PEPTIDES AS HOSTS

*UTEROGLOBIN-LIKE CAVITANDS

J.Am.Chem.Soc.113,5049-5050(1991)
J.Org.Chem.58,6319-6328(1993)
J.Am.Chem.Soc.115,11663-11670(1993)

PEPTIDE / PEPTIDE INTERACTIONS

*PALINDROMIC PEPTIDES

*UTEROGLOBIN FOLDING

*D-AMINO ACIDS IN PROTEIN 'DE 'NOVO' DESIGN

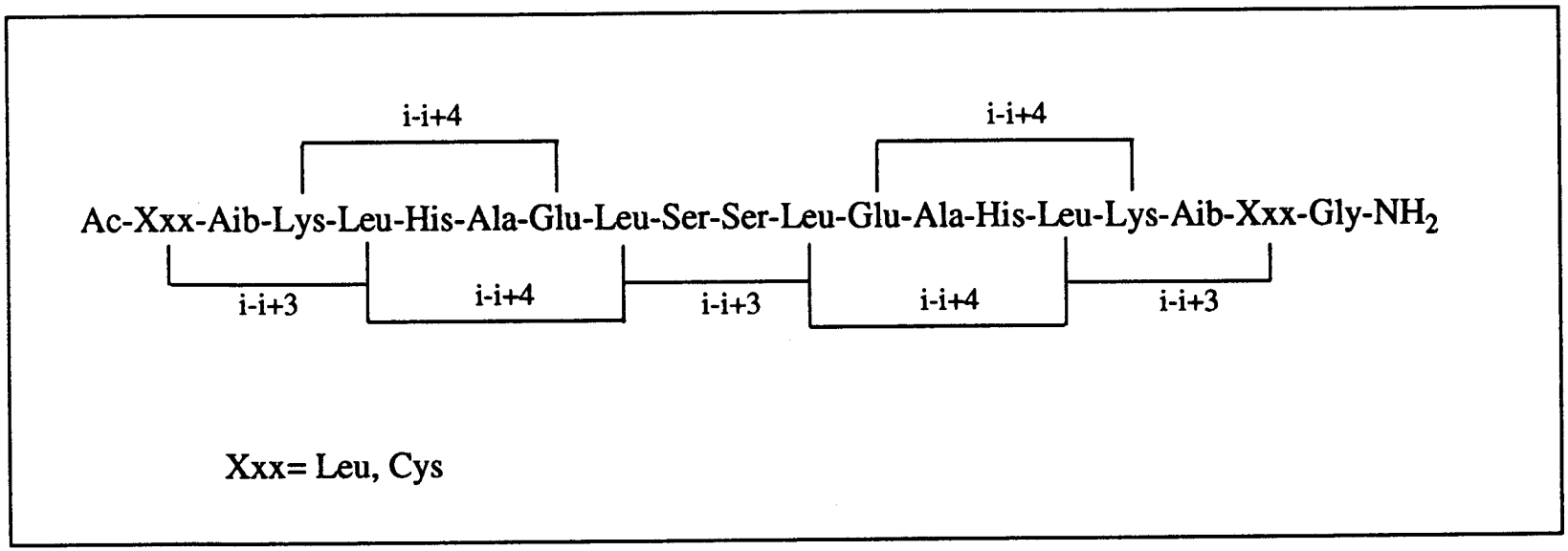
PEPTIDES AS GUESTS

*SYNTHETIC VACCINES AGAINST FMDV

*3D STRUCTURE OF KALIOTOXIN

*PEPTIDE-TETRAGUANIDINIUM INTERACTIONS

J.Am.Chem.Soc.118,277-278(1996)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i-i+4
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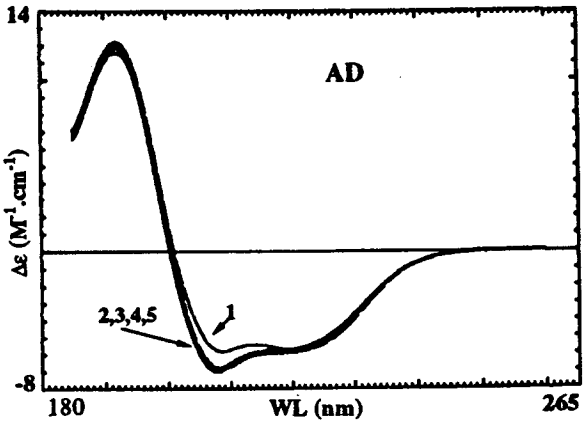
i-i+3
i-i+4
i-i+3
i-i+4
i-i+3
Xxx= Leu, Cys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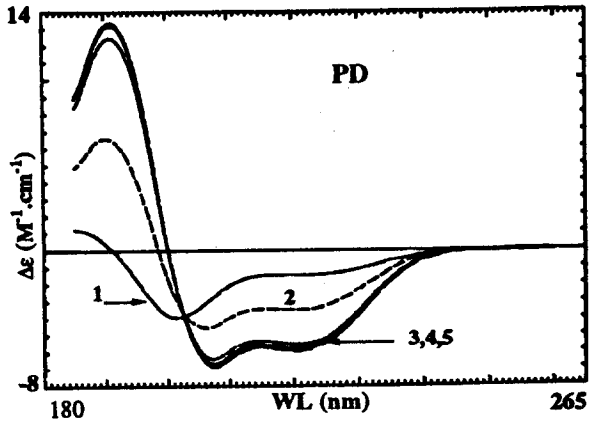
14
-8
Δε (M⁻¹.cm⁻¹)
2,3,4,5
1
180
WL (nm)
265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14
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Δε (M⁻¹.cm⁻¹)
1
2
3,4,5
180
WL (nm)
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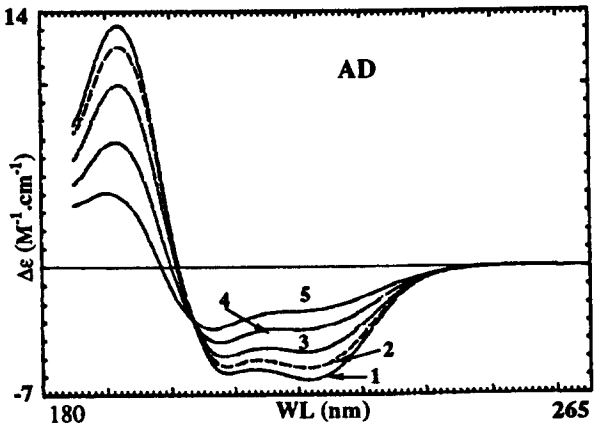
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-7
Δε (M⁻¹.cm⁻¹)
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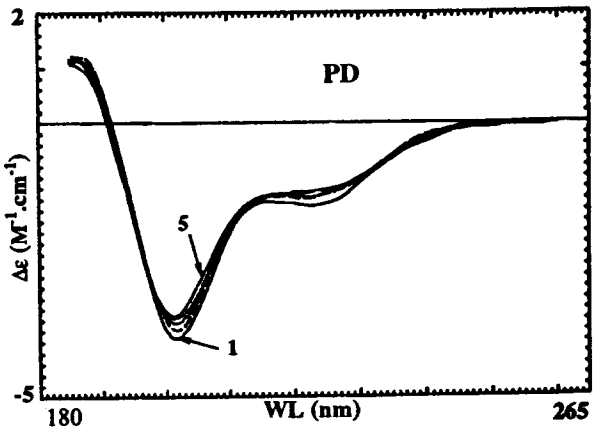
5
3
2
1
180
WL (nm)
265


PD
2
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Δε (M⁻¹.cm⁻¹)
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1
180
WL (nm)
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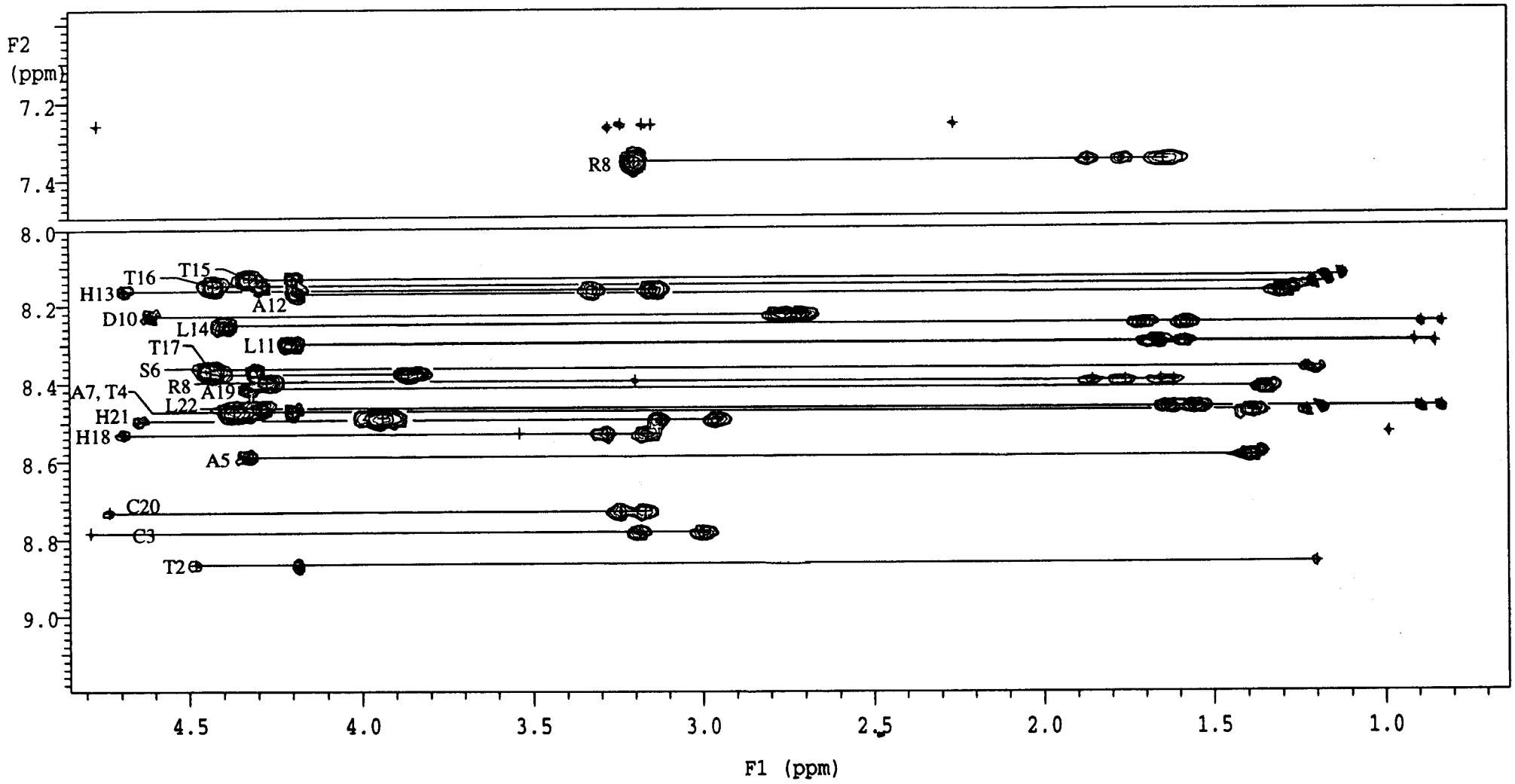
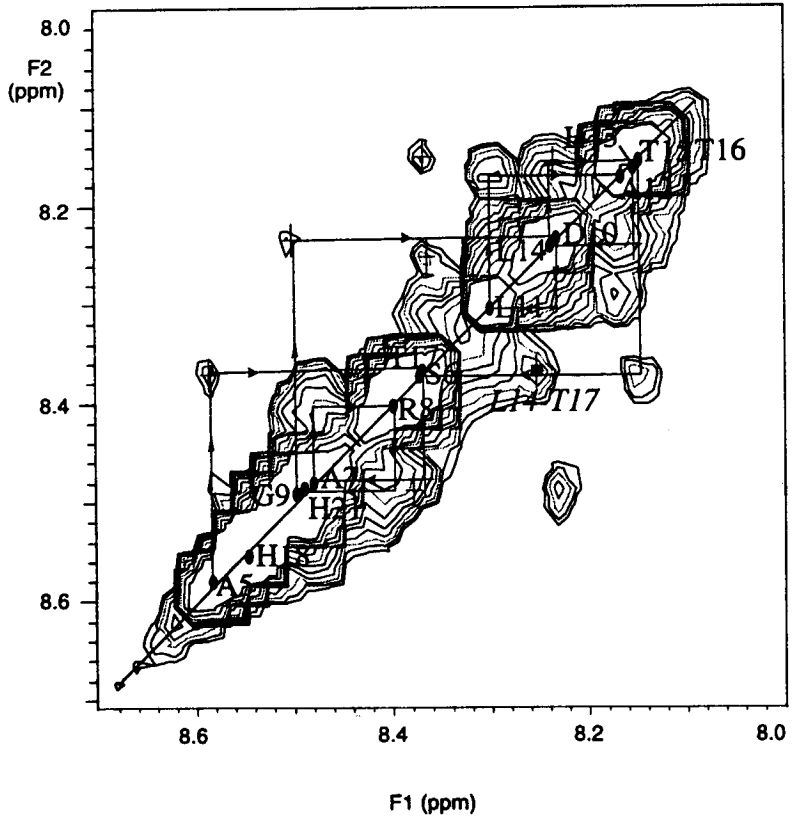
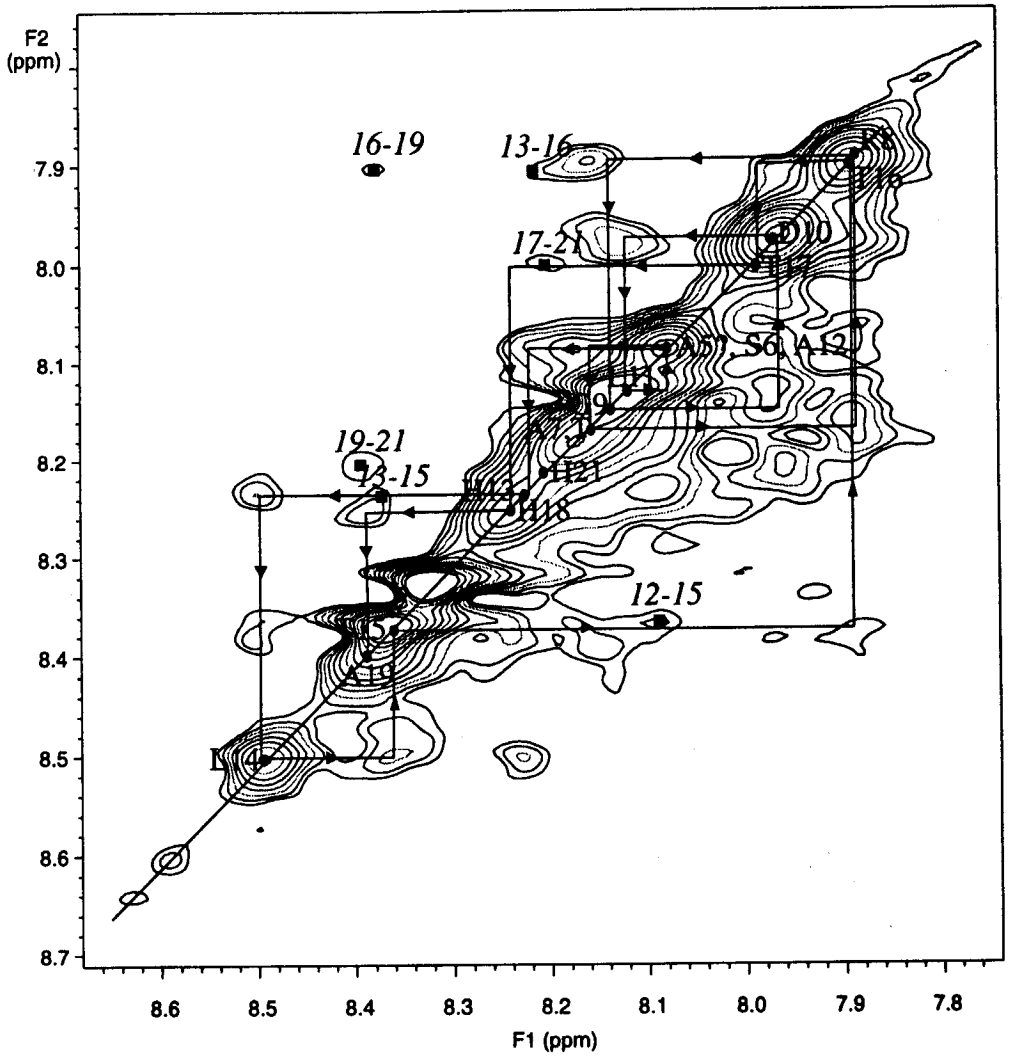
TOCSY - spectrum of of the antigenic site of VP1 (134-155; Y137C, R153C) in water at 5°C

