# STEREOISOMERS OF LUTEINIZING HORMONE-RELEASING HORMONE

Yoshihiro Hirotsu, David H. Coy*, Esther J. Coy, and Andrew V. Schally

Department of Medicine, Tulane University School of Medicine, and
Veterans Administration Hospital, New Orleans, Louisiana 70146

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Summary: Six decapeptide analogs of the luteinizing hormone-releasing hormone (LH-RH) were prepared with the D-isomers of pyroglutamic acid, histidine, tryptophan, tyrosine, leucine, and arginine successively replacing the corresponding L-amino acids of the hormone. [D-pGlu$^1$]-LH-RH and [D-His$^2$]-LH-RH had appreciable LH-releasing activities, 8% and 9%, respectively. The remaining analogs were less potent, having the following activities: [D-Trp$^3$]-LH-RH, 0.1%; [D-Tyr$^5$]-LH-RH, 0.1%; [D-Leu$^7$]-LH-RH, 1.0%; [D-Arg$^8$]-LH-RH, 0.3%.

The incorporation of D-amino acids into a peptide can have profound effects on the overall conformation of the molecule and, hence, its biological activity. In the case of LH-RH this has been dramatically demonstrated by the synthesis of [D-Ala$^6$]-LH-RH (1) and [D-Ala$^6$, desGly$^{10}$]-LH-RH ethylamide (2). These peptides are much more active than the natural hormone in a variety of assay systems. It was, therefore, of interest to synthesize and examine LH-RH analogs in which certain other residues were replaced by D-amino acids. In the study described here pyroglutamic acid, histidine, tryptophan, tyrosine, leucine, and arginine were replaced by the corresponding D-amino acids.

## Synthesis and Characterization

With the exception of D-pyroglutamic acid, which was synthesized by the method of Flouret et al. (3), all L- and D-amino acid derivatives were purchased from Bachem, Inc., Marina del Rey, California. The automated

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* Reprint requests should be directed to D.H. Coy.

Table I

Properties of LH-RH Stereoisomers

| LH-RH Isomer | [α]D (degrees) in 0.1 M AcOH | $R_f$(I)[a] | $R_f$(II) | $R_f$(III) | $R_f$(IV) |
|---|---|---|---|---|---|
| D-pGlu$^1$ | -35 (c 0.92,25°) | 0.15 | 0.38 | 0.49 | 0.62 |
| D-His$^2$ | -48 (c 0.71,24°) | 0.14 | 0.35 | 0.48 | 0.62 |
| D-Trp$^3$ | -40 (c 0.62,27°) | 0.14 | 0.38 | 0.50 | 0.64 |
| D-Tyr$^5$ | -44 (c 0.97,18°) | 0.14 | 0.39 | 0.50 | 0.63 |
| D-Leu$^7$ | -26 (c 1.08,24°) | 0.15 | 0.38 | 0.49 | 0.65 |
| D-Arg$^8$ | -23 (c 1.05,25°) | 0.14 | 0.36 | 0.49 | 0.62 |

[a] The following t.l.c. solvents systems were used:

$R_f$(I), n-BuOH:AcOH:$H_2O$ (4:1:5, upper phase)
Rf(II), i-PrOH: 1 M AcOH (2:1)
$R_f$(III), n-BuOH:AcOH:$H_2O$:EtoAc (1:1:1:1)
$R_f$(IV), EtoAc:Pyridine: AcOH:$H_2O$ (5:5:1:3)

Samples sizes of <u>ca</u>. 30 μg were spotted on Brinkmann Silplates and solvent fronts allowed to travel 10-15 cm. Spots were visualized by exposure to iodine vapor, ninhydrin reagent (all compounds negative), and Ehrlich reagents.

solid-phase procedure used for the syntheses of protected peptide intermediates has been described previously (4). Amino acids with reactive side-chains were protected as follows: D- and L-histidine, dinitrophenyl; serine, benzyl; D- and L-tyrosine, benzyl; D- and L-arginine, tosyl. Protected peptides were cleaved from their resin support as amides by treatment with ammonia in methanol (5) and deprotected with hydrogen fluoride containing 20% anisole. Free decapeptide amides were purified by gel filtration and partition chromatography techniques described previously (2). They were homogeneous by thin layer chromatography in several solvent systems (Table I) and gave correct amino acid ratios after analysis (Table II). Specific rotations are given in Table I.

