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Synthesis of a Biologically Active Pentadecapeptide Corresponding to an Altered Sequence in the Adrenocorticotropin (ACTH) Structure

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The synthesis of a pentadecapeptide with a structure consisting of the first ten NH_2-terminal residues linked with a sequence of positions 15 to 19 in the ACTH structure, namely, L-seryl-L-tyrosyl-L-seryl-L-methionyl-L-glutamyl-L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophyl-glycyl-L-lysyl-L-lysyl-L-arginyl-L-arginyl-L-proline, has been described. The product was found to exhibit the full lipolytic potency of the natural ACTH, but it had less than 1 U.S.P. unit of adrenal-stimulating potency per mg. The synthetic peptide had only one-hundredth of the melanocyte-stimulating activity of the native hormone. This report represents the first synthesis of a biologically active peptide in which the natural sequence of the ACTH structure has been altered.

Whereas the nonessentiality of the acidic carboxyl-terminal portion of ACTH, composed of amino acid residues 25–39, for the manifestation of the observed biological activities of the hormone has been established by chemical and enzymic degradative studies,[1] synthetic endeavor[2–5] has provided evidence for the importance of certain structural features of the NH_2-terminal half of the molecule for hormonal function. Comparison of the adrenal-stimulating potency of the synthetic nonadecapeptide[2] and the synthetic heptadecapeptide[3] corresponding to the first nineteen and seventeen amino acid residues of ACTH, respectively, as assayed by both *in vitro* and *in vivo* procedures, revealed that the integrity of the core of basic amino acids occurring in positions 15–18 is essential for steroidogenesis. Synthetic studies by Hofmann and coworkers[6] showed that the decapeptide seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine[7] is less potent a melanocyte-stimulating agent by nearly three orders of magnitude than the tridecapeptide amide seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valine amide. This indicated the importance of the sequence lysyl-prolyl-valine amide for melanophore-stimulating activity. Hence, it appeared interesting to see if we could obtain a peptide possessing high adrenal-stimulating potency and/or lipolytic activity but having low melanocyte-stimulating activity, by combining the NH_2-terminal decapeptide with the peptide sequence lysyl-lysyl-arginyl-arginyl-proline, omitting the tetrapeptide lysyl-prolyl-valyl-glycine occurring in the native molecule. The pentadecapeptide[8] with the altered sequence is designated as $\alpha^{(1-10)+(15-19)}$-ACTH.

For the synthesis of this altered pentadecapeptide (see Fig. 1), a protected derivative of the NH_2-terminal decapeptide, namely, carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine (XII), was employed. The synthesis of this crystalline derivative has been described.[9,10]

Two protected derivatives of the pentapeptide lysyl-lysyl-arginyl-arginyl-proline were prepared. The synthesis is schematically presented in Fig. 2. Both involved the use of the same partially protected tripeptide N^G-tosyl-arginyl-N^G-tosyl-arginyl-proline *t*-butyl ester (V), which was prepared by stepwise reaction of N^α-carbobenzoxy-N^G-tosyl-arginine[11a] (I) with proline *t*-butyl ester,[11b] with the use of N-ethyl-5-phenylisoxazolium 3′-sulfonate[12] (II). The dipeptide III was obtained in crystalline form. The carbobenzoxy group was removed from III by catalytic hydrogenolysis. Subsequent reaction with I by the use of II yielded the amorphous tripeptide N^α-carbobenzoxy-N^G-tosyl-arginyl-N^G-tosyl-arginyl-proline *t*-butyl ester (IV) in analytically pure form; IV also behaved homogeneously in paper chromatography in two solvents. Catalytic hydrogenolysis of IV gave V which, again, was found to be homogeneous by the aforementioned criteria.

Stepwise reaction of V with N^α-carbobenzoxy-N^ϵ-*t*-butyloxycarbonyl-lysine *p*-nitrophenyl ester[13] yielded the protected pentapeptide VII. Both VII and the protected tetrapeptide intermediate were purified by countercurrent distribution in the toluene system.

The pentapeptide derivative in which the ϵ-amino functions of lysine were masked with the *p*-toluenesulfonyl group was synthesized in two different ways. In the first, N^α-carbobenzoxy-N^ϵ-tosyl-lysyl-N^ϵ-tosyl-lysine hydrazide[9] was converted to the azide and allowed to react with V to yield X in 77% yield after purification through countercurrent distribution in the toluene system. X was also prepared by stepwise elongation of the peptide chain by the reaction of V with N^α-carbobenzoxy-N^ϵ-tosyl-lysine *p*-nitrophenyl ester.[14] The carbobenzoxy group was removed from VII and X by catalytic hydrogenolysis to yield VIII and XI, respectively. Both VIII and XI were purified by countercurrent distribution in the toluene system and found to be homogeneous by paper chromatography in two solvents and by elemental analysis. Table I lists the values for partition coefficients (K) of the various peptides in the toluene system.

