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Conformational study of cyclo[D-Trp-D-Asp-Pro-D-Val-Leu], an endothelin-A receptor-selective antagonist

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The conformation of cyclo[D-Trp-D-Asp-Pro-D-Val-Leu], (BQ123), an endothelin-A receptor-selective antagonist, has been studied in 20% acetonitrile in water by CD and NMR spectroscopy. CD studies showed the peptide adopted a similar, constrained conformation in both water alone and 20% acetonitrile in water. NMR spectra showed the proline residue to be in the *trans* conformation and 2 of the NH protons to exchange slowly with the solvent, indicating hydrogen bonding. Structural constraints derived from the NMR spectra were used to define the conformation in molecular dynamics simulations. A single backbone conformation is observed for the cycle, comprising a β type II turn and a γ' turn.

Endothelin; Nuclear magnetic resonance; Circular dichroism; Conformation; Molecular modeling

1. INTRODUCTION

The endothelins, a family of bicyclic, 21 amino acid peptides, whose first member was isolated and characterized from porcine aortic endothelial cells [1], are potent vasoconstrictors in mammals and may play important roles in hemodynamic regulation and/or modulation of the cardiovascular system. Specific endothelin binding sites are present in many tissues including the central nervous system [2], although not all of these binding sites necessarily represent functional endothelin receptors.

To date, 5 different sequences for endothelin receptors have been reported. These receptors form 2 different receptor subtypes, A and B, which can be distinguished by their affinities for the various endothelins. The endothelin A receptor has a greater affinity for endothelins 1 and 2 than for endothelin 3 [3–5], while the B-subtype has about equal affinity for all the endothelins [6–8].

It has been reported that the acyclic analog [$Ala^{1,3,11,15}$]endothelin-1 is a weak, but selective, ET_B receptor ligand [9]. However, due to the flexibility of the molecule, conformational studies of this linear peptide [10] have shed little insight into the critical features necessary for a selective receptor interaction. Recently BQ123, a small cyclic peptide (cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]), has been reported to be an ET_A receptor-selective antagonist [11]. The constraints from cyclization and the small number of residues should yield a molecule with limited conformational flexibility. Thus a study of this peptide may provide important information regarding the structural and conformational features important for receptor recognition and binding.

Abbreviations: FMOC, fluorenylmethoxy carbonyl; FAB-MS, Fast atom bombardment-mass spectroscopy; CD, Circular dichroism; NMR, Nuclear magnetic resonance spectroscopy; DQF COSY, 2-D Double quantum filtered correlated spectroscopy; TOCSY, 2-D totally correlated spectroscopy; NOESY, 2-D nuclear Overhauser enhancement spectroscopy; ROESY, rotating frame NOESY; ppm, parts per million; Standard 3-letter abbreviations for the amino acids are used throughout.

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2. MATERIALS AND METHODS

2.1. *Peptide synthesis*

D-Trp-D-Asp(OBzl)-Pro-D-Val-Leu was synthesized by standard solid-phase synthetic techniques using an automated peptide synthesizer and FMOC chemistry on a 2-methoxy-4-alkoxybenzyl alcohol resin (Sasrin Resin, Bachem, Switzerland). The peptide was removed from the resin with 1% trifluoroacetic acid in CH_2Cl_2 and cyclized at high dilution in DMF with diphenylphosphoryl azide at −20°C for 4 days. Cyclo[D-Trp-D-Asp(OBzl)-Pro-D-Val-Leu] was deprotected by catalytic transfer hydrogenation using ammonium formate as the hydrogen source and purified by C_{18} RP HPLC (FAB-MS $[MH]^+$ = 611.3, calc = 611.33).

