Adrenocorticotropins. XXXIII. Synthesis of a Biologically Active Hexacosapeptide Corresponding to the First 26 Residues of Bovine ACTH[1]

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The synthesis of a biologically active hexacosapeptide, Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asp-Gly, which has an amino acid sequence identical with the first 26 residues from the NH_2 terminus of bovine adrenocorticotropin (α_b-ACTH), is described. The synthetic product possesses adrenal-stimulating activity comparable with that of the naturally occurring hormone on a molar basis. The melanocyte-stimulating activity of the synthetic peptide is also comparable to that of the native ACTH on a molar basis.

During the course of the synthetic investigations[2,3] of the relationship of the structure of adrenocorticotropin (ACTH)[4] to its biological activities, it became apparent that the structural features essential for the manifestation of the hormonal activities reside in the amino terminal half of the molecule. Thus, the synthetic nonadecapeptide[1,5] corresponding to the first 19 residues of ACTH was found to exhibit all the biological activities associated with the natural hormone, although in varying degrees. Whereas the nonadecapeptide was found to be as potent as ACTH in its ability to darken the skin of amphibians, the adrenal-stimulating potency of the synthetic peptide was found to be less than half that exhibited by the natural hormone on a molar basis, when measured *in vitro* as well as *in vivo*. Peptides corresponding to the first 20,[6] 23,[7] and 24[8] amino acid residues of ACTH have been synthesized in other laboratories. However, it has not been possible to compare the biological activities of these latter peptides with those synthesized in our laboratory because of the differences in the assay procedures employed. In order to obtain a more reliable picture of the relationship of chain length to the biological activities of ACTH, the various synthetic peptides must be compared with the highly purified natural hormone by the same bioassay procedures under identical conditions. To achieve this aim, we have now synthesized a hexacosapeptide corresponding to the first 26 NH_2-terminal residues of beef ACTH,[9] in order to provide a peptide of intermediate chain length between the nonadecapeptide and the native hormone. This paper describes the details of the synthesis of this peptide, namely, seryltyrosylseryl-methionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginyl-prolylvalyllysylvalyltyrosylprolylaspartylglycine.[10]

The steps involved in the synthesis of the hexacosapeptide are shown in Figure 1. Carbobenzoxy-β-*t*-butylaspartylglycine *t*-butyl ester was synthesized from carbobenzoxy-β-*t*-butylaspartic acid[11] and glycine *t*-butyl ester[12] by the use of N-ethyl-5-phenylisoxazolium 3′-sulfonate[13] (NEPIS) (III). The dipeptide, however, was obtained as an oil and could not be crystallized. Hence, it was hydrogenolyzed to remove the carbobenzoxy group and the product was isolated as the crystalline hydrochloride. The dipeptide *t*-butyl ester hydrochloride (IV) was obtained in 88% over-all yield.

Carbobenzoxyvalyl-N$^{\epsilon}$-tosyllysylvalyltyrosylproline (II) was obtained by saponification of the protected pentapeptide benzyl ester[14] (I). After purification by countercurrent distribution in the toluene system, II was isolated in 84% yield and was found to be homogeneous in paper chromatography. Peptide II was activated by the use of III and allowed to react with the dipeptide *t*-butyl ester IV. The heptapeptide, carbobenzoxyvalyl-N$^{\epsilon}$-tosyllysylvalyltyrosylprolyl-β-*t*-butylaspartylglycine *t*-butyl ester (V), was isolated and purified by countercurrent distribution in the toluene system. After 100 transfers V could be isolated from the peak with $K = 0.45$ and crystallized readily. Peptide V was obtained in 51% yield.

The protected nonapeptide N$^{\alpha}$-carbobenzoxy-N$^{\epsilon}$-tosyllysylprolylvalylglycyl-N$^{\epsilon}$-tosyllysyl-N$^{\epsilon}$-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline (VI) was readily obtained by treating the corresponding *t*-butyl ester with trifluoroacetic acid. The synthesis of the nonapeptide *t*-butyl ester has already been described.[5] Peptide VI, which was obtained in high yield (95%), was found to be homogeneous by the criteria of paper chromatography in two solvents and by elemental analysis.

The next step in the synthesis involved the linking of VI to the heptapeptide base Va. For this purpose V

(1) For papers XXX, XXXI, and XXXII of this series, see *J. Am. Chem. Soc.*, **86**, 2703–2718 (1964).

(2) (a) C. H. Li, J. Meienhofer, E. Schnabel, D. Chung, T-B. Lo, and J. Ramachandran, *ibid.*, **82**, 5760 (1960); **83**, 4449 (1961); (b) C. H. Li, J. Ramachandran, D. Chung, and B. Gorup, *ibid.*, **84**, 2460 (1962); **86**, 2703 (1964).

(3) C. H. Li, J. Ramachandran, and D. Chung, *ibid.*, **85**, 1895 (1963); **86**, 2711 (1964).

