Polyfluoroalkylamine Derivatives of Luteinizing Hormone-Releasing Hormone†

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ABSTRACT: A series of C-terminally fluorinated analogs of luteinizing hormone-releasing hormone (LH-RH) was synthesized by a combination of solid-phase and classical techniques and found to have interesting properties when assayed for LH- and FSH-releasing activities over a prolonged period of time in immature male rats. One of the peptides, desGly^{10}-LH-RH-2,2,2-trifluoroethylamide, was found to be about nine times more effective than LH-RH in releasing LH, or approximately twice as active as the corresponding alkylamide peptide, desGly^{10}-LH-RH-ethylamide. However, desGly^{10}-LH-RH-2,2,3,3,3-pentafluoropropylamide was only slightly more active than LH-RH and considerably less active than the corresponding propylamide analog. A family of peptides was also prepared containing D-alanine in position six of the chain in conjunction with the C-terminal modifications. D-Ala^6,desGly^{10}-LH-RH-2,2,2-trifluoroethylamide, surprisingly, gave patterns of gonadotropin release which were only as intense and virtually identical with those obtained with D-Ala^6,desGly^{10}-LH-RH-ethylamide which in turn releases about 20 times more LH than a similar dose of LH-RH. D-Ala^6,desGly^{10}-LH-RH-propylamide also gave almost identical patterns of gonadotropin release. D-Ala^6,desGly^{10},LH-RH-2,2,3,3,3-pentafluoropropylamide was considerably less potent, being only five times more effective than LH-RH.

The close similarity in the sizes of the hydrogen and fluorine atoms and the greatly increased electronegativity of the latter often result in the production of intense biochemical effects when fluorine is introduced into a wide range of biologically active compounds. Possibly because of difficulties associated with the synthesis of fluorinated derivatives of the amino acids, comparatively little work has been published concerning peptides containing fluorine. Reports have been mainly limited to the substitution of *p*-fluorophenylalanine for the phenylalanine residues of bradykinin (Nicolaides et al., 1963), angiotensin (Bumpus and Smeby, 1968; Marshall and Merrifield, 1972; Vine et al., 1973), and the gastrin tetrapeptide (Morley, 1968). Coy et al. (1974a) have also recently reported the synthesis and biological activity of the 5-fluorotryptophan[3] analog of LH-RH.[1]

The synthesis of two C-terminally modified LH-RH analogs, desGly^{10}-LH-RH-ethylamide and propylamide (Fujino et al., 1972), and the subsequent discovery of their high gonadotropin-releasing properties (Arimura et al., 1974a), suggested to us that the insertion of fluoroalkyl groups into the end of the molecule might lead to peptides with interesting biological properties. Furthermore, it seemed probable that simple synthetic methods were available for introducing fluorine into a part of the analog molecule which was obviously exerting profound effects on biological activity. In this paper, we describe the successful syntheses and some biological properties in the rat of several desGly^{10}-LH-RH analogs containing the 2,2,2-trifluoroethylamide and 2,2,3,3,3-pentafluoropropylamide groups at their C-terminus.

Materials and Methods

Amino acid derivatives were of the L configuration and were purchased from Bachem, Inc., Marina del Rey, Calif. Fluorinated amines were purchased from PCR, Inc., Gainesville, Fla.

Amino acid analyses were performed on a Beckman 119 amino acid analyzer 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 method was used in which buffer 1 (pH 3.40) contained 1% 1-propanol, buffer 2 (pH 4.25), 0.2% 1-propanol, and buffer 3 (pH 6.4), 4% 1-propanol. Buffer change times were set at 75.3 and 134.3 min. 2,2,2-Trifluoroethylamine and 2,2,3,3,3-pentafluoropropylamine eluted 5 and 9 min before histidine, respectively. Propylamine emerged 43 min after arginine.

