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The solution structure of a cyclic endothelin antagonist, BQ-123, based on ^1H–^1H and ^{13}C–^1H three bond coupling constants

Michael D. Reily, Venkataraman Thanabal, Diana O. Omecinsky, James B. Dunbar Jr., Annette M. Doherty and Patricia L. DePue

Chemistry Department, Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA

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A cyclic pentapeptide endothelin antagonist, cyclo(dTrp-dAsp-Pro-dVal-Leu), recently reported (K. Ishikawa et al., 13th Am. Pept. Symp., Cambridge MA, 1991) has been studied by NMR spectroscopy and molecular modeling. A stable structure has been determined without the use of nuclear Overhauser effects and is based primarily on homonuclear and heteronuclear three bond coupling constants. The ^{13}C-edited TOCSY experiment is demonstrated at natural abundance and ~30 mM peptide concentrations. Three bond ^{13}C–^1H coupling constants obtained by this method are shown to reduce the ambiguity in ϕ angle determination which exists when only interproton coupling constants are used. Three out of four ϕ angles were determined uniquely by this method and the fourth was reduced to two possible values. The proline ϕ angle was determined to be −78° based on the $^3J_{H\alpha,H\beta}$ and $^3J_{H\alpha,H\beta}$ coupling constants. Comparison of amide proton temperature dependence, chemical shifts and vicinal proton coupling constants in a 20% acetonitrile/80% water solvent mixture and in $(CD_3)_2SO$ indicates that the structure is similar in both solvents.

Endothelin antagonist; Solution structure; Heteronuclear coupling constant; Cyclic peptide

1. INTRODUCTION

In the past decade, NMR methods originally developed by Wüthrich [1] that rely heavily on nuclear Overhauser effects (NOEs) and homonuclear coupling constants for determining the solution structures of small proteins are well-established and have become common practice in many laboratories. In small peptides, however, the number and intensity of NOEs are diminished by several factors including the relatively large surface area and flexibility of such molecules. An important development in NMR structure determination has been the ability to measure heteronuclear coupling constants at natural abundance in peptides, when concentrations greater than about 20 mM can be achieved [2]. The combined use of homonuclear and heteronuclear coupling constants can often rule out ambiguities that arise from the periodic nature of the Karplus relationship [3] and can therefore help define various peptide backbone angles.

In this report, we describe the solution structure of a cyclic peptide, cyclo(dTrp-dAsp-Pro-dVal-Leu), a selective ET_A receptor antagonist that has recently been reported by Ishikawa et al. [4]. The structure was generated using molecular dynamics constrained only by experimental heteronuclear $^3J_{NH,C\beta}$ and homonuclear $^3J_{NH,H\alpha}$ values. Other NMR measurements support the derived structure.

Abbreviations: ROESY, rotating frame nuclear Overhauser spectroscopy; TOCSY, total correlated spectroscopy; FMOC, fluorenylmethyloxycarbonyl; DCC, 1,3-dicyclohexylcarbodiamide; HOBT, 1-hydroxybenzotriazole hydrate; TFA, trifluoroacetic acid.

Correspondence address: M.D. Reily, Chemistry Department, Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, USA. Fax: (1) (313) 998 2716.

