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REVIEW ARTICLE

Protein Design as a Challenge for Peptide Chemists

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Abstract: All efforts to turn the ultimate goal in protein *de novo* design into reality – the construction of new macromolecules with predetermined three-dimensional structure and well-defined functionality – failed because the mechanism of folding has still to be unravelled. In the present review, various attempts to apply synthetic tools for inducing native-like structural features in peptides in order to bypass the folding problem are described. Besides well-established methods for the nucleation and stabilization of secondary structures, e.g. α-helices, β-sheets and β-turns, topological templates as 'built-in' folding devices have more recently become the key elements for the induction of protein-like folding units (template-assembled synthetic proteins, TASP). Progress in the synthetic strategy and structural characterization of this new type of macromolecules opens the way for the design of functional TASP molecules.

Keywords: Protein *de novo* design; topological templates; template-assembled synthetic proteins; artificial proteins; chemoselective ligations

Introduction

Peptide synthesis is no longer regarded as an intellectual challenge for chemists, mainly due to the immense progress achieved over the last decades in solid-phase synthesis and peptide purification. Ironically, innovations in the methodology of chemical synthesis made peptide synthesis almost 'routine work', depriving the synthetic chemist from his key role in the landscape of peptide chemistry. Even worse, the synthesis of proteins – a long-standing final goal in the perfection of chemical peptide synthesis – has become the domain of DNA recombinant techniques. Rather than persisting in a state of inactivity, the peptide chemist should take the opportunity to rediscover the most fundamental task, i.e. the creation of novel compounds in a joint venture of existing knowledge and innovative ideas.

Indeed, the construction of novel proteins exhibiting newly designed structural and functional properties opens up a most rewarding domain for peptide chemistry today. In view of the hurdles encountered in protein design in copying nature's way of synthesizing and folding a linear polypeptide to its unique three-dimensional structure, the need for alternative strategies to construct protein-like macromolecules is obvious. Having almost no restrictions in the choice of building elements (e.g. amino acids) and the construction plan (e.g. folding mechanism), the peptide chemist contributes with a broad palette of synthetic tools another key element in the design process and thus finds a most innovative and creative new role: *the synthetic chemist as designer*. In the present article, this fascinating aspect is brought into focus by reviewing some representative activities in the field of protein *de novo* design, with special emphasis on topological template molecules as a synthetic device for the onset of tertiary structure formation.

Secondary Structures by Design

The assembly of medium-sized peptide blocks adopting amphiphilic secondary structures in solution to a more complex folding topology represents a common feature in all strategies for protein design. Consequently, a detailed knowledge of the process of

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secondary structure formation and stability is essential. Numerous studies using homo-oligopeptides [1] or host–guest techniques [2] contributed to our present knowledge of the critical chain length, solvent and sequence dependence of helix and β-sheet formation. With regard to their use in protein design, the induction and stabilization of short secondary structure blocks by synthetic devices is an example of a most attractive tool (Fig. 1).

The incorporation of C^{α}-alkylated amino acid residues into an oligopeptide chain is a commonly used strategy for stabilizing helical conformations [9];

