Eur J Med Chem (1997) 32, 927–940

Structure elucidation and conformational analysis of gonadotropin releasing hormone and its novel synthetic analogue [Tyr(OMe)^5, D-Lys^6, Aze^{9}NHEt]GnRH: The importance of aromatic clustering in the receptor binding activity†

JM Matsoukas[1]*, M Keramida[1], D Panagiotopoulos[1], T Mavromoustakos[2], HLS Maia[3], G Bigam[4], D Pati[5], HR Habibi[5,6], GJ Moore[6,7]

[1]*Department of Chemistry, University of Patras, Patras 26110;*
[2]*National Hellenic Research Foundation, Institute of Organic and Pharmaceutical Chemistry, Athens 11635, Greece;*
[3]*Department of Chemistry, University of Minho, Gualtar, P-4700 Braga, Portugal;*
[4]*Department of Chemistry, University of Alberta, Edmonton;*
[5]*Department of Biological Sciences, University of Calgary;*
[6]*Department of Pharmacology and Therapeutics, University of Calgary, Calgary, Alberta, T2N 1N4, Canada;*
[7]*Department of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD, UK*

(Received 15 November 1996; accepted after revision 14 May 1997)

Summary — The conformational properties of gonadotropin releasing hormone (GnRH) in dimethylsulfoxide–d_6 were investigated by nuclear Overhauser effect (nOe) enhancement studies and were compared with the conformational properties of its analogue [Tyr(OMe)^5]GnRH resulting after methylation of the tyrosine hydroxyl. Assignment of all backbone and side-chain protons was possible by combining information from intraresidue nOe studies with two-dimensional correlated spectroscopy (COSY/TOCSY) studies. Saturation of distinct proton resonances of the three aromatic residues Tyr, His, Trp, in clear areas of the NMR spectrum of GnRH resulted in interresidue enhancements of aromatic resonances indicating the proximity of the three aromatic rings. This spatial proximity is not observed in [Tyr(OMe)^5]GnRH and is correlated with a lower receptor binding affinity in the rat pituitary (K_d = 1.53 ± 0.35 x 10^{-6} M) compared with that exerted by GnRH (K_d = 3.69 ± 0.89 x 10^{-9} M). However, substitution of Gly at position 6 of [Tyr(OMe)^5]GnRH with D-Lys^6 and further replacement of Pro at position 9 with the more rigid Aze residue [Tyr(OMe)^5, D-Lys^6, Aze^{9}NHEt]GnRH significantly improved the binding affinity (K_d = 0.689 ± 10.15 x 10^{-9}) and this may be due to the restoration of the ring cluster. Overall, the clustering of the aromatic rings observed in GnRH was not seen in [Tyr(OMe)^5]GnRH and this conformational difference may be responsible for receptor recognition and higher binding of the parent peptide.

gonadotropin releasing hormone (GnRH) / conformational analysis / [Tyr(OMe)^5]GnRH / molecular graphics

Introduction

Gonadotropin-releasing hormone (GnRH: pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2; pGlu: 2-pyrrolidone-5-carboxylic acid) is a linear hypothalamic decapeptide which regulates the release and synthesis of the pituitary luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). These hormones in turn regulate the gonadal function including steroidogenesis and gametogenesis in males and females. In addition, GnRH peptides are known to act at various extrapituitary sites such as gonads, and are known to impair the growth of various cancer cell types. Extensive studies over the past 15 years have identified various analogs of GnRH which are currently in therapeutic use in clinical syndromes such as hypogonadotropic hypogonadism, precocious puberty, criptorchidism, endometriosis, steroid-dependent neoplasia, and contraception [1, 2]. In addition, GnRH analogues are currently used for the induction of ovulation in fish and other farm animals [3, 4].

Several conformational models for GnRH have been suggested based on NMR and other physicoche-

†Part of this work is incorporated in the PhD thesis of Maria Keramida.
*Correspondence and reprints

mical methods [5–10]. A feature common to most models of GnRH is the β-turn Tyr[5]-Gly[6]-Leu[7]-Arg[8], and support for the presence of this turn in the bioactive conformation of GnRH comes from the activities of analogues with D-amino acid substitution for Gly[6] which stabilizes this conformation [11]. Proline at position 9 is also an important residue for protecting peptides against enzymatic degradation and stabilizing the conformational integrity of the peptide hormone.

In this study, we have synthesized by the Fmoc solid-phase methodology, using the acid-sensitive 2-chlorotrityl chloride resin, an analogue of GnRH-containing Tyr(OMe)[5] ([Tyr(OMe)[5]]GnRH) [12]. In addition, we synthesized an analogue of [Tyr(OMe)[5]]-GnRH with stabilized β-turn and *C*-terminus protection ([Tyr(OMe)[5], D-Lys[6], Aze[9]-NHEt]GnRH). The synthesized analogues were tested in terms of receptor binding activity in the rat pituitary. We also determined the conformational properties of native GnRH peptide and [Tyr(OMe)[5]]GnRH in DMSO by 2D correlated spectroscopy (2D-COSY/TOCSY) and 1D-nuclear Overhauser effect (1D-nOe) measurements. In previous studies we used DMSO as the solvent for our NMR studies because its dielectric constant ($\varepsilon \approx 45$) is lower than that of water ($\varepsilon \approx 80$) and permits a more ordered peptide structure [11–14] in the relatively nonpolar 'receptor-stimulating' environment provided by this solvent. The higher viscosity of DMSO also allows a stronger buildup of the nuclear Overhauser effect which is the fractional change in intensity of one NMR line when another resonance is saturated. For this reason, GnRH and [Tyr(OMe)[5]]GnRH were subjected to COSY and 1D-nOe NMR studies suitable for resonance assignment and distance information. Ring proton resonances for the three aromatic residues (Tyr, His, Trp) are easily recognized in the one-dimensional NMR spectrum of GnRH and its analogues and provide distinct probes in clear areas of the peptide spectrum for studying the spatial relationship of the three rings with other groups and in particular with each other. A similar approach was recently used for studying conformational characteristics of angiotensin II [14]. The present study provides novel information on GnRH conformation and backbone differences between GnRH and [Tyr(OMe)[5]]GnRH analogues which may explain the structural requirements for better receptor recognition.

Experimental protocols

Peptide synthesis

The syntheses of [Tyr(OMe)[5]]GnRH and [Tyr(OMe)[5], D-Lys[6], Aze[9]NHEt]GnRH were carried out by the Fmoc methodology, utilizing the 2-chlorotrityl chloride resin as solid support reported by Barlos et al [12]. Fmoc-blocked amino acids were synthesized in our laboratories as previously described [15–17] or were purchased. More specifically, Fmoc-His(Trt)-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Ser(^{t}Bu)-OH and Fmoc-D-Lys(Boc)-OH were purchased from Novabiochem, Bachem and CBL (Chemical and Biopharmaceutical Laboratories of Patras). The *N*-terminus was pGlu and Fmoc-L-azetidine-2-carboxylic acid (Fmoc-Aze-OH) was attached to the resin by a simple, fast and racemization-free reaction using diisopropylethylamine (DIPEA) in a dichloromethane (DCM) solution at room temperature.

Preparation of N^{α}-Fmoc-Aze-2-chlorotrityl resin

2-Chlorotrityl chloride resin (1 g, 1.4–1.6 mequiv of Cl^-/g of resin) in dry dichloromethane (10 mL) was stirred in a round-bottom flask. Diisopropylethylamine (DIPEA) (0.545 mL, 3.2 mmol) and N^{α}-Fmoc-amino acid (Fmoc-Aze-OH) (1 mmol) were added, and the solution was stirred for 20 min at room temperature. MeOH (3 mL) and DIPEA (0.5 mL) were then added, and the mixture was stirred for another 10 min at room temperature. The Fmoc-Aze-resin was filtered and subsequently washed with dimethylformamide (DMF) (3 x 10 mL), iso-propanole (*i*PrOH) (2 x 10 mL) and diethylether (1 x 10 mL) and dried in vacuo for 24 h at room temperature. The loading of the amino acid per gram of substituted resin (mmol AA/g of resin) was calculated by a quantitative Kaiser test.

