Investigation of the Role of Tryptophan in the Luteinizing Hormone-Releasing Hormone†

David H. Coy,* Esther J. Coy, Yoshihiro Hirotsu, Jesus A. Vilchez-Martinez, Andrew V. Schally,‡ Johannes W. van Nispen, and Godefridus I. Tesser

ABSTRACT: The tryptophan residue, critical for the biological activity of luteinizing hormone-releasing hormone (LH-RH), was replaced successively by pentamethylphenylalanine, 5-fluorotryptophan, *p*-aminophenylalanine, *p*-nitrophenylalanine, and histidine in an attempt to ascertain those factors responsible for its contribution to the biological activity of the hormone. Pentamethylphenylalanine[3]-LH-RH was found to have FSH and LH releasing potencies of 34–70%, when compared to the natural hormone. Since pentamethylphenylalanine resembles tryptophan only in its ability to form π–π electron complexes with certain aromatic molecules, the high level of activity of this analog suggests that one of the principal criteria for the maintenance of physiological activity in LH-RH peptides is an efficient electron transfer from a group in position 3 of the peptide chain to an electron acceptor on the pituitary receptor site with the formation of a loose bond. The potencies of the other analogs are discussed in relation to this concept.

The synthesis and determination of biological activities of analogs of LH-RH[1] have revealed that tryptophan in position 3 of the decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is one of the most important and interesting residues. Thus, analogs (Yanaihara *et al.*, 1973) in which tryptophan is deleted or replaced with amino acids containing simple hydrocarbon side chains have virtually no LH-releasing activity. When tryptophan is replaced by aromatic amino acids such as phenylalanine (Yanaihara *et al.*, 1973) and tyrosine (Coy *et al.*, 1973a), however, significant, albeit very low, levels of LH-RH activity remain.

Carrión and coworkers (1968) found that D(L)-pentamethylphenylalanine, although structurally and chemically dissimilar to tryptophan, resembled this amino acid in interacting with phthalimides to form colored charge-transfer complexes. If high levels of biological activity were found to exist in a peptide in which tryptophan was replaced by Me_5Phe, then it would be possible to theorize that the importance of these residues lies in their ability to share electrons and to complex with a suitable portion of the hormonal receptor. The recent synthesis and resolution (Tesser *et al.*, 1973) of Me_5Phe enabled the desired analog to be synthesized.

An analog incorporating 5-fluorotryptophan, in which a hydrogen atom in the aromatic ring system is replaced by a fluorine atom of similar size, was synthesized in the expectation that effects on biological activity might be explained by electronic rather than conformational factors. In addition, two analogs were prepared containing derivatives of phenylalanine with substituents in the benzene ring. Histidine[3]-LH-RH was also synthesized in order to examine the effects an aromatic imidazole nucleus might exert in this position.

Materials and Methods

Unless otherwise stated, amino acids and their derivatives were of the L variety.

Boc-Me_5Phe. This derivative was prepared by a modification of the Schnabel method (Schnabel, 1967). L-Me_5Phe-$HCl\cdot H_2O$ (Tesser *et al.*, 1973) (2.9 g, 10 mmol) was dissolved with warming in 0.1 M NaOH (300 ml). Dioxane (100 ml) followed by Boc azide (25 ml, 180 mmol) were added with stirring and the pH was maintained at 11.0 with 1.0 M NaOH by autotitration. The reaction took place under nitrogen and, after 16 hr, the slightly turbid solution was extracted twice with ether to remove excess Boc azide and acidified with 2.0 M $KHSO_4$. A precipitate which formed disappeared on addition of ether. The aqueous layer was extracted twice with ether and the combined organic layers were washed with water and saturated NaCl solution, dried, and evaporated to dryness to give a white powder (3.6 g, 92%); mp 163–165°; $[\alpha]^{21}D$ −9.6° (*c* 2, MeOH), $[\alpha]^{21}D$ −27° (*c* 2, AcOH); R_F (silica, *n*-BuOH–AcOH–H_2O; 10:1:3), 0.80; R_F (silica, $CHCl_3$–MeOH–AcOH; 95:20:3), 0.80. *Anal.* Calcd for $C_{19}H_{29}NO_4$: C, 68.03; H, 8.71; N, 4.18. Found: C, 68.12; H, 8.71; N, 4.07.

