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Novel cyclic azo-bridged analogs of gonadotropin-releasing hormone‡

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Abstract: Five linear analogs of GnRH containing a *p*-aminophenylalanine (Pap) residue in their sequence and their six corresponding azo-bridged cyclic derivatives were synthesized. The precyclic peptides were prepared on solid-support, while azo-cyclization was performed in solution by diazotization of the *p*-aminophenylalanine residue followed by intramolecular coupling of the formed diazo salt with either tyrosine or histidine side chains present in the sequence. All peptides were examined for their binding ability to the GnRH receptor expressed on rat pituitary membranes and for their LH-release activity from dispersed rat pituitary cells. Linear analogs **1** i.e [Pap^5] GnRH and **3,** i.e. [Tyr^3, Pap^5] GnRH, were found to bind to the GnRH receptors only slightly less avidly than native GnRH. Their cyclization, however, led to a marked reduction in the binding capacity, i.e. from IC_{50} of 10^{-9} M to the 10^{-7} M range, and in biopotency, i.e. LH-release. All other linear and cyclic peptides were found to bind selectively to the GnRH receptor only in the low μM range. Only peptide **1** was found comparable to native GnRH in respect to LH-release activity and thus may potentially be a good agonist of the parent peptide. Peptides **1**–**4**, the most potent GnRH receptor binders, were examined for their conformational properties using CD. Cyclic-azo peptides **2** and **4** were further evaluated by NMR spectroscopy in solution combined with molecular modeling. The structural information obtained explains in part the GnRH-like biological activity observed.

Keywords: azo-cyclization; GnRH analogs; NMR

INTRODUCTION

Gonadotropin-releasing hormone (GnRH; pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is a key integrator between the neural and the endocrine systems and plays a pivotal role in the regulation of the reproductive system. GnRH is synthesized in hypothalamic neurosecretory cells and is released in a pulsatile manner into the hypothalamo–hypophyseal portal circulation. This pattern of GnRH secretion provokes the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary, which, in turn, stimulates gonadal steroidogenesis and gametogenesis [1,2].

Synthetic GnRH analogs, agonists as well as antagonists, have attracted remarkable interest because of their most significant clinical potential and actual applications for the treatment of reproductive-related diseases such as prostate and breast cancer, and their possible use as contraceptives [3,4]. Thus, in these respects, numerous and versatile GnRH-like peptides, peptido–mimetic and nonpeptide derivatives have been designed, prepared and evaluated. Noteworthy among these compounds are cyclic peptidic molecules, some of which were found potent in the low nanomolar range [e.g. 5,6]. Cyclization of GnRH, primarily achieved through an intramolecular amide bond formation, was first reported by Goodman and coworkers in 1977 [7]. It is well documented that cyclization, which renders peptides less flexible and conformationally constraint, often results in the peptides' enhanced metabolic stability and increased selectivity [8]. Thus, cyclization might have very significant pharmacological implications on biologically active linear peptides.

An approach to prepare cyclic peptides through intramolecular azo-bond formation, between diazotized *p*-aminophenylalanine and histidyl or tyrosyl residues, was recently described [9]. Using this novel methodology, several cyclic peptides, including two GnRH analogs, were prepared. The purpose of the present study was threefold: first, to evaluate the generality of the azo-cyclization method to different ring-size

Abbreviations: BOC, tert-butoxycarbonyl; BSA, bovine serum albumin; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; MS, mass spectrometry; Rink amide MBHA resin, 4-(2',4'-dimetoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-4-methylbenzhydrylamine polystyrene; TFA, trifluoroacetic acid; r.m.s.d. root mean squared deviation; Trt, trityl.

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formations; second, to examine possible His/Tyr selectivity in azo cyclizations and third, to attempt the synthesis of a potent, conformationally constrained, cyclic GnRH derivative.

MATERIALS AND METHODS

All chemicals and reagents were of analytical grade. Rink amide resin, Fmoc-protected amino acids derivatives and all the reagents for solid-phase synthesis were obtained from Novabiochem (Läufelingen, Switzerland). Side-chain protecting groups employed were as follows: Arg, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf); His, Trt; Trp and *p*-aminophenylalanine, BOC; Ser and Tyr, *tert*-butyl (*t*-Bu). Reversed-phase HPLC was performed on a Spectra-Physics SP-8800 liquid chromatography system equipped with an applied Biosystems 757 variable wavelength absorbance detector. HPLC prepacked columns used were: Lichrocart, containing Lichrospher RP-18 (250 × 10 mm; 7 μm) for semipreparative purification and Lichrospher 100 RP-18 for analytical purposes (250 × 4 mm; 5 μm), Merck (Darmstadt, Germany). HPLC purification and analyses were achieved by using gradients formed from 0.1% TFA in water as solvent A and 0.1% TFA in 75% aqueous MeCN as solvent B. Eluent composition was 10% B in A for the first 10 min and increased linearly to 100% B, 50 min after injection time. Flow rates were 1 ml/min and 10 ml/min for analytical and preparative purposes, respectively. The column effluents were monitored by UV absorbance at 214 nm. Mass analyses were performed using MALDI-TOF and ESI-MS techniques (Bruker-Reflex-Reflection model, Germany; and, VG-platform-II electrospray single quadruple mass spectrometer, Micro Mass, U.K., respectively). For amino acid composition evaluations peptides were hydrolyzed in 6N HCl at 100 °C for 24 h under vacuum, and the hydrolyzates were analyzed with a Dionex Automatic Amino Acid Analyzer. Circular dichroism (CD) spectra were recorded using 0.1 mM peptide solutions at 25 °C on Aviv-202 CD spectrometer (Lakewood, NJ, USA). Peptides were dissolved in phosphate buffer solution (pH = 7.4; 5 mM). Spectra are the average of 6 scans. Scanning speeds were 1 nm/sec. The length of the optical path was 0.1 cm. Baseline was recorded and subtracted after each spectrum. Data is expressed in molar ellipticity: $[\theta] = 10^{-3}$ deg cm^2/dmol.

