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Pseudosymmetry in the Structure of Luteinizing Hormone-Releasing Hormone Studies on a Series of Novel Analogs

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Pseudosymmetry in the LH-RH structure is described. Eleven analogs of LH-RH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) have been synthesized by the fragment condensation method and the repetitive excess mixed anhydride method. Multiple substitutions have been made in the LH-RH sequence, which retain the pseudosymmetry of the LH-RH molecule, while presenting fewer problems of synthesis than the corresponding residues in the natural decapeptide. Thus Trp^3, Ser^4, Tyr^5, Gly^6, Leu^7, and Arg^8 residues were replaced by amino acids having similar properties to the residues that they replace. In all but one of the peptides the Gly^{10}-NH_2 residue was replaced by ethylamide, while in the remaining peptide, 1-methyl-5-aminomethyltetrazole (AMT-Me) was substituted at position 10. The compounds were assayed *in vitro* and *in vivo*. The following analogs had *in vivo* and *in vitro* activities in the range 1–28% relative to LH-RH: I, <Glu-His-Phe-Ala-Tyr-Gly-Leu-Arg-Pro-NHEt; II, <Glu-His-Phe-Gly-Tyr-Gly-Leu-Arg-Pro-NHEt; VII, <Glu-His-Phe-Ala-Tyr-Gly-Phe-Arg-Pro-NHEt; IX, <Glu-His-Phe-Ala-Tyr-D-Ala-Leu-Arg-Pro-NHEt; XI, <Glu-His-Phe-Gly-Tyr-Gly-Leu-Arg-Pro-AMT-Me.

Following the isolation[1] and characterization[2] of luteinizing hormone-releasing hormone (LH-RH) as the decapeptide amide, <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, many analogs have been synthesized and assayed to evaluate structure–activity relationships.[3] Recently we have observed a pseudosymmetry apparent in the LH-RH

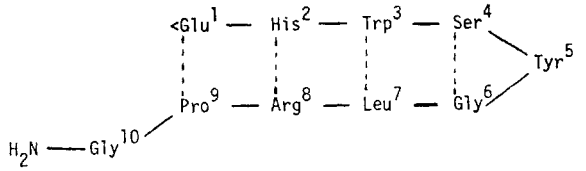


Figure 1. The sequence of LH-RH showing the symmetrical amino acid pairings.

molecule and have related the presence or absence of pseudosymmetry with the reported *in vitro* activities of a number of LH-RH analogs.

Symmetry in the LH-RH Molecule. When the amino acid sequence of the LH-RH molecule is written in the form shown in Figure 1, it is seen that, with the exception of Gly^{10}-NH_2, the various residues are distributed in pairs symmetrically about the tyrosine residue. The amino acids in each pair have similar properties, which distinguish them from the members of other pairs (see Table I).

It is not inconceivable that the activity of LH-RH is related to this marked pseudosymmetry and that the symmetrical structure has evolved in order to optimize the complementarity between the hormone and its receptor site. One would therefore anticipate that the substitution of paired amino acids by other natural amino acids having similar characteristics would lead to a retention of LH-releasing activity. Conversely, substitution by residues of markedly different character would lead to a loss of activity, since such residues would be unable to participate fully in interactions with the receptor.

A detailed analysis of the structure–activity relationships of LH-RH analogs, in which *single substitutions* by common L-amino acids have been made, was carried out. No variant in this group is more active than LH-RH itself. However, those peptides which retain almost complete activity have substitutions by structurally similar amino acids, *e.g.*, $Tyr^5 \rightarrow$ Phe; $Leu^7 \rightarrow$ Ile; $Leu^7 \rightarrow$ Val, and hence they retain the observed pseudosymmetry.

We now wish to describe a series of novel analogs of LH-RH, designed for two specific reasons. The first, to test the concept of pseudosymmetry in the LH-RH molecule further, by making multiple substitutions with amino acid residues having similar characteristics to those that they replace. The second, to produce analogs which, while still retaining a practical level of activity, present fewer problems synthetically (Table II).

Synthesis. Analogs I–VI and XI were synthesized by the stepwise assembly of partial structures as suitably protected peptides, followed by fragment condensation and final deprotection. The preparation of analog I is outlined in Scheme I.

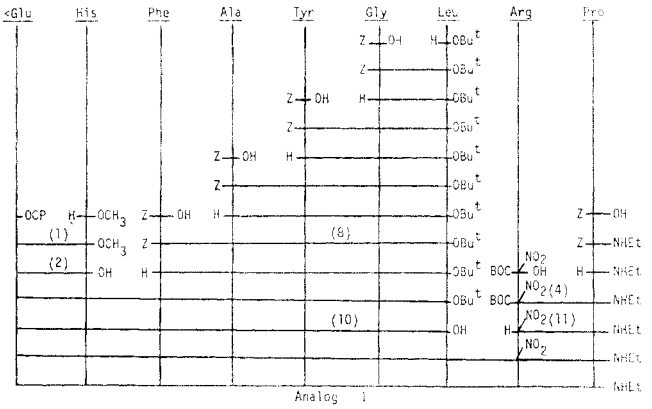
The dipeptide **2**, in which the imidazole ring of histidine was left unprotected, was obtained by the careful saponification of the corresponding methyl ester **1**. Synthesis of the pentapeptide **8** proceeded by the conventional solution method using activated esters of the appropriate benzyloxycarbonyl amino acids. The coupling of **2** and deprotected **8** was mediated by dicyclohexylcarbodiimide (DCC) in the presence of hydroxybenzotriazole[5] (HOBT). Reaction between Boc-Arg-(NO_2)-OH and H-Pro-NHEt (DCC coupling) was accompanied by the formation of the lactam of Boc-Arg(NO_2)-OH, and purification of the intermediate protected dipeptide **4** was necessary. Coupling heptapeptide **10** with **11**, again by the DCC–HOBT procedure, followed by removal of the remaining protecting groups by prolonged hydrogenolysis gave the crude analog I. This was first purified by column chromatography on silica gel and then by gradient elution chromatography on carboxymethylcellulose. The product obtained in this way was chromatographically pure and had the correct ratios of amino acids. Analogs II–IV and XI were prepared by analogous methods, details of which are to be found in the Experimental Section.

Table I. Characteristics of the Amino Acid Pairs in LH-RH (see Figure 1)[a]

Pair	General features
Ser^4, Gly^6	Small side chain, hydrophilic and neutral character
Trp^3, Leu^7	Large side chain, hydrophobic and neutral character
His^2, Arg^8	Basic side chain, hydrophilic character
Glu^1, Pro^9	Five-membered ring containing two main-chain atoms; character intermediate and neutral

[a] The Tyr^5- and Gly^{10}-NH_2 residues have no formal partners.