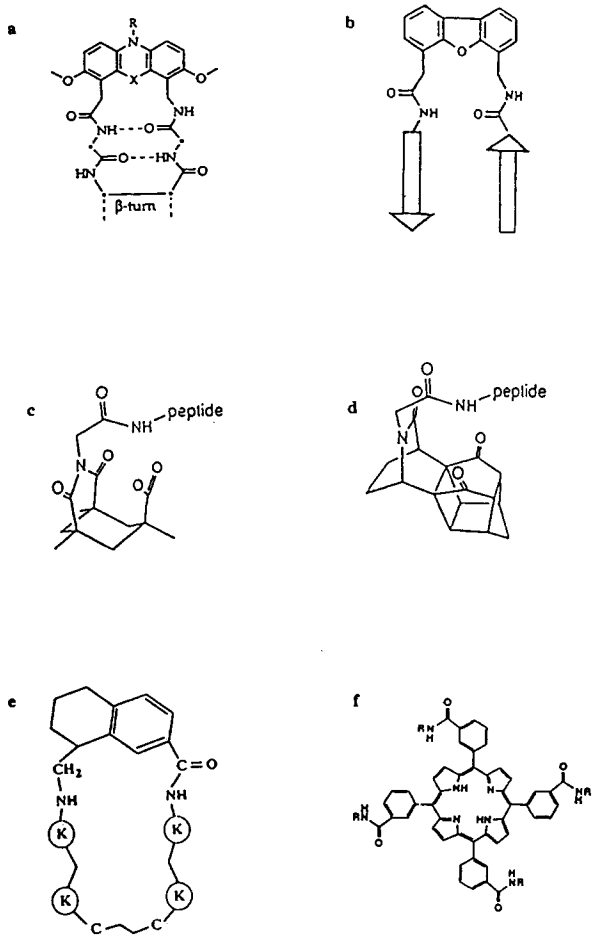


Fig. 1. Topological templates used for the nucleation of secondary structures: dibenzofuran based peptidomimetics (a) and xanthene derivatives (b) induce and stabilize antiparallel β-sheet structure and β-turns, a triacid (c) and derivatives (d) thereof initiate α-helix formation [3–6]; cyclic peptides containing a β-turn mimetic (e) and the tetraphenyl porphyrin system (f) are examples of templates used for the onset of tertiary structure, e.g. for the construction of 4-helix bundle molecules [7, 8].

in particular, systematic studies on the differential propensity of individual C^{α}-methylated amino acids have given valuable insights for stabilizing the right-handed helical structure [10]. As an alternative, the incorporation of trifunctional amino acids (e.g. His, Cys at position i and $i+4$) into the peptide chain allows for helix stabilization via complexation of amino acid side-chains with a transition metal [11]. Furthermore, the use of conformationally constrained molecules as templates by geometrically fixing the first amino acid in the proper orientation for helix or β-sheet initiation is a promising approach to bypass the entropically unfavourable nucleation step in secondary structure formation. The groups of Kemp and Müller described recently the synthesis and structural features of such topological templates feasible for the nucleation of α-helical structures [3, 4]. By covalent attachment of medium-sized model peptides via their N-terminal residues to properly designed template molecules, a substantial increase in the degree of helicity could be observed. Similarly, β-sheet formation can be induced well below the critical chain length observed for the onset of β-sheet structures in solution by fixing the peptide to rigid template molecules [5, 6]. Here, the use of tricyclic heteroaromatic systems (e.g. dibenzofurans, xanthenes) proved to be versatile templates for inducing β-sheets as well as β-turns or loops in short peptide sequences. The amphiphilic character of such stabilized helical or β-sheeted peptide blocks is the prerequisite for self-association in solution and the major driving force for formation of more complex packing topologies typical for proteins.

De novo design makes use of the repetitive alignment of hydrophobic and hydrophilic amino acid residues to create amphiphilic secondary structures [12]. Most notably, the amphiphilic pattern appears to be the dominating factor in determining the specific nature of the ordered structure. For example, using a limited set of amino acid residues, the preference for α-helix and/or β-sheet formation could be directed exclusively by rearranging the amino acid sequence (but not the amino acid composition) to result in a different amphipatic pattern [13]. Protein designers extensively use this fundamental principle by selecting a minimum number of amino acid residues for the construction of amphiphilic helices or β-sheets: thus, DeGrado designed a membrane channel forming α-helical peptide using only leucine (hydrophobic) and serine (hydrophilic) residues [14].

Possibly the most consequent application of this 'amphiphilic principle' with an immense impact on protein design represents the design of polypeptide sequences with potential for 4α-helix bundle formation by using a binary code as a general design strategy [15]. The construction of random amino acid libraries comprising a set of hydrophilic and hydrophobic residues seems to be further support of the binary code approach in protein design [16].

In view of the close relationship between preferred conformation and functional properties, the design of peptides able to undergo distinct conformational transitions are of considerable practical relevance. Again, by designing bis-amphiphilic peptide sequences (Fig. 2) according to the amphipatic pattern of secondary structures, medium-induced transitions of the type α-helix to β-sheet could be observed in this novel class of peptides ('switch peptides') [17].

An interesting variation of this design principle is based on a redox-controlled conformational transition of a peptide containing methionine [18].