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

(5) C. H. Li, D. Chung, and J. Ramachandran, *J. Am. Chem. Soc.*, **86**, 2715 (1964).

(6) K. Hofmann, H. Yajima, T-Y. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *ibid.*, **84**, 1054 (1962); **84**, 4481 (1962).

(7) K. Hofmann, H. Yajima, T-Y. Liu, and N. Yanaihara, *ibid.*, **83**, 487 (1961); **84**, 4475 (1962).

(8) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1136 (1961); **46**, 1550 (1963).

(9) C. H. Li, J. S. Dixon, and D. Chang, *J. Am. Chem. Soc.*, **80**, 2587 (1958).

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

(11) R. Schwyzer and H. Dietrich, *Helv. Chim. Acta*, **44**, 2003 (1961).

(12) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

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

(14) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **28**, 173 (1963).

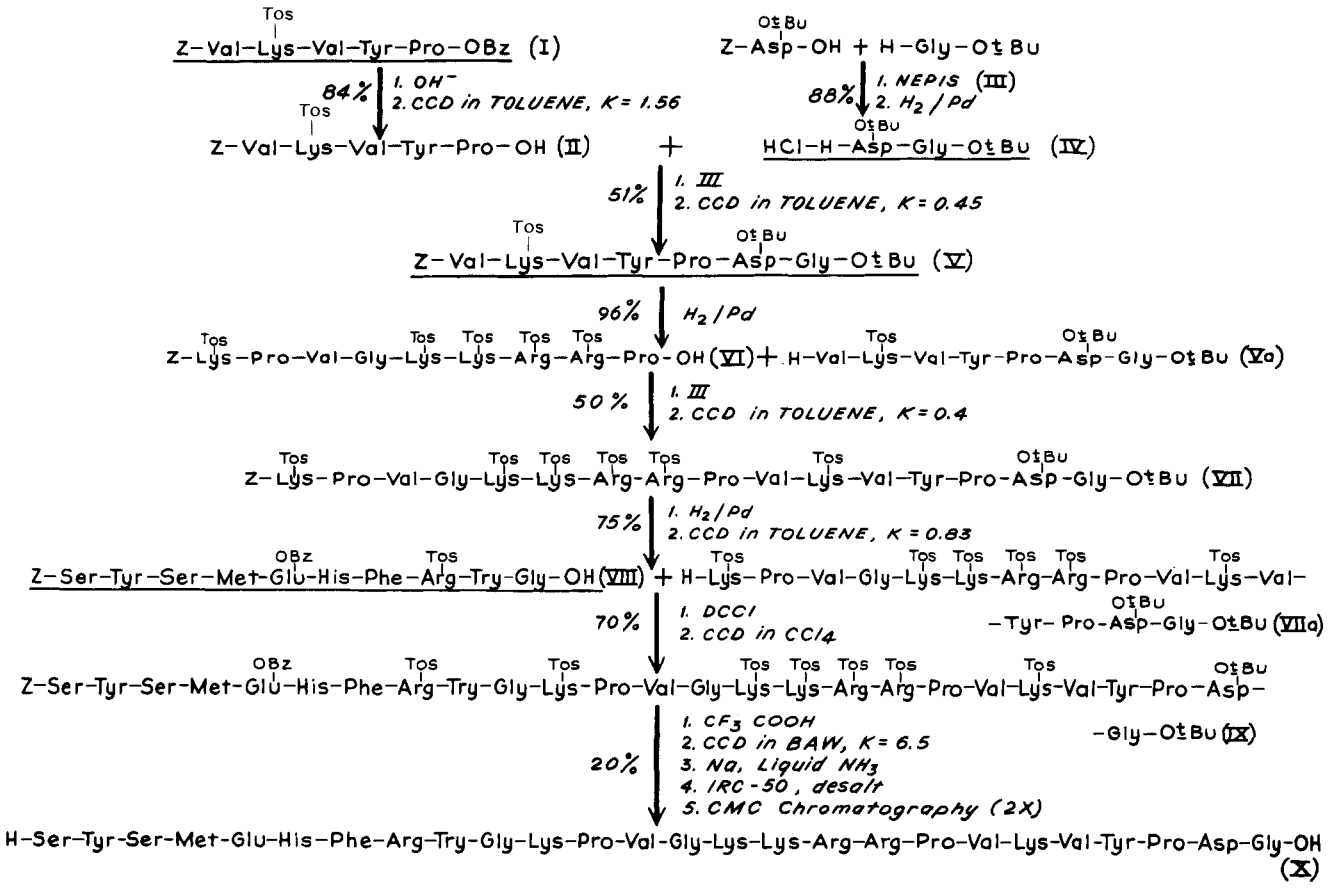

Figure 1. Synthetic scheme of the hexacosapeptide (X): Z, carbobenzoxy; TOS, *p*-toluenesulfonyl; Bz, benzyl; *t*-Bu, *t*-butyl.

was catalytically hydrogenolyzed to yield Va. The nonapeptide VI was activated, again by the use of III, and allowed to react with Va. Subsequent isolation and purification by countercurrent distribution, especially in the toluene system, has been of immense value in obtaining these long chain peptide intermediates in a state of high purity. The carbobenzoxy group was cleaved from the hexadecapeptide by means of catalytic hydrogenolysis. The product VIIa was also submitted to countercurrent distribution in the toluene system. Peptide VIIa exhibited a partition coefficient (K) of 0.83 in this system, whereas VII has a K of 0.4.

