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New Protein Mimetics: The Zinc Finger Motif as a Locked-In Tertiary Fold

Gabriele Tuchscherer,* Christian Lehmann, and Marc Mathieu

Dedicated to Professor Murray Goodman on the occasion of his 70th birthday

De novo protein design aims to mimic some of the structural and functional properties of native proteins.[1–6] One of the most intriguing hurdles in this rapidly growing field is the complexity of the folding mechanism, that is, the pathway by which a linear polypeptide chain adopts its unique three-dimensional structure. To bypass this well-known protein-folding problem,[7–9] the concept of template assembled synthetic proteins (TASP) was introduced, in which topological template molecules direct the folding of covalently attached peptide blocks into a predetermined packing arrangement with a branched-chain architecture.[3, 10–12]

Recent progress in the synthetic methodology for assembling peptides allows us now to access the full potential of the TASP approach. For example, by conceptually separating the structural and functional domains of native proteins, we have recently proposed the use of topological templates as structure-supporting scaffolds for the assembly of receptor binding loops.[13] Here we extend the TASP concept of protein design to the construction of proteinlike packing topologies with multiply branched, oligocyclic chain architectures ("locked-in folds"). These synthetic macromolecules exhibit unique physicochemical and folding properties and serve as versatile scaffolds in protein design and mimicry.

[*] Dr. G. Tuchscherer, Dr. C. Lehmann, Dr. M. Mathieu
Institute of Organic Chemistry, University of Lausanne
BCH-Dorigny, CH-1015 Lausanne (Switzerland)
Fax: (+41) 21-692-3955
E-mail: gabriele.tuchscherer@ico.unil.ch

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As shown in Figure 1, the envisioned structural motif is based on the principles of a molecular kit, in which building blocks such as α helices, β sheets, and loops are assembled by means of templates or spacer molecules to give covalently cross-linked multiply bridged ("locked-in") tertiary folds. Folding into a predetermined three-dimensional structure is

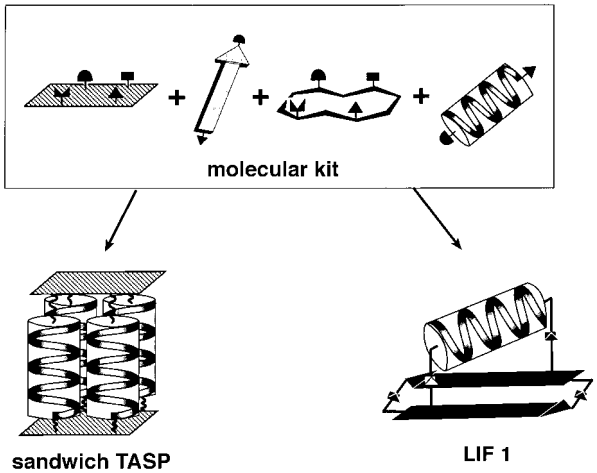

Figure 1. Molecular kit approach in de novo protein design: helices, β sheets, loops, and templates with chemoselectively addressable functional groups (e.g., aldehyde and aminooxy groups; depicted as solid symbols) are the constituents of a molecular kit. A variety of cheomoselective bond-forming methods allows the assembly of these building blocks into locked-in folds (LIF), and the folding problem of linear peptide chains can thus be bypassed.

achieved by the enforced intramolecular association of secondary structure forming peptide blocks to give highly constrained tertiary folds of increased thermodynamic stability. Most importantly, the large number of alternative folding pathways and packing arrangements of linear polypeptide chains is drastically reduced due to the confined conformational space. The proposed concept relies strongly on new methodologies in synthetic peptide chemistry such as chemoselective ligation procedures[14–17] and orthogonal protecting group techniques,[12] which allow the specific assembly of unprotected peptide segments as building blocks according to a molecular kit approach.

Our first aim was to mimic some structural and functional features of a zinc finger motif (finger 1 of Zif268) by a locked-in fold with an overall topology similar to that of the native molecule. Zinc finger proteins contain one of the most interesting structural motifs for the separation of structural and functional domains[13] and for the modulation of DNA-binding specificities.[18, 19] The consensus sequence Xaa_2-Cys-Xaa_{2-4}-Cys-Xaa_{12}-His-Xaa_{2-4}-His-Xaa_4 (Xaa represents variable amino acids)[20, 21] folds in the presence of Zn^{II} ions into a $\beta\beta\alpha$ folded unit, which binds through its helical face to three base pairs of the DNA. Multimeric zinc finger proteins consisting of several zinc finger (Zif) modules play a key role in controlling gene expression.[18, 19]

