Nuclear Magnetic Resonance Analysis and Conformational Characterization of a Cyclic Decapeptide Antagonist of Gonadotropin-Releasing Hormone†

Edmund L. Baniak, II,‡ Jean E. Rivier,§ R. Scott Struthers,§,∥ Arnold T. Hagler,§,∥ and Lila M. Gierasch*,‡

Department of Chemistry, University of Delaware, Newark, Delaware 19716, Peptide Biology Laboratory, The Salk Institute, La Jolla, California 92037, and The Agouron Institute, La Jolla, California 92037

Received August 13, 1986; Revised Manuscript Received December 2, 1986

ABSTRACT: Two-dimensional proton nuclear magnetic resonance spectroscopy at 500 MHz has been carried out on the cyclic decapeptide antagonist of gonadotropin-releasing hormone: *cyclo*-(Δ^3-Pro1-D-pClPhe2-D-Trp3-Ser4-Tyr5-D-Trp6-NMeLeu7-Arg8-Pro9-β-Ala10). The antagonist exists in two slowly interconverting conformations. All data are consistent with the conclusion that one form has all-trans peptide bonds and the other has a cis β-Ala10–Δ^3-Pro1 bond. With the use of sequential assignment methods, chemical shift assignments were obtained for all backbone and side-chain protons of both conformational isomers except for the serine and tyrosine hydroxyl groups and the C^γ, C^δ, and guanidinium group protons of the arginine. Temperature dependence of spectral parameters and magnitudes of observed nuclear Overhauser effects support the interpretation that both conformers of the antagonist consist of two β-turns (type II′, D-Trp6-NMeLeu7; type II, Δ^3-Pro1-D-pClPhe2) connected by extended antiparallel β-like strands.

Gonadotropin-releasing hormone, pGlu1-His2-Trp3-Ser4-Tyr5-Gly6-Leu7-Arg8-Pro9-Gly10-NH_2, is a linear decapeptide hormone that is secreted by the hypothalamus and acts on the pituitary gland where it stimulates the release of luteinizing hormone and follicle-stimulating hormone. These hormones in turn act at the gonads as regulators of ovulation or spermatogenesis (Vander et al., 1970; Matsuo et al., 1971; Burgus et al., 1972). Hence, GnRH[1] has been a target of analogue design for potential reproductive therapy or contraceptive agents (Vickery et al., 1984; Karten & Rivier, 1986). While many of these analogues are highly active either as antagonists or superagonists, in most cases their design has not been based on conformational principles. Relatively little is known of the structural requirements of the hormone and its analogues for receptor binding.

Like many peptide hormones, GnRH is a highly flexible molecule that exists in solution as an equilibrium mixture of several conformers (Sprecher & Momany, 1979; Kopple, 1981a,b, 1983). Nonetheless, working models for its preferred conformation that may coincide with its receptor-bound conformation have emerged. A feature common to most models of GnRH is a β-turn at Gly6-Leu7, originally suggested by Momany from empirical conformational energy calculations (Momany, 1976a,b). Support for the presence of this turn in the bioactive conformation of GnRH comes from the activities of analogues with D-amino acid substitutions for Gly6 (Chandrasekaren et al., 1973; Monahan et al., 1973), an analogue with an *N*-methylleucine for Leu7 (Ling & Vale, 1975; Tonelli, 1976), and a lactam-bridged analogue that forces the ψ angle of Gly6 and the ϕ angle of Leu7 to values characteristic of a β-turn (Freidinger et al., 1980).

Recently, active analogues of GnRH that are conformationally constrained via cyclization have been synthesized. A cyclic hexapeptide, *cyclo*-(Tyr-D-Trp-Leu-Arg-Trp-Pro), while

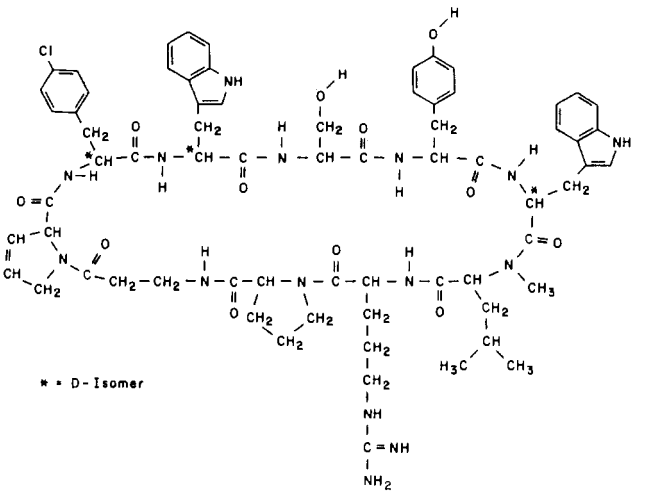


FIGURE 1: Schematic representation of the cyclic decapeptide GnRH antagonist.

a relatively low potency antagonist, retains the region of the native hormone around the β-turn and clearly demonstrates the capacity of this region for receptor binding (Freidinger et al., 1985). Rivier and co-workers (Rivier et al., 1981, 1985) have succeeded in designing and synthesizing a highly potent series of cyclized GnRH analogues. One family of these bioactive analogues, which includes the antagonist *cyclo*-(Δ^3-Pro1-D-pClPhe2-D-Trp3-Ser4-Tyr5-D-Trp6-NMeLeu7-Arg8-Pro9-β-Ala10),[2] hereafter referred to as the cyclic decapeptide, is actively under investigation with a combination of molecular dynamics (Struthers et al., 1983, 1984) and

† This research has been supported in part by NIH Grant GM 27616 (to L.M.G.) and NIH Contract N01-HD 42833 (to J.E.R.). L.M.G. is a Fellow of the A. P. Sloan Foundation (1984–1986).

‡ University of Delaware.
§ The Salk Institute.
∥ The Agouron Institute.

[1] Abbreviations: NMR, nuclear magnetic resonance; GnRH, gonadotropin-releasing hormone; ppm, parts per million; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; COSY, two-dimensional correlated spectroscopy; ppb, parts per billion; CPK, Corey–Pauling–Koltun; Boc, *tert*-butyloxycarbonyl; AcOH, acetic acid; DMF, dimethylformamide; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

[2] The numbering scheme for the cyclic antagonist is based on the analogous residue positions in the native (linear) hormone.

0006-2960/87/0426-2642$01.50/0

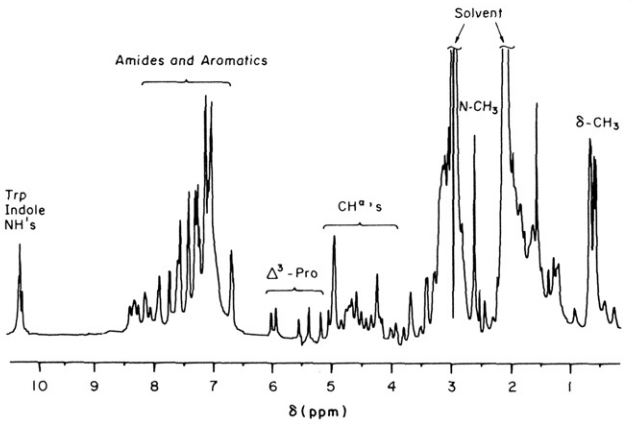


FIGURE 2: One-dimensional 500-MHz proton spectrum of the cyclic decapeptide GnRH antagonist in sulfolane.

NMR spectroscopy to determine its preferred conformation(s). The resulting three-dimensional structures can then be used to yield information on one or more possible topological requirements of the interaction of the native hormone with its receptor. Additionally, the three-dimensional structures determined by molecular dynamics simulations have already guided the design of further analogues (Rivier et al., 1985). In this paper, we describe results of a very high field NMR study of the cyclic decapeptide antagonist (shown schematically in Figure 1). Our data enable development of a conformational model for this peptide that is currently being refined by quantitative NOE analysis and comparison to molecular dynamics results.

Experimental Procedures

Synthesis. The following protected peptidoresin was prepared from a Merrifield-type resin: H-Δ^3-Pro-D-pClPhe-D-Trp-Ser(OBzl)-Tyr(Bzl)-D-Trp-NMeLeu-Arg(Tos)-Pro-β-Ala–carboxymethyl–resin. Boc-β-Ala was coupled to the chloromethylated resin according to the method of Horiki et al. (1978). A substitution of 0.7 mmol of Boc-β-Ala/g of resin was obtained. The peptide was assembled by protocols previously used in our laboratory (Marki et al., 1981). Boc protection was used throughout; couplings were achieved in CH_2Cl_2 or in a mixture of DMF/CH_2Cl_2 with dicyclo-

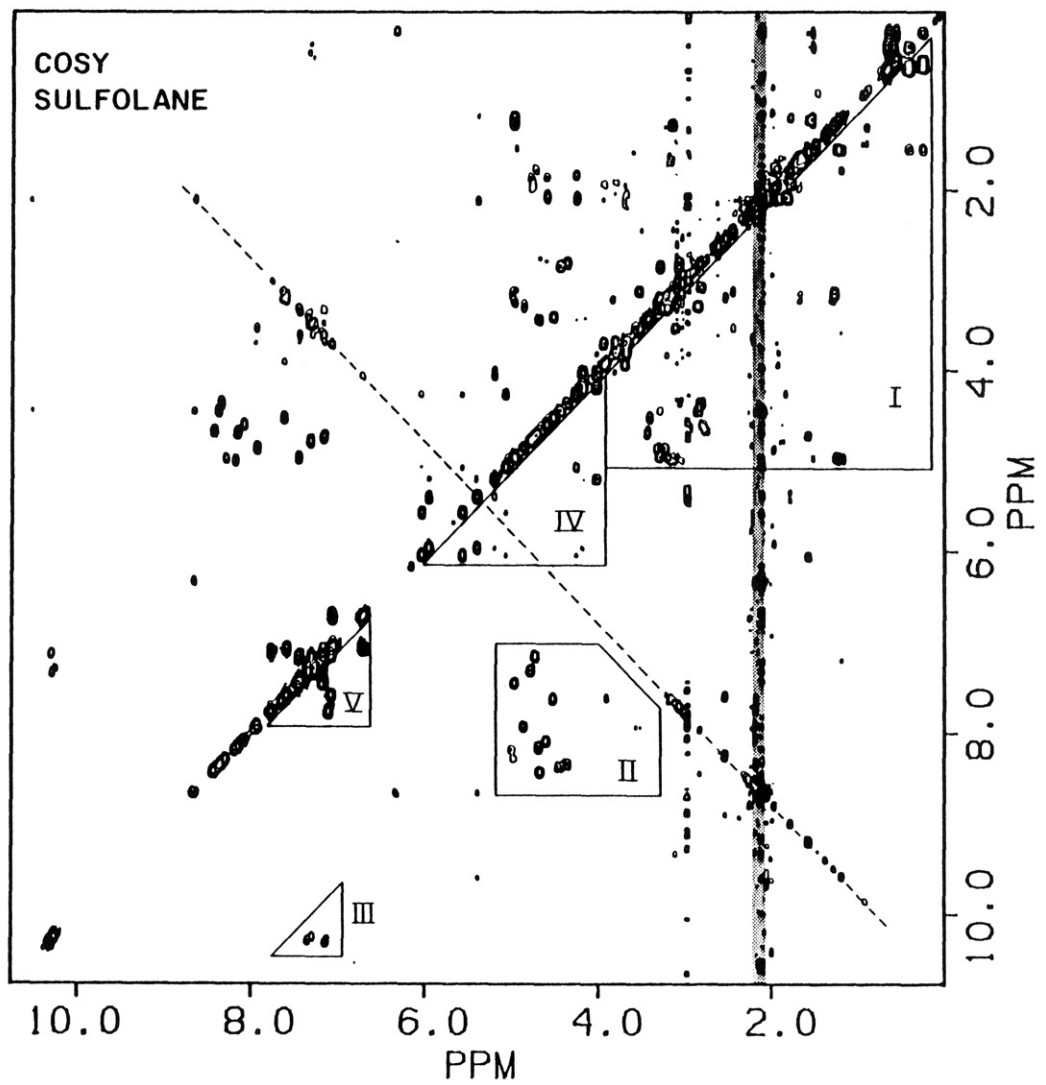


FIGURE 3: Contour map of a 500-MHz ^{1}H COSY spectrum of the cyclic decapeptide antagonist in sulfolane. The sample contained 6.07 mg of peptide, 0.5 mL of 98% [$^2H_8$]sulfolane, and 0.15 mL of 100% [$^2H_6$]dimethyl sulfoxide. The spectrum was recorded in 6 h. The outlined regions represent five unique J-coupled regions: area I, the C^α to C^β to C^γ etc. side-chain connectivities; area II, the NH to C^α connectivities; area III, the indole NH of tryptophan; area IV, the connectivities of the Δ^3-Pro1 residue; area V, the aromatic resonances. Peaks that appear on the solid line (—) are due to t_1 noise and symmetrization artifacts (Baumann et al., 1981), and those that fall along the dashed line (– –) are due to the counterdiagonal.

