Application of alicyclic β-amino acids in peptide chemistry

Ferenc Fülöp,*[a] Tamás A. Martinek[a] and Gábor K. Tóth[b]

Received 5th November 2005
First published as an Advance Article on the web 19th January 2006
DOI: 10.1039/b501173f

The self-organizing β-peptides have attracted considerable interest in the fields of foldamer chemistry and biochemistry. These compounds exhibit various stable secondary structure motifs that can be exploited to construct biologically active substances and nanostructured tertiary structures. The secondary structures can be controlled *via* the β-amino acid sequence, and cyclic β-amino acid residues play a crucial role in the design. The most important procedures for the preparation of cyclic β-amino acid monomers and peptides are discussed in this *tutorial review*. Besides the secondary structure design principles, the methods of folded structure detection are surveyed.

1 Introduction

The interest in cyclic β-amino acids has increased exponentially in the past few years and they have become a hot topic in synthetic and medicinal chemistry.[1–5] Some of the cyclic β-amino acids exhibit strong antibacterial activity (*e.g.* 1*R*,2*S*-2-aminocyclopentanecarboxylic acid, *cispentacin*); one (BAY 10-8888, PLD-118) is currently being investigated in clinical studies for the treatment of yeast infections.[6] These compounds can be widely applied as building blocks in peptide chemistry, in drug research, and in heterocyclic and combinatorial chemistry.[2]

[a]*Institute of Pharmaceutical Chemistry, University of Szeged, H-6720 Szeged, Eötvös u. 6, Hungary*
[b]*Department of Medical Chemistry, University of Szeged, H-6720 Szeged, Dóm tér 8, Hungary*

The synthesis of peptide oligomers that adopt designed compact conformations has become a challenging task in recent years. The peptidomimetic approach in drug research has significant potential. β-Amino acids, among them cyclic β-amino acids, have a broad range of use as building blocks for the preparation of modified analogues of biologically active peptides. In cyclic β-amino acids, the amino and carboxyl functions are situated on neighbouring atoms, and thus these compounds can exist as *R* or *S* isomers, with a total of 4 possible enantiomers. The availability of a vast number of

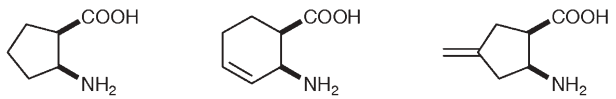


Fig. 1 Cyclic amino acids that exhibit strong antibacterial activity.

Ferenc Fülöp

Ferenc Fülöp was born in Szank, Hungary, in 1952. He received his MSc in Chemistry in 1975 and his PhD in 1979, from József Attila University, Szeged, Hungary, under the supervision of Professor Gábor Bernáth. In 1990, he received his DSc from the Hungarian Academy of Sciences in Budapest. After different teaching positions, he was appointed full professor at the Institute of Pharmaceutical Chemistry in Szeged, and since 1998 has been head of the Institute. He has a wide range of research interests in heterocyclic chemistry, including isoquinolines and fused skeleton saturated 1,3-heterocycles. His studies on the ring-chain tautomerism of 1,3-oxazines and oxazolidines in the 1990s led to interesting results. His recent activities have focused on the use of amino alcohols and β-amino acids in enzymatic transformations, asymmetric syntheses and combinatorial chemistry, with a view to the development of pharmacologically active compounds.

Tamás A. Martinek

Tamás A. Martinek was born in Szeged, Hungary, in 1973. After obtaining his MSc in Chemistry from University of Szeged, Hungary in 1996, he earned his PhD degree at the same university in 2001 under the supervision of Ferenc Fülöp and with guidance of Frank G. Riddell at the University of St. Andrews, UK. Currently, he is a post-doctoral research fellow, and head of the Structural Analysis Laboratory of the Institute of Pharmaceutical Chemistry. His research focuses on the designed secondary and tertiary structures of β-peptides, and on the development of 3D-QSAR methods for flexible molecules.

stereo- and regio-isomers, together with the possibility of ring-size expansion and further substitutions on the ring, significantly extends the structural diversity of β-amino acids, thereby providing enormous scope for molecular design. The insertion of a cyclic β-amino acid in place of an α-amino acid of a naturally occurring pharmacologically active peptide can not only result in potent biological activity, but also enhance the stability; it is currently believed that β-peptides are resistant to enzymatic degradation.

The aims of the present Tutorial Review are (i) to discuss the syntheses of β-amino acid monomers, (ii) to discuss the syntheses of peptides containing an alicyclic β-amino acid unit, and (iii) to review an extremely rapidly developing field: the stereochemical control of the secondary and tertiary structure by alicyclic β-amino acids.

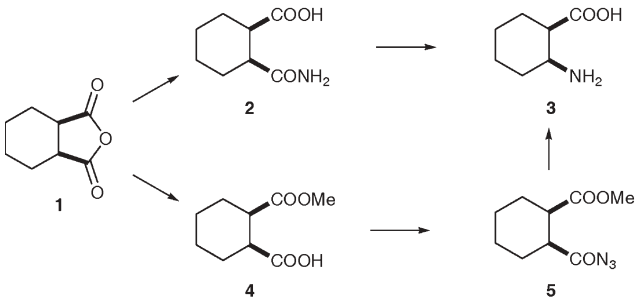
2 Syntheses of β-amino acid monomers

A number of reviews dealing with the syntheses of β-amino acids have been published in recent years, but cyclic amino acids are mentioned in only special cases. Very few articles have focused exclusively on the synthesis and applications of cyclic β-amino acids, where the amino and carboxyl functions are in the β position on a cycloalkane ring.

This section will summarize the most typical recent methods for the synthesis of alicyclic β-amino acid units. The focus will be placed on the methods of practical importance, where the gram-scale synthesis for either racemic or enantiomeric forms is easy to perform.

Most typical racemate syntheses

A number of diastereomerically pure 1,2-dicarboxylic anhydrides are cheap commercial products and can easily be prepared by the Diels–Alder reaction of, for example, butadiene and maleic anhydride. The resulting tetrahydroanthranilic anhydride is readily reducible to the hexahydro analogue. The first selective synthesis of *cis*-2-aminocyclohexanecarboxylic acid (ACHC) **3** was performed from anhydride **1** after amidation, followed by Hofmann degradation with hypobromite. This protocol is used to prepare a number of homologues and analogues. For double bond-containing derivatives, modified Hofmann degradation with hypochlorite has been applied in order to avoid bromine addition. After esterification and isomerization, anhydrides can be recyclized to *trans* anhydrides, following the above protocol, these give the *trans*-substituted amino acids. After Hofmann degradation, ion-exchange chromatographic purification is the method usually applied.



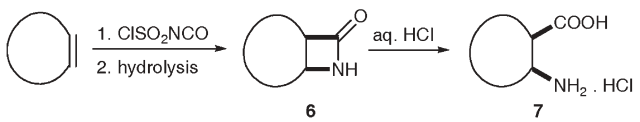
Scheme 1 Classical Hofmann and Curtius degradation protocols to *cis*-2-aminocyclohexanecarboxylic acid.

Anhydride **1** can be esterified and selectively monohydrolysed to **4**, which is a suitable starting substance after Curtius degradation for the production of cyclic β-amino acids. This method is widely applied for the preparation of enantiomeric amino acids since the desymmetrization of diesters is a relatively easy process (see later).

The 1,2-dipolar cycloaddition of chlorosulfonyl isocyanate (CSI) to different cycloalkenes is a well-known route for the synthesis of β-lactams[2] and therefore for alicyclic β-amino acids, after hydrochloric acid treatment of the resulting lactams. The best solvents in the CSI addition reaction are abs. dichloromethane and diethyl ether; the reaction temperature ranges from −78 °C to reflux temperature. The present authors suggest that the reactions should be performed at near to room temperature, since the low-temperature reactions may lead in very few cases to small explosions in gram-scale experiments. The addition always takes places regio- and stereospecifically, in accordance with the Markovnikov orientation rule. The hydrochloric acid ring opening of β-lactams results in amino acid hydrochlorides in an exothermic reaction (Scheme 2).

