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Peptidomimetics for Bridging Structure and Function: Pseudo-Prolines (ΨPro) in Peptide Synthesis, Molecular Recognition, and Drug Design

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Abstract: The central issue of bioorganic chemistry is to unravel the structural and functional complexity of living systems by designing synthetic models that mimic essential features of biomolecules. In view of the expected exponential growth of knowledge within the next decade about structure–activity relationships in bioactive compounds as well as about mechanisms of molecular recognition in cellular communication, conversion of the design of therapeutically relevant molecules currently provides one of the most fascinating challenges for synthetic organic chemistry. Independent of evolutionary restrictions in creating the molecules of life, the chemist may even go a step further in extending Nature's pool of biomolecules for studying biochemical processes. One way of doing this is illustrated in the present article. Taking proline (Pro) as a unique building block in peptides and proteins, we have explored its particular role in a variety of biological processes by tuning its intrinsic structural and functional properties using readily accessible proline mimetics ('pseudo-prolines', ΨPro). In enhancing and extending well-known Pro effects, *i.e. cis-trans* amide bond isomerization, conformational restriction or specific receptor interaction, ΨPro derivatives are useful as synthetic tools in molecular recognition studies and for modulating the physicochemical, pharmacokinetic and biological properties of peptide and protein ligands. Selected examples from our ongoing research program in peptidomimetic chemistry demonstrate that synthetic tools can substantially contribute to our understanding of fundamental principles underlying biological processes and serve as a first step in accessing molecules of therapeutic relevance.

Keywords: *Cis-trans* isomerization · Drug design · Molecular recognition · peptidomimetics · Pseudo-prolines (ΨPro)

Introduction

A fundamental principle in bioorganic chemistry is the application of synthetic organic tools to study complex biochemical and biological processes. The creation and redesign of biomolecules exhibiting novel properties has consequently found wide attention [1]. While Nature has optimized structure-function relationships during evolution, the chemist is not bound to follow the rules imposed by evolutionary principles, *e.g.* the genetic code and protein folding pathways for creating biomolecules resembling and interacting with natural systems. Taking this challenge as a guideline for protein design and mimicry, we have focused our research over the past decade on the understanding of protein secondary and tertiary structure formation applying the tools of synthetic organic chemistry. In creating protein-like macromolecules exhibiting nonnative architectures termed Template Assembled Synthetic Proteins (TASP), we aimed to bypass the most critical hurdle in today's protein *de novo* design, *i.e.* the well-known protein folding problem of linear polypeptide chains [2]. The state-of-the-art of this increasingly accepted concept has been subject of a recent review in this journal [3]. Here, we focus on our ongoing research in peptidomimetic chemistry, with emphasis on the pseudo-proline concept aiming to decipher the unique role of proline (Pro) in peptide structure formation, molecular recognition and in the design of therapeutically relevant molecules. The rapidly growing number of publications over the last few years on the multiple roles of proline demonstrates that Nature strongly relies on this exceptional amino acid for directing molecular recognition and function [4–11]. Given this dominant role of proline, we centered our interest on the question: Can we go beyond evolution in tailoring or enhancing the intrinsic structural properties of Pro by the redesign of proline-like building blocks and thus tackle fundamental questions in biological processes, molecular recognition and drug design? We will show in this article that readily accessible proline mimetics of high structural,

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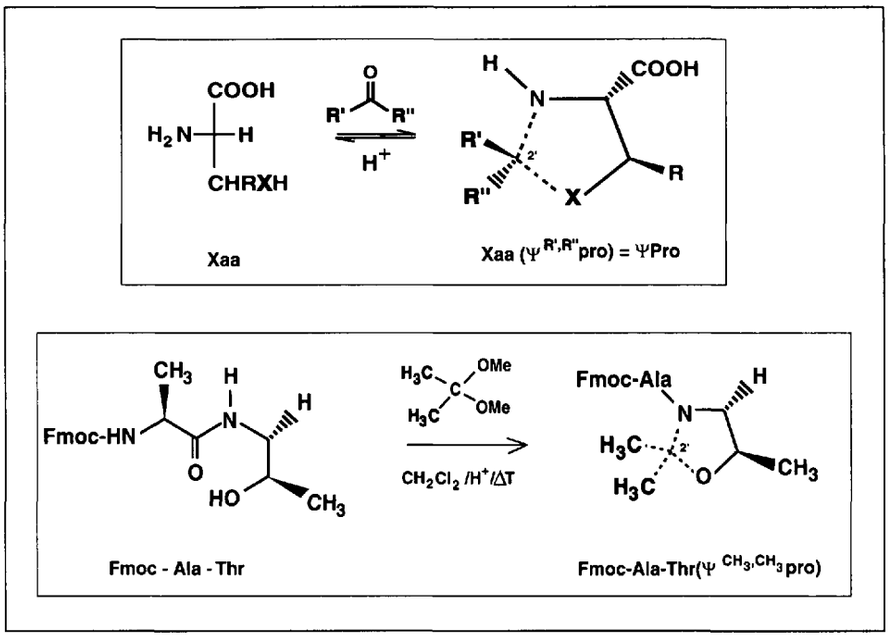
chemical and functional versatility, termed pseudo-prolines (ΨPro), prove to be attractive candidates for answering this question (Scheme 1). Before we discuss the synthesis and physico-chemical properties of some prototype ΨPro and their potential in bioorganic chemistry, we first have to gain an appreciation of the special role of natural prolines in a variety of biological processes.