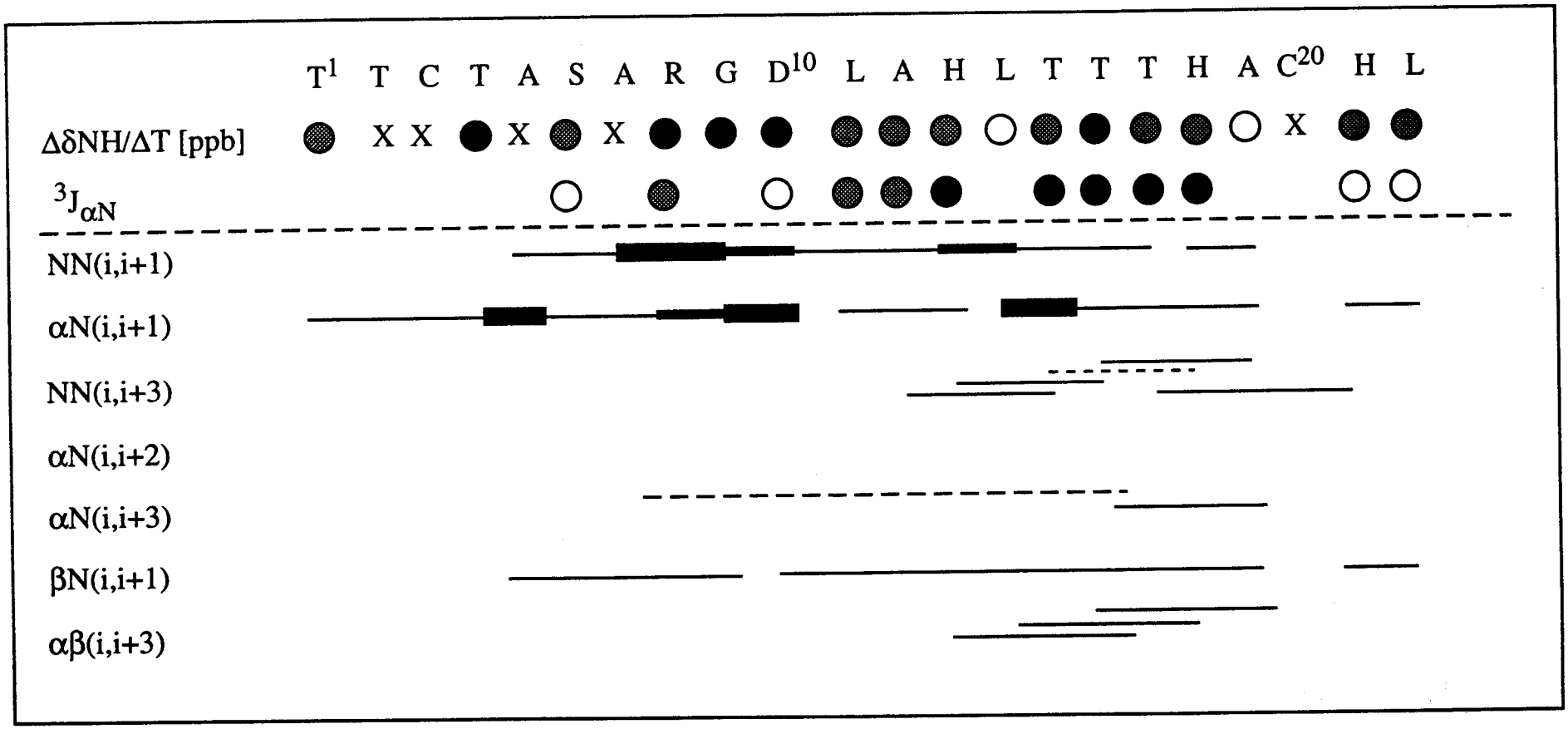



8.0
F2
(ppm)
8.2
8.4
8.6
8.6
8.4
8.2
8.0
F1 (ppm)
T16
D10
T17
R8
G9
H3
A5

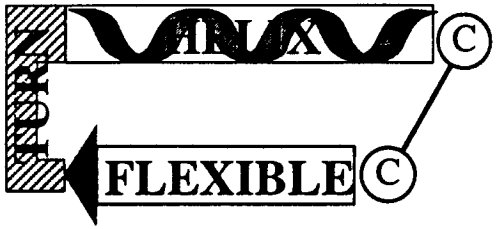
A


F2
(ppm)
7.9
8.0
8.1
8.2
8.3
8.4
8.5
8.6
8.7
16-19
13-16
17-21
19-21
13-15
12-15
8.6
8.5
8.4
8.3
8.2
8.1
8.0
7.9
7.8
F1 (ppm)

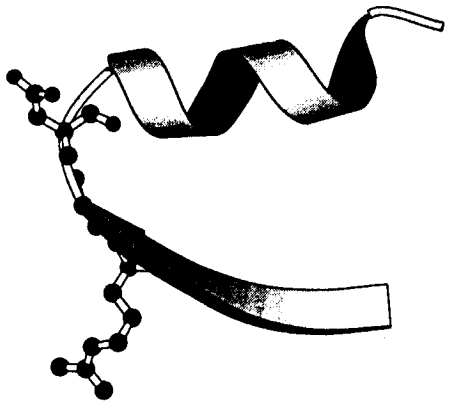
B


T1 T C T A S A R G D10 L A H L T T T H A C20 H L
ΔδNH/ΔT [ppb]
3JαN
NN(i,i+1)
αN(i,i+1)
NN(i,i+3)
αN(i,i+2)
αN(i,i+3)
βN(i,i+1)
αβ(i,i+3)

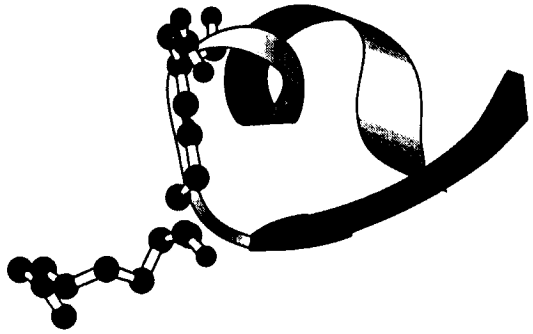
A

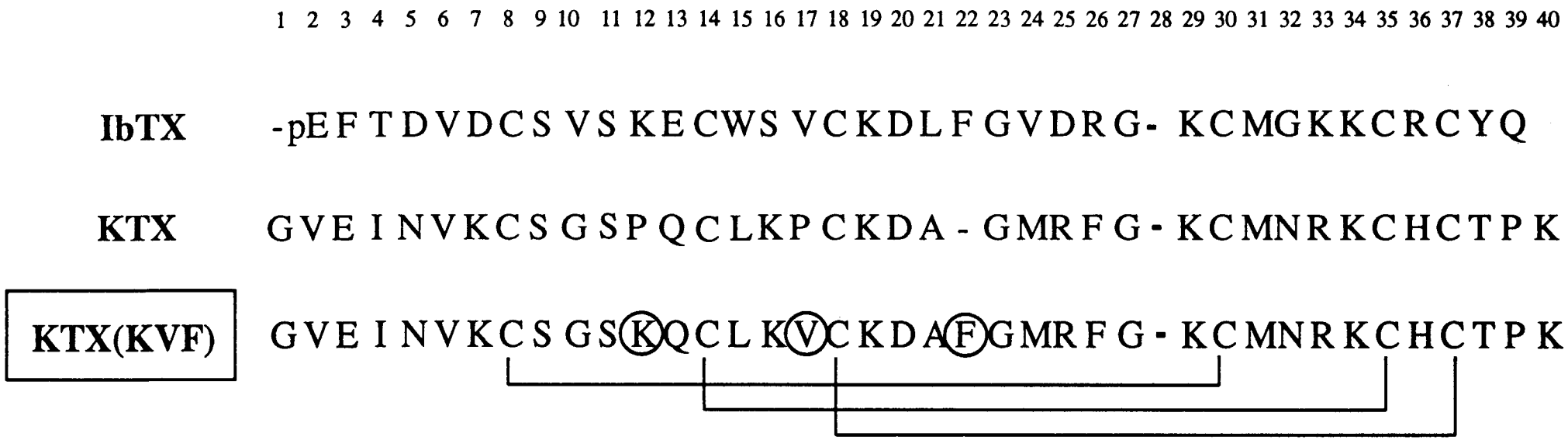


B



C




1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
IbTX -pEFTDVDCSVSKECWSVCKDLFGVDRG- KCMGKKCRCYQ
KTX GVEINVKCSGSPQCLKPCKDA-GMRFG-KCMNRKCHCTPK
KTX(KVF) GVEINVKCSGSKQCLKVCKDAFGMRFG-KCMNRKCHCTPK

Anion Helicates: Double Strand Helical Self-Assembly of Chiral Bicyclic Guanidinium Dimers and Tetramers around Sulfate Templates

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Received September 22, 1995

Synthetic molecules possessing helicity derived from their primary or secondary structures could be related to polypeptides in the α-helix conformation.[1] On the other hand, compounds related to double-helical nucleic acids arise from the spontaneous self-assembly of molecular strands, usually promoted by metal templates (helicates). Double-stranded helicates have been reported with tetrahedral,[2] octahedral,[3] or nondirectional[4] transition metals, whereas triple-stranded helices result from octahedral coordination metals[5] or lanthanides.[6] Here, we describe the first example of a double-helical structure folding around anions.

Enantiomerically pure chiral bicyclic guanidinium salts[7] have been successfully employed for the molecular recognition of carboxylates (amino acids)[8] or phosphates (nucleotides).[9] Consequently, strands of tetraguanidinium salts **1** (chlorides and sulfates) as well as their diguanidinium precursors **2** and **3** were designed for the complexation and transport of oligonucleotides across biological membranes. In our design, the CH_2SCH_2 spacer unit was chosen to span the distance between two adjacent monoanions in the phosphodiester chain. In the case of a divalent sulfate counterion, the spacer is simply too short to wrap two guanidinium subunits in orthogonal planes around a single anion. Therefore, two strands of **1** are forced to fold in a double-helical structure of the predictable handedness imposed by the chiral nature of the receptor. In addition, and unlike with the cation helicates previously mentioned, neutral helices of guanidinium sulfate are obtained.

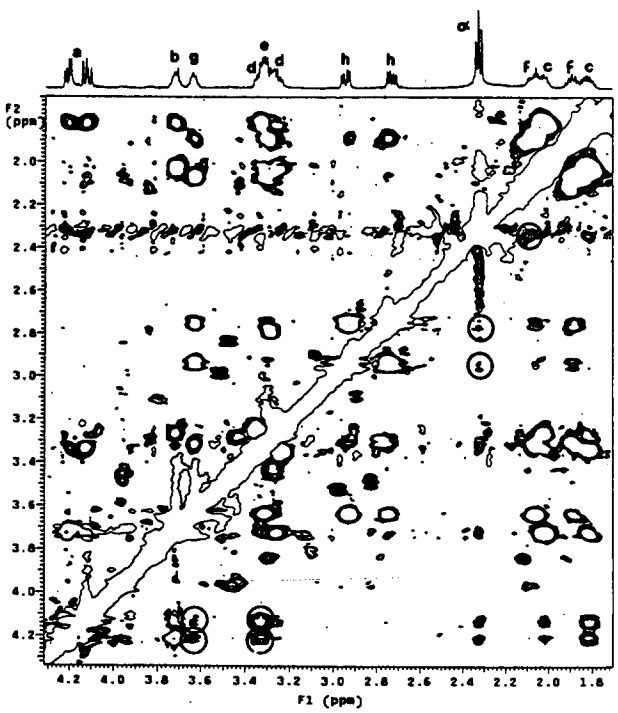
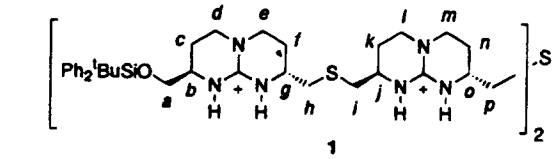
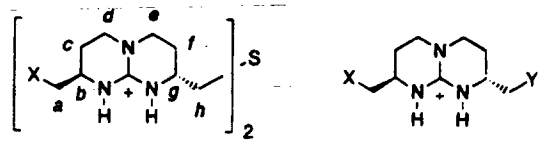


Figure 1. ROESY spectrum of **3** sulfate in $CDCl_3$ (mixing time, 400 ms). Circles show contacts depicted in text and Figure 2a.

