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An Easy Entry to a New High-Symmetry, Large Molecular Framework for Molecular Recognition Studies and de Novo Protein Design. Solvent Modulation of the Spontaneous Formation of a Cyclic Monomer, Dimer, or Trimer from a Bis-cysteine Peptide

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Abstract: Spontaneous oxidation of a short palindromic peptide with the sequence Ac-C-X-K-L-H-A-E-L-S-S-L-E-A-H-L-K-B-C-G-NH_2 (X = Aib) provides up to three different cyclic products: a monomer with an intrachain disulfide bond, an antiparallel dimer, and a trimer with two parallel and an antiparallel chain. Control over the relative amount of the different products can be achieved by adding different amounts of TFE that modulate the population of the α-helix conformation during the reaction. Regioselective disulfide formation affords also the parallel dimer, which was not formed spontaneously, as well as the three possible noncyclic dimers with two protected cysteines and a single disulfide bond. CD spectroscopic studies of these dimers as well as NMR and CD studies of a peptide with the two cysteines replaced by Leu provide the basis for understanding the control of the spontaneous oxidation by TFE and the strong discrimination between parallel and antiparallel dimers. The special topological properties of this trimer and the fact that is formed spontaneously in high yield from the monomer provide a potentially very useful building block for the construction of peptidic receptors as well as minimalist models for α-helical packing in globular proteins.

Introduction

Self-assembly processes are at the heart of many attempts to build the large structures needed to act as receptors for molecules of even moderate size.[1]

Self-assembly is usually driven by complementary groups capable of forming hydrogen bonds placed on a rigid structural backbone. Intramolecular hydrogen-bond formation is prevented, and the geometry of the backbone combined with the directionality requirements for hydrogen bonding determines the topology of the aggregate.[2] The use of hydrogen bonding alone to direct self-assembly requires the use of nonpolar solvents and therefore is incompatible with aqueous solutions. On the other hand, the need for a rigid backbone may complicate the synthetic accesibility of derivatives with different functionalities, and in general, a complete redesign of the synthetic approach will be required to prepare conceptually similar receptors containing different sets of functional groups.

Peptide-based receptors provide an attractive alternative as the whole range of functions present in natural amino acids can be introduced synthetically using standarized methods, and furthermore, it may be possible to produce the desired peptide in large quantities by expression of synthetic genes in suitable organisms.

The self-assembly concept is equally useful for the preparation of de novo designed peptides, and a number of water-soluble synthetic receptors have been produced on the basis of the spontaneous formation of four-helix bundles by amphipathic helices.[3] Helix association is driven mainly by hydrophobic effects although side chain packing, and electrostatic interactions play an important role in determining the type of assemblies formed.[4]

Disulfide-bond formation has been used to direct or to probe the topology of the assembly and often leads to considerably higher stability.[5]

Bis-cysteine peptides can form cyclic molecules by spontaneous oxidation, and we had previously shown that they can be designed to act as ion receptors.[6] To produce larger peptide frameworks we are studying bis-cysteine peptides combining helical and loop regions. In this communication, we report the spontaneous formation of cyclic monomers, dimers, or trimers

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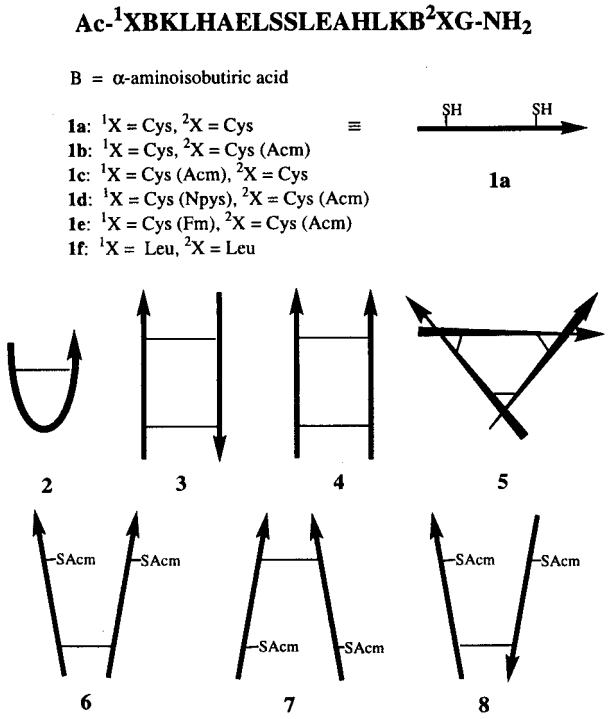

Figure 1. Structures of the peptides studied.

from a bis-cysteine peptide capable of adopting a bent helical conformation and we show how solvent-induced conformational changes can be used to modulate the spontaneous formation of different cyclic molecules. Finally, we explore the basis of the topological selectivity observed. The peptides studied are shown in Figure 1.

Peptides **1a,f** have a palindromic sequence (except for the C-terminal glycine). These peptides have therefore pseudo-C_2 symmetry disregarding the directionality of the peptide backbone, and the same types of residues face each other in both parallel and antiparallel assemblies, increasing the effective symmetries of the cyclic oligomers.

Leucine residues in positions 4, 8, 11, and 15 make the helix amphipathic and along with Cys 1 and 18 form a continuous 3−4 repeat that would place them on the same side of a continuous helical structure. However, in a distorted helix, the two cysteines will no longer be in phase. Serine has only a moderate propensity to occur in the interior of a helix but is one of the best residues in the N-terminal position[7] and is also often located in turns. Histidine residues in positions 5 and 14 were introduced as a tool for future studies as (i) they offer a possibility for modulating easily the total charge of the peptide, (ii) their pK_a values are a probe for electrostatic interaction, and (iii) they could provide complexation points for transition metals.

To improve helix formation in the resulting [19]-peptide, Lys-Glu and Glu-Lys pairs at the i and $i + 4$ positions were introduced. Although both pairs are not equivalent and may interact differently with the helix macrodipole, when introduced in model compounds, they caused similar stabilization.[8] Unfavorable interactions with the helix dipole were prevented by acetylation of the N-terminus and amidation of the C-terminus. Further stabilization of the helix was introduced by placing α-aminoisobutyric acid (Aib) residues near both ends. This α,α-dialkylamino acid has ϕ and ψ angles restricted to the right-handed (and left-handed) α-helix regions of the Ramachandran map and was expected to reduce the entropic cost associated with helix nucleation.[9]

Results

Spontaneous Oxidation of 1a. Solutions of **1a** at different concentrations (75, 150, and 300 μM) and in different solvent systems (0%, 15%, 30%, and 50% TFE in aqueous buffer) were allowed to react under oxidizing conditions (air, pH = 7.9−8.1). The reaction was followed by HPLC until the starting peptide had disappeared completely. Only three products could be detected: cyclic monomeric peptide (**2**), antiparallel cyclic dimer (**3**), and cyclic trimer (**5**). Co-injection of the reaction mixture with authentic samples of parallel and antiparallel cyclic dimers (Figure 2) showed the complete absence of parallel dimer (**4**) under any of the experimental conditions tested. Quantification of the different products in each reaction accounted for the total amount of peptide added, showing that substantial polymerization was not taking place.

All the peptides were studied by electrospray ionization mass spectrometry (ESI-MS).[10] Following careful calibration of the instrument, deconvolution afforded average masses of 6145.3 ± 0.3 for **5** and 2048.3 ± 0.2 for **2**. The calculated average mass for a cyclic trimer with three disulfide bonds is 6145.3 and that of a cyclic monomer 2048.0. The average mass of an open trimer with only two disulfide bonds and two free cysteine residues is 6147.4. Mass spectrometry indicates therefore that **5** is a cyclic trimer with three disulfide bonds. Additional chemical evidence for the absence of free cysteine residues can be summarized as follows: (a) The isolated trimer was stable for over 48 h at room temperature in the presence of atmospheric oxygen in 20 mM phosphate buffer (pH 9.0) without TFE. Under these conditions free cysteines are expected to react either by polymerization or by causing scrambling of existing disulfide bonds. (b) The Ellman test was negative, even in the presence of urea.[11] (c) After long treatments (>10 days) with excess iodoacetamide at room temperature in 6 M guanidinium chloride, the unmodified product was recovered.

The topology of **5** was determined by enzymatic digestion with immobilized trypsin. Digestion fragments were analyzed by mass spectrometry and amino acid analysis. Results are presented in Table 1. Fragments characteristic of both parallel and antiparallel disulfide-bonded peptide chains could be detected. This indicates that the trimer has parallel−antiparallel topology.[12]

The evolution of the concentration of the different products during the oxidation of **1a** at a concentration of 75 μM and in

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(10) Electrospray mass spectrometry of oligomeric species is hindered by the fact that multiple charged species, with correspondingly reduced mass-to-charge ratios, are detected. Unequivocal characterization of the aggregation state, however, can be based on the presence of peaks corresponding to species with a number of charges that is not a multiple of the number of units in the oligomer. Thus, while a trimer with six charges (m/z = (3M + 6)/6) has the same mass-charge ratio of a monomer with two charges or a dimer with four charges, a trimer with seven charges has a m/z that does not correspond to any charged state of smaller oligomers. An independent confirmation of the charge state of peaks observed in ES-MS came for the observation of clusters with a separation of 22/z amu in spectra run from sodium-containing solutions. These clusters can be explained by deprotonation of glutamic acid side chains and formation of an ion pairs with sodium. This process preserves the charge state of the species which can be unambiguously determined even at relatively low resolution from the separation between individual peaks within a cluster.

(11) Control experiments showed that urea did not interfere significantly with detection of free thiols.