Enantiomers by enzyme-catalysed kinetic resolutions

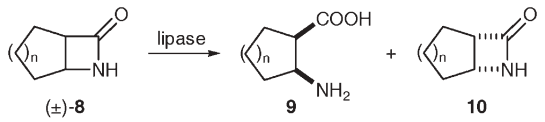
Kanerva *et al.*[7] developed an excellent lipase PS-catalysed protocol for the acylation of ten different cyclic β-amino acid ethyl esters. The best results were obtained in diethyl ether



Scheme 2 β-Lactam route to β-amino acids.

Gábor K. Tóth

Gábor K. Tóth was born in Szentes, Hungary, in 1954. He received his MSc in Chemistry in 1977 and his PhD in 1980, from József Attila University, Szeged, Hungary. In 2001, he received his DSc from the Hungarian Academy of Sciences in Budapest. After different teaching positions, in 2002 he was appointed as full professor at the Department of Medical Chemistry in Szeged, and since 2005 has been head of the Department. His major research interests are the following: synthesis of different biologically active peptides, improvement of synthetic methods, structure–activity relationships, synthetic vaccines, application of synthetic peptides in immunology, postranslationally modified peptides, peptidomimetics, modulation of the 3D structure of biologically active peptide analogues.



Scheme 3 Lipase-catalysed ring opening of cycloalkane-fused β-lactams.

when 2,2,2-trifluoroethyl chloroacetate was used as acylating agent. The acylation was performed on both *cis* and *trans* derivatives, and always took place at the *R* stereogenic centre.

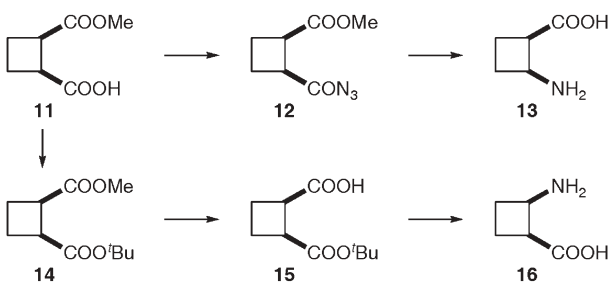
Recently, a highly applicable and efficient method was developed by Forró *et al.*[8] β-Lactams **8** were opened with high enantioselectivity by using lipase B from *Candida antarctica* in diisopropyl ether at 60 °C (Scheme 3). The resolved products **9** and **10** were obtained in good yields and could be easily separated by a simple water–organic solvent extraction. The method was successfully applied for a number of further analogues.

Enantiomers of cyclic *cis*-β-hydroxy esters can easily be prepared by the Baker's yeast-mediated reduction of the corresponding β-keto esters or by lipase-catalysed *R*-selective *O*-acylation of the corresponding hydroxyl esters. Inversion of the enantiomeric *cis* esters to azides, followed by reduction, results in *trans* amino acids.[9,10]

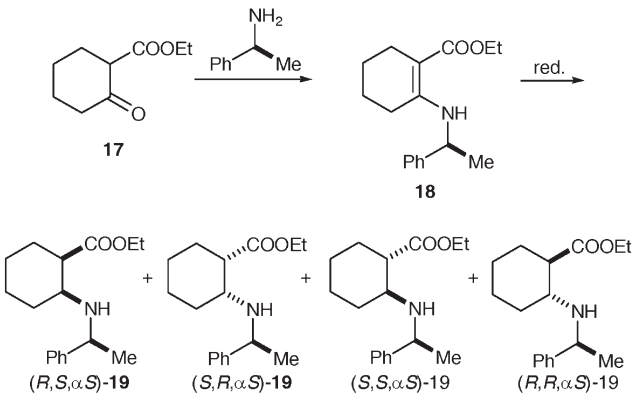
Enantiomers by desymmetrization reactions

The desymmetrization of a *meso*-anhydride or *meso*-dicarboxylate is a very useful process with which to generate chiral hemiesters containing two stereogenic centres. *meso*-Dicarboxylates can be hydrolysed to hemiesters with high selectivity through the use of pig liver esterase. The cinchona alkaloid-mediated quinine or quinidine-catalysed ring opening of prochiral anhydrides at low temperature is an effective protocol for the formation of chiral hemiesters.[11] These hemiesters can easily be converted to amino acids *via* the Curtius degradation protocol. An effective enantiodivergent method is shown in Scheme 4. Ortuno *et al.*[12] used the hemiester **11** for the synthesis of both enantiomers of 2-aminocyclobutanecarboxylic acid (ACBC), by using selective esterification and hydrolysis, followed by Curtius degradation.

A new desymmetrization process was described by Shibasaki *et al.*[13] The reaction is based on the ring opening of *meso*-aziridines in a trimethylsilyl cyanide-catalysed reaction, using a new chiral gadolinium complex. The resulting aminonitriles can be efficiently transformed to cyclic β-amino acids.



Scheme 4 Synthesis of (1*R*,2*S*)- (**13**) and (1*S*,2*R*)-2-ACBC (**16**) by desymmetrization, followed by Curtius degradation.



Scheme 5 Asymmetric reductive amination with (*S*)-α-methylbenzylamine.

Enantiomers from enamines

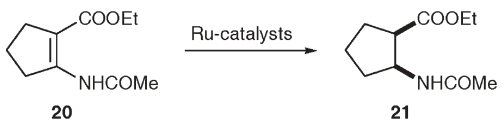
The reduction of enantiopure β-enaminoesters with sodium borohydride in acetic takes place with high diastereo- and enantioselectivity when α-methylbenzylamine is used as chiral side-chain. The major diastereomer is always *cis*-configurated. The protocol was scaled-up by Xu *et al.*[14] to the multi-gram scale (Scheme 5). The major diastereomer (*R*,*S*,α*S*) was isolated by a simple crystallization protocol as the HBr salt. The chiral products **19** can be efficiently converted to amino acids by hydrolysis and debenzylation. When sodium cyanoborohydride was used as reducing agent, the main diastereomer was *trans*-configurated.[10] The main product is also *trans* when the *cis* esters are isomerized with sodium ethylate.

The first catalytic asymmetric synthesis of cyclic β-amino acids was described by Zhang *et al.*[15] Ruthenium catalysts combined with chiral biaryl ligands such as C3-TunaPhos were found to be efficient in catalysing the reduction of **20**, with ee = 99% (Scheme 6). The method was used for higher homologues up to the 8-membered cycle, but in this case the selectivity dropped to ee = 44%. Boc-protected compounds were also applied successfully in the asymmetric catalytic reduction.

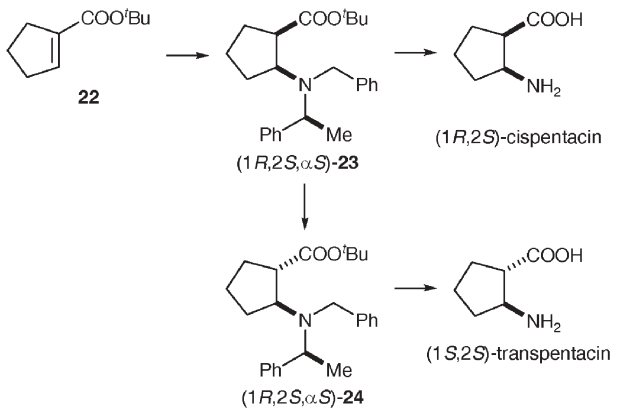
Enantiomers from cycloalkenecarboxylic acids

An excellent review by Davies *et al.*[5] on the above conjugate addition of enantiomeric lithium amides as chiral ammonia equivalents has appeared during the preparation of this manuscript. The scope and limits of its widespread applications in syntheses are discussed in detail.

