234, 1525 (1959).
(10) G. Brawerman, D. A. Hufnagel, and E. Chargaff, *Biochim. Biophys. Acta*, 61, 340 (1962).
(11) H. G. Zachau, *Angew. Chem., Int. Ed. Engl.*, 8, 711 (1969).
(12) E. C. Miller and J. A. Miller, *Pharmacol. Rev.*, 18, 805 (1966).
(13) E. K. Weisburger and J. H. Weisburger, *Advan. Cancer Res.*, 5, 331 (1958).
(14) A. Giner-Sorolla, S. A. O'Bryant, C. Nanos, M. R. Dollinger, A. Bendich, and J. H. Burchenal, *J. Med. Chem.*, 11, 521 (1968).
(15) A. Giner-Sorolla, *ibid.*, 12, 717 (1969).
(16) A. Giner-Sorolla and A. Bendich, *J. Amer. Chem. Soc.*, 80, 3932 (1958).
(17) J. H. Burchenal, M. Dollinger, J. Butterbaugh, D. Stoll, and A. Giner-Sorolla, *Biochem. Pharmacol.*, 16, 423 (1967).
(18) J. W. Jones and R. K. Robins, *J. Amer. Chem. Soc.*, 82, 3773 (1960).
(19) R. Shapiro, *ibid.*, 86, 2948 (1964).
(20) F. R. Benson, L. W. Hartzel, and E. A. Otten, *ibid.*, 76, 1858 (1954).
(21) J. A. Johnson, Jr., H. J. Thomas, and H. J. Schaeffer, *ibid.*, 80, 699 (1958).
(22) A. S. Jones and R. T. Walker, *J. Gen. Microbiol.*, 31, 187 (1963).
(23) R. Shapiro and S. Shiuey, *Biochim. Biophys. Acta*, 174, 403 (1969).
(24) Houben-Weyl, "Methoden der Organischen Chemie," Vol. X, no. 1, Georg Thieme Verlag, Stuttgart, 1971, p 1053.
(25) E. Fischer, *Justus Liebigs Ann. Chem.*, 236, 198 (1886).
(26) E. Fischer, *ibid.*, 239, 249 (1887).
(27) A. Giner-Sorolla, L. Medrek, and A. Bendich, *J. Med. Chem.*, 9, 143 (1966).
(28) F. Feigl, "Spot Tests in Organic Analysis," 6th ed, Elsevier, Amsterdam, 1960, p 165; *cf.* ref 1.
(29) G. V. Bruning, *Justus Liebigs Ann. Chem.*, 253, 5 (1889).
(30) L. Maaskant, *Recl. Trav. Chim. Pays-Bas*, 65, 211 (1937).
(31) J. Attenburrow, *et al.*, *J. Chem. Soc.*, 1094 (1952).
(32) A. Giner-Sorolla, *J. Heterocycl. Chem.*, 8, 651 (1971).
(33) G. B. Brown, K. Sugiura, and R. M. Creswell, *Cancer. Res.*, 25, 986 (1965).
(34) K. Sugiura and G. B. Brown, *ibid.*, 27, 925 (1967).

Synthesis and Biological Activity of the Hypothalamic LH- and FSH-Releasing Decapeptide

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The decapeptide <Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 has been synthesized by solution methods in a stepwise fashion and by the solid-phase method. All protected intermediates were purified by silica gel chromatography. Removal of the blocking groups with liquid HF and a two-stage purification on Sephadex G-25 and G-15 yielded a homogeneous synthetic product with the same chromatographic properties and the same ability to release LH and FSH, from rat pituitaries *in vitro*, as the naturally occurring hormone.

The release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary is under control of the hypothalamus which secretes a controlling substance designated LH-releasing hormone (LH-RH)-FSH-releasing hormone (FSH-RH).[1] Evidence has been presented that both LH and FSH are under control of the same hypothalamic releasing hormone.[2,3] The structure of this hormone, LH-RH/FSH-RH, was described as the decapeptide <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I) for the porcine species.[4]

In order to provide independent confirmation of the validity of structure I for the hormone, we synthesized[5] this decapeptide and attained as well a second goal of providing a convenient route to substantial amounts of decapeptide for the more extensive studies which are required to ascertain its biological role. Several syntheses have been reported[6-11] independently of ours, with yields which are very low or not stated.

For the synthesis of I, the desired triprotected decapeptide <Glu-His-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (X) was synthesized by solution and by solid-phase methods.

In one approach, a stepwise method of synthesis was employed, starting from glycinamide and using mainly *tert*-butyloxycarbonyl (Boc) amino acid active esters.[12] The Boc group was removed from intermediate peptides without affecting side-chain protecting groups by employing trifluoroacetic acid (TFA)-CH_2Cl_2 (1:1),[13] except for tryptophyl peptides, in which case 1% mercaptoethanol was added. All intermediates were purified by column chromatography on silica gel and the structure was corroborated from their nuclear magnetic resonance spectra. The yields at each step were usually high (70–95%) and the overall yield of X was 10% based on glycinamide.

