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THE TASP CONCEPT : MIMETICS OF PEPTIDE LIGANDS, PROTEIN SURFACES AND FOLDING UNITS

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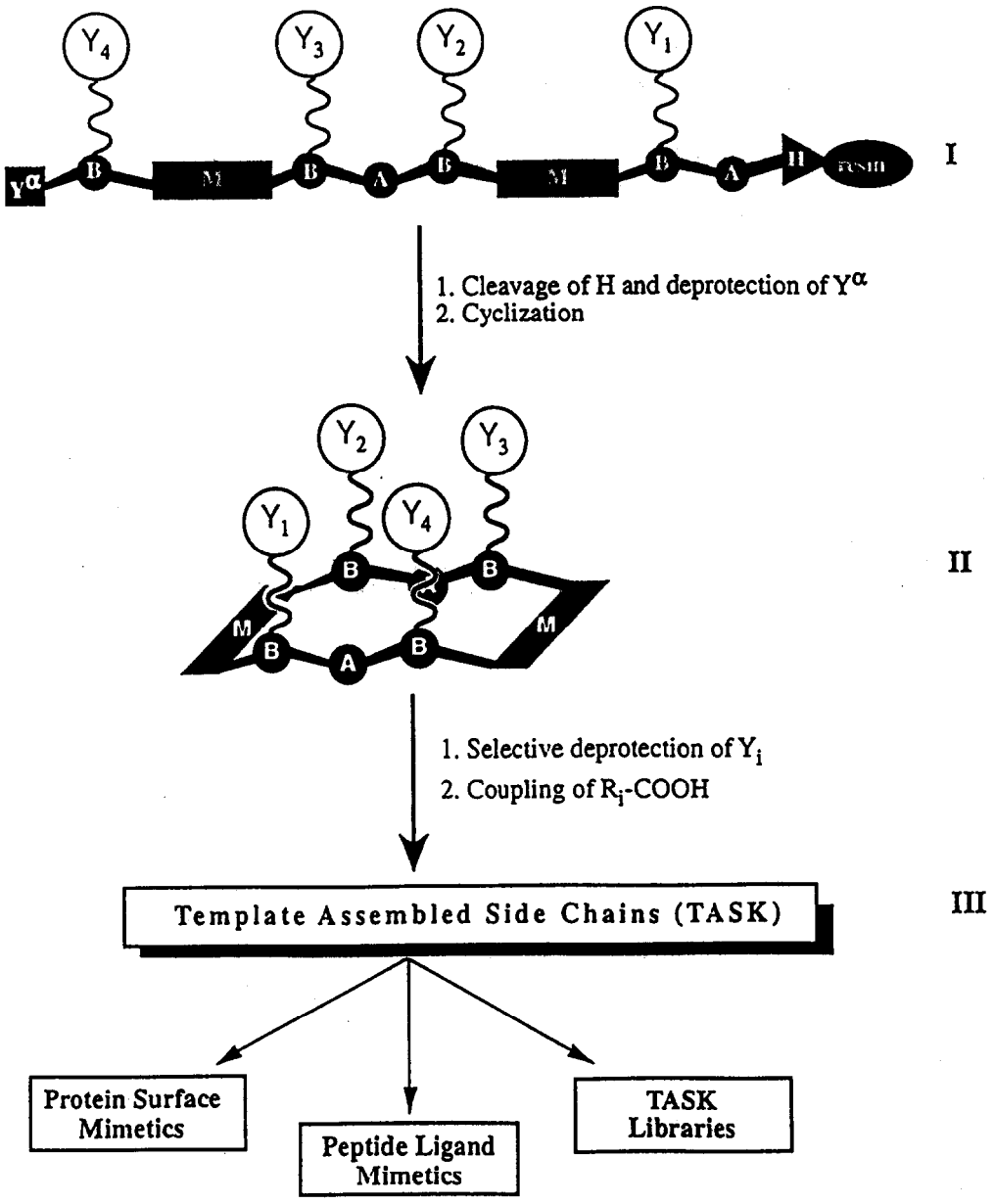
Abstract : *The TASP (Template Assembled Synthetic Protein) concept in protein de novo design has been introduced for the construction of protein-like molecules exhibiting tailor-made functional properties. After establishing some synthetic and conformational foundations in TASP strategy, we describe a 4α - helix bundle TASP molecule mimicking immunological properties of human MHC-I. Furthermore, we focus on structural motifs (e.g. topological templates) disposing functional groups in defined spatial positions as candidates to mimic structural and conformational features of peptides and proteins for molecular recognition studies.*

Introduction

Peptide and protein mimickry aims to transfer some of the complex structural and functional properties of this important class of bioactive molecules to simplified, synthetically accessible compounds. A most versatile approach represents the design of spatially well defined structural motifs (e.g. template molecules) to mimic bioactive conformations of peptides or to induce native-like folding topologies. The TASP (Template Assembled Synthetic Proteins) concept has been introduced a few years ago[1] to overcome the most intriguing hurdle in protein de novo design[2-5], i.e. the protein folding problem[6]. As a key feature of this approach, a topological template molecule serves to reinforce and direct the intramolecular folding of covalently fixed amphiphilic secondary structure blocks into a predetermined three-dimensional conformation. Similar conceptual approaches were described for the construction of protein-like molecules exhibiting tailor-made functional properties[7]. More recently, topological templates were proposed for initiating secondary structure formation[8] and for mimicking bioactive conformations of peptide ligands[9]. For molecular recognition studies, topological template molecules disposing functional groups (e.g. interacting with an acceptor) in defined spatial positions are ideal candidates to mimic structural and conformational features of peptides and proteins. In a series of TASPs with 4α-helical bundle topology prepared by stepwise SPPS the basic principles of this approach were established[10-12]. Here we focus on some new developments of the TASP concept : firstly, we describe a strategy that allows the selective and independent functionalization of structural motifs (topological templates), exhibiting spatially well defined localizations of the functional sites. Secondly, convergent strategies for the chemical total synthesis of TASP molecules exhibiting 4α-helix bundle packing topologies are evaluated. Finally, the design, synthesis and properties of a MHC-TASP model serve as an example for the application of the TASP approach to achieve functional properties.



