major peak, 1540 OD units (97% of the total recovered OD units). A white powder was obtained by working up this peak in the manner previously described for the DEAE-cellulose column effluent peak. After drying for 3 hr at 100° over $P_2O_5$ *in vacuo*, the mixed oxy-CoA isomers were obtained as the trilithium salt trihydrate in a yield of 78 mg (52%).

Venom phosphodiesterase digestion of this material produced spots identical with oxypantetheine-4′ phosphate and adenosine-2′(3′),5′ phosphate when subjected to paper chromatography.

*Anal.* Calcd for $C_{21}H_{33}N_7O_{17}P_3Li_3 \cdot 3H_2O$: C, 30.64; H, 4.78; N, 11.91. Found: C, 30.24; 30.51; H, 4.93, 5.14; N, 12.40.

The ultraviolet absorption spectrum, at pH 7.0, was determined on a Cary Model 14 spectrophotometer: $\lambda_{max}$ 259 mμ, $\lambda_{min}$ 230 mμ; absorbency ratios, 280/260 = 0.15, 250/260 = 0.81; the extinction coefficient (calculated from the spectrum of the trilithium salt trihydrate, mol wt 823) was $\epsilon_{259m\mu}$ $14.8 \times 10^3$.

*Anal.*[25] Calcd for $C_{21}H_{33}N_7O_{17}P_3Li_3 \cdot 3H_2O$ (adenosine:P): P, 11.3; A:P = 1:3. Found: P, 11.6; A:P = 1:3.09.

**Separation of Oxy-Coenzyme A (VII) from Isooxy-Coenzyme A (VIII).** The isooxy-coenzyme A–oxy-coenzyme A mixed isomers (53 mg, 825 OD units) were dissolved in 0.003 *N* HCl (25 ml) and applied to a 1.8 × 80 cm TEAE-cellulose (chloride form) column. The column was washed with 0.003 *N* HCl (150 ml) and then a linear gradient of lithium chloride in 0.003 *N* HCl was applied. The mixing vessel contained 4 l. of 0.025 *M* LiCl in 0.003 *N* HCl and the reservoir contained 4 l. of 0.055 *M* LiCl in 0.003 *N* HCl. Fifteen-milliliter fractions were collected and the optical density (260 mμ) of each fraction was determined. The effluent did not rise above 0.05 OD until fraction 164. Then two well-defined peaks with a slight overlapping area were obtained (Figure 2). Isooxycoenzyme A was present in peak A, fractions 166–193 inclusive (320 OD units). The overlapping fractions, 194–202, contained 62 OD units. Peak B, fractions 203–240, contained oxy-coenzyme A (374 OD units).

The total recovery in fractions 166 to 240 inclusive was 756 OD units (92%). Each of the peaks was characterized by venom phosphodiesterase degradation. The only detectable nucleotides after enzyme digestion and paper chromatography were adenosine-2′,5′ diphosphate from the peak A and adenosine-3′,5′ diphosphate from peak B.

**3′-Dephosphooxy-Coenzyme A.** 4-Morpholine N,N′-dicyclohexylcarboxamidinium adenosine-5′ phosphoromorpholidate[15] (153 mg, 0.2 mmole) was dried by three evaporations with anhydrous pyridine (5 ml each). Separately, D-oxypantetheine-4′ phosphate dilithium salt (223 mg, 0.48 mmole) was converted to the pyridinium salt and dried as described in the preparation of oxy-CoA. The D-oxypantetheine-4′ phosphate in anhydrous pyridine (10 ml) was added to the morpholidate and the mixture evaporated to an oil *in vacuo*. The residue was dissolved in anhydrous pyridine (10 ml) and permitted to react overnight at room temperature in a tightly stoppered flask. Pyridine was subsequently removed by several evaporations *in vacuo* with water; the residue was dissolved in water (25 ml) and applied to a 2.2 × 30 cm DEAE-cellulose (chloride form) column. The column was washed well with water and the adsorbed compounds were eluted by application of an acidic lithium chloride linear gradient. The reservoir contained 2.0 l. of 0.07 *N* lithium chloride in 0.003 *N* hydrochloric acid and the mixing vessel contained 2.0 l. of 0.003 *N* hydrochloric acid. Fifteen-milliliter fractions were collected. The 3′-dephosphooxy-CoA was found in fractions 75–93. Only oxypantetheine-4′ phosphate and adenosine-5′ phosphate were detected on paper chromatograms of the venom phosphodiesterase digestion mixture. 3′-Dephosphooxy-coenzyme A dilithium salt tetrahydrate (78 mg, 52% yield) was obtained as a fine, white powder after working up the peak in the manner described in the oxy-coenzyme A preparation and drying *in vacuo* over $P_2O_5$ at room temperature overnight.

The sample submitted for analysis was dried an additional 3 hr *in vacuo* over $P_2O_5$ at 100°.

*Anal.* Calcd for $C_{21}H_{33}N_7O_{14}P_2Li_2 \cdot 4H_2O$: C, 33.39; H, 5.47; N, 12.98. Found: C, 33.66; H, 5.88; N, 12.60.

**Venom Phosphodiesterase Degradations.** Degradations were accomplished by incubating 1–2 mg of the coenzyme A analogs, 100 μmoles of Tris-HCl buffer[26] (pH 9.0), and 0.2 mg of venom phosphodiesterate in a total volume of 0.4 ml for 90 min at 38°. Aliquots were directly spotted on sheets of chromatography paper and developed with the various solvent systems.

**Phosphotransacetylase Inhibition.** Phosphotransacetylase assays were performed by the method of Bergmeyer, *et al.*[17] The components of the system were: 235 μmoles of Tris-HCl buffer, pH 7.4; 1.5 mg of glutathione; 8 mg of acetylphosphate (lithium salt); 30 μmoles of ammonium sulfate; 0.33 μg of phosphotransacetylase; 0.134 to 0.670 μmole of CoA; inhibitor (Figure 3); and 3.0 ml total volume.