The triprotected decapeptide X was also assembled by the solid-phase method[14] employing 4 *M* HCl–dioxane for the removal of Boc groups.[15] An aliquot of the peptide-resin was retained as octapeptide-resin VIIIa, and the remainder of the material was converted to the decapeptide-resin Xa. The completed Xa was ammonolyzed and purified by chromatography on silica gel yielding triprotected decapeptide X in good yield (35–40%),† identical with X prepared by the stepwise method. Thus, the solid-phase method appeared to be a convenient method for obtaining X rapidly and in good yield. Ammonolysis of VIIIa and chromatography of the crude product led to pure octapeptide VIII of comparable quality to the octapeptide made by the stepwise method.

A fragment-condensation method was also employed for making the octapeptide VIII. This method was of interest in order to develop flexible synthetic routes for analogs involving amino acid substitutions in either of the fragments being condensed. The synthesis of VIII was accomplished by a condensation involving the C-terminal tetrapeptide IV

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†From early chromatographic fractions the faster moving methyl ester precursor of X was isolated, thus indicating some transesterification in NH_3-MeOH. The ester structure was confirmed by nmr, which shows sharp and distinct methyl protons, and by conversion of the ester to X by ammonolysis.

and the tetrapeptide Boc-Trp-Ser(Bzl)-Tyr(Bzl)-Gly, the latter being obtained by saponification of the tetrapeptide methyl ester XII, assembled by the active ester method starting from glycine methyl ester.

The removal of the protecting groups from the triprotected decapeptide amide X was accomplished by treatment with anhydrous hydrogen fluoride.[16] The crude decapeptide was subjected to a two-step Sephadex gel filtration[17] purification yielding I in 25–30% yield from X. The elemental analysis was consistent with the structure of I. Acid hydrolysis of I followed by amino acid analysis[18] showed the presence of all amino acids in the expected molar ratios, except for tryptophane which is destroyed under the conditions of hydrolysis. The optical rotation was identical with that reported recently by Geiger.[7,‡] Two-dimensional thin-layer electrophoresis (tle) followed by tlc showed the decapeptide to move as one component and with a mobility similar to that of a natural porcine-hormone standard.

The decapeptide I was tested biologically by *in vitro* incubation with male rat hemipituitaries, assay where the release for both FSH[19] and LH[20] was measured and compared with the activity of a synthetic standard shown previously at Abbott and in the laboratories of A. V. Schally[21] to be equal to the pure natural hormone.§ The results of the present *in vitro* experiments demonstrate that I has equal LH- and FSH-releasing activity to the synthetic standard. These data and the recent observation[22] that the essentially pure natural releasing hormone stimulates the release of LH and FSH *in vivo* as well as *in vitro* in rats support the concept that both LH and FSH are under control of the same hypothalamic hormone.

Experimental Section#

N-Benzyloxycarbonylprolylglycinamide (II). A solution of glycinamide hydrochloride (25.0 g) in a mixture of MeOH (500 ml) and H_2O (100 ml) was treated with Rexyn 201 (OH^-) (450 g). The suspension was filtered, the resin was washed with three 500-ml portions of MeOH, and the combined filtrates were concentrated to an oil *in vacuo*. To a solution of the glycinamide (19 g), prepared as described above, in DMF (150 ml) was added Cbz-Pro-ONp (56 g) and the resulting solution allowed to stand at room temperature overnight. The solvent was removed under reduced pressure and the residue purified by chromatography on silica gel (800 g) with MeOH–$CHCl_3$ as the eluting solvent. As detected by tlc (Cl_2–tolidine), fast-moving components were eluted with 5% MeOH–$CHCl_3$, whereas II was eluted with 15% MeOH–$CHCl_3$. The desired fractions were combined and concentrated to a solid residue which was crystallized from EtOH to yield 43.73 g (96%): mp 145–146°; $[\alpha]^{24}D$ −31° (*c* 1, DMF); R_f^{II} 0.4 on tlc, showing a single Cl_2–tolidine spot. The nmr (CD_3COOD) was consistent with the structure. *Anal.* ($C_{15}H_{19}N_3O_4$) C, H, N.

N-Benzyloxycarbonyl-N^8-nitroarginylprolylglycinamide (III). Hydrogenation of II (1.22 g) in EtOH–MeOH with 5% Pd/C (0.45 g) and H_2 was conducted for 4 hr. A tlc of this reaction mixture revealed complete reaction. The free dipeptide had R_f^{II} 0.1 as shown with ninhydrin or Cl_2–tolidine color reactions. The reaction mixture was filtered, the filtrate was evaporated to a residual oil which was dissolved in Py (8 ml) containing Cbz-Arg(NO_2) (1.06 g), and the resulting solution was treated at room temperature with DCC (619 mg). After 16 hr the reaction mixture was filtered to remove DCU and the filtrate was evaporated to a residual oil which was purified by chromatography with silica gel as described for II. The desired protected peptide fractions with R_f^{II} 0.4 (Cl_2–tolidine) were combined and evaporated to a solid which was dried *in vacuo* over P_2O_5 yielding 1.32 g (87%), $[\alpha]^{23}D$ −25.4° (*c* 1, DMF). The nmr spectrum (CD_3COOD) was consistent with the structure. A tlc analysis showed III to move as one component (R_f^{II} 0.4). *Anal.* ($C_{21}H_{30}N_8O_7$) C, H; N: calcd, 22.1; found, 21.6.

