A combinatorial approach towards pharmaceutically relevant cyclic peptides
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Chapter 1

General Introduction

Abstract: This chapter contains a brief introduction to some of the main topics of this thesis. The first paragraph will outline the concepts of peptides and cyclic peptides. The second paragraph will introduce the difficulties encountered in the synthesis of cyclic peptides. After explaining the concept of combinatorial chemistry, the fourth paragraph will display the combinatorial synthesis of cyclic peptides and the different approaches towards these structures. The final paragraph will give an outline this thesis.
1.1 Cyclic peptides

Peptides are among the important structures in life and a numerous physiological and biochemical functions are influenced by these compounds. Peptides are formally polymers of amino acids linked by amide bonds (also called peptide bonds) (Figure 1.1). They are abundant in all natural sources, but the isolation and characterization has been found to be problematic due to the low concentrations requiring highly sensitive screening assays. Peptide research has remained limited, until the 1960’s after which screening techniques were improved and the role of many peptides in important physiological processes was identified. To date not only a vast number of peptides have been identified from natural sources, but also their synthesis now has reached the stage that peptides up to 50 residues may be prepared in an automated fashion using solid phase peptide synthesis (SPPS). Several peptides are on the market as drugs for various diseases. However, the use of peptides as drugs has been limited owing to their poor drug-like properties. Besides their metabolic instability the charged termini of peptides prevent proper passive membrane transport. Secondly, because of the highly conformational flexibility a proper spatial positioning of the pharmacophoric moiety cannot be maintained, leading to a lower selectivity and activity.

Figure 1.1 General representation of amino acids, peptides and cyclic peptides.

These drawbacks of peptides may be tackled by the introduction of conformational constraints. One such solution is ring-closure of peptides leading to cyclic peptides. These structures lack the charged termini resulting in a higher bioavailability and a lower biodegradability. More important, cyclization leads to a lower flexibility, placing the side chains of the amino acids at well defined positions in space. This generally leads to improved pharmacodynamic and -kinetic properties.

Since the discovery of the first cyclic peptide, the antibiotic gramicidin S, many cyclic peptides have been identified from natural sources in various ring sizes and structures. Alternatively, many cyclic peptides have been synthesized based on natural sources or de novo incorporating not only the 20 proteinogenic amino acids, but also many non-proteinogenic analogues. Cyclic peptides are divided in two main classes: homodetic cyclic peptides and heterodetic cyclic peptides (Figure 1.2). Homodetic peptides are peptides only linked by amide bonds. In heterodetic cyclic peptides at least one link is not an...
amide bond, but a different functional group. These functional groups can be introduced in the final macrocyclization or can be incorporated during the synthesis of the cyclization precursor.

**Figure 1.2** General classification of cyclic peptides.

Cyclic peptides are divided in three main topologies: head-to-tail cyclic peptides, side chain-to-tail (or head) and side chain-to-side chain. Head-to-tail cyclic peptides generally have the highest conformational strain, caused by the multiple amide bonds. Heterodetic cyclic peptides allow a greater degree of saturation and consequently could be less constrained. Examples of such heterodetic linkages include esters (depsipeptides), thioethers, oxazoles and thiazoles, all of them often encountered in natural peptides. In addition, synthetic peptides incorporate alkenes (derived from cross metathesis reactions or enyne cycloisomerization), imidazoles, triazoles (derived from azide-alkyne cycloaddition reactions) and tetrazoles.