**Acknowledgments.** The authors are indebted to Dr. Grant R. Bartlett for aiding with the phosphorus analyses, and to Mr. Bruce Chassy for assisting in the early stages of the synthetic work.

(25) Phosphorus was determined by the Fiske–SubbaRow method as modified by G. R. Bartlett, *J. Biol. Chem.*, 234, 466 (1959).

(26) Tris = 2-amino-2-hydroxymethyl-1,3-propanediol.

# Synthesis of the Carboxyl-Terminal Heptapeptide Sequence of Bovine Pancreatic Ribonuclease

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**Abstract:** The carboxyl-terminal heptapeptide sequence of ribonuclease A, L-valyl-L-histidyl-L-phenylalanyl-L-aspartyl-L-alanyl-L-seryl-L-valine, has been synthesized. The hexapeptide was prepared by the sequential addition of carbobenzoxy derivatives of amino acids to L-valine *t*-butyl ester by the mixed carbonic anhydride procedure. The final residue was added as the *p*-nitrophenyl ester.

The synthesis of peptide sequences from bovine pancreatic ribonuclease has been reported from two laboratories, those of Hofmann[1] in the United States and of Rocchi, *et al.*,[2] in Italy. Hofmann's work has concerned itself so far with the amino terminal eicosapeptide sequence cleaved from the native enzyme by subtilisin.[3] This sequence contains one of the two

(1) (a) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *J. Am. Chem. Soc.*, 87, 611 (1965); (b) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *ibid.*, 87, 620 (1965); (c) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *ibid.*, 87, 631 (1965); (d) K. Hofmann, R. D. Wells, M. J. Smithers, R. Schmiechen, Y. Wolman, and G. Zanetti, *ibid.*, 87, 640 (1965); (e) F. M. Finn and J. Hofmann, *ibid.*, 87, 645 (1965).

(2) R. Rocchi, F. Marchiori, and E. Scoffone, *Gazz. Chim. Ital.*, 93, 823 (1963).

histidine residues of ribonuclease which have been implicated in the active site of the enzyme;[4] there are four histidines altogether per molecule of enzyme.[5] The second histidine of the active site is located at the opposite end of the polypeptide chain, in position 119. The exact sequence of the naturally occurring form of the protein is not required for the enzymatic activity according to the work of Anfinsen.[6] He has shown that the C-terminal residue, valine-124, and the penultimate residue serine-123 are not required for the reactivation of modified S-protein by intact S-peptide.[3] Inhibition of RNase by iodoacetic acid has been shown by Crestfield, *et al.*,[4] to result from alkylation of one or the other of the two active-site histidine residues; both 3-carboxymethylhistidine-12-ribonuclease and 1-carboxymethylhistidine-119-ribonuclease were isolated and characterized. There is also evidence that lysine-41 is involved in the catalytic functioning of the enzyme.[6b,7] Anfinsen had previously shown that when the four C-terminal residues, aspartic acid-121 through valine-124, were removed from native RNase A by pepsin, the resulting product was inactive.[6a] Hence, the carboxyl terminus of RNase is just as interesting as the amino terminus, although the latter has received most of the attention to date. We wish to report the synthesis of a series of peptides related to the amino acid sequence at the carboxyl terminus of bovine pancreatic ribonuclease A. These peptides specifically reproduce the sequence around histidine-119.

We have generally followed the scheme suggested by Bodanszky and du Vigneaud[8] for the stepwise synthesis of polypeptides. However, we used the mixed anhydrides[9] prepared from isobutyl chloroformate in most instances. In this sequential procedure, the classical carbobenzoxy group of Bergmann and Zervas[10] was used to protect the $\alpha$-amino functions, and the *t*-butyl group[11,12] was used to block carboxyl and aliphatic hydroxyl groups. The carbobenzoxy group was removed by catalytic hydrogenolysis over palladium oxide at each step in the synthesis, which is outlined in Figure 1.

Although Anderson's synthesis of N-carbobenzoxy-$\beta$-O-*t*-butyl-L-serine[12] is generally satisfactory, we were attempting synthesis of this compound through a slightly different intermediate at the time his work appeared in the literature. Hence, it has been convenient for us to continue using our method. The *p*-nitrobenzyl ester of carbobenzoxyserine was prepared by the usual method from *p*-nitrobenzyl bromide. The product inevitably contains at least a trace of *p*-nitrobenzyl bromide when purified by crystallization from either ethyl acetate–hexane or ethanol–water; however, a chromatographically and analytically pure product is

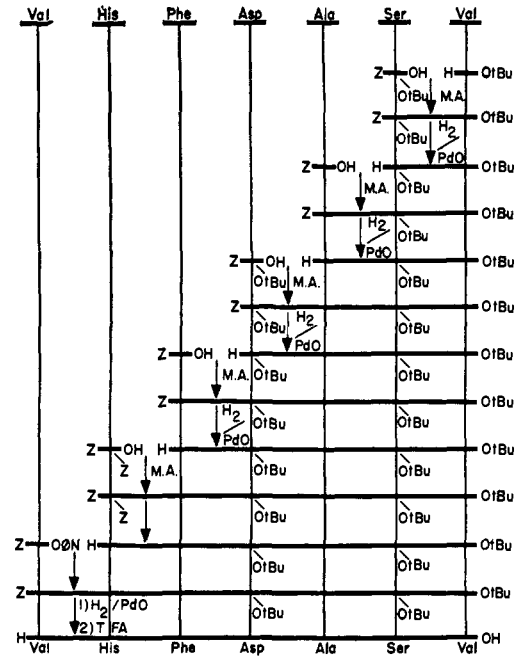


Figure 1. Synthetic scheme. Abbreviations: O$\phi$N, *p*-nitrophenyl ester; MA, mixed anhydride method; TFA, trifluoroacetic acid; O-t-Bu, *t*-butyl ester or ether; H, free amino group; OH, free carboxyl group.

obtained on recrystallization from hot benzene. Conversion of N-carbobenzoxy-L-serine *p*-nitrobenzyl ester to the $\beta$-O-*t*-butyl ether was carried out in methylene chloride–isobutylene with sulfuric acid as catalyst to give a crystalline product, N-carbobenzoxy-$\beta$-O-*t*-butyl-L-serine *p*-nitrobenzyl ester, mp 69–71°. This compound gives the free acid on saponification, mp 85–87°. In all cases where a second crop was recovered from the mother liquors on crystallization of the crude free acid, the material so obtained was found to be the DL compound, mp 59–60°.

