An Approach to the Elucidation of Metabolic Breakdown Products of the Luteinizing Hormone-Releasing Hormone

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Metabolic breakdown of the luteinizing hormone-releasing hormone (LH-RH) could lead to the following fragments containing pyroglutamic acid: pyroglutamic acid (**1**), pGlu-His (**2**), pGlu-His-Trp (**3**), pGlu-His-Trp-Ser (**4**), etc., and finally pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (**10**). We have synthesized fragments **2–10** and successfully separated all ten metabolites and LH-RH by high-performance liquid chromatography (HPLC) with a μBondapak C_{18} column. In a test of the viability of the method, cochromatography of fragments **1–10** and LH-RH with the products of chymotryptic digestion of tritiated LH-RH showed radioactive peaks corresponding to the expected products, fragments **3** and **5**. Analysis of the products of incubation of a rat kidney homogenate supernatant with LH-RH showed fragments **1–4** and LH-RH. The finding of breakdown at position 4 uncovers a new site of LH-RH breakdown and points the way to the design of potential LH-RH antagonists and agonists where the 4 position would be substituted with unnatural amino acids to prevent breakdown.

The fundamental role of the luteinizing hormone-releasing hormone (LH-RH)[1c] in the physiology of ovulation[2] has prompted metabolic breakdown studies in various organs, with the aim of determining enzymatic pathways possibly contributing to its regulation. Of additional impetus has been the belief that the understanding of the mode of LH-RH enzymatic breakdown would be invaluable in the design of metabolically resistant, and hence potentially longer acting, agonists and antagonists of this hormone for use in fertility control.

Studies in various laboratories have attempted to elucidate the mode of breakdown of LH-RH using homogenates or isolated enzymes derived from mammalian organs. Thus, LH-RH has been reported to undergo breakdown at pGlu[1]-His[2] by a pyroglutamate aminopeptidase[3] and at Pro[9]-Gly-NH_2[10] by a postproline cleaving enzyme,[4-6] both enzymes isolated from brain and pituitary. An endopeptidase cleaving at His[2]-Trp[3] and Tyr[5]-Gly[6] has been isolated from pituitary.[7] Cleavages at Tyr[5]-Gly[6] by liver homogenates[8] and at Gly[6]-Leu[7] by brain, hypothalami, pituitary, and liver extracts[9-11] have also been reported. In none of these studies, however, was it possible to determine the full profile of breakdown products and their rate of appearance, on account of the slow methodology used (e.g., separation by electrophoresis or open column chromatography, followed by amino acid analysis).

Analogues of LH-RH have been prepared with unnatural amino acids as substituents, in attempts to prevent enzymatic breakdown at the various susceptible sites or to devise antagonists by replacing amino acids probably involved in the hormonal response. Examples of such analogues are [D-Phe[2]]LH-RH, which was one of the earliest antagonists discovered,[12] and [D-pGlu[1]]-, [D-Ala[6]]-, [(*N*-Me)Leu[7]]-, [aza-Gly[10]]-, and [Pro-ethylamide[9]]LH-RH, which are about 8,[13,14] 450,[15] 102,[16] 100,[17] and 500%[18] as active, respectively, as LH-RH. Incorporation of several of these types of modifications into the same analogue has successfully led to superagonists such as [D-Trp[6], Pro-ethylamide[9]]LH-RH, which is 144 times as active as LH-RH,[19] and [Ac-D-Phe[1],D-(*p*-Cl)Phe[2],D-Trp[3,6]]LH-RH, which is a highly potent antagonist.[20] Incubation of rat brain homogenates with analogues bearing unnatural amino acids or *N*-ethylamide C termini[21,22] has provided direct

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evidence of the reduced susceptibility of these analogues to enzymatic breakdown. The high biological activity of some of these analogues has been attributed, at least in part, to their enhanced affinity for receptors and in part to resistance to metabolic breakdown.[23]

It is well documented that the kidney is one of the most important sites of breakdown for peptide hormones.[24] There is evidence that the kidney may also be involved in the metabolism and clearance of LH-RH. In this connection, it has been found that upon injection of ^{3}H-labeled LH-RH into female rats the highest concentration of the ^{3}H-label was located in the kidney and that an hour after the injection of ^{3}H-labeled LH-RH into man 48% of the radioactivity appeared in the urine.[25] Furthermore, it has been observed that renal-failure patients have higher LH-RH levels, slower LH-RH metabolic clearance rates, and longer half-times of LH-RH disappearance.[26] Consequently, the determination of the products of LH-RH degradation in kidney tissue could be invaluable in the design of LH-RH analogues.

