Two Syntheses of the Luteinizing Hormone Releasing Hormone of the Hypothalamus†

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pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 has been synthesized by two procedures. The first procedure involved, in principle, the synthesis of the tripeptide, pGlu-His-Trp, by classical reactions and then coupling with the protected heptapeptide corresponding to Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 which had been separately obtained by solid-phase reactions. The second procedure involved total solid-phase synthesis. The products of both syntheses were subjected to exhaustive countercurrent distribution, and the yields were disregarded in the interest of establishing purity, the distribution coefficient, and the hormonal activity and potency of the decapeptide. All assayed samples released the luteinizing hormone (LH) in the rat assay. A dosage of only 500 pg, *in vivo*, was effective. A dose–response relationship was obtained for several dose levels as expected for a hormone. This synthetic pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, which releases LH *in vivo*, is the luteinizing hormone releasing hormone of the hypothalamus as based on the data of Matsuo, *et al.* [H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura, and A. V. Schally, *Biochem. Biophys. Res. Commun.*, 43, 1334 (1971)] for the structure of this hormone.

Currie, *et al.*,[1] and Bogentoft, *et al.*,[2] found evidence for the presence of 4 amino acids in the hypothalamic-releasing hormone for the luteinizing hormone (LH) of the anterior pituitary gland. These amino acids are: pyroglutamic acid (pGlu), arginine (Arg), tyrosine (Tyr), and tryptophan (Trp). The amino acid moieties were found by inactivation studies on preparations of the luteinizing hormone releasing hormone (LHRH or LRH) from both bovine and porcine tissue. The inactivation studies were performed with chemical reagents and with enzymes in order to detect as broadly as feasible the presence or absence of certain amino acids in the structure of the hormone. Schally, *et al.*,[3] had earlier reported the absence of Trp by inactivation experiments on porcine LRH.

Syntheses of the 6 possible tetrapeptides containing the 4 amino acids, pGlu, Arg, Tyr, and Trp, were next achieved by Chang, *et al.*[4] Bioassay of these 6 tetrapeptides led to the discovery that the synthetic peptide having the sequence pGlu-Tyr-Arg-Trp-NH_2 actually releases the luteinizing hormone from the anterior lobe of the pituitary. The account by Chang, *et al.*,[4] appears to describe the first peptide to be synthesized which exhibits the activity of LRH. The 5 other tetrapeptides containing these 4 amino acids showed no hormonal activity for the release of LH when assayed at the same dose level.

Schally, *et al.*,[5] reported that LRH from porcine tissue yielded on acid hydrolysis the following 8 amino acids: 1 Glu, 1 Arg, 1 Tyr, 1 His, 1 Ser, 1 Leu, 1 Pro, and 2 Gly, and referred to the hormone as a nonapeptide. It was also reported that essentially pure porcine LRH[3,5] released both the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) from the pituitary.

In agreement with our findings[2,4] Matsuo, *et al.*,[6] and Baba, *et al.*,[7] found a Trp moiety in porcine LRH. The addition of Trp to the earlier list of nine amino acid residues[5] led to their report[6] that LRH is a decapeptide, and the following sequence was proposed: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I). Later, additional evidence was described by Baba, *et al.*,[7] which supported the proposed sequence:

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
1 2 3 4 5 6 7 8 9 10

I (LRH)

In the initial study on the hormonal activity of the tetrapeptide, pGlu-Tyr-Arg-Trp-NH_2, Bowers, *et al.*,[8] found that this peptide releases LH both *in vivo* and *in vitro*, but no increase in the level of FSH was observed under the same conditions. On the basis that the decapeptide, according to Schally, *et al.*,[9] is the hypothalamic regulator for the release of both LH and FSH, then it appears that the functionalities of the decapeptide which release LH and FSH may have been separated since the tetrapeptide, pGlu-Tyr-Arg-Trp-NH_2, appears to release only LH. The discovery of this tetrapeptide which appears to be specific for the release of LH from the anterior lobe pituitary may be important and useful in continuing research on the gonadatropin releasing hormones.

Sievertsson, *et al.*,[10] reported 2 syntheses of the decapeptide, which had been proposed as the structure[6] of LRH, and their initial bioassay data showing that the synthetic decapeptide releases LH.

After writing this paper on the details and additional information about our 2 syntheses of LRH, the report of Monahan, *et al.*,[11] on their total solid-phase synthesis of the same decapeptide reached us.

Chemistry. The 2 syntheses of the decapeptide amide I, were accomplished by a combination of classical methods and the solid-phase peptide synthesis procedure of Merrifield,[12] as well as by a total solid-phase synthesis.

