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Conformational analysis of the nonapeptide leuprorelin using NMR and molecular modeling

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Summary

The nonapeptide Leuprorelin, one of the LHRH agonists, was studied by means of 2D nuclear magnetic resonance spectroscopy and molecular modeling. NOESY spectra in aqueous/deuterated methanol solution (50% H_2O/CD_3OD) at low temperature (268 K) revealed short-range nOe connectivities (i, i+1), characteristic of flexibility of the molecule. The H^N–H^N sequential connectivities observed provide evidence that the sequence has the propensity to form a bend involving residues 5 and 6 and the N-terminal segment. The α-proton chemical shifts compared to random coil and additional data from the amide proton temperature coefficients support this assumption. One long-range nOe cross peak between H_2^{α}–H^{NEth} is indicative of proximity between C- and N-termini.

Abbreviations: NMR, nuclear magnetic resonance; LHRH, luteinizing hormone-releasing hormone; 2D, two dimensional; 1D, one dimensional; Fmoc, 9-fluorenylmethoxycarbonyl; DIC, *N,N'*-diisopropylcarbodiimide; HOAt, 1-hydroxy-7-azabenzotriazole; DMF, dimethylformamide; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; UV, ultraviolet; TLC, thin layer chromatography; MS, mass spectrometry; ES-MS, electrospray mass spectrometry; TPPI, time proportional phase incrementation; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; ppm, parts per million; DQF-COSY, double quantum filtered correlation spectroscopy; DSS, 5,5-dimethylsilapentanesulfonate; MC/LMOD, Monte Carlo/Low mode; LMOD, low-frequency modes; MCMM, Monte Carlo multiple minimum; nOe, nuclear Overhauser effect; GB/SA, generalized Born/surface area; TNCG, truncated Newton conjugated gradient; ppb, parts per billion.

Introduction

Mammalian luteinizing hormone-releasing hormone (LHRH) is a linear hypothalamic decapeptide, that acts in the pituitary gland to stimulate the release of luteinizing hormone and follicle-stimulating hormone, which in turn regulates ovulation and spermatogenesis in the gonads [1]. Because of the native hormone key role in fertility, about 5000 analogues have been synthesized worldwide and tested *in vitro* or *in vivo* and a number of them are currently in therapeutic use against prostate cancer, endometriosis and other hormone dependent diseases. The definition of the bioactive conformation of the peptide is important in the design of new analogues with optimized activity. However, like many peptide hormones LHRH is a highly flexible molecule that can adopt many interconverting conformations in solution, and the assignment of a predominant bioactive conformation is very difficult. Several conformational models for LHRH have

been suggested based on NMR and theoretical studies and have been reviewed recently [2].

Early theoretical studies using model building and energy calculations, suggested a β-turn between 4–7 residues [3] or between 5—8 [4]. This assumption was supported by the substitution of the segment Tyr5–Arg8 by a β-lactam [5], as well as by D-amino acid substitution studies [6, 3]. Further 2D-NMR investigations [7] of the native hormone in various solvents showed that the molecule adopts randomly extended conformations. A systematic conformational build-up procedure by means of energy minimization performed for the LHRH molecule, showed a very high flexibility of the backbone, which varies among the fragments of the molecule [8]. The most rigid fragments of LHRH are the 4–7 and 5–8 central tetrapeptides and some of them resemble the II′ β-turn, in agreement with the experimental data. An extensive exploration in the conformational space using Monte-Carlo and Simulated Annealing, also showed that the most populated family of conformers adopts a turn involving residues 5–8 [9]. In the same study, the authors proposed that the Arg8 side chain is related to the peptide backbone conformation. This is in agreement with a recent ^{17}O-NMR investigation, showing an interaction between the Arg side chain with the carbonyl main chain at the C-terminus of the sequence [10].

Cyclization was used in the hope of reducing the number of allowed conformations [11]. A series of 4–10 bridged analogues exhibited interesting antagonistic activity, having as a common structural feature a II′ β-turn around residues 6 and 7 [12]. The conformational analysis of another cyclic compound with good activity [13], in which the side chains of the amino acids at position 5 and 8 formed part of the ring system, led to a conformation with residues 3–6 in a β-turn configuration and residue 10 coming very close to residue 1 of the peptide. A II β-like turn at residues 5 and 6 and a I′ β-turn at residues 6 and 7 is adopted by a bicyclic compound (4–10/5,5′–8) studied by Rizo and co-workers [14], suggesting a relative flexibility of the peptide despite the double restriction.

Further investigations show that the N- and C-termini of the peptide seem to play an important role in receptor binding [2]. Recent studies on bicyclic compounds (1–5/4–10) suggest that the structure of the N-terminal tripeptide is important for the activity, proposing a type II β-turn about residues 3 and 4 [15d].

The nonapeptide Leuprorelin (5oxo-Pro1-His2-Trp3-Ser4-Tyr5-DLeu6-Leu7-Arg8-Pro9-NHC$_2$H$_5$) is a LHRH agonist currently in therapeutic use, having a DLeu at position 6 in the place of Gly of the native hormone (Leuprorelin primary sequence is shown in Figure 1). Although Leuprorelin has the highest turnover on the market, no structural studies have been reported. In the current study, we present the preferred conformations of Leuprorelin as were determined by 2D nuclear magnetic resonance spectroscopy and theoretical calculations.

