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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.

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
1 5 10

Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-
15 20

Lys-Val-Tyr-Pro- - - - - - - - - - - -

- - - - - -Ala-Phe-Pro-Leu-Glu-Phe-OH
35 39

Figure 1. Partial structure of adrenocorticotropins.

Figure 2. Partition chromatography of crude peptide II on Sephadex G-25 in the system 1-butanol–pyridine–0.1 *N* ammonium hydroxide–acetic acid (400:240:960:0.96); column, 2.2 × 55 cm. The eluent volume per tube was 4.3 ml and the peptide was measured by the method of Lowry, *et al.*[32]

Figure 3. (A) The ultraviolet spectrum of peptide III in 0.001 *N* HCl at a concentration of 0.58 mg/ml. (B) The ultraviolet spectrum of N^{i}-formyltryptophan hydrochloride salt plus tyrosine, each at 1.74×10^{-4} *M* concentration in 0.001 *N* HCl.

The third analog, [Lys[8],Phe[9]]-ACTH-(1–19) (IV), was synthesized for reasons described in the next section and was obtained in a highly purified form after straightforward synthesis.

Biological Activity. The results of biological assays of peptides II, III, and IV, together with the parent peptide I, are summarized in Table I. The most notable result is the significant diminution of steroidogenic potency upon the replacement of tryptophan by β-(1-naphthyl)alanine and N^{i}-formyltryptophan in peptides II and III, respectively. Although neither of these two amino acids is exactly isosteric with tryptophan, their structures are close enough to that of tryptophan to lead us to conclude that an important role in steroidogenic activity is played by the indolyl N–H function of tryptophan, perhaps as a donor or acceptor in a hydrogen bonding interaction.

The *in vitro* lipolytic activities of peptides I and II parallel their *in vitro* steroidogenic activities. The same observation is made on the melanotropic activities of these peptides, except that the melanotropic activity is not as sensitive to changes or modifications of the tryptophan residue as are the steroidogenic and lipolytic activities. This is similar to the result observed when phenylalanine was substituted for tryptophan in [Gln[5]]-ACTH-(1–20) amide[2a] and NPS-Trp was substituted for tryptophan in ACTH;[3] both lead to peptides whose melanotropic activity is higher than would be expected from the steroidogenic activity. It is of interest to note that substituting phenylalanine for histidine-6 in ACTH-(1–19),[16] and lysine for arginine-8 in ACTH-(1–17) amide,[17] gave peptides whose melanotropic activity is equal to or slightly greater than would be expected from their steroidogenic activity.

A comparison of lipolytic activity and steroidogenic activity in this series gave inconsistent results. Substituting lysine for arginine-8 in ACTH-(1–17) amide gave a comparable decrease of lipolysis and steroidogenic activities (in isolated fat cells),[18] but the substitution of lysine for arginine-8 in a similar peptide gave a much greater decrease in lipolytic activity (in rat fat pads) than steroidogenic activity.[19] The discrepancy between these results is presumably due to the difference in the method of lipolytic assay. The substitution of phenylalanine for histidine-6 in ACTH-(1–19) gave a much greater decrease in lipolytic activity than in steroidogenic activity.[16] The situation for the substitution for tryptophan at position 9 was even more complicated. Thus, substituting β-(1-naphthyl)alanine for tryptophan in ACTH-(1–19) gave a comparable decrease in *in vitro* lipolytic and *in vitro* steroidogenic activities, but the substitution of NPS-Trp for tryptophan in ACTH gave a larger decrease in lipolytic activity[5] than steroidogenic activity.[2c]

Peptides II and III were synthesized with the hope that one of them would be able to inhibit the steroidogenic activity of ACTH. The original report that [NPS-Trp[9]]-ACTH inhibited cAMP production by ACTH in isolated rat adrenal cells[2c] has been followed by a report of similar inhibitory action of adenylyl cyclase activity in bovine adrenal cortical plasma membranes[20] by [Gln[5],Phe]-ACTH-(1–20) amide, [N^{α}-Me-Trp[9]]-ACTH-(1–24), and [Lys[8]]-ACTH-(1–24). Sayers, Schwyzer, and their coworkers[21–23] have reported several peptides which inhibit ACTH induced steroidogenesis in isolated adrenal cells, including [NPS-Trp[9]]-ACTH. Other workers[2c,24] have been unsuccessful in obtaining an inhibition of steroidogenesis in the same system.