**Figure 1.3** Cyclic peptides in different sizes.
In contrast to the easily accessible diketopiperazines (Figure 1.3, e.g. 1)\textsuperscript{48,49} derived from two \(\alpha\)-amino acids, their seven-membered homodiketopiperazine homologues are much more challenging. These seven-membered ring bis(lactams) are made up of \(\alpha\)- and \(\beta\)-amino acids and display interesting biological activities (e.g. TAN-1057 C 2).\textsuperscript{50} Cyclic tripeptides are generally very difficult to address because of the instability of these compounds due to a transannular collapse into diketopiperazines.\textsuperscript{51} For this reason, alkyalted amide bonds are usually present,\textsuperscript{52,53} preventing this rearrangement of the cyclic tripeptides.\textsuperscript{54} Cyclotetrapeptides represent an abundant class of cyclic peptides with many examples from nature.\textsuperscript{55} Most of these peptides are isolated from fungi, marine sources, micro organisms or higher plants and their biosynthesis occurs via non-ribosomal pathways.\textsuperscript{56} A broad class of the cyclic tetrapeptides comprise the histone deacetylase inhibitors (e.g. apicidin, HC-toxin and chlamydocin 4),\textsuperscript{57-62} which contain substituted 2-amino-8-oxodecanoic acids (Aodas) as one of their constituents.\textsuperscript{63} A second class consists of cyclic tetrapeptides containing two opposite proline units (e.g. cyclo-[Pro−Leu−Pro−Leu]),\textsuperscript{51,64-68} including tyrosinase inhibitors (cyclo-[Pro−Val−Pro−Tyr] 3).\textsuperscript{69} Different cyclic pentapeptides have been isolated from plants and showed interesting biological activities (e.g. segetalin B 5).\textsuperscript{70-75}

A vast number of cyclic peptides are known containing a higher number of amino acids in the ring, ranging from hexapeptides up to a 31 amino acids containing natural cyclic peptide, cyclopsychotride A, isolated from a tropical plant.\textsuperscript{76} However, by increasing the number of amino acids in the ring, these cyclic peptides also increase the conformational flexibility, reducing their use as effective potential drugs or scaffolds.

1.2 Difficulties in peptide cyclizations

Due to the limited amount of cyclic peptides available from natural sources, the synthesis of cyclic peptides has been an increasingly important task.\textsuperscript{77} Generally, the formation of the macrocycle is the limiting step in the synthesis of cyclic peptides and is accompanied by competing intermolecular reactions leading to oligomerization and polymerization. The main reason behind this problem is the rigidity of the intermediate amide bonds.

Scheme 1.1 Torsion angles \(\varphi, \chi, \omega\) and \(\psi\) and resonance stabilization and cis/trans isomerization of peptide bonds.
The configuration of the amide bond can be described in torsion angles $\varphi$, $\chi$, $\omega$ and $\psi$ (Scheme 1.1).\(^7\) Rotation about the $C\text{-}N$ amide bond in the peptide ($\omega$) is drastically hindered by the strong double bond character (the rotational barrier is $\sim 105$ KJ.mol\(^{-1}\)) of which the *transoid* ($\omega = 180^\circ$) conformation is energetically favoured over the *cisoid* ($\omega = 0^\circ$) conformation by 8 KJ.mol\(^{-1}\).

A second problem in the synthesis of cyclic peptides is the site of ring closure. This phenomenon is also referred to as sequence dependency. This was illustrated by Schmidt *et al.*,\(^7\) who synthesized the cyclic pentapeptide cyclo-[Ala–Phe–Leu–Pro–Ala] 6 starting from all the five possible linear precursors (Figure 1.4). It was shown that depending on the ring closure site, yields of the final cyclic peptides ranged from 0% up to 21%, accompanied in some cases by extensive dimerization.

**Figure 1.4** Synthesis of cyclo-[Ala–Phe–Leu–Pro–Ala] 6 at different ring closure sites.

However, although the choice of the correct linear precursor that will provide the cyclic product is important, this is very difficult to predict. Several factors can influence ring-closure, such as steric hindrance and kinetic competition with the dimerization reaction or the transition-state energy. Molecular modelling experiments were performed on chlamydocin to investigate all these factors\(^8\) and comparison with experimental data revealed that the transition-state energy proved to be the limiting factor in peptide cyclizations. The best linear precursor for the cyclization was found to be the one providing the least strain in the transition-state. This work was extended to the prediction of the best linear precursor of several other cyclic peptides.\(^9\)

