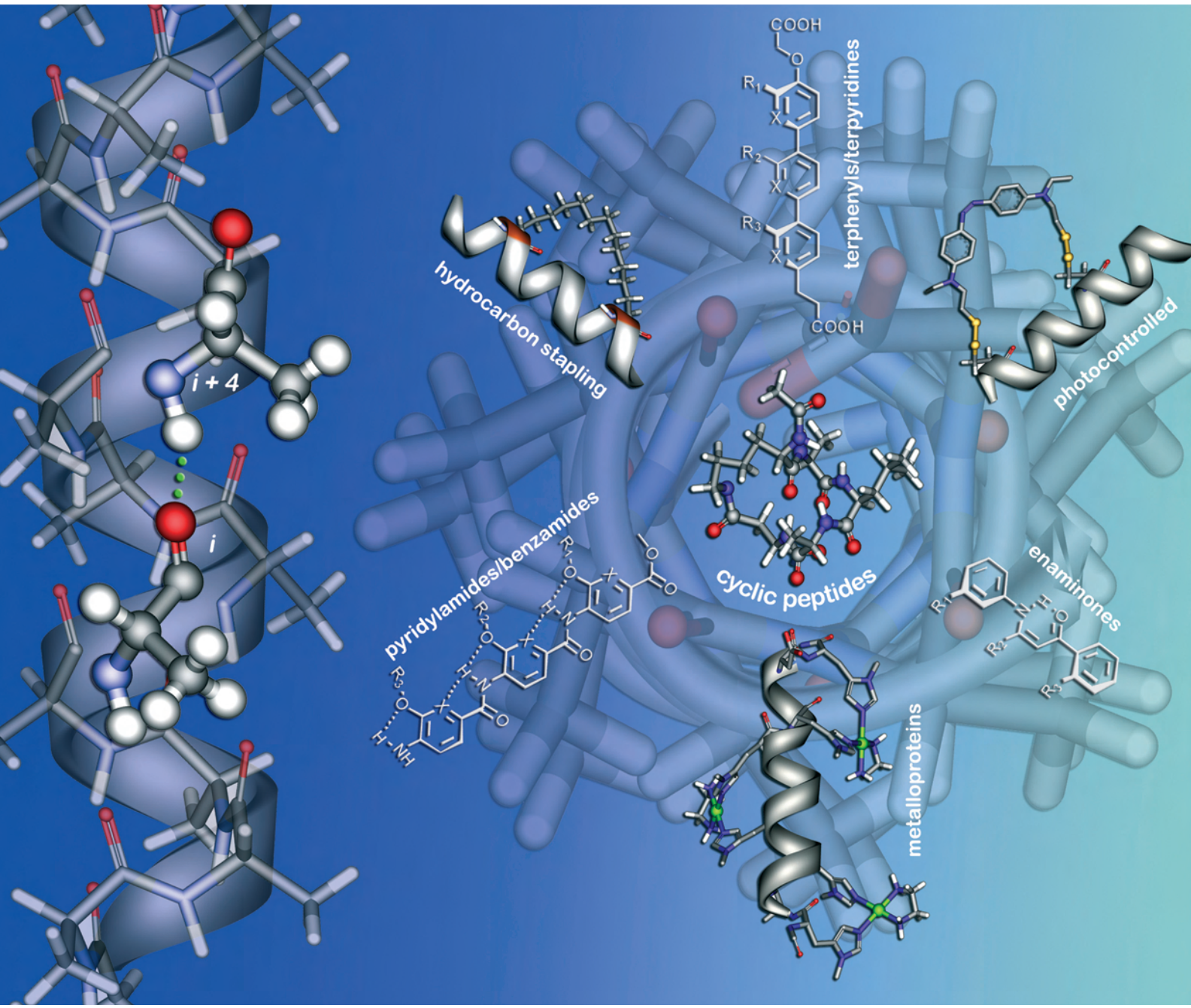
Organic & Biomolecular Chemistry

www.rsc.org/obc

Volume 5 | Number 22 | 21 November 2007 | Pages 3557–3720



ISSN 1477-0520

RSC Publishing

PERSPECTIVE
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Chemical Science

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1477-0520(2007)5:22;1-O

Design and synthesis of α-helical peptides and mimetics

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Received 9th July 2007
First published as an Advance Article on the web 7th September 2007
DOI: 10.1039/b710425a

The α-helix is the most abundant secondary structural element in proteins and is an important structural domain for mediating protein–protein and protein–nucleic acid interactions. Strategies for the rational design and synthesis of α-helix mimetics have not matured as well as other secondary structure mimetics such as strands and turns. This perspective will focus on developments in the design, synthesis and applications of α-helices and mimetics, particularly in the last 5 years. Examples where synthetic compounds have delivered promising biological results will be highlighted as well as opportunities for the design of mimetics of the type I α-helical antifreeze proteins.

1. Introduction

The α-helix is the most predominant secondary structure unit in proteins. Within protein structures, helices are important shape- and sequence-selective recognition motifs for protein–protein and protein–nucleic acid interactions.[1–3] However, the removal of these short polypeptide recognition motifs, which are typically 15–25 residues in length, from the stabilising tertiary structure of proteins generally results in peptides that adopt only random coil structures in water or peptides that adopt only low populations of conformations containing α-helical secondary structure.[4] In addition, the efficacy of short polypeptides corresponding to the α-helical regions in proteins is compromised *in vivo* due to an increased susceptibility to proteolytic degradation and a reduction in cell wall permeability.[4]

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Significant interest in the design and synthesis of short α-helical peptides and α-helical mimetics stems from (i) understanding the role of α-helices in protein folding, (ii) the key role of α-helices in mediating protein–protein, protein–DNA and protein–RNA interactions, and (iii) the remarkable biological activity exhibited by some polypeptide α-helices. Strategies that have been investigated to stabilise short peptides in α-helical conformations include the use of helix-nucleating templates, incorporation of unnatural amino acids, and the introduction of noncovalent and covalent sidechain constraints. More recently the development of non-peptidic scaffolds, and modified peptide backbones that mimic the recognition properties of α-helices, as well as miniature proteins and synthetic foldamers, have delivered promising lead compounds for biological applications.