N-Carbobenzoxy-$\beta$-O-*t*-butyl-L-serine was coupled by the mixed anhydride procedure[9] with L-valine *t*-butyl ester hydrochloride[11] in tetrahydrofuran (THF) in the presence of triethylamine to give N-carbobenzoxy-$\beta$-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester (I) in 80% yield, mp 75.5–77.5°. This peptide was hydrogenated to give the partially deprotected peptide $\beta$-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester as a syrup. When this was converted to the hydrochloride salt, it could be crystallized only by trituration with absolute ether. Attempts to recrystallize it from mixed solvent systems were unsuccessful, so the hydrochloride stage was omitted in the syntheses. Reaction of the hydrogenated dipeptide with the mixed anhydride of N-carbobenzoxy-L-alanine[10] in THF gave the protected tripeptide N-carbobenzoxy-L-alanyl-$\beta$-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester (II) in 80% yield, mp 101–103°. Thin layer chromatography of peptides I and II on silica gel G indicated that both were homogeneous.

Hydrogenation of II and coupling with N-carbobenzoxy-$\beta$-O-*t*-butyl-L-aspartic acid[13] by the mixed anhydride method gave the tetrapeptide N-carbobenzoxy-$\beta$-O-*t*-butyl-L-aspartyl-L-alanyl-$\beta$-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester (III) in 79% yield, mp 163–166°. Thin layer chromatography showed no impuri-

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(5) C. H. W. Hirs, S. Moore, and W. H. Stein, *ibid.*, **235**, 633 (1960).
(6) (a) C. B. Anfinsen, *ibid.*, **221**, 405 (1956); (b) C. B. Anfinsen, *Brookhaven Symp. Biol.*, **15**, 184 (1962); (c) J. T. Potts, Jr., D. M. Young, C. B. Anfinsen, and A. Sandoval, *J. Biol. Chem.*, **239**, 3781 (1964).
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(10) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).
(11) R. Roeske, *Chem. Ind.* (London), 1121 (1959).
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(13) R. Schwyzer and H. Dietrich, *Helv. Chim. Acta*, **44**, 2003 (1961).

ties. The process was repeated using N-carbobenzoxy-L-phenylalanine[14] to give the protected pentapeptide N-carbobenzoxy-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester (IV) in 61% yield, mp 206–208°, which appeared homogeneous by thin layer chromatography in four systems. In one case, the hydrogenated tetrapeptide crystallized and could be recrystallized from aqueous ethanol, mp 134.5–137°. This hydrogenation was always apparently complete, and no contamination of IV by III was detected by thin layer chromatography. When pentapeptide IV was partially deprotected and converted to the hydrochloride salt, mp 197–199°, the product showed a low carbon content on elemental analysis, indicating 1 mole of water of hydration per mole. The free base of pentapeptide IV could be crystallized from aqueous alcohol, mp 146–148°.

$N^{\alpha}$,$N^{IM}$-Dicarbobenzoxy-L-histidyl-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester (V) was prepared by a similar mixed anhydride condensation of dicarbobenzoxyhistidine[15] and the hydrochloride of the hydrogenated pentapeptide L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester. The product was obtained as a crystalline solid from aqueous ethanol in a yield of 63%, mp 181.5–182.5°. The protected hexapeptide was clearly distinguishable from the protected pentapeptide by thin layer chromatography in ethyl acetate–hexane mixtures in the ratios 7:3 and 9:1. By this method the hexapeptide was judged free of peptide IV; however, it showed a faint trace of a slow-moving contaminant, $R_f$ *ca.* 0.05, which also gave a positive reaction with Pauly's reagent. This was probably the monocarbobenzoxy derivative of the hexapeptide.

The heptapeptide was prepared by treating the hydrogenated hexapeptide with N-carbobenzoxy-L-valine *p*-nitrophenyl ester.[16] The product was obtained in 76% yield, mp 218–220°. Thin layer chromatography showed a single, Pauy-positive component in two systems, but a third system showed a trace of material probably corresponding to the monocarbobenzoxy derivative of V. The protected heptapeptide VI was converted to the free heptapeptide by hydrogenation in warm ethanol–acetic acid, followed by treatment with trifluoroacetic acid. The free peptide was homogeneous on paper chromatography, and appeared to be fully degraded to the constituent amino acids by treatment with leucine aminopeptidase. The peptide is soluble in aqueous acetic acid and in base at room temperature, but is only slightly soluble in neutral solution even at 100°.

In some instances, hydrogenation of the carbobenzoxy derivatives of N-terminal histidine peptides led to difficulties. One run of the hexapeptide V was hydrogenated over palladium oxide in methanol to give a mixture of starting material, free tris-*t*-butyl hexapeptide, and what may have been the monocarbobenzoxy hexapeptide. Subsequent purification by countercurrent distribution in the system *n*-butyl alcohol–1% acetic acid led to extensive decomposition. Considerable emulsion formation required an extended period of time for the distribution process. A poor yield of the desired product was obtained from this procedure, 29% of free base, mp 188–191°, from aqueous methanol. The irregular hydrogenations do not occur in all or even in many runs, and do not seem to be such a problem when histidine is in position 2, as in peptide VI. It has not been possible to identify the cause of the complications to date. As an illustration, the run in which peptide V was hydrogenated only to a limited extent is described in the Experimental Section; the sample of VI prepared from that particular run of V was slightly off-white in color and had a slightly lower melting point.