For the synthesis of the protected pentadecapeptide

(1) C. H. Li, *Advan. Protein Chem.*, **11**, 101 (1956).

(2) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T-B. Lo, and J. Ramachandran, *J. Am. Chem. Soc.*, **82**, 5760 (1960); **83**, 4449 (1961).

(3) C. H. Li, D. Chung, J. Ramachandran, and B. Gorup, *ibid.*, **84**, 2460 (1962).

(4) K. Hofmann, H. Yajima, T-Y. Liu, and N. Yanaihara, *ibid.*, **84**, 4475 (1962).

(5) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **46**, 1550 (1963).

(6) K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).

(7) All amino acids occurring in the peptides mentioned in this paper are of the L-configuration, with the exception of glycine.

(8) A preliminary report of this work has appeared in *J. Am. Chem. Soc.*, **85**, 1895 (1963).

(9) C. H. Li, J. Ramachandran, D. Chung, and B. Gorup, *ibid.*, **86**, 2703 (1964).

(10) C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

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(12) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961).

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(14) M. Bodanszky, J. Meienhofer, and V. du Vigneaud, *J. Am. Chem. Soc.*, **82**, 3195 (1960).

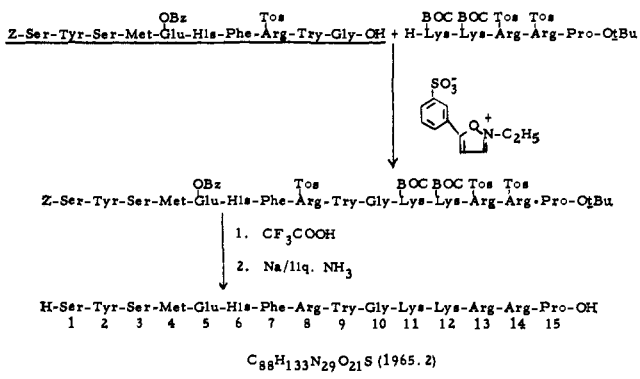

Fig. 1.—Outline of the synthesis of $\alpha^{(1-10)+(15-19)}$-ACTH.

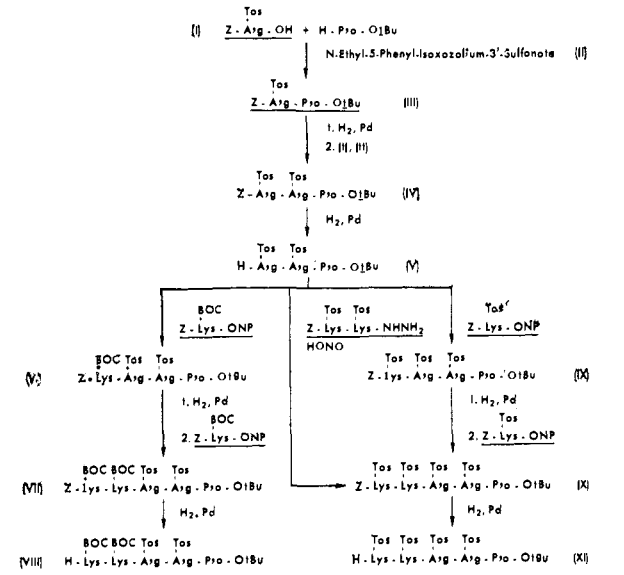

Fig. 2.—Outline of the synthesis of two pentapeptide derivatives: Z, carbobenzoxy; TOS, *p*-toluenesulfonyl; *t*-Bu, *t*-butyl; BOC, *t*-butyloxycarbonyl; ONP, *p*-nitrophenoxy.

XIII,[15] the crystalline decapeptide XII was activated with II and allowed to react with VIII. The product was isolated by countercurrent distribution in the carbon tetrachloride system in which XII had a K of 1 and the by-products resulting from II have much

TABLE I

DISTRIBUTION COEFFICIENTS OF SOME SYNTHETIC PEPTIDE INTERMEDIATES IN THE TOLUENE SYSTEM

Peptide	K	Peptide	K
VI	0.33	IX	0.33
VII	.25	X	.30
VIII	.77	XI	.94

higher partition coefficients. Compound XIII and unreacted VIII remain near the origin since both these peptides have very low K-values in this system. Thus countercurrent distribution in this solvent system removes all contaminants from XIII except VIII.

(15) The synthesis of the pentadecapeptide by the reaction of XII with the pentapeptide derivative XI, in which all the basic side chains are blocked with the tosyl group, proved more cumbersome because of the problems encountered in the removal of unreacted XI. As before, countercurrent distribution in the carbon tetrachloride system yielded a material free of unreacted XII but containing some traces of a ninhydrin-positive contaminant. Thorough washing with methanol removed the ninhydrin-positive substance; however, this resulted in the dissolution of some protected pentadecapeptide also. Since the purification of XIII through the partially deblocked intermediate XIV proved easier and resulted in better yields, the approach employing the fully tosylated pentapeptide XI was not pursued further.

