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Synthesis of the Luteinizing-Releasing Hormone of the Hypothalamus and the 8-Lysine Analog†

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An advantageous synthesis of the hypothalamic-releasing hormone (LRH), which is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, has been achieved by "classical reactions." In principle, the 1-6 sequence, pGlu-His-Trp-Ser-Tyr-Gly, and the 7-10 sequence, Leu-Arg-Pro-Gly, are separately synthesized and then coupled to give the decapeptide. There were appropriate protective groups; good yields, helpful stepwise purifications, and new improvements are evident. Since the Arg^8 moiety of the decapeptide is presumably important to hormonal activity and potency, this new synthesis provides the flexibility needed to replace Arg^8 with new moieties in the relatively small 7-10 sequence. For example, this synthesis was modified to obtain the 8-lysine-luteinizing-releasing hormone. Lys^8-LRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Lys-Pro-Gly-NH_2) was found to release significant levels of the luteinizing hormone in the rat assay, but at nanogram dose levels which were not much higher than that required for LRH. Thus, the relative importance of a guanidino group over an amino group in the 8-moiety for potent activity is evident.

Sievertsson, *et al.*,[1] and Folkers‡[2] described our first 2 syntheses of the luteinizing- (hormone) releasing hormone of the hypothalamus which is the decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2. In the first synthesis, the tripeptide, pGlu-His-Trp, was obtained by "classical reactions" and then coupled with the protected heptapeptide corresponding to Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which had been separately synthesized by the Merrifield solid-phase technique. The second synthesis was entirely by solid-phase coupling. The decapeptides from both syntheses were identical according to the results of extensive countercurrent distribution, hormonal assays, and other comparative data. This decapeptide, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, is the hypothalamic-releasing hormone (LRH) which regulates the luteinizing hormone of the anterior pituitary gland.

The background for these 2 syntheses of LRH was described so recently[1] that it is not repeated herein, but citations to synthesis of the first LRH-active peptide, pGlu-Tyr-Arg-Trp-NH_2, by Chang, *et al.*,[3] and a synthesis of LRH by a solid-phase procedure described by Monahan, *et al.*,[4] are particularly relevant.

We described our third synthesis of the luteinizing-re-

†Hypotholamic Hormones. 36.
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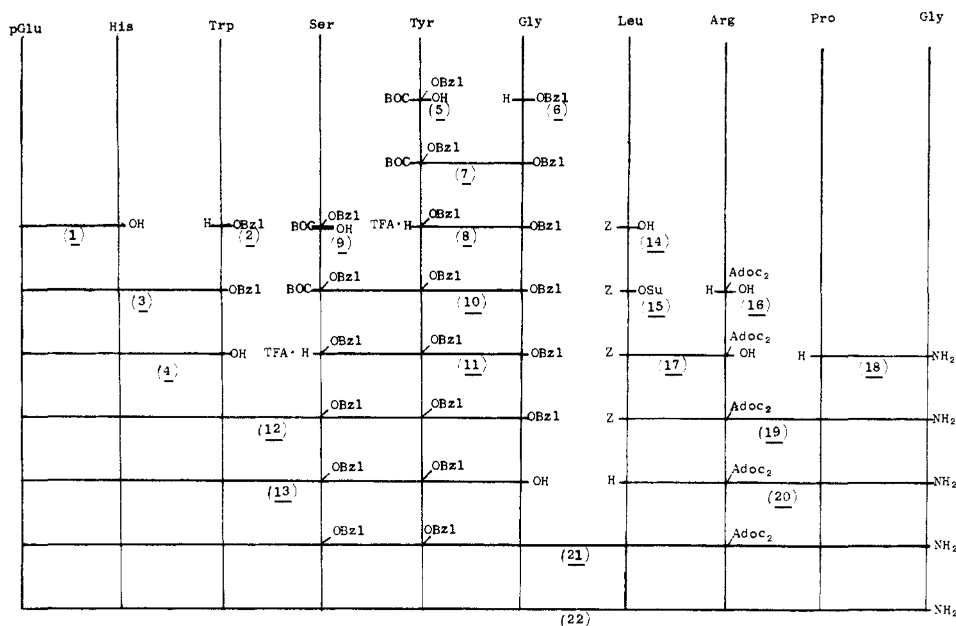

Figure 1. The synthesis of luteinizing-releasing hormone.