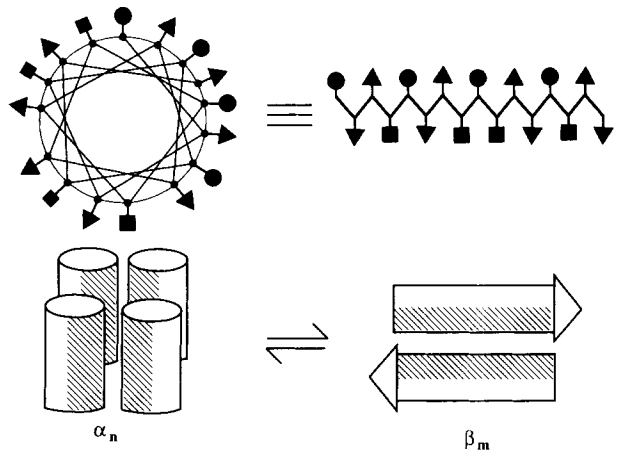


Fig. 2. Bis-amphiphilic oligopeptides, peptides with equal propensity for α-helix and β-sheet formation are able to undergo medium-induced conformational transition (switch); the depicted amino acid sequence shows amphiphilicity in the helical wheel representation (circles resemble hydrophilic, squares hydrophobic and triangles indifferent residues) and an alternating hydrophilic–hydrophobic pattern in the representation of a β-sheet structure. The amphiphilic character of the peptide blocks leads to self-association to form a helical bundle (*m*, *n* = number of blocks) that 'switches' from an α-helix to a β-sheet structure upon change of solvent or pH [17].

Topological Templates as a 'Built-in' Folding Device in Protein Design

In the absence of a detailed knowledge of the folding mechanism of natural proteins, a general design strategy of polypeptide sequences with a high propensity to fold in a predetermined three-dimensional structure appears to be still out of reach. In order to bypass this notorious folding problem, we proposed a conceptually different approach in protein *de novo* design: the template-assembled synthetic proteins (TASP) concept [19]. As a key element, a topological template serves as a 'built-in' device to direct the covalently attached amphipatic peptide blocks to a well-defined packing topology.

The use of templates to direct organic synthesis has a long-standing history [20]. Currently, topological templates have become a versatile tool in peptide mimicry and their full potential is only about to be recognized [21]. In view of the expanding areas of applications and functions, topological templates may be generally characterized as synthetic devices, that orient functional groups or structural units in well-defined spatial arrangements. Typically, template molecules represent structural motifs such as constrained peptides, cyclodextrines or polycyclic systems disposing selectively addressable functional groups in a well-defined manner (Fig. 3).

Templates exhibiting a predetermined backbone conformation as host for the selective attachment of functional sites (e.g. amino acid side-chains or peptides) represent a conceptually new approach in molecular recognition studies and peptide mimicry. As depicted in Fig. 4, these topological templates with appropriately oriented functional sites for interaction with an acceptor molecule are ideal candidates to mimic bioactive conformations of peptide ligands [22] or protein surfaces, e.g. discontinuous epitopes, binding and catalytic sites. A number of most encouraging applications of this concept have been reported recently. For example, using β-D-glucose as a template ('scaffold'), Hirschmann *et al.* were able to design a non-peptidic mimic of the somatostatin agonist SRIF, exhibiting a new functional profile [23]. In an alternative application of template molecules, the group of Hruby describes the use of tetrahydroisoquinoline derivatives as stable backbone templates ('topographical templates') for controlling the spatial orientation of amino acid side-chains of bioactive peptides thought to interact with the receptor molecule [24].

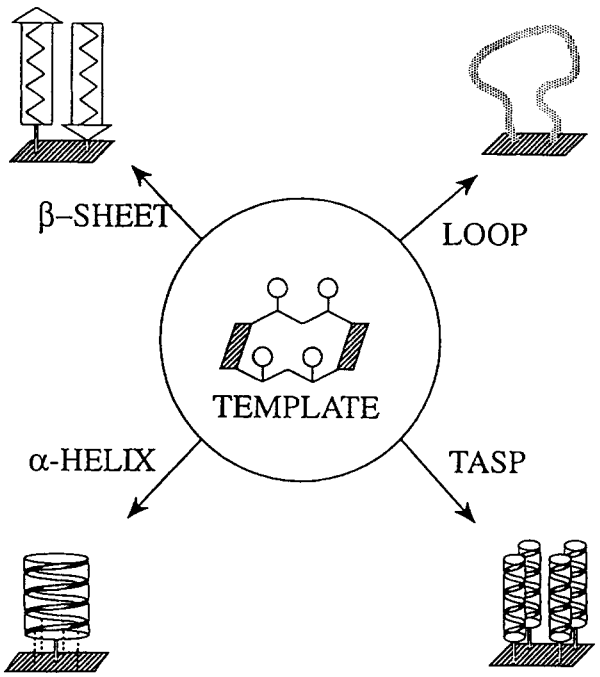


Fig. 3. Schematic representation of the concept of topological templates as device for induction and stabilization of protein folds such as α-helices, β-sheets, loops and for the construction of TASP molecules.