Peptide Synthesis

The precyclic linear peptides were prepared on solid-support (Rink amide MBHA resin), with an AMS-422 multiple peptide synthesizer (ABIMED, Langenfeld, GmbH, Germany) using Fmoc chemistry, following the company's protocols [10]. All synthesized peptides were simultaneously deprotected/cleaved from the resin using a solution of TFA : triethylsilane : anisole : water (17 : 1 : 1 : 1) [10]. After 2 h at room temperature, the cleaved mixtures were filtered and the peptides were precipitated from the solutions with peroxide-free dry ether at 0 °C. Precipitated peptides were washed with cold dry ether, dissolved in water and lyophilized. The crude peptides (>85% pure) were directly used, without further purification, for azo-cyclization [9]. Briefly, the corresponding lyophilized peptide powders (5–50 mg), were dissolved in 0.1 M HCl (50 μl per mg peptide) and the solutions cooled to 0 °C. Cooled aqueous sodium nitrite solution (0.1 M) was then added, portionwise, to achieve a 1 : 1 peptide/nitrite molar ratio, and the reaction mixture was allowed to stand with occasional mixing for 10 min at 0 °C. It was then added, portion-wise, to an ice-cold 0.1-M $KHCO_3$ solution (2 ml per 1 mg peptide), and the reaction mixture, following adjustment of the pH to 8.0 by adding 1 M K_2CO_3, was kept for 3–4 h in the cold, followed by overnight incubation at room temperature. The homogeneous solution gradually turned brownish–orange or yellowish upon standing. Lyophilization resulted in yellowish–brown powdered crude cyclic peptides, which were further purified by semipreparative HPLC. Isolated peptides were evaluated by analytical HPLC, amino acid analysis and mass spectroscopy. Analyses revealed high purities (≥97%).

Binding to Pituitary GnRH Receptors

[D-Lys^6]-GnRH was iodinated by the chloramines T method [11] and the resulting ^{125}I-[D-Lys^6]-GnRH (1700 μCi/nmol) was purified by an analytical HPLC procedure [12]. Binding assay was performed as described [12]. Briefly, pituitary membranes (25 μg protein/tube) prepared from Wistar-derived proestrous rats were incubated for 90 min at 4 °C with 50 000 cpm (23.5 pM) ^{125}I-[D-Lys^6]-GnRH, with or without various concentrations of unlabeled peptides, in a total volume of 0.5 ml of the assay buffer (10 mM Tris-HCl containing 0.1% BSA). The reaction was terminated by rapid filtration through Whatman GF/C filters, which were then washed three times with ice-cold assay buffer and counted in an Auto-Gamma Counting System (Packard, Meriden, CT). Assays were performed in triplicates. Nonspecific binding was defined as the residual binding in the presence of [D-Lys^6]-GnRH (1 μM). Specific binding was calculated by subtracting the nonspecific binding values from the maximal binding determined in the absence of any competing peptide. IC_{50} values were calculated using the curve-fitting software program Enzfitter (Elsevier Biosoft, Cambridge, U.K.).

LH Release from Dispersed Pituitary Cells

Cells from 21-day-old Wistar-derived female rats were dispersed as previously described [11], and incubated in 96-well plates (50 000 cells/well) at 37 °C in M-199 medium containing 5% horse serum. After 48 h, cells were washed with M-199 medium containing 0.1% BSA and incubated for 4 h at 37 °C with M-199/0.1% BSA (0.25 ml) containing the desired concentrations of the tested peptides (four wells/experimental group). The incubation was terminated by removing the medium and diluting it by an equal volume of 1% BSA in phosphate-buffered saline PBS solution. Three different aliquots from each sample were analyzed for LH levels by radioimmunoassay (RIA) [13], using the kit kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Program. Results are expressed in terms of the LH- RP-3 reference preparation [11].

Proteolysis of GnRH and Analogs

Peptides degradation by rat anterior pituitary-derived enzymes was performed as previously reported [14].

NMR Measurements

NMR samples were prepared by dissolving the lyophilized powder peptides in an aqueous solution (pH 2.9–3.5) containing 5.5% (v/v) D_2O at final concentrations of ~2.2 mM. NMR measurements were performed on a Bruker Avance-600 MHz spectrometer (Bruker, Germany) operating at the proton frequency of 600.13 MHz and the carrier frequency was set on the water signal at the chemical shift of 4.66 ppm relative to trimethylsilylpropionate (sodium salt, Cambridge Isotope Laboratories, UK). Structural data was collected at 282 K and mixing time in the NOESY spectra was set to 300 msec [15]. All experiments were carried out in the phase-sensitive mode with 4 K complex data points in t_2 dimension with 400 t_1 increments [16]. Spectra were processed and analyzed with the XWINNMR and Aurelia software packages (Bruker Analytisch Messtechnik, GmbH, Germany, version 2.7) on Silicon Graphics Indigo^2 R10000 workstation. Resonance assignment was based on the TOCSY and NOESY spectra measured under the same experimental conditions, according to the sequential assignment methodology developed by Wüthrich [17]. The NOE distance restrains were calibrated relative to fixed distances between two adjacent protons of the tyrosine aromatic ring and between the two β-protons. For the structural calculation, a total of 137 restraints were used for peptide **2**, among which, 82 were intraresidual, 44 sequential and 11 nonsequential signals. Peptide **4** gave 143 restraints comprising 78 intraresidual, 48 sequential and 17 nonsequential. The restrains were classified into strong (1.8–2.5 Å), medium (1.8–3.5 Å) and weak (1.8–5.0 Å). The three-dimensional structures of the peptides were calculated by the hybrid distance geometry–dynamical simulated annealing method using X-PLOR version 3.856 [18]. The pGlu and Pap residues parameters were generated using XPLO2D (version 2.1) with manual modifications for the protons [19]. The NOE energy was introduced as a square well potential with a force constant of 50 kcal/mol × A^2 that was kept constant throughout the protocol. Each round of simulating annealing refinement consisted of 1500 3-fs steps at 1000 K and 3000 1-fs steps during cooling to 300 K. Finally, the structures were minimized using conjugate gradient energy minimization for 4000 iterations. The NMR-derived structures were analyzed using InsightII (Molecular Modeling System version 97.0, Molecular Simulations, Inc.) while their quality was assessed by PROCHECK statistic analysis [20].