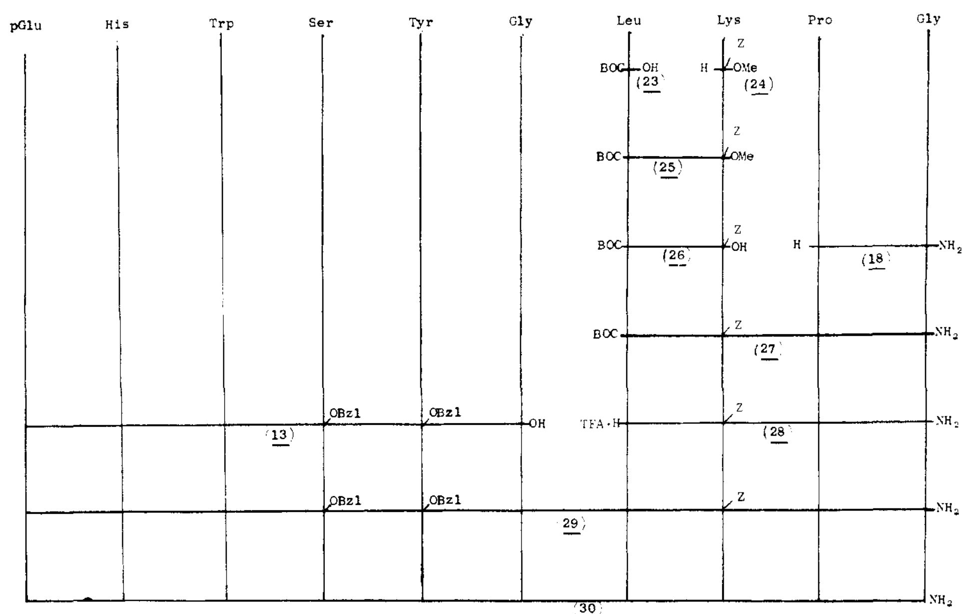

Figure 2. The synthesis of 8-lysine-luteinizing-releasing hormone.

leasing hormone of the hypothalamus which was achieved entirely by "classical reactions" and is a very good synthesis in terms of high yields, minimal racemization, and the advantage of stepwise purifications. Also, this synthesis has unique flexibility since, in principle, it involves the coupling of the 1-6 sequence, pGlu-His-Trp-Ser-Tyr-Gly, with the 7-10 sequence, Leu-Arg-Pro-Gly, to give the synthetic decapeptide or LRH. The flexibility involves the confinement of the apparently important Arg^8 moiety to the smaller sequence of a tetrapeptide. For example, it became easily feasible and was of immediate interest to replace Arg^8 with Lys^8 to determine the effect on hormonal activity. Consequently, we also describe a similar synthesis to obtain the exemplary 8-lysine-luteinizing releasing hormone (Lys^8-LRH) and the data on its hormonal activity.

Chemistry. The synthesis of the luteinizing- (hormone) releasing hormone (LRH) and its analog, 8-lysine-luteinizing-releasing hormone (Lys^8-LRH), have been accomplished by the reactions which are depicted in Figures 1 and 2.

Pyroglutamylhistidine[5] (**1**) was coupled with tryptophan benzyl ester (**2**)·HCl using 1-hydroxybenzotriazole and dicyclohexylcarbodiimide[6] (DCI) to afford pGlu-His-Trp benzyl ester (**3**) in high yield. The tripeptide (**3**) was subjected to hydrogenation using 5% Pd/C catalyst to remove the benzyl group of the ester. The resulting tripeptide, pGlu-His-Trp[1] (**4**), was then condensed with *O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-OBzl (**11**) trifluoroacetate in about 70% yield by DCI[6] to give pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-OBzl (**12**). To hydrolyze **12**, it was convenient to use a solution of NaOH in MeOH, and the corresponding acid **13** was obtained in 84% yield.

In order to prepare relatively large amounts of **11**, the mixed anhydride method was employed with the O functional groups of serine and tyrosine protected by benzyl groups to eliminate certain side reactions and also to increase the solubilities of the amino acids. The starting

amino acid, Boc-*O*-Bzl-Tyr-OH (**5**), in the form of its mixed anhydride[7] (ethyl chloroformate) was allowed to react with Gly-OBzl (**6**) tosylate to give Boc-*O*-Bzl-Tyr-Gly-OBzl (**7**) which was deblocked by F_3CCO_2H. The corresponding *O*-Bzl-Tyr-Gly-OBzl (**8**) trifluoroacetate was coupled with the mixed anhydride[7] (ethyl chloroformate) of Boc-*O*-Bzl-Ser-OH (**9**). Deprotection of the resulting tripeptide **10** was with F_3CCO_2H, and the desired tripeptide, **11**·trifluoroacetate, was obtained in a total yield of 68%.

