Antagonists of the Luteinizing Hormone Releasing Hormone with Emphasis on Amino Acids in Position Five

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Seventeen analogs of the luteinizing hormone releasing hormone (LHRH) have been synthesized, bioassayed, and compared for antiovulatory activity (AOA) in rats. The emphasis of design was replacement of Tyr5 of LHRH. Position 5 has not been extensively studied. [N- Ac-D-2-Nal5, D- pCIPhe5, D-3-Pal5, D- Arg5, D- Ala5]-LHRH was the baseline for new designs. Comparison of the AOA's of the 17 analogs with the baseline revealed the two peptides with Phe5 and 3-Pal5 had equivalent AOA's, and were the best of the 17, and about 45% more potent than the baseline. Analogs with pCIPhe5, oCIPhe5, o-MepCIPhe5, 2-Nal5, Trp5, and His5 were less potent than the Phe5- and 3-Pal5- analogs. Based on the Phe5-analog, eight other analogs were synthesized with changes in positions 1, 2, 3 and 7 and although none were better than the baseline, 5/8 showed 20–60% AOA's at 250 ng and revealed optimum positions for new designs.

Introduction

Investigators since about 1971, have synthesized and bioassayed about 1800 analogs of the luteinizing hormone releasing hormone (LHRH) toward achieving super-agonists and effective antagonists for the control of conception. This very large number of analogs stemmed largely from international and academic investigators; the very large number of analogs which have been industrially achieved is unknown. The present goals for antagonists, having antiovulatory activity, are even higher potency, effective duration of action, and no toxicity. From this enormous literature, the following investigations are cited to exemplify recent progress, and to provide background for the new analogs herein.

Folkers et al. [1] found that antagonists of LHRH with pyridyl-alanines completely inhibited ovulation in rats at ng dosage. D-3-Pal was frequently superior to D-Trp. Analogs having a moiety of P-(3-quinolyl)-D-α-alanine in positions 3 and 6 were synthesized. D-3-Qalβ was as effective as D-Trpβ in certain analogs; Folkers et al. [2].


Experimental

Materials

The amino acid derivatives were from Peninsula Laboratories, San Carlos, CA. α-Amino functions were protected by the Boc-group. Side-chains were protected by benzyl for serine and tyrosine, and tosyl for arginine and histidine. L- and D-Boc-β-(2-naphthyl)-alanine, L- and D-Boc-β-p-chlorophenylalanine, L- and D-Boc-β-(3-pyridyl)-alanine and Boc-3,4-di-chloro-D-phenylalanine were from the Southwest Foundation for Research and Education, San Antonio, TX. Boc-β-methyl-p-chlorophenylalanine was kindly supplied by the Department of Biochemistry, Indiana University School of Medicine. a-Methyltyrosine was from Sigma Chemical Company, St. Louis, MO., and its Boc-derivative was prepared in this laboratory. DL-α-Chlorophenylalanine was from Chemalog, Chemical Dynamics Corporation, South Plainfield, NJ. It was resolved as described [7]. Dicyclohexylcarbodiimide, triethylamine, and the solvents were distilled prior to use. Benzyldihydroxylamine (BHA) resin hydrochloride was from Beckman Bioproducts, Palo Alto, CA. The milliequivalent was 0.845/g.

Synthesis

The peptides were synthesized (solid-phase) with a Beckman Model 990 Peptide Synthesizer. The first
amino acid was attached to the resin, and the coupling programs were essentially as described [8, 9]. The resin, after the first amino acid was attached, was acetylated by 25% acetic anhydride in dichloromethane in the presence of pyridine in a reaction time of 20 min. The peptides were removed from the resin by treatment with doubly distilled (CoF$_3$) HF containing ca. 10% anisole for 1 h at 0 °C.