We have recently proposed the use of topological templates for mimicking conformational epitopes of proteins ('surface mimetic') and bioactive conformations of peptides [22].

In protein design, topological templates serve to direct the attached peptide blocks to a predetermined three-dimensional packing arrangement. Over the last few years, a number of 4α-helical bundle TASP molecules have been synthesized [7, 19, 25] applying

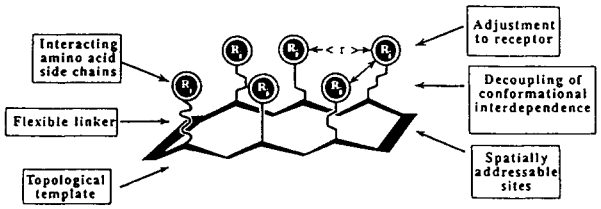


Fig. 4. General features of topological templates used in the TASP design. Conformationally constrained structural motifs (e.g. cyclic peptides or peptidomimetics) dispose functional groups (R_i) in a defined spatial arrangement (r = distance). Orthogonal protection techniques grant selective cleavage and flexible linkers allow for differential adjustment upon acceptor interaction.

stepwise solid-phase peptide synthesis [26] or convergent strategies [27]. After extensive purification both procedures resulted in TASP molecules of high chemical and structural integrity. With respect to the elaborated protection chemistry in peptide synthesis, the use of combined solid-phase techniques (e.g. for the synthesis of the peptide blocks and the orthogonally protected cyclic peptides as templates) with solution methods (for the selective condensation of the blocks to the template) turns out to be more efficient and provides a higher flexibility in the synthesis of complex packing topologies, e.g. the anti-parallel assembly of different peptide blocks. From the results obtained up to now some general properties of TASP molecules can be delineated:

(i) TASP molecules are readily accessible by chemical peptide synthesis and are soluble in aqueous and/or organic solvent systems.
(ii) The template enhances secondary structure formation by enforcing the intramolecular folding to a monomeric, compact structure.
(iii) 4α-helix bundle molecules show a highly cooperative denaturation behaviour similar to natural proteins.
(iv) Conformational energy calculations are in harmony with the hypothetical structures [28].

Several approaches for template-based *de novo* design of artificial proteins have been published. Sasaki and Kaiser proposed the use of a porphyrine derivative as a template for the construction of a 4-helix bundle, thought to mimic some functional properties of hemeproteins [8].

An elegant alternative represents the use of metal complexation for the induction of a helix bundle formation. Here, a transition metal is used as a template to assemble amphiphilic peptide blocks in a well-defined spatial orientation by complexation via N-terminal ligands. In a further elaboration of this approach, a heterodinuclear three-helix bundle metalloprotein was built up, resulting in an artificial protein of increased thermodynamic stability [29].

Over the last few years, several attempts have been made to design artificial proteins 'from scratch', mimicking some structural and functional properties of natural proteins. Though different in the ultimate goal, 'design for structure' and 'design for function' have the same conceptual base and should be regarded as a common effort in protein *de novo* design. Having synthetic access to an increasing number of chain topologies and template molecules,

Table 1 Representative Examples of Topological Templates Used in Protein *de novo* Design

Metal ion assisted self-assembly of a polypeptide into a triple-helix bundle protein using nicotinic acid as ligand	M. R. Ghadiri, 1990 [11]
Synporins–TASP molecules with a proposed 4-helix bundle conformation form ionic channels in lipid bilayers	A. Grove, 1993 [30]
Helichrome–a tetraphenylporphyrin system as template for a designed hemeprotein	T. Sasaki, 1989 [8]
Tempate-assembled melittin with ion channel properties	H. Vogel, 1994 [31]
The TASP concept for mimetics of peptide ligands, protein surfaces and folding units	G. Tuchscherer, 1993 [22]
Design of a heme-binding four-helix bundle	W. DeGrado, 1994 [32]

the variety of potential applications in biomimetic chemistry is about to be recognized (Table 1).