The utility of N^δ,N^ω-bis(adamant-1-yloxycarbonyl) as protecting group, according to Jäger and Geiger,[8] for arginine was applied successfully to prepare the tetrapeptide **19**. Thus, Z-Leu-OH (**14**), as its hydroxysuccinimide ester[9] **15**, was coupled with N^δ,N^ω-bis(adamant-1-yloxycarbonyl)-arginine to afford the dipeptide acid **17** in 87% yield. This peptide, obtained in crystalline form, was then allowed to react with Pro-Gly-NH_2[10] by DCI[6] to give Z-Leu-N^δ,N^ω-$Adoc_2$-Arg-Pro-Gly-NH_2 (**19**) in 45% yield. Hydrogenolysis of this protected tetrapeptide (**19**) in the presence of 5% Pd/C catalyst yielded Leu-N^δ,N^ω-$Adoc_2$-Arg-Pro-Gly-NH_2 (**20**).

The protected decapeptide **21** was obtained from the coupling of the hexapeptide **13** with the tetrapeptide **20** by DCI.[6] Hydrogenation of **21** over 5% Pd-$BaSO_4$ in MeOH containing AcOH and cleavage of the N^δ,N^ω-bis(adamant-1-yloxycarbonyl) group with F_3CCO_2H afforded the free decapeptide **22**, which was then purified on CMC using pH 6.9 NH_4OAc buffers of increasing ionic strength as eluting agents.

Similarly, Lys^8-LRH (**30**) was also synthesized. Boc-Leu-OH (**23**) was coupled with N^ϵ-Z-Lys-OMe (**24**) by the mixed anhydride[7] (ethyl chloroformate) method to give Boc-Leu-N^ϵ-Z-Lys-OMe (**25**). Hydrolysis of **25** with aqueous NaOH and acidification gave the dipeptide acid **26** which was allowed to react with Pro-Gly-NH_2[10] using DCI to afford the tetrapeptide **27** in a total yield of 53%.

Acid hydrolysis of the tetrapeptide **27** was carried out with F_3CCO_2H to remove the *N-tert*-butyloxycarbonyl group. The corresponding tetrapeptide (**28**) trifluoroacetate was then treated with the hexapeptide **13** using DCI to afford the protected decapeptide **29** which was then subjected to hydrogenolysis in the presence of 5% Pd-$BaSO_4$ catalyst. The free decapeptide was also purified by chromatography on CMC to give Lys^8-LRH.

Endocrinology. The data on the hormonal evaluation of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 from our third synthesis and the corresponding Lys^8-LRH or pGlu-His-Trp-Ser-Tyr-Gly-Leu-Lys-Pro-Gly-NH_2 are in Table I. The bioassay on these 2 synthetic peptides for release of the luteinizing hormone in the pituitary were carried out

Table I. Release of Luteinizing Hormone (LH) in a Rat Assay by the Synthetic Luteinizing-Releasing Hormone (LRH) and the 8-Lysine Analog

Sample	Dose, ng	LH levels, ng/ml of serum Before	After
Synthetic LRH	5	10.2	236
		4.0	>285
	25	13	>285
		4	>285
Lys^8-LRH	100	<4	140
		4	111
	300	<4	>285
		<4	172
	900	6	>285
		<4	>285

with Sprague-Dawley female rats after ovariectomy. Before injection of the test samples, the rats were treated with 50 μg of estradiol benzoate and 25 mg of progesterone 72 hr before the assay by the method of Ramirez and McCann.[11] Blood was taken from the jugular vein of the rat, under anesthesia, and the test solutions were injected into this vein. The assays for LH in the serum were carried out in duplicate by the double antibody radioimmuno assay of Niswender, *et al.*[12] The levels of LH in the serum are expressed in terms of ng/ml of LER-1240-2-0.60 NIH-LH-SI units/mg.

The data in Table I show that the synthetic LRH from this third synthesis released LH in this rat assay to a degree comparable with that which was observed for the decapeptide products from the previous two syntheses.[1]

The hormonal activity of the Lys^8-LRH, according to the data in Table I, shows that this analog is apparently considerably less active than LRH. Maximal release of LH was observed for Lys^8-LRH at a dosage of 300 ng. Although the availability of extensive assay data has not been feasible, it appears that the Lys^8-LRH has activity even in the nanogram range of dosage.