RESULTS AND DISCUSSION

Peptide Design

Five linear analogs of GnRH containing a *p*-aminophenylalanine residue in their sequence and their six corresponding azo-bridged cyclic derivatives were synthesized (Table 1).

The cyclic peptides were designed based on two major considerations. Maintaining minimal deviation from the composition and sequence of the parent native GnRH (Table 1) and utilizing structural data of known active cyclic derivatives. Accordingly, only *p*-aminophenylalanine was incorporated as a cyclization tool, and minor site alterations were performed unlike many of the known cyclic GnRH analogs that contain several non-natural amino acids in their sequence.

Table 1 Physical characteristics and binding ability of the synthetic peptides to pituitary GnRH receptors

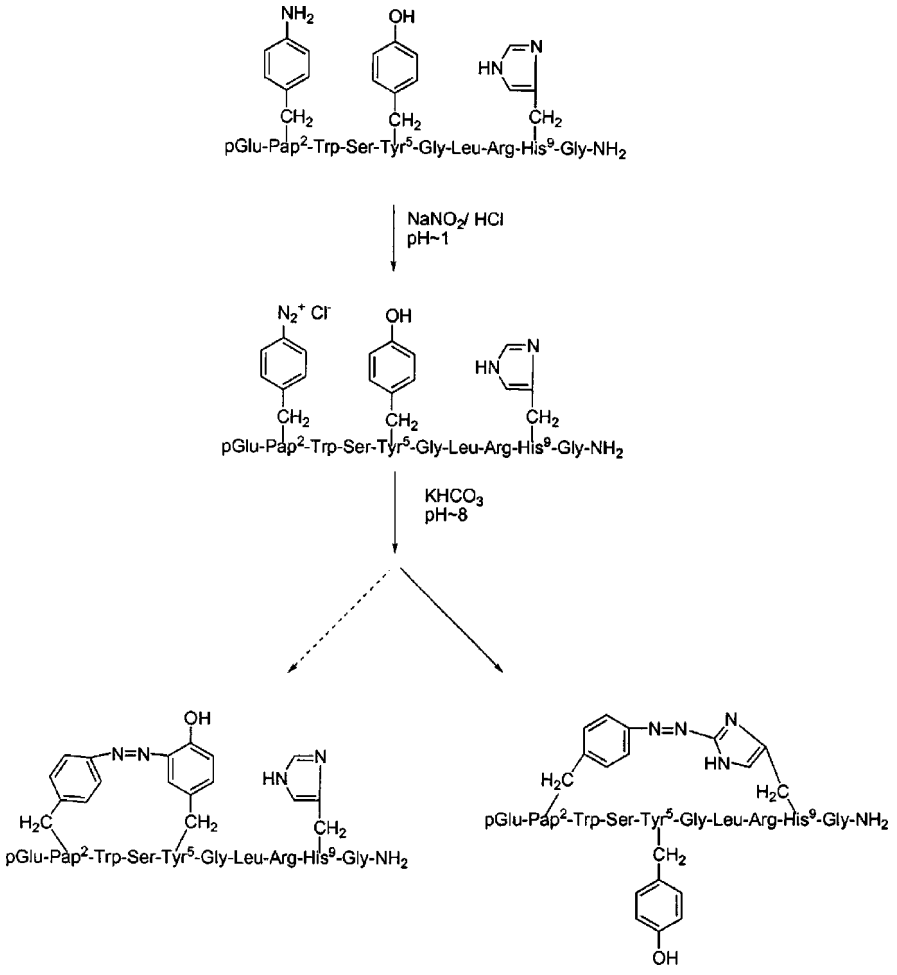
Peptide no.	Compound[a]	Ring size (no. atoms)	Calcul. MH^+	Found MH^+	IC_{50} (M)[b]
GnRH[c]	pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2	—	n.d	n.d	2×10^{-9}
1	pGlu-His-Trp-Ser-**Pap^5**-Gly-Leu-Arg-Pro-Gly-NH_2	—	1181.33	1181.50	4×10^{-9}
2	pGlu-**His^2**-Trp-Ser-**Pap^5**-Gly-Leu-Arg-Pro-Gly-NH_2	22	1192.30	1192.59	3×10^{-7}
3	pGlu-His^2-**Tyr^3**-Ser-**Pap^5**-Gly-Leu-Arg-Pro-Gly-NH_2	—	1158.32	1157.60	5×10^{-9}
4	pGlu-**His^2**-**Tyr^3**-Ser-**Pap^5**-Gly-Leu-Arg-Pro-Gly-NH_2	22	1169.30	1169.35	5×10^{-7}
5	pGlu-**Pap^2**-Trp-Ser-Tyr^5-Gly-Leu-Arg-**His^9**-Gly-NH_2	—	1247.37	1247.40	2×10^{-6}
6	pGlu-**Pap^2**-Trp-Ser-**Tyr^5**-Gly-Leu-Arg-**His^9**-Gly-NH_2	21	1258.35	1258.01	3×10^{-6}
7	pGlu-**Pap^2**-Trp-Ser-Tyr-Gly-Leu-Arg-**His^9**-Gly-NH_2	34	1258.35	1258.62	5×10^{-6}
8	pGlu-His^2-Trp-Ser-**Phe^5**-Gly-Leu-Arg-**Pap^9**-Gly-NH_2	—	1231.40	1231.07	3×10^{-6}
9	pGlu-**His^2**-Trp-Ser-**Phe^5**-Gly-Leu-Arg-**Pap^9**-Gly-NH_2	34	1242.42	1242.04	3×10^{-6}
10	pGlu-His^2-Trp-Ser-**Phe^5**-**dAla**-Leu-Arg-**Pap^9**-Gly-NH_2	—	1255.38	1256.01	3×10^{-6}
11	pGlu-**His^2**-Trp-Ser-**Phe^5**-**dAla**-Leu-Arg-**Pap^9**-Gly-NH_2	34	1244.40	1245.02	2×10^{-6}