In the present study we describe a method for the elucidation of metabolic breakdown products of LH-RH by a novel, rapid, and convenient approach employing HPLC.[27] We also describe the products of LH-RH degradation by homogenates of rat kidney as an example of the practical application of this method.

Results and Discussion

Since we used [pGlu-3,4-^{3}H]LH-RH in incubations with mammalian enzyme systems, we hypothesized that there would be only ten possible breakdown fragments bearing pyroglutamic acid (assuming only conventional modes of enzymatic hydrolysis) namely: pyroglutamic acid (**1**), pGlu-His (**2**), pGlu-His-Trp (**3**), pGlu-His-Trp-Ser (**4**), pGlu-His-Trp-Ser-Tyr (**5**), pGlu-His-Trp-Ser-Tyr-Gly (**6**), pGlu-His-Trp-Ser-Tyr-Gly-Leu (**7**), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg (**8**), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro (**9**), and pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (**10**). Except for **1**, which was purchased, we prepared **2**, **3**, and **7** by solution methods and **4**–**10** and hormone by solid phase (SP) methods. Great difficulty was experienced in obtaining significant yields of **7** by the standard SP methodology. We suspect that during deprotection and coupling of the Gly-Leu-resin sequence to Boc-Tyr(Bzl) substantial amount of peptide was probably lost as diketopiperazine; precedent for such side reaction during SP synthesis has been documented by Rothe and Mazanek.[28] Hence, **7** was prepared successfully by a modified SP synthesis which circumvented the potential formation of diketopiperazine by the use of Boc-Tyr-(Bzl)-Gly (prepared by solution methods) and by an independent synthesis in which pGlu-His-Trp was coupled to Ser(Bzl)-Tyr(Bzl)-Gly-Leu-OBzl (prepared by solution methods).

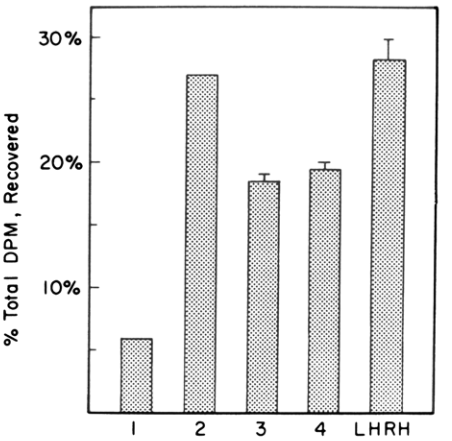

Figure 1. LH-RH and metabolites produced upon incubation with a 105000*g* supernatant of a renal homogenate as determined by HPLC. Quantities are expressed as percent of total radioactivity in renal homogenate incubation sample recovered. Bar is the mean of two experiments. Standard error is represented by the vertical lines except when the standard error is imperceptible. Numbers 1–4 indicate metabolic fragments as defined in the text. Total radioactivity injected = 569059.2 ± 1580.9 dpm (mean ± SE). The percentage of radioactivity recovered averaged 90%.

Employing TLC or TLE analysis, we were unable to devise a solvent system capable of adequately separating all ten peptides and LH-RH on cellulose or on silica gel, even though more than 20 solvent systems were tried. In general, peptides **3**–**7** moved as one group, and **8**–**10** and hormone, the arginine containing peptides, moved as another group. These experiments underscore the great difficulty encountered in attempting to separate breakdown products by conventional techniques and then in elucidating the structure of the apparently "homogeneous" products obtained, as often described in LH-RH breakdown studies.

As described under Experimental Section, we accomplished the separation of our set of peptides by high-performance liquid chromatography (HPLC) with an analytical μBondapak C_{18} column.

