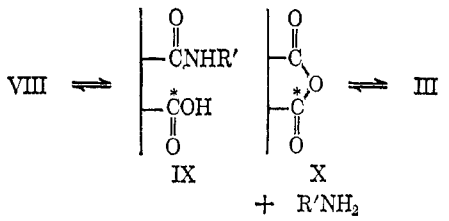

One last mechanistic point should be made. In Schemes I and II we have assumed that one of the reactive groups on the enzyme with a pK of 0.75 is active in its dissociated form while the other with a pK of 2.67 is active in its associated form. However, there is another rather different possibility that is also in agreement with our kinetic results. This is the hypothesis that a catalytically important group on the enzyme with a pK of 0.75 is active in its associated form while the other group with pK = 2.67 is active in its dissociated form. This hypothesis can be shown to give rise to the same kinetic results as would be expected from the previously considered models. There are some difficulties, however. In particular, it is not clear what the group of pK = 0.75 could be. The protonation of amide groups is frequently significant[23] in the pH range near 1, and one could suggest that a protonated amide species is catalytically important in pepsin's mechanism of action. It is hard to envisage though why a protonated amide group would be likely to be catalytically important, unless perhaps it is required to hold pepsin in a reactive conformation.

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Adrenocorticotropins. XXXVII. The Synthesis of Lysine^{8}-α^{1-17NH_2}-ACTH and Its Biological Properties[1]

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Abstract: The synthesis of the heptadecapeptide amide corresponding to the first 17 amino acid residues in the adrenocorticotropin (ACTH) molecule with the replacement of arginine in position 8 by lysine (Lys^{8}-α^{1-17NH_2}-ACTH) is described. The synthetic product possesses much lower adrenal-stimulating and melanocyte-stimulating activities as compared with that of the parent molecule. This indicates that arginine position 8 is essential for the biological function of adrenocorticotropins.

Earlier work has established some biologically nonessential features of the amino-terminal section of the adrenocorticotropins (ACTH) by synthetic means. Li, Schwyzer, and their co-workers[2,3] have synthesized the amino-terminal nonadecapeptide α^{1-19}-ACTH with glutamine substituted for glutamic acid in amino acid residue number 5 (see Figure 1) with no marked change in biological activities. Similarly, Hofmann, *et al.*,[4] have replaced methionine in position 4 by α-amino butyric acid in α^{1-20NH_2}-ACTH. Geiger, *et al.*,[5] have made glycine,[1] phenylalanine,[2] alanine,[3] and deserine[1] analogs in α^{1-23NH_2}-ACTH; and Otsuka, *et al.*,[6] have made a glycine[1] analog of α^{1-17NH_2}-ACTH. When these analogs were assayed and compared with the parent

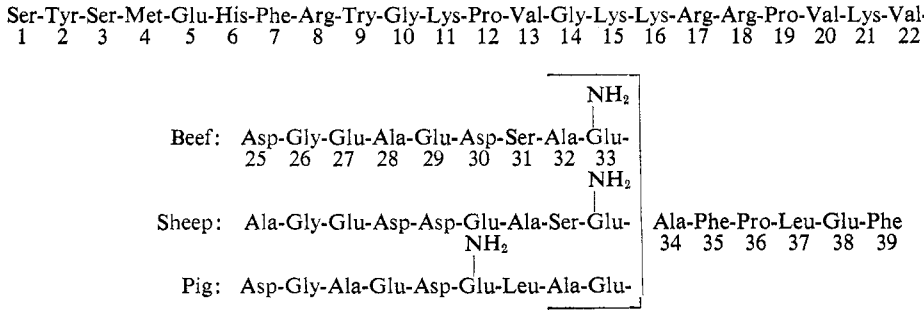

Figure 1. Structure of adrenocorticotropins.

(1) For paper XXXVI, see W. Oelofsen and C. H. Li, *J. Am. Chem. Soc.*, 88, 4254 (1966). All amino acids occurring in the peptides mentioned in this paper are of the L configuration with the exception of glycine.