N-tert-Butyloxycarbonylleucyl-N^8-nitroarginylprolylglycinamide (IV). To a solution of III (10.1 g) in AcOH (90 ml) was added 32% HBr–AcOH (90 ml) and the clear solution was allowed to stand for 1 hr. The product was precipitated by addition of the reaction solution to Et_2O (1 l.) followed by decantation and washing with five additional 1-l. portions of Et_2O. The product thus obtained was dried over P_2O_5 and KOH pellets *in vacuo*. The dried monoprotected tripeptide amide hydrobromide was dissolved in MeOH (100 ml), treated with Rexyn 201 (OH^-) (100 g), and filtered and the resin was washed with MeOH and with 10% AcOH–MeOH. The filtrate and washings were combined and concentrated to an oil which was dissolved in DMF (20 ml) and treated with Boc-Leu-ONp (8.5 g). After 16 hr, the DMF was removed under reduced pressure and the oil obtained was chromatographed on silica gel as described for II. The protected tetrapeptide amide IV was eluted with 15% MeOH–$CHCl_3$. A tlc analysis revealed one component with R_f^{II} 0.25. The appropriate fractions were combined and evaporated to a solid which after drying *in vacuo* over P_2O_5 weighed 7.52 g (65%), $[\alpha]^{23}D$ −44.7° (*c* 1, DMF). The nmr spectrum (CD_3COOD) was consistent with the structure. *Anal.* ($C_{24}H_{43}N_9O_8 \cdot CH_3OH$) C, H, N.

N-tert-Butyloxycarbonylglycylleucyl-N^8-nitroarginylprolylglycinamide (V). A solution of IV (7.04 g) in 50% TFA–CH_2Cl_2 (120 ml) was allowed to stand at room temperature for 20 min. The solvents were removed *in vacuo*, the residue was treated six times with 50-ml portions of CH_2Cl_2, and the mixture was concentrated to dryness under reduced pressure. The solid residue was dissolved in MeOH (150 ml) and the solution treated with Rexyn 201 (OH^-) as described for III, yielding the monoprotected aminotetrapeptide. The product was dissolved in DMF (12 ml) and Boc-Gly-ONp (4.62 g) was added. The resulting solution was allowed to stand for 16 hr, the DMF removed *in vacuo*, and the product purified by column chromatography on silica gel as described for II. The desired product was eluted with 15% MeOH–$CHCl_3$ and after evaporation of the solvent and drying over P_2O_5 *in vacuo* there was obtained 6.04 g (78%), $[\alpha]^{24}D$ −29.9° (*c* 1, DMF). A tlc analysis showed one component (R_f^{II} 0.2) with Cl_2–tolidine or ninhydrin color reactions. The nmr spectrum (CD_3COOD) was consistent with the structure. *Anal.* ($C_{26}H_{46}N_{10}O_9$) C, H, N.

N-tert-Butyloxycarbonyl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (VI). Deprotection of V (6.04 g) in 50% TFA–CH_2Cl_2, coupling of the aminopentapeptide with Boc-Tyr(Bzl)-ONp (5.56 g), and purification of the crude product by column chromatography on silica gel, by methods similar to those described for IV, gave VI as an amorphous solid: 7.82 g (93%); $[\alpha]^{24}D$ −26.6° (*c* 1, DMF). A tlc analysis showed one component (R_f^{II} 0.3) with Cl_2–tolidine or ninhydrin. The nmr spectrum (CD_3COOD) was consistent with the structure. *Anal.* ($C_{42}H_{61}N_{11}O_{11}$) C, H, N.

N-tert-Butyloxycarbonyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (VII). Removal of the Boc group of VI (4.69 g) in 50% TFA–CH_2Cl_2, coupling of the aminohexapeptide with Boc-Ser(Bzl)-ONp (7.02 g), and purification of the coupling product by silica gel chromatography, by methods described for IV, afforded 5.07 g (83%) of VII, $[\alpha]^{23}D$ −28.5° (*c* 1, DMF). The nmr spectrum (CD_3COOD) was similar to that of VI showing the distinct Boc group and, in addition, the characteristic benzylic and aromatic protons of the Boc-Ser(Bzl) moiety. The tlc of VII showed one component (R_f^{II} 0.45) with Cl_2–tolidine and ninhydrin. *Anal.* ($C_{52}H_{72}N_{12}O_{13}$) C, H, N.

N-tert-Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (VIII). Removal of the Boc group of VII (5.0 g) was accomplished by the methods described for IV. The triprotected aminoheptapeptide amide was coupled with Boc-Trp-ONp (2.58 g). The product was purified by

‡ R. Geiger, *et al.*, reported a positive optical rotation for I possibly through a typographical error.

§ Fraction AVS 77-33 (no. 215-269) obtained through the courtesy of Dr. A. V. Schally, Endocrine and Polypeptide Laboratory, Veterans Administration Hospital, New Orleans, La. Abbott's synthetic standard for LH-RH was prepared by the solid-phase method as described in this paper. This standard, labeled 125 c, was exhaustively compared by Dr. Schally to natural LH-RH AVS-77-33 no. 215-269 (see ref 21). Our standard lot 146 is identical with the one submitted to Dr. Schally as sample 125 c.