This perspective will focus on developments in the design, synthesis and applications of α-helices and mimetics, particularly in the last 5 years, and will highlight examples where synthetic compounds have delivered promising biological results. Several excellent recent reviews have covered the general principles for

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the design of peptide-mimetics,[4] and synthetic molecules for the inhibition of protein–protein interactions.[3,5,6] Hence these studies will only be briefly mentioned in this article.

2. *De novo* design of α-helices: helix propensities of amino acids

Fundamental to the *de novo* design of α-helical peptides is a complete understanding of the relationship between amino acids and secondary and tertiary structure. The choice of amino acid, the helix dipole and the presence of *N*- and *C*-capping sequences are all recognised as factors that contribute to the stability of helical peptides, and these aspects of peptide design have been covered elsewhere.[4]

In 1974, Chou and Fasman developed a predictive method for the structure of proteins based on a statistical analysis of proteins of known structure.[7,8] This analysis resulted in the classification of amino acids as helix-stabilising or helix-destabilising. The characterisation of more protein structures lead to an improved classification system, and research in the 1990's applied these concepts to the design of short α-helices of 15–20 amino acid residues, in order to improve the understanding of helices in protein folding and to assist in the *de novo* design of proteins.[9–11]

While early model studies concluded that Ala is helix-stabilising, conflicting results were obtained with nucleated peptides that concluded that Ala is helix-indifferent (see for example ref. 10–12). More recently, a study of polypeptides that lack short terminal sidechains showed that the α-helix propensity of Ala is intrinsic and that the helix content of Ala-based peptides is not derived from neighbouring amino acids.[13] Independent studies on templated nucleated peptides also concluded that Ala is helix-stabilising and showed that the helical content of polypeptides is influenced strongly by the properties of the template–helix junction.[14]

The wealth of data now available on protein structures in the PDB data-bank has permitted a much more rigorous analysis of amino acid propensities in the last 5 years.[15–17] Analyses of α-helices in proteins demonstrated that the helix propensities of amino acids are strongly position-dependent throughout the entire length of most helices, indicating that the protein chain tends to enter and exit from the solvent-accessible side of surface helices of proteins.[15] A more detailed analysis of α-helices within the same structural subclasses of proteins (all-α, all-β and α/β-proteins), provided improved predictive tools for helix propensities.[16] The importance of pairs of amino acid neighbours in α-helices has also been demonstrated by analysis of amino acid pairs in 342 proteins.[17] Finally, given the wide use of CD as a tool for measuring the "helix content" of designed peptides, it should be noted that accurate calibration standards for measuring the helicity of peptides have been recently reported.[18]

3. Cyclic peptides

The incorporation of disulfide linkages *via* oxidation of Cys residues and the formation of amide bonds between the sidechains of Lys and Asp/Glu residues have been the most common methods of constraining the conformational flexibility of peptides and locking segments of these peptides into a helical conformation. The periodicity of a single turn of the α-helix positions the sidechains of the *i*, *i* + 4, *i* + 7 and *i* + 11 residues on the same face of the helix, and hence pairs of these residues are amenable to synthetic modification. In the case of disulfides, this approach requires the incorporation of D-Cys at the *i* residue and L-Cys at either the *i* + 4 or *i* + 7 residues. The corresponding L,L-analogue had a marginal effect on stabilising helicity and the L,D-analogue resulted in a random coil or β-sheet peptide.[19]

The limitation of these methods is that the use of a single covalent bridge constrains only a limited section of the polypeptide between either residues *i* and *i* + 4 or residues *i* and *i* + 7, leaving the remainder of the polypeptide chain flexible. However, several examples of the use of multiple covalent bridges to stabilise longer lengths of polypeptides have been reported. The effectiveness of incorporation of three covalent bridges into a polypeptide is exemplified by modification of the 37 *N*-terminal residues of the human parathyroid hormone (hPTH) to deliver therapeutic osteogenic agents.[20] Introduction of three successive lactam bridges between Lys and Asp residues located at positions *i*, *i* + 4 along the polypeptide afforded a highly constrained peptide in which residues 13–30 were forced into a helical conformation (Fig. 1). This constraint delivered a high level of potency compared to the parent linear sequence.

A more recent study has compared the effectiveness of disulfide *versus* lactam constraints to stabilise the consensus sequence LXXLL found in nuclear receptor proteins, in order to develop selective inhibitors of steroid receptor–coactivator interactions.[21,22] In agreement with molecular modelling studies, an *i*, *i* + 3 disulfide-linked peptidomimetic oestrogen receptor modulator was significantly more potent than the linear 13 residue peptide or the *i*, *i* + 4 bridged cyclic lactam derivative. Based on these promising results, further tailoring of the sequence to include both D- and L-Cys, homocysteine, penicillamine and Leu residues delivered more potent and selective inhibitors.[23]

Despite numerous studies, there remains a lack of consensus as to the optimal residue combinations, ring sizes, and sequences that can effectively stabilise peptides using lactam bridges.[24] Furthermore, disulfides and amide bridges, as well as the polypeptide backbone, are susceptible to oxidative and proteolytic degradation *in vivo* in short peptides, and these properties limit the application of these cyclic peptides in biological systems. A major exception in the field is the discovery that simple cyclic pentapeptides tethered by an amide to form a 20-membered macrocycle deliver highly helical structures.[24] The pentapeptides Ac-(*cyclo*-1,5)[KxxxD]-NH_2 **1** (Fig. 2a) and Ac-(*cyclo*-2,6)R[KxxxD]-NH_2 are the smallest α-helical peptides in water. Furthermore, these cyclic peptides are remarkably stable, even under protein-denaturing conditions (temperature, guanidinium hydrochloride, plasma) and proteolytic cleavage by trypsin. This framework offers major advantages in the design of conformationally stable peptidomimetics in which one face of the helix is involved in recognition. Fairlie and co-workers extended this initial discovery to link several cyclic pentapeptide α-turn units together by amide bonds in a modular fashion to deliver highly stable, conformationally rigid, α-helical peptides.[25]