Solid-phase peptide synthesis

Solid-phase peptide synthesis of the two GnRH analogues were carried out using a manually handled reaction vessel (2 x 12 cm) equipped with a porous G filter (size 2) and tap at the bottom connected with a water vacuum aspirator. A vibrator was used for shaking the reaction vessel throughout the several steps. The protocol used for the peptide synthesis of GnRH analogues is previously described [16]. After preactivation with 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC), a 2.5-fold molar excess of Fmoc amino acids were used. A second coupling was employed in cases of incomplete coupling which could be verified by the ninhydrin test. Deprotection steps (5 and 20 min) utilized 20% (v/v) piperidine in dimethylformamide (DMF). The obtained peptide-resin was dried in vacuo, and treated with the splitting solution, dichloromethane-acetic acid-2,2,2 trifluoroethanol (7:1:2, v/v/v, 15 mL/g resin) for 1 h at room temperature to remove the peptide from the resin. The mixture was filtered off, and the resin was washed with the splitting mixture and DCM several times. The solvent was removed on a rotary evaporator and the obtained oily material was precipitated upon addition of cold diethylether. The protected peptide pGlu-His(Trt)-Trp-Ser(tBu)-Tyr(OMe)-D-Lys(Boc)-Leu-Arg(Pmc)-Aze-OH was coupled with ethylamine after preactivation with HOBt and DCC. Removal of the protecting grounds (His→Trt, Arg→Pmc, D-Lys→Boc, Ser→^{t}Bu) was achieved by treatment of the protected peptide with trifluoroacetic acid (TFA) in DCM (65%, v/v, 1 mL/0.1 g peptide) in the presence of 1,2-ethanedithiol as a scavenger. The solvent was removed on a rotary evaporator, and the crude product was isolated by trituration with cold diethylether and filtration. Finally, the crude product was dissolved in 7% acetic acid (10 mL), clarified by centrifugation, and purified by preparative HPLC. The identity of the synthesized two GnRH analogues was confirmed by amino acid analysis and FAB-MS.

Preparative reversed-phase HPLC

The two GnRH analogues were purified on a Varian HPLC system equipped with a Vista 401 microprocessor, using a Bio-Rad Hi-Pore 318 reversed-phase preparative column (25.0 x 2.15 cm). Separations were achieved with a stepped linear gradient of acetonitrile in 0.1% TFA at a flow rate of 7.5 mL/min. Peptides (18 mg) were injected through a nitrogen-pressurized Rheodyne injector with a 2.0 mL sample loop. One-fifth of the total sample was injected during each run for a 0.1 min injection period and by lowering the flow rate to 4.0 mL/min. One cycle consisted of the following events:

0 → 10 min, 7.5 mL/min, 90% H_2O/10% of 1% aqueous CF_3CO_2H;

10 → 11 min, → 4.0 mL/min;

11 → 11.1 min, 'inject';

11.1 → 13 min, → 7.5 mL/min, → 70% H_2O/20% CH_3CN/10% of 1% CF_3CO_2H;

13 → 30 min, → 45% H_2O/45% CH_3CN/10% of 1% CF_3CO_2H;

30 → 42 min, → 90% CH_3CN/10% of 1% CF_3CO_2H;

42 → 50 min, → 100% H_2O. Fractions were collected at 0.1 min intervals, and the elution time of the major product was 26–29 min. Elution of the peptide was determined simultaneously from the absorbance at 254 nm (Varian UV-1) and 230 nm (Kratos SF 769Z). Fractions containing the major peptide were pooled, and acetonitrile was removed on a rotary evaporator. After lyophilization, the product was stored at −20 °C.

Amino acid analysis, TLC and FAB-MS

Amino acid analysis was performed on a Beckman G300 high-performance analyzer. Compositional analysis data were collected from 6 M HCl hydrosylates (150 °C, 1 h). The peptide content was ~71% and was taken into account in various assays. The purity of products was established by analytical HPLC reruns (> 96% pure, 214 nm, Techsil C-18, 250 x 4.6 mm) and by thin-layer chromatography (TLC). Two solvent systems were used: *n*-butanol–pyridine–acetic acid–water (15:10:3:6) (BPAW) and chloroform–methanol–acetic acid–water (15:10:2:3) (CMAW). The identity of the desired products was established by FAB MS. FAB spectra were run on a AEI M29 mass spectrometer. The FAB gun was run at 1 mA discharge current and 8 KV. The FAB matrix used was a mixture of dithiothreitol/dithioerythritol (6:1) (Cleland Matrix) [18].

NMR experiments

NMR experiments were carried out using a Bruker 400 MHz NMR spectrometer. GnRH (5 mg) was dissolved in 0.4 mL of DMSO–d_6 and the chemical shifts were reported relative to the undeuterated fraction of the methyl group of DMSO–d_6 at 2.50 ppm with respect to TMS. One-dimensional spectra were recorded with a sweep width of 4500 Hz and 32 K (zero filled to 64 K) data points and by methods previously described [17]. One-dimensional nOe experiments were carried out in the difference mode using multiple irradiation. This procedure used a very low decoupler power setting (typically 35 L, lower than for a standard nOe experiment) so that it was possible to avoid partial saturation of resonances in close proximity. The selected lines were irradiated 20 times for 100 ms (total irradiation time 2.0 s). Each line required a total of 1000 scans and the relaxation time was 5.0 sec. The acquisition time for each transient was ~ 2.7 s. We used nOe experimental conditions (low power, different T preirradiation times for saturation of control areas) so that spin diffusion and partial saturation could be visibly minimized for the discussed interactions. The methods used for the COSY and nOe experiments were similar to those previously described [11, 13, 19, 20]. A TOCSY experiment was also carried out. The TOCSY sequence employed two power levels giving 90° pulses of 8.5 μs for the excitation pulse and 17 μs for the MLEV-17 spin locking pulse.

Molecular modeling methods

Theoretical calculations were performed on a Silicon Graphics 4D/35 using QUANTA version 3.3 of Molecular Simulations. The structure of GnRH was built using the Sequence Builder. It was then minimized using steepest-descent (SD) and adopted-basis Newton–Raphson algorithms in order to reach a local minimum structure. Finally, a combination of dynamics with minimization was applied to get the lowest energy structures. In the obtained structures molecular dynamics was performed at 1000 K using 1 ps, 1 ps and 1 ps time frame for heating, equilibration and simulation steps. One hundred structures from the simulated ones were minimized using iteration steps and SD algorithm. Details of the above techniques have been recently described [21]. [Tyr(OMe)^5] was built from the lowest energy conformer of GnRH. The resulting structure was energy-minimized and subjected to a dynamics experiment using the same experimental conditions as with GnRH.

GnRH receptor binding

Rats were anaesthetized and killed by spinal transsection. Pituitaries were rapidly dissected out and transferred into ice-cold assay buffer (10 mM tris[hydroxymethyl] aminomethane-[tris]-HCl), containing 1 mM dithiothreitol and 0.5% BSA [fraction-V] (pH ~ 7.4). The pituitaries were then homogenized by a glass teflon homogenizer, followed by centrifugation at 500 *g* for 5 min (4 °C). The supernatant was further centrifuged at 17000 *g* for 30 min at 4 °C, and the pellet (crude membrane preparations) was separated, rinsed gently and resuspended in the assay buffer (50–80 mg protein per 100 mL). In all experiments, the membrane preparation was used within 60 min in a radioreceptor assay. Iodinated [D-Lys^6]-GnRH was prepared using lactoperoxidase as described previously [22]. The monoiodinated [D-Lys^6]-GnRH (^{125}I-[D-Lys^6]GnRH) was purified by reverse-phase high-pressure liquid chromatography using a Beckman C-18 column eluted with a linear gradient of acetonitrile and water (10–50%) in 0.1% TFA. Fractions (250 mL) were collected and aliquots counted for radioactivity to determine the elution profile. The specific activity of ^{125}I-[D-Lys^6]-GnRH (1000 mCi/mg) was determined by self-displacement in a radioreceptor assay; half of the maximal effective dose (ED_{50}) value (mCi) of the saturation curve was equated with the ED_{50} value (mg) of a displacement curve obtained in the same assay [23]. The binding assay was based on techniques described previously [24]. Briefly, incubations (in triplicate) were carried out in polypropylene microcentrifuge tubes (500 μL) precoated overnight with 2.5% BSA. Membrane preparations (40 to 50 μg of protein) were incubated with varying amounts of unlabeled hormone (~ 22–30 fmol) and centrifuged at 30.000–40.000 cpm of ^{125}I-[D-Lys^6]-GnRH in a final volume of 250 μL at 4 °C. The incubations were terminated after 40 min (for equilibrium binding studies) at 4 °C by centrifugation (23000 *g*) for 5 min at 4 °C, followed by aspiration of the supernatant. The bottom parts of the centrifuge tubes containing the pellet were cut off and transferred into clean tubes for determination of radioactivity using a gamma counter. Parallel incubations were

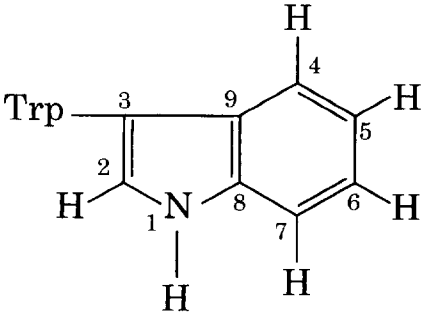
carried out in all experiments for determination of nonspecific binding in the absence of follicular membrane to determine binding to the tubes (< 0.5% of added radioactivity) and with follicular membrane in the presence of excess (10^{-6} M) unlabeled peptide for the determination of nonspecific binding to the tissue.