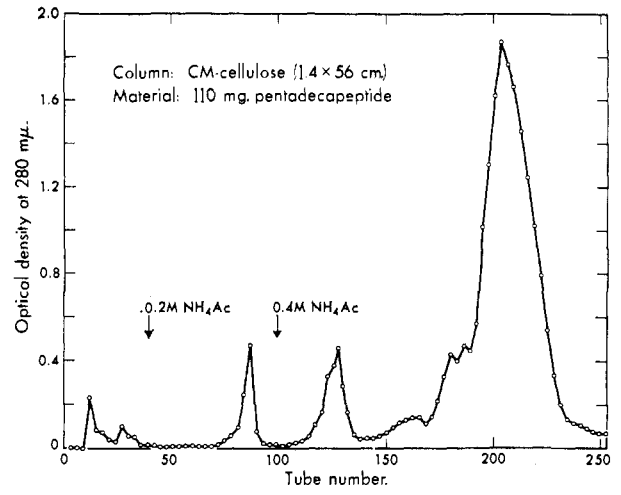

Fig. 3.—CMC chromatography of crude $\alpha^{(1-10)+(15-19)}$-ACTH (XV). The initial buffer was 0.01 M ammonium acetate of pH 4.5. After 3–4 hold-up volumes (4 cc./tube) had been collected, a gradient with respect to pH and concentration was started by introducing 0.2 M ammonium acetate buffer of pH 6.7 through a 500-ml. mixing flask containing the starting buffer. Later, the gradient was increased by substituting 0.4 M ammonium acetate of pH 6.7 as the solution flowing into the mixing flask.

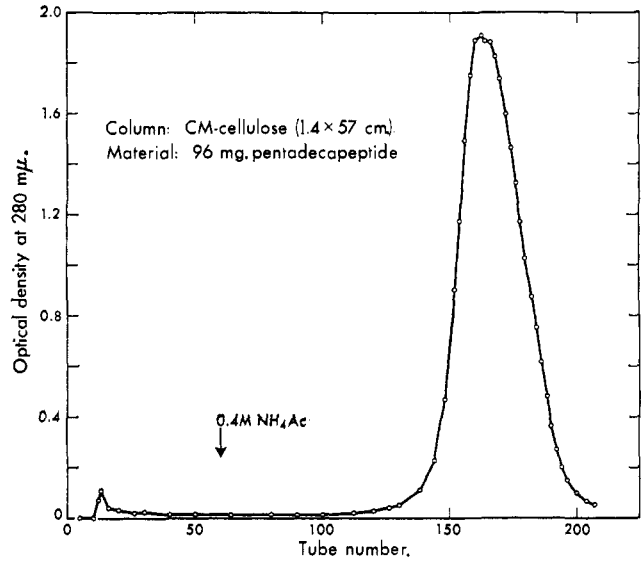

Fig. 4.—Rechromatography of XV on CMC; conditions as Fig. 3.

The material isolated in this manner was washed with methanol to remove the unreacted pentapeptide VIII. This treatment, which was quite successful for removing unreacted heptapeptide in the synthesis of the heptadecapeptide,[9] was not quite satisfactory in the present case since some of the pentadecapeptide XIII was also dissolved by methanol, as could be seen on paper chromatograms of the washings (with the aid of the Pauly reagent). The presence of the *t*-butyloxycarbonyl groups and the *t*-butyl ester group may account for the differences in solubilities of the protected heptadecapeptide[9] and XIII. Therefore XIII was treated with trifluoroacetic acid in order to remove the *t*-butyloxycarbonyl groups and the *t*-butyl group. The partially deblocked pentadecapeptide XIV was submitted to countercurrent distribution in the system composed of butanol–acetic acid–water in the ratio 4:1:5. In this solvent, XIV (K = 6.3, located by ultraviolet absorption and ninhydrin reagent) could be

TABLE II

AMINO ACID COMPOSITION OF $\alpha^{(1-10)+(15-19)}$-ACTH

	Ser	Tyr	Met	Glu	His	Phe	Arg	Try	Gly	Lys	Pro
Theoretical	2	1	1	1	1	1	3	1	1	2	1
Chromatographic	1.76	0.99	0.95	1.00	1.00	1.02	2.88	1.04[a]	0.97	2.00	1.05
Microbiological	1.86	1.04	0.98		1.04	1.00	2.98	..	1.28	1.97	0.92

[a] Determined spectrophotometrically.

TABLE III

BIOLOGICAL ACTIVITIES OF ACTH AND $\alpha^{(1-10)+(15-19)}$-ACTH

Biological effect	α_s-ACTH	$\alpha^{(1-10)+(15-19)}$-ACTH
In vitro adrenal steroidogenesis, I.U./mg.	135	0.42
In vivo adrenal steroidogenesis, u./mg.	113	0.11
In vitro MSH activity, u./g.	6.4×10^7	1.9×10^6
In vivo MSH activity,[a] μg.	0.2	20
In vitro release of nonesterified fatty acids by rabbit perirenal adipose tissue,[b] μg.	0.0064	0.0037

[a] The dose produces a change in melanophore index in hypophysectomized *Rana pipiens* of from 1+ to 3+ within 1 hr. [b] Minimum effective dose as defined in ref. 23.

conveniently separated from the partially deblocked pentapeptide lysyl-lysyl-N^G-tosyl-arginyl-N^G-tosyl-arginyl-proline (K = 0.7, located by ninhydrin reagent) after 80 transfers.

