 (ddd, ^{2}J=10.4 Hz, ^{3}J= 10.4 Hz, ^{3}J= 6.4 Hz, 1H, H$^\varepsilon$ (β-hPro)), 3.57-3.65 (m, 2H, H$^\alpha$ (Gly), H$^\varepsilon$ (β-hPro)), 3.82 (dd, ^{2}J= 13.5 Hz, ^{3}J= 5.3 Hz, 1H, H$^\alpha$ (Gly)), 3.90-3.98 (m, 2H, H$^\beta$ (β-hPro), H$^\alpha$ (Ala)), 4.35 (dd, ^{3}J=9.3 Hz, ^{3}J=3.3 Hz, 1H, H$^\alpha$ (Val)), 4.54 (ddd, ^{3}J=11.1 Hz, ^{3}J=8.7 Hz, ^{3}J=2.8 Hz, 1H, H$^\alpha$ (Phe)), 6.99 (d, J^3=9.4 Hz, 1H, H^N (Val)), 7.16-7.30 (m, 5H, H^{ar}), 7.86 (d, ^{3}J= 8.8 Hz, 1H, H^N (Phe)), 8.41 (dd, ^{3}J=5.4 Hz, ^{3}J=2.1 Hz, 1H, H^N (Gly)), 8.90 (d, ^{3}J=7.0 Hz, 1H, H^N (Ala)).

c-(-Ala-Pro-Phe-Gly-) (37)

$C_{19}H_{24}N_4O_4$ (372.43 g/mol).

The linear peptide H-Ala-Pro-Phe-Gly-OH **(31)** is synthesized on 290 mg (0.2 mmol) Fmoc-Gly-Sasrin (loading 0.69 mmol/g), cleaved with 1 % TFA in DCM (protocol 9), and purified by preparative HPLC.

Yield (linear peptide **(31)**): 47 mg, 0.12 mmol, 59 %.

HPLC (anal. method 2): t_R=12.0 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 391.20 $[M+H]^+$, 413.18 $[M+Na]^+$, 429.15 $[M+K]^+$

Found: 391.17 $[M+H]^+$, 413.13 $[M+Na]^+$, 429.14 $[M+K]^+$

The linear peptide **(31)** (47 mg, 120 μmol) is dissolved in 1.5 l DMF and cyclized following protocol 7, method A.

1.1 equiv HATU (130 μmol, 50mg).

3 equiv DIPEA (270 μmol, 34 mg, 46 μl).

The cyclic peptide **(37)** is purified by reverse phase preparative HPLC after evaporation of the solvent.

Yield (cyclic peptide **(37)**): 1 mg, 2 μmol, 2 %.

HPLC (anal. method 2): t_R=16.4 min, 90 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 373.19 $[M+H]^+$, 395.17 $[M+Na]^+$, 411.14 $[M+K]^+$

Found: 373.53 $[M+H]^+$, 395.51 $[M+Na]^+$, 411.50 $[M+K]^+$

c-(-Ala-β-hPro-Phe-Gly-) (38)

$C_{20}H_{26}N_4O_4$ (386.46 g/mol).

The linear peptide H-Ala-β-hPro-Phe-Gly-OH **(32)** is synthesized on 200 mg (150 μmol) Wang resin previously loaded with Fmoc-Gly-OH (loading 0.76 mmol/g), cleaved with reagent B during 2 h (protocol 10) and purified by preparative HPLC.

Yield (linear peptide **(32)**): 36 mg, 89 μmol, 59 %.

HPLC (anal. method 1): t_R=13.40 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 405.21 $[M+H]^+$, 427.20 $[M+Na]^+$, 443.17 $[M+K]^+$

Found: 405.48 $[M+H]^+$, 427.44 $[M+Na]^+$, 443.43 $[M+K]^+$

The linear peptide **(32)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(32)** (36 mg, 89 μmol) in 15 ml DMF.

Second syringe: Solution of HATU (1.5 equiv, 133 μmol, 51mg) in 15 ml DMF.

Addition rate for both syringes: 0.66 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 267 μmol, 34 mg, 46 μl) in 20 ml DMF.

The cyclic peptide **(38)** is purified by reverse phase preparative HPLC after evaporation of the solvent.

Yield (cyclic peptide **(38)**): 15 mg, 37 μmol, 41 %.

HPLC (anal. method 1): t_R=19.5 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 387.20 $[M+H]^+$, 409.19 $[M+Na]^+$, 425.16 $[M+K]^+$

Found: 387.47 $[M+H]^+$, 409.41 $[M+Na]^+$, 425.28 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 1.30 (d, ^{3}J=6.9 Hz, 3H, H^β (Ala)), 1.63 (m, 1H, H^γ (β-hPro)), 1.80-1.90 (m, 3H, $2H^\delta$ (β-hPro), H^γ (β-hPro)), 2.07 (dd, ^{2}J=13.5 Hz, ^{3}J=7.2 Hz, 1H, H^α (β-hPro)), 2.45 (dd, ^{2}J=13.5 Hz, ^{3}J=1.6 Hz, 1H, H^α (β-hPro)), 2.82 (dd, ^{2}J=13.8 Hz, ^{3}J=8.2 Hz, 1H, H^β (Phe)), 2.93 (dd, ^{2}J=14.1 Hz, ^{3}J=7.9 Hz, 1H, H^β (Phe)), 3.03 (dd, ^{2}J=14.7 Hz, ^{3}J=5.3 Hz, 1H, H^α (Gly)), 3.24 (m, 1H, H^ε (β-hPro)), 3.56 (m, 1H, H^ε (β-hPro)), 3.86 (dd, ^{2}J=14.4 Hz, ^{3}J=8.2 Hz,1H, H^α (Gly)), 4.20-4.40 (m, 2H, H^α (Ala), H^α (Phe)), 4.57 (m, 1H, H^β (β-hPro)), 6.91 (br, 1H, H^N (Ala)), 7.15-7.30 (m, 5H, H^{ar}), 8.56 (d, ^{3}J=5.7, 1H, H^N (Phe)), 8.66 (br, 1H, H^N (Gly)).

6.4.5.1. CD and FTIR Measurements

Circular dichroism:

Circular dichroism spectra are recorded in a 0.02 cm cell at room temperature, c~0.5-1 mg/cm^3 of peptide. Spectra are smoothed by the Savitzky-Golay algorithm.

Fourier transform infrared spectroscopy (FTIR):

Infrared spectroscopic measurements (at a resolution of 2 cm^{-1}) are performed at room temperature using 0.020-0.025 cm liquid cells with CaF_2 windows. The contribution of the H-O-H deformation band to the amide I region which appears at about 1633 cm^{-1} in TFE due to traces of water is removed on the basis of the combination band of the O-H stretching and H-O-H deformation. The amide I region of the spectra is decomposed into individual bands by the Levenberg-Marquardt nonlinear curve-fitting method using weighted sums of Lorentz and Gauss functions. The choice of the starting parameters is assisted by Fourier self-deconvolution (FSD). Both curve fitting and FSD procedures are part of the instrument's software package (OPUS, version 2.2).

Description of cation binding experiments:

In general, the CD spectra show a continuous change up to peptide : Ca^{2+} ration of 1:2 in TFE. No significant spectral shifts are recorded in aqueous solution. Comparative CD and FTIR measurements were performed in TFE, c≅1mM at peptide : Ca^{2+} ration of 1:1 and 1:2 on all the model peptides.

6.4.6. Synthesis of Cyclic Analogs of the Binding Epitope of VCAM-1

c-(-Ile-Asp-Ser-Pro-Leu-Asn-) (39)

$C_{28}H_{45}N_7O_{10}$ (639.72 g/mol).

Cyclization on resin:

The linear peptides H-Ser(t-Bu)-Pro-Leu-Asn(Trt)-Ile-Asp(Wang)-ODmb **(71)** and H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-Asn(Rink amide)-ODmb are synthesized using standard protocol (Table 30, P. 114) and cyclized following protocol 6 using different coupling reagents (Table 31). After deprotection and cleavage from the resin with different cleavage reagents (Table 31), cyclic peptide **(39)** is purified by reverse phase preparative HPLC.

Table 31: On resin synthesis of c-(-Ile-Asp-Ser-Pro-Leu-Asn-) (39)

Resin	n/ mmol	Coupling reagent	t/ min	Cleavage reagent	t/ h	Yield	HPLC/ %
Wang 135 mg	0.10	1.1 equiv PyBOP 3.0 equiv DIPEA	2x45	Reagent B	0.5	4 mg 4 μmol 4 %	56[a]
Wang 135 mg	0.10	2.0 equiv PyAOP 3.0 equiv DIPEA	45	Reagent B	2	4 mg 7 μmol 7 %	100[a]
Rink amide 100 mg	0.06	3.0 equiv HATU 6.0 equiv DIPEA	45	Reagent K	0.5	0	0
Wang 400mg	0.20	3.0 equiv HATU 6.0 equiv DIPEA	45	TFA 48 % DCM 48 % TIS 4 %	2	8 mg 9 μmol 4 %	70[a]

[a] The same HPLC analytical method and the retention time as for the cyclization in solution.

Cyclization in solution:

a) The linear peptide H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(72)** is synthesized on 139 mg (100 μmol) Fmoc-Leu-Sasrin (loading 0.72 mmol/g) and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(72)**): 95 mg, 65 μmol, 65 %.

HPLC (anal. method 1): t_R=31.3 min, 68 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1012.58 $[M+H]^+$, 1034.56 $[M+Na]^+$, 1050.53 $[M+K]^+$

Found: 1012.97 $[M+H]^+$, 1034.63 $[M+Na]^+$, 1050.78 $[M+K]^+$

The linear peptide **(72)** is dissolved in 1 l DMF and cyclized following protocol 7, method A with different coupling reagents (Table 32). The cyclic protected peptide **(73)** is purified by reverse phase preparative HPLC.

Table 32: Cyclization of H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH (72)

Linear peptide		Coupling reagents	Time/ h	Yield		
mg	μmol			mg	μmol	%
68	47	1.1 equiv PyAOP 3.0 equiv DIPEA	2x1	18	18	38
27	19	2.0 equiv DIC 1.0 equiv HOAt	24	-	-	-
27	19	1.1 equiv HATU 3.0 equiv DIPEA	0.5	5	5	26

HPLC (anal. method 1): t_R=37.0 min.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 994.57 $[M+H]^+$, 1016.55 $[M+Na]^+$, 1032.52 $[M+K]^+$

Found: 1016.86 $[M+Na]^+$, 1032.87 $[M+K]^+$

The combined quantities of cyclic protected peptide **(73)** are completely deprotected with the mixture TFA/H_2O/TIS = 95 : 2.5 :2.5 for 3 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(39)**): 10 mg, 17 μmol, 74 %.

HPLC (anal. method 2): t_R=17.3 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 640.33 $[M+H]^+$, 662.31 $[M+Na]^+$, 678.29 $[M+K]^+$

Found: 640.45 $[M+H]^+$, 650.63 $[M+Na]^+$, 678.44 $[M+K]^+$

b) The linear peptide H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(72)** is synthesized on 2-ClTrt resin (295 mg, 200 μmol) previously loaded with Fmoc--Leu-OH (loading 0.68 mmol/g) and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(72)**): 213 mg, 173 μmol, 86 %.

HPLC (anal. method 1): t_R=31.3 min, 82 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z: see above.

The linear peptide **(72)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(72)** (213 mg, 173 μmol) in 20 ml DMF.

Second syringe: Solution of HATU (1.2 equiv, 200 μmol, 76 mg) in 20 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (3.4 equiv, 585 μmol, 76 mg, 100 μl) and HOAt (1.2 equiv, 200 μmol, 27 mg) in 10 ml DMF.

The cyclic protected peptide **(73)** is purified by filtration through a C_{18} plug after evaporation of the solvent.

Yield (cyclic protected peptide **(73)**): 205 mg, 126 μmol, 73 %.

HPLC (anal. method 1): t_R=37 min, 61 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z: see above

The cyclic protected peptide **(73)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(39)**): 30 mg, 46 μmol, 37 %.

HPLC (anal. method 2): t_R=17.3 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z: see above.

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): two set of signals (ratio 3 : 1).

Major conformer: 0.77-0.86 (m, 12H, 3H$^{\gamma}$ (Ile), 3H$^{\delta}$ (Ile), 6H$^{\delta}$ (Leu)), 1.18 (ddq, ^{2}J=13.6 Hz, ^{3}J=7.1 Hz, ^{3}J=7.1 Hz, 1H, H$^{\gamma}$ (Ile)), 1.41-1.55 (m, 4H, H$^{\gamma}$ (Ile), 2H$^{\beta}$ (Leu), H$^{\gamma}$ (Leu)), 1.55-1.75 (m, 2H, H$^{\beta}$ (Ile) H$^{\beta}$ (Pro)), 1.82 (m, 1H, H$^{\gamma}$ (Pro)), 1.93 (m, 1H, H$^{\gamma}$ (Pro)), 2.23 (m, 1H, H$^{\beta}$ (Pro)), 2.44 (dd, ^{2}J=16.3 Hz, ^{3}J=8.3 Hz 1H, H$^{\beta}$ (Asx)), 2.67 (dd, ^{2}J=16.6 Hz, ^{3}J=2.0 Hz, 1H, H$^{\beta}$ (Asx)), 2.78 (dd, ^{2}J=16.6 Hz, ^{3}J=7.8 Hz 1H, H$^{\beta}$ (Asx)), 2.88 (dd, ^{2}J=16.0 Hz, ^{3}J=5.3 Hz 1H, H$^{\beta}$ (Asx)), 3.54 (br, 1H, H$^{\delta}$ (Pro)), 3.65 (dd, ^{2}J=15.1 Hz, ^{3}J=3.1 Hz, 1H, H$^{\beta}$ (Ser)), 3.79 (m, 1H, H$^{\delta}$ (Pro)), 3.84 (dd, ^{3}J=11.9 Hz, ^{3}J=5.0 Hz 1H, H$^{\alpha}$ (Ile)), 3.88 (dd, ^{2}J=14.8 Hz, ^{3}J=7.2 Hz 1H, H$^{\beta}$ (Ser)), 4.12 (m, 1H, H$^{\alpha}$ (Leu)), 4.16 (dd, ^{3}J=9.7 Hz, ^{3}J=7.9 Hz, 1H, H$^{\alpha}$ (Pro)), 4.34 (ddd, ^{3}J=7.5 Hz, ^{3}J=6.6 Hz, ^{3}J=1.9 Hz, 1H, H$^{\alpha}$ (Asx)), 4.54 (ddd, ^{3}J=8.0 Hz, ^{3}J=8.0 Hz, ^{3}J=4.6 Hz, 1H, H$^{\alpha}$ (Asx)), 4.68 (m, 1H, H$^{\alpha}$ (Ser)), 7.40 (s, 1H, H^{N} (Asn)), 7.54 (d, ^{3}J=6.3 Hz, 1H, H^{N} (Asx)), 7.61 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 7.69 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Leu)), 7.81 (s, 1H, H^{N} (Asn)), 8.31 (br 1H, H^{N} (Asx)), 8.73 (d, ^{3}J=5.0 Hz, 1H, H^{N} (Ile)), 12.3 (br, 1H, H^{coo} (Asp)).

Minor conformer 0.77-0.86 (m, 9H, $3H^{\gamma}$ (Ile), $3H^{\delta}$ (Ile), $3H^{\delta}$ (Leu)), 0.93 (d, ^{3}J=6.3 Hz, 3H, H^{δ} (Leu)), 1.07 (ddq, ^{2}J=13.6 Hz, ^{3}J=7.2 Hz, ^{3}J=2.1 Hz, 1H, H^{γ} (Ile)),1.31 (ddq, ^{2}J=13.3 Hz, ^{3}J=6.4 Hz, ^{3}J=3.7 Hz, 1H, H^{γ} (Ile)), 1.41-1.55 (m, 2H, H^{γ} (Leu), H^{γ} (Pro)), 1.64 (m, 1H, H^{β} (Leu)), 1.76-1.87 (m, 3H, H^{β} (Leu), H^{β} (Pro), H^{γ} (Pro)), 1.88-1.99 (m, 2H, H^{β} (Ile), H^{β} (Pro)), 2.37 (dd, ^{2}J=16.0 Hz, ^{3}J=7.2 Hz, 1H, H^{β} (Asx)), 2.42 (dd, ^{2}J=16.3 Hz, ^{3}J=3.8 Hz, 1H, H^{β} (Asx)), 2.58-2.75 (m, 2H, H^{β} (Asx)), 3.39 (dt, ^{2}J=11.1 Hz, ^{3}J=5.7 Hz, 1H, H^{δ} (Pro)), 3.44-3.63 (br, 3H, H^{δ} (Pro), $2H^{\beta}$ (Ser)), 3.88 (m, 1H, H^{α} (Ile)), 4.06 (ddd, ^{3}J=11.6 Hz, ^{3}J=10.1 Hz, ^{3}J=2.5 Hz, 1H, H^{α} (Leu)), 4.31 (m, 1H, H^{α} (Ser)), 4.41 (ddd, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, 1H, H^{α} (Asx)), 4.48 (ddd, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, 1H, H^{α} (Asx)), 4.67 (m, 1H, H^{α} (Pro)), 7.07 (s, 1H, H^{N} (Asn)), 7.12 (d, ^{3}J=6.3 Hz, 1H, H^{N} (Asx)), 7.35 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Ile)), 7.47 (s, 1H, H^{N} (Asn)), 8.19 (d, ^{3}J=3.8 Hz, 1H, H^{N} (Asx)), 8.53 (d, ^{3}J=10.1 Hz, 1H, H^{N} (Leu)), 8.57 (br, 1H, H^{N} (Ser)), 12.3 (br, 1H, H^{COO} (Asp)).

c-(-Ile-Asp-Ser-β-hPro-Leu-Asn-) (40)

$C_{29}H_{47}N_7O_{10}$ (653.74 g/mol).

Cyclization on resin:

Linear peptides H-Ser(t-Bu)-β-hPro-Leu-Asn(Trt)-Ile-Asp(Wang)-ODmb **(74)** and H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Leu-Asn(Rink amide)-ODmb are synthesized following the standard protocol (Table 30, P. 114) and cyclized on resin following protocol 6.

Table 33: On resin synthesis of c-(-Ile-Asp-Ser-β-hPro-Leu-Asn-) (40)

Resin	n/ mmol	Coupling reagent	Cleavage reagent	Time/ h	Yield	HPLC/ %
Wang (300 mg)	0.20	3.0 equiv HATU 6.0 equiv DIPEA	TFA/H_2O/TIS 95 : 2.5 : 2.5	2	1 mg 2 μmol 1 %	100[a]
Rink amide (170 mg)	0.10	3.0 equiv HATU 6.0 equiv DIPEA	Reagent B	1	0	0

[a] The same HPLC analytical method and the retention time as for the cyclization in solution.

After deprotection and cleavage from the resin with different cleavage reagents (Table 33) the cyclic peptide **(40)** is purified by reverse phase preparative HPLC:

Cyclization in solution:

The linear peptide H-Asn(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Leu-OH **(75)** is synthesized on 280 mg (0.20 mmol) Fmoc–Leu-Sasrin (loading 0.72 mmol/g) and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(75)**): 134 mg, 0.12 mmol, 60 %.

HPLC (anal. method 1): t_R=29.8 min, 90 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1026.59 $[M+H]^+$, 1048.57 $[M+Na]^+$, 1064.55 $[M+K]^+$

Found: 1026.94 $[M+H]^+$, 1048.87 $[M+Na]^+$, 1065.91 $[M+K]^+$

The linear peptide **(75)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(75)** (180 mg, 0.12 mmol) in 10 ml DMF.

Second syringe: Solution of HATU (3.0 equiv, 0.36 mmol, 137 mg) in 10 ml DMF.

Addition rate for both syringes: 0.04 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.72 mmol, 93 mg, 123 µl) and HATU (0.1 equiv, 12 µmol, 5 mg) in 10 ml DMF.

The cyclic protected peptide **(76)** is purified by reverse phase preparative HPLC after evaporation of the solvent.

Yield (cyclic protected peptide **(76)**): 72 mg, 72 µmol, 60 %.