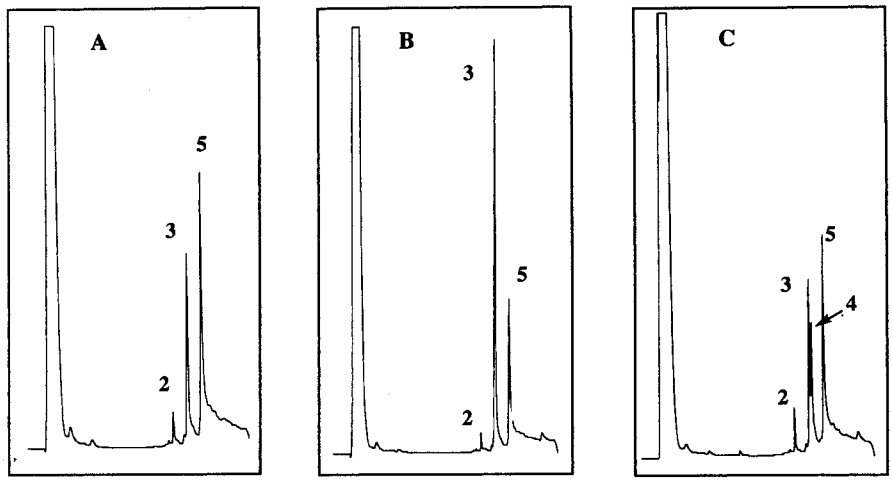

Figure 2. HPLC traces of (a) the final reaction mixture after oxidation of **1a** in 50% TFE; (b) the same mixture co-injected with antiparallel cyclic dimer **3**; and (c) the same mixture co-injected with parallel cyclic dimer **4**. Chromatographic conditions: Linear gradient from 5% to 40% B in 30 min (see the Experimental Section).

Table 1. Fragments from Tryptic Hydrolysis of Trimer

retn time[a] (min)	fragment assignment	topological assignment	exptl (calcd) mass of fragment
5.90	(*Ac*-CXK-*OH*)-*S*-*S*-(*H*-XCG-NH_2)	antiparallel	637.2 (636.27)[b]
8.57	(*Ac*-CXK-*OH*)$_2$	parallel	751.4 (750.36)[b]
15.13	*H*-LHAELSSLEAHLK-*OH*		1446.75 (1446.77)[c]
15.74	(*Ac*-CXKLHAELSSLEAHLK-*OH*)-*S*-*S*-(*H*-XCG-NH_2)	antiparallel	2065.0 (2065.04)[c]
17.49	(*H*-LHAELSSLEAHLKXCG-NH_2)$_2$	parallel	3380.4 (3379.84)[c,d]

[a] HPLC conditions: linear gradient 5 to 65% B in 30 min. Flow: 1 mL/min (A: 0.045% TFA in H_2O; B: 0.036% TFA in CH_3CN). [b] Determined by FAB-MS (glycerol). Observed mass corresponds to $[M + H]^+$. [c] Determined by ESI-MS. Observed mass is the result of deconvolution and corresponds to the unprotonated species. [d] MS performed on products with the same retention time and amino acid analyses obtained from tryptic digestion of a dimer of the appropriate topology.

different solvents is shown in Figure 3, and the compositions of the mixtures at the end of the experiment are presented in Table 2.

The percentage of TFE present during the oxidation of **1a** clearly determines the outcome of the reaction. With 0−15% TFE, cyclic monomer **2** and antiparallel dimer **3** are exclusively formed and the composition of the mixture does not depend significantly on peptide concentration. On the other hand, in 30% and 50% TFE, monomer formation is strongly reduced and the main products are dimer **3** and trimer **5** in proportions that depend on the total peptide concentration.

In the absence of TFE, formation of **2** starts immediately while formation of dimer shows a lag phase, indicating that formation of the two disulfide bonds is not simultaneous. Interestingly, while the final proportion of monomer and dimer are the same in 15% and 0% TFE, the evolutions of the two reaction mixtures over a period of time are quite different. The presence of TFE causes a 2-fold increase in the rate of conversion of the starting product and eliminates the lag period before dimer formation is detected. This indicates that both disulfide bonds are formed nearly simultaneously as would be expected in a coiled coil structure.

Trimer formation at 30% and 50% TFE generally involves a lag phase but then becomes the dominant process. In 30% TFE, **5** is kinetically favored but it is slowly converted to dimer, which becomes the dominant product at the end of the reaction. This process requires the presence of free thiols as an isolated sample of trimer was stable in buffer, without TFE, for more than 48 h. In 50% TFE formation of trimer is both kinetically and thermodinamically favored. A reaction mixture in 50% TFE did not change its composition even after 30 days.

Spontaneous Formation of Open Dimers. An equimolar mixture of **1b,c**, both with a free cysteine and the second one protected as an acetamidomethyl derivative, was allowed to react with air in 20 mM phosphate buffer (pH = 8) or in a mixture of the same buffer with 30% TFE. HPLC analysis of the mixtures formed after complete disappearance of the starting products showed the presence of dimers **6**, **7**, and **8** (characterized by comparison with authentic samples) in a ratio of 0.80: 0.95:2.25, independent of the solvent used. These ratios represent only a ca. 10% excess of antiparallel dimer with respect to the statistical mixture.

Regiospecific Synthesis of Cyclic Dimers. Parallel and antiparallel cyclic dimers with two disulfide bonds were unequivocally prepared from peptides of the same sequence, but containing different side-chain protecting groups for the cysteine.[13] All of these peptides have been synthesized by solid-phase methodology, using either Boc/benzyl or Fmoc/*t*Bu approaches with similar results.[14] The retrosynthetic analysis used to identify the appropriate protected peptides and the experimental conditions used in the synthetic process are shown in Figure 4.

(12) A possible contamination by a completely antiparallel trimer would not be detected by enzymatic digestion. However, **5** gives a single HPLC peak under different gradient conditions and is found to be homogeneous also by capillary electrophoresis.

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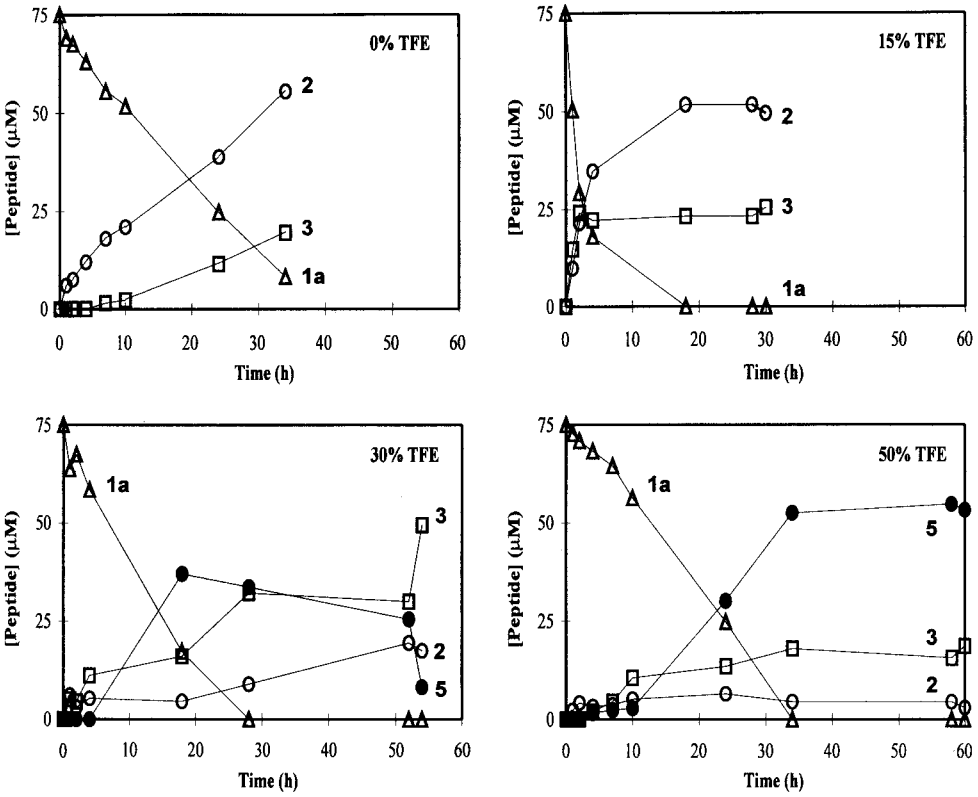

Figure 3. Time evolution of the air oxidation of **1a** (75 mM) dissolved in 20 mM sodium phosphate buffer (pH 8) containing different amounts (v/v) of TFE. Concentrations of all species are referred to monomeric chains and were obtained by integration of the areas of HPLC peaks from samples withdrawn at different times.

Table 2. Percentage of Monomer (**2**), Dimer (**3**), and Trimer (**5**) after Complete Oxidation of **1a**

	75 μM [**1a**]			150 μM [**1a**]			300 μM [**1a**]		
TFE (%)	**2** (%)	**3** (%)	**5** (%)	**2** (%)	**3** (%)	**5** (%)	**2** (%)	**3** (%)	**5** (%)
0	75	25		80	20		77	23	
15	66	34		79	21		63	37	
30	23	66	11	11	64	25	8	41	51
50	4	25	71	4	28	68	2	18	80

The preparation of the parallel dimer requires a single peptide containing two different side-chain protecting groups for the cysteine.[6,15] These protecting groups should be removed independently by different chemical mechanisms. In a first synthesis, Fm[16] and acetamidomethyl (Acm)[17] were chosen for the protection of ^{1}Cys and ^{18}Cys, respectively. Treatment of the bis-cysteine-protected peptide with piperidine−DMF (1:1) afforded easily the open dimer **6**.[18] On the other hand, formation of the second disulfide bond requires a careful choice of experimental conditions. In this case, the conditions chosen were I_2 (10−30 equiv) in 20% aqueous HOAc for 2 h at 25 °C. If less I_2 is used, cyclic monomer is formed together with the parallel dimer and traces of antiparallel dimer. With 3 equiv of I_2, the proportion of cyclic monomer to parallel dimer was 45:55. Similar results were obtained with the regioisomeric open dimer **7**, which contains a disulfide bond between both Cys residues in position 18, and therefore Cys in position 1 is protected with Acm.[19]

Regioselective formation of the first disulfide bond in an antiparallel dimer requires the synthesis of two peptides with different protecting schemes.[15] One Cys of each monomer is protected with the Acm group, and the second one is unprotected in one of the peptides and protected with 3-nitro-2-pyridinesulphenyl (Npys)[20] in the other.[21] Npys is also a leaving group directing the formation of the first disulfide bond with the free thiol of the other monomer. Once the first disulfide bridge has been formed, the second one is formed from the Acm-protected cysteines using a standard protocol.