A most encouraging application of the TASP concept represents the construction of artificial membrane channels. Several authors have reported on selective membrane channel formation using topological templates to define and orient membrane spanning helical segments [14, 30, 31]. As a striking common feature, the membrane channel forming TASP molecules exhibit single channel conductance, ion selectivity and high thermodynamic stability (Fig. 5).

Numerous activities in the template-based design of functional molecules are being observed in the field of immunology. For example, Tam established the 'multiple antigenic peptide' (MAP) approach using branched oligo-lysines as template for the attachment of antigenic peptides [33]. Here, the template acts merely as a support to increase the immunogenicity rather than as a structure-inducing device. Kaumaya and his group report on the design of TASP molecules as prototypes for synthetic vaccines [34]. We have used the TASP approach for mimicking some of the structural and immunological properties of discontinuous epitopes derived from natural proteins. For example, the helical segment of lysozyme 87–99 [19] as well as the MHC I derived helical segments α_1 (58–74) [35] were assembled on a topological template to 4α-helical bundle TASP molecules. Specific anti-TASP antibodies could be raised that did recognize the corresponding native protein, confirming the preservation of the conformational epitope in the 4-helix bundle TASP molecule.

Probably the most challenging goal in protein *de novo* design represents the design of molecules exhibiting enzyme-like catalytic properties [8, 36]. In principle, the construction of a catalytic site requires a precise mutual positioning of functional groups and thus a detailed knowledge of the folding topology of the designed molecule. In view of the limited structural data obtained so far on designed proteins, the correlation of observed catalytic activities with structural features must be treated with caution. Consequently, functional properties are generally of limited value when considered as a

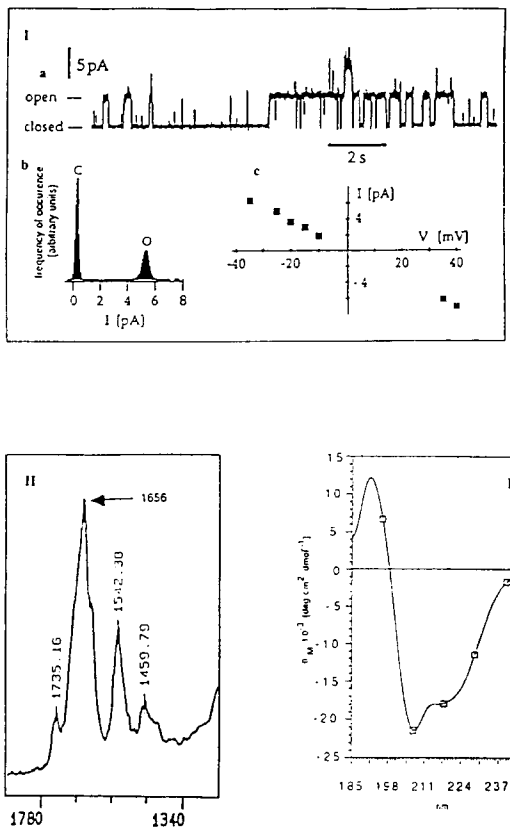


Fig. 5. (a) Single channel conductivity on small patch membranes is one property characteristic for template-assembled melittin, T_4-(4α_{26}) as a membrane channel-forming protein: (b) typical segments of current recordings of the channel states are depicted (closed or open states are indicated by C and O respectively); (c) current–voltage characteristics of the single state; markers represent mean single channel currents. ATR-FTIR in planar membrane and circular dichroism studies in TFE indicate helical conformation.

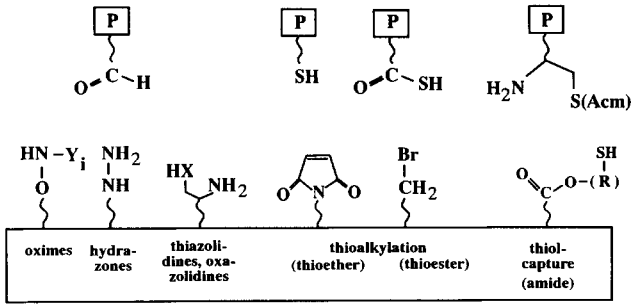
for the hypothetical three-dimensional structure. However, the more we learn about the structural properties of newly designed macromolecules, the more far-reaching will be the conclusions that can be derived with respect to the structure–function relationship of natural and non-natural proteins.