2.2. *CD spectroscopy*

CD studies were performed with an Aviv Model 62D Spectropolarimeter. Spectra were recorded using a 1.5 nm bandwidth, 0.5 nm step, and a time constant of 4 s. A total of 5 scans were averaged for both sample and solvent. After correction of the sample spectrum for solvent and cell contributions, the data were fitted by nonlinear regression analysis. The peptide was dissolved in water (9–147 μM at pH 5.3) or in 20% CH_3CN in water (12 μM–2.7 mM) and the concentration estimated from the UV absorbance of the tryptophan residue at 280 nm [12]. Temperature studies between 4 and 60°C were made with water-jacketed, dichroically neutral circular quartz cuvettes.

2.3. *Nuclear magnetic resonance spectroscopy*

2.3.1. Sample preparation

A sample was prepared for NMR studies by dissolving ca. 2.5 mg BQ123 in 500 μl 20% CD_3CN in H_2O to give a final concentration of ca. 8 mM. The sample was lyophilized and dissolved in 20% CD_3CN in D_2O (pH 5.4) for NH exchange experiments and for recording spectra without exchangeable protons. A further sample was prepared containing 140 μM BQ123 in H_2O containing 10% D_2O. Sodium (trimethylsilyl) propionate-*d4* was used as an internal reference set at 0.00 ppm.

2.3.2. NMR experiments

All NMR spectra were recorded at 25°C and 500 MHz on a Bruker AM500 spectrometer equipped with an Aspect 3000 computer and a digital phase shifter.

1D spectra were acquired over 16K data points, using a spectral width of 6024 Hz. Solvent suppression was achieved by irradiation of the H_2O resonance during the relaxation time (2.0 s). Coupling constants were measured directly from the 1D spectra without correction for linewidths.

All 2D experiments (DQF COSY [13], TOCSY [14–16], NOESY [17–18] and ROESY [19]) were acquired in the phase sensitive mode using time-proportional phase incrementation [20], with the transmitter set on the H_2O resonance and using CYCLOPS phase cycling [21]. NOESY spectra were recorded with mixing times of 200, 400 and 700 ms, and with random variation of the mixing time (typically 10%) to eliminate zero-quantum transfer [22]. TOCSY and ROESY experiments used a radio-frequency field strength of 5–7 kHz and mixing times of 50 and 150 ms, respectively. Typically, 512 t_1 increments were acquired for each experiment with 16 transients and 2 dummy transients per increment. In t_2, 2048 data points were acquired over a spectral width of 6024 Hz. All spectra were processed offline on a Bruker X32 workstation, using the manufacturer's program, UXNMR. Prior to Fourier transformation in t_2, the free induction decays were multiplied by a Gaussian function (for NOESY, ROESY and TOCSY experiments) or a shifted sine-bell function (for the DQF COSY experiments). The data were multiplied by a cosine-bell function (for NOESY, ROESY and TOCSY experiments) or a shifted sine-bell function (for the DQF COSY experiment) in t_1 and zero-filled to 2048 data points before the second Fourier transform. For each spectrum, the baseline was fitted with a third order polynomial in both dimensions and subtracted. All spectra are shown unsymmetrized with the F_2 dimension horizontal and the contour levels spaced logarithmically.

2.3.3. NH exchange

1D spectra were recorded at intervals over the 3 h following dissolution of the lyophilized peptide in 20% CD_3CN in D_2O, and again after 16 h. A ROESY and a DQF COSY experiment were recorded as described above.

2.3.4. Structural data

The intensity of crosspeaks in the ROESY spectra were assessed as being strong, medium or weak and converted into upper limits on interproton distances as follows: strong – 0.3 nm; medium – 0.4 nm; weak – 0.5 nm. Since the modeling software did not allow the specification of pseudoatoms, distances to the methylene groups of Pro-3 and the methyl groups of Val-4 and Leu-5 were specified to the appropriate C atom and a correction added.