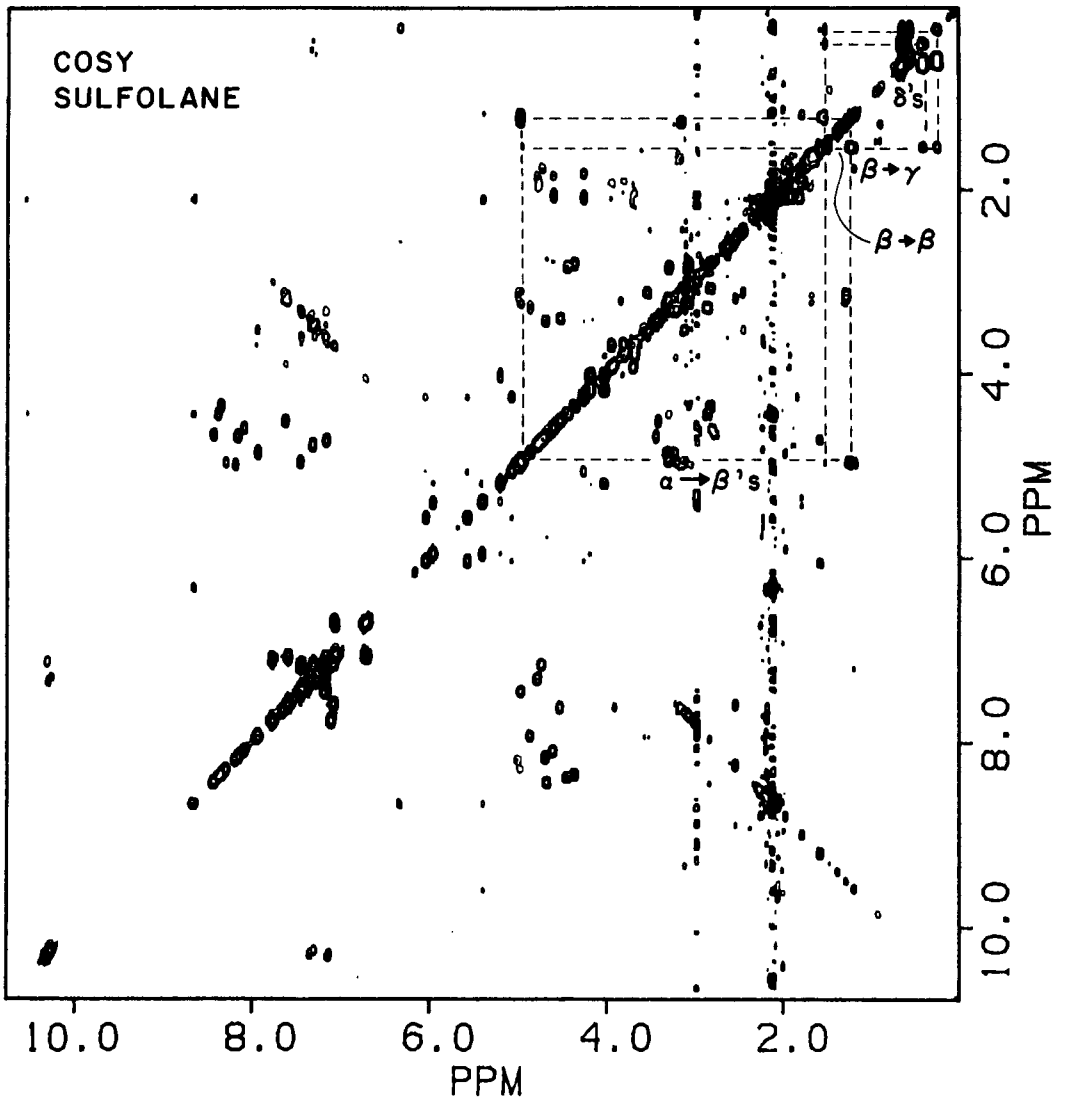


FIGURE 4: Contour map of a 500-MHz ^{1}H COSY spectrum of the cyclic decapeptide antagonist with the NMeLeu7 residue (--) coupling pattern indicated.

hexylcarbodiimide. TFA was used for the removal of the Boc group. The partially protected peptide resin shown above was hydrazinolyzed with a large excess of anhydrous hydrazine in DMF/CH_2Cl_2 (1/1) for 100 h at room temperature. The resin was then removed by filtration, and the solution was evaporated to dryness. The solid product was washed with water to yield the partially protected crude 10-peptide hydrazide (4.87 g). Partially protected peptide was cyclized by the azide coupling procedure at low temperature and high dilution in DMF. Azide was formed in DMF (50 mL/g of peptide) with 4 M HCl in dioxane (5-fold excess) and isoamyl nitrite (1.2 eq) at −25 °C. Neutralization was carried out with diisopropylethylamine, and cyclization was allowed to proceed over 4 days after further dilution in DMF (650 mL/g of peptide hydrazide). DMF was rotoevaporated, and the protecting groups were cleaved in HF. The crude unprotected cyclic peptide had one major component that was isolated by preparative HPLC (Rivier et al., 1984). The cyclic peptide product (greater than 98% pure) had the expected amino acid composition and $[\alpha]_D = -101.3°$ (*c* 1, AcOH/H_2O, ratio 1/1, uncorrected). Fast atom bombardment mass spectrometry showed the expected molecular ion.

NMR Spectroscopy. Sequential resonance assignments were obtained by using standard two-dimensional correlated spectroscopy (Aue et al., 1976; Nagayama et al., 1980; Bax, 1982) and two-dimensional nuclear Overhauser spectroscopy (Jeener et al., 1979; Kumar et al., 1981; Macura et al., 1981) pulse sequences. In order to enhance the NOE's, the sample was dissolved in a viscous solvent, tetramethylene sulfone (also referred to as sulfolane): mp 27.4 °C; viscosity (at 30 °C) 10.34 cP. This puts the molecule in the negative NOE regime where the NOE's can be up to double the size of those in the fast motion (extreme narrowing) regime (Noggle & Schirmer, 1971). Previous investigations have shown that the solvation properties of sulfolane are similar to those of chloroform; i.e., it does not solvate amide protons strongly and therefore promotes the formation of intramolecular hydrogen bonding where possible (Kartha et al., 1984; Gierasch et al., 1985). To check whether spin diffusion is occurring, several mixing times were employed.

The sample was prepared by dissolving 6.07 mg of the cyclic decapeptide in 0.5 mL of 98% [$^2H_8$]tetramethylene sulfone (MSD Isotopes) and 0.15 mL of 100% [$^2H_6$]dimethyl sulfoxide, yielding a ca. 6.9 mM solution. The sample was degassed and placed under a nitrogen atmosphere. Chemical shifts are quoted relative to the β-methylene resonance of the solvent (used as an internal reference), which was found to resonate at 2.1 ppm (at 30 °C) downfield of tetramethylsilane. Unless noted, all data were collected at 30 °C.

The COSY NMR spectra at 500 MHz were recorded on

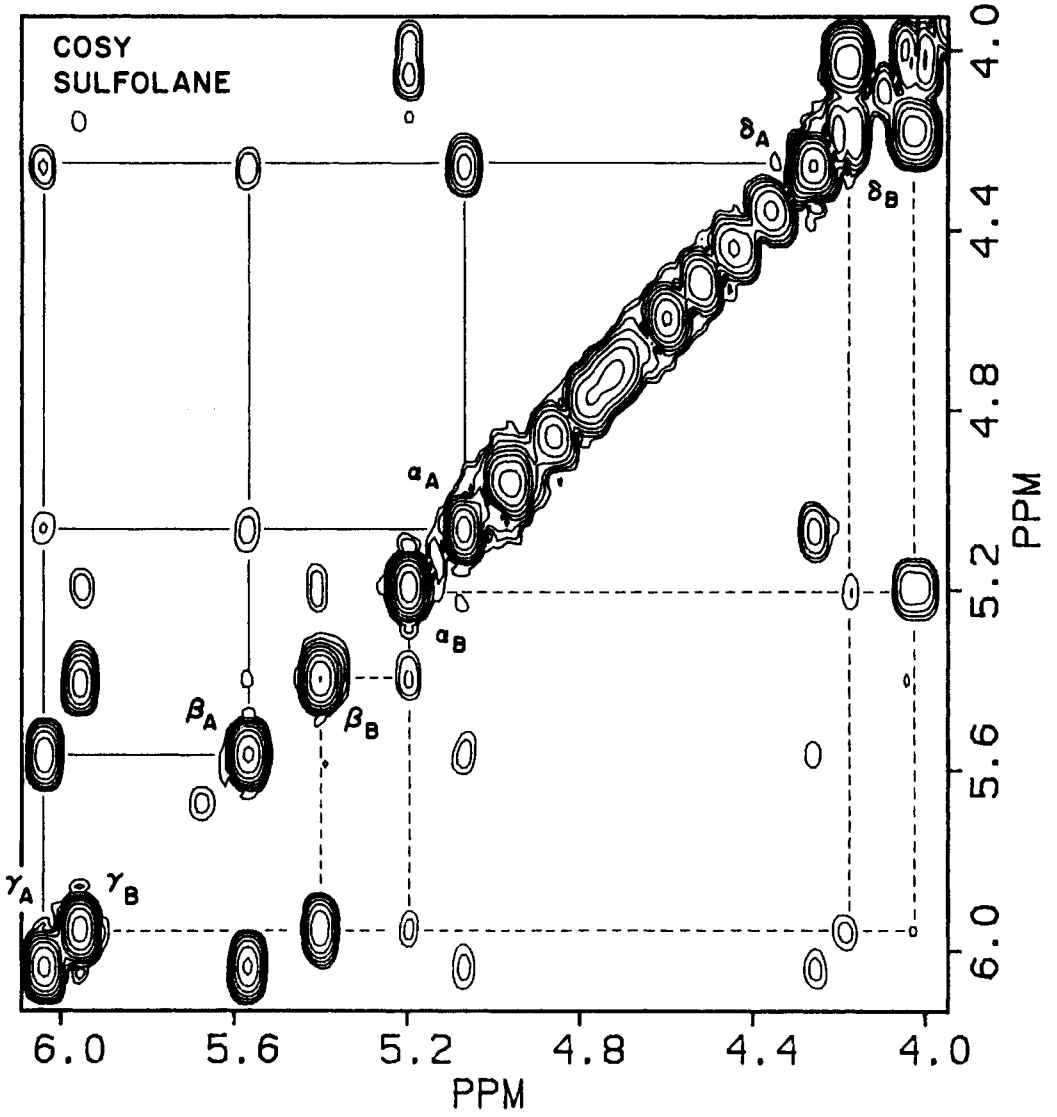


FIGURE 5: Isolated region of the spectrum in Figure 4 that shows the couplings of the Δ^3-Pro1 residue of the all-trans conformer (—) and the one-cis conformer (--). This region is presented on an expanded scale.

a Bruker WM500 spectrometer (Yale University, New Haven, CT). The spectra were obtained with 2048 points for each value of t_1. There were 256 t_1 measurements of 64 transients each with values of t_1 from 100 μs to 46 ms. The downfield solvent peak (ca. 2.5 ppm) of the tetramethylene sulfone was suppressed by gated irradiation at its observed resonance frequency. This solvent resonance was selected to be irradiated since fortuitously the residual methyl protons of the dimethyl sulfoxide also resonate at this frequency. Several COSY spectra were collected at different temperatures in order to assess the effect of temperature on the amides of the peptide backbone and its structural implications. The final processed data matrix of 1024 × 1024 points in the frequency domain was obtained by zero filling and eliminating the imaginary components. This results in a digital resolution of 5.4 Hz/point.

