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A Potent Cyclolinopeptide A Analogue: Solid State and Solution Conformation of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$

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Abstract: The conformational analysis of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$, in the solid state and in solution, has been carried out by X-ray diffraction and NMR spectroscopy. The structure of the orthorhombic form obtained from dioxane-water-acetonitrile mixture [a = 9.994(1) Å, b = 21.846(5) Å, c = 37.357(9) Å, space group $P2_12_12_1$; Z = 4] shows the presence of four intramolecular NH–CO hydrogen bonds, with formation of two β turns (one of type I and one of type II) and two C_{14} ring structures. All peptide units are *trans*. The solution structure, as determined by NMR, indicates that, at room temperature, the peptide is conformationally homogeneous; the structure determined is perfectly symmetrical and topologically similar to that found in the solid state. The cyclodecapeptide exhibits similar biological activity to cyclolinopeptide A.

Introduction

The natural cycloamanides antamanide (AA),[1] cyclolinopeptide A (CLA),[2] and, to a lesser extent, the peptide hormone somatostatin[3,4] are characterized by the common capacity to inhibit the uptake of bile salts into hepatocytes. CLA and AA share the same postulated active sequence Pro-Pro-Phe-Phe, while in somatostatin only adjacent aromatic amino acids are present. This feature is thought to represent the necessary prerequisite for bioactivity which, as in CLA and in AA, is highly increased by the preceding proline residue. The molecular mechanism of the biological activity of these peptides is represented by their strong interaction with the multispecific transport system of the hepatocyte membrane, responsible for uptake into those cells of bile salts as well as of several structurally different substances.[5,6] AA is one of the most investigated natural bioactive cyclopeptides; its biological, structural, and conformational properties have been studied in the native peptide and in a large number of synthetic analogs.[7–9] Among other structural requisites, the presence of two adjacent Phe residues[10] and of a hydrophobic residue in position 1 (Val in the natural peptide) have been established as essential for biological function. In the AA series a striking coincidence between bioactivity and ion complexing ability has been observed.[11,12] This capability could reflect the readiness of the molecule to assume the proper conformation for binding to the substrate or, as suggested by I. Karle et al., the metal ion could induce the proper shape in the AA molecule.[13] In their research on somatostatin and related peptides, Kessler and co-workers inserted the postulated bioactive sequence Pro-Pro-Phe-Phe into small sized synthetic cyclopeptides, in order to identify the structural parameters and the conformational requirements able to increase bioactivity. Significant insight into the SAR of this class of compounds has been gained through conformational studies, carried out by means of NMR and X-ray techniques on very potent inhibitors of the hepatic cholate uptake, like *cyclo*[D-Pro-Phe-Thr-Lys(Z)-Trp-Phe], so called "008", or on totally inactive products such as *cyclo*(Pro-Pro-Phe-Phe-Gly).[14,15]

The determination of the X-ray structure and of an NMR analysis of CLA, in our laboratories,[16–18] prompted us to be involved in a research project based on the synthesis, biological, structural, and conformational studies of CLA analogs and

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related compounds.[19-29] In solution, at room temperature, CLA exhibits marked conformational mobility leading to the presence of a mixture of several conformers. A suitable NMR analysis of CLA has been carried out in $CDCl_3$ at low temperature (214 K). In these conditions CLA exists as a single "frozen" conformer,[17] whose structure is consistent with that found in the solid state. This conformation, however, cannot be regarded as the bioactive one since it corresponds to that exhibited, both in the solid state and in solution at room temperature, by the less active constrained *cyclo*(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val).[19,20,28] The necessary structural parameters suggested by others authors[14] have been confirmed by our studies on CLA analogs, carried out by replacing in turn each residue of the postulated active sequence with a L-Ala residue.[21,22] This led us to identify the "minimal active sequence" Pro-Phe-Phe. The insertion of this sequence into small cyclic cystinyl peptides gave compounds with different activity, depending on their ring size.[24-26] Our studies suggest that the inhibitory potency of peptides containing the bioactive sequence Pro-Phe-Phe seems somehow to be independent from the composition and size of the remaining portion of the molecule, provided its conformational freedom is preserved.[24,25]

As a further development of our studies in this field, we have designed a series of CLA related homodetic cyclopeptides. In this paper we report on the synthesis, and conformational properties, as determined by X-ray, NMR, and molecular dynamics simulation, of the dimeric decapeptide *cyclo*[-Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$, called cDECA, that shows biological activity similar to CLA.[17]

Experimental Section

Synthesis. Z-Glu(OtBu)-Pro-Phe-Phe-OMe (**1**). The Boc group was removed by treating 2.61 g (5 mmol) of Boc-Pro-Phe-Phe-OMe with 20 mL of TFA for 1 h at room temperature. The solution was evaporated to dryness, and the residue was repeatedly triturated and washed with ether. The resulting TFA salt was dissolved in 30 mL of methylene chloride, deprotonated with NMM, and added to a MA solution prepared by stirring 10 min at −10 °C 1.68 g (5 mmol) of Z-Glu(OtBu)-OH, 0.68 g (5 mmol) of iBCCl, and 0.5 g (5 mmol) of NMM in 30 mL of methylene chloride. After stirring 3 h at room temperature the reaction mixture was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$, and water, dried over Na_2SO_4, and evaporated to give 2.50 g of pure (**1**) as a foam: (yield 96%) R_f 0.7 in ethyl acetate.