HPLC (anal. method 1): t_R=35.8 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1008.58 $[M+H]^+$, 1030.56 $[M+Na]^+$, 1046.54 $[M+K]^+$

Found: 1030.42 $[M+Na]^+$, 1046.72 $[M+K]^+$

The cyclic protected peptide **(76)** is completely deprotected with reagent K during 4 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(40)**): 47 mg, 71 µmol, 99 %.

HPLC (anal. method 2): t_R=16.7 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 654.35 $[M+H]^+$, 676.33 $[M+Na]^+$, 692.30 $[M+K]^+$

Found: 654.52 $[M+H]^+$, 676.56 $[M+Na]^+$, 692.50 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two set of signals (ratio 7 : 1).

0.80 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.82 (dd, ^{3}J=7.2 Hz, ^{3}J=7.2 Hz, 3H, H^δ (Ile)), 0.84 (d, ^{3}J=6.3 Hz, 3H, H^γ (Ile)), 0.86 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 1.19 (ddq, ^{2}J=15.5 Hz, ^{3}J=7.1 Hz, ^{3}J=7.2 Hz, 1H, H^γ (Ile)), 1.37-1.48 (m, 2H, H^γ (Ile), H^β (Leu)), 1.54-1.64 (m, 2H, H^γ (Leu), H^γ (β-hPro)), 1.69-1.87 (m, 4H, H^β (Ile), H^β (Leu), 2H^δ (β-hPro)), 2.06 (dddd, ^{2}J=11.9 Hz, ^{3}J=6.4 Hz, ^{3}J=6.4 Hz, ^{3}J=5.4 Hz, 1H, H^γ (β-hPro)), 2.16 (dd, ^{2}J=14.4 Hz, ^{3}J=1.9 Hz, 1H, H^α (β-hPro)), 2.50-2.56 (m, 2H, H^β

(Asx), H^{α} (β-hPro)), 2.67-2.73 (m, 2H, $2H^{\beta}$ (Asx)), 2.83 (dd, ^{2}J=15.4 Hz, ^{3}J=4.1 Hz, 1H, H^{β} (Asx)), 3.43 (dd, ^{2}J=10.4 Hz, ^{3}J=8.5 Hz, 1H, H^{β} (Ser)), 3.51 (dd, ^{2}J=10.4 Hz, ^{3}J=4.7 Hz, 1H, H^{β} (Ser)), 3.52 (m, 1H, H^{ε} (β-hPro)), 3.59 (ddd, ^{2}J=10.1 Hz, ^{3}J=7.9 Hz, ^{3}J=7.9 Hz, 1H, H^{ε} (β-hPro)), 3.69 (dd, ^{3}J=5.0 Hz, ^{3}J=3.6 Hz, 1H, H^{α} (Ile)), 4.11 (dddd, ^{3}J=7.5 Hz, ^{3}J=7.5 Hz, ^{3}J=6.3 Hz, ^{3}J=1.9 Hz, 1H, H^{β} (β-hPro)), 4.25 (ddd, ^{3}J=8.6 Hz, ^{3}J=6.4 Hz, ^{3}J=3.0 Hz, 1H, H^{α} (Leu)), 4.53-4.56 (m, 2H, H^{α} (Asx), H^{α} (Ser)), 4.72 (ddd, ^{3}J=8.2 Hz, ^{3}J=4.7 Hz, ^{3}J=4.7 Hz, 1H, H^{α} (Asx)), 7.1 (d, ^{3}J=7.5 Hz, 1H, H^{N} (Ser)), 7.19 (s, 1H, H^{N} (Asx)), 7.62 (s, 1H, H^{N} (Asx)), 7.79 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Leu)), 8.14 (d, ^{3}J=9.4 Hz, 1H, H^{N} (Asx)), 8.17 (d, ^{3}J=3.2 Hz, 1H, H^{N} (Ile)), 8.48 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Asx)).

c-(-Ile-Asp-β-hSer-Pro-Leu-β-hAsn-) (41)

$C_{30}H_{49}N_7O_{10}$ (667.77 g/mol).

Cyclization on resin:

The linear peptide H-β-hSer-Pro-Leu-β-hAsn(Trt)-Ile-Asp(Wang)-ODmb **(77)** is synthesized on Wang resin (170 mg, 90 μmol), and cyclized following protocol 6 (HATU as coupling reagent). After complete deprotection and cleavage from the resin with the cleavage cocktail, TFA/DCM/TIS = 48 : 48 : 4 during 2 h, (protocol 10), the cyclic peptide **(41)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(41)**): 3 mg, 4 μmol, 4 %.

HPLC (anal. method 2): t_R=14.5 min, 85 area %.

Cyclization in solution:

The linear peptide H-β-hAsn(Trt)-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-Leu-OH **(78)** is synthesized on 280 mg (0.20 mmol) Fmoc–Leu-Sasrin (loading 0.72 mmol/g) and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(78)**): 180 mg, 0.14 mmol, 70 %.

HPLC (anal. method 1): t_R=29.2 min, 80 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1040.61 $[M+H]^+$, 1062.59 $[M+Na]^+$, 1078.56 $[M+K]^+$

Found: 1040.79 $[M+H]^+$, 1062.73 $[M+Na]^+$, 1078.75 $[M+K]^+$

The linear peptide **(78)** is cyclized following protocol 7, method B.

First syringe: Solution of linear peptide **(78)** (180 mg, 0.14 mmol) in 15 ml DMF.

Second syringe: Solution of HATU (1.1 equiv, 0.15 mmol, 59 mg) in 15 ml DMF.

Addition rate for both syringes: 0.02 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 0.42 mmol, 54 mg, 72 μl) and HATU (0.5 equiv, 70 μmol, 27 mg) in 20 ml DMF.

The cyclic protected peptide **(79)** is purified by reverse phase preparative HPLC after evaporation of the solvent.

Yield (cyclic protected peptide **(79)**): 78 mg, 76 μmol, 54 %.

HPLC (anal. method 1): t_R=34.5 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1022.60 $[M+H]^+$, 1044.58 $[M+Na]^+$, 1060.55 $[M+K]^+$

Found: 1044.67 $[M+Na]^+$, 1060.88 $[M+K]^+$

The cyclic protected peptide **(79)** is completely deprotected with the cleavage mixture TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 1 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(41)**): 33 mg, 49 μmol, 65 %.

HPLC (anal. method 2): t_R=14.5 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 668.36 $[M+H]^+$, 690.34 $[M+Na]^+$, 706.32 $[M+K]^+$

Found: 668.64 $[M+H]^+$, 690.62 $[M+Na]^+$, 706.58 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two set of signals (ratio 6 : 1)

0.76 (d, ^{2}J=6.9 Hz, 3H, H^{δ} (Leu)), 0.82 (dd, ^{3}J=7.5 Hz, ^{3}J=7.5 Hz, 3H, H^{δ} (Ile)), 0.86 (d, ^{3}J=6.9 Hz, 3H, H^{γ} (Ile)), 0.87 (d, ^{3}J=6.3 Hz, 3H, H^{δ} (Leu)), 1.23 (m, 1H, H^{γ} (Ile)), 1.33-1.63 (m, 5H, H^{γ} (Ile), 2H^{β} (Leu), H^{γ} (Leu), H^{γ} (Pro)), 1.73 (m, 1H, H^{β} (Ile)), 1.83 (m, 1H, H^{γ} (Pro)), 2.00-2.11 (m, 2H, H^{β} (Pro)), 2.22-2.31 (m, 2H, H^{α} (β-hAsn), H^{γ} (β-hAsn)), 2.33-2.40 (m, 2H, H^{γ} (β-hAsn), H^{β} (Asp)), 2.57 (dd, ^{2}J=16.9 Hz, ^{3}J=10.7 Hz, 1H, H^{α} (β-hSer)), 2.63 (dd, ^{2}J=15.7 Hz, ^{3}J=5.7 Hz, 1H, H^{α} (β-hAsn)), 2.75 (dd, ^{2}J =16.6 Hz, ^{3}J =2.8 Hz, 1H, H^{α} (β-hSer)), 2.86 (dd, ^{2}J=16.0 Hz, ^{3}J=11.6 Hz, 1H, H^{β} (Asp)), 3.30 (ddd, ^{2}J=11.3 Hz, ^{3}J=9.7 Hz, ^{3}J=9.4 Hz, 1H, H^{δ} (Pro)), 3.34 (dd, ^{2}J=11 Hz, ^{3}J=3.4 Hz, 1H, H^{γ} (β-hSer)), 3.42 (ddd, ^{2}J=11.2 Hz, ^{3}J= 11.2 Hz, ^{3}J= 7.1 Hz, 1H, H^{δ} (Pro)), 3.67 (dd, ^{2}J=10.7 Hz, ^{3}J=3.8 Hz, 1H, H^{γ} (β-hSer)), 3.74 (dd, ^{3}J=3.8 Hz, ^{3}J=3.8 Hz, 1H, H^{α} (Ile)), 4.04 (m, 1H, H^{α} (Asp)), 4.12 (ddd, ^{2}J=4.2 Hz, ^{2}J=7.1 Hz, ^{2}J= 11.2 Hz, 1H, H^{α} (Leu)), 4.25 (m, 1H, H^{β} (β-hAsn)), 4.40-4.49 (m, 2H, H^{β} (β-hSer), H^{α} (Pro)), 7.19 (s, 1H, H^{N} (β-hAsn)), 7.45 (d, ^{3}J=7.5 Hz, 1H, H^{N} (Asp)), 7.63 (d, ^{3}J=8.8 Hz, 1H, H^{N} (β-hAsn)), 7.66 (m, 2H, H^{N} (β-hAsn), H^{N} (Leu)), 8.34 (d, ^{3}J=3.8 Hz, 1H, H^{N} (Ile)), 8.40 (d, J=7.5 Hz, 1H, H^{N} (β-hSer)).

c-(-Val-Asp-Ser-Pro-Leu-Asn-) (42)

$C_{27}H_{43}N_7O_{10}$ (625.69 g/mol).

Cyclization in solution:

The linear peptide H-Asn(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(80)** is synthesized on 2-ClTrt resin (300 mg, 0.20 mmol) previously loaded with Fmoc--Leu-OH (loading 0.67 mmol/g), cleaved with 1 % TFA in DCM (protocol 9), and purified by preparative HPLC.

Yield (linear protected peptide **(80)**): 90 mg, 72 μmol, 36 %.

HPLC (anal. method 1): t_R=29.1 min, 80 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 998.56 $[M+H]^+$, 1020.54 $[M+Na]^+$, 1036.52 $[M+K]^+$

Found: 998.66 $[M+H]^+$, 1020.56 $[M+Na]^+$, 1036.58 $[M+K]^+$

The linear peptide **(80)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(80)** (90 mg, 72 μmol) in 10 ml DMSO.

Second syringe: Solution of HATU (1.0 equiv, 72 μmol, 27mg) in 10 ml DMSO.

Addition rate for both syringes: 0.02 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 216 μmol, 28 mg, 37 μl) and HOAt (0.1 equiv, 7 μmol 10 mg) in 10 ml DMSO.

After cyclization, the cyclic protected peptide **(81)** is precipitated with 200 ml H_2O and filtered through a C_{18} plug. The peptide is washed with water (3x) and Et_2O (3x) and eluted from the plug with DCM.

Yield (cyclic protected peptide **(81)**): 75 mg, 61 μmol, 85 %.

HPLC (anal. method 1): t_R=34.5 min, 34.8 min, 80 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 980.55 $[M+H]^+$, 1002.53 $[M+Na]^+$, 1018.51 $[M+K]^+$

Found: 1002.30 $[M+Na]^+$, 1018.59 $[M+K]^+$

The cyclic protected peptide **(81)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(42)**): 29 mg, 47 μmol, 77 %.

HPLC (anal. method 2): t_R=16.0 min, 16.6 min, 100 area %, epimers ratio 65:35.

For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 626.31 $[M+H]^+$, 648.30 $[M+Na]^+$, 664.27 $[M+K]^+$

Found: 626.56 $[M+H]^+$, 648.54 $[M+Na]^+$, 664.52 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): 0.79 (d, ^3J=6.3 Hz, 3H, H^{δ}(Leu)), 0.85-0.90 (m, 6H, $3H^{\delta}$(Leu), $3H^{\gamma}$(Val)), 0.92 (d, ^3J=6.9 Hz, 3H, H^{γ}(Val)), 1.49 (m, 1H,

H^{β} (Leu)), 1.60-1.69 (m, 2H, H^{β} (Leu), H^{γ} (Leu)), 1.71-1.86 (m, 2H, H^{β} (Pro), H^{γ} (Pro)), 1.89-2.07 (m, 3H, H^{β} (Pro), H^{γ} (Pro), H^{β} (Val)), 2.30 (dd, ^{2}J=16.0 Hz, ^{3}J=2.8 Hz, 1H, H^{β} (Asx)), 2.45 (dd, ^{2}J=15.4 Hz, ^{3}J=8.8 Hz, 1H, H^{β} (Asx)), 2.76 (dd, ^{2}J=15.4 Hz, ^{3}J=5.3 Hz, 1H, H^{β} (Asx)), 2.82 (dd, ^{2}J=15.7 Hz, ^{3}J=8.8 Hz, 1H, H^{β} (Asx)), 3.48 (ddd, ^{2}J=9.7 Hz, ^{3}J=9.7 Hz, ^{3}J=6.3 Hz, 1H, H^{δ} (Pro)), 3.53 (dd, ^{2}J=11.3 Hz, ^{3}J=4.4 Hz, 1H, H^{β} (Ser)), 3.61 (dd, ^{2}J=11.3 Hz, ^{3}J=4.4 Hz, 1H, H^{β} (Ser)), 3.73 (ddd, ^{2}J=10.1 Hz, ^{3}J=7.2 Hz, ^{3}J=2.5 Hz, 1H, H^{δ} (Pro)), 3.77 (dd, ^{3}J=7.5 Hz, ^{3}J=5.7 Hz, 1H, H^{α} (Val)), 4.04 (ddd, ^{3}J=10.9 Hz, ^{3}J=8.2 Hz, ^{3}J=2.5 Hz, 1H, H^{α} (Leu)), 4.18 (dd, ^{3}J=8.5 Hz, ^{3}J=7.2 Hz, 1H, H^{α} (Pro)), 4.45 (ddd, ^{3}J=8.6 Hz, ^{3}J=8.6 Hz, ^{3}J=2.3 Hz, 1H, H^{α} (Asx)), 4.52 (ddd, ^{3}J=6.6 Hz, ^{3}J=4.7 Hz, ^{3}J=4.7 Hz, 1H, H^{α} (Ser)), 4.65 (ddd, ^{3}J=8.8 Hz, ^{3}J=8.8 Hz, ^{3}J=5.0 Hz, 1H, H^{α} (Asx)), 7.24 (s, 1H, H^{N} (Asn)), 7.35 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 7.65 (s, 1H, H^{N} (Asn)), 8.10 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Asx)), 8.43 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Asx)), 8.74 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Leu)), 9.07 (d, ^{3}J=5.7 Hz, 1H, H^{N} (Val)), 12.32 (br, 1H, H^{COO} (Asp)).

c-(-D-Val-Asp-Ser-Pro-Leu-Asn-) (43)

$C_{27}H_{43}N_7O_{10}$ (625.69 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-Pro-Leu-Asn(Trt)-D-Val-Asp(Wang)-ODmb **(82)** is synthesized on Wang resin (400 mg, 0.20 mmol) and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as the cleavage reagents). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 48 : 48 : 4 during 2 h, (protocol 10) the cyclic peptide **(43)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(43)***:* 9 mg, 14 μmol, 7 %.

HPLC (anal. method 2): t_R=16.3 min, 100 area %.

Cyclization in solution:

The linear peptide H-Leu-Asn(Trt)-D-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(83)** is synthesized on 2-ClTrt resin (260 mg 0.20 mmol) previously loaded with Fmoc-Pro-OH (loading 0.76 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(83)**): 133 mg, 99 μmol, 50 %.

HPLC (anal. method 1): t_R=31.5 min, 74 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 998.56 $[M+H]^+$, 1020.54 $[M+Na]^+$, 1036.52 $[M+K]^+$

Found: 998.74 $[M+H]^+$, 1020.74 $[M+Na]^+$, 1036.77 $[M+K]^+$

The linear peptide **(83)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(83)** (133 mg, 99 μmol) in 15 ml DMF.

Second syringe: Solution of HATU (2.0 equiv, 200 μmol, 76 mg) in 15 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 600 μmol, 77 mg, 100 μl) and HOAt (3.0 equiv, 200 μmol, 27 mg) in 20 ml DMF.

The cyclic protected peptide **(84)** is purified by filtration through a C_{18} plug after removing the DMF.

Yield (cyclic protected peptide **(84)**): 59 mg, 60 μmol, 61 %.

HPLC (anal. method 1): t_R=35.7 min, 77 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 980.55 $[M+H]^+$, 1002.53 $[M+Na]^+$, 1018.51 $[M+K]^+$

Found: 1002.45 $[M+Na]^+$, 1018.53 $[M+K]^+$

The cyclic protected peptide is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(43)**): 32 mg, 51 μmol, 85 %.

HPLC (anal. method 2): t_R=16.3 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 626.31 $[M+H]^+$, 648.30 $[M+Na]^+$, 664.27 $[M+K]^+$

Found: 626.59 $[M+H]^+$, 648.45 $[M+Na]^+$, 664.48 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 6 : 1).

0.78 (d, ^3J=6.3 Hz, 3H, H^γ (D-Val)), 0.79 (d, ^3J=6.3 Hz, 3H, H^δ (Leu)), 0.86 (d, ^3J=6.3 Hz, 3H, H^δ (Leu)), 0.91 (d, ^3J=6.3 Hz, 3H, H^γ (D-Val)), 1.44-1.55 (m, 3H, $2H^\beta$ (Leu), H^γ (Leu)), 1.64 (m, 1H, H^β (Pro)), 1.80-1.90 (m, 2H, H^γ (Pro), H^β (D-Val)), 1.94 (m, 1H, H^γ (Pro)), 2.18 (m, 1H, H^β (Pro)), 2.23 (dd, ^2J=15.1 Hz, ^3J=3.8 Hz, 1H, H^β (Asx)), 2.48 (br, 1H, H^β (Asx)), 2.74 (dd, ^2J=15.1 Hz, ^3J=10.0 Hz, 1H, H^β (Asx)), 2.78 (dd, ^2J=16.6 Hz, ^3J=4.1 Hz, 1H, H^β (Asx)), 3.55 (dd, ^3J=9.7 Hz, ^3J=5.3 Hz, 1H, H^α (D-Val)), 3.63-3.71 (br, 2H, H^δ (Pro)), 3.83 (dd, ^2J=11.9 Hz, ^3J=3.8 Hz, 1H, H^β (Ser)), 3.93 (dd, ^2J=11.6 Hz, ^3J=6.6 Hz, 1H, H^β (Ser)), 4.01 (dd, ^3J=8.5 Hz, ^3J=7.9 Hz, 1H, H^α (Pro)), 4.08 (ddd, ^3J=8.6 Hz, ^3J=8.6 Hz, ^3J=5.8 Hz, 1H, H^α (Leu)), 4.49 (ddd, ^3J=9.6 Hz, ^3J=5.5 Hz, ^3J=4.2 Hz, 1H, H^α (Asx)), 4.57 (ddd, ^3J=10.2 Hz, ^3J=8.7 Hz, ^3J=3.9 Hz, 1H, H^α (Asx)), 4.72 (ddd, ^3J=9.6 Hz, ^3J=6.1 Hz, ^3J=3.6 Hz, 1H, H^α (Ser)), 6.83 (s, 1H, H^N (Asn)), 6.97 (d, ^3J=5.7 Hz, 1H, H^N (Asx)), 7.22 (s, 1H, H^N (Asn)), 7.44 (d, ^3J=8.8 Hz, 1H, H^N (Leu)), 7.95 (d, ^3J=9.4 Hz, 1H, H^N (Ser)), 8.7

(d, ^{3}J=8.2 Hz, 1H, H^N (Asx)), 8.7 (d, ^{3}J=5.7 Hz, 1H, H^N (D-Val)), 12.30 (br, 1H, H^{COO} (Asp)).

c-(-Val-Asp-Ser-β-hPro-Leu-Asn-) (44)

$C_{28}H_{45}N_7O_{10}$ (639.72 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-β-hPro-Leu-Asn(Trt)-Val-Asp(Wang)-ODmb **(85)** is synthesized and cyclized (protocol 6) on Wang resin. After complete deprotection and cleavage from the resin with different cleavage cocktails (Table 34, protocol 10) the cyclic peptide **(44)** is purified by reverse phase preparative reverse phase HPLC:

Table 34: On resin cyclization of H-Ser(t-Bu)-β-hPro-Leu-Asn(Trt)-Val-Asp(Wang)-ODmb (85)

Resin	n/ mmol	Coupling reagent	t/ min	Cleavage reagent	t/ h	Yield	HPLC / %
Wang 280 mg	0.20	3.0 equiv HATU 6.0 equiv DIPEA	45	Reagent B	5	8 mg 6 μmol 3 %	50
Wang 400 mg	0.20	3.0 equiv HATU 6.0 equiv DIPEA	45	TFA 48 DCM 48 TIS 4	2	10 mg 16 μmol 8 %	100

HPLC (anal. method 2): t_R=17.0 min.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 640.33 $[M+H]^+$, 662.31 $[M+Na]^+$, 678.29 $[M+K]^+$

Found: 640.42 $[M+H]^+$, 662.55 $[M+Na]^+$, 678.45 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): two sets of signals (ratio 8 : 1).