Materials and Methods

Peptide synthesis

9-Fluorenylmethoxycarbonyl-protected amino acids and peptide reagents were obtained from Bachem AG and Nova Biochem, while 1-hydroxy-7-azabenzotriazole was purchased from Applied Biosystems. All solvents and reagents used for solid-phase synthesis were of analytical quality.

The nonapeptide was synthesized by Fmoc solid phase methodology [16] utilizing Sieber Ethylamide resin (ethylamino-xanthen-3-yloxy-Merrifield resin) to provide C-terminal ethylamide [17]. Fmoc-protected amino acids were used with the following side chain protection groups: trityl (His); *t*-butyloxycarbonyl (Trp); *t*-butyl (Ser, Tyr); 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg). Stepwise synthesis of the peptide was achieved with *N,N′*-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole (DIC/HOAt) in *N,N′*-dimethylformamide as coupling agent. Couplings were performed with 3 molar excess of Fmoc-amino acid, 3.3 molar excess DIC and 4.5 molar excess HOAt as coupling agents in DMF for 3 hr at room temperature. The progress of the reaction was monitored by the Kaiser test [18]. For the incorporation of the first amino acid (Fmoc-Pro-OH) extended coupling time (18 hr) and double coupling are necessary. The Fmoc groups were removed by treatment with 20% piperidine/*N,N′*-dimethylformamide for 30 min at room temperature. Cleavage of the peptide-linker bond and removal of the side chain protecting groups were accomplished in 4 hr at room temperature using a solution (15 ml/g peptide resin) of trifluoroacetic acid/1,2-ethanedithiol/anisole/water (90:3.5:2:4.5, v/v). The free peptide was precipitated after partial removal of the solvent and addition of diethyl ether.

The peptide was desalted by gel chromatography on a Sephadex G-15 column eluted with aqueous

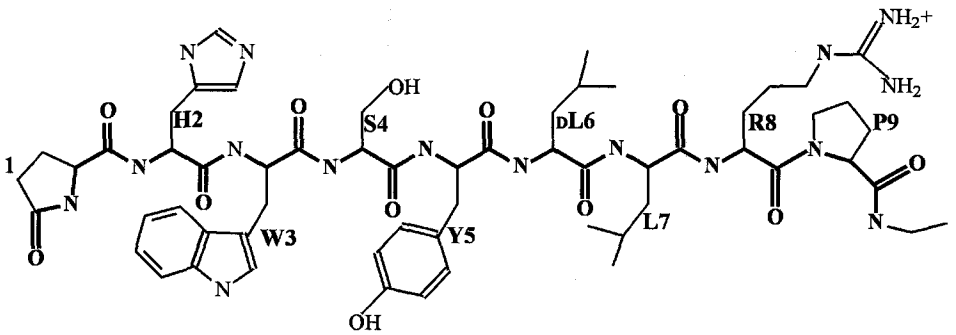

Figure 1. Primary structure of Leuprorelin peptide.

acetic acid (15%) at a flow rate of 6 ml/hr. Final purification was achieved by preparative HPLC (Pharmacia LKB-2248) on reversed-phase support C-18 (Lichrosorb RP) with a linear gradient from 20 A to 80% B over 30 min (A, 0.1% TFA in H_2O; B, 0.1% TFA in acetonitrile) at a flow rate of 2 ml/min and UV detection at 220 and 254 nm. The product gave single spots on TLC (Merck precoated silica gel plates, type G_{60}-F_{254}) in the solvent systems: (A) 1-butanol-acetic acid-water (4:1:5 upper phase) and (B) 1-butanol-acetic acid-water-pyridine (15:3:10:6). Analytical HPLC (Pharmacia LKB-2248) equipped with a C-18 Nucleosil 100 column produced single peaks with at least 98% of the total peptide peak integrals. Electrospray MS was in agreement with the expected results ($[M+H]^+$ found 1210.7, peptide molecular weight calculated 1209.6).

NMR experiments

NMR experiments were performed on a 400 MHz Bruker DRX-Avance spectrometer. Leuprorelin (2 mg) was dissolved in 600 μl of 50% CD_3OD/H_2O solution. All 2D spectra were recorded in the phase sensitive mode using time proportional phase incrementation (TPPI) [19].

NOESY spectra [20] were recorded with mixing times of 80, 150, 200, 250 and 400 ms. In the ROESY experiment the spin lock time was 250 ms at 268 K. TOCSY spectra [21] were recorded using the MLEV-17 spin lock sequence, with mixing time of 70–80 ms. Typically, the sweep width was 12 ppm and spectra were collected into 2048 points in the t_2 dimension, and 64 transients were co-added for each of 512 t_1 points. Double quantum filtered correlation experiment (DQF-COSY) [22] was recorded into 8192 points in t_2 and 1024 increments in t_1, with 24 scans per t_1 experiment. The relaxation delay (D1) in all the experiments was fixed to 2 s.

Solvent suppression was achieved, either by presaturation during the relaxation and mixing period (NOESY and ROESY experiments), or by the WATERGATE gradient module [23].

2D spectra were Fourier transformed after applying phase-shifted squared sine-bell functions in both dimensions (optimal phase shift was 60°). t_1 data were zero filled to 1024 points (2048 points for the DQF-COSY experiment).