L-*5-Fluorotryptophan.* Chloroacetic anhydride (3.3 g, 25 mmol) and 2.0 M NaOH (12.5 ml, 25 mmol) were added to a solution of D(L)-5-fluorotryptophan (Aldrich Chemical Co.) (4.4 g, 20 mmol) in 2.0 M NaOH (10 ml, 20 mmol). The mixture was stirred (1.5 hr) and 6 M H_2SO_4 was added until a strong blue color was obtained with Congo dye. On cooling, the large crystals were ground to a powder, washed with ice-water, and recrystallized from water to give chloroacetyl-D(L)-5-fluorotryptophan (3.1 g, 52%), mp 169–171°. *Anal.* Calcd for $C_{13}H_{12}O_3N_2FCl$: C, 52.26; H, 4.05; N, 9.39. Found: C, 52.19; H, 4.11; N, 9.31.

Chloracetyl-D(L)-5-fluorotryptophan (6.9 g, 23 mmol) was suspended in water (300 ml) and 2.0 M LiOH was added to dissolve the crystals and establish a pH between 7.0 and 7.2. Bovine pancreatic carboxypeptidase (100 mg) was added and

† From the Department of Medicine, Tulane University School of Medicine and the Veterans Administration Hospital, New Orleans, Louisiana 70146 (D. H. C., E. J. C., Y. H., J. A. V.-M., and A. V. S.), and the Department of Organic Chemistry, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands (J. W. van N. and G. I. T.). *Received April 8, 1974.* Supported by National Institutes of Health, Contract NICHD 72-2741, U. S. Public Health Service Grant AM 07467, The Population Council, New York, N. Y., and the Veterans Administration.

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[1] Abbreviations used are: LH, luteinizing hormone; FSH, follicle stimulating hormone; RH, releasing hormone; Boc, *tert*-butyloxycarbonyl; Me_5Phe, pentamethylphenylalanine.

TABLE I: Properties of LH-RH Analogs.

Substituent in Position 3	Yield[a] (%)	[α]D (deg) in 0.1 M AcOH	R_F (I)[b]	R_F (II)	R_F (III)	R_F (IV)
Me_5Phe	43	−38 (*c* 0.56, 24°)	0.16	0.36	0.52	0.32
5-F-Trp	29	−41 (*c* 1.16, 26°)	0.16	0.35	0.47	0.60
p-NO_2-Phe	75	−38 (*c* 0.94, 24°)	0.14	0.36	0.19	0.62
p-NH_2-Phe	27[c]	−53 (*c* 0.73, 24°)	0.04	0.19	0.07	0.49
His	42	−51 (*c* 0.88, 24°)	0.02	0.03	0.08	0.51

[a] Yields based on protected peptides. [b] The following tlc solvent systems were used: R_F (I), *n*-BuOH–AcOH–H_2O (4:1:5, upper phase); R_F (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). Sample sizes of *ca.* 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 apart from (*p*-NH_2-Phe)^3–LH-RH) and Pauly reagent in succession. [c] Yield based on (*p*-NO_2-Phe)^3–LH-RH precursor.

the mixture kept at 38° (24 hr). A further 50 mg of enzyme was then added and the digestion continued for 24 hr. Acetic acid was added to pH 5, the mixture boiled for 5 min, and insoluble protein filtered off. The filtrate was concentrated *in vacuo* and the residue applied to a column (2 × 40 cm) of Dowex AG 1X2 (acetate form), which was then eluted with water. Fractions containing ninhydrin positive material were combined and dried, and the white powder was crystalized from water–ethanol to give L-5-fluorotryptophan (1.5 g, 68%): mp 158–163° dec; $[\alpha]^{23}D$ −8.3° (*c* 2.5, 1 M NaOH). *Anal.* Calcd for $C_{11}H_{11}O_2N_2F$: C, 59.44; H, 4.99; N, 12.62. Found: C, 59.54; H, 4.96; N, 12.62.