What is Special about Proline?

Nature evolved twenty amino acids for creating peptides and proteins of enormous structural and functional complexity to sustain life. Out of this pool, Pro is unique in exhibiting a cyclic side chain constraining the N-C^{α} bond (Φ) to a fixed value. Due to this exceptional structural feature, the imidic Xaa_{i-1}-Pro_i peptide bond readily undergoes *cis-trans* isomerization within a peptide backbone. Unlike regular amino acids, which show a strong preference for *trans* amide bonds, the *cis* and *trans* states of Xaa-Pro units are of similar energies, resulting in a mixture of two isomers in the absence of conformational constraints as imposed, for example, by the framework of proteins of defined three-dimensional structure.

Due to these unique conformational properties, Pro residues do not occur in regular α-helices or β-sheet structures. Rather, they play a specific structural role as N-terminal caps to α-helices [4], as helix termination signals [5] or as corner residues in β-turn sequences [6]. Most notably, however, prolines are key players in many biologically essential processes, such as protein folding, signal transduction, protein-protein recognition, or receptor-ligand binding of Pro-containing peptides. Consequently, elucidation of the molecular origin in which *cis-trans* isomerization is vital for diverse biological processes has recently become an attractive topic of research, documented by an exponential increase of publications dedicated to this subject (for recent reviews see [14][15]).

Trans to *cis* isomerizations as observed in Pro-containing peptides and proteins result in dramatic changes in the overall shape of the polypeptide backbone due to the induction of chain reversals (see later Fig. 2a and Scheme 3). Chemical fixation of a Xaa_{i-1}-Pro_i imide bond in a given target peptide/protein can help unravel the complex and underlying structure function relationships in these systems.



Scheme 1. Pseudoprolines Xaa($\Psi^{R',R"}$pro) = ΨPro are obtained by condensation reaction of aldehydes and ketones with Xaa = Ser or Thr, (X = O; R = H or CH_3) and Cys (X = S; R = H) [12][13]. Upon treatment with acid the ΨPro ring is cleaved and the parent amino acid restored (upper panel). Example for the direct insertion ('post-insertion') of ΨPro systems into dipeptide derivatives as used in peptide synthesis (lower panel).

From Proline to Pseudo-Proline (ΨPro)

To date, chemists have proposed a number of Pro analogues for constraining an amide bond in a *cis* conformation [16–18]. For example, bulky substituents at C(5) of the cyclic Pro system result in *cis*-amide bond formation; however, the need for chemical synthesis of these Pro-surrogates and their incorporation into peptide or protein backbones hampers this strategy from becoming a routinely applied tool in biomimetic chemistry. During our ongoing work on peptidomimetics for tuning secondary structure formation, we have noticed that the temporary transformation of serine (Ser), threonine (Thr) and cysteine (Cys) residues by established procedures [19][20] results in proline-like compounds (Pseudo-Prolines, ΨPro (Scheme 1) [12][13]. The physical, chemical and conformational properties of the resulting oxazolidine- or thiazolidine systems prove to be strongly dependent upon the character of the C(2') substituents of the cyclic ring. For example, as shown by a chymotrypsin coupled assay and NMR-studies using ΨPro containing model peptides, the *cis*-content of Xaa_{i-1}-Pro_i amide bonds can be tailored by varying the aldehyde or ketone component (*i.e.* R^1, R^2) in the cyclization reaction (Table 1) [21][22]. As the use of bulky substituents at C(2') (*e.g.* dialkyl groups) induces up to 100% *cis* conformation, this technique may serve to target bioactive conformations in Pro-containing peptide ligands (see below). Similarly, the chemical stability of ΨPro systems strongly depends on the electronic effects of the C(2') substituents, ranging from highly acid labile (*e.g.* electron donating substituents in oxazolidines) to completely acid stable (*e.g.* unsubstituted thiazolidines) derivatives (Fig. 1).

The facile synthetic accessibility of ΨPro building blocks exhibiting differential chemical stabilities and *cis/trans* ratios allows us to mimic and even to enhance the unique properties of Pro in natural peptides and proteins, thus opening a broad palette of applications in bioorganic chemistry.

Ψ-Prolines to Prevent Aggregation in Peptide and Protein Synthesis

Due to enormous progress in the methodologies of synthesis, purification and characterization, the chemical synthesis of peptides of nearly any sequence or chain length up to small proteins is within reach and represents a major facet in structural and functional biology today [23][24]. However, the routine synthesis of large peptides or proteins is often limited by the poor solvation during solid-phase peptide synthesis (resulting in decreased coupling yields) or the limited solubility of fully protected peptide frag-

