ride, the solvent was removed by distillation under reduced pressure, and the residue was crystallized by trituration with petroleum ether. Recrystallization of the solid from isopropyl alcohol–ether gave a small yield of solid, m.p. 146–148°. The solid gave a negative Beilstein test for halogen and positive sodium fusion tests for nitrogen and sulfur. The compound appeared to be a 3-benzoyl-1-methyl-4-phenyldihydropyridinesulfinic acid (XVI), plus oxygen or water.

Anal. Calcd. for $C_{18}H_{16}NO_4S$: C, 64.00; H, 5.10. Found: C, 64.58; H, 5.21.

(e) **The Reaction of the Methobromide V with Sodium Cyanide.**—A 1.0-g. sample of V was dissolved in 50 ml. of water and 0.5 g. of sodium cyanide was added. A small amount of yellow precipitate formed. The addition of an additional 3.0 g. of sodium cyanide caused further precipitation. The solid was removed by filtration and washed with water. During this process the solid turned brown. Recrystallization of the solid was accompanied by a darkening in color. Thus analyses were determined on crude material, m.p. 75–80°. The analytical data suggest that this compound is a 3-benzoyl-1-methyl-4-phenylcyano-1,4-dihydropyridine (XVII), plus oxygen or water.

Anal. Calcd. for $C_{20}H_{16}N_2O_2$: C, 76.00; H, 5.07. Found: C, 75.25; H, 5.10.

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The Synthesis of L-Valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline[1]

JANAKIRAMAN RAMACHANDRAN AND CHOH HAO LI

Hormone Research Laboratory, University of California, Berkeley, California

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The peptide sequence, L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline, which occurs in adrenocorticotropins, has been synthesized by the reaction of carbobenzoxy-L-valyl-N$^{\epsilon}$-tosyl-L-lysine azide with L-valyl-L-tyrosyl-L-proline benzyl ester, followed by hydrogenation and treatment with sodium in liquid ammonia. The protected pentapeptide was obtained in crystalline form. A side reaction was observed when carbobenzoxy-L-valine *p*-nitrophenyl ester was coupled with L-tyrosine methyl ester hydrochloride in the presence of *excess* triethylamine. The by-product was identified as O-(carbobenzoxy-L-valyl-)-L-tyrosine methyl ester. The disubstituted by-product, N,O-di(carbobenzoxy-L-valyl)-L-tyrosine methyl ester, was also isolated from this reaction.

The pentapeptide L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline (VI) occurs at position 20–24 in the amino acid sequence[2–4] of adrenocorticotropins (ACTH) isolated from pituitary glands of various species. It is generally assumed[2,5] that the adrenocorticotropic activity resides in the sequence consisting of the first twenty-four or twenty-eight amino acid residues. Indeed, we have recently reported the synthesis[6] of a nonadecapeptide corresponding to the first nineteen amino acid residues of adrenocorticotropins and have shown that it possesses approximately 50% of the potency of the natural product. Subsequently, other investigators[7–9] have described briefly the synthesis of ACTH analogues consisting of 19, 20, 23, and 24 amino acid residues. In the course of the synthesis of the tetracosapeptide, we have obtained peptide VI and crystalline L-valyl-N$^{\epsilon}$-tosyl-L-lysyl-L-valyl-L-tyrosyl-L-proline (V).

The scheme for the synthesis of VI is given in Fig. 1. The benzyl group was employed for the protection of the C-terminus in order to avoid saponification at the end of the synthesis. The protected dipeptide (I) carbobenzoxy-L-valyl-L-tyrosine methyl ester[10,11] was obtained in good yield by coupling carbobenzoxy-L-valine and tyrosine methyl ester *via* the dicyclohexylcarbodiimide (DCCI) procedure.[12] Carbobenzoxy-L-valyl-L-tyrosyl-L-proline benzyl ester (II)[14] was prepared in 75% yield by the reaction of carbobenzoxy-L-valyl-L-tyrosine azide with L-proline benzyl ester. The use of dicyclohexylcarbodiimide with carbobenzoxy-L-valyl-L-tyrosine was avoided because of the reported racemization with this combination in an analogous synthesis.[13] A sample of the protected tripeptide was hydrogenated exhaustively in the presence of palladium. The free tripeptide L-valyl-L-tyrosyl-L-proline was isolated and crystallized from methanol–water.

Carbobenzoxy-L-valyl-N$^{\epsilon}$-tosyl-L-lysine methyl ester[16] was prepared by the reaction of carbobenzoxy-L-

(1) Taken from a portion of the Ph.D. thesis of J. Ramachandran at the University of California, 1962.

(2) K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies, and P. H. Bell, *J. Am. Chem. Soc.*, **77**, 3419 (1955); R. G. Shepherd, S. D. Wilson, K. S. Howard, P. H. Bell, D. S. Davies, S. B. Davis, E. A. Eigner, and N. E. Shakespeare, *ibid.*, **78**, 5067 (1956).