The chemical shifts were referenced to internal DSS at 0.0 ppm.

For the determination of the amide proton temperature coefficients, the chemical shifts of the H^Ns were obtained from TOCSY spectra acquired at 268, 272, 276, 280, 284, 295 and 300 K.

Structure calculations

The ^1H–^1H distances for structure determination were deduced from nOe cross-peak intensities in the 2D-NOESY spectrum obtained with 200 ms mixing time. The intra-residue cross peaks $H^{\varepsilon 3}$–$H^{\eta 2}$ (4.338 Å) and $H^{\varepsilon 3}$–$H^{\zeta 3}$ (2.494 Å) of Trp3, as well as $H^{\delta 2}$–$H^{\delta 3}$ (1.817 Å) of Pro9 cross peak, were selected as references to calibrate the intensities against known distances, using AURELIA software (ver. 2.0, Bruker Analytische Messtechik GmbH).

All computations were carried out on a single SGI O2 R5k processor.

Theoretical conformational searching, as applied in the current study, was achieved by using the Monte Carlo/Low mode (MC/LMOD) [24] mixed-mode search strategy, which involves the application of either systematic search of the low-frequency modes (LMOD), or torsional Monte Carlo mode (as implemented in the MCMM – Monte Carlo Multiple

Minimum – command in MacroModel/BatchMin, ver. 6.5) for structure perturbation [25]. The protocol comprised 10 000 steps of MC/LMOD search, using AMBER* force field [26]. Structures selected for perturbation by the usage-directed protocol during any stage of the search, were subjected to either a LMOD step or a MCMM step. The MCMM step involved the simultaneous rotation of up to three randomly selected dihedral angles from a total of 36 rotable bonds of the peptide, that were allowed to vary by 0 to 180°.

A series of alternative ways of conformational searches was implemented concerning the usage or not of nOe restraints, in vacuum or in solvent.

^1H–^1H distances derived from AURELIA calculations were implemented as energetic restraints in the conformational search protocol. The command used from the MacroModel package provides flat-bottom energetic restraint wells, with a force constant value of 500 kJ/Å^2 and half-width of flat bottom part of the potential well varying to the extent of 0.5 Å.

Solvent molecules were not explicitly introduced in the computations; instead, their presence was simulated either by using a value of 40 as appropriate for dielectric constant, or the generalized Born/surface area (GB/SA) continuum solvation model [27]. In the final step of conformational search, the resulting structures were subjected to energy minimization with truncated Newton conjugated gradient (TNCG) minimizer [28].

The initial structure for the conformational searches was generated by the computer model incorporating the extended chain conformation and all amide bonds were fixed to be *trans*, as suggested by the nOe patterns. The starting structure resulted after energy minimization, using the TNCG method for 1500 steps.

Results and Discussion

NMR

The assignment of proton resonances was achieved through the combined analysis of 2D TOCSY experiments, in the range of 268–300 K, and 2D NOESY experiments with different mixing times (80–400 ms) using the standard protocol for sequential assignment [29].

The part of a 2D TOCSY spectrum, illustrating the backbone amide connectivities to the side chain protons, in 50% CD_3OD/H_2O solution recorded at 268 K, is provided in Figure 2. Chemical shift values of identified protons of Leuprorelin at 268 K, are listed in Table 1.

The fingerprint region and the assignment of a NOESY spectrum (t_m = 250 ms), is shown in Figure 3. The H_i^β–H_i^N and H_i^β–H_{i+1}^N connectivities were used to identify certain overlapping H_i^α–H_i^N and H_i^α–H_{i+1}^N cross-peaks, as Ser4, Tyr5, Leu7 and the inter-residue H_4^α–H_5^N.

The cross-peak patterns in the aromatic region of TOCSY and NOESY spectra, and the nOe correlations with the intra-residue α and β protons, allowed the assignment of all the aromatic signals.

The relative nOe intensities of the sequential backbone and β protons are depicted in Figure 4.

The inter-residue nOe peaks connecting α, β and γ protons of 5oxo-Pro1 residue to the side chain amide proton of His2 are indicative of their spatial proximity and suggest that the aromatic ring is directed backward the backbone, toward the 5oxo-Pro ring. The orientation of His2 ring is further confirmed by the intra-residue nOes H_2^α–$H_2^{\delta 2}$ and $H_2^{\varepsilon 1}$–H_2^N nOes.

The strong nOe among Tyr5 aromatic protons and DLeu6 $H^{\delta 1,2}$, as well as the upfield shift experienced by DLeu6 protons, provide evidence for the proximity of the Tyr5 ring and the DLeu6 side chain. The correlation peaks among Tyr5 $\delta 1$, $\delta 2$ and $\varepsilon 1$, $\varepsilon 2$ protons with $\delta 1$-, $\delta 2$-methyl protons of DLeu6, appear strong, even in NOESY spectra with the shortest mixing time (80 ms, data not shown).

Strong H_i^N–H_{i+1}^N nOe correlation peaks are observed between H_2^N–H_3^N and H_6^N–H_7^N, even at 80 ms, suggestive of a propensity of the peptide chain to form a local structure in these regions of the sequence. Other H_i^N–H_{i+1}^N nOe connectivities, such as Trp3–Ser4, and Leu7–Arg8 are observed at longer mixing time experiments.