All optically active amino acids are of L configuration. A Thomas-Hoover apparatus was used for melting point determinations in capillary melting tubes. Where analyses are indicated only by symbols of the elements, analytical results were obtained for the elements within ±0.4% of the theoretical value. Where tlc was used to determine purity of intermediates and products, silica gel G plates were used. The solvent systems employed were: I, 2% MeOH–CH_2Cl_2; II, 15% MeOH–$CHCl_3$; III, 33% MeOH–$CHCl_3$; and IV, 66% MeOH–$CHCl_3$. The nmr spectra were obtained at 100 MHz using a Varian Associates HA-100 spectrometer. The following abbreviations were used throughout the text: DCC = dicyclohexylcarbodiimide; DCU = dicyclohexylurea; TFA = trifluoroacetic acid; TEA = triethylamine; ONp = *p*-nitrophenyl ester; Py = pyridine.

column chromatography on silica gel and crystallization from MeOH–EtOH yielding 4 g (76%) of VIII: mp 147–149°; $[\alpha]^{24}D$ −23.9° (*c* 1, DMF). The nmr spectrum (CD_3COOD) showed the incorporation of the Boc-tryptophyl residue. The tlc analysis showed one component (R_f^{II} 0.3) with Cl_2–tolidine, ninhydrin, and Ehrlich's color reactions. *Anal.* ($C_{63}H_{82}N_{14}O_{14}$) H, N; C: calcd, 60.1; found, 59.6.

N-tert-**Butyloxycarbonylhistidyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (IX).** The Boc group of VIII (1.43 g) was removed as described for V, except for the addition of 1% mercaptoethanol to the 50% TFA–CH_2Cl_2.[23] The triprotected aminooctapeptide amide was dissolved in a solution containing Boc-histidine (296 mg) in DMF (2 ml) and DCC (240 mg) in CH_2Cl_2 (2 ml) was added. After 16 hr the reaction mixture was filtered to remove DCU, the filtrate evaporated under reduced pressure, and the residue purified by silica gel chromatography as described above for IV, yielding IX: 1.17 g; $[\alpha]^{24}D$ −19.4° (*c* 1, AcOH). The nmr spectrum (CD_3COOD) showed the incorporation of the Boc-histidyl residue. Analysis of IX by tlc showed one spot (R_f^{II} 0.2) with Cl_2–tolidine, ninhydrin, and Ehrlich and Pauly's spray. *Anal.* ($C_{69}H_{89}N_{17}O_{15}$) N.

Pyroglutamylhistidyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (X). The Boc group of IX (900 mg) was removed as described in the preparation of IX. The triprotected aminononapeptide amide was coupled with pentachlorophenyl pyroglutamate (755 mg) and the coupling product was purified by column chromatography on silica gel. The faster moving components, as detected by tlc (33% MeOH–$CHCl_3$) with Ehrlich's color spray, were eluted with 5 and 15% MeOH–$CHCl_3$, while the product was eluted with 33% MeOH–$CHCl_3$. The desired fractions were combined and concentrated to a solid which was dissolved in boiling MeOH; the solution was concentrated and allowed to cool to room temperature. The triprotected decapeptide precipitated from the solution as an amorphous hard solid, yielding 612 mg: mp 166–169° dec; $[\alpha]^{24}D$ −25.2° (*c* 1, AcOH). The nmr spectrum (CD_3COOD) was identical with that of X made by the solid-phase method. Analysis by tlc showed X to have only one component (R_f^{III} 0.3) with Cl_2–tolidine and Ehrlich and Pauly's color sprays. *Anal.* ($C_{69}H_{86}N_{18}O_{15}$) C, H, N.

N-tert-**Butyloxycarbonyl-*O*-benzyltyrosylglycine Methyl Ester.** To a stirred mixture of 1.26 g (10 mmol) of Gly-OMe·HCl, 20 ml of CH_2Cl_2, and 1.0 g (1.4 ml) of TEA at 10° was added a solution of 3.8 g (7.7 mmol) of Boc-Tyr(Bzl)-ONp in 20 ml of CH_2Cl_2. Stirring was continued for 1 hr at 10° and then for 39 hr at room temperature. Evaporation of the solvent left a residue which, after EtOAc extraction, washing with a citric acid solution, H_2O, $NaHCO_3$ solution, and H_2O, concentration, and crystallization from Et_2O gave 3.08 g (90%) of product: mp 118–120°; $[\alpha]^{24}D$ −7° (*c* 1, DMF). *Anal.* ($C_{24}H_{30}N_2O_6$) C, H, N.

A sample of the above product, upon hydrolysis with an aqueous MeOH solution of 1 equiv of NaOH at room temperature for 3 hr, and subsequent acidification gave a sample of Boc-Tyr(Bzl)-Gly, which separated from EtOH–Et_2O as white crystals, mp 151–152°. *Anal.* ($C_{23}H_{28}N_2O_6$) C, H, N.

In another experiment in which Gly-OBzl was employed, there was obtained the corresponding Boc-Tyr(Bzl)-Gly-OBzl as a waxy amorphous solid. This product, upon ester exchange with anhydrous MeOH and a basic resin, gave the corresponding methyl ester, mp 119–120°.