Data analysis

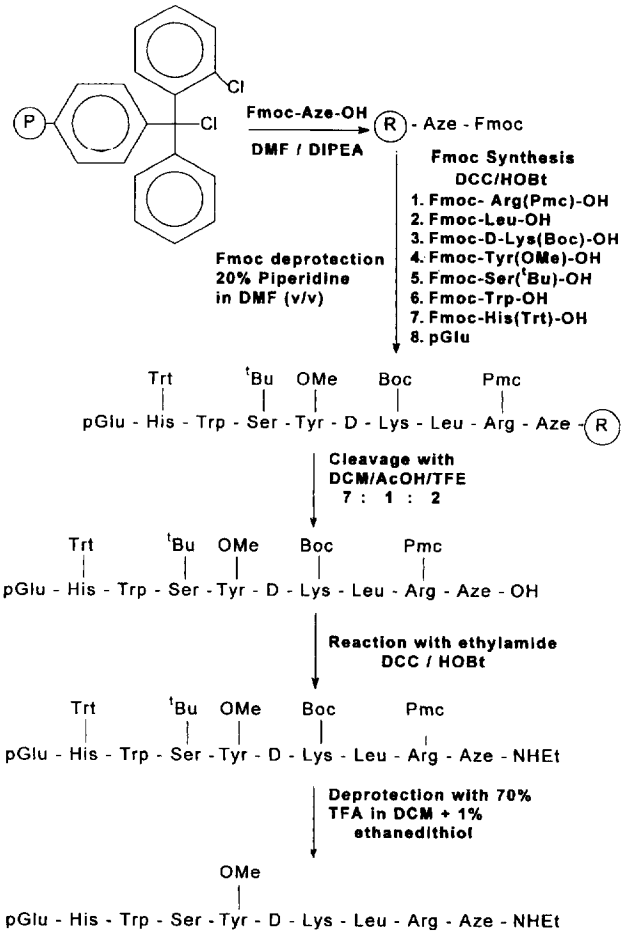
The equilibrium association constant (K_d; reciprocal affinity) was estimated from a Scatchard plot of competition curves using a computerized nonlinear least-square curve-fitting program (LIGAND) as described by Munson and Rodbard [25]. The displacement curves were fitted for single classes of binding sites by computer-calculated lines as indicated previously [24].

Results and discussion

Scheme 1 shows the tryptophan ring with carbons numbered for the purpose of notating NMR investigations of Trp ring protons in GnRH and [Tyr(OMe)^5]-GnRH by nuclear Overhauser effect enhancements. Scheme 2 shows the synthesis of [Tyr(OMe)^5, D-Lys^6, Aze^9-NHEt]GnRH, using the acid-sensitive 2-chlorotrityl resin and suitably protected amino acids. GnRH produced by the Fmoc solid-phase methodology had the same binding affinity to the rat pituitary GnRH receptors as the commercially purchased GnRH synthesized by the Boc methodology. For the synthesis of GnRH (for NMR purposes) and the two GnRH analogues we resorted to the Fmoc methodology utilizing the 2-chlorotrityl-resin as solid support as reported by Barlos et al [12]. The acid-sensitive 2-chlorotrityl chloride resin allows splitting of the peptide from the resin by mild conditions, affording a clean protected product in high yield which, after incorporation of the *C*-terminal residue, is finally deprotected with



Scheme 1. Tryptophan sidechain with ring protons numbered.



Scheme 2. General synthetic procedure for the synthesis of [Tyr(OMe)^5, D-Lys^6, Aze^{9}NHEt]GnRH.

trifluoroacetic acid (TFA) in dichloromethane (DCM) (65%, v/v) to afford the desired product. The *N*-^{t}Boc methodology, when applied to these analogues, resulted in crude material of very poor purity and yields for the desired products as estimated by HPLC. The two synthesized analogues were also tested for binding to GnRH receptors in the rat pituitary.

GnRH receptor binding

The two GnRH analogues were tested in terms of their binding to the rat pituitary, using ^{125}I-[D-Lys^6]GnRH as labeled ligand (figs 1 and 2). [Tyr(OMe)^5]GnRH

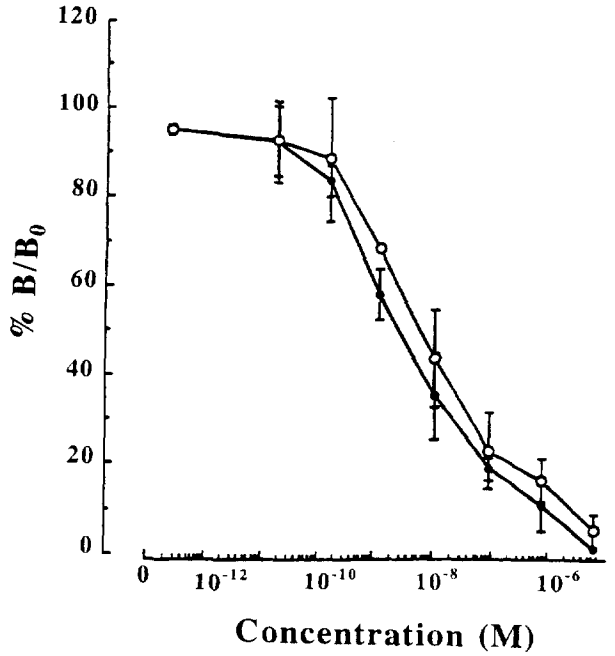


Fig 1. Receptor binding of a sample of GnRH prepared using novel 2-chlorotrityl resin and Fmoc methodology (○) and a commercial sample (Sigma GmbH, Germany) of GnRH (●) in the rat pituitary.

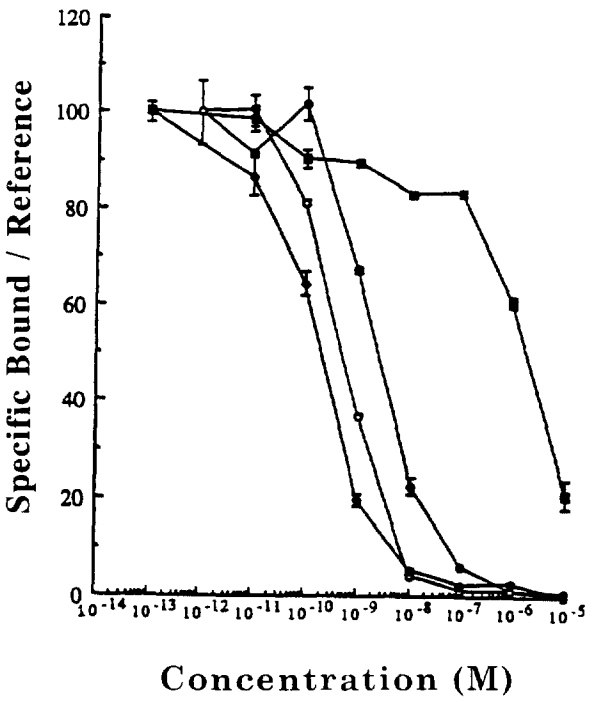


Fig 2. Displacement curves for the binding of ^{125}I-[D-Lys^6]GnRH to rat pituitary membranes in the presence of GnRH (●), [Tyr(OMe)^5]GnRH (■), [D-Lys^6]GnRH (◇) and [Tyr(OMe)^5, D-Lys^6, Aze^9-NHEt]GnRH (∩).

was found to have a significantly lower binding affinity (K_d = 1.53 ± 0.35 x 10^{-6} M) compared to GnRH (K_d = 3.69 ± 0.89 x 10^{-9} M). However, modifications at positions 6 and 9 ([Tyr(OMe)^5, D-Lys^6, Aze^9-NHEt]GnRH) significantly improved its binding affinity (K_d = 0.689 ± 0.15 x 10^{-9} M) in the rat pituitary which almost approached that of [D-Lys^6]GnRH (K_d = 0.19 ± 0.04 x 10^{-9} M).

Conformational properties of GnRH

In this study, the proton structural assignments of GnRH and [Tyr(OMe)^5]GnRH were based on 1D and 2D NMR experiments and their conformational characteristics by nuclear Overhauser effect enhancement spectroscopy. Figure 3 shows the one-dimensional NMR spectrum of GnRH in DMSO-d_6 in which all proton resonances have been assigned. This was possible by combined information from the COSY, TOCSY (fig 4), one-dimensional nOe experiments (table I) and on the basis of previous assignments of resonances in GnRH and its analogues [6, 7]. Ring proton resonances for the three aromatic residues Tyr, His, Trp of GnRH have been readily assigned and were used as probes to study their spatial location. Saturation of the Tyr meta proton at δ = 7.00 ppm, overlapped with the Trp C_6 proton at δ = 7.01 ppm, resulted in enhancements of the Tyr ortho proton (6.60 ppm, 33.28%), the Trp C_2 proton (7.10 ppm, 12.20%), the Trp C_5 proton (6.95 ppm, 18.84%), the Trp C_7 proton (7.29 ppm, 16.42%) and the His C_4 proton (6.75 ppm, 6.14%). Except the His C_4 proton enhancements, the others may result from intraresidue interactions originating from saturation of the overlapping Tyr meta and the Trp C_6 proton resonances. The His C_4 proton enhancement presents an interesting finding since it establishes an interresidue interaction of the His ring with either the Trp or Tyr rings. Proximity of the three rings and in particular of the His/Trp rings was furthermore indicated upon saturation of the His C_2 proton resonance at δ = 7.47 ppm. Enhancements of the Trp N_1, C_2, C_4 and C_7 proton resonances at δ =