The synthesis of the antiparallel dimer **3** is much more straightforward than that of parallel dimer **4**, providing a first indication of the completely different stability of both topoisomers that will be discussed below.

The resulting peptides were characterized by HPLC, mass spectrometry, and amino acid analysis. The expected topology

(15) (a) Ruiz-Gayo, M.; Albericio, F.; Pons, M.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1988**, *29*, 3845−3848. (b) Ruiz-Gayo, M.; Royo, M.; Fernandez, I.; Albericio, F.; Giralt, E.; Pons, M., *J. Org. Chem.* **1993,** *58*, 6319−6328.

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(18) This reaction takes place with much better yields when the monomeric protected peptide is still anchored on the solid support. If this strategy is followed, the open dimer is obtained after release of peptide from the resin. See: (a) Albericio, F.; Hammer, R. P.; García-Echeverría, C.; Molins, M. A.; Chang, J. L.; Munson, M.; Pons, M.; Giralt, E.; Barany, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 402−413. (b) Munson, M. C.; Lebl, M.; Slaninova, J.; Barany, G. *Pept. Res.* **1993**, *6*, 155−159.

(19) This open dimer was also prepared using a Fmoc/*t*Bu strategy, using Acm for the protection of Cys in postion 1 and Trt for those in position 18. After the release of the peptide from the resin with TFA in the presence of scavengers, the first disulfide bridge was produced by air oxidacion.

(20) (a) Matsueda, R.; Kimura, T.; Kaiser, E. T.; Matsueda, G. R. *Chem. Lett.* **1981**, 737−740. (b) Bernatowicz, M. S.; Matsueda, R.; Matsueda, G. R. *Int. J. Pept. Protein Res.* **1986**, *28*, 107−112. (c) Albericio, F.; Andreu, D.; Giralt, E.; Navalpotro, C.; Pedroso, E.; Ponsati, B.; Ruiz-Gayo, M. *Int. J. Pept.Protein Res*. **1989**, *34*, 124−128.

(21) These two peptides have been synthesized using a Boc/Bzl strategy, using Acm, Npys, and *p*-MeBzl. The latter is removed during the final acidolysis with HF to give the free thiol.

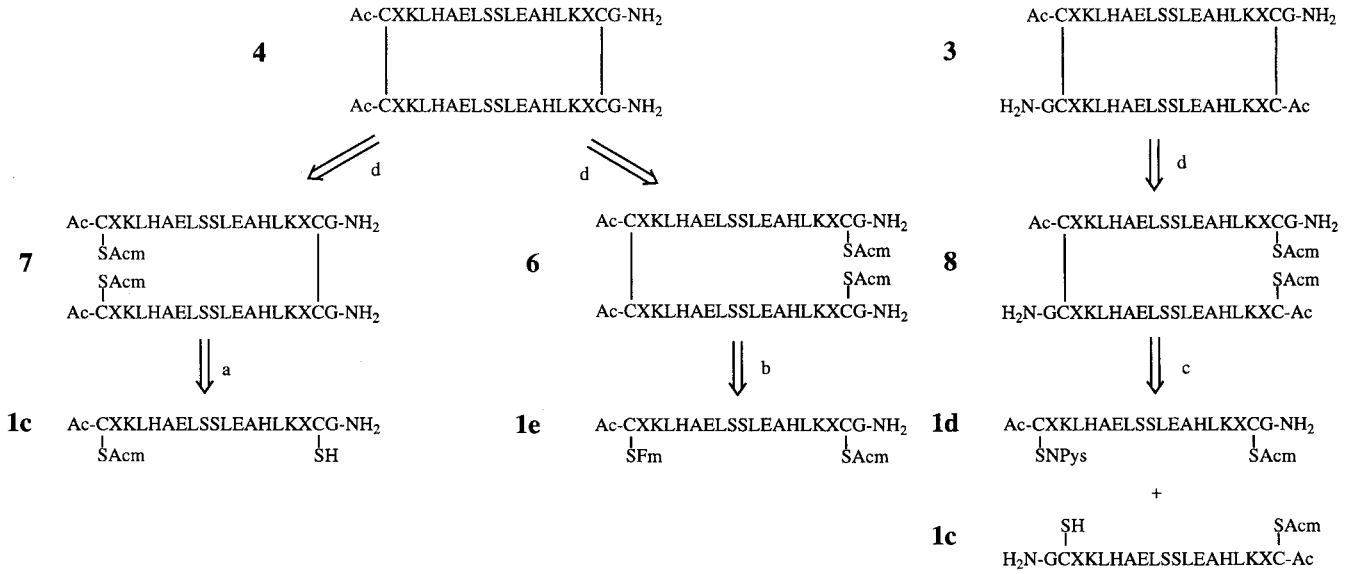

Figure 4. Retrosynthetic analysis for the univocal preparation of dimers parallel (**4**) and antiparallel (**3**). Experimental conditions for the synthesis are (a) 0.01 M Tris pH 8, air; (b) i. piperidine−DMF (1:1 v/v); ii. HF; (c) HOAc−H_2O (9:1 v/v); (d) I_2, HOAc−H_2O (8:2 v/v).

could be demonstrated in each case by tryptic digestion and analysis of the resulting fragments.

Conformational Analysis by CD. A freshly dissolved sample[22] of peptide **1f** in phosphate buffer, pH = 7.02, 25 °C, shows CD spectra typical of an α-helix, with minima at 222 and 205 nm and a maximum at 190 nm. The amount of helix present[23] varies between 27% (5 °C) and 11% (95 °C). Addition of TFE causes an increase in the helicity until a plateau is reached around 30% v/v TFE. CD spectra in buffer are concentration independent in the range between 3.6 and 150 μM. Ultracentrifugation studies carried out at 75 μM confirmed that **1f** is a monomer under these conditions. CD spectra recorded at low concentrations of TFE depend on peptide concentration, but this effect is attenuated at higher percentages of TFE. Thus, in 14% TFE, the helix population of **1f** changes from 35 to 54% when the peptide concentration is increased from 20 to 150 μM, but in 50% TFE, the helix content is 79 and 83% at the same concentrations.

CD spectra of the oxidation products of **1a** display features characteristic of an α-helix, enhanced by TFE addition, with minima at 222 and ca. 200−205 nm and a maximum around 190 nm. All spectra are concentration independent both in the presence and in the absence of TFE. The maximum helicity that can be induced by TFE varies among the members of the family. Table 3 summarizes the amount of helix present at 25 °C in different peptides.

Peptides **3** and **5** are the most helical in buffer. However, although addition of TFE does not increase the helix content of **3**, it does induce more than 90% helix in **5**. The minimum amount of helix is found in the cyclic monomer **2** both in the presence and in the absence of TFE.

Noncyclic dimers **6**, **7**, and **8** provide good models to study the effect of individual disulfide bonds on the amount of helix formed by the different dimers. The three compounds have similar CD spectra in phosphate buffer and the calculated helix content ranges from 21 to 24%. Addition of TFE however causes different increases in helix content in the three compounds. Peptide **6**, with a ^{1}Cys−$^{1'}$Cys disulfide bond is only 61% helical above 50% TFE. On the other hand, peptide **7** with a ^{18}Cys−$^{18'}$Cys bond can adopt 91% helix in the same solvent. The helix content in 50% TFE of antiparallel dimer **8**, with a disulfide bond between ^{1}Cys and $^{18'}$Cys, is 70%, close to the mean value of the helix population of the two parallel dimers.

Table 3. Helix Population of Different Peptides in Buffer and in 50% TFE

peptide	[peptide] (μM)[a]	0% TFE	50% TFE[b]
1f[c]	75	20	77
1f	150	23	83
2	30	13	28
3	75	53	53
4	81	14	56
5	75	39	93
6	75	21	61
7	150	23	91
8	75	24	70

[a] Referred to monomer. Conditions were 5 mM sodium phosphate, pH 7.02. 25 °C. [b] Prepared by mixing equal amounts of peptide solutions in buffer and TFE of the same concentration. [c] Sample annealing at pH 7.02 in the absence of TFE reduces the helix population to 11%

Comparison of Parallel and Antiparallel Cyclic Dimers. The CD spectrum of the antiparallel cyclic dimer **3** (Figure 5a) has a ratio of the intensities of the bands at 222 and 208 nm very close to that observed in typical coiled-coil dimers.[24] The helix content in buffer at 5 °C is 58%, the highest value for any peptide of this family in the absence of TFE. Addition of TFE does not change the intensity of the band at 222 nm but makes the band at 208 nm more negative. As a result, the ratio $\Delta\epsilon_{222}/\Delta\epsilon_{208}$ changes from 1.01 in buffer to 0.79 in 50% TFE while the helix content calculated from $\Delta\epsilon_{222}$ remains constant.

CD spectra of parallel cyclic dimer (**4**) in phosphate buffer (Figure 5b) present minima at 222 and 201 nm and a positive

(22) At pH 7.02 **1f** experiences a slow transition to a different conformation characterized by a lower $|\Delta\epsilon|$ at 222 nm. CD spectra of this form are only slightly temperature dependent in the range from 5 to 95 °C. Addition of TFE to aged or freshly prepared samples give similar CD spectra.

(23) The amount of helix present was calculated assuming that a 100% helical peptide of this size would have $\Delta\epsilon_{222} = -10.51$ M^{-1} cm^{-1} (Chen, Y. H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, *8*, 4108−4116). The absolute helicity observed using this method should only be taken just as a qualitative indication of the amount of helix present. However, it may also be considered as a useful parameter reflecting changes in the CD spectra under different conditions.