In the combined classical and solid-phase synthesis, the 1–3 sequence of the hormone was separately obtained by classical procedures, and the 4–10 sequence was prepared by the solid-phase technique. The combination of these two peptides was then accomplished by classical procedures. The advantages of this synthesis was the purification of the 4–10 sequence from the solid-phase synthesis and the fact that the peptide was never exposed to treatment with HF which is often used for removal of protective groups in solid-phase synthesis,[13] and which might have a destructive effect on the acid-labile indole nucleus of the Trp moiety.

In the total solid-phase synthesis of the hormone, all of the protecting groups of the amino acid side chains could be removed in one step by HF. The Trp moiety in the hormone seemed to be sufficiently protected in the HF cleav-

†Hypothalamic Hormones. 35. The scheme of these two syntheses were included in the presentation on Hypothalamic Neurohormones by K. Folkers on July 28, 1971 at the XXIII International Congress of Pure and Applied Chemistry in Boston, Mass.

age by adding anisole and an excess of methionine (Met) to the reaction. In a similar deprotection experiment when Met was replaced by Trp, the loss of Trp in the molecule of the hormone seemed to be greater.

tert-Butyloxycarbonylglycine (*tert*-Boc-Gly) was attached to a chloromethylated resin.[13] To the *tert*-Boc-Gly resin (2.1 m*M* of Gly), the following *tert*-Boc protected amino acids were successively introduced, using dicyclohexylcarbodiimide (DCI) as coupler: proline (Pro), nitroarginine [Arg(NO_2)], leucine (Leu), glycine (Gly), *O*-benzyltyrosine [Tyr(Bzl)], and *O*-benzylserine [Ser(Bzl)]. A 2.5 molar excess of each amino acid was used.

The *tert*-Boc group was removed by 4 *N* HCl in dioxane. A prewash with the same solvent was performed to avoid dilution of the HCl with the dioxane from the preceding wash. The coupling reactions were performed in CH_2Cl_2, except for *tert*-Boc-Arg(NO_2) when DMF was used. The coupler, DCI, was added in CH_2Cl_2 throughout the synthesis. The reaction time for each coupling was 4 hr, and the progress of the coupling was monitored on small samples of the resin by the semiquantitative ninhydrin method of Kaiser, *et al.*[14]

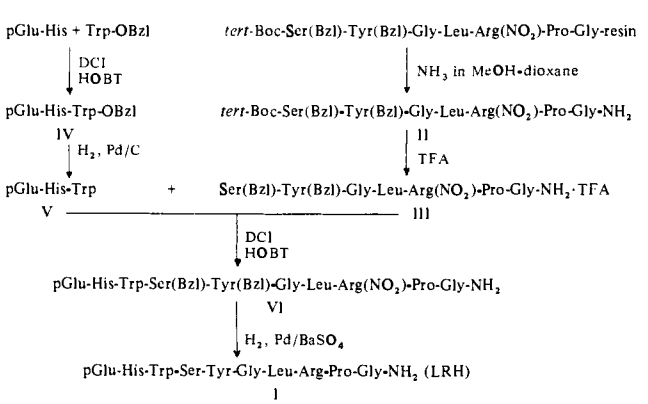
The synthesis of the hormone was accomplished as described in Scheme I. The *tert*-Boc-Ser-(Bzl)-Tyr-(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-resin was suspended and stirred in MeOH–dioxane saturated with NH_3 to cleave the protected heptapeptide as the amide, *tert*-Boc-Ser-(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (II), from the resin. II was readily purified by column chromatography on silica gel G using MeOH–$CHCl_3$ as solvents.

In order to remove the *tert*-Boc group, the heptapeptide amide, II, was treated with trifluoroacetic acid (TFA), and the TFA salt of Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (III) was obtained.

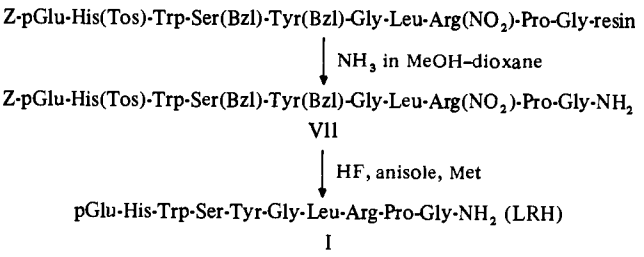
The 1–3 sequence was prepared by classical peptide synthesis. pGlu-His[15] was coupled with Trp-OBzl by using DCI. The resulting tripeptide, pGlu-His-Trp-OBzl (IV), was subjected to catalytic hydrogenolysis to yield pGlu-His-Trp (V).