(3) C. H. Li, I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris, and J. S. Dixon, *Nature*, **176**, 687 (1955).

(4) C. H. Li, J. S. Dixon, and D. Chung, *J. Am. Chem. Soc.*, **80**, 2587 (1958); *Biochem. Biophys. Acta*, **46**, 324 (1961).

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

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

(7) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, **23**, 915 (1960); H. Kappeler and R. Schwyzer, *Helv. Chim. Acta*, **44**, 1136 (1961).

(8) K. Hofmann, H. Yajima, N. Yanaihara, T. Y. Liu, and S. Lande, *J. Am. Chem. Soc.*, **83**, 487 (1961).

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

(10) W. Rittel, B. Iselin, H. Kappeler, B. Riniker, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(11) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

(12) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955).

(13) B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **44**, 658, 674, 677, 685 (1961).

(14) Pannemann,[15] *et al.*, reported the synthesis of II by the use of ethoxyethane; carbobenzoxy-L-valyl-L-tyrosine reacted with L-proline benzyl ester hydrochloride by refluxing with ethoxyethene in moist ethyl acetate for 2.5 hr. The protected tripeptide was obtained in 61% yield.

(15) H. J. Pannemann, A. F. Marx, and J. F. Arens, *Rec. trav. chim.*, **78**, 487 (1959).

(16) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **41**, 1582 (1958).

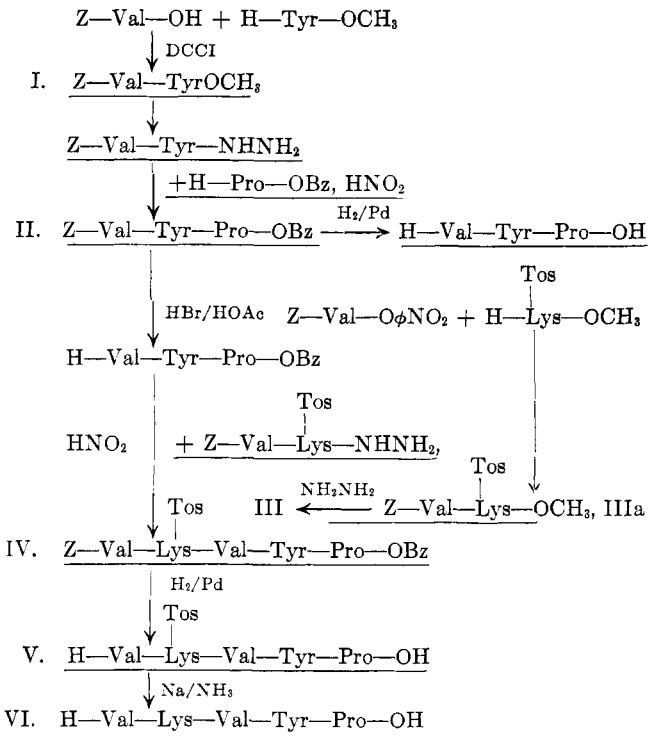

Fig. 1.—Outline of the synthesis of L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline; Z, carbobenzoxy; Bz, benzyl; Tos, *p*-toluenesulfonyl. Underlines indicate crystalline products.

valine *p*-nitrophenyl ester with N^{ϵ}-tosyl-L-lysine methyl ester. The protected dipeptide was converted to the hydrazide (III). The azide of the protected dipeptide was prepared and allowed to react in ethyl acetate with L-valyl-L-tyrosyl-L-proline benzyl ester, which was prepared by treatment of II with hydrogen bromide in glacial acetic acid. It was advantageous to use the free tripeptide ester in this reaction instead of the hydrobromide,[17] since the free tripeptide ester was readily soluble in ethyl acetate whereas the hydrobromide could be dissolved in this solvent only by the addition of some dimethylformamide besides an equivalent of triethylamine. Thus, when the reaction in ethyl acetate was performed with the free tripeptide ester, the protected pentapeptide (IV) separated in crystalline form. IV was isolated in 75% yield and recrystallized from ethyl acetate.

Exhaustive hydrogenation of IV in the presence of palladium yielded the crystalline peptide V, L-valyl-N^{ϵ}-tosyl-L-lysyl-L-valyl-L-tyrosyl-L-proline monohydrate. Removal of the tosyl group was accomplished by reduction with sodium in liquid ammonia. The free pentapeptide L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline (VI) was isolated in 69% yield by desalting on an IRC-50 column.[18] VI was found to be homogeneous in paper chromatography in three different solvents and by paper electrophoresis in a buffer of pH 3.7. Amino acid analysis of an acid hydrolysate of the pentapeptide VI by the procedure of Spackman,[19] *et al.*, gave the following ratios of the amino acids: $Val_{2.0}Lys_{0.94}Tyr_{1.0}Pro_{1.0}$.