Crystalline derivatives of peptides containing several *t*-butyl protecting groups have been obtained. In fact, it was found that the free bases obtained by hydrogenation of peptides III and IV could be induced to crystallize, even though this was not necessary in the sequential synthesis of the longer peptide. Moreover, excellent yields can be achieved in synthesis of small peptides by the stepwise method[8] when isobutyl mixed carbonic anhydrides are substituted for active esters.

The incorporation of histidine into a peptide by methods consistent with the general technique of stepwise synthesis is not yet possible with all the ease one might wish. There are really only two methods of application here, the use of $N^{\alpha}$-carbobenzoxyhistidine azide[17] and the combination of $N^{\alpha}$,$N^{IM}$-dicarbobenzoxyhistidine[15] with any of a variety of coupling reagents. The azide gives only moderate yields, while the dicarbobenzoxy derivative worked well with the mixed anhydride method. There is always a trace of the monocarbobenzoxy derivative accompanying any preparation of a dicarbobenzoxyhistidyl peptide, and attempts at purification by recrystallization only serve to generate more. Fortunately, this is a benign impurity. It is to be expected that hydrogenation of monocarbobenzoxyhistidyl peptides will occasionally encounter the same difficulties as we have observed at times with the dicarbobenzoxy compounds.

## Experimental Section

**N-Carbobenzoxy-L-serine *p*-Nitrobenzyl Ester.** N-Carbobenzoxy-L-serine (17.2 g, 0.072 mole) and 16.2 g (0.075 mole) of *p*-nitrobenzyl bromide were dissolved in 500 ml of ethyl acetate and 9.75 ml (0.070 mole) of triethylamine. The solution was refluxed for 22 hr, cooled, and filtered. The filtrate was washed once with water, twice with 2 *N* potassium bicarbonate, and twice again with water. The organic layer was dried with anhydrous magnesium sulfate and filtered, and the filtrate was evaporated under vacuum. The material was crystallized from benzene. The crystalline product was dried under vacuum at room temperature (0.10 mm) over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets, yielding 2.14 g (82%), mp 115–118°.

For analysis, a sample was recrystallized twice from hot benzene and dried under vacuum at room temperature over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets, mp 117.5–118.5°, $[\alpha]^{21}D$ −9.8° (*c* 3.41, 95% ethanol). Before analysis, a sample was dried under vacuum at 100° (0.25 mm) for 16 hr over phosphorus pentoxide.

*Anal.* Calcd for $C_{18}H_{18}N_2O_7$ (374.35): C, 57.75; H, 4.85; N, 7.48. Found: C, 57.59; H, 5.00; N, 7.32.

**N-Carbobenzoxy-β-O-*t*-butyl-L-serine *p*-Nitrobenzyl Ester.** N-Carbobenzoxy-L-serine *p*-nitrobenzyl ester (20.0 g, 0.0534 mole) was divided into four portions and each was placed in a 250-ml pressure bottle. To each portion was added 50 ml of methylene chloride, *ca.* 0.5 ml of concentrated sulfuric acid, and *ca.* 50 ml of

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(15) A. Patchornik, A. Berger, and E. Katchalski, *J. Am. Chem. Soc.*, **79**, 6416 (1957).
(16) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).
(17) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

liquid isobutylene. The bottles were closed and then shaken at room temperature for 12 hr. The bottles were cooled to +5° before opening. The contents of the opened bottles were diluted with ethyl acetate; then part of the isobutylene was allowed to evaporate in the hood. Finally, the solutions were combined, treated with potassium carbonate solution, and washed three times with water. The organic layer was dried with anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The residue was crystallized from hot 95% ethanol, the yield of crude product being 19.2 g (84%), mp 68.5–69.5°. This material was suitable for conversion to the free acid, even though it contained a little polyisobutylene.

For analysis, a sample was purified by dissolving it in warm 95% ethanol, treating with decolorizing carbon, and filtering through Celite. The product crystallized on cooling, and was recrystallized once again from ethanol without further decolorizing treatment, mp 69–71°, $[\alpha]^{22}D$ −17.1° (*c* 3.05, dimethylformamide). Before analysis, a sample was dried under vacuum at 55° (0.5 mm) for 16 hr over phosphorus pentoxide.

*Anal.* Calcd for $C_{22}H_{26}N_2O_7$ (430.46): C, 61.38; H, 6.09; N, 6.51. Found: C, 61.16; H, 6.03; N, 6.37.

**N-Carbobenzoxy-β-O-*t*-butyl-L-serine.** N-Carbobenzoxy-β-O-*t*-butyl-L-serine *p*-nitrobenzyl ester (42.2 g, 98.4 mmoles) was dissolved in 250 ml of methanol by warming. To this solution was added 110 ml of 1 *N* sodium hydroxide. The solution turned yellow and an oil precipitated which dissolved on stirring. After 30 min, the methanol was removed under vacuum and 50 ml of water was added to the residue. The aqueous solution was extracted four times with ether, which was then washed once with 1 *N* sodium hydroxide and twice with water. The aqueous solutions were combined, acidified with citric acid, and extracted with ethyl acetate. The organic phase was washed once with citric acid solution, twice with water, and dried over magnesium sulfate. The solution was filtered and evaporated under vacuum, and the residual oil was crystallized from ethyl acetate by addition of hexane, the crude yield being 14.4 g (56%). The product was dissolved in hot carbon tetrachloride, filtered to remove some insoluble citric acid, and crystallized by cooling, yielding 9.6 g (38%), mp 85–87°, $[\alpha]^{21}D$ +21.2° (*c* 2.82, 95% ethanol). A second crop of 1.8 g (7%), mp 83–86°, was recovered from mother liquors.