The presence of H_8^α–H_9^δ nOe cross peaks, combined with the absence of any H_8^α–H_9^α correlation, indicate that Pro9 is in the *trans* conformation [30].

In the NOESY experiments with mixing time >150 ms, a H_8^δ–H_9^α cross peak was detected, providing additional information on Arg8 side chain orientation. In longer mixing time NOESY experiments, H_8^β–H_9^δ and H_8^γ–H_9^δ connectivities were also detected.

The experimental data (nOes and chemical shifts) imply the absence of any ring stacking.

Only one long range nOe was detected, connecting His2 H^α to the amide proton of the C-terminal N-ethylamide of the peptide sequence (Figure 3).

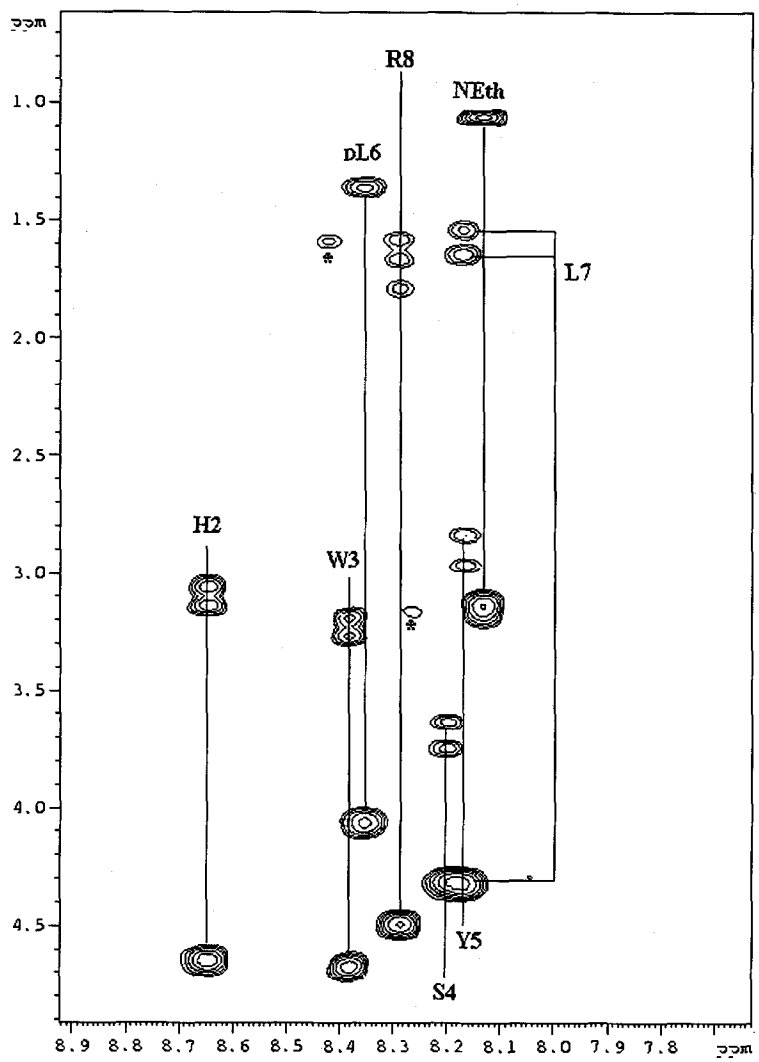

Figure 2. Part of a 400 MHz TOCSY spectrum recorded at 80 ms of Leuprorelin (2 mg) in 50% CD_3OD/H_2O solution at 268 K, illustrating the amide resonance connectivities to H^{α} and side chain proton resonances. Cross peaks marked with an asterisk arise from small impurities.

This nOe was also detected in the 2D ROESY spectrum recorded under the same conditions (mixing time 250 ms). This long range nOe may imply the intramolecular proximity of the peptide termini. Alternatively, the nOe may be intermolecular due to the formation of dimeric species. In order to verify one of these hypotheses the following experiments were performed.

2D NOESY spectra were acquired at different dilutions (2, 0.7 and 0.2 mM) at 268 K. The H_2^{α}–H^{NEth} nOe cross peak was present at all concentrations suggesting that its presence is not concentration depended. Supporting evidence was provided by ES-MS, which indicated that there was no dimer formation in peptide 50% CD_3OD/H_2O solutions, at the concentration of 2 mM.

Chemical shifts analysis

H^{α} chemical shift analysis (Figure 5) was performed using Wishart and coworkers' values for the same amino acids in random coil peptides [31]. His2, Trp3, Leu7 and Arg8 at 300 K difference values are characteristic of random coil and only in the central region of the peptide sequence three consecutive residues (Ser4, Tyr5, DLeu6) exhibit negative values <–0.1 ppm, suggesting the existence of a β-turn conformation [32].