Z-Glu(OtBu)-Pro-Phe-Phe-Ala-OMe (2). For saponification 3.58 g (4.82 mmol) of **1** were dissolved in 50 mL of methanol and reacted overnight at room temperature with 0.250 g (6.23 mmol) of solid NaOH in 10 mL of water. The organic solvent was evaporated in vacuo, and the aqueous phase was acidified with 0.5 M $KHSO_4$ to pH 3 and extracted several times with ethyl acetate to afford 3.2 g (4.4 mmol, 94% yield) of Z-Glu(OtBu)-Pro-Phe-Phe-OH. To a solution of that tetrapeptide in 50 mL of methylene chloride were added 0.906 g (4.4 mmol) of DCCI and 0.453 g (4.4 mmol) of H-Ala-OMe. After stirring 1 h at 0 °C and overnight at room temperature, the solution was cleared of dicyclohexylurea by filtration, and the filtrate was worked up as described in the preceding paragraph to give a residue that was chromatographed on a silica gel column (2.5 × 70 cm) in chloroform−methanol (96:4) as eluent yielding 2.6 g of **2** as a TLC pure foam (64% yield): R_f 0.5 in chloroform−methanol (95:5).

H-Glu(OtBu)-Pro-Phe-Phe-Ala-OH (3). The removal of methyl ester and of the benzyloxycarbonyl protecting group from **2** were achieved by saponification and catalytic hydrogenation, respectively.

For saponification 2.6 g (3.31 mmol) of **2** were dissolved in 50 mL of methanol and treated overnight at room temperature with a solution of 0.16 g (4 mmol) of NaOH in 10 mL of water. After workup as previously described, 2.2 g of TLC pure Z-Glu(OtBu)-Pro-Phe-Phe-Ala-OH was obtained. This product was hydrogenated in 100 mL of methanol over 0.3 g of 10% Pd on charcoal for 24 h to remove the Z protecting group, yielding 1.7 g of **3**.

***cyclo*[-Pro-Phe-Phe-Ala-Glu(OtBu)-]$_2$ (4).** For cyclization, a solution of 1.7 g (2.55 mmol) of **3** in 300 mL of DMF and 200 mL of THF were treated while stirring at −10 °C with 0.347 g (2.55 mmol) of iBCCl. After 10 min stirring at −10 °C a precooled solution of 0.505 g of NMM in 800 mL of DMF and 400 mL of THF was added, and the reaction mixture was stirred 1 h at 0 °C and overnight at room temperature. The organic solvent was evaporated under vacuum, and the residue, dissolved in 100 mL of methylene chloride, was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$, and water, dried over Na_2SO_4, and evaporated in vacuo. The resulting residue was chromatographed on a silica gel column (2.5 × 100 cm) in chloroform methanol (97:3) as eluant to obtain 0.240 g of TLC pure **4** (7% yield): R_f 0.4 in chloroform−methanol (95:5).

Further purification by preparative HPLC using a C_{18} RP column eluted by linear gradient H_2O 0.1% TFA and CH_3CN 0.1% TFA gave the pure cyclopeptide **4**.

FABMS of **4** was 1329 as expected. The amino acid analysis was satisfactory. The use of chiral stationary phases for determination of amino acids optical purity showed the absence of racemization during the peptide synthesis.[30]

X-ray Diffraction. Crystals of the decapeptide were grown by slow evaporation from dioxane/water/acetonitrile mixture. An irregular prism of 0.5 × 0.3 × 0.4 mm was mounted on a glass fiber with epoxy. Preliminary oscillation and Weissenberg photographs were taken to establish the crystal symmetry and the preliminary space group. Determination of cell constants was obtained by a least squares procedure on the angular settings of 25 reflections in the θ range 21−25°. The crystallographic data are reported in Table 1. Data were measured on an Enraf-Nonius CAD-4 diffractometer equipped with graphite monochromated CuKα radiation (λ = 1.5418 Å).

Intensity data were collected in the ω-2θ scan mode, with a scan angle $\Delta\omega = (1.1 + 0.16 \tan \theta)°$; background counts were taken in an additional area of $\Delta\omega/4$, on both sides of the main scan, using the same scan speed. Prescan runs were made at a speed of 5°/min. Reflections with a net intensity $I > 0.5\sigma(I)$ were measured at lower speed depending on the $\sigma(I)/I$ value, in the range 1:4°/min. Two intensity control reflections were measured every 60 min of X-ray exposure time in order to monitor the crystal and the electronic stability; no significant change in intensity was observed during data collection. Orientation matrix checks were made with respect to the scattering vectors of two well-centered reflections every 300 reflection. All reflections were corrected for Lorentz and polarization effects. In a range 1 to 70° of θ 8561 reflections were collected; 3383 of which having a net intensity greater than $2.0\sigma(I)$ were considered observed and used for further calculation.

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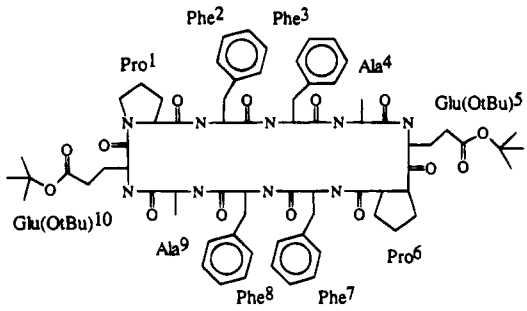
Table 1. Crystal Data of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$

molecular formula	$C_{70}H_{90}N_{10}O_{14}\cdot 2C_4H_8O_2$
molecular wt (amu)	1471.8
crystal system	orthorombic
space group	$P2_12_12_1$
Z, molecules/unit cell	4
a (Å)	9.994 (1)
b (Å)	21.846 (5)
c (Å)	37.357 (9)
V (Å^3)	8156 (3)
D_{calc} (g/cm^3)	1.198
radiation (Å)	Cu Kα (1.5418)
measured reflcns	8561
independent reflcns	8519
obsd reflcns (with $I > 2.0\sigma(I)$)	3383
R factor	0.084
R factor, weighted	0.081
no. parameters refined	896
S	2.345
$(\Delta\varrho)$max (e Å^{-3})	0.270
$(\Delta\varrho)$min (e Å^{-3})	−0.084
temp (K)	293
solvent of crystallization	dioxane/water/acetonitrile