V and III were then combined by DCI. 1-Hydroxybenzotriazole (HOBT) was used in the coupling as described by König and Geiger[16] to minimize racemization and side reactions. The protected decapeptide amide, pGlu-His-Trp-Ser-(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-NH_2 (VI), was purified by preparative tlc. After catalytic hydrogenolysis at atm pressure using Pd on $BaSO_4$, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I) was obtained. This catalytic reduction was accompanied by some side reactions depending on the conditions which are necessary to remove completely the NO_2 group from the Arg moiety and, at the same time, keep the Trp moiety intact. A reaction time of 40 hr using MeOH–AcOH seemed to be satisfactory. The product, synthetic LRH (I), showed high hormonal activity; at a dose level of 25 ng; there was a 30-fold average increase in the level of LH when I was tested *in vivo* in rats.[11]

Scheme I. Synthesis of the Luteinizing-Releasing Hormone (LRH)



Scheme II. Alternate Synthesis of the Luteinizing-Releasing Hormone (LRH)



In the synthesis of the entire decapeptide amide, I, by the solid-phase method, we started with *tert*-Boc-Gly-resin (1.4 m*M*); after addition of the seven *tert*-Boc protected amino acids described above, *tert*-Boc-Trp, *tert*-Boc-N^{im}-tosylhistidine [*tert*-Boc-His(Tos)], and *N*-carbobenzoxy-pyroglutamic acid (Z-pGlu)[4] were successively introduced. The coupling of *tert*-Boc-Trp was performed in DMF while *tert*-Boc-His(Tos) and Z-*p*Glu were coupled in CH_2Cl_2. After introduction of the Trp moiety, the removal of the *tert*-Boc group, using 4 *N* HCl in dioxane, was performed in the presence of 1,4-butanedithiol. Otherwise, the same conditions as described for II were used. From the protected decapeptide resin, Z-pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-resin, the corresponding protected decapeptide amide, VII, was liberated by ammonolysis (Scheme II). I was then obtained by deprotection of VII using HF. For protection of the Trp moiety, 10% anisole and a 10-fold molar excess of Met were added to the HF.

The purification of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (I) from these two different syntheses was accomplished by countercurrent distribution (CCD).

The decapeptide amide, I, prepared from these two different syntheses showed identical mobilities on tlc. Furthermore, the *K* values in countercurrent distribution appear to correspond to the reported value of natural LRH in the corresponding system.[5] An additional and important characterization of the decapeptide amide, I, is its hormonal activity and potency. As seen in Table I, synthetic LRH from the 2 different syntheses releases LH *in vivo*. Activity at a dose of 0.5 ng was observed, which is a potency that can be expected for a hypothalamic hormone. Also, this activity is compatible with that which was reported for synthetic thyrotropin-releasing hormone (TRH).[17]

Countercurrent Distribution. The product of the synthesis of Scheme I (sample 1) was subjected to countercurrent distribution involving 100 transfers. The 2-phase system used was *n*-BuOH–pyridine–0.1% AcOH (5:3:11). A protein analysis of every second tube according to the Folin–Lowry method[18] indicated the presence of several products in all the tubes; the contents of tubes 60–88 (maximum at $K \sim 1.8$) showed a peak. After removal of the solvent from these combined fractions, the material was redistributed in the same solvent system. The apparatus was set on a recycling operation to permit a 200-transfer distribution.

The center fractions of the peak representing tubes 147–159 were combined and assayed as sample 3. Further purification of sample III by 100 transfers in H_2O–*n*-BuOH–HOAc (5:4:1) was achieved. The center fractions represent-

Table I. Release of Luteinizing Hormone in a Rat Assay by the Synthetic Luteinizing-Releasing Hormone

Sample	Dose, ng	LH levels, ng/ml of serum Before	After	Sample	Dose, ng	LH levels, ng/ml of serum Before	After
I	25	<4	134	IV	1	4.2	68
		<4	127			<4	34
	50	<4	>285		5	4	120
		<4	>285			5.4	218
					25	<41	166
II	1	5	12			5.6	262
		4	13				
	5	<4	42	V	0.5	5	17
		<4	38			<4	10
	10	4	262		1	<4	16
						4	23
III	5	<4	146		5	4	83
		4	65			<4	114
		<4	85				
	10	3.2	113	VI	1	5.5	9
		5.4	176			5	6
					5	4	18
						4	28
					25	6	220
						4	>284

ing tubes 29-36 were combined and assayed as sample 4. This material was taken for a final distribution in 100 transfers in the same solvent. Tubes 18-25 (peak at $K \sim 0.24$) yielded sample 5.