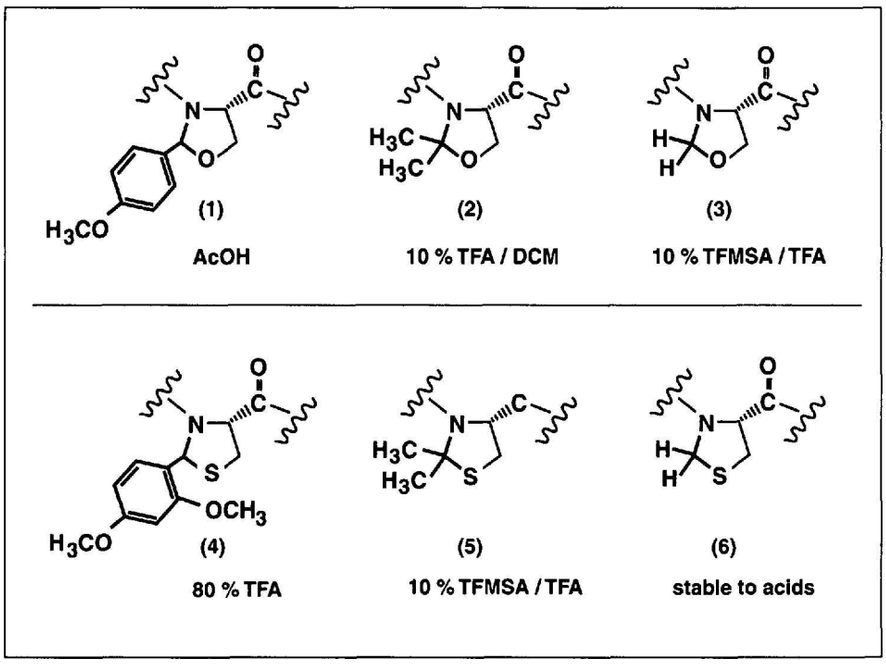


Fig. 1. Differential stability of various ΨPro systems towards acids [20]. Depending on the substituents at C(2'), harsher conditions are needed to cleave the ring. In general, thiazolidines (**4–6**) are more stable than oxazolidines (**1–3**).

Table 1. *Cis* content of peptides Ac-Ala-Xaa[$\Psi^{R1,R2}$pro]-NHMe (see Scheme 1) as determined by NMR in DMSO [21].

R_1	R_2	Xaa	X	cis [%][a]
Me	Me	Ser	O	~ 100
Me	Me	Thr	O	~ 100
Me	Me	Cys	S	~ 100
H	H	Ser	O	~ 33
Biphenyl	H	Ser	O	~ 63
–	–	Pro	CH_2	~ 18

ments in convergent strategies. Even recently introduced chemoselective ligation methods for the assembly of small proteins from completely side chain unprotected peptide fragments in aqueous solution suffer from the tendency of secondary structure forming peptide segments to self-associate. The elucidation of the relationship between the preferred conformation of a growing peptide chain and its physicochemical properties reveals that β-sheet or helix-bundle formation is often paralleled by a significant decrease in solvation and reaction kinetics [25–27]. Hence, much attention has been devoted in recent years to this common problem in the various strategies of peptide synthesis [23][24][28]. Due to the differential acid lability (Fig. 1), ΨPro systems are versatile protecting groups for Ser, Thr, or Cys in chemical peptide synthesis and are preferentially introduced as N-protected $\Psi^{Me,Me}$Pro-dipeptide derivatives (Fmoc-Xaa_{i-1}-$\Psi^{Me,Me}Pro_i$, see Scheme 1) in standard Fmoc-based peptide synthesis [29]. As indicated above, the induction of a *cis*-imide bond results in a 'kink'-conformation of the peptide backbone, thus preventing the onset of regular secondary structures (Fig. 2). In particular, the disruption of β-sheet conformations as nuclei for peptide aggregation renders ΨPro a strongly solubilizing protection technique in the synthesis of potentially insoluble peptides. In applying this strategy, the yields and purity of peptides prepared by multistep syntheses have been significantly improved (see Fig. 3) [30]; most notably, previously 'inaccessible' peptide sequences have become accessible to structural and functional investigation [27][31]. In addition, the cyclization tendency of ΨPro containing peptides was considerably enhanced due to the induction of chain reversals (*e.g.* type VI β-turns) in linear peptide precursors [32][33]. In the context of modern techniques for peptide assembly, the Ψ-proline strategy has become a rather efficient tool for bypassing some significant limitations, and thus helps to close the gap between chemical and biological methods in peptide and protein synthesis.

Ψ-Prolines as Versatile Tools for Studying Molecular Recognition Processes

Recognition processes between biomolecules are essential in living systems, and understanding these complex processes at a molecular level is the ultimate goal of work at the interface of chemistry and biology. Within the next decade, in the so-called 'post-genomic era', a vast body of structural data of proteins will emerge, substantiating and expanding our present knowledge about structure-function relationships, *i.e.* about the mechanism of cellular communication. In view of these fascinating perspectives, the question arises: How can we convert this structural information into fundamental principles for the rational design of functional, therapeutically potent molecules? With respect to the enormous variety of novel synthetic building blocks which are available to chemists, Nature uses a surprisingly small set of monomers (α-amino acids, nucleic acids, monosaccharides) for creating the biomolecules of life in their unsurpassed structural