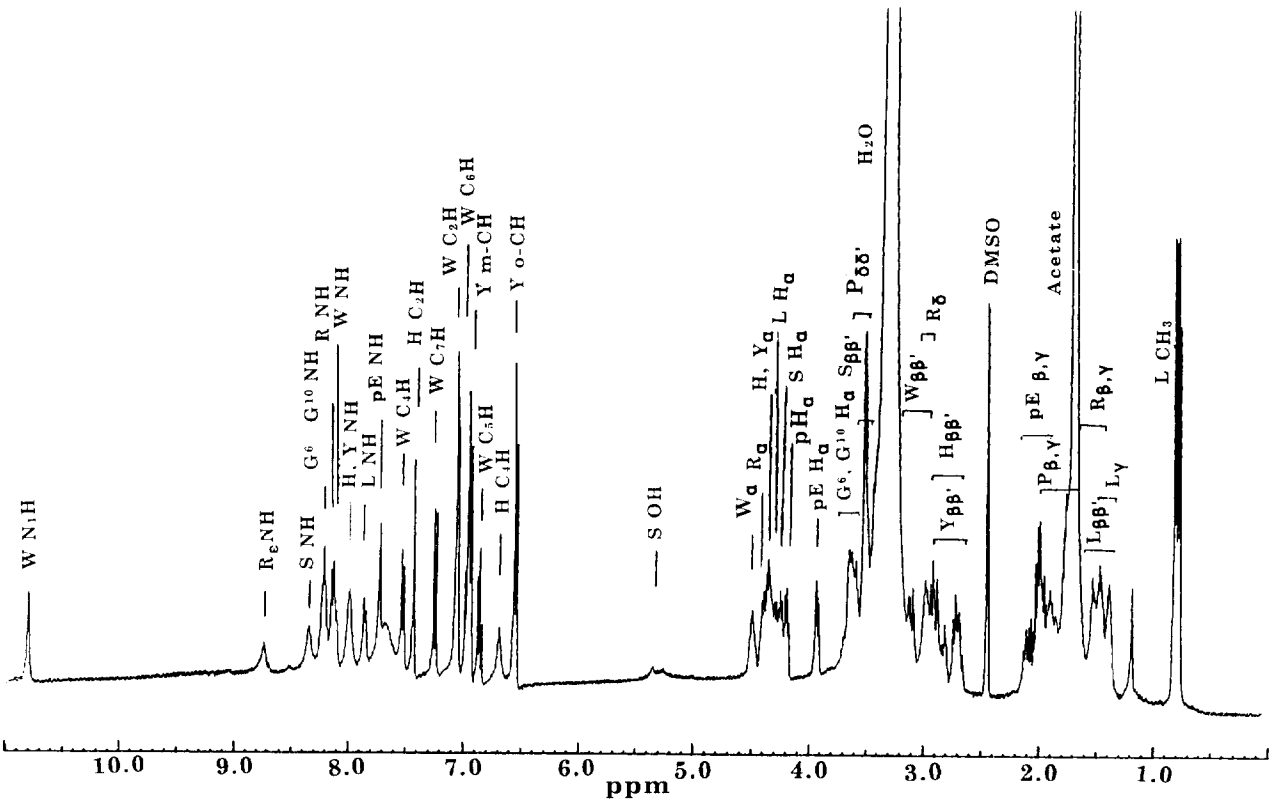


Fig 3. 400 MHz NMR spectrum of GnRH in DMSO–d_6.

10.80 ppm (4.70%), 7.10 ppm (2.28%), 7.58 ppm (16.20%) and δ = 7.29 ppm (1.82%), confirm proximity of the His/Trp rings. Enhancements of the pGlu N proton resonance at δ = 7.29 ppm (4.16%) and the Arg N_ε proton resonance at δ = 8.45 ppm (2.51%) indicate also proximity of the Arg guanidino group and the pGlu ring to the His ring. As seen in figure 5, the nOe difference spectrum which resulted after saturation of the Tyr ortho proton at δ = 6.60 ppm showed an enhancement for the His C_4 proton at δ = 6.75 ppm. This interaction was measured to be 11.70% and was found to be a reverse phenomenon. Thus, the nOe difference spectrum which resulted after saturation of the His C_4 proton resonance resulted in enhancement of the Tyr ortho proton validating proximity and interaction of the His/Tyr rings. Figure 6 shows the nOe difference spectra resulting after saturation of the Trp ring protons. In particular, we saturated the Trp C_5 proton at δ = 6.95 ppm, the Trp N_1 proton at δ = 10.80 ppm, the Trp C_4 proton at δ = 7.29 ppm and the Trp C_6 proton at δ = 7.01 ppm overlapped with the Tyr meta proton at δ = 7.00 ppm.

Except for the Trp C_6 proton, the Trp C_4 (doublet), the Trp C_5 (triplet), the Trp C_7 (doublet) and the Trp N_1 (singlet) proton resonances appear as distinct signals at non-overlapping areas and represent very good probes for studying the spatial location of the Trp ring to other side chains. As shown in figure 6 and table I, saturation of the Trp C_5 proton resonance resulted in enhancements of the Tyr ortho proton at δ = 6.60 ppm (2.13%) and the His C_4 proton at δ = 6.75 ppm (9.87%), indicating proximity of the three aromatic rings. Saturation of the Trp C_7 proton resonance at δ = 7.29 ppm resulted also in intraresidue resonance enhancements of the Trp N_1 proton at δ = 10.80 ppm (3.7%), the Trp C_2 proton at δ = 7.10 ppm (4.08%) and the Trp C_6 proton at δ = 7.01 ppm (12.12%) as well as in interresidue resonance enhancements of the His C_2 proton at δ = 7.47 ppm (6.72%), the pGlu N proton at δ = 7.75 ppm (3.90%) and the Arg N_ω proton at δ = 7.52 (20.15%). These enhancements, while confirming the Trp/His proximity, indicate also that the Trp ring is closely spaced to the Arg guanidino group and the pGlu ring.

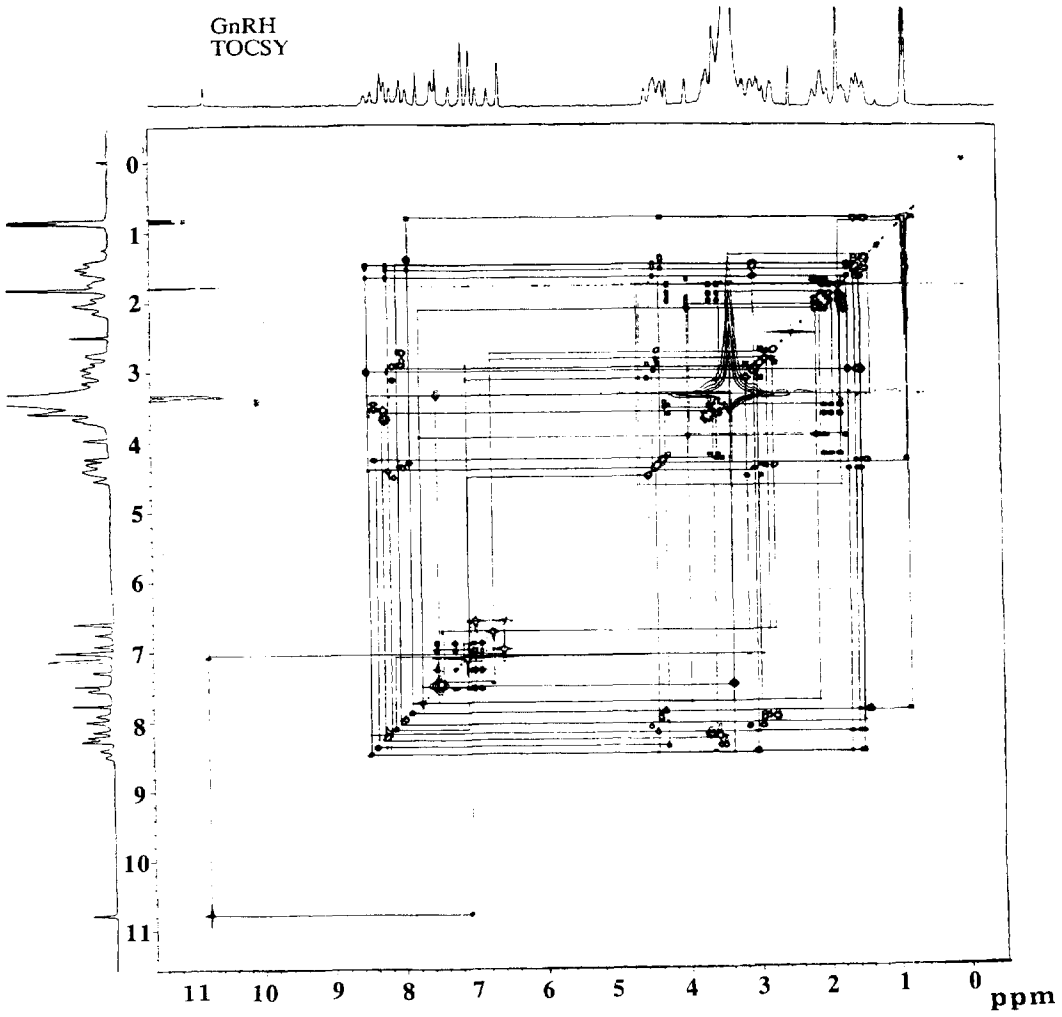


Fig 4. TOCSY spectrum for GnRH in DMSO–d_6.