Chart 1



The structure was solved by means of direct methods, using SIR92.[31] The best solution revealed all non-hydrogen atoms of cDECA. Subsequent difference Fourier analysis revealed the two dioxane molecules. The hydrogen atoms were introduced in fixed, stereochemically expected positions with an isotropic temperature factor equal to B_{eq} of the heavy atom to which they are linked. Full-matrix least-squares procedures were used and converged to a final R factor of 0.084 and R_w of 0.081, using anisotropic temperature factors for the non-hydrogen atoms of the peptide molecule and isotropic temperature factors for the solvent atoms. Refinement was ended when the shifts in the atomic coordinates and temperature factors were less than 1/5 and 1/3 of the corresponding standard deviations, respectively. The extimated standard deviations for all parameter and metrics can be underestimated based on this level of refinement and the lack of completeness for the data at high resolution. All refinements were carried out using the SDP package.[32] Atomic scattering factors for all atomic species were calculated from Cromer and Waber.[33] The final atomic parameters for all non-hydrogen atoms are reported in the supporting information, the numbering of the atoms follows the recommendations of the IUPAC-IUB Commission[34] (Chart 1).

NMR Studies. NMR spectra were recorded on Bruker AMX 500 spectrometer, in 99.98% isotopically pure deuterated acetonitrile (Carlo Erba, Milano, Italy). The peptide concentration was 2 mM. All chemical shifts in part per millions (ppm) are referred to acetonitrile peak at 2.0 ppm. One-dimensional (1D) spectra have been acquired using typically 16−32 scans with 16 K data size. For the two-dimensional (2D) experiments, pulse programs of the standard Bruker software library were used. All 2D experiments have been acquired in the phase sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increment (TPPI).[35] Typically 256 experiments of 64 scans each were performed: relaxation delay 1 s; size 2 k; 6024-Hz spectral width in F2; zero filling to 1 k in F1; apodization in both dimensions with squared cosine function in both dimensions. NOESY spectra have been acquired with mixing times in the interval 150−350 ms. The interprotonic distances have been calculated according to the method of Esposito and Pastore.[36]

Computational Details. All the computations were performed using a Silicon Graphics Iris 4D35 GT Turbo workstation. The package INSIGHT/DISCOVER (Biosym Technologies, San Diego, CA, USA) with the consistent valence force field (CVFF)[37−39] was used for energy minimization, restrained molecular dynamics (RMD), and molecular dynamics (MD) simulations of the model derived from NMR data.

The RMD and MD simulations were performed at a temperature of 300 K *in vacuo*. The equations of motion were solved using the Leapfrog integration algorithm, with a time step of 0.5 fs.[40] For each simulation the computational conditions were chosen to avoid boundary effects.[20] The distance restraints were given by a skewed biharmonic potential added to the total energy of the system.[41]

Starting models for the computational studies were built using geometrical parameters for amino acid residues derived from solid state structure, using structural parameters from NMR measurements (e.g., interproton distances from NOE values or H-bonds from temperature coefficients). These structures were subjected to a simulated annealing procedure using the NOE effects to bias the trajectory into a reduced area. The structures best satisfying distances and dihedral angles derived from NMR data were subjected to the RMD with the following procedure. Each system was relaxed to eliminate hot spots, performing 100 steps of energy minimizations using the conjugate gradient method, before RMD were run on resulting models. The energy-minimized structures were used as the initial structures for the RMDs *in vacuo* at 300 K. The RMD simulations were carried out for 40 ps in the equilibration phase and for 80 ps without velocity rescaling since the temperature remained constant around 300 K. The average structures were then checked for consistency with all observable NOEs.

Unconstrained MD simulations were also carried out on the NMR average structures. In these simulations, the system was equilibrated for 50 ps, then an additional 100 ps of simulation without velocity rescaling was carried out, since it was observed that energy conservation as well as the average temperature remained constant around the target value of 300 K.

Coordinates and velocities for the simulations were dumped to a disk every 20 step during the last 20 ps of the two simulations. The dumped data were used for the statistical analysis.

Result and Discussion

X-ray Analysis. The molecular model of cDECA, as derived from solid state analysis, is reported in Figure 1. All the amino acid residues are in L-configuration. The geometrical parameters for all residues are near the expected values[42,43] and should be considered unexceptional. The decapeptide presents all peptide bonds *trans*, with a range of values of 170−180° and an average value of 176°.

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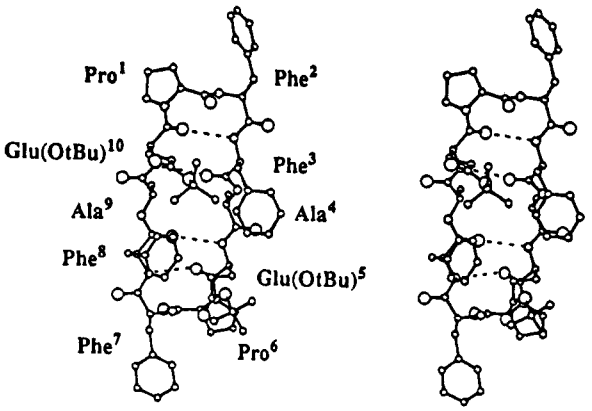

Figure 1. Stereo view of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ as obtained from X-ray analysis viewed along the *c* direction with residue numbering. The intramolecular hydrogen bonds are reported as dashed lines.