In order to establish proof of purity of the decapeptide, the theoretical distribution was calculated and compared to the peak obtained for the last distribution. Calculations were made on the assumption that the fraction at the center of the peak had the highest purity, and the K value would be the most accurate.

The substance in the tube with the distribution maximum was equated to the observed optical density of 0.446, and the concentrations in the other tubes were expressed as OD values which were calculated[19] from the expression

$$T_{n,r} = \left[\frac{n!}{r!(n-r)!}\right]\left[\frac{1}{\epsilon+1}\right]^n (\epsilon)^r$$

with r = number of tube, n = number of transfers, ϵ = extraction factor = $K[(V_{\text{upper phase}})/(V_{\text{lower phase}})]$, $T_{n,r}$ = fraction in the rth tube in a distribution of n transfers. In this way, the theoretical and the experimental curves have been adjusted to equal heights which permits comparison of the shape of both curves.

Table II shows the theoretical and experimental values of the optical densities for $K = 0.24$.

The curves are in good agreement at both sides of the maximum. The experimental concentrations on the outer parts of the slopes are higher than the calculated ones, probably because of diminishing accuracy of the protein determination with the very small quantity of substance.

The product of synthesis by Scheme II was purified in 3 steps. After the first 100-transfer distribution in *n*-BuOH-pyridine-0.1% AcOH, 200 transfers were made in the same solvent system using the material in tubes 64-92.

Five fractions (tubes 130-141, 142-154, 155-161, 162-175, and 176-186) were chemically assayed by tlc. The plates indicated the presence of a second product contained in increasing quantities in tubes 142-186. Tubes 130-154 were combined, and the material was distributed in 100 transfers in *n*-BuOH-HOAc-H_2O, and the contents of tubes 18-25 were combined and assayed as sample 6.

Release of Luteinizing Hormone. The *in vivo* bioassays for release of LH were performed in Sprague-Dawley female rats after ovariectomy. The rats were injected with 50 μg of estradiol benzoate and 25 mg of progesterone 72 hr before injection of the test samples according to the method of Ramirez and McCann.[20] Under anesthesia, blood was collected from the jugular vein, and the test samples were injected into the same vein. Serum assays for LH were performed in duplicate by the double antibody radioimmuno assay of Niswender, *et al.*[21] The LH results are expressed in terms of ng/ml of LER-1240-2-0.60 NIH-LH-SI units/mg. The data for samples I-VI are in Table I.

Table II. CCD Data on Synthetic LRH

Tube No.	OD exp	OD calcd
14	0.083	0.008
16	0.128	0.023
18	0.147	0.069
20	0.199	0.156
22	0.264	0.277
24	0.374	0.391
26	0.446	0.446
28	0.397	0.414
30	0.337	0.316
32	0.269	0.200
34	0.193	0.105
36	0.134	0.047
38	0.111	0.017

Experimental Section

Melting points were performed on a Thomas-Hoover melting point apparatus and are uncorrected. Microanalyses were performed by the Mikroanalytishes Laboratorium, Bonn, West Germany. R_f^1, R_f^2, R_f^3, values refers to the systems: *n*-BuOH-glacial HOAc-EtOAc-H_2O (1:1:1:1); $CHCl_3$-MeOH-concd NH_4OH (60:45:20); and EtOH-H_2O (7:3), respectively. The nmr spectra were recorded on a Varian Associates A60 spectrometer (Me_4Si as internal standard). The optical rotations were measured on a Perkin-Elmer Model 141 digital readout polarimeter using a microcell. All of the amino acids which were used were purchased as pure L isomers. A solid-phase peptide synthesis was carried out with a Beckman Model 990 peptide synthesizer, and the amino acid analyses were done with a Beckman Model 121 amino acid analyzer which was connected to a Beckman Model 126 data analyzer. All of the samples for amino acid analysis were hydrolyzed with 4% thioglycolic and 6 *N* HCl for 3 hr, in an evacuated sealed tube at 138°.

All distributions were carried out with a 100-tube automatic countercurrent fractionator of E-C Apparatus Corporation. Each tube contained 10 ml of solvent in the lower phase and 13-15 ml in the upper phase. A distribution in more than 100 tubes could be attained by setting the apparatus on a recycling operation. At the end of each distribution, aliquots from the tubes were taken for the

protein analysis according to Folin and Lowry.[18] The fractions were then lyophilized to remove the solvents. All solvents were of reagent grade and were redistilled before use.