The NOESY NMR spectra at 500 MHz were recorded on the same instrument as above in the phase-sensitive mode and utilized a modified four-phase CYCLOPS phase cycling routine (States et al., 1982) to effect quadrature detection in the second time dimension. The spectra were obtained with 2048 points for each value of t_1. There were 512 t_1 measurements taken of 80 transients each. The t_1 values ranged from 0.9 μs to 46 ms. Additionally, the downfield solvent peak was suppressed as above. The data were symmetrized about the diagonal with a resulting digital resolution of 5.4 Hz/point (Baumann et al., 1981).

Both the COSY and NOESY data matrices were processed on a VAX/11-780 computer using a FORTRAN program, FTNMR, version 3.5, written by Dennis Hare (copyright 1984). The COSY data were apodized in t_2 with a sine-bell-squared function phase-shifted 3°. The NOESY data were apodized in t_2 with a Gaussian function. Since Bruker experimental methodology is to sample real and imaginary points at different times, the data were corrected according to the method of Redfield and Kunz (1975). Both the COSY and NOESY data were apodized in t_1 with an unshifted sine-bell function. Spectral simulations were also done with Dennis Hare's FTNMR program.

Results and Discussion

Identification of the Amino Acid Side-Chain Spin Systems. As seen in Figure 2, making initial resonance assignments is complicated by significant spectral overlap, especially in the regions of the C^α and C^β protons. In the 500-MHz COSY spectrum of the cyclic decapeptide antagonist (Figure 3), the off-diagonal peaks can be grouped into five general categories depending upon their connectivities as indicated by the boxes

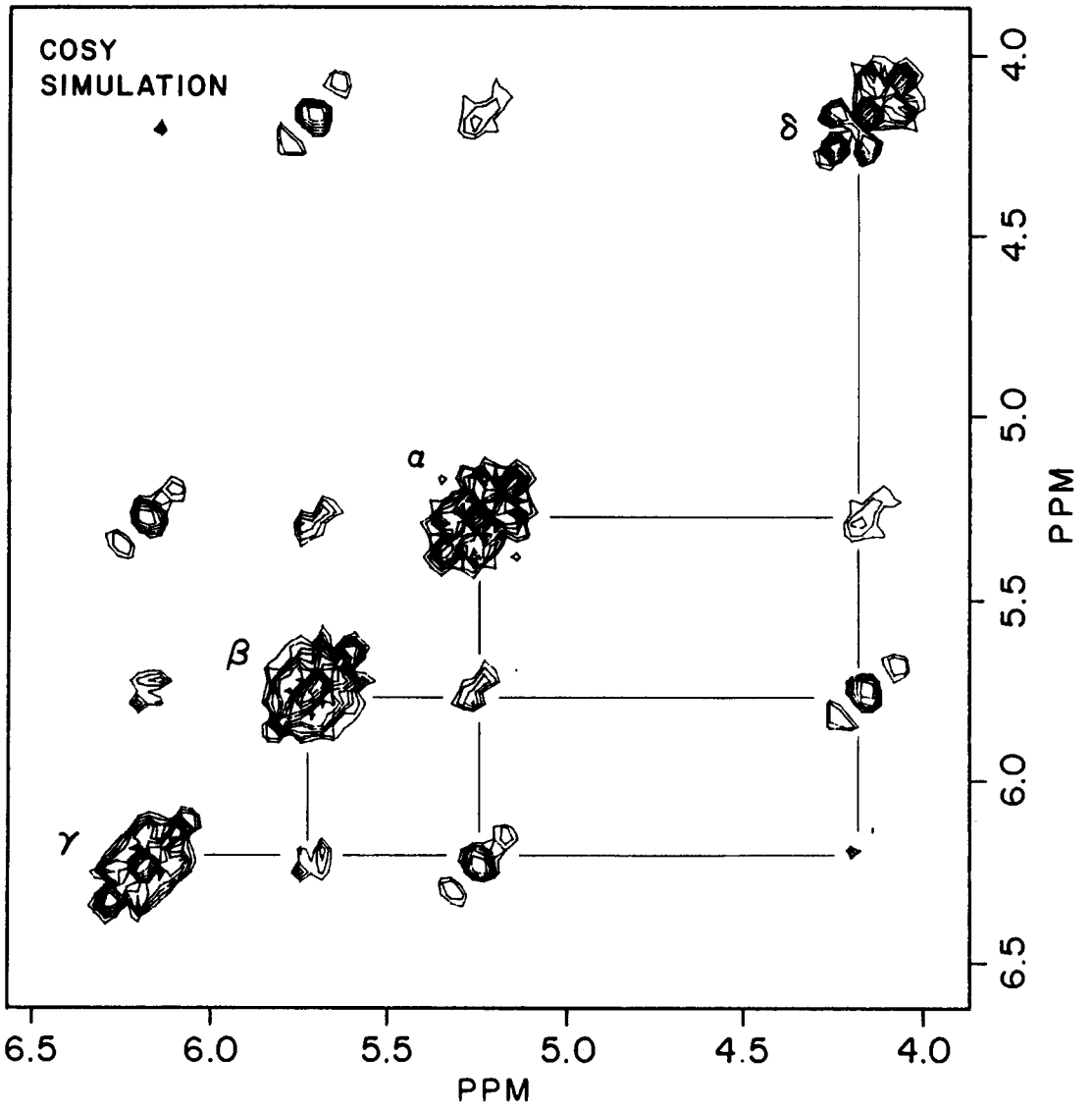


FIGURE 6: Two-dimensional spin simulation of an isolated Δ^3-Pro residue. The calculations were done with a program written by Dennis Hare. Chemical shifts were obtained experimentally and correspond to those of the all-trans conformer. All the coupling constants were set to 1.5 Hz, and t_2 was 0.1 s.

in the figure. Area I represents the coupling of the C^α to C^β to C^γ protons etc. of the amino acid side chains. Unambiguous assignments are difficult in this region due to the presence of large solvent peaks and the t_1 noise that often accompanies large peaks. Area II shows the off-diagonal peaks from through-bond J connectivities between NH and C^α proton backbone resonances. Area III includes coupling of the tryptophan indole NH to the adjacent CH of the indole ring. Area IV comprises coupling from the unusual Δ^3-proline residue. Area V delimits the aromatic resonances.

In accounting for the total number of amides in the molecule, one would expect to find seven or eight NH to C^α proton connectivities [the latter if the ϵ-NH of the guanidinium group of arginine (Wuethrich, 1976; Wider et al., 1982) is included]. Even if the amide of the β-Ala10 is split separately by each of the C^α protons (analogous to the splitting found in a glycine residue), which are themselves nonequivalent, it is clear that several more cross-peaks exist in area II of Figure 3 than are expected. The fifteen observed cross-peaks in this region are consistent with the presence of two slowly interconverting conformers of the cyclic decapeptide as would result from the cis/trans isomerization about one of the Xxx–Pro peptide bonds.

Sequential Connectivities via Short ^1H–^1H NOE Interactions. In the cyclic decapeptide, sequential assignment of the residues can begin from either of two readily assigned resonances: the *N*-methylleucine or the Δ^3-proline residues. In the former, the δ-methyls appear at ca. 0.6 ppm while in the latter the vinylic protons are found between 5 and 6 ppm. Figure 4 shows the J coupling pattern for the *N*-methylleucine. The C^β protons are magnetically inequivalent and show off-diagonal peaks (ca. 1.5 and 1.2 ppm; both conformers). These in turn each have different coupling constants (note the intensity differences in the off-diagonal peaks) with the C^α proton (4.95 ppm; both conformers) and the C^γ proton (0.43 and 0.27 ppm; for the two respective conformers). The COSY pattern (expanded scale) of the Δ^3-proline with two entirely distinct sets of resonance assignments is shown in Figure 5, lending support to the possible existence of two conformers.

The Δ^3-proline residue shows a high degree of coupling, as evidenced by COSY cross-peaks between *all protons* of the proline ring (Figure 5). To corroborate the assignments within the Δ^3-proline residue, a simulated COSY spectrum of a four-spin system with chemical shifts corresponding to the experimental values (for the all-trans conformer) was generated (Figure 6). For the purposes of this calculation, all of

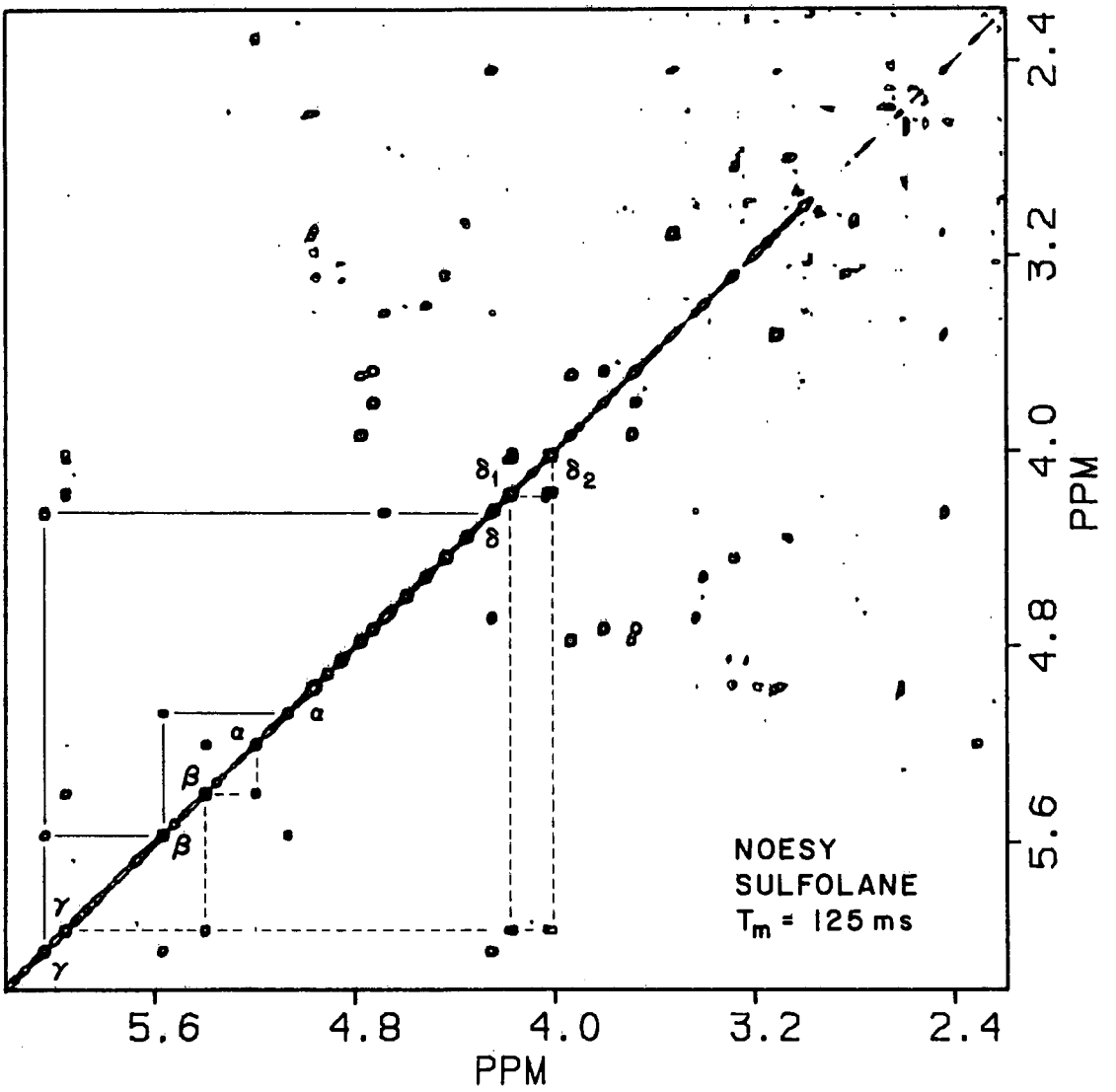


FIGURE 7: Contour map of a 500-MHz ^{1}H phase-sensitive NOESY spectrum of the cyclic decapeptide (C^α to C^β to C^γ region). The spectrum was recorded in 13.5 h with a mixing time of 125 ms and a random modulation of amplitude 10 ms (Macura et al., 1981). The digital resolution is 5.4 Hz/point. NOE connectivities within the Δ^3-Pro1 residue are shown for the all-trans conformer (—) and the one-cis conformer (– –).

the coupling constants were set to 1.5 Hz; consequently, the relative intensities of the cross-peaks cannot be interpreted quantitatively.

