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-

(1) Supported in part by Grant A1260 from the U. S. Public Health Service. With the technical assistance of J. Senderowitz and J. P. Watson.
(2) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(3) R. B. Merrifield, *Science*, **150**, 178 (1965).
(4) K. Hoffmann, F. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaihara, *J. Am. Chem. Soc.*, **85**, 833 (1963).
(5) E. B. Ong, E. Shaw, and G. Schoellmann, *J. Biol. Chem.*, **240**, 694 (1965).
(6) K. A. Walsh, D. L. Kauffmann, K. S. V. S. Kumar, and H. Neurath, *Proc. Natl. Acad. Sci. U. S.*, **51**, 301 (1964).
(7) D. Findlay, D. G. Herries, A. P. Mathias, B. R. Robin, and C. A. Ross, *Nature*, **190**, 781 (1961).
(8) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2421 (1963).
(9) D. W. Woolley, J. W. B. Hershey, and I. H. Koehelik, *Proc. Natl. Acad. Sci. U. S.*, **48**, 709 (1962).
(10) Y. Shalitin and S. Bernhard, *J. Am. Chem. Soc.*, **86**, 2291 (1964).
(11) H. N. Rydon, *Nature*, **183**, 928 (1958).

ity. These were not unknown in the planning of this work. However, it was felt that a start should be made in an effort to construct synthetic model enzymes, and that the one suggested might serve some useful purpose. It would, for example, show whether a relatively large peptide with aspartic acid and serine in proper sequence, and with histidine residues suitably disposed, might in fact possess activity.

The plan was to test for specific chymotryptic activity, not to test for ability to catalyze the hydrolysis of nitrophenyl esters. Although the study of the hydrolysis of nitrophenyl acetate by chymotrypsin has in the past yielded valuable information about the mechanism of enzyme action,[12,13] it must not be forgotten that many nonenzymic proteins, and even imidazole itself, will catalyze the hydrolysis of this unstable ester. The substrate specificity, so characteristic of enzymes, is lacking in the action of chymotrypsin on nitrophenyl acetate. The importance of this consideration can be seen from the fact that, although the pentapeptide Thr-Ala-Ser-His-Asp which includes the "active" serine of phosphoglucomutase will catalyze the hydrolysis of nitrophenyl acetate somewhat better than imidazole itself will, the pentapeptide has none of the ability of the entire enzyme to act catalytically on the specific substrate for this enzyme.[14]

The hexadecapeptide mentioned earlier in this paper was synthesized and obtained in relatively pure condition. Chromatographically and electrophoretically it appeared to be a single substance. It did not show chymotryptic activity against either acetylphenylalanine ethyl ester or serum albumin.

Experimental Section

Synthesis of the Hexadecapeptide Resin. The peptide was made by a modification of the solid-phase method as described by Merrifield[2,3] for the synthesis of bradykinin. The modifications were those described by Stewart and Woolley.[15]

Chloromethylated polystyrene (1.5 g) was prepared and esterified with *t*-BOC-L-phenylalanine[16] (400 mg.) according to the general method of Merrifield.[2] The product was found to contain 0.73 mmole of phenylalanine when hydrolyzed (20 hr) in refluxing 50% dioxane–water, 6 *M* with respect to HCl, and analyzed according to Moore, *et al.*[17] The *t*-BOC group was removed by treatment of the resin in a shaker for 30 min with 10 ml of 4 *M* HCl in dry dioxane, followed by filtration and manifold washing with dioxane and then chloroform. The resin was then shaken for 10 min with 10 ml of 10% triethylamine in chloroform, followed by filtration and thorough washing with chloroform. The resin was then suspended in 4 ml of dry DMF (freshly distilled under reduced pressure to remove formic acid and dimethylamine) containing 1.26 g (5 equiv) of *t*-BOC-benzyl-L-histidine. After the solution had been allowed to penetrate into the resin for 10 min, a solution of 752 mg (5 equiv) of dicyclohexylcarbodiimide in 2 ml of dry DMF was added in four portions, 2 min apart, with good agitation. The suspension was shaken for 2 hr, and the resin was filtered and washed thoroughly, first with DMF and then with dry dioxane. It was then ready for the next cycle of deprotection, liberation of the free amino group from its hydrochloride, and coupling with the next amino acid residue, *viz.* *t*-BOC-L-phenylalanine.

In this way, each amino acid residue in turn was added to the growing peptide chain on the resin. All *t*-BOC amino acids were of the L configuration. DMF was required for coupling with *t*-BOC-benzylhistidine. However, the solvent used during each of the other coupling steps was methylene chloride. The serines were added in the form of *t*-BOC-O-benzyl-L-serine, and aspartic acids in the form of *t*-BOC-L-aspartic-β-benzyl ester. The tyrosine was added as N-carbobenzoxy-O-benzyl-L-tyrosine and was used as one residue in preference to phenylalanine in order to provide a marker to increase the accuracy of interpretation of the amino acid analysis.