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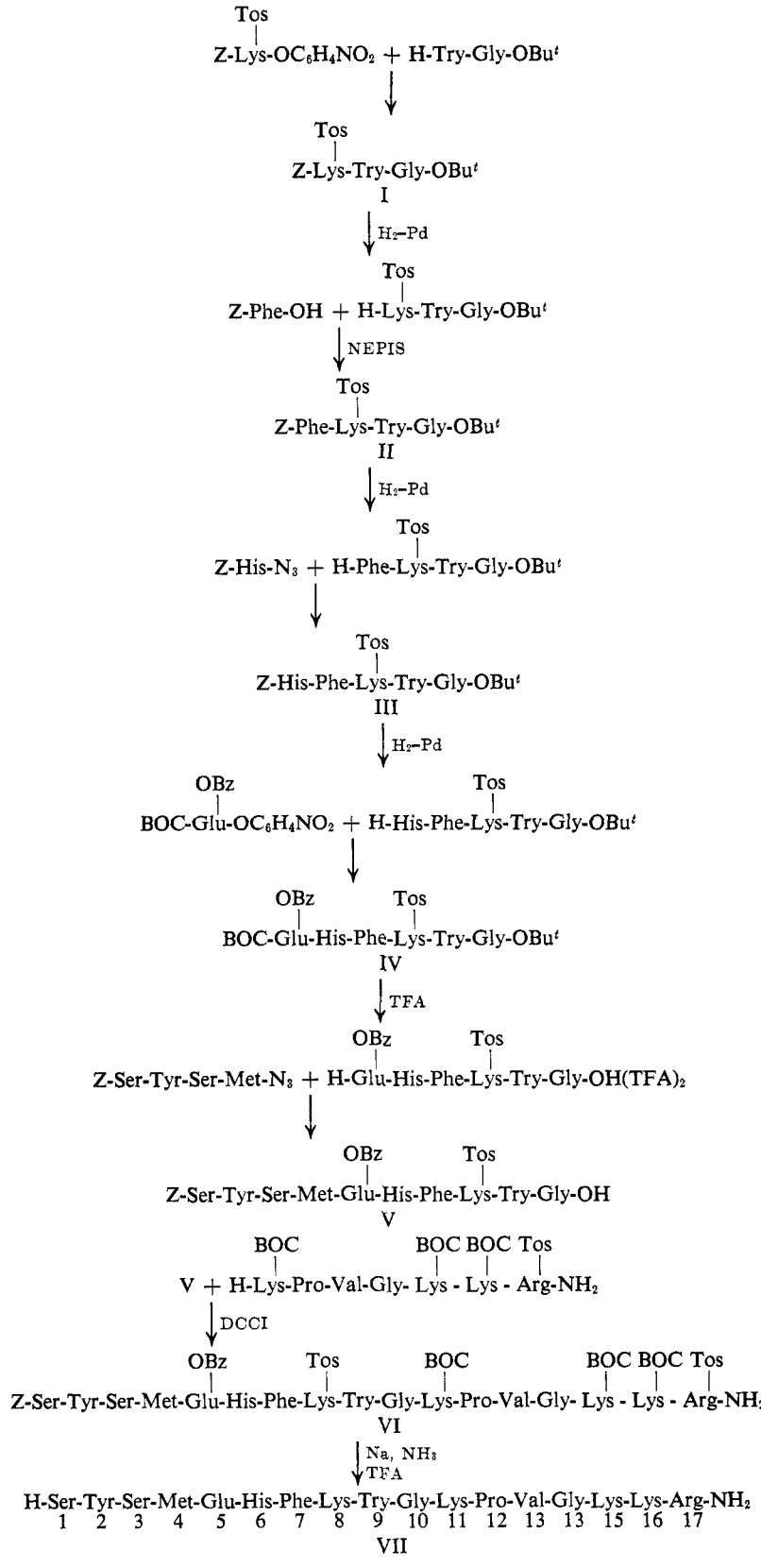

Figure 2. Synthetic scheme for lysine^{8}-$\alpha^{1-17}NH_2$-ACTH: Z, carbobenzoxy; OBz, benzyl ester; NEPIS, N-ethyl-5-phenylisoxazolium-3′-sulfonate; Tos, *p*-toluenesulfonyl; OBu^t, *t*-butyl ester; BOC, *t*-butyloxycarbonyl; TFA, trifluoroacetic acid; DCCI, dicyclohexylcarbodiimide.

molecules, no significant alterations in biological activities were observed. The "basic" portion of the ACTH molecule (position 15–18, Figure 1) has been substituted with other basic amino acids (ornithine^{17,18}-α^{1-24}-ACTH) and no significant loss of activity was apparent as shown by Tesser and Schwyzer.[7] Boissonnas, *et al.*,[8] recently reported the synthesis of a highly

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(8) R. A. Boissonnas, S. Guttmann, and J. Pless, *Experientia*, **22**, 526 (1966).

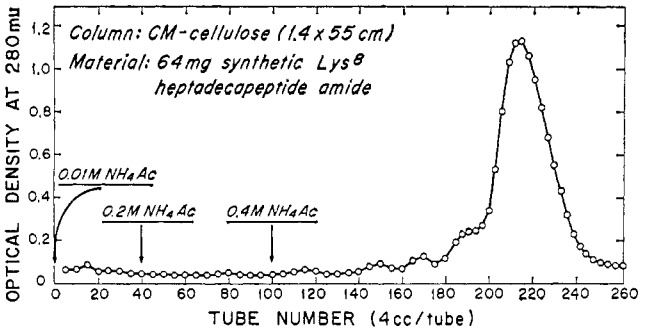

Figure 3. Carboxymethylcellulose chromatography of crude lysine^{8}-α^{1-17NH_2}-ACTH. The material was applied to the column in 0.01 *M* ammonium acetate buffer of pH 4.5. After 40 aliquots (4 cc/tube) has been collected, a pH and concentration gradient was started by introducing a 0.2 *M* ammonium acetate solution (pH 6.7) into the column through a mixing chamber containing 500 cc of the starting buffer. When a total of 100 aliquots of eluate had been collected, the gradient was further increased by introducing a 0.4 *M* ammonium acetate solution through the mixing chamber for the remainder of the experiment.

potent analog of ACTH, D-serine^{1},norleucine^{4},valine^{25}-α^{1-25NH_2}-ACTH.