N-tert-**Butyloxycarbonyl-*O*-benzylseryl-*O*-benzyltyrosylglycine Methyl Ester.** A mixture of 2.08 g (4.7 mmol) of Boc-Tyr(Bzl)-Gly-OMe and 18 ml of 4 *M* HCl in anhydrous dioxane was stirred at room temperature for 40 min and then concentrated to a residue. The residue was treated with 5 ml of Et_2O and the solvent removed *in vacuo*. The product was dissolved in 25 ml of anhydrous MeOH and treated with Rexyn 201 (OH^-) and filtered, and the filtrate was concentrated to 1.8 g of amorphous Tyr(Bzl)-Gly-OMe. A mixture of the above 1.8 g of monoprotected aminodipeptide methyl ester, 1.18 g of Boc-Ser(Bzl) (4 mmol), and 15 ml of CH_2Cl_2 was stirred and cooled to 5°. To this mixture was added 0.87 g (4 mmol) of DCC dissolved in 3 ml of CH_2Cl_2. The reaction mixture was stirred in the cold for 30 min and for 16 hr at room temperature, followed by filtration to remove crystalline DCU by-product. The CH_2Cl_2 filtrate was washed with H_2O, citric acid solution, H_2O, $NaHCO_3$ solution, and H_2O. The resulting CH_2Cl_2 solution was dried (Na_2SO_4) and concentrated *in vacuo* to a white solid froth. The product was crystallized from MeOH–Et_2O yielding 1.40 g, mp 127–128°, and a second crop, 0.89 g, mp 121–125°. These crops were equivalent by tlc analysis (solvent I), $[\alpha]^{26}D$ −13.4° (*c* 1, DMF). *Anal.* ($C_{34}H_{41}N_3O_8$) C, H, N.

In a similar fashion from Boc-Ser(Bzl) and Tyr(Bzl)-Gly-OBzl there was obtained the corresponding Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OBzl: mp 78° (variable with rate of heating), $[\alpha]^{25}D$ −15° (*c* 1, DMF). *Anal.* ($C_{40}H_{45}N_3O_8$) C, H, N.

N-tert-**Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycyl Methyl Ester (XII).** The Boc group of 1.24 g (2 mmol) of Boc-Ser(Bzl)-Tyr(Bzl)-Gly-OMe was removed as described above. A mixture of 1.04 g of the triprotected aminotripeptide, 0.544 g (1.8 mmol) of Boc-tryptophane, and 9 ml of CH_2Cl_2 was stirred at 0° while 0.45 g of DCC was added. This mixture was stirred without cooling for 16 hr and filtered to remove solid DCU by-product, and the latter was washed with CH_2Cl_2. The CH_2Cl_2 solution was washed with H_2O, citric acid solution, H_2O, $NaHCO_3$ solution, and H_2O and concentrated *in vacuo* yielding a solid. This material was recrystallized by dissolving in hot MeOH, concentrating to about half-volume, and cooling very slowly. The crystalline product weighed 1.29 g (88%), mp 168–170°. An analytical sample, obtained by an additional crystallization from MeOH, had mp 172°, $[\alpha]^{25}D$ −15° (*c* 1, DMF). *Anal.* ($C_{45}H_{51}N_5O_9$) C, H, N.

N-tert-**Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycine (XI).** A solution of 0.4 g (0.5 mmol) of the above tetrapeptide ester XII in 2 ml of CH_2Cl_2 containing 4 ml of MeOH was concentrated to a volume of 3.5 ml. To this solution was added 0.5 ml of H_2O and 0.5 ml of 1 *N* NaOH while stirring at room temperature. The turbid mixture was stirred for 1 hr. Concentration *in vacuo* without heating followed by shaking the residue with aqueous citric acid solution gave a solid product, which was collected on a filter and washed with citric acid solution, H_2O, and several times with Et_2O. The crude product, 0.34 g, mp 120–125°, was shown by tlc to be a mixture of the desired acid plus the starting ester. Separation was effected by chromatography on a silica gel column conditioned with CH_2Cl_2–MeOH (98:2). Elution was begun with the same solvent followed by gradual increase to 15% MeOH–CH_2Cl_2. Early fractions gave 0.096 g of recovered methyl ester, while later fractions gave 0.22 g of crystalline acid. The acid was recrystallized from MeOH–Et_2O yielding 0.204 g of a white crystalline powder: mp 133–135°; $[\alpha]^{25}D$ −14.7° (*c* 1, DMF). A comparison of the nmr spectra (CD_3OD) of the methyl ester XII and the acid XI showed the difference to be essentially that of a CH_3 proton peak at 222 Hz (TMS, 60 MHz), attributable to the methyl ester.

N-tert-**Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycinamide (VIII).** A sample of 0.193 g (0.33 mmol) of IV was converted to the amorphous monoprotected aminotetrapeptide amide (126 mg) as described for V. The material thus obtained was dissolved in 0.5 ml of CH_2Cl_2 plus 0.5 ml of Py and mixed with a suspension of 0.25 mmol (198 mg) of the acid XI in 1 ml of CH_2Cl_2. This mixture was stirred at 20° while 62 mg (0.30 mmol) of DCC was added with 2 ml of CH_2Cl_2. Stirring at room temperature for 20 min gave a nearly clear solution. After 40 hr the mixture was concentrated, and the residue was dissolved in 5 ml of a solvent mixture of CH_2Cl_2–MeOH (85:15) and chromatographed on 20 g of silica gel. The mid-fractions gave 58 mg of crude octapeptide, identified by tlc methods, and later fractions contained 0.18 g of by-product peptides, from which a small amount of the starting acid was recovered. The 58 mg of crude product (18%) was purified further by chromatography and crystallization from MeOH to obtain the purified tetraprotected octapeptide amide as white crystals: mp 144–147°; 35 mg (11%). By tlc and melting point comparisons this product appeared to be identical with VIII made by the solid-phase method or by the stepwise method. The nmr spectra (CD_3COOD) for VIII prepared by any of these methods were identical.