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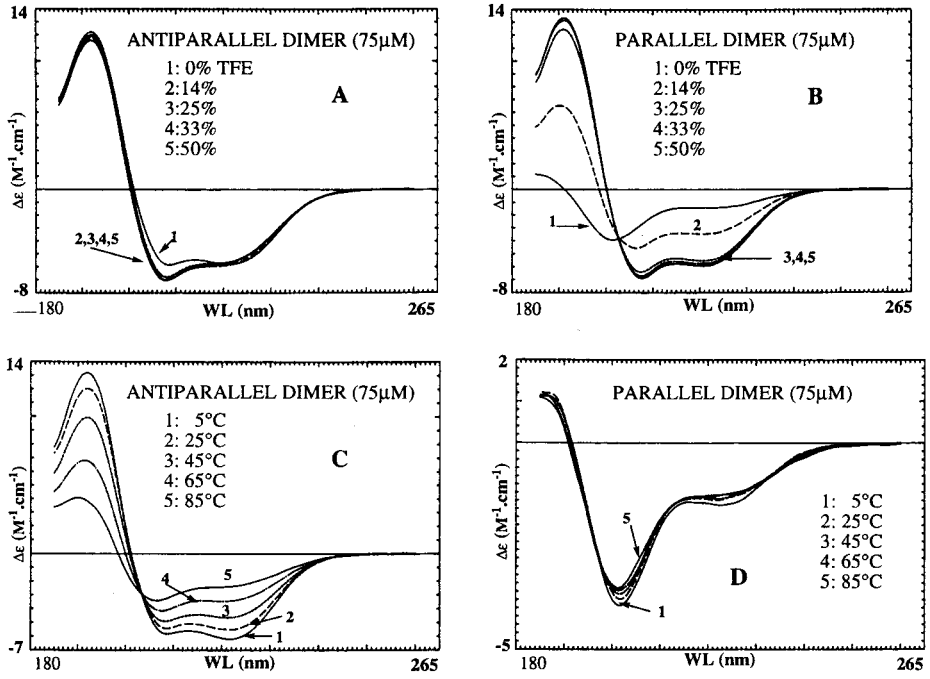

Figure 5. Circular dichroism spectra of antiparallel dimer **3** (a, c) and parallel dimer **4** (b, d) in 20 mM sodium phosphate buffer pH 7.02, 25 °C, with different amounts of TFE (v/v) (a, b) and in the same buffer, without TFE at different temperatures (c, d).

band at 187 nm indicative of a helical structure. However the value of $\Delta\epsilon_{222}$ corresponds to an helicity of only 14% at 5 °C. Addition of TFE causes an increase in helix content, and a plateau of 56% helix is attained around 25−30% TFE.

Increasing the temperature causes a reversible decrease in the amount of secondary structure of **3** in buffer, but even at 85 °C, the helix content is still of 22−24% (Figure 5c). Likewise, addition of urea (data not shown) also causes denaturation of **3**, and in 8 M urea, the helix content is reduced to 14%. The CD spectra of **4** in buffer change only very slightly with temperature (Figure 5d): at 85 °C, the calculated helicity is still 12%, very close to the 14% calculated at 5 °C.

Conformational Analysis by NMR. NMR spectra of **1f** recorded in the presence of 20% HFIP (pH 3.03) show two helical regions defined by a number of *i*,*i*+3 and *i*,*i*+4 NOEs and negative conformational shifts (Figure 6). Helix ends can be structurally defined either by the first/last residue in the expected position according to the regularity of the helix, by the first/last residue with ϕ and ψ dihedral angles in the correct range, or by a combination of both criteria. Loosely, the first criterion would correspond to the observation of *i*,*i*+3 and/or *i*,*i*+4 NOEs and the second to the observation of negative conformational shifts. Following the first criterion, the two helices span residues 1−8 and 10−17, while according to conformational shift arguments, the second helix starts at residue 12. One should keep in mind however that, while *i*,*i*+3 and *i*,*i*+4 NOEs are strong indications of a helical conformation, conformational shifts may be affected by a number of interactions including aromatic ring shifts and, to a smaller extent, solvent effects.

In freshly prepared solutions of **1f** in H_2O/D_2O (pH 3.58), *i*,*i*+3 and *i*,*i*+4 NOEs are only observed between residues 13−14 and 17, while conformational shifts suggest a single helical region, less populated than in 20% HFIP, starting at residue 12 (Figure 7b). After annealing of the sample at pH 3.94[25] by repeated heating and cooling, additional *i*,*i*+3 and *i*,*i*+4 NOEs

(25) The form annealed at pH 7.02 is very insoluble, preventing its study by NMR.

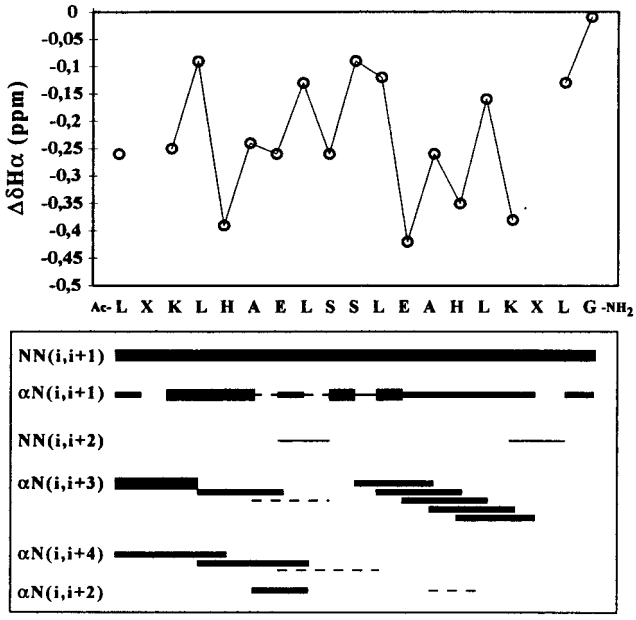

Figure 6. NMR information obtained for **1f** in d_2HFIP−D_2O−H_2O (20:8:72), pH 3.03. Top: CH^{α} conformational shifts. Bottom: observed NOEs. The width of the line indicates the intensity of the cross-peak.

could be observed in the C-terminal half of the peptide (10−13, 11−14, 10−14, 13−17, 16−19) and also in the N-terminal region (1−4, 2−5, 3−6) as well as some *i*,*i*+2 NOEs in both regions (1−3, 2−4, 14−16, and 16−18). CH^{α} conformational shifts indicate that the N-terminal helix is less populated than the C-terminal one.

NMR spectra of antiparallel dimer **3** obtained by oxidation of **1a** show only one set of resonances in agreement with the expected C_2 symmetry. The similarity in the chemical shifts of **3** in 30% TFE with those of **1f** in 20% HFIP (Figure 7a) suggests that the helix present in the dimer is similar to that found in **1f**. In the absence of fluoro alcohols, residues from ^{10}Ser to the C-terminus have similar conformational shifts. However, significant differences are observed in the N-terminal

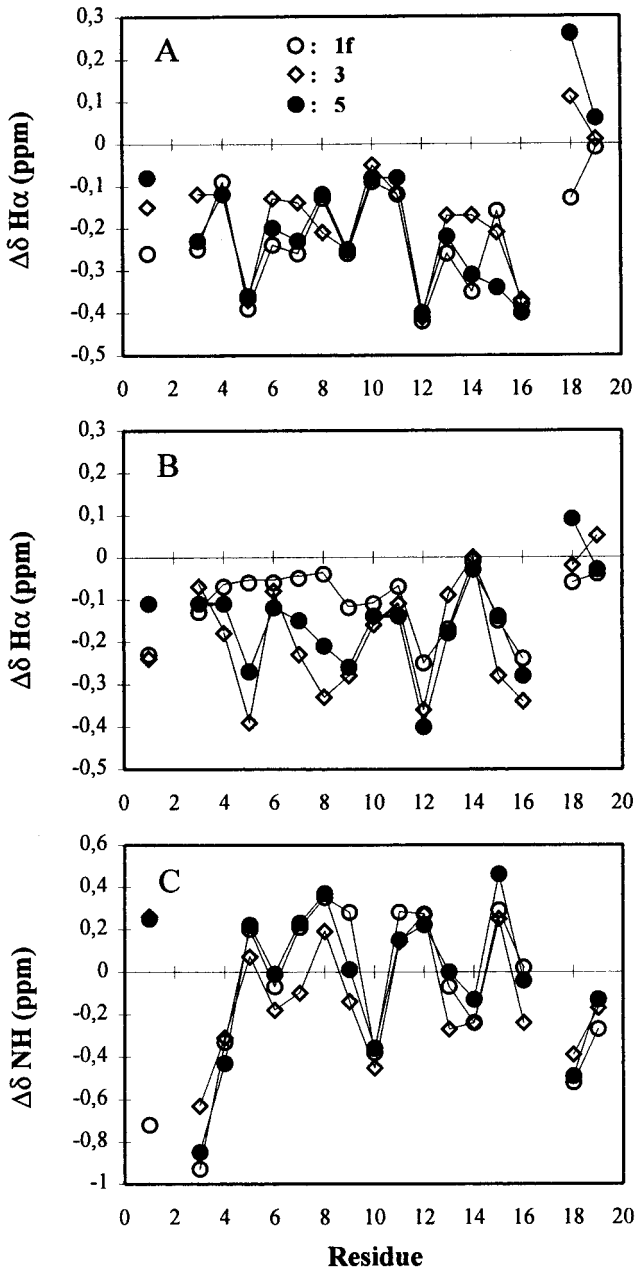

Figure 7. (a) CH^{α}-conformational shifts of **1f** in 20% HFIP and **3** and **5** in 30% TFE. (b) CH^{α}-conformational shifts in D_2O:H_2O (9:1 v/v). Symbols are the same as for panel a. (c) NH conformational shifts of **1f** in 20% HFIP and **3** and **5** in 30% TFE. Symbols are the same as for panel a.

half of the molecule which is much more helical in **3** than in **1f**. (Figure 7b).

