λ_{min} 303 nm (ϵ 1688), 396 (5980). For riboflavin, λ_{max} 267 nm (ϵ 32,270), 374 (10,420), 445 (12,310); λ_{min} 306 nm (ϵ 1120), 402 (6790). *Anal.* ($C_{17}H_{19}BrN_4O_6$) C, H, Br, N.

7-Bromo-8-ethyl-10-(1′-D-ribityl)isoalloxazine (XVI). 2-Nitro-4-bromo-5-ethyl-*N*-D-ribitylaniline (XIV, 5.00 g, 0.0132 mol) and approximately 5 g of Raney nickel in 350 ml of EtOH was hydrogenated as described for XIII. At the completion of the hydrogenation the catalyst was removed by filtering the *o*-phenylenediamine solution directly into a hot solution of 2.55 g of alloxan and 5.10 g of boric acid in 200 ml of HOAc. The solution was heated under refluxing conditions for 5 min and stored in the dark for 2 days. The solution was evaporated to dryness *in vacuo*; the crystalline product was suspended in 50 ml of H_2O and filtered. The product was recrystallized from 1 l. of 2% HOAc solution to produce 2.62 g (43%) as orange crystals: mp 259–261° dec (uncor); λ_{max} (H_2O) 270 nm (ϵ 37,110), 360 (9410), 447 (12,300); λ_{min} 305 nm (ϵ 1630), 394 (4515). *Anal.* ($C_{17}H_{19}BrN_4O_6$) C, H, Br, N.

Biological. The rats used were CFN (Carworth, Inc., New City, N. Y.). *L. casei* 7469 was obtained from American Type Culture Collection, Rockville, Md. The procedures used for the biological evaluation of the new analogs in rats and *L. casei* were the same as those used by us on earlier occasions.[3,4]

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Synthetic Luteinizing Hormone Releasing Factor. Short Chain Analogs

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The synthesis by solid phase on a benzhydrylamine resin of two series of analogs of the luteinizing hormone releasing factor, LRF (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), is described. The new oligopeptide amides described in the first series have the LRF sequence, have an acetylated N terminus, and are successively shortened from the N terminus by 1 (*e.g.*, Ac-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) 2, 3, etc., amino acids. The second series includes all the nonapeptides having the LRF sequence minus one amino acid. These peptide amides were purified by ion-exchange and partition chromatography and were characterized by amino acid analysis and, when possible, mass spectrometry after derivatization. Their specific rotations are reported. Homogeneity of these peptides was tested by thin-layer chromatography in seven different solvent systems and electrophoresis. The *in vitro* and *in vivo* LRF and follicle-stimulating hormone releasing activities (FRF) of these peptides are compared to that of the synthetic LRF. Structure–activity relationship is discussed. None of these peptides have any thyrotropin hormone releasing activity.

In a preceding paper[1] we have reported the synthesis of a series of LRF homologous amides where the chain was shortened by 1, 2, etc., amino acids from the C terminus. Toward the same goal of finding the shortest peptide with LRF biological activity, we synthesized another series of LRF homologs amides where the chain was successively shortened from the N terminus by 1 (*e.g.*, Ac-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), 2, 3, etc., amino acids (see Table I). The N-terminal amino acid was acetylated to replace the blocked N-terminal pyroglutamyl residue found in LRF.

In order to investigate the importance of each amino acid to the biological activity of LRF, we report another series of analogs in which each amino acid has been successively deleted. We will compare it with two other series where each amino acid has been (1) either replaced by glycine[2] or (2) replaced by alanine (Monahan, *et al.*, in preparation). Compounds **10–18** are also of interest since they are the most probable contaminants of LRF synthesized on a benzhydrylamine resin (failure sequences). One of them, des-His²-LRF, was found to be a competitive inhibitor of LRF devoid of intrinsic biological activity *in vitro* as has been described elsewhere.[3] The ability of des-Arg⁸-LRF,[4] des-Trp³-LRF,[5] and des-Leu⁷-LRF[6] to release LH has been reported by others while this systematic study was being carried out.

Synthesis, Purification, and Characterization. The details of the synthesis have been reported elsewhere.[1] It was found, however, that coupling time could be reduced to 30 min and the amount of reagents needed could be lowered (three times molar excess) with no apparent change in yield.