Scheme I



The homoarginine[8] peptides V and VI were prepared by the direct guanylation of analogs III and IV by reaction with 1-guanyl-3,5-dimethylpyrazole nitrate.[6] Gradient elution chromatography on carboxymethylcellulose was used to separate the products from unchanged lysine[8] peptides.

The syntheses of analogs VII–X all proceeded from the common precursor Boc-Arg(NO_2)-Pro-NHEt (**4**). In each case, the next five coupling steps were carried out using the repetitive excess mixed anhydride (REMA) procedure described by Tilak[7] and by Beyerman.[8,9] This technique was highly satisfactory, giving pure intermediates in high yields at each stage. The simple work-up procedure after each coupling allowed the synthesis to proceed rapidly and without complications. Each intermediate step was checked for purity by tlc, but crystallization and subsequent characterization of the intermediate peptides were not undertaken in the interests of high yields and speed. Beyerman[9] reports duplicate syntheses of a pentapeptide by the REMA method, with and without purification of intermediates. He found the product of each synthesis to be identical according to melting point, optical rotation, and chromatographic behavior. We regularly obtained coupling yields of 85–95% according to the precise nature of the work-up procedure used. The tyrosine residue in each of these analogs was incorporated as Boc-Tyr(Bzl)-OH, and careful washing was required to remove the excess of this compound from the isolated peptide. The heptapeptides corresponding to residues **3–9** of analogs VII–X were then each coupled with <Glu-His-OH by DCC–HOBT mediated reactions. Despite the use of a 20% excess of <Glu-His-OH, difficulty was experienced in obtaining complete coupling with each of the four heptapeptides. However, the purification of the products was readily achieved by gradient elution chromatogra-

Table II. Structure and Activities of LH-RH Analogs

	Residue no.										Activities *in vivo*[c]		Activities *in vitro*,[e]
	1	2	3	4	5	6	7	8	9	10	ED_{50}, μg/hamster	Rel potency, %	rel potency, %
LH-RH	<Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2	0.31	100	100
I	<Glu	His	*Phe*	*Ala*	Tyr	Gly	Leu	Arg	Pro	*NHEt*	4.14	7.5	6.04 (3.35–10.89)
II	<Glu	His	*Phe*	*Gly*	Tyr	Gly	Leu	Arg	Pro	*NHEt*	6.10	5.1	2.81 (1.51–5.24)
III	<Glu	His	*Phe*	*Ala*	Tyr	Gly	Leu	*Lys*	Pro	*NHEt*	56.87	0.6	0.12 (0.031–0.45)
IV	<Glu	His	*Phe*	*Gly*	Tyr	Gly	Leu	*Lys*	Pro	*NHEt*	*d*		0.091 (0.025–0.33)
V	<Glu	His	*Phe*	*Gly*	Tyr	Gly	Leu	*Har*[a]	Pro	*NHEt*	*d*		0.0013 (0.00086–0.0018)
VI	<Glu	His	*Phe*	*Ala*	Tyr	Gly	Leu	*Har*[a]	Pro	*NHEt*	*d*		0.0019 (0.0014–0.0027)
VII	<Glu	His	*Phe*	*Ala*	Tyr	Gly	*Phe*	Arg	Pro	*NHEt*	3.82	8.1	1.59 (1.00–2.53)
VIII	<Glu	His	*Tyr*	*Ala*	*Phe*	Gly	Leu	Arg	Pro	*NHEt*	161.13	0.2	0.24 (0.14–0.41)
IX	<Glu	His	*Phe*	*Ala*	Tyr	D-*Ala*	Leu	Arg	Pro	*NHEt*	1.10	28.3	2.13 (1.34–3.40)
X	<Glu	His	*Phe*	D-*Ala*	Tyr	Gly	Leu	Arg	Pro	*NHEt*	100.89	0.3	0.24 (0.14–0.42)
XI	<Glu	His	*Phe*	*Gly*	Tyr	Gly	Leu	Arg	Pro	*AMT-Me*[b]	22.29	1.4	1.18 (0.56–2.48)

[a]Har = homoarginine. [b]AMT-Me = 1-methyl-5-aminomethyltetrazole. [c]The data, analyzed by the parallel line probit method, showed no significant nonparallelism. Although there was some departure from linearity this has been allowed for in the error. The data were derived from two to four different dosages (4–6 hamsters each). The approximate standard error of the log ED_{50}'s was ±0.19, corresponding to p_{95} limits of 40–240%. [d]Inactive at 128 μg per hamster. [e]95% confidence limits are shown in parentheses. The data were derived by the method of parallel line assay and combination of estimates.[4]

phy, after the removal of the remaining protecting groups by hydrogenation.

Biological Testing. LH Release *in Vitro.* The release of LH from anterior pituitaries of normal male rats (250 g) was determined using a modification of the incubation procedure of Mittler and Meites.[10] It was found desirable to increase the preincubation period to 2 hr with three changes of medium (TC 199) to ensure a steady base line. The length of the test incubation was 1 hr, after which the incubation medium was collected, snap-frozen, and stored at −20°. The medium was assayed for LH radioimmunologically with reagents provided by the NIH Pituitary Hormone Program (NIAMD, rat LH-RPI; NIAMD, anti-rat LH serum S2; and NIAMD, rat LH-I-1). All glassware was autoclaved and surfaces exposed to peptide solutions were siliconed. Peptide solutions were protected from light. A comprehensive description of this procedure will be published elsewhere.

LH Release *in Vivo.* The hamster ovulation test of Arimura, *et al.*,[11] was used. Mature female hamsters (90–120 g) were maintained in individual cages under controlled lighting conditions (lights on at 03.00 and off at 17.00) with food and water *ad libitum.*

An ip injection of 13 mg/100 g of body weight phenobarbitone sodium (1 ml of 0.9% saline/100 g for controls) was given at 11.30 on the day of prooestrous, and LH-RH or analog or saline was given sc in a volume of 0.25 ml at 13.30 and 14.30.

After killing the hamsters the next morning the oviducts were removed and the ova counted.

The ED_{50} of LH-RH was 0.31 μg/hamster, and the activities of the analogs shown in Table II are related to this dose.