0.79 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.86 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.88 (d, ^{3}J=6.9 Hz, 3H, H^γ (Val)), 0.90 (d, ^{3}J=6.9 Hz, 3H, H^γ (Val)), 1.44 (ddd, ^{2}J=13.5 Hz, ^{3}J=11.9 Hz, ^{3}J=3.5 Hz, 1H, H^β (Leu)), 1.53-1.62 (m, 2H, H^γ (Leu), H^γ (β-hPro)), 1.69-1.85 (m, 3H, H^β (Leu), 2H^δ (β-hPro)), 1.98-2.09 (m, 2H, H^γ (β-hPro), H^β (Val)), 2.16 (dd, ^{2}J=14.4 Hz, ^{3}J=1.9 Hz, 1H H^α (β-hPro)), 2.49 (br, 1H, H^α (β-hPro)), 2.52 (dd, ^{2}J=16.3 Hz, ^{3}J=10.0 Hz, 1H, H^β (Asx)), 2.67 (dd, ^{2}J=15.7 Hz, ^{3}J=3.8 Hz, 1H, H^β (Asx)), 2.69 (dd, ^{2}J=15.1 Hz, ^{3}J=5.7 Hz, 1H, H^β (Asx)), 2.85 (dd, ^{2}J=15.4 Hz,

^{3}J=4.1 Hz, 1H, H$^{\beta}$ (Asx)), 3.42 (m, 1H, H$^{\beta}$ (Ser)), 3.47-3.54 (m, 2H, H$^{\varepsilon}$ (β-hPro), H$^{\beta}$ (Ser)), 3.57 (m, 1H, H$^{\varepsilon}$ (β-hPro)), 3.61 (dd, ^{3}J=4.1 Hz, ^{3}J=4.7 Hz, 1H, H$^{\alpha}$ (Val)), 4.10 (m, 1H, H$^{\beta}$ (β-hPro)), 4.24 (ddd, ^{3}J=11.9 Hz, ^{3}J=8.8 Hz, ^{3}J=3.1 Hz, 1H, H$^{\alpha}$ (Leu)), 4.52-4.61 (m, 2H, H$^{\alpha}$ (Asx), H$^{\alpha}$ (Ser)), 4.73 (ddd, ^{3}J=7.9 Hz, ^{3}J=4.7 Hz, ^{3}J=4.7 Hz, 1H, H$^{\alpha}$ (Asx)), 4.87 (br, 1H, H^{O} (Ser)), 7.11 (d, ^{3}J=7.5 Hz, 1H, H^{N} (Ser)), 7.24 (s, 1H, H^{N} (Asn)), 7.67 (s, 1H, H^{N} (Asn)), 7.82 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Leu)), 8.16 (d, ^{3}J=3.8 Hz, 1H, H^{N} (Val)), 8.17 (d, ^{3}J=10.1 Hz, 1H, H^{N} (Asx)), 8.5 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Asx)), 12.33 (br, 1H, H^{COO} (Asp)).

c-(-D-Val-Asp-Ser-β-hPro-Leu-Asn-) (45)

$C_{28}H_{45}N_7O_{10}$ (639.72 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-β-hPro-Leu-Asn(Trt)-D-Val-Asp(Wang)-ODmb **(86)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as the cleavage reagents) on Wang resin (330 mg, 0.20 mmol). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 47 : 47 : 6 during 2 h, (protocol 10) the cyclic peptide **(45)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(45)***):* 13 mg, 30 μmol, 15 %.

HPLC (anal. method 2): t_R=16.4 min, 100 area %.

MALDI-ToF MS$_{monoisotopic}$, m/z:

Calcd.: 640.33 [M+H]$^{+}$, 662.31 [M+Na]$^{+}$, 678.29 [M+K]$^{+}$

Found: 640.74 [M+H]$^{+}$, 662.73 [M+Na]$^{+}$, 678.69 [M+K]$^{+}$

^{1}H NMR (500 MHz, DMSO-d$_6$), δ (ppm): Two sets of signals (ratio 6 : 1).

0.77 (d, ^{3}J=6.3 Hz, 3H, H$^{\gamma}$ (D-Val)), 0.79 (d, ^{3}J=6.3 Hz, 3H, H$^{\delta}$ (Leu)), 0.85 (d, ^{3}J=6.9 Hz, 3H, H$^{\delta}$ (Leu)), 0.92 (d, ^{3}J=6.9 Hz, 3H, H$^{\gamma}$ (D-Val)), 1.34 (ddd, ^{2}J=13.7 Hz, ^{3}J=11.5 Hz, ^{3}J=3.0 Hz, 1H H$^{\beta}$ (Leu)), 1.48-1.59 (m, 2H, H$^{\gamma}$ (Leu), H$^{\gamma}$ (β-hPro)), 1.67 (ddd, ^{2}J=13.7 Hz, ^{3}J=10.2 Hz, ^{3}J=3.3 Hz, 1H, H$^{\beta}$ (Leu)), 1.73-1.87 (m, 3H, 2H$^{\delta}$ (β-hPro), H$^{\beta}$ (D-Val)), 2.04 (dddd, ^{2}J=12.1 Hz, ^{3}J=6.1 Hz, ^{3}J=6.1 Hz, ^{3}J=6.1 Hz, 1H, H$^{\gamma}$ (β-hPro)), 2.15 (d, ^{2}J=14.5 Hz, 1H, H$^{\alpha}$ (β-hPro)), 2.25 (dd, ^{2}J=15.1 Hz, ^{3}J=7.5 Hz, 1H, H$^{\alpha}$ (β-hPro)), 2.34-2.47 (m, 3H, H$^{\beta}$ (Asx)), 2.79 (dd, ^{2}J=16.6 Hz, ^{3}J=3.5 Hz, 1H, H$^{\beta}$ (Asx)), 3.50 (dd, ^{3}J=9.4 Hz, ^{3}J=5.0 Hz, 1H, H$^{\alpha}$ (D-Val)), 3.55-3.62 (m, 2H, H$^{\varepsilon}$ (β-hPro), H$^{\beta}$ (Ser)), 3.70 (ddd, ^{2}J=9.7 Hz, ^{3}J=7.5 Hz, ^{3}J=4.7 Hz, 1H, H$^{\varepsilon}$ (β-hPro)), 3.83 (ddd, ^{2}J=11.0 Hz, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, 1H, H$^{\beta}$ (Ser)), 4.06 (m, 1H, H$^{\beta}$ (β-hPro)), 4.25 (ddd, ^{3}J=11.5 Hz, ^{3}J=8.3 Hz, ^{3}J=3.3 Hz, 1H, H$^{\alpha}$ (Leu)), 4.49 (ddd, ^{3}J=9.7 Hz, ^{3}J=9.0 Hz, ^{3}J=3.5 Hz, 1H, H$^{\alpha}$ (Asx)), 4.68 (ddd, ^{3}J=9.1 Hz, ^{3}J=7.2 Hz,

^{3}J=7.2 Hz, 1H, H$^\alpha$ (Ser)), 4.74 (ddd, ^{3}J=9.3 Hz, ^{3}J=6.7 Hz, ^{3}J=4.6 Hz, 1H, H$^\alpha$ (Asx)), 4.80 (dd, ^{3}J=6.3 Hz, ^{3}J=6.3 Hz, 1H, H^O (Ser)), 6.84 (s, 1H, H^N (Asn)), 7.23 (s, 1H, H^N (Asn)), 7.74 (d, ^{3}J=6.3 Hz, 1H, H^N (Asx)), 7.66 (d, ^{3}J=8.2 Hz, 1H, H^N (Leu)), 7.95 (d, ^{3}J=8.8 Hz, 1H, H^N (Ser)), 8.50 (d, ^{3}J=5.0 Hz, 1H, H^N (D-Val)), 8.60 (d, ^{3}J=8.8 Hz, 1H, H^N (Asx)), 12.29 (br, 1H, H^{COO} (Asp)).

c-(-Asp-Ser-Pro-Leu-Asn-) (46)

$C_{22}H_{34}N_6O_9$ (526.55 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-Pro-Leu-Asn(Trt)-Asp(Wang)-ODmb **(87)** is synthesized and cyclized following protocol 6 on Wang resin. After complete deprotection and cleavage from the resin (Table 35, protocol 10) cyclic peptide **(46)** is purified by reverse phase preparative HPLC.

Table 35: On resin cyclization of H-Ser(t-Bu)-Pro-Leu-Asn(Trt)-Asp(Wang)-ODmb (87).

Resin	n/ mmol	Coupling reagent	t/ min	Cleavage Reagent	t/ h	Yield	HPLC/ %
Wang 135 mg	0.10	1.1 equiv HATU 3.0 equiv DIPEA	45	Reagent B	5	2 mg 2 μmol 2 %	57[a]
Wang 130 mg	0.10	3.0 equiv HATU 6.0 equiv DIPEA	2x45	TFA 47 DCM 47 TIS 6	2	13mg 22 μmol 22 %	90[a]

[a] The same HPLC analytical method and the retention time as for the cyclization in solution.

Cyclization in solution:

The linear peptide H-Leu-Asn(Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-OH **(88)** is synthesized on 2-ClTrt resin (240 mg 0.20 mmol) previously loaded with Fmoc--Pro-OH (loading 0.84 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(88)**): 101 mg, 90 μmol, 45 %.

HPLC (anal. method 1): t_R=27.8 min, 80 area %.

MALDI-ToF MS$_{monoisotopic}$, m/z:

Calcd.: 899.49 $[M+H]^+$, 921.47 $[M+Na]^+$, 937.45 $[M+K]^+$

Found: 921.51 $[M+Na]^+$, 937.53 $[M+K]^+$

The linear peptide **(88)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(88)** (101 mg, 90 μmol) in 11 ml DMF.

Second syringe: Solution of HATU (1.5 equiv, 135 μmol, 51 mg) in 11 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 270 μmol, 35 mg, 46 μl) and HOAt (1.0 equiv, 90 μmol, 12 mg) in 20 ml DMF.

After evaporation of DMF the cyclic protected peptide **(89)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(89)**): 90 mg, 60 μmol, 68 %.

HPLC (anal. method 1): t_R=31.3 min, 60 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 881.48 $[M+H]^+$, 903.46 $[M+Na]^+$, 919.44 $[M+K]^+$

Found: 903.52 $[M+Na]^+$, 919.55 $[M+K]^+$

The cyclic protected peptide **(89)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(46)**): 79 mg, 60 μmol, 100 %.

HPLC (anal. method 2): t_R=15.0 min, 40 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 527.25 $[M+H]^+$, 549.23 $[M+Na]^+$, 565.20 $[M+K]^+$

Found: 549.49 $[M+Na]^+$, 565.47 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 0.82 (d, ^{3}J=6.3 Hz, 3H, H^{δ} (Leu)), 0.85 (d, ^{3}J=6.3 Hz, 3H, H^{δ} (Leu)), 1.28-1.43 (m, 3H, $2H^{\beta}$ (Leu), H^{γ} (Leu)), 1.66 (m, 1H, H^{γ} (Pro)), 1.81-1.93 (m, 2H, H^{β} (Pro), H^{γ} (Pro)), 2.10 (dd, ^{2}J=15.7 Hz, ^{3}J=4.4 Hz, 1H, H^{β} (Asx)), 2.19 (m, 1H, H^{β} (Pro)), 2.35 (m, 1H, H^{β} (Asx)), 2.38 (dd, ^{2}J=12.2 Hz, ^{3}J=9.7 Hz, 1H, H^{β} (Asx)), 2.58 (dd, ^{2}J=15.4 Hz, ^{3}J=9.1 Hz, 1H, H^{β} (Asx)), 3.40-3.50 (m, 3H, H^{δ} (Pro), $2H^{\beta}$ (Ser)), 3.55 (ddd, ^{2}J=11.2 Hz, ^{3}J=8.0 Hz, ^{3}J=3.3 Hz, 1H, H^{δ} (Pro)), 4.12 (ddd, ^{3}J=9.7 Hz, ^{3}J=9.7 Hz, ^{3}J=3.5 Hz, 1H, H^{α} (Asx)), 4.34 (ddd, ^{3}J=9.1 Hz, ^{3}J=9.1 Hz, ^{3}J=6.3 Hz, 1H, H^{α} (Leu)), 4.47 (ddd, ^{3}J=8.6 Hz, ^{3}J=4.9 Hz, ^{3}J=3.9 Hz, 1H, H^{α} (Ser)), 4.51 (dd, ^{3}J=8.8 Hz, ^{3}J=2.5 Hz, 1H, H^{α} (Pro)), 4.83 (ddd, ^{3}J=9.1 Hz, ^{3}J=9.1 Hz, ^{3}J=4.1Hz, 1H, H^{α} (Asx)), 4.95 (dd, ^{3}J=6.3 Hz, ^{3}J=5.0 Hz, 1H, H^{O} (Ser)), 6.72 (s, 1H, H^{N} (Asn)), 7.01 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Ser)), 7.24 (s, 1H, H^{N} (Asn)), 7.39 (d, ^{3}J=9.4 Hz, 1H, H^{N} (Leu)), 7.75 (d, ^{3}J=10.0 Hz, 1H, H^{N} (Asx)), 8.35 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Asx)), 12.5 (br, 1H, H^{COO} (Asp)).

c-(-Asp-Ser-Pro-Leu-β-hAsn-) (47)

$C_{23}H_{36}N_6O_9$ (540.58 g/mol).

Cyclization in solution:

The linear peptide H-β-hAsn(Trt)-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Leu-OH **(90)** is synthesized on 280 mg (0.20 mmol) Fmoc-Leu-Sasrin (loading 0.72 mmol/g) and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(90)**): 167 mg, 0.15 mmol, 75 %.

HPLC (anal. method 2): t_R=29.5 min, 82 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 913.51 $[M+H]^+$, 935.49 $[M+Na]^+$, 951.46 $[M+K]^+$

Found: 913.33 $[M+H]^+$, 935.45 $[M+Na]^+$, 951.39 $[M+K]^+$

The linear peptide **(90)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(90)** (180 mg, 0.14 mmol) in 9 ml DMF.

Second syringe: Solution of HATU (1.5 equiv, 0.22 mmol, 84mg) in 9 ml DMF.

Addition rate for both syringes: 0.02 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.90 mmol, 116 mg, 154 µl) and HATU (0.1 equiv, 15 µmol, 6 mg) in 20 ml DMF.

After evaporation of DMF the cyclic protected peptide **(91)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(91)**): 94 mg, 0.11 mmol, 70. %.

HPLC (anal. method 1): t_R=31.8 min, 100 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 895.50 $[M+H]^+$, 917.48 $[M+Na]^+$, 933.45 $[M+K]^+$

Found: 917.57 $[M+Na]^+$, 933.74 $[M+K]^+$

The cyclic protected peptide **(91)** is completely deprotected with a cleavage cocktail TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(47)**): 25 mg, 46 µmol, 44 %.

HPLC (anal. method 2): t_R=13.0 min, 13.6 min, 100 area %, epimers ratio 40:60.

For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 541.26 $[M+H]^+$, 563.24 $[M+Na]^+$, 579.22 $[M+K]^+$

Found: 541.27 $[M+H]^+$, 563.28 $[M+Na]^+$, 579.47 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 0.82 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 0.89 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 1.37-1.63 (m, 4H, H^β (Leu), H^γ (Leu), $2H^\gamma$ (Pro)), 1.86-1.98 (m, 2H, H^β (Leu), H^β (Pro)), 2.15 (dd, ^{2}J=13.2 Hz, ^{3}J=3.1 Hz, 1H, H^γ (β-hAsn)), 2.19

(m, 1H, H^{β} (Pro)), 2.28 (dd, ^{2}J=14.4 Hz, ^{3}J=6.9 Hz, 1H, H^{α}(β-hAsn)), 2.35 (dd, ^{2}J=14.4 Hz, ^{3}J=5.7 Hz, 1H, H^{α}(β-hAsn)), 2.42-2.46 (m, 2H, H^{β} (Asp)), 2.59 (dd, ^{2}J=13.2 Hz, ^{3}J=11.9 Hz 1H, H^{γ}(β-hAsn)), 3.31 (ddd, ^{2}J=11.8 Hz, ^{3}J=8.9 Hz, ^{3}J=2.4 Hz, 1H, H^{δ} (Pro)), 3.34-3.51 (m, 2H, H^{δ} (Pro), H^{β} (Ser)), 3.62 (dd, ^{2}J=10.1 Hz, ^{3}J=5.7 Hz, 1H, H^{β} (Ser)), 4.09-4.19 (m, 2H, H^{β}(β-hAsn), H^{α} (Ser)), 4.32 (ddd, ^{3}J=9.6 Hz, ^{3}J=9.6 Hz, ^{3}J=4.9 Hz, 1H, H^{α} (Leu)), 4.55 (ddd, ^{3}J=8.9 Hz, ^{3}J=7.7 Hz, ^{3}J=6.8 Hz, 1H, H^{α}(Asp)), 4.64 (dd, ^{3}J=8.2 Hz, ^{3}J=1.0 Hz, 1H, H^{α} (Pro)), 6.82 (s, 1H, H^{N} (β-hAsn)), 7.31 (s, 1H, H^{N} (β-hAsn)), 7.52 (d, ^{3}J=7.5 Hz, 1H, H^{N} (β-hAsn)), 7.96 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Asp)), 8.04-8.09 (m, 2H, H^{N} (Leu), H^{N} (Ser))

c-(-β-hAsp-Pro-Leu-Asn-) (48)

$C_{20}H_{31}N_5O_7$ (453.50 g/mol).

Cyclization in solution:

The linear peptide H-Asn(Trt)-β-hAsp(Ot-Bu)-Pro-Leu-OH **(92)** is synthesized on 2-ClTrt resin (280 mg, 0.20 mmol) previously loaded with Fmoc-Leu-OH (loading 0.72 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(92)**): 129 mg, 0.14 mmol, 68 %.

HPLC (anal. method 1): t_R=26.5 min, 80 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 770.41 $[M+H]^+$, 792.39 $[M+Na]^+$, 808.37 $[M+K]^+$

Found: 770.82 $[M+Na]^+$, 792.81 $[M+K]^+$

The linear peptide **(92)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(92)** (129 mg, 0.14 mmol) in 10 ml DMF.

Second syringe: Solution of HATU (3.9 equiv, 0.53 mmol, 200 mg) in 10 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (8.5 equiv, 1.2 mmol, 151 mg, 200 µl) in 10 ml DMF.

After evaporation of DMF cyclic protected peptide **(93)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(93)**): 51 mg, 48 µmol, 35 %.

HPLC (anal. method 1): t_R=30.5 min, 70 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 752.40 $[M+H]^+$, 774.38 $[M+Na]^+$, 790.36 $[M+K]^+$

Found: 774.38 $[M+Na]^+$, 790.48 $[M+K]^+$

The cyclic protected peptide **(93)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(48)**): 16 mg, 28 µmol, 58 %.