The final steps of the synthesis involved the reaction of the protected amino terminal decapeptide, carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^G-tosylarginyltryptophylglycine[2] (VIII), with VIIa. This was achieved by the use of dicyclohexylcarbodiimide.[15] The fully protected hexacosapeptide was isolated from this reaction and partially purified by countercurrent distribution in the system composed of carbon tetrachloride–chloroform–methanol–0.01 M ammonium acetate (1:3:3:1). The product was obtained in 70% yield. However, this material was contaminated with the acylurea[16] of the decapeptide which could be conveniently removed during the purification of the deblocked peptide. The *t*-butyl ester groups masking the carboxyl-terminal glycine and the β-carboxyl of aspartic acid were removed by treatment with trifluoroacetic acid. At this stage the partially deblocked hexacosapeptide was submitted to countercurrent distribution in the system composed of 1-butanol–acetic acid–water (4:1:5). A single peak with $K = 6.5$ was seen; the acylurea of the decapeptide could not be removed at this stage. The remaining protecting groups, namely, seven tosyl groups, one carbobenzoxy group, and a benzyl group, were removed by treatment with sodium in liquid ammonia.[17] The product was desalted on IRC-50 resin[18] and then purified by chromatography on carboxymethylcellulose[19] using gradient elution with ammonium acetate. The results are shown in Figure 2A. Peak B was found to correspond to the required hexacosapeptide by amino acid analysis. The material from peak B was isolated and rechromatographed on carboxymethylcellulose. The hexacosapeptide X was isolated after rechromatography and found to be homogeneous by electrophoresis on paper. The amino acid composition of the synthetic hexacosapeptide after acid hydrolysis for 20 and 40 hr., determined by the chromatographic method,[20] is given in Table I and is seen to be in good agreement with the expected values.

Peak A was found to have the amino acid composition similar to that of the decapeptide; this contaminant

(15) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, 77, 1067 (1955).
(16) H. G. Khorana, *Chem. Ind.* (London), 1087 (1955).
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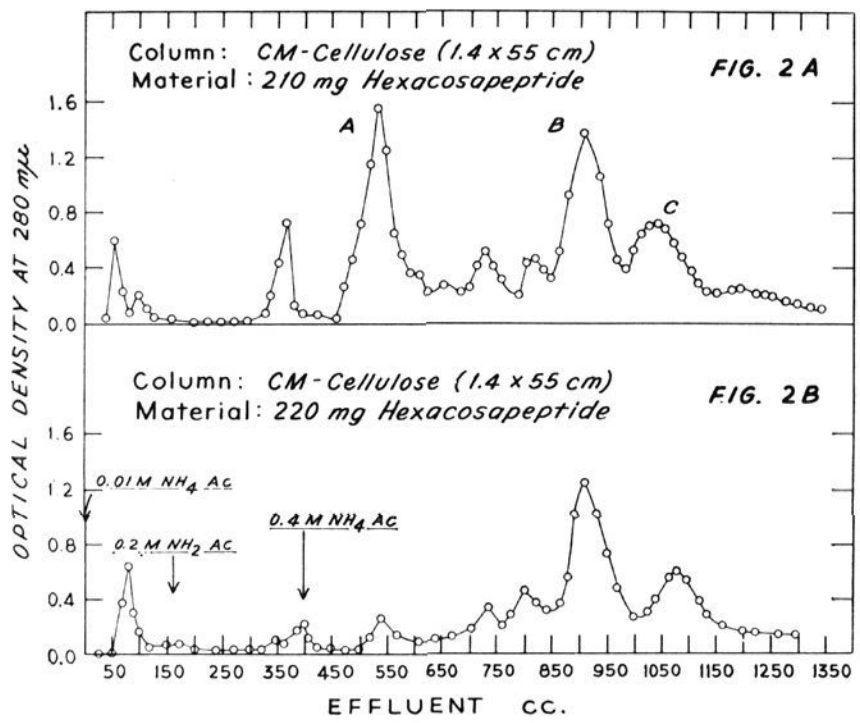

Figure 2. Carboxymethylcellulose chromatography of the hexacosapeptide (X). The initial buffer was 0.01 *M* ammonium acetate, pH 4.5. After 3–4 hold-up volumes had been collected, a gradient with respect to pH and concentration of salt was started by introducing 0.2 *M* ammonium acetate buffer of pH 6.7 through a 500-cc. 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.