Results and Discussion

Fujino, *et al.*,[12,13] have shown that the replacement of Gly^{10}-NH_2 by a suitable alkylamine does not decrease the binding affinity of the hormone, and, as is indicated in Table I, the Gly^{10}-NH_2 residue has no formal partner. Therefore, as a first step toward simplification it was decided to incorporate this modification in our series of analogs. In analog I, a further variation of the structure was obtained by replacing Trp^3 by Phe. The choice of Phe was based on the fact that it has a large neutral aromatic side chain of hydrophobic character, a feature which is also present in Trp. Thus the pairing of residues **3** and **7** is maintained. Tryptophan is readily oxidized, particularly in the acid media commonly used in peptide synthesis for the removal of protecting groups, and colored by-products are produced, for example, during the work-up of the reaction mixture after palladium-catalyzed hydrogenation in acid media.[14] Thus, its replacement has synthetic advantages. Also in analog I, the Ser^4 residue has been replaced by alanine. Thus the preparations of *O*-benzylserine, which necessitates a resolution,[15,16] and of an N-protected *O*-benzylserine as an acylating agent are avoided. Furthermore, the subsequent deblocking of the hydroxyl group is avoided. We have previously experienced some difficulty in removing the benzyl group from serine residues by hydrogenolysis. Thus in analog I the synthesis has been simplified by introducing three modifications, and in two of these the concept of pseudosymmetry was applied. As can be seen from the data in Table II these replacements have resulted in a structure which retains LH-releasing activity both *in vivo* and *in vitro.*

The replacement of Ala^4 in analog I by Gly (analog II) caused slight reductions in the levels of *in vivo* and *in vitro* activities. Although analog II has a higher degree of symmetry than I, its lower level of activity may indicate that there is a minimum size requirement for the residue in position 4.

To test the concept of symmetry still further, the structures of analogs I and II were modified by replacement of the Arg^8 residue by Lys. The choice of Lys (large, basic hydrophilic side chain) allowed the pairing of residues **2** and **8** to be maintained. However, this substitution provided comparatively low activities for the analogs III and IV. Subsequently, the guanidine function was reintroduced into the structure by guanylation[17] of both III and IV to yield the two corresponding homoarginine[8] peptides V and

VI. However, homologation did not restore activity to the levels observed in I and II. The low levels of LH release recorded for analogs III–VI are in general agreement with the results obtained by other workers[18–20] for LH-RH analogs substituted in position 8, and they emphasize the importance of the precise nature and dimensions of the basic side chain in this position for optimum hormone-receptor binding.

To establish the effect of the replacement of Leu^7 in analog I by Phe, analog VII was synthesized. In this peptide a very high degree of symmetry is maintained by virtue of the fact that positions 3 and 7 are both occupied by Phe. Its activity *in vivo* is of the same order as that of analog I. Thus in analog VII (<Glu-His-Phe-Ala-Tyr-Gly-Phe-Arg-Pro-NHEt), the LH-RH molecule has been modified in four positions, with a retention of activity which is 8% that of the natural decapeptide.

Further manipulations based on the structure of analog I were tried. The Phe^3 and Tyr^5 residues were transposed to yield analog VIII. However, this resulted in a very considerable decrease in the level of activity. This is unlikely to reflect the importance of a phenolic function at position 5, for maximum binding of the hormone at the receptor site, because of the high activity of [Phe^5]-LH-RH.[21,22] It is more likely to indicate the undesirability of a hydrophilic or sterically hindering group at position 3.

The replacement of the Gly^6 residue in LH-RH by D-Ala has been recently reported by Monahan, *et al.*[23] This conformational change resulted in a 350–450% increase in releasing activity, the corresponding L-Ala^6 peptide having only 4% the activity of LH-RH. The authors state that *in vivo* and *in vitro* tests are in agreement, and hence they argue that the wide differences in activity are not due to difference in rates of clearance or serum inactivation. They suggest that the results are best interpreted as a change in conformation leading to an increased binding affinity due to the stabilization of a conformation favorable for binding. As a result of these interesting observations, it was considered desirable to incorporate D-Ala in position 6 of the active analog I, giving structure IX.

This substitution resulted in a fourfold increase in the *in vivo* activity over that of I, whereas the *in vitro* activity decreased slightly. Thus, these results suggest that the increase in activity arising from the incorporation of D-Ala may not be caused by increased binding affinity due to a favorable conformation but rather that the D-Ala^6 residue increases the resistance of the peptide to enzymic degradation, and this fact is reflected in the relatively high activity in the *in vivo* test system. Chymotryptic digestion of LH-RH has been shown to result in the cleavage of the Tyr-Gly bond, liberating the C-terminal fragment Gly-Leu-Arg-Pro-Gly-NH_2.[24] Furthermore, when the pairing of the residues in positions 4 and 6 is considered, it is seen that the degree of pseudosymmetry in IX is higher than that of I, thus supporting the concept that pseudosymmetry is an important feature of the hormone–receptor interaction.

In an earlier series of peptides related to LH-RH (unpublished), we had observed that when the terminal Gly^{10}-NH_2 group in LH-RH was replaced by 5-aminomethyltetrazole, activity was completely lost. The acidic nature of the tetrazole function probably affects the ionic interactions between the hormone and receptor site. In view of these observations analog XI was synthesized, in which the neutral 1-methyl-5-aminomethyltetrazole occupied the C-terminal position. Such a derivative might exhibit prolongation of *in vivo* activity due to a possible increased resistance to enzymatic degradation. The *in vivo* activity, as measured by the hamster ovulation test, was decreased by a factor of about 4. The reduction in the *in vivo* activity may indicate that the methyltetrazole residue is, in fact, split off more readily than the ethylamide group, leading to inactivation.

Conclusions

This work has indicated that it is possible to design analogs of LH-RH in which multiple substitutions of amino acids have been made, which retain useful levels of LH-releasing activity, maintain the pseudosymmetry of the molecule, and provide considerable advantages synthetically.

Experimental Section

The purity of intermediates and products was checked by tlc on silica gel G plates. The solvent systems employed were S1, $CHCl_3$–MeOH–0.880 ammonia (60:45:20); S2, $CHCl_3$–MeOH–32% aqueous acetic acid (60:45:20); S3, *n*-BuOH–AcOH–H_2O–EtOAc (1:1:1:1); S4, $CHCl_3$–MeOH (8:2). The amino acid compositions of acid hydrolysates (6 *N* HCl at 110° for 24 hr in evacuated sealed tubes) were determined with a Beckman-Spinco Model 120C amino acid analyzer. No corrections were made for the decomposition of tyrosine. Optical rotations were determined on a Bendix-N.P.L. automatic polarimeter. Evaporations were carried out with a rotary evaporator.