2. EXPERIMENTAL

2.1. *Peptide Synthesis*

The peptide, BQ-123, was synthesized by solid-phase peptide synthesis techniques on an Applied Biosystems Model 430A peptide synthesizer using an N-alpha FMOC protection scheme with a SASRIN resin (Bachem Bioscience, Inc.). All other reagents and amino acids were obtained from Applied Biosystems. The aspartic acid side chain was protected as the *t*-butylester. The peptide was FMOC deprotected with 20% piperidine in *N*-methylpyrrolidone, followed by cleavage from the resin with 1% TFA in dichloromethane. The peptide was cyclized with diphenylphosphorylazide and triethylamine [5], followed by deprotection of the butylester with TFA/thioanisole/ethanedithiol/anisole (90:5:3:2). The peptide was purified on reverse phase C_{18} preparative scale Vydac column (2.2 × 25.0 cm) eluting at 15 ml/min with a linear gradient of 90: 10 0.1% TFA in H_2O: 0.1% TFA in acetonitrile to 40:60 0.1% TFA in H_2O: 0.1% TFA in acetonitrile. The purified peptide was isolated after lyophilization as a white powder. The homogeneity of the peptide was determined by analytical HPLC and TLC. HPLC on RP C_{18} analytical scale Vydac column eluting at 1.5 ml/min with a linear gradient of 90:10 0.1% TFA in H_2O:0.1% TFA in acetonitrile over 20 min shows a single peak with retention time of 16.7 min. The peptide was characterized by amino acid analysis, FAB mass spectrometry (MH^+ = 611.2), ^1H NMR spectroscopy and elemental analysis.

Alternatively, the compound was synthesized using standard DCC/

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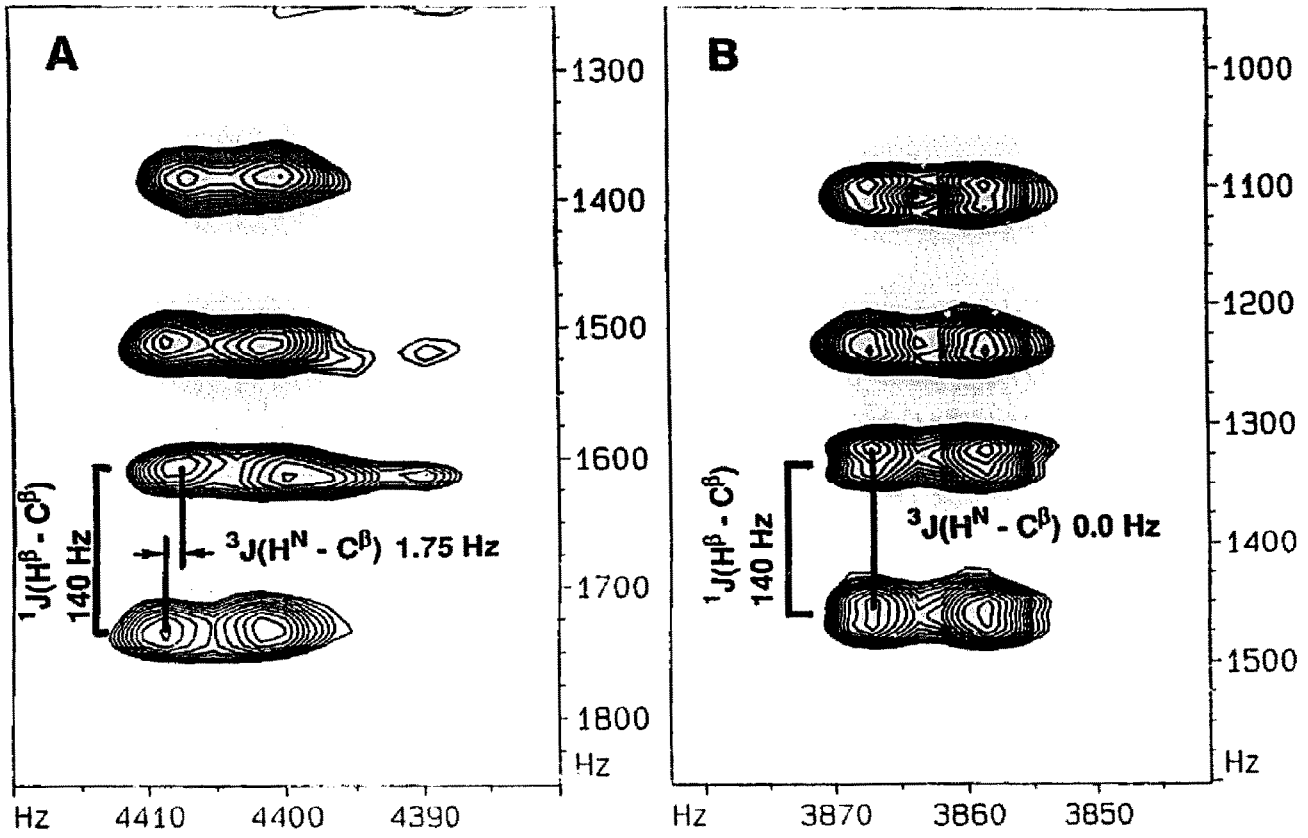