On applying the general concept of locked-in folds, the $\beta\beta\alpha$ framework of the Zif motif immediately suggests the use of a strategy based on the principles of a molecular kit. The constituent elements are a cyclic β-sheet template that mimics

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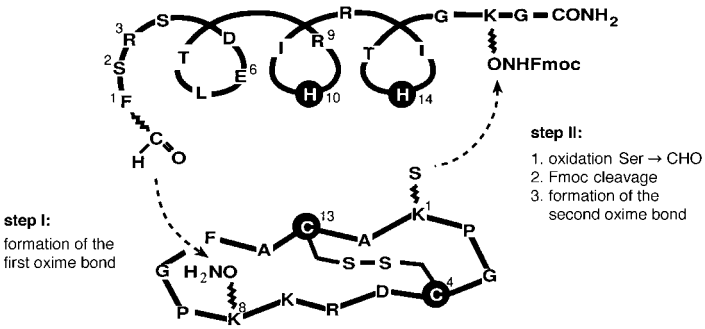
the β strand/turn/β strand motif (structural part) and a helical block (functional part). For the assembly of these building blocks into a locked-in fold (LIF1, Figure 1), the helix is covalently attached at both chain ends ("sticky ends") through nonpeptidic linker groups to a cyclic template consisting of 14 amino acids to result in a highly branched packing arrangement (Scheme 1). The binding site for the



Scheme 1. Selective oxime bond formation for the construction of LIF1.

complexation of Zn^{II} ions was introduced into the LIF1 molecule by incorporating two cysteine and two histidine residues in the template and the helix, respectively. The final zinc finger model was relaxed in the MAB force field.[22] The geometry of tetrahedrally coordinated Zn^{II} center was taken from the X-ray structure of a native zinc finger (1zaa.pdb) and kept constant during the entire optimization cycle. The geometry of the linker $HN(CO)HC{=}NOCH_2$ in the engineered molecule was initially chosen to be *trans*, on the basis of the geometries of a corresponding substructural fragment in a set of well-resolved crystal structures. Additional constraints included hydrogen bonds within the α-helical and the β-sheet regions.

Apart from the overall $\beta\beta\alpha$ topology, important features of a native zinc finger were also taken into account, for example the incorporation of a lypophilic pocket, which is created by the N-terminal phenylalanine residue of the helix. This increases the thermodynamic stability of LIF1, as shown by molecular dynamics studies,[23] and plays a crucial role in the folding and stability of linear zinc finger molecules.[6] Furthermore, a Thr-Gly moiety at the C terminus (Figure 2) was retained to favorably support the intrahelical hydrogen-bond network.

The synthesis of LIF1 (Scheme 1) began with the preparation of the individual building blocks, that is, the template molecule and the helical segment, by stepwise solid-phase synthesis.[24] After cleavage from the resin and reverse-phase HPLC purification, the side-chain deprotected peptides containing chemoselectively addressable groups as attachment sites were subjected in aqueous solution to a two-step condensation process in which the helical 18-mer peptide was attached to the cyclic template by selective oxime formation.[14, 15] After oxidation of the serine residue at the N terminus of the helical block, the resulting aldehyde readily formed an oxime bond with the aminooxyacetyl group of the template. The reaction was monitored by analytical HPLC (Figure 3a). In the second step, the serine residue attached to the ε-NH_2 group of lysine in the template was oxidized, the Fmoc protecting group of the aminooxy functionality at the C terminus of the helical fragment removed, and the second oxime bond formed. The final chemoselective reaction— the attachment of the C-terminal end of the helix to the underlying template—was very fast and proceeded in high yield.

As suggested by molecular modeling studies, LIF1 preserves essential structural features of the native Zif molecule. This is confirmed by the absorption and circular dichroism (CD) spectra and by the zinc-complexation properties. The CD spectrum (Figure 3b) indicates an increase in helicity upon complexation of Zn^{II} ions, but in contrast to the native linear polypeptide, LIF1 retains its secondary structural elements even in the absence of the zinc complex (Figure 3b). This reflects the onset of tertiary interactions in LIF1 enforced by C and N capping of the helical block on the template.

It is interesting to note that a Zn^{II}-independent folding was described for a Zif-derived linear $\beta\beta\alpha$ motif after iterative redesign of the native sequence.[6] In the case of LIF1, the trapped architecture results in stabilization of the helix and higher thermodynamic stability of the Zif folding motif, as indicated by the denaturation behavior (insert in Figure 3b). Molecular dynamics calculations and NMR studies showed[25] that this type of template preferentially adopts a β-sheet structure in which the side chains of the Zn^{II}-complexing site and the helix attachment sites are located on the same face of the template plane.