Synthesis of Intermediate Peptides. <Glu-His-OMe (1). His-OMe · HCl (9.7 g, 40 mmol) was dissolved in 80% aqueous methanol (100 ml) and stirred with Dowex 2 X8 (OH^-) resin (100 ml) until Cl^- could no longer be detected in the supernatant. The resin was filtered and washed with methanol and the combined filtrates were concentrated *in vacuo*. The residual oil, after reevaporation twice from ethanol, was dissolved in redistilled dimethylformamide (DMF, 50 ml). <Glu-OCP (12.35 g, 40 mmol) was added and the reaction mixture was stirred at room temperature for 24 hr. After removal of solvent, the residue was crystallized from MeOH–Et_2O to give colorless prisms: 7.7 g (70%); mp 205–206°; $[\alpha]^{26.5}D$ −50.84° (*c* 1.0, AcOH). *Anal.* ($C_{12}H_{16}N_4O_4$) C, H, N.

<Glu-His-OH (2). The methyl ester 1 (8.24 g) was dissolved in 2 *N* NaOH (15 ml) and after 1 hr at 20° the solution was neutralized with 1 *N* HCl and concentrated *in vacuo*. The residue was extracted with hot DMF, filtered to remove sodium chloride, and concentrated *in vacuo*. Crystallization from MeOH gave **2** as colorless prisms: 7.5 g (96%); mp 213–215° dec; $[\alpha]^{28}D$ +10.5° (*c* 1.0, AcOH). *Anal.* ($C_{11}H_{14}N_4O_4$) C, H, N.

Z-Pro-NHEt (3). The dicyclohexylamine salt of Z-Pro-OH (43.0 g, 0.1 mol) was suspended in EtOAc (500 ml) and stirred vigorously with a solution of $KHSO_4$ (20.4 g) in 250 ml of H_2O. When all of the solid had dissolved the layers were separated and the aqueous layer was washed once with EtOAc (250 ml). The combined organic phases were washed with H_2O (2 × 100 ml), dried, and concentrated *in vacuo*. The residual oil in $CHCl_3$ (200 ml) was treated with Et_3N (13.8 ml) and cooled to −10°, and isobutyl chloroformate (13.65 g, 0.1 mol) was added dropwise. After 30 min a precooled solution of ethylamine (9.02 g, 0.2 mol) in $CHCl_3$ (100 ml) was added over 10 min. The mixture was stirred at room temperature overnight. After evaporation of solvent the residue was partitioned between EtOAc (300 ml) and water (100 ml). The EtOAc layer was separated and washed with 5% Na_2CO_3 solution (50 ml), 5% citric acid solution (50 ml), and water (50 ml). After drying and removal of the solvent the product was crystallized from EtOAc–petroleum ether to give **3**: 19.31 g (70%); mp 104–106°. *Anal.* ($C_{15}H_{20}N_2O_3$) C, H, N.

Boc-Arg(NO_2)-Pro-NHEt (4). H-Pro-NHEt was obtained from 3 (9.10 g, 33 mmol) by hydrogenation in MeOH (200 ml) in the presence of 10% palladium on charcoal catalyst (1.5 g). After 3.5 hr the catalyst was removed by filtration and the solution evaporated *in vacuo*. The residual oil was reevaporated twice from CH_3CN. Boc-Arg(NO_2)-OH (10.55 g, 33 mmol) was dissolved in DMF (20 ml) and diluted with redistilled CH_3CN (200 ml), and the solution was cooled to −10°. Separate solutions of the H-Pro-NHEt in DMF (5 ml) and CH_3CN (45 ml) and dicyclohexylcarbodiimide (DCC, 6.80 g) in CH_3CN (25 ml) were added in five equal portions at 0.5-hr intervals, the temperature being maintained at −10° throughout the additions. The mixture was then stirred at 4° overnight. After removal of dicyclohexylurea (DCU) and evaporation of solvents, the residue was dissolved in $CHCl_3$ (400 ml) and washed with 5% Na_2CO_3 solution (3 × 60 ml), 5% citric acid solution (3 × 60 ml), and finally saturated NaCl solution (2 × 60 ml). Removal of the $CHCl_3$ and trituration of the residue with ether and petroleum ether yielded 13 g of a mixture of the protected di-

peptide and the lactam of Boc-Arg(NO_2)-OH. This mixture was dissolved in EtOAc (150 ml) and extracted thoroughly with H_2O (5 × 150 ml). The combined aqueous extracts were back-washed once with EtOAc, concentrated *in vacuo* to reduce the volume, and then freeze-dried. The material obtained in this way was dried *in vacuo* over P_2O_5 at 55°: yield 6.25 g (43%); $[\alpha]^{23}D$ −52.5° (*c* 1, AcOH). Tlc gave a single spot at R_f^{S4} 0.58 (lactam R_f^{S4} = 0.71). *Anal.* ($C_{18}H_{33}N_7O_6$) C, H; N: calcd, 22.12; found, 21.66. Peptides containing Arg(NO_2) have been reported to give low N values.[25]

Z-Pro-AMT-Me (5). 1-Methyl-5-aminomethyltetrazole hydrochloride (2.99 g, 20 mmol) was suspended in DMF (20 ml) and cooled in ice. Et_3N (2.76 ml) was added and the mixture was stirred in ice for 10 min before filtering off the $Et_3N \cdot HCl$. Z-Pro-ONp (8.14 g, 22 mmol) was then added and the reaction mixture was stirred at room temperature for 24 hr. After removing the solvent the residue was dissolved in EtOAc (100 ml) and treated with dimethylaminopropylamine (0.6 ml) for 1 hr. The EtOAc solution was washed with 5% citric acid solution (2 × 25 ml), 5% Na_2CO_3 solution (2 × 20 ml), and water (2 × 25 ml). After drying and evaporation *in vacuo*, a residue was obtained which crystallized from EtOAc–Et_2O: yield 4.91 g (71%); mp 91–92°; $[\alpha]^{25}D$ −51.5° (*c* 1, MeOH). *Anal.* ($C_{16}H_{20}N_6O_3$) C, H, N.