Table 2. Intra- and Intermolecular Hydrogen Bonds of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$[a]

Intramolecular			
donor	acceptor	distance (Å) D−A	angle (deg) N−O=C′
N_3	O_{10}	3.24	125
N_{10}	O_3	3.06	169
N_5	O_8	3.15	176
N_8	O_5	3.43	119

Intermolecular				
donor	acceptor	distance (Å) D−A	angle (deg) N−O=C′	symmetry operation
N_4	O_9	3.07	161	$1 + x, y, z$
N_9	O_4	3.03	159	$x - 1, y, z$
N_2	O_{6S}	2.97		$^1/_2 + x, ^3/_2 - y, -z$

[a] Esd deviations are of the order of 0.02 for H-bond lengths and 1° for angles.

Structures of cyclodecapeptides exhibit in most cases an antiparallel β-sheet type hydrogen bond pattern connecting adjacent pentapeptide strands.[8,44,45] This feature is also shown by the crystal structure of cDECA. The overall structure can be described as formed by antiparallel β-strands linked by Pro-Phe moieties and stabilized by four intramolecular H-bonds. A very similar shape can be observed in the X-ray structure of the cyclodecapeptide gramicidin S.[44]

Details on intra- and intermolecular hydrogen bonds are listed in Table 2. The backbone conformation of cDECA is stabilized in the solid state by the following hydrogen bonds:

(i) an intramolecular 4 → 1 H-bond, involving the NH of Phe^3 and the C=O of Glu(OtBu),^{10} with the formation of a type I β turn;

(ii) a week intramolecular 4 → 1 H-bond in the opposite corner of the molecule involving the NH of Phe^8 and the C=O of Glu(OtBu)^5, with the formation of a distorted β-turn of type II;

(iii) two intramolecular H-bonds, the first between the NH of Glu(OtBu)^{10} and the C=O of Phe^3, the second involving the NH of Glu(OtBu)^5 and the C=O of Phe^8, lead to the formation of two C_{14} ring structures or of two C_{22} ring structures counting the atoms of the remaining portion of the cyclic molecule.

Different schemes of H-bonds are found in the solid state of cyclodecapeptides.[8,44,45] The intramolecular H-bonds network reported for gramicidin S-urea complex[44] presents four H-bonds,

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Table 3. Torsional Angles in deg of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ as Obtained from X-ray Analysis[a]

residue	φ	ψ	ω	$\chi^{1,1}$	$\chi^{2,1}$	$\chi^{2,2}$	χ^3	χ^4
Pro^1	−58	127	172	−29	42			
Phe^2	65	18	179	−51	−65	114		
Phe^3	−156	123	180	−169	−83	96		
Ala^4	−133	138	−179					
Glu(OtBu)^5	−149	176	−175	65	148		74	−171
Pro^6	−61	−32	−172	−32	42			
Phe^7	−94	−15	−178	−77	−78	102		
Phe^8	−166	162	170	58	−99	85		
Ala^9	−128	143	176					
Glu(OtBu)^{10}	−147	177	−179	69	175		159	172

[a] Esd deviations are of the order of 2° for the torsion angles.

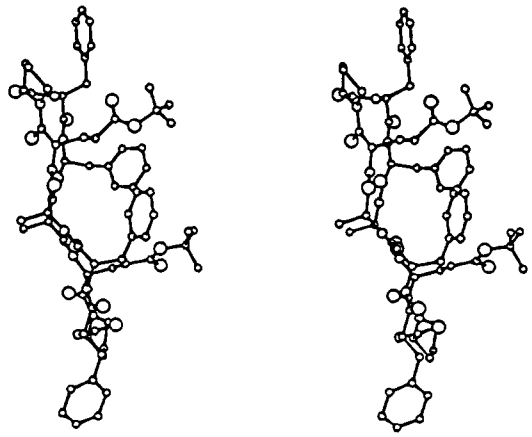

Figure 2. Stereoview of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ as obtained from X-ray analysis viewed along the *a* direction.

quite similar to that observed for cDECA, with an additional intramolecular H-bond between one ornithine side-chain nitrogen atom and D-Phe carbonyl oxygen. In the symmetric [Phe^4-Val^6]AA,[8] the structure presents two intramolecular α-turns, while in the cyclo-bis-elastin[45] two type III β-turns are present in each pentapeptide Val-Pro-Ala-Val-Gly segment.

In Table 3 the solid state torsional angles for cDECA are summarized. All values for the ϕ and ψ angles fall within the allowed region of L-residues in the Ramachandran map.

The conformational parameters of the Pro-Phe-Phe sequence are quite different from those of the similar sequence found in the solid state structures of CLA[17] and of antamanide.[13] Comparison of the Glu(OtBu)^5-Pro^6-Phe^7-Phe^8 moiety with the Val^9-Pro^1-Pro^2-Phe^3 segment of the Ba^{2+}−CLA solution complex,[23] characterized by all trans peptide bonds, shows a significant structural similarity, with a root mean square (RMS) deviation of 0.37 Å for the backbone atoms.