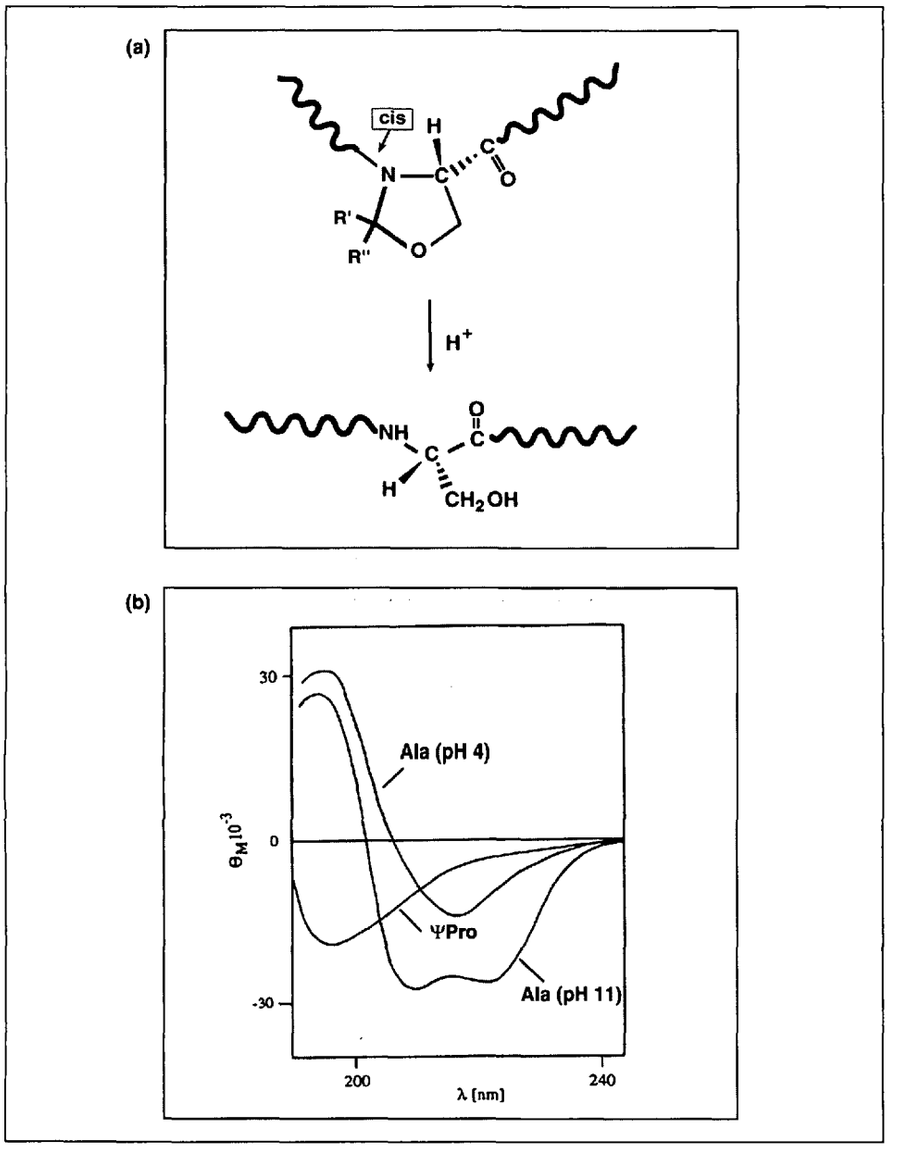


Fig. 2. a) Due to the formation of *cis*-imide bonds, ΨPro induces a 'kink' in the peptide backbone resulting in the disruption of regular secondary structures [21][22]. Ring cleavage by acid restores the parent amino acid and consequently, the *trans* amide bond. b) CD spectra of a 16-peptide that shows pH dependent secondary structure formation [27]. The reference peptide contains Ala at position 10 and shows the characteristic bands of an α-helix at pH 11 and of a β-sheet at pH 4; replacement of Ala^{10} by ΨPro^{10} results in an disordered conformation independent of pH.

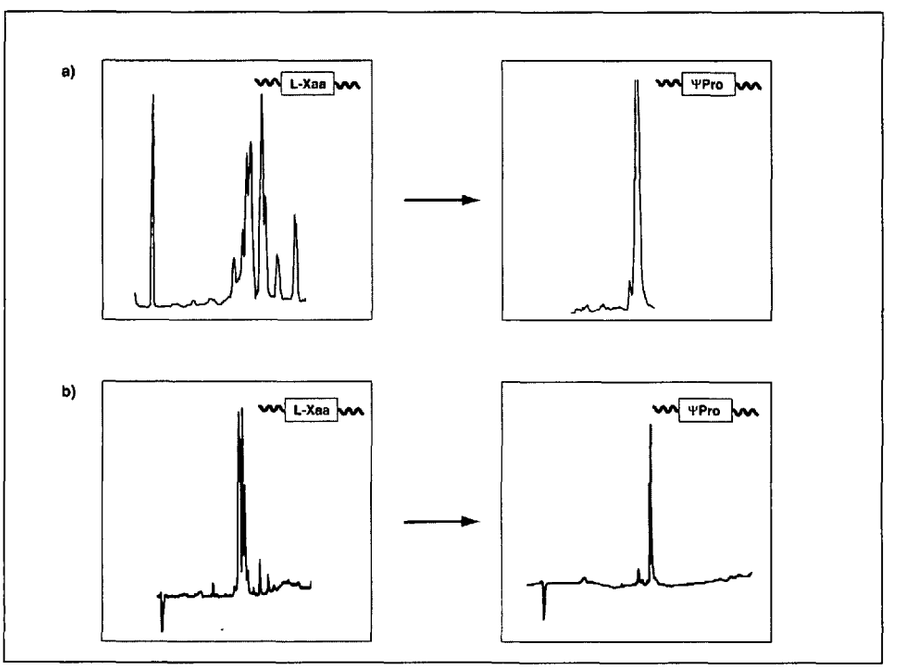


Fig. 3. ΨProlines used as temporary protection for Ser, Thr, and Cys, disrupt secondary structure formation during solid-phase peptide synthesis and significantly improve the synthesis of 'difficult' peptides as a result of a better solvation of the growing peptide chain [27]. a) HPLC profiles of a crude 14-mer model peptide without (upper left) and with (upper right) the use of a Ser-derived ΨPro derivative; b) HPLC profiles of a crude 15-mer model peptide without (lower left) and with (lower right; taken from [30]) the use of a Thr-derived ΨPro derivative.