Boc-Arg(NO_2)-Pro-AMT-Me (6). Compound **5** (2.40 g, 7 mmol) was hydrogenated in MeOH (50 ml) in the presence of 10% palladium charcoal (0.5 g). After the usual work-up, the resulting oil, together with Boc-Arg(NO_2)-OH (2.24 g, 7 mmol), was dissolved in DMF (15 ml). The solution was cooled to −10° and DCC (1.44 g, 7 mmol) was added. The reaction mixture was stirred at 4° for 24 hr, filtered to remove DCU, and concentrated *in vacuo*. The oil was dissolved in $CHCl_3$ (100 ml) and washed with 5% Na_2CO_3 (2 × 15 ml), 5% citric acid (2 × 15 ml), and finally saturated NaCl solution (2 × 15 ml). The $CHCl_3$ was removed *in vacuo* to give an oil. Tlc showed the presence of a second component which corresponded to the lactam of Boc-Arg(NO_2)-OH (see above). Therefore the crude product was taken up in EtOAc (60 ml) and extracted with water (5 × 40 ml). The combined aqueous extracts were backwashed once with EtOAc, concentrated *in vacuo* to reduce the volume, and then freeze-dried to afford **6**: 890 mg (25.5%); $[\alpha]^{25}D$ −44.68° (*c* 1, MeOH). *Anal.* ($C_{18}H_{33}N_{11}O_6$) C, H; N: calcd, 30.86; found, 29.06.[25]

Z-Lys(Boc)-Pro-NHEt (7). Compound **3** (2.76 g, 10 mmol) was hydrogenated as described in the preparation of **4**, and the resulting H-Pro-NHEt was coupled with Z-Lys(Boc)-OCP (6.15 g, 11 mmol) in DMF (20 ml). After 24 hr at room temperature the DMF was removed *in vacuo* and the oil dissolved in EtOAc (20 ml) and treated with dimethylaminopropylamine (0.5 ml). The solution was diluted to 200 ml with EtOAc and washed with 5% citric acid solution (2 × 50 ml), 5% Na_2CO_3 solution (2 × 50 ml), and H_2O (2 × 50 ml). The oil obtained by evaporation of the EtOAc crystallized on trituration with ether: yield 2.45 g (49%); mp 72–75°; $[\alpha]^{25}D$ −53.57° (*c* 1, MeOH); tlc pure in S3. *Anal.* ($C_{26}H_{40}N_4O_6$) C, H, N.

Z-Phe-Ala-Tyr-Gly-Leu-*t*-OBu (8). Z-Gly-OH (26.1 g, 0.125 mol) and H-Leu-*t*-OBu (23.4 g, 0.125 mol) were dissolved in a mixture of CH_2Cl_2 (250 ml) and DMF (50 ml). The solution was cooled in ice–salt and treated with DCC (25.75 g, 0.125 mol) and the reaction mixture stirred overnight at 4°. After filtration the solvent was removed *in vacuo* and the residual oil dissolved in ether. A further quantity of DCU was filtered off and the solution reevaporated to dryness. The resulting oil solidified on trituration with water yielding 44.4 g (94%) of Z-Gly-Leu-*t*-OBu, mp 40–43°. This compound (22.0 g, 58 mmol) was hydrogenated in MeOH (250 ml) in the presence of 10% palladium-on-charcoal catalyst (3 g). After the usual work-up the resulting oil and Z-Tyr-OH (21.28 g) were coupled in CH_2Cl_2 (150 ml) in the presence of DCC (13.9 g). Filtration followed by evaporation of the solvent gave an oil which was dissolved in EtOAc and washed with 5% Na_2CO_3 solution, 5% citric acid solution, and water. After drying over $MgSO_4$ and concentration, the product was solidified by trituration with petroleum ether to give **Z-Tyr-Gly-Leu-*t*-OBu** (25.2 g, 69%). Tlc in S1 gave a single spot, positive to Pauly reagent. This protected tripeptide (25.2 g, 46.6 mmol) was hydrogenated and then coupled in CH_2Cl_2 (150 ml) with Z-Ala-OH (10.4 g, 46.6 mmol) in the presence of DCC (9.60 g, 46.6 mmol). After stirring overnight at 4° the reaction mixture was worked up as described above to yield **Z-Ala-Tyr-Gly-Leu-*t*-OBu** (25.9 g, 91%): tlc pure in S3. The protected tetrapeptide (25.9 g, 42.4 mmol) was hydrogenated and then coupled with Z-Phe-OH (12.69 g, 42.4 mmol) in CH_2Cl_2 (200 ml) in the presence of DCC (8.74 g, 42.4 mmol). The product obtained by the usual work-up procedure crystallized from 2-propanol and diisopropyl ether to give **8**: 23.7 g (74%); mp 157–160°; $[\alpha]^{26}D$ −40.09° (*c* 1, MeOH); tlc, single Pauly positive spot in S3 and S4. *Anal.* ($C_{41}H_{53}O_9N_5$) C, H, N.

Z-Phe-Gly-Tyr-Gly-Leu-*t*-OBu (9). H-Tyr-Gly-Leu-*t*-OBu (3.95 g, 9.7 mmol) was dissolved in DMF (25 ml) and allowed to react with Z-Gly-OCP (3.77 g, 9.7 mmol) at 4° for 60 hr. After removal of the DMF *in vacuo*, the residue was dissolved in EtOAc (50 ml) and treated with dimethylaminopropylamine (0.5 ml) for 30 min at room temperature. The solution was washed with 5% citric acid solution (2 × 50 ml), 5% Na_2CO_3 solution (2 × 50 ml), and water (2 × 50 ml). After drying and removal of solvent the product was obtained as a gum. Tlc (S1 and S4) revealed the presence of some trichlorophenol. The gum was taken up in MeOH (100 ml) and hydrogenated in the presence of 10% Pd/C (0.5 g). The deprotected tetrapeptide was distributed between 1% acetic acid (150 ml) and ether (100 ml). The aqueous layer was separated off and washed with ether (100 ml) and then EtOAc (75 ml). After partial evaporation *in vacuo* the aqueous layer was freeze-dried to give **H-Gly-Tyr-Gly-Leu-*t*-OBu** (3.87 g, 86%): tlc pure in S1 and S4. This partially deprotected tetrapeptide (3.73 g, 8.05 mmol) was coupled with Z-Phe-OCP (3.86 g, 8.05 mmol) in the way described above. The crude product obtained after removal of DMF was dissolved in EtOAc and treated with dimethylaminopropylamine (0.5 ml), and the solution was washed with 5% citric acid and water. After drying and removal of solvent, the residue was crystallized from EtOAc–Et_2O to give **9**: 2.05 g (34%); mp 188–189°; $[\alpha]^{25}D$ −10.0° (*c* 1, MeOH); tlc pure in S3 and S4 (Pauly positive). *Anal.* ($C_{40}H_{51}N_5O_9$) H, N; C: calcd, 64.4; found, 63.73.