VI was digested with leucine aminopeptidase[20] (LAP), trypsin, and chymotrypsin. Paper chromatography of aliquots of these digests in the solvent system BPAW (*n*-butyl alcohol–pyridine–acetic acid–water) may be seen in Fig. 2. It was found that the LAP digest gave three spots which showed a positive reaction to ninhydrin: valine (R_f 0.42) and lysine (R_f 0.11) were identified by controls. The third ninhydrin-positive spot (R_f 0.55) also gave a positive reaction with the Pauly reagent.[21] No free tyrosine or free proline was detected. Quantitative amino acid analysis of an aliquot of the digest by the Spinco amino acid analyzer showed the molar ratio of valine to lysine to be exactly 2:1 and no tyrosine or proline were detected. Hence the third ninhydrin-positive spot (R_f 0.55) must represent L-tyrosyl-L-proline.

The tryptic digest revealed two ninhydrin-positive spots in chromatography on paper in the system BAW (*n*-butyl alcohol–acetic acid–water). As expected, one of these spots was also positive to the Pauly reagent and corresponded to L-valyl-L-tyrosyl-L-proline. The spot with the lower mobility corresponded to L-valyl-L-lysine. As may be seen in Fig. 2, chymotrypsin caused no splitting of the peptide bonds.

A Side Reaction in Peptide Synthesis with *p*-Nitrophenyl Esters.[22]—In an attempt to prepare I, carbobenzoxy-L-valine *p*-nitrophenyl ester was allowed to react with tyrosine methyl ester hydrochloride in the presence of two equivalents of triethylamine. The progress of the reaction was followed by paper chromatography in the solvent system BAW. The disappearance of ninhydrin positive material (tyrosine methyl ester) was followed. After eight hours' reaction, the chromatograms revealed the presence of unchanged tyrosine methyl ester, which gave a positive reaction with ninhydrin and the Pauly reagent; in addition, a second ninhydrin-positive spot with a high R_f was observed. This latter spot failed to give any reaction with the Pauly reagent (Fig. 3). The reaction mixture was worked up by removing the solvent *in vacuo*, redissolving the residue in ethyl acetate and removing the triethylamine hydrochloride crystals by filtration. When hydrogen chloride in ethyl acetate was added to the filtrate, a crystalline material separated. Paper chromatography in BAW showed that this crystalline product corresponded to the unknown spot which was positive to ninhydrin but negative to the Pauly reagent.[21] This compound could be recrystallized from methanol and microanalysis confirmed the structure of O-(carbobenzoxy-L-valyl)-L-tyrosine methyl ester hydrochloride (VII).

When VII was treated with 0.1 *N* sodium hydroxide for five minutes at room temperature, carbobenzoxy-L-valine and tyrosine methyl ester were formed, both of which were identified by paper chromatography. Further proof of the structure of the product was obtained

(17) When the hydrobromide of the tripeptide benzyl ester reacted with carbobenzoxy-L-valyl-N^{ϵ}-tosyl-L-lysine azide in the presence of an equivalent of triethylamine, considerable amounts of unchanged tripeptide benzyl ester remained even after a week and could be removed from the product only by countercurrent distribution in the system consisting of chloroform–toluene–methanol–water (5:5:8:2, by volume). The protected pentapeptide IV was obtained from the peak with K = 0.21 in 38% yield and unchanged L-valyl-L-tyrosyl-L-proline benzyl ester was recovered from the peak with K = 9.

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

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

(20) R. L. Hill and E. L. Smith, *J. Biol. Chem.*, **228**, 577 (1957).

(21) H. Z. Pauly, *Physiol. Chem.*, **42**, 508 (1904); **94**, 427 (1915).

(22) A preliminary account of this observation has been reported by one of us.[23]

(23) J. Ramachandran, abstract of paper presented at the 140th National Meeting of the American Chemical Society, Chicago, Ill., September, 1961.

from an examination of the ultraviolet absorption spectrum. In Fig. 4 are shown the ultraviolet absorption spectra of an authentic sample of carbobenzoxy-L-valyl-L-tyrosine methyl ester, of O,N-dicarbobenzoxy-L-tyrosine, and of VII. It is evident that the characteristic absorption maximum of tyrosine at 275 mμ is absent from VII. The effect of alkali on the absorption spectra of these three compounds is shown in Fig. 5. With regeneration of the hydroxyl group in the presence of alkali, all three compounds exhibit the typical tyrosine absorption at 295 mμ. Now after acidification, the absorption maxima shift to shorter wave lengths (275 mμ) and all the compounds exhibit nearly identical spectra.

After the isolation of VII, the mother liquors yielded a small amount of another crystalline product that gave negative reactions with both ninhydrin and the Pauly reagent. Analysis established its structure as O,N-di(carbobenzoxy-L-valyl)-L-tyrosine methyl ester. This was further confirmed by its ultraviolet absorption spectra and by the results of mild alkali treatment. The latter gave rise to carbobenzoxyvalyltyrosine methyl ester and carbobenzoxyvaline, both of which were identified by paper chromatography.