All the remaining protecting groups were removed from XIV by treatment with sodium in liquid ammonia. The product XV was desalted and purified by chromatography on carboxymethylcellulose as described earlier.[9] A typical chromatogram and rechromatography of the major peak are shown in Fig. 3 and 4. The peptide XV, isolated in this manner, was found to be homogeneous by electrophoresis on paper as well as on polyacrylamide gel[16] (Fig. 5).

Amino acid analysis by the chromatographic method[17] as well as by microbiological means[18] showed that the constituent amino acids were present in the expected ratio (Table II).

By bioassay procedures both *in vivo*[19] and *in vitro*,[20] XV was found to exhibit an adrenal-stimulating potency of less than 1 unit/mg. By the frog skin assay *in vitro*,[21] peptide XV was shown to possess an MSH activity of 1.9×10^6 units/g., whereas natural α-ACTH exhibited a potency of 6.4×10^7 units/g. Furthermore, in the hypophysectomized frog assay *in vivo*,[22] the pentadecapeptide was only one-hundredth as active as ACTH. However, XV was found to be as potent a lipolytic agent as natural ACTH when assayed *in vitro*[23] with perirenal adipose tissues of the rabbit. The biological activities of XV are summarized and compared with ACTH in Table III. The synthetic pentadecapeptide is the first instance where a separation of the MSH and lipolytic activities has been achieved.

(16) S. Raymond and L. Weintraub, *Science*, **130**, 711 (1959); R. A. Reisfeld, U. J. Lewis, and D. E. Williams, *Nature*, **195**, 281 (1962).

(17) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(18) The microbiological assay was carried out by the Shankman Laboratories, Los Angeles, Calif.

(19) H. S. Lipscomb and D. H. Nelson, *Endocrinology*, **71**, 13 (1962).

(20) M. Saffran and A. V. Schally, *ibid.*, **56**, 523 (1955).

(21) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *ibid.*, **54**, 553 (1954).

(22) L. T. Hogben and D. Slome, *Proc. Roy. Soc.* (London), **B108**, 10 (1931).

(23) A. Tanaka, B. T. Pickering, and C. H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962).

Experimental[24]

N^α-Carbobenzoxy-N^G-tosyl-arginyl-proline *t*-Butyl Ester (III). —Carbobenzoxy-proline *t*-butyl ester,[11b] (0.335 g., 1.1 mmoles) was dissolved in 20 cc. of methanol and submitted to catalytic hydrogenolysis with the use of a Vibro-mixer[28] in the presence of palladium freshly prepared from 0.5 g. of $PdCl_2$. After the evolution of CO_2 had ceased[29] (2 hr the palladium was removed by filtration and the methanol was removed *in vacuo* at room temperature. The residue, proline *t*-butyl ester, was dissolved in 10 cc. of acetonitrile. N^α-Carbobenzoxy-N^G-tosyl-arginine[11a] (I, 0.462 g., 1 mmole) was dissolved in warm acetonitrile (10 cc.) and cooled to 0°. Triethylamine (0.14 cc., 1 mmole), and 0.254 g. (1 mmole) of N-ethyl-5-phenylisoxazolium 3′-sulfonate[12] (II) were added and the mixture stirred for 1 hr. at 0°. The solution of proline *t*-butyl ester in acetonitrile was then added and the mixture was stirred overnight (16 hr.) at room temperature. Acetonitrile was removed *in vacuo*, the residue was redissolved in wet ethyl acetate (40 cc.), and the solution was extracted successively with 5% citric acid, water, 5% sodium bicarbonate, and water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was found to be homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.79, $R_{f\ SBA}$ 0.89. Compound III crystallized from ethyl acetate–petroleum ether to yield 0.464 g. (76%), m.p. 112–114°. A sample was recrystallized from ethyl acetate; m.p. 115–118°, $[\alpha]^{25}D$ −42.5° (*c* 1, methanol).

Anal. Calcd. for $C_{30}H_{41}O_7N_5S$ (615.7): C, 58.5; H, 6.71; N, 11.4. Found: C, 58.4; H, 6.67; N, 11.4.