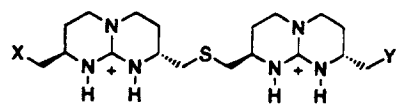


1



2 X = OSi^tBuPh_2
3 X = O(C=O)α-$CH_2C_6H_{13}$

4 X = OSi^tBuPh_2, Y = Br
5 X = OH, Y = Br
8 X = OSi^tBuMe_2, Y = OSi^tBuPh_2



6 X = OSi^tBuPh_2, Y = OH
7 X = OSi^tBuPh_2, Y = Br

Sulfate salts of **1**–**3** were readily prepared from known compounds, in both enantiomeric forms. Thus, reaction of the chiral bicyclic guanidinium monobromo derivative **4**[10,11] with sodium sulfide (1 equiv) afforded diguanidinium **2**[10] in 87% yield. Silyl ethers were quantitatively removed by 12 N HCl/methanol (1:1), and the resulting diol was esterified with octanoic acid and carbonyldiimidazole as coupling agent to give

(1) Meurer, K. P.; Vögtle, F. *Top. Curr. Chem.* **1985**, *127*, 1–76. For a recent example of helicenes, see: Deshayes, K.; Broene, R. D.; Chao, I.; Knobler, C. B.; Diederich, F. *J. Org. Chem.* **1991**, *56*, 6787–6795 and references therein.

(2) (a) Struckmeier, G.; Thewalt, U.; Fuhrhop, J.-H. *J. Am. Chem. Soc.* **1976**, *98*, 278–279. (b) Lehn, J.-M.; Rigault, A.; Siegel, J.; Harrowfield, J.; Chevrier, B.; Moras, D. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 2565–2569. (c) Piguet, C.; Bernardinelli, G.; Williams, A. F. *Inorg. Chem.* **1989**, *28*, 2920–2925. (d) Garrett, T. M.; Koert, U.; Lehn, J.-M.; Rigault, A.; Meyer, D.; Fischer, J. *J. Chem. Soc., Chem. Commun.* **1990**, 557–558. (e) Koert, U.; Harding, M. M.; Lehn, J.-M. *Nature* **1990**, *346*, 339–342. (f) Dietrich-Buchecker, C. O.; Guilhem, J.; Pascard, C.; Sauvage, J.-P. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 1154–1156. (g) Evans, D. A.; Woerpel, K. A.; Scott, M. J. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 430–432.

(3) (a) Constable, E. C.; Ward, M. D.; Tocher, D. A. *J. Am. Chem. Soc.* **1990**, *112*, 1256–1258. (b) Constable, E. C.; Chotalia, R. *J. Chem. Soc., Chem. Commun.* **1992**, 64–66.

(4) Bell, T. H.; Jousselin, H. *Nature* **1994**, *367*, 441–444.

(5) (a) Libman, J.; Tor, Y.; Shanzer, A. *J. Am. Chem. Soc.* **1987**, *109*, 5880. (b) Williams, A. F.; Piguet, C.; Bernardinelli, G. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1490–1492. (c) Krämer, R.; Lehn, J.-M.; De Cian, A.; Fischer, J. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 703–706. (d) Enemark, E. J.; Stack, T. D. P. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 996–998.

(6) Piguet, C.; Bünzli, J.-C. G.; Bernardinelli, G.; Hopfgartner, G.; Williams, A. F. *J. Am. Chem. Soc.* **1993**, *115*, 8197–8206.

(7) (a) Echavarren, A.; Galán, A.; de Mendoza, J.; Salmerón, A.; Lehn, J.-M. *Helv. Chim. Acta* **1988**, *71*, 685–693. (b) Kurzmeier, H.; Schmidtchen, F. P. *J. Org. Chem.* **1990**, *55*, 3749–3755.

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(9) (a) Schmidtchen, F. P. *Tetrahedron Lett.* **1989**, *30*, 4493–4496. (b) Andreu, C.; Galán, A.; Kobiro, K.; de Mendoza, J.; Park, T. K.; Rebek, J., Jr.; Salmerón, A.; Ussman, N. *J. Am. Chem. Soc.* **1994**, *116*, 5501–5502 and references therein. (c) Schiessl, P.; Schmidtchen, F. P. *J. Org. Chem.* **1994**, *59*, 509–511.

(10) Peschke, W.; Schiessl, P.; Schmidtchen, F. P.; Bissinger, P.; Schier, A. *J. Org. Chem.* **1995**, *60*, 1039–1043. Improved procedures for the syntheses of **2** and **4** were employed. No disulfide formation was detected in the preparation of **2** with sodium sulfide. On the other hand, the yield of pure **4** as a colorless solid was enhanced from 59% to 84% by reaction of the corresponding guanidinium alcohol (Kato, Y.; Conn, M. M.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1994**, *116*, 3279–3284) with carbon tetrabromide and tetraphenylphosphine instead of thionyl bromide.

0002-7863/96/1518-0277$12.00/0

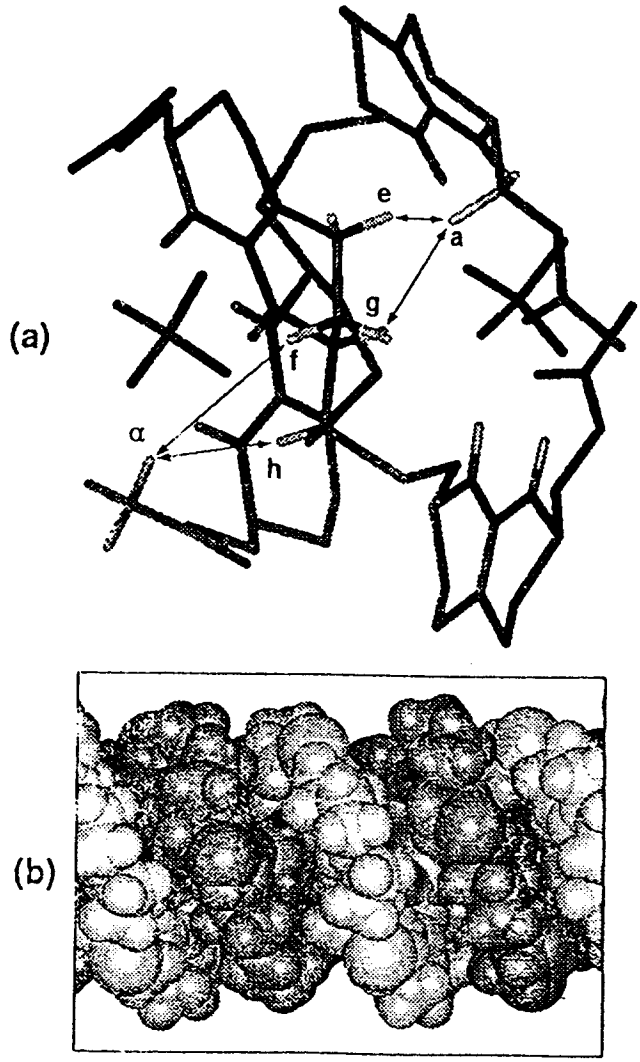

Figure 2. (a) Energy-minimized[13] molecular model of a diacetate analogue of **3** (arrows show ROE contacts observed in **3**). (b) CPK model of a polyguanidinium right-handed double helix made from two strands of subunits of *(R,R)* configuration.

3 in 72% yield. Similarly, tetramer **1** was prepared by reaction of bromoguanidinium **4** with thiourea, followed by treatment of the resulting thiouronium salt with cesium carbonate and bromo alcohol **5** (obtained quantitatively from **4** by acid hydrolysis), to afford the monoprotected diguanidinium alcohol **6** in 53% yield. Alcohol **6** was then transformed into bromide **7** with carbon tetrabromide and triphenylphosphine (80% yield). Treatment of **7** with 1 equiv of sodium sulfide gave **1** in 82% yield.

Counterions strongly influenced the ^{1}H NMR spectra ($CDCl_3$, 500 MHz) of the salts. As in previously reported guanidinium oxoanion complexes,[8,9] strong downfield shifts of ~1 ppm were observed for the guanidinium NH protons when the anion changed from chloride to sulfate, accounting for the formation of two strong salt-bridged N—H···O hydrogen bond pairs. Additionally, in diester **3**, the A_2X pattern of the CH_2OCO group (labeled a) changed to an ABX system.[12] Particularly diagnostic for helix formation was the comparison between ROESY spectra of the chloride and sulfate salts of compound *(R,R)*-**3** (Figure 1). In the sulfate, contacts were observed between protons α and f, α and h, or between protons a and e, and a and g. Since distances between either α or a and each of the above-mentioned

(11) Unless specified, counterions refer to chloride. The synthetic scheme was performed either with guanidines of *(R,R)* or *(S,S)* configuration, although only compounds with *(R)* configuration are shown in the formulae. All new compounds were characterized by a full complement of high-resolution spectra and elemental microanalyses for C and H which are within 0.40% theory.

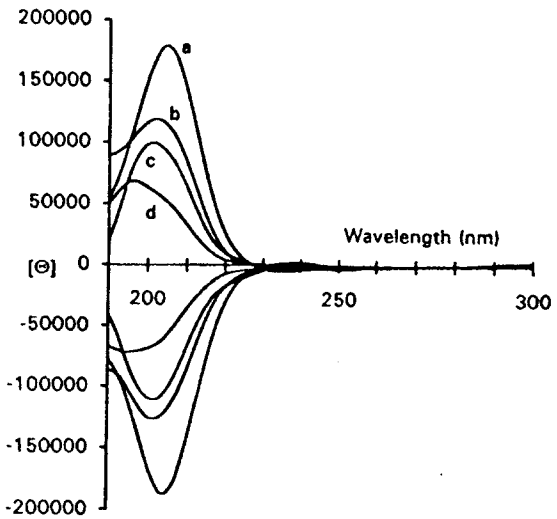

Figure 3. CD spectra in acetonitrile at 4 °C (molar ellipticities in deg cm^2 dmol^{-1} for 0.5 mM solutions): (a) *(S,S)*-**1** sulfate, (b) *(S,S)*-**1** chloride, (c) *(S,S)*-**2** sulfate, and (d) *(S,S)*-**2** chloride.

protons of the same chain are simply much too long for any cross peak to be observed, the signals must correspond to intermolecular contacts between *different* chains. This is precisely the situation when the two chains wrap around each other as shown in Figure 2. For tetramer *(R,R)*-**1**, a similar situation applies, although because of the increased complexity of the molecule, some assignments were only tentative.

Finally, CD spectra of diguanidinium dichloride **2** and tetraguanidinium tetrachloride **1** were registered in acetonitrile, showing in each case almost complete mirror image spectra for both enantiomers (Figure 3, curves d and b, respectively). The higher ellipticities observed for the tetramer are due to the increased number of bicyclic guanidinium subunits. Similar shapes resulted from the corresponding sulfate salts (Figure 3, curves c and a), but in this case significant increases in ellipticity with respect to the chlorides were observed for the same number of bicyclic guanidinium subunits, accounting for the highly structured helical conformation. No such differences were observed for chlorides and sulfates of parent guanidinium **8**, whose CD spectra per cationic subunit were almost superimposable. Thus, changes in ellipticity are related not to the different anions employed but to the helical conformations of the corresponding polycations.

Presumably, tetraguanidinium chains like **1** could also wrap around the phosphate chains of double-stranded nucleic acids, giving rise to triple or quadruple helices with modified properties. This could find application in the delivery of the antisense nucleotides and ribozymes or eventually of therapeutic agents such as phosphorylated analogs of AZT and DDI across biological membranes.[9b]

Acknowledgment. We thank DGICYT for financial support (Grants PB93-0283 and PB92-0864), Comunidad de Madrid and CNDCT (Brazil) for predoctoral fellowships (to J.S.-Q. and I.D., respectively), and Dr. Miquel Feliz (Serveis Científico-Tècnics, Universitat de Barcelona) for technical assistance in the NMR experiments.

Supporting Information Available: Copies of spectral and physical data, 1D and 2D NMR as well as CD spectra for new guanidinium chlorides and sulfates (19 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(12) NMR spectral assignments were performed using 1D and 2D COSY and 2D ROESY experiments (mixing times, 150, 400, and 650 ms) at 25 °C in $CDCl_3$ (10 mg/mL). The additional cross peaks present in the ROESY spectra of *(R,R)*-**3** sulfate, compared to the ROESY spectra of the dichloride, do not disappear on dilution of the sample, excluding the possibility of these being due to molecular contacts.

(13) Molecular mechanics calculations were made in vacuo, with a dielectric constant $\epsilon = 2r_{ij}$, with Insight-II 2.3.0/Discover packages (Biosym) using AMBER force field. CHARMM parameters and Spartan 3.0 electrostatic charges at the 6-31G* level were used for sulfate.

Reprinted from The Journal of Organic Chemistry, 1993, 58.

Unequivocal Synthesis and Characterization of a Parallel and an Antiparallel Bis-Cystine Peptide†

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Received May 20, 1993•

Using an unambiguous methodology we have synthesized the two topological isomers (parallel and antiparallel) of a cyclic peptide dimer containing two disulfide bridges, and we have characterized them spectroscopically using circular dichroism and NMR. We have shown that the interactions between the two chains play a dominant role in determining the different conformational properties of both dimers. Although both molecules are flexible, the ensemble of conformations available to them is clearly different: the antiparallel dimer gives extended structures while the parallel dimer gives mainly folded conformations. Interchain NOEs between symmetry-related residues could be differentiated from the trivial intraresidue NOE using a selective TOCSY–NOESY experiment, and this assignment was in agreement with the predictions made from a conformational search using molecular dynamics without experimental constraints.