The above-cited findings are in agreement with the previously reported Tyr[5]-Gly[6]-Leu[7]-Arg[8] β-turn and a conformation of GnRH in which the aromatic nuclei of the tryptophan and tyrosine are in close proximity [3]. Disturbance of this interaction results in marked reduction of activity. Our nOe studies on GnRH, which are an extension of previously reported conformational studies, indicate that not only the Trp and Tyr rings are in close proximity but that all three rings Tyr, Trp, His cluster together, which may account for the expression of maximum activity. It is interesting that similar conformational features, ie, ring clustering, has also been observed in the peptide hormone angiotensin [11–14].

Conformational differences between GnRH and [Tyr(OMe)[5]]GnRH

Turning to the [Tyr(OMe)[5]]GnRH analogue, different conformational properties were revealed for this peptide molecule compared to GnRH. Saturation of distinct aromatic resonances of the Tyr, His and Trp residues did not produce interresidue aromatic enhancements indicating the absence of a ring cluster in [Tyr(OMe)[5]]GnRH. Since the main difference between the ^{1}H-NMR spectra of GnRH and [Tyr(OMe)[5]]GnRH is the singlet at $\delta = 3.65$ ppm for the Tyr-OMe protons, assignment of all aromatic resonances in [Tyr(OMe)[5]]GnRH was a simple task, faci-

Table I. NOe enhancements of GnRH protons.

Protons/ saturated	*Chemical shift*	*Enhancement*	*Chemical shift*	*% Proton*
Tyr-o	6.60	His C_4	6.75	11.70
Tyr-o	6.60	Tyr-m	7.00	33.30
Tyr-m (Trp C_6)	7.00 (7.01)	Tyr-o	6.60	33.28
Trp C_6 (Tyr-m)	7.00 (7.01)	Trp C_5	6.91	18.87
Tyr-m (Trp C_6)	7.00 (7.01)	Trp C_2	7.10	12.20
Trp C_6 (Tyr-m)	7.00 (7.01)	Trp C_7	7.29	16.42
His C_4	6.75	Tyr-o	6.60	10.40
His C_2	7.47	Trp C_4	7.58	16.20
His C_2	7.47	Trp C_2	7.10	2.28
His C_2	7.47	Trp C_7	7.29	1.82
Trp N_1	10.80	Trp C_2	7.10	12.52
Trp C_5	6.95	Trp C_6 (Tyr-m)	7.00 (7.01)	47.83
Trp C_5	6.95	Trp C_4	7.58	11.61
Trp C_4	7.58	His C_2	7.47	12.30
Trp C_4	7.58	Trp C_5	6.95	8.85
Trp C_7	7.29	Trp C_6	7.01	12.12
Trp C_7	7.29	His C_2	7.47	6.72
pGlu N	7.75	pGlu C_α	3.96	3.45
pGlu C_α	3.96	pGlu N	7.75	7.58
pGlu C_α	3.96	His NH	8.00	8.94
pGlu C_α	3.96	Arg N_ω	7.52	6.40
Arg N_ε	8.45	Arg N_ω	7.52	4.25

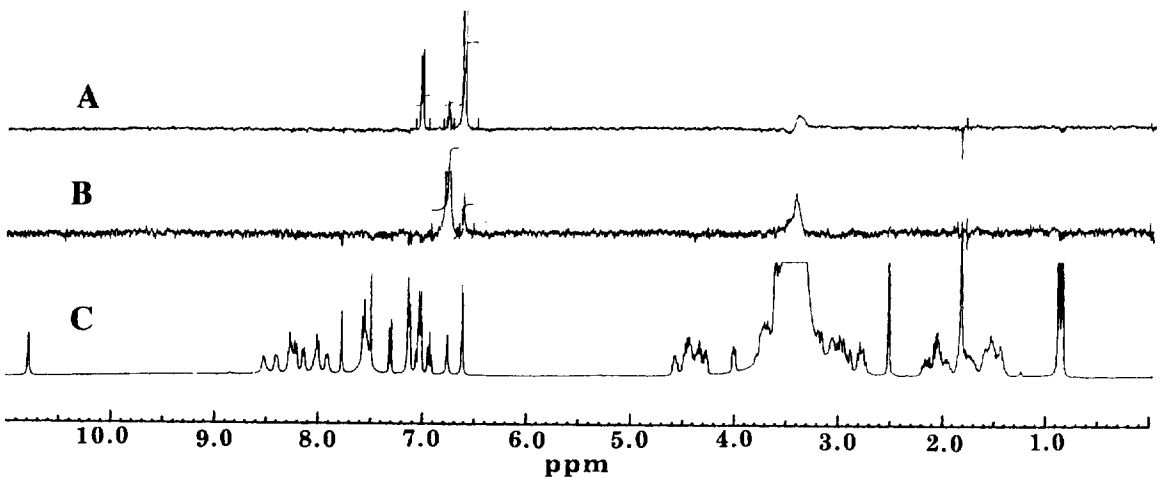


Fig 5. Reference spectrum (C) and nOe difference spectra for GnRH in DMSO–d_6 obtained upon saturation of the Tyr ortho proton line (A) and the His C_4H (B).

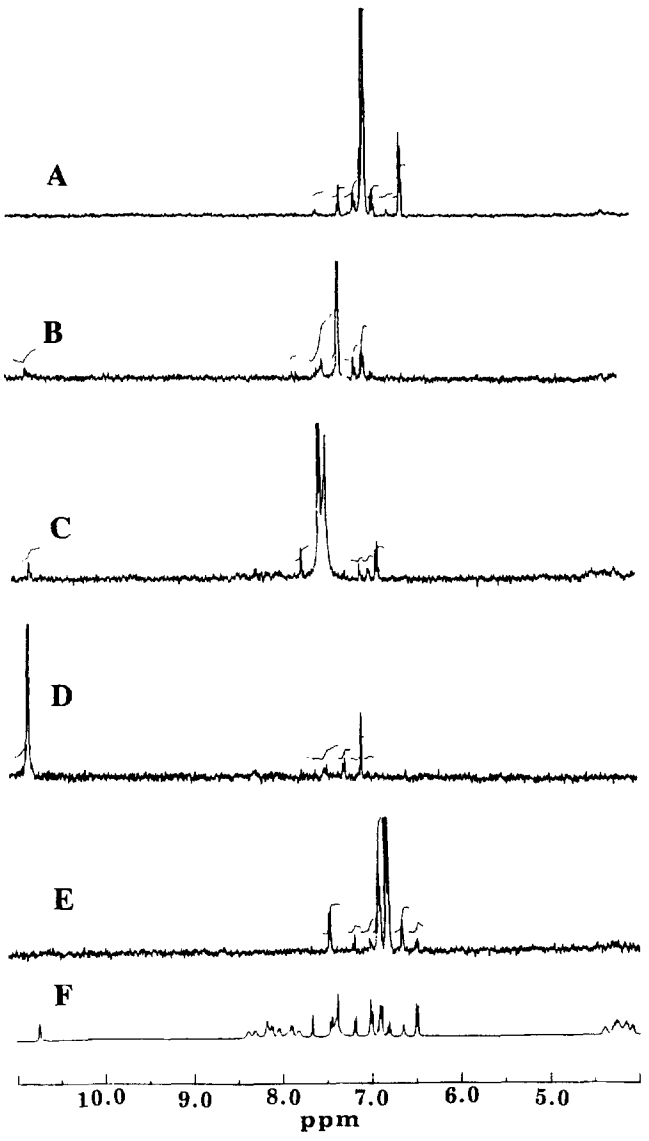


Fig 6. Reference spectrum (F) and nOe difference spectra for GnRH in DMSO–d_6 obtained upon saturation of proton lines for Trp C_6H (A), Trp C_7H (B), Trp C_4H (C), Trp N_1H (D), Trp C_5H (E).

litating the nOe experiments (tables II and III, figs 7 and 8). COSY/TOCSY and nOe information confirmed the assignments. The Tyr-OMe singlet at δ = 3.65 ppm as well as the Tyr ortho and meta protons provide distinct probes for studying the spatial relationship of the Tyr ring with other groups. While in GnRH the Tyr ring was found to be in close proximity with the His and Trp rings, no such proximity was observed for [Tyr(OMe)^5]GnRH. Thus, saturation of the Tyr-OCH_3 singlet at δ = 3.65 ppm, except the intraresidue Tyr ortho (6.76 ppm) enhancement (3.8%), did not produce other aromatic interresidue enhancements for the His and Trp ring proton resonances, indicating that the Tyr ring methyl group is not in close proximity with the other two rings. Furthermore, saturation of the Tyr ortho proton at δ = 6.76 ppm resulted as expected in an enhancement of the adjacent meta proton at δ = 7.15 ppm (12.3%) but not in other interresidue ring proton enhancements. An enhancement of the nearby His C_4 proton at δ = 6.68 ppm must be the result of partial saturation of this proton due to close proximity of the two Tyr ortho (6.76 ppm) and His C_4 (6.68 ppm) proton resonances in the NMR spectrum. The distant location of the Tyr and His rings is also suggested when the His C_4 proton at δ = 6.68 ppm is saturated. A clean nOe difference spectrum is obtained (fig 8) without enhan-

Table II. Chemical shifts of [Tyr(OMe)^5] GnRH.