Fig. 1. Expansions of the ^{13}C-edited TOCSY spectrum of 10 mg of cyclo(dTrp-dAsp-Pro-dVal-Leu) in 0.5 ml of $(CD_3)_2SO$ at 310K. Shown are the NH, Hβ crosspeaks of (a) dTrp and (b) dAsp. Horizontal displacements of the indicated crosspeaks arise from the ca. 140 Hz one bond Cβ–Hβ coupling and vertical displacement corresponds to the three bond NH–Cβ coupling.

HOBT coupling techniques in solution phase synthetic strategy. It was cyclized, deprotected and purified similarly to the methods described above for the resin-bound peptide.

2.2. *NMR measurements*

Unless otherwise noted, experiments were carried out with either 4.0 mg of the peptide dissolved in 0.5 ml of a 20% CD_3CN/8% D_2O/72% H_2O mixture at 298K or with 10 mg of the peptide dissolved in 0.5 ml of $(CD_3)_2SO$ at 295K. Assignments were based on the TOCSY [6] experiment acquired (with solvent presaturation in the aqueous solvent) into 512 t1 blocks of 2048 t2 data points. A 7 kHz MLEV17 [7] spin-lock field was applied at the transmitter frequency (ca. 4.8 ppm) for 65 ms. A trim pulse of 2.5 ms was applied prior to and after the spinlock pulse train (the trailing trim pulse was omitted in the aqueous solution). A ROESY [8] experiment was acquired on the aqueous sample into 512 t1 blocks of 2048 t2 data points. A 4 kHz continuous wave spin-lock field was applied during the 200 ms mixing time at the transmitter frequency. The temperature dependence of the proton spectrum was determined by acquiring spectra between 280 and 300K in the aqueous solution and at 295K, 300K and 310K in $(CD_3)_2SO$. Homonuclear coupling constants were measured directly from spectra that were resolution enhanced by application of a gaussian function (GB=0.5, LB=−5) to the free induction decay prior to Fourier transformation. Heteronuclear coupling constants were measured from ^{13}C-edited TOCSY spectra as described elsewhere [2]. The pulse sequence includes a BIRD type heteronuclear editing to select protons attached to ^{13}C nuclei followed by a conventional TOCSY pulse sequence.

2.3. *Molecular modeling*

Molecular dynamics were run in the gas phase using a Boltzmann weighted starting velocity with the Tripos implementation of the Kollman AMBER [9] force field in the modeling package Sybyl [10] version 5.5 beta. Dynamics runs were carried out for 100 ps at 600K (without constraints) or for 100 ps at 900K (with NMR-derived torsion angle constraints). Constrained molecular dynamics runs were done with ϕ angles restrained to a designated value with a force constant of 0.01, a dielectric constant of 4, and a distance-dependent dielectric function.

3. RESULTS AND DISCUSSION

Fig. 1 shows selected expansions of the NH–Hβ region of the ^{13}C edited homonuclear TOCSY experiment. The crosspeaks have one-bond ^{13}C–^1H coupling (~140 Hz) in f1 and the long-range ^{13}C–^1H coupling in f2. In this case, the center of the two components are displaced in f2 by $^3J_{NH,C\beta}$. Three bond NH–Cβ coupling constants can be measured to ± 0.5 Hz by this method. These are listed in Table I together with the $^3J_{NH,H\alpha}$, the corresponding derived ϕ angles and the temperature coefficients of the amide protons in both solvent systems. For Pro, the $^3J_{H\alpha-H\beta(pro-R)}$ was ~0 Hz and $^3J_{H\alpha,H\beta(pro-S)}$ was ~8 Hz, indicating a ϕ of ca. −75° in both solvent systems.