VII was obtained in 40–50% yield. The reaction was then repeated under different conditions in order to establish the cause of the side reaction. No trace of VII was found when carbobenzoxy-L-valine *p*-nitrophenyl ester was allowed to react with tyrosine methyl ester in the absence of triethylamine; only traces of VII appeared on paper chromatograms when the reaction with tyrosine methyl ester hydrochloride was repeated with exactly one equivalent of triethylamine. Hence, it is apparent that the excess triethylamine removes the proton from the phenolic hydroxyl group, thus leaving a highly nucleophilic phenoxide ion which in preference to the amino group, is attacked by the *p*-nitrophenyl ester.

The incidence of the side reaction when other protected amino acid *p*-nitrophenyl esters are allowed to react with tyrosine methyl ester was then investigated. The appearance of a new ninhydrin-positive, Pauly-negative spot on the chromatograms in BAW was taken as an indication that the side reaction had occurred. The *p*-nitrophenyl esters of N^{α}-carbobenzoxy-N^{ϵ}-tosyl-L-lysine, N^{α}-carbobenzoxy-L-glutamine and N^{α}-carbobenzoxy-S-benzyl-DL-cysteine gave rise to O-substituted products when allowed to react with tyrosine methyl ester hydrochloride in the presence of more than one equivalent of triethylamine.

Experimental[24]

Carbobenzoxy-L-valyl-L-tyrosine Methyl Ester (I).—L-Tyrosine (45.3 g., 250 mmoles) was esterified with methanol (300 ml.) by the thionyl chloride method[25] to yield 46 g. (79%) of L-tyrosine methyl ester hydrochloride, m.p. 189–190°. L-Tyrosine methyl ester was prepared in 66% yield from the hydrochloride by treatment with alcoholic potassium hydroxide (m.p. 135°). L-Tyrosine methyl ester (3.9 g., 20 mmoles) was dissolved in 150 cc. of acetonitrile by warming. Carbobenzoxyl-L-valine (5.02 g., 20 mmoles) was added and the mixture cooled to 0°. Dicyclohexylcarbodiimide (4.12 g., 20 mmoles) was added and the reaction mixture was stirred for 4 hr. at 4° and for 15 hr. at room temperature. Dicyclohexylurea was filtered off and washed with 100 ml. of hot acetone. The filtrate and washings were concentrated *in vacuo* and the crystalline residue was recrystallized from 60

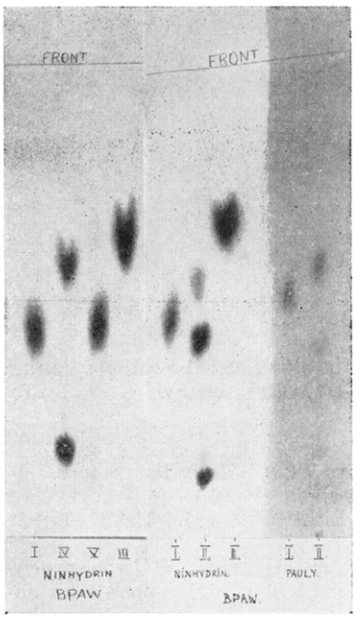

Fig. 2.—Enzymic digestion of the pentapeptide L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline.

I. L-valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline
II. I + leucine aminopeptidase
III. L-valyl-L-tyrosyl-L-proline
IV. I + trypsin
V. I + chymotrypsin

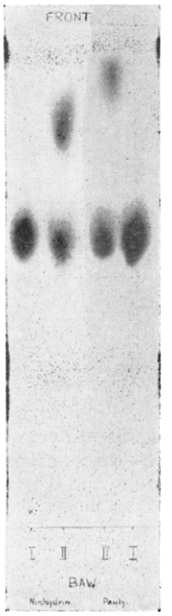

Fig. 3.—Reaction of carbobenzoxy-L-valine *p*-nitrophenyl ester with L-tyrosine methyl ester.

I. L-tyrosine methyl ester
II. I + carbobenzoxy-L-valine *p*-nitrophenyl ester + triethylamine

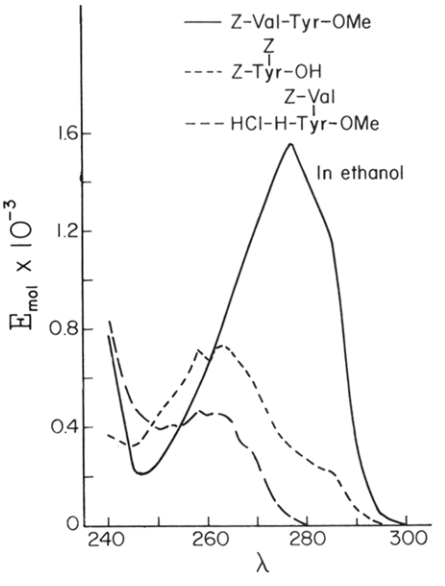

Fig. 4.—Ultraviolet absorption spectrum of O-(carbobenzoxy-L-valyl)-L-tyrosine methyl ester hydrochloride, and derivatives.