Since hydrogen atoms in sequentially adjoining residues and aromatic residues are separated by four or more covalent bonds, off-diagonal COSY peaks are usually not observed. Therefore, through-space interactions are required for sequential assignments (and complete side-chain assignments). These distances are a function of the dihedral angles of the residues, and stereochemical considerations show that for all sterically allowed conformations at least one interresidue distance is shorter than 3.0 Å (Leach et al., 1977; Kuo & Gibbons, 1979; Billeter et al., 1982; Wuethrich, 1983). By alternating between COSY and NOESY spectra, successive residues in an amino acid sequence can then be identified stepwise.

Figure 7 displays the C^α to C^β to C^γ region of the NOESY spectrum of the cyclic decapeptide. NOE connectivities within the Δ^3-proline residue are shown for both conformers. Despite the highly coupled region of the Δ^3-Pro residue seen in the COSY spectrum (Figure 5), *specific* assignments of resonances within each Δ^3-Pro1 residue are obtained from the NOESY spectrum (Figure 7) due to the limited spatial arrangement of each proton with respect to the others.

The connectivities between the NH_{i+1} and C^α_i protons are presented in a combined COSY–NOESY connectivity diagram (Figure 8) (Wagner et al., 1981; Wider et al., 1982; Wuethrich et al., 1982). The combined contour map shows the through-space NOE connectivities between the NH and the C^α proton of the residue preceding it, while the COSY portion accounts for the through-bond *J* connectivities between the NH and the C^α within the same residue. Connectivities between the COSY and NOESY moieties of Figure 8 are through a virtual diagonal, exactly analogous to connectivities through a real diagonal in a complete COSY or NOESY spectrum. In order to extract assignments from Figure 8, for example, begin with the C^α proton of D-Trp6 at 4.95 ppm (see arrows in the figure). A horizontal line toward the left gives the D-Trp6 NH on the virtual diagonal (COSY). A vertical line from this point leads to the C^α proton (NOESY) cross-peak of Tyr5. A horizontal line toward the right yields the Tyr5 CH^α on the diagonal, and another vertical line goes to the NH COSY connectivity of Tyr5, etc. The analyses of spectral assignments with Figure 8 are incomplete due to the presence of the two prolines and the *N*-methylleucine (all of which contain no amide protons). Additionally, the NH to NH NOE interactions between D-Trp3 and D-pClPhe2 (Figure 9) confirmed these amide resonance assignments obtained from

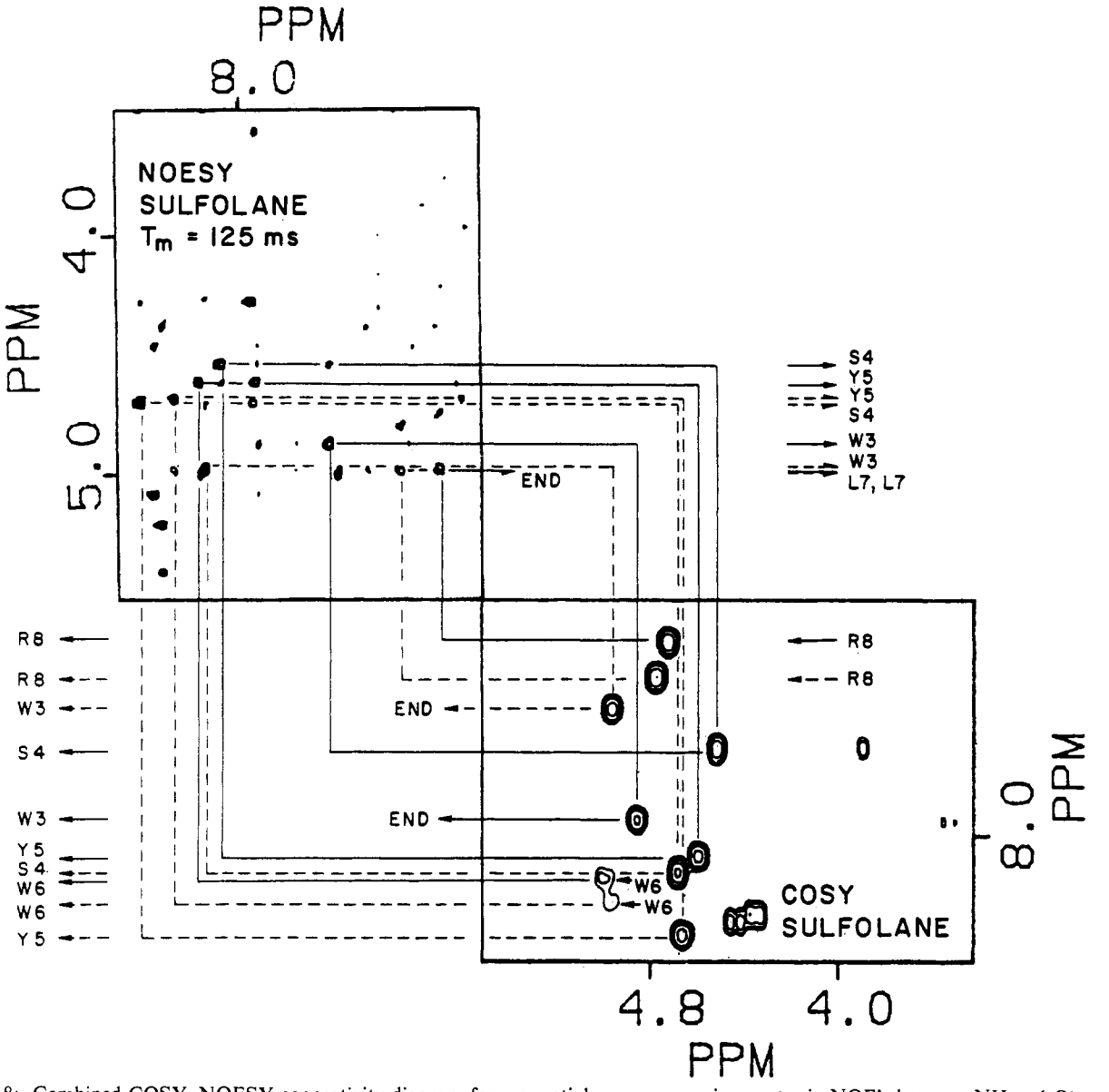


FIGURE 8: Combined COSY–NOESY connectivity diagram for sequential resonance assignments via NOE's between NH and C^{α} protons of the preceding residue. In the upper left is the region 3.42–5.50 ppm × 6.96–8.54 ppm from the 500-MHz ^1H NOESY spectrum of the cyclic decapeptide in sulfolane. In the lower right is the corresponding COSY region. The straight lines represent connectivities between neighboring residues in the peptide: all-trans conformer (—) and one-cis conformer (– –). The arrows identify the starting points. Unlabeled COSY peaks are isolated NH to C^{α} proton connectivities (i.e., no NOESY interaction to the rest of the backbone). Unlabeled NOESY peaks are predominantly due to C^{α} and side-chain aromatic ring proton interactions.

the procedure outlined in Figure 8 and were the first indication of secondary structure (see below).

The Pro9 assignments were made by first finding the NOESY cross-peak between the C^{α} proton of Arg8 and the C^{δ} protons of Pro9. Then the COSY spectrum allowed for the remaining assignments of the proline. Once the β-Ala10 NH and Pro9 C^{α} NOESY interactions were found, the COSY spectrum then allowed for the assignment of the β-Ala10 C^{α} and C^{β} protons.

We have completed all assignments of the ^1H NMR spectrum of both conformers of the cyclic decapeptide antagonist of GnRH (Table I) except for the hydroxyl moieties of both the Ser4 and Tyr5 residues and the guanidinium group of Arg8. Additionally, due to overlap with the solvent, the C^{γ} and C^{δ} protons of Arg8 could not be identified.

NH Resonances: Environment and Accessibility. The local environment around an NH resonance significantly affects the observed chemical shift of the NH. Changes in temperature can alter an NH resonance position and may be interpreted as an indication of that amide's availability for participation in intermolecular hydrogen bonding. A resonance that remains relatively unchanged as a function of temperature fluctuations is sequestered from solvent and may be intramolecularly hydrogen bonded. A resonance that is significantly affected by changes in environmental conditions is regarded as an exposed NH. This argument is valid only under the assumption that major conformational changes are absent as the perturbation occurs. The lack of discontinuous shifts in resonance values in response to these perturbations argues for a rigid molecule that does not undergo major conformational rearrangement as temperature is changed (Rose et al., 1984).

The temperature dependences of the NH resonances of the two species of the cyclic decapeptide in sulfolane are shown in Figure 10. The monotonic and nearly linear dependence of these resonances in response to temperature suggests no major conformational change and allows the slopes ($\Delta\delta/\Delta T$)