Many methodologies have been developed to improve the cyclization of peptides, with the ideal synthesis being sequence independent, free of racemization and high yielding. The traditional lactamization between the $C$-terminus and the $N$-terminus in solution is still the most applied method for the synthesis of cyclic peptides, although as outlined before the result of the final lactamization reaction is difficult to predict. The reactions have to performed at high dilution ($10^{-2}$ to $10^{-3}$ M) to avoid competing oligomerization and polymerization. Many peptide coupling reagents have been developed for the activation of the
C-terminal carbonyl group,\textsuperscript{82-84} including carbodiimides,\textsuperscript{85} phosphonium,\textsuperscript{86} uronium,\textsuperscript{87} immonium,\textsuperscript{88} imidazolium,\textsuperscript{89} triazine,\textsuperscript{90} organophosphorus\textsuperscript{91} and acid halogenating reagents.\textsuperscript{92,93} Some coupling reagents result in better cyclization yields than others, although the outcome differs in individual cases. No predictions can be made on the best results and optimization of the lactamization is just a matter of trial and error. However, some additives and coupling reagents derived from HOAt have been shown to promote cyclization by preorganization of the linear precursor (Scheme 1.2) and suppress side reactions such as racemization.\textsuperscript{94}

**Scheme 1.2** Preorganization by HOAt-derived coupling reagents.

![Scheme 1.2](image)

Primarily, prolonged existence of the activated carbonyl group of the amino acids or peptides A (Scheme 1.3)\textsuperscript{83} gives $O$-attack of the amide on the activated ester resulting in the formation of an oxazolone B, which under mildly basic conditions undergoes racemization via the formation of tautomer C. The resulting oxazolone stereoisomer mixture of A and D directly reacts with a nucleophile, explaining the loss of stereochemical homogeneity of the coupled material E and F. Consequently, peptide coupling should always proceed at the $N$-terminus and mildly activating coupling conditions are needed in combination with protective groups preventing oxazolone formation such as carbamates.

**Scheme 1.3** Mechanism of the racemization during peptide coupling via oxazolones.

![Scheme 1.3](image)

To overcome the limitations of the classical lactamization strategies, different alternative approaches have been developed. Preorganization of the linear precursor and strain relief of the linear precursor are main pivots on which these methods hinge. Different auxiliary-based methods have been developed for the synthesis of several cyclic peptides, based on the original strategy by Meutermans \textit{et al.} (Scheme 1.4). Salicylaldehyde derived linkers are
incorporated into linear peptides, inducing the cyclization by positioning of the $C$- and $N$-termini in close proximity, after which a transannular $O\rightarrow N$ acyl transfer reaction delivers the final lactams.

**Scheme 1.4** Auxiliary-mediated synthesis of cyclic peptides.

This approach has been successfully applied in the synthesis of all-L cyclic pentapeptides and tetrapeptides. The auxiliary was attached at the $N$-terminus of the linear peptide. However, extensive $C$-terminal racemization occurred during the formation of the macrolactone.$^{95-97}$ To overcome these problems, incorporation of the auxiliary in between the linear peptide resulted in the formation of bis(lactams) without extensive racemization of the $C$-terminus in a sequence independent manner.$^{98-100}$

The synthesis of cyclic peptides has also been described by means of an intramolecular Staudinger ligation (Scheme 1.5).$^{101,102}$ Coupling of a borane-protected auxiliary to azido acid dipeptides resulted in the formation of the linear cyclization precursors $7$ and $8$, after which the intramolecular Staudinger ligation could be effected after liberation of the phosphane. The 1,4-diazepine-2,5-dione $9$ was obtained from both linear precursors in reasonable yield.

**Scheme 1.5** Synthesis of 1,4-diazepine-2,5-dione by intramolecular Staudinger ligation.

Similar to the synthesis of cyclic peptides in nature by non-ribosomal peptide synthetase (NRPS), an isolated terminal thioesterase domain (TE) of this multienzymatic complex catalyzed the cyclization of a decapeptide to form gramicidin S.$^{103}$ Other groups have used a similar strategy for the synthesis of medium-sized cyclic peptides (9-14 residues).$^{104-109}$
Core-functionalized dendrimers have been used for the synthesis of cyclic peptides (Scheme 1.6). Several generations of carbosilane dendrimers were functionalized at the core by carbodiimides and used in the synthesis of small bis(lactams). Induced by the site-isolation effect exerted within the dendrimeric core, intramolecular reactions were favoured over intermolecular reactions and resulted in formation of the bis(lactams) in good yields compared to usual carbodiimides.

Scheme 1.6 Encapsulation of the linear peptide prevents intermolecular reactions.

Solid phase chemistry has also been applied for the synthesis of cyclic peptides. In this case, the pseudo-dilution effect exerted within the resins favours the intramolecular reaction over intermolecular resins, especially with low resin loadings. Examples of different strategies for the solid phase synthesis of cyclic peptides will be outlined in the next two sections.