Scheme 1 : General strategy for the construction of TASK molecules H : handle; M : structure inducing residue, e.g. turn mimetic, non natural amino acid; A : amino acid; B : N^{α}, N^{ω}-diaminoacid, e.g. lysine, diaminobutyric acid; Y_i : N^{ω} - protecting group of B_i; Y^{α} : N^{ω} - protecting group; R_i : natural or non natural amino acid side chain.

Strategy	$Y\alpha$ / Y_4	$Y\alpha$ / Y_3	$Y\alpha$ / Y_2	$Y\alpha$ / Y_1	Handle[10]
A	Trt / Fmoc	Fmoc / Boc	Fmoc / Aloc	Fmoc / Z(Cl)	Sasrin
B	Aloc / Trt	Trt / Boc	Boc / Fmoc	Boc / Z(Cl)	Allyl (Hycram™)

Table 1 : Orthogonal protection techniques for solid-phase peptide synthesis of TASK molecules (Scheme 1).

The independent attachment of groups R_i responsible for molecular interaction results for example in mimetics of

(i) discontinuous epitopes or binding sites of proteins ("surface mimetics"[2, 14, 17]);

(ii) bioactive conformations of peptide ligands.

Making use of today's potential of amino protecting groups (Table 1), topological templates carrying up to eight independently addressable sites are conceivable. Moreover, by varying the structural parameters of the template, as well as the orientational flexibility and chemical nature of the functional groups, this new type of molecules can serve as lead in the rational search for biologically active compounds. Most notably, the preparation of TASK libraries according to strategies described for linear peptides[18] appears to be of considerable practical interest[19]. As a challenging application of the TASP concept, we are presently using topological template molecules for the presentation of protein surfaces ("surfmics"[14-20]). As shown schematically in Fig. 1, amino acid side chains of discontinuous reactive sites (epitopes, substrates, binding or catalytic centres) are assembled on a conformationally constrained template, thus mimicking the topological features of a surface segment.

As exemplified in Fig. 2, the epitope HEL10 (left)[21] is represented by a "surfmic" (right) in which the distances of the selectively addressable attachment sites in the template for fixing the residue side chains 21, 96, 97 and 101 are comparable to those in the native lysozyme. Optimal interactions with the monoclonal antibody anti-HEL10 are ensured by a linker allowing for independent conformational adjustment of the individual side chains to the receptor molecule. Explorative studies indicate[22] that this application of the TASP concept represents a powerful new tool in studying the principles of molecular recognition, and in searching for nonpeptidic drugs.

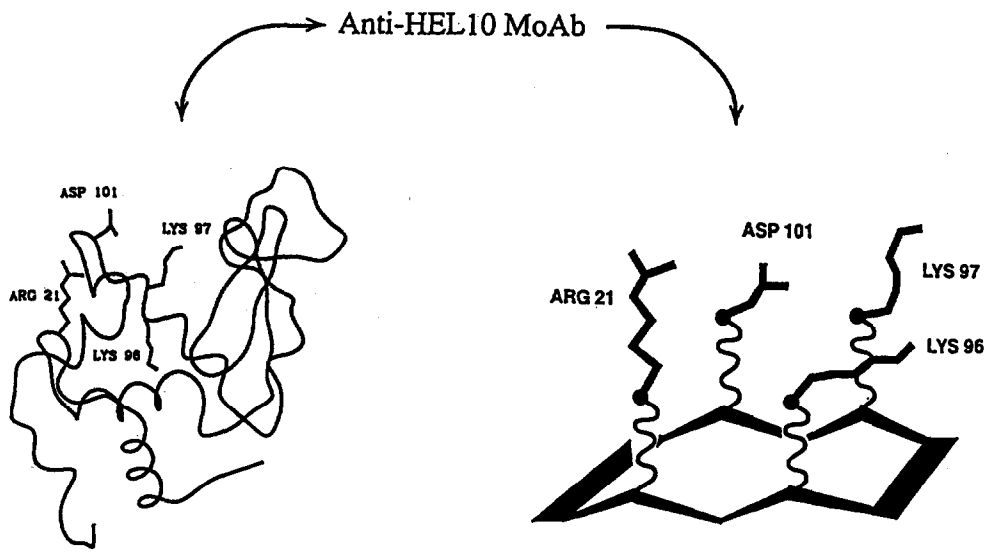


Figure 2 : Schematic representation of the structural features of a surface mimetic ("surfmic") as derived from the TASP concept. Receptor binding is promoted by the reduced conformational entropy of the "surfmic" molecule.

TASP Molecules

Convergent Strategies for TASP Synthesis

TASP molecules designed to exhibit tailor-made functional properties were synthesized by SPPS[11-12] and - as an alternative approach - by fragment condensation of the helical block α to the cyclic templates (Fig. 3a)[23].