The negative deviation of 5oxo-Pro1 H^{α} resonance is due to its position on the N-terminal of the peptide

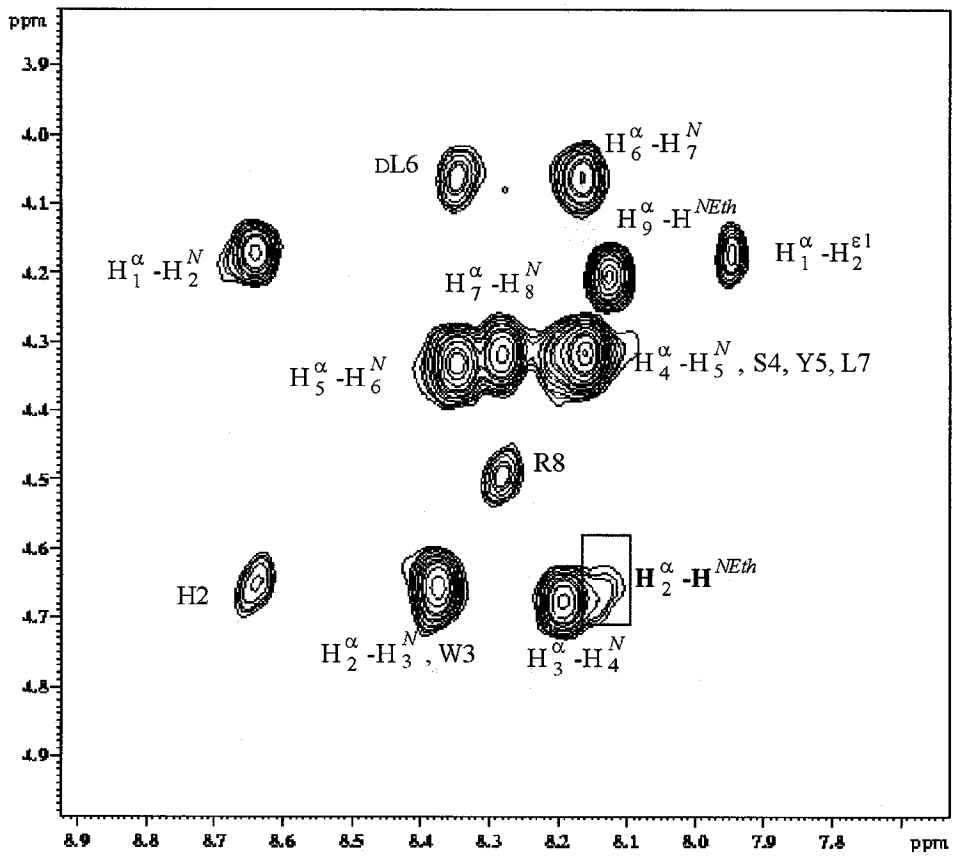

Figure 3. H^{N}–H^{α} region of a 400 MHz NOESY spectrum (250 ms, 268 K) of 2 mg of Leuprorelin in 50% CD_3OD/H_2O solution at 268 K showing H^{α}_{i}–H^{N}_{i}, H^{α}_{i}–H^{N}_{i+1} and the long range (H^{α}_{2}–H^{NEth}) nOe connectivities.

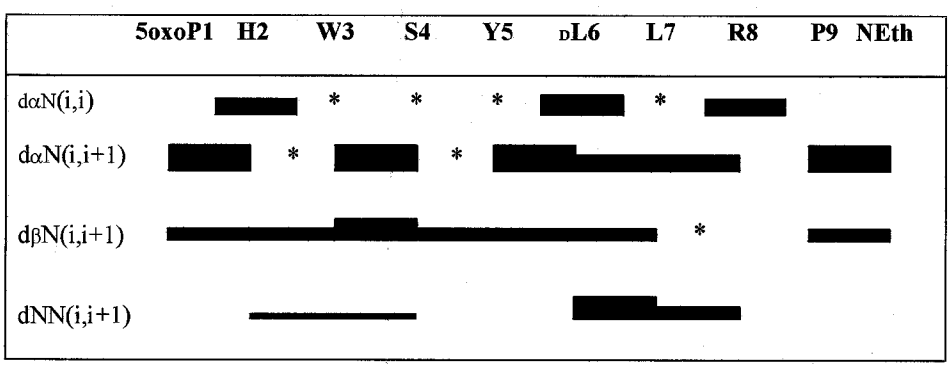

Figure 4. Summary of observed intra- and inter-residual nOes involving H^{N}, H^{α} and H^{β} protons. Data were taken from NOESY spectra (mixing time 150 ms) acquired in CD_3OD/H_2O at 268 K. The intensities of nOes are reflected by the thickness of the lines. When peak overlap prevented assignments, nOe connectivities are marked by an asterisk.