Scheme 7 presents merely one example of its application. The conjugate addition of lithium (*S*)-*N*-benzyl-*N*-α-methylbenzylamide to *tert*-butylcyclopentene-1-carboxylate gives **23** with 98% de. Selective epimerization yields the thermodynamic epimer **24**. *N*-Deprotection followed by ester hydrolysis gives cispentacin and transpentacin with high enantiomeric purity.



Scheme 6 Ruthenium-catalysed enantioselective hydrogenation.



Scheme 7 Asymmetric syntheses of 2-ACPC stereoisomers.

This method is widely applicable for a number of substituted cycloalkenes.

Cyclic amino acids from natural sources

The readily available chiral terpenes are excellent starting substances for β-amino acids **25–27** (Fig. 2). CSI addition takes place with full regio- and stereoselectivity to give α-pinene and 3-carene enantiomers.[16] Hydrolysis of the resulting lactams can be performed only after Boc activation of the lactams, due to the less reactive crowded nitrogen.

3,4,5-Trihydroxy-2-aminocyclopentanecarboxylic acid enantiomers **28** and **29** were prepared through a strategically new protocol: from D-glucose or from L-iodose.[17] The strategy is based on the transformations of nitrofuranose to cyclopentylamine derivatives *via* an intramolecular cyclization to a 2-oxabicyclo[2.2.1]heptane derivative, followed by selective ring opening.

Cyclic amino acids *via* further functionalizations

The readily available β-amino acids **30–32**, containing a cycloalkene skeleton, are excellent starting substances for the introduction of polar substituents, *e.g.* hydroxy or amino groups, to the cycloalkane ring.[18–20] Amino acids **30–32** and their homologues, for example, can be prepared in enantiomerically pure form from readily available α-amino acid building blocks by a ring-closing metathesis-based approach.[21] From **30–32**, the 1,3-oxazine and γ-lactone strategies are excellent ways to produce hydroxylated products with total regio- and diastereoselectivity. The methods shown in Scheme 8

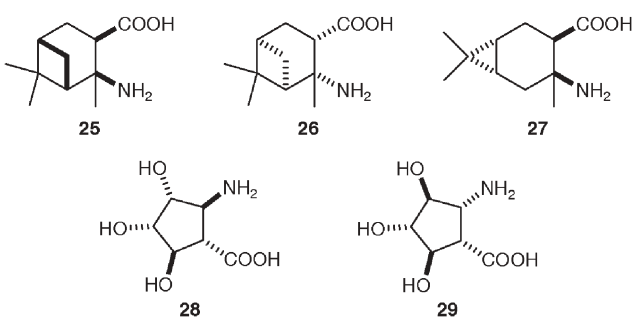
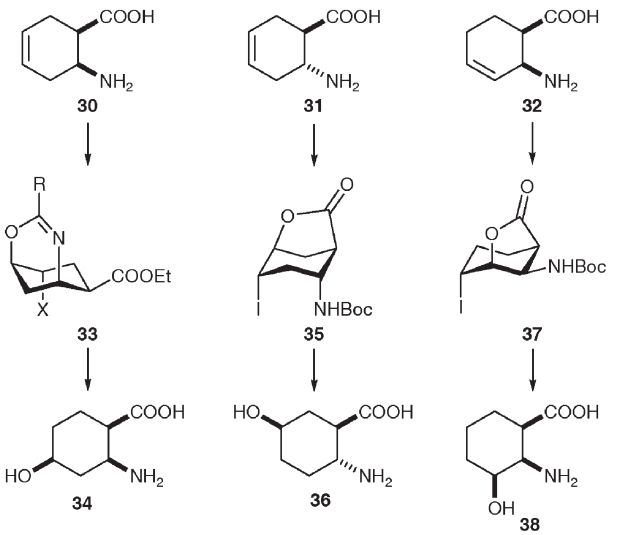


Fig. 2 Exotic amino acids from natural sources.



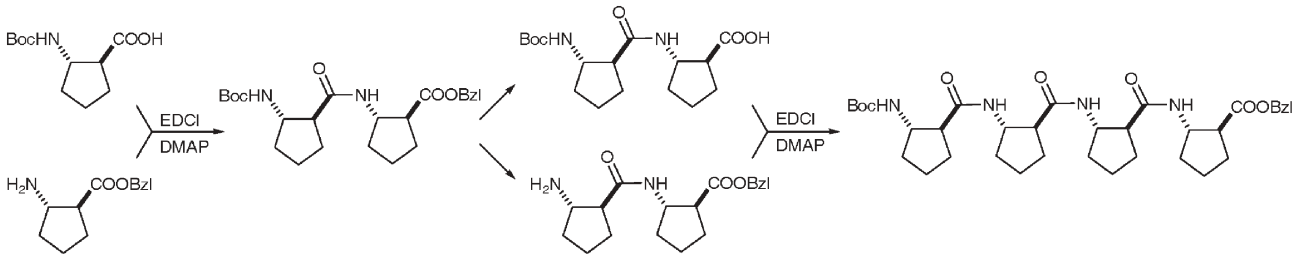
Scheme 8 Examples of transformation to hydroxylated ACHC enantiomers.

have been successfully used for both racemate and enantiomeric functionalizations. In several steps, the hydroxylated amino acids can be transformed to orthogonally protected diamino carboxylic acids.[18]

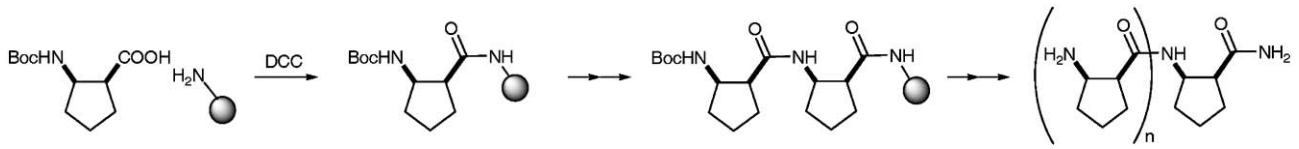
3 Peptide syntheses

The incorporation of the β-amino acids into peptides, in a similar manner as for α-amino acids, has wide variety. The well-known tactics and strategies for α-peptides are applicable for β-peptides too. Additionally, the greater distance between the amino and carboxyl functions can increase the number of activation methods, *e.g.* the application of β-lactams.

Although the homologation of *N*-protected α-amino acids by various methods was described several decades ago, the application of β-amino acids in peptide chemistry is relatively new: the first papers reporting the syntheses of β-amino acid-containing peptides appeared only within the past ten years. In contrast with the interesting physicochemical and biological features of these building blocks, only a few descriptions are to be found in the literature, and even they contain very limited details. The pioneering work of Gellman[22] involved the homo-oligomers of cyclic β-amino acids. As he worked with aminocyclopentane- and aminocyclohexanecarboxylic acids, no side-chain protection was necessary. The first synthesis of short ACPC oligomers was carried out in the liquid phase, Boc protecting groups being applied for the β-amino function and the benzyl ester for the α-carboxyl. The activation was achieved with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP). The syntheses are outlined in Scheme 9. The fragment condensation approach for the homo-oligomers seems favourable, but this method suffers from several difficulties, *e.g.* the carbodiimide activation of the β-carboxyl function leads to an increased probability of racemization, and the decreased solubility of the longer protected fragments would result in complex products containing truncated sequences.



Scheme 9 Boc-benzyl strategy and liquid-phase synthesis of protected *trans*-ACPC oligomers.