As a test of the viability of our method, we restudied the chymotryptic cleavage[29,30] of LH-RH using [pGlu-3,4-^{3}H]LH-RH. We had previously isolated the breakdown products, **3** and **5**, by gel filtration and had verified their structure by amino acid analysis.[30] In the present study we analyzed the chymotryptic products by injecting into our HPLC system an aliquot of the incubate plus "cold" fragments **1**–**10** and hormone (approximately 1 μg each), in order to test the relative retention time for each possible fragment. In confirmation of the previous findings,[30] we detected only radioactive peptides which coeluted with standards of fragments **3** and **5**.

Similar HPLC analysis of a rat kidney homogenate 105000*g* supernatant after incubation with ^{3}H-labeled LH-RH showed recovered hormone, as well as fragments **1**–**4** (Figure 1). All of the radioactive products observed in the above degradation studies coeluted with standards, thus corroborating our expectation of a conservative mode of peptide breakdown.

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The use of retention times to characterize the breakdown products from hormone labeled with high specific activity (38 Ci/mmol) allows the detection of metabolites in the picomole range. This is well beyond the capabilities of more conventional methods of peptide identification. We believe this unique and sensitive technique is suitable for determining the rates of breakdown and the identity of metabolites of LH-RH not only in renal but other pertinent tissues involved in LH-RH breakdown.

The products obtained with kidney homogenates could arise via cleavage by separate endopeptidases or, alternatively, via cleavage at Ser^4-Tyr^5 to yield fragment **4**, followed by stepwise cleavage of **4** by carboxypeptidases to yield successively **3**, **2**, and **1**. In either case, cleavage at position 4 has not been reported to date in any mammalian tissue.

It has been proposed that analogues of LH-RH with D-amino acids in positions 1–3 and 6 are good antagonists on account of their high affinity for pituitary receptors and, at least in part, on account of their resistance to metabolic breakdown at position 6.[23] The profile of LH-RH breakdown products determined in kidney homogenates suggests that metabolic resistance to renal degradation at positions 1–3 may be an additional factor in making these analogues long lasting and, hence, more active. Furthermore, our studies suggest that LH-RH agonists or antagonists should be prepared featuring the substitution of L-serine with an unnatural amino acid lacking the natural L configuration. The latter property would render the **4**–**5** bond much less susceptible to breakdown by the kidney enzyme, possibly leading to analogues which would be longer acting in vivo.

Experimental Procedures

Optical rotations were measured with a Rudolph polarimeter (precision ±0.1°). Thin-layer electrophoresis (TLE) was performed on Eastman Chromagram, silica gel, thin-layer sheets and Quantagram silica gel plates with 0.1 M Py–AcOH (pH 3.3 to 6) at 500 V for 45 min to 7.5 h, by means of a Desaga-Brinkman apparatus. Thin-layer chromatography (TLC) was performed on Quantagram silica gel plates. When partition solvent systems were used, the lower phase was sprayed on the plate and development was accomplished with the upper phase. Solvent systems used for column chromatography or TLC were (A) 15% MeOH–$CHCl_3$, (B) 33% MeOH–$CHCl_3$, (C) 66% MeOH–$CHCl_3$, (D) *n*-BuOH–AcOH–H_2O (4:1:5), (E) *n*-BuOH–AcOH–H_2O (8:1:2), (F) *n*-BuOH–AcOH–H_2O (4:1:1), and (G) *n*-BuOH–EtOH–Py–1% AcOH (14:2:5:24). For detection of peptides, Cl_2–tolidine or Ehrlich's or Pauly reactions were used. For gel permeation chromatography we employed a Sephadex G-25 (Pharmacia) column 2.4 × 117 cm) unless otherwise indicated. Partition chromatography was accomplished on Sephadex G-25 by the method of Yamashiro.[31] Column elution profiles were determined by the Folin method with the Lowry modification.[32] For deprotection of synthetic peptides with liquid hydrogen fluoride,[33] an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan) was used. Boc amino acids were purchased from Bachem Inc. (Torrance, CA) and Vega Biochemicals (Tucson, AZ). Pyroglutamic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). For high-performance liquid chromatography (HPLC) we used a Waters Associates apparatus consisting of the UK6 injector, two Model 6000A solvent pumps, a 660 solvent programmer, a Model 450 variable-wavelength detector, a Houston Instruments dual pen recorder, and a μBondapak C_{18} column 30 × 0.39 cm). UV absorbance is expressed as AUFS (absorbance units full scale) and, unless otherwise indicated, it was determined at 220 nm, AUFS = 0.1. The solvents used were glass distilled (Burdick and Jackson). Water used was purified by passing institutionally deionized and distilled water through a D5041 mixed ion-exhcange cartridge (Barnstead, Sybron Corp., Boston, MA) and distilling in an all-glass still. [pGlu-3,4-^{3}H]LH-RH was purchased from New England Nuclear Corp. For amino acid analyses, peptides were hydrolyzed for 24 h at 110 °C with 6 N HCl and then were analyzed in a JEOL amino acid analyzer by the method of Spackman et al.[34] Tryptophan peptides were hydrolyzed by the method of Simpson et al.,[35] which gives good yields of this amino acid. All tryptophan peptides gave positive Ehrlich's reaction and had expected UV absorptions corresponding to aromatic structures, suggesting an intact tryptophane ring. α-Chymotrypsin was purchased from Sigma Chemical Co.