The NMR spectra of the trimer show only one set of resonances both in 30% TFE (pH 4.7) and in water (pH 4.0 and 7.0). As in the case of **3**, chemical shifts of **5** and **1f** are very similar in the presence of fluorinated alcohols, while in water, residues 3−9 are more helical in **5** than in **1f** (Figures 7a,b). The similarity in conformational shifts between **5** and **3** is much higher than that between any of them and **1f** both in the presence or in the absence of fluorinated alcohols.

The apparent symmetry observed by NMR does not reflect the parallel−antiparallel topology of **5** found by enzymatic digestion, which should lead to three sets of signals. Backbone resonances in helices are only weakly sensitive to tertiary structure. Alternatively, because no side chain complementarity has been built into the molecule, it is very likely that **5** adopts a molten globule-like structure.

The central part of the molecule, including the two Ser residues, acts as a hinge. The exact conformation of this region could not be completely determined in our NMR experiments in any of the samples studied. In particular, of the NOEs defining the reciprocal side-chain−main-chain interactions described[26] in a peptide with a Ser-X-X-Glu sequence, only those between CH^{α} of ^{9}Ser and the β protons of ^{12}Glu could be observed for **1f** in 20% HFIP and for **3** in water but not in TFE. Spectral overlap prevents its observation in the case of **5**. NH conformational shifts in fluorinated alcohols are very sensitive to hydrogen-bond lengths.[27] Interestingly, in the three peptides studied by NMR, the NH conformational shift of ^{10}Ser is significantly lower than that of other residues in the center of the peptide (Figure 7c). This residue is probably involved in a hydrogen bond longer than the average for a helical structure, indicating the presence of a bend in the center of the helix.

Discussion

Hairpin versus Extended Helical Conformations. Serine residues have a low helical propensity but are often found in turns or in the N-cap position of helices.[28] The sequence Ser-X-X-Glu has been identified as a capping box by Harper and Rose.[29]

The peptides studied by NMR show two conformationally distinct regions separated by Ser residues. Both regions can be helical, but the C-terminal helix is more stable both in water and in the presence of HFIP. Both helices span a *i*,*i*+4 ion pair but with inverted charges. The different interaction of both ion pairs with the helix macrodipole could explain the higher stability of the C-terminal helix which is also expected to be stabilized by N-capping effects. TFE and HFIP are known to stabilize secondary structures, especially—but not only—α-helices, in many peptides.[30] Addition of TFE or HFIP increases the amount of helix observed by CD in all the peptides except **3**. NMR studies in 20% HFIP show that this is the consequence of the stabilization and extension of the two helices observed in water.

The central region, around the two Ser residues, shows little helical character in water, suggesting that this region acts as a hinge between the C-terminal helical region and the N-terminus which can be more or less helical depending on the peptide and experimental conditions. A hairpin conformation in buffer is supported by the results of spontaneous oxidation of **1a** that give cyclic monomer **2** as the main product, without detectable polymerization, suggesting that ^{1}Cys and ^{18}Cys are close in space.

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In 20% HFIP, the CH^{α} conformational shifts of ^{10}Ser and ^{11}Leu in **1f** are still less negative than for the rest of the internal residues; however, ^{9}Ser shows conformational shifts characteristic of a helical conformation and a weak NOE links its NH with the CH^{α} of ^{6}Ala and its CH^{α} with the β protons of ^{12}Glu, suggesting that the conformation of **1f** in 20% HFIP is best described as a bent helix instead of a hairpin. In agreement with this interpretation, formation of **2** from **1a** is strongly inhibited in the presence of TFE while formation of dimer and trimer, resulting from intermolecularly aggregated species, is increased in a concentration-dependent manner. The conformational shifts and temperature coefficient of the NH proton of ^{10}Ser in 20% HFIP suggest that the corresponding hydrogen bond is located on the convex side of the bend.

Helix Stability in Open Dimers: The Effect of Disulfide Bonding. Dimers with a single disulfide bond, **6**−**8**, have similar amounts of helix in buffer and are consistently more helical than the model compound **1f** with two leucines replacing both ^{1}Cys and ^{18}Cys. This is consistent with a stabilization of the helical structure and a destabilization of the hairpin conformation by intermolecular contacts. In 50% TFE the three open dimers form different amounts of helix. Dimer **7**, with only ^{18}Cys participating in disulfide bonding, is nearly completely (91%) helical, but dimer **6**, with a disulfide bond involving ^{1}Cys, shows only 61% helix in the same conditions. Antiparallel dimer **8**, with a disulfide bond between the two nonequivalent cysteine residues, gives an intermediate helix content (70%). In 50% TFE peptide **1f** forms 80% helix. The fact that **7** has a higher helix content than **1f** despite the replacement of leucine by cysteine indicates that disulfide-bond formation stabilizes the helix in peptide **7**. This is not the case of peptides **6** and **8** and suggests that the disulfide bond involving two Cys residues in position 1 is actually destabilizing. Zhou et al.[12] have shown that a disulfide bond linking *a* positions in the interior of a coiled coil causes destabilization with respect to a peptide with Leu in the corresponding positions. The fact that **8** has a helix content very close to the mean of the values found in **6** and **7** suggests an additive effect and that destabilization is the result of the conformation forced upon ^{1}Cys by the formation of an interhelical disulfide bond.

Helix Stability in Cyclic Dimers. The presence of two disulfide bonds stabilizes the helix in the antiparallel cyclic dimer in buffer. This is clearly seen by comparing the amount of helix present in **3** (ca. 50%) and in **1f** and **6**−**8**, all of which form around 20% helix. On the other hand, in a helix inducing solvent, the helix content of **3** does not change, while that of acyclic dimers **6**−**8** and the model compound **1f** increase to the range 60−91%. From the analysis of acyclic dimers **6**−**8,** it has been shown that disulfide bonds involving ^{1}Cys are helix-destabilizing, and therefore, it is not surprising to find that **3**, with two such disulfide bonds, is less helical in 50% TFE than **8**.

Parallel cyclic dimer **4** presents only 14% helix in buffer, but it can reach 56% helix in 50% TFE. This value is close to the maximum helix that can be induced in peptide **6** with a ^{1}Cys−$^{1'}$Cys disulfide bond (58%). This indicates that formation of *two* disulfide bonds by itself does not prevent helix formation.

CD spectra of **4** indicate a low helix content (11−14%) in buffer that does not decrease at high temperatures. On the other hand it can be increased by addition of TFE. On the contrary, **3** is more helical in buffer but the amount of helix does not increase upon TFE addition and is reduced at higher temperatures. The ratios $\Delta\epsilon_{222}/\Delta\epsilon_{208}$ in CD spectra recorded in buffer at 5 °C are in the range 1.02−1.07 but are reduced to 0.7−0.9 at high temperatures and in the presence of 50% TFE. Those features indicate that **3** forms a coiled-coil in buffer stabilized by two disulfide bonds. On the other hand, the similarities in the CD spectra of **4** with those of cyclic monomer **2** suggest that **4** has a hairpin conformation in buffer.

The completely different stability of the parallel and antiparallel cyclic coiled-coils could be the result of electrostatic interactions involving either charged side chains or the helix dipole or could be the result of packing effects. The palindrome character of the peptides under study should compensate for differential electrostatic contributions from charged side chains between both dimers. Packing effects and the interaction of helix dipoles are the remaining interactions. While the former are expected to be very different in cyclic and acyclic dimers, macrodipole interactions should be comparable and should become more important as the helix content is increased and in solvents of lower polarity. The fact that **7** has higher helicity than **8** in 50% TFE suggests that interaction between macrodipoles is not the dominant effect and that the different conformational preferences of **3** and **4** are probably due to packing effects.

The sequences of **1a**−**f** have some of the characteristics of coiled-coil forming peptides and can be analyzed as a heptad repeat *(abcdefg)$_2$abcde* with leucine or cysteine residues at positions *a* and *d*. The two hydrophobic positions in the repeat are not equivalent in coiled-coils. Considering the dimers as coiled-coils, a different hydrophobic packing is expected for **3** and **4.** While the former would involve only *ad* contacts, *aa* and *dd* pairs would alternate along the dimer interface in the parallel dimer. Leucine residues, in contrast to β-branched residues, show only a weak discrimination for different types of knobs-into-holes packing,[31] and therefore, it is unlikely that this difference can account for the complete absence of parallel dimer in the oxidation products of **1a**, although simultaneous formation of two disulfide bonds in a relatively short peptides may enhance the packing differences. Recently, the study of the narrowest part of low-angle helix pairs has shown a strong preference for antiparallel pairing. The sequences involved a form that has been described as the alacoil motif,[32] with small residues in positions *a* and *d* of an heptad repeat. The interhelical separation in alacoil dimers is 7.5−8.5 Å, while typical parallel coiled-coils are separated by 9.6 Å. The heptads in ferritin-type alacoils are offset by 0.25 of an heptad, while in ROP-type alacoils, the offset is half an heptad, i.e., a complete helix turn.

While alacoil-type packing effects could explain the preference for antiparallel versus parallel cyclic dimers they do not explain the specific formation of a cyclic trimer from **1a** in the presence of high percentages of TFE.

Spontaneous Disulfide-Bond Formation from Unprotected Cysteine-Containing Peptides. Oxidation of equimolar mixtures of peptides containing an unprotected Cys residue at either position 1 or 18 gives open dimers in proportions similar to those expected in an statistical mixture, and this result is not affected by the addition of TFE. On the other hand, simultaneous formation of two disulfide bonds is very selective, both with respect to the number of peptide molecules involved in the formation of the cyclic product and their relative orientation. The composition of the final reaction mixture is strongly dependent on the presence of TFE and also on the concentration

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of the starting peptide, but only one of the possible dimers or trimers is apparently formed.

The control of product formation can be rationalized using a model based on the conformational preferences of this particular sequence. At low concentrations of TFE intramolecular cyclization is the dominant process, in agreement with other evidences suggesting a hairpin conformation in the absence of TFE. Intermolecular disulfide-bond formation is a competitive process but does not lead immediately to cyclic dimer as a presumably slow conformational rearrangement to a coiled-coil-type conformation would be required. Addition of small amounts of TFE causes a transition from a hairpin to a bent helix conformation in which intramolecular disulfide-bond formation is prevented.