In this paper we wish to describe the synthesis of the lysine^{8} analog of α^{1-17NH_2}-ACTH[9] and its biological properties. The synthetic scheme may be seen in Figure 2. Crystalline tryptophanylglycine *t*-butyl ester[10] was treated with the *p*-nitrophenyl ester of N^{α}-carbobenzoxy-N^{ϵ}-tosyllysine[11] to yield the crystalline protected tripeptide I. The protected tripeptide I was decarbobenzoxylated by catalytical hydrogenolysis and treated with carbobenzoxyphenylalanine activated with N-ethyl-5-phenylisoxazolium-3′ sulfonate[12] to form the crystalline protected tetrapeptide II. After catalytical hydrogenolysis, the free base of tetrapeptide II was treated with the azide formed from the hydrazide of carbobenzoxyhistidine[13] to obtain the crystalline protected pentapeptide III. Pentapeptide III was catalytically decarbobenzoxylated and treated with the *p*-nitrophenyl ester of *t*-butyloxycarbonyl-γ-benzylglutamic acid[10] to yield the crystalline hexapeptide IV. On treatment with trifluoroacetic acid, the protected hexapeptide was partially deblocked and subsequently treated with the azide formed from N-carbobenzoxyseryltyrosylserylmethionine hydrazide[14] to yield the crystalline protected decapeptide V. With the aid of dicyclohexylcarbodiimide[15] this decapeptide V was linked to the free base heptapeptide N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylargininamide[9] to form the protected heptadecapeptide amide VI. After treatment with trifluoroacetic acid and sodium reduction in liquid ammonia[16] the free heptadecapeptide amide VII (Lys^{8}-α^{1-17NH_2}-ACTH) was

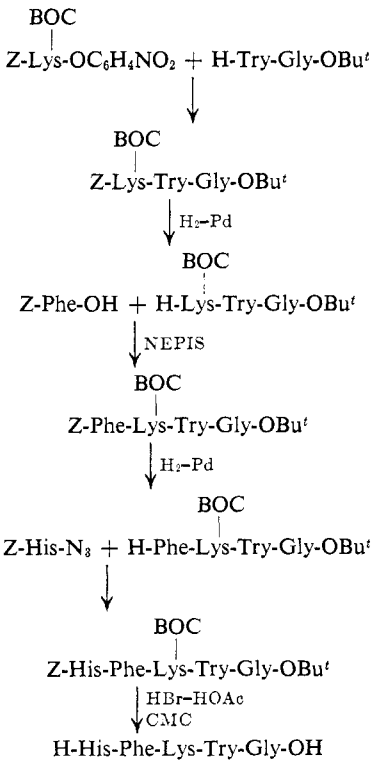

Figure 4. Synthetic scheme for lysine^{8}-α^{6-10}-ACTH.

isolated and purified by chromatography on carboxymethylcellulose[17] using gradient elution with ammonium acetate (Figure 3).

The free peptide VII behaved as a single component in paper electrophoresis and yielded the expected amino acid residues on acid hydrolysis.[18] To establish the stereochemical purity of the synthesized peptide, the decapeptide V was deblocked by sodium reduction in liquid ammonia and the free decapeptide was purified by carboxymethylcellulose chromatography. Complete enzymic digestion of the purified decapeptide by trypsin, chymotrypsin, and leucine aminopeptidase[19] indicated that the amino acids were probably of the L configuration. Since the heptapeptide of the carboxyl terminal was previously characterized[14] and the coupling of the amino terminal decapeptide to a heptapeptide involved the carboxyl group of a glycine residue, it is reasonable to assume that the optical purity of the amino acids was retained in the synthesis.

The *in vitro* ACTH activity of the lysine^{8}-α^{1-17NH_2}-ACTH was determined by the method of Saffran and Schally,[20] and the result showed an activity of less than 1.0 USP unit/mg. Since the α^{1-17NH_2}-ACTH has an activity of ~44 USP units/mg,[9] it is clear that the substitution of lysine for arginine in position 8 greatly diminished, if not abolished, the steroidogenic function of the ACTH molecule. The *in vitro* melanocyte-stimulating (MSH) activity[21] was also markedly reduced when lysine was substituted for arginine in position 8 of α^{1-17NH_2}-ACTH: 1×10^{7} units/g as compared with

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2×10^8 units/g. The free decapeptide lysine^8-α^{1-10}-ACTH was also assayed for *in vitro* MSH activity; it had a value of less than 2×10^4 units/g as compared with the α^{1-10}-ACTH[14] value of 1.5×10^6 units/g. From these data it can be inferred that the arginine in position 8 of the ACTH molecule cannot be replaced by lysine without a drastic reduction of its adrenocorticotropic and melanocyte-stimulating activities.