2.4. *Modeling and structural calculations*

All model-building, energy minimization and molecular dynamics simulations were performed in vacuo, using the SYBYL molecular modeling package (Tripos Associates, St. Louis, MO, version 5.4). A model of BQ123 was built with all peptide bonds in the *trans* conformation. Initial calculations were performed without any NMR constraints. The structure built using SYBYL was energy-minimized and subjected to a molecular dynamics simulation of 80 ps at 300K, 80 ps at 500K, 80 ps at 1000K and a further 80 ps at 300K. Structures were stored every 1 ps and energy-minimized. NMR distance constraints were added to the 25 structures with the lowest energies and 1 structure with the highest energy, and these were energy-minimized once again. Five structures, including the best and the worst of the set of 25 structures, and the high energy structure, were used as starting structures for molecular dynamics simulations. Runs were performed for 40 ps at 300K, 40 ps at 500K and a further 80 ps at 300K. ϕ, ψ and χ_1 angles were analyzed for changes in conformation. The ϕ and χ_1 angles which were consistent with measured coupling constants and which were observed to vary about a single value in the 5 runs were added to the constraint list. One run was continued with the updated constraint list, for 40 ps at 300K, 40 ps at 500K, 40 ps at 300K, 40 ps at 500K and 40 ps at 300K. The ϕ, ψ and χ_1 angles were analyzed using the graphical facilities in SYBYL.

3. RESULTS AND DISCUSSION

3.1. *CD spectra*

The far UV CD spectra of BQ123 in water (9.5 μM) and 20% CH_3CN/water (2.4 mM) are shown in Fig. 1. The spectra were nearly identical and the slight differences in intensities that are observed are probably due to scaling errors arising from inaccuracies in the determination of concentrations and path lengths. In addition, no differences were observed in the CD spectra of BQ123 obtained over a wide range of concentrations in either solvent. These similarities suggest that the peptide adopts the same conformation in both solvents.

In the near UV region, the CD spectrum of the indole ring of tryptophan gives rise to a broad, negative band with a characteristic shoulder. The L_b transition, distinguished by its vibrational fine structure in the absorbance spectrum (insert, Fig. 1), occurs at 289 nm, and the

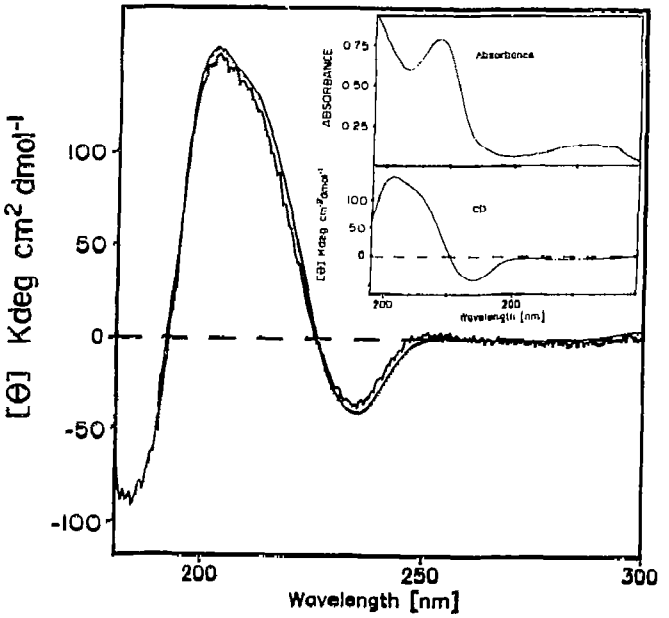

Fig. 1. CD spectrum of BQ123 in water (9 μM, noisy spectrum) and in 20% CH_3CN in water (2.7 mM), both at 20°C. The 2 spectra are very similar indicating that the peptide assumes a similar conformation in both solvents. (Insert) The absorbance and CD spectra of BQ123 in 20% CH_3CN in water.

L_a transition is at 281 nm. Again, no differences in the spectra of the peptide in water or aqueous CH_3CN were observed in this region (data not shown).

In the far UV region, the CD spectra are characterized by a minimum at 185 nm, a broad maximum at 204 nm that contains a shoulder at about 212 nm, and another minimum at 234 nm. While it is difficult to assign the bands to a particular conformation, it is clear from the molar ellipticity of the $n \rightarrow \pi^*$ transitions of the amide bond that the peptide is highly constrained.