Introduction

A growing number of peptides of great biological interest are found to be dimers linked by two disulfide bridges. Examples are atrial natriuretical peptides,[1] factor F_2 from Locusta migratoria,[2] and the proteins uteroglobin[3] and fibronectin.[4] In all of these cases the dimers are antiparallel. The hinge region of immunoglobulins contains a parallel dimer.[5] The study of the interaction of two identical chains in a topologically well-defined fashion is therefore a structural problem of great interest. The synthesis of model compounds by direct oxidation of bis-cysteine peptides very often leads to mixtures of the two possible topoisomers or to ambiguities as to the topology of the dimers obtained. We have developed a methodology based on the use of two or three orthogonal protecting groups[6,7] for the four cysteines involved that allows the unambiguous synthesis of both topological isomers. Peptides **1** and **2**, obtained with this methodology, have been studied by circular dichroism (CD) and nuclear magnetic resonance (NMR).

In spite of the fact that **1** and **2** have the same sequence and only differ in their topology, they show very different conformational properties arising from the different interaction of the two chains in both dimers.

H-Gly-Cys-Phe-Val-Pro-Cys-Gly-OH
|
H-Gly-Cys-Phe-Val-Pro-Cys-Gly-OH

1

H-Gly-Cys-Phe-Val-Pro-Cys-Gly-OH
|
HO-Gly-Cys-Pro-Val-Phe-Cys-Gly-H

2

The comparison has been extended also to the noncyclic dimers **3** and **4**, synthetic precursors of **1** and **2**.

H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH
|
H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH

3

H-Gly-Cys(Acm)-Phe-Val Pro-Cys-Gly-OH
|
H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH

4

The central residues, which along with the cysteines form the cyclic part of the antiparallel dimer, were chosen to have the sequence of [Phe[4]-Val[6]]antamanide,[8] an analogue with C_2 symmetry of the antitoxin antamanide from Amanita phaloides.[9]

Molecular mechanics calculations without experimental constraints provide an explanation for the observed behavior and a model for the conformation of the target molecules as ensembles of structures. Experimental data were used to check the predictions of conformationally relevant properties, such as the presence of long-distance

† Abbreviations used: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; CD, circular dichroism spectroscopy; DCC, *N,N'*-dicyclohexylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; FABMS, mass spectrometry by fast atom bombardment; Fm, fluorenylmethyl; hANP, human atrial natriuretic peptide; HOAc, acetic acid; HPLC, high-performance liquid chromatography; MeBzl, *p*-methylbenzyl; MPLC, medium-pressure liquid chromatography; NMR, nuclear magnetic resonance; Npys, 3-nitro-2-pyridinesulfenyl; Pab–resin, phenylacetamidobenzyl–resin; pip, piperidine; TFA, trifluoroacetic acid; TFE, trifluoroethanol. Amino acid symbols denote the L configuration where applicable.

• Abstract published in *Advance ACS Abstracts*, October 1, 1993.

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(3) (a) Popp, R. A.; Foresman, K. R.; Wise, L. D.; Daniel, J. C. *Proc. Natl. Acad. Sci. U.S.A.* 1978, *75*, 5516–5519. (b) Tancredi, T.; Temussi, P. A.; Beato, M. *Eur. J. Biochem.* 1982, *122*, 101–104. (c) Morize, I.; Surcouf, E.; Vaney, M. C.; Epelboin, Y.; Buehrer, M.; Fridlanski, F.; Milgrom, E.; Mornon, J. P. *J. Mol. Biol.* 1987, *194*, 725–739.

(4) (a) Petersen, T. E.; Thogersen, H. C.; Skorstengaard, K.; Vibe-Pedersen, K.; Sahl, P.; Sottrup-Jenesen, L.; Magnusson, S. *Proc. Natl. Acad. Sci. U.S.A.* 1983, *80*, 137–141. (b) Very recently fibronectin has been proven to be antiparallel by NMR: Seong Soo A. An; Jimenez-Barbero, J.; Petersen, T. E.; Llinas, M. *Biochemistry* 1992, *31*, 9927–33.

(5) (a) Roitt, I. M.; Brostoff, J.; Male, D. K. *Immunology*; Gower Medical Publishing, Ltd.: London, 1985. (b) Kessler, H.; Mronga, S.; Müller, G.; Moroder, L.; Huber, R. *Biopolymers* 1991, *31*, 1189–1204.

(6) Ruiz-Gayo, M.; Albericio, F.; Pons, M.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* 1988, *31*, 3845–3848.

(7) An orthogonal system has been defined as a set of completely independent classes of protecting groups, such that each class of groups can be removed in any order and in the presence of all other classes; see: (a) Barany, G.; Merrifield, R. B. *J. Am. Chem. Soc.* 1977, *99*, 7363–7365. (b) Barany, G.; Albericio, F. *J. Am. Chem. Soc.* 1985, *107*, 4936–4942.

(8) (a) Wieland, T.; Dungen, A. V.; Birr, C. *FEBS Lett.* 1971, *14*, 299–300. (b) Karle, I.L. *Biochemistry* 1974, *13*, 2155–2162.

(9) Wieland, Th.; Lueben, G.; Ottenheym, H.; Faesel, J.; DeVries, J. X.; Konz, W.; Prox, A.; Schmid, J. *Angew. Chem.* 1968, *6*, 209–212.

0022-3263/93/1958-6319$04.00/0

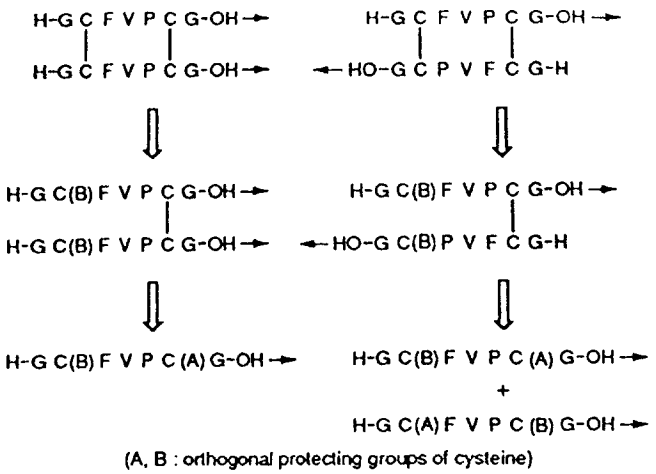
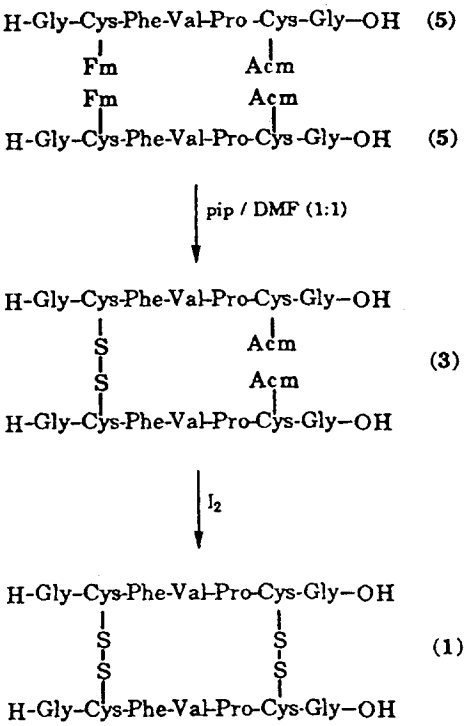


Figure 1. Retrosynthetic analyses of the parallel and antiparallel cyclic dimers. Parallel dimers can be obtained from a single monomeric precursor while at least two differently protected precursors are needed for the preparation of the antiparallel dimer. The formation of the first disulfide bridge of the antiparallel dimer has to be directed to avoid the simultaneous formation of parallel dimer.

Scheme I



NOEs, made from the ensemble of structures using the average relaxation matrix. We believe that this is a general approach to the study of flexible peptides that does not use the concept of "average" conformation.

Results and Discussion

Synthesis of the Parallel Dimer. The retrosynthetic analyses of parallel and antiparallel dimers of bis-cysteine peptides are shown in Figure 1. The parallel dimer can be prepared from a single peptide with two orthogonal protecting groups for both residues of cysteine and we suggest the combined use of fluorenylmethyl (Fm)[10] and acetamidomethyl (Acm)[11] (Scheme I). These two groups are stable to all the usual reagents used in solid-phase

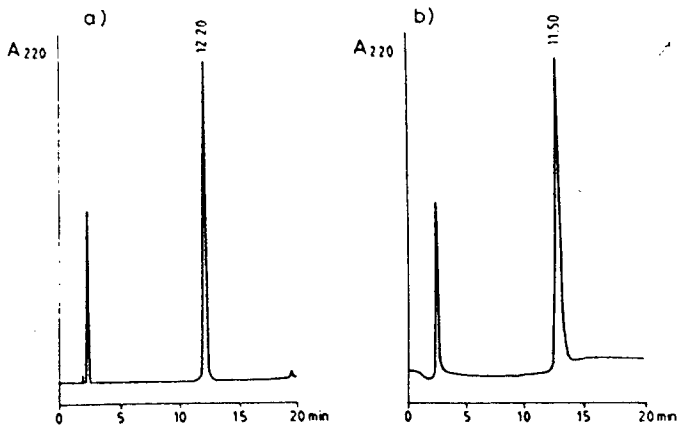


Figure 2. HPLC chromatograms of (a) pure parallel dimer 1 and (b) pure antiparallel dimer 2. Chromatographic conditions are the following: Nucleosil C-18 column, Particle size 5 μ. Linear gradient from 5–80% B in 20 min. Solvent systems: A: H_2O 0.045% TFA., B: CH_3CN 0.036% TFA.

synthesis including the HF reaction. Although both can be removed in any order, we prefer to eliminate first the Fm and then the Acm in order to overcome solubility problems of the linear dimer intermediate due to the high hydrophobicity of the Fm group. Thus, to the end of the synthesis,[12] the peptide–resin was subjected to the HF reaction and the crude product washed with CH_2Cl_2 and used without further purification to obtain pure parallel dimer (>99% as judged by HPLC, Figure 2a) by treatment with piperidine followed by oxidation with I_2[13,14] and purification by MPLC. The overall yield from Boc-Gly-resin was 17%.

Synthesis of the Antiparallel Dimer. For this isomer, the successive disconnections of the two disulfide bridges led to two versions of the same monomeric precursor which differ only in the position of the cysteine thiol protection group (A and B in Figure 1). The synthesis of these molecules can be done either in solid-phase or in solution, but an especially attractive alternative is the simultaneous synthesis of both peptides based on the joint use of polystyrene–1%-divinylbenzene and Kel-F–*g*-styrene[15] as polymeric supports. These resins have different densities and can, therefore, be separated by flotation[16] before the incorporation of the first protected cysteine. Then, the two peptide–resins are mixed again and the synthesis

(10) (a) Bodanszky, M.; Bednarek, M. A. *Int. J. Pept. Protein Res.* 1982, *20*, 434–437. (b) Albericio, F.; Nicolás, E.; Rizo, J.; Ruiz-Gayo, M.; Pedroso, E.; Giralt, E. *Synthesis* 1990, 119–122.

(11) Veber, D. F.; Milkowski, J. D.; Varga, S. L.; Denkewalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* 1972, *94*, 5456–5461.

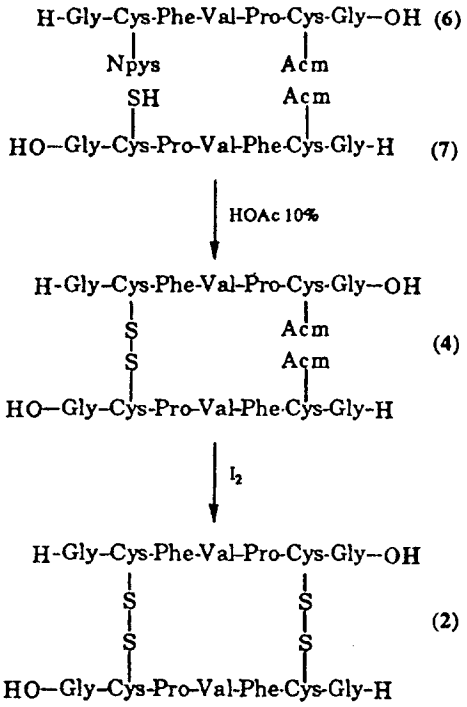
(12) Solid-phase peptide synthesis of all the monomers was carried out on a (chloromethyl)-Pab-resin (see: Giralt, E.; Andreu, D.; Miró, P.; Pedroso, E. *Tetrahedron* 1983, *39*, 3185–3188) with Boc group for α-amino protection using a conventional synthetic protocol.

(13) Kamber, B., Hartmann, A., Eisler, K., Riniker, B., Rink, H., Sieber, P., and Rittel, W., *Helv. Chim. Acta* 1980, *63*, 899–915.

(14) In order to avoid overoxidations of the thiol function to sulfonic acid during the deprotection step with I_2 or its workup, we use an adequate excess of I_2 (10 equiv) in concentrated aqueous solution of HOAc (80%), and after the formation of the disulfide bridge (120 min), we eliminate the excess iodine by extractions with carbon tetrachloride. Use of zinc to quench the I_2 oxidation leads to dimer peptides containing important amounts of zinc (different samples of parallel and antiparallel dimers show 5–14% of zinc by atomic absorption). Other reductors like absorbic acid or sodium thiosulfate lead to a rapid iodine regeneration and the consequent overoxidation of disulfide bridges; see: Ruiz-Gayo, M.; Albericio, F.; Royo, M.; García-Echeverría, C.; Pedroso, E.; Pons, M.; Giralt, E. *Anal. Quim.* 1989, *85*, 116-118.