Solid-Phase (SP) Synthesis of Peptides. The starting Boc-amino acid-resin, prepared by standard methodology[36] or by the cesium salt esterification method,[37] was taken manually through the required number of coupling cycles by the SP method of synthesis,[38] as modified in a previous synthesis of LH-RH by Flouret et al.[39] In each cycle, the Boc group was removed with 25% trifluoroacetic acid in CH_2Cl_2 and, after neutralization of the resin with triethylamine (TEA), coupling was performed with a 4 mol excess of the desired amino acid and dicyclohexylcarbodiimide (DCC). For the protection of side chain functionalities Boc-Ser(Bzl), Boc-Tyr(Bzl), Boc-Arg(Tos), and Boc-His(DNP) were used. Completion of the coupling was monitored by means of the ninhydrin test of Kaiser et al.[40] After introduction of the histidyl residue, the DNP group was removed by treatment of the protected peptide resin with thiophenol (20 mmol/mmol of Boc-amino acid-resin) in DMF,[41] and the synthesis was then continued. The final peptide was removed by treatment with liquid HF–anisole as described by Flouret et al.[39] for a previous synthesis of LH-RH.

Pyroglutamic Acid (1). This amino acid was recrystallized from H_2O, and was homogeneous on solvent systems A–C.

pGlu-His (2). This peptide was prepared in crystalline form by saponification of its methyl ester as described previously.[42,43]

pGlu-His-Trp (3). This tripeptide was prepared by hydrogenolysis of its benzyl ester.[44]

pGlu-His-Trp-Ser (4). Boc-Ser(Bzl)-resin (0.53 mmol of Boc-Gly/g, 3.8 g) was converted to peptide **4**, 337 mg, by the SP method. For purification, tetrapeptide was dissolved in the upper phase (3 mL) of solvent system D and subjected to partition chromatography on Sephadex G-25. Fractions corresponding to R_f 0.22 were pooled and lyophilized, yielding 181 mg: $[\alpha]^{24}_D$ −11° (*c* 1, 50% AcOH); homogeneous and Ehrlich's reagent positive on TLE and on TLC. Amino acid analysis (Glu = 1.00): His, 0.98; Ser, 0.91; Glu, 1.00; Trp, 0.76.

pGlu-His-Trp-Ser-Tyr (5). Boc-Tyr(Bzl)-resin (0.43 mmol of Boc-Tyr(Bzl)/g, 2.33 g) was converted to 0.6 g of peptide **5** by the SP method. For purification, 500 mg of this material was chromatographed on a silica gel column with the upper phase of solvent D as the eluent. The effluent, monitored by TLC with system F, had a main component with R_f^F 0.31. After lyophilization of appropriate fractions, we obtained 53 mg of peptide **5**: homogenous on TLE and on TLC; $[\alpha]^{24}_D$ −9.8° (*c* 1, 50% AcOH).