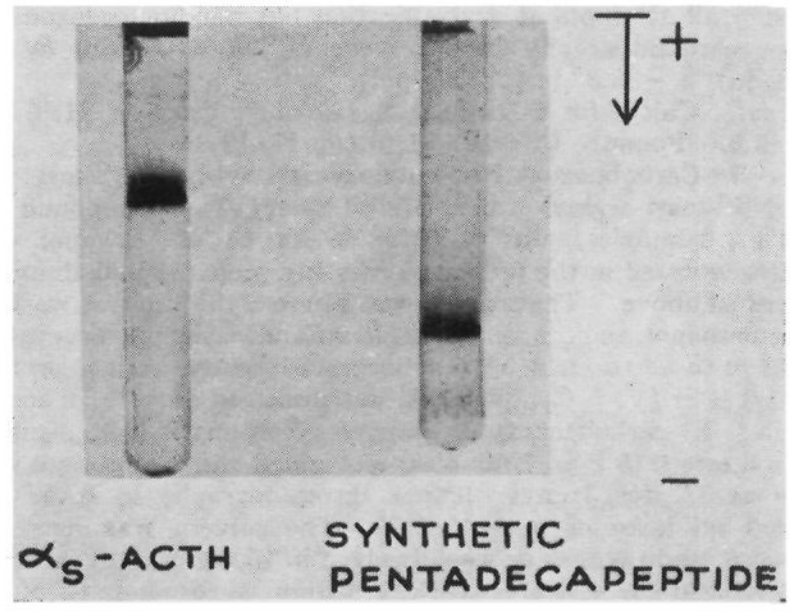

Fig. 5.—Disk electrophoresis of XV on polyacrylamide gel (0.8 × 6.5 cm.) at pH 4.5, 220 v., for 30 min.

(24) Melting points were determined on a Fisher–Johns block and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. All samples for microanalysis were dried in an Abderhalden drying pistol with P_2O_5 at 77° for 16 hr. at 0.3 mm. pressure. Paper chromatography was carried out on Whatman No. 1 filter paper at room temperature; the solvents used were 1-butanol–acetic acid–water (BAW) in a ratio of 4:1:1, 2-butanol–10% ammonia (SBA) in a ratio of 85:15, and 1-butanol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24, all by volume. Peptide spots were located by the ninydrin reagent, chlorine method,[25] Pauly reagent,[26] and the Ehrlich reagent.[27] Countercurrent distribution was performed using either the toluene system (toluene–chloroform–methanol–water, 5:5:8:2, by volume) or the carbon tetrachloride system (carbon tetrachloride–chloroform–methanol–0.01 *M* ammonium acetate, 1:3:3:1, by volume).

(25) H. Zahn and E. Rexroth, *Z. anal. Chem.*, **148**, 181 (1955).

(26) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(27) J. Smith, *Nature*, **171**, 43 (1953).

(28) Vibro-mixer, A. G. Fuer Chemie-Apparatebau, Zurich, Model E1.

(29) Completion of hydrogenolysis was routinely checked by paper chromatography in the BAW system.

N^{α}-Carbobenzoxy-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (IV).—Compound III (0.68 g., 1.1 mmoles) was dissolved in 20 cc. of methanol and submitted to catalytic hydrogenolysis as described above. The solvent was removed *in vacuo* after the CO_2 evolution had ceased and the residue N^{G}-tosyl-arginyl-proline *t*-butyl ester was dissolved in 10 cc. of acetonitrile. Compound I (0.462 g., 1 mmole) was dissolved in 10 cc. of warm acetonitrile and cooled to 0°. Triethylamine (0.14 cc., 1 mmole) and 0.25 g. (1 mmole) of II were added and the mixture was stirred at 0° for 1 hr. The solution of N^{G}-tosyl-arginyl-proline *t*-butyl ester was added and stirring continued at room temperature for 16 hr. The solvent was removed *in vacuo;* the residue was dissolved in 40 cc. of wet ethyl acetate and washed successively with 5% citric acid, water, 5% sodium bicarbonate, and water. The ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* to yield 0.653 g. (71%) of IV as an amorphous product, m.p. 100–120°. Peptide IV resisted all attempts at crystallization but was homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.88, $R_{f\ SBA}$ 0.86, $[\alpha]^{25}D$ −33.3° (*c* 1, methanol).

Anal. Calcd. for $C_{43}H_{59}N_9O_{10}S_2$ (926.1): C, 55.8; H, 6.42; N, 13.6. Found: C, 55.6; H, 6.19; N, 13.8.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (VI).—Compound IV (5.6 g., 6 mmoles), was dissolved in 100 cc. of methanol and hydrogenolyzed in the presence of freshly prepared palladium as described above. The solution was filtered, the catalyst washed with methanol, and the combined filtrate and washings were evaporated *in vacuo* to yield N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-butyl ester (V). Compound V was dissolved in 60 cc. of acetonitrile; N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyl-lysine *p*-nitrophenyl ester[13] (3.5 g., 7 mmoles) was added and the mixture was kept at 37° for 3 days. Paper chromatography in BAW revealed no trace of unreacted V. The solvent was removed *in vacuo*, and the residue was dissolved in 100 cc. of ethyl acetate and washed successively with 5% sodium bicarbonate (3 × 40 cc.) and water (2 × 40 cc.). The organic phase was dried over anhydrous sodium sulfate, concentrated to a small volume, and precipitated into 400 cc. of anhydrous ether. The white precipitate was filtered, washed with more ether, and dried to yield 6.7 g. (94.5%) of VI, m.p. 108-112°, $[\alpha]^{25}D$ −33.8° (*c* 1, methanol). Compound VI was found to be homogeneous in paper chromatography, $R_{f\ BAW}$ 0.87, $R_{f\ SBA}$ 0.89, and by countercurrent distribution in the toluene system, K = 0.33.