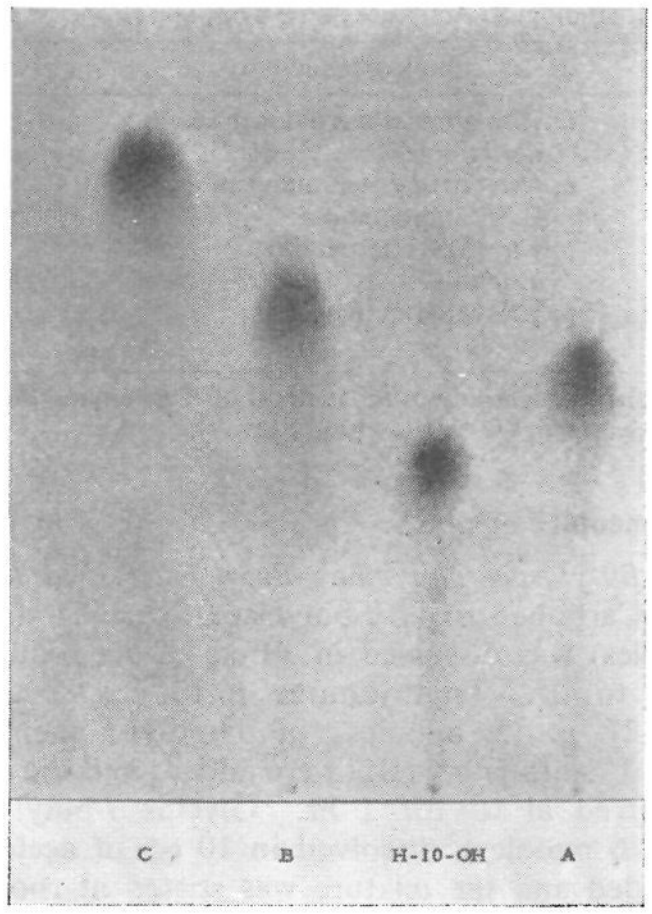

Figure 3. Electrophoresis of peaks A, B, and C, and the N-terminal decapeptide (Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly) on Whatman No. 3 filter paper in pyridine acetate buffer, pH 3.7, at 400 volts for 6 hr. The peptides were revealed by the ninhydrin reagent.

is probably the acylurea of the decapeptide. In paper electrophoresis the material from peak A moved faster than an authentic sample of the decapeptide. Further proof that peak A is a contaminant derived from the decapeptide was obtained by repeating the synthesis of the hexacosapeptide using N-ethyl-5-phenylisoxazolium 3′-sulfonate (III) in the final condensation step. During the countercurrent distribution in the carbon tetrachloride system, all the by-products resulting from the condensing agent III are conveniently removed from the protected hexacosapeptide because of their high *K* values in this solvent system. When IX prepared in this manner was deblocked by treatment with trifluoroacetic acid followed by sodium–liquid ammonia reduction, and the product was desalted and chromatographed on carboxymethylcellulose, the pattern shown in Figure 2B was obtained. It is apparent that peak A is missing. Peak C which appears in both cases was obtained in apparently homogeneous form after rechromatography on carboxymethylcellulose. Amino acid analysis indicated that all the amino acids found in the hexacosapeptide were present, although aspartic acid, glycine, tyrosine, lysine, and valine were low.[21] Paper electrophoresis at pH 3.7 showed that C migrates faster toward the cathode than B (Figure 3).

Table I. Amino Acid Composition of the Synthetic Hexacosapeptide

Amino acid	Theor.	Chromatographic 20 hr.	Chromatographic 40 hr.
Serine	2	1.66	1.70
Tyrosine	2	1.83	1.78
Methionine	1	0.70	0.57
Glutamic acid	1	1.00	1.00
Histidine	1	0.94	0.96
Phenylalanine	1	1.11	1.02
Tryptophan	1	1.03[a]	..
Arginine	3	3.01	3.12
Glycine	3	2.97	2.70
Lysine	4	3.66	3.76
Proline	3	2.97	2.85
Valine	3	2.93	2.88
Aspartic acid	1	0.86	0.88

[a] Determined spectrophotometrically.

The hexacosapeptide X was assayed for adrenal-stimulating potency *in vitro* by a modification of the Saffran and Schally procedure[22] and *in vivo* by the method of Lipscomb and Nelson.[23,24] It can be seen from Table II that, on a molar basis, the steroidogenic activity of the hexacosapeptide is comparable to that of the native hormone and higher than that of the nonadecapeptide.[5] The melanocyte-stimulating activity of X, *in vitro*[25a] and *in vivo*,[25b] was also found to be comparable to that exhibited by ACTH. These results are discussed in more detail in the following paper.