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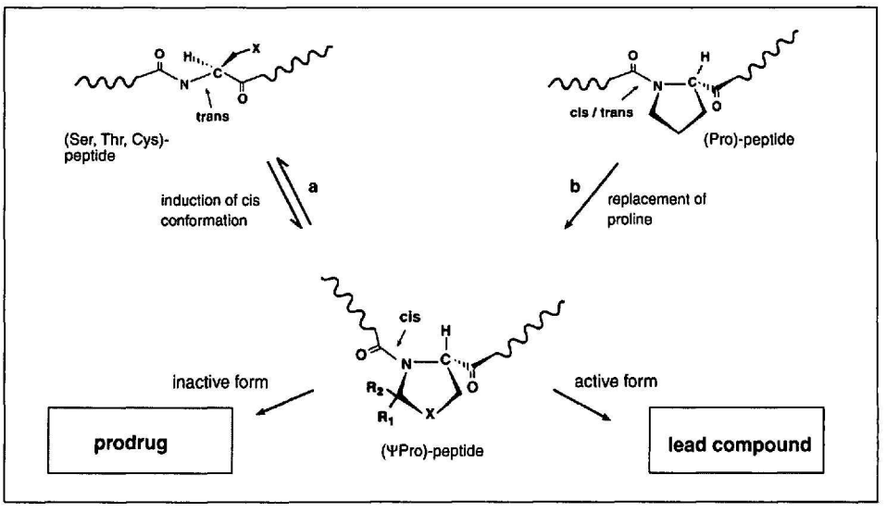
and functional complexity. On the other hand, and even more puzzling, macromolecules exhibiting protein-like properties such as cooperative folding and unfolding have been constructed from scratch with a substantially smaller subset of naturally occurring amino acids (see *e.g.* the 'binary code' principle [34]). Obviously, evolution has left us a large degree of freedom for biomolecular tinkering of alternative molecular worlds, capable of mimicking and interacting with natural systems.

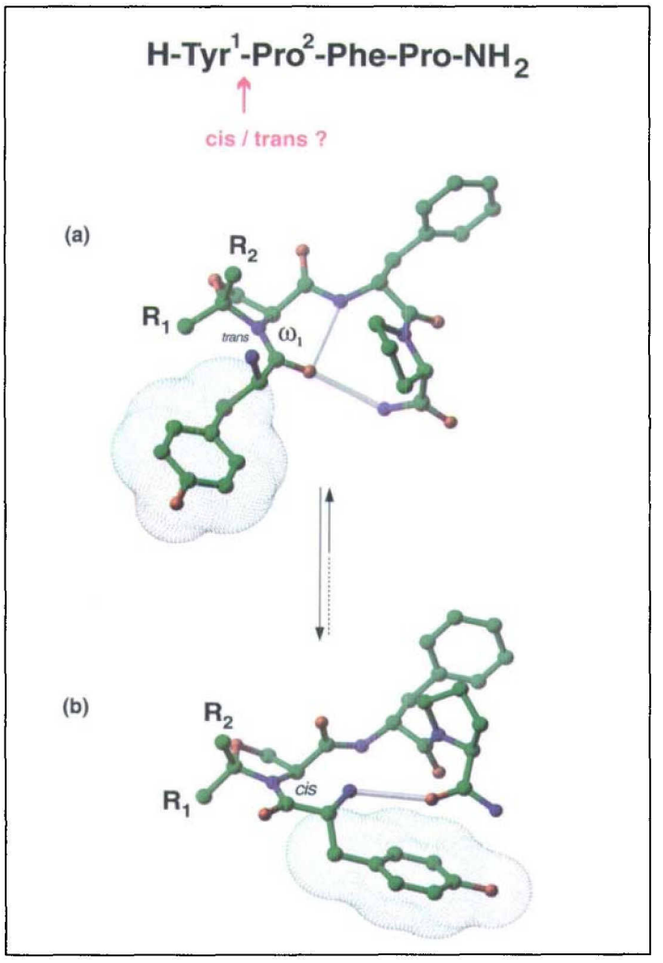
As we have seen above, Nature uses proline for switching the shape of a potentially bioactive molecule – thus modulating its affinity to a given receptor, or alternatively, directing the pathway of protein folding. The clue to this 'chimeric behavior' of Pro lies in the *cis-trans* isomerization of imidic bonds. Taking this lesson from Nature, we aimed to use the pseudo-proline concept to explore the potential of this unique building block in molecular recognition processes.



Scheme 2. The ΨPro concept in molecular recognition and drug design. a) Direct insertion of ΨPro units into biologically active, all-*trans* peptide sequences containing Ser, Thr, or Cys allows modulation of the physico-chemical, biological and pharmacokinetic properties, for example in prodrug design; b) in Pro-containing peptides, the dynamic process of *cis-trans* isomerization can be shifted towards *cis* by replacing Pro by ΨPro, thus targeting bioactive conformations for use in lead finding.