(24) All melting points were performed on a Fisher–Johns melting point apparatus and are uncorrected. Microanalyses were performed by the Microanalytical Laboratory of Department of Chemistry of this University. All samples for microanalyses were dried in an Abderhalden drying pistol with phosphorus pentoxide 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 *n*-butyl alcohol–acetic acid–water (BAW) in a ratio of 4:1:1 (by volume), *sec*-butyl alcohol–10% ammonia (SBA) in a ratio of 85:15 (by volume), *n*-butyl alcohol–pyridine–acetic acid–water (BPAW) in a ratio of 30:20:6:24 (by volume), and 3% ammonia–*sec*-butyl alcohol (ASB) in a ratio of 44:100 (by volume). Zone electrophoresis on paper (Whatman 3 MM) was performed at room temperature in a Spinco apparatus for 8 hr. at 400 volts with a pyridine–acetic acid buffer of pH 3.7.

(25) M. Brenner and W. Huber, *Helv. Chim. Acta*, **36**, 1114 (1953).

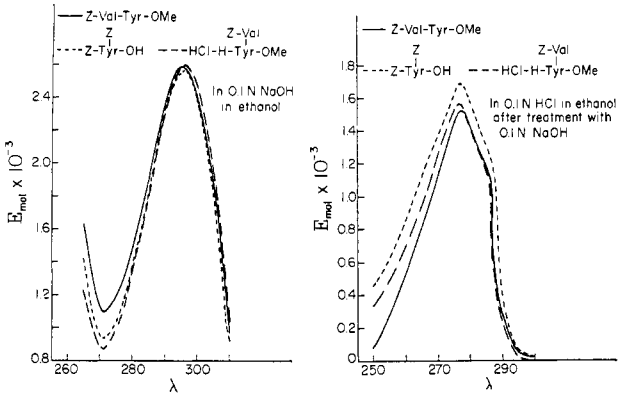

Fig. 5.—Effect of alkali on the ultraviolet absorption spectrum of O-(carbobenzoxy-L-valyl)-L-tyrosine methyl ester hydrochloride, and derivatives.

ml. of hot ethyl acetate to give 7 g. (81%) of the protected dipeptide ester, m.p. 150°; $[\alpha]^{25}D$ + 12.1° (*c* 1, pyridine); R_f BAW 0.83; R_f SBA 0.91. Lit.,[11] m.p. 155.5–156°; $[\alpha]^{25}D$ + 10.2° (*c* 4.8, pyridine).

Carbobenzoxy-L-valyl-L-tyrosine Hydrazide.—Carbobenzoxy-L-valyl-L-tyrosine methyl ester (2.91 g., 6.8 mmoles) was dissolved in 20 ml. of methanol, and 0.48 g. of hydrazine (15 mmoles) was added. Crystals appeared after an hour. The reaction mixture was left overnight, then stirred with methanol and water, filtered, and washed with water. Recrystallization from hot methanol (150 ml.) gave 2.6 g. (90%) of carbobenzoxy-L-valyl-L-tyrosine hydrazide, m.p. 247–248°. Lit.,[11] m.p. 239–241°.

L-Proline Benzyl Ester Hydrochloride.—L-Proline (22.8 g., 200 mmoles) was added to a mixture of 300 ml. of benzyl alcohol and 50 g. of thionyl chloride (prepared by adding the thionyl chloride to the alcohol at −5°). The mixture was stirred at room temperature for 48 hr., hydrochloric acid was removed *in vacuo*, and the residual solution was poured into 1 l. of anhydrous ether. The white crystalline material was filtered, washed with ether, and dried. Recrystallization from hot ethanol yielded 30 g. (64%) of L-proline benzyl ester hydrochloride, m.p. 148–149°. $[\alpha]^{25}D$ −43.3° (c 1, methanol). R_f BAW 0.62; R_f ASB 0.86. Lit.,[26] m.p. 148–148.5°; $[\alpha]^{28}D$ −41.6° (*c* 1.4, ethanol).

Carbobenzoxy-L-valyl-L-tyrosyl-L-proline Benzyl Ester (II).—Carbobenzoxy-L-valyl-L-tyrosine hydrazide (8.6 g., 20 mmoles) was dissolved in a mixture of 50 ml. of 2 *N* hydrochloric acid, 30 ml. of glacial acetic acid, and a few drops of ethyl acetate. This mixture was cooled to −2° and stirred vigorously with a vibro-mixer.[27] Sodium nitrite (1.4 g., 20 mmoles) was added in small portions over a period of 20 min. Stirring was continued for 30 min. Further operations were conducted in the cold room with reagents and glassware precooled to 0° for at least 2 hr. The azide was extracted into 60 ml. of ethyl acetate, and the solution was washed with water, 5% sodium bicarbonate, and then again with water. The organic layer was dried over anhydrous sodium sulfate. L-Proline benzyl ester hydrochloride (7.32 g., 30 mmoles) was suspended in 50 ml. of ethyl acetate, cooled to 0°, and stirred with 4.2 ml. of triethylamine (30 mmoles) for 60 min. The proline ester was filtered from triethylamine hydrochloride and washed with a few ml. of ethyl acetate. The azide solution was added and the volume of the mixture was reduced to half by evaporation *in vacuo* at 0°. Crystals appeared after 4 hr. The mixture was kept at 4° for 2 days and the protected tripeptide ester was filtered and washed with ethyl acetate to yield 9 g. (75%) of the product, m.p. 188–190°. Recrystallization from hot ethyl acetate gave a product that melted at 191–192°. $[\alpha]^{25}D$ −38.8° (*c* 1.2, pyridine). R_f BAW 0.83; R_f SBA 0.87. Lit.,[15] m.p. 186–188°; $[\alpha]^{21.5}D$ −40.6° (*c* 1, pyridine).