The following TLC solvent systems were used to demonstrate the homogeneity of final peptides: R_f^1, 1-butanol–acetic acid–water (4:1:5, upper phase); R_f^2, 2-propanol–1 *M* acetic acid (2:1); R_f^3, 1-butanol–acetic acid–water–ethyl acetate (1:1:1:1); R_f^4, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Sample sizes of about 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 reagent in succession.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHNH$_2$ (*I*). Boc-protected amino acids (3.0 mmol) were coupled in a Beckman 990 automatic peptide synthesizer to a 1% cross-linked poly(styrene–divinylbenzene–proline) resin (2.78 g, 1.0 mmol of proline) in the presence of dicyclohexylcarbodiimide (3.0 mmol) by a process which has been described

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

previously (Coy et al., 1973a). After each coupling, Boc protecting groups were removed by treatment with 25% trifluoroacetic acid in methylene chloride. Amino acids with reactive side chains were protected as follows: histidine, dinitrophenyl (Coy et al., 1973b); serine, benzyl; tyrosine, 2-bromobenzyloxycarbonyl (Yamashiro and Li, 1973); arginine, tosyl (Coy et al., 1973c).

The completed protected peptide resin (1.8 g) was stirred with anhydrous hydrazine (1 ml) in dimethylformamide (10 ml) at 0° for 6 hr and the volatile components were then removed in vacuo at 30°. The residue was extracted with methanol and the protected nonapeptide (309 mg) precipitated by the addition of ethyl acetate.

Part of this material (287 mg) in anisole (6 ml) was stirred (30 min) with anhydrous hydrogen fluoride (24 ml) (Sakakibara et al., 1965) at 0°. Hydrogen fluoride was removed as rapidly as possible (75 min) in vacuo and the peptide–anisole mixture distributed between 0.1 *M* acetic acid and ethyl acetate. The aqueous layer (50 ml) was extracted three times with ethyl acetate and lyophilized to a powder which was first applied to a column (2.5 × 95 cm) of Sephadex G-25 fine and eluted with 0.2 *M* acetic acid giving a major peak (elution volume 310–380 ml). The partially purified peptide was then subjected to partition chromatography (Yamashiro, 1964) on a column (2.5 × 95 cm) of Sephadex G-25 fine previously equilibrated with the lower followed by the upper phase of a system of 1-butanol–acetic acid–water (4:1:5). Elution with the upper phase yielded pure peptide I (70 mg) (R_f 0.23–0.15); $[\alpha]^{26}D$ −45° (*c* 1.07, 0.1 *M* AcOH); R_f^1, 0.24; R_f^3, 0.55; R_f^4, 0.51. Amino acid analysis gave: Glu, 1.05; His, 0.95; Trp, 0.85; Ser, 0.98; Tyr, 1.00; Gly, 1.00; Leu, 1.01; Arg, 0.96; Pro, 0.98.

*pGlu-His-Trp-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHNH*$_2$ (*II*). A protected nonapeptide resin was prepared using the reagents and conditions described in the synthesis of peptide I, except that Boc-D-Ala was used instead of Boc-Gly in cycle 3. Part of this resin (2.0 g) was stirred with anhydrous hydrazine (1 ml) in dimethylformamide (12 ml) at 0° for 6 hr and the solvent was then removed in vacuo. The residue was dissolved in methanol and the protected peptide intermediate precipitated with ethyl acetate to yield 485 mg of powder.

Peptide obtained from the hydrogen fluoride deprotection of a part of this material (380 mg) was subjected to gel filtration (peak at 300–360 ml) and partition chromatography (R_f 0.34–0.23) to give 170 mg of homogeneous peptide II: $[\alpha]^{23}D$ −45° (*c* 0.54, 2 *M* AcOH); R_f^1, 0.20; R_f^3, 0.54; R_f^4, 0.65. Amino acid analysis gave: Glu, 1.03; His, 0.95; Trp, 0.89; Ser, 0.96; Tyr, 1.00; Ala, 1.01; Leu, 1.01; Arg, 0.94; Pro, 0.98.

*pGlu-His-Trp-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHCH*$_2$*C-H*$_2$*CH*$_3$ (*III*). Protected peptide resin (3.98 g), prepared in the synthesis of peptide II, was stirred with propylamine (15 ml) at 0° for 6 hr. Excess amine was removed in vacuo; the residue was dissolved in methanol, and precipitated by the addition of ethyl acetate to yield 490 mg of powder.