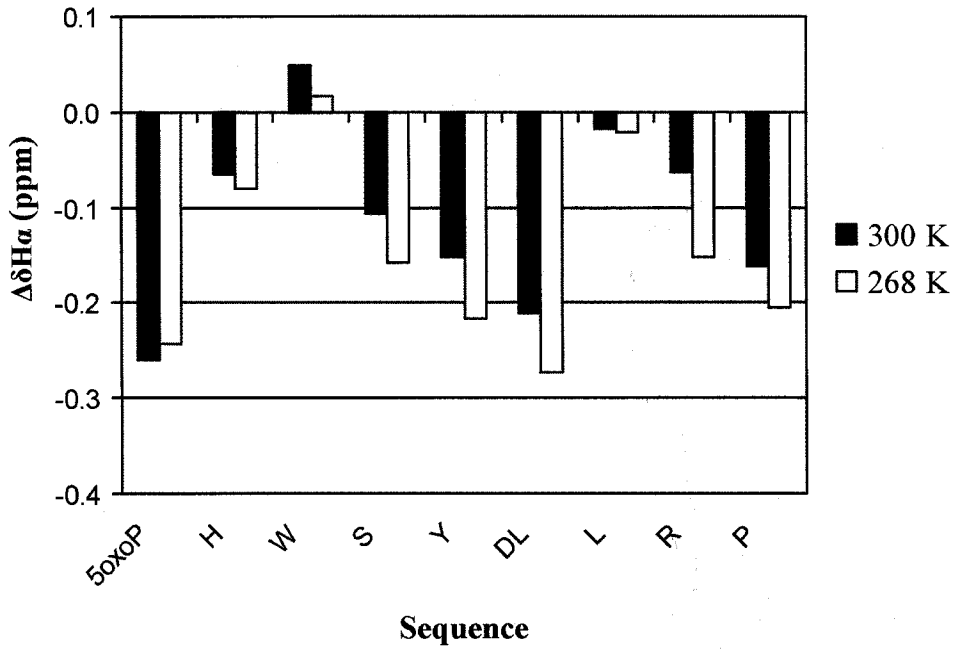

Figure 5. Chemical shift differences of the H^{α} ($\Delta\delta H^{\alpha}$) from random coil values at 268 and 300 K.

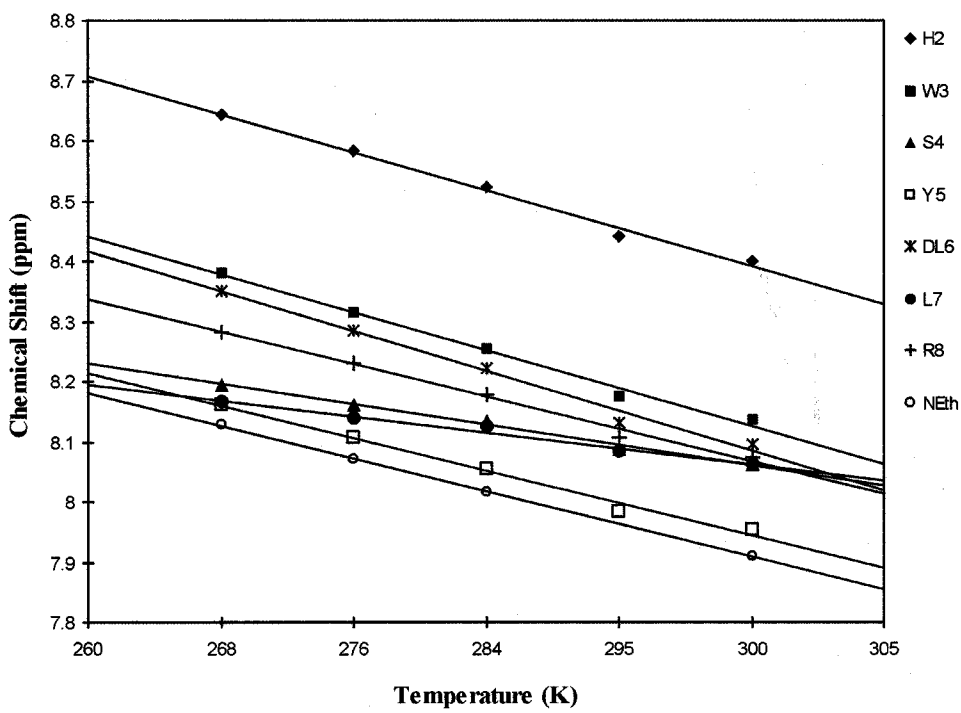

Figure 6. Variation of chemical shift of the amide resonances of Leuprorelin with temperature. The slope of the line of best fit between the points constitutes the temperature coefficient of the resonance. Lines drawn on the figure are computed fits obtained by the method of least squares.

Table 1. ^{1}H chemical shift values for Leuprorelin in 50% CD_3OD/H_2O at 268 K[a]

Residue	H^N	H^α	H^β	Others (ppm)
5oxo-Pro1	–	4.18	2.31	H^γ 1.63
His2	8.64	4.65	3.15, 3.06	$H^{\varepsilon 1}$ 8.58; $H^{\delta 2}$ 7.16; $H^{\delta 1}$ 7.95
Trp3	8.38	4.68	3.27, 3.20	$H^{\delta 1}$ 7.22; $H^{\varepsilon 3}$ 7.56; H^{J3} 7.06
				$H^{\eta 2}$ 7.18; H^{J2} 7.45; $H^{\varepsilon 1}$ 10.31
Ser4	8.20	4.31	3.74, 3.64	
Tyr5	8.16	4.33	2.97, 2.85	H^δ 7.07; H^ε 6.77
DLeu6	8.35	4.07	1.37	H^γ 1.37; $H^{\delta 1}$ 0.77, $H^{\delta 2}$ 0.68
Leu7	8.17	4.32	1.65	H^γ 1.55; $H^{\delta 1}$ 0.91, $H^{\delta 2}$ 0.83
Arg8	8.28	4.50	1.80, 1.68	H^γ 1.59; H^δ 3.12
				H^ε 7.37; $H^{\eta 1}$ 7.13, $H^{\eta 2}$ 6.64
Pro9	–	4.21	2.15, 1.84	H^γ 1.99; $H^{\delta 2}$ 3.67, $H^{\delta 3}$ 3.50
NEth	8.13		CH_2 3.15	CH_3 1.06

[a] Chemical shifts are reported in ppm relative to internal DSS.

sequence, but negative values of Pro9 and Arg8 at low temperature, in combination with the long range H_2^α–H^{NEth} nOe, could imply the presence of a bend localized at the end of the peptide sequence.