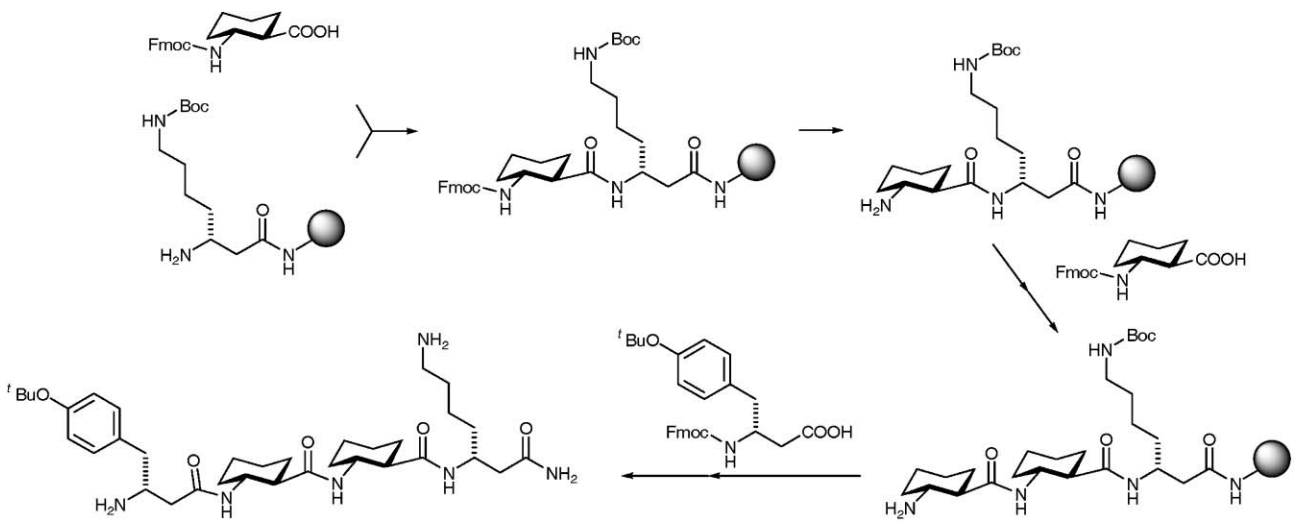


Scheme 10 Boc solid-phase synthesis of oligo *cis*-ACPC amides, applying the MBHA resin carrier and DCC as coupling agent.

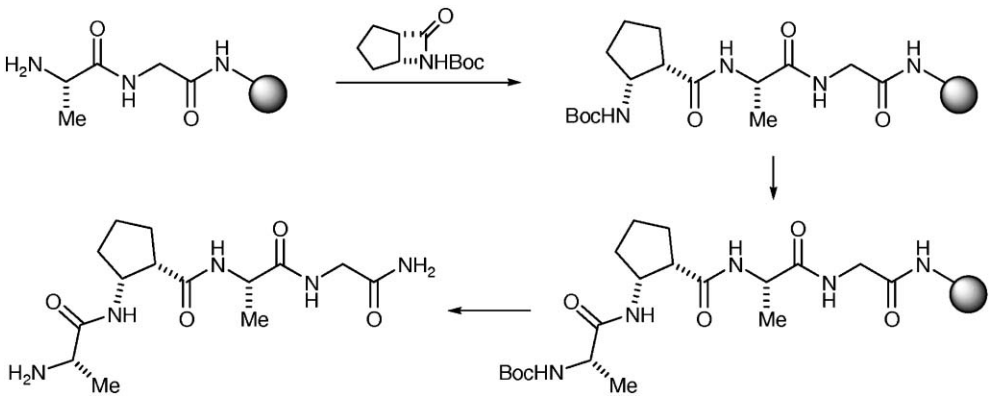
The presence of the protecting groups in the final products may increase the stability and the solubility of the investigated target compounds, but the results differ from those for the free oligomers. The deprotection and purification of the oligo β-amino acids was not described originally.[22] β-Peptides such as the foregoing ones can be conveniently prepared by conventional solid-phase methods, although the coupling and deprotection steps require more time than that for the analogous steps in α-peptide synthesis.[23,24] There are two main modes of application: the older methodology involving the Boc protecting group, and Fmoc chemistry.

The latter mode is recently more favoured in α-peptide chemistry, but the deprotection of protected β-amino acids is rather problematic; even the use of stronger bases such as DBU and an elevated deprotection time led to incomplete removal of the Fmoc protecting groups.[25] Only an elevated temperature (60 °C) was sufficient for the complete deprotection step. However, this modification led to an increased tendency to racemization. Another attempt was the direct application of the Boc-protected cyclic β-amino acid derivatives (the precursors of the Fmoc-protected derivatives too) for the solid-phase incorporation of the *trans*-ACHC and *cis*-ACPC derivative into the appropriate homo-oligomers.[23,24]

The main difficulties are as follows: completion of the coupling step is difficult, probably because of steric hindrance. Visualization of the incompleteness of the coupling steps is much more complicated as compared with the well-known methods for α-peptides, and the final products can be contaminated by truncated sequences. The recently described improvements, *e.g.* the application of new, more active coupling reagents such as fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate, 2-(7-aza-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, *etc.*, or microwave-assisted coupling conditions,[26] can lead to a significant increase in the quality of the final products. The second question is how to purify the final product after the HF deprotection step (this question, of course, is valid for all the applied methods). The dramatically lower water solubility of the long, hydrophobic cyclic β-amino acid oligomers does not allow the RP-HPLC purification of



Scheme 11 Fmoc solid-phase synthesis applying different β-amino acids.



Scheme 12 Incorporation of β-amino acids into an α-peptide chain, using Boc-protected β-amino acid lactams.

9–10-residue containing ACHC and ACPC oligomers. In consequence of the amphiphilic character of these peptides, the application of normal-phase silica chromatography is feasible for only shorter oligomers. Moreover, the separating power of classical silica chromatography is lower than that of the reverse-phase method. The Boc methodology has been applied successfully for the synthesis of numerous ACHC and ACPC oligomer amides.[23,24,27]

The β-lactam method,[28] in which Boc-protected cyclic β-lactams are used in the solid phase opens up an entirely new synthetic route for the preparation of β-amino acid-containing peptides.

The introduction of trifunctional β-amino acids into peptide chemistry raised the further question of: how to protect the side-functions. Several attempts have been made as regards modified the alicyclic β-amino acids *trans*-3-aminopyrrolidine-4-carboxylic acid and nipecotic acid, where no extra protection is necessary, 3-aminomethyl-ACPC, where the aminomethyl function is protected by Boc while the β-amino groups holds an Fmoc function,[29] and 4,4-disubstituted-ACPC derivatives bearing a *tert*-butyl ether and ester protection.[30] The major problem is usually the preparation of the appropriately protected building block; its incorporation is generally less problematic. The protection of the amino and hydroxy functions in the side-chain has been more or less solved, but the protection of other interesting functional groups such as guanidine or thiol has not yet been reported.