**Purification and purity**

After HF-cleavage, the peptides were lyophilized; the yield was about 300-400 mg from 0.5 g of resin. The crude peptide (200 mg), was purified on a column packed with 50 g silica gel. Elution was with either n-BuOH:HoAc:H$_2$O (4:1:5), upper phase or n-BuOH: n-BuOH:HoAC:H$_2$O (2:8:2:3). The purified peptides (ca. 50 mg), as detected by silica gel TLC, were further purified by high pressure liquid chromatography on column of µ-Bondpak C$_{18}$ in various linear gradients of buffer A (0.1 m ammonium acetate pH 5.0) and buffer B (20% buffer A+80% acetonitrile), or of buffer A (0.1 m ammonium formate pH 4.0) and buffer B (10% buffer A+90% acetonitrile).

The peptides, after HPLC purification, were visualized as single-spots on silica gel TLC (Merck) plates in the following solvent systems:

1. n-BuOH:EtoAc:HoAc:H$_2$O (5:5:1:3);
2. n-BuOH:HoAc:H$_2$O (4:1:5) upper phase;
3. n-BuOAc:n-BuOH:HoAc:H$_2$O (2:8:2:3);
4. n-BuOH:HoAc:H$_2$O (2:8:2:3);

Purity was determined by analytical high pressure liquid chromatography in the following solvent systems:

1. A. 0.01 M KH$_2$PO$_4$ pH 3.0; B. 20% A+80% CH$_3$CN, linear gradient 40-100% B/20 min.
2. A. 0.01 M KH$_2$PO$_4$ pH 3.0; B. 20% A+80% CH$_3$CN, linear gradient 0-100% B/20 min.
3. A. 0.1 M NH$_4$OAc pH 5.0; B. 40% A+60%, CH$_3$CN, linear gradient 40-100% B/25 min.
4. A. 0.01 M KH$_2$PO$_4$ pH 3.0; B. 20% A+80% CH$_3$CN, linear gradient 40-100% B/15 min.

**Amino acid analyses**

The amino acid analyses, after conventional acid hydrolyses, were performed on a Beckman 118 CL Automatic Amino Acid Analyzer with a Hewlett Packard 3390 A intergrator. Tryptophan and unnatural amino acids were qualitatively determined. The chemical data, and the amino acid data on the peptides are in Tables II and III.

**Biological assays**

The peptides were bioassayed for activity to inhibit ovulation in rats [10]. The data from the bioassays are in Table I.

<table>
<thead>
<tr>
<th>No.</th>
<th>[N—Ac—D-2-Nal$^1$, D-pClPhe$^2$, D-3-Pal$^3$, Tyr$^5$, D-Arg$^6$, D-Ala$^{19}$]-LHRH*</th>
<th>Dose (µg)</th>
<th>AOA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>[N—Ac—D-2-Nal$^1$, D-pClPhe$^2$, D-3-Pal$^3$, Tyr$^5$, D-Arg$^6$, D-Ala$^{19}$]-LHRH*</td>
<td>0.5</td>
<td>100</td>
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<tr>
<td>2.</td>
<td>a-Me—Tyr$^5$</td>
<td>0.25</td>
<td>57</td>
</tr>
<tr>
<td>3.</td>
<td>pClPhe$^5$</td>
<td>0.25</td>
<td>83</td>
</tr>
<tr>
<td>4.</td>
<td>a-Me-pClPhe$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>5.</td>
<td>o-ClPhe$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>6.</td>
<td>Phe$^5$</td>
<td>0.25</td>
<td>83</td>
</tr>
<tr>
<td>7.</td>
<td>2-Nal$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>8.</td>
<td>3-Pal$^5$</td>
<td>0.25</td>
<td>83</td>
</tr>
<tr>
<td>9.</td>
<td>Trp$^5$</td>
<td>0.25</td>
<td>12</td>
</tr>
<tr>
<td>10.</td>
<td>His$^5$</td>
<td>0.25</td>
<td>11</td>
</tr>
<tr>
<td>11.</td>
<td>Phe$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>12.</td>
<td>Phe$^5$</td>
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<tr>
<td>13.</td>
<td>Phe$^5$</td>
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<td>0</td>
</tr>
<tr>
<td>14.</td>
<td>Phe$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>15.</td>
<td>Phe$^5$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>16.</td>
<td>[N—Ac-3,4-diCl—D—Phe$^1$</td>
<td>0.25</td>
<td>43</td>
</tr>
<tr>
<td>17.</td>
<td>D—Phe$^3$</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td>18.</td>
<td>D—Phe$^3$</td>
<td>0.25</td>
<td>0</td>
</tr>
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</table>