Conformationally constrained templates were prepared by incorporation of β-turn mimetics such as 8-aminomethyl-5,6,7,8-tetrahydro-2-naphthoic acid (AMTA) in different enantiomeric forms employing classsical solution chemistry. DCC/HOBt activation, Boc strategy and Z protection for the N^{ε} amino groups of the lysine side chains were applied for the synthesis of the linear octapeptides. Cyclization in solution (DPPA, DMA, 24h) and deprotection resulted in the cyclic template (Fig. 3b) in overall yields of 76%. Molecular dynamics calculations and successive ^{1}H-NMR investigations on these conformationally constrained template molecules indicated the spatial proximity of the two aromatic dipeptide mimetics in aqueous solution due to hydrophobic interactions; most importantly, the lysine side chains adopted almost ideal orientations for the covalent attachment of the peptide blocks in this low energy conformation of the template backbone. As a representative example, a helical block α_{14}(α_{14}=Ac-D-L-Aib-T-A-L-Aib-N-L-Aib-K-K-L-G-OH, a modelled 87-97 helix of hen egg-white lysozyme[7]) has been synthesized on a Sasrin-resin,

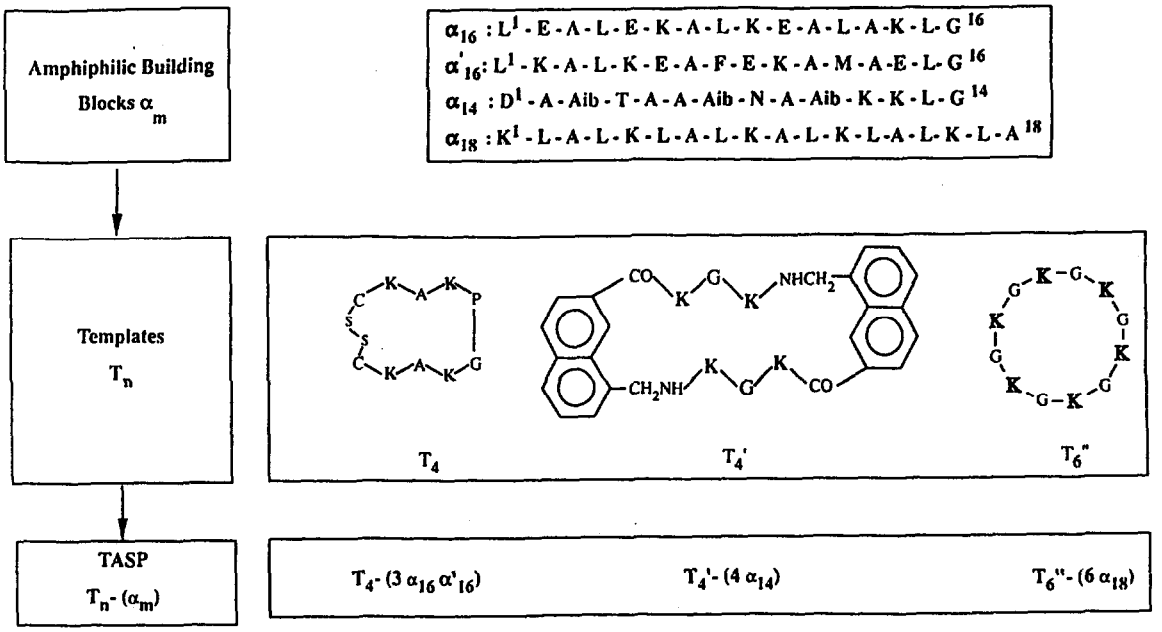


Figure 3a : TASP molecules T_n-(α_m) synthesized by SPPS and by fragment condensation techniques.

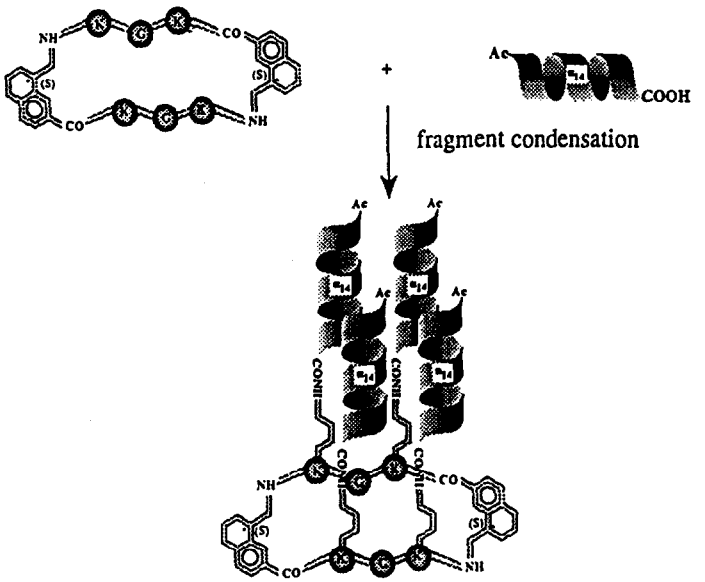


Figure 3b : Fragment condensation of a typical helical block to a cyclic template.

applying tBu, Trt and Boc protecting groups for the lysine side chains and Fmoc strategy. The TFA salt of the (S,S)-AMTA template was reacted with 2-fold excess (with respect to the amino groups on the template) of fully protected fragment acid activated with BOP, HOBt, DIEA in DMA. The fragment condensation reaction proceeded at 40°C and pH 8 to 9. After 1h the reaction was close to completion as monitored by HPLC. Deprotection with TFA for 5h and purification by RP-HPLC led to the desired product (MW = 6860.33) in 43% overall yield. The chemical identity of the TASP molecule was confirmed by LDI-MS. In conclusion, convergent strategies represent efficient alternative routes to stepwise SPPS[12] for the construction of TASP molecules.