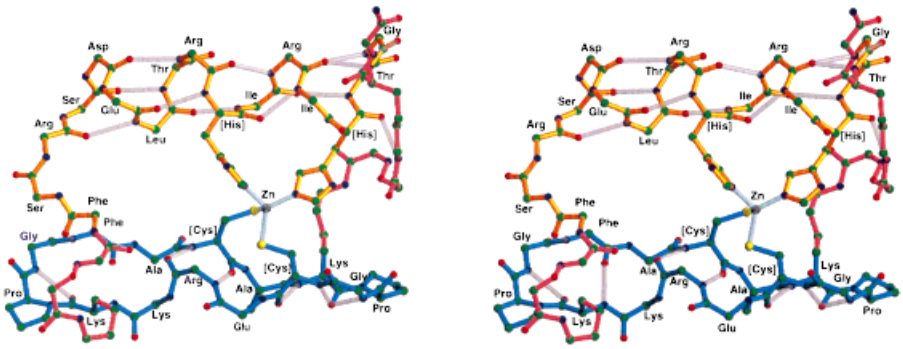


Figure 2. Stereoprojection of a strain-free, energy-minimized conformation of the locked-in fold LIF1 derived from the consensus sequence of finger 1 of Zif268. Covalent bonds between the ends of the helix (yellow) and the underlying template (blue) are shown in red. Only the side chains of the complex-forming amino acid residues are shown.

Cobalt(II) was used to investigate the metal-binding site of LIF1 due to its distinctive optical absorption spectra. The UV/Vis absorption spectrum (Figure 3c) indicates the formation of a Zif-like structure in the presence of Co^{II} ions. After reduction of the disulfide bond and addition of $CoCl_2$ the UV/Vis spectrum of LIF1 (Figure 3c) exhibits the characteristic absorption bands of a tetrahedral complex (as is present in native Zif modules) near 310 nm and 640 nm