Synthesis of Luteinizing-Releasing Hormone. Scheme I. *tert*-Boc-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-NO_2-Arg-Pro-Gly-NH_2 (II). The protected heptapeptide amide (II) was prepared by the solid-phase peptide synthesis procedure of Merrifield.[12] *tert*-Boc-Gly was attached to a Bio-Beads SX-1 chloromethylated resin (200–400 mesh) with a capacity of 1.75 mequiv of Cl/g of resin by refluxing in EtOH in the presence of Et_3N as described.[12] An aliquot of the *tert*-Boc-resin was deprotected with HCl in dioxane and then hydrolyzed. Gly was determined in the amino acid analyzer to be 0.7 m*M*/g of resin. *tert*-Boc-Gly-resin (3.0 g; 2.1 m*M* of Gly) was added to the reaction vessel and the following steps were used to couple each new amino acid: (a) washing with dioxane (3 × 24 ml); (b) prewash with 4 *N* HCl in dioxane (24 ml); (c) removal of the *tert*-Boc group using 4 *N* HCl in dioxane (24 ml) for 30 min; (d) washing with dioxane (3 × 24 ml); (e) washing with CH_2Cl_2 (3 × 24 ml); (f) prewash with 10% Et_3N in CH_2Cl_2 (24 ml); (g) neutralization of the HCl salt with 10% Et_3N in CH_2Cl_2 (24 ml) for 10 min; (h) washing with CH_2Cl_2 (3 × 24 ml); (i) addition of 5.25 m*M* of the appropriate protected *tert*-Boc amino acid in CH_2Cl_2 (17 ml) and mixing for 10 min; (j) addition of DCI (5.25 m*M*) in CH_2Cl_2 (4 ml) followed by a reaction period of 4 hr; (k) washing with CH_2Cl_2 (3 × 24 ml). Each prewashing period was for 1.5 min. The following *tert*-Boc protected amino acids were successively added, Pro, Arg(NO_2), Leu, Gly, Tyr(Bzl), Ser(Bzl). When *tert*-Boc-Arg (NO_2) was introduced, step h was followed by a wash with DMF (3 × 24 ml), and the *tert*-Boc-Arg (NO_2) was dissolved in DMF. Step i was then followed by another DMF wash (3 × 24 ml).

The success of the coupling reactions was monitored by the semiquantitative ninhydrin reaction as described by Kaiser *et al.*[14] The *tert*-Boc-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg(NO_2)-Pro-Gly-resin was dried *in vacuo* overnight; 4.8 g of material was obtained, which was suspended in MeOH–dioxane (4:1 v/v) (50 ml) saturated with NH_3 at −2°. The mixt was stirred in a tightly stoppered flask at room temp for 36 hr. After filtration and evaporation of the solvent *in vacuo*, the residue was purified by column chromatography on silica gel G. Starting with $CHCl_3$ as elution solvent and by increasing the polarity of the solvent by using MeOH, the protected heptapeptide amide, II, was eluted with $CHCl_3$–MeOH (9:1 v/v). After precipitation from MeOH–Et_2O, II was obtained; 797 mg (35% yield based on attached Gly to the resin), $[\alpha]^{22}D$ −43.4 (*c* 1.0 MeOH) R_f^1 0.80, R_f^2 0.97, R_f^3 0.73, single spot to Cl-tolidine reagent; nmr (MeOH-d_4) τ 2.62 and 2.70 (ArH, 5 H each), 4.98 ($ArCH_2$, 2 H), 6.25 ($ArCH_2$, 2 H), 8.70 [$C(CH_3)_3$, 9 H], 9.10 [$CH(CH_3)_2$ 6 H]. Amino acid analyses were Glu 0.90; Tyr 0.28; Gly 2.00; Leu 0.93; Arg(NO_2) 0.62 (uncorrected); Pro 1.06. Tyr is known to give a low value in the presence of Arg(NO_2).[22]

***O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-NO_2-Arg-Pro-Gly-NH_2·TFA Salt (III).** Compd II (322 mg) was dissolved in TFA (3 ml) for 5 min. The solvent was then evaporated and the product was dried over KOH *in vacuo* for 12 hr; tlc showed a single product positive to the ninhydrin and Cl-tolidine reagents with R_f^1 0.73, R_f^3 0.68.