Synthesis of the LH-RH Analogs. <Glu-His-Phe-Ala-Tyr-Gly-Leu-Arg-Pro-NHEt (Analog I). Compound **8** (2.31 g, 3.04 mmol) was hydrogenated in MeOH (50 ml) with 10% Pd/C catalyst (0.3 g). After 3 hr the catalyst was removed and the MeOH evaporated *in vacuo*. The residue was evaporated twice from benzene and then dissolved in DMF (15 ml). A solution of <Glu-His-OH (**2**, 810 mg) in water (6.5 ml) was added, together with HOBT (410 mg) in DMF (4.5 ml). A further 5 ml of DMF was added and the mixture was cooled in ice–salt. DCC (626 mg) in DMF (9 ml) was then added and the mixture was stirred at 4° for 72 hr. The reaction mixture was filtered and the filtrate concentrated to dryness. The residue was triturated with ether until solid, filtered, and washed thoroughly with $CHCl_3$. After drying the solid was washed with 5% Na_2CO_3 solution and finally with water. The dried product weighed 2.15 g. The heptapeptide ester (2.15 g) was suspended in redistilled anisole (14 ml) and treated with trifluoroacetic acid (40 ml) at room temperature for 1 hr. Concentration *in vacuo* and trituration with ether gave 2.35 g of crude product, which was purified by dry-column chromatography on silica using the solvent mixture $CHCl_3$–MeOH–0.880 ammonia (60:45:20). The yield of chromatographically pure **<Glu-His-Phe-Ala-Tyr-Gly-Leu-OH (10)** was 1.90 g (76% based on compound **8**). Boc-Arg(NO_2)-Pro-NHEt (**4**, 221 mg, 0.5 mol) was deprotected in 1 *N* HCl–AcOH (7.5 ml) and the product obtained dissolved in DMF (3 ml), cooled in ice, and treated with Et_3N (0.075 ml). After 10 min the mixture was filtered and the filtrate evaporated *in vacuo* to remove the excess Et_3N. The residue was redissolved in DMF (8 ml) together with compound **10** (409 mg, 0.5 mmol) and HOBT (67.5 mg). The mixture was cooled to −10°, treated with DCC (103 mg) in DMF (2 ml), and stirred at 4° for 72 hr. The crude material obtained after removal of DCU and evaporation of the DMF was dissolved in a mixture of MeOH (10 ml), water (2 ml), and AcOH (2 ml) and hydrogenated in the presence of Pd/C catalyst (250 mg) for 20 hr: yield 595 mg of crude I. This was chromatographed on a 3 × 40 cm dry column of silica, developed with the solvent system $CHCl_3$–MeOH–0.880 ammonia (60:45:20). Fractions corresponding to the major peak (positive reactions with Pauly and Sakaguchi reagents) were collected and evaporated to give 275 mg of partially purified material. This was dissolved in water (10 ml) and applied to a 2.5 × 30 cm column of carboxymethylcellulose. The column was eluted with pH 5.1 NH_4OAc buffer [first gradient, 0.005 *M* (500 ml)–0.2 *M* (500 ml); second gradient, 0.2 *M* (500 ml)–0.5 *M* (500 ml)]. A peak corresponding to the heptapeptide **10** eluted during the first gradient, and the main peak eluted during the second gradient. The appropriate fractions were pooled and lyophilized twice to remove the buffer. The yield of analog I was 150 mg (25%): tlc pure in S1, S2, and S3; Pauly and Sakaguchi reactions positive; $[\alpha]^{24}D$ −70.1° (*c* 1, 1% AcOH). Amino acid ratios after acid hydrolysis: Glu 1.08, His 1.02, Phe 1.00, Ala 1.02, Tyr 1.00, Gly 1.03 Leu 1.03, Arg 0.96, Pro 0.99; recovery 91%.

<Glu-His-Phe-Gly-Tyr-Gly-Leu-Arg-Pro-NHEt (Analog II). This compound was prepared by exactly the same procedure

as described for analog I. Thus <Glu-His-OH (1) was coupled with H-Phe-Gly-Tyr-Gly-Leu-*t*-OBu (from **9**) and the resulting heptapeptide ester deprotected and coupled with H-Arg(NO_2)-Pro-NHEt(4). After final deblocking, the crude peptide was purified first by silica gel column chromatography and then by gradient elution chromatography to yield 160 mg of analog II: tlc pure in S1, S2, and S3 (Pauly and Sakaguchi reagents positive); $[\alpha]^{24}D$ −50.2° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.04, His 0.99, Phe 0.97, Gly 2.01, Tyr 0.97, Leu 1.00, Arg 0.92, Pro 0.95; recovery 94%.

<Glu-His-Phe-Ala-Tyr-Gly-Leu-Lys-Pro-NHEt (Analog III). Compound **7** (252 mg, 0.5 mmol) was hydrogenated in MeOH (10 ml) in the presence of 10% Pd/C catalyst (50 mg). After 4 hr the catalyst was filtered off and the filtrate evaporated to dryness. The residue was reevaporated twice from benzene and then dissolved in DMF (5 ml). <Glu-His-Phe-Ala-Tyr-Gly-Leu-OH (409 mg, 0.5 mmol) (see under analog I) was added together with HOBT (67.5 mg). The solution was cooled to −10° and DCC (103 mg) was added. The reaction was allowed to proceed at 4° for 72 hr and then worked up in the usual way to give 650 mg of crude protected nonapeptide. The final deprotection was carried out in 1 *N* HCl–AcOH (10 ml) for 45 min at room temperature. The product was purified first by dry-column chromatography in the system $CHCl_3$–MeOH–0.880 ammonia (60:45:20) and then by gradient elution chromatography (0.005 *M*, 500 ml–0.2 *M*, 500 ml) on carboxymethylcellulose. The appropriate fractions were pooled and lyophilized twice to give 200 mg (34%) of analog III: tlc pure in S1, S2, and S3; $[\alpha]^{24}D$ −63.0° (*c* 0.33, 1% AcOH). Amino acid ratios: Glu 1.00, His 0.97, Phe 0.96, Ala 0.99, Tyr 0.96, Gly 1.04, Leu 1.00, Lys 0.98, Pro 0.93; recovery 90%.

<Glu-His-Phe-Gly-Tyr-Gly-Leu-Lys-Pro-NHEt (Analog IV). Compound **7** was hydrogenated and coupled with <Glu-His-Phe-Gly-Tyr-Gly-Leu-OH (see under analog II) in precisely the manner described for analog III: $[\alpha]^{24}D$ −50.8° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.03, His 1.02, Phe 0.98, Gly 2.03, Tyr 0.98, Leu 1.00, Lys 1.02, Pro 0.88; recovery 90%.