Targeting Bioactive Conformations: A First Step toward Lead Finding

Rational drug design is strongly hampered by the inherently high flexibility of linear peptides in solution. For the elucidation of the receptor-bound, bioactive state, co-crystallization of receptor-ligand complexes or sophisticated NMR techniques represent powerful, but elaborate and often unsuccessful methodologies. Similarly, theoretical predictions together with molecular dynamics simulations are still only of limited practical value. Thus, the introduction of conformational constraints by chemical means, *e.g.* by cyclization, use of peptidomimetics or amino acid substitution, represents an attractive alternative in structure-activity relationship studies. A representative example is depicted in Scheme 2b. By replacing a Pro residue by C(2')-disubstituted ΨPro in a bioactive peptide, the preceding imide bond can no longer switch between *cis* and *trans*. Consequently, if the binding capacity of the ΨPro-analogue to a given receptor is retained, the bioactive conformation of the ligand is concluded to be *cis*, and this information can be directly exploited in further lead optimization. We have successfully applied this strategy for the example of the opioid peptide morphiceptin, where the Tyr^1-Pro^2 imide bond was shown to be *cis* in the receptor-bound state, thus resolving a highly disputed controversy (Scheme 3) [35].

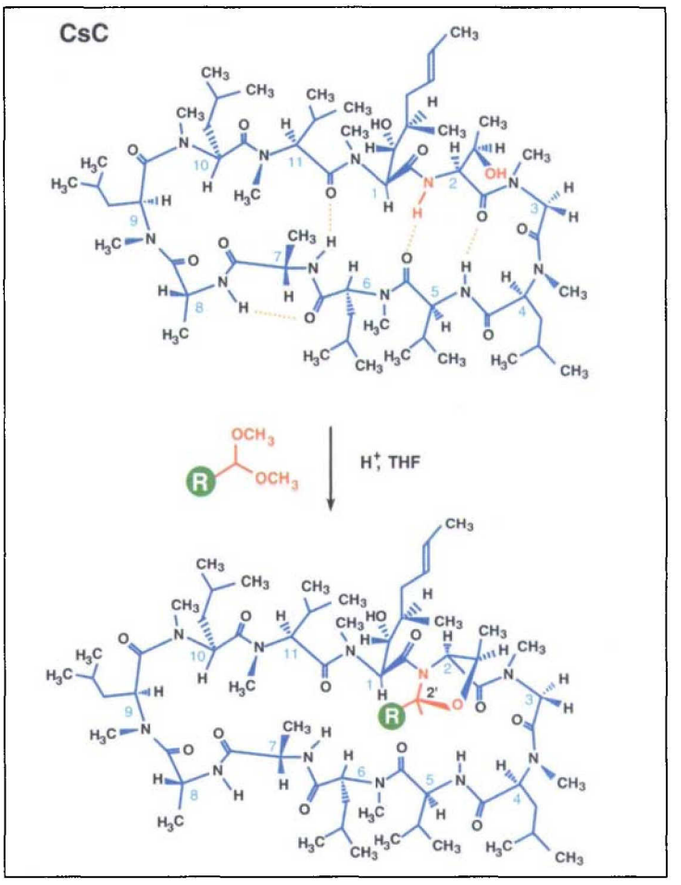


Scheme 3. ΨPro-containing analogues of morphiceptin for probing the *cis* Tyr^1-Pro^2 amide bond hypothesized to be the bioactive conformation [35]. The presence of a *cis*-amide bond as preferred conformation (b) results in a chain reversal of the extended form (a).

The ΨPro Concept in Drug Design: Opportunities and Surprises

Taking for granted that lead finding is just the first step on the adventurous road leading to a therapeutically potent drug, any novel tool – synthetic or biological – for unraveling the complex interplay between structure and function of native molecules may contribute invaluably to the process of drug design. Here, the cyclic undecapeptide cyclosporin (CsA) may serve as paradigm for extensive structure-function studies to modulate its biological and pharmacokinetic properties. Our early interest in this therapeutically important peptide – known primarily for its immunosuppressive activity preventing graft rejection and commercially available under the tradename Sandimmun® and Neoral® – is due to the fact that some members of this natural peptide family contain the amino acids serine and threonine, thus being potential candidates for applying our ΨPro concept for altering its pharmacokinetic profile. Furthermore, as an extension, the chemical introduction of ΨPro at any given position of CsA by multistep syntheses would provide a broad array of analogues and represents an ongoing project in our laboratory. But first the question had to be addressed: Is it possible to directly insert the ΨPro-system into Ser, Thr, or Cys-containing peptides of high structural complexity such as cyclosporine?

Starting from the Thr[2]-containing analog CsC, we succeeded in the one-step insertion of ΨPro moieties featuring a variety of C(2') substituents (Scheme 4). As shown in Table 2, ΨPro monosubstituted derivatives of CsC were obtained in good yields by condensation of a series of dimethylacetals with CsC [36]. Surprisingly, the condensation reaction proceeds with high stereo- and regioselectivity [37–39], possibly originating from specific conformational constraints within the cyclic peptide backbone. In view of the drastic structural changes induced by the ΨPro system in the direct vicinity of the receptor (cyclophiline A, CypA) binding site, we expected a complete loss of binding activity to CypA for the various ΨPro-containing analogues. The unexpected finding that some ΨPro-derivatives of CsC retain their CypA binding capacity can only be rationalized by the induction and stabilization of bioactive conformations. As shown in Scheme 2b, we envision applying the ΨPro concept to prodrug design by temporarily inserting ΨPro-systems into bioactive compounds featuring Ser, Thr or Cys in the active site. Most notably, in tailoring the physicochemical properties of the C(2') substituents R, the bioavailability of the peptide ligand can be modulated over a wide range, giving access to prodrugs with tailored pharmacokinetic properties. For example, CsA analogues containing solubilizing C(2') substituents (*e.g.* PEG, lipids) have been prepared in our laboratory [36]. In applying the ΨPro concept to analogues such as D-Ser[8]-CsA or Thr[4]-CsA [37][40], we are presently exploring further structure-activity relationships for lead finding and prodrug development of this therapeutically important peptide family.