N-tert-**Butyloxycarbonylglycyl-resin.** This intermediate was prepared by usual methods[24] starting from polystyrene resin 2% crosslinked with divinylbenzene (100 g, Cyclo, 1.4 mequiv of Cl/g of resin). The final dried resin weighed 105 g. Amino acid analysis of an acid hydrolysate of the Boc-glycyl-resin indicated a content of 0.40 mmol of glycine/g of resin.

N-tert-**Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^8-nitroarginylprolylglycyl-resin (VIIIa).** A sample of 17.85 g of Boc-glycyl-resin was placed in a custom-made glass vessel mounted on a mechanical shaker. The following steps were performed for each amino acid coupling cycle. The resin was treated with (1) three 100-ml portions of dioxane; (2) three 100-ml portions of 4 *M* HCl–dioxane for 0.5 hr; (3) six 100-ml portions of dioxane; (4) three 100-ml portions of EtOH; (5) three 100-ml portions of $CHCl_3$; (6) three 100 ml-portions of TEA in $CHCl_3$ (1:10) for 10 min; (7) three 100-ml portions of $CHCl_3$; (8) four 100-ml portions of CH_2Cl_2. (9) The resin was then suspended in a solution of Boc-proline (8.61 g, 40 mmol) in 60 ml of CH_2Cl_2. After 10 min a solu-

Table I. Effect of Synthetic Decapeptide on *in Vitro* FSH and LH Release from Male Rat Hemipituitaries[a]

Treatment	Dose/pit., ng	LH release/pit.,[b] μg	FSH release/pit.,[c] μg
Control		0.31	41.2
Standard (lot 146)	1.76	1.52	131.6
Standard (lot 146)	7.04	2.42	220.9
Synthetic decapeptide (lot 049)	1.6	1.49	120.6
Synthetic decapeptide (lot 049)	6.4	2.56	172.9
Relative potency of 049 *vs.* 146 (95% confidence limits)		1.14 (2.41–0.55)	0.65 (1.19–0.33)

[a]Ten randomly selected hemipituitaries per flask incubated 6 hr in 4 ml of a tissue culture media containing 0.5% lactalbumin hydrolyzate. [b]Bioassay method of Parlow,[20] 5 assay rats per treatment group. [c]Bioassay method of Steelman and Pohley,[19] 5 assay rats per treatment group.

tion of DCC (8.65 g) in 10 ml of CH_2Cl_2 was added and the coupling reaction was allowed to proceed overnight. (10) The resulting peptide-resin was washed with six 100-ml portions of MeOH in $CHCl_3$ (1:2) and a final washing with three 100-ml portions of EtOH. The repetition of this cycle with substitution of the appropriate Boc-amino acids (40 mmol) for Boc-proline gave the desired protected octapeptide-resin ester VIIIa (28.72 g). The arginine and tryptophane derivatives were coupled in CH_2Cl_2–DMF (1:1) and the other amino acids were coupled in CH_2Cl_2.

Pyroglutamylhistidyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^{α}-nitroarginylprolylglycyl-resin (Xa). A sample of 5.743 g of the completed octapeptide-resin was taken through two cycles as described in the preceding experiment, with Boc-histidine and finally pyroglutamic acid added in DMF solution at step 9. During these latter two cycles, 1% mercaptoethanol was added to the dioxane washes and the 4 *M* HCl–dioxane solution. The completed decapeptide-resin was dried over P_2O_5 in a vacuum desiccator.

N-tert-Butyloxycarbonyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^{α}-nitroarginylprolylglycinamide (VIII). A sample of 17.230 g of the octapeptide-resin VIIIa was suspended in 500 ml of MeOH–DMF (1:1). The suspension was saturated with NH_3 and stirred magnetically for 20 hr at room temperature. The material was filtered and washed with DMF and MeOH, and the filtrate concentrated *in vacuo*. The semisolid product was twice purified by column chromatography on silica gel (80 g) with MeOH–$CHCl_3$ (1:2) as the eluent. The peptide containing fractions were analyzed by tlc (solvent II, Ehrlich reagent). The purified product (VIII) was isolated as a white solid from MeOH yielding a first crop: 3.529 g; mp 145–147°; $[\alpha]^{25}D$ −24° (*c* 1, DMF). *Anal.* ($C_{63}H_{82}N_{14}O_{14}$) H, N; C: calcd, 60.1; found, 59.6. A second crop (0.63 g, mp 147–148°) was obtained by concentration of the mother liquors and precipitation of the residue from MeOH.

Pyroglutamylhistidyltryptophyl-*O*-benzylseryl-*O*-benzyltyrosylglycylleucyl-N^{α}-nitroarginylprolylglycinamide (X). The entire amount of completed decapeptide-resin Xa was ammonolyzed as described for the octapeptide. The crude product was twice purified by column chromatography and finally precipitated from MeOH yielding a first crop: 0.943 g; mp 169° dec; $[\alpha]^{25}D$ −23.8° (*c* 1, AcOH). *Anal.* ($C_{69}H_{86}N_{18}O_{15}$) C, H, N. A second crop (0.083 g, mp 172°) was obtained by concentration of the mother liquor and precipitation of the residue from MeOH.