The two steps of purification used on these peptides consisted of ion-exchange chromatography on carboxymethylcellulose and partition chromatography on Sephadex as previously described.[1]

After purification these peptides were characterized by amino acid analysis (Table II) and mass spectrometry† when possible. These new compounds were homogeneous in seven different thin-layer chromatography (on silica gel) systems and electrophoresis on paper (see Table III).

Biological Activity. The ability of the peptides to stimulate LH and FSH secretion *in vitro*[8] and *in vivo*[9]

† Due to the growing interest in peptide sequencing by mass spectrometry, this series was analyzed in a successful attempt to sequence LRF; see ref 7.

Table I. Specific Biological Activity of LRF Analogs[a]

	Analogs	% specific act. of LRF with confidence limits		
		In vitro	*In vivo*	FSH[b]
1	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	100	100	*[c]
2	Ac-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	0.2 (0.06–1.5)	0.5 (0.18–3.8)	*
3	Ac-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.01[e]	–[d]
4	Ac-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.01	–
5	Ac-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.01	–
6	Ac-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.01	–
7	Ac-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.01	–
8	Ac-Arg-Pro-Gly-NH$_2$	<0.001	<0.01	–
9	Ac-Pro-Gly-NH$_2$	<0.001	<0.01	–
10	pGlu-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.001[e]	–
11	pGlu-His-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.001	–
12	pGlu-His-Trp-Tyr-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.001	–
13	pGlu-His-Trp-Ser-Gly-Leu-Arg-Pro-Gly-NH$_2$	<0.001	<0.001	–
14	pGlu-His-Trp-Ser-Tyr-Leu-Arg-Pro-Gly-NH$_2$	0.1 (0.06–0.17)	0.01 (0.001–0.03)	*
15	pGlu-His-Trp-Ser-Tyr-Gly-Arg-Pro-Gly-NH$_2$	0.005 (0.002–0.01)	0.001 (0.0004–0.004)	–
16	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Pro-Gly-NH$_2$	<0.001	<0.001	–
17	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Gly-NH$_2$	0.11 (0.06–0.2)	0.03 (0.01–0.05)	*
18	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NH$_2$	11 (6–20)	10 (3–25)	*

[a]All the analogs were tested *in vitro* at concentrations up to 100 μM and *in vivo* at doses up to 10–100 nmol. [b]Release of FSH *in vivo* and *in vitro* different from controls. [c]*, significant; no discrepancies were observed between the potencies of these peptides with regard to their ability to release LH and FSH. [d]–, not significant. [e]Slight activity at high doses, response nonparallel with response to LRF; maximum response less than that due to low dose of LRF standard.

Table II. Amino Acid Analysis of LRF Analogs

Compd	Amino acid ratios									
1	Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH$_2$
2	Ac	1.0	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.0
3		Ac	0.9	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.0
4			Ac	0.9	0.9	1.0	1.0	1.1	1.0	1.0	1.2
5				Ac	0.9	1.0	1.0	1.0	1.0	1.0	1.0
6					Ac	1.0	1.0	1.0	1.1	1.0	0.9
7						Ac	1.0	1.0	1.0	1.0	1.1
8							Ac	0.9	1.0	1.0	0.9
9								Ac	0.9	1.0	0.9
10	1.0	X	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0
11	1.0	0.9	X	0.9	1.0	1.0	1.0	1.0	1.1	1.0	1.1
12	1.0	0.9	0.9	X	1.0	1.0	1.0	0.9	1.0	1.0	1.0
13	1.0	1.0	1.0	0.9	X	1.0	1.0	1.0	1.0	1.0	1.0
14	1.0	0.9	1.0	0.9	1.0	X	1.0	0.9	1.0	1.0	1.0
15	1.0	1.0	1.0	0.9	0.9	1.0	X	0.9	1.0	1.0	1.4
16	1.0	0.9	1.0	0.9	1.0	1.0	1.0	X	1.0	1.0	1.0
17	1.0	0.9	1.0	0.9	0.9	1.0	1.0	1.0	X	1.0	1.0
18	1.0	0.9	0.9	0.9	1.0	0.9	1.0	1.0	1.1	X	1.0

as well as to antagonize LRF activity *in vitro*[3] was determined and is shown in Tables I and IV, respectively. The ability of the peptides to stimulate TSH secretion has also been investigated according to the procedure reported by Vale, *et al.*[10] LRF standards were given *in vitro* over a range of 0.1–10 nM and *in vivo* from 2.5 to 80 ng. Analogs were tested *in vitro* at concentrations up to 100 μM and *in vivo* at doses up to 100 nmol.