In a continuous straight helix ^{1}Cys and ^{18}Cys would be placed on the same side of the helix. This would favor the formation of dimers, but little discrimination between parallel and antiparallel dimers would be expected even considering the small angular displacement of the two Cys residues caused by the fact that they are separated by a fractional number of heptad repeats. Helix disruption at the center of the peptide has the effect of altering the relative phase of the two helical moieties, and therefore, the two cysteines will be pointing in different directions around the helix axis. Simultaneous bending of this axis has the result of preventing the simultaneous formation of two disulfide bonds in a parallel arrangement but not if the two helices are antiparallel.

Therefore, after the formation of the first disulfide bond under conditions favoring the formation of helices, the antiparallel open dimer yields a cyclic antiparallel dimer but the parallel open dimer cannot produce cyclic dimer. Addition of a third molecule however allows the formation of a cyclic trimer with parallel antiparallel topology.

Interplay between cyclization strain and helix stability is illustrated by the comparison of **3** and **4**. Both parallel and antiparallel cyclic dimers can span the same range of helix content under different experimental conditions. However, **3** forms the maximum amount of helix in water and the addition of TFE cannot increase it any further. On the other hand, **4** in buffer has the minimum amount of helix and heating of the sample does not decrease the amount of helix observed. We believe that the two limits are set by the cyclization constraints. The upper limit probably arises from a restriction on the relative orientation of the cysteine residues around the helix and the lower one by supercoiling of the cycle or interchain contacts as the helix present in the cyclic dimer is denatured.

Interestingly, the main oxidation product of **1a** in different solvents can be related to the amount of helix that can be accommodated in the cyclic molecules and that adopted by **1f**, taken as a model for **1a** (cf. Table 3). Peptide **1f** in 50% TFE forms more helix than the maximum compatible with the cyclization constrains induced by the disulfide bonds in the cyclic monomer and the antiparallel dimer but not in the trimer, and **5** is the dominant species in this solvent. Likewise, in the absence of TFE, the helicity of **1f** agrees with that of **2,** which is the main product under these conditions. Finally, at intermediate percentages of TFE, the antiparallel dimer is favored in agreement with the intermediate helix content of **1f** and **3** in 30% TFE.

Cyclic Trimer 5. Symmetry and Topology. Coiled-coil trimers have been described for a number of model coiled-coils[33] and also in natural proteins.[34] However, simultaneous formation of three disulfide bonds is incompatible with classical helix bundles either parallel or antiparallel in which helix axes form a small angle and have an overall cylindrical shape. To accommodate the three disulfide bonds, **5** should have a more spherical shape with helices crossing at a wider angle. In natural proteins the relative orientations of packed proteins have a distribution with a major peak at −50° and a minor peak at +20°.[35] Helix assemblies in many globular proteins can be embodied in a simple geometrical model, the "quasi-spherical polyhedron model",[36] based on the realization that the packing geometry of α-helices around a core can be described by polyhedra. The packing of three helices can be described by an octahedron in which helical axes run along three nonintersecting edges (two enantiomorph forms are possible). Helices in this model cross at an angle of −60°, close to the optimal of −50°. The modeling of natural proteins with this fold shows that three interhelical disulfide bonds forming a cyclic trimer can indeed be introduced without major disturbances in the packing. Figure 8 shows a schematic diagram of one of the possible quasi-spherical folds of the trimer.

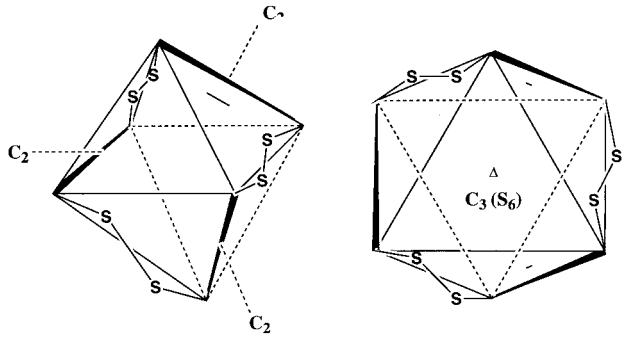

Figure 8. Schematic representations of the quasi-spherical arrangement of the three helical peptide chains in a cyclic trimer. Helices, comprising a complete peptide chain, are represented by a straight bold lines and drawn on three nonintersecting edges of an octahedron. Disulfide bonds linking the three peptide chains forming an overall cyclic structure are indicated. Pseudo-symmetry elements apply to a trimer of a palindromic peptide and do not consider the intrinsic directionality of the peptide bond. The S_6 pseudo-axis could exist only in a peptide made of nonchiral (e.g., Aib) or a combination of D- and L-amino acids. Only one of the enantiomorph forms is shown.

The high symmetry of the parent octahedron is certainly reduced in the helix assembly. By modeling each peptide chain as a cylinder (i.e., not considering the directionality of the amide bonds and the chirality of the constituent amino acids and of the α-helix), the resulting object would have an S_6 and three C_2 symmetry axes. The chiral nature of the peptide helices reduces the S_6 axis to C_3. The directionality of the peptide backbone destroys the C_2 axes, and the symmetry of the completely antiparallel trimer would be just C_3. Finally, the parallel antiparallel topology of **5** gives a completely asymmetrical molecule.

The use of a palindromic sequence, as here, alleviates the problem of the directionality of the peptide bond and increases the effective symmetry of the trimer, making it more suitable as a molecular framework from which new receptors can be built.

We are presently studying the sequence determinants of the spontaneous trimerization of **1a** and the exact three-dimensional

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structure of **5** to be able to modulate the residues located in the interior of such a cavity. This could open the way to a family of de novo designed proteins with a quasi-spherical globular fold which could include new receptor molecules of considerable structural complexity and versatility but that could be produced easily by chemical or even biotechnological methods.

Experimental Section

Materials. *p*-Methylbenzhydrylamine hydrochloride resin was obtained from Novabiochem (Läufelfingen, Switzerland). Boc-Cys-(Acm)-OH and Boc-Cys(Fm)-OH were from Bachem-California (Torrance, CA). Boc-Cys(Npys)-OH was obtained from Kokusan (Japan), and the rest of Boc-amino acids and Fmoc-amino acids were purchased from Novabiochem and Advanced Chemtech. Agarose-bound trypsine [E.C. 3.4.21.2] was from Sigma (St. Louis, MO). Solvents for peptide synthesis (DMF and DCM) and HPLC (MeCN) were from Scharlau (Barcelona, Spain). HPLC was carried out on a Shimadzu apparatus with a SIL-6B automatic injector, a model SCL-6B system controller, a SPD-6A UV spectrophotometric detector, two LC-6A liquid chromatography pumps, and a C-R6A plotter. Samples were injected onto a Vydac C18 reversed-phase column (5 μm, 250 × 50 mm) from The Separations Group (Hesperia,CA), developed with linear gradients (gradient 1: from 5% B to 65% B in 20 min; gradient 2: from 10% B to 50% B in 30 min) of B [MeCN (+0.036% TFA)] into A [H_2O (+0.045% TFA)], flow rate 1 mL/min and detection at 220 nm. Reverse-phase MPLC was carried out using a Duramat pump, a 757 Applied Biosystems absorbance detector, a Gilson FC 205 automatic fraction collector, and a Pharmacia LKB-REC 101 plotter. Samples were loaded on the top of a Vydac C18 column (15−20 μm, 270 × 27 mm (column 1) or 100 × 25 mm (column 2)). Unless stated otherwise a convex gradient was used starting from H_2O−MeCN−TFA (85:15:0.1, 400 mL) taken to H_2O−MeCN−TFA (60:40:0.1, 400 mL), flow rate 2.5 mL/min, detection at 220 nm. Preparative HPLC was carried out using a Labomatic apparatus comprising a Labomat VS-200 system controller, a Labomatic HD-200 high-pressure metering pump, a Labocord 700 UV/vis detector, a Labocol Roto 100 automatic fraction collector, and a Servoscribe 1s plotter. Amino acid analyses of peptide hydrolysates (6 N HCl, 150 °C 90 min) were run in a Beckman 6300 autoanalyzer. Aib and Cys were not quantified. Analytical capillary electrophoresis was performed in a Applied Biosytems 270A instrument using a fused silica 72-cm capillary (50 μm i.d., 50 cm to detector) at 275 V/cm, 30 °C, in 20 mM sodium citrate buffer (pH 2.5), and detection at 220 nm. MS (ES and FAB) were recorded in a VG Quattro apparatus from Fisons Instruments or in the facilities of the University of Minnesota (Minneapolis).

Solid-Phase Peptide Synthesis. The peptides (**1a**−**f**) were synthesized by solid-phase methodology on a ABI 430A peptide synthesizer on a *p*-methylbenzhydrylamine resin (0.25 mmol, $f = 0.81$ mmol of NH_2/g of resin). All couplings were carried out with symmetrical anhydride activation of the Boc-amino acids, previously preformed with Boc-amino acid (1 mmol, 4 equiv) and DCC (0.5 mmol, 2 equiv). Syntheses were carried out using the standard protocols dictated by the manufacturer. The final acetylation was performed manually with Ac_2O−DCM (1:9, v/v). The peptides were cleaved from the resin and deprotected by treatment with HF−anisole (9:1, v/v) for 60 min at 0 °C.

Tryptic Hydrolysis. A suspension of trypsin [E.C. 3.4.21.2] (25−50 μL) attached to agarose (1−2 UE) was added to 1 mL of Na_2HPO_4−NaH_2PO_4 0.02 M (pH = 7.9−8) buffer, and the supernatant was removed by centrifugation. The enzyme was washed three more times with phosphate buffer solution, and then 1−2 μmol of peptide dissolved in 1.5 mL of phosphate buffer solution was added. The tryptic digestion was carried out at 38 °C. Aliquots of the solution were removed at different times and analyzed by HPLC. Fractions corresponding to different fragments were collected, lyophilized, and characterized by MS (FAB or ESI) and gave correct amino acid analysis.