The absorption and CD spectra for BQ123 in 20% aqueous CH_3CN are shown in the insert to Fig. 1. The ^1B_b band for the indole ring of tryptophan is clearly evident in the absorption spectrum at about 222 nm, while its CD band apparently contributes to the broad positive ellipticity seen in the region between 225 and 190 nm. The negative band centered at 234 nm is somewhat unusual and may be a ^1C transition in the indole [23].

3.2. *Nuclear magnetic resonance*

3.2.1. *Assignment*

The NMR spectrum was found to be well-dispersed with little overlap of the resonances from the 5 residues. The spectrum of BQ123 was assigned largely by direct inspection of the DQF COSY spectrum (Fig. 2). Crosspeaks in the ROESY spectrum were used to identify the C_7H resonance of Trp-1 and to distinguish between the AMX spin systems of Trp-1 and Asp-2. Stereo-assignments could be made for the $C\beta H_2$ protons of Trp-1 and Asp-2, based on $^3J_{C\alpha H\text{-}C\beta H}$ coupling constants and ROESY crosspeak intensities [24], taking into account

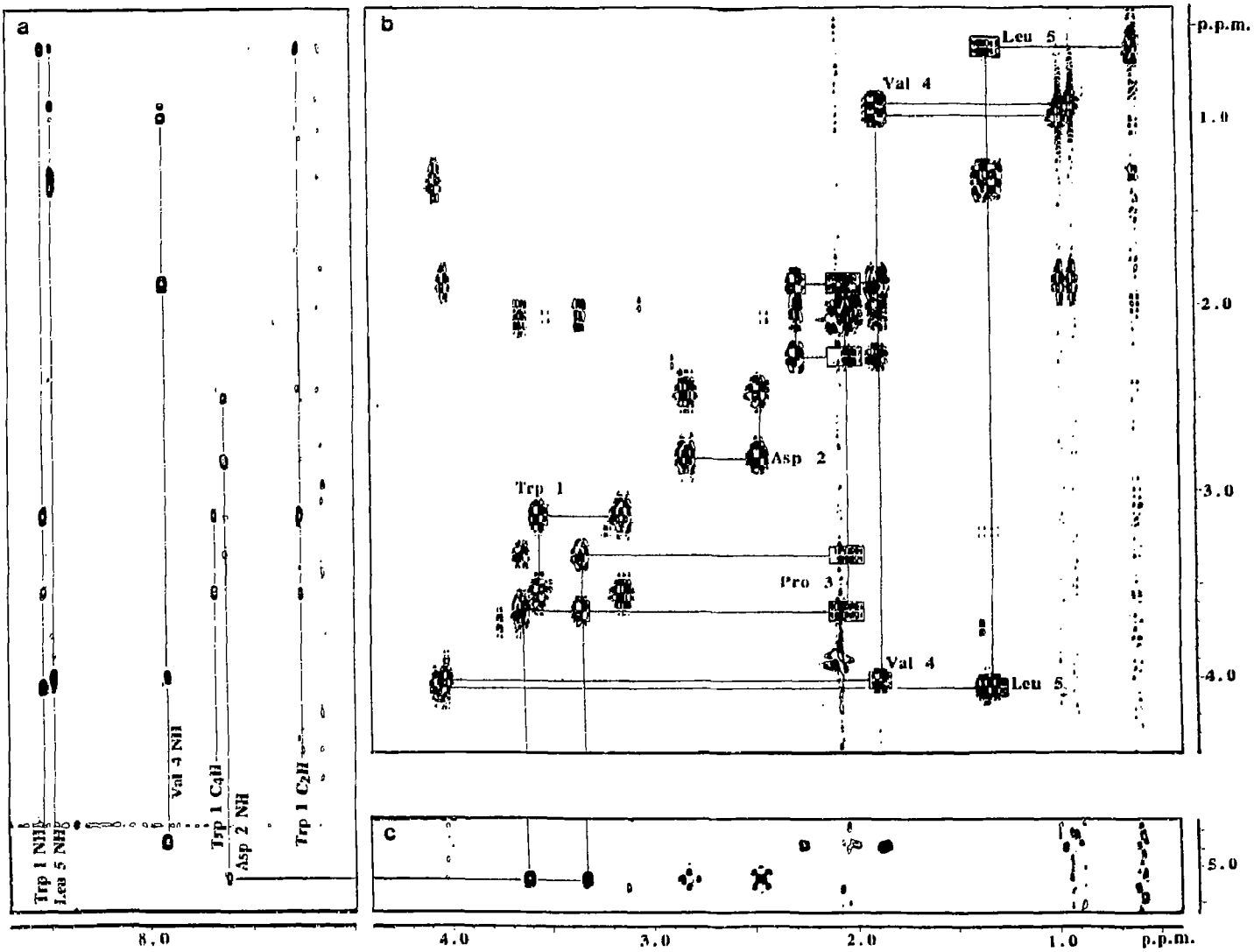

Fig. 2. NMR spectra of BQ123. (a) Portion of the ROESY spectrum recorded in 20% CD_3CN in H_2O, showing crosspeaks from the backbone NH resonances and aromatic resonances of Trp-1. For clarity, only negative contour levels are plotted. (b) The aliphatic region of the DQF COSY spectrum recorded in 20% CD_3CN in D_2O, showing the spin systems of the 5 residues of BQ123. (c) Portion of the ROESY spectrum recorded in 20% CD_3CN in D_2O, showing sequential $C\alpha H_i$–$C\delta H_{i+1}$ crosspeaks between Asp-2 and Pro-3. Only negative contour levels are plotted.