<Glu-His-Phe-Gly-Tyr-Gly-Leu-Har-Pro-NHEt (Analog V). 1-Guanyl-3,5-dimethylpyrazole nitrate[6] (24 mg) was dissolved in DMF (1 ml) and the pH adjusted to 9.5 by the addition of 10% Et_3N in DMF. A solution of IV (70 mg, 0.059 mmol) in DMF (1 ml) was added and the mixture was allowed to stand at room temperature for 4 days. The reaction mixture was evaporated to dryness and triturated with ether to give 85 mg of a mixture of IV and V. This mixture was dissolved in a small volume of water and applied to a column of carboxymethylcellulose. Elution by the two-gradient system described above resolved the two peptides: yield of V 20 mg; tlc single spot in S1, S2, and S3, positive to Pauly and Sakaguchi reagents. Unchanged starting material (30 mg) was also isolated (Sakaguchi reaction negative). Amino acid ratios: Glu 1.04, His 0.98, Phe 0.99, Gly 1.95, Tyr 0.98, Leu 1.00, Har 0.97, Pro 0.96 (lysine was absent from the hydrolysate); recovery 85%.

<Glu-His-Phe-Ala-Tyr-Gly-Leu-Har-Pro-NHEt (Analog VI). This was prepared from 70 mg of analog III in precisely the way described above: yield 28 mg; tlc pure in three systems (Pauly and Sakaguchi positive reactions). Amino acid ratios: Glu 1.13, His 0.96, Phe 0.98, Ala 1.01, Tyr 0.96, Gly 0.96, Leu 1.00, Har 0.99, Pro 0.98 (no lysine was detected in the hydrolysate).

<Glu-His-Phe-Ala-Tyr-Gly-Phe-Arg-Pro-NHEt (Analog VII). Compound **4** (2.215 g, 5 mmol) was deprotected in 1 *N* HCl–AcOH (30 ml) for 30 min at room temperature. The solvent was removed *in vacuo* at 35° and the residue solidified by trituration with ether. The deprotected peptide was collected by filtration, dried thoroughly over P_2O_5 and NaOH pellets, and then dissolved in DMF (15 ml). A solution of *N*-methylmorpholine (505 mg, 5 mmol) in DMF (2.5 ml) was added and the mixture cooled to −15°. The *mixed anhydride* was prepared by dissolving Boc-Phe-OH (1.99 g, 7.5 mmol) in tetrahydrofuran (THF, 10 ml) and adding a solution of *N*-methylmorpholine (755 mg, 7.5 mmol) in THF (2.5 ml). This mixture was cooled to −15° with stirring and a solution of isobutyl chloroformate (953 mg, 7.0 mmol) in THF (2.5 ml) was added. After 2 min at −15° the DMF solution of the amino component was added and the mixture stirred at −15° for 2.5 hr. The temperature was then raised to 0° and the excess mixed anhydride decomposed by the addition of 2 *M* $KHCO_3$ solution (7.5 ml). After 30 min stirring at 0°, the mixture was poured onto 50% saturated NaCl solution (200 ml) and the oily product extracted into EtOAc (2 × 150 ml). The combined EtOAc extracts were washed with 50% NaCl solution (40 ml) and then with water (40 ml). After drying over $MgSO_4$ and evaporation of the solvent, the product was solidified by trituration with ether to give Boc-Phe-Arg(NO_2)-Pro-NHEt, 2.50 g (85%). The purity of this material was confirmed by tlc in S1, S2, and S3. By an alternating sequence of deprotections and excess mixed anhydride couplings, carried out in exactly the manner described above, the following intermediate peptides were prepared in chromatographically pure form: **Boc-Gly-Phe-Arg(NO_2)-Pro-NHEt** (86.5% yield), **Boc-Tyr(Bzl)-Gly-Phe-Arg(NO_2)-Pro-NHEt** (84% yield), **Boc-Ala-Tyr(Bzl)-Gly-Phe-Arg(NO_2)-Pro-NHEt** (99% yield). The only variation in technique was in the method of work-up after decomposition of the excess mixed anhydride. In the cases of the hexa- and heptapeptides, the reaction mixture was poured onto water and the solid product filtered off, washed, and dried. The deprotected heptapeptide H-Phe-Ala-Tyr(Bzl)-Gly-Phe-Arg(NO_2)-Pro-NHEt · HCl (1.054 g, 1 mmol) was dissolved in DMF (10 ml) and neutralized with *N*-methylmorpholine (101 mg) in DMF (1 ml). Compound **2** (319 mg, 1.2 mmol) in water (4 ml) was added together with HOBT (162 mg, 1.2 mmol) in DMF (2.5 ml). DMF (6 ml) was added and the mixture was cooled to −15° before the addition of DCC (247 mg, 1.2 mmol). The reaction mixture was stirred at −15° for 2 hr and then at 4° for 48 hr. AcOH (0.1 ml) was added and the temperature raised to 20° for 30 min before filtering off the DCU. The filtrate was concentrated *in vacuo* and the residue triturated successively with ether, 5% Na_2CO_3 solution, and water. The weight of crude protected nonapeptide was 1.15 g. The side-chain protecting groups were removed by hydrogenation for 24 hr in MeOH (30 ml), AcOH (6 ml), and water (3 ml) in the presence of 10% Pd/C catalyst (0.5 g). The crude deprotected peptide was dissolved in 2% AcOH (50 ml), filtered to remove some DCU, and diluted to 100 ml with water before lyophilization to give 915 mg of crude VII. This material (500 mg) was purified on a 2.5 × 30 cm column of carboxymethylcellulose, eluting with the following gradients of NH_4OAc, pH 5.1: first gradient, 0.005 *M* (500 ml)–0.2 *M* (500 ml); second gradient, 0.2 *M* (500 ml)–0.5 *M* (500 ml). The required peptide eluted during the second gradient and was isolated by pooling the appropriate fractions and repeated lyophilization to remove NH_4OAc: yield 190 mg of VII; tlc pure in three systems; $[\alpha]^{24}D$ −44.5° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.03, His 0.97, Phe 2.00, Ala 0.95, Tyr 1.04, Gly 0.97, Arg 0.94, Pro 0.96; recovery 95%.

Analogs VIII, IX, and X were prepared from peptide **4** using the REMA method as far as the heptapeptide stage and then by DCC–HOBT coupling with dipeptide **2** precisely as has been detailed above for analog VII.

