Syntheses and Biological Activities of 7-Ethyl-8-bromo-10-(1′-D-ribityl)isoalloxazine and 7-Bromo-8-ethyl-10-(1′-D-ribityl)isoalloxazine, Analogs of Riboflavin†

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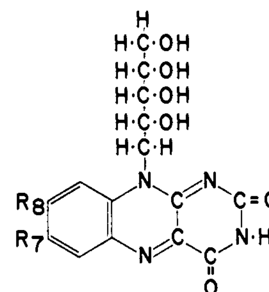
The riboflavin analogs 7-ethyl-8-bromo-10-(1′-D-ribityl)isoalloxazine and 7-bromo-8-ethyl-10-(1′-D-ribityl)isoalloxazine have been synthesized starting with *o*-aminoethylbenzene. The former analog is devoid of activity in the riboflavin-deficient rat and it was found to be a weak antagonist of riboflavin in *Lactobacillus casei*. The latter analog produced a growth response equivalent to approximately 2% of the activity of an equivalent amount of riboflavin, and it also caused an improvement in the visually observable signs of the riboflavin deficiency. Administration of large amounts of this analog was lethal to the animals. The latter analog also inhibited the utilization of administered riboflavin and since the reduced utilization of riboflavin and the lethality of the analog could be counteracted by the administration of sufficient riboflavin, the analog is a reversible antagonist of riboflavin. The latter analog was found to be a moderately strong, reversible antagonist of riboflavin in *L. casei*.

In a recent report[1] we discussed the essential structural features required in an analog of riboflavin to ensure biological activity in a form resembling riboflavin in action or in a form antagonistic to the action of riboflavin. In our efforts to explore the limits of structural modifications consistent with some form of biological activity in the rat and *Lactobacillus casei*, we found that the structure of riboflavin (A) could be modified to the extent of replacing the methyl group at either position 7 or 8 with a chloro group[2-4] (7-chloro-8-methylflavin, B; 7-methyl-8-chloroflavin, C) or with a bromo group[5,6] (7-bromo-8-methylflavin, D; 7-methyl-8-bromoflavin, E) with the retention of some forms of biological activity. Further, we have found that if the methyl group at either position 7 or 8 is replaced with an ethyl group and the methyl group in the other position is replaced by a chloro group[1] (7-chloro-8-ethylflavin, F; 7-ethyl-8-chloroflavin, G), reduced but still significant biological activity is retained.

Since we had not reached the limits of structural modification consistent with biological activity, we were prompted to make one last effort to discover these limits through the synthesis and evaluation of 7-ethyl-8-bromo-10-(1′-D-ribityl)isoalloxazine (7-ethyl-8-bromoflavin, XV) and 7-bromo-8-ethyl-10-(1′-D-ribityl)isoalloxazine (7-bromo-8-ethylflavin, XVI). While it is clear that the limits of structural modification have not yet been reached, the approach is near enough so that we do not plan to pursue the subject of still further, similar modifications (Chart I).

Chart I. Basic Isoalloxazine or Flavin Structure



	R_7	R_8	trivial name
A	CH_3-	CH_3-	riboflavin
B	Cl-	CH_3-	7-chloro-8-methylflavin
C	CH_3-	Cl-	7-methyl-8-chloroflavin
D	Br-	CH_3-	7-bromo-8-methylflavin
E	CH_3-	Br-	7-methyl-8-bromoflavin
F	Cl-	C_2H_5-	7-chloro-8-ethylflavin
G	C_2H_5-	Cl-	7-ethyl-8-chloroflavin
XV	C_2H_5-	Br-	7-ethyl-8-bromoflavin
XVI	Br-	C_2H_5-	7-bromo-8-ethylflavin

In addition to 7-bromo-8-methylflavin and 7-methyl-8-bromoflavin, the only bromine-containing analogs of riboflavin to have been described are 7,8-dibromo-10-(1′-D-ribityl)isoalloxazine and the two isomers containing the polyhydric alcohol groups L-arabityl or D-ducityl in the 10 position in place of D-ribityl.[7] These latter three flavins have not been tested in the rat or *L. casei*, but since 7,8-dichlororiboflavin[3,8] was found to be devoid of activity in the rat[3] and *L. casei*,[3,9] it is reasonable to assume that these dibromoflavins would be inert in these organisms also.