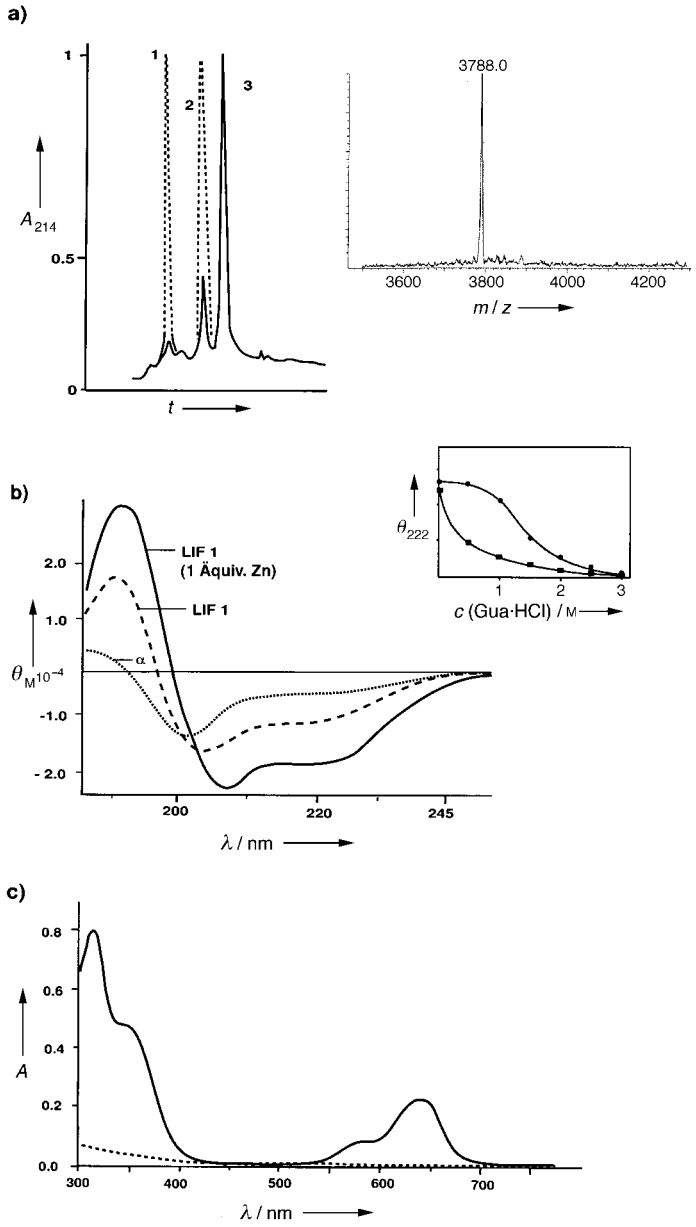


Figure 3. Characterization of LIF1. a) Left: RP-HPLC chromatogram after the first condensation step; the peaks of the starting materials (template (1), helix (2)) are dotted; peak 3 corresponds to the condensation product LIF1 after 18 h reaction time. Right: corresponding ESI-MS spectrum (m/z 3788.0). b) Circular dichroism (CD) spectra of LIF1 with the reduced disulfide bond in the absence of metal (– – –) and after the addition of stoichiometric amounts of Zn^{II} ions (——). The helicity increases moderately upon complexation of Zn; the spectrum of the completely unprotected helical block with an N-terminal serine residue (••••) reflects a predominantly unordered conformation. The spectra were recorded in 5 mM Tris buffer (pH 7) at a concentration of 10^{-5} M and normalized to the mean ellipticities per amino acid residue Θ_M. Insert: chemical denaturation (Gua · HCl) of LIF1 (●; $c = 0.25$ mg of peptide per mL; Θ_M at $\lambda = 222$ nm) and of the single helical block α (■; $c = 0.25$ mg mL^{-1}). c) UV/Vis spectra of LIF1 ($c = 150\,\mu$M) after addition of 150 μM $CoCl_2$ (——) and after addition of 150 μM $ZnCl_2$ (- - - -).

with a shoulder at 580 nm.[26, 27] Addition of one equivalent of a Zn^{II} salt to LIF1 readily displaces Co^{II} from the complex, even in the presence of a 50-fold excess of Co^{II}, with formation of the more stable Zn^{II} complex, which is indicated by the disappearence of the absorption maxima (Figure 3c). Titration curves with $CoCl_2$ and $Zn(BF_4)_2$ gave dissociation constants in the range of 10^{-6} M (Co^{II}) and 10^{-9} M (Zn^{II}),[28] which are comparable with those of natural zinc finger proteins such as transcription factor III A.[18, 26]

In the search for Zif mimetics with novel DNA-binding properties, the present approach offers some unique features:

1) The conformationally constrained template molecule serves as a stable scaffold that mimicks the β strand/turn/β strand motif of Zif.
2) The variable helical block is readily accessible by solid-phase peptide synthesis, and the incorporation of non-proteinogenic building blocks offers a wide range of structural and functional modifications.
3) The assembly of the constituent blocks to give tertiary locked-in folds by a single condensation step provides facile access to numerous analogues, including LIF libraries.
4) The chemical synthesis of multimeric locked-in folds with tailor-made DNA-binding specificities should become a powerful tool for studying protein DNA interactions.

In conclusion, the further elaboration of modern methodologies for peptide synthesis will rapidly expand the scope of the present approach and allow the construction of locked-in folds of even greater structural complexity. The concept elaborated here represents a chemical way to bypass the protein-folding problem in the design of synthetic proteins and opens interesting perspectives for the development of molecules of therapeutic relevance.

Experimental Section

The $\beta\beta\alpha$-Zif mimetic is based on the consensus sequence of finger 1 of Zif268.[29] The building blocks, helix and template, were synthesized according to standard procedures of solid-phase peptide synthesis[24] by the Fmoc strategy on Rink-MBHA (4-methylbenzhydrylamine)[30] or Sasrin (super acid sensitive resin).[31] The chemoselectively addressable sites were introduced as orthogonally protected aminooxy derivatives or serine residues.[13–15] After assembly of the linear template sequence Trt-K(Boc-S(*t*Bu))-P-G-C(Trt)-D(*t*Bu)-R(Pmc)-R(Pmc)-K(Dde)-P-G-F-A-C(Trt)-A (Trt: triphenylmethyl; Boc = *tert*-butoxycarbonyl; Pmc = 2,2,5,7,8-pentamethylchromane 6-sulfonyl; Dde = (4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl) on a Sasrin resin, the peptide was cleaved with 1% trifluoroacetic acid (TFA) and cyclized with PyBOP (benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate) in dimethylformamide (DMF). After selective removal of the Dde protecting group with 10% $H_2NNH_2 \cdot H_2O$ in DMF (20 min), Boc-aminooxyacetic acid succinimide ester (Boc-$NHOCH_2COOSu$) was coupled to the ε-amino group of the lysine residue. Subsequently, all acid-labile protecting groups were removed with TFA/TIS/H_2O (95/2.5/2.5; TIS = triisopropylsilane), and the disulfide bridge was formed. The template molecule was purified by RP-HPLC (67% overall yield) and characterized by electrospray ionization mass spectrometry (ESI-MS: m/z: 1647).