Protons	*Shifts*
Tyr (OMe)	3.65 ppm (s)
Ser OH	5.43 ppm (b)
His C_4H	6.68 ppm (s)
Tyr o-CH	6.76 ppm (d)
Trp C_5H	6.92 ppm (t)
Trp C_6H	7.02 ppm (t)
Tyr m-CH	7.15 ppm (d)
Trp C_2H	7.15 ppm (s)
Trp C_7H	7.29 ppm (d)
His C_2H	7.42 ppm (s)
Trp C_4H	7.57 ppm (d)
pGlu NH	7.85 ppm (s)
Arg Nε	9.38 ppm (bs)
Trp N_1H	10.88 ppm (s)

Table III. NOe enhancements for [Tyr(OMe)^5]GnRH aromatic protons.

Protons saturated	*Chemical shift (ppm)*	*Enhancement*	*Chemical shift (ppm)*	*% Proton*
Tyr-OMe	3.65	Tyr o-CH	6.76	3.8
Tyr o-CH	6.76	Tyr m-CH	7.15	12.3
Tyr o-CH	6.76	Tyr o-CH_3	3.65	1.92
Trp C_4H	7.57	Trp C_5H	6.92	3.9
Trp C_5H	6.92	Trp C_4H	7.57	5.9
Trp C_5H	6.92	Trp C_6H	7.02	15.6
Trp N_1H	10.88	Trp C_2H	7.15	10.3
His C_2H	7.42	–	–	–
His C_4H	6.68	–	–	–

cement of any Tyr or Trp aromatic proton resonance. Similarly, saturation of the His C_2 proton resonance at $\delta = 7.42$ ppm did not produce any enhancement of Tyr or Trp aromatic proton resonances, furthermore indicating distant location of the three rings from each other. Saturation of the Trp C_4H at $\delta = 7.57$ ppm resulted as expected in enhancement of the Trp C_5 proton resonance at $\delta = 6.92$ ppm. However, no other intrarresidue or interresidue enhancements were observed, indicating that this Trp ring proton is not in close proximity with the Tyr or His rings. No interresidue ring enhancements were observed upon saturation of the Trp C_5 proton at $\delta = 6.92$ ppm, furthermore indicating distant location of the Trp ring from the Tyr and His rings. Expected intraresidue enhancements of the Trp C_4 and C_6 proton resonances at $\delta = 7.57$ ppm (5.9%) and $\delta = 7.02$ ppm (15.6%) serve as control experiment validating our nOe results. Saturation of the Trp ring N_1 proton at $\delta = 10.88$ ppm resulted only in enhancement of the adjacent Trp C_2 proton at $\delta = 7.15$ ppm (10.3%). These nOe data obtained upon saturation of distinct Tyr, His and Trp ring proton resonances, indicate that the three aromatic rings are not in close proximity as is the case in the GnRH peptide molecule which is characterized by clustering of the three rings. This conformational difference may be essential in receptor recognition and potency of GnRH agonists.

Molecular dynamics

The minimized structure of GnRH was manipulated in order to fulfill the observed nOe's shown in table I. Thus, pGlu was rotated in such a way as to communicate in space with Arg. Trp was moved into a position to interact in space with His. His is situated in a position where it can be in spatial proximity with both Trp and Tyr residues. After the construction of a preliminary model, distance constraints were applied using the obtained nOe data in order to further refine it. Thus, first the GnRH structure was minimized with the distance constraints, and then dynamics was applied. The calculations were carried out at 1000 K and in a dielectric environment of $\varepsilon \approx 45$, corresponding to that of DMSO. 1/10 of the resulting trajectory files of simulated conformations were minimized

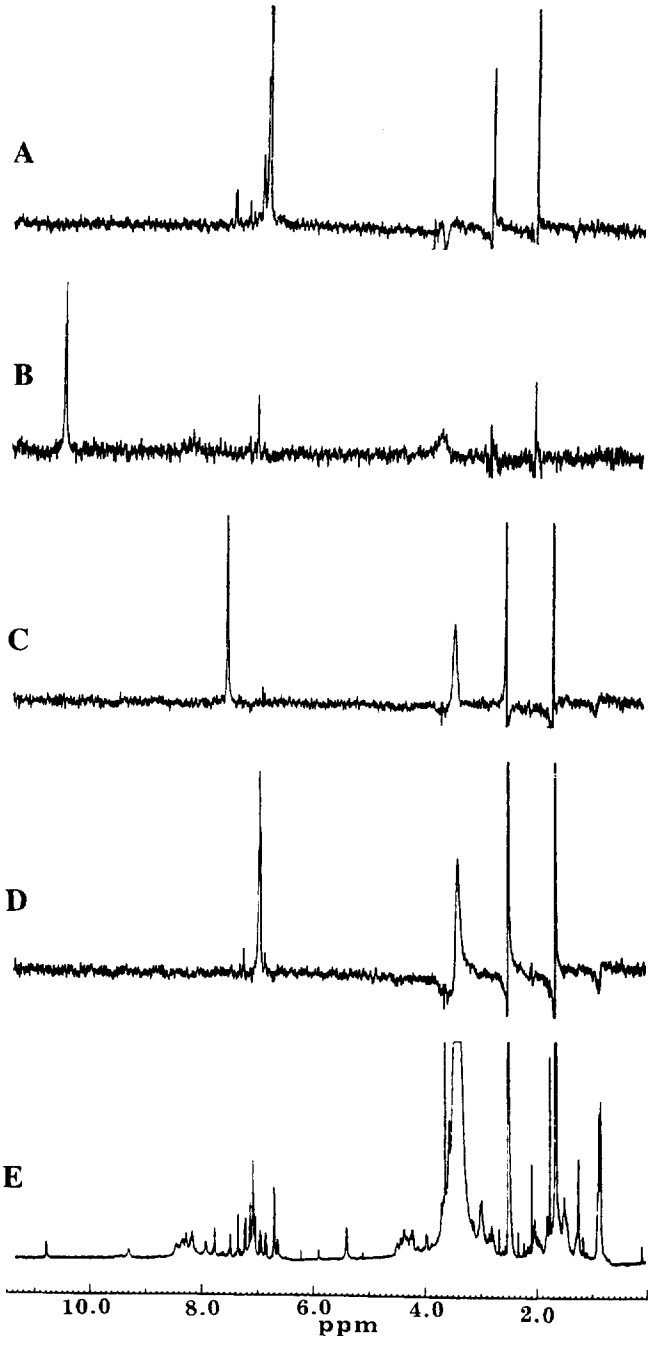


Fig 7. Reference spectrum (E) and nOe difference spectra for [Tyr(OMe)^5]GnRH in DMSO–d_6 obtained upon saturation of proton lines for Trp C_6H (A), Trp N_1H (B), Trp C_4H (C), Trp C_5H (D).