Although peptides II and III have low steroidogenic activities, their potencies are still too high to make them attractive possibilities as inhibitors. Consequently, we synthesized [Lys[8],Phe[9]]-ACTH-(1–19) (IV). This peptide, which differs from peptide I by conservative replacements at positions 8 and 9, was expected to have a structure very similar to that of peptide I. The assay data in Table I show that peptide IV possesses less than 0.1% of the steroidogenic and lipolytic potency of peptide I and 4% of the melanotropic potency of peptide I.

To study inhibition, the *in vitro* steroidogenic dose–response curve of ACTH-(1–19) (I) was determined in the presence of peptide IV (at a concentration 40 times that of the concentration of I at half-maximal response) and in the absence of peptide IV. The two curves were essentially the same and peptide IV did not inhibit steroidogenic activity of ACTH-(1–19) in isolated adrenal cells (Figure 4, upper). Similarly, no inhibition of *in vitro* lipolysis was observed

Table I. Biological Activity of the Synthetic Peptides

	Steroidogenic act.			
	In vivo (units/μmol)[a]	*In vitro* (mol/l.)[b]	Melanocyte-stimulating act. *in vitro* (units/mmol)[c]	Lipolytic act. *in vitro* (mol/l.)[b]
ACTH	454	6.6×10^{-10}	45×10^7	4×10^{-9}
ACTH-(1–19) (I)	215 (178–285) [3]	8.4×10^{-10}	23×10^7 (7.0–30×10^7) [3]	1.8×10^{-9}
[Nal^9]-ACTH-(1–19) (II)	16 (11–22) [3]	370×10^{-10}	9.2×10^7 (3.5–17×10^7) [3]	120×10^{-9}
[For-Trp^9]-ACTH-(1–19) (III)	38 (26–57) [3]	*d*	11×10^7 (6.2–28×10^7) [4]	33×10^{-9}
[Lys^8, Phe^9]-ACTH-(1–19) (IV)	*d*	2×10^6	10×10^6 (5.2–20×10^6) [3]	$>4 \times 10^{-6}$

[a]Activity was measured against highly purified sheep ACTH with a potency of 454 units/μmol. Numbers in parentheses represent the 95% confidence limits; numbers in brackets represent the number of assays. [b]Concentration at half-maximal response; result of duplicate assays. [c]Activity was measured against sheep ACTH. [d]Not determined.

for a concentration of IV which was 100 times that of the concentration of I at half-maximal response (Figure 4, lower).

Experimental Section

N^α-Trifluoroacetyl-β-(1-naphthyl)-DL-alanine. β-(1-Naphthyl)-DL-alanine[6] (13.44 g) was dissolved in 150 ml of cold trifluoroacetic anhydride. The solution was stirred at 0° for several minutes until a thick precipitate formed. After 5 min further standing at 0°, the reaction mixture was evaporated *in vacuo* and the residue was dissolved in 200 ml of ethyl acetate. The ethyl acetate solution was washed with three 50-ml portions of cold water, dried, and evaporated to a crystalline residue of 10.6 g (54%): mp 188–191°; tlc [chloroform–methanol (1:1, v/v)] R_f 0.6; tlc [1-butanol–acetic acid–water (4:1:1, v/v)] R_f 0.8. *Anal.* ($C_{15}H_{12}F_3NO_3$) C, H, N.

β-(1-Naphthyl)-L-alanine. NaOH (2 *N*) was slowly added to a stirring mixture of 10.45 g of N^α-trifluoroacetyl-β-(1-naphthyl)-DL-alanine, 200 ml of 0.2 *M* sodium bicarbonate, and 500 ml of water until a stable pH of 7.0 was attained. The mixture was filtered to remove insolubles and 160 mg of carboxypeptidase A (COA-DFP, Worthington) was added to the filtrate. The solution was stirred at room temperature for 4 hr and 0.1 *N* HCl was periodically added to maintain the pH at 7.0. Then, 6 *N* hydrochloric acid was added to bring the pH to 5.4. The resulting mixture was chilled, decanted, and filtered. The precipitate was washed with water and then suspended in 10 ml of water. Aqueous NaOH (2 *N*) was added until most of the solid had dissolved; after filtration to remove some insolubles, the pH of the filtrate was adjusted to 5.0 by adding 6 *N* HCl. The mixture was chilled and filtered, and the precipitate was washed with water and dried: yield 1.19 g (33%); mp 210–216° dec; tlc [1-butanol–acetic acid–water (4:1:1, v/v)] R_f 0.7; paper electrophoresis (pH 2.1, 2 kV, 1 hr) showed a single ninhydrin positive spot at R_f^{Gly} 0.42; $[\alpha]^{22}D$ +24° (*c* 1, 1 *N* NaOH); $\lambda_{max}^{0.1\,N\,NaOH}$ 282 nm (ϵ 6900). *Anal.* ($C_{13}H_{13}NO_2$) H, N; C: calcd, 72.5; found, 71.4.