Anal. Calcd. for $C_{54}H_{78}N_{11}S_2O_{13}$ (1154): C, 56.2; H, 6.90; N, 13.4; S, 5.56. Found: C, 56.0; H, 6.82; N, 13.2; S, 5.48.

N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyl-lysyl-N^{ϵ}-*t*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (VII).—Compound VI, (6 g., 5.2 mmoles) was dissolved in 100 cc. of methanol and decarbobenzoxylated by catalytic hydrogenolysis. The catalyst was removed by filtration and washed with methanol, and the filtrate was evaporated to dryness. The residue was dissolved in 60 cc. of acetonitrile and treated with N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyl-lysine *p*-nitrophenyl ester[13] (3 g., 6 mmoles) at 37° for 48 hr. Paper chromatography in BAW revealed no ninhydrin-positive material. The solvent was removed *in vacuo*, the residue was redissolved in 200 cc. of ethyl acetate, washed successively with water (2 × 50 cc.), ice-cold 5% citric acid (3 × 50 cc.), water (2 × 50 cc.), 5% sodium bicarbonate (3 × 50 cc.), water (2 × 50 cc.), and saturated NaCl (50 cc.). The organic layer was dried over anhydrous sodium sulfate, concentrated to a small volume, and precipitated from 400 cc. of anhydrous ether to yield 6.9 g. of VII. This material was further purified by countercurrent distribution in the toluene system for 100 transfers. The material from the peak with K = 0.25 was isolated; wt. 5.5 g. (72.4%), m.p. 105–110°, $[\alpha]^{25}D$ −36.5° (*c* 1, methanol). Compound VII behaved as a homogeneous component in paper chromatography, $R_{f\ BAW}$ 0.93, $R_{f\ SBA}$ 0.93.

Anal. Calcd. for $C_{65}H_{99}N_{13}S_2O_{16}$ (1383): C, 56.4; H, 7.22; N, 13.2; S, 4.64. Found: C, 56.2; H, 7.33; N, 13.0; S, 4.69.

N^{ϵ}-*t*-Butyloxycarbonyl-lysyl-N^{ϵ}-*t*-butyloxycarbonyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (VIII).—Peptide VII (4.49 g., 3.25 mmoles), was dissolved in 100 cc. of methanol and submitted to catalytic hydrogenolysis in the presence of freshly prepared Pd (from 1 g. of $PdCl_2$). After CO_2 evolution had stopped (5 hr.), the catalyst was filtered and washed with methanol. The filtrate and washings were evaporated to dryness *in vacuo*. Paper chromatography of the residue in BAW revealed a single ninhydrin- and chlorine-positive spot, R_f 0.81. However, since the R_f values of VII and VIII were too similar, a further check on the homogeneity of VIII was made by means of countercurrent distribution in the toluene system for 100 transfers. A single symmetrical peak with K = 0.77 was seen (VII has a K of 0.25 in this system). Compound VIII was obtained in 78% yield (3.15 g.) after countercurrent distribution, m.p. 108–115°, $[\alpha]^{25}D$ −33.7° (*c* 1, methanol).

Anal. Calcd. for $C_{57}H_{93}N_{13}S_2O_{14}$ (1248.5): C, 54.8; H, 7.51; N, 14.6. Found: C, 54.6; H, 7.30; N, 14.5.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (IX).—Compound V (2.63 g., 3.3 mmoles) was dissolved in 30 cc. of acetonitrile and allowed to react with 1.85 g. (3.3 mmoles) of N^{α}-carbobenzoxy-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester[14] at 37° for 48 hr. The solvent was removed *in vacuo*, and the residue was dissolved in wet ethyl acetate (100 cc.) and washed with 5% sodium bicarbonate (4 × 50 cc.) and water (2 × 50 cc.). The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated to dryness. Since paper chromatography in BAW and SBA revealed the presence of traces of ninhydrin-positive material and nitrophenol, the residue was further purified by countercurrent distribution in the toluene system for 100 transfers. Compound IX was isolated from the peak with K = 0.33; wt. 3.5 g. (92%), m.p. 120–125°, $[\alpha]^{25}D$ −32.2° (*c* 1, methanol). Compound IX was found to be homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.92, $R_{fS\ BA}$ 0.92.