The potential of the cyclic pentapeptide scaffold **1** (Fig. 2a) in medicinal chemistry has been demonstrated by the design of an effective mimic of the contiguous α-turns of a respiratory syncitial virus (RSV) protein. The RSV protein mediates fusion of viral and cell membranes, enabling entry into cells.[26] A cyclic peptide which contains the key amino acid sidechains in a fragment

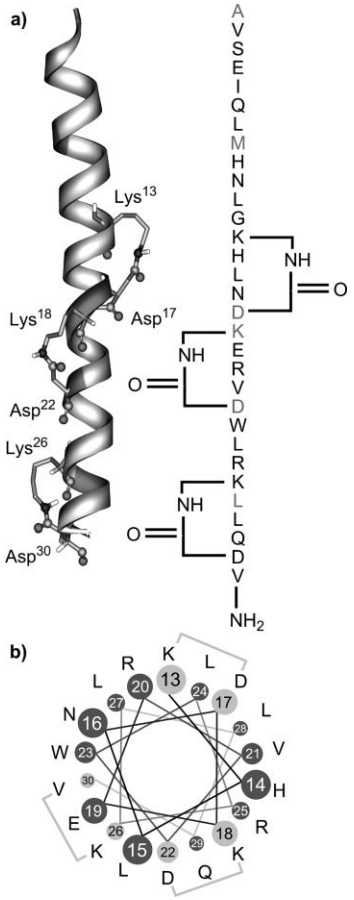


Fig. 1 (a) A sketch of the truncated mimetic comprising 31 *N*-terminal residues of the human parathyroid hormone (PDB coordinates 1et1) stabilised by three successive lactam bridges inserted between Lys13–Asp17, Lys18–Asp22 and Lys26–Asp30.[20] Residues which were mutated from the native sequence are shaded dark grey. Sidechain hydrogens are omitted for clarity. (b) A helical wheel diagram of residues 13–30 illustrating the *i*, *i* + 4 facial periodicity of the residues selected to be linked by lactam bridges (shaded light grey).

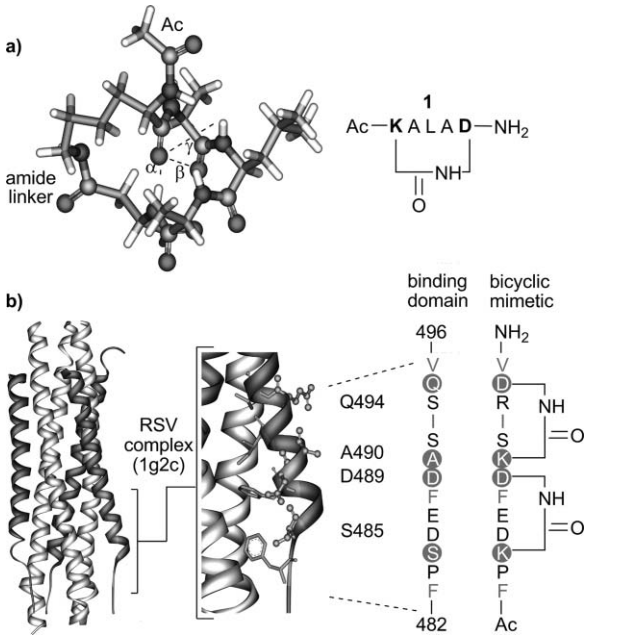


Fig. 2 (a) The cyclic pentapeptide motif that forms that smallest α-helical peptide in water.[24] (b) A potent RSV inhibitor based on this motif.[26] Structure generated used PDB coordinates 1g2c. Solvent-exposed residues are indicated by grey circles/ball-and-sticks, and binding residues by grey text/sticks.

of the RSV protein (13 of 39 residues), shown schematically in Fig. 2b, exhibited highly potent antifusion and antiviral activity. In contrast, the unconstrained acyclic peptide had no helical character in water and exhibited poor antiviral activity. While the peptidomimetic shown in Fig. 2b was highly potent, it was noted from the NMR solution structure that a key Phe residue was not in the optimal orientation for binding. Hence further modification of the framework to more closely align the Phe sidechain in the binding pocket has the potential to deliver even higher potency.[26]

4. Photocontrolled α-helices

In a series of papers, Woolley, Alleman and co-workers have reported a mechanism for controlling the stability of α-helices *via* the use of photoisomerisable azobenzene cross-linking agents (Fig 3a).[27–32] Azobenzenes have been used previously to modulate the relative orientation of two α-helical peptides to facilitate sequence-specific DNA binding.[33] In order to induce formation of an α-helical conformation in peptides, the diphenylazo group