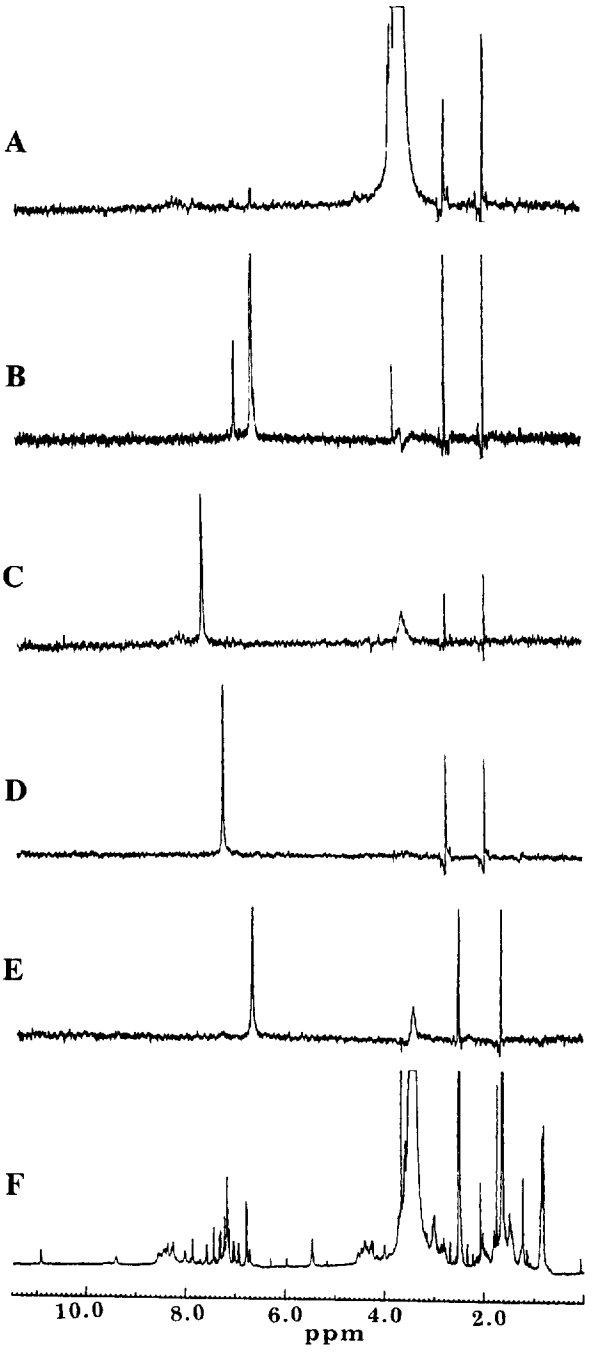


Fig 8. Reference spectrum (F) and nOe difference spectra for [Tyr(OMe)^5]GnRH in DMSO–d_6 obtained upon saturation of proton lines for Tyr(OMe) (A), Tyr-ortho H (B), pGlu NH (C), His C_2H (D), His C_4H (E).

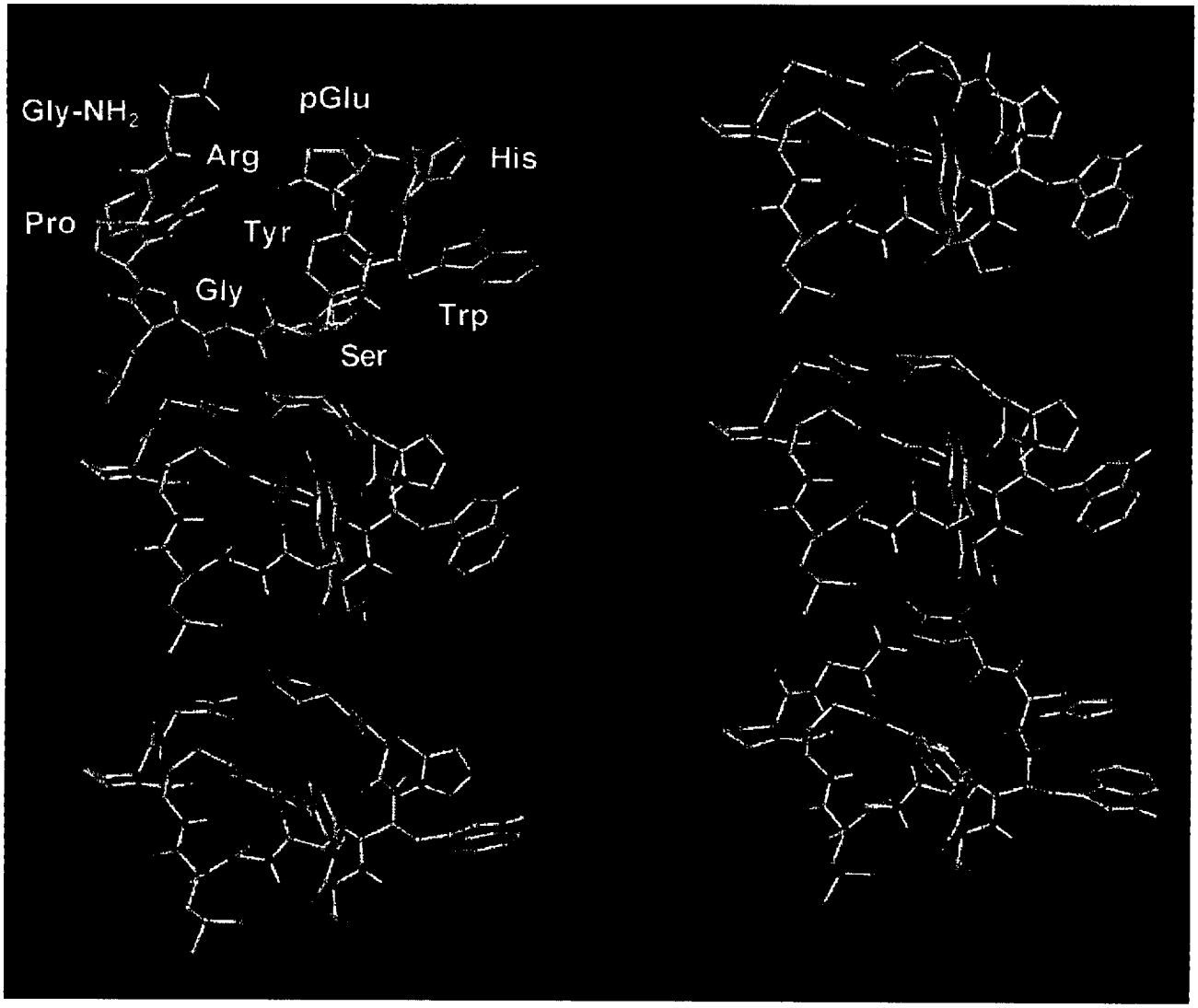


Fig 9. Representative lowest-energy conformers of GnRH derived from six family structures obtained after performing dynamics at a simulated temperature of 1000 K.

using the steepest descent algorithm (1000 iterations). The resulting conformers were clustered using the torsion angle criterion (20°) and RMS values ranged between 0.9–49.7. The lowest energy conformers from six family structures are shown in figure 9. Distance geometries have been calculated based on nOe intensities (table I) using the interproton distances Tyr o/m (2.5 Å) and available α/β information (2.3 Å min–3.0 Å max) for calibration. NOe intensity values smaller than 2% are considered insignificant and were not incorporated into models generated by molecular dynamics simulations. These conformers reveal that His and Trp remain in spatial proximity. Tyr shares its spatial interactions with both Arg and His sidechains. On the other hand, Arg and His interact in space with pGlu and Trp sidechains. In addition, pGlu interacts with Gly, imposing a cyclic conformation on GnRH. Thus, the proposed confor-

mations shown in figure 10 are stabilized by pGlu^{1}···Gly^{10} (A), Arg^{8}···Tyr^{5}···His^{2}···Trp^{8} (B), Arg^{8}···pGlu^{1} (C), Tyr^{5}···pGlu^{1} (D) plausible hydrogen bondings and clustering of guanidinium (Arg), imidazole (His), phenol (Tyr) and indole (Trp) rings. This model can be used as a basis for drug design and may lead to GnRH non-peptide mimetics for drug therapy [26–27].

Six family structures for [Tyr(OMe)^{5}]GnRH were also achieved (fig 11). Interestingly, the dynamics experiment revealed low-energy conformers with new features. Due to the lack of a phenolic hydroxyl group the cluster between Arg/His/Tyr/Trp is no longer energetically favored. This is due to the inability of methylated Tyr to stabilize any spatial interaction with His. Consequently, His and Trp are free to adopt energetically favored positions that do not allow any spatial vicinity. The methylation of Tyrosine permits Arg to interact and protrude towards Gly and pGlu. This cascade of molecular changes in aminoacid moieties of [Tyr(OMe)^{5}]GnRH explains the fact that no nOes were observed between the aminoacids Tyr/His/Trp.

In conclusion, GnRH and [Tyr(OMe)^{5}]GnRH appear to potentially adopt a cyclic form (see figs 9 and 11, top left conformers), stabilized by Arg^{8}···pGlu^{1} electrostatic interactions. A cluster between Arg/Tyr/His/Trp is only observed in GnRH lowest-energy conformers. The Arg^{8}···pGlu^{1} hydrogen bonding is predominant in the lowest-energy conformers of [Tyr(OMe)^{5}]GnRH.

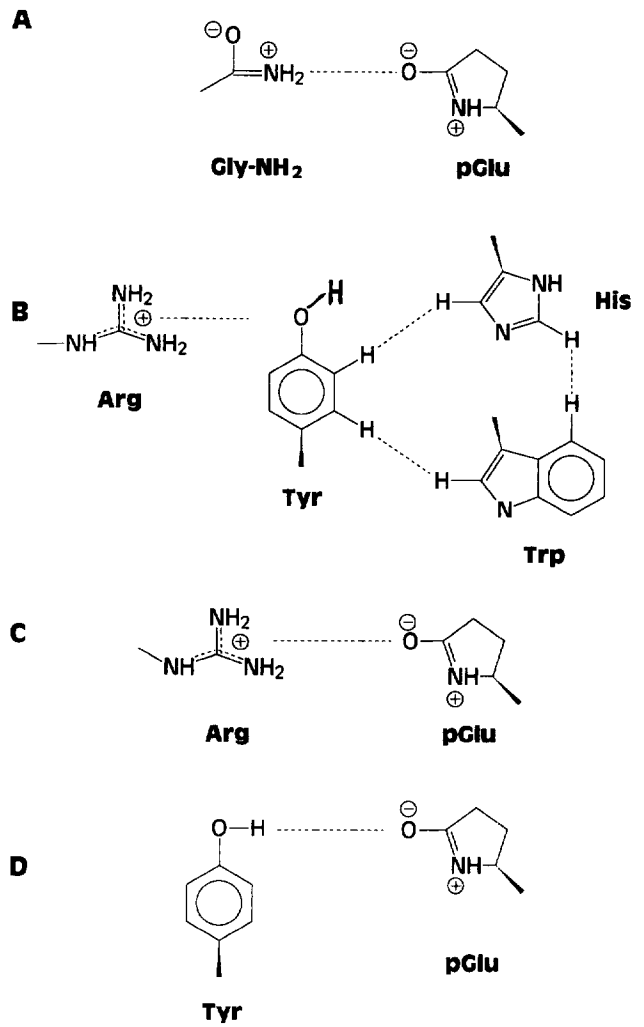


Fig 10. Plausible hydrogen bondings observed in the lowest-energy conformers of GnRH and [Tyr(OMe)^{5}]GnRH derived after performing dynamics at a simulated temperature of 1000 K.