Boc-L-5-Fluorotryptophan. The free amino acid (0.44 g, 2 mmol) was converted by the Schwyzer method (Schwyzer *et al.*, 1959) to its Boc derivative (0.54 g, 84%): mp 157–158° dec; $[\alpha]^{23}D$ −6.1° (*c* 2.1, AcOH). *Anal.* Calcd for $C_{16}H_{19}O_4N_2F$: C, 59.62; H, 5.94; N, 8.69. Found: C, 59.51; H, 5.90; N, 8.57.

Boc-p-nitrophenylalanine. The preparation of this compound has been described previously (Coy *et al.*, 1973b).

Peptide Synthesis. For all the peptides described in this work, Boc-protected amino acids (3.0 mmol) were coupled in a Beckman Model 990 automatic peptide synthesizer to a 2% cross-linked, poly(styrene–divinylbenzeneglycine) resin (1.0 mmol of glycine) in the presence of dicyclohexylcarbodiimide (3.0 mmol) by a process which has been described previously (Coy *et al.*, 1973a,b). Boc protecting groups were removed by treatment with 1 M HCl in glacial acetic acid. In each case reactive side chains were protected as follows: histidine, dinitrophenyl; serine, benzyl; tyrosine, *O-o*-chlorocarbenzoxy (Yamashiro and Li, 1973); arginine, tosyl. Protected peptides were cleaved from the resin by ammonolysis, whereupon the dinitrophenyl group of histidine was simultaneously removed (Coy *et al.*, 1973a). Yields of protected peptide amides ranged from 80 to 90% and 150–200-mg amounts were deprotected by treatment with hydrogen fluoride containing 20% anisole at 0° (30 min). (*p*-NH_2Phe)^3-LH-RH was prepared from purified (*p*-NO_2-Phe)^3-LH-RH by hydrogenation of the latter over 10% palladium on charcoal in 0.1 M acetic acid for 12 hr.

Purification of Peptides. Hydrogen fluoride deprotected peptides were first eluted on a column (2.5 × 95 cm) of Sephadex G-25 (medium) in 0.2 M acetic acid and emerged as major peaks between elution volumes of 300–400 ml.

In a final purification step, (5-F-Trp)^3-LH-RH was eluted between 1070 and 1150 ml on a column (1.4 × 95 cm) of CM-cellulose using a buffer gradient system already described (Coy *et al.*, 1973a). The other peptides were fractionated by partition chromatography on a column (1.4 × 95 cm) of Sephadex G-25 (medium) previously equilibrated with the lower phase, followed by the upper phase, of a system of 1-butanol–acetic acid–water (4:1:5). Elution of peptides with the upper phase yielded peaks with R_F's of: Me_5Phe^3-LH-RH, 0.29–0.22; (*p*-NO_2-Phe)^3-LH-RH, 0.17–0.08; (*p*-NH_2-Phe)^3-LH-RH, 0.054–0.045; His^3-LH-RH, 0.054–0.045.

Yields of final peptides, based on protected peptides, are given in Table I together with optical rotations and R_F's in several solvent systems.

Amino acid analyses were performed in a Beckman Model 119 amino acid analyser equipped with a System AA computing integrator on samples which were hydrolyzed (110°, 18 hr) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (Moore, 1972). A modified single column methodology

TABLE II: Amino Acid Ratios of LH-RH Analogs.

Substituent in Position 3	Glu	His	X^3	Ser	Tyr	Gly	Leu	Arg	Pro	NH_3
Me_5Phe	1.00	1.00		0.92	1.02	2.02	1.05	1.03	1.06	0.97
5-F-Trp	1.00	0.95	0.98	0.99	1.00	1.99	1.01	1.00	0.97	1.45
p-NO_2-Phe	1.03	0.98	0.97	0.92	0.98	2.03	1.00	0.96	1.01	1.25
p-NH_2-Phe	1.06	0.99[a]	0.99[a]	0.98	1.00	2.01	1.03	0.98	1.00	1.48
His^3	1.10	1.90		1.00	1.00	1.98	1.00	0.96	0.95	1.38

[a] Value is ½ (His + *p*-NH_2-Phe) since these amino acids eluted together.