pGlu-His-Trp Bzl Ester (IV). Pyroglutamylhistidine[15] (723 mg), 1-hydroxybenzotriazole[16] (250 mg), tryptophan benzyl ester hydrochloride (900 mg), and Et_3N (0.35 ml) in dry DMF (25 ml) were treated with DCI (560 mg) at 0°. After being stirred at room temp during 48 hr, the reaction mixt was evaporated *in vacuo* to dryness. The residue was partitioned with $CHCl_3$ and H_2O, and an oily product was separated between these 2 solvents. The oily product was collected and purified by recrystallization from MeOH–EtOAc to afford the tripeptide 1.16 g (yield 79%), mp 235–238° dec, $[\alpha]^{22}D$ −6.8° (*c* 1.54, glacial HOAc), R_f^1 0.72, R_f^2 0.89, and R_f^3 0.59, single spot with Pauly, Ehrlich, Cl-tolidine, and I_2 reagents. *Anal.* $C_{29}H_{30}N_6O_5 \cdot H_2O$: C, H, N.

pGlu-His-Trp (V). Pyroglutamylhistidyltryptophan benzyl ester (780 mg) in MeOH (30 ml) and glacial HOAc (10 ml) with 5% Pd/C as catalyst was hydrogenated during 2 hr at room temp at 1 atm. The reaction mixt was filtered and evaporated to give the tripeptide; 422 mg (yield 64%), mp 188–190° dec, R_f^1 0.58, R_f^2 0.64, R_f^3 0.50, single spot with Pauly, Ehrlich, and Cl-tolidine reagents.

pGlu-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-NO_2-Arg-Pro-Gly-NH_2 (VI). Pyroglutamylhistidyltryptophan (147 mg) was mixed with 1-hydroxybenzotriazole[16] (45 mg) and DCI (62 mg) in dry DMF (5 ml) and the mixt was stirred at 0° for 1 hr; the stirring was then continued at room temp for 1 hr. Compd III (326 mg) and Et_3N (0.045 ml) were then added at 0° and the reaction mixt was left at room temp for 5 hr. After evaporation of the solvent *in vacuo* (40°), the residue was purified by preparative tlc on silica gel G using MeOH–$CHCl_3$ (3:7 v/v) as the developing solvent. This procedure gave 180 mg (yield 45%) of VI; R_f^1 0.65, R_f^2 0.84, R_f^3 0.63, single spot positive to Pauly, Ehrlich, and Cl-tolidine reagents. Amino acid analyses were Glu 1.0; His 1.1; Ser 0.80; Tyr 0.61; Gly 2.0; Leu 0.98; Arg(NO_2) (corr) 1.0; Pro 1.1. Arg(NO_2) is corrected for Orn formed in the hydrolysis.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LRH) (I). Compd VI (30 mg) was dissolved in MeOH–glacial AcOH (9:1 v/v) (10 ml) and was hydrogenated over Pd/$BaSO_4$ (60 mg) at room temp and atm pressure for 40 hr. Evaporation of the solvent *in vacuo* yielded I; 15 mg (yield 60%) R_f^1 0.64, R_f^3 0.37, positive to Pauly, Ehrlich, Sakaguchi, and Cl-tolidine reagents. The product was purified by countercurrent distribution. Amino acid analyses of sample 5 from distribution were Glu 1.00; His 1.00; Trp 0.92; Ser 0.98; Tyr 0.78; Gly 2.06; Leu 0.88; Arg 1.07; Pro 1.16; NH_3 1.00.