Chemistry. The syntheses of 7-ethyl-8-bromo- and 7-bromo-8-ethyl-10-(1′-D-ribityl)isoalloxazine required 2-bromo-4-nitroethylbenzene and 2-bromo-5-nitroethylbenzene, respectively. Our experience with the preparation of the corresponding chloro isomers[1] provided us with satisfactory procedures for their preparation. The syntheses of the flavins were accomplished as outlined in Schemes I and II. The many steps in the syntheses were essentially like those familiar to us from earlier work[1,2,10] and only significant differences are noted in the Experimental Section.

Biology. Table I shows the results of the rat growth studies in response to 7-ethyl-8-bromoflavin and to 7-bromo-8-ethylflavin with and without added riboflavin. It is clear that 7-ethyl-8-bromoflavin is devoid of activity in the riboflavin-deficient rat, except for a small effect on improving the appearance of the deficient animal.

When 7-bromo-8-ethylflavin was administered in quantities of 25 or 100 μg per day to riboflavin-deficient rats, it stimulated growth but the appearance of the animals was not improved as consistently as was observed when the corresponding 7-bromo-8-methylflavin was given. When the analog was administered in quantities of 250, 500 μg, and 1 mg per day, 30, 90, and 100% of the animals died before the end of the 28-day test period; yet, when these quantities were administered, all of the animals showed prompt return to normal appearance if they lived as long as 7 days. The administration of 10 or 20 μg per day of riboflavin with 500 μg per day of 7-bromo-8-ethylflavin did not protect the animals against the lethal properties of this analog; 40 μg per day of riboflavin did provide protection against 500 μg per day of the analog. It is reasonable to assume that the toxicity of 7-bromo-8-ethylflavin, as well as that of 7-chloro-8-ethylflavin,[1] is due to the specific displacement of riboflavin from the kidney, as was found to be true in the case of the related 7-chloro-8-methylflavin.[11]

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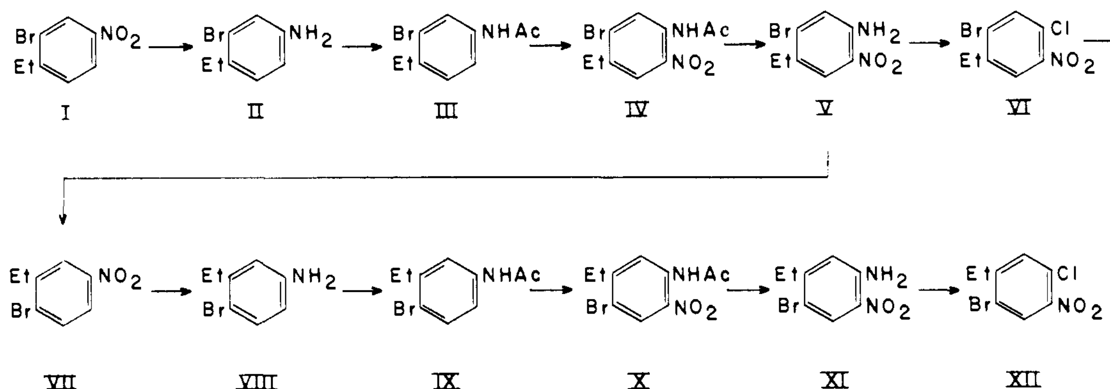
Table I. Growth of Rats Administered 7-Ethyl-8-bromoflavin, 7-Bromo-8-ethylflavin, Riboflavin, or Mixtures of 7-Bromo-8-ethylflavin and Riboflavin

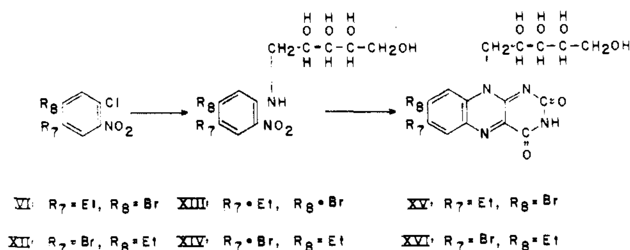
Compd	Quant flavin admin, μg equiv[b]	Wt gained, g	P[a]	Animals surviving, %
Flavin deficient	0	9 ± 2[c]		100
7-Ethyl-8-bromoflavin	25	11 ± 1	0.461	100
	100	12 ± 2	0.322	100
	250	13 ± 2	0.164	100
	500	16 ± 3	0.052	100
7-Bromo-8-ethylflavin	25	28 ± 2	0[d]	100
	100	42 ± 3	0	100
	250	48 ± 4	0	70[e]
	500	57[f]		10[g]
	1000			0[h]
7-Bromo-8-ethylflavin	500A + 10R			0[i]
(A) plus riboflavin (R)	500A + 20R	39[j]		10
	500A + 40R	83 ± 8	0	100
Riboflavin	5	50 ± 3	0	100
	10	74 ± 3	0	100
	20	109 ± 4	0	100
	40	122 ± 4	0	100