1.3 Combinatorial chemistry

Traditionally, new chemical lead structures for pharmaceutical research relied on the isolation and modification of natural products or on the mechanism-based or structure-based design of potential drugs. With the emerging field of high-throughput screening (HTS) devices in 1980s, these traditional methods no longer fulfilled the need for large amounts of molecules. Thus, large numbers of molecules were needed for screening purposes. As an answer, combinatorial chemistry was developed in the early nineties addressing the need by the construction of chemical libraries from a collection of building blocks, and systematically combining these to create a vast amount of molecules.111-115

The potential of even a small set of building blocks is substantial and can lead to a large set of molecules. Traditionally molecule A and molecule B react in a single vessel to form the product AB. Applying the building block concept of combinatorial chemistry a set of
molecules $A_1$-$A_n$ react with a set of molecules $B_1$-$B_m$ resulting in the formation of any product combination $A_1B_1$ to $A_nB_m$. With the basic set of twenty encoded amino acids a tripeptide would have $20^3 = 8,000$ library entities, a tetrapeptide $20^4 = 160,000$ library entities and a pentapeptide even $20^5 = 3,2$ million library entities. The advantage of combinatorial chemistry is its efficiency, being faster and cheaper than the orthodox chemistry.

The construction of a large amount of molecules can be performed as mixtures, or can be performed in parallel as single compounds, either in solution phase or on solid supports. Although a diverse set of solid supports has been developed with different linker units and swelling properties in particular solvents, reactions on solid supports afford clean products after cleavage and reactions can be driven to completion by the use of excess of reagents, the supports are not compatible with heterogeneous reagents or solid side products. Solution phase techniques have become increasingly popular and can employ existing reactions and more harsh reaction conditions, but the purification of the products becomes more important. Solid phase extractions or chromatographic techniques have been used to deal with this issue, but more important are the use of immobilized reagents or scavengers to selectively trap side products.

The early experiments with combinatorial chemistry were mainly in peptide chemistry. These sets of building blocks were easily available and the chemistry of peptide coupling and protection was well documented. A pioneer in the field of peptide synthesis was Merrifield, introducing synthesis on solid supports. Most of the methods nowadays still rely on this original synthesis. Initial experiments with supports with the so-called split-and-mix method resulted in large quantities of mixtures of compounds, from which individual activities were difficult to deconvolute and false positives and negatives were commonly encountered. To circumvent the synthesis and screening of mixtures, peptides can be synthesized on pins arranged on microtitre plates. Another alternative is the so-called ‘teabag’ method where resins in sealed bags are placed in activated monomer solutions and result in single peptide sequences in each bag. Simultaneous coupling of mixtures of activated monomers results in the formation many peptides, but the extent of coupling of each monomer is difficult to control. A very elegant method for the identification of single library members is based on the optical encoding by laser etched grids in polypropene blocks grafted with polystyrene resins.

1.4 Combinatorial synthesis of cyclic peptides

The main goal of the pharmaceutical industry nowadays has shifted from the careful investigation of a few well documented targets towards the screening of a large amount of molecules on a diverse and diffuse set of target structures using advanced methods, such as bioinformatics, proteomics, genomics, combinatorial chemistry/HTS. However, especially the latter two techniques have not fulfilled the initial expectations. Although the techniques have matured to a state in which a large number of molecules can be synthesized and screened, it is becoming more and more important which molecules are selected as input.
It has been observed in HTS, that a number of molecules have a significantly higher hit-rate compared to other molecules. The term ‘privileged structures’ has been coined for these kind of molecules by Evans in reference to the benzodiazepine scaffold. These molecules have the ability to bind to a large set of receptors.

Cyclic peptides are also considered as being part of these privileged structures. Although these molecules have not been identified as ‘drug-like’, some cyclic peptides have been exploited as drugs and are currently on the market. However, a library of these molecules would serve as a perfect molecular toolkit to study pharmacophoric properties. This library would be optimally diverse, both in terms of conformational space and R-group diversity. This is due to the possibility of introducing not only different side chains of the amino acids, but also introducing both enantiomers. The exploration of this library could consequently lead to valuable information on the structural properties of these pharmacophores and could subsequently lead to lead optimization programs.