Liberation of the Hexadecapeptide from the Resin. The dried hexadecapeptide resin (2.7 g) was suspended in trifluoroacetic acid (20 ml), and dry HBr was bubbled through the suspension for 90 min. The resin was filtered off and washed with trifluoroacetic acid, and the solution so obtained was immediately concentrated under reduced pressure to dryness. Excess HBr was removed by solution of the residue in trifluoroacetic acid and evaporation.

The benzyl groups were removed from the histidine residues of the peptide by solution of it in liquid NH_3 and treatment with Na (blue for 5 min). An equivalent of NH_4Cl was then added, and the NH_3 was allowed to evaporate. Preliminary experiments had shown that two of the five benzyl groups could be removed by catalytic hydrogenation in 80% ethanol for 4 days with a large excess of freshly prepared palladium black. The other three benzyl groups could not be so removed.

The desired hexadecapeptide was isolated from the reaction mixture after the reduction with Na by means of countercurrent distribution. The solvent system used was *n*-butyl alcohol–water–trifluoroace ic acid, 1:1:0.01, and 99 transfers were made. The maximum of the desired peptide was in tube 86 as shown by measurement of dry weight and tyrosine. The shape of the distribution curve indicated that the pep ide was homogeneous. The pure peptide was finally obtained as a fluffy powder by lyophilization of a solution of it in glacial acetic acid. The yield was 375 mg (19% over-all).

Analysis of the Hexadecapeptide. In paper chromatography in the solvent system *n*-propyl alcohol–water–concentrated NH_4OH, 50:48.5:1.5, the peptide showed a single spot visible with ninhydrin of R_f 0.85. In paper electrophoresis at pH 5.0 in 0.1 *M* pyridine acetate it moved as a spot toward the cathode with migration relative to histidine of 0.26. There was considerable streaking, probably due to the limited solubility of the peptide at pH 5.0. For elemental and amino acid analyses a sample was dried at 78° over P_2O_5 *in vacuo*. Amino acid analyses were performed by the method of Moore, *et al.*,[17] on hydrolysates. The analyses indicated that the substance was the salt with 5 equiv of trifluoroacetic acid.

Anal. Calcd for $C_{112}H_{119}N_{16}O_{35}F_{15}$: C, 50.0; H, 4.5; N, 13.5. Found: C, 50.2; H, 5.1; N, 13.5.

The ratio of amino acids in the hydrolysate was Asp, 3.3; His, 5.1; Phe, 5.2; Ser, 1.8; Tyr, 1.0. These values were not corrected for possible destruction during hydrolysis. The calculated ratios were: 3.0, 5.0, 5.0, 2.0, 1.0.

Chymotryptic Activity. The hexadecapeptide showed no catalytic activity in the hydrolysis of acetyl-L-phenylalanine ethyl ester when present at 0.5 mg/ml anywhere from pH 8.5 to 3.0. Measurements were made with a Radiometer pH stat at 30.0° by determination of the rate of consumption of 0.1 *N* NaOH. Crystalline chymotrypsin was detectable under the same conditions at pH 7.9 at 0.03 μg/ml. Similarly, no proteolytic activity against serum albumin was detected with 0.7 mg of the peptide per milliliter at a variety of pH's. Measurements were made by the Northrop method.[18]

(12) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **56**, 288 (1954).
(13) B. Zerner, R. Bond, and M. Bender, *J. Am. Chem. Soc.*, **86**, 3674 (1964).
(14) P. Cruickshank and J. C. Sheehan, *ibid.*, **86**, 2070 (1964).
(15) J. M. Stewart and D. W. Woolley, *Nature*, **206**, 619 (1965).
(16) *t*-BOC means *t*-butyloxycarbonyl. Optically active *t*-BOC amino acids were prepared according to the methods of R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959), or were purchased from Cyclo Chemical Co., Los Angeles, Calif. Whenever commercial preparations were used, their authenticity was checked by comparison with authentic materials in thin layer chromatography on plates of silicic acid H with either the solvent system chloroform–acetic acid, 95:5, or chloroform–methanol–acetic acid, 85:10:5. Occasionally the commercial *t*-BOC-benzyl-L-histidine was supplied as its salt with an alkaline cation instead of as the free acid. When this occurred it could be detected readily because the salt contained ash and was not soluble in DMF.
(17) S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, *1185 (1958)*.
(18) J. H. Northrop, "Crystalline Enzymes," Columbia University Press, New York, N. Y., 1939.