Peptide obtained from the hydrogen fluoride deprotection of part of this material (350 mg) was subjected to gel filtration (peak at 340–390 ml) and partition chromatography (R_f 0.45–0.34) and gave 204 mg of pure propylamide (III): $[\alpha]^{23}D$ −44° (*c* 0.69, 2 *M* AcOH); R_f^1, 0.18; R_f^2, 0.48; R_f^3, 0.58; R_f^4, 0.81. Amino acid analysis gave: Gly, 1.04; His, 0.98; Trp, 0.95; Ser, 0.96; Tyr, 1.00; Ala, 1.01; Leu, 1.00; Arg, 0.95; Pro, 1.00; $CH_3CH_2CH_2NH_2$, 0.99.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH$_2$*CF*$_3$ (*IV*). Attempts were made to prepare the fluorinated peptides via the cleavage of intermediate protected nonapeptides from their resin support by direct reaction with the fluoroalkylamines. The peptide–resin ester linkage, however, was not cleaved under these conditions, presumably because of the weak basicity and nucleophilicity of the amines due to the electron-withdrawing fluorinated groups. All of the fluorinated analogs were, therefore, synthesized by the azide coupling method.

DesGly^{10}-LH-RH hydrazide (I) (50 mg, 50 μmol) was dissolved in dimethylformamide (1.5 ml) and cooled to −20°. Hydrogen chloride (5.4 *M*) in dioxane (57 μl, 300 μmol) followed by isoamyl nitrite (14 μl, 300 μmol) were added and stirred at −20° (10 min). After the addition of 2,2,2-trifluoroethylamine (30 μl), the solution was kept at −10° for 1 hr and 4° overnight. Dimethylformamide was removed in vacuo and the residue eluted directly on a small partition column (1.5 × 95 cm) under the conditions already described. Homogeneous peptide IV (21 mg) was recovered with an R_f of 0.50–0.35; $[\alpha]^{23}D$ −64° (*c* 0.39, 2 *M* AcOH); R_f^1, 0.30; R_f^2, 0.55; R_f^3, 0.60; R_f^4, 0.63. Amino acid analysis gave: Glu, 1.05; His, 1.05; Trp, 0.94; Ser, 0.94; Tyr, 0.96; Gly, 1.05; Leu, 1.00; Arg, 1.00; Pro, 0.99; $CF_3CH_2NH_2$, 0.93.

pGlu-His-Trp-Ser-Tyr-D-Ala-Leu-Arg-Pro-NHCH$_2$*C-F*$_3$ (*V*). D-Ala^6,desGly^{10}-LH-RH hydrazide (II) (50 mg, 50 μmol) was converted to its azide and reacted with trifluoroethylamine under the conditions described for peptide IV. Partition chromatography gave homogeneous peptide V (25 mg): R_f 0.42–0.30; $[\alpha]^{23}D$ −31° (*c* 0.53, 2 *M* AcOH); R_f^1, 0.27; R_f^2, 0.51; R_f^3, 0.60; R_f^4, 0.72. Amino acid analysis gave: Glu, 1.01; His, 0.95; Trp, 0.88; Ser, 0.95; Tyr, 0.98; Ala, 1.00; Leu, 0.98; Arg, 0.97; Pro, 0.96; $CF_3CH_2NH_2$, 0.97.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHCH$_2$*CF*$_2$-*CF*$_3$ (*VI*). DesGly^{10}-LH-RH hydrazide (I) (50 mg, 50 μmol) was converted to the azide and reacted with 2,2,3,3,3-pentafluoropropylamine (45 μl) under the conditions described under the synthesis of peptide IV. Partition chromatography of the products gave homogeneous peptide VI (29 mg): R_f 0.59–0.41; $[\alpha]^{23}D$ −51° (*c* 0.52, 2 *M* AcOH); R_f^1, 0.20; R_f^2, 0.54; R_f^3, 0.57; R_f^4, 0.67. Amino acid analysis gave: Glu, 1.05; His, 0.97; Trp, 1.03; Ser, 0.94; Tyr, 1.02; Gly, 1.00; Leu, 1.02; Arg, 1.00; Pro, 1.00; $CF_3CF_2CH_2NH_2$, 1.01.