L-Valyl-L-tyrosyl-L-proline Benzyl Ester.—Peptide II (3.32 g., 5.5 mmoles) was finely ground in a glass mortar and stirred vigorously with 10 ml. of 4 *N* hydrobromic acid in glacial acetic acid for 15 min. at room temperature with the exclusion of moisture. The tripeptide ester hydrobromide was precipitated by the addition of 250 ml. of anhydrous ether. The precipitate was washed twice with ether by decantation. The residue was dissolved in 60 ml. of cold water that had been saturated with ethyl acetate and then was washed with ether (2 × 30 ml.). The aqueous phase was then brought to pH 8 with cold 5% sodium bicarbonate and the tripeptide base was extracted into ethyl acetate (2 × 50 ml.). The organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was dried overnight over phosphorus pentoxide to yield 2 g. (77.8%) of L-valyl-L-tyrosyl-L-proline benzyl ester, m.p. 50–60°; $[\alpha]^{25}D$ −41.9° (*c* 0.6, methanol). R_f BAW 0.70; R_f SBA 0.83. Lit.,[15] $[\alpha]^{25}D$ −42.6° (*c* 1, methanol).

Anal. Calcd. for $C_{26}H_{33}N_3O_5$ (467.6): C, 66.8; H, 7.11; N, 8.98. Found: C, 66.5; H, 7.32; N, 8.77.

L-Valyl-L-tyrosyl-L-proline.—Carbobenzoxy-L-valyl-L-tyrosyl-L-proline benzyl ester (0.5 g., 0.83 mmole) was dissolved in a mixture of 20 ml. of glacial acid and 20 ml. of methanol, and hydrogenated for 6 hr. in the presence of freshly prepared palladium. The catalyst was filtered and washed and the filtrate and washings were evaporated *in vacuo*. The residue was crystallized from methanol-water to give 0.25 g. (75%) of L-valyl-L-tyrosyl-L-proline, m.p. 176–178°. The tripeptide was recrystallized from methanol-water, m.p. 177–178°; $[\alpha]^{25}D$ −27.4° (*c* 0.6, water). Lit.,[15] m.p. of the one-half hydrate, 206–208°, $[\alpha]^{22}D$ −29° (*c* 1, water). The difference in m.p. may be due to differences in water of crystallization. The tripeptide was found to be homogeneous in paper chromatography in three solvents and gave a positive reaction with ninhydrin and Pauly reagent; R_f BAW 0.53; R_f SBA 0.11; R_f BPAW 0.68. C, H, and N analyses indicated that the tripeptide crystallized with 1.25 moles of water.

Anal. Calcd. for $C_{19}H_{27}N_3O_5 \cdot 1.25\ H_2O$ (400): C, 57.1; H, 7.44; N, 10.5. Found: C, 57.0; H, 7.33; N, 10.8.

Paper electrophoresis revealed a single spot positive to ninhydrin and the Pauly reagent; mobility 0.23 with respect to lysine.

Carbobenzoxyl-L-valyl-N$^{\epsilon}$-tosyl-L-lysine Methyl Ester.—N$^{\epsilon}$-Tosyl-L-lysine methyl ester[16] hydrochloride (8.77 g, 25 mmoles) was suspended in 50 ml. of ethyl acetate, and the suspension was cooled in ice and stirred vigorously with 3.5 ml. of triethylamine (25 mmoles) for 30 min. The ethyl acetate solution was filtered into a flask containing 9.13 g. (25 mmoles) of carbobenzoxy-L-valine *p*-nitrophenyl ester,[28] and the precipitate of triethylamine hydrochloride was washed with 30 ml. of ethyl acetate. The reaction mixture was stirred at room temperature for 4 days. The protected dipeptide ester crystallized and was filtered and washed with ether. Yield 11.5 g. (83%), m.p. 131–132°; $[\alpha]^{25}D$ −18.8° (*c* 0.8, methanol); R_f BAW 0.91; R_f ASB 0.94. Lit.,[16] m.p. 130° $[\alpha]^{18}D$ −8° (*c* 1, acetic acid).