* Reference [6].
Results and Discussion

The data in Table I are on 17 analogs of LHRH which were synthesized and bioassayed for antiovulatory activity (AOA) in rats. The primary emphasis in the design of these analogs was to make substitutions for the intrinsic Tyr\(^5\) of LHRH. As the base line, analog 1 was selected which has the intrinsic Tyr\(^5\) of LHRH, and is one of the most potent known antagonists; it caused 100% AOA at 0.5 \(\mu\)g and 57% AOA at 0.25 \(\mu\)g. It is known in this field of research that antagonists which show 100% AOA in the range of 0.25—0.5 \(\mu\)g are recently among the best. In this field, substitutions in position 5 of highly potent antagonists have been less explored than those for positions 1, 2, 3, 6, 10, etc.

The L configuration was maintained for the substitutions in position 5 of the 17 analogs. McDermott et al. [11] investigated the mechanism of degradation of LHRH by synaptosomal peptidases. The synaptosomes were isolated from rat hypothalamus cortex. It was found that LHRH was cleaved at Tyr\(^5\)—Gly\(^6\) and Pro\(^9\)—Gly\(^10\).
The introduction of α-MeTyr$^5$ toward surprising enzymic cleavage between positions 5 and 6 decreased activity to about one-half in comparison with the base line antagonist. Roeske et al. [12] found that an antagonist with D-α-Me-Trp$^3$ had only 10% of the activity of the parent compound. Also, the presence of α-Me-D-Phe$^2$ and α-Me-D-Trp$^3$ and α-Me-Phe$^2$ and α-Me-Trp$^3$ in two antagonists had significantly decreased activity.

Introducing pClPhe$^5$ was less effective than Tyr$^5$; 100% AOA at 1 μg. α-MepClPhe$^5$ was less effective than pClPhe$^5$; zero AOA at 1 μg. oClPhe$^5$ showed zero AOA at 0.25 μg. Phe$^5$ was superior to Tyr$^5$; 83%/0.25 μg. Phe$^5$ was superior to pClPhe$^5$, oClPhe$^5$ and α-MepClPhe$^5$.

2-Nal$^5$ was less effective, zero AOA/0.25 μg, than Tyr$^5$ or Phe$^5$, but 3-Pal$^5$ was equivalent to Phe$^5$ which was better than Tyr$^5$.

Trp$^5$ and His$^5$ were less effective than Tyr$^5$ but retained activity; 11–12% AOA/0.25 μg.

Analogs 10–17 maintained the effective Phe$^5$, but included other substitutions in positions 1, 2, 3 and 7.

Analogs 11–15 maintained Phe$^5$, but the intrinsic Leu$^7$ of LHRH was replaced by Phe$^7$, Trp$^7$, 3-Pal$^7$, Ile$^7$ and Val$^7$. The Phe$^5$–Phe$^7$ analog 11 showed zero AOA at 0.25 μg, but the other four analogs showed AOA ranging from 20–60% at 0.25 μg, and are a basis for still newer designs. The best of these five analogs had the substitution of Phe$^5$, Ile$^7$. Comparison of analogs 6, 14 and 15 shows that Ile$^7$ and Val$^7$ are less effective than the intrinsic Leu$^7$ of LHRH.

In the background of this research, N–Ac-3,4-diCl–D–Phe$^1$ has sometimes been comparable to N–Ac–D-2-Nal$^1$, but comparison of the baseline analog 1 with analog 16 shows that N–Ac-3,4-diCl–D–Phe$^1$ was much less effective. D–Phe$^5$, Phe$^5$ unit was less effective than D-3-Pal$^5$, Phe$^5$ on the basis of 43% AOA vs. 83%/0.25 μg. The combination of D–Phe$^5$, Phe$^5$, Phe$^7$ was not effective; zero AOA/0.25 μg.

In summary, of all the substitutions in position 5 of the 18 analogs including the baseline, Phe$^5$ was ca. 45% more effective than the intrinsic Tyr$^5$; 83% AOA vs. 57%.

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