*pGlu-His-Trp-Ser-Tyr-*D*-Ala-Leu-Arg-Pro-NHCH*$_2$*C-F*$_2$*CF*$_3$ (*VII*). D-Ala^6,desGly^{10}-LH-RH hydrazide (II) (50 mg, 50 μmol) was converted to the azide and reacted with pentafluoropropylamine in the usual manner. Partition chromatography of the products gave homogeneous peptide VII (24 mg): R_f 0.59–0.40; $[\alpha]^{23}D$ −44° (*c* 0.52, 2 *M* AcOH); R_f^1, 0.21; R_f^2, 0.59; R_f^3, 0.54; R_f^4, 0.75. Amino acid analysis gave: Glu, 1.04; His, 0.98; Trp, 1.03; Ser, 0.93; Tyr, 1.00; Ala, 1.00; Leu, 1.00; Arg, 1.00; Pro, 0.98; $CF_3CF_2CH_2NH_2$, 0.99.

Bioassays. LH- and FSH-releasing activities of the four fluorinated analogs, D-Ala^6,desGly^{10}-LH-RH-propylamide (III), and D-Ala^6,desGly^{10}-LH-RH-ethylamide (Coy et al., 1974b) were measured over a prolonged period of time in 25 day-old male rats. Peptides and LH-RH were dissolved in 0.1% gelatin/0.9% saline solution in concentrations of 50 ng/0.2 ml and these amounts were injected subcutaneously into immature Sprague-Dawley rats (6 per group). Control groups were injected with the carrier solution only. Serum gonadotropin levels were measured by radioimmunoassay

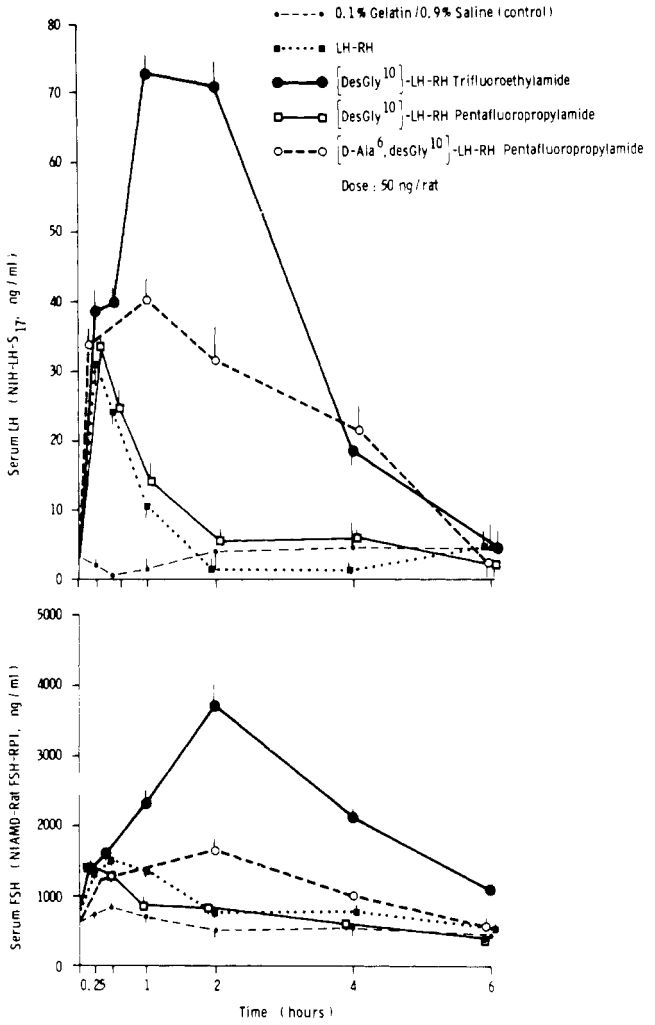

FIGURE 1: Serum LH and FSH concentrations at 0.25–6 hr after sc injection of immature male rats with 50-ng amounts of LH-RH, desGly[10]-LH-RH-trifluoroethylamide (IV), desGly[10]-LH-RH-pentafluoropropylamide (VI), and D-Ala[6],desGly[10]-LH-RH-pentafluoropropylamide (VII).