Experimental Section

Melting points were performed on a Thomas-Hoover capillary melting point apparatus and are uncor. Microanalyses were performed by the Mikroanalytisches Laboratorium, Bonn, Germany. Where analyses are indicated only by symbols of the elements, analytical results obtd for those elements were within ±0.4% of the theoretical values. On tlc (silica gel G), $R_f^1, R_f^2, R_f^3, R_f^4$, and R_f^5 refer to the systems of *n*-BuOH-glacial HOAc-EtOAc-H_2O (1:1:1:1), $CHCl_3$-MeOH-NH_4OH (60:45:20), EtOH-H_2O (7:3), $CHCl_3$-MeOH (9:1), and EtOAc-pyridine-glacial HOAc-H_2O (5:5:1:3), respectively. The nmr spectra were measured at 60 Hz on a Varian Associates A-60 spectrometer (Me_4Si or sodium 2,2-dimethyl-2-silapentane-5-sulfonate) and the chemical shifts are expressed in τ values. The optical rotations were measured on a Pekin-Elmer Model 141 digital readout polarimeter. All amino acids used as starting material were purchased as the pure L isomers. Amino acid analyses of the final peptides were carried out with a Beckman Amino Acid Analyzer Model 120 on samples which were hydrolyzed with 4% thioglycolic acid in 6 *N* HCl for 3 hr in an evacd sealed tube at 138°.

Synthesis of the Luteinizing-Releasing Hormone. pGlu-His-Trp-OBzl (3). pGlu-His-OH[5] (1) (6.9 g) and 1-hydroxybenzotriazole[6] (3.8 g) in dry DMF (50 ml) were treated with DCI (5.3 g) at 0°. After being stirred during 1 hr, Trp-OBzl·HCl (8.55 g) and Et_3N (3.6 ml) were added. After 72 hr at room temp, the reaction mixt was filtered and the filtrate was evapd *in vacuo* to dryness. The residue was purified by recrystn twice from EtOH-EtOAc to afford the tripeptide (3): 10.2 g; yield 72%; mp 235-238° dec; $[\alpha]^{22}D$ −6.8° (*c* 1.54, glacial HOAc); R_f^1, 0.72; R_f^2, 0.89; R_f^3, 0.59; single spot with the Pauly, Ehrlich, and I_2 reagents. *Anal.* ($C_{29}H_{30}N_6O_5 \cdot H_2O$) C, H, N.

pGlu-His-Trp-OH[1] (4). pGlu-His-Trp-OBzl (3) (7.1 g) in MeOH (150 ml) and glacial HOAc (50 ml) with 5% Pd/C as catalyst was hydrogenated during 5 hr at room temp (1 atm). The reaction mixt was filtered and evapd *in vacuo* to give 4: 5.9 g, quant yield; mp 188-190° dec; R_f^1, 0.58; R_f^2, 0.64; R_f^3, 0.50; single spot with the Pauly, Ehrlich, and I_2 reagents. A portion of this free acid was recrystd from EtOH-Et_2O for analysis. *Anal.* ($C_{22}H_{24}N_6O_5 \cdot C_2H_5OH$) C, H, N.

Boc-*O*-Bzl-Tyr-Gly-OBzl (7). Boc-*O*-Bzl-Tyr-OH (5) (27.6 g) in dry THF (100 ml) at 0° was treated successively with Et_3N (10.3 ml), ethyl chloroformate[7] (7.1 ml) and, after 1 hr, Gly-OBzl tosylate (6) (25 g) and Et_3N (10.3 ml) in dry THF (100 ml). After being stirred at room temp during 16 hr, the reaction mixt was evapd to dryness. The residue was dissolved in EtOAc (400 ml) and the soln was washed with 5% citric acid (2 × 50 ml), 5% $NaHCO_3$ soln (2 × 50 ml), and H_2O (2 × 50 ml). The organic layer was dried (Na_2SO_4) and evapd. The residue was crystd from MeOH-*n*-hexane to afford 7; 35.7 g; yield 93%; mp 101-104°; $[\alpha]^{22}D$ +1.2° (*c* 1.29, MeOH); R_f^4, 0.91; single spot with Cl-tolidine and I_2 reagents; τ_{CDCl_3} 2.65

(d, 10 H, benzylic aromatic protons), 4.86 (s, 2 H), 4.98 (s, 2 H, benzylic CH_2), and 8.64 (s, 9 H, butyloxy protons). *Anal.* ($C_{30}H_{34}N_2O_6$) H, N, C: Calcd, 69.48; found, 68.98.