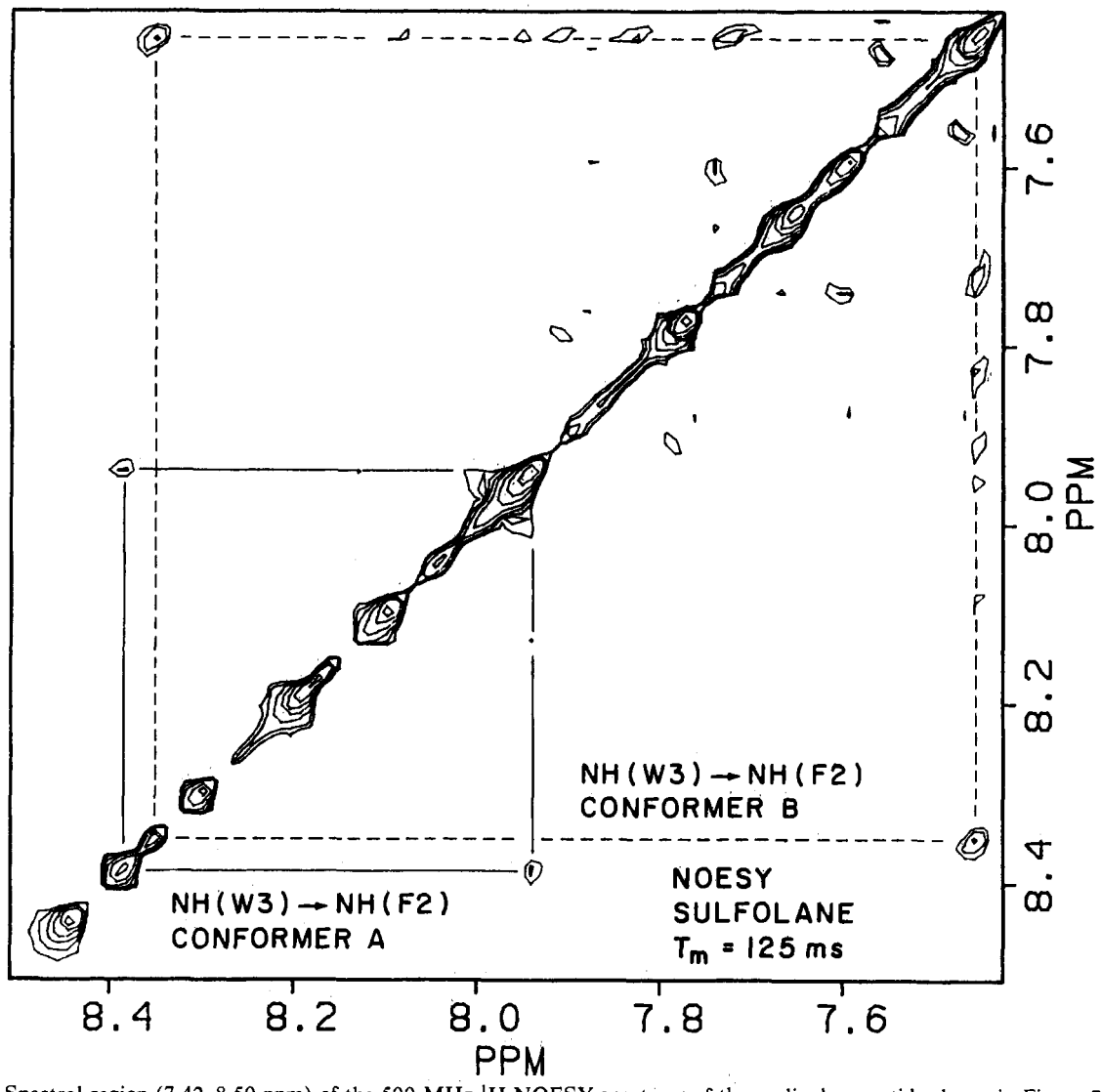


FIGURE 9: Spectral region (7.42–8.50 ppm) of the 500-MHz ^{1}H NOESY spectrum of the cyclic decapeptide shown in Figure 7. The solid line (—) shows the NH to NH connectivities between D-Trp3 and D-pClPhe2 for the all-trans conformer. The dashed line (– –) shows the same for the one-cis conformer.

Table I: Chemical Shifts (ppm) of the Protons of the Cyclic Decapeptide GnRH Antagonist[a]

amino acid residue	NH	H^{α}	H^{β}	H^{γ}	other
			All-Trans Conformer		
Δ^3-Pro1		5.07	5.57	6.04	H^{δ} 4.26, 4.21
D-pClPhe2	8.37	4.44	3.29, 2.85		ring[b] (H2,6) 7.27, (H3,5) 7.33
D-Trp3	7.92	4.86	3.30, 3.23		ring[b] (N1) 10.24, (H2) 7.29, (H4) 7.08, (H5) 7.59, (H6) 7.16, (H7) 7.30
Ser4	7.62	4.52	3.42[c]		OH[d]
Tyr5	8.09	4.60	2.80[c]		ring[b] (H2,6) 7.07, (H3,5) 6.68; OH[d]
D-Trp6	8.18	4.99	3.23, 3.13		ring[b] (N1) 10.29, (H2) 7.13, (H4) 7.71, (H5) 7.09, (H6) 7.18, (H7) 7.45
NMeLeu7	2.62[e]	4.95	1.54, 1.25	0.43	CH^{δ} 0.69, 0.60
Arg8	7.16	4.73	1.77, 1.95	[f]	H^{δ};[f] guanidinium[d]
Pro9		4.60	1.87, 2.08	1.93, 2.07	H^{δ} 3.81, 3.70
β-Ala10	7.93	3.54, 3.12	2.45[c]		
			One-Cis Conformer		
Δ^3-Pro1		5.19	5.40	5.95	H^{δ} 4.18, 4.03
D-pClPhe2	8.35	4.37	3.07, 2.82		ring[b] (H2,6) 7.14, (H3,5) 7.42
D-Trp3	7.44	4.96	3.30, 3.20		ring[b] (N1) 10.28, (H2) 7.33, (H4) 7.66, (H5) 7.06, (H6) 7.27, (H7) 7.44
Ser4	8.16	4.68	3.46[c]		OH[d]
Tyr5	8.43	4.67	2.78[c]		ring[b] (H2,6) 7.05, (H3,5) 6.72; OH[d]
D-Trp6	8.29	4.96	3.26, 3.11		ring[b] (N1) 10.30, (H2) 7.16, (H4) 7.76, (H5) 7.10, (H6) 7.18, (H7) 7.46
NMeLeu7	2.62[e]	5.00	1.59, 1.23	0.27	H^{δ} 0.66, 0.57
Arg8	7.32	4.78	1.87, 1.97	[f]	H^{δ};[f] guanidinium[d]
Pro9		4.26	1.85, 2.10	1.95, 2.10	H^{δ} 3.95, 3.82
β-Ala10	7.95	3.70	2.22[c]		

[a] Chemical shifts relative to upfield sulfolane resonance (2.1 ppm): temperature 30 °C; concentration 6.92 mM. [b] Numbering scheme based on IUPAC standards. [c] Two methylene protons may have identical shifts, or one of the resonances may not be identified. [d] Exchangeable protons not assigned. [e] *N*-Methyl resonance assignment. [f] Resonances not assigned—obscured by solvent peaks.

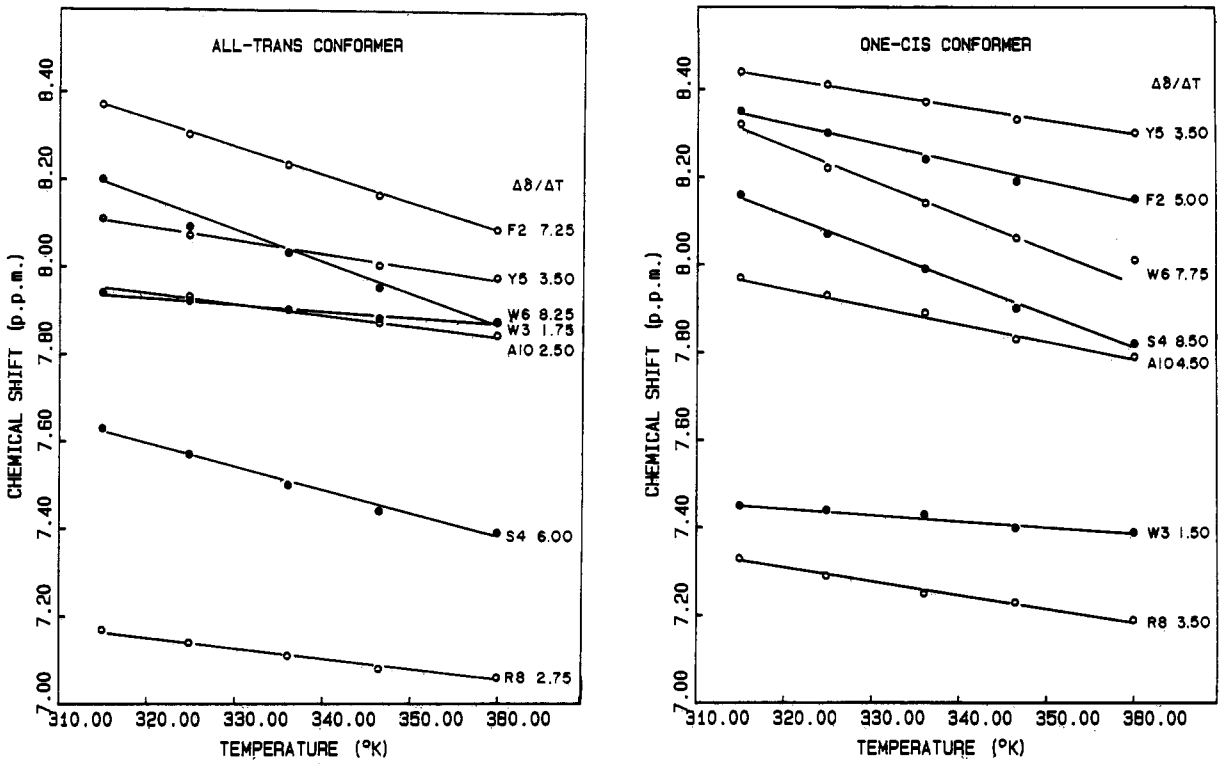


FIGURE 10: Temperature dependence of the amide resonances in the all-trans conformer and the one-cis conformer. Data were collected from temperature-dependent COSY spectra of the cyclic decapeptide antagonist in sulfolane. Experimental conditions were identical with those used to collect data in Figure 4. Slopes ($\Delta\delta/\Delta T$) are reported in units of ppb/deg.

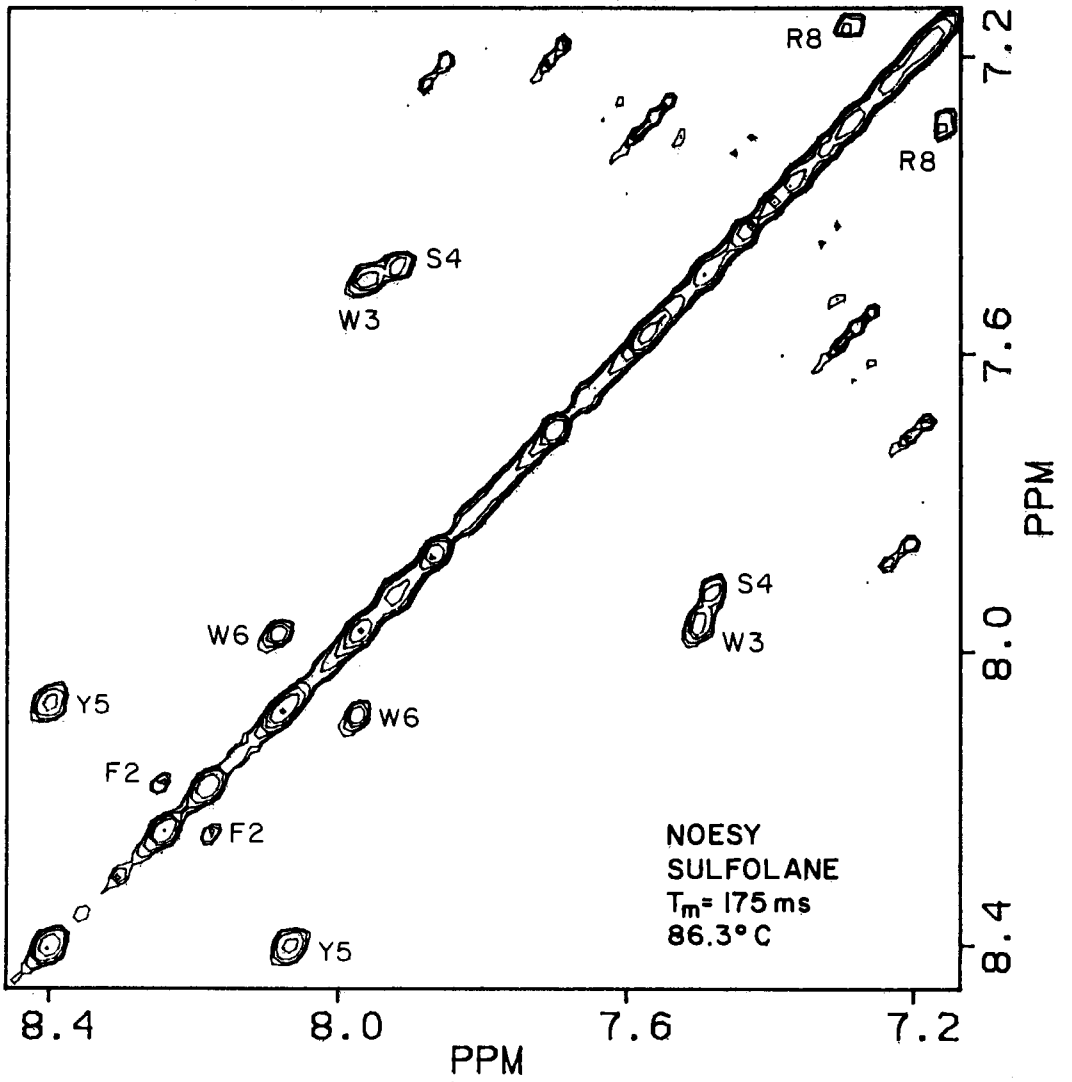


FIGURE 11: Spectral region (7.03–8.35 ppm) of the 500-MHz ^{1}H NOESY spectrum of the cyclic decapeptide at 86.3 °C. Off-diagonal peaks represent chemical exchange NOE's between the same amides of the two different conformations. The unlabeled cross-peaks are due to the aromatic moieties in both conformers.