The expected temperature coefficient for a fully solvent-exposed amide proton is 10 ppb/K in H_2O and 5 ppb/K in $(CD_3)_2SO$ [12]. Taking this difference into

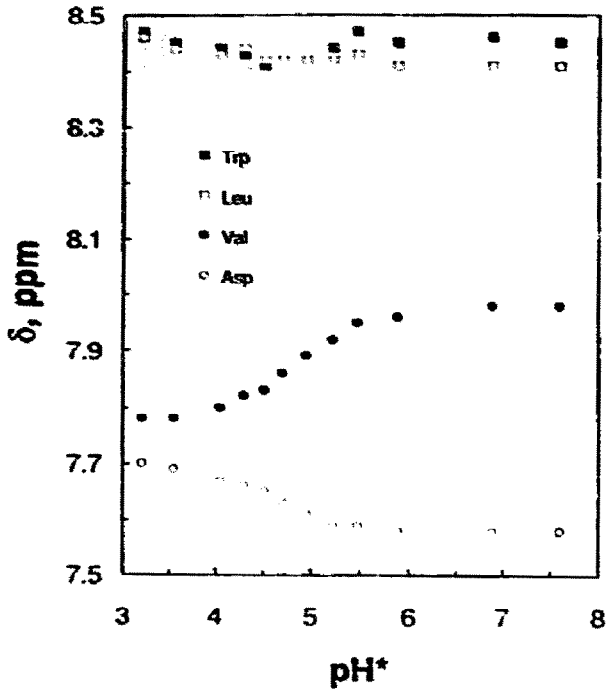

Fig. 2. The pH dependence of the amide chemical shifts of 3 mg of cyclo(dTrp-dAsp-Pro-dVal-Leu) in 0.625 ml of 20% CD_3CN/8% D_2O/72% H_2O at 298K. The chemical shifts are measured relative to internal TSP and the pH values are uncorrected meter readings.

consideration, the rank order of solvent accessibility of the amide protons is identical and the relative solvent accessibility is similar in both solvents. Furthermore, the proton-proton coupling constants vary only slightly between the two solvents. This suggests a similar conformation of the peptide in both aqueous and $(CD_3)_2SO$ solutions.

The proton NMR chemical shifts of the peptide in aqueous and $(CD_3)_2SO$ solutions are listed in Table II. Major shift differences between the two solvents include the Leu Hγ, one of the Leu δ-CH_3 resonances and the dVal NH and Hα signals. In the more polar aqueous solvent system, the Leu sidechain is apparently more closely associated with the dTrp aromatic ring and both methyl protons are shifted upfield by ca. 0.2 ppm from their expected random-coil chemical shifts. This is also consistent with nuclear Overhauser (ROESY) effects in aqueous solution which show close contacts between the dTrp benzene ring protons and the Leu methyl protons. Possibly, this hydrophobic region of the molecule is better solvated in $(CD_3)_2SO$, diminishing the interaction between the Leu and dTrp sidechains. A small relocation of the average position of the dTrp sidechain could explain the solvent dependent differences observed. In $(CD_3)_2SO$ the dAsp sidechain is protonated and the dVal amide proton appears significantly upfield-shifted with respect to the peptide in aqueous solution at pH 6.9 (Table II), where the dAsp sidechain is ionized. To investigate the effect of protonation state on the chemical shifts, ^1H NMR spectra were recorded in 20% CD_3CN/8% D_2O/72% H_2O as a function of pH over the range 3.2 to 7.6. The pH dependence of the amide protons are shown in Fig. 2. As the pH was decreased, the dVal NH shifted upfield ca. 0.2 ppm toward a chemical shift more similar to that observed in $(CD_3)_2SO$. This shift is sigmoidal with an inflection near pH 4.8%, consistent with dAsp sidechain protonation. Changing the pH does not, however, significantly affect the temperature dependence of the amide proton chemical shifts (Table I). Thus, the chemical shift of the dVal NH proton is strongly influenced by the protonation state of the dAsp sidechain, whereas its apparent hydrogen bonding ability is not. The origin of this effect on the chemical shifts of the dVal NH is unclear but may be due to a slight repositioning of the anisotropic groups in the dTrp or dAsp sidechains. Despite the observed differences in chemical shifts, the similarities in the temperature dependence of the amide proton chemical shifts and the homonuclear coupling constants