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Amino acid analysis (Glu = 1.00): His, 1.01; Ser, 0.98; Glu, 1.0; Tyr, 1.03; Trp, 0.48 (partially destroyed during acid hydrolysis).

pGlu-His-Trp-Ser-Tyr-Gly (6). Boc-Gly-resin (0.7 mmol of Boc-Gly/g, 1.43 g) was converted to **6**, 512 mg, by the SP method. For purification, 210 mg of this material was subjected to gel permeation on Sephadex G-25. Fractions corresponding to the main peak, after lyophilization, yielded 45 mg of product. The latter was purified once again by gel permeation, yielding 35 mg of peptide **6**: $[\alpha]^{24}_D$ −13° (*c* 1, 50% AcOH); homogeneous on TLE and on TLC. Amino acid analysis (Glu = 1.00): His, 0.98; Ser, 0.89; Glu, 1.0; Gly, 1.01; Tyr, 1.00; Trp, 0.79 (partially destroyed by acid hydrolysis).

Boc-Tyr(Bzl)-Gly-OEt. Boc-Tyr(Bzl) (22.27 g, 30 mmol) was added at 0 °C to a suspension of Gly-OEt·HCl (9.2 g, 30 mmol) in CH_2Cl_2 (60 mL) containing TEA (8.4 mL, 30 mmol); after 10 min, DCC (12.4 g, 30 mmol) was added. After 16 h, the reaction mixture was filtered, the filtrate was evaporated to dryness, and the residue crystallized from EtOAc–petroleum ether, yielding 24.6 g (90%) of the dipeptide: mp 124–125 °C; $[\alpha]^{25}_D$ −7.65° (*c* 1, DMF). Anal. ($C_{25}H_{32}N_2O_6$) C, H, N.

Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OEt. The above protected dipeptide ester (9.13 g, 20 mmol) was treated with 4 M HCl–EtOAc (100 mL); after 20 min, the resulting hydrochloride was collected and washed with Et_2O. The peptide hydrochloride was dissolved in CH_2Cl_2 (40 mL), neutralized with TEA (2.8 mL, 20 mmol), and then coupled to Boc-Ser(Bzl) (5.9 g, 20 mmol) with DCC (4.13 g, 20 mmol) and HBT (3.07 g, 20 mmol) at 0 °C. The reaction mixture was filtered after 16 h, the filtrate was evaporated to dryness, and the residue was dissolved in EtOAc; the latter solution was washed with citric acid, H_2O, and $NaHCO_3$, dried ($MgSO_4$), and evaporated to dryness. The solid residue thus obtained was crystallized from Et_2O–petroleum ether, yielding 11.6 g (91%) of the tripeptide: mp 85–89 °C; $[\alpha]^{25}_D$ −11.6° (*c* 1, DMF). Anal. ($C_{35}H_{43}N_3O_8$) C, H, N.

Boc-Ser(Bzl)-Tyr(Bzl)-Gly. A solution of the preceding tripeptide ester (5.7 g, 9 mmol) in MeOH (10 mL) was cooled to 0 °C and treated with 4 N KOH (2.5 mL). After 50 min, the clear solution was acidified with citric acid and extracted with EtOAc (50 mL, 3 times). The EtOAc extracts were washed with H_2O, dried ($MgSO_4$), and evaporated to an oil. This residual oil was dissolved in EtOAc, and dicyclohexylamine (DCHA; 1.8 mL, 9 mmol) was added. The crystals which formed were collected and washed with EtOAc, yielding 6.35 g (90%) of the DCHA salt: mp 171–173 °C; $[\alpha]^{25}_D$ −8.56° (*c* 1, DMF). Anal. ($C_{45}H_{62}N_4O_8$) C, H, N.

The DCHA salt (3.29 g, 4.18 mmol) was converted to the free acid by treatment with citric acid solution and extraction with EtOAc. Evaporation of solvent left a solid residue, which was recrystallized from EtOAc to yield 2.33 g (92%) of the tripeptide: mp 133–135 °C; $[\alpha]^{24}_D$ −5° (*c* 2, Py), −12.2° (*c* 2, EtOH).

Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-OBzl. A solution of the above tripeptide DCHA salt (3.08 g, 3.92 mmol) was converted to the free acid. Leu-OBzl *p*-toluenesulfonate salt (1.54 g, 3.92 mmol) was converted to free leucine benzyl ester by treatment with Na_2CO_3 solution and extraction with EtOAc. A solution of the above Leu-OBzl in CH_2Cl_2 (8 mL) was added to the above tripeptide acid, and the resulting solution was cooled to 0 °C and treated with HBT (1 g, 7.4 mmol) and then with DCC (0.85 g, 4.1 mmole). After 16 h, the reaction mixture was filtered to remove dicyclohexylurea, and the filtrate was evaporated to dryness. The solid residue was dissolved in EtOAc, and the solution was washed with citric acid, H_2O, Na_2CO_3, and H_2O, dried ($MgSO_4$), and evaporated to dryness. Material thus obtained was crystallized from EtOAc–petroleum ether, yielding 2.11 g (76%) of the tetrapeptide: mp 100–102 °C; $[\alpha]^{24}_D$ −25° (*c* 2, EtOH). Anal. ($C_{46}H_{56}N_4O_9$) C, H, N.

Boc-Tyr(Bzl)-Gly-OBzl. This peptide was prepared in 82% yield by the methods described for the preparation of the ethyl ester: mp 101–104 °C; $[\alpha]^{24}_D$ +2° (*c* 2, EtOH). Anal. ($C_{30}H_{34}N_2O_6$) C, H, N.

Boc-Tyr(Bzl)-Gly. To a solution of the above dipeptide ester (10.4 g, 20 mmol) in EtOH (20 mL) was added 4 N KOH (10 mL) over a period of 2 min. After 5 min, the reaction mixture was acidified with citric acid to pH 3–4 and extracted with EtOAc (100 mL, 3 times); the EtOAc extract was dried (Na_2SO_4), the solvent was removed under vacuum, and the residue was crystallized from EtOAc–petroleum ether, yielding 7.51 g (88%) of product: mp 146–148 °C; $[\alpha]^{24}_D$ −5° (*c* 2, Py). Anal. ($C_{23}H_{28}N_2O_6$) C, H, N.

pGlu-His-Trp-Ser-Tyr-Gly-Leu (7). (a) Fragment Condensation Method. A solution of Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-OBzl (790 mg, 1 mmol) in 50% F_3AcOH–CH_2Cl_2 (10 mL) was allowed to stand at room temperature; after 15 min, the solvent was evaporated under vacuum, the residue was dissolved in CH_2Cl_2, and the solution was washed with Na_2CO_3 and H_2O, dried ($MgSO_4$), and evaporated to dryness. The residue was dissolved in DMF (10 mL), and the solution was treated at 0 °C with HBT (300 mg, 2.2 mmol), pGlu-His-Trp (450 mg, 1 mmol), and DCC (210 mg, 1 mmol). After 20 h, the dicyclohexylurea was collected by filtration, and the filtrate was evaporated under vacuum to an oil. Product thus obtained was purified by preparative TLC on silica gel with 33% MeOH–$CHCl_3$; material with R_f 0.46 (Ehrlich's reaction) was eluted with MeOH, yielding the protected peptide as a gum (500 mg). Heptapeptide thus obtained was deprotected with liquid HF, yielding 400 mg of product. This material was purified by preparative silica gel TLC with *n*-BuOH–Py–H_2O–AcOH–benzene (15:10:12:3:4), R_f 0.25. The product obtained was homogeneous on TLE and on TLC: $[\alpha]^{24}_D$ −20° (*c* 1, 50% AcOH). Amino acid analysis (His = 1.00): Glu, 1.00; His, 1.00; Trp, 0.86; Ser, 0.87; Tyr, 1.00; Gly, 1.03; Leu, 0.99.

(b) Solid-Phase Method. Boc-Leu-resin (0.636 mmol of Boc-Leu/g, 3.15 g) was converted to **7**, 0.59 g, by the SP method. Tyr and Gly were introduced by coupling Boc-Tyr(Bzl)-Gly. For purification, 200 mg of this material was subjected to gel filtration with 10% AcOH as the eluent; the main peak (elution volume, 270 mL) was lyophilized, yielding 63 mg of the desired heptapeptide, identical with the sample prepared by solution methods on TLC, on TLE, and on HPLC (to be described below).