TABLE III: LH-Releasing Activity of LH-RH Analogs as 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		5.3 ± 1.5	
LH-RH	1.0	24.5 ± 2.4	
	5.0	47.0 ± 4.6	
Me_5Phe^3	5.0	37.5 ± 9.9	69 (30–392)
	25.0	98.6 ± 14.8	
Saline		5.4 ± 0.4	
LH-RH	0.5	11.2 ± 0.6	
	2.5	44.9 ± 11.6	
$(5\text{-F-Trp})^3$	10.0	16.8 ± 1.5	6 (3–14)
	50.0	45.8 ± 3.6	
Saline		4.8 ± 0.8	
LH-RH	1.0	20.2 ± 5.8	
	5.0	57.4 ± 9.8	
$(p\text{-}NO_2\text{-Phe})^3$	1000	4.9 ± 0.6	0.01 (0.001–0.03)
	5000	10.4 ± 2.0	
$(p\text{-}NH_2\text{-Phe})^3$	200	30.6 ± 6.9	0.59 (0.05–3)
	1000	53.5 ± 11.3	
His^3	100	5.7 ± 0.7	<0.05 (no limits)
	500	4.6 ± 0.3	

[a] Natural LH-RH accepted as 100%; all analogs showed log-linear dose response curves parallel to the response elicited by LH-RH.

was employed. Buffer 1 (pH 3.4) contained 1% 1-propanol, buffer 2 (pH 4.25) 0.15% 1-propanol, and buffer 3 (pH 6.4) 5% 1-propanol. Buffer change times were set at 76 and 134 min. 5-Fluorotryptophan was eluted 20 min after arginine, *p*-NO_2-Phe 10 min after histidine, and *p*-NH_2-Phe with histidine. Me_5Phe could not be eluted from the column under a variety of conditions and appears to stick tightly to the ion-exchange resin. Its presence in Me_5Phe^3-LH-RH was, therefore, demonstrated by thin-layer chromatography of a hydrolysate of the peptide on silica gel using $CHCl_3$–MeOH–17% NH_3(2:2:1) as the developing medium. Me_5Phe gave a ninhydrin positive spot (R_F 0.52) well separated from the other amino acids, the fastest moving of which was leucine (R_F 0.42).

The amino acid ratios appear in Table II.

Bioassays. LH-RH activities of the five analogs were determined *in vivo* at two dose levels by the stimulation of LH release in ovariectomized rats (four per group) pretreated with estrogen and progesterone (Ramirez and McCann, 1963; Schally *et al.*, 1971) followed by radioimmunoassay (Niswender *et al.*, 1968) for rat LH. Serum LH levels 30 min after injection of samples were compared with those present after administration of saline and of two doses of pure, natural LH-RH. LH and FSH activities of Me_5Phe^3-LH-RH were also determined by an assay based on a 4-hr infusion (Arimura *et al.*, 1972) of the peptide into immature male rats (six per group). The radioimmunoassay for FSH was carried out by the method of Daane and Parlow (1971). LH was expressed as NIH-LH-S_{17} and FSH in terms of NIAMD-Rat-FSH-RP-1.

Results

Biological Activities. In the ovariectomized rats pretreated with steroids Me_5Phe^3-LH-RH, $(5\text{-F-Trp})^3$-LH-RH, $(p\text{-}NO_2$-Phe$)^3$-LH-RH, $(p\text{-}NH_2$-Phe$)^3$-LH-RH, and His^3-LH-RH were found to have LH-releasing activities of 69, 6, 0.01, 0.6, and *ca.* 0.05%, respectively, when compared to LH-RH itself (Table III). In the infusion assay (Table IV), Me_5Phe^3-LH-RH showed an LH-releasing potency of 41% and an FSH-releasing potency of 34%. These values fall within the fiducial limits of the potency obtained in the first assay (Table III). No dissociation of LH and FSH releasing activities was observed.

TABLE IV: FSH-RH and LH-RH Activity of Me_5Phe^3–LH-RH When Infused into Immature Male Rats for 4 Hr.