Anal. Calcd. for $C_{54}H_{79}N_{11}O_{13}S_2$ (1154.4): C, 56.2; H, 6.90; N, 13.4. Found: C, 56.0; H, 6.61; N, 13.1.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (X). (a) By the *p*-Nitrophenyl Ester Method.—Compound IX (3.45 g., 3 mmoles) was dissolved in 100 cc. of methanol and subjected to catalytic hydrogenolysis as described. After CO_2 evolution had ceased, the catalyst was removed by filtration and washed with methanol, and the filtrate and washings were evaporated to dryness. The residue was dissolved in 60 cc. of acetonitrile and treated with N^{α}-carbobenzoxy-N^{ϵ}-tosyl-lysine *p*-nitrophenyl ester[14] for 3 days at room temperature. The solvent was removed *in vacuo*, the residue dissolved in 100 cc. of wet ethyl acetate, and washed with 5% sodium bicarbonate and water. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was further purified by countercurrent distribution in the toluene system for 100 transfers. The peak with K = 0.3 was isolated to yield 3.5 g. (78.3%) of X. The peptide was found to be homogeneous in paper chromatography in two solvents: $R_{f\ BAW}$ 0.90, $R_{f\ SBA}$ 0.92, m.p. 110-115°, $[\alpha]^{25}D$ −30.9° (*c* 1, methanol).

(b) By the Azide Procedure.—N^{α}-Carbobenzoxy-N^{ϵ}-tosyl-lysyl-N^{ϵ}-tosyl-lysine hydrazide,[9] 1.6 g., 2.2 mmoles, was dissolved in a mixture of 6 cc. of glacial acetic acid and 10 cc. of 1 *N* hydrochloric acid. The solution was cooled to −2° and 0.14 g. g. (2 mmoles) of sodium nitrite was added slowly while stirring vigorously with a Vibro-mixer.[28] Stirring was continued at 0° for 30 min. Ice-cold water (30 cc.) was added and the azide extracted into precooled ethyl acetate (3 × 20 cc.). The ethyl acetate layer was washed in the cold room (4°) with cold 5% sodium bicarbonate until neutral and then with water. The organic layer was dried over anhydrous sodium sulfate and filtered into a solution of 1.6 g. (2 mmoles) of V in 10 cc. of ice-cold dimethylformamide. The mixture was stirred at 4° for 2 days and then at room temperature for 1 day. The solvent was removed *in vacuo* and the residue purified by countercurrent distribution in the toluene system. A major peak with K = 0.3 and a small peak with K = 2.8 were seen. The peak with the K of 0.3 was isolated, and the residue was dissolved in methanol and precipitated into ether to yield 2.3 g. (77.1%) of X, m.p. 110–115°, $[\alpha]^{25}D$ −28.7 (*c* 1, methanol). Compound X was found to be homogeneous in paper chromatography in two solvents.

Anal. Calcd. for $C_{69}H_{95}N_{13}S_4O_{16}$ (1491): C, 55.6; H, 6.42; N, 12.2; S, 8.60. Found: C, 55.4; H, 6.54; N, 12.1; S, 8.69.

N^{ϵ}-Tosyl-lysyl-N^{ϵ}-tosyl-lysyl-N^{G}-tosyl-arginyl-N^{G}-tosyl-arginyl-proline *t*-Butyl Ester (XI).—Compound X, 2 g., was dissolved in 40 cc. of methanol and hydrogenolyzed in the presence of Pd freshly prepared from 1 g. of $PdCl_2$ until no more CO_2 evolved (8 hr.). The catalyst was removed by filtration and washed with methanol, and the filtrate and washings were evaporated to dryness. The residue was purified by countercurrent

distribution in the toluene system for 100 transfers. A single peak with $K = 0.94$ was observed. The material isolated from this peak behaved as a single component in paper chromatography: $R_{f\,BAW}$ 0.77, $R_{f\,SBA}$ 0.79, yield 1.6 g. (87.9%), m.p. 104–110°, $[\alpha]^{25}D$ −30.0° (*c* 1, methanol).

Anal. Calcd. for $C_{61}H_{89}N_{13}S_4O_{14}$ (1357): C, 54.0; H, 6.61; N, 13.4. Found: C, 54.2; H, 6.69; N, 13.7

Carbobenzoxy-seryl-tyrosyl-seryl-methionyl-γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycyl-N^ϵ-*t*-butyloxycarbonyl-lysyl-N^ϵ-*t*-butyloxycarbonyl-lysyl-N^G-tosyl-arginyl-N^G-tosyl-arginyl-proline *t*-Butyl Ester (XIII).—The decapeptide[9] XII (0.35 g., 200 mmoles) was dissolved in 2 cc. of dimethylformamide, and the solution was cooled in ice and stirred with 0.1 cc. of triethylamine. Compound II (0.076 g., 200 mmoles) was added and stirring was continued for 1 hr. at 0°. Compound VIII (0.25 g., 200 mmoles) was then added and the mixture stirred at room temperature for 24 hr. The reaction mixture was then kept at 37° for another 24 hr. The solvent was removed *in vacuo* at 40°, and the residue was dissolved in the lower phase of the carbon tetrachloride system and submitted to countercurrent distribution for 70 transfers. The material in tubes 0–20 was pooled, evaporated to dryness, washed with water, and triturated with methanol to yield 0.305 g. of XIII, which, however, was contaminated with traces of ninhydrin-positive material (unreacted VIII). A sample of XIII was prepared for analysis by exhaustive washing with methanol until all traces of VIII were removed; $[\alpha]^{25}D$ −51.5° (*c* 2.4, dimethylformamide).