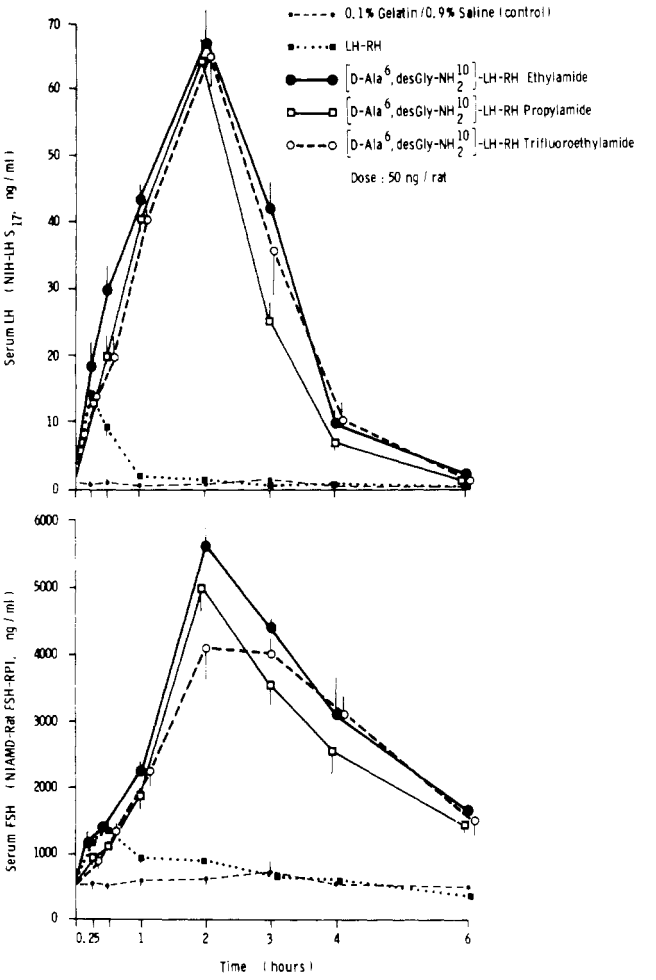

FIGURE 2: Serum LH and FSH concentrations at 0.25–6 hr after sc injection of immature male rats with 50-ng amounts of LH-RH, D-Ala[6],desGly[10]-LH-RH-ethylamide, D-Ala[6],desGly[10]-LH-RH-propylamide (III), and D-Ala[6],desGly[10]-LH-RH-trifluoroethylamide (V).

(Niswender et al., 1968; Daane and Parlow, 1971) after injection. LH was expressed as NIH-LH-S_{17} and FSH in terms of NIAMD-RAT-FSH-RP-1. Mean serum gonadotropin levels in all groups at each time interval were calculated and plotted on arithmetic graphs against time (Figures 1 and 2). The gonadotropin-releasing activity of an analog was then considered to be proportional to the integral of the corresponding curve.