Conformational Properties

The high purity of the TASPs as established by HPLC, CZE and MS allowed for detailed CD and NMR investigations[11-12]. Figure 4 shows the amide proton region of the 1D NMR spectra of the cyclic template molecule (a), the single peptide block Ac-Lys (α_{15}-Ac)NH_2 (b) and the TASP molecule T_4-(4α_{15}-Ac) (d).

Figure 4 (c) shows the superposition in a 4:1 ratio of spectra (b) and (a). The actual NMR spectrum of T_4-(4α_{15}-Ac) 4(d) is distinctly different from the spectrum depicted in 4(c) and does not represent a simple superposition of the spectra of its constituent components. Most notably, the folding into the hypothetical 4α -helix bundle structure of T_4-(3α_{16},α'_{16}) (Fig. 3a and Fig. 4e) is strongly supported by at least three separate crosspeaks in the NOESY spectrum between F8 in α', and leucine residues in α or α' (Fig. 5). Furthermore, denaturation experiments (Fig. 6) pointed to a considerable template-induced increase in the thermodynamic stability of the helix bundles, the structural features of the various templates being of notable importance for this stabilizing effect.

TASP Molecules with Functional Properties

MHC class I - TASP model

We have recently focused on the design of TASP molecules of a four-α-helix bundle topology, in which antigenic helical segments of protein surface domains are assembled on suitable templates[23-25]. The MHC class I molecule can be regarded as an interesting candidate of considerable immunological relevance for modeling according to the TASP concept. Most notably, the three-dimensional structure of human class I MHC[26-28] reveals a characteristic folding topology that can serve as a structural motif in protein de novo design. For example, the extracellular portion of the heavy chain is composed of three domains α_1, α_2 and α_3, each composed of about 90 amino acid residues; the α_1 and α_2 domains form a deep groove of about 25 Å long and 10 Å wide, considered to be the binding site for antigenic peptides[29]. The construction of TASP molecules exhibiting some of the characteristic structural features could contribute to the elucidation of the functional relevance of the individual building blocks of MHC and serve as a synthetic antigen for obtaining MHC-specific antibodies.

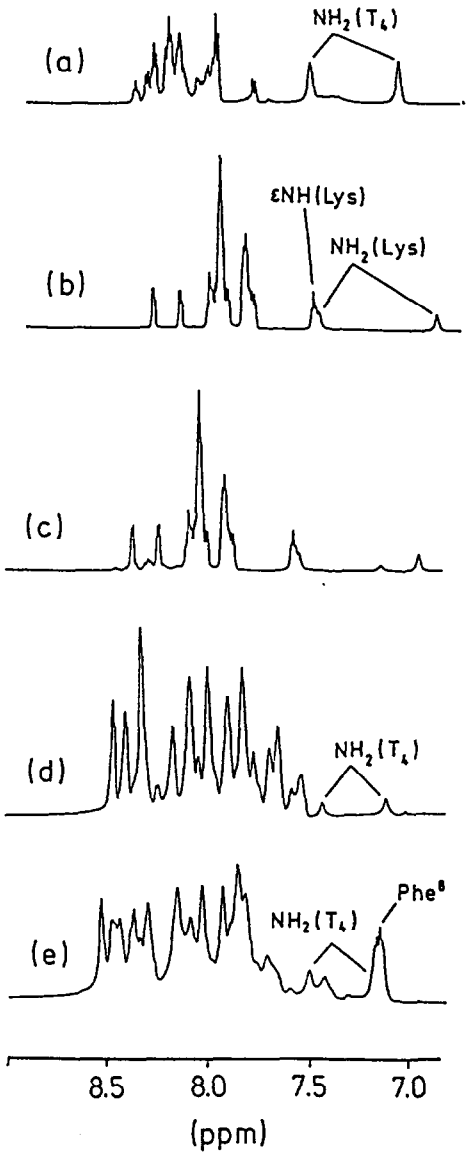


Figure 4 : Amide region of the 1D ^1H NMR spectra in water, pH 3.0 of a cyclic template (a), single peptide block Ac-Lys (α_{15}-Ac)NH_2 (b), superposition of (b) and (a) in a 4:1 ratio (c), T_4-(4α_{15}-Ac) (d), and T_4-(3α_{16}, α'_{16}-Ac_d) (e).

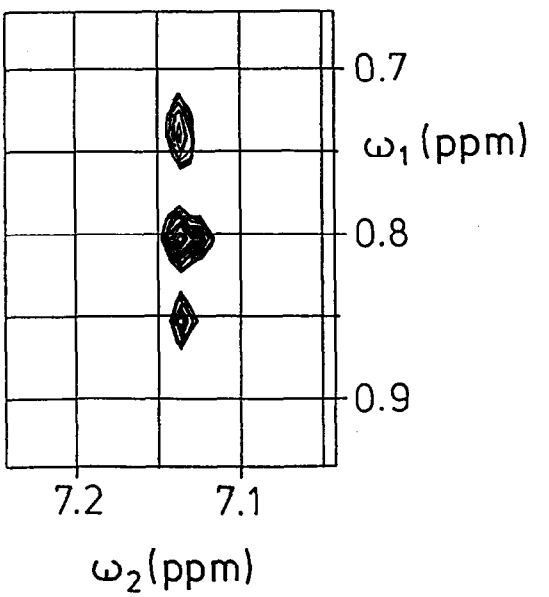


Figure 5 : Aromatic (ω_2) versus (ω_1) region of a NOESY spectrum of T_4-(3α_{16},α'_{16}) recorded in H_2O, pH 3.0.