The solid phase synthesis of libraries of cyclic peptides has been performed by three main strategies. The peptides may be synthesized by the anchoring of the different side chains of the amino acids to a solid support. The peptide can also be linked to the solid support via the nitrogen of a backbone amide in the so-called backbone amide linkage strategy. Finally, the peptide can be synthesized immobilised via the C-terminus either with an activated linker or with a ‘safety catch’ linker.

Attachment via the side chain of the amino acids of the peptide is the most common method for the synthesis of cyclic peptide libraries (Figure 1.5). The synthesis requires an orthogonal protection of the C- and N-termini and is usually performed by N-Fmoc and CO2All protection. The final macrolactamization is performed on the resin and cleavage of the cyclic peptide and removal of the side chain protective groups is accomplished in one step after cyclization. A disadvantage of this method is the requirement of specific residues in the design of the cyclic peptide.

Figure 1.5 Side chain attachment via different amino acids.

Obviously the amine functionality of lysine together with the acid functionality in the glutamic and aspartic acid side chains serve as perfect resin attachment sites. The
tyrosine phenolic hydroxyl group has been anchored to a solid support via a Mitsunobu reaction\(^{159}\) and histidine has been attached via its imidazole ring to trityl-resins and both have been used for the synthesis of cyclic peptides.\(^{160,161}\) Besides this, an elegant traceless method based on arylsilanes has been developed for the immobilization of phenylalanine and the subsequent synthesis of sansalvamide A and scytalidamide A.\(^{162,163}\) Using the side chain attachment method, a self-deconvolution cyclic pentapeptides library based on the natural peptide BQ-123 designed to produce 82,944 cyclic peptides, was prepared.\(^{153}\) At four variable positions a dozen residues were scanned and although no analogues more active than the natural peptide were found, this example nicely shows the potency of cyclic peptide libraries.

A second method which has been employed for the synthesis of cyclic peptides is by means of the so called backbone amide linkage strategy (Figure 1.6). This has the advantage over the earlier described method of side chain attachment that no specific residues are required. The backbone amide linker can be placed at any residue except for proline. Besides this, \(N\)-amide alkylation has been speculated to prevent aggregation\(^{164}\) of the growing peptide chain and can aid the final macrocyclization by favouring the \textit{cisoid} character of the amide bond.\(^{165}\) Different types of backbone amide linkers have been described depending on the cleavage conditions. Poly(alkoxy)benzylamine linkers (PAL) can be efficiently removed from the final product by treatment with TFA.\(^{166-169}\) Monoalkoxybenzylamine type linkers require harsh condition for their removal, like treatment with HF, but have been successfully applied in the synthesis of library of cyclic pentapeptides based on somatostatin.\(^{170}\) Different to the benzylamine derived linkers was the use of a hydroxyamine type linker, from which the peptides could be removed by treatment with SmI\(_2\).\(^{171}\)

\textbf{Figure 1.6 Different backbone amide linkers.}

Although attachment via the side chains and backbone amide linkers attached via the amide provide robust procedures, further purification of the peptides after cleavage is usually required to remove the remainings of the side chain protection and cleavage cocktails. A more ideal type of linker would eliminate final purification and would render pure cyclic peptides. Initially these types of linkers were activated linkers, attached via the \(C\)-terminus to the peptide (Scheme 1.7). During the usually \(N\)-Boc based peptide synthesis, this bond was designed to be stable to acidic conditions and nucleophilic attack during the coupling procedures, but at the end be susceptible for cleavage through head-to-tail lactamization of the final peptides.
Nitro-substituted phenols have been used for the synthesis of cyclotetrapeptides, activated by the neutralization of the end amine groups with triethylamine.\textsuperscript{172} Osapay \textit{et al.}\textsuperscript{173,174} applied Kaiser’s oxime resin for the synthesis of several peptides.\textsuperscript{175,176} A series of cyclic penta-, hexa- and heptapeptides was synthesized using the thioester linkage in a type of intramolecular native chemical ligation strategy.\textsuperscript{109,177} Finally polymer-bound HOBt was used for the synthesis of a series of small lactams.\textsuperscript{178}

However, the initial strategies suffered from substantial loss of the peptides during the synthesis due to the labile activated bond. Moreover, complete side chain protection was still necessary during the cyclization. A second generation of these types of linkers were based on the so-called ‘safety catch’ principle (Scheme 1.8). These linkers were attached to the peptide by a stable bond. After completion of the synthesis, this bond is transformed to a labile bond inducing the peptide cyclization.