Synthesis of Luteinizing-Releasing Hormone. Scheme II. *N*-Cbz-*p*Glu-*N*^{im}-Tos-His-Trp-*O*-Bzl-Ser-*O*-Bzl-Tyr-Gly-Leu-NO_2-Arg-Pro-Gly-NH_2 (VII). *tert*-Boc-Gly resin (2.0 g, 1.4 m*M* Gly) was used and the same reaction cycle as described for II was utilized. The first 7 amino acids were introduced as described for II; then, *tert*-Boc-Trp, *tert*-Boc-His(Tos), and Z-pGlu were added. The *tert*-Boc-amino acids (3.5 m*M* of each) were dissolved in CH_2Cl_2 (11 ml) except for *tert*-Boc-Arg(NO_2) and *tert*-Boc-Trp when DMF (11 ml) was used as the solvent. DCI (3.5 m*M*) was added in CH_2Cl_2 (3 ml). After the introduction of *tert*-Boc-Trp, 1% 1,4-butanedithiol was added in step b. The Z-pGlu-His(Tos)-Trp-Ser(Bzl)-Tyr(Bzl)-Gly-Leu-Arg-(NO_2)-Pro-Gly-resin was dried *in vacuo* overnight, and was then suspended in MeOH–dioxane–NH_3, as described for II, and the mixture was stirred for 40 hr at room temp. Evaporation and drying *in vacuo* overnight yielded crude VII which was purified by recrystallization from MeOH giving 790 mg (yield 33%, based on Gly attached to the resin). Single spot in tlc to Ehrlich and Cl-tolidine reagents. Amino acid analyses after HCl hydrolyses were Glu 0.98; His(Tos) 0.99; Ser 0.79; Tyr 0.45; Gly 2.0; Leu 0.92; Arg(NO_2) 0.98 (corr. for Orn); Pro 1.0.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (LRH) (I). Compd VII (220 mg), anisole (1.5 ml), and a 10-fold molar excess of Met were mixed and dry HF (15 ml) was then distd into the reaction vessel kept at −60°. The temp was then adjusted to 0°, and the reaction mixt was stirred for 45 min. The HF and the anisole were removed *in vacuo*, and the residue was dissolved in 1% HOAc and lyophilized. The material was purified by CCD. The product showed identical R_f values in tlc as for the decapeptide from Scheme I; single product positive to Pauly, Ehrlich, Sakaguchi, and Cl-tolidine reagents. Amino acid analyses on sample 6 from countercurrent distribution were Glu 0.84, His 0.92, Ser 0.74, Tyr 0.84, Gly 2.00, Leu 0.86, Arg 1.03, Pro 1.28.

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References

(1) B. L. Currie, H. Sievertsson, C. Bogentoft, J.-K. Chang, K. Folkers, C. Y. Bowers, and R. F. Doolittle, *Biochem. Biophys. Res. Commun.*, **42**, 1180 (1971).
(2) C. Bogentoft, B. L. Currie, H. Sievertsson, J.-K. Chang, K. Folkers, and C. Y. Bowers, *ibid.*, **44**, 403 (1971).
(3) A. V. Schally, Y. Baba, A. Arimura, T. W. Redding, and W. F. White, *ibid.*, **42**, 50 (1971).
(4) J.-K. Chang, H. Sievertsson, C. Bogentoft, B. L. Currie, K. Folkers, and C. Y. Bowers, *ibid.*, **44**, 409 (1971).
(5) A. V. Schally, A. Arimura, Y. Baba, R. M. G. Nair, H. Matsuo, T. W. Redding, L. Debeljuk, W. F. White, *ibid.*, **43**, 393 (1971).
(6) H. Matsuo, Y. Baba, R. M. G. Nair, A. Arimura, and A. V. Schally, *ibid.*, **43**, 1334 (1971).
(7) Y. Baba, H. Matsuo, and A. V. Schally, *ibid.*, **44**, 459 (1971).
(8) C. Y. Bowers, J.-K. Chang, H. Sievertsson, C. Bogentoft, B. L.

Currie, and K. Folkers, *ibid.*, 44, 414 (1971).
(9) A. V. Schally, A. Arimura, A. Kastin, H. Matsuo, Y. Baba, T. W. Redding, R. M. G. Nair, and L. Debeljuk, *Science*, 173, 1036 (1971).
(10) H. Sievertsson, J.-K. Chang, C. Bogentoft, B. L. Currie, K. Folkers, and C. Y. Bowers, *Biochem. Biophys. Res. Commun.*, 44, 1566 (1971).
(11) M. Monahan, J. Rivier, R. Burgus, M. Amoss, R. Blackwell, W. Vale, and R. Guillemin, *C. R. Acad. Sci., Ser. D*, 273, 508 (1971).
(12) R. B. Merrifield, *J. Amer. Chem. Soc.*, 85, 2149 (1963).
(13) J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis," W. H. Freeman and Co., San Francisco, Calif., 1969.
(14) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. T. Cook, *Anal. Biochem.*, 34, 595 (1970).
(15) J.-K. Chang, H. Sievertsson, B. L. Currie, K. Folkers, and C. Y. Bowers, *J. Med. Chem.*, 14, 484 (1971).
(16) W. König and R. Geiger, *Chem. Ber.*, 103, 788 (1970).
(17) F. Enzmann, J. Boler, K. Folkers, C. Y. Bowers, and A. V. Schally, *J. Med. Chem.*, 14, 469 (1971).
(18) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).
(19) B. Williamson and L. C. Craig, *ibid.*, 168, 687 (1947).
(20) V. D. Ramirez and S. M. McCann, *Endocrinology*, 73, 193 (1963).
(21) G. D. Niswender, A. R. Midgley, Jr., S. E. Monroe, and L. E. Reichert, Jr., *Proc. Soc. Exp. Biol. Med.*, 128, 807 (1968).
(22) M. Moritz and R. Wade, *Anal. Biochem.*, 41, 446 (1971).