Sample	Dose (ng/rat)	Mean Plasma LH[a] (ng/ml ± S.E.)	Mean Plasma FSH (ng/ml ± S.E.)
Saline		0.36 ± 0.12	594.02 ± 6.93
LH-RH	100	9.42 ± 1.60	1003.33 ± 87.73
	300	45.17 ± 3.31	4342.00 ± 132.10
Me_5Phe^3	100	1.84 ± 0.02	793.93 ± 34.81
	300	13.99 ± 1.97	1560.00 ± 131.65
		FSH-RH Potency (%) with 95% Confidence Limits	LH-RH Potency (%) with 95% Confidence Limits
Me_5Phe^3		34 (28–40)	41 (31–52)

[a] Plasma samples were taken 30 min after the cessation of infusion.

Discussion

Me_5Phe^3-LH-RH. The potency of this peptide is far greater than that reported for any other analog of LH-RH with modifications in the three position. It is, at first sight, remarkable that replacement of tryptophan by an amino acid with a side chain so dissimilar both structurally and chemically to the indole ring system should result in retention of such high levels of activity. Since the only observable similarity in the properties of these two amino acids, apart from aromaticity, is their ability to donate π electrons to phthalyl-, 4-nitrophthalyl-, and tetrachlorophthalylimides (Carrión *et al.*, 1968), resulting in the formation of charge-transfer complexes, it is probable that similar electronic interactions between tryptophan or Me_5Phe, and a suitably oriented aromatic moiety on the pituitary receptor, represents one of the principal criteria for the maintenance of biological function in LH-RH peptides, either by ensuring binding of the hormone to the receptor, participation in the mechanism promoting LH release, or a combination of both factors. Hofmann *et al.* (1974) recently found that analogs of ACTH in which tryptophan is replaced by phenylalanine are almost devoid of biological activity and yet exhibit high affinity for ACTH receptor(s). This suggests that tryptophan, in addition to being involved in the binding process, is also in some way involved in the mechanism of corticosteroid release *via* the adenylyl cyclase system. It is tempting to surmise that, in this example, as well as in the case of LH-RH, the essential feature of the tryptophan residue for maintaining hormonal action is its electron-donating properties. Essential functionality of the indole NH group in the LH-release triggering mechanism can be ruled out.

The resistance of derivatives of Me_5Phe to enzymatic hydrolysis (Tesser *et al.*, 1973) suggested the possibility that Me_5Phe^3-LH-RH might be more resistant to enzymatic inactivation than LH-RH itself, and, therefore, have more prolonged activity. This was checked by infusing the peptides over a 4-hr period into immature male rats, an assay which has been

shown (Coy *et al.*, 1974a,b) to reveal this phenomenon quite well. It is apparent from the lack of increase of activities (Table IV) that there is probably no prolongation of activity.

(5-F-Trp)^3*-LH-RH.* The replacement of the hydrogen atom in position 5 of the aromatic nucleus of tryptophan by a fluorine atom of a similar atomic radius might be expected to have little effect on the stereochemistry of the molecule. Nevertheless, the LH releasing activity of the fluorinated peptide was drastically reduced. It is possible that the electron-withdrawing effect of fluorine, although in competition with an opposing electron-donating resonance effect, might reduce the electron density of the aromatic ring system and hence its ability to complex with the receptor site.

(p-NO_2-Phe)^3*-, (p-NH_2-Phe)*^{3}*-, and His*^{3}*-LH-RH.* Since Phe^3-LH-RH (Yanaihara *et al.*, 1973) and Tyr^3-LH-RH (Coy *et al.*, 1973a) have significant, although low, LH-releasing activities, it was of interest to prepare analogs with substituents in the benzene ring of phenylalanine. (*p*-NO_2-Phe)^3-LH-RH had much diminished activity as compared to Phe^3-LH-RH, whereas (*p*-NH_2-Phe)^3-LH-RH showed a slightly higher potency than the latter compound. With these peptides it is more difficult to attribute changes in biological activity to the electronic effects of ring substituents since other factors such as size, acid–base behavior, and hydrophobicity might have overriding effects. However, the *p*-NO_2-Phe analog, containing the strongly electron-withdrawing nitro group, is by far the least active of the phenylalanine analogs. The activity of His^3-LH-RH was also very low, which is not surprising since the physical and chemical properties of this peptide are very different from LH-RH.

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