*Anal.* Calcd for $C_{15}H_{21}NO_5$ (295.34): C, 61.00; H, 7.17; N, 4.74. Found: C, 61.21; H, 7.08; N, 5.03.

**N-Carbobenzoxy-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (I).** N-Carbobenzoxy-β-O-*t*-butyl-L-serine (2.85 g, 9.66 mmoles) was dissolved in 50 ml of tetrahydrofuran. Triethylamine (1.34 ml, 9.66 mmoles) was added, and the solution was cooled in an ice–isopropyl alcohol bath. The solution was stirred magnetically and treated with 1.24 ml (9.50 mmoles) of isobutyl chloroformate. Stirring and cooling were continued for 25 min. At that time, 1.8 g (10.5 mmoles) of *t*-butyl-L-valinate was added. (Use of the ester hydrochloride and 1 equiv of triethylamine did not alter yields or properties of the dipeptide.) The reaction mixture was stirred in the cold bath and allowed to come to room temperature gradually overnight. The precipitated triethylammonium chloride was removed by filtration and the filtrate was evaporated under vacuum. The residual oil was dissolved in ethyl acetate and washed twice with 5% citric acid solution, once with water, twice with 2 *N* potassium bicarbonate solution, and twice with water. The aqueous washes were in turn washed with a portion of fresh ethyl acetate. The ethyl acetate solutions were combined, dried with anhydrous magnesium sulfate, and filtered. The solvent was evaporated under vacuum. The residual oil was crystallized from hexane by cooling in Dry Ice. The resulting solid was filtered, air dried, and recrystallized from aqueous ethanol by seeding with solid from the hexane product. In subsequent runs, the oil was crystallized directly from ethanol–water or from warm hexane with the assistance of seed crystals. The product was dried in a desiccator under vacuum over phosphorus pentoxide, sodium hydroxide pellets, and paraffin wax, yielding 2.54 g (59%), mp 75.5–77.5°. A second crop was obtained by evaporation of the combined mother liquors from above, treatment with decolorizing charcoal, and crystallization from ethanol–water, yielding 0.87 g (20%), mp 66–70°.

A sample was recrystallized with decolorization for analysis, mp 73.5–76.5°, $[\alpha]^{21}D$ −2.2° (*c* 2.54, 95% ethanol).

Thin layer chromatography of the product purified for analysis showed only one component: $R_f$ 0.75 in ethyl acetate–hexane (9:1), $R_f$ 0.69 in ethyl acetate–hexane (7:3). Before analysis, the material was dried under vacuum for 16 hr at 65° (0.15 mm) over phosphorus pentoxide.

*Anal.* Calcd for $C_{24}H_{38}N_2O_6$ (450.58): C, 63.98; H, 8.50; N, 6.22. Found: C, 63.83; H, 8.32; N, 6.25.

**β-O-*t*-Butyl-L-seryl-L-valine *t*-Butyl Ester Hydrochloride.** The protected dipeptide I (7.37 g, 16.3 mmoles) was hydrogenated in 150 ml of methanol over palladium oxide. When the catalyst had coagulated, the mixture was filtered and evaporated under vacuum. The residual oil was dissolved in ether and treated with 4.8 ml of 3.5 *N* methanolic hydrogen chloride. On concentration to dryness under vacuum, the residual material crystallized. These crystals were triturated with ether, filtered, and dried in a desiccator under vacuum over phosphorus pentoxide, paraffin wax, and sodium hydroxide pellets, yielding 5.19 g (90%), mp 170–173°, $[\alpha]^{21}D$ −15.5° (*c* 3.42, 95% ethanol).

*Anal.* Calcd for $C_{16}H_{33}N_2O_4Cl$ (352.90): C, 54.45; H, 9.43; N, 7.34; Cl, 10.05. Found: C, 54.21; H, 9.25; N, 7.62; Cl, 9.99.

**Carbobenzoxy-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (II).** The protected dipeptide I (3.71 g, 8.23 mmoles) was dissolved in 110 ml of methanol. Palladium oxide (0.41 g) was added, and the system was flushed with nitrogen for about 5 min. Then hydrogen was bubbled through the solution with magnetic stirring for about 2 hr. Midway through the hydrogenolysis, 0.10 g of fresh palladium oxide was added to the reaction mixture. When all the catalyst had precipitated as palladium black, the system was again flushed with nitrogen and the catalyst removed by filtration. The filtrate was evaporated under vacuum and the residual syrup dissolved in 50 ml of tetrahydrofuran.

Carbobenzoxy-L-alanine[10] (2.01 g, 9.0 mmoles) was dissolved in 50 ml of tetrahydrofuran. Triethylamine (1.2 ml, 8.6 mmoles) was added, and the solution was cooled in an ice–2-propanol bath and stirred magnetically. To the cold solution was added 1.08 ml (8.23 mmoles) of isobutyl chloroformate. After having reacted for 15 min, the solution of mixed anhydride was treated with the dipeptide ester. The reaction mixture was allowed to come to room temperature overnight.

The product was worked up exactly as for the dipeptide, but the hexane crystallization was omitted and the product was crystallized directly from ethanol and water. The crystalline material was dried in a desiccator as in the case of the dipeptide, yielding 3.01 g (81%), mp (90°) 96–100°.

For analysis, a sample was recrystallized twice from ethanol–water and dried under vacuum for 25 hr at 76° (0.10 mm) over phosphorus pentoxide, mp 101–103°, $[\alpha]^{21}D$ −25.0° (*c* 2.89, 95% ethanol).

Thin layer chromatography of the analytical sample showed only one component: $R_f$ 0.62 in ethyl acetate–hexane (9:1), $R_f$ 0.46 in ethyl acetate–hexane (7:3).