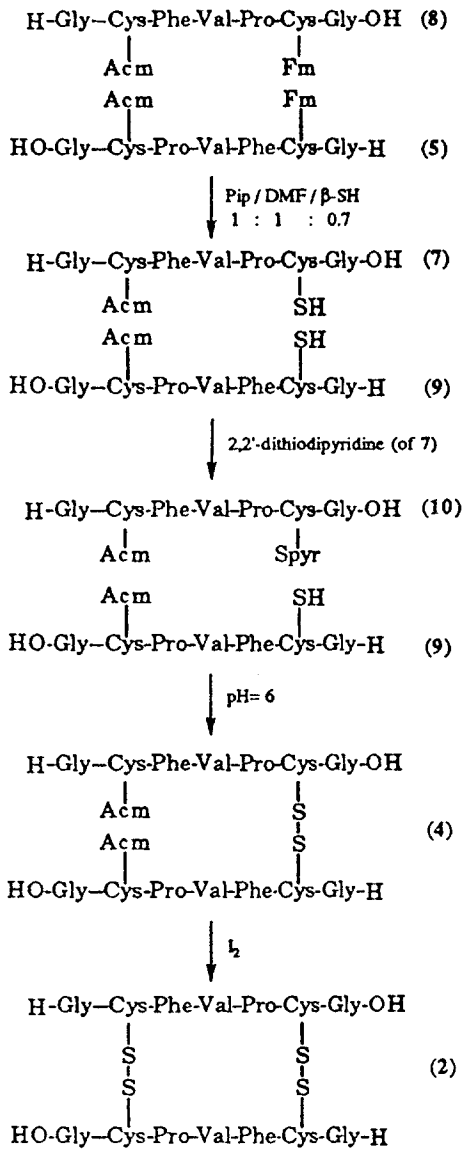
(15) Battaerd, H. A. J.; Tregear, G. W. In *Graft Copolymer*; John Wiley & Sons, Interscience: New York, 1967.

Scheme II



jointly continued until the second cysteine residue where the process is repeated. After incorporation of the last residue, the two peptide–resins were separately treated with HF in the presence of *p*-cresol. The appropriate choice of the solvent is of great importance for the separation. For these two polymers, we found that a mixture of CH_2Cl_2–TFE (8:2) provides the best conditions (less than 0.1% of cross-contamination as judged by HPLC). In this work, the antiparallel dimer has been obtained by two alternative strategies using the Kel-F–polystyrene procedure above described. The first one (Scheme II) involves the use of only two protecting groups: Fm and Acm. The Fm group of both peptides was removed with piperidine in presence of β-mercaptoethanol in order to obtain both peptides with the thiol function free.[17] One of them was allowed to react with dithiodipyridine and after purification by Sephadex G-10, and MPLC was mixed with the other peptide to form the first disulfide bridge. The final oxidation with I_2 and purification lead to the antiparallel dimer (>99% pure as judged by HPLC, Figure 2b) with an overall yield of 15% from Boc-Gly–resin. The second strategy (Scheme III), similar to the one described by Sakakibara *et al.*[18] for the synthesis of β-hANP, involves the preparation of a peptide with 3-nitro-2-pyridylsulfenyl (Npys)[19] and Acm as protecting groups for the cysteine and a second one with a

Scheme III



free thiol and Acm. The latter was protected during the solid-phase assembly of this peptide with a *p*-methylbenzyl (MeBzl) group, which is removed during the HF treatment. Equivalent amounts of both peptides were mixed and the pure antiparallel dimer (>99% as judged by HPLC) was obtained by oxidation with I_2 as before with an overall yield of 11% from Boc-Gly–resin.

Chemical Characterization of the Dimers. The identification and characterization of parallel and antiparallel dimers constitutes a problem not totally solved. Classical techniques like mass spectrometry by fast atom bombardment (FABMS) fail to characterize this kind of compounds as in both dimers the first bonds broken are the two S–S, giving in both cases the same fragments. Therefore, we have used enzymatic hydrolysis[20] to prove the identity of **1** and **2**. In this case, we have taken advantage of the relative specificity of chymotrypsin for

(16) (a) Tregear, G. W. In *Chemistry and Biology of Peptides*; Meienhofer, J., Ed., Ann Arbor Sci. Publi.: Ann Arbor, 1972; pp 175–178. (b) Albericio, F.; Ruiz-Gayo, M.; Pedroso, E.; Giralt, E. *Reactive Polymers* **1989**, *10*, 259–268.

(17) Considering that the possible formation of a saline bridge between both extremes, C- and *N*-terminal, of the peptides could direct the formation of heterodimers, an attempt of undirected oxidation was carried out with these two free thiol peptides at pH 8. The disminution of free thiol as determined by the Ellman test (see: Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70-77) was concomitant with the appearance of three peaks in the HPLC with relative intensities 1:1:2, which were identified by coelution with real samples of both homodimers and the heterodimer. The same experiment carried out in CH_3OH led to a mixture of both homodimers in a proportion of 1:1.

(18) Chino, N.; Yoshizawa-Kumagaye, K.; Noda, Y.; Watanabe, T. X.; Kimura, T.; Sakakibara, S. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 665–672.

(19) (a) Matsueda, G. R.; Aiba, K. *Chem. Lett.* **1978**, 951–952. (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.

(20) Other authors have used similar methods to characterize dimers, e.g.: Hiskey, R.G.; Muthu-Kumaraswamy, N.; Vunnan, R. *J. Org. Chem.* **1975**, *40*, 950-953.

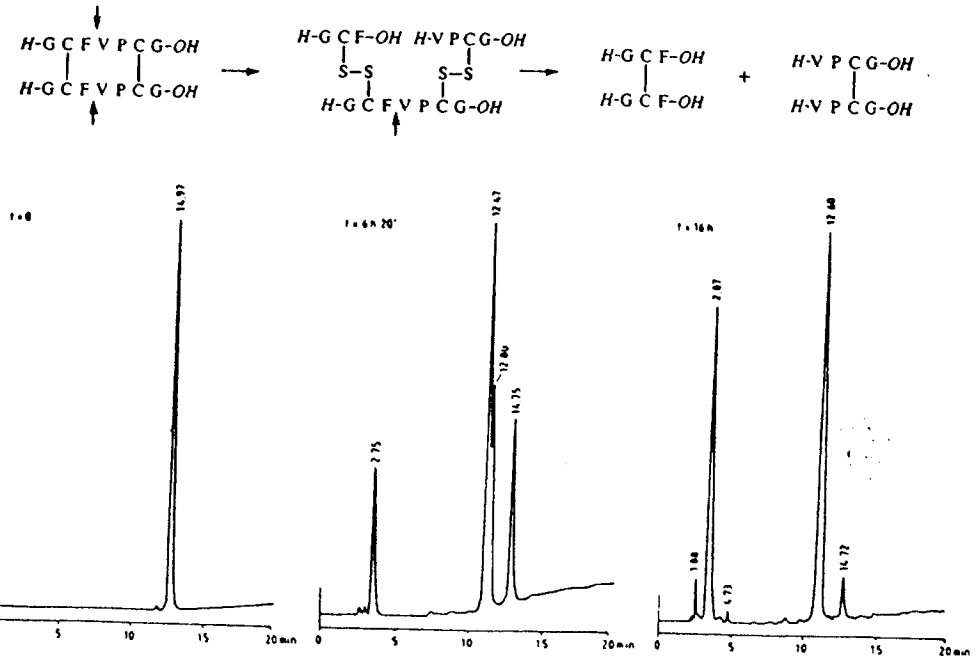


Figure 3. Characterization of the parallel dimer **1** by enzymatic hydrolysis using immobilized chymotripsin. Chromatographic conditions: Nucleosil C-18, particle 5μ. Linear gradient from 10–60% B in 30 min. Solvent systems: A: H_20 0.045% TFA, B: CH_3CN 0.036% TFA.

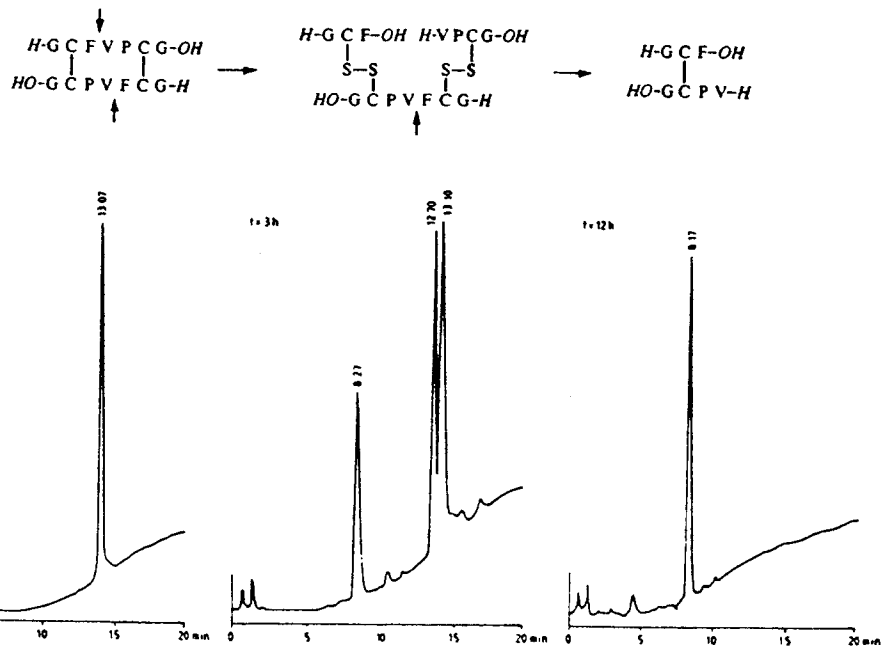


Figure 4. Characterization of the antiparallel dimer **2** by enzymatic hydrolysis using immobilized chymotripsin. Chromatographic conditions are the same as in Figure 3.

the peptidic bonds where the carboxyl component contains an aromatic side chain (Phe, Trp, or Tyr).[21] The mild conditions of the hydrolysis do not affect the disulfide bonds, and therefore two different fragments are expected after the complete hydrolysis of the target peptide bonds of the parallel dimer while only one fragment will be produced from the antiparallel dimer. Indeed, after 16 h of incubation with immobilized chymotrypsin the parallel dimer (Figure 3) gave two fragments, with HPLC retention times of 2.7 and 12.5 min, that had the correct amino acid analyses for (H-Val-Pro-Cys-Gly)$_2$ and (H-Gly-Cys-Phe)$_2$, respectively. The antiparallel dimer (Figure 4) after 12 h of reaction gave a single fragment with a retention time of 8.2 min. At shorter reaction times an extra HPLC peak appears in both cases (at 12.4 min for the parallel and 12.7 min for the antiparallel dimers). These peaks disappear as the reaction proceeds and correspond to the products of the hydrolysis of the Phe-Val bond of only one chain. This is the expected behavior considering the cyclic nature of the dimers.

CD Study. The CD spectrum of the parallel dimer **1** in TFE/H_2O (9:1) is shown in Figure 5a. It contains a

(21) In order to simplify this process we have employed α-chymotrypsine immobilized on a support of (carboxymethyl)cellulose. This allows the separation of the enzyme after the reaction simply by a centrifugation step.

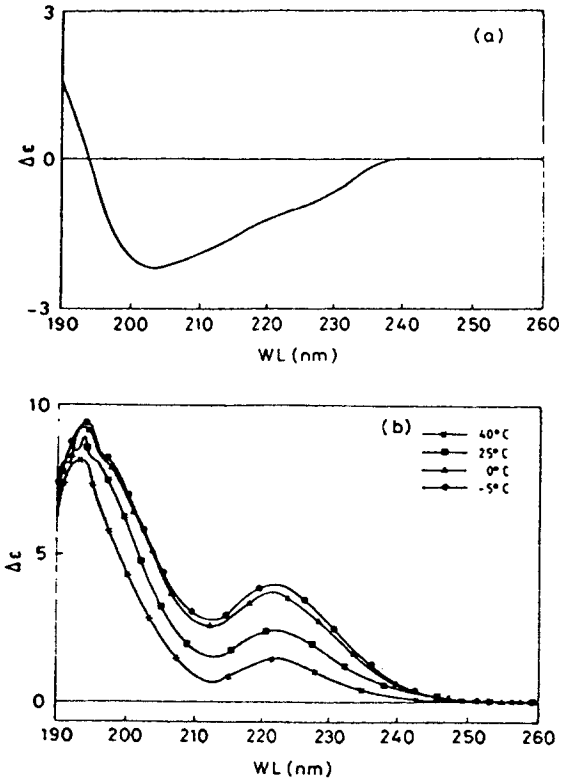


Figure 5. CD spectra of (a) 1 at 298 K (spectra from 268 K to 298 K are superimposable) and (b) 2 at different temperatures. In both cases the solvent was TFE:H_2O (9:1 v/v).

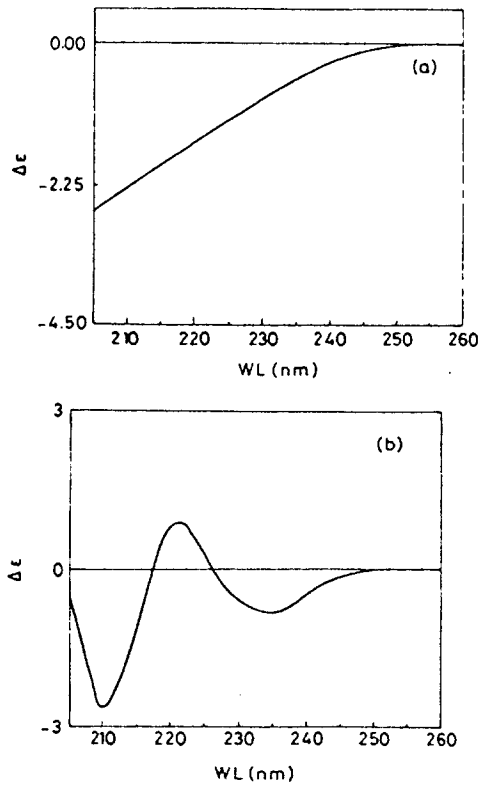


Figure 6. CD spectra of open dimers 3 (a) and 4 (b) in TFE:H_2O (9:1 v/v) and 298 K.

positive band on the short wavelength end of the spectrum followed by a broad negative band with a minimum at 201–205 nm. Between these two bands a zero-crossing point appears at 193 nm. On the long-wavelength side of the negative band there is a shoulder at *ca.* 226 nm. This spectrum is independent of the concentration in the range from 2 to 0.06 mg mL^{-1} and it is also independent of the temperature in the range +25 to −5 °C. This could be described as a slightly red-shifted type-C spectrum[22] usually assigned to type-I β-turns.[23] Above 250 nm (data not shown) there is a broad weak negative band with an extreme at 295 nm, corresponding either to the disulfide bridge absorption or to a weak band from the aromatic ring.