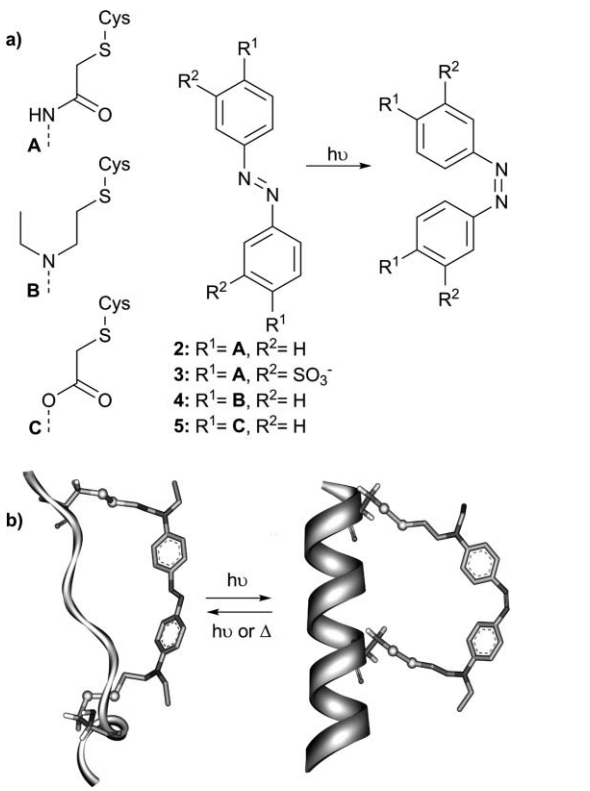


Fig. 3 (a) Photochemistry of azobenzene linkers that have been incorporated into peptides and (b) a sketch of the reversible photochemical conversion of a random coil peptide into an α-helix.[31] Disulfide atoms are shown as ball-and-stick. Hydrogen atoms of the azo-linker have been omitted for clarity.

was introduced *via* i, $i + 7$ Cys residues, as modelling suggested that under photochemical conditions this spacing would permit the disordered peptide to reversibly form an α-helix upon photoswitching from the *trans* to *cis* isomer (Fig. 3b). Applications of this chemistry include the introduction of an azobenzene-derived photo-cross-linker into the DNA-recognition helix of the muscle-specific transcriptional activator MyoD, which belongs to a family of transcription factors that rely on a basic helix–turn–helix motif for DNA binding. The DNA-binding activity of the resultant photoMyOD derivative was able to be controlled by light pulses.[27] Similar reversible photocontrol of DNA binding was achieved by incorporation of an azobenzene cross-linker into the leucine zipper region of the well-characterised bZIP DNA-binding domain of the yeast transcriptional activator GCN4.[30] The increased helical conformation favoured dimerisation and hence DNA binding.

While this article has focused on applications to α-helical peptides, it should be noted that azobenzenes have also been used as conformational switches to regulate other structural units including β-hairpins and small peptides with relevance to understanding protein folding.[34]

5. Hydrocarbon-stapled peptides

As highlighted above, while disulfide and lactam bridges are effective in stabilising α-helices, such mimetics are not always stable in cells and are generally susceptible to degradation. To address this limitation, all hydrocarbon cross-links, introduced *via* a ring-closing metathesis reaction using Grubbs chemistry,[35,36] have been investigated to deliver metabolically stable α-helical peptides (Fig. 4). Shafmeister *et al.* carried out a systematic investigation of the stereochemistry of unnatural amino acids bearing alkyl tethers of various lengths at the α-carbon.[37] Given the helix-stabilising effects of α,α-disubstituted amino acids,[38] α-methyl groups were also incorporated into the design of the Fmoc-protected unnatural amino acids. Incorporation of the unnatural (*R*)-amino acid **6** (Fig. 4a) at position i, and the (*S*)-amino acid **7** at $i + 7$, resulted in an 11-membered cycle, which gave the most pronounced helix-stabilising effects. Comparative cleavage experiments performed with the acyclic and cyclic peptides using trypsin confirmed the enhanced stability of the hydrocarbon-stabilised helix. While incorporation of the amino acids at positions i, $i + 7$ without cross-linking decreased the cleavage rate of the unmodified control peptide by 5-fold, metathesis and subsequent hydrogenation produced further stabilisation.[37]

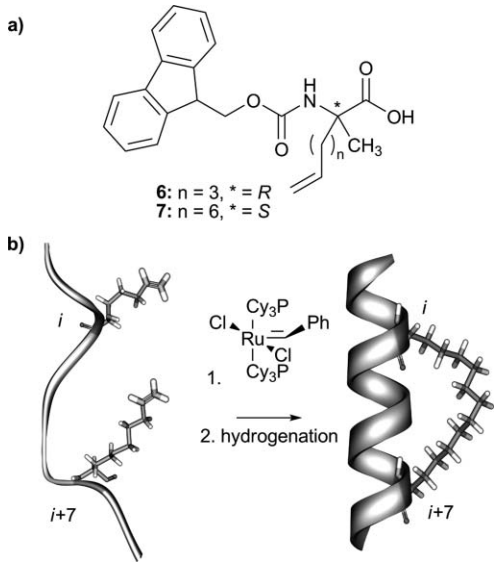


Fig. 4 (a) The α-methyl Fmoc-protected unnatural amino acids **6** and **7** required for the introduction of hydrogen staples and (b) sketch of the introduction of a staple into peptide Ac-EWAEXAAAKFLYAHA-NH_2 [X = **6**, Y = **7**] *via* a ring-closing metathesis reaction with Grubbs' catalyst followed by hydrogenation.[40]

Applications of this hydrocarbon stapling methodology to the formation of stabilised α-helical peptides derived from the human B-cell lympoma-2 (Bcl-2) family of proteins and the therapeutic use of these derivatives for modulating cell death has been patented.[39] Introduction of hydrocarbon tethers in 23-residue helical mimics of the minimal death domain BH3 of the pro-apoptotic sub-family of proteins increased the helicity of the peptides significantly and enhanced stability and both *in vitro* and *in vivo* activity.[40] A very recent report has used the same approach to deliver a stabilised α-helix based on the 15-residue α-helical transactivation domain of the transcription factor protein p53, that mediates the p53–hDM2 protein interaction. The hydrocarbon-stapled peptide exhibited a high affinity for hDM2 and readily entered cells through an active uptake mechanism.[41]

6. Metallopeptides and proteins

Metal ions play an important role in stabilising α-helices in Nature. For example, the well-characterised zinc-finger proteins are assembled by tetrahedral coordination of imidazole nitrogens in His residues and/or thiol coordination to Cys residues to form highly structured DNA binding motifs incorporating α-helices.[42]

Early studies on simple peptides demonstrated that transition metal ions are able to stabilise helical peptides *via* coordination to His and/or Cys residues located on one face of the helix.[43,44] The use of kinetically inert metals including ruthenium(II) delivered stable metallopeptides, thus addressing the lability of transition metal ions such as zinc(II) and cadmium(II). However, most examples were restricted to the stabilisation of only a few residues within a polypeptide with the coordinating His and Cys residues positioned at residues i, $i + 4$.