Table 1

Homonuclear and heteronuclear coupling constants, φ values and NH solvent accessibility determined from NMR data for cyclo(dTrp-dAsp-Pro-dVal-Leu)[a].

Residue	$^3J_{HN,C\beta}$ (Hz)	$^3J_{HN,H\beta}$ (Hz)	ϕ_{HN}[b]				$\Delta\delta/\Delta T$[c]		
							pH=6.5	pH=3.2	
dTrp	1.75±0.25	7.7 (8.6[d])	150	90	40	80	10.5	11.3	(5.9)
dAsp	0.00±0.25	9.1 (8.9)	150	90	75	45	4.5	3.7	(2.0)
Pro			75						
dVal		9.9 (10.5)	120	60			2.0	1.8	(0.1)
Leu	1.75±0.25	5.1 (5.2[d])	20	100	70	170	9.0	9.9	(4.3)

[a] Unless otherwise noted, data were acquired in 20% CD_3CN/80% H_2O, pH 6.5 or in $(CD_3)_2SO$ (in parentheses).
[b] Determined by comparison with empirical ^1H-^1H and theoretical ^{13}C-^1H relationships (see [3]). The only values consistent with both ^1H-^1H and ^{13}C-^1H coupling constants are underlined. The Pro φ angle was determined from Hα-Hβ coupling constants (see text).
[c] Values at pH 6.5 and 3.2 were measured between 280 and 300K and 288 and 298K, respectively. The pH 3.2 solvent was 20% CD_3CN/8% D_2O/72% H_2O. $(CD_3)_2SO$ solutions were measured between 300 and 310K.
[d] Measured at 295K, all other values measured at 280K in aqueous solution and at 310K in $(CD_3)_2SO$.