[a]Probability. The numbers represent the P values for the differences between the group in that line and the flavin-deficient group. [b]The symbol μg equiv means microgram equivalents. The molecular weights of the analogs and riboflavin are 455 and 376, respectively. Thus, 100 μg equiv of analog is equal to 121 μg of the analog but equimolar with 100 μg of riboflavin. [c]Average weight gains for animals surviving the 28-day test period ± S.E.M. All animals in groups flavin-deficient and 7-ethyl-8-bromoflavin had average starting weights of 61 ± 2 g, those in the 7-bromo-8-ethylflavin groups, 60 ± 2 g, those in the 7-bromo-8-ethylflavin plus riboflavin groups, 65 ± 5 g, and those in the riboflavin groups, 59 ± 2 g. [d]The symbol 0 means that the P value is less than 0.0001. [e]The animals died from day 14 to 23; those gaining weight most rapidly died first. [f]Average weight gained by two surviving rats of 20 used. [g]Animals died from day 3 to 25. [h]Animals died from day 5 to 14. [i]Animals died from day 4 to 25. [j]One surviving animal.

Scheme I



Scheme II



The growth response when 250 μg per day of the analog was administered was approximately equivalent to the growth response elicited by 5 μg per day of riboflavin, indicating that the growth-promoting activity of the analog is equivalent to approximately 2% of that of riboflavin.

The administration of 500 μg per day of analog with 40 μg per day of riboflavin reduced the growth response to the vitamin to approximately the response which would have been elicited by 12–13 μg per day of riboflavin, if administered alone.

The response of *L. casei* to 7-ethyl-8-bromoflavin is shown in Figure 1. As a replacement for riboflavin in the metabolism of this microorganism, the analog is inert. As an antagonist of the utilization of riboflavin by this organism, the analog is a weak, reversible inhibitor; I.I. = 248.‡

The response of *L. casei* to 7-bromo-8-ethylflavin is shown in Figure 2. This analog is inert as a replacement for riboflavin in this microorganism. As an antagonist of the utilization of riboflavin by this organism, the analog is a moderately strong, reversible inhibitor; I.I. = 141.

More acid is produced by *L. casei* in the presence of a mixture of small amounts of either analog and riboflavin than could have been produced by the utilization of the riboflavin alone. Presumably, this additional acid was produced by the mutant strain of *L. casei* which we have discovered and isolated.[4,12,13] This property of permitting the emergence of the mutant strain under specified conditions was found to be true also for 7-chloro-8-methylflavin,[4] 7-bromo-8-methylflavin,[6] 7-chloro-8-ethylflavin,[1] and 7-ethyl-8-chloroflavin.[1]

7-Ethyl-8-bromoflavin, as was found to be true for 7-methyl-8-chloroflavin,[3] 7-methyl-8-bromoflavin,[5] and 7-

‡Inhibition index (I.I.) = μg of analog which in the presence of 0.3 μg of riboflavin reduces acid production to half that produced in the presence of (0.3 μg of riboflavin alone/0.3 μg of riboflavin)(mol wt of riboflavin/mol wt of analog).

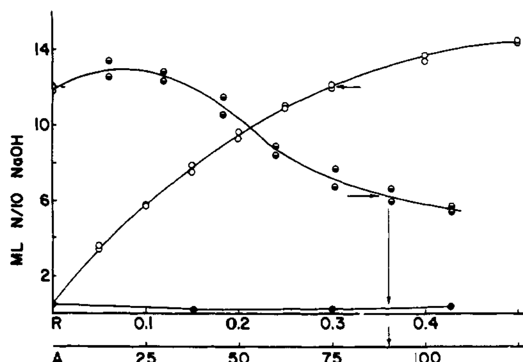


Figure 1. Lactic acid production by *L. casei* grown in a culture medium containing riboflavin (empty circles), 7-ethyl-8-bromoflavin (filled circles), and mixtures of riboflavin and the analog (half-filled circles). R, riboflavin; A, analog; the quantities of flavins are in μg per tube. For the mixed flavin curve (half-filled circles) all tubes contained 0.3 μg of R and from 0 to 107 μg of A. The response to 0.3 μg of R was reduced to 50% when 90 μg of A was also present in the tube. I.I. = (90/0.3)(376/455) = 248.