Table Ia

Assignment of the resonances of BQ123 (20% CD_3CN in H_2O or D_2O, 25°C, pH 5.4)

Residue	NH	CαH	CβH1, CβH2	CγH	CδH	Other
Trp-1	8.53	4.69	3.50, 3.09		7.26 (C_2H)	10.15 (N_1H) 7.68 (C_4H) 7.14 (C_5H) 7.22 (C_6H) 7.49 (C_7H)
Asp-2	7.63	5.05	2.80, 2.46			
Pro-3	–	4.86	2.22, 1.82	2.01	3.59, 3.30	
Val-4	7.92	3.97	1.82	0.93, 0.87		
Leu-5	8.48	4.02	1.29	1.29	0.56	

Table Ib

Coupling constants and torsion angles

Residue	Coupling constant	Value (Hz)	Torsion angle	Value
Trp-1	$^3J_{NH-C\alpha H}$	8.1	ϕ	160°, <u>80°</u>, −60°
	$^3J_{C\alpha H-C\beta H}$	2.9, 11.8	χ_1	<u>60°</u>
Asp-2	$^3J_{NH-C\alpha H}$	8.8	ϕ	<u>150°</u>, 90°, −60°
	$^3J_{C\alpha H-C\beta H}$	10.3, 4.4	χ_1	180°
Val-4	$^3J_{NH-C\alpha H}$	10.3	ϕ	<u>120°</u>
	$^3J_{C\alpha H-C\beta H}$	9.6	χ_1	60°
Leu-5	$^3J_{NH-C\alpha H}$	5.2	ϕ	−170°, <u>−70°</u>, 20°, 100°

the inverted chirality of these residues. The resonance assignments are listed in Table Ia.

3.2.2. *NH exchange*

Two of the four NH protons were observed to exchange slowly into the deuterated solvent. Of these, the NH resonance of Asp-2 was seen to vanish 30 min after dissolution, while that of Val-4 persisted for 3 h.

3.2.3. *Structural data*

Measured coupling constants are listed in Table Ib. All possible values of ϕ angles were derived from Karplus curves for L- and D-amino acids [25]. χ_1 values for the predominant rotamers were determined from the coupling constants (Table Ib).