[a] Bold letters represent the residues substituted in GnRH and those associated with azo-bridged formation; Pap = L-*p*-aminophenylalanine.
[b] Results are the mean of two-binding-competition experiments carried out in triplicate with standard deviations of ±10%.
[c] Commercial GnRH (Sigma, St. Louis, MO) was employed.

It is generally accepted that GnRH assumes a definitive β-turn at the Gly^6-Leu^7 site [21–23]. The existence of this unique structural entity in a cyclic GnRH analog was also reported [24,25]. Further, NMR studies of GnRH revealed close proximity of residues 4 and 9, and, indeed 4–9 amide-bridged derivatives were found to possess substantial receptor binding affinities [26], 4–10 bridging yielded even more avidly binding derivatives, i.e. antagonists [25]. A 5–8 bridged analog of GnRH revealed, an internal β-turn configuration as inferred from NMR and molecular dynamics calculations, as well as close proximity of amino acids residues 1–10 [27]. Adjacency between residues 1 and 3 within the *N*-terminal of a constrained cyclic analog of GnRH was reported [5]. Finally, an association of the side chains of residues 2, 5 and 8 in native GnRH was suggested, based on fluorescence measurements [28].

In view of the above, introduction of constraint at the *N*-terminal domain of GnRH was performed through 2–5 azo-cyclization (peptides **2**, **4** and **6**, Table 1), whereas *N*- and *C*-terminals proximity resulted through 2–9 azo-cyclization (peptides **7**, **9** and **11**, Table 1). Further, a d-Ala residue was introduced at position 6 of peptide **10**, to enhance possible β-turn formation.

In a preceding publication, a GnRH analog containing both His and Tyr as well as *p*-aminophenylalnine residues, i.e. pGlu-His^2-Tyr^3-Ser-Pap^5-Gly-Leu-Arg-Pro-Gly-NH_2 was prepared as a precyclic derivative for azo-bridge cyclization [9]. Potentially, both His^2-Pap^5 and Tyr^3-Pap^5 azo conjugates were anticipated. However, cyclization yielded primarily the His^2-Pap^5 cyclic derivative while only traces of the Tyr^3-Pap^5 cyclic compound was detected. The preferred cyclization through the His residue was attributed to a possible unique ring-sized dependence. Thus, a 22-atom ring in the former peptide was more easily formed than an 18-atom ring of the latter. In the present study, we have repeated the synthetic procedure and revealed the exclusive formation of a His^2-Pap^5 cyclic peptide (number **4** in Table 1) in high yield. To further investigate the above issue peptide **5** (Table 1), i.e. pGlu-Pap^2-Trp-Ser-Tyr^5-Gly-Leu-Arg-His^9-Gly-NH_2 was prepared. Its azo-cyclization yielded the two cyclic products, **6** and **7** (Table 1) containing Pap^2-Tyr^5 (21 atoms ring) and Pap^2-His^9 (34



Scheme 1 Schematic representation of azo-cyclization of linear peptide **5**, to obtain peptide **6** (dashed line) and peptide **7** (full line). The full line indicates that this is the favored route with 60% yield, whereas the dashed line denotes that this route is less favored with only 40% yield.

atoms ring) azo conjugates in 40% and 60% yields, respectively (Scheme 1). It seems therefore that azo-ring formation is indeed dependant, among other factors, on ring-size parameters, and no His/Tyr selective azo cyclization prevailed.

Peptide Synthesis

The linear peptide chains were assembled, automatically, by the solid-phase strategy. Following simultaneous detach from the polymeric support and deprotection, the crude peptides (as a rule >85% purity) were cyclized (post lyophylization) in an aqueous solution through diazotization of the aromatic *p*-amino moiety and intramolecular coupling of the resulting diazonium salt with either His or Tyr residues present within the peptide chain. Conversions of the parent precyclic compounds to the corresponding cyclic ones were rather quantitative. The various linear as well as cyclic peptides were purified by semipreparative HPLC prior to the physico–chemical and biological evaluations. Purity was ascertained by analytical HPLC, and further substantiated by MS and amino acid analysis. Thus, >98% purity was revealed by HPLC and m/z values were found as calculated (Table 1). Amino acid ratios were found to be very close to the expected values, while those of His and Tyr at the corresponding azo-joints in the cyclic peptides were of background levels (Table 2).