N^α-*tert*-Butyloxycarbonyl-β-(1-naphthyl)-L-alanine Dicyclohexylamine Salt. A mixture of 1.133 g of β-(1-naphthyl)alanine, 26 ml of dimethyl sulfoxide, 2.65 ml of diisopropylethylamine, and 1.60 ml of Boc azide was stirred at room temperature for 18 hr. Then, 0.2 ml of Boc azide was added and the mixture was stirred for 1 additional hr and distributed between 120 ml of cold water and 60 ml of ether. Sodium hydroxide was added to ensure a pH of 10 in the aqueous layer. The aqueous layer was washed with 50 ml of ether, cooled, acidified to pH 4, and extracted with two 75-ml portions of ethyl acetate. The combined organic extracts were washed with water, dried, and evaporated to a volume of *ca.* 3 ml. Adding 1 ml of dicyclohexylamine, followed by the addition of petroleum ether, gave 1.615 g (61%) of a crystalline product: mp 154–156°; tlc chloroform–acetic acid (15:1, v/v)] R_f 0.5; $[\alpha]^{23}D$ −28° (*c* 1, methanol). *Anal.* ($C_{30}H_{44}N_2O_4$) C, H, N.

H-Ala-Nal-Gly-OH. *tert*-Butyloxycarbonylglycyl resin (200 mg, 0.04 mmol) was subjected to the standard procedure of solid-phase peptide synthesis (see below). Coupling was achieved with 4 equiv of Boc-Nal (and Boc-Ala) and 4 equiv of dicyclohexylcarbodiimide in methylene chloride. The tripeptide resin (150 mg) was treated with 0.2 ml of anisole and 3 ml of liquid HF at 0° for 30 min. The mixture was evaporated at 0°, dried *in vacuo*, and stirred with 5 ml of trifluoroacetic acid for 5 min. Filtration and evaporation of the filtrate gave a residue which was distributed between 5 ml of 0.2 *N* acetic acid and 3 ml of ether. The aqueous layer was lyophilized to give 6.7 mg of peptide. Paper electrophoresis (pH 2.1, 2 kV, 1 hr) showed a single ninhydrin positive spot at R_f^{Gly} 0.52. The ultraviolet spectrum of the peptide in 0.1 *N* NaOH was the same as that for L-Nal, λ_{max} 282 nm. Amino acid analysis[25] of an acid hydrolysate gave $Ala_{0.97}$ $Nal_{1.01}$ $Gly_{1.00}$. Nal was determined on the short column of the amino acid analyzer where it was eluted 6 min after ammonia. Paper electrophoresis (as described above) of a leucine aminopeptidase (Worthington) digest (18 hr at 37°; enzyme–peptide, 1:25) showed only three ninhydrin-positive spots corresponding to Gly, Ala, and Nal.