The first types of these linkers were developed by Marshall and Flannigan and relied on the oxidation from sulphide to sulphone, but major drawbacks were caused by cysteine residues and methionines.\textsuperscript{179,180} A hydrazide-activated linker was used for the synthesis of stylostatin 1, activated by oxidation.\textsuperscript{181} The best known safety catch linker was developed by Kenner\textsuperscript{182} and was used in the synthesis of many cyclic peptide libraries.\textsuperscript{183-185} For example a 192-
membered library of cyclic decapeptides was constructed on the basis of the natural products tyrocidine, streptocidin and loloatin. Screening identified nine analogues with potencies increased up to nine-fold compared to the natural products. Alkylation of side chains could again be a potential problem, especially employing cysteine and tryptophan. Finally, a catechol type linker was described that is deactivated by protection of one of the hydroxyl groups with a benzyl group. Activation is employed by treatment with TFMSA.

In the synthesis of cyclic peptides problems can be encountered within the growing peptide chain. The incorporation of difficult sequences, generally consisting of sterically hindered building blocks or $\alpha,\alpha$-disubstituted amino acids, has initiated the development of different coupling reagents and coupling strategies. A second problem is the back-folding of the growing peptide chain, inhibiting proper couplings of the next amino acids. The choice of solvent and polymer support is crucial in these cases, but also repetitive alkylation of the amides of the growing peptide chain inhibits unwanted back-folding. Diketopiperazine formation during coupling of the third amino acid on the peptide depends strongly on the coupling conditions and the linkage to the solid support, but is still encountered as a persisting side reaction (Scheme 1.9).

**Scheme 1.9** Diketopiperazine formation during peptide synthesis.

![Scheme 1.9](image)

Although different methods exist for the combinatorial synthesis of cyclic peptides, the ultimate strategy, independent of the sequence design of the linear precursor, should be high yielding and free from competing side reactions. Therefore, several new strategies will be presented for the combinatorial synthesis of cyclic peptides, each with their own advantages.

### 1.5 Outline of the thesis

The main goal of the research described in this thesis was to develop combinatorial approaches towards the synthesis of small homodetic and heterodetic cyclic peptides. This work would build on the already existing methods developed in our group for the synthesis of small homodetic cyclic peptides and triazole-containing cyclic peptides.

In Chapter 2 the existing auxiliary developed for the synthesis of bis(lactams) was refined to make it more sequence independent and robust. The improved auxiliary was further modified to make it suitable for solid supported reactions.

A backbone amide linker strategy for the solid phase synthesis of triazole-containing cyclic pseudotetrapeptides and pentapeptides has been described in Chapter 3. The method was
optimized for the synthesis of a triazole analogue of the cyclic tetrapeptide cyclo-
[Pro–Val–Pro–Tyr] and used for the synthesis of a library of tetrapeptides. An analogue of
the cyclic pentapeptide segetalin B was made and the effects of the introduction of the triazole
linkage on the conformation of the peptide were elucidated.

Chapter 4 describes a new strategy for the introduction of 1,5-connected triazoles in cyclic
peptides. These proposed cisoid amide bond surrogates were introduced into the cyclic
tetrapeptide cyclo-[Pro–Val–Pro–Tyr] at different sites. The effects of the introduction on the
final macrolactamization was investigated.

The usefulness of a combination of the Ugi multicomponent reaction and the copper-
catalyzed azide-alkyne cycloaddition reaction for the synthesis of small cyclic peptides is
described in Chapter 5. The method was optimized for small cyclic pseudopeptides and cyclic
pseudotetrapeptides and used for the synthesis of libraries of triazole-containing cyclic
pseudopeptides. Finally, this methodology was applied to the synthesis of an analogue of the
biologically relevant cyclic tetrapeptide chlamydacin.

In the final Chapter 6 a retrospection on the different topics of this thesis is presented together
with a discussion on the future of the topics outlined in this thesis.

This thesis ends with summaries in English and Dutch.
1.6 References and notes


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