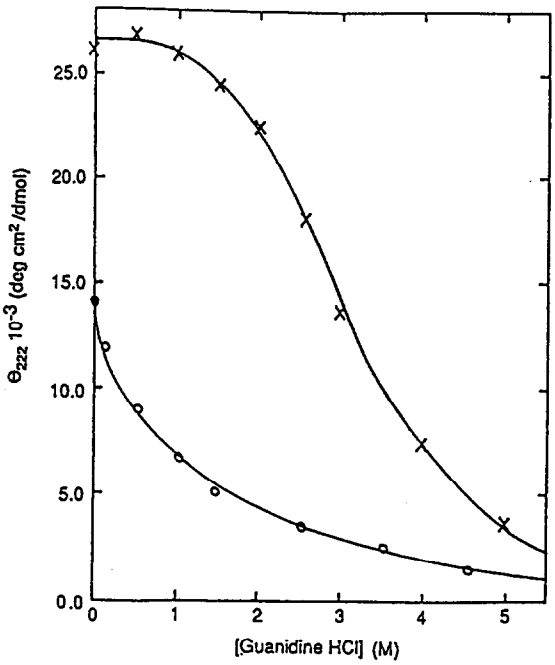


Figure 6 : Denaturation of T_4-(4α_{15}-H) (x) and of the single helix block (o) (concentration = 1 mg peptide / mL) by increasing concentrations of guanidine hydrochlorid (guanidine HCl).

Design

Previous CD and NMR studies showed that template assembled four-α-helix bundle molecules indeed adopt the proposed folding pattern in aqueous solutions [1,14,23,24]. A cyclic decapeptide of the sequence

Ac-C-K-A-K-P-G-K-A-K-C-NH_2

was used to induce a four-α-helix bundle formation of the attached peptide blocks. Molecular dynamics calculations suggest that this molecule adopts an antiparallel β-sheet connected by a β-turn type II (P-G) and cyclized via an S-S bond[13(a)], as schematically shown in Figure 7. In this low energy conformation, the four attachment sites of the helices (ε-amino groups of K) are oriented on the same side of the template plane, enforcing helix bundle formation. Starting from the native sequence of the α_1 heavy chain domain 58-84 of HLA-A2[26], we decided to split this helical sequence in two shorter, overlapping fragments (designated as α_{17} and α'_{17}, respectively), each comprising 17 amino acid residues (Fig. 8).

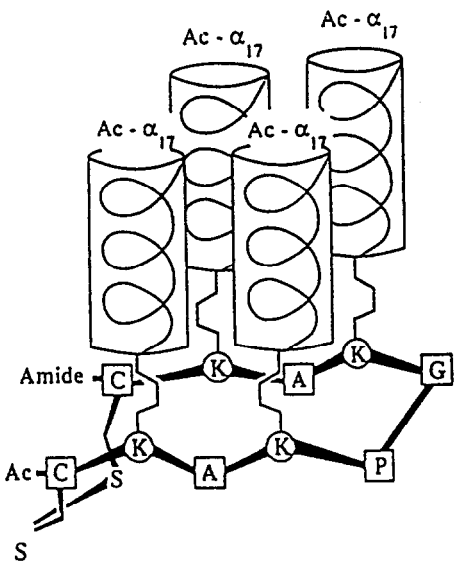
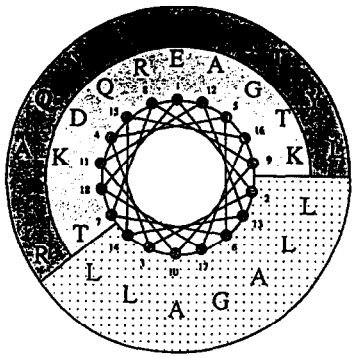


Figure 7 : Schematic representation of the modeled 4-α-helical bundle TASP molecule, T_4-(4α_{17}) (MHC-TASP). T_4, cyclic template containing four attachment sites (ε-NH_2 groups of the lysine [K] side chains; Ac-α_{17}, 17-mer peptide. The helical peptides Ac-α_{17} are covalently linked to the template via amide bond formation as described in Figure 9.

The redesign of the helical peptides was performed under the following considerations : (1) Residues that were not thought to be involved in the interaction of the MHC class I molecule with the TCR[30] were replaced by helix-forming residues (Ala, Leu). (2) In order to enhance hydrophobic interactions in the postulated four-α-helical bundle, Leu residues were inserted in the hydrophobic side of the helical wheel[31], thus, increasing the amphiphilic character and the propensity for packing into a hydrophobic core of the helical sequences as described recently in a strategy to design a topographical determinant to the protein antigen LDH-C4[32]. (3) Amino acids that may raise problems in the stepwise solid-phase synthesis, such as Trp, His and Arg, are replaced by Leu or Ala residues as far as these substitutions are consistent with (1) and (2). The resulting sequences of the two overlapping 17-peptides (N-terminal sequence, α_{17} and C-terminal sequence, α'_{17}) are depicted in Fig. 8. The resulting helical wheel presentation (Fig. 8) indicates that the modeled helices show a clear segregation into a hydrophilic and a hydrophobic part; the present design results in a remarkable increase in hydrophobicity, allowing the formation of a hydrophobic core as a major driving force for the onset of a four-α-helix bundle conformation.