HPLC (anal. method 2): t_R=16.5 min, 82 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 454.23 $[M+H]^+$, 476.21 $[M+Na]^+$, 492.19 $[M+K]^+$

Found: 454.62 $[M+H]^+$, 476.61 $[M+Na]^+$, 492.57 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): 0.85 (d, ^3J=6.3 Hz, 3H, H^{δ} (Leu)), 0.88 (d, ^3J=6.9 Hz, 3H, H^{δ} (Leu)), 1.36-1.45 (m, 2H, H^{β} (Leu), H^{γ} (Leu)), 1.49 (ddd, ^2J=13.7 Hz, ^3J=6.4 Hz, ^3J=6.4 Hz, 1H, H^{β} (Leu)), 1.69 (dddd, ^2J=12.7 Hz, ^3J=6.3 Hz, ^3J=6.3 Hz, ^3J=6.3 Hz, 1H, H^{β} (Pro)), 1.85-1.92 (m, 2H, H^{γ} (Pro)), 2.17 (dddd, ^2J=12.6 Hz, ^3J=8.8 Hz, ^3J=7.1 Hz, ^3J=7.1 Hz, 1H, H^{β} (Pro)), 2.26 (dd, ^2J=14.4 Hz, ^3J=8.8 Hz, 1H, H^{β} (Asn)), 2.45 (dd, ^2J=10.1 Hz, ^3J=6.3 Hz, 1H, $H^{\alpha(\gamma)}$ (β-hAsp)), 2.47-2.52 (m, 2H, H^{β} (Asn), $H^{\alpha(\gamma)}$ (β-hAsp)), 2.56 (dd, ^2J=16.0 Hz, ^3J=3.5 Hz, 1H, $H^{\alpha(\gamma)}$ (β-hAsp)), 2.81 (dd, ^2J=15.7 Hz, ^3J=12.6 Hz, 1H, $H^{\alpha(\gamma)}$ (β-hAsp)), 3.47 (ddd, ^2J=9.4 Hz, ^3J=6.9 Hz, ^3J=6.9 Hz, 1H, H^{δ} (Pro)), 3.77 (ddd, ^2J=9.7 Hz, ^3J=6.6 Hz, ^3J=6.6 Hz, 1H, H^{δ} (Pro)), 4.09 (dd, ^3J=8.8 Hz, ^3J=5.8 Hz 1H, H^{α} (Pro)), 4.14 (m, 1H, H^{β} (β-hAsp)), 4.36 (ddd, ^3J=10.1 Hz, ^3J=9.7 Hz, ^3J=6.9 Hz, 1H, H^{α} (Leu)), 4.44 (ddd, ^3J=9.1 Hz, ^3J=9.1 Hz, ^3J=5.7 Hz, 1H, H^{α} (Asn)), 6.95 (s, 1H, H^{N} (Asn)), 7.11 (d, ^3J=10.2 Hz, 1H, H^{N} (Leu)), 7.14 (d, ^3J=6.9 Hz, 1H, H^{N} (β-hAsp)), 7.49 (s, 1H, H^{N} (Asn)), 7.66 (d, ^3J=10.1 Hz, 1H, H^{N} (Asn)), 12.31 (br, 1H, H^{coo} (β-hAsp)).

c-(-Thr-Gln-Ile-Asp-Ser-Pro-) (49)

$C_{27}H_{43}N_7O_{11}$ (641.69 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Wang)-ODmb **(94)** is synthesized and cyclized following protocol 6, using different coupling reagents (Table 36), on Wang resin. After complete deprotection and cleavage from the resin with different cleavage cocktails (Table 36) the cyclic peptide **(49)** is purified by reverse phase preparative HPLC.

Table 36: On resin cyclization of H-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Wang)-ODmb (94)

Resin	n/ mmol	Coupling reagent	t/ min	Cleavage Reagent	t/ h	Yield	HPLC /%
Wang 300 mg	0.20	3.0 equiv HATU 6.0 equiv DIPEA	45	Reagent K	5	0	0
Wang 130 mg	0.10	2.0 equiv PyBOP 6.0 equiv DIPEA	60	Reagent K	2	0	0
Wang 130 mg	0.1	1.1 equiv PyAOP 3.0 equiv DIPEA	2x30	TFA 95 % H_2O 2.5 % TIS 2.5 %	2	0	0
Wang 500 mg	0.2	3.0 equiv HATU 6.0 equiv DIPEA	45	TFA 95 % H_2O 2.5 % TIS 2.5 %	2	12 mg 15 μmol 8 %	80[a]

[a] The same HPLC analytical method and the retention time as for the cyclization in solution.

Cyclization in solution:

The linear peptide H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-OH **(95)** is synthesized on 2-ClTrt resin (300 mg, 0.20 mmol) previously loaded with Fmoc-Gln(Trt)-OH (loading 0.67 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(95)**): 209 mg, 0.12 mmol, 58 %.

HPLC (anal. method 1): t_R=29.5 min, 60 area %.

MALDI-ToF $MS_{monoisotopic}$*, m/z:*

Calcd.: 1070.62 $[M+H]^+$, 1092.60 $[M+Na]^+$, 1108.57 $[M+K]^+$

Found: 1092.82 $[M+Na]^+$, 1108.76 $[M+K]^+$

The linear peptide **(95)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(95)** (209 mg, 0.12 mmol) in 10 ml DMF.

Second syringe: Solution of HATU (4.5 equiv, 0.53 mmol, 200 mg) in 10 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (10.0 equiv, 1.17 mmol, 151 mg, 200 μl) in 20 ml DMF.

After cyclization, DMF is evaporated and the protected cyclic peptide **(96)** is purified by filtration through a C_{18} plug.

Yield (cyclic protected peptide **(96)**): 37 mg, 35 μmol, 30 %.
HPLC (anal. method 1): t_R=35.5 min, 35.9 min, 100 area % sum of epimers.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1052.61 $[M+H]^+$, 1074.59 $[M+Na]^+$, 1090.56 $[M+K]^+$
Found: 1074.56 $[M+Na]^+$, 1090.81 $[M+K]^+$

A similar procedure is used for the synthesis of the same cyclic peptide **(96)** from different linear precursors **(97)**-**(99)**. Results are shown in Table 15, P. 68.
The cyclic protected peptide **(96)** is complete deprotected with reagent K during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.
Yield (cyclic deprotected peptide **(49)**): 11 mg, 17 μmol, 49 %.
HPLC (anal. method 2): t_R=12.0 min, 13.3 min, 100 area %, epimers ratio 37:63.
For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 642.31 $[M+H]^+$, 664.29 $[M+Na]^+$, 680.27 $[M+K]^+$
Found: 642.57 $[M+H]^+$, 664.68 $[M+Na]^+$, 680.58 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 2 : 1).
Major conformer 0.79 (d, ^{3}J=6.9 Hz, 3H, H^γ (Ile)), 0.80 (dd, ^{3}J=7.5 Hz, ^{3}J=7.5 Hz, 3H, H^δ (Ile)), 1.06 (m, 1H, H^γ (Ile)), 1.15 (d, ^{3}J=5.7 Hz, 3H, H^γ (Thr)), 1.32 (m, 1H, H^γ (Ile)), 1.54 (m, 1H, H^γ (Pro)), 1.70-1.83 (m, 2H, H^β (Ile), H^β (Pro)), 1.83-1.91 (m, 2H, H^γ (Pro), H^β (Gln)), 2.06-2.12 (m, 2H, H^β (Gln), H^γ (Gln)), 2.17 (m, 1H, H^γ (Gln)), 2.25 (m, 1H, H^β (Pro)), 2.36 (dd, ^{2}J=16.0 Hz, ^{3}J=7.9 Hz, 1H, H^β (Asp)), 2.54 (dd, ^{2}J=15.9 Hz, ^{3}J=5.7 Hz, 1H, H^β (Asp)), 3.40 (br, 1H, H^δ (Pro)), 3.45 (ddd, ^{2}J=11.3 Hz, ^{3}J=11.3 Hz, ^{3}J=6.6 Hz, 1H, H^δ (Pro)), 3.54 (m, 1H, H^β (Ser)), 3.66 (dd, ^{2}J=10.1 Hz, ^{3}J=5.7 Hz, 1H, H^β (Ser)), 3.81 (dd, ^{3}J=7.5 Hz, ^{3}J=4.4 Hz, 1H, H^α (Ile)), 4.16 (dd, ^{3}J=10.1 Hz, ^{3}J=2.5 Hz, 1H, H^α (Pro)), 4.28-4.38 (m, 2H, H^α (Gln), H^α (Ser)), 4.48-4.58 (m, 3H, H^α (Asp), H^α (Thr), H^β (Thr)), 5.04 (br, 1H, H^O), 5.38 (br, 1H, H^O), 6.77 (d, ^{3}J=6.3 Hz, 1H, H^N (Asp)), 6.87 (s, 1H, H^N (Gln)), 7.25 (m, 1H, H^N (Ser)), 7.37 (s, 1H, H^N (Gln)), 8.47 (d, ^{3}J=4.4 Hz, 1H, H^N (Ile)), 8.74 (d, ^{3}J=10.1 Hz, 1H, H^N (Thr)), 8.84 (br, 1H, H^N (Gln)), 12.32 (br, 1H, H^{COO} (Asp)).

Minor conformer 0.83 (d, ^{3}J=6.9 Hz, 3H, H^γ (Ile)), 0.85 (dd, ^{3}J=7.5 Hz, ^{3}J=7.5 Hz, 3H, H^δ (Ile)), 0.97 (d, ^{3}J=6.3 Hz, 3H, H^γ (Thr)), 1.06 (m, 1H, H^γ (Ile)), 1.32 (m, 1H, H^γ (Ile)), 1.76 (m, 1H, H^β (Ile)), 1.82-1.91 (m, 3H, $2H^\beta$ (Gln), H^γ (Pro)), 1.90-2.03 (m, 3H, $2H^\beta$ (Pro), H^γ (Pro)), 2.22-2.31 (m, 2H, H^γ (Gln)), 2.65 (dd, ^{2}J=16.3 Hz, ^{3}J=8.2 Hz, 1H, H^β (Asp)), 2.92 (dd, ^{2}J=16.3 Hz, ^{3}J=5.0 Hz, 1H, H^β (Asp)), 3.54 (m, 1H, H^β (Ser)), 3.61 (ddd, ^{2}J=10.7 Hz, ^{3}J=9.7 Hz, ^{3}J=3.1 Hz, 1H, H^δ (Pro)), 3.77-3.84 (m, 2H, H^α (Gln), H^δ (Pro)), 3.97 (dd, ^{3}J=8.8 Hz, ^{3}J=8.2 Hz, 1H, H^α (Ile)), 3.91 (m, 1H,

H^{β} (Ser)), 4.18-4.25 (m, 2H, H^{α} (Thr), H^{β} (Thr)), 4.33 (m, 1H, H^{α} (Asp)), 4.69-4.76 (m, 2H, H^{α} (Pro), H^{α} (Ser)), 4.93 (br, 1H, H^{O}), 5.38 (br, 1H, H^{O}), 6.88 (s, 1H, H^{N} (Gln)), 7.21-7.30 (m, 2H, H^{N} (Gln), H^{N} (Ile)), 7.49 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Thr)), 7.77 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 8.19 (d, ^{3}J=5.0 Hz, 1H, H^{N} (Gln)), 8.59 (br, 1H, H^{N} (Asp)), 12.32 (br, 1H, H^{COO} (Asp)).

c-(-Thr-Gln-Ile-Asp-Ser-β-hPro-) (50)

$C_{28}H_{45}N_7O_{11}$ (655.71 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-β-hPro-Thr(t-Bu)-Gln(Trt)-Ile-Asp(Wang)-ODmb **(100)** is synthesized and cyclized (3 equiv HATU and 6 equiv DIPEA as coupling reagents), on Wang resin (400 mg, 0.20 mmol) following protocol 6. After cleavage from the resin and deprotection (protocol 10), the cyclic peptide **(50)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(50)**): 6 mg, 8 μmol, 4 %.

HPLC (anal. method 2): t_R=13.4 min, 90 area %.

Cyclization in solution:

The linear peptide H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Thr(t-Bu)-Gln(Trt)-OH **(101)** is synthesized on 2-ClTrt resin (300 mg, 0.20 mmol) previously loaded with Fmoc-Gln(Trt)-OH (loading 0.67 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(101)**): 190 mg, 0.15 mmol, 74 %.

HPLC (anal. method 1): t_R=29.2 min, 84 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1084.63 $[M+H]^+$, 1106.62 $[M+Na]^+$, 1122.59 $[M+K]^+$

Found: 1084.82 $[M+H]^+$, 1106.83 $[M+Na]^+$, 1122.69 $[M+K]^+$

The linear peptide **(101)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(101)** (190 mg, 0.15 mmol) in 15 ml DMF.

Second syringe: Solution of HATU (1.8 equiv, 0.26 mmol, 100 mg) in 15 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.88 mmol, 113 mg, 150 μl) and HOAt (1.0 equiv, 0.15 mmol, 20 mg) in 20 ml DMF.

After evaporation of DMF the cyclic protected peptide **(102)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(102)**): 114 mg, 0.11 mmol, 73 %.

HPLC (anal. method 1): t_R=35.8 min, 36.4 min, 100 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1066.62 $[M+H]^+$, 1088.60 $[M+Na]^+$, 1104.58 $[M+K]^+$

Found: 1088.52 $[M+Na]^+$, 1104.88 $[M+K]^+$

The cyclic protected peptide **(102)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(50)**): 36 mg, 55 μmol, 51 %.

HPLC (anal. method 2): t_R=12.4 min, 13.7 min, 100 area %, epimers ratio 43:57.

For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 656.32 $[M+H]^+$, 678.31 $[M+Na]^+$, 694.28 $[M+K]^+$

Found: 656.37 $[M+H]^+$, 678.38 $[M+Na]^+$, 694.29 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 2 : 1).

Major conformer 0.80 (dd, ^3J=7.2 Hz, ^3J=7.2 Hz, 3H, H^{γ} (Ile)), 0.82 (d, ^3J=6.3 Hz, 3H, H^{δ} (Ile)), 0.99 (d, ^3J=6.3 Hz, 3H, H^{γ} (Thr)), 1.18 (m, 1H, H^{γ} (Ile)), 1.30 (m, 1H, H^{γ} (Ile)), 1.67-2.15 (m, 8H, $2H^{\beta}$ (Gln), H^{γ} (Gln), H^{β} (Ile), $2H^{\gamma}$ (β-hPro), $2H^{\delta}$ (β-hPro)), 2.43-2.53 (br, 2H, H^{γ} (Gln), H^{α} (β-hPro)), 2.61 (dd, ^2J=17.3 Hz, ^3J=9.1 Hz, 1H, H^{β} (Asp)), 2.73 (dd, ^2J=17.3 Hz, ^3J=3.5 Hz, 1H, H^{β} (Asp)), 2.93 (m, 1H, H^{α} (β-hPro)), 3.26 (ddd, ^2J=11.3 Hz, ^3J=8.2 Hz, ^3J=3.5 Hz, 1H, H^{ε} (β-hPro)), 3.20-3.45 (br, 2H, H^{ε} (β-hPro), H^{β} (Ser)), 3.56 (dd, ^2J=10.1 Hz, ^3J=8.1 Hz, 1H, H^{β} (Ser)), 3.85 (m, 1H, H^{β} (Thr)), 4.08 (dd, ^3J=8.8 Hz, ^3J=4.4 Hz, 1H, H^{α} (Ile)), 4.26 (ddd, ^3J=6.8 Hz, ^3J=6.8 Hz, ^3J=6.8 Hz, 1H, H^{α} (Gln)), 4.31 (dd, ^3J=7.9 Hz, ^3J=4.7 Hz, 1H, H^{α} (Thr)), 4.39 (m, 1H, H^{β} (β-hPro)), 4.55 (ddd, ^3J=7.9 Hz, ^3J=7.2 Hz, ^3J=6.6 Hz, 1H, H^{α} (Ser)), 4.74 (ddd, ^3J=9.3 Hz, ^3J=9.3 Hz, ^3J=3.3 Hz, 1H, H^{α} (Asp)), 6.74 (s, 1H, H^N (Gln)), 7.28 (s, 1H, H^N (Gln)), 7.42 (d, ^3J=8.2 Hz, 1H, H^N (Thr)), 7.50 (d, ^3J=9.4 Hz, 1H, H^N (Asp)), 8.1 (d, ^3J=8.8 Hz, 1H, H^N (Ile)), 8.35 (d, ^3J=6.9 Hz, 1H, H^N (Ser)), 8.46 (d, ^3J=6.9 Hz, 1H, H^N (Gln)), 12.30 (br, 1H, H^{COO} (Asp)).

Minor conformer 0.78 (dd, ^3J=6.9 Hz, ^3J=6.9 Hz, 3H, H^{γ} (Ile)), 0.82 (d, ^3J=6.3 Hz, 3H, H^{δ} (Ile)), 1.02 (d, ^3J=6.3 Hz, 3H, H^{γ} (Thr)), 1.05 (m, 1H, H^{γ} (Ile)), 1.39 (m, 1H, H^{γ} (Ile)), 1.67-2.15 (m, 8H, $2H^{\beta}$ (Gln), H^{γ} (Gln), H^{β} (Ile), $2H^{\gamma}$ (β-hPro), $2H^{\delta}$ (β-hPro)), 2.25 (dd, ^2J=14.1 Hz, ^3J=1.0 Hz, 1H, H^{α} (β-hPro)), 2.43-2.53 (br, 3H, H^{β} (Asp), H^{γ} (Gln), H^{α} (β-hPro)), 2.93 (dd, ^2J=16.3 Hz, ^3J=5.7 Hz, 1H, H^{β} (Asp)), 3.20-3.45 (br, 3H, $2H^{\varepsilon}$ (β-hPro), H^{β} (Ser)), 3.49 (m, 1H, H^{β} (Ser)), 3.90 (dd, ^3J=9.7 Hz, ^3J=8.5 Hz, 1H, H^{α} (Ile)), 3.95 (m, 1H, H^{β} (Thr)), 4.08 (m, 1H, H^{α} (Asp)), 4.15 (dd, ^3J=7.5 Hz, ^3J=3.8 Hz, 1H, H^{α} (Thr)), 4.39 (m, 1H, H^{β} (β-hPro)), 4.44 (ddd, ^3J= 8.6 Hz, ^3J=4.6 Hz, ^3J=4.6 Hz, 1H, H^{α} (Gln)), 4.61 (ddd, ^3J= 8.5 Hz, ^3J=6.3 Hz, ^3J=6.3 Hz, 1H, H^{α} (Ser)), 6.71 (s, 1H, H^N (Gln)), 6.73 (d, ^3J=8.8 Hz, 1H, H^N (Ser)), 7.14 (s, 1H, H^N

(Gln)), 7.29 (d, ^{3}J=7.5 Hz, 1H, H^N (Thr)), 7.98 (d, ^{3}J=8.8 Hz, 1H, H^N (Gln)), 8.12 (d, ^{3}J=8.2 Hz, 1H, H^N (Ile)), 8.80 (d, ^{3}J=7.5 Hz, 1H, H^N (Asp)), 12.30 (br, 1H, H^{COO} (Asp)).

c-(-Thr-β-hGln-Ile-Asp-β-hSer-Pro-) (51)

$C_{29}H_{47}N_7O_{11}$ (669.74 g/mol).

Cyclization on resin:

The linear peptide H-β-hSer(t-Bu)-Pro-Thr(t-Bu)- β-hGln-Ile-Asp(Wang)-ODmb **(103)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents), on Wang resin (200 mg, 92 μmol). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 47 : 47: 6 during 2 h (protocol 10) the cyclic peptide **(51)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(51)**): 3 mg, 5 μmol, 5 %.

HPLC (anal. method 2): t_R=15.3 min, 100 area %.

Cyclization in solution:

a) The linear peptide H-β-hGln-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-Thr(t-Bu)-OH **(104)** is synthesized on 2-ClTrt resin (125 mg, 0.10 mmol) previously loaded with Fmoc-Thr(t-Bu)-OH (loading 0.80 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9). The linear protected peptide **(104)** is not purified.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 856.54 $[M+H]^+$, 878.52 $[M+Na]^+$, 894.50 $[M+K]^+$

Found: 856.74 $[M+H]^+$, 878.55 $[M+Na]^+$, 894.62 $[M+K]^+$

The linear peptide **(104)**, is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(104)** in 7 ml DMF.