The antiparallel cyclic dimer 2 has a completely different CD pattern in TFE/H_2O (9:1) (Figure 5b). Starting from the short wavelength side of the spectra a strong positive band appears below 190 nm and extends up to *ca.* 210 nm. In some of the spectra a shoulder is visible around 200 nm (about the same wavelength where the main negative band appeared in the parallel dimer). A well-resolved positive band of low intensity appears at 222 nm, about the same wavelength of the low intensity negative band found in the parallel dimer. The appearance of n–π^* positive bands is not common in homochiral peptides. This feature has been recently described in an acyclic dimer linked by a single disulfide bridge and reported to form an antiparallel β-sheet in solution.[24] A positive band in the n–π^* region also appears in both the parallel and the antiparallel cyclic dimers of Boc-L-Cys-L-Cys-OMe.[25]

The CD spectrum of 2 is strongly temperature dependent. The intensity of the long-wavelength band at 222 nm increases at low temperature suggesting that this band arises from a more structured form of the peptide.

The large differences found in the CD spectra of the two dimers contrasts with the observations described for the Boc-L-Cys-L-Cys-OMe dimers in which the CD spectra in TFE of the parallel and antiparallel dimers are very similar.[23] This suggests that in our case the two peptide chains, at least in one of the dimers, may be strongly interacting. In order to investigate that point we also recorded the spectra of two open dimers containing only one disulfide bond and two Acm-protected Cys residues.

The spectra from the linear dimer 3, a synthetic precursor of the parallel cyclic dimer, in 90% TFE (Figure 6a) consists of a broad, featureless negative band extending from 250 nm down to the shortest wavelength studied. On the other hand, the CD spectra of dimer 4, an intermediate in the synthesis of the antiparallel dimer, in 90% TFE (Figure6b)has a positive band of medium intensity at 221 nm about the same wavelength at which the cyclic antiparallel dimer had a characteristic positive band. At both sides of the 221 positive band the CD spectrum of 4 shows negative minima which are not present in the cyclic dimer. These may be interpreted as arising from a broad negative band analogous to that found in the CD spectra of the open parallel dimer. The CD spectra of the open antiparallel dimer, therefore, indicate the presence of disordered conformations indistinguishable by CD from those found in the open parallel dimer, in equilibrium

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(23) Perczell, A.; Hollósi, M.; Foxman, B. M.; Fasman, G. D. *J. Am. Chem. Soc.* **1991**, *113*, 9772–9784.
(24) Raj, P. A.; Soni, S. D.; Ramasubbu, N.; Bhandary, K. K.; Levine, M.J. *Biopolymers* **1990**, *30*, 73–85.
(25) Capasso, S.; Mazzarella, L.; Tancredi, T.; Zagari, A. *Biopolymers* **1984**, *23*, 1085–1097.

Table I. Chemical Shifts of Dimer Peptides in DMSO-d_6[a]

	1[b]	2[b]	3[b]	4A[c]	4B[c]
^{1}Gly NH$_3$	7.97				
^{1}Gly CH$_\alpha$	3.57	3.71	4.19	3.49	3.51
^{2}Cys NH	8.59	8.64	8.50	8.60	8.69
^{2}Cys CH$_\alpha$	4.73	4.80	4.15	4.60	4.63
^{2}Cys CH$_\beta$	2.76/3.10	2.74/2.88	2.68/2.88	2.52/2.85	2.74/3.04
Acm CH$_3$				1.90	
Acm NH				8.56	
Acm CH$_2$				4.05/4.36	
^{3}Phe NH	8.48	8.78	8.60	8.08	8.24
^{3}Phe CH$_\alpha$	4.86	4.97	4.54	4.56	4.57
^{3}Phe CH$_\beta$	2.79/2.96	2.69/2.87	2.77/2.96	2.70/2.92	2.70/2.89
^{3}Phe CH$_{arom}$	7.20	7.09	7.19	7.39	7.39
^{4}Val NH	8.27	8.20	8.09	8.23	8.26
^{4}Val CH$_\alpha$	4.49	4.59	4.35	4.30	4.30
^{4}Val CH$_\beta$	2.01	2.13	2.00	1.85	1.85
^{4}Val CH$_\gamma$	0.85/0.91	0.81/0.90	0.88	0.81	0.81
^{5}Pro CH$_\alpha$	4.41	4.41	4.35	4.30	4.34
^{5}Pro CH$_\beta$	1.95	1.82/2.06	2.00	2.00	2.00
^{5}Pro CH$_\gamma$	1.81/1.95	1.83/1.97	1.93	2.00	2.00
^{5}Pro CH$_\delta$	3.60/3.67	3.64/3.65	3.77	3.72	3.72
^{6}Cys NH	7.86	8.18	8.28	8.26	8.18
^{6}Cys CH$_\alpha$	4.56	4.41	4.37	4.39	4.39
^{6}Cys CH$_\beta$	2.90/3.08	2.88/3.13	2.77/2.96	2.89/3.11	2.70/2.85
Acm CH$_3$			1.83		1.90
Acm NH			8.60		8.48
Acm CH$_2$			4.21		4.20
^{7}Gly NH	8.20	8.25	7.81	8.19	8.13
^{7}Gly CH$_\alpha$	3.74	3.69	3.48/3.61	3.69	3.71

[a] Chemical shifts have been measured at 298 K from the coordinates of cross-peaks in 2D NMR spectra and are reported downfield from TMS. [b] The molecule has effective C_2 symmetry at this temperature. [c] The two peptide chains are not equivalent. Assignment of the two chains was initially based on the different intensities of the cross-peaks from equivalent residues in the two chains in DQF–COSY or TOCSY experiments. This originates from the longer relaxation time of protons situated near the end of the peptide chain opposite to the one linked by the disulfide bridge. This was also corrobrated by correlation with ^{13}C-NMR spectra via a heteronuclear multiple quantum correlation (HMQC) experiment, as C_β carbons of cystine appear at higher field than those of Acm-protected cysteine.

with more ordered conformations related to those that originate the CD spectra of the cyclic antiparallel dimer. This behavior suggests that in the antiparallel cyclic dimer the second disulfide bond simply fixes an interaction that occurs spontaneously between the two peptide chains when they are held together by the first disulfide bond.

NMR Study. The NMR study was restricted to the two cyclic dimers. Both have C_2 symmetry in the NMR time scale and show a single set of signals for the two chains. Sequence-specific assignments could be readily made from DQF–COSY and ROESY or NOESY experiments using the standard protocol.[26] The observed chemical shifts are listed in Table I. The chemical shifts of two open chain dimers are also included for reference. The proline residues have been found to be trans in both cyclic peptides using ^{13}C-NMR (data not shown) and also considering the strong NOE between the δ protons of Pro and the CHα of Val.

The temperature coefficients of the amide protons of both cyclic dimers in DMSO-d_6 range from −2.5 to −6.7 ppb K^{-1} indicating intermediate to full solvent exposure. The lowest coefficient in both peptides corresponds to the amide proton of ^{2}Cys (−3.7 for 1 and −2.5 ppb K^{-1} for 2). The NH proton of ^{6}Cys has the second lowest temperature coefficient (−3.9 ppb K^{-1}) in the antiparallel dimer but the largest in the parallel dimer (−6.1 ppb K^{-1}). The antiparallel dimer shows temperature-dependent chemical shifts also for nonamide protons indicating rapid exchange between different conformations of similar energy. The same behavior was observed for another antiparallel dimer peptide of identical size derived from fibronectin.[4]

(26) Wüthrich, K. In *NMR of Proteins and Nucleic Acids*; John Wiley and Sons: New York, 1986.

Table II. NOESY Cross-Peak Volumes of Backbone Protons

proton pair				normalized NOESY cross-peak volumes (rigid pair distance)[a]	
				parallel dimer	antiparallel dimer
CH$_\alpha$	G1	NH	C2	8.0(2.7)	2.9(3.2)
NH	C2	CH$_\alpha$	C2	4.6(3.0)	3.8(3.1)
CH$_\alpha$	C2	NH	F3	22.7(2.3)	24.7(2.3)
NH	F3	CH$_\alpha$	F3	6.9(2.8)	5.1(3.0)
CH$_\alpha$	F3	NH	V4	22.4(2.3)	27.2(2.2)
NH	V4	CH$_\alpha$	V4	11.2(2.6)	7.7(2.8)
CH$_\alpha$	V4	CH$_\beta$	P5	17.2(2.4)	41.9(2.1)
CH$_\alpha$	P5	NH	C6	20.8(2.3)	*b*
NH	C6	CH$_\alpha$	C6	7.9(2.7)	*b*
CH$_\alpha$	C6	NH	G7	17.1(2.4)	13.0(2.5)
NH	G7	CH$_\alpha$	G7	7.0(2.8)	6.8(2.8)

[a] Cross-peak volumes are reported as a percentage of the volume of the cross-peaks between geminal protons of proline. The mixing time was 200 ms. The rigid pair distance in Å is the one calculated from the cross-peak volumes assuming isolated spin pairs and a unique rigid conformation an is included only as a qualitative aid to evaluate the differences between the topological isomers. [b] Overlapping signals.

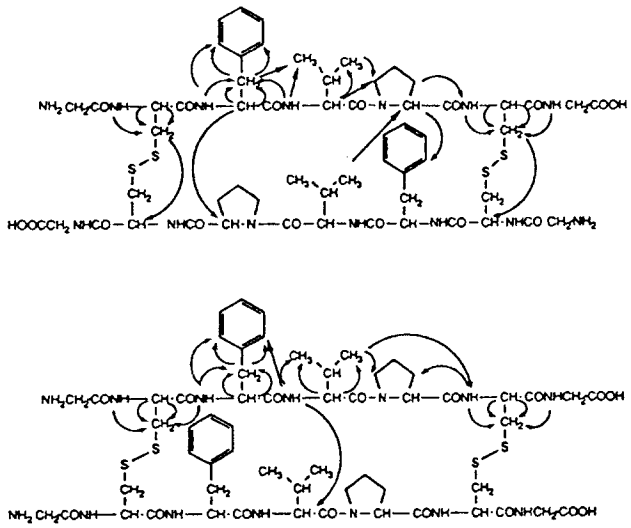

Figure 7. Observed NOEs for 1 and 2. in DMSO-d_6 using a mixing time of 200 ms. NOEs in 2 tentatively assigned as interchain are compatible with the covalent structure and, if assigned as intrachain, would be incompatible with other evidence suggesting basically extended chains. The interchain NOE in 1 has been proven using a selective-TOCSY-NOESY experiment. Sequential and intraresidue NOEs involving NH and CH$_\alpha$ protons are not shown.

The NOEs observed for peptides 1 and 2 are summarized in Table II and Figure 7. Although both are flexible molecules, and therefore, deriving a single structure from the NOEs is not justified, the patterns of NOE in both isomers differ, indicating different conformational preferences. Table II shows the volumes of the cross-peaks between backbone protons. In order to allow a comparison between the two molecules, the volumes were normalized by measuring the volume of the cross-peaks between geminal protons in proline. In the case of rigid molecules this would also allow the derivation of the interproton distances listed in parentheses. For each molecule the sequential NOEs are more intense than the ones within a residue. This is generally found in peptide molecules

that sample a large proportion of the allowed regions of the Ramachandran map, basically the β (extended) and the α_R region. However, when the two sets are compared, the sequential $CH_\alpha NH$ NOEs are stronger in the antiparallel isomer while the corresponding intraresidue NOEs are weaker. This points to a higher population of conformers in the extended conformation in the antiparallel molecule.

The long-range NOEs and those involving the side chains are summarized in Figure 7. While the symmetry of the molecule introduces some ambiguity, the assignment of the NOEs between CH_α of ^{6}Cys and CH_β of ^{2}Cys, CH_α of ^{3}Phe and CH_α of ^{5}Pro, CH_γ of ^{4}Val and CH_α of ^{5}Pro, and an aromatic proton of ^{3}Phe and CH_α of ^{5}Pro as interchain NOEs is consistent with the assumption of an extended conformation in the antiparallel dimer. The corresponding cross-peaks were not found in the parallel isomer. On the other hand, the NOE between $CH\gamma$ of ^{4}Val and NH of ^{6}Cys is consistent with the presence of folded conformations in the parallel dimer.

The observation of long distance NOEs in the parallel peptide is much more difficult because sequential or intraresidue cross-peaks could mask long-range NOEs between residues situated in symmetry-related positions in both chains that, for the parallel dimer, involve residues in equivalent sequence positions. In order to investigate this point, the recently described selective TOCSY–NOESY experiment[27] was performed in both a fully protonated and a partially deuterated sample of the parallel dimer, and the intensities of the $NHCH_\alpha$ cross-peaks were compared.

This experiment differentiates pairs of protons that present an NOE and that are scalarly coupled from those that also present an NOE and do not belong to the same spin system. In the former case the intensity of the cross-peaks, normalized with respect to the intensity of the amide protons, is not reduced by partial deuteration of the exchangeable protons while in the latter a strong decrease of the intensity is expected. For the problem at hand this experiment allows the identification of NOEs involving the NH of one residue and the CH_α of the residue located in the same sequence position in the second chain. The results are presented in Table III. Clearly, the NH of Val in one chain and the CH_α of Val in the second chain present an interchain NOE in the parallel dimer while no such interchain contribution can be detected to the other CH_α-NH cross-peaks.[28]

Molecular Modeling. It is obvious from the experiments presented that both dimers are flexible molecules and therefore the observed properties represent averages over a number of conformations. The particular conformations involved cannot be deduced from the NMR or CD data alone, and it is necessary to postulate reasonable candidates from an independent source. For that reason and in order to try to understand the different behavior of both dimers, their conformational space was explored using high-temperature molecular dynamics in vacuo. The absence of solvent, imposed by computational reasons, will not allow a quantitative comparison between the calculated and observed properties but it has certain advantages in the present case as it favors compact structures and emphasizes the competition between intra- and interchain interactions.