The Phe^2 and Phe^7 side chains have $\chi1$ and $\chi2$ values close to −60° (g^- conformation) and 90°, respectively; the Phe^3 and Phe^8 side chains present $\chi1$ conformational angles close to 180° (t conformation) and 60° (g^+), respectively, and $\chi2$ close to 90°. The conformation of the two Glu(OtBu) side chains can be described as g^+, t for the $\chi1$ and $\chi2$ angles. The two prolyl residues, characterized by negative $\chi1$ and $\chi3$ values and positive $\chi2$ and $\chi4$ values, adopt a C^γ-exo type conformation. It is noteworthy that in the crystalline structure all side chains, except the alanines, point in the same direction with respect to the plane of the backbone, giving rise to an overall shape characterized by a well-defined hydrophobic side (Figure 2). This feature can be also observed in the solution structure of the CLA−Ba^{2+} complex.[23] A further difference between the structure of cDECA and those of CLA and antamanide is found in the Pro-Phe side-chain mutual orientation. The stacking of the side chains of these two residues, which occurs in all antamanide

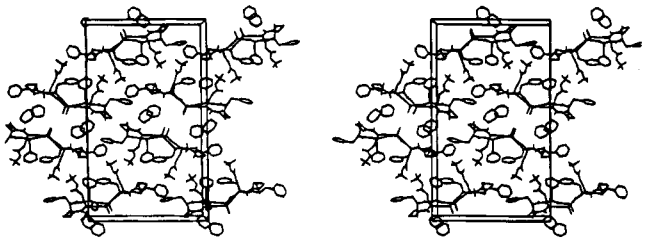

Figure 3. Stereoview of molecular packing of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ view along the *a* axis.

structures,[8,13] in CLA[17] and in other peptides containing the Pro-Phe sequence,[9,15] has not been observed in the cDECA structure.

The molecules in the solid state are held together in the *ab* plane by a network of hydrogen bonds, as described in Table 2 and in Figure 3. All distances of the type N−H··O and O-H··O are within expected values.[46] In this plane, the molecules translated along the *x* axis are linked by intermolecular hydrogen bonds, connecting directly parallel β-strands. In particular two NH groups are intermolecularly H-bonded: the NH of Ala^4 is bonded to the C=O of Ala^9 and the NH of Ala^9 is H-bonded to C=O of Ala^4. Consequently, a layer of cDECA molecules, almost parallel to the *ab* plane, is formed.

The two independent dioxane molecules fill the space between cDECA molecules symmetry related along the *z* direction, giving a motif in which the solvent is encapsulated between the hydrophilic sides of the peptide molecules. Moreover, one of dioxane molecule oxygens (O6s) acts as an H-bond acceptor from the NH of Phe^2, stabilizing the crystal structure and concomitantly distorting the type II β-turn. The structure is further stabilized by van der Waals contacts between side-chain hydrophobic groups. In conclusion, the crystal packing of cDECA shows alternate hydrophobic and hydrophilic regions, perpendicular to the *z* direction, in the *ab* plane.

NMR Analysis. The solution NMR study of cDECA has been carried out in acetonitrile on a 2 mM sample at a temperature of 320 K. This temperature value has been chosen in order to remove the degeneracy, present at room temperature, of the chemical shifts of two NHs, later identified as the amide protons of Phe^2 and Phe^3. To be sure that the increase in temperature did not alter significantly the peptide structure, control NOESY spectra at 300 and 320 K have been collected: they show almost identical results.

The 1D spectrum of the peptide in Figure 4 shows, at a first glance, only one set of sharp signals. In particular, it is possible to distinguish only four doublets in the low field region of the spectrum attributable to amidic protons. This behavior, confirmed by the remaining regions of the spectrum, indicates that the structure of the dimer in solution is perfectly symmetric, i.e., only one set of resonances is obtained for the protons of the i and the i+5 residues. Since they are indistinguishable, in the NMR analysis section of this paper we will therefore use the abbreviation Pro^{1,6} to indicate both Pro^1 and Pro^6 residues, and so on.

The main features of the spectrum are indicative of a complete conformational homogeneity. In fact, all diastereotopic proton pairs exhibit distinct chemical shifts (the $\Delta\delta$ between the β protons of Phe^{2,7} is 0.11 ppm, while that for Phe^3 is 1.0 ppm). The NH and α proton resonances are spread out, and even the aromatic proton resonances of the two Phe residues are nonequivalent. It is possible to exclude both the presence of slowly interconverting *cis*−*trans* isomers (around the Glu-Pro bond) and of fast interconversion among two or more isomers.

(46) Ramakrishan, C.; Prasad, N. Int. *J. Pept. Protein Res.* **1971**, *3*, 209−231

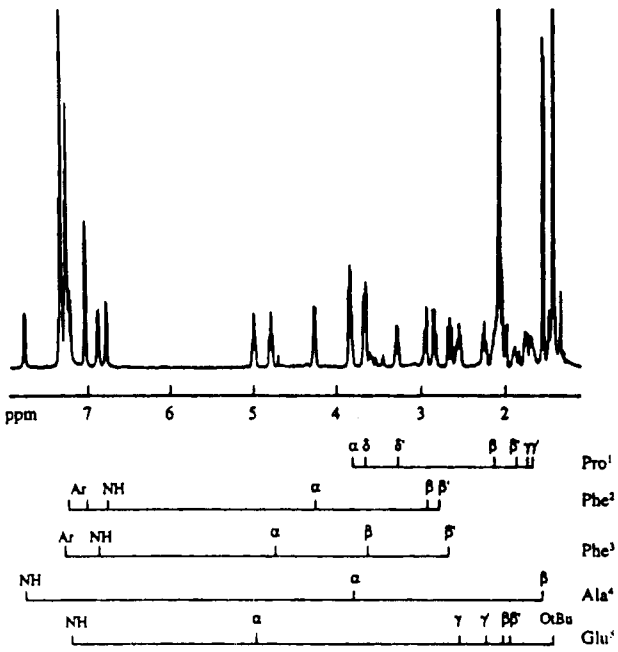

Figure 4. 500-MHz ^{1}H NMR spectum of 2 mM *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ in CD_3CN at 320 K, together with subspectra of all amino acid residues. Greek letters identify different types of protons of each residue.

Further strong support for this argument comes from the conformationally outstanding values shown by some of the $^3J_{NHC\alpha H}$ and of the temperature coefficient of the NHs (see Table 4).