Besides the relatively widely used cyclopentane and cyclohexane ring-containing amino acids, a report recently appeared on the synthesis of cyclobutane β-dipeptides in which *N*-benzyloxycarbonyl and methyl ester protection and carbodiimide activation were applied.[12] Probably the most variable and versatile application of the cyclic β-amino acids relates to the synthesis of hetero-oligomers, using either linear β-amino acids or α-amino acids and the alicyclic derivatives.[31] Antimicrobial, cell-penetrating, agonistic or antagonistic analogues were synthesized in this way. The synthetic protocols applied include the automated Fmoc solid-phase synthesis,[32] the classical liquid-phase synthesis and the Boc solid-phase synthesis.[33] Cyclic β-amino acid oligomers were recently used as scaffolds.[34]

4 β-Peptides with cyclic side-chains: a toolkit with which to construct foldamers

Secondary structure variants and their nomenclature

Similarly to the natural α-peptides, the β-peptides belong in the class of polymers which have a strong propensity to fold into stable, well-determined and compact conformations.[35] These materials have recently been designated as foldamers,[36] this notation relating to an important generalization of the complex conformational behaviour observed for biopolymers. At first glance, the major difference between β-peptides and α-peptides in general is that the backbone contains an additional carbon atom in each β-residue (Fig. 3). The increased chain length per residue means a larger conformational space, which in turn theoretically hinders the formation of the folded structures because of the higher conformational entropy to be overcome during the self-organization process. Fortunately, designed β-peptides have been observed to fold readily, and the secondary structure formation is particularly pronounced for β-peptides with cyclic side-chains.[22] In fact,

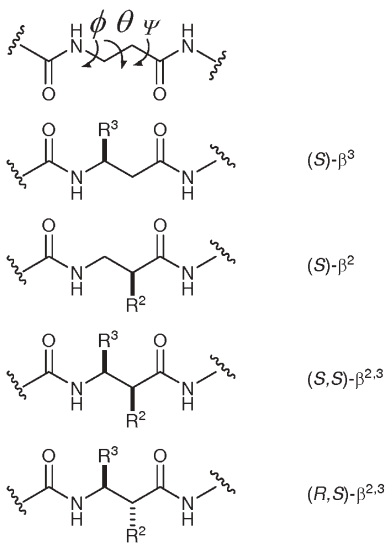


Fig. 3 General constitution, definition of the backbone torsions and designation of the substitution pattern of β-amino acid residues.

these polymers exhibit a rich diversity of secondary structures such as various helices, strands and turns.[37]

The first step toward the nomenclature of the secondary structure variants of β-peptides is to consider the conformational properties of the β-amino acid residues. These building blocks possess three potentially flexible torsions in the backbone, which are defined as CO–N–C^3–C^2, C^3–C^2–CO–N and N–C^3–C^2–CO, designated ϕ, ψ and θ, respectively. The three dihedrals allow a generalized 3D Ramachandran plot, which is a useful tool with which to locate the periodic regular conformations available for β-peptides, but it is rather difficult to view them in print. Therefore, a set of 2D projections is utilized[28] to simplify the representation. As regards the nomenclature of the secondary structures, there are a number of naming conventions depending on the research groups and the scope of the study. The most recognized nomenclatures[35,37] are basically confined to the helices and use of the size of the H-bonded pseudorings and/or the number of residues contained in one helical turn to describe the secondary structures. In this way, it is difficult to describe the experimentally observed strand-like or non-internal H-bond-stabilized periodic structures, and therefore the strand notation was added later. According to the definition created earlier, periodic conformations stabilized by backbone H-bonds with an angle greater than 120° are classified as non-polar strands, because the peptide bonds display alternating orientation and are exposed to intermolecular/long-range interstrand interactions.[38] The expression polar strand designates an elongated conformation with uniform peptide bond orientation. Probably the most general nomenclature was introduced recently in a theoretical work.[39] Although this nomenclature breaks with the strand notation, despite the obvious and useful analogy with the β-strand of α-peptides, all the experimentally observed helical and strand types, together with the theoretically predicted periodic conformations, are mapped into the full 3D ϕ, ψ, θ space. We summarize the wide variety of β-peptide periodic secondary structures found in the literature with high-resolution experimental geometry (Fig. 4).

The β-peptide secondary structures have several interesting features. Because of the limited reference space available in the present tutorial, the reader is referred to recent reviews[35,37,38] as regards the first occurrence in the literature and a detailed description of the secondary structure variants. Here, only the most important features will be discussed. First, the helices can have the backbone H-bond donor to acceptor direction either parallel or antiparallel with respect to the chain direction from the N-terminal to the C-terminal, a property which is completely missing for α-peptides. The parallel orientation is observed in the H14 and H10 helices, while the H12 and H8 helices are stabilized by antiparallel H-bonds. Interestingly, a helix type with alternating H-bond orientation also exists; it is the H10/12, which has zero dipole moment. An important feature of the β-peptide helices is that the direction of the helicity on going from the N-terminal to the C-terminal can be clockwise (*M*) or counter-clockwise (*P*), which can readily be controlled by the absolute stereochemistry of the β-amino acid residues. In this review, only the *M*-helicity is presented; all the *P*-type counterparts can be reconstructed by multiplying the given dihedrals by −1. As concerns the strand-like secondary

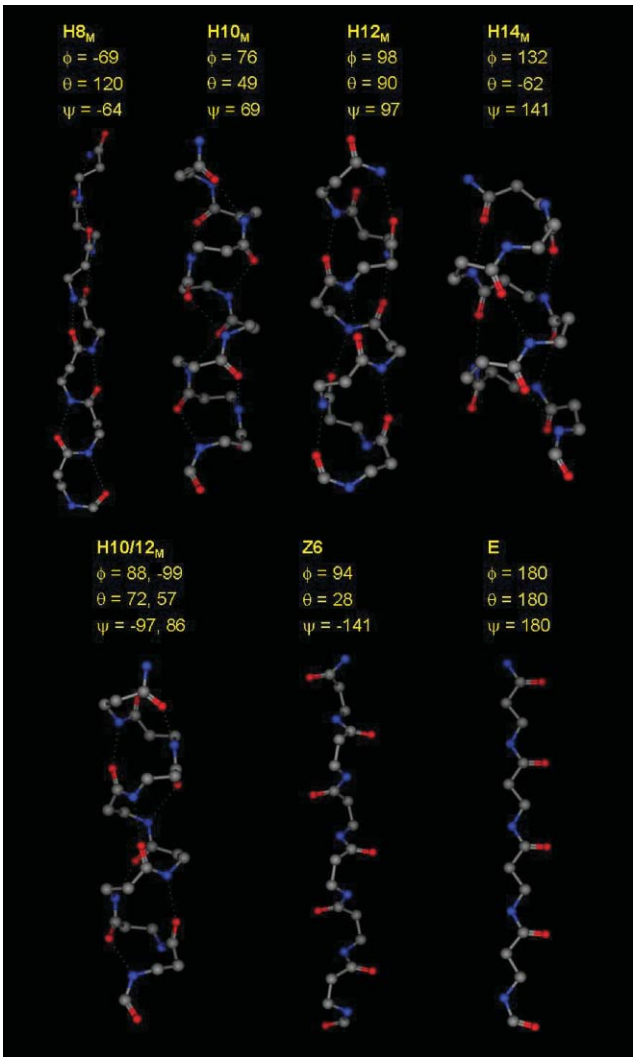


Fig. 4 The backbone geometry of the experimentally observed helices and strands with right-handed helicity. The representative backbone torsions are given in degrees.

structures, the possibility of uniform and alternating peptide bond directions results in a polar strand and a non-polar strand, respectively. The latter is a close analogue of the β-strand; alternatively, it can be designated a zig-zag conformation (Z). The polar strand, alternatively referred to as elongated (E), is a completely new strand type that is not available for α-peptides; it has a net dipole moment. Turn nucleating structural elements for β-peptides are known as well. A β-peptidic turn can be stabilized with both 10- and 12-membered H-bonded rings.