The replacement of the pyroglutamyl residue (pGlu) by an acetyl grouping, which, if it does not have the steric or chemical properties of the lactam ring, does, however, block the N terminus of histidine, yielded a nonapeptide having 0.2% of LRF biological activity. It is noteworthy that deletion of the pGlu[11] to give des-pGlu[1]-LRF with a free N-terminal histidine residue or replacement of pGlu[12] by proline ([Pro[1]]-LRF) has given two compounds having less than 1/1000 of LRF biological activity. This stresses the importance of the blocked N terminus. Similarly, a neutral amidated C terminus has been shown by Chang, *et al.*,[4] to be critical; LRF-OH has only 0.2% of LRF biological activity. It is of interest to mention that similar results were obtained for the thyrotropin releasing factor TRF (pGlu-His-Pro-NH$_2$).[13] All the other members of that series (**3**–**9**) are inactive agonists at the doses tested.

Compounds **2**, **14**, and **17** have shown some ability to trigger the release of LH *in vitro* and *in vivo* at very high doses, thus showing an ability to recognize and bind to the receptor site, a property not shared by most peptides of this series. In all cases the active agonist peptides have shown dose–response curves parallel to that of LRF with similar maximum response, which can be interpreted as an altered affinity of the analogs for the LRF receptor. The biological activities of the analogs in which one amino acid has been successively replaced by glycine[2] or alanine[14] are greater than that of the corresponding analogs of the series **10**–**18**, indicating that conservation of the peptide backbone is necessary for high affinity for the receptor. Chang, *et al.*,[4] Grant and Vale,[15] and Monahan, *et al.*,[14] have recently proposed the existence of a preferred tertiary structure for LRF. The fact that all nonapeptides with the exception of **18** show very little biological activity as well as our current work on nmr analysis (manuscript in preparation) is consistent with this hypothesis.

Compounds **3** and **10** have shown antagonistic activity in an *in vitro* system recently reported[3] and describing the two analogs [Gly[2]]-LRF and des-His[2]-LRF. The former, [Gly[2]]-LRF, had some partial agonistic activity and therefore an altered dose–response curve, whereas the latter,

Table III. Physical Constants and Yield of LRF Analogs

Compd	Tlc system[a]							Electro-		Yield,[d]
	BPA	EPAW	IaPW	IpN	IpA	BAW	BIpNE	phoresis[b]	$[\alpha]^{20}D^{c}$	%
2	0.65	0.85	0.65	0.75	0.80	0.50	0.40	0.50	−47.0	15
3	0.70	0.90	0.70	0.75	0.85	0.60	0.40	0.23	−38.7	22
4	0.65	0.85	0.65	0.70	0.80	0.55	0.35	0.33	−67.5	30
5	0.70	0.85	0.70	0.75	0.80	0.60	0.40	0.35	−42.0	28
6	0.50	0.80	0.60		0.70	0.55	0.20	0.47	−79.0	29
7	0.60	0.80	0.60		0.75	0.60	0.35	0.50	−72.5	25
8	0.25	0.65	0.37		0.55	0.40	0.10		−66.6	25
9	0.40	0.66	0.40	0.56	0.59	0.35	0.49		−64.0	35
10	0.57	0.77	0.56	0.78	0.85	0.45	0.42	0.24	−47.0	35
11	0.48	0.63	0.54	0.80	0.75	0.27	0.30	0.56	−55.7	29
12	0.56	0.72	0.54	0.75	0.76	0.36	0.42	0.49	−57.2	23
13	0.52	0.67	0.54	0.72	0.69	0.28	0.34	0.54	−55.6	27
14	0.55	0.72	0.59	0.75	0.76	0.35	0.41	0.47	−60.2	32
15	0.49	0.64	0.51			0.23	0.35	0.49	−46.2	22
16	0.63	0.75	0.76	0.87	0.83	0.39	0.53	0.24	−51.3	26
17	0.58	0.74	0.61	0.72	0.77	0.34	0.35	0.48	−30.2	21
18	0.52	0.66	0.51	0.72	0.80	0.33		0.48	−53.5	19

