# SYNTHETIC ANALOGS OF LUTEINIZING HORMONE RELEASING HORMONE (LH-RH) SUBSTITUTED IN POSITION 6 AND 10

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SUMMARY: A series of peptide analogs of luteinizing hormone releasing hormone (LH-RH), altered at position 6 and 10, was synthesized and evaluated *in vivo* for the ability to induce ovulation in the diestrous rat and *in vitro* for ability to release pituitary luteinizing hormone and follicle stimulating hormone. All the analogs with D-amino acid substitutions at position 6, even those with large bulky side chain, exhibited an amazingly high potency compared with the parent hormone, LH-RH. On the basis of the biological activities, structure-activity relationships in the central part of this molecule were discussed in detail.

This paper is part of a continuing investigation on the biological effects of amino acid replacement in the molecule of luteinizing hormone releasing hormone (LH-RH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$ (1)). We have recently reported that analogs based on the highly potent des-$Gly^{10}$-LH-RH-ethylamide [I](2), with D-Ala in position 6 and a hydrophobic amino acid in position 5, i.e., des-$Gly^{10}$-[D-$Ala^6$]-LH-RH-ethylamide [II], des-$Gly^{10}$-[$Phe^5$, D-$Ala^6$]-LH-RH-ethylamide [III] and des-$Gly^{10}$-[$Ile^5$, D-$Ala^6$]-LH-RH-ethylamide [IV], possessed surprizingly intense biological activity when evaluated by ovulation-inducing index (3). Independently, Coy *et al.*(4) have reported similar results on the *in vivo* LH-

release activity of compound II. Monahan et al.(5) reported previously that [D-Ala$^6$]-LH-RH [V] exhibited 350 - 450% of the potency of the parent hormone in in vivo and in vitro assays, although analogs having a D-amino acid with a bulky side chain in the position 6 (D-Val, D-Pro and D-Leu) were less potent than LH-RH itself. Monahan et al. explained this by steric consideration of intermolecular interactions and/or interaction at the binding site.

In our independent studies on structure-activity relationships of the LH-RH molecule, we had synthesized [DL-Leu$^6$]-LH-RH and des-Gly$^{10}$-[DL-Leu$^6$]-LH-RH-ethylamide (T. Fukuda et al., unpublished data) and found that these analogs exhibited very high ovulation-inducing activity, approximately 8 and 20 times that of LH-RH standard, respectively. These findings led us to systematic studies on the synthesis and biological evaluation of LH-RH analogs having D-amino acids at position 6 and ethylamine at position 10.

This paper describes the synthesis and the biological potency of seven new analogs of LH-RH, des-Gly$^{10}$-[D-α-amino-n-butyric acid$^6$]-LH-RH-ethylamide [VI], des-Gly$^{10}$-[D-norvaline$^6$]-LH-RH-ethylamide [VII], des-Gly$^{10}$-[D-Leu$^6$]-LH-RH-ethylamide [VIII], des-Gly$^{10}$-[Phe$^5$, D-Leu$^6$]-LH-RH-ethylamide [IX], des-Gly$^{10}$-[Ile$^5$, D-Leu$^6$]-LH-RH-ethylamide [X], des-Gly$^{10}$-[Phe$^5$, D-Phe$^6$]-LH-RH-ethylamide [XII] and des-Gly$^{10}$-[D-Ser$^6$]-LH-RH-ethylamide [XIII].

Synthesis of peptides. The essential strategy for making the peptides in this series has been described previously (3). The C-terminal fragments, H-$A_6$-Leu-Arg($NO_2$)-Pro-$NHCH_2CH_3$ [$A_6$=D-amino acids], were prepared by acylation with Z- or BOC-amino acid activated esters of H-Leu-Arg($NO_2$)-Pro-$NHCH_2CH_3$ (2,6), and followed by removal of the protecting group on the amino function. The resulting C-terminal fragments were coupled with pGlu-His-Trp-Ser-$A_5$-OH [$A_5$=Tyr, Phe or Ile] (3) by the HONB/DCC method (7) to minimize

undesirable racemization during the coupling reactions. The crude protected peptides were purified by column chromatography on Amberlite XAD-2 in a manner similar to that described for our synthesis of other LH-RH analogs (6,8). The purified mono-protected peptides were subjected to reduction with stannous chloride in 60% formic acid for 2 hr at 80 - 85° which is a modification of the method of Hayakawa et al.(9). The crude peptides thus obtained were further purified by column chromatography on CMC in a manner similar to that described previously (6,7,8). All the analogs obtained were chromatographically homogeneous and gave the correct amino acid ratios. The data for characterization of the key intermediates and the final products are listed in Table I and II, respectively.