Preliminary Biological Studies of Several Aliphatic Amino Acid Analogs

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The synthesis of 27 unnatural amino acids (14 are new compounds) and 30 hydantoins (19 are new compounds) was carried out. Biological investigation has shown that 5 of the amino acids are toxic to *Escherichia coli* 9723 (4 are methionine analogs and 1 is an analog of threonine and isoleucine); one of the compounds (VI-A) is a competitive inhibitor of methionine. None of the amino acids showed any toxicity to growing mice. No cancer regression was noted with any of the amino acids in the cases of sarcoma 180, carcinoma 755, Walker carcinoma 256, and leukemia L-1210.

It has been known for decades that certain metabolic analogs interfere with the normal life functions of some microbes and yet have no harmful effect in mammals; this is well exemplified in the classic work of Woods.[1] Mammals get most of their amino acid requirements from foodstuff; in contrast, many microbes biosynthesize them. In hopes of obtaining substances that would control microbial growth and yet not harm mammals, a series of aliphatic unnatural amino acids was synthesized. Of the 27 DL-amino acids prepared and investigated, 14 are new compounds. All of these were prepared from the corresponding ketones *via* the hydantoins; the latter were also isolated and characterized (19 are new compounds). Three of the hydantoins (XXVIII-B, XXIX-B, and XXX-B) resisted all attempts at hydrolysis. Of all the unnatural amino acids investigated, it was found that 5 of them (VI-A, XI-A, XIII-A, XV-A, and XXII-A) showed appreciable toxicity to *Escherichia coli* 9723. Of the 18 natural amino acids investigated as reversal agents, it was found that in smallest quantities, methionine reversed the toxicities of VI-A, XI-A, XV-A, and XXII-A, and either threonine or isoleucine reversed the toxicity of XIII-A.

Compound VI-A was studied in greater detail since it was the most toxic. The antagonism between VI-A and methionine seemed to be a competitive one, with the antibacterial index (VI-A/methionine) being 33,000.

It was observed that an increase of the inoculum annuls the inhibitive effect of the analogs. Perhaps this phenomenon is due to the presence of a substance (X), produced by *E. coli*, which reverses the inhibition; thus the concentration of X increases as the concentration of the bacteria in the inoculum increase. This substance (X) could well be a metabolite which acts in very small quantities. Results of this work actually indicate that one or several diffusible substances, capable of reversing the toxicity, are excreted into the incubation mixture.

In order to further elucidate the action of VI-A in *E. coli*, the compound was prepared containing $[^{14}C]CO_2H$. Results indicate that VI-A is not actively accumulated within the cells and that even penetration by simple diffusion is difficult. Figure 1 illustrates the results. The existence of prior accumulated L-methionine in *E. coli* in no way affects the entrance of VI-A; conversely, prior accumulated VI-A in *E. coli* has no effect on entrance of L-methionine into the cells. It has been shown that L-methionine added to the media does not displace VI-A from the cells; conversely VI-A does not displace L-methionine from *E. coli*. It has been noted that the same quantitity of VI-A enters the cells in presence or absence of L-methionine in the media. It would appear that the toxicity of VI-A and its reversal are not related to permeation phenomena.

E. coli, allowed to grow in a concentration of VI-A which causes a retardation in growth, will subsequently show permanent resistance to the effect of the same concentration of VI-A. This seems to indicate that a resistant strain has been developed (see Figure 2). It has been observed that the resistant strain of *E. coli* cultured on agar develops rough colonies compared to the smooth colonies of the wild strain.

The ketone and hydantoin intermediates used in the synthesis of VI-A are both nontoxic to *E. coli* at their maximum solubility.

In preliminary experiments, it was shown that the pathogen, *Salmonella typhimurium*, is equally subject to the toxic action of VI-A.

By means of a series of ip injections, it was shown that none of the analogs exhibits any toxicity to growing mice. Likewise, none of the analogs showed any antitumor effect on animals bearing sarcoma 180, carcinoma 755, Walker carcinoma 256, and leukemia L-1210.

By use of isotopic labeling and a metabolic cage, it was shown that VI-A on injection into mice is excreted unchanged relatively quickly by way of the renal system. It is apparently unmetabolized and causes no tissue damage. Radioautography has shown that, on injection, VI-A accumulates in the pancreas and is subsequently excreted *via* the urine.