Slight racemization of D-amino acid derivatives during the syntheses

Table II

Amino Acid Ratios in Acid Hydrolysates[a] of LH-RH Stereoisomers

| LH-RH Isomer | Glu | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro |
|---|---|---|---|---|---|---|---|---|---|
| D-pGlu$^1$ | 1.00 | 0.97 | 0.89 | 0.85 | 0.92 | 1.99 | 1.00 | 1.00 | 1.02 |
| D-His$^2$ | 1.02 | 1.03 | 1.01 | 0.82 | 0.98 | 2.05 | 1.00 | 1.03 | 1.05 |
| D-Trp$^3$ | 1.00 | 1.00 | 0.85 | 0.83 | 0.97 | 2.07 | 1.00 | 1.03 | 1.10 |
| D-Tyr$^5$ | 1.10 | 1.00 | 0.85 | 0.82 | 0.95 | 2.06 | 1.08 | 1.05 | 1.07 |
| D-Leu$^7$ | 1.02 | 1.01 | 0.88 | 0.84 | 0.96 | 1.93 | 1.02 | 0.98 | 1.00 |
| D-Arg$^8$ | 1.00 | 0.91 | 0.85 | 0.83 | 0.92 | 2.05 | 0.99 | 0.91 | 0.99 |

[a] Amino acid analyses were performed on a Beckman Model 119 analyser on samples which were hydrolysed (110°, 18 hr.) in 6 M HCl, containing 4% thioglycolic acid.

would result in contamination of analogs with LH-RH itself and hence, would produce anomalously high biological activities. Therefore, each analog was hydrolysed in 6 M HCl containing 4% thioglycolic acid and the liberated amino acids digested with L-amino acid oxidase under the conditions described by Jorgensen *et al.* (6). Amino acid analyses of the digests (Table III) indicate that, within the limits of detectability, little racemization of tyrosine, leucine, and arginine occurred. Because of the instability of tryptophan under the conditions of hydrolysis and digestion, the analysis showed a rather low level of this amino acid in [D-Trp$^3$]-LH-RH. L-Glutamic acid was not attacked by the enzyme to any observable extent during the 24 hour digest so that possible racemization of pyroglutamic acid could not be detected. However, previous incorporations of D-pyroglutamic acid in TRH analogs (3) revealed no racemization of this amino acid. Serine and proline were also stable to oxidation by the enzyme. The well-known tendency of histidine to racemize during solid-phase synthesis led us to examine both

Table III

A. Amino Acid Ratios in L-Amino Acid Oxidase Digests of Acid Hydrolysates of LH-RH Stereoisomers

| LH-RH Isomer | Glu | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro |
|---|---|---|---|---|---|---|---|---|---|
| D-pGlu$^1$ | 1.03 | 0.04 | 0.00 | 0.79 | 0.01 | 1.90 | 0.02 | 0.02 | 1.00 |
| D-His$^2$ | 1.00 | 0.95 | 0.00 | 0.83 | 0.01 | 2.00 | 0.02 | 0.04 | 1.06 |
| D-Trp$^3$ | 1.00 | 0.05 | 0.86 | 0.79 | 0.01 | 1.95 | 0.02 | 0.06 | 1.15 |
| D-Tyr$^5$ | 1.01 | 0.02 | 0.00 | 0.73 | 0.93 | 2.03 | 0.03 | 0.07 | 1.00 |
| D-Leu$^7$ | 0.99 | 0.04 | 0.00 | 0.81 | 0.03 | 1.90 | 1.00 | 0.03 | 1.03 |
| D-Arg$^8$ | 1.00 | 0.05 | 0.00 | 0.86 | 0.02 | 2.00 | 0.02 | 1.00 | 1.06 |

B. Amino Acid Ratios in a D-Amino Acid Oxidase Digest of an Acid Hydrolysate of [D-His$^2$]-LH-RH

| LH-RH Isomer | Glu | His | Trp[a] | Ser | Tyr | Gly | Leu | Arg | Pro |
|---|---|---|---|---|---|---|---|---|---|
| D-His$^2$ | 1.00 | 0.05 | --- | 0.75 | 1.00 | 1.90 | 1.03 | 0.90 | 1.00 |

[a] Tryptophan not determined.