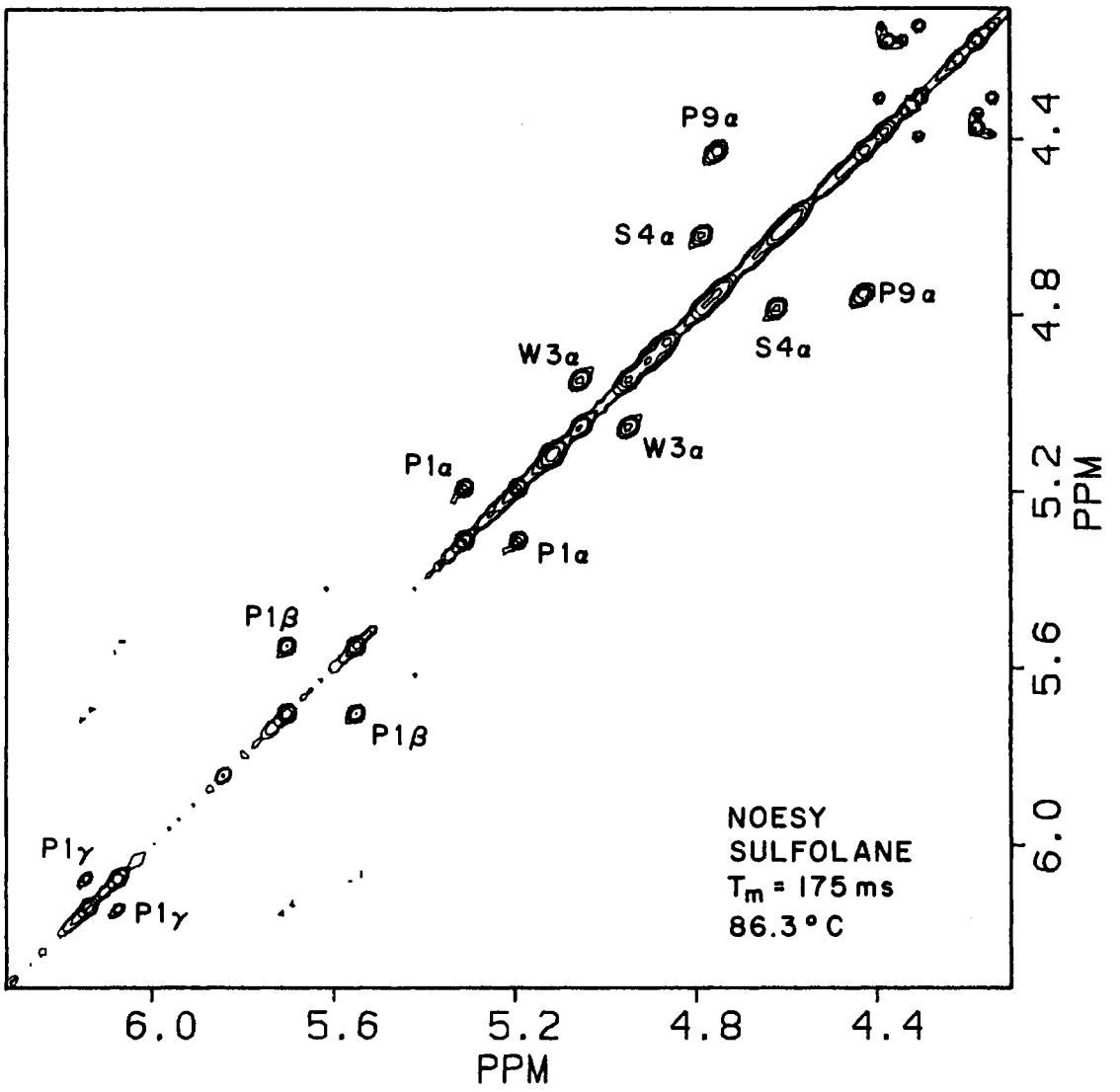


FIGURE 12: Spectral region (4.03–6.17 ppm) of the 500-MHz ^{1}H NOESY spectrum of the cyclic decapeptide at 86.3 °C. Off-diagonal peaks represent chemical exchange NOE's between the same proton of the two different conformations.

to be compared. In both species, residues that have a relatively large slope include D-pClPhe2, Ser4, and D-Trp6, which thus can be concluded to be exposed to the solvent to a greater extent than the remaining amides. The latter, which include those of the D-Trp3, Tyr5, Arg8, and β-Ala10, have smaller slopes, which suggest their reduced accessibility to the environment. Both of these observations are consistent with the proposed structures of the molecule (see below).

The chemical shift of an amide can generally be used as an indication of the strength of its hydrogen bonding, in the absence of magnetic anisotropic effects. If the NH is sequestered from intermolecular interactions (low $\Delta\delta/\Delta T$), then its chemical shift qualitatively reflects the strength of intramolecular hydrogen bonds. Among the lowest field resonances in this molecule are the D-Trp3, Tyr5, and β-Ala10, all of which are proposed to be involved in β-turn or cross-ring (β-sheet) hydrogen bonding (see below), consistent with their small slopes as discussed above. The D-Trp3 NH resonance undergoes a significant upfield chemical shift (ca. 0.45 ppm) from the trans to cis conformations. This phenomenon together with its small change in $\Delta\delta/\Delta T$ between the two conformations argues that it is *buried* in both conformers but intramolecularly hydrogen bonded only in the trans conformation. This is consistent with cis/trans isomerization arguments discussed later. The highest field resonance, Arg8, although buried ($\Delta\delta/\Delta T$ of ca. 3.0 ppb/deg) appears not to be involved in strong hydrogen bonding.

Evidence for Cis/Trans Conformers. Although three potential sites (the two prolines and the *N*-methylleucine) for cis/trans isomerization are present within the molecule, experimental evidence strongly supports the conclusion that the two conformers arise from isomerization about the β-Ala10–Δ^3-Pro1 peptide bond exclusively. The strongest evidence is manifested in the NOESY cross-peaks, namely, the cross-peaks between the C^δ protons of Δ^3-Pro1 and the C^β protons of β-Ala10. Only a trans arrangement of the intervening peptide bonds would yield a significant cross-peak between these protons (as is observed in *one* conformer). However, were a cis isomer about the β-Ala10–Δ^3-Pro1 peptide bond to exist, there would be no NOE cross-peak between these protons due to the 180° rotation of the carbonyl of the peptide bond and its subsequent location between the protons in question. The presence of this carbonyl between the protons therefore increases their internuclear distance beyond the 4–5 Å maximal limit detectable by the NOESY experiment. Computer modeling and simple geometric calculations show that these protons are less than 2.8 Å apart in a trans peptide bond while they are greater than 4.4 Å apart in a cis peptide bond.

Additionally, both conformations exhibit NOE cross-peaks between the C^δ protons of Pro9 and the C^α proton of Arg8 as

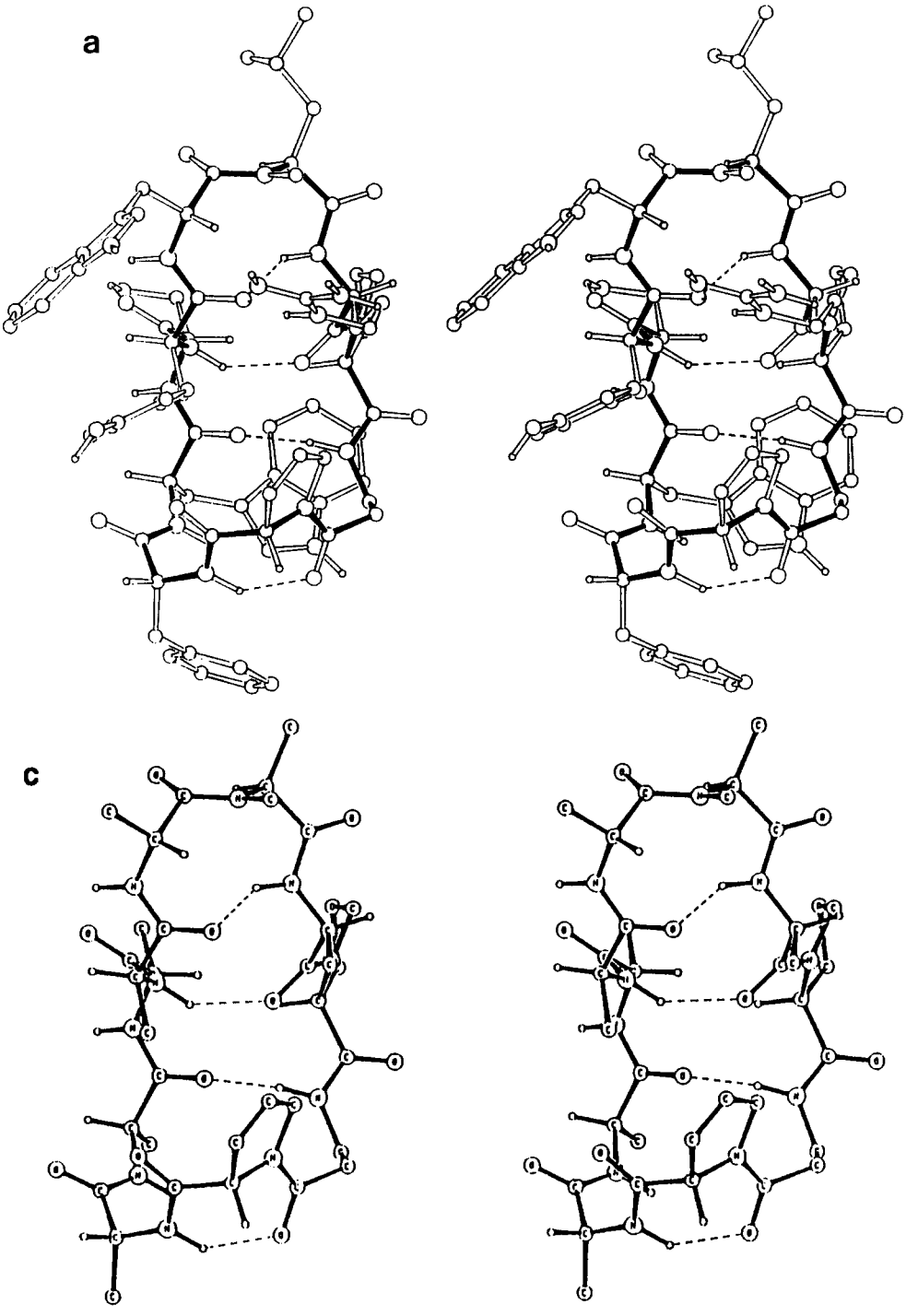
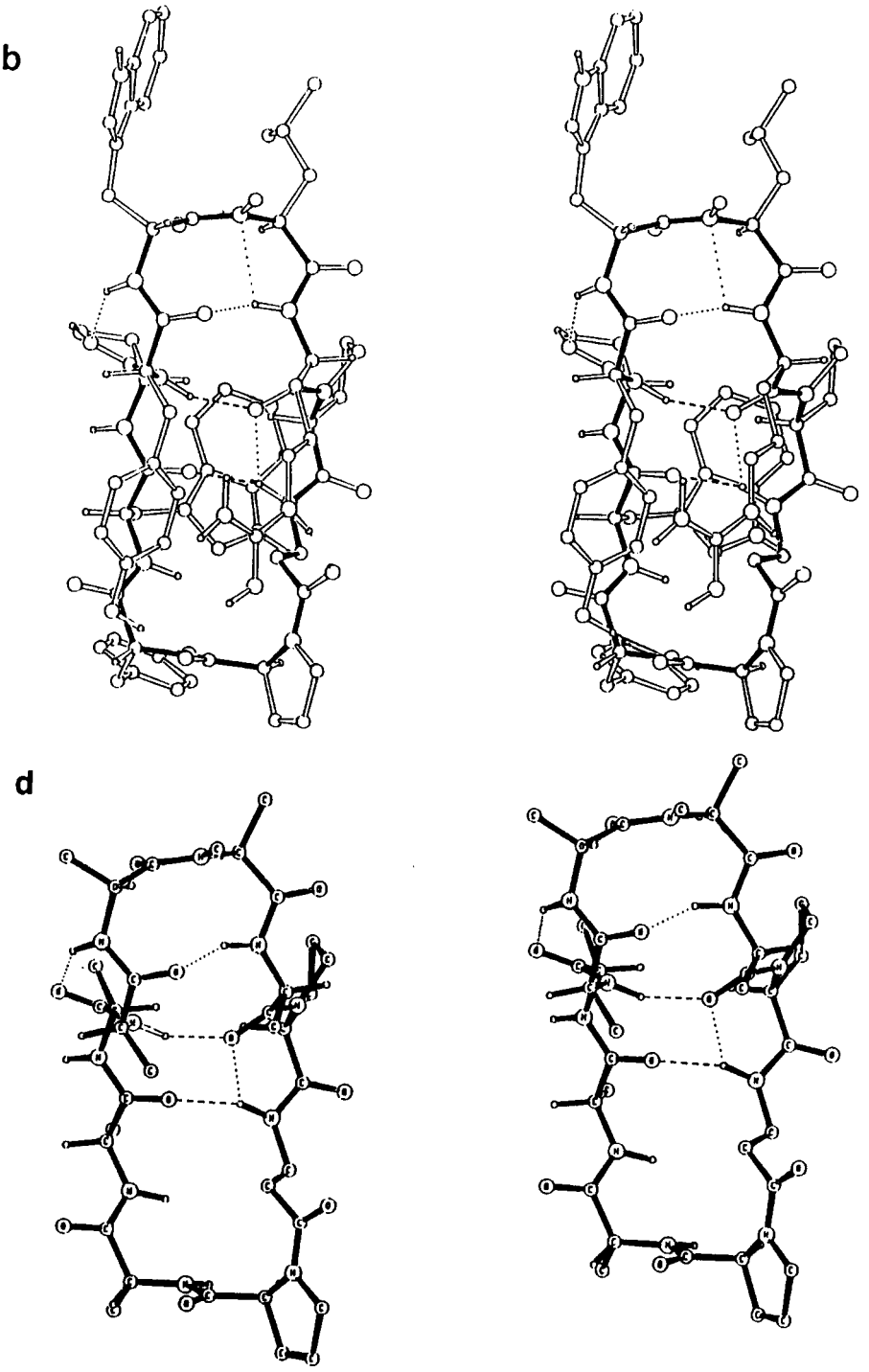