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg (8). Boc-Arg(Tos)-resin [0.43 mmol of Boc-Arg(Tos)/g resin, 2.33 g] was converted to **8**, 889 mg, by the SP method. For purification, 400 mg of this material was chromatographed on a silica gel (80 g) column with solvent system E; the slower moving main peak yielded 85 mg of partially purified product. A second purification by gel permeation on Sephadex G-25 yielded 55 mg of peptide **8**: $[\alpha]^{24}_D$ −17.2° (c 1, 50% AcOH). Amino acid analysis (Gly = 1.00): His, 0.96; Ser, 0.90; Glu, 0.98; Gly, 1.00; Tyr, 0.93; Trp, 0.75 (partially destroyed by acid hydrolysis). Heptapeptide is homogeneous on TLC and TLE.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro (9). Boc-Pro-resin (0.5 mmol of Boc-Pro/g resin, 4 g) was converted to peptide **9**, 1.26 g, by SP synthesis. The latter product, 0.63 g, was subjected to partition chromatography on Sephadex G-25 with solvent system D. Appropriate fractions, R_f 0.21, were lyophilized, and the solid obtained, 0.27 g, was purified by gel permeation chromatography, which yielded 0.26 g of peptide 9: homogenous on TLC and TLE. Amino acid analysis (Glu = 1.0): His, 0.95; Arg, 1.01; Ser, 0.84; Glu, 1.0; Pro, 1.0; Gly, 1.04; Leu, 0.97; Tyr, 1.03; Trp, 0.85.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly (10). This decapeptide was prepared as described earlier.[45]

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LH-RH). The preparation of this hormone has been previously described.[39]

Analysis of Peptides by HPLC. The following solvent systems were used: (A) 99% of 0.1% H_3PO_4 (pH 2.4)–1% CH_3CN; (B) 60% CH_3CN–40% of 0.1% H_3PO_4 (pH 2.4). The use of phosphoric acid[46] provided better separation of peptides than the use of organic acids. Solvents were filtered and degassed prior to use. Detection of peptides was accomplished at 220 nm. For analysis of a sample of ^3H-labeled LH-RH after incubation with a 105000*g* supernatant of a rat kidney homogenate or with α-chymotrypsin, a standard mixture of LH-RH, fragments **1–10**, and an aliquot of the incubation medium were injected simultaneously and were eluted with solvent A (2 mL/min). After fragments **1** and **2** emerge unresolved with the solvent front, a

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linear gradient elution from 0 to 20% solvent B (2 mL/min) for 3 min elutes, and suitably resolves, the other peptides in the following order (capacity factor indicated in parentheses): **4** (k_4 = 6.2), **3** (k_3 = 7.7), **6** (k_6 = 11.0), **5** (k_5 = 15), **8** (k_8 = 21), LH-RH (k_{LH-RH} = 24), **10** (k_{10} = 27), **9** (k_9 = 44), **7** (k_7 = 60). The capacity factor k_n is a measure of the column retention volume for eluted compound n, as defined by the equation $k_n = (V_n - V_0)/V_0$, where V_n = retention volume from the time of injection to the peak maximum and V_0 = void volume or volume from the time of injection to the unretained solvent peak. In a separate experiment, isocratic elution with H_2O (0.4 mL/min) results in the emergence of suitably resolved fragments **1** (k_1 = 0.4) and **2** (k_2 = 2.0). The other peptides are strongly retained on the column and are removed by a 10-min gradient from 0 to 100% solvent B. To determine the LH-RH breakdown pattern, the eluent is collected in 1-min samples, except when **3** and **4** are eluted, at which time 0.5-min samples are collected. The radioactivity is measured by counting with Aquasol (New England Nuclear). Each radioactive peak obtained was assigned the identity of the peptide standared with which it cochromatographed.