All spin systems have been identified by means of TOCSY[47] and DQF-COSY.[48] The sequential assignment of the resonances of the pair of identical Phe residues relies on NOESY spectra.[49] Under the chosen experimental conditions at 320 K, the 500 MHz NOESY spectra do show positive NOEs, i.e., the systems in the extreme narrowing limit. Figure 5 shows the NOESY spectrum collected at 320 K with a mixing time of 200 ms. The Pro^{1,6} spin system has been assigned via Glu^{10,5}-Pro^{1,6} $\delta\alpha\delta$ and $\delta\alpha\delta'$ and Phe^{3,8} resonances via Phe^{3,8}-Ala^{4,9} $\delta\alpha$N and δNN.

The conformational analysis of the peptide backbone has been performed taking into account the chemical shift values, the temperature coefficients of the NH resonances and the quantitative evaluation of the vicinal coupling constants $^3J_{NHC\alpha H}$ and NOEs.

The very first structural indication comes from the presence in the spectrum, of only one set of resonances for the protons belonging to the i and the i+5 residues. This observation implies that the solution structure of the dimeric peptide cDECA is perfectly symmetrical. Thus the dissymetry of the cycle present in the solid state structure, due to the different arrangement of the two turns closing the β-strand, is relaxed.

The NH−CαH coupling constants of all NH-bearing residues have been measured directly from the one-dimensional spectrum, although they were also double checked in the DQF-COSY. Table 4 shows all experimental $^3J_{NHC\alpha H}$ values, corrected for electronegativity, with the corresponding allowed ϕ ranges.[50] Two of the four $^3J_{NHC\alpha H}$ values, those pertaining to residues Phe^{3,8} and Glu^{5,10}, are high enough to be compatible with a very narrow set of ϕ angle values, ranging from ca. −110° to ca.

(47) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528.

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(50) Bystrov, V. F. *Prog. NMR. Spectrosc.* **1976**, *10*, 41−62.

Table 4. Vicinal $^3J_{NHC\alpha H}$ (Hz) of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ in Acetonitrile at 320 K, Corresponding ϕ Angles, Temperature Dependence of the NH Chemical Shifts

	Phe^2	Phe^3	Ala^4	Glu^5
$^3J_{NH\text{-}\alpha CH}$	6.1	10.8	7.5	11.2
Φ	−170, −80, 25, 95	−110 ÷ −130	−160, −85, 35, 85	−110 ÷ −130
$\Delta\delta/\Delta T$ (ppb/K)	−4.6	−0.6	−2.4	−0.6

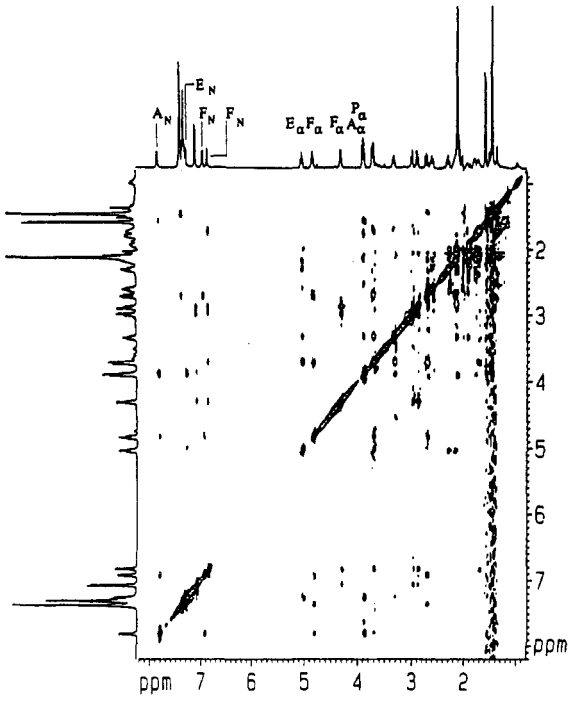

Figure 5. 500-MHz NOESY spectrum of 2 mM *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$ in CD_3CN at 320 K, performed with a 200-ms mixing time. Standard single-letter code is used to identify amino acid residues.

−130°, corresponding to a *trans* arrangement of the HN−CαH moiety. The $^3J_{NHC\alpha H}$ value of Phe^{2,7} is compatible with the ϕ value characteristic of a residue in the i+2 position of a type I β-turn. The stereochemistry of the Glu^{10,5}-Pro^{1,6} peptide bond was determined on the basis of NOEs based on the strong cross peaks connecting Glu^{10,5} CαH to the δ,δ' protons of Pro^{1,6} which indicate a *trans* arrangement of the Glu-Pro peptide bonds.

The temperature coefficients of the NHs can provide information on the intramolecular hydrogen bond pattern. Table 4 shows the NH $\Delta\delta/\Delta T$ values measured in the range 295−325 K. In this range dependence of all NH chemical shifts on the temperature is linear, indicating that no conformational transition is taking place. The temperature coefficients of Phe^{3,8} and Glu^{5,10} residues exhibit a very low value (−0.6 ppb) indicating that these NHs are involved in stable H-bonds throughout the temperature interval considered. This behavior is consistent with the H-bond pattern found in the solid state structure.

NOEs have been converted to interproton distances using the method of Esposito and Pastore.[36] The resonances of the two β protons of Phe^3, corresponding to an interproton distance of 1.8 Å, have been chosen as reference cross peaks. Distances have been calculated from the NOESY spectrum obtained with a mixing time of 200 ms. The calculated correlation time of the molecule under these conditions is 0.53 ns. Table 5 shows the calculated distances for the indicated pairs of protons.