(21) It is not known whether the lower amounts of these amino acids in C is the result of treatment with trifluoroacetic acid or due to the sodium–liquid ammonia reduction. The pentapeptide valyl-N$^{\epsilon}$-tosyl-lysylvalyltyrosylproline has been previously deblocked[14] by treatment with sodium in liquid ammonia in 69% yield and the product was obtained directly in homogeneous form without recourse to chromatography. Peak C is being further investigated.

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(23) H. S. Lipscomb and D. H. Nelson, *ibid.*, **71**, 13 (1962).

(24) The assays *in vivo* were kindly performed by Dr. G. W. Liddle and associates at Vanderbilt University.

(25) (a) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954); (b) L. T. Hogben and D. Slome, *Proc. Roy. Soc.* (London), **B108**, 10 (1931).

Table II. Biological Activities of the Synthetic Hexacosapeptide

Biological activity	$\alpha_b{}^{1-26}$-ACTH	α_s-ACTH
in vitro adrenal steroidogenesis,[22] I.U./μmole	279 (369–210)	618 (917–418)
in vivo adrenal stereoidogenesis,[23,24] U.S.P. units/μmole	390 (237–636)	481 (318–722)
in vitro MSH activity,[25a] u./μmole	4.8×10^5	2.9×10^6
in vivo MSH activity,[a] μmole	6.6×10^{-5}	4.4×10^{-5}

[a] Performed according to the method of Hogben and Slome[25b]; the dose produces a change in melanophore index in hypophysectomized *Rana pipiens* from 1+ to 3+ within 1 hr.

Experimental[26]

β-t-Butylaspartylglycine t-Butyl Ester Hydrochloride (IV). Carbobenzoxy-β-*t*-butylaspartic acid[11] (1.62 g., 5 mmoles) was dissolved in 30 cc. of acetonitrile and cooled to 0°. Triethylamine (0.70 cc., 5 mmoles) and 1.27 g. (5 mmoles) of N-ethyl-5-phenylisoxazolium 3′-sulfonate[13] (III) were added, and the mixture was stirred at 0° for 1 hr. Glycine *t*-butyl ester[12] (0.8 g., 6 mmoles), dissolved in 10 cc. of acetonitrile, was added and the mixture was stirred at room temperature for 16 hr. The solvent was removed *in vacuo*, the residue was again dissolved in wet ethyl acetate (40 cc.), and the solution was washed successively with water, ice-cold 0.1 *N* HCl, water, 5% sodium bicarbonate, and water. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The protected dipeptide ester was obtained as an oil which was found to be homogeneous in paper chromatography in BAW and SBA; 2.1 g.

This oil was dissolved in 30 cc. of ethyl acetate and was submitted to catalytic hydrogenolysis in the presence of Pd freshly prepared[31] from 1 g. of $PdCl_2$ in the apparatus described by Meienhofer.[30] The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The dipeptide base was found to be homogeneous in paper chromatography, $R_{f_{BAW}}$ 0.68. This material was redissolved in 20 cc. of ethyl acetate and 2.5 cc. of a solution of 2 *N* HCl in ethyl acetate (5 mmoles) was added. The solvent was again removed *in vacuo*, the residue was dissolved in ether (30 cc.), and enough petroleum ether was added to make the solution faintly cloudy. The hydrochloride of the dipeptide *t*-butyl ester crystallized upon standing; 1.5 g. (88%), m.p. 126–128°, $[\alpha]^{25}D$ 9.3° (*c* 1, methanol).

Anal. Calcd. for $C_{14}H_{26}N_2O_5Cl$ (338.8): C, 49.6; H, 8.04; N, 8.27. Found: C, 49.8; H, 7.87; N, 8.41.

Carbobenzoxyvalyl-N$^\epsilon$-tosyllysylvalyltyrosylproline (II). Carbobenzoxy-valyl-N$^\epsilon$-tosyllysylvalyltyrosylproline benzyl ester[14] (1.97 g., 2 mmoles) was dissolved in 30 cc. of methanol and stirred at room temperature with 10 cc. of 2 *N* sodium hydroxide (20 mmoles) for 90 min. The solution was diluted with 100 cc. of water, filtered free from traces of insoluble material, and acidified to pH 2 with 6 *N* hydrochloric acid. The precipitate was kept at 4° for 2 hr., filtered, washed with water, and dried to yield 1.7 g. of white powder, m.p. 125–130°. This powder was submitted to countercurrent distribution in the toluene system. A large peak with $K = 1.56$ and a very small peak with a lower *K* value (0.59) were seen after 100 transfers. The material in the major peak was isolated to yield 1.5 g. (84%) of II; m.p. 133–135°; $[\alpha]^{25}D$ −52.5° (*c* 1, methanol); $R_{f_{BAW}}$ 0.88; $R_{f_{SBA}}$ 0.49.

Anal. Calcd. for $C_{45}H_{60}N_6O_{11}S$ (893.0): C, 60.5; H, 6.77; N, 9.41; S, 3.59. Found: C, 60.1; H, 6.71; N, 9.34; S, 3.73.