The Peptide Chemist as Protein Designer

Protein *de novo* design represents a new field in biomimetic chemistry and contributes to our understanding of the complex interplay between primary sequence, structure and function of natural proteins. The concept of template-based design strategies of peptidomimetics and artificial proteins combines elements of synthetic organic chemistry, structural biochemistry and computer-aided molecular design.

The peptide chemist will play an essential role in the further development of this fascinating field. It is only now that he can become aware of the unlimited potential of the synthetic tools in protein design. For example, the access of chemoselective ligation [25, 37] methods for assembling side-chain unprotected peptide blocks on spatially addressable template molecules (Scheme 2) opens the way for constructing TASP molecules of much higher structural and functional complexity (Fig. 6).

Furthermore, protein design will directly benefit from progress in peptide methodology, such as orthogonal protection techniques, peptide solubilization or the development of novel mimetics, templates and building blocks for structure induction and stabilization. In this perspective, the construction of



Scheme 1. Chemoselective ligation methods that allow for the covalent attachment of unprotected peptide fragments to appropriately functionalized template molecules in the TASP concept; P = peptide; Y = protecting group; X = O, S.

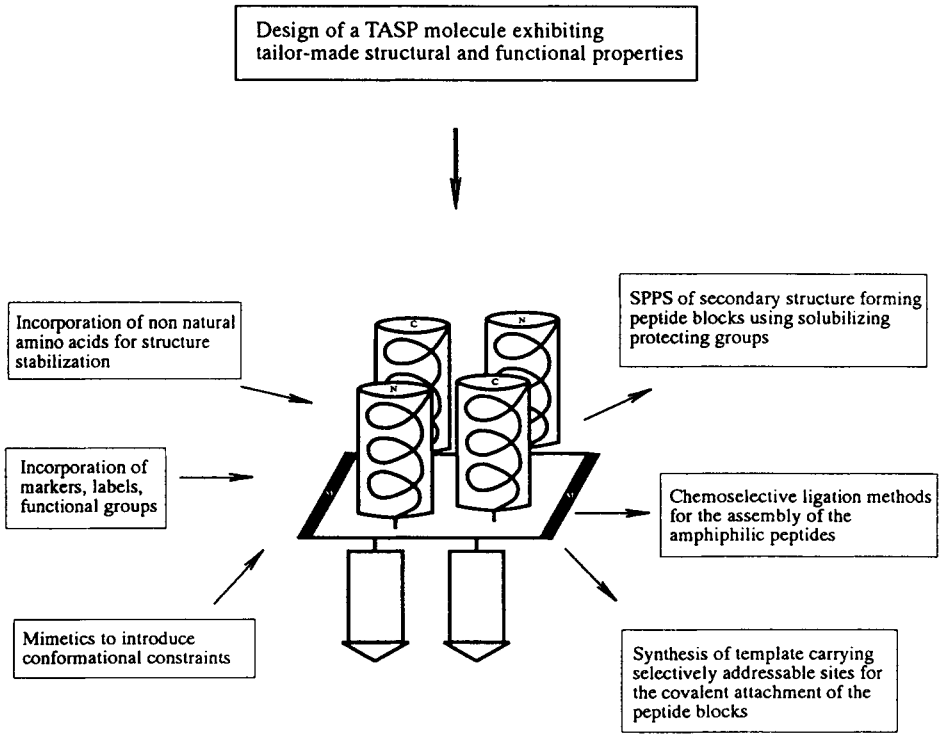


Fig. 6. An almost inexhaustible pool of various construction elements (e.g. non-natural amino acids, functional groups, conformational constraints) and methods for the synthesis (solid-phase peptide synthesis, chemoselective ligation, orthogonal protecting groups, etc.) of native-like macromolecules manifests the chemist's new role in the *de novo* design of proteins with tailormade structural and functional properties.

artificial proteins exhibiting tailormade functional properties represents an intellectually challenging and ambitious 'masterpiece' for peptide chemists and may reflect the high art of today's synthetic peptide chemistry.

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