Conclusion

The present study demonstrates that there is clustering of the three aromatic rings Tyr, His and Trp in GnRH which becomes minimal after methylation of the tyrosine hydroxyl. This slackening of the ring cluster interaction in [Tyr(OMe)^{5}]GnRH is accompanied by a decrease in receptor binding affinity and biological potency in the rat pituitary. Clustering of the three aromatic rings in GnRH may be important to stabilize conformation necessary for receptor recognition.

Acknowledgment

This work was supported by European Community (EC) grants (BIOMED programme No 920038, BIOMED-PECO No 930158, and COPERNICUS No 940238) and the Ministry of Energy and Technology of Greece. We also acknowledge NATO support (Grant CRG 940612).

References

1 Karten M, Rivier J (1986) *Endocrine Reviews* 7, 44–46
2 Coy D, Coy E, Schally A (1973) *J Med Chem* 16, 827–829
3 Chang JK, Williams RH, Humphries et al (1972) *Biochem Biophys Res Commun* 47, 727–732
4 a) Keramida M, Matsoukas JM, Agelis G et al (1994) In *Peptides 1994*, Proceedings of the 23rd European Peptide Symposium, Braga, Portugal (H Maia, Ed), ESCOM, Leiden
b) Keramida M, Matsoukas JM, Panagiotopoulos D, Alexopoulos K et al (1996) *Lett Peptide Sci* 3, 257–262
5 Baniak EL, Rivier JE, Struthers RS et al (1987) *Biochem* 26, 2642–2650
6 Chary KVR, Srivastava S, Hosur R et al (1986) *Eur J Biochem* 158, 323–332
7 Rizo J, Koerber SC, Bienstock RJ et al (1992) *J Am Chem Soc* 114, 2852–2859

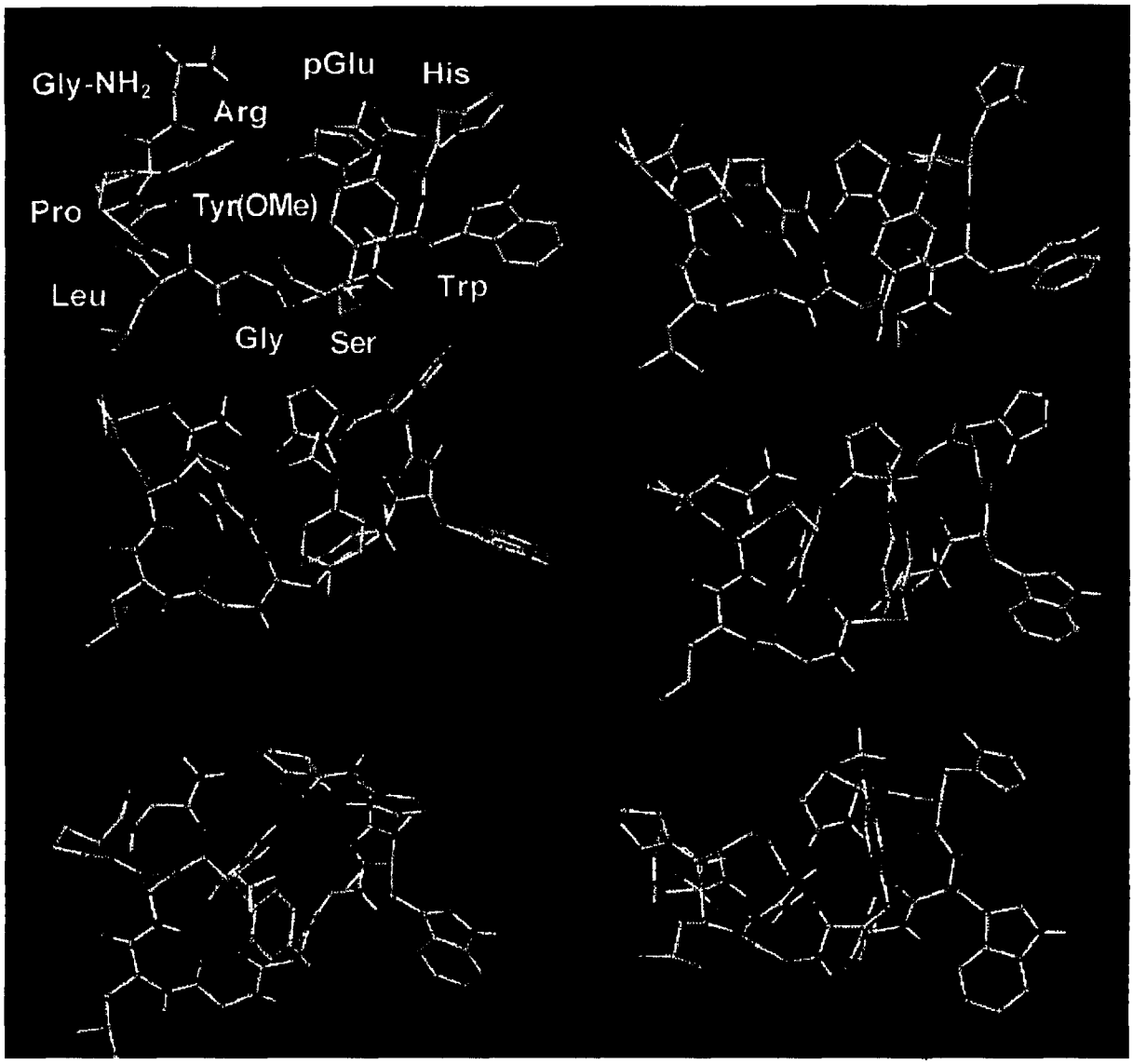


Fig 11. Representative lowest-energy conformers of [Tyr(OMe)^5]GnRH derived from six family structures obtained after performing dynamics at a simulated temperature of 1000 K.

8 Rizo J, Koerber SC, Bienstock RJ et al (1992) *J Am Chem Soc* 114, 2860–2871
9 Freidinger RM, Veber DF, Perlow DS et al (1989) *Science* 210, 656–658
10 Monahan MW, Amoss MS, Anderson et al (1973) *Biochem* 12, 4616–4620
11 Matsoukas JM, Bigham G, Zhou N et al (1990) *Peptides* 11, 359–366
12 Barlos K, Gatos D, Hondrelis J et al (1989) *Liebigs Ann Chem* 951–955
13 Matsoukas JM, Yamdagni R, Moore GJ (1990) *Peptides* 11, 367–374
14 a) Matsoukas JM, Hondrelis J, Keramida M et al (1994) *J Biol Chem* 269, 5303–5312
b) Matsoukas JM, Agelis G, Wahhab A et al (1995) *J Med Chem* 38, 4660–4669
15 Bolin DR, Sytwu II, Humjec F et al (1989) *Int J Pept Protein Res* 33, 353–359
16 Matsoukas JM, Agelis G, Hondrelis J (1993) *J Med Chem* 36, 904–911
17 Matsoukas JM, Hondrelis J, Agelis G (1994) *J Med Chem* 37, 2958–2669
18 Hogg AM (1983) *Int J Mass Spectrom Ion Phys* 49, 25–34
19 Noggle JH, Shirmer BE (1991) *The Nuclear Overhauser Effect*, Academic Press, New York
20 Marion D, Wuthrich K (1983) *Biochem Biophys Res Commun* 113, 967–974
21 Mavromoustakos T, Yang DP, Theodoropoulou E (1995) *Eur J Med Chem* 30, 227–234
22 Pati D, Habibi HR (1995) *Endocrinology* 136, 75–84
23 Habibi HR, Marchant TA, Nahorniak CS et al (1989) *Biol Reprod* 40, 1152–1161
24 Habibi HR, Peter RE, Nahorniak CS et al (1992) *Regulatory Peptides* 37, 271–284
25 Munson PM, Rodbard D (1980) *Anal Biochem* 107, 220–239
26 Moore GJ (1994) *Trends Pharmacol Sci* 15, 124–129
27 Moore GJ, Smith JR, Baylis B et al (1995) *Adv Pharmacol* 33, 91–141