[a]BPA, 1-butanol–pyridine–0.1 *N* acetic acid (5:3:11, upper phase); EPAW, ethyl acetate–pyridine–acetic acid–water (5:5:1:3); IaPW, isoamyl alcohol–pyridine–water (7:7:6); IpN, 2-propanol–1 *N* ammonia (2:1); IpA, 2-propanol–1 *N* acetic acid (2:1); BAW, butanol–acetic acid–water (4:1:5, upper phase); butanol–2-propanol–1 *N* ammonia–ethyl acetate (1:1:1:2.5). Uv, I_2, ninhydrin spray, phenanthrenequinone [S. Yamada and H. A. Itano, *Biochem. Biophys. Acta*, **130,** 538 (1966)], and Pauly reagents were successively used. Loads varied from 20 to 40 μg per spot. [b]R_f values relative to lysine and alanine, 100-μg loads on Whatman 3MM (pH 4.7 buffer containing 2.5% acetic acid, 2.5% pyridine, 5% 1-butanol, and 90% water) at 3500 V, 2.5 hr run. [c]Concentration in 1% AcOH = 1. [d]Peptide yields are calculated on the basis of m*M* of peptides isolated after final purification relative to the total m*M* of starting *tert*-butyloxycarbonylamino acid, *viz.*, as resin amide.

Table IV. Antagonistic Activity of **3** *in Vitro*

No.	Addition	LH secreted per dish, ng ± S.E.M.	*p* difference from **1**
1	Saline	180 ± 26	
2	1.0 n*M* LRF	1550 ± 160	<0.01
3	100 μ*M* **3**	190 ± 20	<0.05
4	100 μ*M* **3** + 1.0 n*M* LRF	395 ± 66	>0.05
Multiple comparisons test of Duncan **2** *vs.* **4**			<0.01

des-His[2]-LRF, had no agonistic activity at doses ten times higher than those required to inhibit the response to LRF by more than 70% in that *in vitro* system.[3] Compound **3** in which the 1 and 2 positions have been replaced by an acetyl group shows some antagonistic properties toward LRF at higher doses than those required by des-His[2]-LRF (Table IV). It would appear that while the 2 position is involved in the mechanism of activation of the receptor site,[16] the entire molecule including the 2 position is involved in the binding to the receptor site. Fujino, *et al.*,[17] describing a series of analogs where Gly[10]-NH_2 had been replaced by NCH_3, NCH_2CH_3, and $NCH_2CH_2CH_3$ (biological activity: 80–100, 500, and 200–300%, respectively), arrived at an even more stringent conclusion, namely that "the total length of the LRF molecule might have a very important role in the binding of the hormone to its receptor(s) at the target organ, pituitary."

With the exception of des-Gly[10]-LRF, none of the nonapeptides nor any of the C-terminus shortened analogs described here exhibit more than 1/500 of LRF-agonist activity; further, in the assay systems reported here, they are not antagonists at ratios of analog/LRF = 10.000/1. Thus, if traces of those peptides (the most probable contaminants in our solid-phase synthetic approach for LRF[1]) were present in small quantities in an LRF preparation, they would not significantly alter its biological properties.

Experimental Section

Mass spectra were obtained at 70 eV with a Varian CH-5; data were recorded with the Varian 620 I computer. High-resolution mass spectrometry was used to characterize the final products as such for Ac-Pro-Gly-NH_2 (calcd mass, 213.111; found, 213.111) and derivatized[18] Ac-N^{δ}-2-(4,6-dimethylpyrimidyl)-Orn-Pro-Gly-NH_2 for Ac-Arg-Pro-Gly-NH_2 (calcd mass, 433.244; found, 433.249). Amino acid analyses were performed on peptide hydrolysates using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. Hydrolyses were performed in 6 *N* HCl containing 0.5 and 5% thioglycolic acid at 110° in evacuated sealed tubes for 20 hr. A marked improvement in the Trp values was observed with the higher concentration of scavenger.[19] No corrections were made for decomposition of serine and tyrosine.

Starting Materials. The benzhydrylamine resin used was obtained according to the procedure reported elsewhere.[1] The substitution in amino groups was 0.15 mequiv/g of resin for the penta- to nonapeptides and 0.30 mequiv/g of resin for the shorter peptides. *tert*-Butyloxycarbonylamino acids‡ were bought from BACHEM and further purified when necessary. All amino acids were of the L configuration.§

Synthesis. Deblocking and coupling procedures have been described in detail. The coupling time in this series of syntheses was reduced to 0.5 hr, an equimolar amount of *tert*-butyloxycarbonylamino acid and DCC (3 mequiv/mequiv of free amino group on the resin) being added simultaneously. Acetylation was performed on the deblocked peptide resin with an excess (50-fold) of acetic anhydride‡ in CH_2Cl_2 for 0.5 hr. Cleavage from the resin and ion-exchange chromatography followed by partition chromatography were described previously.[1] The procedure was scaled up to obtain 175–250 mg of pure peptide from 3–4 g of peptide resin.