Second syringe: Solution of HATU (110 μmol, 42 mg) in 7 ml DMF.

Addition rate for both syringes: 0.005 ml/min.

Flask: Solution of DIPEA (300 μmol, 39 mg, 51 μl) and HATU (50 μmol, 19 mg) in 10 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(105)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(105)**): 15 mg, 18 μmol, 18 % (Calculated on resin loading).

HPLC (anal. method 1): t_R=23.7 min, 100 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 838.53 $[M+H]^+$, 860.51 $[M+Na]^+$, 876.48 $[M+K]^+$

Found: 860.83 $[M+Na]^+$, 876.83 $[M+K]^+$

The cyclic protected peptide **(105)** is completely deprotected with the cleavage mixture TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(51)**): 4 mg, 5.5 μmol, 31 %.

HPLC (anal. method 2): t_R=13.6 min, 14.1 min, 92 area %, epimers ratio 1:1.

b) The linear peptide H-Thr(t-Bu)-β-hGln-Ile-Asp(Ot-Bu)-β-hSer(t-Bu)-Pro-OH **(106)** is synthesized on 2-ClTrt resin (265 mg, 0.20 mmol) previously loaded with Fmoc-Pro-OH (loading 0.76 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9). The linear protected peptide **(106)** is purified by filtration through a C_{18} plug.

*Yield (linear protected peptide **(106)**:* 180 mg, 174 μmol, 87 %.

HPLC (anal. method 1): t_R=22.6 min, 83 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 856.54 $[M+H]^+$, 878.52 $[M+Na]^+$, 894.50 $[M+K]^+$

Found: 856.78 $[M+H]^+$, 878.59 $[M+Na]^+$, 894.60 $[M+K]^+$

The linear peptide **(106)**, is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(106)** (180 mg, 174 μmol) in 20 ml DMF.

Second syringe: Solution of HATU (2.3 equiv, 0.4 mmol, 152 mg) in 20 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (4.5 equiv, 0.8 mmol, 103 mg, 136 μl) and HOAt (2.3 equiv, 0.4 mmol, 54 mg) in 20 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(105)** is purified by filtration through C_{18} plug.

Yield (cyclic protected peptide **(105)**): 92 mg, 66 μmol, 38 %.

HPLC (anal. method 1): t_R=23.7 min, 60 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 838.53 $[M+H]^+$, 860.51 $[M+Na]^+$, 876.48 $[M+K]^+$

Found: 860.81 $[M+Na]^+$, 876.83 $[M+K]^+$

The cyclic protected peptide **(105)** is completely deprotected with reagent K during 5 h, (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(51)**): 14 mg, 21 μmol, 32 %.

HPLC (anal. method 2): t_R=15.3 min, 100 area %. No isomers detected.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 670.34 $[M+H]^+$, 692.32 $[M+Na]^+$, 708.30 $[M+K]^+$

Found: 670.43 $[M+H]^+$, 692.45 $[M+Na]^+$, 708.39 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): two sets of signals (ratio 3 :1).

Major conformer 0.84 (dd, ^{3}J=6.9 Hz, ^{3}J=6.9 Hz, 3H, H$^{\delta}$ (Ile)), 0.86 (d, ^{3}J=6.3 Hz, 3H, H$^{\gamma}$ (Ile)), 1.00 (d, ^{3}J=6.3 Hz, 3H, H$^{\gamma}$ (Thr)), 1.22 (ddq, ^{2}J=13.0 Hz, ^{3}J=8.2 Hz, ^{3}J=6.9 Hz, 1H, H$^{\gamma}$ (Ile)), 1.42 (ddq, ^{2}J=13.3 Hz, ^{3}J=6.7 Hz, ^{3}J=6.3 Hz, 1H, H$^{\gamma}$ (Ile)), 1.56-1.78 (m, 4H, 2H$^{\gamma}$ (β-hGln), H$^{\beta}$ (Ile), H$^{\gamma}$ (Pro)), 1.83 (m, 1H, H$^{\gamma}$ (Pro)), 2.00-2.10 (m, 4H, 2H$^{\delta}$ (β-hGln), 2H$^{\beta}$ (Pro)), 2.32 (dd, ^{2}J=15.1 Hz, ^{3}J=3.1 Hz, 1H, H$^{\alpha}$ (β-hGln)), 2.37 (dd, ^{2}J=16.9 Hz, ^{3}J=3.8 Hz, 1H, H$^{\alpha}$ (β-hSer)), 2.58 (dd, ^{2}J=15.1 Hz, ^{3}J=6.9 Hz, 1H, H$^{\alpha}$ (β-hGln)), 2.64 (dd, ^{2}J=16.6 Hz, ^{3}J=9.7 Hz, 1H, H$^{\beta}$ (Asp)), 2.77 (dd, ^{2}J=17.0 Hz, ^{3}J=3.5 Hz, 1H, H$^{\beta}$ (Asp)), 2.80 (dd, ^{2}J=16.6 Hz, ^{3}J=11.6 Hz, 1H, H$^{\alpha}$ (β-hSer)), 3.18-3.66 (br, 4H, 2H$^{\delta}$ (Pro), 2H$^{\gamma}$ (β-hSer)), 3.69 (dd, ^{3}J=4.4 Hz, ^{3}J=4.4 Hz, 1H, H$^{\alpha}$ (Ile)), 3.82 (m, 1H, H$^{\beta}$ (β-hGln), 3.94 (dd, ^{3}J=7.9 Hz, ^{3}J=4.1 Hz, 1H, H$^{\alpha}$ (Thr)), 4.07-4.15 (m, 2H, H$^{\beta}$ (β-hSer), H$^{\beta}$ (Thr)), 4.38 (ddd, ^{3}J=10.2 Hz, ^{3}J=7.1 Hz, ^{3}J=3.3 Hz, 1H, H$^{\alpha}$ (Asp)), 4.44 (dd, ^{3}J=5.0 Hz, ^{3}J=5.0 Hz, 1H, H$^{\alpha}$ (Pro)), 4.89-5.16 (br, 2H, H^{O} (β-hSer), H^{O} (Thr)), 6.85 (s, 1H, H^{N} (β-hGln)), 7.30 (d, ^{3}J=9.4 Hz, 1H, H^{N} (β-hSer)), 7.31 (s, 1H, H^{N} (β-hGln)), 7.44 (d, ^{3}J=7.5 Hz, 1H, H^{N} (Thr)), 7.69 (d, ^{3}J=8.8 Hz, 1H, H^{N} (β-hGln)), 8.06 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Asp)), 8.22 (d, ^{3}J=3.8 Hz, 1H, H^{N} (Ile)), 12.33 (br, 1H H^{COO} Asp).

Minor conformer 0.78-0.88 (m, 6H, 3H$^{\gamma}$ (Ile), 3H$^{\delta}$ (Ile), 1.03 (d, ^{3}J=6.3 Hz, 3H, H$^{\gamma}$ (Thr)), 1.10 (m, 1H, H$^{\gamma}$ (Ile)), 1.39 (m, 1H, H$^{\gamma}$ (Ile)), 1.74 (m, 1H, H$^{\beta}$ (Ile)), 1.58-1.71 (m, 2H, H$^{\gamma}$ (Pro)), 1.81-1.91 (m, 3H, 2H$^{\gamma}$ (β-hGln), H$^{\beta}$ (Pro)), 2.12 (m, 1H, H$^{\beta}$ (Pro)), 2.45 (m, 1H, H$^{\alpha}$ (β-hSer)), 2.47-2.53 (br, 2H, H$^{\beta}$ (Asp)), 2.61-2.67 (m, 2H, H$^{\delta}$ (β-hGln)), 2.72-2.81 (m, 2H, H$^{\alpha}$ (β-hGln)), 2.93 (dd, ^{2}J=16.0 Hz, ^{3}J=7.2 Hz, 1H, H$^{\alpha}$ (β-hSer)), 3.00 (m, 1H, H$^{\delta}$ (Pro)), 3.18-3.66 (br, 3H, 2H$^{\gamma}$ (β-hSer), H$^{\delta}$ (Pro)), 3.73-3.85 (m, 2H, H$^{\beta}$ (β-hGln), H$^{\beta}$ (β-hSer)), 3.94 (m 1H, H$^{\alpha}$ (Ile)), 4.03 (dd, ^{3}J=8.6 Hz, ^{3}J=2.5 Hz, 1H, H$^{\alpha}$ (Thr)), 4.20-4.28 (m, 2H, H$^{\alpha}$ (Pro), H$^{\beta}$ (Thr)), 4.42 (m, 1H, H$^{\alpha}$ (Asp)), 4.89-5.16 (br, 2H, H^{O} (β-hSer), H^{O} (Thr)), 6.78 (s, 1H, H^{N} (β-hGln)), 7.08 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Thr), 7.18 (s, 1H, H^{N} (β-hGln)), 7.55-7.59 (m, 2H, H^{N} (Asp), H^{N} (β-hGln)), 7.77 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ile)), 7.89 (d ^{3}J=6.9 Hz, 1H, H^{N} (β-hSer)), 12.33 (br, 1H H^{COO} Asp).

c-(-Thr-Gln-Val-Asp-Ser-Pro-) (52)

$C_{26}H_{41}N_7O_{11}$ (627.66 g/mol).

Cyclization on resin:

The linear peptide H-Ser-Pro-Thr(t-Bu)-Gln(Trt)-Val-Asp(Wang)-ODmb **(107)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents), on Wang resin (330 mg, 0.2 mmol). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 48 : 48: 4 during 2 h (protocol 10), the cyclic peptide **(52)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(52)**): 6 mg, 10 μmol, 5 %.

HPLC (anal. method 2): t_R=13.07 min, 100 area %.

Cyclization in solution:

The linear peptide H-Gln(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-OH **(108)** is synthesized on 2-ClTrt resin (250 mg, 0.2 mmol) previously loaded with Fmoc-Thr(t-Bu)-OH (loading 0.80 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9). The linear protected peptide **(108)** is neither purified by reverse phase preparative HPLC nor with filtration through C_{18} plug.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1056.60 $[M+H]^+$, 1078.58 $[M+Na]^+$, 1094.56 $[M+K]^+$

Found: 1078.69 $[M+Na]^+$, 1094.67 $[M+K]^+$

The linear peptide **(108)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(108)** in 7 ml DMF.

Second syringe: Solution of HATU (0.22 mmol, 84 mg,) in 7 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (0.66 mmol, 85 mg, 113 μl) and HATU (0.10 mmol, 37 mg) in 10 ml DMF.

After evaporation of the solvent the protected cyclic peptide **(109)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(109)**): 16 mg, 15 μmol, 8 % (calculated on resin loading).

HPLC (anal. method 1): t_R=32.0 min, 100 area % sum of epimers.

Also 164 mg, 0.16 mmol of the linear peptide **(108)** is isolated by reverse phase preparative HPLC.

Repeated cyclization following protocol 7, method B:

First syringe: Solution of linear peptide **(108)** (164 mg, 0.16 mmol) in 7 ml DMF.

Second syringe: Solution of HATU (2.0 equiv, 0.32 mmol, 117 mg,) in 7 ml DMF.

Addition rate for both syringes: 0.02 ml/min.

Flask: solution: DIPEA (6.0 equiv, 0.93 mmol, 120 mg, 159 µl) and HATU (1.0 equiv, 0.16 mmol, 59 mg) in 10 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(109)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(109)**): 35 mg, 34 µmol, 22 %.

HPLC (anal. method 1): t_R=32.0 min, 100 area % sum of epimers.

MALDI-ToF MS$_{monoisotopic}$, m/z:

Calcd.: 1038.59 $[M+H]^+$, 1060.57 $[M+Na]^+$, 1076.55 $[M+K]^+$

Found: 1060.70 $[M+Na]^+$, 1076.85 $[M+K]^+$

In the reaction mixture linear protected peptide is again detected with MALDI-ToF MS.

The combined cyclic protected peptide **(109)** (51 mg, 49 µmol) is completely deprotected with the cleavage mixture TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(52)**): 19 mg, 30 µmol, 61 %.

HPLC (anal. method 2): t_R=13.1 min, 100 area %, sum of epimers.

For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).

MALDI-ToF MS$_{monoisotopic}$, m/z:

Calcd.: 628.29 $[M+H]^+$, 650.28 $[M+Na]^+$, 666.25 $[M+K]^+$

Found: 650.19 $[M+Na]^+$, 666.26 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 1.5 : 1).

Major conformer 0.78 (d, ^{3}J=6.3 Hz, 3H, H^γ (Val)), 0.82 (d, ^{3}J=6.9 Hz, 3H, H^γ (Val)), 1.14 (d, ^{3}J=6.3 Hz, 3H, H^γ (Thr)), 1.56 (m, 1H, H^γ (Pro)), 1.80-1.90 (m, 4H, $2H^\beta$ (Gln), H^β (Pro), H^γ (Pro)), 1.90-2.02 (m, 2H, H^β (Pro), H^β (Val)), 2.06-2.31 (m, 2H, H^γ (Gln)), 2.36 (dd, ^{2}J=15.7 Hz, ^{3}J=8.2 Hz, 1H, H^β (Asp)), 2.55 (dd, ^{2}J=15.7 Hz, ^{3}J=5.7 Hz, 1H, H^β (Asp)), 3.32-3.38 (m, 2H, $2H^\delta$ (Pro)), 3.52 (br, 1H, H^β (Ser)), 3.65 (dd, ^{2}J=10.4 Hz, ^{3}J=5.3 Hz, 1H, H^β (Ser)), 3.80 (ddd, ^{3}J=6.4 Hz, ^{3}J=6.4 Hz, ^{3}J=3.8 Hz, 1H, H^α (Gln)), 3.90 (m, 1H, H^α (Val)), 4.33 (m, 1H, H^α (Ser)), 4.47 (ddd, ^{3}J=7.9 Hz, ^{3}J=7.2 Hz, ^{3}J=5.9 Hz, 1H, H^α (Asp)), 4.50-4.56 (m, 2H, H^α (Thr), H^β (Thr)), 4.69 (d, ^{3}J=8.8 Hz, 1H, H^α (Pro)), 4.90-5.10 (br, 2H, H^O (Ser), H^O (Thr)), 6.73 (d, ^{3}J=6.9 Hz, 1H, H^N (Asp)), 6.85 (s, 1H, H^N (Gln)), 7.20-7.26 (m, 2H, H^N (Ser), H^N (Val)), 7.45 (s, 1H, H^N (Gln)), 8.49 (d, ^{3}J=3.8 Hz, 1H, H^N (Gln)), 8.71 (d, ^{3}J=8.8 Hz, 1H, H^N (Thr)), 12.31 (br, 1H, H^{COO} (Asp)).

Minor conformer 0.86 (d, ^{3}J=6.9 Hz, 3H, H^γ (Val)), 0.92 (d, ^{3}J=6.9 Hz, 3H, H^γ (Val)), 0.96 (d, ^{3}J=6.3 Hz, 3H, H^γ (Thr)), 1.74 (m, 1H, H^β (Pro)), 1.80-1.90 (m, 2H, H^β (Gln)), 1.90-2.02 (m, 3H, $2H^\gamma$ (Pro), H^β (Val)), 2.06-2.31 (m, 3H, $2H^\gamma$ (Gln), H^β

(Pro)), 2.91 (dd, ^{2}J=16.3 Hz, ^{3}J=5.0 Hz, 1H, H^{β} (Asp)), 2.64 (dd, ^{2}J=16.0 Hz, ^{3}J=7.9 Hz, 1H, H^{β} (Asp)), 3.30-3.67 (br, 3H, $2H^{\delta}$ (Pro), H^{β} (Ser)), 3.69 (m, 1H, H^{α} (Val)), 3.80 (ddd, ^{3}J=6.4 Hz, ^{3}J=6.4 Hz, ^{3}J=3.8 Hz, 1H, H^{α} (Gln)), 3.90 (m, 1H, H^{β} (Ser)), 4.15 (dd, ^{3}J=9.4 Hz, ^{3}J=1.9 Hz, 1H, H^{α} (Pro)), 4.18-4.24 (m, 2H, H^{α} (Thr), H^{β} (Thr)), 4.30 (m, 1H, H^{α} (Asp)), 4.72 (ddd, ^{3}J=6.8 Hz, ^{3}J=3.3 Hz, ^{3}J=3.3 Hz, 1H, H^{α} (Ser)), 4.90-5.10 (br, 2H, H^{O} (Ser), H^{O} (Thr)), 6.88 (s, 1H, H^{N} (Gln)), 7.24 (m, 1H, H^{N} (Asp)), 7.45 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Thr)), 7.78 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 8.22 (d, ^{3}J=5.0 Hz, 1H, H^{N} (Val)), 8.45 (br, 1H, H^{N} (Gln)), 8.83 (s, 1H, H^{N} (Gln)), 12.31 (br, 1H, H^{COO} (Asp)).

c-(-Thr-Gln-Val-Asp-Ser-β-hPro-) (53)

$C_{27}H_{43}N_7O_{11}$ (641.69 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-β-hPro-Thr(t-Bu)-Gln(Trt)-Val-Asp(Wang)-ODmb **(110)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents) on Wang resin (330 mg, 0.2 mmol). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 48 : 48: 4 during 2 h (protocol 10), the cyclic peptide **(53)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(53)**): 10 mg, 16 μmol, 8 %.

HPLC (anal. method 2): t_R=13.0 min, 100 area %.

Cyclization in solution:

The linear peptide H-Gln(Trt)-Val-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Thr(t-Bu)-OH **(111)** is synthesized on 2-ClTrt resin (250 mg, 0.20 mmol) previously loaded with Fmoc-Thr(t-Bu)-OH (loading 0.80 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(111)**): 160 mg, 0.13 mmol, 67 %.

HPLC (anal. method 1): t_R=min, 90 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1070.62 $[M+H]^+$, 1092.60 $[M+Na]^+$, 1108.57 $[M+K]^+$

Found: 1092.61 $[M+Na]^+$

The linear peptide **(111)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(111)** (160 mg, 0.13 mmol) in 10 ml DMF.

Second syringe: Solution of HATU (3.0 equiv, 0.4 mmol, 153 mg,) in 10 ml DMF.

Addition rate for both syringes: 0.04 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.80 mmol, 104 mg, 137 µl) and HATU (0.1 equiv, 13 µmol, 5 mg) in 20 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(112)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(112)**): 84 mg, 50 µmol, 37 %.

HPLC (anal. method 1): t_R=35.8 min, 62 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1052.61 $[M+H]^+$, 1074.59 $[M+Na]^+$, 1090.56 $[M+K]^+$

Found: 1074.72 $[M+Na]^+$

The cyclic protected peptide **(112)** is completely deprotected with reagent K during 2 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(53)**): 27 mg, 42 µmol, 84 %.

HPLC (anal. method 2): t_R=11.4 min, 11.7 min, 100 area %, epimers ratio 1:1.

For the biological tests and the NMR analysis the epimers were separated by reverse phase preparative HPLC (preparative method 3).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 642.31 $[M+H]^+$, 664.29 $[M+Na]^+$, 680.27 $[M+K]^+$

Found: 642.54 $[M+H]^+$, 664.68 $[M+Na]^+$, 680.58 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 3 : 1).