The combined use of all structural parameters derived from NMR data allowed us to build a rather detailed model of the structure in solution of cDECA. We have already seen that Glu^{5,10} and Phe^{3,8} NHs are involved in hydrogen bonds; the NOEs involving these amidic protons, namely the Glu^{10}-Phe^3 and the Glu^5-Phe^8 pairs, confirm that, as in the solid state

Table 5. Comparison of Experimental (NOE) and Calculated (RMD) Interprotonic Distances for *cyclo*[Pro-Phe-Phe-Ala-Glu-(OtBu)]$_2$, in Å

	NOE	RMD
d[NHPhe^3-NHAla^4]	2.6	2.6
d[αPhe^3-NHAla^4]	3.1	2.8
d[αAla^4-NHAla^4]	2.3	2.3
d[αAla^4-NHGlu^5]	2.5	2.5
d[αGlu^5-NHGlu^5]	3.2	3.0
d[δPro^1-NHPhe^2]	3.0	3.1
d[αPhe^2-NHPhe^2]	2.9	2.9
d[βPhe^2-NHPhe^2]	3.2	2.7
d[β′Phe^2-NHPhe^2]	2.7	2.4
d[αPhe^3-NHPhe^3]	3.1	3.0
d[β′Phe^3-NHPhe^3]	2.6	2.8
d[αGlu^5-δPro^1]	2.2	2.2
d[αGlu^5-δ′Pro^1]	3.2	3.9
d[αPro^1-NHPhe^2]	3.5	3.5
d[NHPhe^3-NHGlu^5]	3.4	3.8

structure, they are involved in interstrand H-bonds, with the Phe^3 NH linked to the Glu^{10} carbonyl and the Phe^3 carbonyl linked to the NH of Glu^{10}. Similar interactions characterize the pair Glu^5-Phe^8. The ϕ values calculated from the $^3J_{NHC\alpha H}$ values for the residues Glu^{5,10} and Phe^{3,8}, shown in Table 4, are in accord with a β strand arrangement of the backbone of these residues. The two β strands are interconnected in the solution structure by two type I β turns. In fact, in Table 5 it is shown that the distance between the NH and the α proton of Phe^{2,7} is 2.9 Å and that those between the NH of Phe^{2,7} and the δ and α protons of Pro^{1,6} are 3.0 and 3.5 Å, respectively. These values are in good agreement with those characteristic of a canonical type I β turn.[51]

The solution structure of cDECA differs from the solid state one in two more features: the conformation around the Ala residues and the mutual orientation of the side-chains in the -Pro-Phe-Phe- part of the sequence. The calculated distances involving Ala protons and the ϕ values compatible with the $^3J_{NHC\alpha H}$ value do not fit well the solid state ones; they are more representative of a local structure resembling a sort of inverse γ turn.

As far as the conformation of the Phe side chains is concerned, in the X-ray structure the fragment CHα-CH$\beta\beta'$ of Phe^{2,7} is in the g$^-$ conformation and that of Phe^{3,8} in the *trans* one. In solution the situation is almost reversed. $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ values can provide information[52] on the relative populations of the three staggered conformations g$^-$, *trans*, and g$^+$. The g$^-$ and *trans* conformers can be distinguished from one another provided a stereoselective assignment for the two β geminal protons is made. Considering first the Phe^{3,8} residues; the vicinal coupling constants measure 11.6 and 3.4 Hz, corresponding to a 10% population of the g$^+$ conformer and to a 2% and 88% population of the remaining two staggered conformers. The stereoselective assignment of the two methylene protons of the Phe^{3,8} residues can be made on the basis of the peculiar NOE pattern involving the NH and the $\beta\beta'$ protons. In fact, the NOESY spectra show the existence of only one such NOE,

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(52) Pachler, K. G. R. *J. Magn.Reson.* **1972**, *8*, 183−187

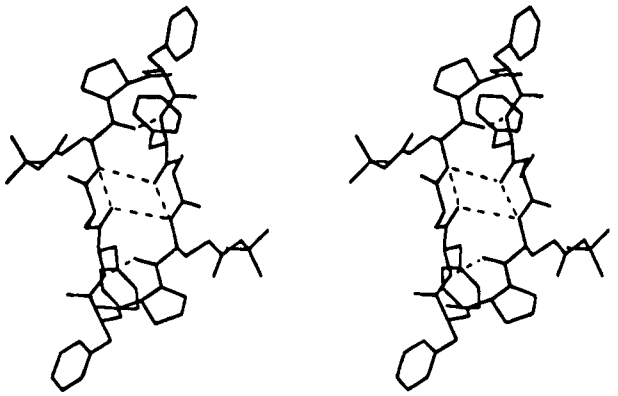

Figure 6. Stereoview of average solution structure of *cyclo*[Pro-Phe-Phe-Ala-Glu(OtBu)]$_2$, as obtained from RMD simulation *in vacuo* at 300 K. The intramolecular hydrogen bonds are reported as dashed lines.

Table 6. Averages of the Dihedral Angles in the cDECA Main Chain as Obtained from the RMD Simulation *in vacuo* at 300 K

residue	φ	ψ	ω
Pro^1	−59.4	−33.0	165.6
Phe^2	−73.2	−37.2	−171.4
Phe^3	−120.3	50.9	−164.9
Ala^4	−79.2	76.2	179.2
Glu^5	−119.8	148.2	−169.8

that linking amide and β' protons. This NOE accounts for an interproton distance of 2.6 Å. A much higher distance, based on the lack of the corresponding NOE, must be hypothesized for the NH−CHβ pair. This situation is only compatible with a prevailing presence in solution of the g$^-$ conformer, identifying the β' proton as the Pro-S one.