Characterization. Amino acid analyses of these LRF analogs are reported in Table II. Specific rotations and yields are also reported (Table III). Homogeneity of the peptides was demonstrated by electrophoresis and by tlc on Eastman Chromatogram sheets (6061 silica gel without fluorescent indicator) in seven solvent systems (see Table III). On the basis of the loads applied and the sensitivity of the color or uv tests, one can ascertain that these peptides had less than 5% impurity.

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‡ Boc-His(Tos), Boc-Ser(Bzl), Boc-Tyr(Bzl), Boc-Gly, Boc-Leu, Boc-Pro. Boc-Arg(NO_2) contained an unidentified amino acid, probably *tert*-butyloxycarbonylornithine, eliminated by repeated extraction in boiling $CHCl_3$. *tert*-Butyloxycarbonyltryptophan was freed from an unknown contaminant by repeated extractions in boiling CCl_4. Acetic anhydride was of reagent grade.

§ The starting materials were found to be optically pure by determination of their optical rotation.

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Adrenocorticotropin.† 45. Synthesis and Steroidogenic Activity of [1-Alanine]adrenocorticotropin-(1–20)-amide, [1-D-Alanine]adrenocorticotropin-(1–20)-amide, and [[1-^{14}C]Alanine]adrenocorticotropin-(1–20)-amide

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Three biologically active peptides, [1-alanine]-α-adrenocorticotropin-(1–20)-amide (I), [1-D-alanine]-α-adrenocorticotropin-(1–20)-amide (II), and [[1-^{14}C]alanine]-α-adrenocorticotropin-(1–20)-amide (III), have been synthesized by the solid-phase method. All of the synthetic peptides exhibited high steroidogenic potency, and peptide II was shown to have prolonged steroidogenic activity *in vivo*.

The synthesis of the NH_2-terminal nonadecapeptide of α-ACTH and its analogs[2,3] has recently been achieved by the solid-phase method.[4] This communication reports the synthesis and adrenal-stimulating activity of [Ala^1]-α-ACTH-(1–20)-NH_2 (I), [D-Ala^1]-α-ACTH-(1–20)-NH_2 (II), and [^{14}C-Ala^1]-α-ACTH-(1–20)-NH_2 (III). The structure of I is shown in Figure 1.

The decision to synthesize these peptides was based upon several considerations. (i) Since the NH_2-terminal amino acid in ACTH is serine,[5] whose hydroxyl group must be protected in the solid-phase method, and since the side chain of serine does not contribute significantly to the steroidogenic activity of the hormone,[6,7] alanine was used to replace serine at the NH_2 terminus. (ii) The synthesis of II was carried out in order to determine whether D-alanine would enhance the biological activity as does D-serine.[8] (iii) It is desirable to synthesize a peptide whose biological potency is as close as possible to natural ACTH. Since α-ACTH-(1–19)-amide possesses greater steroidogenic activity than its free acid,[9] we decided to synthesize the amide instead of the free acid. (iv) For synthesis of a peptide amide, it was advisable to employ a phenyl ester linkage of the peptide to the polystyrene resin as demonstrated by our success in the synthesis of α-melanotropin[10] whose COOH-terminal amino acid is valine. Since the 20th amino acid in ACTH is valine,[5] we decided to synthesize I instead of the nonadecapeptide amide. Finally, for convenience in handling radioactive materials, it was deemed advisable to attach the radioactive label in the NH_2-terminal alanine.

Peptides I-III were synthesized in a manner analogous to that used for α-melanotropin.[10] The Boc-Val-O-C_6H_4-CH_2CO_2-resin was subjected to the standard procedure of the solid-phase method.[4] The side-chain protecting groups are described in the Experimental Section, but we note here the use of the *tert*-butyl group for protection of the side chain of glutamic acid. Although N^{α}-Boc protection

†For paper 44, see ref 1. All asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration unless otherwise indicated. Abbreviations used are: ACTH, adrenocorticotropic hormone; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenylyl)isopropyloxycarbonyl; DCC, dicyclohexylcarbodiimide; CMC, carboxymethylcellulose.