Biological evaluations and discussion. The ovulation-inducing activity of these synthetic analogs was determined in the diestrous

Table I. Physicochemical Properties of Intermediates

$R\text{-}A_6\text{-}Leu\text{-}Arg(NO_2)\text{-}Pro\text{-}NHCH_2CH_3$

| Compound R-$A_6$ | Formula[a] | Mp (°C) | $[\alpha]_D^{23}$ (conc., solvent) | Rf[b] |
|---|---|---|---|---|
| Z-D-Abu[c] | $C_{28}H_{51}O_8N_9 \cdot 0.5H_2O$ | 111-114 | -51.2°(0.5, DMF) | 0.58 |
| Z-D-Nva[c] | $C_{32}H_{51}O_8N_9$ | 105-107 | -51.2°(1.0, MeOH) | 0.57 |
| Z-D-Leu | $C_{33}H_{53}O_8N_9$ | 133-134 | -47.3°(0.5, MeOH) | 0.59 |
| Z-D-Phe | $C_{36}H_{51}O_8N_9$ | 162-164 | -64.2°(1.0, MeOH) | 0.67 |
| BOC-D-Ser | $C_{27}H_{49}O_9N_9 \cdot H_2O$ | 136-139 | -17.6°(1.0, DMF) | 0.45 |

a) All compounds listed gave the correct analytical values (C, H, N). b) Solvent system (Merck's precoated silica gel plate F 254): $CHCl_3$-MeOH-AcOH (9:1:0.5). c) Abu = α-amino-n-butyric acid, Nva = norvaline

Table II. Chemical and Physical Properties of LH-RH Analogs

Analog VI: $[\alpha]_D^{23}$ -43.6°(c 0.5, 5% AcOH); TLC[a] $Rf^2$ 0.063, $Rf^3$ 0.67, $Rf^4$ 0.61, $Rf^5$ 0.69; Amino acid anal.[b] His 0.97, Arg 0.97, Trp 1.00, Ser 0.94, Glu 1.00, Pro 1.01, Abu 1.03, Leu 1.03, Tyr 1.03 (86%)[c].

Analog VII: $[\alpha]_D^{24}$ -39.8°(c 0.5, 5% AcOH); TLC[a] $Rf^2$ 0.063, $Rf^3$ 0.68, $Rf^4$ 0.63, $Rf^5$ 0.70; Amino acid anal.[b] His 0.92, Arg 0.96, Trp 0.92, Ser 0.81, Glu 1.00, Pro 1.01, Nva 1.04, Leu 1.01, Tyr 1.00 (84.5%)[c].

Analog VIII: $[\alpha]_D^{24}$ -32.7°(c 0.55, 5% AcOH); TLC[a] $Rf^2$ 0.070, $Rf^3$ 0.65, $Rf^4$ 0.65, $Rf^5$ 0.72; Amino acid anal.[b] His 1.00, Arg 0.96, Trp 0.96, Ser 1.00, Glu 1.00, Pro 1.04, Leu 2.00, Tyr 1.00 (82%)[c].

Analog IX: $[\alpha]_D^{25}$ -38.5°(c 0.5, 5% AcOH); TLC[a] $Rf^2$ 0.082, $Rf^3$ 0.69, $Rf^4$ 0.72, $Rf^5$ 0.74; Amino acid anal.[b] His 1.00, Arg 1.04, Trp 0.98, Ser 0.92, Glu 1.00, Pro 1.00, Leu 2.04, Phe 1.01, Ethylamine 1.02(87%)[c].

Analog X: $[\alpha]_D^{24}$ -50.3°(c 0.6, 5% AcOH); TLC[a] $Rf^2$ 0.082, $Rf^3$ 0,69, $Rf^4$ 0.70, $Rf^5$ 0.73; Amino acid anal.[b] His 0.98, Arg 1.00, Trp 0.97, Ser 0.98, Glu 1.01, Pro 1.00, Leu 2.02, Ile 0.98 (89%)[c].

Analog XII: $[\alpha]_D^{26}$ -71.8°(c 0.2, 5% AcOH); TLC[a] $Rf^2$ 0.082, $Rf^3$ 0.70, $Rf^4$ 0.64, $Rf^5$ 0.74; Amino acid anal.[b] His 1.01, Arg 0.97, Trp 0.92, Ser 0.98, Glu 1.00, Pro 0.99, Leu 1.00, Phe 2.03 (87%)[c].

Analog XIII: $[\alpha]_D^{27}$ -46.4°(c 0.5, 5% AcOH); TLC[a] $Rf^2$ 0.038, $Rf^3$ 0.64, $Rf^4$ 0.42, $Rf^5$ 0.65; Amino acid anal.[b] His 1.00, Arg 1.04, Trp 1.00, Ser 1.82, Glu 1.00, Pro 1.00, Leu 1.04, Tyr 0.96 (76%)[c].

a) Solvent systems and sheets employed were: $Rf^2$ (silica gel), EtOAc-pyridine-AcOH-$H_2O$ (60:20:6:10); $Rf^3$ (silica gel), EtOAc-BuOH-AcOH-$H_2O$ (1:1:1:1); $Rf^4$ (cellulose), n-BuOH-AcOH-$H_2O$ (4:1:1); $Rf^5$ (silica gel), n-BuOH-pyridine-AcOH-$H_2O$ (30:20:6:24). b) Acid hydrolysate (5.7 N HCl, 105°, 30 hr., in the presence of thioglycolic acid)(14).
c) Peptide content.