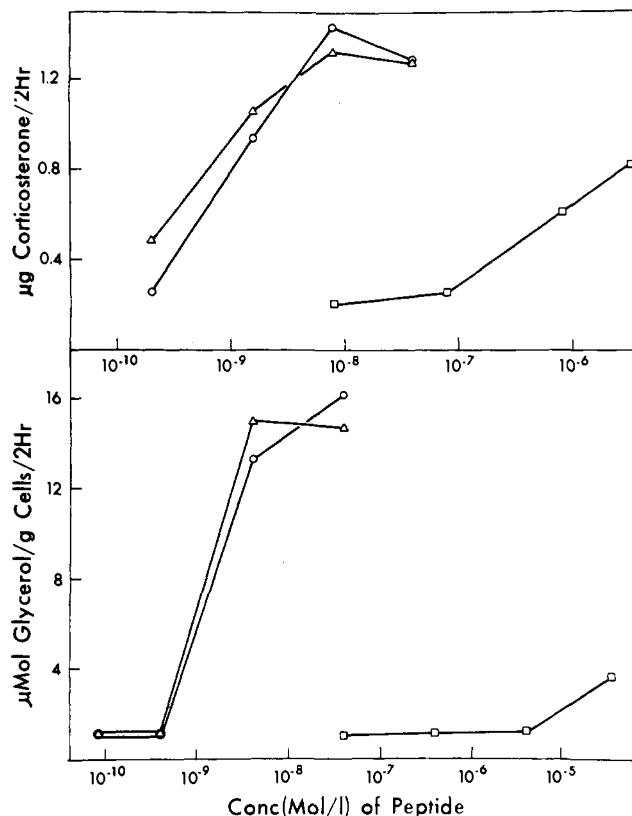

Figure 4. Upper: the production of corticosterone in isolated rat adrenal cells upon incubation with the synthetic peptides: peptide I (–○–); peptide IV (–□–); peptide I at the indicated concentrations plus peptide IV at a constant concentration of 4×10^{-8} mol/l. (–△–). Lower: the release of glycerol from isolated rat fat cells upon incubation with the synthetic peptides: peptide I (–○–); peptide IV (–□–); peptide I at the indicated concentrations plus peptide IV at a constant concentration of 2×10^{-7} mol/l. (–△–).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Nal-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-OH(II). *tert*-Butyloxycarbonylprolyl resin (3.08 g, 1.05 mmol), prepared by the modified Loffet procedure,[26,27] was treated as follows: (a) washed with three 40-ml portions of methylene chloride; (b) treated with 50 ml (total volume) of trifluoroacetic acid–methylene chloride (1:1, v/v) for 15 min; (c) washed with three 40-ml portions of methylene chloride, three 40-ml portions of ethanol–methylene chloride (1:2, v/v), and

three 40-ml portions of methylene chloride; (d) treated with 40 ml of methylene chloride and 5 ml of diisopropylethylamine for 5 min; (e) washed with six 40-ml portions of methylene chloride; (f) treated with 4.0 mmol of N^{α}-*tert*-butyloxycarbonyl-N^{G}-*p*-toluenesulfonylarginine in 23 ml of methylene chloride and 1.8 ml of dimethylformamide for 5 min; (g) an addition of 4.0 mmol of dicyclohexylcarbodiimide in 5 ml of methylene chloride and shaken for 3 hr; and (h) washed with three 40-ml portions of dimethylformamide and three 40-ml portions of ethanol.

The peptide resin was subjected to 17 additional cycles of synthesis as described above. N^{α}-Protection for the amino acid derivatives was by the Boc group, except for Met, Glu, and His which were coupled as their N^{α}-Bpoc derivatives. Deblocking of the N^{α}-Bpoc group was achieved in 0.05 *N* HCl in methylene chloride as previously described.[28] Side-chain protecting groups were as follows: Arg, N^{G}-*p*-toluenesulfonyl; Lys, N^{ϵ}-*o*-bromobenzyloxycarbonyl; Ser, *O*-benzyl; Tyr, *O*-*o*-bromobenzyloxycarbonyl; His, N^{im}-Boc; Glu, γ-benzyl ester.

A portion of the dried nonadecapeptide resin (0.95 g, 0.15 mmol) was treated with 2.8 ml of anisole and 15 ml of liquid HF for 45 min at 0°. The HF was evaporated at 0° and the peptide resin was dried *in vacuo*. The resulting mixture was stirred for 10 min with 10 ml of trifluoroacetic acid and filtered. The filtrate was evaporated to a residue that was distributed between 25 ml of 0.2 *N* acetic acid and 12 ml of ether, and the aqueous layer was washed with two 12-ml portions of ether. Evaporation of the aqueous layer to a 5-ml volume and chromatography on Sephadex-G-25 in 0.5 *N* acetic acid gave a peptide material that was rechromatographed on the same column to give 110 mg of peptide. Chromatography on CMC as previously described,[9] followed by rechromatography of the major peak, gave 38.5 mg of peptide. Finally, 21.5 mg of peptide was subjected to partition chromatography on Sephadex G-25 (Figure 2). The material corresponding to the major peak at R_f 0.39 was pooled, diluted with two volumes of water, evaporated to a 3-ml volume, and chromatographed on Sephadex G-10 in 0.5 *N* acetic acid. The peptide eluted from Sephadex G-10 was isolated by lyophilization to give 7.1 mg of peptide II (peptide content was 77% by OD_{280}, 7% yield based on starting *tert*-butyloxycarbonylprolyl resin).

Paper electrophoresis (400 V, 3 hr) of peptide II at pH 3.7 and 6.9 gave single ninhydrin-positive, Pauly positive spots at R_f^{Lys} 0.90 and 0.67, respectively. Amino acid analysis of an acid hydrolysate gave $Lys_{2.8}$ $His_{0.9}$ $Nal_{1.0}$ $Arg_{2.8}$ $Ser_{1.9}$ $Glu_{1.0}$ $Pro_{2.1}$ $Gly_{2.1}$ $Val_{1.0}$ $Met_{1.0}$ $Tyr_{1.0}$ $Phe_{1.0}$. A solution of 0.5 mg of peptide II in Tris buffer (pH 8.5, 0.01 *M* Mg^{2+}) was incubated with 10 μg of trypsin and 10 μg of chymotrypsin for 22 hr at 37°. The solution was boiled for 15 min, cooled, and then incubated with 20 μg of leucine aminopeptidase for 42 hr at 37°. Amino acid analysis of the digest gave $Lys_{2.7}$ $His_{0.9}$ $Nal_{0.9}$ $Arg_{2.8}$ $Ser_{1.8}$ $Glu_{1.0}$ $Pro_{1.6}$ $Gly_{1.9}$ $Val_{1.1}$ $Met_{0.9}$ $Tyr_{1.0}$ $Phe_{1.0}$.