Boc-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-OBzl (10). A soln of the protected dipeptide ester 7 (10 g) in TFA (15 ml) was stirred at room temp during 15 min. The reaction mixt was evapd *in vacuo* to afford the dipeptide trifluoroacetate 8. This was neutralized with Et_3N and added to the mixed anhydride soln of Boc-*O*-Bzl-Ser (9) (5 g) which was prepd as described for the prepn of 7. After being stirred at room temp during 16 hr, the reaction mixt was evapd and the residue was treated with cold H_2O. The ppt was collected and washed well with 5% citric acid, 5% $NaHCO_3$ soln, and H_2O, respectively. The crude product was purified by crystn from EtOAc-petr ether to afford the tripeptide 10; 9.8 g; yield 83%; mp 92-95°; $[\alpha]^{22}D$ -3.2° (*c* 1.59, $CHCl_3$); R_f^4, 0.92; single spot with Cl-tolidine and I_2 reagents; τ_{CCl_4} 2.76 and 2.82 (15 H, benzylic aromatic protons), 4.95 (s, 2 H), 5.12 (s, 2 H), and 5.60 (s, 2 H, benzylic CH_2), and 8.65 (s, 9 H, *N-tert*-butyloxy group). *Anal.* ($C_{40}H_{45}N_3O_8$) C, H, N.

O-Bzl-Ser-*O*-Bzl-Tyr-Gly-OBzl·TFA (11). Boc-tripeptide 10 (9.5 g) was treated with TFA (20 ml) at 0°. After being stirred at room temp during 10 min, the reaction mixt was evapd *in vacuo* to dryness. The residue was treated with anhyd Et_2O, and the white ppt was collected and purified by crystn from MeOH-Et_2O to afford 11: 8.5 g; yield 88%; mp 143-145° dec; $[\alpha]^{22}D$ +2.9° (*c* 0.92, MeOH); R_f^4, 0.84; single spot with ninhydrin and Cl-tolidine reagents. *Anal.* ($C_{35}H_{37}N_3O_6\cdot CF_3COOH$) C, H, N.

pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-OBzl (12). The tripeptide[1] 4 (5.4 g) and 1-hydroxybenzotriazole[6] (1.82 g) in dry DMF (50 ml) were treated with DCI (2.7 g) at 0°. After being stirred at room temp during 2 hr, the tripeptide 11 (8.5 g) and Et_3N (1.7 ml) in dry DMF (50 ml) were added. After 36 hr, the reaction mixt was filtered and the filtrate was evapd *in vacuo*. The residue was washed with H_2O and then recrystd from EtOH to give 12: 8.3 g; yield 69%; mp 197-200° dec; $[\alpha]^{22}D$ -2.8° (*c* 1.23, glacial HOAc); R_f^1, 0.78; R_f^2, 0.95; R_f^3, 0.66; single spot with Pauly, Ehrlich, and I_2 reagents. *Anal.* ($C_{57}H_{59}N_9O_{10}$) C, H, N.

pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-OH (13). The hexapeptide ester 12 (2.6 g) in EtOH (50 ml) was treated with NaOH soln (8 *N*, 5 ml). After 20 min at room temp, the reaction mixt was acidified with glacial HOAc, and evapd *in vacuo* to dryness. The resulting ppt was rinsed with H_2O and purified by crystn from EtOH to afford 13: 2 g; yield 84%; mp 171-175° dec; $[\alpha]^{22}D$ -0.9° (*c* 0.68, glacial HOAc); R_f^1, 0.71; R_f^2, 0.81; R_f^3, 0.59; single spot with Pauly, Ehrlich, and I_2 reagents. *Anal.* ($C_{50}H_{53}N_9O_{10}$) C, H, N.

Cbz-Leu-N^δ,N^ω-Adoc$_2$-Arg-OH (17). *N*-Hydroxysuccinimido-*N*-benzyloxycarbonylleucinate[9] (15) (700 mg) was added to a soln of N^δ,N^ω-Adoc$_2$-Arg-OH[8] (16) (1 g) and Et_3N (0.3 ml) in dry DMF (20 ml). After 16 hr, the reaction mixt was evapd *in vacuo*, and the residue was crystd from MeOH-H_2O to afford 17: 1.6 g; yield 87%; mp 93-96°; $[\alpha]^{22}D$ -7.2° (*c* 2.63, MeOH); R_f^4, 0.58; single spot I_2 reagent. *Anal.* ($C_{42}H_{59}N_5O_9$) H, N; C: Calcd, 64.84; found, 64.37.