Pyroglutamylhistidyltryptophylseryltyrosylglycylleucylarginylprolylglycinamide (I). A 0.500-g sample of the purified protected decapeptide X, obtained by the solid-phase method, was placed in a Kel-F reaction vessel similar to the one designed by Sakakibara[16] and treated with 10 ml of liquid hydrogen fluoride in the presence of 1.0 ml of anisole for 1 hr at 0°. The HF was removed with a stream of N_2 and the residue was dried *in vacuo* over P_2O_5 and KOH pellets. The dried product was dissolved in 0.1 *M* AcOH and passed through a column of ion-exchange resin AG 1-X2 (AcO^-). The effluent and washings were lyophilized yielding 450 mg of product. Purification was achieved by passage of the crude decapeptide through a column of Sephadex G-25 as shown in Figure 1. The central portion of the main peak was lyophilized and rechromatographed on Sephadex G-15. Removal of trace impurities from opposite sides of the main peak resulted in a product which was judged to be pure by two-dimensional tlc using 0.1 *N* Py–AcOH, pH 6.5, followed by tlc in *n*-BuOH–AcOH–H_2O (4:1:1). The yield through the two stages of Sephadex purification was 25%. The fractions in each stage were cut conservatively. The material corresponding to the central portion of the peak gave the following analyses, $[\alpha]^{24}D$ −50.5° (*c* 1, 1% AcOH). *Anal.* Calcd for $C_{55}H_{75}N_{17}O_{13}$: C, 55.89; H, 6.40; N, 20.15. Found: C, 52.18; H, 6.24; N, 17.55. Calcd for $C_{55}H_{75}N_{17}O_{13}\cdot 2AcOH\cdot 3H_2O$: C, 52.21; H, 6.62; N, 17.55. Amino acid analysis molar ratios with histidine taken as 1.00: Glu, 1.01; His, 1.00; Ser, 0.91; Tyr, 0.98; Gly, 2.00; Leu, 1.01; Arg, 0.92; Pro, 1.02; NH_3, 1.19.

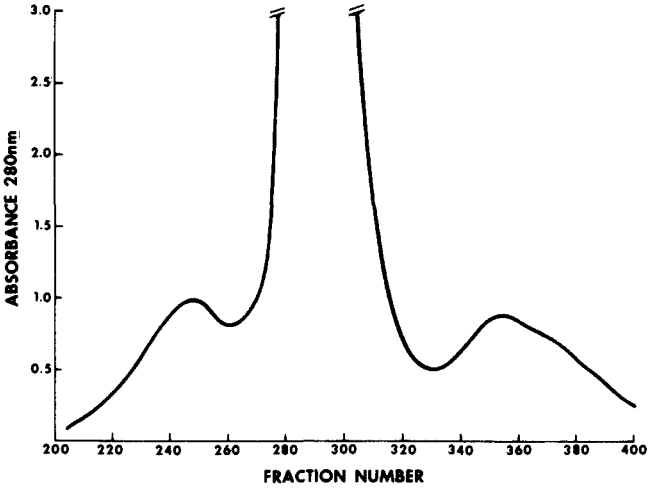

Figure 1. Purification of crude decapeptide on Sephadex G-25.

Biological Results. Table I shows the results of an *in vitro* test of the decapeptide amide in comparison with a standard which has been exhaustively compared with the pure natural porcine hormone.[21,§]

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References

(1) A. V. Schally, A. Arimura, C. Y. Bowers, A. J. Kastin, S. Sawano, and T. W. Redding, *Recent Progr. Horm. Res.*, 24, 497 (1968).
(2) W. F. White, "Mammalian Reproduction," H. Gibian and E. J. Plotz, Ed., Springer-Verlag, New York, N. Y., 1970, p 84.
(3) A. V. Schally, A. Arimura, A. J. Kastin, H. Matsuo, Y. Baba, T. W. Redding, R. M. G. Nair, L. Debeljuk, and W. F. White, *Science*, 173, 1036 (1971).
(4) A. V. Schally, A. Arimura, Y. Baba, R. M. G. Nair, H. Matsuo, T. W. Redding, and W. F. White, Abstracts of the 53rd Meeting of the Endocrine Society, San Francisco, Calif., June 1971, Abstract No. 55.
(5) *Chem. Eng. News*, 49, 7 (June 28, 1971).
(6) H. Matsuo, A. Arimura, R. M. G. Nair, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, 45, 822 (1971).
(7) R. Geiger, W. König, H. Wissmann, K. Geisen, and F. Enzmann, *ibid.*, 45, 767 (1971).
(8) H. Sievertsson, J. K. Chang, A. von Klaudy, C. Bogentoft, B. Currie, K. Folkers, and C. Y. Bowers, *J. Med. Chem.*, 15, 222 (1972); J. K. Chang, H. Sievertsson, B. L. Currie, C. Bogentoft, and K. Folkers, *ibid.*, 15, 623 (1972).
(9) M. Monahan, J. Rivier, R. Burgus, M. Amoss, R. Blackwell, W. Vale, and R. Guillemin, *C. R. Acad. Sci. Paris*, 273, 508 (1971).
(10) P. Rivaille, A. Robinson, M. Kamen, and G. Milhaud, *Helv. Chim. Acta*, 54, 2772 (1971).