Carbobenzoxy-L-valyl-N$^{\epsilon}$-tosyl-L-lysine Hydrazide (III).—The protected dipeptide methyl ester described above (2.74 g., 5 mmoles) was dissolved in 30 ml. of methanol by warming and allowed to react with 0.5 ml. of hydrazine at room temperature. Crystals appeared after 3 hr. These were filtered after 12 hr. and washed with methanol to give 2.5 g. (91%) of III, m.p. 212°; $[\alpha]^{25}D$ −14.6° (*c* 4, acetic acid).

Anal. Calcd. for $C_{26}H_{37}N_5O_6S$ (547.7): C, 57.0; H, 6.81; N, 12.9; S, 5.85. Found: C, 57.3; H, 6.89; N, 12.8; S, 5.56.

Carbobenzoxy-L-valyl-N$^{\epsilon}$-tosyl-L-lysyl-L-valyl-L-tyrosyl-L-proline Benzyl Ester (IV).—III (2 g., 3.66 mmoles) was dissolved in a mixture of 16 ml. of 1 *N* hydrochloric acid and 10 ml. of glacial acetic acid containing a few drops of ethyl acetate. The mixture was cooled to −2° and stirred vigorously with a Vibro-mixer,[27] and 0.276 g. of sodium nitrite was then added in small portions over a period of 20 min. Stirring was continued for another 30 min. Further operations were carried out in the cold room with equipment and reagents precooled to 0° for at least 2 hr. An equal volume of ice water was added and the azide was extracted into 40 ml. of ethyl acetate. The organic phase was washed with water, then with 5% sodium bicarbonate until neutral, and again with water. The ethyl acetate extract was dried over anhydrous sodium sulfate and filtered into a solution of 1.404 g. (3 mmoles) of L-valyl-L-tyrosyl-L-proline benzyl ester in 20 ml. of ethyl acetate at 0°. The total volume was reduced to about 50 ml. *in vacuo* at 0°, and the reaction mixture was kept at 4° for 3 days. A gelatinous precipitate was formed. After 3 days the solvent was removed *in vacuo*, the residue was again dissolved in fresh ethyl acetate, washed with 1 *N* hydrochloric acid, water, 5% sodium bicarbonate, and water, and, finally, the washed solution was dried over anhydrous sodium sulfate. The ethyl acetate

(26) R. E. Neuman and E. L. Smith, *J. Biol. Chem.*, **93**, 97 (1951).

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

(28) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

solution was filtered and concentrated to a volume of 50 ml. The protected pentapeptide benzyl ester IV crystallized on standing; yield 2.2 g. (75%); m.p. 149–151°. Recrystallization from hot ethyl acetate improved the m.p. to 152.5–153.5°. $[\alpha]^{25}D$ −25.7° (*c* 1, dimethylformamide). R_f BAW 0.91; R_f ASB 0.94.

Anal. Calcd. for $C_{52}H_{69}N_6O_{11}S$ (983.2): C, 63.5; H, 6.77; N, 8.55; S, 3.26. Found: C, 63.4; H, 6.72; N, 8.80; S, 3.41.

L-Valyl-N$^{\epsilon}$-tosyl-L-lysyl-L-valyl-L-tyrosyl-L-proline (V).—Carbobenzoxy-L-valyl-N$^{\epsilon}$-tosyl-L-valyl-L-tyrosyl-L-proline benzyl ester (0.5 g., 0.5 mmole) was dissolved in a mixture of 20 ml. of glacial acetic acid and 20 ml. of methanol, and the solution was hydrogenated for 8 hr. in the presence of freshly prepared palladium. The catalyst was filtered and washed with methanol. The filtrate and washings were evaporated to dryness to yield a crystalline residue, m.p. 215–218° dec. Recrystallization from hot methanol-water yielded 0.32 g. (84%) of V monohydrate, m.p. 240–241° dec.; $[\alpha]^{25}D$ −33.2° (*c* 1, acetic acid); R_f BAW 0.74; R_f ASB 0.67. C, H, and N analysis indicated that the pentapeptide crystallized with 1 mole of water.

Anal. Calcd. for $C_{37}H_{53}N_6O_8S \cdot H_2O$ (759.9) C, 58.5; H, 7.30; N, 11.1. Found: C, 58.6; H, 7.52; N, 11.1.