Major conformer 0.87 (d, ^{3}J=6.3 Hz, 3H, H^{γ} (Val)), 0.94 (d, ^{3}J=6.3 Hz, 3H, H^{γ} (Val)), 1.00 (d, ^{3}J=6.9 Hz, 3H, H^{γ} (Thr)), 1.61 (dddd, ^{2}J=12.4 Hz, ^{3}J=7.9 Hz, ^{3}J=7.9 Hz, ^{3}J=7.5 Hz, 1H, H^{γ} (β-hPro)), 1.72-1.88 (m, 4H, $2H^{\beta}$ (Gln), $2H^{\delta}$ (β-hPro)), 1.92 (m, 1H, H^{β} (Val)), 2.07 (dddd, ^{2}J=12.2 Hz, ^{3}J=6.2 Hz, ^{3}J=6.2 Hz, ^{3}J=6.2 Hz, 1H, H^{γ} (β-hPro)), 2.11-2.18 (m, 2H, H^{γ} (Gln)), 2.21 (dd, ^{2}J=14.8 Hz, ^{3}J=1.6 Hz, 1H, H^{α} (β-hPro)), 2.49 (br, 1H, H^{β} (Asp)), 2.67-2.76 (m, 2H, H^{β} (Asp), H^{α} (β-hPro)), 3.26-3.68 (br, 5H, $2H^{\varepsilon}$ (β-hPro), $2H^{\beta}$ (Ser), H^{α} (Val)), 4.11 (m, 1H, H^{β} (β-hPro)), 4.19 (dd, ^{3}J=9.4 Hz, ^{3}J=1.3 Hz, 1H, H^{α} (Thr)), 4.36-4.47 (m, 2H, H^{α} (Asp), H^{β} (Thr)), 4.55 (ddd, ^{3}J=7.2 Hz, ^{3}J=7.2 Hz, ^{3}J=5.0 Hz, 1H, H^{α} (Ser)), 4.63 (ddd, ^{3}J=9.1 Hz, ^{3}J=10.1Hz, ^{3}J=2.2 Hz, 1H, H^{α} (Gln)), 6.82 (s, 1H, H^{N} (Gln)), 7.00 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 7.07 (s, 1H, H^{N} (Gln)), 7.49 (d, ^{3}J=10.0 Hz, 1H, H^{N} (Thr)), 7.97 (d J=8.8 Hz, 1H, H^{N} (Asp)), 8.18 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Gln)), 8.28 (d, ^{3}J=3.1 Hz, 1H, H^{N} (Val)), 12.33 (br, 1H, H^{COO} (Asp)).

Minor conformer 0.77 (d, ^{3}J=6.9 Hz, 3H, H^{γ} (Val)), 0.81 (d, ^{3}J=6.9 Hz, 3H, H^{γ} (Val)), 1.08 (d, ^{3}J=6.3 Hz, 3H, H^{γ} (Thr)), 1.72-1.98 (m, 6H, $2H^{\gamma}$ (β-hPro), $2H^{\delta}$ (β-hPro), $2H^{\beta}$ (Gln)), 2.11-2.24 (m, 3H, $2H^{\gamma}$ (Gln), H^{β} (Val)), 2.47-2.57 (br, 2H, H^{β} (Asp)), 2.39 (dd, ^{2}J=14.4 Hz, ^{3}J=8.2 Hz, 1H, H^{α} (β-hPro)), 2.99 (dd, ^{2}J=14.1 Hz, ^{3}J=2.2 Hz, 1H, H^{α} (β-hPro)), 3.17 (ddd, ^{2}J=11.8 Hz, ^{3}J=8.3 Hz, ^{3}J=8.3 Hz, 1H, H^{ε} (β-hPro)), 3.26-3.68

(br, 3H, H^{ε} (β-hPro), $2H^{\beta}$ (Ser)), 3.86 (dd, ^3J=8.8 Hz, ^3J=6.9 Hz, 1H, H^{α} (Val)), 3.97 (ddd, ^3J=7.9 Hz, ^3J=5.7 Hz, ^3J=5.7 Hz, 1H, H^{α} (Gln)), 4.29-4.35 (m, 2H H^{α} (Thr), H^{β} (Thr)), 4.42 (m, 2H, H^{α} (Asp), H^{β} (β-hPro)), 4.76 (ddd, ^3J=8.5 Hz, ^3J=6.3 Hz, ^3J=6.3 Hz, 1H, H^{α} (Ser)), 6.81 (s, 1H, H^{N} (Gln)), 7.27 (d, ^3J=6.9 Hz, 1H, H^{N} (Asp)), 7.30 (s, 1H, H^{N} (Gln)), 7.46 (d, ^3J=8.8 Hz, 1H, H^{N} (Val)), 7.67 (d, ^3J=9.4 Hz, 1H, H^{N} (Thr)), 8.25 (d, ^3J=5.7 Hz, 1H, H^{N} (Gln)), 8.43 (d, ^3J=6.3 Hz, 1H, H^{N} (Ser)), 12.33 (br, 1H, H^{COO} (Asp)).

c-(-Thr-Gln-D-Val-Asp-Ser-Pro-) (54)

$C_{26}H_{41}N_7O_{11}$ (627.66 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-D-Val-Asp(Wang)-ODmb **(113)** is synthesized and cyclized following protocol 6 (HATU as coupling reagent) on Wang resin (400 mg, 0.20 mmol). The cyclic peptide **(54)** is completely deprotected, cleaved from the resin with the cleavage cocktail DCM/TFA/TIS = 48 : 48 : 4 (protocol 10), and purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(54)**): 10 mg 14 μmol, 7 %.

HPLC (anal. method 2): t_R=9.9 min, 90 area %.

Cyclization in solution:

The linear peptide H-D-Val-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Thr(t-Bu)-Gln(Trt)-OH **(114)** is synthesized on 2-ClTrt resin (400 mg, 0.20 mmol) previously loaded with Fmoc-Gln(Trt)-OH (loading 0.50 mmol/g), cleaved with 1 % TFA in DCM (protocol 9), and purified by preparative HPLC.

Yield (linear protected peptide **(114)**): 160 mg, 0.13 mmol, 65 %.

HPLC (anal. method 1): t_R=29.2 min, 85 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1056.60 $[M+H]^+$, 1078.58 $[M+Na]^+$, 1094.56 $[M+K]^+$

Found: 1056.42 $[M+H]^+$, 1078.31 $[M+Na]^+$, 1094.45 $[M+K]^+$

The linear peptide **(114)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(114)** (160 mg, 0.13 mmol) in 15 ml DMF.

Second syringe: Solution of HATU (1.1 equiv, 53 mg, 0.14 mmol) in 15 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (3.0 equiv, 0.39 mmol, 50 mg, 67 μl) and HOAt (1.0 equiv, 0.13 mmol, 17 mg) in 20 ml DMF.

After evaporation of the solvent the epimers are separated by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(115)**): 52 mg, 47 μmol, 36 %.

HPLC (anal. method 1): t_R=32.8 min, 93 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1038.59 $[M+H]^+$, 1060.57 $[M+Na]^+$, 1076.55 $[M+K]^+$

Found: 1060.29 $[M+Na]^+$, 1076.85 $[M+K]^+$

The cyclic protected peptide **(115)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(54)**): 27 mg, 43 μmol, 92 %.

HPLC (anal. method 2): t_R= 9.9 min, 100 area %. No isomers observed.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 628.29 $[M+H]^+$, 650.28 $[M+Na]^+$, 666.25 $[M+K]^+$

Found: 628.27 $[M+H]^+$, 650.32 $[M+Na]^+$, 666.21 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 5 : 1).

0.85 (d, ^3J=6.3 Hz, 3H, H^γ (D-Val)), 0.97 (d, ^3J=6.9 Hz, 3H, H^γ (D-Val)), 1.01 (d, ^3J=6.9 Hz, 3H, H^γ (Thr)), 1.68-1.81 (m, 2H, H^β (Gln), H^β (Pro)), 1.83-2.02 (m, 6H, $2H^\gamma$ (Gln), H^β (Gln), $2H^\gamma$ (Pro), H^β (D-Val)), 2.24 (m, 1H, H^β (Pro)), 2.45 (dd, ^2J=16.8 Hz, ^3J=9.0 Hz, 1H, H^β (Asp)), 2.83 (dd, ^2J=16.6 Hz, ^3J=4.7 Hz, 1H, H^β (Asp)), 3.56 (dd, ^3J=10.1 Hz, ^3J=5.0 Hz, 1H, H^α (D-Val)), 3.68 (ddd, ^2J=9.7 Hz, ^3J=9.7 Hz, ^3J=6.3 Hz, 1H, H^δ (Pro)), 3.76 (ddd, ^2J=9.9 Hz, ^3J=8.0 Hz, ^3J=2.4 Hz, 1H, H^δ (Pro)), 3.82 (dd, ^2J=11.9 Hz, ^3J=2.5 Hz, 1H, H^β (Ser)), 3.95 (m, 1H, H^β (Thr)), 4.00 (dd, ^2J=11.6 Hz, ^3J=6.0 Hz, 1H, H^β (Ser)), 4.04 (dd, ^3J=9.4 Hz, ^3J=3.8 Hz, 1H, H^α (Thr)), 4.07 (dd, ^3J=9.1 Hz, ^3J=7.9 Hz, 1H, H^α (Pro)), 4.32 (ddd, ^3J=7.7 Hz, ^3J=5.2 Hz, ^3J=4.2 Hz, 1H, H^α (Gln)), 4.67 (ddd, ^3J=9.0 Hz, ^3J= 9.0 Hz, ^3J= 4.6 Hz, 1H, H^α (Asp)), 4.74 (ddd, ^3J=8.6 Hz, ^3J=5.8 Hz, ^3J=2.7 Hz, 1H, H^α (Ser)), 4.91 (br, 1H, H^O (Thr)), 5.29 (br, 1H, H^O (Ser)), 6.63 (br, 1H, H^N (Gln)), 6.70 (d, ^3J=5.0 Hz, 1H, H^N (Gln)), 6.99 (d, ^3J=9.4 Hz, H^N (Thr)), 7.16 (br, 1H, H^N (Gln)), 7.99 (d, ^3J=8.2 Hz, 1H, H^N (Ser)), 8.68 (d, ^3J=8.8 Hz, H^N (Asp)), 8.87 (d, ^3J =5.0 Hz, H^N (Val)), 12.84 (br, 1H, H^{COO} (Asp)).

c-(-Gln-Ile-Asp-Ser-Pro-) (55)

$C_{23}H_{36}N_6O_9$ (540.58 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-Pro-Gln(Trt)-Ile-Asp(Wang)-ODmb **(116)** is synthesized and cyclized following protocol 6, with different coupling reagents (Table 37), on Wang resin. After complete deprotection and cleavage from the resin, with different cleavage cocktails (Table 37), the cyclic peptide **(55)** is purified by reverse phase preparative HPLC.

Table 37: On resin cyclization of H-Ser(t-Bu)-Pro-Gln(Trt)-Ile-Asp(Wang)-ODmb (116)

Resin	n/ mmol	Coupling reagent	t/ min	Cleavage Reagent	t/ h	Yield	HPLC/ %
Wang 135 mg	0.10	1.1 equiv HATU 3.0 equiv DIPEA 2.0 equiv HOAt	45	Reagent B	5	6 mg 8 μmol 8 %	70[a]
Wang 130 mg	0.10	3.0 equiv HATU 6.0 equiv DIPEA	40	TFA 47 DCM 47 TIS 6	2	3 mg 6 μmol 6 %	100[a]

[a] The same HPLC analytical method and the retention time as for the cyclization in solution.

Cyclization in solution:

The linear peptide H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Gln(Trt)-OH **(117)** is synthesized on 2-ClTrt resin (300 mg, 0.20 mmol) previously loaded with Fmoc-Gln(Trt)-OH (loading 0.66 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(117)**): 150 mg, 89 μmol, 45 %.

HPLC (anal. method 1): t_R= 26.9 min, 54 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 913.51 $[M+H]^+$, 935.49 $[M+Na]^+$, 951.46 $[M+K]^+$

Found: 913.33 $[M+H]^+$, 935.28 $[M+Na]^+$, 951.36 $[M+K]^+$

The linear peptide **(117)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(117)** (150 mg, 89 μmol) in 10 ml DMF.

Second syringe: Solution of HATU (6.0 equiv, 0.53 mmol, 200 mg) in 10 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (13.0 equiv, 1.17 mmol, 151 mg, 200 μl) in 20 ml DMF.

After evaporation of the solvent the epimers are separated by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(118)**): 29 mg, 26 μmol, 29 %.

HPLC (anal. method 1): t_R= 31.8 min, 81 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 895.50 $[M+H]^+$, 917.48 $[M+Na]^+$, 933.45 $[M+K]^+$

Found: 917.28 $[M+Na]^+$, 933.45 $[M+K]^+$

The protected cyclic peptide **(118)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(55)**): 15 mg, 24 μmol, 92 %.

HPLC (anal. method 2): t_R= 11.0 min, 85 area %. No isomers observed.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 541.26 $[M+H]^+$, 563.24 $[M+Na]^+$, 579.22 $[M+K]^+$

Found: 541.49 $[M+H]^+$, 563.69 $[M+Na]^+$, 579.71 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 3 : 1).

Major conformer: 0.81 (d, ^{3}J=6.3 Hz, 3H, H^{γ} (Ile)), 0.82 (dd, ^{3}J=7.5 Hz, ^{3}J=7.5 Hz, 3H, H^{δ} (Ile)), 0.98 (ddq, ^{2}J=13.8 Hz, ^{3}J=7.2 Hz, ^{3}J=1.3 Hz, 1H, H^{γ} (Ile)), 1.38 (m, 1H, H^{γ} (Ile)), 1.65-1.79 (m, 2H, H^{β} (Gln), H^{γ} (Pro)), 1.79-1.92 (m, 3H, H^{β} (Gln), H^{β} (Ile), H^{γ} (Pro)), 1.92-2.09 (m, 3H, $2H^{\gamma}$ (Gln), H^{β} (Pro)), 2.21 (dddd, ^{2}J=10.4 Hz, ^{3}J=9.8 Hz, ^{3}J=7.5 Hz, ^{3}J=2.8 Hz, 1H, H^{β} (Pro)), 2.33 (dd, ^{2}J=16.9 Hz, ^{3}J=11.3 Hz, 1H, H^{β} (Asp)), 2.71 (dd, ^{2}J=16.6, ^{3}J=2.2 Hz, 1H, H^{β} (Asp)), 3.36 (dd, ^{2}J=10.1 Hz, ^{3}J=5.0 Hz, 1H, H^{β} (Ser)), 3.39-3.56 (br, 2H, H^{δ} (Pro), H^{β} (Ser)), 3.60 (ddd, ^{2}J=11.5 Hz, ^{3}J=7.7 Hz, ^{3}J=3.6 Hz, 1H, H^{δ} (Pro)), 4.11 (ddd, ^{3}J=10.8 Hz, ^{3}J=10.8 Hz, ^{3}J=2.6 Hz, 1H, H^{α} (Asp)), 4.30 (ddd, ^{3}J=9.9 Hz, ^{3}J=9.9 Hz, ^{3}J=5.5 Hz, 1H, H^{α} (Gln)), 4.40 (ddd, ^{3}J=8.6 Hz, ^{3}J=8.6 Hz, ^{3}J=5.2 Hz, 1H, H^{α} (Ser)), 4.47 (dd, ^{3}J=9.1 Hz, ^{3}J=9.1 Hz, 1H, H^{α} (Ile)), 4.51 (dd, ^{3}J=8.8 Hz, ^{3}J=2.5 Hz, 1H, H^{α} (Pro)), 6.77 (s, 1H, H^{N} (Gln)), 6.94 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Ser)), 7.28 (s, 1H, H^{N} (Gln)), 7.54 (d, ^{3}J=10.0 Hz, 1H, H^{N} (Gln)), 7.84 (d, ^{3}J=10.7 Hz, 1H, H^{N} (Asp)), 8.35 (d, ^{3}J=9.4 Hz, 1H, H^{N} (Ile)), 12.51 (br, 1H, H^{COO} (Asp)).

Minor conformer 0.78 (d, ^{3}J=6.3 Hz, 3H, H^{γ} (Ile)), 0.79-0.83 (m, 3H, H^{δ} (Ile)), 1.04 (m, 1H, H^{γ} (Ile)), 1.42 (m, 1H, H^{γ} (Ile)), 1.70 (m, 1H, H^{γ} (Pro)), 1.79-1.92 (m, 4H, $2H^{\beta}$ (Gln), H^{β} (Ile), H^{γ} (Pro)), 1.92-2.09 (m, 4H, $2H^{\gamma}$ (Gln), $2H^{\beta}$ (Pro)), 2.74 (dd, ^{2}J=16.0 Hz, ^{3}J=6.0 Hz, 1H, H^{β} (Asp)), 2.80 (dd, ^{2}J=16.0 Hz, ^{3}J=7.5 Hz, 1H, H^{β} (Asp)), 3.39-3.56 (br, 3H, $2H^{\delta}$ (Pro), H^{β} (Ser)), 3.69 (dd, ^{2}J=10.1 Hz, ^{3}J=4.4 Hz, 1H, H^{β} (Ser)), 3.75 (dd, ^{3}J=8.7 Hz, ^{3}J=8.5 Hz, 1H, H^{α} (Ile)), 4.10 (m, 1H, H^{α} (Ser)), 4.19-4.27 (m, 2H, H^{α} (Asp), H^{α} (Gln)), 4.59 (dd, ^{3}J=6.6 Hz, ^{3}J=2.2 Hz, 1H, H^{α} (Pro)), 6.82 (s, 1H, H^{N} (Gln)), 7.33 (s, 1H, H^{N} (Gln)), 7.66 (d, ^{3}J=3.8 Hz, 1H H^{N}

(Ser)), 7.73 (d, ^{3}J=8.8 Hz, 1H H^N (Gln)), 7.94 (d, ^{3}J=8.2 Hz, 1H H^N (Ile)), 8.02 (d, ^{3}J=8.8 Hz, 1H H^N (Asp)), 12.51 (br, 1H, H^{COO} (Asp)).

c-(-Gln-Ile-Asp-Ser-β-hPro-) (56)

$C_{24}H_{38}N_6O_9$ (554.61 g/mol).

Cyclization on resin:

The linear peptide H-Ser(t-Bu)-β-hPro-Gln(Trt)-Ile-Asp(Wang)-ODmb **(119)** is synthesized and cyclized following protocol 6 (HATU as coupling reagent), on Wang resin (125 mg, 50 μmol), After complete deprotection and cleavage from the resin with cocktail mixture TFA/DCM/TIS = 48 : 48 : 4 (protocol 10), the cyclic peptide **(56)** is purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(56)**): 4 mg, 7.2 μmol, 14 %.

HPLC (anal. method 2): t_R= 13.1 min, 100 %.

Cyclization in solution:

The linear peptide H-β-hPro-Gln(Trt)-Ile-Asp(Ot-Bu)-Ser(t-Bu)-OH **(120)** is synthesized on 2-ClTrt resin (200 mg, 0.20 mmol) previously loaded with Fmoc--Ser(t-Bu)-OH (loading 1.00 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (protected linear peptide **(120)**): 74 mg, 72 μmol, 36 %.

HPLC (anal. method 1): t_R= 28.1 min, 90 %.

MALDI-ToF $MS_{monoisotopic}$, m/z

Calcd.: 927.52 $[M+H]^+$, 949.51 $[M+Na]^+$, 965.50 $[M+K]^+$

Found: 927.49 $[M+H]^+$

The linear peptide **(120)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(120)** (74 mg, 72 μmol) in 10 ml DMF.

Second syringe: Solution of HATU (3.0 equiv, 0.22 mmol, 82 mg) in 10 ml DMF.

Addition rate for both syringes: 0.05 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.43 mmol, 56 mg, 74 μl) and HATU (0.1 equiv, 7 μmol 3 mg) in 10 ml DMF.

After evaporation of the solvent the the epimers are separated by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(121)**): 40 mg, 26 μmol, 36 %.