Fairlie and co-workers have demonstrated that the electrophilic $Pd(en)^{2+}$ metal clip can induce the folding of short unstructured peptides in water (Fig. 5).[45–47] Thus, $Pd(en)^{2+}$-induced helicity in 5-, 10- and 15-residue non-helical peptides corresponding to the Zn(II)-binding α-helix of the active site of thermolysin.[45] The resultant Pd-macrocycles formed helical structures in solution that were analogous to those in the thermolysin crystal structure. However, studies were restricted to DMF, as much less helicity (<40%) was observed in water. Further studies in water with unstructured octapeptides containing several possible metal chelation sites permitted the detection and characterisation of kinetic and thermodynamic metallomacrocycles. This study demonstrated that both solvent and metal-coordination sites can regulate the rate and extent of α-helix folding, and suggests the possible transient involvement of transition metal ions in the generation of kinetically labile turn structures during protein folding.[46] The use of $Pd(en)^{2+}$ metal clips to induce full helicity in free peptides that are essentially random coils is noteworthy,

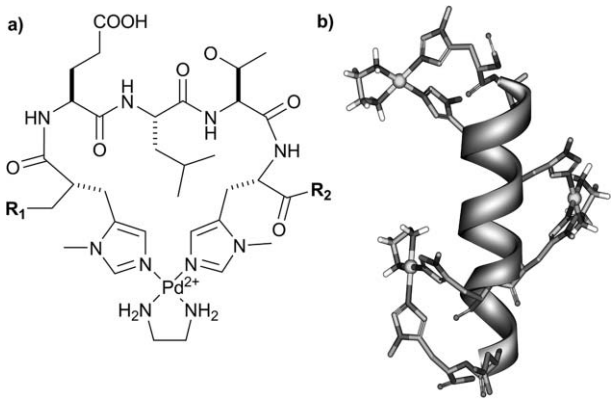


Fig. 5 (a) General structure of metallopentapeptides that mimic the α-helix in the active site of thermolysin.[45] (b) Sketch of the induced helicity in a 15-residue non-helical peptide by the use of three $Pd(en)^{2+}$ metal clips.[47] Pd indicated by ball-and-sticks. Sidechain hydrogens of protein omitted for clarity.

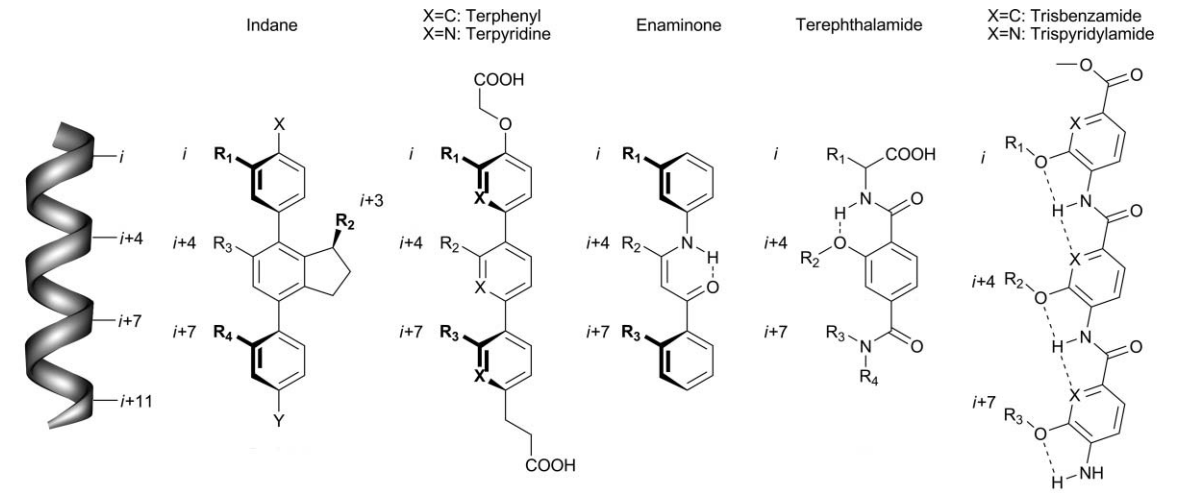
as in almost all other studies with metal ions the peptides studied have contained a significant population of molecules in an α-helical conformation.

Metals have also been used to direct the assembly of well-defined peptide structures including α-helices. For example, Cd(II) binding was used to mediate the assembly of a two-helix-bundle from a random coil peptide by incorporation of a metal binding Cys-X-X-Cys motif.[48] The *de novo* design of metallopeptides has also provided insight into the role of metal ions in directing folding of partially folded and random coil peptides into helices.[49,50] By appropriate positioning of Cys residues, site selectivity in biomolecules can be encoded into short α-helical sequences, thus removing the requirement for extensive protein scaffolds to stabilise the peptides.[50]

7. Synthetic backbone scaffolds

The first entirely non-peptidic scaffold that could be synthesised in a modular fashion and project sidechain functionality with similar distances and angular relationships to those found in α-helices was reported by Hamilton and co-workers in 2001.[51] In a series of papers, successive modifications to the initial terphenyl scaffold design were made to improve synthetic accessibility, solubility and flexibility (Fig. 6).[52–59] The overall features of these scaffolds and their applications to disrupt protein–protein interactions have been reviewed recently,[3] and hence detailed analysis of these mimetics will not be presented in this article. Fig. 6 summarises the key structural features of the different classes of scaffolds that have been reported, and their applications as inhibitors of protein–protein interactions.