The NOESY spectra were found to contain little information but crosspeaks were observed in the ROESY spectrum (Fig. 2). Most notably, these show Pro-3 to be in the *trans* conformation and the NH proton of Asp-2 to be close to the NH protons of both Trp-1 and Val-4.

3.3. *Structural calculations*

Molecular dynamics runs, using the 5 starting structures from unconstrained molecular dynamics simulations (see Materials and Methods) and including NMR distance constraints, showed that each ϕ angle varied about a single value, close to one of the set of possible angles derived from the $^3J_{NH-C\alpha H}$ coupling constants (underlined in Table I). The run with the high energy starting structure had an initial ϕ angle for Trp-1 around −60°C which, during the run, flipped to a value around 80°. Only one χ_1 angle (Trp-1) was seen to be well-defined around 60° throughout the runs, in accordance with the predominant rotamer, indicated by the coupling constants and ROESY crosspeaks. The χ_1 angles of Asp-2 and Val-4 were seen to vary between all 3 rotamers (−60°, 60°, 180°) in the 5 runs while that for Leu-5 had only values around −60° and 180°. While coupling constant data suggests predominant rotamer populations for Asp-2 at 180° and Val-4 at 60°, these were not added to the constraint list.

From the extended molecular dynamics run, with all constraints derived from NMR (including 4 ϕ and 1 χ_1 angle constraints), average values of the ϕ and ψ angles, together with the possible χ_1 angles could be derived (Table II). Structures from the last 15 ps of the run were energy-minimized and 6 of these are shown in Fig. 3a.

Table II

Average, ϕ, ψ and χ_1 angles

Residue	ϕ	ψ	χ_1
Trp-1	80°	50°	60°
Asp-2	140°	−90°	60°, −60°, 180°
Pro-3	−75°	85°	30°
Val-4	100°	−100°	60°, −60°, 180°
Leu-5	−80°	100°	−60°, 180°