Carbobenzoxyvalyl-N$^\epsilon$-tosyllysylvalyltyrosylprolyl-β-t-butylaspartylglycine t-Butyl Ester (V). Peptide II (1.34 g., 1.5 mmoles) was dissolved in a mixture of 40 cc. of acetonitrile and 10 cc. of dimethylformamide and the solution was cooled to 0°. Triethylamine (0.21 cc., 1.5 mmoles) and III (0.38 g. 1.5 mmoles) were added and the mixture was stirred at 0° for 1 hr. Meanwhile, IV (0.68 g., 2 mmoles) was suspended in 50 cc. of a mixture of ethyl acetate and ether (1:1) and stirred at room temperature for 20 min. with 0.28 cc. (2 mmoles) of triethylamine. The precipitate of triethylamine hydrochloride was filtered and washed with ether. The filtrate and washings were evaporated to dryness *in vacuo* at 20°. The residue was dissolved in 5 cc. of acetonitrile and added to the activated reaction mixture. Stirring was continued at room temperature for 48 hr. The solvent was removed *in vacuo* and the residue was purified by countercurrent distribution in the toluene system. After 100 transfers the material in the peak with $K = 0.45$ was isolated to yield V, which crystallized when triturated with ethyl acetate; 0.9 g. (51%); m.p. 211–213°; $[\alpha]^{24}D$ −37.0° (*c* 1, dimethylformamide). Peptide V was found to be homogeneous in paper chromatography, $R_{f_{BAW}}$ 0.90; $R_{f_{SBA}}$ 0.91.

Anal. Calcd. for $C_{59}H_{84}O_{15}N_8S$ (1177): C, 60.2; H, 7.19; N, 9.52. Found: C, 59.9; H, 7.16; N, 9.45.

Valyl-N$^\epsilon$-tosyllysylvalyltyrosylprolyl-β-t-butylaspartylglycine t-Butyl Ester (Va). Peptide V (1.18 g., 1 mmole) was dissolved in 40 cc. of dimethylformamide and submitted to catalytic hydrogenolysis in the

(26) 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. 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 and 2-butanol–10% ammonia (SBA) in a ratio of 85:15, both by volume. Peptide spots were located by the ninhydrin reagent, chlorine method,[27] Pauly reagent,[28] and Ehrlich reagent.[29] Countercurrent distribution was performed in the toluene system (toluene–chloroform–methanol–water, 5:5:8:2 by volume). Catalytic hydrogenolysis was performed in the apparatus described by Meienhofer[30] and the completion of hydrogenolysis was checked by paper chromatography.

(27) H. Zahn and E. Rexroth, *Z. Anal. Chem.*, **148**, 181 (1955).

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

(30) J. Meienhofer, *Chimia*, **16**, 385 (1962).

(31) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54**, 128 (1921).

presence of Pd freshly prepared[31] from 1 g. of $PdCl_2$ until the evolution of CO_2 ceased. The catalyst was filtered off and washed with dimethylformamide, and the filtrate and washings were evaporated to dryness *in vacuo*. The residue was dissolved in methanol (15 cc.) and precipitated from 300 cc. of ether to yield 1.0 g. of Va (96%); m.p. 108–112°; $[\alpha]^{25}D$ −62° (*c* 1, methanol). Peptide Va was found to be homogeneous in paper chromatography, R_{fBAW} 0.85; R_{fsBA} 0.90.

Anal. Calcd. for $C_{51}H_{78}N_8O_{13}S$ (1043): C, 58.8; H, 7.54; N, 10.8. Found: C, 58.1; H, 7.46; N, 10.8.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysylprolylvalylglycyl-N^{ϵ}-tosyllysyl-N^{ϵ}-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline (VI). N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysylprolylvalylglycyl-N^{ϵ}-tosyllysyl-N^{ϵ}-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylproline *t*-butyl ester[5] (1.4 g., 0.69 mmole) was dissolved in 20 cc. of anhydrous trifluoroacetic acid and kept at room temperature for 90 min. The solvent was removed *in vacuo* and the residue was dried overnight over P_2O_5 and NaOH. The residue was then dissolved in 10 cc. of methanol and precipitated from 400 cc. of ether to yield 1.3 g. (95%) of VI, m.p. 126–132°; $[\alpha]^{25}D$ −27.8° (*c* 0.5, dimethylformamide). Peptide VI was found to be homogeneous in paper chromatography in two solvents: R_{fBAW} 0.88; R_{fsBA} 0.56.

Anal. Calcd. for $C_{90}H_{127}N_{18}O_{22}S_5$ (1973): C, 54.8; H, 6.49; N, 12.8. Found: C, 54.6; H, 6.46; N, 12.6.