FIGURE 13: Proposed three-dimensional structures (stereoviews) of the cyclic decapeptide antagonist of GnRH. Conformations shown are the lowest energy ones obtained by energy minimization of structures occurring along a molecular dynamics trajectory with all amide bonds trans (a) (Struthers et al., 1983; 1984) or during a similar trajectory with the β-Ala10–Δ^3-Pro1 bond in the cis arrangement (b) (T. Solmajer,

well as between the *N*-methyl protons of NMeLeu7 and the C^{α} proton of D-Trp6. These observations eliminate the possible isomerization about these peptide bonds. Examination of molecular models shows that the NMeLeu7 residue is not affected by the β-Ala–Δ^3-Pro isomerization as is evidenced by the degeneracy of the chemical shifts of this residue between the two conformers. This degeneracy is unlikely to be possible if there were isomerization at the Arg8–Pro9 or the D-Trp6–NMeLeu7 peptide bonds. Yet isomerization centered around the β-Ala10–Δ^3-Pro1 peptide bond would be expected to cause a greater perturbation in the chemical shifts of neighboring residues (D-pClPhe2, D-Trp3), as observed. This analysis from model building is fully supported by calculations of preferred one-cis conformations of the cyclic decapeptide (T. Solmajer, R. S. Struthers, and A. T. Hagler, unpublished results). In addition, the presence of the β-carbon of β-Ala10 within the backbone appears to afford a relatively facile transition from cis to trans conformations of the molecule without greatly affecting the spatial orientation of the remaining backbone atoms.

The dramatic change in chemical shift of the D-Trp3 amide is also consistent with isomerization about the β-Ala10–Δ^3-Pro1 peptide bond. When this bond is trans, the carbonyl of β-Ala10 is oriented toward the amide of D-Trp3 and is available for hydrogen bonding. However, when this bond is cis, the CO is directed away from the center ring of atoms



R. S. Struthers, and A. T. Hagler, unpublished results). The backbone is shown alone for the all-trans conformer (c) and the one-cis conformer (d) in order to clearly demonstrate their similarities except at the point of isomerization.

forming the backbone and is no longer available for hydrogen bonding to the NH of D-Trp3. This rearrangement would be expected to cause a large upfield shift of this amide, as observed (ca. 0.45 ppm, Figure 10). Of additional note is the constancy of the very low $\Delta\delta/\Delta T$ of this residue. Despite the change in hydrogen-bonding environments between the cis and trans conformations, the NH remains buried ($\Delta\delta/\Delta T$ of ca. 1.6 ppb/deg).

Chemical exchange NOE data in Figures 11 and 12 clearly demonstrate the existence of one molecular species that slowly exchanges between two conformations. Figure 11 is a high-temperature (86.3 °C) phase-sensitive NOESY spectrum of the amide region. The major off-diagonal peaks are labeled that show chemical exchange occurring between corresponding resonances from the two conformers. In this example, all the amide protons exhibit this exchange except for the β-Ala10 residue, possibly due to broadening and to its close proximity to the diagonal. Figure 12 shows the C^{α} proton region of the spectrum with a few of the resonances showing chemical exchange between like moieties of the two different conformers.

Conformational Interpretation. The most significant spectral data for deducing the overall shape of the molecule in three dimensions are the semiquantitative NOE data. The conformational constraints imposed by cyclization coupled with a series of individual NOE distance measurements quickly begin to place the molecule in some sort of restricted con-

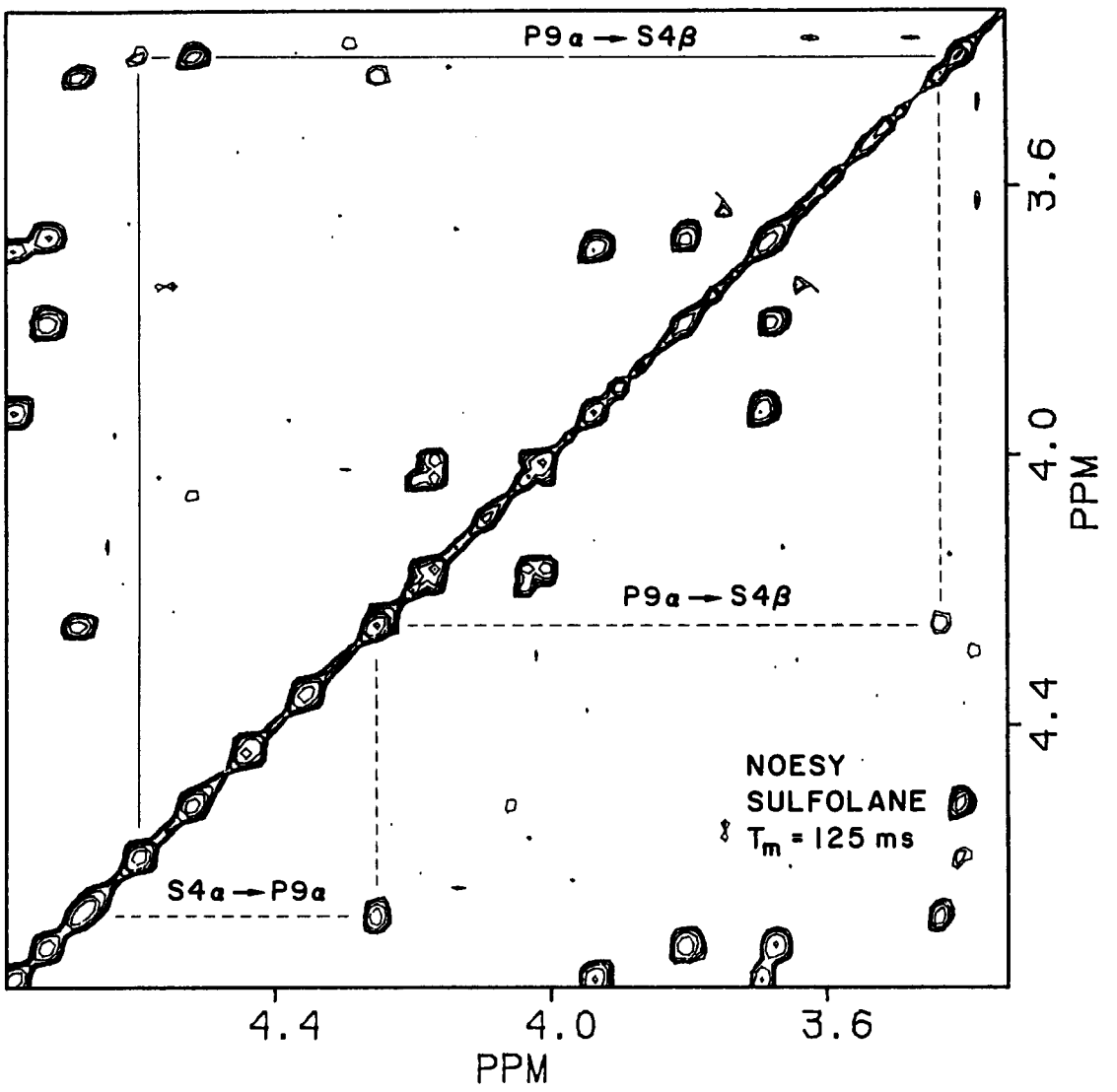


FIGURE 14: Spectral region (3.32–4.82 ppm) of the 500-MHz ^{1}H NOESY spectrum of the cyclic decapeptide shown in Figure 7. The solid line (—) shows the cross-ring connectivities for the all-trans conformer. The dashed line (– –) shows the same for the one-cis conformer.

formation. The primary assumption made in NOE analysis is that the same correlation time, τ_c, exists for all parts of the molecule. The NH accessibility data presented above support the models proposed.

The proposed conformations consist of an all-trans peptide bond structure in one case and a structure with one cis peptide bond (β-Ala10–Δ^3-Pro1) in the other. Both conformers contain a type II′ β-turn (D-Trp6 to NMeLeu7) and a type II β-turn (Δ^3-Pro1 to D-pClPhe2). A weak hydrogen bond between the Arg8 NH and the Tyr5 CO in the former turn site and a stronger one between the D-Trp3 NH and the β-Ala10 CO in the latter turn site (all-trans conformer only) are postulated. Two additional transannular hydrogen bonds are proposed, one from the NH of Tyr5 to the CO of Arg8 and the other from the NH of β-Ala10 to the CO of D-Trp3. Chemical shift data suggest that all the hydrogen bonds are of nearly the same strength in the two conformers.

Conformations consistent with the NMR results were generated by molecular dynamics calculations on the cyclic decapeptide GnRH antagonist (Struthers et al., 1983, 1984; T. Solmajer, R. S. Struthers, and A. T. Hagler, unpublished results); Figure 13 shows the lowest energy conformers obtained by energy minimization of structures occurring along molecular dynamics trajectories for the antagonist, either with all peptide bonds trans (a) or with a cis β-Ala10–Δ^3-Pro1 peptide bond (b). (Side-chain positions differ since they are relatively unconstrained in the calculation; furthermore, NMR data were not used to deduce preferred side-chain rotamers in this work.) For comparison, (c) and (d) show the respective conformers with the side chains removed. Note the striking similarity in the backbone conformation in all regions except the site of peptide bond isomerization.