Ac-Leu-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Leu-Gly-NH_2 (1f). The crude peptide (65 μmol) was initially purified by semipreparative HPLC on a HD-Sil-100C C-18 (5μ,_250 × 20 mm) column, at a flow rate of 25 mL/min, using a linear gradient starting from 20% B taken to 45% B over 30 min. Fractions containing the title peptide as a major component were combined giving 22.3 μmol (34% yield). A second purification by MPLC using column 1 gave pure **1f** with a yield of 45%. The purity (>99%) was determined by analytical HPLC using three different linear gradients (gradient 1, 20% to 50% B over 30 min and 20% to 40% B in 30 min) and HPCE. Amino acid analysis: Ala 1.79(2), Glu 2.18-(2), Gly 0.81(1), His 1.93(2), Leu 6.08(6), Lys 1.99(2), Ser 1.72(2). FAB-MS: 2071.1 $[M + H]^+$ (2071.5). Tryptic digestion: H_2N-Aib-L-G-NH_2 (FAB-MS: 273.1 $[M+H]^+$), Ac-L-Aib-K-COOH (FAB-MS: [387.3 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (FAB-MS: 1447.6 $[M + H]^+$), H_2N-L-H-A-E−L-S-S-L-E-A-H-L-K-Aib-L-G-NH_2 (FAB-MS: 1700.9 $[M + H]^+$).

Ac-Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Cys-Gly-NH_2 (1a). The crude peptide was treated with an aqueous solution of 10 mM DTT (60 mL) at room temperature for 2 h. The mixture was acidified to pH 3−4 and lyophilized. The crude was desalted by gel filtration (Sephadex G-10, 900 × 30 mm) with HOAc−H_2O (9:1, v/v) giving 53 μmol (90% recovery peptide). Desalted product (33 μmol) was purified by MPLC (column 1) giving 11 μmol (34% yield) of **1a**. The purity (>98%) was determined by analytical HPLC (gradients 1 and 2) and HPCE. Amino acid analysis: Ala 1.82(2), Glu 2.16(2), Gly 0.94(1), His 1.93(2), Leu 4.03-(4), Lys 2.03(2), Ser 1.74(2). FAB-MS: 2051.1 $[M + H]^+$ (2051.4). Tryptic digestion: H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (ESI-MS: 1206.4 ± 0.1), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS: 1447.8 ± 0.1).

Ac-Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Cys(Acm)-Gly-NH_2 (1b). The crude peptide was treated with DTT and desalted as above, giving 53 μmol of peptide (81% recovery peptide). Purification by MPLC (column 1) afforded 21 μmol (66% yield) of **1b**. The purity (>99%) was determined by analytical HPLC (gradients 1 and 2) and HPCE. Amino acid analysis: Ala 2.04-(2), Glu 2.21(2), Gly 1.04(1), His 1.78(2), Leu 3.78(4), Lys 1.92(2), Ser 1.88(2). FAB-MS: 2122.7 $[M + H]^+$ (2122.5). Tryptic digestion: H_2N-Aib-C(Acm)-G-NH_2, H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1205.6 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS:: 1447.8 ± 0.1), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C(Acm)-G-NH_2 (FAB-MS: 1762.1 $[M + H]^+$).

Ac-Cys(Acm)-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Cys-Gly-NH_2 (1c). The crude peptide (80 μmol) was treated with 10 mM DTT (60 mL) at room temperature for 2 h, acidified to pH 3−4 and lyophilized. The crude product was desalted by gel filtration (Sephadex G-10, 900 × 30 mm) with HOAc−H_2O (9:1, v/v) giving 53 μmol (90% recovery peptide). 32 μmol of desalted peptide were purified by MPLC (column 1) giving 21 μmol (66% recovery) of **1c**. The purity (>95%) was determined by analytical HPLC (gradients 1 and 2) and HPCE. Amino acid analysis: Ala 2.11-(2), Glu 2.2(2), Gly 0.91(1), His 1.75(2), Leu 3.86(4), Lys 1.9(2), Ser 1.68(2). FAB-MS: 2122.6 $[M + H]^+$ (2122.3). Tryptic digestion: H_2N-Aib-L-G-NH_2, Ac-C(Acm)-Aib-K-COOH (FAB-MS: 447.2 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (FAB-MS: 1447.6 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C-G-NH_2 (FAB-MS: 1700.9 $[M + H]^+$).

Ac-Cys(Npys)-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Cys(Acm)-Gly-NH_2 (1d). The crude peptide (14 μmol) was purified by MPLC using column 1 with a convex gradient from A (400 mL, H_2O−MeCN−TFA (70:30:0.1)) taken to B (400 mL, H_2O−MeCN−TFA (65:35:0.1)). Fractions containing pure peptide were combined, giving 4.5 μmol (33% yield) of **1d**. The purity (>95%) was determined by analytical HPLC (gradient 1) and HPCE. Amino acid analysis: Ala 2.1(2), Glu 2.14(2), Gly 0.92(1), His 1.92-(2), Leu 3.96(4), Lys 1.86(2), Ser 1.66(2). FAB-MS: 2276.2 $[M + H]^+$ (2276.6). Tryptic digestion: H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1206.4 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (FAB-MS: 1447.9 $[M + H]^+$).

Ac-Cys(Fm)-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-Cys(Acm)-Gly-NH_2 (1e). The crude peptide (47 μmol) was purified by MPLC (column 1) using a convex gradient starting from A (400 mL, H_2O−MeCN−TFA (80:20:0.1)) taken to B (400 mL, H_2O−MeCN−TFA (60:40:0.1)). Fractions were combined

and lyophilized giving 7 μmol (14% yield) of **1e**. The purity (>95%) was determined by analytical HPLC (gradient 1 and a linear gradient from 20% to 60% B in 30 min) and HPCE. Amino acid analysis: Ala 1.78(2), Glu 2.06(2), Gly 0.99(1), His 1.78(2), Leu 3.93(4), Lys 1.98(2), Ser 1.78(2). FAB-MS: 2301.1 $[M + H]^+$ (2300.7). Tryptic digestion: H_2N-Aib-C(Acm)-G-NH_2, H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1205.7 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS: 1448.0 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C(Acm)-G-NH_2 (FAB-MS: 1763.6 $[M + H]^+$). Ac-Cys(Fm)-Aib-K-COOH (FAB-MS: 555.3 $[M + H]^+$)

(^{1}Cys-$^{1'}$Cys)-bis(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys(Acm)-Gly-NH_2) (6). 1e peptide resin (0.55 g) (f = 0.18 mmol/g of peptide resin) was washed with DCM (5 × 4 mL) and DMF (3 × 4 mL) and suspended in 4 mL of piperidine:DMF solution (1:1, v/v) during 3 h at room temperature. The resin was washed with DCM (3 × 4 mL), DMF (3 × 4 mL), and DCM (3 × 4 mL). This resin (0.29 g, 49.8 μmol) was cleaved with anhydrous HF, giving 48 μmol (96% yield) of crude product. This product (39 μmol) was purified by MPLC (column 1) using a convex gradient from A (300 mL, H_2O−MeCN−TFA (90:10:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). Fractions containing pure peptide were collected and lyophilized, giving 5 μmol (12% yield). The purity (>90%) was determined by analytical HPLC (gradients 1 and 2) and HPCE. Amino acid analysis: Ala 1.86(2), Glu 1.94(2), Gly 0.83(1), His 1.89(2), Leu 4.03(4), Lys 1.91(2), Ser 1.74(2). ESI-MS: 4240.4 ± 0.3 (4240.6). Tryptic digestion: H_2N-Aib-C(Acm)-G-NH_2, (Ac-C-Aib-K-COOH)$_2$ (FAB-MS:751.4 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1205.7 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS: 1446.9), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C(Acm)-G-NH_2 (FAB-MS: 1762.9 $[M + H]^+$).

(^{18}Cys-$^{18'}$Cys)-bis(Ac-^{1}Cys(Acm)-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys-Gly-NH_2) (7). 1c (6.5 μmol) was dissolved in 3 mL of Tris-HCl (0.01 M pH = 8), and the pH was adjusted with a solution of Tris-HCl (0.2 M) and stirred vigorously. After complete disappearance of the starting material (by HPLC and Ellman test), the reaction was stopped by addition of HOAc until pH = 3−4. Lyophilization afforded 4.25 μmol of product (65% yield). The crude dimer was desalted by gel filtration (Sephadex G-15, 750 × 25 mm) eluted with 0.1 N HOAc (65% yield) and purified by MPLC using column 2 with a convex gradient from A (300 mL, H_2O−MeCN−TFA (85:15:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). Relevant fractions were combined and lyophilized giving 2.41 μmol (57% yield) of pure **7**. The purity (>99%) was determined by analytical HPLC (gradients 1 and 2) and HPCE. Amino acid analysis: Ala 2.01(2), Glu 2.1(2), Gly 0.99(1), His 1.62(2), Leu 3.75-(4), Lys 1.92(2), Ser 1.78(2). FAB-MS: 4241.6 $[M + H]^+$ (4241.6). ESI-MS: 4240.9 ± 0.6 (4240.6). Tryptic digestion: Ac-C(Acm)-Aib-K-COOH (FAB-MS:448.2 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (ESI-MS: 1206.3) H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS: 1447.3), (^{18}Cys-$^{18'}$Cys)(H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-^{18}C-G-NH_2)(H_2N-Aib-$^{18'}$C-G-NH_2) (ESI-MS: 1952.47), (H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-^{18}C-G-NH_2)$_2$ (ESI-MS: 3379.9).