Synthesis and Conformational Properties

The strategy for the synthesis of the MHC-TASP molecule T_4-(4α_{17}) follows the principles of the Boc/Fmoc-



α_{17} : Ac-^{1}E L L D G A T R K A K A L L Q T G^{17}

^{58}E Y W D G E T R K V K A H S Q T H R V D L G T L R G Y G^{85}

α_{17}' : Ac-^{1}A L L Q T A R V L A A T L L G Y G^{17}

Figure 8 : Helical wheel presentation of the overlapping 17-mer peptides α_{17} (N-terminal sequence, inner ring), α'_{17} (C-terminal sequence, outer ring) of HLA-A1 (58-85) (overlapping region in the native sequence : residues 69-74). Pointed area of the helical wheel shows the hydrophobic core.

based protection techniques of solid-phase peptide synthesis[33] as described in detail in Experimental Part (Fig. 9). Subsequent to the completion of the linear template molecule on a MBHA resin and removal of the Nε-protecting groups for the lysine side chains, the α-helical segments α_{17} were built up in a parallel fashion as depicted in Figure 9. The complete peptides were simultaneously deprotected and cleaved from the resin by treatment with liquid HF. The crude material was directly cyclized with $K_3(Fe(CN)_6)$ under conditions of infinite dilution. Figure 9 shows a schematic representation of the individual synthesis steps leading to the TASP molecule T_4-(4α_{17}).

After cyclization of the crude T_4-(4α_{17}) and subsequent concentration on a cation-exchange column, an analytical reversed-phase HPLC shows a major peak that was isolated by preparative HPLC. In the final purification step a 0.1% TFA buffer/CH_3CN solvent was used on the same column. A conservative estimate based on amino acid analysis, analytical HPLC, and mass spectrometry (Fig. 10a) indicates a purity for the target molecule T_4-(4α_{17}) of >90%.

As depicted by the CD spectra in Figure 10b, the TASP T_4-(4α_{17}) exhibits a pronounced tendency for α-helix formation in different solvents. The CD spectra in the helix-promoting solvent TFE (mimicking a more hydrophobic environment) is characterized by two negative cotton effects at 207 nm (π-π^* transition) and at 222 nm (n-π^*), typical for the onset of a helical structure (Fig. 10b). As expected, the degree of helicity decreases by addition of an aqueous buffer solution (50 mM phosphate). For example, the θ_{222}-value in an aqueous buffer solution containing 20% TFE (Fig. 10b) is consistent with a helical degree of about 50%,

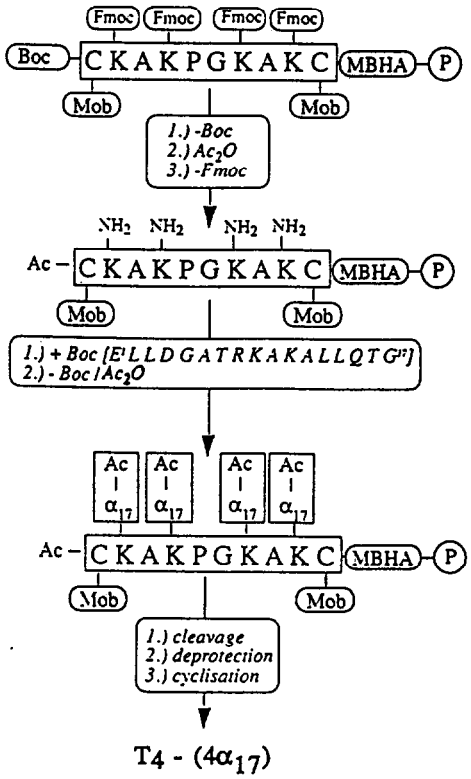


Figure 9 : Schematic representation of the solid-phase synthesis of the MHC-TASP T_4-($4\alpha_{17}$).

whereas the CD spectra in pure aqueous phosphate buffer solution points to a helical degree of only about 20% (Fig. 10b).

Similar to previous findings, the CD spectra of the free peptide (not shown here) exhibits no significant (<5%) helical content under identical conditions. As a major general feature of the TASP approach, the covalent attachment of potentially α-helix-forming peptide blocks to a topological template molecule results in a substantial increase in the helical structure compared to the free peptide. This has also been shown for a number of partial sequences of proteins as well as model peptides of comparable structure[1, 24, 31]. From these data, it can be concluded that the MHC-TASP molecule does not adopt a stable helical bundle conformation in purely aqueous solutions; however, in a more hydrophobic medium or in interaction with a receptor molecule, the helical conformation can easily be induced as indicated by the CD data obtained in TFE-containing solutions.

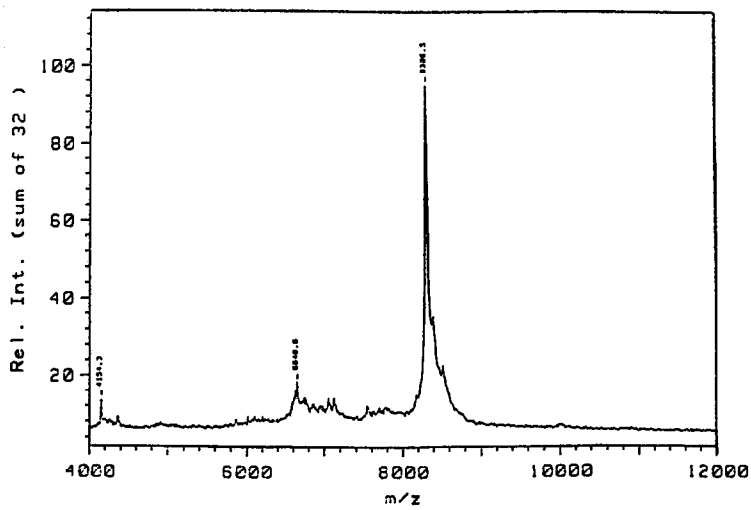


Figure 10a : LDI mass spectrum of T_4-(4α_{17}) (expected molecular weight, 8,306 Da).