Elements of secondary structure design

The design principles of the β-peptide secondary structure motifs were recently summarized.[35,38] Here, we give a brief overview of the most important design tools, with focus on what can be done with the cyclic β-amino acid residues. First, the local structuring effect of β^2 and/or β^3 substitution should be mentioned, whereas β^3 substituents have a more pronounced influence on the local torsional space. Both *S*-β^3 and

S-β^2 substitution reduce the available dihedral angles for ϕ and ψ to the region 60°–180°, and to a narrow well of higher energy level at −60°.[40,41] Inspection of the characteristic torsions of secondary structure types readily reveals that such a substitution pattern strongly promotes helix formation ($H14_M$, $H12_P$ and $H10_M$) in general. Another requirement for the helical structure is the gauche conformation along θ. The cyclic β-amino acid residues with *S*-β^3-*S*-β^2 (*R*-β^3-*R*-β^2 for the opposite helicity) configuration exhibit all the advantageous factors for helix formation: (i) ϕ and ψ are confined to the preferred region and (ii) θ is covalently fixed in the gauche position. In line with these rules, the β-peptides with cyclic side-chains were among the first to be observed to form stable helices. In connection with the covalently fixed θ, a word of caution is in order. The helix type can be controlled by the nature of the cyclic side-chain; *trans*-ACHC, *trans*-ACPC and oxetane side-chains promote H14, H12 and H10 helices, respectively. It might be concluded that the smaller the alicyclic side-chain, the bigger is the value of θ, which allows increasing pitch height for the helix. Fig. 4 clearly demonstrates that θ increases in the following sequence: $H10_M < H14_M < H12_P$, which means that the greatest pitch height requires the lowest θ, and the trend is not monotonous. Moreover, the residual flexibility of the alicyclic moiety for *trans*-ACHC allows the H10 motif in the event of a shorter peptide chain length. The useful structuring effect of the cyclic β-residues can be exploited in a nucleation approach. If a single *trans*-ACHC residue is incorporated in the middle of the β-peptidic chain, its H14-stabilizing effect can be observed, and if the number of such building blocks is increased, the influence can be scaled up. Similar investigations on the *trans*-ACPC and *trans*-APC residues revealed a strong nucleating effect for the H12 helix. Thus far, stabilization of the H10/12 helix by using cyclic side-chains has not been attained, probably because of the homochirality of the cyclic residues applied.

$\beta^{2,3}$-Disubstitution not only affects the torsional preferences along ϕ and ψ for the non-cyclic monomers, but also has a major influence on θ. When the residues are *S*-β^3-*R*-β^2-disubstituted (or *R*-β^3-*S*-β^2), the pattern strongly stabilizes the antiperiplanar arrangement for θ, which is the very orientation necessary for the polar strand (elongated). Cyclic β-amino acids do not tend to occupy this conformation, and the elongated structure is therefore unavailable for them. For a synclinal θ and the above-mentioned propensities for ϕ and ψ, it remains to consider the non-polar strand (zig-zag) structure, which resembles the natural β-strand. Indeed, by using *cis*-ACPC residues, the 6-membered H-bond-stabilized non-polar strand can be constructed. For the chain lengths attainable for β-peptides, detection of the strand conformations is not a trivial task. While non-polar strands created by using *cis*-ACPC are self-stabilizing, despite the rather weak internal H-bonds, the polar strands are not fixed by such electrostatic interactions, and thus interstrand H-bonds should be induced by using turn nucleating segments. A rigid cyclic β-residue enters the picture here, nipecotic acid, which is a close analogue of proline. A dinipecotic segment with devised stereochemistry serves as an effective turn stabilization fragment by generating an ideal 12-membered H-bonded ring.[37]

Attention must be paid to the long-range side-chain interactions between residues separated by a turn of the helix. The extra stabilization energy may stem from van der Waals, electrostatic or solvent-driven hydrophobic interactions. The structures of the H14 and H12 helices disclose the necessary juxtapositions, while H10 and H10/12 lacks these side-chain contacts. In the case of H14, all the β^2 and β^3 substituents with appropriate spatiality are adjacent, at positions i and (i + 3) in the chain. For H12, the proximity can be observed between the (i)β^3 − (i + 2)β^2 and the (i)β^2 − (i + 3)β^3 pairs. Cycloaliphatic side-chains expose a massive hydrophobic surface, which is advantageous for these helix types in terms of solvent-driven attractive forces. On the other hand, the incorporation of heteroatom functionalities into the cyclic side-chains facilitates solubility in water.[42] It must be mentioned, however, that an aqueous medium destabilizes the helical fold.

In connection with cyclic side-chains, it can be concluded that their strong preference for the periodic secondary structure motifs gives a valuable tool for the creation of secondary structure. At the same time, this property gives rise to the possibility of an overly preorganized residue pattern, preventing a real self-organization process. The answer to this question is that short β-peptide oligomers with cyclic side-chains display chain length-dependent folding behaviour, which rules out the preorganization issue.[24]

Towards tertiary structures of β-peptides

When biopolymers are considered, their interesting structural features and biological functions are closely related to their highly hierarchical conformation, which affords tertiary and quaternary structures. For β-peptides, a 'Holy Grail' to find is a controlled tertiary structure formation which would contribute to the final validation of the generalization embodied in the notion 'foldamer'. While the construction of protein-sized β-peptides (β-protein) would require tremendous effort at the present level of β-peptide chemistry, the synthesis of self-assembling secondary structure units may pave the way for the construction of tertiary structure motifs. As an indication of the severe difficulties encountered in this field, only a few successful attempts has been published so far.

The tertiary structure motifs potentially available for β-peptides are closely analogous with those well known for natural proteins: the helix bundle and pleated sheet sandwich (Figs. 5 and 6).

Signs of the formation of helix bundles were observed on the analytic ultracentrifugation of amphiphilic 10-residue cyclic side-chain stabilized H14 helices in an aqueous solvent,[43] and on the diffusion-ordered NMR investigation of hydrophobic H14 helices in methanol.[24] The measurements suggest helix oligomers of tetramer-hexamer size, where the hydrophobic attractive forces drive the self-assembly. H14 helices with designed hydrophobic and ionic surface features stapled together by disulfide bonds exhibited a thermal unfolding curve sufficiently steep to render 2-helix formation likely, which in turn suggests that the structural motif is stabilized by long-range interactions.[44] A radically different approach was introduced when the covalent functionalization of every third 14-helix side-chain with nucleobases was used for a reversible

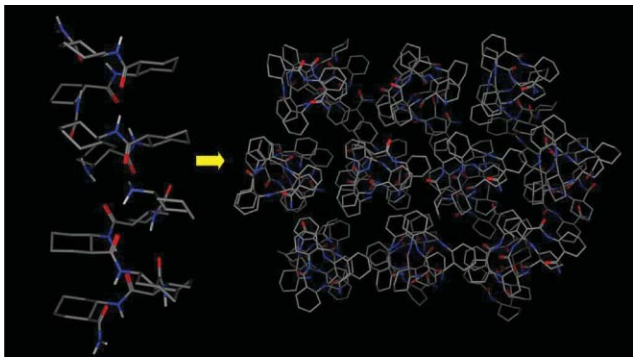


Fig. 5 Helix-bundle tertiary structure motif for β-peptides.