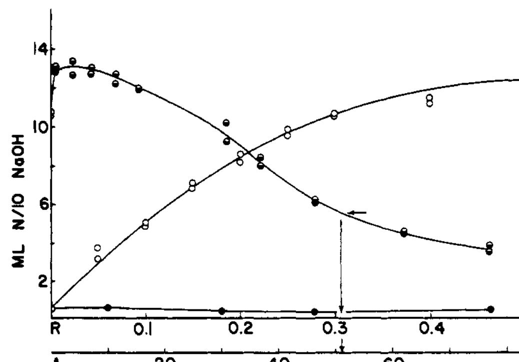


Figure 2. Lactic acid production by *L. casei* grown in a culture medium containing riboflavin (empty circles), 7-bromo-8-ethylflavin (filled circles), and mixtures of riboflavin and the analog (half-filled circles). R, riboflavin; A, analog, the quantities of flavins are in μg per tube. For the mixed flavin curve (half-filled circles) all tubes contained 0.3 μg of R and from 0 to 77 μg of A. The response to 0.3 μg of R was reduced to 50% when 51 μg of A was also present in the tube. I.I. = (51/0.3)(376/455) = 141.

ethyl-8-chloroflavin,[1] possesses an E'_0 which is sufficiently positive so that it is reduced to a "poised" rhodoflavin state when autoclaved with the basal medium used for the growth of *L. casei.*[3]

Experimental Section

Chemistry. Melting points were determined in open Pyrex capillary tubes in an electrically heated, modified Drechsel-type bath and are corrected (thermometers calibrated against U.S.P. melting point reference standards). Boiling points are uncorrected. Decomposition points of the two flavins were obtained by immersing the capillary tube into the preheated bath (225°) and then heating rapidly to the decomposition point (uncorrected). Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theroretical values. Uv spectra were determined in H_2O solution on a Beckman DU equipped with a Gilford photometric indicator unit.

2-Bromo-4-nitroethylbenzene (I). Practical grade *o*-ethylaniline (Eastman P-3066) was purified by vacuum fractionation, conversion to the acetanilide of mp 111-113° (lit.[14] 111-112°) and then hydrolysis to the pure aniline. The latter was nitrated[15] to produce 2-ethyl-5-nitroaniline (84%) as yellow crystals: mp 63-64° (EtOH) (lit.[15] 63-64°). Lots of 109 g (0.66 mol) of 2-ethyl-5-nitroaniline were converted to I as described.[15] Purification was accomplished by steam distillation, then vacuum distillation, and then recrystallization from EtOH to produce an average yield of 111 g (73%) of yellow prisms: bp 156-158° (18 mm); mp 27°. The latter product was then recrystallized from *n*-hexane to produce 60 g (39%): mp 30-31° (lit.[16] 31-32°). I was also prepared from *p*-nitroethylbenzene.[16]

3-Bromo-4-ethylacetanilide (III). I (130 g, 0.57 mol) was reduced with Fe filings and HCl to yield 3-bromo-4-ethylaniline (II). The reaction mixture was steam distilled to remove traces of unreduced I, made alkaline with NH_3, and then steam distilled to remove the aniline II in an average yield of 102 g (90%) as a colorless liquid: bp 138-140° (14 mm); 152-156° (26 mm). II (102 g, 0.51 mol) was immediately converted to the acetanilide III with Ac_2O in average yield of 94 g (77%) as white plates: mp 111-112° (benzene). *Anal.* ($C_{10}H_{12}BrNO$) C, H, Br, N.

2-Nitro-4-ethyl-5-bromoacetanilide (IV). III (91.4 g, 0.40 mol) was nitrated by the procedure described for the preparation of 4,5-diethyl-2-nitroacetanilide[17] to produce an average yield of 69 g (64%) of IV as yellow needles: mp 115-117° (EtOH). *Anal.* ($C_{10}H_{11}BrN_2O_3$) C, H, Br, N.

2-Nitro-4-ethyl-5-bromoaniline (V). IV (116 g, 0.40 mol) was hydrolyzed as described for 2-nitro-4-methyl-5-chloroacetanilide[2] to produce an average yield of 88 g (90%) of V as orange needles: mp 112-114° (EtOH). *Anal.* ($C_8H_9BrN_2O_2$) C, H, Br, N.

2-Chloro-4-bromo-5-ethylnitrobenzene (VI). V (65.4 g, 0.267 mol) was converted to VI as described for the preparation of 4,6-dichloro-3-nitrotoluene[2] to produce 51 g (72%) as yellow prisms: mp 43-45° (*n*-hexane). *Anal.* ($C_8H_7BrClNO_2$) C, H, Br, N.