While this manuscript was being prepared, Yajima, *et al.*,[22] reported a synthesis and *in vitro* MSH activity of the pentapeptide lysine^8-α^{6-10}-ACTH. The activity was reported to be 1×10^4 units/g, and the authors interpreted this to mean almost complete retention of activity since the natural arginine^8-α^{6-10}-ACTH has an activity of $\sim 3 \times 10^4$ units/g. We have also synthesized lysine^8-α^{6-10}-ACTH and found it to be inert *in vitro* at the level of 1×10^4 units/g, and inert *in vivo*[23] at 250-μg level MSH assays (arginine^8-α^{6-10}-ACTH is active *in vivo* at 50 μg). These data plus those of the above lysine decapeptide and heptadecapeptide amide analogs indicating the essential nature of arginine in position 8 are in variance with the findings of Yajima, *et al.*[22]

Figure 4 outlines the synthesis of lysine^8-α^{6-10}-ACTH to yield a product which was completely degraded by leucine aminopeptidase, indicating optical homogeneity of the product. The synthesis is identical with that of the pentapeptide III above except that N^{α}-carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[24] was used instead of the N^{ϵ}-tosyl derivative. The protected pentapeptide was deblocked by hydrogen bromide in glacial acetic acid[25] and the free peptide was purified by carboxymethylcellulose chromatography. The peptide was homogeneous in paper electrophoresis and paper chromatography. We have also resynthesized ornithine^8-α^{6-10}-ACTH[26] in a similar manner and found it to be inert, as reported by Bodanszky, *et al.*,[27] and Yajima, *et al.*[22]

Previously[26] we had reported an *in vitro* MSH activity of 3×10^4 units/g for the ornithine^8-α^{6-10}-ACTH. However, that initial synthesis involved (a) saponification of peptide esters and (b) coupling of peptide fragments with dicyclohexylcarbodiimide, both of which can lead to a racemized product. The incomplete enzymatic digestion data seem to indicate this to be the case. Thus its activity can be explained in terms of the well-known alkaline or prolongation effect on MSH peptides involving the phenylalanine.[22] Because of the discrepancy arising from the work of others[22,27] who found this pentapeptide to be inert, we resynthesized this ornithine pentapeptide *via* one by one condensation to suppress racemization as outlined for the lysine^8-α^{6-10}-ACTH analog. We obtained crystalline intermediates as well as the crystalline protected product, and the deblocked pentapeptide was completely degraded by leucine aminopeptidase, indicating optical purity of the product.

From the data above and from the results of Bodanszky, *et al.*,[27] it appears that spatial arrangement as well as a basic center is necessary for the melanocyte-stimulating activity of the naturally occurring pentapeptide α^{6-10}-ACTH. The lysine and ornithine analogs with their positive charge are not active because perhaps spatially they do not "fit" into any proposed receptor site. On the other hand, the citrulline analog[27] spatially resembles the guanidino function of arginine, but it is not active presumably because of the lack of a positive charge. The absence of or greatly diminished adrenocorticotropic activity of the lysine^8-α^{1-17NH_2}-ACTH can perhaps be interpreted in a like manner.

Experimental Section

General. Melting points were determined on a Fisher–Johns block and are uncorrected. All samples for microanalysis[28] were dried in an Abderhalden pistol with phosphorus anhydride at 77° (0.3 mm) for 16 hr. Paper chromatography was carried out on Whatman No. 1 filter paper with 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. Peptide spots were located by the ninhydrin reagent, the Pauly reagent,[29] the Ehrlich reagent,[30] and the chlorine method.[31] Countercurrent distribution (CCD) was performed on a 100-tube, all-glass apparatus with a capacity of 10 cc for each phase. Analysis of the material was by ultraviolet absorption. Catalytic hydrogenolysis was performed in the presence of palladium freshly prepared[32] from palladium chloride, by means of a Vibro-Mixer[33] in the apparatus described by Meienhofer.[34]

N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysyltryptophanylglycine *t*-Butyl Ester (I). N^{α}-Carbobenzoxy-N^{ϵ}-tosyllysine *p*-nitrophenyl ester[11] (5.55 g, 10 mmoles) was added to a solution of 3.17 g (10 mmoles) of tryptophanylglycine *t*-butyl ester[10] in 100 cc of ethyl acetate. The solution was stirred overnight (16 hr) at room temperature after which the ethyl acetate was removed by evaporation *in vacuo*. The peptide I was isolated by precipitation from ethyl acetate–ethyl ether to yield 6.61 g (90%), mp 84–88°. Purification by countercurrent distribution (CCD, 100 transfers) in the toluene system (toulene–chloroform–methanol–water, 5:5:8:2 by volume) yielded 5.07 g (69%) of peptide I ($K = 0.33$) which was crystallized from methanol; mp 98–100°; R_{fBAW} 0.82; R_{fSBA} 0.91; $[\alpha]^{25}D$ −19.2° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{38}H_{47}O_8N_5S$ (733.87): C, 62.2; H, 6.46; N, 9.54. Found: C, 61.6; H, 6.69; N, 9.90.