Phe^{2,7} residues show vicinal coupling constants of 9.2 and 6.0 Hz, corresponding to an 8% abundance of the g$^+$ conformer and 63% and 29% of the remaining two. In this case, the distances CHα → CH$\beta\beta'$, calculated by means of NOEs, are 2.7 and 3.2 Å, values consistent with the presence in solution of a *trans* conformer. The *trans* conformation of this side chain is also in agreement with two other considerations. A g$^-$ conformation would in fact position the Phe^{2,7} aromatic rings close to the Pro^{1,6} side chain protons. This proximity would induce a high field ring current shift of at least one of these protons, which is not present in our case. On the other hand, a *trans* conformation can explain the high field shift experienced by the 2,6 aromatic protons of Phe^{2,7}, due to the ring current of the adjacent phenyl ring of the Phe^{3,8} almost frozen in the g$^-$ conformation.

Computational Results. The molecular modeling procedure, used to build a particular peptide model of cDECA matching all experimental data, showed that a single conformer could satisfy all NMR structural parameters. It exhibits exact twofold symmetry with two repeating sequences -Pro^1-Phe^2-Phe^3-Ala^4-Glu(OtBu)^5- linked together by turns with the Pro-Phe residues in the corners. This conformer, satisfying distance, temperature coefficient, and coupling constant constraints was subjected to restrained molecular dynamics simulation (RMD) *in vacuo* at 300 K, as described in the Experimental Section. Table 6 shows the dihedral angles of the average structure of cDECA, as obtained from RMD simulation. A stereoview of the model, evidencing all intramolecular hydrogen bonds, is presented in Figure 6. This symmetric structure presents all *trans* peptide bonds and is stabilized by internal transannular hydrogen bonds involving Phe^{3,8} and Glu(OtBu)^{5,10} NHs. In particular, it presents two type I β-turns with the Pro-Phe residues in the i+1 and i+2 position, respectively, and the Phe^{3,8} amide hydrogen bonded to the C=O of Glu(OtBu)^{5,10}. In addition, NHs of Glu(OtBu)^{5,10} are involved both in interstrand and intrastrand hydrogen bonds with the carbonyl oxygens of Phe^{3,8}. The first one, though weaker, corresponds to that one found in the solid state structure. The second one, absent in the X-ray structure, forms an equatorial γ-turn. A rough estimate of the correctness of the proposed structure could be made by comparing the average RMD structural parameters with the corresponding values derived from the solution conformation of cDECA, i.e., the corresponding φ dihedral angles and the experimental interproton NOE distances. These data are listed in Table 6.

The RMD average model has been used as a starting structure for a molecular dynamics simulation *in vacuo* without constraints at 300 K. This simulation shows that all trajectories converge to a structure consistent with the NMR data.

Comparison of the X-ray and the solution structures of cDECA shows a similar overall shape. The major differences, as revealed by the interproton NOE-derived distances, are located on the atoms of the Phe-Ala peptide bonds (Cα-C'O-N). An approximately 20° rotation of these planes in the CLA structure stabilizes the γ-turn more than the C$_{14}$ ring structures.

Finally, it is interesting to note that the solution structures of the Glu(OtBu)-Pro-Phe-Phe moiety of cDECA and of the Val-Pro-Pro-Phe segment of the Ba^{2+}−CLA complex[23] are structurally similar. The rms deviation of the backbone atom superposition is 0.34 Å. In the solution structure of cDECA all side chains, except the alanine ones, point in the same direction with respect to the plane of the backbone, giving rise to a well defined hydrophobic side. This feature has also been observed in the solution structure of the CLA−Ba^{2+} complex.[23]

Conclusions

The homodetic cyclopeptide *cyclo*[-Pro-Phe-Phe-Ala-Glu-(OtBu)]$_2$ (cDECA) is an active synthetic analog of CLA. Both CLA[17] and cDECA exhibit similar CD$_{50}$[5,6] [peptide concentration required to inhibit the cholate uptake in hepatocytes by 50%] values, 0.8 and 0.6 mM, respectively (Ziegler, K. private comunication), but are characterized by different conformational behavior, both in the solid state and in solution.

The X-ray structure of CLA is stabilized by five strong transannular hydrogen bonds, with the formation of one C$_7$, two C$_{10}$, one C$_{13}$, and one C$_{17}$ ring structures; the Pro-Pro peptide bond is *cis*, with all remaining peptide bonds in the *trans* conformation.[17] The X-ray structure of cDECA exhibits an all-*trans* peptide skeleton, with two C$_{10}$ ring structures connecting two almost extended strands. The structure is characterized by a clear separation of two distinct surfaces, one hydrophilic and the other hydrophobic. The latter contains all side chains but the alanine ones. The cDECA structure is topologically similar to that of gramicidin-S[44] (which contains two D-residues in the corners) but differs from the structures of antamanide and related analogs (containing all L-residues) which, both in their free and metal-complexed forms,[53] present two *cis* peptide bonds.

In solution the high degree of flexibility of CLA and the use of polar solvents always prevented the observation of a single conformer. Only in chloroform at 214 K has it been possible to solve by NMR methods[17] the solution structure of CLA. Under those conditions, it is practically identical to the solid state form.

Addition of bivalent metal cations to the CLA solution induces drastic structural changes. In particular, Ba^{2+} ions stabilize a well defined equimolar cation/peptide complex. The complexed peptide structure is characterized by all *trans* peptide bonds and two clearly distinct surfaces, a polar one hosting Ba^{2+}

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ion and the other predominantly apolar.[23] cDECA behaves, with respect to CLA, as a more rigid peptide: its solution structure is in fact substantially consistent with the solid state one.

On the basis of these findings it seems reasonable to conclude that the solution structure of cDECA, sharing some features of the barium-complexed structure of CLA, could be regarded as a bioactive conformation.

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Supporting Information Available: An additional seven tables with fractional atomic coordinates, bond angles, bond distances, torsional angles, structure factors, general displacement parameter expressions, and 1H chemical shifts (17 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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