Table III. Normalized Cross-Peak Intensities in a Selective TOCSY–NOESY Experiment

pathway[a]	(^{n}NH^{n}CH) > ^{n}NH		(^{n}NH^{n}CH) > $^{n+1}$NH		$^{n+1}$NH > ^{n}CH	
sample	A	B	A	B	A	B
^{1}Gly			6.1[c]	3.8[c]	11.1[c]	12.9[c]
^{2}Cys	2.4	2.5	5.0	3.7	35.4	41.3
^{3}Phe	2.9	3.8	12.2	10.4	21.7	25.5
^{4}Val	8.1	6.8				
^{5}Pro					32.4	36.7
^{6}Cys	4.4	5.6	14.6	8.6	7.8	7.8
^{7}Gly	3.7	3.9				

[a] Magnetization transfer pathways are represented as follows: The leftmost NH corresponds to the selectively excited proton. TOCSY mixing occurs between the protons enclosed in parentheses and the > sign represents NOE transfer in the direction indicated. The cross-peak ($a > b$) appears at frequencies $\omega_1 = a$, $\omega_2 = b$. [b] Sample A: 64 mg of peptide was dissolved in 0.7 mL of DMSO-d_6. Sample B was obtained from A by partial exchange with D_2O. Degree of deuteration: 31%. Spectra were obtained in a Varian VXR-500 instrument with a 10 dB fixed attenuator at the output of the transmitter. The 270° Gaussian pulse was 2 ms, and the 90° pulse width was 17 ms. TOCSY mixing: 30 ms (6.5 kHz). NOESY mixing: 300 ms. [c] Normalized to the $^{n+1}$NH intensity. Otherwise, normalized with respect to ^{n}NH intensity. Units are percent of the intensity of the reference peak. Intensities are the average of two experiments with the same samples. Reproducibility is better than 10%.

Structures were obtained by systematic energy minimization of points separated by 0.5 ps along a dynamic trajectory running at 750 K with periodic reinitialization of the velocities as described in the Experimental Section. In order to have a reference calculation, the conformations of the monomeric peptide (*H*-GCFVPCG-*OH*) were sampled during a 170-ps trajectory starting from a fully extended structure, 78 distinct[29] conformations were found with energies, after minimization, ranging from 156.2 to 178.2 kcal mol^{-1}. Table IV contains the conformations of the monomer having energies less than 5 kcal mol^{-1} over the global energy minimum. The large number of conformations of low energy is indicative of the flexibility expected from a short linear peptide.

Minimization of the structures sampled along a 130-ps trajectory of the antiparallel dimer starting from an extended structure yielded 263 conformations with energies ranging from 289.1 to 324.7 kcal mol^{-1}.

The conformations of the parallel dimer were sampled along a 200-ps trajectory yielding 383 conformations with energies ranging from 290.9 to 324.0 kcal mol^{-1}. Some of these conformations had cis amide bonds[30] and were not analyzed further as this was not detected experimentally. The all-trans lowest energy conformations ($\Delta E < 5$ kcal mol^{-1}) of 1 and 2 are listed in Table IV.

The energy of interaction between the two chains in vacuo is clearly negative for both dimers, and its magnitude,

(27) Pons, M.; Giralt, E. *J. Am. Chem. Soc.* 1991, *113*, 5049–550.

(28) The lower intensity of the NH CHα (i,i) cross-peaks on the antiparallel cyclic dimer prevented the analysis of these peaks to try to identify long-range interactions. The selective-TOCSY–NOESY experiment does not differentiate between true sequential cross-peaks and their interchain counterparts as in both cases the protons involved belong to different spin systems. The increase in the normalized intensity of the true intraresidue cross-peaks can be explained by the elimination of competing relaxation pathways by deuteration.

(29) Conformations were considered distinct only if the main-chain dihedral angles of the internal residues (i.e., excluding the *N*- and *C*-terminal glycine residues) belonged to different regions of the Ramachandran map according to the usual classification (Zimmerman, S. S.; Pottle, M. S.; Némethy, G.; Scheraga, H. A. *Macromolecules* 1977, *10*, 1–9).

(30) While in the simulations of 2 all the amide bonds remained trans without the need of forcing potentials, it was very difficult to avoid isomerizations during the simulation of 1. The best results were obtained starting the exploration of the conformational space from an structure generated by gradually decreasing the S–S distance between two monomers while forcing all the amide bonds to be trans.

Table IV. Low-Energy Conformations of H-GCFVPCG-OH and Its Cyclic Bis-Cystine Dimers[a]

monomer	ΔE^b (kcal/mol)	parallel cyclic dimer 1	ΔE^b (kcal/mol)	antiparallel cyclic dimer 2	ΔE^b (kcal/mol)
CC*CAC	0.00 (156.23)	CECCA/ECDAA	0.00 (290.86)	CCCCC/CCCCC	0.00 (289.09)
CC*FAC	0.47	CECCA/ECCAA	3.24	CCDCC/CCCCC	0.60
CC EAA	2.30	CCCCA/ECCAA	4.54	DCDCC/DECCC	2.08
CE CAC	2.88			CDDAD/CDDCC*	2.63
CC*CAA	2.98			CCCAC/CDDCB	3.72
CC DAC	3.17			CCEAD/CDDFA	4.39
CE CCC	3.59				
CC CAC	3.67				
CC DCC	4.22				
CC EAC	4.27				
CC FAC	4.62				
CC CAA	4.69				
CC*CBG	5.00				

[a] Conformations with energies within 5 kcal/mol above the lowest energy found are listed using the classification of Zimmerman et al. (ref 29) for the $\geq$ and ψ angles of the five central residues. The conformational search was carried out by molecular dynamics as described in the Experimental Section. The length of the trajectory and the total number of distinct conformations found were: 170 ps (78 structures) for the monomer, 200 ps (383 structures) for 1, and 130 ps (263 structures) for 2. [b] The energy of the lowest minimum (cvff force field) is given in parentheses.

estimated from the comparison between the energy of the different conformers of the dimer and twice the energy of the lowest energy conformation of the monomer,[31] is comparable to the largest energy difference observed between conformations of the monomer and therefore indicates that the observed conformations will mainly reflect the optimization of the interaction between the two chains rather than the intrinsic conformational preferences of the monomer peptide.

As expected, the number of accesible conformational states is reduced in both dimers as compared to the monomer although both peptides retain considerable flexibility. The number of conformers found within a given energy range is lower in the parallel dimer indicating that it is probably more rigid.

In 2 the optimal interaction seems to take place between fully extended chains as in a β-sheet structure although, in our model, intrachain hydrogen bonding seems to predominate over the interchain one.[32] Interchain hydrogen bonds involving a valine and the cysteine in position 2 of the other chain were observed in several low-energy structures including the minimum with the lowest energy found. In one of the minima the NH of ^{2}Cys was also involved in a hydrogen bond with a sulfur atom in the bridge. These observations agree with the observation that in the ^{1}H-NMR spectra the NH proton of ^{2}Cys gives a broad signal even at the highest temperature studied and the apparent temperature coefficient is the lowest measured (−2.5 ppb K^{-1}). The extended structure is consistent with a higher intensity of cross-peaks between residues i, i+1 in the fingerprint region of the NOESY spectra of 2 and is also in agreement with the CD spectra.

The peptide chains in the low-energy conformations of 1,on the other hand, tend to be more folded than in the antiparallel dimer specially near the C-terminus where several residues adopt dihedral angles in the α-helical region of the Ramachandran map (A). This is consistent with the observation of a type C CD spectrum for 1 in 90% TFE and with more intense intraresidue cross-peaks in the fingerprint region of its NOESY spectra in DMSO-d_6.

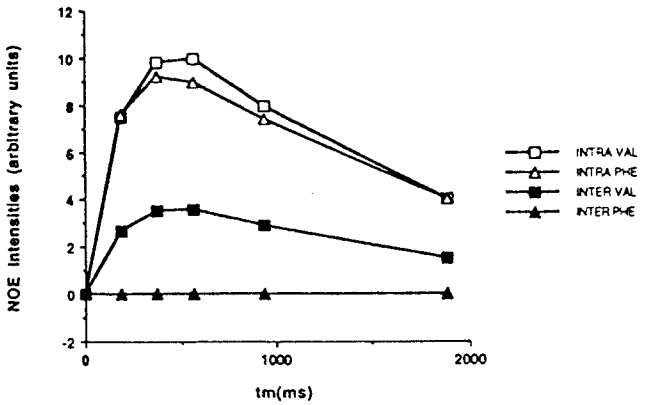

Figure 8. Calculated NOE buildup curves between NH and CHα protons of valine (squares) and phenylalanine (triangles). Open symbols refer to the interaction between an NH and a CH$_\alpha$ proton that belong to the same residue. Closed symbols indicate that the interaction involves an NH and a CH$_\alpha$ protons that belong to different, although symmetry related, residues.

We evaluated the ensemble of conformations found in the modeling study by calculating the evolution of the cross-peak intensities corresponding to the intraresidue and interchain NH to CHα NOE of Val and Phe in 1. Cross-relaxation and autorelaxation rates were calculated from a weighted average of the inverse sixth power of the distance between the relevant pairs of protons in 340 conformations that had either a distance between the two protons smaller than 4 Å or an energy less than 10 kcal mol^{-1} above the global minimum. Cross-peak intensities at different mixing times were calculated using a relaxation matrix approach.[33] The curves representing the growth of intra- and interchain NOE for the two types of residues are shown in Figure 8. The calculations indicate that the intensity of the interchain NOE involving the two Val residues should be significantly higher than that involving the two Phe residues, in good agreement with the experimental result.

Conclusions

Our CD, NMR, and molecular modeling studies on two peptide dimers with identical sequence but different

(31) The energy associated with breaking the SH bonds and the formation of the disulfide bond can be estimated by comparing the minimized energies of cysteine and cystine in vacuo with the force field used, and its value is less than 1 kcal mol^{-1}.

(32) This is most probably an artifact coming from the absence of solvent during the simulations. For that reason we assimilate the C region of the Ramachandran map with an extended structure.

(33) Ernst, R. R.; Bodenhausen, G.; Wokaun, A. *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*; Oxford Science Publications: New York, 1990.

topology indicates that although both peptides retain considerable flexibility in solution they show different conformational preferences which are clearly identified experimentally. According to our calculations these differences arise mainly from the optimization of the interactions between the two chains. Given the flexibility of both peptides it has not been considered appropriate to try to present a single structure that may be considered the "average structure". Rather, the conformational ensemble, derived from the calculations, has been shown to explain the most distinctive experimental features, and it can be considered a realistic picture of these flexible dimers in solution.

Experimental Section

Kel-F–*g*-styrene (containing 6% of styrene) and polystyrene–1%-divinylbenzene (200–400 mesh) were obtained from ICI (Australia) and Bio-Rad Labs (Richmond, CA), respectively. Benzhydrylamine resins were synthesized according to the method of Pietta and Marshall.[34] (Chloromethyl)-Pab-resins were prepared as reported,[11] *N*-α-Boc-*S*-Npys-L-cysteine and *N*-α-Boc-*S*-Fm-L-cysteine were synthetized from L-cysteine according to previously described procedure 61.[10b,18c] Other Boc-amino acids were from Peninsula (Belmont, CA) and Research Fundation (Japan). α-Chymotrypsin immobilized on (carboxymethyl)-cellulose contained 1 unit of enzyme per 2 mg of support and was obtained from Sigma (St. Louis). Dichloromethane was distilled over anhydrous sodium carbonate. *N,N*-Dimethylformamide was distilled over P_2O_5 and stored over 4-Å molecular sieves. All others solvents and chemicals were analytical grade and were used without further purification.

Peptide–resins and peptides were hydrolyzed in vacuum-degassed tubes at 130 °C with 12 N HCl–propionic acid (1:1) for 3 h and with 6 N HCl for 1 h, respectively. HF reactions were performed in a Toho-Kasei apparatus. Analytical HPLC columns used were Nucleosil C18 (4 × 250 mm, 5 mm) and Spherisorb C18 (4 × 300 mm, 5 mm). Preparative medium pressure liquid chromatography (MPLC) was performed on a Vydac C18 (25 × 300 mm) column.

FABMS were obtained in a VG 70E-HF instrument with thioglycerol or glycerol matrices. The latter had to be used in the case of peptides with sulfenyl-type protection of the cysteine as thioglycerol leads to thiol–disulfide interchange.

General Procedure for Solid-phase-peptide Synthesis. (i) Anchoring of the First Amino Acid on Pab-Resins. Cesium Boc-glycinate[35] (1.5 equiv) was added to (chloromethyl)-Pab-resin (1 equiv) in DMF and the suspension stirred overnight at 50 °C. The resin was then filtered and washed with DMF, 5 × 10 mL; DMF–H_2O (1:1), 5 × 10 mL; DMF, 5 × 10 mL; and CH2Cl2, 5 × 10 mL. The Boc was removed, and picric acid titration[36] revealed yields of 86%.

(ii) General Method for Solid-Phase Peptide Assembly. All peptides were synthesized in 20-mL polypropylene syringes fitted with a porous polyethylene filter disk. All couplings were carried out as follows: (1) CH_2Cl_2, 4 × 10 mL, 30 s; (2) TFA–CH_2Cl_2 (3:7), 1 × 10 mL, 1 min, 1 × 10 mL, 30 min; (3) CH_2Cl_2, 5 × 10 mL, 30 s; (4) DIEA–CH_2Cl_2 (1:19), 3 × 10 mL, 1 min; (5) CH_2Cl_2, 5 × 10 mL, 30 s; (6) Boc-amino acid (2.5 equiv) in CH_2Cl_2, 5 mL (for Boc-Cys(Acm)-OH, CH_2Cl_2–DMF (4:1) was required), after 1 min add equivalent amount of DCC in CH_2Cl_2, 5 mL, shake for 45 min; (7) CH_2Cl_2, 5 × 10 mL, 30 s. Ninhydrin test[37] was negative after each coupling.