Carbobenzoxyphenylalanyl-N^{ϵ}-tosyllysyltryptophanylglycine *t*-Butyl Ester (II). Tripeptide I (5.07 g, 6.9 mmoles) was dissolved in 150 cc of methanol and 50 cc of dimethylformamide and submitted to catalytic hydrogenolysis with palladium prepared from 1 g of palladium chloride After the cessation of carbon dioxide liberation (~6 hr), the palladium was filtered, and the solvents were removed by evaporation *in vacuo*. The residue gave a single spot on paper chromatography, R_{fBAW} 0.71.

Carbobenzoxyphenylalanine (2.06 g, 6.9 mmoles) was dissolved in 50 cc of acetonitrile. The solution was cooled to 0° and 1.0 cc of triethylamine (7.0 mmoles) and 1.75 g (6.9 mmoles) of N-ethyl-5-phenylisoxazolium-3′-sulfonate[12] were added, and the mixture was stirred at 0° for 1 hr. The above free base tripeptide I was dissolved in 50 cc of acetonitrile and then added to the activated carbobenzoxyphenylalanine solution, and the mixture was stirred at room temperature overnight. The acetonitrile was removed by evaporation *in vacuo* and the residue redissolved in ethyl acetate–water. The ethyl acetate solution of II was washed with 0.1 *M* acetic acid, water, 5% sodium bicarbonate, and water and then dried over anhydrous sodium sulfate. Removal of the ethyl acetate *in vacuo* yielded 5.57 g (92%) of the crude peptide II. Purification by CCD (100 transfers, toluene system) yielded ($K = 0.20$) peptide

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(34) J. Meienhofer, *Chimia* (Aarau), **16**, 385 (1962).

II which was crystallized from warm ethyl acetate to give 4.79 g (79%); mp 140–141°; R_{fBAW} 0.88; R_{fSBA} 0.90; $[\alpha]^{25}D$ −26.0° (*c* 1, methanol).

Anal. Calcd for $C_{47}H_{56}O_9N_6S$ (881.04): C, 64.1; H, 6.41; N, 9.54. Found: C, 63.8; H, 6.28; N, 9.38.

N^{α}-Carbobenzoxyhistidylphenylalanyl-N^{ϵ}-tosyllysyltryptophanylglycine *t*-Butyl Ester (III). Tetrapeptide II (4.4 g, 5.0 mmoles) was dissolved in 150 cc of methanol and decarbobenzoxylated as above with palladium from 1 g of palladium chloride. The residue, after removal of palladium and methanol, gave a single spot on paper chromatography, R_{fBAW} 0.78.

N^{α}-Carbobenzoxyhistidine hydrazide (3.02 g, 10.0 mmoles) was converted to its azide[13] at 0° and added (in ethyl solution) to the above tetrapeptide dissolved in 100 cc of dimethylformamide. The ethyl acetate was removed *in vacuo* at 0°, and the remaining dimethylformamide solution was kept at 0° for 2 days. Purification by CCD (150 transfers; toluene system) yielded peptide III as a single component; K = 0.61; R_{fBAW} 0.87. Crystallization from methanol yielded 4.27 g (84%); mp 161–163°; $[\alpha]^{25}D$ −38.8° (*c* 1, methanol).

Anal. Calcd for $C_{53}H_{63}O_{10}N_9S$ (1018.18): C, 62.5; H, 6.24; N, 12.4. Found: C, 62.1; H, 6.14; N, 12.3.

***t*-Butyloxycarbonyl-γ-benzylglutamylhistidylphenylalanyl-N^{ϵ}-tosyllysyltryptophanylglycine *t*-Butyl Ester (IV).** Pentapeptide III (4.0 g, 3.9 mmoles) was decarbobenzoxylated as described above using 1 g of palladium chloride. The free base pentapeptide was found to be homogeneous, R_{fBAW} 0.63, and was dissolved in 50 cc of dimethylformamide. *t*-Butyloxycarbonyl-γ-benzylglutamic acid *p*-nitrophenyl ester[10] (1.97 g, 4.3 mmoles) was added and the mixture stirred at room temperature for 24 hr. The dimethylformamide was removed by evaporation *in vacuo* and the residue triturated with ethyl ether to yield 4.34 g (92%) of crude peptide IV. Crystallization from hot methanol provided 3.31 g (70%) of the desired peptide IV; mp 160–162°; R_{fBAW} 0.79; $[\alpha]^{25}D$ −27.4° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{62}H_{78}O_{13}N_{10}S$ (1203.4): C, 61.9; H, 6.53; N, 11.6. Found: C, 61.9; H, 6.47; N, 11.3.

Carbobenzoxyseryltyrosylserylmethionyl-γ-benzylglutamylhistidylphenylalanyl-N^{ϵ}-tosyllysyltryptophanylglycine (V). Hexapeptide IV (2.97 g, 2.46 mmoles) was dissolved in 10 cc of trifluoroacetic acid under a nitrogen atmosphere. After 30 min at room temperature evaporation *in vacuo* yielded a residue that was homogeneous in paper chromatography, R_{fBAW} 0.55. The partially deblocked hexapeptide (free base and free acid) salt was dissolved in 50 cc of dimethylformamide, cooled to 0°, and neutralized by the addition of 0.69 cc (4.92 mmoles) of triethylamine.