Several significant conformation-directing NOE's exist in both conformers. First, the interactions between the *N*-methyl of NMeLeu7 and the NH of Arg8 and between the NH's of D-Trp3 and of D-pClPhe2 are both indications of locations where the chain turns back upon itself, namely, β-turns. There also exist significant cross-ring NOE interactions (Figure 14) between the C^α proton of Pro9 and the C^β of Ser4 in the all-trans case and between the C^α of Pro9 and both the C^α and C^β protons of Ser4 in the one-cis case. Both of these Pro9/Ser4 NOE observations argue for a stable overall ring structure.

The goal of our conformational analysis is to relate NMR results to possible receptor-binding conformations of GnRH and its analogues. The choice of solvent for this type of correlation is highly problematical. The solvent used in this study, sulfolane, is less strongly interacting with peptide solutes than is water, in which the hormone resides prior to receptor binding. Yet upon interaction with the receptor, the hormone experiences the largely nonaqueous environment of the protein

receptor, which is itself membrane resident. There is no effective way to replace these interactions by a bulk solvent in which intramolecular factors (not solvation) will play a primary role in determining favored conformations. This choice facilitates comparisons with structures emerging from molecular dynamics simulations carried out in the absence of solvent but is nonetheless limited as a means of deducing receptor-binding conformations. This limitation is most serious for the flexible native hormone; indeed, the problem of conformational change in response to different environments was one of the major motivations for studying a bioactive, conformationally *constrained* analogue of GnRH.

Our data support the conclusion that the cyclic decapeptide antagonist of GnRH strongly prefers a conformation with two strands of antiparallel β-structure connected by β-turns. The segment of the sequence of the cyclic antagonist from Tyr5 to Arg8, which forms a type II′ β-turn, corresponds to a region of the native hormone that has been proposed to be critical for receptor interaction and to exist in a β-turn when bound [Karten and Rivier (1986) and references cited therein]. The present results therefore lend support to the model that GnRH interacts with its receptor in a folded conformation. The closing of the antagonist sequence in a ten-residue cyclic structure clearly contributed to the stability of the β-type structure. In comparison, other cyclic peptides of $2(n + 1)$ residues have been found to have similar structures made up of β-turns connected by extended β-structure. An analogous structure was originally proposed for the cyclic decapeptide gramicidin S, *cyclo*-(Val-Orn-Leu-D-Phe-Pro)$_2$ (Schwyzer & Ludescher, 1968; Stern et al., 1968; Ovchinnikov et al., 1970; Urry et al., 1975; Rae et al., 1977). The β-turns in gramicidin S, with its twice repeated pentapeptide sequence, are both type II′ (D-Phe-Pro), and four NH's (both of the Val and Leu residues) are strongly intramolecularly hydrogen bonded. The presence of a Pro in the extended region of the GnRH antagonist may disrupt the transannular hydrogen bond array.

CONCLUSIONS

All nonlabile protons of the cyclic decapeptide antagonist of GnRH were observed and assigned with the exception of the C^{γ} and C^{δ} protons of the Arg8 residue, which are overlapped with solvent peaks. The antagonist exists nearly equally distributed between two slowly interconverting conformations that differ only by cis/trans isomerization about the β-Ala10–Δ^3-Pro1 bond. Each conformer contains two β-turns and four (all-trans case) or three (one-cis case) transannular hydrogen bonds consistent with the presence of extended antiparallel β-like strands that join the two turn regions. This overall conformation is consistent with previous molecular dynamics simulations (Struthers et al., 1983, 1984). Additional experiments are in progress to determine more quantitative interproton distances and to relate these proposed three-dimensional structures derived from experimental data to theoretical structures obtained by molecular dynamics calculations.

ACKNOWLEDGMENTS

We thank Dennis Hare and Luciano Mueller for their helpful discussions and suggestions regarding NMR techniques and data processing, R. Galyean, J. Varga, and G. Kupryszewski for synthesizing the cyclic antagonist, and Tom Solmajer for providing calculated structures of the one-cis conformer of the decapeptide.

Registry No. Cyclic decapeptide, 77060-24-3; LH-RH, 9034-40-6.

REFERENCES

Aue, W. P., Bartholdi, E., & Ernst, R. R. (1976) *J. Chem. Phys. 64*, 2229–2246.

Baumann, R., Wider, G., Ernst, R. R., & Wuethrich, K. (1981) *J. Magn. Reson. 44*, 402–406.

Bax, A. (1982) *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Reidel, Boston.

Billeter, M., Braun, W., & Wuethrich, K. (1982) *J. Mol. Biol. 155*, 321–346.

Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., & Guillemin, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 278–282.

Chandrasekaren, R., Lakshminarayanan, A. V., Pandya, U. V., & Ramachandran, G. N. (1973) *Biochim. Biophys. Acta 303*, 14–27.

Freidinger, R. M., Veber, D. F., Perlow, D. S., Brooks, J. R., & Saperstein, R. (1980) *Science (Washington, D.C.) 210*, 656–658.

Freidinger, R. M., Colton, C. D., Randall, W. C., Pitzenberger, S. M., & Veber, D. F. (1985) in *Peptides: Structure and Function*, Proceedings of the 9th American Peptide Symposium (Deber, C. M., Hruby, V. J., & Kopple, K. D., Eds.) pp 549–552, Pierce Chemical Co., Rockford, IL.

Gierasch, L. M., Rockwell, A. L., Thompson, K. F., & Briggs, M. S. (1985) *Biopolymers 24*, 117–135.

Horiki, K., Igano, K., & Inouye, K. (1978) *Chem. Lett.*, 165–168.

Jeener, J., Meier, B. H., Bachmann, P., & Ernst, R. R. (1979) *J. Chem. Phys. 71*, 4546–4553.

Karten, M., & Rivier, J. (1986) *Endocr. Rev. 7*, 44–66.

Kartha, G., Bhandary, K. K., Kopple, K. D., Go, A., & Zhu, P.-P. (1984) *J. Am. Chem. Soc. 106*, 3844–3850.

Kopple, K. D. (1981a) *Biopolymers 20*, 1913–1920.

Kopple, K. D. (1981b) in *Peptides: Synthesis-Structure-Function*, Proceedings of the 7th American Peptide Symposium (Rich, D. H., & Gross, E., Eds.) pp 295–298, Pierce Chemical Co., Rockford, IL.

Kopple, K. D. (1983) *Int. J. Pept. Protein Res. 21*, 43–48.

Kumar, A., Wagner, G., Ernst, R. R., & Wuethrich, K. (1981) *J. Am. Chem. Soc. 103*, 3654–3658.

Kuo, M., & Gibbons, W. A. (1979) in *Peptides: Structure and Biological Function*, Proceedings of the 6th American Peptide Symposium (Gross, E., & Meienhofer, J., Eds.) pp 226–232, Pierce Chemical Co., Rockford, IL.

Leach, S. J., Nemethy, G., & Scheraga, H. A. (1977) *Biochem. Biophys. Res. Commun. 75*, 207–215.

Ling, N., & Vale, W. (1975) *Biochem. Biophys. Res. Commun. 63*, 801–805.

Macura, S., Huang, Y., Suter, D., & Ernst, R. R. (1981) *J. Magn. Reson. 43*, 259–281.

Marki, W., Spiess, J., Tache, Y., Brown, M., & Rivier, J. E. (1981) *J. Am. Chem. Soc. 103*, 3178–3185.

Matsuo, H., Baba, Y., Nair, R. M. G., Arimura, A., & Schally, A. V. (1971) *Biochem. Biophys. Res. Commun. 43*, 1374–1439.

Momany, F. A. (1976a) *J. Am. Chem. Soc. 98*, 2990–2996.

Momany, F. A. (1976b) *J. Am. Chem. Soc. 98*, 2996–3000.

Monahan, M. W., Amoss, M. S., Anderson, H. A., & Vale, W. (1973) *Biochemistry 12*, 4616–4620.

Nagayama, K., Kumar, A., Wuethrich, K., & Ernst, R. R. (1980) *J. Magn. Reson. 40*, 321–334.

Noggle, J. H., & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect*, Academic, New York.

Ovchinnikov, Y. A., Ivanov, V. T., Bystrov, V. F., Miroshnikov, A. I., Shepel, E. N., Addullaev, N. D., Efremov, E. S., &

Senyavina, L. B. (1970) *Biochem. Biophys. Res. Commun. 39*, 217–225.
Rae, I. D., Stimson, E. R., & Scheraga, H. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 225–229.
Redfield, A. G., & Kunz, S. D. (1975) *J. Magn. Reson. 19*, 250–254.
Rivier, J., Rivier, C., Perrin, M., Porter, J., & Vale, W. (1981) in *LHRH Peptides as Female and Male Contraceptives* (Zatachni, G. I., Shelton, J. D., & Sciarra, J. J., Eds.) pp 13–23, Harper and Row, Philadelphia, PA.
Rivier, J., McClintock, R., Galyean, B., & Anderson, H. (1984) *J. Chromatogr. 288*, 303–328.
Rivier, J., Varga, J., Porter, J., Perrin, M., Haas, Y., Corrigan, A., Rivier, C., & Vale, W. (1985) in *Peptides: Structure and Function*, Proceedings of the 9th American Peptide Symposium (Deber, C. M., Hruby, V. J., & Kopple, K. D., Eds.) pp 541–544, Pierce Chemical Co., Rockford, IL.
Rose, G. D., Gierasch, L. M., & Smith, J. A. (1984) *Adv. Protein Chem. 37*, 1–109.
Schwyzer, R., & Ludescher, U. (1968) *Biochemistry 7*, 2519–2522.
Sprecher, R. F., & Momany, F. A. (1979) *Biochem. Biophys. Res. Commun. 87*, 72–77.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.
Stern, A., Gibbons, W. A., & Craig, L. C. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 734–741.
Struthers, R. S., Rivier, J., & Hagler, A. (1983) *Conformationally Directed Drug Design—Peptides and Nucleic Acids as Templates or Targets* (Vida, J. A., & Gorden, M., Eds.) pp 239–261, American Chemical Society, Washington, DC.
Struthers, R. S., Rivier, J., & Hagler, A. (1984) *Ann. N.Y. Acad. Sci. 439*, 81–96.
Tonelli, A. E. (1976) *Biopolymers 15*, 1615–1622.
Urry, D. W., Long, M. M., Mitchell, L. W., & Okamoto, K. (1975) in *Peptides: Chemistry, Structure, Biology*, Proceedings of the 4th American Peptide Symposium (Walter, R. A., & Meienhofer, J., Eds.) pp 113–126, Ann Arbor Science, Ann Arbor, MI.
Vander, A. J., Sherman, J. H., & Luciano, D. S. (1970) in *Human Physiology, the Mechanisms of Body Function*, pp 443–471, McGraw-Hill, New York.
Vickery, B. H., Nestor, J. J., Jr., & Hafez, E. S. E., Eds. (1984) *LHRH and Its Analogs: Contraceptive and Therapeutic Applications*, MTP Press, Lancaster, England.
Wagner, G., Kumar, A., & Wuethrich, K. (1981) *Eur. J. Biochem. 114*, 375–384.
Wider, G., Lee, K. H., & Wuethrich, K. (1982) *J. Mol. Biol. 155*, 367–388.
Wuethrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland, Amsterdam.
Wuethrich, K. (1983) *Biopolymers 22*, 131–138.
Wuethrich, K., Wider, G., Wagner, G., & Braun, W. (1982) *J. Mol. Biol. 155*, 311–319.

CORRECTION

Anti-Sense Peptide Recognition of Sense Peptides: Direct Quantitative Characterization with the Ribonuclease S-Peptide System Using Analytical High-Performance Affinity Chromatography, by Yechiel Shai,* Michael Flashner, and Irwin M. Chaiken*, Volume 26, Number 3, February 10, 1987, pages 669–675.

Page 672. In Figure 2, the absorbance units of the ordinate axis in panels A and B should be in thousandths instead of hundredths; also the assignment of elution curves in panels A and B to concentrations of soluble S-peptide competitor should be reversed.