[For-Trp[9]]-ACTH-(1-19) (III). Peptide III was synthesized as described for peptide II. The peptide resin was worked up similarly and the final product was obtained from CMC chromatography: yield 11% based on starting *tert*-butyloxycarbonylprolyl resin. The ultraviolet spectrum of peptide III in 0.001 *N* HCl is shown in Figure 3.

Paper electrophoresis (400 V, 3 hr) at pH 3.7 and 5.8 gave single ninhydrin-positive, Pauly positive spots at R_f^{Lys} 0.88 and 0.71, respectively. Amino acid analysis of an acid hydrolysate gave $Lys_{3.0}$ $His_{1.0}$ $Arg_{2.8}$ $Ser_{1.7}$ $Glu_{0.9}$ $Pro_{2.1}$ $Gly_{2.0}$ $Val_{1.1}$ $Met_{1.0}$ $Tyr_{0.9}$ $Phe_{0.9}$. Amino acid analysis of an enzyme digest as described for peptide II gave $Lys_{2.1}$ $His_{0.9}$ $Arg_{2.3}$ $Trp_{0.8}$ $Ser_{2.1}$ $Glu_{1.0}$ $Pro_{1.6}$ $Gly_{2.0}$ $Val_{1.2}$ $Met_{0.9}$ $Tyr_{1.0}$ $Phe_{1.0}$. The presence of Trp in the digest is due to deformylation during digestion.

[Lys[8],Phe[9]]-ACTH-(1-19) (IV). Peptide IV was synthesized as described for peptide II, except that N^{α}-protection was by the Boc group for all the amino acid residues. The peptide resin was worked up exactly as described for peptide II. Partition chromatography on Sephadex G-25 gave a single symmetrical peak at R_f 0.26. The yield of peptide IV was 6% based on starting *tert*-butyloxycarbonylprolyl resin.

Paper electrophoresis (400 V, 3 hr) at pH 3.7 and 6.9 gave single ninhydrin-positive, Pauly positive spots at R_f^{Lys} 0.96 and 0.72, respectively. Amino acid analysis of an acid hydrolysate gave $Lys_{4.0}$ $His_{0.9}$ $Arg_{1.9}$ $Ser_{1.8}$ $Glu_{1.0}$ $Pro_{2.1}$ $Gly_{2.0}$ $Val_{1.0}$ $Met_{1.0}$ $Tyr_{1.0}$ $Phe_{2.0}$. Amino acid analysis of an enzyme digest as described for peptide II gave $Lys_{3.8}$ $His_{0.9}$ $Arg_{2.0}$ $Ser_{2.0}$ $Glu_{1.0}$ $Pro_{1.9}$ $Gly_{2.1}$ $Val_{1.1}$ $Met_{1.0}$ $Tyr_{1.0}$ $Phe_{2.0}$.

Biological Assay. *In vivo* steroidogenic activity was determined in the rat by the method of Vernikos-Danellis, *et al.*[29] Potency was measured against sheep ACTH standard. *In vitro* steroidogenesis in isolated rat adrenal cells was measured by the method described by Moyle, *et al.*[2c] *In vitro* lipolytic activity in isolated rat fat cells was measured by the method of Ramachandran and Lee.[5] *In vitro* melanotropic activity was determined by the method of Shizume, *et al.*[30] Potency was measured against sheep ACTH standard.[31]

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References and Notes

(1) For paper 46, see J. Blake and C. H. Li, *Int. J. Peptide Protein Res.*, **6**, 141 (1974). All asymmetric amino acids occurring in the peptides mentioned in this paper are of the L configuration. Abbreviations: ACTH, adrenocorticotropin; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenylyl)isopropyloxycarbonyl; Nal, β-(1-naphthyl)alanine; For-Trp, N^{i}-formyltryptophan; NPS, *o*-nitrophenylsulfenyl; NPS-Trp, *o*-nitrophenylsulfenyltryptophan; tlc, thin layer chromatography; CMC, carboxymethylcellulose.

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