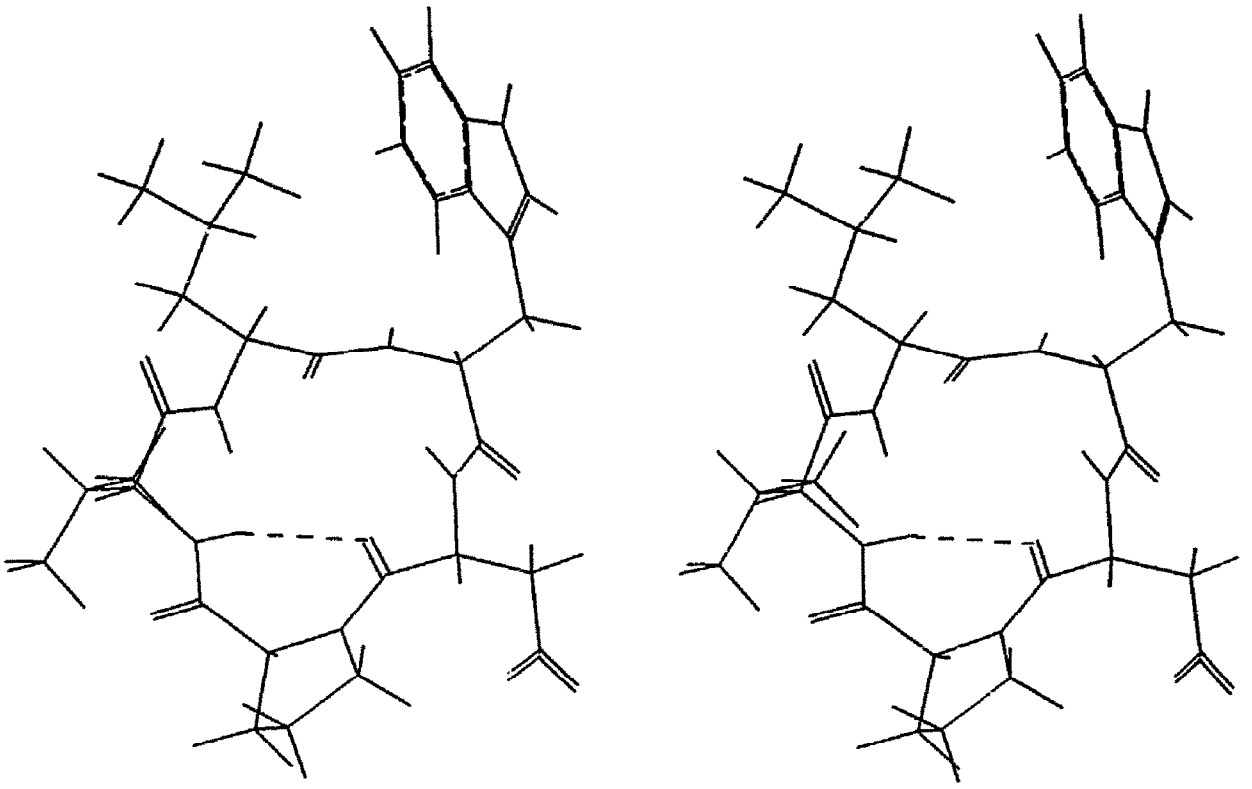

Fig. 3. Stereoviews of the structure of cyclo(dTrp-dAsp-Pro-dVal-Leu) derived from experimentally determined ϕ angles. The dashed line indicates a possible hydrogen bond. The average RMSD of the Cα atoms from ten independently calculated structures was 0.18 Å.

Table II

Chemical shifts of cyclo(dTrp-dAsp-Pro-dVal-Leu) in different solvents[a]

Residue	NH	α	β	Other
20% CD_3CN/8% D_2O/72% H_2O, pH 6.89, 298K:				
dAsp	7.58	5.05	2.77, 2.43	
Pro	–	4.86	2.22, 1.85	2.00 (γ), 2.05 (γ'), 3.31 (δ), 3.62 (δ')
dVal	7.98	3.97	1.85	0.88 (γ), 0.94 (γ')
Leu	8.41	4.01	1.32, 1.26	1.32 (γ), 0.57 (δ), 0.59 (δ')
dTrp	8.46	4.64	3.51, 3.08	7.26 (δ1), 7.68 (ε3), 7.14 (ζ3), 7.23 (η2), 7.49 (ζ2), 10.11 (ε1)
20% CD_3CN/8% D_2O/72% H_2O, pH 3.21, 298K:				
dAsp	7.70	5.09	2.90, 2.56	
Pro	–	4.85	2.24, 1.81	2.03 (γ), 2.03 (γ'), 3.28 (δ), 3.53 (δ')
dVal	7.78	3.98	1.81	0.87 (γ), 0.93 (γ')
Leu	8.47	3.98	1.27, 1.32	1.27 (γ), 0.55 (δ), 0.58 (δ')
dTrp	8.46	4.62	3.50, 3.10	7.25 (δ1), 7.66 (ε3), 7.13 (ζ3), 7.22 (η2), 7.48 (ζ2), 10.13 (ε1)
$(CD_3)_2SO$, low pH, at 295K:				
dAsp	7.73	4.98	2.80, 2.36	
Pro	–	4.77	2.25, 1.92	1.60 (γ), 1.75 (γ'), 3.15 (δ), 3.30 (δ')
dVal	7.50	4.15	1.69	0.82 (γ), 0.88 (γ')
Leu	8.79	3.92	1.22, 1.15	1.00 (γ), 0.60 (δ), 0.75 (δ')
dTrp	8.81	4.26	3.35, 2.90	7.15 (δ1), 7.56 (ε3), 6.95 (ζ3), 7.05 (η2), 7.32 (ζ2), 10.82 (ε1)