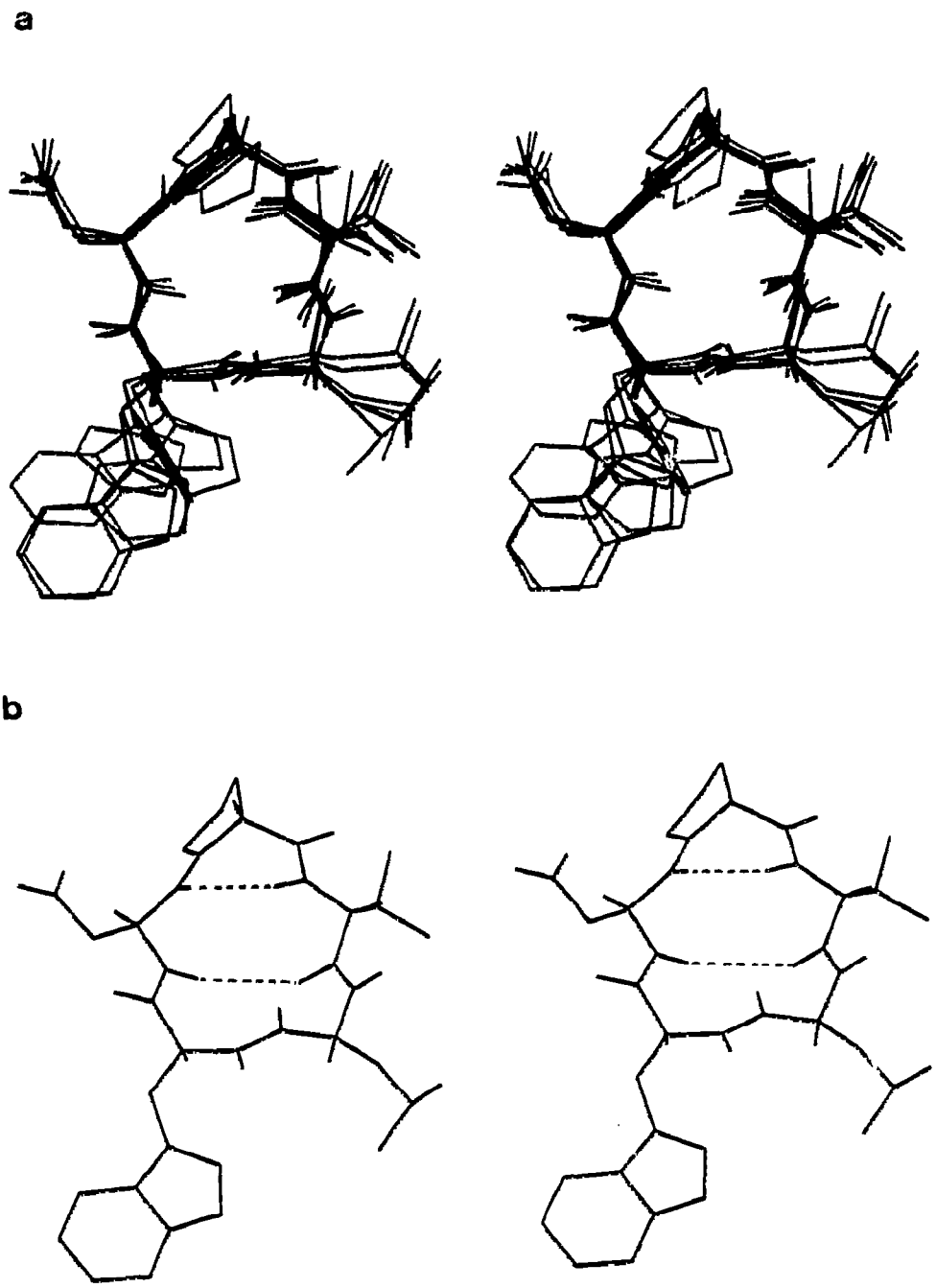

Fig. 3. Stereoviews of calculated structures of BQ123. (a) Six superimposed structures, energy-minimized from the last 15 ps of the extended molecular dynamics run (see text for details). For clarity, hydrogen atoms are not shown on the sidechains. (b) A single structure from the set of six, showing the β type II turn and the γ' turn.

The set of 15 structures have an average pairwise rmsd value of 0.050 nm and the lowest energy structure has just 2 residual violations of the distance constraints of 0.052 and 0.016 nm (both internal to the Trp residue).

Molecular dynamics simulations in the absence of experimental constraints show that the backbone can adopt only a limited number of conformations, while the sidechains may populate all 3 major rotamers. Experimentally-derived NMR constraints show that the structure in solution fluctuates about a unique backbone conformation (Table II, Fig. 3a) with the χ_1 angle being defined for Trp-1. These results are consistent with the CD spectra where the large molar ellipticity in the far UV indicates that the molecule assumes a well-defined structure. The observation of the L_a and L_b bands of tryptophan in the near UV CD spectrum also

supports a constrained conformation for this residue. The backbone structure comprises a type II β-turn formed by residues Val-4:Leu-5:Trp-1:Asp-2, and a γ' turn formed by Asp-2:Pro-3:Val-4, and contains both the expected hydrogen bonds (Fig. 3b). The type II β-turn observed in the calculated structures contains a hydrogen bond between Asp-2 NH and Val-4 CO, while the γ' turn is stabilized by a stronger hydrogen bond between Val-4 NH and Asp-2 CO. BQ123 is a tightly constrained cycle forming a structure which is quite flat, and in which the hydrophobic sidechains lie largely in the plane of the cycle. The proline residue makes an angle to the plane of the cycle and is seen to fluctuate by ±30° about a mean position during the molecular dynamics simulations.

4. CONCLUSIONS

The conformation of BQ123, an endothelin-A receptor-selective antagonist, has been determined in solution. CD results show the cycle to be highly constrained. NMR data define the structure as comprising a type II β-turn and a γ' turn. The determined structure may be used as the basis for the design of further peptidic and/or non-peptidic selective antagonists.

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