N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysylprolylvalylglycyl-N^{ϵ}-tosyllysyl-N^{ϵ}-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylprolylvalyl-N^{ϵ}-tosyllysylvalyltyrosylprolyl-β-t-butylaspartylglycine t-Butyl Ester (VII). Compound VI (1.2 g., 0.6 mmole) was dissolved in 10 cc. of dimethylformamide and cooled to 0°. Triethylamine (0.1 cc., 0.7 mmole) and III (0.18 g., 0.7 mmole) were added and the mixture was stirred at 0° for 1 hr. Peptide Va (0.73 g., 0.7 mmole) was added and the reaction mixture was stirred at room temperature for 24 hr., and then kept at 37° for another 36 hr. The solvent was removed *in vacuo* and the residue was submitted to countercurrent distribution in the toluene system. After 100 transfers the material from the peak with K = 0.4 was isolated to yield 1.08 g. (50%) of VII; m.p. 132–136°; $[\alpha]^{25}D$ −30.4° (*c* 1, dimethylformamide). Peptide VII was found to be homogeneous in paper chromatography to two solvent systems; R_{fBAW} 0.85; R_{fsBA} 0.83.

Anal. Calcd. for $C_{141}H_{203}N_{26}O_{34}S_6$ (2999): C, 56.5; H, 6.82; N, 12.1. Found: C, 56.3; H, 6.89; N, 11.8.

N^{ϵ}-Tosyllysylprolylvalylglycyl-N^{ϵ}-tosyllysyl-N^{ϵ}-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylprolylvalyl-N^{ϵ}-tosyllysylvalyltyrosylprolyl-β-t-butylaspartylglycine t-Butyl Ester (VIIa). Peptide VII (0.85 g., 0.28 mmole) was dissolved in 30 cc. of dimethylformamide and hydrogenolyzed in the presence of Pd freshly prepared from 1 g. of $PdCl_2$ until the evolution of CO_2 ceased (8 hr.). The catalyst was filtered off and washed with dimethylformamide, and the filtrate and washings were evaporated to dryness *in vacuo*. The residue was subjected to countercurrent distribution in the toluene system for 100 transfers. A single peak with K = 0.83 was observed. The material from this peak was pooled, evaporated to dryness, redissolved in methanol (10 cc.), and precipitated into 300 cc. of ether to yield 0.6 g. of VIIa (75%) as a white powder, m.p. 128–132°; R_{fBAW} 0.83; R_{fsBA} 0.81.

Carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^{G}-tosylarginyltryptophylglycyl-N^{ϵ}-tosyllysylprolylvalylglycyl-N^{ϵ}-tosyllysyl-N^{ϵ}-tosyllysyl-N^{G}-tosylarginyl-N^{G}-tosylarginylprolylvalyl-N^{ϵ}-tosyllysylvalyltyrosylprolyl-β-t-butylaspartylglycine t-Butyl Ester (IX). (a) via the DCCI Method. The decapeptide VIII[2b] (0.168 g., 0.1 mmole) and the hexadecapeptide VIIa (0.235 g., 0.082 mmole) were dissolved in 2 cc. of dimethylformamide and cooled to 0°. DCCI (0.021 g., 0.1 mmole) was added and the mixture was stirred at 4°. After 24 hr., more DCCI (0.021 g.) was added. The reaction mixture was kept at 4° for 3 more days. Crystals of dicyclohexylurea were removed by filtration and washed with dimethylformamide. The filtrate and washings were evaporated to dryness and the residue was purified by countercurrent distribution in the system composed of carbon tetrachloride–chloroform–methanol–0.01 ammonium acetate, pH 4.5 (1:3:3:1). After 100 transfers, the contents of tubes 0–13 were pooled, evaporated to dryness, washed with water, triturated with methanol, filtered, and dried to yield 0.26 g. of IX (70%).

(b) By the Use of N-Ethyl-5-phenylisoxazolium 3′-Sulfonate. Peptide VIII (0.168 g., 0.1 mmole) was dissolved in 2 cc. of dimethylformamide, and the solution was cooled to 0° and stirred with 0.03 cc. of triethylamine and 0.04 g. of III for 1 hr. Peptide VIIa (0.285 g., 0.1 mmole) was added and the mixture was stirred for 24 hr. at room temperature and 96 hr. at 37°. The solvent was then removed *in vacuo* and the residue was purified by countercurrent distribution for 100 transfers in the carbon tetrachloride system mentioned above. Tubes 0–20 were pooled and worked up to yield 0.36 g. of IX.

Seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvalyllysylvalyltyrosylprolylaspartylglycine (X). The protected hexacosapeptide IX from the DCCI reaction (0.26 g.) was dissolved in 15 cc. of trifluoroacetic acid and kept at room temperature for 90 min. The solvent was then removed *in vacuo* and the residue was dried over P_2O_5 and NaOH. The product was submitted to countercurrent distribution in the system composed of 1-butanol–acetic acid–water (4:1:5) for 90 transfers. The material in the single peak with K = 6.5 was pooled and the solvent was removed *in vacuo*. The residue was dissolved in dimethylformamide and then evaporated to dryness again *in vacuo* at 40°. The glassy residue was dried overnight over P_2O_5 *in vacuo*. This material was then dissolved in 200 cc. of liquid ammonia freshly distilled from sodium and treated with small pieces of sodium until the blue color was maintained for 30 min. without further addition of sodium. About 0.08 g. of sodium was required. Ammonia was allowed to evaporate and the residue was freed of all traces of ammonia *in vacuo* over concentrated H_2SO_4 and P_2O_5. The residue was dissolved in 15 cc. of ice-cold 10% acetic acid and desalted on IRC-50 resin. The peptide was eluted with pyridine–6% acetic acid (3:7). The solvent was removed *in vacuo* at 30° and the peptide

was dissolved in water and lyophilized to yield 0.21 g. of material, which was then purified by chromatography on carboxymethylcellulose using gradient elution with ammonium acetate. The conditions of chromatography were the same as described previously.[2b,3,5]

Three peaks, A, B, and C (Figure 2A), were isolated. Peak A (0.02 g.) was identified as the acylurea of the N-terminal decapeptide on the basis of electrophoretic mobility (Figure 3) and the amino acid composition of an acid hydrolysate: $Ser_{1.8}Tyr_{1.13}Met_{1.02}Glu_{1.00}His_{0.91}Phe_{1.01}Arg_{0.84}Gly_{0.61}$. Peak B (0.06 g.) was rechromatographed on carboxymethylcellulose to yield 0.045 g. of the hexacosapeptide X (peptide content based on ultraviolet absorption 68%). Peptide X was found to be homogeneous by electrophoresis on paper (mobility relative to lysine 0.67; pH 3.7, 400 volts, 6 hr.). The amino acid composition of X was determined by the chromatographic method[20] after hydrolysis for 20 and 40 hr. and was found to be in good agreement with theoretically expected values (Table I); $[\alpha]^{25}D$ $-126.4°$ (*c* 1, 0.1 *N* acetic acid) based on peptide content determined by ultraviolet absorption. Peak C (0.04 g.) was rechromatographed on carboxymethylcellulose to yield 0.024 g. of an apparently homogeneous peptide with the following amino acid composition: $Ser_{1.88}Tyr_{1.13}Met_{0.91}Glu_{1.00}His_{0.82}Phe_{1.06}Arg_{2.86}Gly_{2.13}Lys_{3.18}Pro_{2.70}Val_{1.30}Asp_{0.07}$.

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Adrenocorticotropins. XXXIV. Aspects of Structure–Activity Relationships of the ACTH Molecule. Synthesis of a Heptadecapeptide Amide, an Octadecapeptide Amide, and a Nonadecapeptide Amide Possessing High Biological Activities

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The synthesis of a heptadecapeptide amide ($\alpha^{1-17}NH_2$-ACTH), an octadecapeptide amide ($\alpha^{1-18}NH_2$-ACTH), and a nonadecapeptide amide ($\alpha^{1-19}NH_2$-ACTH) corresponding to the first 17, 18, and 19 amino acid residues of adrenocorticotropins (ACTH) has been described. By assay procedures both in vivo and in vitro, the adrenal-stimulating activities of the heptadecapeptide amide and the nonadecapeptide amide were found to be considerably higher than those of their respective acid analogs. The adrenocorticotropic potency of the octadecapeptide amide as estimated by bioassay in vivo is almost identical with that of the nonadecapeptide amide. The melanocyte-stimulating activities of the synthetic products are comparable to that of the natural hormone. The relationship of structure to biological activity with respect to the mechanism of action of the hormone is discussed. Comments on the use of sodium in liquid ammonia for the removal of tosyl groups in peptide synthesis are also presented.

During the past 5 years considerable effort has been directed toward the synthesis of various peptides related to pituitary adrenocorticotropins (ACTH, Figure 1) with the aim of delineating the structural features of the molecule responsible for the manifestation of the biological activities associated with the hormone. Thus, a nonadecapeptide,[1] an eicosapeptide amide,[2] a tricosapeptide,[3] and a tetracosapeptide,[4] all related to the NH_2-terminal half of ACTH, were synthesized in three different laboratories.

When a heptadecapeptide[5] corresponding to the first 17 residues of ACTH was synthesized, it was found to be only 15% as potent a steroidogenic agent as the nonadecapeptide.[1] The heptadecapeptide lacks the dipeptide unit arginylproline found at the COOH terminus of the nonadecapeptide and representing positions 18 and 19 of the ACTH molecule. In view of the concentration of basic amino acid residues in the region between 5 and 22 in the ACTH molecule (see Figure 1), it became of interest to determine if the diminished potency of the heptadecapeptide was the result of the loss of positive charge contributed by arginine in position 18. We have now synthesized three new peptides, namely, seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginine amide[6] (X), seryltyrosylserylmethionylglutamylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginine amide (XV), and seryltyrosylseryl-

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