The helical block Boc-S(*t*Bu)-F-S(*t*Bu)-R(Pmc)-S(*t*Bu)-D(O*t*Bu)-E(O*t*-Bu)-L-T(*t*Bu)-R(Pmc)-H(Trt)-I-R(Pmc)-I-H(Trt)-T(*t*Bu)-G-K(Dde) was synthesized on Rink-MBHA resin. The Dde protecting group was selectively removed with 10% H_2NNH_2 (20 min), and the liberated ε-NH_2 group of the C-terminal lysine residue was functionalized with Fmoc-$HNOCH_2COOSu$. After cleavage of the helix from the resin and simultaneous removal of all acid-labile protecting groups (95% TFA, 1.5 h), mild oxidation of the N-terminal serine residue with $NaIO_4$ (fivefold excess, 5 min) led to the aldehyde. The helical block was purified by RP-HPLC (53% overall yield) and characterized by ESI-MS (m/z: 2458).

To generate the first oxime bond between the N terminus of the helix and the aminooxy function of the lysine side chain in the template, 1.1 equiv of

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the helix was dissolved in sodium acetate buffer (10 mM, pH 4) and added dropwise to a solution of the template in acetate buffer (pH 7.5). The condensation reaction was complete after 18 h, as monitored by analytical HPLC.

In the next step, the serine residue in the ε position of the lysine residue of the template was oxidized to the aldehyde (fivefold excess of $NaIO_4$, 5 min), and the peptide purified by RP-HPLC (56%). Subsequently, the Fmoc protecting group of the aminooxy group at the C-terminal lysine residue of the helix was removed with 20% piperidine in DMF. After precipitation with diethyl ether, the crude peptide was dissolved in 10 mM acetate buffer (pH 4); this resulted in immediate formation of the second oxime bond (>90%). The LIF 1 was purified by RP-HPLC and characterized by ESI-MS (*m*/*z*: 3788).

Optical absorption spectra of the Co^{II} peptide complex were recorded on a Beckmann spectrophotometer. Prior to complex formation, the internal disulfide bond of LIF 1 was reduced with 250 mM dithiothreitol (DTT) at room temperature for 10 h. After lyophilization, LIF 1 (150 μM) was suspended in Tris·HCl buffer (pH 7; degassed with helium) and 150 μM $CoCl_2$ added. The addition of an equimolar amount of $ZnCl_2$ to this solution readily displaces Co^{II} from the complex.

CD spectra were recorded on a Jobin Yvon Marck VI circular dichrometer in quartz cells (path length 0.1 cm). Prior to recording the spectra the internal disulfide bond in LIF 1 was reduced as described above, LIF 1 was taken up in Tris·HCl, and spectra were recorded before and after addition of equimolar amounts of $ZnCl_2$.

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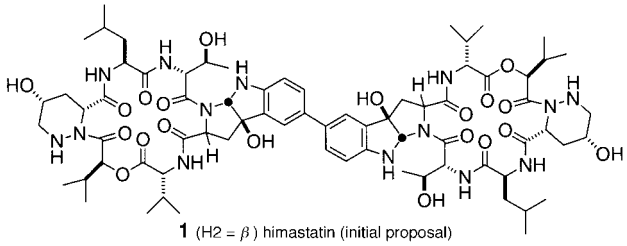
Studies in the Total Synthesis of Himastatin: A Revision of the Stereochemical Assignment**

Theodore M. Kamenecka and Samuel J. Danishefsky*

Dedicated to Professor E. J. Corey

The quest for new antibiotic and antitumor agents prompted scientists at Bristol Myers Squibb to investigate an actinomycete strain (ATCC 53653) from the state of Himachal Pradesh in India. In doing so, they encountered a new compound of formula $C_{72}H_{104}N_{14}O_2$, which they named himastatin.[1] After extensive optimization with the strain, himastatin could be obtained in scales adequate for sustaining chemical and biological investigation. While himastatin has not been developed to the point of clinical trials, its activity against gram-positive microorganisms and a variety of tumor probe systems is impressive. Based on spectroscopic investigations augmented by modest degradative studies, **1** was



1 (H2 = β) himastatin (initial proposal)
20 (H2 = α) himastatin (revised proposal)

[*] Prof. S. J. Danishefsky,[+] T. M. Kamenecka
Laboratory for Bioorganic Chemistry
Sloan-Kettering Institute for Cancer Research
1275 York Avenue, Box 106, New York, NY 10021 (USA)
Fax: (+1) 212-772-8691
E-mail: c-kandell@ski.mskcc.org

[+] Further address:
Department of Chemistry, Columbia University
Havemeyer Hall, New York, NY 10027 (USA)

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