Shortly after the report of the terphenyl scaffold,[51] Jacoby used molecular modelling to identify potential organic scaffolds exhibiting helical character through a chiral axis.[60] Biphenyl **8**, allene **9**, alkylidene cycloalkane **10** and spiranes **11** that included *i*, *i* + 1, *i* + 3 and *i* + 4 sidechain positions were modelled and superimposed upon the highly helical poly-Ala, to examine the



Applications (mimetic / target)	N/A	smMLCK / calmodulin (CAM) p53 / MDM2 Bak BH3 / Bcl-x_L or Bcl-2	N/A	Bak BH3 / Bcl-x_L or Bcl-2	Bak BH3 / Bcl-x_L or Bcl-2
Important Structural Features	'Twisted' Backbone				Planar Backbone
	Potential Atropisomers		Backbone Stabilised by Intramolecular H-bonds		
	Mimics 2 Faces of α-helix		Mimics one Face of an α-helix		
Extendable Scaffold	Y	Y	Y	N	?
Modular Synthesis	Y	Y	N	N	Y
Reference	55	47,49,50,52,53	54	51	48

Fig. 6 Major classes of non-peptidic scaffolds that mimic the backbone of α-helical peptides, showing general structural features and applications.

viability of the scaffolds to deliver mimics of segments of α-helical peptides (Fig. 7). The study identified 2,6,3′,5′-tetrasubstituted biphenyls as the most promising candidates for superimposing the *i*, *i* + 1, *i* + 3 and *i* + 4 sidechains in an α-helix. Since this report in 2002, there have been no applications of these scaffolds in the design of mimetics, presumably due to synthetic issues.

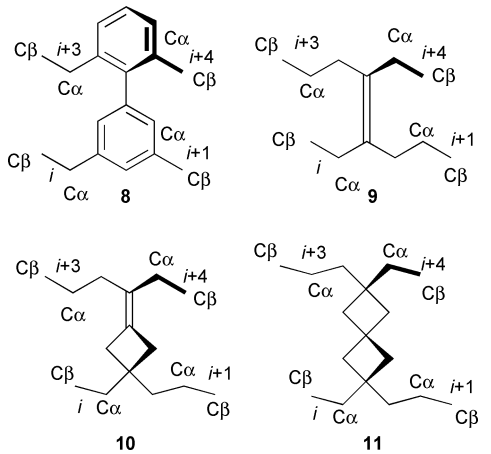


Fig. 7 Hydrocarbon scaffolds showing the relative position of sidechains that mimic the position and orientation of the amino acid sidechains at positions *i*, *i* + 1, *i* + 2, *i* + 3 and *i* + 4 in α-helical peptides.[60]

Two classes of mimetics in which the α-peptide backbone has been modified by replacement of the *N*-terminal *i* and *i* + 4 hydrogen bond with a covalent link have been reported (Fig. 8). Based on the work of Cabezas and Satterwait,[61] who prepared hydrazone-linked peptides **12**, Wang *et al.* have recently introduced a carbon–carbon bond *via* a ring-closing metathesis reaction to give peptides of general structure **13**.[62,63] Incorporation of the carbon–carbon link afforded well-defined short α-helices from sequences that do not spontaneously form helices with minimal perturbations to their molecular recognition surfaces. An attractive feature of this strategy is that all of the amino acid sidechain functionality is retained and there are no significant steric features introduced by the chemistry that may perturb the recognition features of the helix.

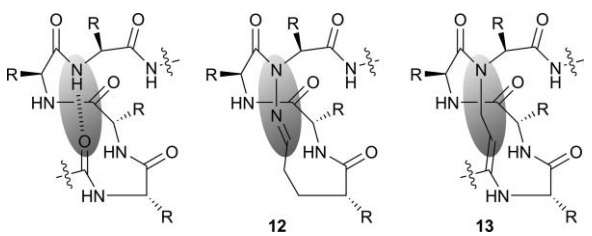


Fig. 8 Partial modification of the peptide backbone by replacement of the hydrogen bond that stabilises the helical backbone with hydrazone (**12**)[61] and carbon–carbon links (**13**).[62]

An elegant but synthetically demanding strategy (18 steps) created α-helix mimetics through *trans*-fused tetracyclic ethers related to marine toxins. The tetracyclic ether scaffold contains two equatorial hydroxyl groups separated by a distance of 4.8 Å, which were functionalised with guanidinium groups, affording a receptor for sequence-selective binding of *i*, *i* + 4 spaced aspartate pairs on the surface of α-helical peptides in aqueous media (structure not shown).[64] While the lengthy synthesis of the scaffold is a limitation of this approach, the authors reported that the development of cell-permeable molecules that bind to a larger area and disrupt protein–protein interactions are planned.

8. Foldamers

The term "foldamers" refers to oligomers that adopt well-defined structures and conformations. A range of building blocks have been used to generate foldamers including α- and β-peptides, alternating α/β-peptides and γ-peptides.[65–67]

Several examples of the *de novo* design of non-natural oligomers that form helical structures have illustrated the potential of foldamer research to deliver functional inhibitors of protein–protein interactions mediated by α-helices. Foldamer scaffolds incorporating α- and β-peptides have been recently designed and synthesised as effective inhibitors of the anti-apoptotic proteins BaK and Bad which are overexpressed in malignant cells and prevent apoptosis.[68] Synthetic peptides comprising β-amino acids are known to favour 12- and 14-helix conformations which are structurally similar to the α-helix,[69] and hence the incorporation of β-amino acids into the design of α-helical mimetics has been investigated. A number of different foldamers that mimic the 16-residue α-helical BH3 domain of BaK and position the critical sidechains in the helix were designed using both α- and β-amino acids. While the binding of the fully β-peptide homologue to Bcl-x_L was poor, a foldamer scaffold containing alternating α- and β-amino acids with a terminal α-peptide segment showed a strong affinity for the α-helix recognising surface of Bcl-x_L.[68] This study suggests that the general strategy of the combination of different foldamer scaffolds has significant potential to deliver small-molecule mimetics.