Scheme 4. Direct insertion ('post-insertion') of ΨPro systems into structurally complex molecules like Cyclosporine C [36][37]. Different C(2') substituents (R) modulate the pharmacokinetic and biological profiles of the peptide.

Table 2. ΨPro C(2') monosubstituted derivatives of CsC (see Scheme 4); IC_{50} values indicate inhibition of calf-thymus cyclophilin A. [a] Separated enantiomers at C(2') [36].

R	reaction time [min]	Yield [%]	IC_{50}/IC_{50CsA}
Ph	45	74	6.0
p-Ph-C_6H_4	30	89	5.8
CH_2=CH	60	75	5.3
p-MeO_2C-C_6H_4	120	55	7.8
[a]*p*-MeO-C_6H_4	60	90	52.1
[a]*p*-MeO-C_6H_4	60	90	15.4
p-Alloc-C_6H_4	50	95	4.0
p-HOOC-C_6H_4	50	75	24.1
PEG	240	20	21.5

Antibodies as 'Detectives' for Critical *cis* Bonds in Peptides and Protein

Cis-trans isomerizations are often the on-off switch for biological activity, as demonstrated by the example of opioid peptides (Scheme 3). Alternatively, the presence of a *cis*-amide bond at the surface of a protein may be crucial for initiating a biochemical chain reaction. Targeting or blocking such key events by a monoclonal antibody therefore represents a potential tool in diagnostics and drug design [41].

As a selected example, the V3 loop of the envelope protein gp 120 of immunodeficiency virus type 1 (HIV-1) has been chosen. It corresponds to the major neutralizing epitope of the virus (Fig. 4). A generally conserved tetrapeptide motif GPGR, situated on the tip of the loop forming a type II β-turn is thought to undergo *trans* to *cis* isomerization towards a type VI β-turn with a *cis* proline peptide bond as a prerequisite for viral infection. For probing the infection-active *cis*-prolyl loop tip conformation, ΨPro building blocks have been introduced in V3 loop analogues to constrain the peptide in a *cis* conformation. A cyclic, ΨPro-containing, *cis*- constrained V3 loop mimetic has been used as an immunogen for the preparation of polyclonal and monoclonal antibodies; the results of immunological studies demonstrate that the antibodies selectively distinguish between the *cis* and *trans* conformation of Xaa-Pro imide bonds in cyclic peptides as well as their linear analogues. Potentially, a *cis*-directed monoclonal antibody (mAb) can be used as a diagnostic tool for the detection of conformational changes during the HIV-1 infection process. Work is in progress to target biologically relevant *cis*-amide bonds in proteins applying a set of *cis*-directed mAbs.

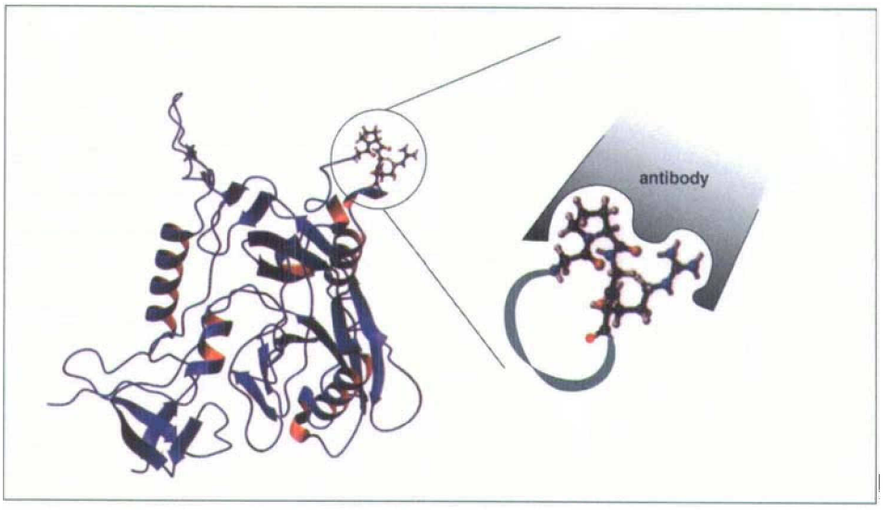


Fig. 4. NMR derived structure of the envelope protein gp 120 of HIV-1. The V3 loop corresponds to the major neutralizing epitope (left, circled). A highly conserved tetrapeptide adopting a type II β-turn is thought to isomerize to give a type VI β-turn with a *cis* proline peptide bond in the infection-active state. For targeting the relevant *cis* conformation, ΨPro-containing, *cis*-constrained V3 loop analogues were prepared and used as immunogens to raise antibodies that selectively recognize *cis* amide bonds (right, close-up) [41].