Cbz-Leu-N^δ,N^ω-Adoc$_2$-Arg-Pro-Gly-NH$_2$ (19). The dipeptide 17 (1.55 g) and 1-hydroxybenzotriazole (0.3 g) in dry DMF (20 ml) were treated with DCI (0.45 g) at 0°. After being stirred during 2 hr, Pro-Gly-NH$_2$[10] (0.35 g) was added. The reaction mixt was stirred during 48 hr, filtered, and evapd, and the resulting peptide was washed with 5% citric acid, 5% $NaHCO_3$ soln, and H_2O. It was purified by prep chromatog on silica gel to furnish 19: 0.84 g; yield 45%; mp 125-130° dec; $[\alpha]^{22}D$ -31.0° (*c* 1.48, MeOH); R_f^4, 0.76; single spot with Cl-tolidine and I_2 reagents; $\tau_{MeOH\text{-}d_4}$ 2.60 (s, 5 H, arom protons), 4.92 (s, 2 H, benzylic CH_2), 7.82 (s), and 8.30 (s, adamantyl group). *Anal.* ($C_{49}H_{70}N_8O_{10}\cdot 1.5H_2O$) C, H, N.

pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-N^δ,N^ω-Adoc$_2$-Arg-Pro-Gly-NH$_2$ (21). The protected tetrapeptide 19 (680 mg) in abs EtOH (30 ml) contg 5% Pd/C catalyst was hydrogenated at 1 atm. After being stirred at room temp during 1 hr, the reaction mixt was filtered and evapd *in vacuo* to afford 20. This product in dry DMF (10 ml) was added to a soln of DMF (20 ml) contg the protected peptide 13 (680 mg), 1-hydroxybenzotriazole (140 mg), and DCI (150 mg). After being stirred at room temp during 16 hr, the reaction mixt was filtered, and the filtrate was evapd *in vacuo*. The resulting ppt was collected by addn of H_2O, 1.2 g. A portion of this material (850 mg) was purified by column chromatography on silica gel with elution by $CHCl_3$-MeOH (8:2 v/v) to afford 21 (380 mg, yield 43%) which was crystd from EtOH: mp 165-170° dec; $[\alpha]^{22}D$ -10.6° (*c* 0.98, glacial HOAc); R_f^1, 0.81; R_f^2, 0.96; R_f^3, 0.67; single spot with Pauly, Ehrlich, and I_2 reagents. *Anal.* ($C_{91}H_{115}N_{17}O_{17}$) C, H, N.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$ (22) (LRH). The protected decapeptide 21 (340 mg), in MeOH (20 ml) and glacial HOAc (2 ml) contg 5% Pd-$BaSO_4$ catalyst, was hydrogenated at 1 atm. After being stirred at room temp during 25 hr, the reaction mixt was filtered and evapd *in vacuo* to dryness. The residue was then treated with TFA (5 ml) at 0°. After 15 min at room temp, the reaction mixt was evapd *in vacuo*. The resulting decapeptide was washed well with anhyd Et_2O and was dissolvd in 1% HOAc (100 ml). This soln was passed through a AG1-X8 (OH^-) (1 × 15 cm) column and the filtrate was evapd *in vacuo* to dryness. The residue was dissolved in H_2O (10 ml). The soln was applied to a CMC column (1 × 20 cm) which was eluted with each of the following pH 6.9 NH_4OAc buffers: 0.005, 0.01, 0.025, 0.05, 0.075, and 0.1 *M*. Individual fractions (3 ml each) were collected at a flow rate of approximately 3 ml/min. The desired peptide 22 was located in the 0.075-*M* eluates by the Pauly reaction. These eluates were pooled and the solvent was evapd. The residue was lyophilized to constant wt with H_2O affording a white powder: 98 mg; yield 42%; $[\alpha]^{22}D$ -33.6° (*c* 1.16, MeOH); R_f^1, 0.64; R_f^3, 0.37; R_f^5, 0.49; single spot with Pauly, Ehrlich, Sakaguchi, and Cl-tolidine reagents. The chromatography mobilities were completely identical with those of the compd derived from the previous series.[1] Amino acid ratios in acid hydrolysate: Glu 1.0, His 1.1, Trp 0.6, Ser 0.8, Tyr 0.9, Gly 2.0, Leu 0.9, Arg 1.0, Pro 1.0, NH_3 1.1. *Anal.* ($C_{55}H_{75}N_{17}O_{13}\cdot 2CH_3COOH$) C, H, N.

Synthesis of the 8-Lysine-Luteinizing-Releasing Hormone. Boc-Leu-N^ϵ-Z-Lys-OMe (25). To a soln of Boc-Leu-OH (700 mg) in dry THF (10 ml) and Et_3N (0.45 ml) there were added successively ethyl chloroformate (0.3 ml) at 0° and, after 30 min, N^ϵ-Z-Lys-OMe·HCl (1 g) in Et_3N (0.45 ml). After being stirred at room temp during 16 hr, the reaction mixt was evapd to dryness. The residue was dissolved in EtOAc, and the soln was washed well with 5% citric acid, 5% $NaHCO_3$ soln, and H_2O, respectively. The organic layer was dried (Na_2SO_4) and evapd to the dipeptide 25: 1.54 g; yield 100%; after recrystn from petr ether, mp 84-87°; $[\alpha]^{22}D$ -20.7° (*c* 0.62, MeOH); R_f^4, 0.88; single spot with Cl-tolidine reagent; τ_{CDCl_3} 2.65 (s, 5 H, arom protons), 4.90 (s, 2 H, CH_2), 6.28 (s, 3 H, CH_3), and 8.58 (s, 9 H, *N-tert*-butyloxy group). *Anal.* ($C_{26}H_{41}N_3O_7$) C, H, N.