2-Bromo-5-nitroethylbenzene (VII). 2-Nitro-4-ethyl-5-bromoaniline (V, 24.5 g, 0.1 mol) was deaminated by the procedure described for the deamination of 2-nitro-4-ethyl-5-methylaniline[10] to produce an average yield of 183 g (80%) of VII as a yellow liquid: bp 148-151° (14 mm); 150-154° (20 mm). *Anal.* ($C_8H_8BrNO_2$) C, H, Br, N.

3-Ethyl-4-bromoacetanilide (IX). VII (54.0 g, 0.20 mol) was reduced by the procedure used to reduce I to II to produce 3-ethyl-4-bromoaniline (VIII) in average yield of 34.0 g (85%) as a colorless liquid: bp 142-146° (14 mm); 149-151° (25 mm). VIII (28.0 g, 0.14 mol) was immediately converted to the acetanilide IX in average yield of 23.0 g (68%) as white plates: mp 110-112° (benzene). *Anal.* ($C_{10}H_{12}BrNO$) C, H, Br, N.

2-Nitro-4-bromo-5-ethylacetanilide (X). IX (42.4 g, 0.175 mol) was nitrated as described for III to produce X. The crude nitration product was dissolved in 100 ml of EtOH to yield, on cooling, 38.0-42.8 g (75-85%) of product as yellow prisms: mp 112-114° (EtOH). *Anal.* ($C_{10}H_{11}BrN_2O_3$) C, H, Br, N.

2-Nitro-4-bromo-5-ethylaniline (XI). X (23.0 g, 0.08 mol) was hydrolyzed as described for IV to produce XI in average yield of 13.5 g (69%) as orange plates: mp 122-124° (EtOH). *Anal.* ($C_8H_9BrN_2O_2$) C, H, Br, N.

2-Chloro-4-ethyl-5-bromonitrobenzene (XII). XI (24.5 g, 0.10 mol) was converted to XII as described for the preparation of VI to produce an average yield of 17.8 g (67%) of yellow liquid: bp 159-162° (12 mm); 188-192° (30 mm). *Anal.* ($C_8H_7BrClNO_2$) C, H, Br, N.

2-Nitro-4-ethyl-5-bromo-*N*-D-ribitylaniline (XIII). 2-Chloro-4-bromo-5-ethylnitrobenzene (VI, 10.0 g, 0.038 mol) and 20 g of D-ribamine in 300 ml of pyridine were heated under refluxing conditions for 10 hr and then processed as described for 2-nitro-4-methyl-5-chloro-*N*-D-ribitylanil ine[2] to produce 5.6 g (38%) of XIII as orange needles: mp 180-181° (MeOH). *Anal.* ($C_{13}H_{19}BrN_2O_6$) C, H, Br, N.

2-Nitro-4-bromo-5-ethyl-*N*-D-ribitylaniline (XIV). 2-Chloro-4-ethyl-5-bromonitrobenzene (XII) was allowed to react with D-ribamine as described for VI. The product was processed as described for XIII to yield 6.15 g (43%) of XIV as orange crystals: mp 161-162° (MeOH). *Anal.* ($C_{13}H_{19}BrN_2O_6$) C, H, Br, N.

7-Ethyl-8-bromo-10-(1'-D-ribityl)isoalloxazine (XV). 2-Nitro-4-ethyl-5-bromo-*N*-D-ribitylaniline (XIII, 4.500 g, 0.0119 mol) and approximately 5 g of Raney nickel in 350 ml of EtOH were hydrogenated at room temperature at approximately 4.2 kg/cm² for 2 hr. The catalyst was removed and the solution evaporated to dryness *in vacuo*. A hot solution of 2.4 g of alloxan and 4.4 g of boric acid dissolved in 225 ml of HOAc was added to the *o*-phenylenediamine solution. The solution was shaken at 50° for 0.5 hr and then stored in the dark for 3 days. The reaction mixture (including the precipitate) was evaporated to dryness *in vacuo*. Absolute EtOH, 100 ml, was added and evaporated; this treatment was repeated three times. The product was suspended in 50 ml of H_2O, collected on a filter, and dried. The product was dissolved in 40 ml of hot concentrated HCl, filtered, and then diluted with 80 ml of H_2O. When cooled, the product was collected and recrystallized from the minimum amount of 2% HOAc solution to yield 3.30 g (62%) of yellow needles: mp 253-254° dec (uncor); λ_{max} (H_2O) 268 nm (ϵ 31,850), 365 (9920), 443 (12,300);

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-His[2]-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-Arg[8]-LRF,[4] des-Trp[3]-LRF,[5] and des-Leu[7]-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.