*Anal.* Calcd for $C_{27}H_{43}N_3O_7$ (521.66): C, 62.17; H, 8.31; N, 8.05. Found: C, 62.13; H, 8.17; N, 7.98.

**Carbobenzoxy-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (III).** N-Carbobenzoxy-β-O-*t*-butyl-L-aspartic acid α-benzyl ester[13] (4.35 g, 10.5 mmoles) was saponified as described by Schwyzer and Dietrich and the syrupy product, N-carbobenzoxy-β-O-*t*-butyl-L-aspartic acid, was dissolved in 100 ml of tetrahydrofuran.

The protected tripeptide II (4.21 g, 9.33 mmoles) was dissolved in 100 ml of 95% ethanol and hydrogenated with 0.45 g of palladium oxide as described for the preparation of the tripeptide from the dipeptide. When the hydrogenolysis was finished, the catalyst was filtered off and the solution was evaporated under vacuum. The residual oil was taken up in about 120 ml of tetrahydrofuran.

For the preparation of the mixed anhydride, the solution of the aspartic acid derivative was treated with 1.30 ml (9.33 mmoles) of triethylamine and cooled in an ice–2-propanol bath. With magnetic stirring, the anhydride was formed by addition of 1.22 ml (9.33 mmoles) of isobutyl chloroformate to the cold solution. After 25 min, the solution of tripeptide ester was added to the mixed anhydride, and the reaction mixture was stirred overnight as the cold bath gradually warmed to room temperature. The product was isolated exactly as in the case of the di- and tripeptides. Before crystallization, the tetrapeptide was treated with decolorizing charcoal. The product was twice crystallized from ethanol–water and was dried in a desiccator under vacuum over phosphorus pentoxide, sodium hydroxide pellets, and paraffin wax, yielding 5.34 g (82%), mp (155°) 163–166°, $[\alpha]^{21}D$ −22.2° (*c* 3.90, 95% ethanol).

For analysis, a sample was dried under vacuum for 16 hr at 100° (0.10 mm) over phosphorus pentoxide.

Thin layer chromatography of a sample showed only one component: $R_f$ 0.76 in chloroform–methanol (19:1); $R_f$ 0.60 in

100% ethyl acetate; $R_f$ 0.59 in ethyl acetate–hexane (9:1); $R_f$ 0.36 in ethyl acetate–hexane (7:3).

*Anal.* Calcd for $C_{35}H_{56}N_4O_{10}$ (692.85): C, 60.67; H, 8.15; N, 8.09. Found: C, 60.55; H, 8.07; N, 8.30.

**N-Carbobenzoxy-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (IV).** Tetrapeptide III (4.56 g, 6.37 mmoles) was dissolved in 100 ml of 95% ethanol. Palladium oxide (0.42 g) was added, and hydrogenolysis was carried out as described for synthesis of II. The mixed anhydride was prepared by cooling a solution of 1.97 g (6.59 mmoles) of N-carbobenzoxy-L-phenylalanine[14] in 50 ml of THF in an ice–acetone bath. To the cold solution were then added 0.82 ml (6.28 mmoles) of isobutyl chloroformate and 0.91 ml (6.59 mmoles) of triethylamine with magnetic stirring. Cooling and stirring were continued for 32 min, at which time the hydrogenated peptide III was taken up in about 50 ml of THF and added to the mixed anhydride. The reaction mixture was stirred magnetically and allowed to come to room temperature overnight. Triethylammonium chloride was removed by filtration and washed with ethyl acetate. The filtrates were combined and evaporated under vacuum. The residue was triturated with ether, filtered, washed on the filter with water, and crystallized from aqueous ethanol. The product was dried in a desiccator under vacuum over phosphorus pentoxide and sodium hydroxide pellets at room temperature, yielding 3.26 g (62%), mp 206–208°.

For analysis, a sample was recrystallized from aqueous ethanol and dried *in vacuo* for 14 hr at 100° (0.10 mm) over phosphorus pentoxide, mp 206–208°, $[\alpha]^{22}D$ −17.4° (*c* 1.47, dimethylformamide).

Thin layer chromatography of the analytical samples showed only one component: $R_f$ 0.72 in $CHCl_3$–methanol (19:1); $R_f$ 0.46 in ethyl acetate (100%); $R_f$ 0.45 in ethyl acetate–hexane (9:1); $R_f$ 0.25 in ethyl acetate–hexane (7:3).

*Anal.* Calcd for $C_{44}H_{65}N_5O_{11}$ (840.03): C, 62.91; H, 7.80; N, 8.34. Found: C, 63.05; H, 7.86; N, 8.39.

*Amino Acid Anal.* Calcd:[18] $Ala_1Asp_1Phe_1Ser_1Val_1$. Found: $Ala_{0.99}Asp_1Phe_{1.05}Ser_{0.90}Val_{0.99}$.

**L-Phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester Hydrochloride.** The protected pentapeptide IV (2.90 g, 3.45 mmoles) was suspended in 200 ml of methanol, 0.23 g of palladium oxide was added, and the hydrogenolysis was carried out as usual. A little dimethylformamide (DMF) was added to help get the peptide into solution. The residue from evaporation of the solvents was taken up in ethyl acetate. Ether was added, followed by 1.0 ml of 3.4 *N* methanolic hydrogen chloride. The product precipitated and was collected. The material was dried overnight at room temperature in a desiccator under vacuum over phosphorus pentoxide, paraffin wax shavings, and sodium hydroxide pellets, yielding 2.41 g (92%), mp *ca.* 190° dec.

For analysis, a sample was recrystallized from ethanol–ethyl acetate by warming, mp 197–199° dec., $[\alpha]^{21}D$ −7.9° (*c* 1.00, dimethylformamide).