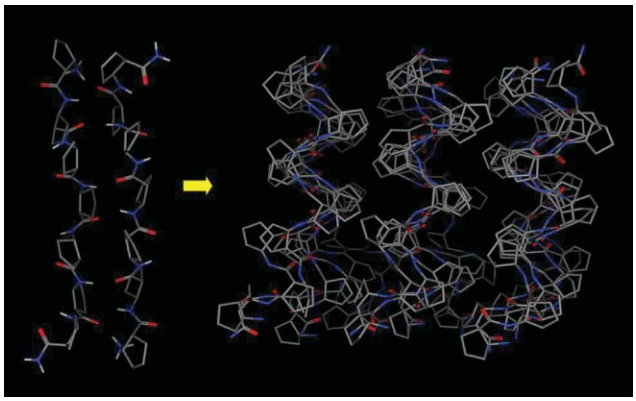


Fig. 6 Pleated sheet sandwich tertiary structure motif for β-peptides.

organization of two helices based on nucleobase pairing.[45] Here, the intrinsic tertiary structure formation of the β-peptide is rather suppressed and thus the helix units serve purely as a preorganized scaffold for duplexes. Nevertheless, the strength of this methodology lies in the future possibility of creating molecular architectures displaying functional mimicry. It may be seen that the helical units have proved to be relatively useful for tertiary structure creation. Because of the long-range or the intermolecular forces necessary to stabilize a pleated sheet, the construction of a pleated sheet sandwich motif is rather difficult. When *cis*-$ACPC_7$ non-polar strand (Z6) units were used, however, self-assembly into a pleated sheet sandwich was detectable in polar solvents (Fig. 7).[46] After incubation for 1 week, ribbon-like fibril formation could be observed, the ribbon height corresponding exactly to the length of the *cis*-$ACPC_7$ strand unit (2.5 nm). The width was 30–50 nm, suggesting 30–40 sheet layers in the ribbon, and the length could reach the μm range. Interestingly, the experiments repeated with *trans*-$ACHC_6$ helix units revealed multilamellar vesicles with a diameter of 60–180 nm. The nano-sized spheres are made up of a continuous 2.2 nm thick helix-bundle membrane, which is sensitive to ultrasound, similarly to phospholipid membranes.

Methods for detection of folded β-peptides

The arsenal necessary for the unequivocal proof of the presence of a certain folded or self-assembled structure of β-peptides contains a number of proven methods that are generally utilized in the conformational analysis of proteins and peptides. In accordance with the scope of this review, we summarize the information content and possible pitfalls of the following experimental methods commonly applied to β-peptides without going into theoretical and experimental details: circular dichroism (CD) spectroscopy, Fourier transform-infrared (FT-IR) spectroscopy, X-ray crystallography, NMR methods, various particle size measurements (dynamic light scattering, analytical ultracentrifugation, pulsed field gradient echo NMR) and electron microscopy.

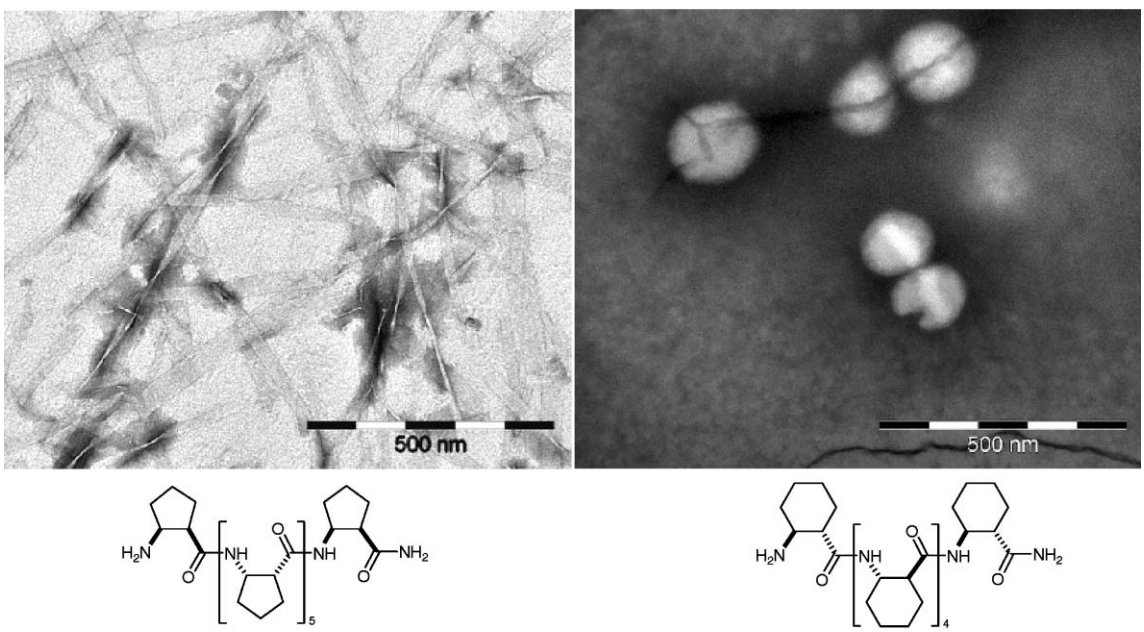


Fig. 7 Nanostructured pleated sheet sandwich fibrils (left) and helix-bundle membrane vesicles (right) observed for β-peptides.

CD spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light by a substance. The most important optically active groups in β-peptides are the amide bonds in the backbone. These amide chromophores generate CD bands in the far-UV region (178–250 nm), which can be analysed for the different types of secondary structures. A series of publications have revealed that the interpretation of the CD spectra of β-peptides requires special care, as they are extremely sensitive to minor variations in the structure that do not affect the overall fold of the molecule.[47] Nevertheless, the spectral data that have accumulated in the literature with concomitant high-resolution structures allow certain assignments between the CD features and the helical patterns. The CD curve of helix H14 exhibits Cotton effects of nearly equal intensity but opposite sign near 200 nm and near 215 nm, or a single broad band at around 215 nm. The intense signal at around 200 nm and a disappearing band at 215 nm usually point to the appearance of helix motifs with larger pitch height: H10 and H12. Unfortunately, CD data on strand-like structures are rare. A non-polar strand (Z6) constructed from 1*R*,2*S*-ACPC showed a negative band at 203 nm. Overall, the CD spectroscopy of β-peptides provides a fast and efficient tool with which to probe the folding propensity, but it is not recommended to rely solely on CD curves.

The folded structures are organized by the backbone H-bonds. FT-IR spectroscopy is a sensitive method that is easily applicable for the detection of vibrational modes influenced by the presence or strength of the H-bonds. In the N–H stretch region (amide A), the free N–H vibrations appear as a sharp signal at 3446–3456 cm^{-1}, while intramolecular H-bonded N–H stretches give rise to a broad band in the region 3285–3344 cm^{-1}. Another important source of information in IR spectra is the amide I region (1600–1700 cm^{-1}), which includes the carbonyl stretching vibrations coupled with the bending of the N–H bond and the stretching of the C–N bond. Measurements on β-peptides unequivocally adopting helical conformations (H10 or H14) reveal a band at around 1650 cm^{-1}, which can be assigned to the H-bonded amides in the helix. Weaker H-bonds result in a slightly increased frequency. The band at 1680 cm^{-1} could be assigned to the terminal $CONH_2$ group. During IR measurements, the presence of any aggregated species should be considered with caution. For self-stabilizing Z6 strands, a band was observed at 1635 cm^{-1}, which is in good correlation with the appearance of pleated sheet oligomers. Attention must be paid to the H-bonding properties of the solvent, *e.g.* the H-bonding propensities of DMSO, and the residual TFA may lead to artefactual assignments.

X-ray crystallography played a pioneering role in determining the high-resolution structure of β-peptide secondary structures. The information content of the electron density map provided by the diffraction pattern is imperative, despite the fact that it might give a more favorable account of secondary structure stability due to the crystal packing forces than would be observed in a solvent. The greatest challenge with X-ray crystallography is the preparation of the single crystals necessary for proper diffraction patterns. Folded structures of short β-peptides exhibit extraordinary stability as compared with those of short α-peptide oligomers, but the conformational polymorphism observed in several cases or the possible disordered conformations may hinder the crystallization process. The self-assembling features of the β-peptides may also lead to the formation of nanostructured materials, where single crystal preparation is not possible; special techniques (*e.g.* fiber diffraction) will be useful here.