Renal Homogenate Preparation and Incubations. Five female rats (Charles River) were decapitated and exsanguinated. The kidneys were removed, washed in buffer, and frozen at -80 °C. The defrosted kidneys were put through a tissue press at 4 °C. Five grams of pressed tissue was homogenized in 50 mL of a 10 mM phosphate buffer, pH 7.2. The homogenate was centrifuged at 105000*g* for 60 min. The supernatant was drawn off and stored at -80 °C. This supernatant was used for breakdown studies. [pGlu-3,4-^{3}H]LH-RH (10 μL, 0.2 mmol) was incubated at 37 °C with 40 μL of supernatant (2.6 mg of protein/mL) for 30 min. The reaction was terminated by the addition of 50 μL of CH_3CN, followed by centrifugation at 17300*g* for 10 min.

Incubation with Chymotrypsin. [pGlu-3,4-^{3}H]LH-RH (5 μL, 0.15 mol) was incubated for 1 h at 25 °C with α-chymotrypsin (40 ng) in a total volume of 50 μL of solution (pH 7.8) containing a final concentration of 0.05 M $CaCl_2$ and 0.04 M Tris-HCl. The reaction was terminated by the addition of CH_3CN (50 μL), followed by centrifugation at 17300*g* for 10 min. The supernatant was drawn off and frozen at -80 °C for later analysis.

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Synthesis of Thyrotropin-Releasing Hormone Analogues with Selective Central Nervous System Effects

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Thyrotropin-releasing hormone (TRH) analogues which show relative selectivity for action in the central nervous system have been recognized. Practical syntheses for three of these TRH analogues which show the greatest selectivity, <Aad-His-Tzl-NH_2 (**5**), <Glu-His-Pip-OMe (**2**), and <Aad-His-Pro-NH_2 (**6**), are described. The first two were prepared by solution methods of peptide synthesis. Compound **6** was prepared by the solid-phase method. Problems of histidine racemization, facile diketopiperazine formation, and instability of acylated thiazolidine carboxylic acid derivatives under acidic conditions have been minimized in order to attain optimal yields. Physical properties such as pK, NMR shifts, and circular dichroism have been examined as they might relate to biological activity and peptide conformation.

In a preliminary communication,[1] we have reported the synthesis and biological properties of a series of thyrotropin-releasing hormone (TRH) analogues. The primary objective of our studies of TRH analogues has been the dissociation of the hormone releasing properties[2] from the direct central nervous system (CNS) effects[3] which were thought to relate to antidepressant activity in humans.[4] The nature of the CNS effects of TRH as they relate to antidepressant activity has not been clear. Initial studies suggested that a dihydroxyphenylalanine (Dopa) potentiation activity might be important.[3] Another study has related CNS responses to enhanced turnover of noradrenalin,[5] while another relates them to cholinergic pathways.[6] These effects and their implications for therapy have been reviewed.[7]

In our initial structure–activity studies, CNS activity was measured by the ability to restore the anticonvulsant effect of methazolamide in reserpine-treated rats, a measure of noradrenergic stimulation.[8] The potency in this test has been compared to the potency of analogues for their ability to stimulate release of T_3 and T_4 using a standard type of in vivo assay.[2] As shown in Table I, structure modification

Table I. Biological Activities of TRH Analogues

analogue	activities[a] hormonal[b]	CNS[c]
<Glu-His-Pro-NH_2 (TRH, **1**)	1	1
<Glu-His-Pip-OMe[d] (**2**)	0.2	1
<Aad-His-Pip-OMe[e] (**3**)	0.02	0.9
Ica-His-Pro-NH_2[f] (**4**)	0.1	0.35
<Aad-His-Tzl-NH_2[g] (MK 771, **5**)	1	35
<Aad-His-Pro-NH_2 (**6**)	1	4
<Glu-His-Tzl-NH_2 (**7**)	0.2	2.3

[a] Potency relative to TRH. [b] Based on measurement of ^{125}I-labeled T_3 and T_4 after administration of varying doses of the analogue.[2] [c] Based on measurement of the ability of varying doses of the analogue to restore the anticonvulsant activity of methazolamide in reserpine-treated animals.[8,17] [d] Pip-OH = L-pipecolic acid. [e] Aad-OH = L-α-aminoadipic acid. [f] Ica-OH = L-2-oxoimidazolidine-4-carboxylic acid. [g] Tzl-OH = L-thiazolidine-4-carboxylic acid.

† Deceased November 12, 1980. The coauthors dedicate this paper to the memory of our friend and colleague.

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