Non-peptidic helical scaffolds have also been developed that can adopt well-defined helical structures. For example, Stadler *et al.* have reported helical supramolecular systems that can undergo dynamic structural changes.[70] The controlled folding of molecular strands into helical forms has provided valuable insight into how the control of molecular interactions in organic frameworks can deliver helical structures whose conformations can be controlled in a predictable manner. While the first-generation scaffolds incorporated a pyridine–pyrimidine motif, the degree of helicity was significantly enhanced by replacement of pyridine with hydrazone. These motifs are similar to that employed for the arylcarboxamide/enaminone strategies shown in Fig. 6, but utilising much larger chain lengths. Oligoarylamides with backbones that are rigidified by hydrogen bonds are also able to adopt well-defined helical structures with the potential to form folding nanotubes.[71]

9. Miniature proteins

An alternate approach to stabilising small α-helices involves the use of proteins to stabilise the conformation of small helices in the same way that Nature does, by grafting a protein onto the face of the α-helix that is not involved in recognition. This concept of using stable folds in small proteins to display functional epitopes has been applied to a number of systems.[72,73] Protein

grafting, shown schematically in Fig. 9, involves identification of the critical α-helical residues in a protein and substituting them onto a protein scaffold.[74] The small stable protein avian pancreatic polypeptide (aPP) has been most commonly used as a protein scaffold to generate different miniature proteins containing the α-helical binding epitopes for a number of targets.[74–77] This protein grafting strategy has resulted in highly potent and specific ligands for human Bcl-2 and Bcl-x_L. Another example described a miniature protein that presents the cAMP-dependent protein kinase (PKA) recognition epitope found within the heat-stable protein kinase inhibitor protein (PKI).[76]

The 35-residue pancreatic polypeptide, peptide YY,[78] and the neurotoxic peptide apamin[79] have also been used as scaffolds to design miniature proteins with catalytic properties. The scaffold was designed to contain reactive Lys residues provided by bovine pancreatic polypeptide and delivered a folded conformationally stable oxaloacetate decarboxylase.

10. Type I α-helical antifreeze proteins

Our group has made major contributions in understanding the mechanism of action of fish antifreeze proteins (AFPs) and glycoproteins (AFGPs).[80–89] The type I AFPs are highly helical Ala-rich peptides comprising three 11-residue $ThrX_2AsxX_7$ repeat units (Fig. 10b) where X is usually Ala or an α-helix-inducing residue.[80] These remarkable molecules are kinetic ice growth inhibitors that have evolved in the blood plasma of Arctic and Antarctic fish allowing them to survive in sub-zero ice-laden waters at temperatures below the freezing temperature of blood plasma.[80,90,91] These properties have attracted significant interest, as there are numerous applications in areas including the frozen food industry, organ and tissue storage and biotechnology, where ice crystal growth is damaging.[92]

The most widely studied type I AFP is TTTT (Fig. 10) found in the winter flounder. After considerable debate, molecular simulations, and structure–activity studies, current evidence is consistent with ice growth inhibition occurring through accumulation of the protein at specific ice–water interfaces through the interaction of the hydrophobic face (Fig. 10a) of the protein.[80,93,94] While it was assumed that the Thr residues were involved in hydrogen bonding in the mechanism of action, the hydrophobic γ-methyl group of the Thr residues provides an important hydrophobic interaction, as replacement of Thr with Val resulted in a derivative with antifreeze activity.[81] However, while the four Thr residues, which are spaced equally along one face of the helix (Fig. 10b), are important residues for activity, surrounding residues on the same surface of the helix are also important and contribute to the overall hydrophobic surface. The length of the polypeptide is important, with at least 25 residues required for antifreeze activity.

The production of type I AFPs has been by solid-phase peptide synthesis[80] or molecular biology techniques.[84,95] The high hydrophobicity of the AFPs presents challenges for synthesis and purification and for this reason, in our structure–activity studies, additional salt bridges were incorporated into the structures on the hydrophilic face of TTTT (Fig. 10a).[81] While the introduction of these charged residues do not affect antifreeze activity, our more recent studies with model membranes suggest that the hydrophilic face of the helix is involved in the protection of cell membranes from damage.[85] Despite the strong interest in the potential applications of AF(G)Ps in medicine and industry, there are no compounds in commercial use.

In light of the advances in the design of stabilised α-helices and mimetics, TTTT presents an ideal target for the design and

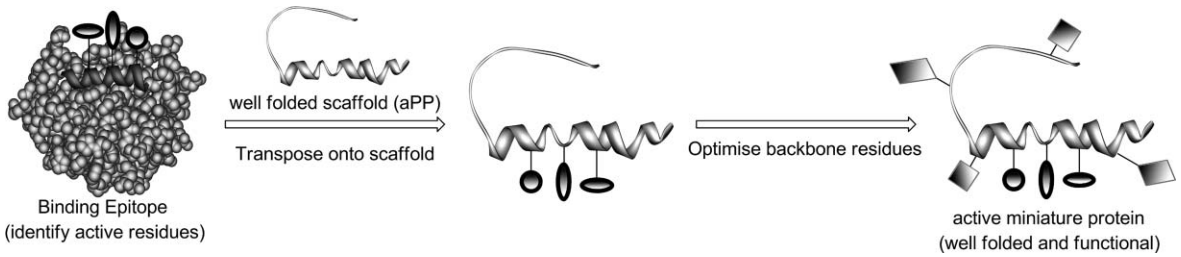


Fig. 9 Schematic of protein grafting, illustrating the mapping of critical α-helical residues (curved shapes) onto a helical scaffold (aPP) to produce an active miniature protein after optimisation (block shapes).