<Glu-His-Tyr-Ala-Phe-Gly-Leu-Arg-Pro-NHEt (analog VIII): tlc pure in three systems; $[\alpha]^{26}D$ −60.7° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.05, His 1.03, Tyr 0.99, Ala 1.02, Phe 1.00 Gly 1.07, Leu 1.00, Arg 0.94, Pro 1.02; recovery 95%.

<Glu-His-Phe-Ala-Tyr-D-Ala-Leu-Arg-Pro-NHEt (analog IX): tlc pure in three systems; $[\alpha]^{28}D$ −51.8° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.02, His 0.95, Phe 0.95, Ala 2.00, Tyr 0.91, Leu 1.00, Arg 0.90, Pro 0.96; recovery 103%.

<Glu-His-Phe-D-Ala-Tyr-Gly-Leu-Arg-Pro-NHEt (analog X): tlc pure in three systems: $[\alpha]^{27}D$ −36.5° (*c* 1, 1% AcOH). Amino acid ratios: Glu 1.02, His 0.99, Phe 0.96, Ala 0.98, Tyr 0.95, Gly 0.98, Leu 1.00, Arg 0.93, Pro 0.99; recovery 100%.

<Glu-His-Phe-Gly-Tyr-Gly-Leu-Arg-Pro-AMT-Me (Analog XI). Compound **6** (337 mg, 0.67 mmol) was deprotected in 1 *N* HCl–AcOH (10 ml) and the isolated product was taken up in DMF (5 ml), cooled in ice, and treated with an excess of Et_3N (0.18 ml). After 10 min the salt was filtered off and the filtrate concentrated *in vacuo* to remove the excess Et_3N. <Glu-His-Phe-Gly-Tyr-Gly-Leu-OH (482 mg, 0.6 mmol) (prepared as described under analog II) was dissolved in DMF (5 ml) and added to the DMF solution of H-Arg(NO_2)-Pro-AMT-Me. The solution was cooled in an ice–salt mixture and treated with HOBT (89 mg), followed by DCC (136 mg). The mixture was stirred at 4° for 48 hr and filtered and the filtrate concentrated to dryness. The weight of crude product obtained after trituration with ether was 805 mg. This material was hydrogenated in a mixture of MeOH (10 ml), AcOH (2 ml), and water (2 ml) in the presence of 10% Pd/C (260 mg). The resulting deprotected peptide was purified by gradient elution chromatography on carboxymethylcellulose exactly as described for analog I: yield 180 mg; tlc pure in S1, S2, and S3 (Pauly and Sakaguchi positive); $[\alpha]^{24}D$ −46.9° (*c* 1, 1% AcOH). Amino acid ratios: Glu 0.97, His 1.03, Phe 1.00, Gly 2.09, Tyr 0.97, Leu 1.02, Arg 0.95, Pro 0.93; recovery 83%.

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Adrenocorticotropin. 47.[1] Synthesis and Biological Activity of Adrenocorticotropic Peptides Modified at the Tryptophan Position

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Three biologically active peptides, [9-β-(1-naphthyl)alanine]-ACTH-(1–19), [9-N^i-formyltryptophan]-ACTH-(1–19), and [8-lysine,9-phenylalanine]-ACTH-(1–19), have been synthesized by the solid-phase method. All of the synthetic peptides showed diminished biological activity compared to ACTH-(1–19). It was also shown that the steroidogenic and lipolytic activities of ACTH-(1–19) were not inhibited by [8-lysine,9-phenylalanine]-ACTH-(1–19).

Structure–activity studies on ACTH (Figure 1) and its related peptides have shown that their steroidogenic activity is particularly sensitive to replacement or modification of the tryptophan residue at position 9. Thus, whereas almost all of the amino acid residues of ACTH may be replaced by amino acids of similar structure without significant loss of activity, the replacement of tryptophan by phenylalanine or N^α-methyltryptophan results in a marked diminution of steroidogenic potency.[2a,b] It has also been reported[2c] that [NPS-Trp[9]]-ACTH contains only 1% of the *in vitro* steroidogenic activity of ACTH but is slightly more potent than ACTH as a melanotropic agent.[3] Of further interest was the observation that [NPS-Trp[9]]-ACTH is able to inhibit cAMP production in isolated rat adrenal cells[2c] and inhibit cyclic AMP production[4] and lipolysis[5] in isolated rat fat cells.

In order to further delineate the structural significance of the tryptophan residue in ACTH-(1–19) (I) we have synthesized [Nal[9]]-ACTH-(1–19) (II), [For-Trp[9]]-ACTH-(1–19) (III), and [Lys[8],Phe[9]]-ACTH-(1–19) (IV) and measured their biological activities. The results are reported here.

Synthesis. Our choice for an α-amino acid whose structure was very similar to that of tryptophan and which was readily available by synthesis was β-(1-naphthyl)alanine, which differs from tryptophan in that the indole moiety is replaced by naphthalene. Alkylation of ethyl acetamidocyanoacetate with 1-chloromethylnaphthalene, followed by alkaline hydrolysis and decarboxylation, gave β-(1-naphthyl)-DL-alanine.[6] Resolution was accomplished by carboxypeptidase digestion of the N^α-trifluoroacetyl derivative.[7] The N^α-Boc derivative was prepared and used for the solid-phase synthesis[8] of the model peptide, H-Ala-Nal-Gly-OH. The stereochemical homogeneity of the resolved product (L-Nal) was demonstrated by the complete digestion of the model peptide by leucine aminopeptidase.

[Nal[9]]-ACTH-(1–19) was synthesized by the standard solid-phase procedure[8] as described for the synthesis[9] of ACTH-(1–19) and as indicated in the Experimental Section. The protected nonadecapeptide resin was treated with liquid hydrogen fluoride[10,11] and the crude peptide was purified by chromatography on Sephadex G-25 and carboxymethylcellulose.[12] Final purification was achieved by partition chromatography[13] on Sephadex G-25 (Figure 2). Paper electrophoresis and amino acid analysis indicated that the product was homogeneous.

The second analog, [For-Trp[9]]-ACTH-(1–19) (III), was suggested by recent work[14,15] in which N^i-formyltryptophan was successfully used in peptide synthesis. Peptide III was synthesized as described for peptide II and the highly purified product was obtained after carboxymethylcellulose chromatography. The ultraviolet spectrum of peptide III is shown in Figure 3 and is in good agreement with that expected for a peptide containing an equimolar content of tyrosine and N^i-formyltryptophan.