Biological Activity

In order to evaluate the selective binding ability of the synthetic linear and cyclic peptides to the GnRH receptors, a competitive binding assay was performed with all peptides on rat pituitary membranes expressing these receptors (not shown). Linear analogs **1** and **3** were found to bind to the GnRH receptors only slightly less avidly than native GnRH (Table 1). It is thus apparent that substitution of Tyr^5 by Pap (peptides **1** and **3**) and Trp^3 by Tyr (peptide **3**) does not significantly alter the association with the receptor. Cyclization, however, led to a marked reduction in the binding capacity, i.e. from 10^{-9} M to the 10^{-7} M range as inferred from the binding activity of the corresponding cyclic derivatives **2** and **4** (Table 1). All other linear and cyclic peptides, listed in Table 1, bind selectively to the GnRH receptor only in the low μM range.

Functionality of binding was asserted through the capacity of the peptides to augment LH release from dispersed rat pituitary cells. Thus, only peptide **1**, i.e. [Pap^5] GnRH was found comparable to native GnRH. Both peptides, at concentration of 10^{-9} M led to 10–15 folds of basal (~5 ng LH/ml; see *Materials and Methods section*) LH release. At 5×10^{-7} M, peptide **1** exhibited activity of >20 folds of basal release (not shown). Cyclization of peptide **1** resulted, however, in a dramatic decrease of activity. The respective cyclic peptide **2** enhanced LH–basal release only ~1.5–2 folds at 10^{-9} M and 5–7 folds at 5×10^{-7} M. The decrease in bioactivity is therefore in line with the substantially diminished binding ability. Similarly, the ability of all other peptides listed in Table 1, to augment LH–basal release, although detectable, was very small (~1.5–2 folds of basal LH release at 5×10^{-7} M) and not significant again, in line with their poor receptor binding ability.

Linear peptide **8**, i.e. [His^2, Phe^5, Pap^9] GnRH exhibited moderate LH-release activity of ~5 folds and ~3.5 folds of basal levels at concentration of 1×10^{-7} M and 1×10^{-9} M, respectively. Azo-cyclization, however, led to a marked reduction of activity as indicated from the LH-release levels of peptide **9** (~1.5 folds of basal level at 5×10^{-7} M). Both peptide **10**, i.e. [Phe^5, d-Ala^6, Pap^9] GnRH, intended to enhance formation of β-structure, as well as its derived cyclic peptide **11** exhibited very low potency.

Comparison of the LH-releasing ability of peptides **1** and **3**, revealed that, although both peptides exhibit high affinity binding to the pituitary receptor i.e. almost

Table 2 Amino acid composition of the synthesized peptides[a]

Peptide No.	Glu	His	Ser	Tyr	Gly	Leu	Arg	Pro	Phe	Ala
1	(1) 1.00	(1) 0.98	(1) 0.94	—	(2) 2.04	(1) 1.00	(1) 1.03	(1) 1.00	—	—
2	(1) 0.95	0.00	(1) 0.80	—	(2) 2.13	(1) 1.01	(1) 1.05	(1) 1.00	—	—
3	(1) 1.00	(1) 0.95	(1) 0.94	(1) 0.93	(2) 2.02	(1) 1.00	(1) 1.03	(1) 1.07	—	—
4	(1) 0.94	(0) 0.07	(1) 0.85	(1) 0.88	(2) 2.16	(1) 1.04	(1) 1.08	(1) 1.00	—	—
5	(1) 1.07	(1) 0.91	(1) 0.94	(1) 0.94	(2) 2.09	(1) 1.00	(1) 1.09	—	—	—
6	(1) 1.00	(1) 0.93	(1) 0.90	(0) 0.04	(2) 2.06	(1) 1.00	(1) 1.09	(1) 0.97	—	—
7	(1) 1.00	(0) 0.00	(1) 0.90	(1) 0.06	(2) 2.04	(1) 1.00	(1) 1.06	(1) 1.06	—	—
8	(1) 1.05	(1) 1.10	(1) 0.94	–	(2) 2.09	(1) 1.00	(1) 1.10	–	(1) 1.00	–
9	(1) 1.07	(0) 0.06	(1) 0.93	–	(2) 2.15	(1) 1.00	(1) 1.10	–	(1) 1.00	–
10	(1) 1.00	(1) 0.97	(1) 0.92	–	(1) 1.01	(1) 1.00	(1) 1.12	–	(1) 1.03	(1) 1.06
11	(1) 0.96	(0) 0.02	(1) 0.97	–	(1) 1.00	(1) 1.03	(1) 1.11	–	(1) 1.09	(1) 1.00

[a] *p*-aminophenylalanine (Pap) and tryptophan were not determined.

as active as the parent GnRH, peptide **3** is practically devoid of LH-release activity, whereas **1** is equipotent to the native peptide in this respect. The only difference between peptides **1** and **3** is present in position three, i.e. Trp3 in peptide **1**, like GnRH, and Tyr3 in peptide **3**. The strict divergence in bioactivity between peptides **1** and **3** stands in line with previous observations that Tyr3-GnRH and Phe3-GnRH possess only 0.1% and 0.3% GnRH-like activity, respectively [29,30]. Thus, it appears that the presence of Trp in position three is crucial for expression of bioactivity, presumably due to its ability to form charge-transfer complexes [28]. This assumption is strengthened by the fact that substitution of Trp3 by pentamethyl-Phe preserves 30–70% of the GnRH activity [29–31]. Cyclization of peptides **1** and **3** led to cyclic-azo **2** and **4**, respectively, both having a 22-member ring. Similar activity trends were observed for the cyclic derivatives; namely, peptide **2** exhibited moderate LH-releasing activity whereas peptide **4** was devoid of this capacity. The rather strong association of peptide **3** with the pituitary receptor may suggest that it has an antagonistic activity. This possibility will be examined in future experiments.