Carbobenzoxyseryltyrosylserylmethionine hydrazide[14] (3.12 g, 4.92 mmoles) was dissolved in a mixture of 45 cc of acetic acid, 4.5 cc of concentrated hydrochloric acid, and 45 cc of water. The solution was cooled to −5° and 0.4 g of sodium nitrite was added with vigorous sitrring. After 30-min mixing at −5°, 200 cc of ice-cold ethyl acetate was added to solubilize the azide derivative. The ethyl acetate solution was extracted with ice-cold 5% sodium bicarbonate until the aqueous extract was no longer acidic. The ethyl acetate solution of the azide was dried over anhydrous sodium sulfate and then added to the above 0° dimethylformamide solution of free base hexapeptide. The ethyl acetate was removed by distillation at 0° and the remaining dimethylformamide solution was kept at 4° for 2 days. The solution was acidified with 1 cc of 5.7 *N* hydrochloric acid and evaporated to dryness *in vacuo*. The residue was dissolved in 25 cc of dimethylformamide and water was added until turbidity appeared (~25 cc). On cooling to 4° and storing for 3 days, the decapeptide V crystallized. The product was filtered, washed with methanol, and dried to yield 3.37 g (83%), mp 174–176°; $[\alpha]^{25}D$ −7.7° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{81}H_{96}O_{20}N_{14}S_2 \cdot HCl$ (1686.1): C, 57.7; H, 5.80; N, 11.6. Found: C, 57.4; H, 6.02; N, 11.5.

Seryltyrosylserylmethionylglutamylhistidylphenylalanyllysyltryptophanylglycyllysylprolylvalylglycyllysyllysylargininamide (VII). Decapeptide V (0.165 g, 0.1 mmole), N^{ϵ}-*t*-butyloxycarbonyllysylprolylvalylglycyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{ϵ}-*t*-butyloxycarbonyllysyl-N^{G}-tosylargininamide[9] (0.127 g, 0.1 mmole), and 0.01 cc of triethylamine (0.1 mmole) were dissolved in 2 cc of dimethylformamide. The solution was cooled to 0° and 0.023 g of dicyclohexylcarbodiimide[15] was added. After being stirred for 1 hr at 0° the solution was allowed to stand at 4° for 4 days. Acetic acid, 0.5 cc, was added, and the solvents were removed by evaporation *in vacuo*. The residue was submitted to CCD, 150 transfers in the system chloroform–carbon tetrachloride–methanol–0.01 *M* ammonium acetate (3:1:3:1 by volume). The crude protected heptadecapeptide amide VI in tubes corresponding to K = 0.05 was isolated by evaporation of the solvents and thoroughly dried. Peptide VI was dissolved in 10 cc of trifluoroacetic acid and allowed to stand for 2 hr at room temperature to remove the *t*-butyloxycarbonyl groups. The trifluoroacetic acid was removed by distillation *in vacuo* and the residual partly deblocked peptide VI thoroughly dried *in vacuo* over sodium hydroxide pellets. The material was dissolved in 150 cc of freshly distilled liquid ammonia, and small pieces of sodium[16] were added at the boiling point of the liquid ammonia (−33°). When the blue color persisted for 0.5 hr, no more sodium was added, and the ammonia was allowed to evaporate at room temperature. The residue was dried under high vacuum over concentrated sulfuric acid and then desalted with IRC-50 resin.[35] The lyophilized crude heptadecapeptide amide VII was finally purified by ammonium acetate gradient chromatography using carboxymethylcellulose[17] (see Figure 3) to yield 0.046 g (22%) of the heptadecapeptide amide VII, $[\alpha]^{25}D$ −84° (*c* 0.69, 0.1 *N* HOAc).

Paper electrophoresis on Whatman No. 3 filter paper in pyridine acetate buffer, pH 3.7, 6 hr, 400 v, shows a single spot, R_{fLys} 0.96. Amino acid analysis[18] following acid hydrolysis gave the following results (calcd, found): Ser (2, 1.7); Tyr (1, 0.9); Met (1, 0.9); Glu (1, 0.9); His (1, 1.0); Phe (1, 0.9); Lys (4, 4.2); Try (1, 0); Gly (2, 1.8); Pro (1, 1.1); Val (1, 0.9); Arg (1, 1.0).

In a similar manner, decapeptide V was completely deblocked by sodium in liquid ammonia and purified by chromatography on carboxymethylcellulose. A sample of the free peptide (0.5 mg) was submitted to total enzyme digestion exactly as described previously.[14] The results were (calcd, found): Ser (2, 2.3); Tyr (1, 1.0); Met (1, 0.8); Glu (1, 1.0); His (1, 0.9); Phe (1, 0.8); Lys (1, 1.1); Try (1, 1.0); Gly (1, 1.2).