(11) J. Rivier, M. Monahan, W. Vale, G. Grant, M. Amoss, R. Blackwell, R. Guillemin, and R. Burgus, *Chimia*, **26**, 300 (1972).
(12) M. Bodanszky, *Nature (London)*, **175**, 685 (1955).
(13) B. Gutte and R. B. Merrifield, *J. Amer. Chem. Soc.*, **91**, 501 (1969).
(14) R. B. Merrifield, *ibid.*, 85, 2149 (1963).
(15) J. M. Stewart and D. W. Woolley, *Nature (London)*, **206**, 619 (1965).
(16) S. Sakakibara and Y. Shimonishi, *Bull. Chem. Soc. Jap.*, 38, 141 (1965); S. Sakakibara, M. Shin, M. Fujino, Y. Shimonishi, S. Inouye, and N. Inukai, *ibid.*, 38, 1522 (1965); S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *ibid.*, 40, 2164 (1967).
(17) J. Porath and P. Flodin, *Nature (London)*, 183, 1310 (1959).
(18) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, 30, 1190 (1958).
(19) S. L. Steelman and F. M. Pohley, *Endocrinology*, **53**, 604 (1953).
(20) A. F. Parlow in "Human Pituitary Gonadotropins," A. Albert, Ed., Charles C Thomas, Springfield, Ill., 1961, p 300.
(21) A. V. Schally, T. W. Redding, H. Matsuo, and A. Arimura, *Endocrinology*, 90, 1561 (1972).
(22) A. V. Schally, A. Arimura, Y. Baba, R. M. G. Nair, H. Matsuo, T. W. Redding, L. Debeljuk, and W. F. White, *Biochem. Biophys. Res. Commun.*, 43, 393 (1971).
(23) G. R. Marshall, International Symposium in Hormonal Polypeptides, Milan, 1967.
(24) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1969.

Synthesis and Biological Evaluation of LH and FSH Releasing Hormone and Its Analogs†

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Details and additional information about another synthesis of LH-RH/FSH-RH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I), are described. In order to obtain enough highly purified material, the synthesis was performed by stepwise elongation according to the conventional method for peptide synthesis. The product was purified effectively by column chromatography on CM-Sephadex C-25. Homogeneity of the purified product was confirmed by tlc as well as by elementary analysis and acid and enzyme hydrolyses. The *in vivo* and *in vitro* activities of the synthetic LH-RH/FSH-RH (I) were of the same order of magnitude as those of pure natural LH-RH/FSH-RH. On the other hand, nonapeptide amide, des-pGlu[1]-LH-RH/FSH-RH (II), and octapeptide amide, des-pGlu[1]-des-His[2]-LH-RH/FSH-RH (III), were, at best, only very weakly active. In addition, I stimulated cyclic AMP formation in rat anterior pituitary, while II and III exhibited no stimulation.

The decapeptide amide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 was prepared by stepwise elongation according to the conventional method for peptide synthesis. The product was purified by column chromatography on CM-Sephadex C-25. Homogeneity of the product was confirmed by tlc as well as elementary analysis and acid and enzymic hydrolyses. Because of lack of the α-amino group at the N terminus of the decapeptide amide, stereohomogeneity of the synthetic peptide was assessed by aminopeptidase-M (AP-M) digestion of nonapeptide amide H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (des-pGlu[1]-LH-RH/FSH-RH). The *in vivo* and *in vitro* LH-RH activities of the synthetic decapeptide amide were of the same order of magnitude as those of pure natural porcine LH-RH/FSH-RH. The *in vitro* FSH-RH activity was also compared.

Studies by Schally and his group[2-5] led to isolation of porcine LH and FSH releasing hormone (LH-RH/FSH-RH) and determination of the structure as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). This structure was confirmed by the synthesis of the decapeptide amide possessing identical properties with those of LH-RH/FSH-RH isolated from porcine hypothalami.[1,6-12] This communication provides details and additional information about another synthesis of the decapeptide amide.

In order to obtain enough material for further investigation of this hormone, we performed the synthesis according to the conventional method for peptide synthesis. The well-planned synthesis excluded any possibility of the presence of by-products in our LH-RH/FSH-RH preparation. LH-RH and FSH-RH activities of our synthetic decapeptide amide (I) were shown to be identical with those of pure natural porcine LH-RH/FSH-RH, while both nonapeptide amide, H-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Des-pGlu[1]-LH-RH/FSH-RH) (II), and octapeptide amide, H-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Des-pGlu[1]-des-His[2]-LH-RH/FSH-RH) (III), were, at best, only very weakly active. The *in vivo* LH-RH activity of II and III was reported by Schally, *et al.*[13]

Synthesis. Synthesis of I was carried out according to the route shown in Scheme I. H-Pro-Gly-NH_2·HBr (IV) was used as the starting material. The mixed anhydride of Z-Arg(NO_2)-OH[14] was coupled with IV to yield V. Decarbobenzoxylation of V with 25% HBr in AcOH followed by coupling with Z-Leu-OSu[15] produced VI, which was decarbobenzoxylated by the same treatment. The resulting partially deblocked material was coupled with Z-Gly-OSu[15] to give protected pentapeptide amide VII. This material was partially deblocked in the same manner as above and then combined with Z-Ser-Tyr-N_3, derived from the corresponding hydrazide,[16] to yield VIII. It has been known that the treatment of a Ser-containing peptide with HBr–AcOH leads to O-acetylation of the Ser residue.[17,18] In addition, this treatment might cause partial destruction of Trp residue. Ac-

†For the preliminary communication, see ref 1. The amino acid residues except glycine are of the L configuration.