rat (sc, injection) by the method of Yamazaki and Nakayama (10) and the *in vitro* LH- and FSH-releasing activities were measured by a modification of the method of Mittler and Meites (11) using rat hemisected anterior pituitaries (12). The details of these assays

Table III. Structures and Relative Activities of Synthetic LH-RH Analogs

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$

5 6 10

| Compound No. | Substituted residue 5 | 6 | 10 | Ovulation-inducing activity (%) | in vitro LH release | in vitro FSH release(%) |
|---|---|---|---|---|---|---|
| LH-RH | | | | 100 | 100 | 100 |
| I[a] | | | $NHCH_2CH_3$ | 672 | 300 | 280 |
| II lot 1[b] | | D-Ala | $NHCH_2CH_3$ | 8,270 | 180 | 300 |
| lot 2 | | | | 5,120 | | |
| III[b] | Phe | D-Ala | $NHCH_2CH_3$ | 5,810 | 270 | 300 |
| IV[b] | Ile | D-Ala | $NHCH_2CH_3$ | 1,340 | 160 | 350 |
| V[b] | | D-Ala | | 2,830 | 570 | 475 |
| VI | | D-Abu[c] | $NHCH_2CH_3$ | 6,520[d] | 180 | 170 |
| VII | | D-Nva[c] | $NHCH_2CH_3$ | 5,810 | 90-400 | 160-290 |
| VIII[e] lot 1 | | D-Leu | $NHCH_2CH_3$ | 8,270 | 275 | 280 |
| lot 2 | | | | 6,900 | | |
| IX | Phe | D-Leu | $NHCH_2CH_3$ | 4,580 | 100 | 90 |
| X | Ile | D-Leu | $NHCH_2CH_3$ | 1,990 | 85 | 67 |
| XI[f] | | D-Leu | | 2,900 | 110 | 100 |
| XII | Phe | D-Phe | $NHCH_2CH_3$ | 5,400 | 140 | 120-520 |
| XIII | | D-Ser | $NHCH_2CH_3$ | 6,140 | 100 | 100 |

a) See Fujino, et al., Ref. 3. b) See Fujino, et al., Ref. 4. c) Abu = α-amino-n-butyric acid, Nva = norvaline. d) The relative potencies were calculated from the $ED_{50}$ values which were reduced from the data of five or six different dosages (5-10 rats each). e) The first synthesis of this analog in our collaborative project was carried out by Dr. John Seely using the solid phase method. The details of the synthesis of this compound by the solid phase method will be published separately by Dr. Seely. f) The synthesis of this analog was carried out by the solid phase method: $[\alpha]_D^{24}$ -31.5°(c 0.55, 5% AcOH)(M. Fujino and C. Kitada, unpublished data).

have been described previously (8). As can be seen from Table III, all the analogs having D-amino acids at position 6, even those with a large bulky side chain possessed an amazingly high potency when

evaluated by the ovulation-inducing index. Moreover, the potencies of a series of D-Leu analogs were closely comparable to those of the corresponding D-Ala analogs (3). These data indicate that the spatial effect of the side-chain of the D-amino acid in position 6 is relatively unimportant in the interaction of the peptide with the binding site. Our ovulation-inducing potencies of [D-Ala$^6$]-analogs as well as the [D-Leu$^6$]-analogs are very different from the *in vivo* LH-releasing activity reported by Monahan *et al.*(5), whereas our *in vitro* results are in good agreement with Monahan's data. The reasons for this apparent discrepancy are not known at the present time. However, dose level studies comparing LH-RH and compound II or VIII show a 50 - 80 fold increase in the integrated LH levels in both rats and sheep (R. H. Rippel *et al.*, in preparation), which is good agreement with the ovulation-inducing activities. This indicates that the discrepancy may be resolved by a comparative time study, since Monahan *et al.*(5) determined the *in vivo* LH-release at only 15 min after administration of the peptides. Moreover, our unpublished results mentioned above indicate that [D-amino acid$^6$]-analogs have generally exhibited a delayed LH-release and also an increased duration of the LH peak. Therefore, the intense ovulation-inducing potencies of these analogs may result from their greater resistance to enzymatic digestion and/or to increased affinity at the receptor. In the incubation of isolated pituitary tissue where catalytic feedback influences are minimal, the activities of [D-amino acid$^6$]-analogs are much lower than those obtained by the ovulation induction test.

It, however, appears that the introduction of a D-amino acid residue in position 6 might generally result in restriction of freedom in the central portion of the molecule. This could result in a conformational change giving rise to a better fit at the

receptor site(s) of the target organ, the pituitary. In this connection, it is interesting to note that Arnold et al,(13) found very low biological activity of [Sarcosine$^{6}$]-LH-RH, which has a methyl group on the peptide nitrogen between residues 5 and 6. This substitution could prevent the attainment of a biological active conformation.

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