(^{1}Cys-$^{18'}$Cys)(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys(Acm)-Gly-NH_2)(Ac-$^{1'}$Cys(Acm)-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-$^{18'}$Cys-Gly-NH_2) (8). 1d (11.4 μmol) was dissolved in 10 mL of HOAc−H_2O (9:1, v/v, pH = 2), and 9.22 μmol of **1c** was added dropwise. Small quantities of Na_2CO_3 were added to adjust the pH to 4.0−4.5. Disappearance of the starting products was followed by HPLC. The crude peptide was lyophilized, giving 9.2 μmol of crude peptide that was purified by MPLC (55% yield) using a convex gradient from A (300 mL, H_2O−TFA (100:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). The purity was determined by analytical HPLC (>90% using gradient 1) and HPCE (75%). Amino acid analysis: Ala 1.89(2), Glu 2.11(2), Gly 0.94(1), His 1.86(2), Leu 4.1-(4), Lys 1.95(2), Ser 1.83(2). FAB-MS: 4241.2 $[M + H]^+$ (4241.6). ESI-MS: 4240.5 ± 0.3 (4240.6). Tryptic digestion: H_2N-Aib-C(Acm)-G-NH_2 (FAB-MS: 356.1 $[M + Na]^+$), (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K-COOH)(H_2N-G-$^{18'}$C-Aib-NH_2) (FAB-MS: 637.1 $[M + H]^+$), Ac-C(Acm)-Aib-K-COOH (FAB-MS: 448.2 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1207.0 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (FAB-MS: 1448.3 $[M + H]^+$), (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH)(H_2N-Aib-$^{18'}$C-G-NH_2) (ESI-MS: 2065.1), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C(Acm)-G-NH_2 (ESI-MS: 1761.0).

(^{1}Cys-$^{1'}$Cys)(^{18}Cys-$^{18'}$Cys)-bis(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys-Gly-NH_2) (4). 7 or **8** (2.23−4.24 μmol) was dissolved in 10−15 mL of HOAc−H_2O (4:1, v/v) (3 μmol Acm/mL) and treated with 15.6−10.9 mg of I_2 (0.035−0.042 mmol, 15−10 equiv) at room temperature. Disappearance of the starting material was followed by HPLC. The reaction mixture was diluted with an equal volume of H_2O, and excess I_2 was eliminated with CCl_4 washes (4 × 10 mL). The aqueous layer was concentrated and lyophilized, giving 1.88−2.54 μmol of peptide (84−60% yield). A 2.54-μmol sample of crude were purified by MPLC using column 1 with a convex gradient from A (300 mL, H_2O−MeCN−TFA (85:15:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). The pure fractions were combined and lyophilized, giving 1.2 μmol (47% yield). The purity was determined by analytical HPLC (>99% using gradient 1 and >96% using a linear gradient from 5% to 50% B in 30 min). Amino acid analysis: Ala 1.77(2), Glu 2.17(2), Gly 1.0-(1), His 1.73(2), Leu 3.67(4), Lys 2.14(2), Ser 1.77(2). ESI-MS: 4096.5 ± 0.4 (4096.8). Tryptic digestion: (Ac-^{1}C-Aib-K-COOH)$_2$ (FAB-MS: 750.7 $[M + H]$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1207.0 $[M + H]^+$), (^{1}Cys-$^{1'}$Cys)(Ac-^{1}C-Aib-K -L-H-A-E-L-S-S-L-E-A-H-L-K-COOH)(Ac-$^{1'}$C-Aib-K-COOH) (ESI-MS: 2065.5), (H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-Aib-C-G-NH_2)$_2$ (ESI-MS: 3380.4).

(^{1}Cys-$^{18'}$Cys)(^{18}Cys-$^{1'}$Cys)-bis(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys-Gly-NH_2) (3). 8 (19μmol) was dissolved in 45 mL of HOAc−H_2O (4:1, v/v) and treated with 33.9 mg of I_2 (0.13 mmol, 7 equiv) at room temperature until complete disappearance of the starting material. After elimination of excess I_2 with CCl_4 washes (4 × 20 mL), the aqueous layer was lyophilized, giving 12.7 μmol of **3** (67% yield). A portion of crude peptide (5 μmol) was purified by MPLC using a convex gradient from A (300 mL, H_2O−MeCN−TFA (90:10:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). The pure fractions were combined, giving 2.32 μmol (46% yield). The purity (>98%) was determined by analytical HPLC (using linear gradients from 5% to 65% B in 30 min and from 5% to 40% B in 30 min) and HPCE. Amino acid analysis: Ala 2.01(2), Glu 1.87(2), Gly 0.95(1), His 1.98(2), Leu 4.09(4), Lys 2.07(2), Ser 1.69(2). ESI-MS: 4097.0 ± 0.1 (4096.8). Tryptic digestion: (^{1}Cys-$^{18'}$Cys)(Ac-1C-Aib-K-COOH)(H_2N-G-$^{18'}$C-Aib-NH_2) (FAB-MS: 637.3 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1206.7 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-COOH (FAB-MS: 999.4 $[M + H]^+$), (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K -L-H-A-E-L-S-S-L-E-A-H-L-K-COOH)(H_2N-Aib-$^{18'}$C−G-NH_2).

Nondirected Formation of Disulfide Bridges. Air oxidation[37] of unprotected cysteine-containing peptides was carried out by vigorous stirring of the peptide solutions dissolved in Na_2HPO_4−NaH_2PO_4 0.02 M (pH = 7.9−8) buffer or in the same buffer with the addition of variable amounts of TFE. To prevent evaporation of TFE, the reaction vessels were kept inside a desiccator saturated with the same buffer−TFE mixture. Reactions were monitored by analytical HPLC and Ellman test. When the starting material disappeared, the reactions were stopped by acidification to pH 3−4 with HOAc. Oxidation products were identified by co-injection with authentic samples.

(^{1}Cys-$^{1'}$Cys)(^{18}Cys-$^{18''}$Cys)($^{18'}$Cys-$^{1''}$Cys)-tris-(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys-Gly-NH_2) (5). 1a (6 μmol) was dissolved in 5 mL of Na_2HPO_4−NaH_2PO_4 (0.02 M) at pH 7.9 and added to 15 mL of a TFE−Na_2HPO_4−NaH_2PO_4 buffer (2:1, v/v). The pH was adjusted to 7.9−8 with 0.0.1 N NaOH. The reaction proceeded under vigorous stirring and was monitored by analytical HPLC. After 72 h the reactions was quenched by addition of HOAc until pH 2−3 and the sample was lyophilized, giving 4.4 μmol (73% yield referred to monomeric chain) of a mixture of **2**, **3**, and **5** with a ratio 5:11:84 (**2**:**3**:**5**). This crude was purified by MPLC using column 2 with a convex gradient from A (300 mL, H_2O−

(37) No catalyst was added during the oxidation. However, the possibility that traces of metal ions can be present has not been explicitly ruled out at this point.

MeCN−TFA (90:10:0.1)) taken to B (300 mL, H_2O−MeCN−TFA (60:40:0.1)). Relevant fractions were combined and lyophilized, giving 1.08 μmol of **5** (25% yield). The purity (>99.9%) was determined by HPLC (gradients 1 and 2 and a linear gradient from 20% B to 40% B in 30 min). Amino acid analysis: Ala 1.96(2), Glu 2.16(2), Gly 0.99(1), His 1.88(2), Leu 3.97(4), Lys 1.96(2), Ser 1.9(2). ESI-MS: 6145.3 ± 0.3 (6145.3). Tryptic digestion: (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K-COOH)-(H_2N-G-$^{18'}$C-Aib-NH_2) (FAB-MS:637.2 $[M + H]^+$), (Ac-C-Aib-K-COOH)$_2$ (FAB-MS:751.4 $[M + H]^+$, H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (FAB-MS:1446.75 $[M + H]^+$), (^{1}Cys-18′Cys)(Ac-^{1}C-Aib-K -L-H-A-E-L-S-S-L-E-A-H-L-K-COOH)(H_2N-Aib-$^{18'}$C-G-NH_2) (ESI-MS: 2065.04).

Cyclo-(^{1}Cys-^{18}Cys)(Ac-^{1}Cys-Aib-Lys-Leu-His-Ala-Glu-Leu-Ser-Ser-Leu-Glu-Ala-His-Leu-Lys-Aib-^{18}Cys-Gly-NH_2) (2). MPLC of the same reaction mixture used for the preparation of **5** afforded also 0.6 μmol of pure **2** as a byproduct. The purity (>99.9%) was determined by analytical HPLC (gradients 1 and 2). Amino acid analysis: Ala 2.1(2), Glu 2.3(2), Gly 1.26(1), His 1.52(2), Leu 3.58(4), Lys 1.75(2), Ser 2.0(2). ESI-MS: 2048.3 ± 0.2 (2048.04). Tryptic digestion: (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K-COOH)(H_2N-G-$^{18'}$C-Aib-NH_2) (FAB-MS: 637.3 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-COOH (FAB-MS: 1207.0 $[M + H]^+$), H_2N-L-H-A-E-L-S-S-L-E-A-H-L-K-COOH (ESI-MS: 1448.3), (^{1}Cys-$^{18'}$Cys)(Ac-^{1}C-Aib-K -L-H-A-E-L-S-S-L-E-A-H-L-K-COOH)(H_2N-Aib-$^{18'}$C-G-NH_2) (ESI-MS: 2065.4).

CD Spectroscopy. CD spectra were recorded in a Jasco J-720 spectropolarimeter using a spectral bandwidth of 2 nm and a time constant of 4−8 s (scan speed 2 nm/min). Baselines were corrected using blank spectra in buffer of TFE recorded under the same conditions and the same cell. Cylindrical quartz cells with a path length of 1 or 0.5 mm were used. Data are presented in $\Delta\epsilon$ per residue ($\epsilon_L - \epsilon_R$) (M^{-1} cm^{-1}). Peptides were dissolved in 5 mM Na_2HPO_4−NaH_2PO_4 (pH 7.02) or in TFE. TFE titrations were carried out by mixing equimolar peptide solutions in buffer and TFE into the cell. Peptide concentrations were calculated by amino acid analysis using an internal standard.

NMR Spectroscopy. NMR spectra were recorded at 500 MHz in a Varian VXR-500S or a Bruker DMX-500 spectrometer using dioxane as internal reference. TOCSY (80 ms of mixing using MLEV-17) and NOESY spectra (100−400 ms mixing) were recorded with 256 × 2048 data points and zero filled to 2K × 4K. Water was suppressed using Watergate[38] or by presaturation. Spectral assignments were carried out using the standard method.[39]

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