Metabolic Stability

In order to evaluate the impact of azo-cyclization on the metabolic stability of the GnRH analogs, the resistance of linear peptides **1** and **3**, their cyclic counterparts **2** and **4** and native GnRH, toward proteolysis by enzymes was examined. This was performed by means of analytical HPLC monitoring of residual amounts of peptides left after various time-intervals of incubation with cytosolic enzymes isolated from pituitary glands of ovariectomyzed female rats. Preliminary results thus obtained reveal that all four Pap[5]-containing peptides were much more proteolytically stable than GnRH. Thus, after 1 h of incubation, residual GnRH and peptides **1**–**4** amounted to 29, 72, 70 and 45%, respectively. Following 3 h of incubation, GnRH was fully degraded, whereas levels of remaining peptides **1**–**4** were 22, 71, 45 and 40%, respectively. Peptides **1** and **3** were not entirely degraded even after 7 h of incubation. The relative stability of peptides **1**–**4** is perhaps not surprising in view of the fact that position 5 of GnRH is known to reside within the proteolytically, i.e. by endopeptidases, sensitive domain of GnRH [32,33]. The fact that linear peptides **1** and **3** are more stable than their cyclic derivatives may indicate that the patterns of their enzymatic degradation are different.

Structure Analyses

CD. Peptides **1** and **3**, the most avid GnRH receptor binding analogs, their corresponding cyclic-azo derivatives peptides **2** and **4**, and native GnRH were examined by CD spectroscopy for their conformational properties in phosphate buffered saline (PBS, pH 7.4). The CD spectra (not shown) reveal that GnRH as well as linear peptide **3** and its derived azo-bridged cyclic peptide **4**, possess an unordered structure. Peptide **1**, pGlu-His-Trp-Ser-Pap-Gly-Leu-Arg-Pro-Gly-NH_2 exhibit a certain bent structure, which is dramatically enhanced upon intramolecular azo-bridging as apparent in a negative band in the region of 218–224 nm ($\Theta = -6$ and -23, respectively) and a positive band at 202–204 nm ($\Theta = +3$ and $+15$, respectively), in the CD spectra of peptide **2**. The rather unexpected structural, and to certain extent functional, differences above between peptides **1** and **3** and their respective cyclic azo-derivatives peptides **2** and **4** have prompted us to investigate the structure of the latter two cyclic compounds by NMR.

NMR. Cyclic analogs **2** and **4** differ only in the identity of the amino acid residue at position 3 of their sequence,

Table 3 Proton chemical shift (ppm) of peptides **2** and **4**

Peptide **2** Residue	HN	Hα	Hβ	Others
pGlu[1]	7.95	4.25	2.41 1.91	γCH_2: 2.33
His[2]	8.90	4.52	3.75 2.98	Not detected
Trp[3]	7.36	4.68	3.01 2.68	2H: 6.53; 4H:7.47; 5H:7.14; 6H:6.85; 7H:7.05; NH:10.00
Ser[4]	7.93	4.34	3.66	
Phe[5]	8.67	4.65	3.35 2.75	2,6H: 7.30, 3,5H:7.57
Gly[6]	8.50	3.89		
Leu[7]	8.35	4.26	1.52	γCH_2: 1.44, δCH_3:0.78
Arg[8]	8.53	4.55	1.72 1.62	γCH_2: 1.55, δCH_2: 3.10, εNH: 7.16
Pro[9]		4.33	2.20 1.83	γCH_2: 1.94, δCH_2: 3.74 3.53
Gly[10]	8.67	3.79		
Peptide **4**				
pGlu[1]	7.97	4.28	1.93	γCH_2: 2.46 2.36
His[2]	8.88	4.55	3.87 3.12	4H: 7.15, 3H: 7.52
Tyr[3]	7.54	4.57	2.83 2.04	2,6H: 6.65; 3,5H:6.58
Ser[4]	7.95	4.37	3.69	
Phe[5]	8.68	4.71	3.40 2.83	2,6H: 7.37, 3,5H:7.68
Gly[6]	8.52	3.94		
Leu[7]	8.35	4.33	1.58	γCH_2: 1.52, δCH_3:0.85
Arg[8]	8.54	4.61	1.80 1.70	γCH_2: 1.62, δCH_2: 3.16, εNH: 7.19
Pro[9]		4.39	2.26 2.00	γCH_2: 1.89, δCH_2: 3.81 3.58
Gly[10]	8.67	3.87		

namely, Trp and Tyr in peptide **2** and **4** respectively. Accordingly their corresponding TOCSY and NOESY spectra showed very similar spectral features with only minor variations in the chemical shifts (Table 3) and the intensities of several NOEs connectivities (Figure 1). The fact that a large number of NOE cross-peaks were detected indicates that both peptides are well structured in solution. All the observed connecting stretches were found between the amino acids located within the ring or in its close vicinity, whereas, no interresidual NOE interactions were detected at the *C*-terminus of both peptides due to the high flexibility of this region. Continuous stretches of $d_{HNH\alpha(i,i+1)}$ connectivities between pGlu[1] and Gly[6] and $d_{HNH\beta(i,i+1)}$ and $d_{HNH\beta(i,i+2)}$ connectivities between pGlu[1] and Leu[7] were observed for both peptide **2** and **4**, whereas the latter had an additional continuous stretch of $d_{HNHN(i,i+1)}$ in the same region. A continues stretch of $d_{HNH\alpha(i,i+2)}$ was detected between Ser[4] and Arg[8] of peptide **2** which may suggest on the presence of a β-turn at this part. However, a $d_{HNHa(i,i+3)}$ connectivity which is generally present in β-turns was not found in this peptide, rather $d_{HNH\beta(i,i+3)}$ cross peaks between Ser[4] and Leu[7], and Pap[5] and Arg[8] were observed. The $^3J_{HNH\alpha}$ coupling constants in this region were similar to the theoretical ones of β-turn type I' [17]. Although similar structural element was not observed for peptide **4**, it showed a $d_{HNH\alpha(i,i+3)}$ cross peak between Pap[5] and Arg[8] with relative intensity between 2.8 Å and 3.8 Å.