*Anal.* Calcd for the anhydrous peptide, $C_{36}H_{60}N_5O_9Cl$ (742.36): C, 58.25; H, 8.15; N, 9.43; Cl, 4.78. Calcd for the monohydrate, $C_{36}H_{62}N_5O_{10}Cl$ (760.37): C, 56.87; H, 8.22; N, 9.22; Cl, 4.67. Found: C, 57.28; H, 8.52; N, 9.47; Cl, 4.74.

**$N^{\alpha}$,$N^{1M}$-Dicarbobenzoxy-L-histidyl-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (V).** $N^{\alpha}$,$N^{1M}$-Dicarbobenzoxy-L-histidine[15] (1.34 g, 3.0 mmoles) was dissolved in 50 ml of THF and cooled in an ice–acetone bath. With magnetic stirring and continued cooling, 0.42 ml (3.0 mmoles) of triethylamine and 0.35 ml (2.7 mmoles) of isobutyl chloroformate were added; the temperature of the reaction mixture was −3°. After 18 min, the mixed anhydride was treated with 0.37 ml (2.7 mmoles) of triethylamine and 2.0 g (2.7 mmoles) of L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-butyl ester hydrochloride. Stirring and cooling were continued for a few minutes, and then the reaction mixture was allowed to stand in the cold room (+5°) overnight. The solvent was evaporated under vacuum and the residue was taken up in ethyl acetate and 5% citric acid solution. The organic layer was washed twice more with fresh portions of 5% citric acid, once with distilled water, twice with 2 *N* potassium bicarbonate, and twice with distilled water. The bicarbonate solutions required centrifugation to break the emulsions which formed on washing. The ethyl acetate solution was dried with anhydrous magnesium sulfate, filtered, and evaporated under vacuum. The residue was crystallized from aqueous ethanol. The product was collected and dried at room temperature in a desiccator at less than 1 mm over phosphorus pentoxide, paraffin wax shavings, and sodium hydroxide pellets, yielding 1.89 g (63%), mp 181.5–182.5°, $[\alpha]^{22}D$ −18.6° (*c* 2.30, dimethylformamide).

Thin layer chromatography indicated that the product contained a trace impurity which showed $R_f$ about 0 for the solvent systems tried; it was presumably the monocarbobenzoxy derivative of the product. The product itself showed the following: $R_f$ 0.065 in ethyl acetate–hexane (7:3); $R_f$ 0.60 in chloroform–methanol (19:1).

*Anal.* Calcd for $C_{58}H_{78}N_8O_{14}$ (1111.30): C, 62.69; H, 7.08; N, 10.08. Found: C, 62.43; H, 7.23; N, 10.16.

*Amino Acid Anal.* Calcd:[18] $Ala_1Asp_1His_1Phe_1Ser_1Val_1$. Found: $Ala_{0.98}Asp_1 His_{0.99}Phe_{1.06}Ser_{0.92}Val_{0.99}$.

**N-Carbobenzoxy-L-valyl-L-histidyl-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-seryl-L-valine *t*-Butyl Ester (VI).** The protected hexapeptide V (1.58 g, 1.42 mmoles) was dissolved in 100 ml of 95% ethanol by warming. Palladium oxide (0.31 g) was added, and the sample was hydrogenated as usual, with hydrogen passed through overnight. The catalyst was filtered off, and the solution was evaporated under vacuum. The residue was slurried up in ethyl acetate, giving an amorphous gel which was filtered, washed on the filter with ether, and air dried, yielding 1.12 g (93%). This material was dissolved in DMF and treated with 0.75 g (2.0 mmoles) of carbobenzoxy-L-valine *p*-nitrophenyl ester[16] at room temperature. After about 24 hr, 5 ml of water and 1 ml of pyridine were added to the reaction mixture, and the solution was heated in a boiling water bath for 1 hr. After cooling, the solution was poured into water and the precipitated product was filtered. The solid was washed on the filter with water and then with ether, the crude yield being 1.27 g (89%). The peptide was recrystallized from hot ethanol by addition of water. The solution was cooled overnight, and the crystalline product was filtered and washed with water. The peptide was dried in a vacuum desiccator over phosphorus pentoxide, sodium hydroxide pellets, and paraffin wax, yielding 1.04 g (72%), mp 218–220°, $[\alpha]^{22}D$ −20.2° (*c* 1.25, DMF). A second crop of 0.06 g (4%) was recovered from mother liquors. The main crop had $R_f$ 0.09 in thin layer chromatography on silica gel plates in the system chloroform–methanol (19:1), and $R_f$ *ca.* 0 in ethyl acetate. The spot gave a positive Pauly test.

*Anal.* Calcd for $C_{55}H_{81}N_9O_{13}$ (1076.3): C, 61.38; H, 7.59; N, 11.71. Found: C, 61.33; H, 7.45; N, 11.59.

*Amino Acid Anal.* Calcd:[18] $Ala_1Asp_1His_1Phe_1Ser_1Val_2$. Found: $Ala_{0.96}Asp_1His_{0.97}Phe_{1.03}Ser_{0.93}Val_{1.81}$.

**L-Valyl-L-histidyl-L-phenylalanyl-L-aspartyl-L-alanyl-L-seryl-L-valine.** The protected heptapeptide VI (0.53 g, 0.49 mmole) was dissolved in 150 ml of 95% ethanol by warming. Palladium oxide (0.10 g) was added and the sample was hydrogenated as usual. Part way through the course of the reaction, 1 ml of acetic acid was added. The hydrogenation was run for 22 hr, then the catalyst was removed by filtration. An aliquot of the filtrate was chromatographed on paper in the systems *n*-butyl alcohol–acetic acid–water (4:1:1) and *n*-butyl alcohol–acetic acid–water–pyridine (30:6:24:20), and showed only one component. The solvent was removed under vacuum and the residue was treated with trifluoroacetic acid at room temperature. The acid was evaporated and the residue was precipitated from water and neutralized with pyridine by addition of acetone. The product was an amorphous gel, yielding 0.21 g (55%), $[\alpha]^{24}D$ −74.3° (*c* 0.498, water).