Results and Discussion

The ratios of the integrated serum gonadotropin concentrations produced by the synthetic analogs and LH-RH are given in Table I. All of the fluorinated analogs were more active in releasing LH than equal doses of LH-RH. The corresponding FSH-releasing activities in most cases were elevated to a lesser extent. DesGly[10]-LH-RH-trifluoroethylamide (IV) released about nine times more LH than LH-RH and was almost twice as active as desGly[10]-LH-RH-ethylamide (Fujino et al., 1972) which has been assayed previously (Arimura et al., 1974a) under similar conditions. Furthermore, peak gonadotropin levels appeared to be considerably displaced from 30 min to 1–2 hr. It is possible that this large increase in activity is a result of the uniquely strong electron-withdrawing properties of the trifluoromethyl group situated at the end of the molecule and exerting a favorable effect on the receptor-site binding conformation of the peptide as a whole. Also, we have noticed in studying other analogs with changes at the C-terminus (Fujino et al., 1973; Coy et al., 1975) that, for groups of a similar size to an ethyl or propyl group, the more lipophilic the end of the peptide chain the greater the biological activity appears to be. The trifluoroethyl group is more hydrophobic than an ethyl group and might, therefore, interact even more favorably with a given portion of the receptor. However, the low activity of desGly[10]-LH-RH-pentafluoropropylamide (VI) containing an even more hydrophobic end group does not support this trend. This peptide, in fact, is considerably less active than desGly[10]-LH-RH-propylamide (Fujino et al., 1972; Arimura et al., 1974a) which displays lower but delayed peak gonadotropin responses and is about as active as the ethylamide peptide. It is unlikely that the increased activities of this type of analog are due to greater physiological half-lives since desGly[10]-LH-RH-ethylamide has a similar rate of disappearance to LH-RH in the human when concentrations are measured by radioimmunoassay (A. Arimura, unpublished observations). It has recently been found that when the ethylamide modification is introduced into peptides with D-alanine and D-leucine in place of glycine in position 6 of LH-RH, analogs of similar and extremely high gonadotropin-releasing activity result (Coy et al., 1975; Vilchez-Martinez et al., 1974; Fuji-

Table I: Activities of Analogs and LH-RH Expressed as Ratios of Integrated Serum Gonadotropin Levels in Figures 1 and 2.

Peptide	Activity LH	Activity FSH
LH–RH	1.0	1.0
DesGly^{10}-LH-RH-ethylamide	5.5[a]	7.0[a]
DesGly^{10}-LH-RH-propylamide	5.5[a]	9.4[a]
DesGly^{10}-LH-RH-trifluoroethylamide	9.3	5.9
DesGly^{10}-LH-RH-pentafluoropropylamide	1.6	0.6
D-Ala^6,desGly^{10}-LH-RH-ethylamide[b]	21	16
D-Ala^6,desGly^{10}-LH-RH-trifluoroethylamide	20	14
D-Ala^6,desGly^{10}-LH-RH-propylamide	17	13
D-Ala^6,desGly^{10}-LH-RH-pentafluoropropylamide	5.5	2.3

[a] Arimura et al. (1974a). [b] Arimura *et. al.* (1974b).

no et al., 1974a, b). It was of interest, therefore, to synthesize D-alanine^6 peptides with the fluorinated C-terminus. In particular, we expected that D-Ala^6,desGly^{10}-LH-RH-trifluoroethylamide (V) would perhaps be the most potent LH-RH analog synthesized thus far. In view of the prolonged activity of desGly^{10}-LH-RH-propylamide, D-Ala^6,desGly^{10}-LH-RH-propylamide (III) was also synthesized and both peptides were assayed against D-Ala^6,-desGly^{10}-LH-RH-ethylamide (Figure 2). Surprisingly, within the limits of experimental error, all three analogs gave identical patterns of gonadotropin release. The failure of the trifluoroethylamide group to enhance the potency of the combination analog is peculiar and possibly provides a clue to explain the high activity of desGly^{10}-LH-RH-trifluoroethylamide. Perhaps the trifluoroethylamide group, in this case, is exerting two favorable effects on the binding affinity of the peptide, one of which is related to that produced by the original ethylamide alteration and the other to that produced by D-alanine in position 6, which is believed to be predominantly conformational in character (Monahan et al., 1973). When D-alanine is incorporated into the same molecule it is conceivable that the resulting rigid and irreversible conformational change overrides the qualitatively similar effect produced by the trifluoroethylamide group. Consequently, the activity of the combination analog is lower than would have been predicted. As expected, D-Ala^6,-desGly^{10}-LH-RH-pentafluoropropylamide (VII) (Figure 1) was considerably less active than the other combination analogs; however, it still exhibited the same type of delayed releasing patterns, levels peaking at 1–2 hr.

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