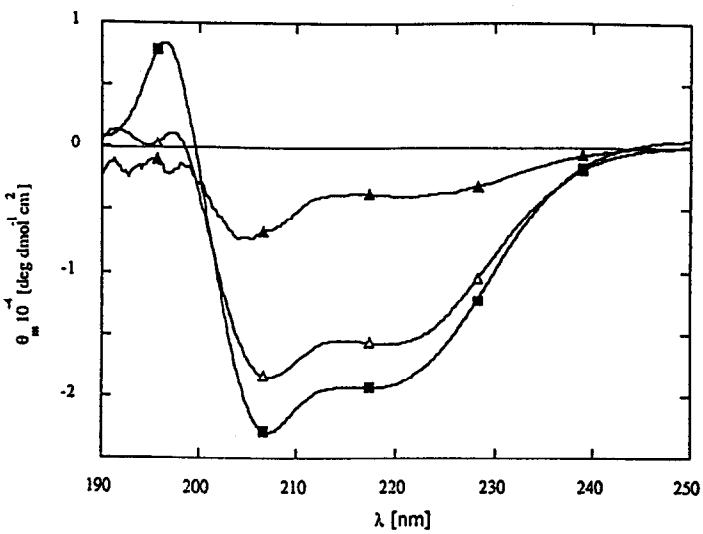


Figure 10b : CD spectra of T_4-(4α_{17}) in 50 mM phosphate buffer, pH 7.0 (-▲-); TFE/50 mM phosphate buffer (20/80), pH7 (-△-); and TFE (-■-); concentrations = 1 mg peptide / mL.

Immunological Properties

The presence of specific antibodies in the sera obtained from mice immunized with the MHC-TASP was detected by ELISA. The antisera obtained from both BALB/c (H-2^d) and C57BL (H-2^b) mice immunized with the MHC-TASP contained specific antibodies able to recognize the MHC-TASP molecule. Most notably, we

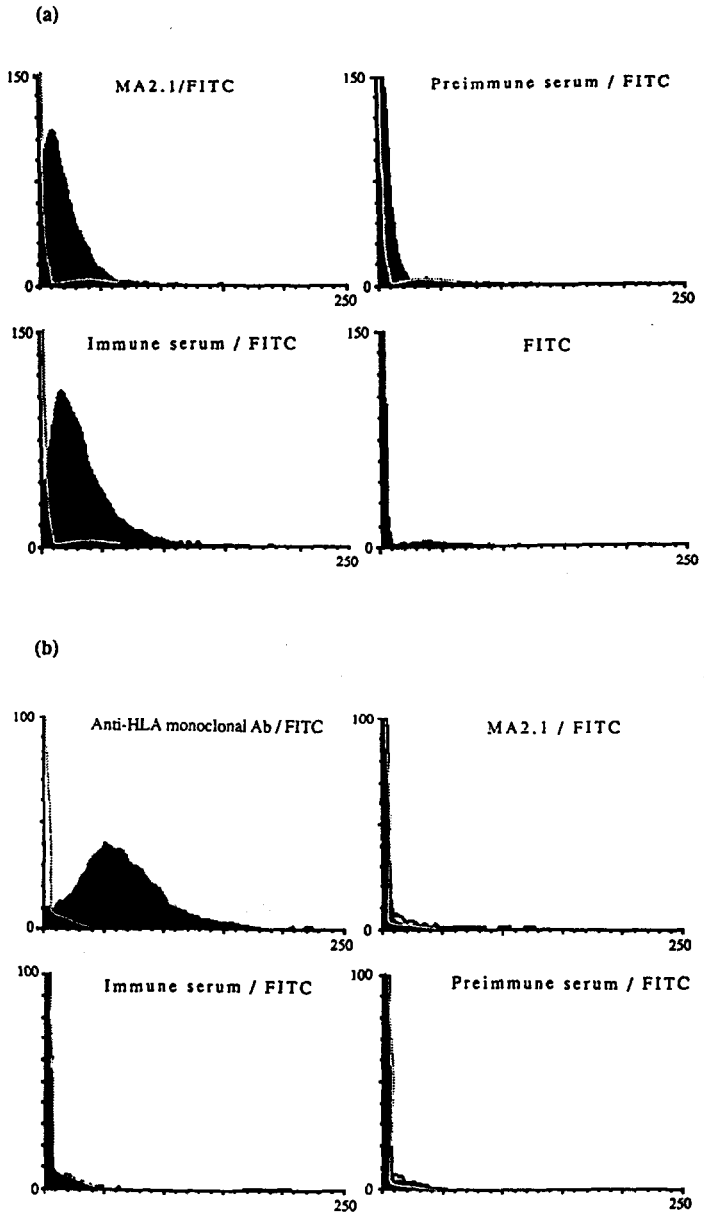


Figure 11 : Flow cytometry analysis of anti-MHC-TASP antibodies on (a) T2 (HLA-A2-positive) cells and (b) KT2 (HLA-A2-negative) cells.

succeeded in raising antibodies against the highly immunogenic MHC-TASP molecule without prior conjugation to a carrier molecule. Both mouse strains produced high antibody titers obtained three weeks after the primary immunization and an increase in the antibody response after boosting, indicating a

memory response. The anti-TASP antibodies proved to be specific for the native HLA-A2 molecule[7(g)] as indicated by the immunoflorescence experiments on the A2-positive T-lymphoblastoid cell line (Fig. 11). The fact that anti-A2-TASP antibodies stain the T2 cells to the same extent as MA2.1 (a monoclonal antibody that recognizes a conformational epitope on folded A2 molecules associated with β_2-microglobulin) indicates that the antibodies obtained recognize the native molecule on the surface of A2-positive cells.