(iii) HF Cleavage of Peptide-Pab–Resins. Peptide–resins were placed in a Kel-F vessel and treated with HF–*p*-cresol (9:1), 10 mL. After 1 h at 0 °C, the HF was removed under vacuum. The residue was washed with diethyl ether, 3 x 10 mL , and CH_2Cl_2, 3 × 10 mL, and then extracted with HOAc–H_2O (1:1), 3 × 10 mL, and H_2O, 3 × 10 mL.

Synthesis and Purification of H-Gly-Cys(Fm)-Phe-Val-Pro-Cys(Acm)-Gly-OH (5). Peptide 5 was synthesized as described above starting from 2.5 g (0.4 mmol Cl/g of resin) of (chloromethyl)-Pab-polystyrene–1%-divinylbenzene. After HF treatement, crude peptide (0.48 mmol, 48% overall yield) was washed with MeOH to provide 0.45 mmol (95% yield) of pure cysteine protected peptide (single peak in HPLC in differents conditions). Amino acid analysis: $Gly_{1.97}$, $Cys_{0.90}$,[38] $Val_{0.98}$, $Phe_{1.05}$, $Pro_{0.90}$. FABMS: *m/z* 931.4.

Synthesis of (H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH)$_2$ (3). A solution of 0.30 mmol of peptide 5 in 15 mL of piperidine–DMF (1:1) was stirred for 2 h at 25 °C. After evaporation of DMF and excess of piperidine, crude material was washed several times with CH_2Cl_2 and the remaining solid separated by centrifugation. The purity was checked by HPLC, and peptide 3 (0.12 mmol, 82% yield) was used without further purification. Amino acid analysis: $Gly_{2.02}$, $Cys_{1.63}$, $Val_{0.99}$, $Phe_{1.02}$, $Pro_{0.98}$. FABMS: *m/z* 753.3.

Synthesis and Purification of the Parallel Cyclic Dimer (1). To a solution of 0.1 mmol of 3 in 100 mL of HOAc–H_2O (4:1) were added 0.25 g (1 mmol) of I_2. After 35 min all product was oxidized as demonstrated by HPLC. The solution was diluted with 50 mL of H_2O, extracted with CCl_4 (4 × 50 mL), and concentrated to remove rests of CCl_4. After lyophilization, the product was purified by MPLC (convex gradient formed from 700 mL each of 0.1 N HCl and acetonitrile–0.1 N HCl (32:64) to provide 28 μmol (28% yield) of pure dimer (single peak in HPLC in different conditions). Amino acid analysis: $Gly_{2.02}$, $Cys_{1.25}$, $Val_{0.99}$, $Phe_{0.99}$, $Pro_{0.90}$. FABMS, *m/z* 1359.3.

Simultaneous Synthesis of H-Gly-Cys(Npys)-Phe-Val-Pro-Cys(Acm)-Gly-OH (6) and H-Gly-Cys(Acm)-Phe-Val-Pro-Cys-Gly-OH (7). Peptides 6 and 7 were synthesized on (chloromethyl)-Pab-Kel-F-*g*-styrene (1 g, 0.14 mmol Cl/g of resin) and (chloromethyl)-Pab-polystyrene–1%-divinylbenzene (0.45 g, 0.4 mmol Cl/g of resin), respectively, following the general protocol. The cysteine 6 of peptide 7 was protected during the synthesis with the MeBzl group. The resins were mixed together and the couplings were carried out simultaneously, only being separated[39] for anchoring of cysteine residues and prior to HF cleavage. HF reaction provides 74 μmol (53% overall yield) of crude peptide 6 and 49 μmol (36% overall yield) of 7 ($Gly_{2.06}$, $Cys_{1.03}$, $Val_{1.03}$, $Phe_{0.96}$, $Pro_{0.87}$). After lyophilization both peptides were purified by MPLC (convex gradient formed from 300 mL each of H_2O–acetonitrile–TFA (95:5:0.05) and H_2O–acetonitrile–TFA (75:25:0.05)) to yield 44 μmol of pure peptide 6 (59% yield; amino acid analysis: $Gly_{2.03}$, $Cys_{1.37}$, $Val_{1.03}$, $Phe_{0.93}$, $Pro_{0.84}$; FABMS *m/z* 907.3) and 35 μmol of peptide 7 (71%; amino acid analysis: $Gly_{2.01}$, $Cys_{0.94}$, $Val_{1.09}$, $Phe_{0.98}$, $Pro_{0.87}$; FABMS *m/z* 753.4).

Simultaneous Synthesis of H-Gly-Cys(Acm)-Phe-Val-Pro-Cys(Fm)-Gly-OH (8) and H-Gly-Cys(Fm)-Phe-Val-Pro-Cys(Acm)-Gly-OH (5). Peptides 8 and 5 were prepared simultaneously on (chloromethyl)-Pab-polystyrene–1%-divinylbenzene) (0.4 g, 0.7 mmol Cl/g of resin) and (chloromethyl)-Pab-Kel-F-*g*-styrene (2 g, 0.14 mmol Cl/g of resin), respectively, as described for peptides 6 and 7. HF treatement of peptide–resins provides 188 μmol (67% overall yield) of peptide 8 (amino acid analysis: $Gly_{1.97}$, $Val_{1.04}$, $Phe_{0.98}$, $Pro_{0.86}$; FABMS *m/z* 931.4) and 188 μmol (67% overall yield) of peptide 5 (amino acid analysis: $Gly_{2.10}$, $Val_{0.97}$, $Phe_{0.94}$, $Pro_{0.86}$; FABMS *m/z* 931.2). These peptides were used without further purification.

Preparation of H-Gly-Cys(Acm)-Phe-Val-Pro-Cys-Gly-OH (7) and H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH (9). To a solution of 100 μmol of peptide 8 or 5 in 1 mL of DMF–β-mercaptoethanol (9:1) was added 1 mL of piperidine under an atmosphere of Ar. The mixture was stirred for 2 h at room temperature, and then DMF and excess of piperidine were removed by rotary evaporation. The residue was treated with 20 mL of ethyl ether containing 0.1 mL of TFA and the remaining

(34) Pietta, P. G.; Marshall, G. R. *J. Chem. Soc., Chem. Commun.* **1970**, 650–651.
(35) Gisin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476–1482.
(36) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248–249.
(37) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.

(38) Cys(Fm) is resistant to the conditions of hydrolysis.
(39) Peptide-resins I and II were suspended in 20 mL of CH_2Cl_2–TFE (8:2) for 1 h. After this time, the upper layer containing resin II was removed by suction with a pipette. The procedure was repeated three times with each of the two resins.

solid separated by centrifugation to yield 84 μmol (84% yield) of peptide 7 ($Gly_{1.94}$, $Cys_{1.16}$, $Val_{1.09}$, $Phe_{0.97}$, $Pro_{0.91}$; FABMS *m/z* 753.3) or 89 μmol (89% yield) of peptide 9 ($Gly_{2.00}$, $Cys_{1.77}$, $Val_{1.09}$, $Phe_{0.97}$, $Pro_{0.91}$; FABMS *m/z* 753.3) or 89 μmol (89% yield) of peptide 9 ($Gly_{2.00}$, $Cys_{1.77}$, $Val_{1.09}$, $Phe_{0.97}$, $Pro_{0.91}$; FABMS *m/z* 753.4) respectively.

Preparation of Gly-Cys(Acm)-Phe-Val-Pro-Cys(Spyr)-Gly-OH (10). Eighty μmol of 8 dissolved in 15 mL of *n*-propanol–0.1 M Tris buffer solution, pH = 8 (1:1), was treated with 27 μmol (88 μmol) of tributylphosphine overnight under Ar atmosphere. After this time, 174 mg (0.8 mmol) of 2,2'-dithiodipyridine in 5 mL of 1-propanol–0.1 M Tris buffer solution pH = 8 (1:1) was added, and the mixture was stirred during 10 min and finally chromatographied through a Sephadex G-10 column (2.3 × 100 cm; in 0.1 M HOAc) to provide 50 μmol (63% yield) of peptide 10 (amino acid analysis: $Gly_{1.90}$, $Cys_{1.50}$, $Val_{1.10}$, $Phe_{0.98}$, $Pro_{0.86}$; FABMS *m/z* 859.[40]

Synthesis and Purification of *S,S'*-(H-Gly-Cys-Phe-Val-Pro-Cys(Acm)-Gly-OH)(H-Gly-Cys(Acm)-Phe-Val-Pro-Cys-Gly-OH) (4). Method i. Thirty μmol of peptide 7 in 10 mL of 10% HOAc was added dropwise to a 10% HOAc solution containing 30 μmol of peptide 6. The mixture was stirred at 25 °C for 3 h and then concentrated and purified by MPLC (convex gradient from A (300 mL; 0.05% TFA in H_2O) to B (300 mL; H_2O–acetonitrile–TFA (75:25:0.05))) to yield 21.4 μmol (77% yield) of pure peptide 4. Amino acid analysis: $Gly_{1.98}$, $Cys_{1.40}$, $Val_{1.04}$, $Phe_{0.94}$, $Pro_{0.87}$. FABMS: *m/z* 1503.9.

Method ii. To a solution of 2 μmol of 10 in 2 mL of acetonitrile–0.1 M Tris buffer solution pH = 8 (1:1) were added 2 mL of a solution of 10% HOAc containing 2 μmol of 9. The pH was adjusted to 6 with 10% sodium carbonate solution and the reaction stopped after 30 min. Crude material was concentrated and purified by MPLC (convex gradient formed from 300 mL each of 0.05% TFA in H_2O and acetonitrile–H_2O–TFA (75:25:0.05)) to yield 1.6 μmol (80% yield) of 4. Amino acid analysis: $Gly_{1.98}$, $Cys_{1.40}$, $Val_{1.04}$, $Phe_{0.94}$, $Pro_{0.87}$. FABMS: *m/z* 1503.9.

Synthesis and Purification of the Antiparallel Cyclic Dimer (2). Twenty μmol of peptide 4 in 20 mL of HOAc–H_2O (8:2) was treated with 50 mg (0.2 mmol) of iodine and the mixture vigorously stirred at room temperature. The reaction was monitored by HPLC (Nucleosil C_{18}; linear gradient of acetonitrile–0.01 N HCl from 5:95 to 90:10) and stopped after 1 h by dilution with 20 mL of H_2O and extraction of the iodine with CCl_4. The aqueous solution was purified after concentration by MPLC (convex gradient from A (300 mL, 0.05% TFA in H_2O) to B (300 mL, H_2O–acetonitrile–TFA (25:75:0.05))) yielding 9 μmol (45% yield) of purified 2. Amino acid analysis: $Gly_{1.90}$, $Cys_{1.40}$, $Val_{1.11}$, $Phe_{0.97}$, $Pro_{0.88}$. FABMS: *m/z* 1361.5.

Enzymatic Hydrolysis. Two mg of α-chymotrypsin immobilized on (carboxymethyl)cellulose (1 unit of enzyme) was suspended in 0.2 mL of H_2O, and after 2 h at 4 °C the supernatant was removed by centrifugation. The enzyme was washed five times with 0.04 M Na_2HPO_4–NaH_2PO_4 buffer solution pH 8.00, and then 2 mg of dimer parallel or antiparallel dissolved in 0.2 mL of phosphate buffer solution was added. Aliquots of the solution were removed at different times and checked by HPLC. Fractions corresponding to different peptides were collected, lyophilyzed, and subjected to acid hydrolysis in order to determine the amino acid composition.

Circular Dichroism Spectroscopy. CD spectra were run at concentrations ranging from 0.2 to 1 mg mL^{-1}. One-mm cells thermostated at 25 °C were used unless otherwise stated.

NMR Spectroscopy. ^1H-NMR spectra were recorded at 200 MHz or 500 MHz using standard sequences for DQF-COSY, ROESY, and TOCSY experiments.

Selective TOCSY–NOESY Experiment. The sequence used is as follows: 270 (Gaussian)-MLEV17-t_1-90-mix-90-Acq. A standard phase cycling for the NOESY experiment was used. The phase of the MLEV pulse train was kept constant.

Spectra were obtained in a Varian VXR-500 instrument equiped with linear amplifiers. A 10 dB fixed attenuator was placed at the output of the transmitter to obtain properly shaped pulses. The 270° Gaussian pulse was 2 ms and was selective for the NH protons. The hard 90° pulse width was 17 μs. Simulation of the NOE cross-peak growth curves was carried out using Mathematica.

Molecular Modeling. Calculations were performed on a Silicon Graphics Personal IRIS 4D/25 using the INSIGHTII and DISCOVER packages.[41] The CVFF[42] force field was used. No Morse potentials or cross terms were used for the high-temperature simulations, and no solvent was included in the calculations. The search protocol was the following: starting from an energy-minimized structure, the temperature was forced to equilibrate for 0.1 ps at 750 K, and a molecular dynamics simulation was run using 0.5-fs integration steps. This time step ensured that the oscillations in the total energy in a simulation where the molecule was decoupled from the thermal bath were of the same level as a standard simulation at 300 K using a 1-fs integration step. Complete thermal equilibration was achieved after further 0.4 ps of free dynamics. Snapshots were taken every 0.5 ps and the corresponding structures minimized and stored. Every 10 ps the dynamics was restarted from the last minimized structure by reasigning the velocities of the atoms according to a Maxwell–Boltzmann distribution corresponding at 750 K, and reequilibrating the system. In an independent study[43] we have found that this procedure provides a more thorough exploration of the conformational space than a continuous dynamic simulation at the same temperature as each reinitialization corresponds to a high-temperature pulse that allows the system access to new regions of the conformational hypersurface.

Acknowledgment. This work was supported in part by funds from the CICYT (Grant Nos. PB89–257 and PB91–283). We thank Dr. Edward Larka (University of Minnesota) for running the FAB-MS, Dr. Ettore Castiglioni (Jasco Europe s.r.l.) for the CD spectra, and Ms. M. Antònia Molins for running some NMR spectra. We acknowledge the use of the facilities of the Serveis Científics i Tècnics of the University of Barcelona.

(40) This *m/z* correspondes to the product obtained by disulfide interchange between the peptide and the thioglycerol matrix.

(41) Commercialy available from Biosym Technologies Inc.

(42) (a) Hagler, A. T.; Lifson, S. *J. Am. Chem. Soc.* **1974**, *96*, 5319–5327. (b) Hagler, A. T.; Dauber, P.; Lifson, S. *J. Am. Chem. Soc.* **1979**, *101*, 5131–5141.

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