The final simulated annealing stage, performed using the XPLOR program, yielded 47 and 85 low-energy structures for peptide **2** and **4**, respectively, which had no NOE restraint violations above 0.5 Å, no bond-length violations above 0.05 Å and no bond-angle violations above 5°. The low-energy structures of peptide **2** could be divided into three ensembles based on the differences in their total energies; namely, one set containing 20 low-energy structures, with r.m.s.d. values of 0.25 and 0.87 Å for the backbone and side chains atoms respectively (Figure 2), and two other sets, which were characterized by rather high total energies that differed from the first set by 15–30 kJ/mol (not shown). Although this cyclic peptide was found to coexist in several energetically different conformations in solution, a common β-turn structure was identified for all ensembles between residues Ser[4]-Leu[7] through comparison of the experimental dihedral ϕ and φ angles and the accepted canonical values. Thus, evidently, peptide **2** exhibits at this part of the sequence similar conformation to native GnRH, which possess a type II' β-turn between residues Tyr[5] and Arg[8] [21–23]. In regard to the azo bond, again, the three ensembles behaved similarly, namely, all NMR-derived conformers indicated clearly that the bond is in its *cis* conformation. Further verification of this aspect by intraresidual connectivity analysis was, however, not possible due to the small ring size. Thus, numerous

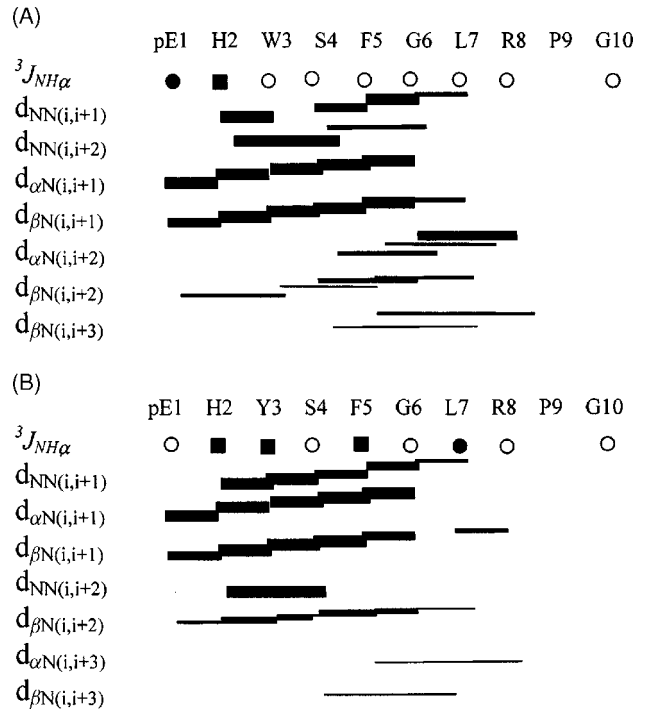


Figure 1 Summary of the NOE connectivities and $^3J_{HNH\alpha}$ coupling constants observed for peptide **2** (panel A) and **4** (panel B) in water. Coupling constants are indicated with *filled circles* for $^3J_{HNH\alpha}$ smaller than 5 Hz, *open circles* for 5 Hz $<^3J_{HNH\alpha}<7.5$, and *filled squares* for $^3J_{HNH\alpha}$ larger than 8 Hz. The width of the NOE connectivities is proportional to its intensity ranging from strong to very weak signals.

interactions of aromatic protons, with the backbone and between themselves were identified. The most prevalent hydrogen bonds were found between the carbonyl oxygen and the backbone amide proton, in the low-energy structures between Ser[4]-Leu[7] and in the high-energy sets between Ser[4]-Gly[6] and Arg[8]-Gly[10]. In the high-energy structures, another hydrogen bond was identified between the azo bond at Pap[5] and Arg[8] side-chain. In both low- and high-energy conformers, the hydrogen bonds stabilize the β-turn structure at Ser[4]-Leu[7].

The 85 low-energy structures of peptide **4** could be divided into two well-defined subsets with a total energy

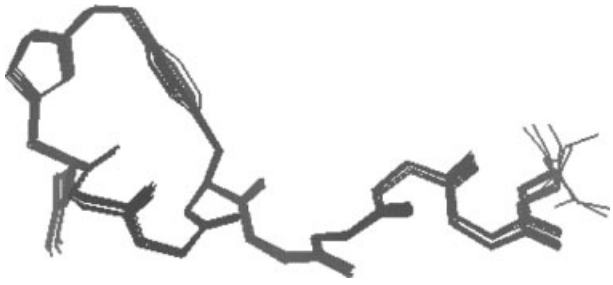


Figure 2 Superposition of 10 low-energy structures of peptide **2**.