HPLC (anal. method 1): t_R= 32.7 min, 60 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 909.51 $[M+H]^+$, 931.49 $[M+Na]^+$, 947.47 $[M+K]^+$

Found: 931.54 $[M+Na]^+$, 947.62 $[M+K]^+$

The cyclic protected peptide **(121)** is completely deprotected with reagent K during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.
Yield (cyclic deprotected peptide **(56)**): 13 mg, 23 μmol, 89 %.
HPLC (anal. method 2): t_R= 13.1 min, 100 area %. No isomers observed.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 555.28 $[M+H]^+$, 577.26 $[M+Na]^+$, 593.23 $[M+K]^+$
Found: 555.26 $[M+H]^+$, 577.08 $[M+Na]^+$, 593.00 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 2 : 1).
Major conformation 0.73-0.88 (m, 6H, $3H^{\gamma}$ (Ile), $3H^{\delta}$ (Ile)), 0.98 (ddq, ^2J=14.4 Hz, ^3J=7.2 Hz, ^3J=7.2 Hz, 1H, H^{γ} (Ile)), 1.35 (m, 1H; H^{γ} (Ile)), 1.62 (m, 1H, H^{γ} (Gln)), 1.76-1.91 (m, 5H, H^{β} (Ile), $2H^{\gamma}$ (β-hPro), $2H^{\delta}$ (β-hPro)), 1.90-2.10 (m, 2H, H^{β} (Gln), H^{γ} (Gln)), 2.17 (m, 1H, H^{β} (Gln)), 2.34 (dd, ^2J=17.3 Hz, ^3J=6.6 Hz, 1H, H^{α} (β-hPro)), 2.55 (dd, ^2J=16.6 Hz, ^3J=8.5 Hz, 1H, H^{β} (Asp)), 2.60 (dd, ^2J=17.6 Hz, ^3J=2.5 Hz, 1H, H^{α} (β-hPro)), 2.73 (dd, ^2J=16.3 Hz, ^3J=5.7 Hz, 1H, H^{β} (Asp)), 3.27-3.55 (br, 2H, H^{ε} (β-hPro)), 3.64 (dd, ^2J=12.6 Hz, ^3J=4.4 Hz, 1H, $H^{\beta\,(Ser)}$), 3.82 (dd, ^2J=12.6 Hz, ^3J=3.1 Hz, 1H, $H^{\beta\,(Ser)}$), 3.87 (dd, ^3J=9.7 Hz, ^3J=9.7 Hz, 1H, H^{α} (Ile)), 4.06 (m, 1H, H^{α} (Gln)), 4.39 (m, 1H, H^{α} (Ser)), 4.50 (ddd, ^3J=8.5 Hz, ^3J=8.5 Hz, 6.3 Hz, 1H, H^{α} (Asp)), 4.60 (m, 1H, H^{β} (β-hPro)), 6.72 (s, 1H, H^{N} (Gln)), 7.22 (s, 1H, H^{N} (Gln)), 7.33 (br, 1H, H^{N} (Gln)), 7.66 (d, ^3J=9.4 Hz, 1H, H^{N} (Ile)), 7.70 (d, ^3J=8.8 Hz, 1H, H^{N} (Asp)), 7.97 (d, ^3J=6.9 Hz, 1H, H^{N} (Ser)), 12.3 (br, 1H, H^{COO} (Asp)).

Minor conformation 0.73-0.88 (m, 6H, $3H^{\gamma}$ (Ile), $3H^{\delta}$ (Ile)), 0.98 (ddq, ^2J=14.4 Hz, ^3J=7.2 Hz, ^3J=7.2 Hz, 1H, H^{γ} (Ile)), 1.35 (m, 1H, H^{γ} (Ile)), 1.48 (m, 1H, H^{δ} (β-hPro)), 1.59-1.76 (m, 2H, H^{β} (Gln), H^{γ} (β-hPro)), 1.76-1.91 (m, 2H, H^{β}(Gln), H^{δ} (β-hPro)), 1.90-2.10 (m, 5H, $2H^{\gamma}$ (Gln), H^{β} (Ile), H^{α} (β-hPro), H^{γ} (β-hPro)), 2.17 (m, 1H, H^{α} (β-hPro)), 2.92 (m, 1H, H^{ε} (β-hPro)), 2.89 (dd, ^2J=16.3 Hz, ^3J=8.8 Hz, 1H, H^{β} (Asp)), 2.95 (dd, ^2J=16.6 Hz, ^3J=5.3 Hz, 1H, H^{β} (Asp)), 3.27-3.55 (br, 2H, H^{ε} (β-hPro), H^{β} (Ser)), 3.58 (dd, ^2J=17.0 Hz, ^3J=6.9 Hz, 1H, H^{β} (Ser)), 3.93-4.01 (m, 2H, H^{α} (Asp), H^{α} (Ile)), 4.12 (ddd, ^3J=9.1 Hz, ^3J=9.1 Hz, ^3J=5.0 Hz, 1H, H^{α} (Gln)), 4.31 (m, 1H, H^{β} (β-hPro)), 4.45 (m, 1H, H^{α} (Ser)), 6.81 (s, 1H, H^{N} (Gln)), 7.33 (br, 1H, H^{N} (Gln)), 7.66 (d ^3J=8.2 Hz, 1H, H^{N} (Ile)), 7.73 (d, ^3J=9.3 Hz, 1H, H^{N} (Gln)), 7.98 (d, ^3J=5.7 Hz, 1H, H^{N} (Ser)), 9.21 (d, ^3J=6.9 Hz, 1H, H^{N} (Asp)), 12.3 (br, 1H, H^{COO} (Asp)).

c-(-Gln-Leu-Asp-Ser-Pro-) (57)

$C_{23}H_{36}N_6O_9$ (540.58 g/mol).

Cyclization in solution:

The linear peptide H-Asp(Ot-Bu)-Ser(t-Bu)-Pro-Gln(Trt)-Leu-OH **(122)** is synthesized on 2-ClTrt resin (278 mg, 0.20 mmol) previously loaded with Fmoc--Leu-OH (loading 0.72 mmol/g), cleaved with 1 % TFA in DCM (protocol 9) and purified by preparative HPLC.

Yield (linear protected peptide **(122)**): 130 mg, 0.14 mmol, 71 %.

HPLC (anal. method 1): t_R= 27.4 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 913.51 $[M+H]^+$, 935.49 $[M+Na]^+$, 951.46 $[M+K]^+$

Found: 935.93 $[M+Na]^+$, 951.52 $[M+K]^+$

The linear peptide **(122)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(122)** (130 mg, 0.14 mmol) in 20 ml DMF.

Second syringe: Solution of HATU (1.1 equiv, 0.16 mmol, 59 mg) in 20 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (2.0 equiv, 0.28 mmol, 37 mg, 49 µl) and HOAt (1.0 equiv, 0.14 mmol 19 mg) in 20 ml DMF.

After evaporation of DMF the cyclic protected peptide **(123)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(123)**): 120 mg, 0.10 mmol, 71 %.

HPLC (anal. method 1): t_R= 31.4 min, 75 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 895.50 $[M+H]^+$, 917.48 $[M+Na]^+$, 933.45 $[M+K]^+$

Found: 895.76 $[M+H]^+$, 917.73 $[M+Na]^+$, 933.70 $[M+K]^+$

The cyclic protected peptide **(123)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(57)**): 32 mg, 44 µmol, 44 %.

HPLC (anal. method 2): t_R= 11.9 min, 75 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 541.26 $[M+H]^+$, 563.24 $[M+Na]^+$, 579.22 $[M+K]^+$

Found: 541.27 $[M+H]^+$, 563.43 $[M+Na]^+$, 579.32 $[M+K]^+$

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 9 : 1).

0.84 (d, ^{3}J=6.9 Hz, 3H, H^δ (Leu)), 0.86 (d, ^{3}J=6.3 Hz, 3H, H^δ (Leu)), 1.15 (ddd, ^{2}J=13.7 Hz, ^{3}J=6.8 Hz, ^{3}J=6.4 Hz, 1H, H^β (Leu)), 1.47 (m, 1H, H^γ (Leu)), 1.62-1.76 (m, 4H, 2H^β (Gln), H^β (Leu), H^γ (Pro)), 1.84 (m, 1H, H^γ (Pro)), 1.88-2.00 (m, 3H, 2H^γ (Gln), H^β (Pro)), 2.19 (dddd, ^{2}J=16.3 Hz, ^{3}J=9.3 Hz, ^{3}J=6.8 Hz, ^{3}J=6.8 Hz, 1H,

H^{β} (Pro)), 2.36 (dd, ^{2}J=16.9 Hz, ^{3}J=2.1 Hz, 1H, H^{β} (Asp)), 2.72 (dd, ^{2}J=16.9 Hz, ^{3}J=11.0 Hz, 1H, H^{β} (Asp)), 3.35 (dd, ^{2}J=10.4 Hz, ^{3}J=5.3 Hz, 1H, H^{β} (Ser)), 3.41-3.49 (m, 2H, H^{δ} (Pro), H^{β} (Ser)), 3.60 (ddd, ^{2}J=11.2 Hz, ^{3}J=8.3 Hz, ^{3}J=3.0 Hz, 1H, H^{δ} (Pro)), 4.12 (ddd, ^{3}J=10.7 Hz, ^{3}J=10.7 Hz, ^{3}J=2.5 Hz, 1H, H^{α} (Asp)), 4.25 (ddd, ^{3}J=9.4 Hz, ^{3}J=9.4 Hz, ^{3}J=5.6 Hz, 1H, H^{α} (Gln)), 4.42 (ddd, ^{3}J=8.6 Hz, ^{3}J=8.6 Hz, ^{3}J=5.5 Hz, 1H, H^{α} (Ser)), 4.50 (dd, ^{3}J=8.5 Hz, ^{3}J=2.2 Hz, 1H, H^{α} (Pro)), 4.67 (ddd, ^{3}J=8.3 Hz, ^{3}J=8.3 Hz, ^{3}J=6.1 Hz, 1H H^{α} (Leu)), 6.78 (s, 1H, H^{N} (Gln)), 7.00 (d, ^{3}J=8.2 Hz, 1H, H^{N} (Ser)), 7.30 (s, 1H, H^{N} (Gln)), 7.46 (d, ^{3}J=9.4 Hz, 1H, H^{N} (Gln)), 7.83 (d, ^{3}J=10.7 Hz, 1H, H^{N} (Asp)), 8.4 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Leu)), 12.54 (br, 1H, H^{COO} (Asp)).

c-(-Ile-Asp-Ser-β-hPro-Leu-) (58)

$C_{25}H_{41}N_5O_8$ (539.64 g/mol).

Cyclization in solution:

The linear peptide H-Ile-Asp(Ot-Bu)-Ser(t-Bu)-β-hPro-Leu-OH **(124)** is synthesized on 280 mg (0.20 mmol) Fmoc-Leu-Sasrin (loading 0.72 mmol/g), and cleaved with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(124)**): 98 mg, 0.13 mmol, 65 %.

HPLC (anal. method 1): t_R= 22.3 min, 88 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 670.44 $[M+H]^+$, 692.42 $[M+Na]^+$, 708.39 $[M+K]^+$

Found: 670.66 $[M+H]^+$, 692.56 $[M+Na]^+$, 708.45 $[M+K]^+$

The linear peptide **(124)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(124)** (98 mg, 0.13 mmol) in 10 ml DMF.

Second syringe: Solution of HATU (3.0 equiv, 0.39 mmol, 148 mg) in 10 ml DMF.

Addition rate for both syringes: 0.04 ml/min.

Flask: Solution of DIPEA (6.0 equiv, 0.78 mmol, 101 mg, 133 µl) and HATU (0.1 equiv, 13 µmol, 5 mg) in 10 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(125)** is purified by reverse phase preparative HPLC.

Yield (cyclic protected peptide **(125)**): 8 mg, 10 µmol, 8 %.

HPLC (anal. method 1): t_R= 26.6 min, 87 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 652.43 $[M+H]^+$, 674.41 $[M+Na]^+$, 690.38 $[M+K]^+$

Found: 674.80 $[M+Na]^+$, 690.79 $[M+K]^+$

The cyclic protected peptide **(125)** is completely deprotected with reagent K during 2 h (protocol 13) and finally purified by preparative HPLC.

Yield (cyclic deprotected peptide **(58)**): 4 mg, 6 mmol, 62 %.
HPLC (anal. method 2): t_R= 21.54 min, 84 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 540.30 $[M+H]^+$, 562.29 $[M+Na]^+$, 578.26 $[M+K]^+$
Found: 540.39 $[M+H]^+$, 562.38 $[M+Na]^+$, 578.25 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): Two sets of signals (ratio 8 : 1).
0.74-0.91 (m, 12H, 3H$^{\gamma}$ (Ile), 3H$^{\delta}$ (Ile), 6H$^{\delta}$ (Leu)), 0.98 (ddq, ^{2}J=14.3 Hz, ^{3}J=7.1 Hz, ^{3}J=7.1 Hz, 1H, H$^{\gamma}$ (Ile)), 1.30-1.53 (m, 4H, H$^{\gamma}$ (Ile), 2H$^{\beta}$ (Leu), H$^{\gamma}$ (β-hPro)), 1.56 (m 1H, H$^{\gamma}$ (β-hPro)), 1.64 (m, 1H, H$^{\delta}$ (β-hPro)), 1.78-1.89 (m, 2H, H$^{\gamma}$ (Leu), H$^{\delta}$ (β-hPro)), 1.95 (m, 1H, H$^{\beta}$ (Ile)), 2.07 (m, 1H, H$^{\alpha}$ (β-hPro)), 2.17 (m, 1H, H$^{\alpha}$ (β-hPro)), 2.49 (br, 1H, H$^{\beta}$ (Asp)), 2.71 (dd, ^{2}J=15.7 Hz, ^{3}J=5.6 Hz, 1H, H$^{\beta}$ (Asp)), 3.50 (m, 1H, H$^{\varepsilon}$ (β-hPro)), 3.53-3.68 (m, 2H, H$^{\varepsilon}$ (β-hPro), H$^{\beta}$ (Ser)), 3.83 (dd, ^{3}J=12.9 Hz, ^{3}J=10.4 Hz, 1H, H$^{\beta}$ (Ser)), 4.03-4.16 (m, 2H, H$^{\alpha}$ (Ile), H$^{\beta}$ (β-hPro)), 4.23 (ddd, ^{3}J=9.4 Hz, ^{3}J=9.1 Hz, ^{3}J=2.8 Hz, 1H, H$^{\alpha}$ (Leu)), 4.38 (m, 1H, H$^{\alpha}$ (Ser)), 4.48 (m, 1H, H$^{\alpha}$ (Asp)), 7.25 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ile)), 7.66-7.74 (m, 2H, H^{N} (Asp), H^{N} (Leu)), 7.98 (d, ^{3}J=6.9 Hz, 1H, H^{N} (Ser)), 12.33 (br, 1H, H^{COO} (Asp)).

6.4.7. Synthesis of Cyclic Analogs of the Binding Epitope of Invasin

c-(-Ser-Asp-Met-Ser-D-Gln-Gly-) (126)

$C_{22}H_{35}N_7O_{11}S$ (605.63 g/mol).
Cyclization on resin:
The linear peptide H-Met-Ser(t-Bu)-D-Gln-Gly-Ser(t-Bu)-Asp(Wang)-ODmb **(128)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents) on Wang resin (250 mg, 0.10 mmol). After deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 47 : 47 : 6 during 2 h (protocol 10) the cyclic peptide **(126)** is purified by reverse phase preparative HPLC.
Yield: 5 mg, 7 μmol, 7 %.
HPLC (anal. method 4): t_R= 5.8 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 606.22 $[M+H]^+$, 628.20 $[M+Na]^+$, 644.18 $[M+K]^+$
Found: 606.45 $[M+H]^+$, 628.49 $[M+Na]^+$, 644.47 $[M+K]^+$

c-(-Ser-Asp-Met-Ser-D-Ala-Gly-) (127)

$C_{20}H_{32}N_6O_{10}S$ (548.58 g/mol).

Cyclization on resin:

The linear peptide H-Met-Ser(t-Bu)-D-Ala-Gly-Ser(t-Bu)-Asp(Wang)-ODmb **(129)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents) on Wang resin (250 mg, 0.10 mmol). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/DCM/TIS = 47 : 47 : 6 during 2 h (protocol 10) the cyclic peptide **(126)** is purified by reverse phase preparative HPLC.

Yield: 2 mg, 3 μmol, 3 %.

HPLC (anal. method 4): t_R= 6.1 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 549.20 $[M+H]^+$, 571.18 $[M+Na]^+$, 587.15 $[M+K]^+$

Found: 549.30 $[M+H]^+$, 571.32 $[M+Na]^+$, 587.32 $[M+K]^+$

c-(-Ser-Asp-Met-Ser-Lys(H-Arg-(β-Ala)$_5$-)-Gly-) (130)

$C_{44}H_{76}N_{16}O_{16}S$ (1117.26 g/mol).

Two segments of this peptide **(131)** and **(132)** are separately synthesized, selectively deprotected and condensed.

Fmoc-Arg(Pbf)-β-Ala-β-Ala-β-Ala-β-Ala-β-Ala-OH **(131)**

$C_{49}H_{65}N_9O_{12}S$ (1004.19 g/mol).

The peptide **(131)** is synthesized on 2-ClTrt resin (330 mg, 0.10 mmol) previously loaded with Fmoc-β-Ala-OH (loading 0.31 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9) and purified by reverse phase preparative HPLC.

Yield: 90 mg, 85 μmol, 85 %.

HPLC (anal. method 1): t_R= 26.7 min, 95 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1004.46 $[M+H]^+$, 1026.44 $[M+Na]^+$, 1042.41 $[M+K]^+$

Found: 1004.64 $[M+H]^+$, 1026.61 $[M+Na]^+$, 1042.67 $[M+K]^+$

c-(-Ser(t-Bu)-Asp(Ot-Bu)-Met-Ser(t-Bu)-D-Lys-Gly-) **(132)**

$C_{35}H_{63}N_7O_{10}S$ (774.00 g/mol).
The linear peptide H-Ser(t-Bu)-Asp(Ot-Bu)-Met-Ser(t-Bu)-D-Lys(Aloc)-Gly-OH **(133)** is synthesized on the 2-Cl-trityl resin (285 mg, 0.20 mmol) previously loaded with Fmoc-Gly-OH (loading 0.70 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9) and purified by reverse phase preparative HPLC.
Yield: 160 mg, 0.13 mmol, 64 %.
HPLC (anal. method 1): t_R= 22.0 min, 70 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 876.48 $[M+H]^+$, 898.46 $[M+Na]^+$, 914.43 $[M+K]^+$
Found: 876.64 $[M+H]^+$, 898.71 $[M+Na]^+$, 914.72 $[M+K]^+$
The linear peptide **(133)** is cyclized following protocol 7, method B:
First syringe: Solution of linear peptide **(133)** (160 mg, 0.13 mmol) in 17 ml DMF.
Second syringe: Solution of HATU (1.5 equiv, 0.20 mmol, 76 mg) in 17 ml DMF.
Addition rate for both syringes: 0.01 ml/min.
Flask: Solution of DIPEA (4.7equiv, 0.60 mmol, 77 mg, 102 µl) and HOAt (1.5 equiv, 0.20 mmol, 27 mg) in 20 ml DMF.
After evaporation of the solvent the cyclic protected peptide **(134)** is purified by reverse phase preparative HPLC.
Yield (cyclic protected peptide **(134)**): 47 mg, 53 µmol, 41 %.
HPLC (anal. method 1): t_R= 25.9 min, 95 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 858.46 $[M+H]^+$, 880.44 $[M+Na]^+$, 896.42 $[M+K]^+$
Found: 880.55 $[M+Na]^+$, 896.59 $[M+K]^+$

Aloc-cleavage:

The Aloc protective group from the c-(-Ser(t-Bu)-Asp(Ot-Bu)-Met-Ser(t-Bu)-D--Lys(Aloc)-Gly-), **(134)** (47 mg, 53 µmol) is cleaved with 0.2 equiv $[Pd(PPh_3)_4]$ (10 µmol, 12 mg) and 24.0 equiv (1.2 mmol, 130 mg, 0.15 ml) phenylsilane in 2 ml DCM (protocol 12). After evaporation of the solvent, the product **(132)** is used for the next reaction without further purification.
HPLC (anal. method 1): t_R= 23.1 min.
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 774.44 $[M+H]^+$, 796.43 $[M+Na]^+$, 812.40 $[M+K]^+$
Found: 774.64 $[M+H]^+$, 796.61 $[M+Na]^+$, 812.59 $[M+K]^+$

Segment condensation reaction:

HATU (60 μmol, 23 mg) and DIPEA (150 μmol, 19 mg 25 μl) are added to the solution of Fmoc-Arg(Pbf)-β-Ala-β-Ala-β-Ala-β-Ala-β-Ala-OH **(131)** (90 mg, 85 μmol) in 3 ml DMF. The reaction mixture is stirred for 5 min at room temperature and added to the solution of c-(-Ser(t-Bu)-Asp(Ot-Bu)-Met-Ser(t-Bu)-D-Lys-Gly-) **(132)** in DCM. After 30 min stirring additional HATU (60 μmol, 23 mg) and DIPEA (150 μmol, 19 mg 25 μl) are added to the reaction mixture. The mixture is stirred overnight at room temperature, DMF is evaporated and the fully protected peptide **(135)** is purified by reverse phase preparative HPLC.