N^{α}-Carbobenzoxy-N^{ϵ}-butyloxycarbonyllysyltryptophanylglycine *t*-Butyl Ester. N^{α}-Carbobenzoxy-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[24] (1.50 g, 3.2 mmoles) was added to a solution of 1.00 g of tryptophanylglycine *t*-butyl ester (3.0 mmoles), and the mixture was then stirred at room temperature for 24 hr. The ethyl acetate was then washed with 0.1 *N* acetic acid (twice), water (twice), 5% sodium bicarbonate (thrice), and water, and dried over anhydrous sodium sulfate. The ethyl acetate was then evaporated *in vacuo* and the residue triturated with ethyl ether to yield 1.59 g (78%) of the crude, crystalline tripeptide, mp 92–97°. The product was then recrystallized from ethyl acetate to yield 1.28 g (63% mp 97–99°; R_{fBAW} 0.88; R_{fSBA} 0.94; $[\alpha]^{25}D$ 27.9° (*c* 1, methanol).

Anal. Calcd for $C_{36}H_{49}O_8N_5$ (679.8): C, 63.6; H, 7.27; N, 10.3. Found: C, 63.5; H, 7.36; N, 10.8.

Carbobenzoxyphenylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysyltryptophanylglycine *t*-Butyl Ester. The above protected tripeptide (1.02 g, 1.5 mmoles) was dissolved in 25 cc of methanol and submitted to catalytic hydrogenolysis with palladium from 0.5 g of palladium chloride. After cessation of carbon dioxide (6 hr), the palladium was filtered and the methanol evaporated *in vacuo*. The free base tripeptide gave a single spot in paper chromatography, R_{fBAW} 0.77, positive to ninhydrin and the Ehrlich reagents. The free base tripeptide was redissolved into 15 cc of acetonitrile. Carbobenzoxyphenylalanine (0.45 g, 1.5 mmoles) was dissolved in 15 cc of acetonitrile; the acetonitrile solution was cooled to 0°, and 0.21 cc of triethylamine (1.5 mmoles) and 0.38 g (1.5 mmoles) of N-ethyl-5-phenylisoxazolium-3′-sulfonate were added. After stirring for 1 hr at 0° the solution of the tripeptide base was added, and the mixture was stirred overnight at room temperature. The acetonitrile was evaporated *in vacuo* and the residue redissolved in wet ethyl acetate and then washed successively with 0.1 *N* acetic acid and water. After drying over anhydrous sodium sulfate the ethyl acetate was evaporated to yield 0.91 g (74%) of the crude crystalline protected peptide, mp 160–175°. Recrystallization from methanol produced 0.87 g (70%), mp 183–184°; R_{fBAW} 0.81; R_{fSBA} 0.88; $[\alpha]^{25}D$ −26.5° (*c* 1, methanol).

Anal. Calcd for $C_{45}H_{58}O_9N_6$ (827.0): C, 65.4; H, 7.07; N, 10.2. Found: C, 65.0; H, 7.29; N, 10.2.

N^{α}-Carbobenzoxyhistidylphenylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysyltryptophanylglycine *t*-Butyl Ester. The preceding protected tetrapeptide (0.81 g, 0.97 mmole) in methanol was decarbobenzoxylated by catalytic hydrogenolysis as described above. The palladium was filtered and the methanol evaporated. The residual free base tetrapeptide was redissolved in 25 cc of dimethylformamide.

(35) H. B. F. Dixon and M. B. Stack-Dunne, *Biochem. J.* **61**, 483 (1955).

N^{α}-Carbobenzoxyhistidine hydrazide (0.60 g, 2.0 mmoles) was converted to its azide and then added (in ethyl acetate solution) to the above dimethylformamide solution of the free base tetrapeptide at 0°. The ethyl acetate was removed by distillation *in vacuo* at 0°, and the remaining dimethylformamide solution was kept at 0° for 48 hr. The dimethylformamide was removed by distillation *in vacuo* at 40–50° and the residue submitted to purification by countercurrent distribution in the toluene system for 120 transfers. The material in tubes 35–63 ($K = 0.67$) was pooled and evaporated. Crystallization of the protected pentapeptide from methanol yielded 0.73 g (78%), mp 172–174°; R_{fBAW} 0.85; $[\alpha]^{25}D$ −32.5° (*c* 1, methanol).

Anal. Calcd for $C_{51}H_{65}O_{10}N_9$ (964.1): C, 63.5; H, 6.80; N, 13.1. Found: C, 63.8; H, 6.96; N, 13.6.