L-Valyl-L-lysyl-L-valyl-L-tyrosyl-L-proline.—L-Valyl-N$^{\epsilon}$-tosyl-L-lysyl-L-valyl-L-tyrosyl-L-proline monohydrate (0.1 g.) was dissolved in 150 ml. of liquid ammonia freshly distilled from sodium, and small pieces of sodium were added with stirring until a blue color persisted for 30 min. Ammonia was allowed to evaporate and the residue was dried over phosphorus pentoxide and sulfuric acid. This material was dissolved in 10 ml. of 1 *M* acetic acid, applied onto an Amberlite ion-exchanger IRC-50 column (3 × 5 cm.) in the acid form, and the column was washed with 300 ml. of 0.1 *M* acetic acid and 300 ml. of water. The peptide was eluted with 60 ml. of pyridine–acetic acid–water (30:4:66). The eluate was evaporated to dryness at room temperature *in vacuo*, and the residue was dissolved in water and lyophilized to yield 62 mg. of the pentapeptide VI. Peptide acetate content was estimated on the basis of ultraviolet absorption at 275 mμ, to be 97.7%. Yield 69%. $[\alpha]^{\circ 25}D$ −67° (*c* 0.6, water) (calcd. for the free peptide). The peptide was found to be homogeneous in three solvent systems in paper chromatography and gave a positive reaction with ninhydrin and the Pauly reagent. R_f BAW 0.26; R_f BPAW 0.50; R_f ASB 0.37. Paper electrophoresis revealed a single spot positive to ninhydrin and the Pauly reagent; mobility 0.53 with respect to lysine.

Anal. Calcd. for $C_{30}H_{48}N_6O_7 \cdot CH_3COOH \cdot 1.5\ H_2O$ (691.9): C, 55.5; H, 8.02; N, 12.1. Found: C, 55.5; H, 7.7; N, 11.9.

One milligram of the peptide was hydrolyzed for 25 hr. at 110° in a sealed evacuated tube, with 0.5 ml. of constant boiling hydrochloric acid. Quantitative analysis in the Spinco amino acid analyzer[19] gave the following ratio for the amino acids: $Val_{2.0}$, $Lys_{0.94}$, $Tyr_{1.0}$, $Pro_{1.0}$.

Enzymic Digestion of VI.—The pentapeptide VI (1.0 mg.) was dissolved in 0.5 ml. of tris(hydroxymethyl)aminomethane (TRIS) buffer, pH 8, containing 0.002 *M* magnesium chloride. To this solution was added 0.1 ml. of LAP solution containing 0.5 mg. of the enzyme (Worthington Biochemical, lot no. 5917). The digestion mixture was kept at 37° for 24 hr.

Crystalline α-chymotrypsin and trypsin were commercial products (Armour); digestion of VI by these enzymes was carried out at 25° for 24 hr. in a solution of pH 8.5, with an enzyme–substrate ratio of 1/100 (w./w.).

Reaction of Carbobenzoxy-L-valine *p*-Nitrophenyl Ester with L-tyrosine Methyl Ester.—L-Tyrosine methyl ester hydrochloride (3.45 g., 15 mmoles) was suspended in 40 ml. of acetonitrile and 2.1 ml. of triethylamine was added. The solid dissolved readily. Carbobenzoxy-L-valine *p*-nitrophenyl ester (5.58 g., 15 mmoles) was added, followed by another 2.1 ml. of triethylamine (15 mmoles). The reaction mixture was stirred at room temperature. Paper chromatograms in BAW, after 8 hr. of reaction, showed the presence of unchanged tyrosine methyl ester (R_f BAW) 0.46, ninhydrin and Pauly positive), and a new ninhydrin positive Pauly negative spot, (R_f BAW 0.68). The solvent was removed *in vacuo* after 48 hr. and the residue was redissolved in 50 ml. of ethyl acetate. The crystalline material was filtered off (1.6 g., m.p. 252–254°), and was identified as triethylamine hydrochloride (mixed m.p. 252–254°). The filtrate was washed with water; the ethyl acetate layer was dried, and 7.5 ml. of 2 *N* hydrochloric acid in ethyl acetate was added. Crystals appeared in a few minutes. The mixture was kept at 4° for 6 hr. and filtered. The crystalline product was washed with ethyl acetate and dried over sodium hydroxide to yield 3 g. of a product which was identified as O-(carbobenzoxy-L-valyl)-L-tyrosine methyl ester hydrochloride (VII), m.p. 183–185°. A sample was recrystallized from methanol–ether, m.p. 186–187°. $[\alpha]^{25}D$ −26.7° (*c* 1, methanol).

Anal. Calcd. for $C_{23}H_{29}N_2O_6Cl$ (464.9): 59.4; H, 6.29; N, 6.03; Cl, 7.63. Found: C, 59.6; H, 6.43; N, 6.31; Cl, 7.90.

The filtrate from VII was washed with water, dried, and concentrated. A second crystalline material separated; it was recrystallized from methanol to yield 0.53 g. of a product which melted at 159–160, and which failed to give any reaction with ninhydrin or the Pauly reagent. The second crystalline product was identified as O,N-di(carbobenzoxy-L-valyl)-L-tyrosine methyl ester, m.p. 160°.

Anal. Calcd. for $C_{36}H_{43}N_3O_9$ (661.7): C, 65.3; H, 6.55; N, 6.35. Found: C, 65.8; H, 6.83; N, 6.55.

Determination of the ultraviolet absorption spectra of VII and other crystalline products in ethanol, alkali and acid was performed in a Beckman Model DU spectrophotometer.

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