The free peptide appeared to be homogeneous on chromatography in the two above solvent systems, with $R_f$ 0.20 in the former and $R_f$ 0.50 in the latter. A sample was digested with leucine amino peptidase, and the digest analyzed by paper chromatography. Only the component free amino acids were observed.

*Anal.* Calcd for $C_{35}H_{51}N_9O_{11}\cdot 4H_2O$ (845.91): C, 49.70; H, 7.03; N, 14.90. Found: C, 50.00; H, 6.38; N, 15.24.

*Amino Acid Anal.* Calcd:[18] $Ala_1Asp_1His_1Phe_1Ser_1Val_2$. Found: $Ala_{1.02}Asp_1His_{0.95}Phe_{0.99}Ser_{0.90}Val_{1.93}$.

**L-Histidyl-L-phenylalanyl-β-O-*t*-butyl-L-aspartyl-L-alanyl-β-O-*t*-butyl-L-valine *t*-Butyl Ester.** Protected hexapeptide V (3.4 g, 3.06 mmoles) was dissolved in 250 ml of ethanol. Palladium oxide (0.65 g) was added and the mixture was hydrogenated for 7 hr in the usual way. Filtration of the reaction mixture and concentration under vacuum gave a residue which showed four components on thin layer chromatography after crystallization from aqueous ethanol. The material was again hydrogenated for 14 hr and worked up, again showing four components, which corresponded to starting material, monocarbobenzoxy hexapeptide, the desired product, and some free base of IV. A sample (1.67 g) was purified by countercurrent distribution in the system *n*-butyl-alcohol–1% aqueous acetic acid. The product was located by paper chromatography of aliquots. Tubes containing the material

(18) Amino acid analysis calculated with respect to aspartic acid. Samples were hydrolyzed in glass-distilled HCl at 110°.

were combined and evaporated under vacuum, and the residue was crystallized from aqueous methanol, yielding 0.75 g (29%), mp 188–191°, $[\alpha]^{23}D$ −32.2° (*c* 2.11, 95% ethanol).

*Anal.* Calcd for $C_{42}H_{66}N_8O_{10}$ (843.04): C, 59.84; H, 7.89; N, 13.29. Found: C, 59.61; H, 8.32; N, 12.83.

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# Solid-Phase Synthesis of a Hexadecapeptide and Test of Its Chymotryptic Activity[1]

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**Abstract:** The synthesis of the hexadecapeptide Tyr-His-His-Phe-Phe-Asp-His-Ser-Asp-Ser-His-Phe-Asp-Phe-His-Phe was carried out by the solid-phase method. This large peptide was obtained in analytically pure condition. It had been conceived as one which might possibly exhibit the specific catalytic action of chymotrypsin on the basis of some recent findings about the groups which may be connected with the enzymic activity of this enzyme. The hexadecapeptide, however, showed no esteratic activity toward acetylphenylalanine ethyl ester and no proteolytic activity against serum albumin. These findings indicated that more is required for specific chymotryptic activity than histidine and serine residues interspersed at frequent and definite intervals along a rather large peptide chain.

The introduction by Merrifield[2,3] of the solid-phase method of peptide synthesis has made it practical to explore by means of synthetic organic models some of the structural features of a protein which give it specific enzymic action. Although earlier methods of peptide synthesis have allowed the preparation of fragments of enzymes such as ribonuclease,[4] these methods are so laborious and costly that to use them to prepare a considerable number of enzyme models composed of peptides with 15 to 30 amino acid residues is quite difficult. In the present paper will be described the ready synthesis in good over-all yield of a hexadecapeptide and the use of it to begin the testing of current concepts of what is required for chymotryptic activity.

Much work during the past decade has indicated that in the enzymic activity of chymotrypsin (and of several other enzymes) one particular serine residue is essential. More recently,[5] a particular histidine residue has also been shown to participate. Perhaps even two histidine residues are required[6] since in ribonuclease, two separate histidines have been directly connected with the enzymic activity[7,8] and the suggestion has been made that the same may possibly hold for chymotrypsin,[6] although there has been no direct experimental demonstration of this with the latter enzyme.

Several other features of the structure of chymotrypsin also have been postulated to be intimately concerned with its specific enzymic activity. Among these is the suggestion that the specificity site may reside in the aromatic (tyrosine) residue at the carboxyl end of one of the peptide chains,[9] and that the aspartic acid residue next to the special serine in the "active center" may play some essential role.[10,11] There is also the suggestion that a large molecular weight may be a necessity for specific enzyme action, although there is as yet no unequivocal evidence to support such a view.

With the advent of the solid-phase method of peptide synthesis, it seemed possible to test some of the ideas about what may be required. For example, it should be possible to synthesize relatively large peptides which incorporate some of the structural features discussed above, and to test these polypeptides for the specific enzymic activity.

The peptide to be described in this paper is L-tyrosyl-L-histidyl-L-histidyl-L-phenylalanyl-L-phenylalanyl-L-aspartyl-L-histidyl-L-seryl-L-aspartyl-L-seryl-L-histidyl-L-phenylalanyl-L-aspartyl-L-phenylalanyl-L-histidyl-L-phenylalanine. It incorporated some of the features of chymotrypsin which are thought to be necessary for its specific activity. For example, it has several histidine residues spaced at such intervals as to have two of them adjacent in the primary structure, and also, if helices were to form, to have histidines one turn apart, and possibly therefore relatively close together in space. It contained aspartic acid and serine residues in the sequence found in chymotrypsin. It had six residues of aromatic amino acids, with one of these at the carboxyl end. The idea here was to provide substrate specificity in this way.

Some objections could be raised against the idea that this hexadecapeptide might exhibit chymotryptic activ-

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