The decrease of $\Delta\delta H^\alpha$ values at 268 K suggests that at lower temperature, a further stabilization of the peptide structure occurs.

Temperature coefficients

The temperature dependence of the amide proton chemical shifts was determined over the range 268 to 300 K. In all cases, the changes of the amide proton chemical shifts varied linearly with temperature (Figure 6), suggesting that no major conformational rearrangements occur in the temperature range studied. While the coefficients for most of the residues were similar, varying from 6.5 to 8.0 ppb/K, the values of the backbone amide proton chemical shift change of Ser4 and Leu7 are 4.0 and 3.2 ppb/K, respectively, suggesting a relatively higher protection from the solvent and indicating a possible participation of the amide proton in an intramolecular hydrogen bond.

These NMR data are consistent with the presence of a type II β-turn involving residues 4–7, with residues 5 and 6 at the corners of the turn. The low temperature dependence of Leu7 H^N chemical shift, the strong NOESY cross peak between DLeu6 H^N and Leu7 H^N and the negative deviation of the H^α chemical shifts of Ser4, Tyr5 and DLeu6 from the random coil values, agree with the formation of a β-turn. This structure is also supported by the presence of the strong nOe peaks among the Tyr5 aromatic ring and the DLeu6 side chain protons and the upfield shift experienced by the side chain proton resonances of DLeu6. The presence of the weaker H_7^N–H_8^N correlation peak suggests that a bend around 6–7 residues cannot be excluded.

The above-mentioned bend of the peptide backbone probably brings the two termini in spatial proximity, as the weak nOe H_2^α–H^{NEth} suggests. Finally, it should be noticed that the H^N–H^N nOe correlations between residues 2–3 and 3–4 suggest that N-terminal peptide part has a propensity to form a more stable structure.

Structure calculations

Theoretical calculations using nOe derived distances, were performed in order to illustrate plausible conformations of the Leuprorelin peptide. Structures were generated starting from the extended conformation previously subjected to unrestrained energy minimization. A total number of 53 inter-residue (sequential: backbone-backbone, backbone-side chain and side chain-side chain nOes, as well as the long range H_2^α–H^{NEth}) and 36 intra-residue restraints were introduced, resulting from the nOe data.

A number of structures resulted from the calculations, using different types of solvation models or dielectric constants. Representative conformations of the generated structures with distinct backbone are depicted in Figure 7. The starting structure (Figure 7a) was derived after constructing the peptide sequence using the Macromodel software, leaving the backbone in extended conformation and subjecting it in simple energy minimization. It should be noticed that, the 4–