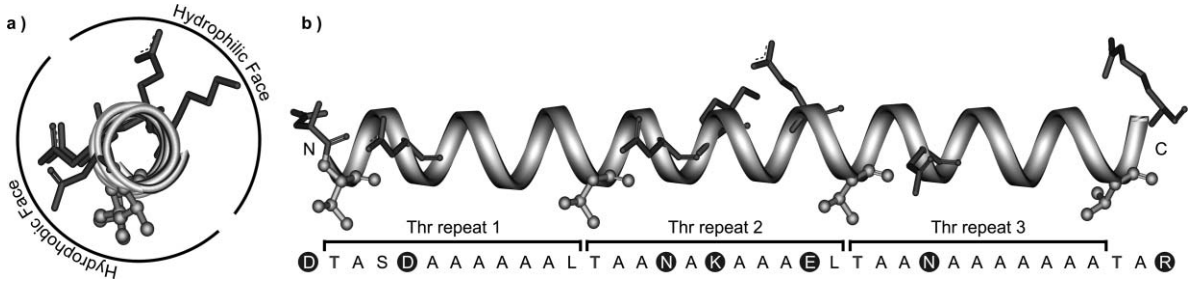


Fig. 10 Two views of the crystal structure of type I antifreeze protein TTTT (generated from PDB coordinates 1wfa) (a) looking down the helix axis (C to N) to show the hydrophobic and hydrophilic face and (b) primary sequence and ribbon structure of TTTT highlighting the four equally spaced Thr residues that are positioned on one face of the helix; Thr indicated by ball-and-sticks, polar residues indicated by sticks. Sidechain hydrogens omitted for clarity.

synthesis of mimetics that are able to be routinely produced in a cost-effective manner for possible commercial applications. The effect of the introduction of an *i*, *i* + 4 lactam or an *i*, *i* + 7 disulfide bridge on a peptide containing only two Thr repeat units (TTT) did not produce active derivatives.[80,96] However, a 15-residue peptide that contained a single Thr repeat plus the *N*- and *C*-capping residues present in TTTT, constrained by an *i*, *i* + 4 lactam bridge, was able to stabilise a pyramidal plane on the surface of growing crystals,[97] suggesting that the design of small structured polypeptides with antifreeze activity may be possible.

While the hydrophobic surface of TTTT contains Thr, Ala and Asx residues, two studies suggest that simple peptides containing only Ala residues along with solubilising charged residues (Asp/Glu and/or Lys) can have antifreeze activity. A random uncharacterised copolymer (~65% Ala, ~35% Asp) gave antifreeze activity approximately one third less than TTTT.[98] Two Ala-rich peptides with regularly spaced Lys residues positioned on the hydrophilic face of helix showed antifreeze activity, but this activity was weaker than that observed with TTTT.[99]

The design of mimetics of TTTT requires a helical scaffold with a hydrophobic face and a hydrophilic face. While the truncated peptides studied to date have not been active, in all cases the shorter peptides have been significantly less helical than TTTT, despite the presence of backbone constraints.[96,97] The design and synthesis of mimetics containing 11–25 residues (*i.e.*, only one or two Thr repeat units plus capping units) that are locked into a fully helical conformation and incorporate hydrophobic sidechains that mirror the recognition face of TTTT has not been reported. The use of non-peptidic scaffolds, and in particular the partially modified scaffolds such as **13**, are attractive platforms for further study. Similarly, the incorporation of multiple helical constraints (lactams, hydrocarbon staples) into truncated peptides that incorporate the general recognition features required for activity would allow the importance of helicity in the design of new antifreezes to be addressed. Research towards these goals is currently underway in our laboratory.

11. Summary

The design and synthesis of α-helical peptides has advanced significantly in the last decade, with the smallest α-helix in water reported only two years ago.[24] Synthetic and bioorganic chemistry have played a major role in delivering mimetics of α-helices that are stable under biological conditions and address the limitations of small peptides that are susceptible to degradation and have poor proteolytic stability. The first non-peptide backbone, reported in 2001,[51] has demonstrated that careful design of organic frameworks that mimic the natural scaffolds present in proteins is a powerful approach to generate highly potent inhibitors of protein–protein interactions.

In comparing the different approaches to helical mimetics, it should be noted that the modifications to sidechains and steric effects introduced through sidechain modifications effectively modifies one face of the helix. In addition, the use of unnatural amino acids may require the replacement of key recognition sidechains in the design of the mimetic. The synthetic scaffolds shown in Fig. 8 are noteworthy in that only the peptide backbone is modified and all sidechains can be positioned on the scaffold in an identical way to the natural peptide. In addition, the peptide dipole is retained.

Comparison of the effectiveness of the different approaches to α-helical mimetics outlined in this article is not straightforward. However, some comparative remarks can be made regarding the relative effectiveness of inhibitors of the protein–protein interaction between Bak BH3/Bid BH3 with Bcl-x_L/Bcl-2 as inhibitors of these protein–protein interactions using synthetic backbone scaffolds, protein grafting, foldamer and hydrocarbon stapling, have been reported. Under the same assay conditions, the foldamer strategy[68] has produced the most effective inhibitors against Bcl-x_L with the synthetic backbone scaffolds delivering inhibitors with micromolar activities.[52,53,55] The effect of modifying the terphenyl scaffold to a trispyridylamide scaffold, which was more amenable to synthesis, resulted in a 10-fold loss of activity. The protein grafting[74] and hydrocarbon stapling[40] strategies produced mimetics with similar inhibitory activities for binding to Bcl-2.

Finally, while much research is still required to solve the holy grail of protein folding, recent research suggests that the study of model metallopeptides[46] may provide some insight into the possible transient involvement of metals in protein folding.

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