Histidylphenylalanyllysyltryptophanylglycine. The protected pentapeptide (96 mg, 0.10 mmole) was dissolved in 4 cc of 4 *N* hydrogen bromide in glacial acetic acid. After standing at room temperature for 30 min, a large volume of ethyl ether was added to precipitate the hydrogen bromide salt of the free pentapeptide. The ether was decanted and the residue washed twice with fresh ether and then dried *in vacuo* over sodium hydroxide pellets. The dried peptide was dissolved in a small volume of 0.01 *M* ammonium acetate, and applied to a 1.4 × 55 cm column of carboxymethylcellulose previously buffered with 0.01 *M* ammonium acetate. After collecting 40 fractions (4 cc each) a gradient was begun with 0.2 *M* ammonium acetate with a 500-cc mixing chamber. The purified peptide was isolated from tubes 85–120 by lyophilization (three times) to remove ammonium acetate and excess water. The yield of the lysine pentapeptide was 62.5 mg (96%). The ultraviolet absorption of the material revealed a peptide content of 76%, thus making the actual yield of free lysine pentapeptide as 73%. This product gave a single spot, positive to ninhydrin, Pauly, Ehrlich, and chlorine, in paper chromatography, BAW, SBA, and paper electrophoresis at pH 7.0, 400 v, 6 hr (R_{fLys} 0.40); $[\alpha]^{25}D$ −15.4° (*c* 0.3, 0.1 *N* acetic acid).

The lysine pentapeptide (0.5 mg) was dissolved in 0.5 cc of Tris, pH 8.0, buffer (0.01 *M* Mg^{2+}) and 0.005 cc of a leucine aminopeptidase solution (0.14 mg of Worthington No. 5917 in 0.025 cc of water) was added and the digest incubated at 37° for 16 hr. Paper electrophoresis revealed no more pentapeptide. The digest was then directly analyzed for amino acid composition in a Spinco amino acid analyzer. The results in mole ratios were: histidine, 0.98; phenylalanine, 1.03; lysine, 1.00; tryptophan, 1.02; and glycine, 0.88.

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Communications to the Editor

Flash Vacuum Pyrolysis. I. Allylcyclopentadiene from Allyl Phenyl Ether

Sir:

Lossing and co-workers[1] have demonstrated in their comprehensive study of free radicals by mass spectroscopy that pyrolysis of certain normally stable organic molecules at temperatures up to the softening point of quartz (about 1200°), low pressures (about 10^{-3} mm) and short contact times (1–10 msec) can efficiently lead to a wide variety of organic free radicals. While searching for an unequivocal method for generating the succinimidyl radical[2] in an environment suitable for physical and chemical characterization, we realized that pyrolysis at the above conditions, which can serve to define the process of flash vacuum pyrolysis[3] (FVP), had great potential for fundamental studies of the chemical and spectroscopic properties of organic free radicals and other unstable, primary pyrolytic intermediates; the possibility of synthetic utility was also envisioned. In this and the following report[4] we describe the techniques and some preliminary results which have evolved in our investigation of FVP.

Our basic apparatus consists of a tubular oven (*radical gun*) which is housed in a vacuum-tight chamber coupled to a large Pyrex dewar (Figure 1). In the present work the radical gun was constructed from a quartz tube which is tightly and closely coiled with Nichrome wire and then encased with fused quartz. The temperature is measured along the axis by a thermocouple encased in a quartz capillary.

In order to determine whether our apparatus could be used to chemically duplicate results of mass spectrometric experiments, we investigated the FVP of allyl phenyl ether. Lossing and co-workers[5] have shown that the phenoxy radicals initially produced decarbonylate near 900° to give the cyclopentadienyl radical. In our first experiments 0.5 or 1.0 g of allyl phenyl ether was pyrolyzed and the products were immediately quenched on the dewar surface kept at −196° (the distance from the tip of the radical gun to the dewar was about 2.5 cm). The dewar was slowly warmed to room temperature and the volatile products were collected on a vacuum manifold attached to the apparatus. These were isolated by preparative glpc and characterized by modern analytical techniques. The results are summarized by the equation given below and the data given in Table I.

Allylcyclopentadiene[6] (**4**) had the following properties: parent peak at *m/e* 106; nmr in $CDCl_3$ (TMS)

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(2) (a) E. Hedaya, R. L. Hinman, and S. Theodoropulos, *J. Am. Chem. Soc.*, **85**, 3052 (1963); (b) E. Hedaya, R. L. Hinman, L. M. Kibler, and S. Theodoropulos, *ibid.*, **86**, 2727 (1964); (c) E. Hedaya, R. L. Hinman, V. Schomaker, S. Theodoropulos, and L. M. Kyle, in press.

(3) The terms "flash pyrolysis" or "flash heating" have been used to describe experiments where flash lamp emission is converted to heat: L. A. Nelson and N. A. Kuebler, *J. Chem. Phys.*, **37**, 47 (1962).

(4) C. L. Angell, E. Hedaya, and D. McLeod, Jr., *J. Am. Chem. Soc.*, **89**, 4214 (1967).

(5) (a) I. P. Fisher, F. F. Palmer, and F. P. Lossing, *ibid.*, **86**, 2741 (1964); (b) R. F. Pottie and F. P. Lossing, *ibid.*, **85**, 269 (1963); (c) A. G. Harrison, T. R. Horner, H. J. Dauben, Jr., and F. P. Lossing, *ibid.*, **85**, 5593 (1963).

(6) A. Renner, U. S. Patent 3,105,839 (Oct 1, 1963).