[a] Chemical shifts reported relative to dimethylsulfoxide-d5 = 2.50 ppm and TSP = 0.0 ppm.

indicate that the peptide adopts comparable structures in both solvents.

An initial structure for cyclo(dTrp-dAsp-Pro-dVal-Leu) was obtained from a molecular dynamics calculations without experimental constraints. This structure contained a β turn with Pro as the i+1 residue and dVal as the i+2 residue. A γ turn was centered on the dTrp. The manifold of structures from the molecular dynamics was consistent with work done on similar cyclic pentapeptides [11].

With the completion of the NMR analysis it was clear that the initial model was not correct and subsequent molecular dynamics runs were carried out utilizing the ϕ values for dTrp, dAsp, dVal and Leu provided by NMR. The hydrogen bond of the N–H in dVal and the Pro ϕ angles were used as secondary filters for examining the output. The initial model was modified to the experimental ϕ values listed in Table I, with dAsp ϕ = −45. To ensure adequate sampling of the conformational space, four dynamics runs with the same conditions were performed with different starting structures. Two of runs constrained dAsp ϕ to −45°. In the first of these, all starting ψ angles were derived from the initial structure (above). In the second run, the starting structure was derived from the initial model with all of the ψ angles inverted 180°. The remaining two dynamics runs were carried out similarily with dAsp ϕ constrained at 150°. Forty conformations were chosen and energy was minimized from the resulting data set, derived from the four different starting conformations. The set of structures from the molecular dynamics run that consistently had the lowest energy had ϕ and ψ values that were compatible with the NMR data and all minimized structures have the observed hydrogen bond for the dVal NH. The ϕ and ψ values (expressed as ϕ,ψ) for the lowest energy structure are dTrp = 84,29; dAsp = 145, −128; Pro = −78,82; dVal = 123, −47; Leu = −166, 100. This structure is shown in Fig. 3. Although hydrogen bonds were not used in the modeling calculations, it is clear from the model that the dVal amide proton is internal and is involved in a transannular hydrogen bond to the carbonyl oxygens of the dAsp residue. This is consistent with the very small temperature coefficient observed for the dVal NH in both solvents.

4. CONCLUSION

The solution structure of BQ-123 was derived exclusively from coupling constant data without NOE or hydrogen bonding constraints. The resulting structure is supported by several additional NMR observations including amide protons likely to be involved in hydrogen bonding, temperature dependent shifts of exposed amide protons and observed NOEs. We have demonstrated that the ^{13}C-edited TOCSY experiment at natural abundance and ~30 mM peptide concentrations can reduce the ambiguity in ϕ angle determination which exists when only homonuclear coupling constants are used. This method should be of general utility in constrained systems such as the peptide analyzed in this study and the structure of BQ-123 derived from these experiments should aid in the development of novel ET_A receptor antagonists.

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Note added in proof

After submission of this manuscript, work describing the structure of BQ-123 appeared (Atkinson, R.A. and Pelton, J.T. (1992) FEBS Lett. 296, 1–6). Of the eleven possible ϕ angles reported by this group to be consistent with the $^3J_{HN,\alpha H}$, only four were consistent with NOE distance constraints. Three of the four ϕ angles are, within experimental error, identical to those measured directly from homonuclear and heteronuclear coupling constants in the present study.