Boc-Leu-N^ϵ-Z-Lys-OH (26). A soln of Me ester 25 (1.54 g) in MeOH (20 ml) contg NaOH soln (7 *N*, 3 ml) was allowed to stand at room temp during 15 min. The reaction mixt was acidified with glacial HOAc and evapd to dryness. The residue was partitioned between EtOAc and H_2O. The organic layer was sepd, dried (Na_2SO_4), and evapd to afford 26: 1.4 g; yield 94%; $[\alpha]^{22}D$ -12.1° (*c* 2.5, MeOH); R_f^4, 0.18; single spot with I_2 reagent. A portion of this free acid was converted into the dicyclohexylamine salt: mp 138-140°; $[\alpha]^{22}D$ -7.4 (*c* 1.27, MeOH). *Anal.* ($C_{25}H_{39}N_3O_7\cdot C_{12}H_{23}N$) H, N, C: Calcd, 65.84; found, 65.33.

Leu-N^ϵ-Z-Lys-Pro-Gly-NH$_2$·TFA (28). The dipeptide 26 (1 g) and 1-hydroxybenzotriazole (250 mg) in dry DMF (30 ml) were treated with DCI (450 mg) at 0°. After being stirred at room temp during 1 hr, a soln of Pro-Gly-NH$_2$[10] (360 mg) in dry DMF (10 ml) was added. After 24 hr, the reaction mixt was filtered and the filtrate was evapd *in vacuo* to dryness. The residue was dissolved in EtOAc and the soln was washed with 5% citric acid, 5% $NaHCO_3$ soln, and H_2O, respectively. The organic layer was dried (Na_2SO_4) and evapd to dryness. The crude product was purified by prep tlc on silica gel with elution by $CHCl_3$-MeOH (97:3 v/v) to afford 27: 720 mg; yield 56%; R_f^4, 0.47; single spot with I_2 reagent; $\tau_{MeOH\text{-}d_4}$ 2.68 (s, 5 H, arom protons), 4.92 (s, 2 H, CH_2), and 8.58 (s, 9 H, *N-tert*-butyloxy group). The deprotection of this tetrapeptide (27) with TFA was carried out as described for the prepn of 11 to afford 28: 650 mg; yield 88%; $[\alpha]^{22}D$ -23.9° (*c* 0.77, MeOH); R_f^4, 0.07; single spot with ninhydrin and I_2 reagents. *Anal.* ($C_{27}H_{42}N_6O_6\cdot CF_3COOH\cdot MeOH$) C, H, N,

pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-N^ϵ-Z-Lys-Pro-Gly-NH$_2$ (29). The hexapeptide 13 (924 mg) and 1-hydroxybenzotriazole (170 mg) in dry DMF (20 ml) were treated successively with DCI (225 mg) and, after 4 hr, with 28 (650 mg) and Et_3N (0.15 ml) in dry DMF (10 ml). After being stirred at room temp during 36 hr, the reaction mixt was filtered and the filtrate was evapd *in vacuo*. The residue was washed well with H_2O and the ppt was collected (1.7 g). A portion of this material (270 mg) was purified by prep tlc on silica gel with elution by $CHCl_3$-MeOH (7:3 v/v) to afford 29: 111 mg; yield 47%; $[\alpha]^{22}D$ -27.9° (*c* 1.34, MeOH); R_f^1, 0.80; R_f^2, 0.93; R_f^3, 0.66; single spot with Pauly, Ehrlich, and Cl-tolidine reagents. *Anal.* ($C_{77}H_{93}N_{15}O_{15}\cdot 2H_2O$) C, H, N: Calcd, 13.96; found, 13.50.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Lys-Pro-Gly-NH$_2$ (30). The pro-