*Yield (Fully protected peptide **(135)**):* 39 mg, 18 μmol, 34 %.

HPLC (anal. method 1): t_R= 29.7 min, 80 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1759.88 $[M+H]^+$, 1781.86 $[M+Na]^+$, 1797.84 $[M+K]^+$

Found: 1759.59 $[M+H]^+$, 1781.42 $[M+Na]^+$, 1797.74 $[M+K]^+$

Cleavage of the protective groups:

To the fully protected peptide **(135)**, obtained by segment condensation reaction, is added a solution of 2 % DBU and 2 % piperidine in DMF. Reaction mixture is shaken at room temperature for 30 min and DMF is evaporated under high vacuum (temperature of the bath must not exceed 30 °C). To the residue are added 150 ml dried diethylether. The ether is decanted, centrifuged 1 h on 0 ºC with 4000 rpm, and again decanted. The residue (after both decantation) is dried in vacuum.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1537.81 $[M+H]^+$, 1559.79 $[M+Na]^+$, 1575.77 $[M+K]^+$

Found: 1559.80 $[M+Na]^+$, 1575.90 $[M+K]^+$

The residue is completely deprotected with reagent K during 3 h (protocol 13) and purified by reverse phase preparative HPLC.

*Yield (peptide **(130)**):* 7 mg, 6.3 μmol, 35 %.

HPLC (anal. method 2): t_R= 12.5 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1117.54 $[M+H]^+$, 1139.52 $[M+Na]^+$, 1155.50 $[M+K]^+$

Found: 1117.94 $[M+H]^+$, 1139.55 $[M+Na]^+$, 1155.74 $[M+K]^+$

6.4.8. Synthesis of Linear Precursors of Tyrocidine A Analogs

H-D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-S-CH_2-CH_2-$NHCOCH_3$ (136)

$C_{70}H_{96}N_{14}O_{14}S$ (1389.70 g/mol).

The peptide Boc-D-Phe-Pro-Phe-D-Phe-Asn(Trt)-Gln(Trt)-Tyr(t-Bu)-Val-Orn(Boc)--Leu-S-CH_2-CH_2-$NHCOCH_3$ **(144)** is synthesized on the "safety catch" resin (240 mg, 0.10 mmol) previously loaded with Fmoc-Leu-OH (protocol 2) and cleaved from the resin with formation of thioester bond (protocol 11).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 2130.09 $[M+H]^+$, 2152.07 $[M+Na]^+$, 2168.05 $[M+K]^+$

Found: 2152.05 $[M+Na]^+$, 2168.29 $[M+K]^+$

Without further purification the peptide **(144)** is completely deprotected (except the N-acetyl group) with reagent B during 1h (protocol 13). Finally the peptide **(136)** is purified by reverse phase preparative HPLC.

Yield: 90 mg, 61 μmol, 62 %.

HPLC (anal. method 1): t_R= 22.0 min, 95 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 1389.70 $[M+H]^+$, 1411.68 $[M+Na]^+$, 1427.66 $[M+K]^+$

Found: 1389.64 $[M+H]^+$

H-D-Phe-β-hPro-β-hPhe-D-β-hPhe-β-hAsn-Gln-Tyr-Val-Orn-Leu-S-CH_2-CH_2--$NHCOCH_3$ (142)

$C_{74}H_{104}N_{14}O_{14}S$ (1445.81 g/mol).

The peptide Boc-D-Phe-β-hPro-β-hPhe-D-β-hPhe-β-hAsn(Trt)-Gln(Trt)-Tyr(t-Bu)--Val-Orn(Boc)-Leu-S-CH_2-CH_2-$NHCOCH_3$ **(145)** is synthesized on the "safety catch" resin (240 mg, 0.1 mmol) previously loaded with Fmoc-Leu-OH (protocol 2) and cleaved from the resin with formation of thioester bond (protocol 11).

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 2186.15 $[M+H]^+$, 2208.13 $[M+Na]^+$, 2224.11 $[M+K]^+$

Found: 2208.27 $[M+Na]^+$, 2224.30 $[M+K]^+$

Without further purification the peptide **(145)** is completely deprotected (except the N-acetyl group) with reagent B during 2h (protocol 13). Finally the peptide **(142)** is purified by reverse phase preparative HPLC.

Yield: 18 mg, 1.1 μmol, 11 %.

HPLC (anal. method 1): t_R= 21.0 min, 90 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 1445.77 $[M+H]^+$, 1467.75 $[M+Na]^+$, 1483.72 $[M+K]^+$
Found: 1445.72 $[M+H]^+$, 1467.70 $[M+Na]^+$

6.4.9. Synthesis of RGD Peptides

c-(Arg-Gly-Asp-D-Phe-β-hPro-) (146)

$C_{27}H_{38}N_8O_7$ (586.66).
Cyclization on resin:
The linear peptide H-D-Phe-β-hPro-Arg(Pbf)-Gly-Asp(Wang)-ODmb **(147)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents) on Wang resin (300 mg, 0.2 mmol), previously loaded with Fmoc-Asp-ODmb (0.68 mmol/g). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 10) the cyclic peptide **(146)** is purified by reverse phase preparative HPLC.
Yield: 30 mg, 51 μmol, 26 %.
HPLC (anal. method 2): t_R= 18.2 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$, m/z:
Calcd.: 587.29 $[M+H]^+$, 609.28 $[M+Na]^+$, 625.25 $[M+K]^+$
Found: 587.57 $[M+H]^+$, 609.53 $[M+Na]^+$
^1H NMR (500 MHz, DMSO-d_6), δ (ppm): 1.36-1.59 (m, 5H, H^β (Arg), 2H^γ (Arg), H^γ (β-hPro), H^δ (β-hPro)), 1.60-1.75 (m, 2H; H^β (Arg), H^γ (β-hPro)), 1.81 (m, 1H, H^δ (β-hPro)), 2.18 (dd, ^{2}J=13.8 Hz, ^{3}J=10.1 Hz, 1H, H^α (β-hPro)), 2.62 (m, 1H, H^β (Asp)), 2.62 (m, 1H, H^β (Asp)), 2.65 (dd, ^{2}J=13.8 Hz, ^{3}J=3.8 Hz, 1H, H^α (β-hPro)), 2.94-3.13 (m, 6H, 2H^δ (Arg), H^β (Asp), 2H^β (Phe), H^ε (β-hPro)), 3.46-3.52 (m, 2H, H^α (Asp), H^ε (β-hPro)), 3.72 (dd, ^{2}J=17.0 Hz, ^{3}J=5.0 Hz, 1H, H^α (Gly)), 3.76 (dd, ^{2}J=17.3 Hz, ^{3}J=5.3 Hz, 1H, H^α (Gly)), 4.16 (m, 1H, H^β (β-hPro)), 4.32 (ddd, ^{3}J=6.9 Hz, ^{3}J=7.9 Hz, ^{3}J=6.9 Hz, 1H, H^α (Arg)), 4.40 (ddd, ^{3}J=6.6 Hz, ^{3}J=7.9 Hz, ^{3}J=7.5 Hz, 1H, H^α (Phe)), 7.25-7.36 (m, 6H, H^N (Asp), 5H^{ar} (Phe)), 7.57 (br, 1H, $H^{\varepsilon N}$ (Arg)), 7.85 (d, ^{3}J=7.5 Hz, 1H, H^N (Phe)), 8.09 (d, ^{3}J=8.2 Hz, 1H, H^N (Arg)), 8.32 (dd, ^{3}J=5.3 Hz, ^{3}J=5.3 Hz, 1H, H^N (Gly)), 12.60 (br, 1H, H^{COO} (Asp)).

c-(-Arg-Gly-Asp-Phe-Cpr(OMe)-) (149)

$C_{27}H_{36}N_8O_9$, (616.64 g/mol).

Cyclization on resin:

The linear peptide H-Cpr-Phe-Arg(Pbf)-Gly-Asp(Wang)-ODmb **(150)** is synthesized and cyclized following protocol 6 (3.0 equiv HATU and 6.0 equiv DIPEA as coupling reagents) on Wang resin (160 mg, 64 μmol), previously loaded with Fmoc-Asp-ODmb (0.40 mmol/g). After complete deprotection and cleavage from the resin with the cleavage cocktail TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 10) the cyclic peptide **(149)** is purified by reverse phase preparative HPLC.

Yield: 4 mg, 5.8 μmol, 9 %.

HPLC (anal. method 2): t_R= 17.0 min, 17.2 min, 90 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 617.27 $[M+H]^+$, 639.25 $[M+Na]^+$, 655.22 $[M+K]^+$

Found: 617.53 $[M+H]^+$, 639.52 $[M+Na]^+$, 655.48 $[M+K]^+$

c-(-Arg-Gly-Asp-Cpr(OMe)-Val-) (151)

$C_{23}H_{36}N_8O_9$, (568.59 g/mol).

Cyclization in solution:

The linear peptide H-Asp(Ot-Bu)-Cpr(OMe)-Val-Arg(Pbf)-Gly-OH **(152)** is synthesized on 2-ClTrt resin (100 mg, 0.06 mmol), previously loaded with Fmoc--Gly-OH (0.60 mmol/g), cleaved from the resin with 1 % TFA in DCM (protocol 9) and purified by reverse phase preparative HPLC.

Yield: 14.3 mg, 16 μmol, 26 %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 895.42 $[M+H]^+$, 917.41 $[M+Na]^+$, 933.38 $[M+K]^+$

Found: 895.50 $[M+H]^+$, 917.88 $[M+Na]^+$, 933.54 $[M+K]^+$

The linear peptide **(152)** (14.3 mg, 16 μmol) is dissolved in 600 ml DMF and cyclized following protocol 7, method A.

1.1 equiv HATU (18 μmol, 7 mg).

3.0 equiv DIPEA (50 μmol, 6 mg, 8.0 μl).

After evaporation of DMF, the protected cyclic peptide **(153)** is purified by filtration through a C_{18} plug.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 877.41 $[M+H]^+$, 899.39 $[M+Na]^+$, 915.37 $[M+K]^+$

Found: 899.13 $[M+Na]^+$, 915.11 $[M+K]^+$

The protected cyclic peptide **(153)** is completely deprotected with the cleavage cocktail TFA/H_2O/TIS = 95 : 2.5 : 2.5 (protocol 13) and the cyclic peptide **(151)** is finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(151)**): 5 mg, 8.8 μmol, 55 % (Calculated from linear protected peptide **(152)**).

HPLC (anal. method 2): t_R= 13.5 min, 13.6 min, 100 area % sum of epimers.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 569.27 $[M+H]^+$, 591.25 $[M+Na]^+$, 607.22 $[M+K]^+$

Found: 569.73 $[M+H]^+$, 591.56 $[M+Na]^+$

c-(-Arg-Gly-Asp-D-1-Nal-) (154)

$C_{25}H_{31}N_7O_6$, (525.61 g/mol).

Cyclization in solution:

The linear peptide H-Asp(Ot-Bu)-D-1-Nal-Arg(Pbf)-Gly-OH **(156)** is synthesized on 2-ClTrt resin (225 mg, 0.20 mmol), previously loaded with Fmoc-Gly-OH (0.90 mmol/g), and cleaved from the resin with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(156)***)*: 184 mg, 80 μmol, 40 %.

HPLC (anal. method 1): t_R= 27.1 min, 37 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 852.40 $[M+H]^+$, 874.38 $[M+Na]^+$, 890.35 $[M+K]^+$

Found: 852.82 $[M+H]^+$, 874.78 $[M+Na]^+$, 890.77 $[M+K]^+$

The linear peptide **(156)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(156)** (184 mg, 80 μmol) in 20 ml DMF.

Second syringe: Solution of HATU (2.0 equiv, 160 μmol, 60 mg) in 20 ml DMF.

Addition rate for both syringes: 0.01 ml/min.

Flask: Solution of DIPEA (5.3 equiv, 420 μmol, 54 mg, 84 μl) and HOAt (2.0 equiv, 160 μmol, 22 mg) in 20 ml DMF.

After evaporation of the solvent the cyclic protected peptide **(157)** is purified by filtration through C_{18} plug.

Yield (cyclic protected peptide **(157)***)*: 123 mg, 75 μmol, 94 %.

HPLC (anal. method 1): t_R= 31.6 min, 51 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 834.39 $[M+H]^+$, 856.37 $[M+Na]^+$, 872.34 $[M+K]^+$

Found: 834.79 $[M+H]^+$, 856.71 $[M+Na]^+$, 872.72 $[M+K]^+$

The cyclic protected peptide **(157)** is completely deprotected with the cleavage cocktail TFA/H_2O/TIS = 95 : 2.5 : 2.5 during 2 h (protocol 13) and finally purified by reverse phase preparative HPLC.

Yield (cyclic deprotected peptide **(154)**): 18 mg, 34 μmol, 45 %.

HPLC (anal. method 2): t_R= 20.5 min, 100 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 526.24 $[M+H]^+$, 548.22 $[M+Na]^+$, 564.20 $[M+K]^+$

Found: 526.50 $[M+H]^+$, 548.51 $[M+Na]^+$ 564.48 $[M+K]^+$

^1H NMR (500 MHz, DMSO-d_6), δ (ppm): 1.31 (m, 2H, $2H^{\gamma}$ (Arg)), 1.40 (m, 1H, H^{β} (Arg)), 1.60 (m, 1H, H^{β} (Arg)), 2.39 (dd, ^2J=16.6 Hz, ^3J=5.3 Hz, 1H, H^{β} (Asp)), 2.72 (dd, ^2J=17.0 Hz, ^3J=9.4 Hz, 1H, H^{β} (Asp)), 2.94-3.06 (m, 2H, H^{δ} (Arg)), 3.14-3.21 (m, 2H, H^{α} (Gly), H^{β} (D-Nal)), 3.54 (dd, ^2J=14.4 Hz, ^3J=8.2 Hz, 1H, H^{β} (D-Nal)), 4.05 (dd, ^2J=13.2 Hz, ^3J=8.8 Hz, 1H, H^{α} (Gly)), 4.24 (ddd, ^3J=9.1 Hz, ^3J=8.8 Hz, ^3J=7.5 Hz, 1H, H^{α} (Arg)), 4.57 (ddd, ^3J=9.1 Hz, ^3J=9.1 Hz, ^3J=5.7 Hz, 1H, H^{α} (Asp)), 4.71 (ddd, ^3J=8.5 Hz, ^3J=8.5 Hz, ^3J=7.2 Hz, 1H, H^{α} (D-Nal)), 7.31-7.40 (m, 2H, H^{ar} (D-Nal)), 7.47 (t, ^3J=5.7 Hz, 1H, $H^{\varepsilon N}$ (Arg)), 7.51 (m, 1H, H^{ar} (D-Nal)), 7.57 (m, 1H, H^{ar} (D-Nal)), 7.76 (d, ^3J=9.4 Hz, 1H, H^{N} (Asp)), 7.77 (m, 1H, H^{ar} (D-Nal)), 7.84 (d, ^3J=9.4 Hz, 1H, H^{N} (Arg)), 7.89-7.93 (m, 2H, H^{N} (Gly), H^{ar} (D-Nal)), 7.98 (d, ^3J=8.8 Hz, 1H, H^{N} (D-Nal)), 8.06 (d, ^3J=8.2 Hz, 1H, H^{ar} (D-Nal)), 12.31 (br, 1H, H^{COO} (Asp)).

c-(-Arg-Gly-Asp-2-β-hNal-) (155)

$C_{26}H_{33}N_7O_6$, (539.63 g/mol).

Cyclization in solution:

The linear peptide H-Asp(Ot-Bu)-2-β-hNal-Arg(Pbf)-Gly-OH **(158)** is synthesized on 2-ClTrt resin (280 mg, 0.20 mmol), previously loaded with Fmoc-Gly-OH (0.72mmol/g,), and cleaved from the resin with 1 % TFA in DCM (protocol 9).

Yield (linear protected peptide **(158)***)*: 128 mg, 114 μmol, 57 %.

HPLC (anal. method 1): t_R= 26.8 min, 77 area %.

MALDI-ToF $MS_{monoisotopic}$, m/z:

Calcd.: 866.41 $[M+H]^+$, 888.39 $[M+Na]^+$, 904.37 $[M+K]^+$

Found: 866.37 $[M+H]^+$, 888.50 $[M+Na]^+$, 904.42 $[M+K]^+$

The linear peptide **(158)** is cyclized following protocol 7, method B:

First syringe: Solution of linear peptide **(158)** (128 mg, 114 μmol) in 20 ml DMF.

Second syringe: Solution of HATU (1.3 equiv, 150 μmol, 57 mg), in 20 ml DMF.
Addition rate for both syringes: 0.01 ml/min.
Flask: Solution of DIPEA (3.9 equiv, 0.45 mmol, 58 mg, 77 μl) and HOAt (1.3 equiv, 0.15 mmol, 20 mg) in 20ml DMF.
After evaporation of the solvent the cyclic protected peptide **(159)** is purified by reverse phase preparative HPLC.
Yield (cyclic protected peptide **(159)***)*: 18 mg, 21 μmol, 18 %.
HPLC (anal. method 1): t_R= 30.7 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$*, m/z:*

Calcd.: 848.40 $[M+H]^+$, 870.38 $[M+Na]^+$, 886.36 $[M+K]^+$
Found: 848.75 $[M+H]^+$, 870.66 $[M+Na]^+$, 886.80 $[M+K]^+$

The cyclic protected peptide **(159)** is completely deprotected with reagent K during 5 h (protocol 13) and finally purified by reverse phase preparative HPLC.
Yield (cyclic deprotected peptide **(155)**): 11 mg, 20 μmol, 97 %.
HPLC (anal. method 2): t_R= 20.5 min, 100 area %.
MALDI-ToF $MS_{monoisotopic}$*, m/z:*

Calcd.: 540.26 $[M+H]^+$, 562.24 $[M+Na]^+$, 578.21 $[M+K]^+$
Found: 540.20 $[M+H]^+$, 562.20 $[M+Na]^+$ 578.10 $[M+K]^+$.

^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm): 1.35-1.50 (m, 3H, H^{β} (Arg), $2H^{\gamma}$ (Arg)), 1.59 (m, 1H, H^{β} (Arg)), 2.32 (dd, ^{2}J=14.4 Hz, ^{3}J=3.8 Hz, 1H, H^{α} (β-hNal)), 2.37 (dd, ^{2}J=14.1 Hz, ^{3}J=12.2 Hz, 1H, H^{α} (β-hNal)), 2.47 (br, 1H, H^{β} (Asp)), 2.75 (dd, ^{2}J=16.3 Hz, ^{3}J=6.3 Hz, 1H, H^{β} (Asp)), 2.97 (dd, ^{2}J=13.2 Hz, ^{3}J=6.3 Hz, 1H, H^{γ} (β-hNal)), 3.02 (dd, ^{2}J=13.2 Hz, ^{3}J=4.4 Hz, 1H, H^{γ} (β-hNal)), 3.00-3.09 (m, 2H, $2H^{\delta}$ (Arg)), 3.54 (dd, ^{2}J=13.8 Hz, ^{3}J=6.9 Hz, 1H, H^{α} (Gly)), 3.70 (dd, ^{2}J=13.8 Hz, ^{3}J=5.0 Hz, 1H, H^{α} (Gly)), 4.01-4.13 (m, 2H, H^{α} (Arg), H^{β} (β-hNal)), 4.41 (ddd, ^{3}J=8.6 Hz, ^{3}J=8.6 Hz, ^{3}J=6.4 Hz, 1H, H^{α} (Asp)), 6.80 (br, 1H, $H^{\varpi N}$ (Arg)), 7.26 (d, ^{3}J=6.9 Hz, 1H, H^{N} (β-hNal)), 7.32 (m, 1H, H^{ar} (β-hNal)), 7.40 (d, ^{3}J=8.8 Hz, 1H, H^{N} (Asp)), 7.44-7.54 (m, 3H, $H^{\varepsilon N}$ (Arg), $2H^{ar}$ (β-hNal)), 7.63 (s, 1H, $H^{ar\ 1}$ (β-hNal)), 7.82-7.91 (m, 4H, H^{N} (Gly), $3H^{ar}$ (β-h Nal)), 8.03 (d, ^{3}J=7.5 Hz, 1H, H^{N} (Arg)), 12.51 (br, 1H, H^{COO} (Asp)).

7. LITERATURE

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