NMR spectroscopy itself provides a wealth of methods for the detection of folding in solution. At first glance, the chemical shift dispersion in a single proton spectrum may provide a hint of the folded state, similarly as for natural peptides and proteins. A folded structure of a β-peptide oligomer can result in a reasonably good dispersion in the amide region, even if a homo-oligomer is studied, but unfortunately this is not general at this size. A fast approach is to gather the spectral information directly related to the organizing H-bonds. By default, amide hydrogens are species that undergo exchange with protic solvents, which means that their signal intensities and chemical shifts are strongly dependent on the nature of the possible solvent–solute interactions. In folded geometry, amide hydrogens are usually buried in H-bonds and sterically shielded by the side-chains, which can inhibit the NH-solvent exchange process considerably. The signal intensity of a free amide hydrogen vanishes immediately when the sample is dissolved in deuteromethanol or D_2O; an NMR spectrum displaying no amide signal after dissolution is a clear indication of the random structure. The lifetime of signals corresponding to more or less shielded amide hydrogens at room temperature in deuteromethanol can extend from 15 min to several months, depending on the stability of the secondary structure and the nature of the side-chains. We should emphasize the role of the self-association phenomena. Secondary structure units with a propensity to self-associate in a polar solvent decrease their surface exposed to the solvent by hydrophobic interactions, which can dramatically decrease the exchange rate. For self-organizing non-polar strands, a concentration-dependent exchange rate is observed, testifying to intermolecular H-bonds. The temperature dependence of the amide signals in ^1H spectra can also be utilized for the detection of protons involved in H-bonds. The temperature coefficients ($\Delta\delta/\Delta T$) should generally not significantly exceed the range 0 to -4 ppb K^{-1} for solvent-inaccessible protons in a protic solvent. An extremely important aspect in connection with measurements of both the exchange rate and the temperature coefficient is the residual trifluoroacetic acid (and other chaotropic substances) content of the β-peptide sample stemming from the purification protocols, since these materials can inhibit the secondary structure formation and catalyse the chemical exchange between the amide and the solvent.

The major advantage of NMR spectroscopy is that high-resolution structural information can be obtained. The first step in geometry refinement is the resonance assignment, which can be performed for β-peptides in a fundamentally similar way as established for small proteins. Sequential assignment of the backbone can be carried out in most cases with the standard homonuclear 2D NMR techniques (COSY, TOCSY and ROESY) and by careful tuning of the solvent and temperature. At the usual sample concentration, which is not

Table 1 Dominant helical fold determination by using NOE interactions

H14	H12	H10
H_N (i)–H_β (i + 3)	H_N (i)–H_β (i − 3)	H_N (i)–H_β (i + 2)
H_N (i)–H_β (i + 2)	H_α (i)–H_β (i − 3)	H_N (i)–H_β (i + 1)
H_α (i)–H_β (i + 3)		H_β (i)–H_α (i + 2)

more than a few mM in order to prevent uncontrolled aggregation, heteronuclear measurements and the related 3D measurements are not very useful. The incorporation of ^{13}C or ^{15}N-labelled residues into the β-peptidic chain, facilitating heteronuclear NMR techniques, is expected in the future. For these oligopeptides, measurement of the rotating frame NOE is recommended in order to avoid the cancellation of cross-relaxation peaks in the laboratory frame due to the intermediate tumbling rate. The backbone resonances usually occur at around 7.5–8.5 ppm, 3.5–4.2 ppm and 2.5–3.5 ppm for H_N, H_β and H_α, respectively. An unprotected N-terminal is more likely to increase the chemical shift dispersion to some extent, alleviating the problem of heavy overlaps. The type of the dominant helical fold can be determined unequivocally by using the resolved non-sequential or long-range NOE interactions along the backbone (Table 1).

Sequential NOEs can also be put to good use, especially when a β-peptide with a cyclic side-chain is analysed. The relative intensities of ROESY cross-peaks for [H_N (i)–H_α (i − 1)]/[H_N (i)–H_β (i)] are extremely sensitive indicators of the orientation around the peptide moieties, due to the r^{-6} dependence of the NOE cross-peak intensity, where r is the internuclear distance. The interpretation of the 3D structures generated by molecular modelling and the NMR distance restraints requires special care for the short β-peptide sequences. It may very well be a common view that the resulting cluster of the lowest-energy structures meeting the NMR restraints is the one and only conformation observable in the solvent. On the other hand, these oligomers are considerably more flexible species than the large peptides for which the NMR refinement methodologies were developed. The conclusion that can safely be drawn is that a geometry obtained in this way will predominate over other low-probability unordered structures, but its overall weight could sometimes be as low as 30% and this ratio still explains the experimental NMR data.[48]

On passing to the controlled self-association of β-peptides, particle size measurements are getting increasingly more emphasis. Analytical ultracentrifugation (AU) has emerged as a powerful technique for characterization of the assembly and disassembly mechanisms of biomolecular complexes. This method was applied for the first time for the quantitative detection of the aggregation state of designed amphiphilic β-peptide helices.[43] The experiment was conducted in the sedimentation equilibrium mode, where the sedimentation velocity is in balance with the diffusion rate. Another useful technique is dynamic light scattering (DLS), which is capable of determining the size distribution of nanosized particles on the basis of the autocorrelation intensity of the back-scattered light influenced by the Brownian tumbling.[46] The movement of particles in a centrifuge demonstrates a stronger size dependence than the diffusion coefficient measured by DLS, and hence the resolution obtained by AU is significantly higher and less model-dependent than in DLS. On the other hand, DLS measurements are easier to carry out. For the lower end of the size region, the oligomerization of β-peptides can readily be observed by using pulsed field gradient echo NMR.[24] The gradient echo intensity decay is influenced by the rate of diffusion of the observed sample. Since the NMR resonance broadening is not sensitive enough to indicate the oligomerization of short β-peptides, this method is recommended to be routinely performed for all NMR samples in order to disclose any aggregation before the high-resolution structural data are evaluated.

Especially when the specific self-association leads to informative morphology on the nanometre scale, electron microscopy is a very effective method with which to acquire images with the necessary resolution. Nanostructured fibrils and membranes of β-peptides have been investigated by utilizing transmission electron microscopy.[46]

5 Conclusions and outlook

Knowing that the conditions on primitive Earth were such as to lead to the formation of β-alanine, and that β-amino acids presumably of cometary or asteroidal origin have also been found (see ref. 46 and the references cited therein), one could question why these substances did not play roles in the construction of living organisms. Since one of the essential prerequisites of life; the ability to fold into highly organized structures, is proven for β-peptides, the reasons why nature chose α-amino acids may rather lie elsewhere (*e.g.* in the autocatalytic properties of *N*-phosphoryl derivatives of α-amino acids).[49] Nevertheless, β-peptides may become invaluable tools for medicinal and biochemists in their quest for moderately sized scaffolds necessary to build molecules that are able to interact beneficially with biomolecules and to inhibit important protein–protein interactions responsible for diseases. This paradigm is augmented by the fact that β-peptides are stable to proteolytic degradation both *in vitro* and *in vivo*.[35] Thus far, a number of promising biological applications have been published where the secondary structure of the designed β-peptides was inevitable: inhibitors of fat and cholesterol absorption,[50] selective antibacterial amphiphiles,[51] RNA binding[52] and inhibitors of the interaction between the tumour suppressor p53 and the sequestering factor hDM2.[34] The future of β-peptides is currently unfolding, and these secondary structures, and later tertiary structure scaffolds, will hopefully provide elegant solutions for a number of problems in chemistry and biology.

To support the above ideas, more research is still necessary at a basic (peptide monomer unit and peptide synthesis) level. Thus, access may be achieved to highly substituted new cyclic β-amino acids, and to the development of simple and non-expensive, scalable routes to enantiomers.

Acknowledgements

This work was supported by Hungarian Research Foundation (OTKA T 034901, F038320 and T048848) and the National

Research and Development Office, Hungary (OMFB-0066/2005-DNT and GVOP-311-2004-05-0255/3.0), for financial support. T.A.M. acknowledges the János Bolyai scolarship of the Hungarian Academy of Sciences.

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