Anal. Calcd. for $C_{138}H_{187}N_{29}S_4O_{33}$ (2908): C, 57.0; H, 6.48; N, 14.0. Found: C, 56.6; H, 6.17; N, 13.8.

Seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-lysyl-arginyl-arginyl-proline (XV).—Compound XIII (0.305 g.) was dissolved in 15 cc. of trifluoroacetic acid in a nitrogen atmosphere and kept at room temperature for 1.5 hr. The trifluoroacetic acid was removed *in vacuo* at room temperature and the residue was dried overnight over P_2O_5–NaOH. The residue was then submitted to countercurrent distribution in the system composed of 1-butanol–acetic acid–water (4:1:5, by volume) for 80 transfers. A fast moving peak, $K = 6.3$ (ultraviolet, Pauly and ninhydrin positive), and a slower moving peak, $K = 0.7$ (ninhydrin positive, ultraviolet and Pauly negative), were seen. The peak with $K = 6.3$ (XIV) was isolated and found homogeneous in paper chromatography; $R_{f\,BAW}$ 0.66, wt. 0.175 g. (yield based on XII is 33%).

Compound XIV (0.16 g.) was dissolved in 200 cc. of freshly distilled liquid ammonia and treated with small pieces of sodium until the blue color persisted for 30–40 min. The ammonia was allowed to evaporate and the residue dried *in vacuo* over P_2O_5–concentrated H_2SO_4. The peptide was desalted[9] on IRC-50 cation exchange resin and eluted with pyridine–acetic acid–water (30:4:66, by volume). The solvent was removed *in vacuo* at room temperature and the residue was dissolved in water and lyophilized to yield 0.11 g. of crude XV. This material was purified by chromatography on a carboxymethylcellulose column (1 × 55 cm.) using continuous gradient elution with ammonium acetate (Fig. 3). The major peak was isolated and rechromatographed on CMC (Fig. 4) to yield, after three lyophilizations, 71 mg. of XV (peptide content determined by ultraviolet absorption at 280 mμ, 87%). Compound XV was found to be homogeneous by electrophoresis on paper at pH 3.7 (mobility relative to lysine, 0.81) and on polyacrylamide gel at pH 4.5 (see Fig. 5). Amino acid analysis is given in Table II; $[\alpha]^{25}D$ −56.8 (*c* 0.5, 0.1 *M* acetic acid).

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A New Synthesis of a Biologically Active Nonadecapeptide Corresponding to the First Nineteen Amino Acid Residues of Adrenocorticotropins

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A completely new method for the synthesis of a nonadecapeptide with an amino acid sequence corresponding to the first nineteen residues of ACTH has been described. The biological and chemical properties of the product are in agreement with those of the synthetic peptide reported by us in 1960.

The synthesis of the nonadecapeptide seryl-tyrosyl-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valyl-glycyl-lysyl-lysyl-arginyl-arginyl-proline,[1,2] in 1960, not only served to establish the structural features of the ACTH molecule essential for eliciting adrenal response, but also proved conclusively that the melanocyte-stimulating activity exhibited by the adrenocorticotropins is an intrinsic property. Subsequent work from our laboratory[3,4] as well as the efforts of other investigators[5–7] has shown that the nonadecapeptide corresponding to the first nineteen residues of ACTH probably represents the active core of this hormone. Hence it appeared important to synthesize this peptide by a completely different route and obtain confirmation of the biological properties previously observed.[1]

In our original synthesis, the final steps involved the reaction of the NH_2-terminal tetrapeptide with the C-terminal pentadecapeptide by the azide procedure. With the availability of the crystalline NH_2-terminal decapeptide carbobenzoxy-seryl-tyrosyl-seryl-methionyl γ-benzyl-glutamyl-histidyl-phenylalanyl-N^G-tosyl-arginyl-tryptophyl-glycine[8] (V), the protected nonadecapeptide was prepared by the reaction with a suitably protected nonapeptide derivative.

Two different derivatives of the nonapeptide were synthesized. In one, the ε-amino groups of lysine were blocked with the *t*-butyloxycarbonyl group and the guanidino functions of the arginine residues were masked with the *p*-toluenesulfonyl group. The protected nonapeptide N^α-carbobenzoxy-N^ϵ-*t*-butyloxycarbonyl-lysyl-prolyl-valyl-glycyl-N^ϵ-*t*-butyloxycarbonyl-lysyl-N^ϵ-*t*-butyloxycarbonyl-lysyl-N^G-tosyl-ar-

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