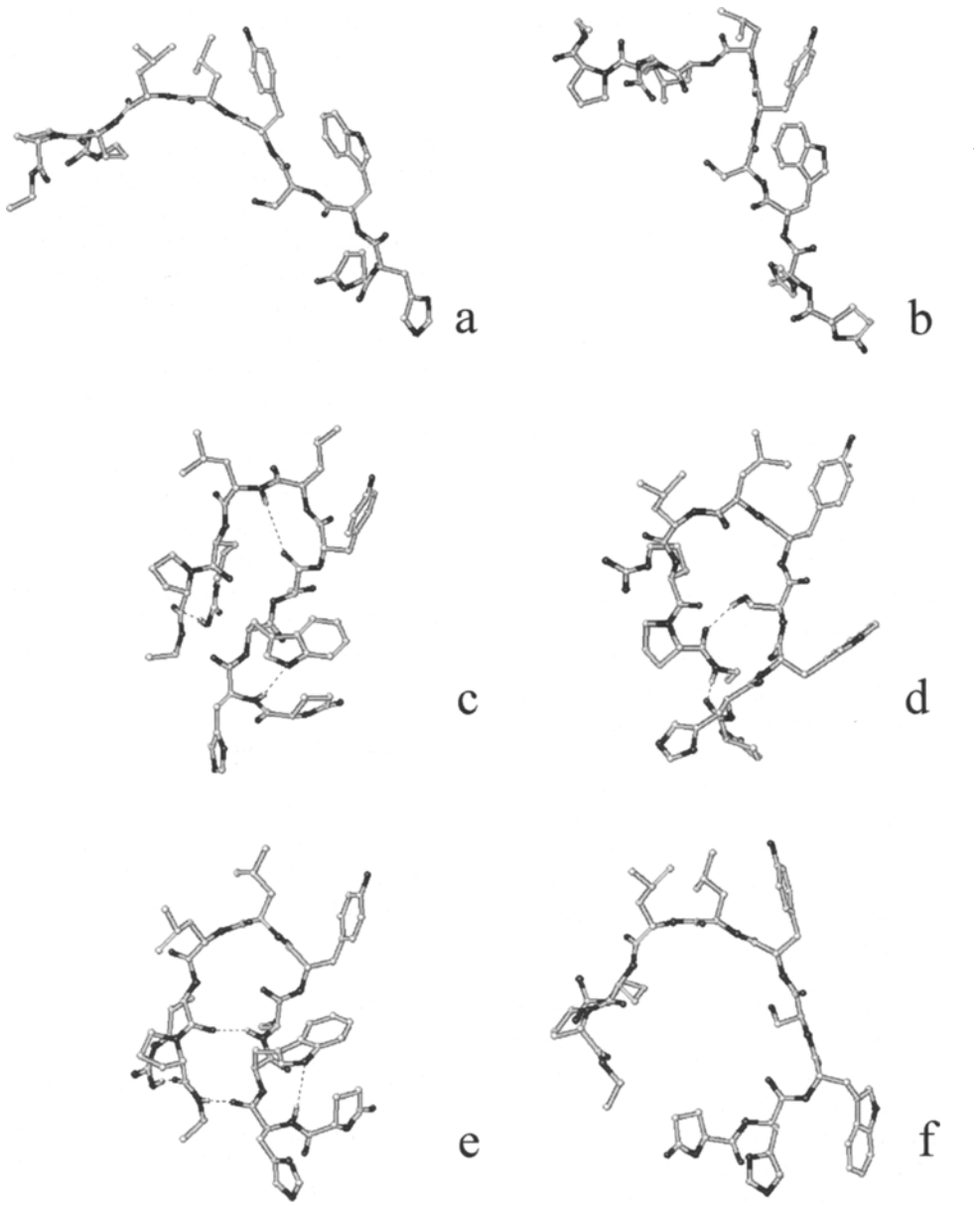

Figure 7. Backbone trace of representative structures obtained from conformational searching.

8 part of the sequence appears to have the tendency to form a large bend induced probably by the presence of DLeu6 in the peptide sequence.

The common characteristic of the generated conformers is a loop like structure formed by the residues Ser4-Leu7. Calculations performed without imposing the long-range restraint, H_2^{α}–H^{NEth}, generated structures like Figure 7b. In this structure a bend around DLeu6 is manifested more clearly, while the other parts of the sequence show random extended conformations. When the long range H_2^{α}–H^{NEth} restraint was taken into account, the resulting structures showed the two termini in proximity, forming a type II β-turn (Figure 7c), or larger loops (Figures 7d–f) around the 4–7 segment of the peptide sequence. In those structures different hydrogen bonds are formed with the participation of the H^N of Ser4 and Leu7 in agreement with the experimental data. A number of other conformers were also generated from these calculations, in which the side chains of Arg8, Trp3 and

His2 residues were interacting with the opposite corresponding terminal segment. These conformers were not considered further, as there is no experimental evidence to justify them.

Conclusions

The conformational analysis of Leuprorelin peptide using NMR and molecular calculations described in this study suggest that the free molecule in solution adopts random coil extended conformations, in agreement with the peptide backbone flexibility proposed from previous studies on LHRH. A propensity to form a β-turn around 4–7 residues was revealed from chemical shift data, temperature coefficients, nOe cross-peak patterns and H_i^N–H_{i+1}^N nOes. The above data imply that the side chains of Tyr5 and DLeu6 are directed towards the same side of the peptide backbone and are aligned almost parallel to each other. The same arrangement of side chains has been previously reported in isolated alanine-based peptides containing (i, i+4) Tyr-Leu or Leu-Tyr pairs and has been shown to stabilize α-helical structure [33]. Similar interactions are seen in protein α-helices with (i, i+3) or (i, i+4) Tyr-Leu or Leu-Tyr pairs, where the Tyr and Leu side chains are in contact. Hydrorphobic contacts between these residues may be important at early stages in directing secondary structure formation [34] as was proposed several years ago [33].

This 4–7 turn, although it was initially proposed [3, 6] based on the assumption that its formation is stabilized by the presence of a D-amino acid in the i+2 position, does not agree with the generally accepted belief that the active LHRH analogues have a β-turn involving residues 5–8. According to our data, the 5–8 turn is not excluded, but probably exists in a minor population compared with the 4–7 conformer. These findings are in agreement with the loose β-bend structures proposed by Guarnieri [13] and the 3–6 bend found by Reddy [13] for the cyclic (5–8) synthetic analogues. It is probable that the active conformation does not depend so much on a specific turn type, as postulated recently by Rivier and co-workers [15a].

Our data also suggest the proximity between the peptide termini, which although surprising for a nonapeptide sequence, is also in agreement with Reddy and co-workers' results [13], and a tendency of the N-terminus to form a stable conformation proposed also by Rivier and co-workers [15].

NMR studies of the native hormone indicated that is essentially a random coil, in three different solvent conditions (aqueous solution, DMSO solution and lipid-bound form in model membranes) [7]. In Leuprolelin, the DLeu6 seems to participate in hydrophobic interactions (Tyr5–DLeu6 side chain interactions), which provide the necessary conditions under which the molecule expresses its inherent structural propensity. It should also be noticed that the experimental conditions used in this study e.g. methanol solution and subzero temperatures, may aid to the stabilization of such a structure.

Given the high *in vivo* affinity of Leuprorelin, the results of the present study, in agreement with previous studies of cyclic analogues, suggest that Leuprorelin, might adopt a functional conformation at the hormone receptor with a bend around residues 4–8.

Additional studies with rigid analogues possessing a bend around the above mentioned peptide region, are needed to further elucidate the bioactive conformation of Leuprorelin.

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