In summary, the stabilization of the native conformation in the fully synthetic TASP molecule gives access to the presentation of conformational epitopes and results in antibodies of high specificity. Contrary to immunizing with peptides conjugated to protein carriers, the approach of using TASP molecules as immunogens provides several advantages : first, the template assembled peptide remains in its original form, modifications of amino or carboxylic side chains, which can occur during conjugation, may alter the antigenic epitope. Second, chemical conjugation of peptides to carrier proteins has been shown to influence protein structure and thus immunogenicity[32]. The data obtained demonstrate that TASP molecules with a predetermined, stabilized tertiary structure can play an important role in studies of protein structure and antigenicity. With the stabilization of potentially secondary structure-forming oligopeptides (and thus, e.g. of conformational epitopes) on topological template molecules as realized in TASP molecules and the presentation of discontinuous reactive sites such as protein surfaces (e.g., epitopes, substrates, binding or catalytic centres) on conformationally constrained templates as realized in "surfmics" and "TASK" molecules, the TASP concept represents a powerful new tool in studying principles of molecular recognition, and in the search for nonpeptidic drugs.

Experimental Part

Solid-phase Synthesis of T_4-($4\alpha_{17}$). (MHC-TASP, Fig. 6)

All amino acids - Boc-Cys(S-p-OMeBzl), Boc-Lys(Fmoc), Boc-Gly, Boc-Pro, Boc-Leu, Boc-Lys(2ClZ), Boc-Ala, Boc-Glu(cHxl), Boc-Asp(Bzl), Boc-Thr, Boc-Arg(Mtr), Boc-Gln, Boc-Tyr(oBzl), and Boc-Val - were purchased from Bachem (Torrance, California) and were used directly without further purification. Solvents were obtained from Aldrich. The synthesis was carried out manually using an MBHA 1% cross-linked polystyrene resin (2.0 g) that was obtained according to published procedures (using *p*-toluoyl chloride instead of benzoyl chloride) with a substitution of 0.45 mmol NH_2/g[12]. The synthesis protocol followed established SPPS methodology[33] for Boc protection except for the Fmoc-protected ε-amino groups of the four template lysine side chains[12]. The linear protected precursor of the cyclic decapeptide template was assembled in a stepwise manner according to the following general procedure : the Boc protecting group was removed with 80% TFA and 1% EDT in CH_2Cl_2 (25 min), washes with 1% EDT in 2-propanol, MeOH, and CH_2Cl_2 ; neutralization with 10% Et_3N/CH_2Cl_2 , coupling with a twofold excess of activated amino acid KT2-(HLA-A2-negative) cells derivative using either diisopropylcarbodiimide (DIC) (with or without 1-hydroxybenzotriazole (HOBT) as additive) or benzotriazol-1-yl-oxytris(dimethylamino) phosphonium hexafluorophosphate (BOP) as activating agents. Depending on the solubility of the Boc amino acid, coupling were performed in CH_2Cl_2 , CH_2Cl_2 / DMF or NMP/DMF mixtures. Each coupling step was monitored by the qualitative ninhydrin test, and recouplings were performed if necessary. After completion of the template molecule using Boc-Lys (Fmoc), the Fmoc groups were removed (20% piperidine/CH_2Cl_2 ; 5 min and 10 min), and the four identical helical building blocks were synthesized in parallel following the

strategy for Boc protection. N-terminal acetylations were performed with 10% Ac_2O in 1% pyridine/CH_2Cl_2. Clevage of the peptide from the resin with anhydrous HF in the presence of anisole (5%) and ethyl methyl sulfide (5%) was carried out using an in-house (Salk Institute)-designed Kel-F line (1.5 g peptide / resin, 0°C, 75 min). After evaporation of HF, the peptide was precipitated with anhydrous ether, collected by filtration, and redissolved in 60 mL water/acetic acid (80:20, v/v). For the cyclization process, the obtained crude product was diluted with 3 L of water and then added dropwise to a vigorously stirred solution of $K_3(Fe(CN)_6)$ (200 mg) and NH_4OAc (10g) in 1 L water (total addition time about 4 h). The pH was kept between 6.8 and 6.9 by simultaneous addition of 10% aqueous NH_4OH. The solution was stirred at 4°C overnight. The pH was adjusted to 5.0 by addition of acetic acid, and a slurry of the anion-exchange resin (10mL; Bio-Rad anion-exchange resin AG3-X4A [analytical grade, 100-200 mesh], chloride form) was added. The mixture was stirred for 10 min, filtered, and the filtrate applied to an ion-exchange column, filled with AG3-X4A (15 mL gel bed volume). The eluate was subsequently loaded on a cation-exchange column, filled with BioRex 70 resin (analytical grade, 100-200 mesh, H^+ form, 30 mL gel bed volume). The peptide that was retained on the column as shown by the absence of any absorbing material by HPLC analysis of the eluate was eluted with 50% acetic acid in water (total volume about 100 mL). After lyophilization the crude T_4-(4α_{17}) Ac was purified by preparative HPLC using three different systems to gain maximum purity : (1) buffer A = 0.1% TEAP, pH 2.25; buffer B = 80% acetonitrile/20% A (50% B to 90% B in 50 min); (2) buffer A = 0.1% TEAP, pH 6.25; buffer B = 80% acetonitrile/20% A (50% B to 90% B in 50 min); (3) buffer A = 0.1% TFA, buffer B = 0.1% TFA in 80% acetonitrile/20% H_2O (50% B to 90% B in 20 min). The amino acid analysis gave the expected amino acid ratios, and mass spectrometric analysis confirmed the calculated molecular weight for the T_4-(4α_{17}) (MHC-TASP).

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