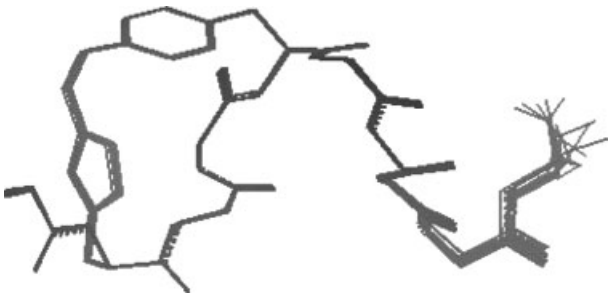


Figure 3 Superposition of 15 low-energy structures of peptide **4**.

difference of 15–35 kJ/mol (Figure 3, low-energy set). Despite this energy gap, both ensembles show excellent convergence, and straightforward comparison of their three-dimensional structures revealed a striking similarity between them, namely, the two subsets differ only in the relative orientation of the four amino acid rings with respect to the linear fragment of the peptide. The r.m.s.d. values calculated for the 34 low-energy set were 0.21 Å for the backbone and 0.60 Å for the side-chains atoms, while the high-energy set showed r.m.s.d. values of 0.47 Å and 1.08 Å for the backbone- and heavy atoms, respectively. In resemblance to peptide **2**, β-turn structures were identified in both ensembles of peptide **4**. The β-turn in the low-energy conformer was located between residues Tyr^3 and Arg^8 and was identified as a type I β-turn on the basis of the observed dihedral angles. Whereas, in the high-energy set, the β-turn was located between residues Ser^4 and Arg^8 and the ϕ and φ angles in this region were found to be very close to the theoretical angles of β-turn type I'. Peptide **4** resembles peptide **2** also in its azo bond conformation. Thus, by the same considerations described for peptide **2**, a cis azo bond is proposed for peptide **4**. Hydrogen bonds were found in the low-energy conformers, between the carbonyl oxygen and the backbone amide proton of Tyr^3-Arg^8, Tyr^3-Pap^5 and Ser^4-Gly^6 while in the high-energy conformers they were found between the carbonyl oxygen and the backbone amide proton of Tyr^3-Pap^5, Ser^4-Leu^7 and Ser^4-Arg^8. The backbone of peptide **4** is folded in a very particular manner. In both conformers it forms three semicircles that cross at residues Ser^4, Gly^6 and Arg^8 (Figure 3). This organization brings the side chains of $pGlu^1$ and Arg^8 of the high energy set to the vicinity of approximately 2.7 Å, thus allowing the formation of a salt bridge between the corresponding CO and NH, which stabilizes this high-energy conformer in a very compact, nearly spherical structure (not shown). On the other hand, in the low-energy set, the linear, *C*-terminus part of the peptide points to the direction opposite to the azo linkage and the total conformer structure is more open, and less constrained (Figures 3 and 4B). Peptide **2** conformers fold in a less defined three-dimensional structure than peptide **4**. However, their molecular organization shows similar trends, the high-energy structures are rather compact since the β-turn brings the four amino acid rings relatively close to the linear part of the peptide (not shown), whereas, the low-energy conformers exhibits an open, elongated structure in which the ring is separated from the *C*-terminus part (Figures 2 and 4A). In this conformer, the bulky tryptophane side-chain is exposed to the solvent such that steric hindrance within the peptide is prevented and the molecular organization stabilized (Figure 4A).

Although, in general, peptides **2** and **4** show similar spatial differences between their low- and high-energy structures, these differences are much more pronounced within peptide **2**. Thus, the low-energy structures of peptide **2** are more open and its residues better exposed to functional binding with the receptor, as compared to the low-energy conformers of peptide **4** (Figure 4). It is likely that these conformational differences are responsible, at least partially, for the difference in bioactivity present between azo-peptides **2** and **4**. However, since the precyclic peptides, which differ only at position 3 and exist in multiple conformations in solution, already exhibit markedly different activity, it is reasonable to believe that the presence of the available Trp at position 3, rather than the difference in spatial organization, plays as the major factor affecting the activity of the cyclic peptides.

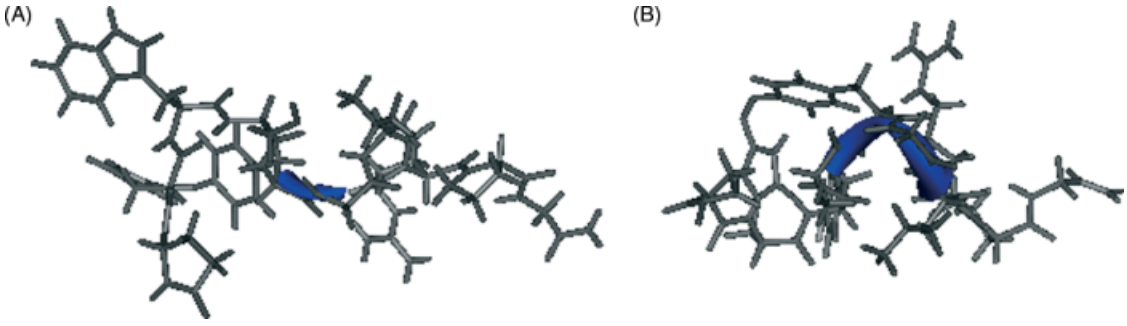


Figure 4 Superposition of the low-energy conformers of peptide **2** (A) and peptide **4** (B). The β-turn is presented by the blue arrow.

CONCLUSIONS

The present study demonstrates the successful application of a novel synthetic route to the synthesis of a new family of cyclic GnRH analogs, i.e. azo-bridged molecules. The method is general and may be employed to extend and diversify libraries of cyclic bioactive peptides. It is clear though, that cyclization, which leads to enhanced molecular rigidity, does not necessarily result in a parallel increase in receptor recognition and higher biopotency. Thus, on the one hand cyclization can force a linear peptide to obtain rather stable well-defined configuration in solution, while on the other, it may lead to loss of flexibility. Consequently, the cyclic peptide can not experience the small structural modulations necessary for efficient receptor binding and activation. Peptide **1**, [Pap^5]GnRH, albeit a linear one, exhibited avid receptor affinity, substantial LH-release activity and high metabolic stability, which suggest that it is a potentially good agonist of the parent peptide. Its implication to prolonged in vivo activity remains to be examined.

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