tected decapeptide 29 (40 mg) in MeOH (10 ml) and glacial HOAc (10 ml) contg 5% Pd-$BaSO_4$ catalyst was hydrogenated at 1 atm. After being stirred at room temp during 24 hr, the reaction mixt was filtered. The filtrate was evapd *in vacuo* to afford the crude peptide. This material was dissolved in H_2O (10 ml), and the soln was added to a CMC column (1 × 15 cm) which was successively eluted with 20 ml each of the following pH 6.9 NH_4OAc buffers: 0.005, 0.01, 0.025, 0.05, 0.075, and 0.1 *M*. Individual fractions (3 ml each) were collected at a flow rate of approximately 3 ml/min. The desired peptide was located in the 0.075-*M* NH_4OAc eluates by the use of Pauly reagent. These fractions were pooled and evapd to a small vol *in vacuo* and the residue was lyophilized to constant wt from small vols of H_2O: 21 mg; yield 67%; $[\alpha]^{22}D$ −27.6° (*c* 0.88, MeOH); R_f^1, 0.65; R_f^3, 0.31; and R_f^5, 0.49; single spot with ninhydrin, Pauly, Ehrlich, and Cl-tolidine reagents. Amino acid ratios in acid hydrolysate: Ser 0.6, Glu 1.0, Pro 1.0, Gly 2.0, Leu 1.0, Tyr 1.0, His 1.0, Lys 1.0, and Trp 0.7. *Anal.* ($C_{55}H_{75}N_{15}O_{13} \cdot 3CH_3COOH$) C, H, N.

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Solid-Phase Synthesis of [6-Arginine]-, [4-Histidine, 6-tyrosine]-, and [8-Isoleucine]angiotensins II†

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To further evaluate the contribution of positions 6 and 8 for agonistic or antagonistic activity, some analogs of angiotensin II have been synthesized by solid-phase procedure with side-chain variations in both these positions. [Ile^8]angiotensin II has been found to be a very potent and specific competitive antagonist of angiotensin II both *in vitro* on isolated rabbit aortic strips and rat uterus and in the *in vivo* pressor assays in rats and cats, while [Arg^6]- and [His^4, Tyr^6]angiotensins were found to be inactive as pressor or antagonistic agents.

[Ile^5, Ala^8]angiotensin II was synthesized earlier in this laboratory[2] and was found to antagonize the action of angiotensin II on strips of guinea pig ileum.[3] Further testing[4] of this analog on isolated rabbit aortic strips and rat colon revealed that this analog had a specific competitive antagonistic effect against angiotensin II at very low concentrations without having a myotropic effect on these smooth muscle preparations. However, when tested on pentobarbital-anesthetized cats, this compound did not antagonize the pressor and intestinal inhibitory effect of angiotensin II. In view of this, it was thought of interest to synthesize analogs of angiotensin in which position 8 was replaced with other aliphatic amino acids, and as a first step, we have synthesized [Ile^8]angiotensin II.

Position 6, occupied by histidine, appears essential for biological activity of angiotensin[5] and has been studied from different points of view.[6-8] Recently Marshall, *et al.*,[9] showed that [Phe^4,Tyr^8]angiotensin II inhibited the response of the isolated rat uterus to angiotensin II when the pH of the medium was 7.4 or less. It is interesting to note that at this pH the imidazole ring of histidine would be significantly protonated.[10] It therefore appears likely that it is in the protonated form that histidine residue may be interacting with the receptor. In order to study the role of positive charge or an aromatic ring in this residue, we synthesized [Arg^6]-, and [His^4, Tyr^6]angiotensins II.

All the analogs reported in this paper were synthesized by the solid-phase procedure,[11] as previously described by Khosla, *et al.*[12,13]

Biological Results.‡ The pressor activity was determined by pressor assay in vagotomized, ganglion-blocked rat,[15] and the myotropic activity was studied on isolated rabbit aortic strips. Inhibition of angiotensin II myotropic response was studied using isolated rabbit aortic strips *in vitro* and by *in vivo* pressor assay in anesthetized cats and rats. The results obtained indicate that [Ile^8]angiotensin II has a specific competitive antagonistic effect against angiotensin II both *in vitro* on isolated rabbit aorta and rat uterus, and *in vivo* on the rat and cat blood pressure. It has a pA_2 value of 9.21 as compared to 8.3 obtained for [Ala^8]angiotensin II, and a log K_2 value of 8.86 at molar concentrations of $10^{-9.34}$ (Schild's parameters). It antagonizes the pressor effect of angiotensin II at a dose level of 5 ng/g body weight in rat and cat. As compared to other analogs such as [Phe^4, Tyr^8]angiotensin II,[9] it appears to be the most potent competitive inhibitor of angiotensin II so far reported. On the other hand, [Arg^6]-, and [His^4, Tyr^6]angiotensins II were

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‡The antagonistic activities were determined by Yamamoto, *et al.*,[14] and the pressor assays by Dr. S. Sen, Cleveland Clinic, Research Division.