L- and D-amino acid oxidase digests of the [D-His$^2$]-analog. The results indicate that very little racemization took place with the dinitrophenyl derivative of histidine used in this synthesis.

## Biological Assays and Discussion

The LH-releasing activities of the peptides (Table IV) were determined by the stimulation of LH release in ovariectomized rats, pretreated (7,8) with estrogen and progesterone (4 per group), followed by radioimmunoassay (9) for LH. Serum LH levels were compared with responses to the administration of saline and 0.5 ng and 2.5 ng doses of natural LH-RH.

[D-pGlu$^1$]-LH-RH had a moderately high activity (8%) of that of the natural hormone. This finding fits in with the assumption that a modification

Table IV

LH-Releasing Activity of LH-RH Stereoisomers Compared with Natural LH-RH in Ovariectomized, Estrogen-Progesterone Treated Rats

| Sample | Dose ng/rat | Mean Plasma LH (ng/ml ± S.E.) | Potency[a] (%) with 95% Confidence Limits |
|---|---|---|---|
| Saline | --- | 4.1 ± 0.4 | |
| LH-RH | 0.5 | 11.2 ± 1.9 | |
| | 2.5 | 32.1 ± 3.4 | |
| D-pGlu$^1$ | 50 | 39.5 ± 8.6 | 7.5 (2.3-56) |
| | 250 | 76.0 ± 13.5 | |
| Saline | --- | 5.3 ± 0.9 | |
| LH-RH | 0.5 | 10.3 ± 1.8 | |
| | 2.5 | 49.8 ± 18.2 | |
| D-His$^2$ | 50 | 18.4 ± 0.4 | 9.4 (0.04 -14.4) |
| | 250 | 39.4 ± 7.5 | |
| Saline | --- | 8.3 ± 1.7 | |
| LH-RH | 0.5 | 15.2 ± 1.4 | |
| | 2.5 | 61.1 ± 1.5 | |
| D-Trp$^3$ | 100 | 6.8 ± 0.2 | 0.13 (0.06-0.20) |
| | 500 | 19.5 ± 5.3 | |
| Saline | --- | 4.6 ± 0.7 | |
| LH-RH | 0.5 | 7.4 ± 0.4 | |
| | 2.5 | 31.4 ± 10.6 | |
| D-Tyr$^5$ | 100 | 4.6 ± 0.5 | <u>ca</u>. 0.1 |
| | 500 | 6.5 ± 0.6 | |
| D-Leu$^7$ | 100 | 12.2 ± 0.2 | 0.99 (0.43-3.90) |
| | 500 | 41.9 ± 3.5 | |
| D-Arg$^8$ | 100 | 3.8 ± 0.2 | 0.27 (0.01-1.49) |
| | 500 | 17.5 ± 1.3 | |

[a] Natural LH-RH accepted as 100%

of the stereochemistry of this N-terminal residue might have a less severe effect on the configuration of the peptide molecule as a whole and, hence, result in some retention of activity. While this work was in progress, another synthesis of $[\text{D-pGlu}^1]$-LH-RH was described (10) and this material exhibited roughly 1% LH-RH activity in an *in vitro* assay system which may have been imprecise. Replacement of L-histidine by its D-isomer also resulted in retention of significant LH-RH activity (9%); furthermore, the activity appears to be mainly due to the D-analog itself rather than to contamination with LH-RH.

The remaining four peptides were all considerably less active, possibly because of the positions occupied by the D-amino acids deeper within the peptide chain. The tyrosine and leucine residues should be intimately involved in the "β-turn" configuration centered around glycine in position six, which has been postulated (1) to be necessary for high levels of biological potency to exist in LH-RH analogs. Thus, changes in the stereochemistry of tyrosine and leucine might be expected to drastically lower biological activity.

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