Going beyond Evolution: The Dual Function of Ψ-Prolines (Super-Prolines)

Often the formation and specific assembly of multicomponent protein complexes is mediated by binding of protein modules to proline-rich peptide sequences of medium size. Such proline-rich ligands adopt a left-handed polyproline II helical conformation (PPII) and bind to a highly conserved patch of aromatic amino acids on, for example, Src homology 3 (SH3) domains. As shown in Fig. 5, Pro residues are essential for this kind of protein-protein interaction by:

- inducing the unique PPII conformation (placing every third Xaa_i residue on top of Xaa_{i-3}, *i.e.* all facing the receptor) and
- allowing specific van der Waals contacts between the cyclic Pro side chain and its complementary receptor site.

It has been argued that the incorporation of more bulky side chains at the receptor site could modulate receptor affinity and/or specificity. In maintaining the structural and functional effects of Pro, N-substituted peptoids have been proposed as Pro-mimetics [43]. We have previously shown that ΨPro-containing oligopeptides exhibit solvent-dependent conformational transitions from an all-*cis* amide bond containing right handed PPI to an all-*trans* amide bond containing left-handed PPII [44]. In comparison to homooligo-prolines, the incorporation of ΨPro enhances this conformational transition significantly, possibly due to a decrease of the *cis* to *trans* activation barrier. Consequently, Ψ-prolines represent ideal candidates for tailoring protein-protein interactions in Pro-rich sequences exerting a dual functionality. They can (i) enhance the onset of the relevant PPII conformation and ii) increase and optimize van der Waals contacts and hydrogen bonding to the receptor molecule, thus modulating affinity and specificity (Fig. 5).

To exploit this principle, two proline residues were replaced in the consensus sequence Pro-Xaa-Xaa-Pro by monoaryl substituted ΨPro in a Sos derived decameric peptide sequence. *In vitro* assessment of the binding activity of model peptides to SH3 containing adapter proteins reveals binding constants in the low micromolar range as is typical for Pro-rich ligands. In particular, the designed ligands have been shown to inhibit protein-protein complex formation, indicating that C(2') substituted ΨPro moieties do indeed exert the postulated dual functionality by enhancing the proline effect in the recognition process [42]. We are presently generating ΨPro libraries with combinatorial methods to tune the affinity and specificity of potential inhibitors of protein-protein interactions.

ΨPro in Perspective: The Story Continues

The concept of Ψ-prolines has developed to a state where the unique intrinsic proline effects, such as *cis-trans* isomerization, can be modulated and enhanced at will. Consequently, our groups are focusing on peptides and proteins in which Pro residues may play a pivotal structural or functional role. The design of 'Super-Prolines', *i.e.* Pro mimetics with additional functionalities for protein-protein interaction studies or prodrug development represents just a first step in this direction. Currently, ΨPro derivatives are introduced by chemical peptide synthe-

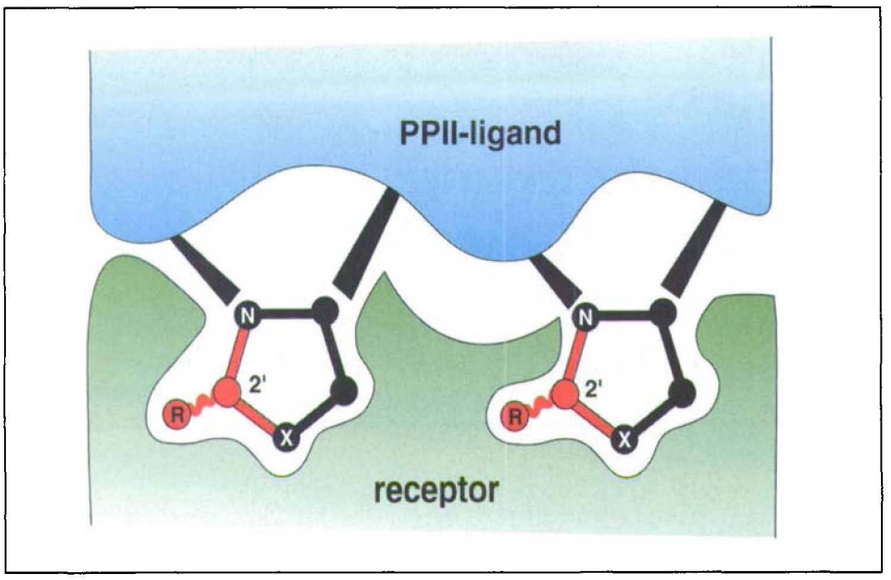


Fig. 5. The dual function of ΨPro is demonstrated with the example of Pro-rich peptides that serve as ligands for Src homology (SH3) domains. ΨPro building blocks induce the relevant PPII conformation of the ligand and additionally allow the modulation of affinity and specificity by tuning van der Waals contacts and hydrogen bonding interactions of the substituents R at C(2') of ΨPro to the receptor molecule [42].

sis. Due to recent progress in chemoselective ligation procedures, even the synthesis of ΨPro containing small protein domains seems to be within reach [23] [24]. However, the further elaboration of recombinant DNA techniques for the incorporation of non-natural building blocks into any given protein sequence may extend the ΨPro concept for addressing fundamental questions of protein folding and interactions.

In summary, the chemical transformation of the naturally occurring amino acids serine, threonine, and cysteine to proline-like building blocks with differential structural, chemical and functional properties has opened an unforeseen palette of applications in biomimetic chemistry, ranging from protection and solubilization techniques in peptide chemistry to molecular recognition studies of biological pivotal processes and finally, to the design of compounds of therapeutic interest. And this is possibly just the beginning of the story...

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