

does not appear to be of a type which can account for enzymic catalysis.

Conclusion

Since most enzymic reactions in which thiamine

pyrophosphate is a cofactor are mechanistically similar to the pyruvate decarboxylase reaction,⁴⁷ our results and the above discussion are relevant to many thiamine pyrophosphate-dependent enzymic reactions.

(47) L. O. Krampitz, *Ann. Rev. Biochem.*, **38**, 213 (1969).

Studies on Polypeptides. XLIV. Potent Synthetic S-Peptide Antagonists¹⁻⁴

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Abstract: The concept that chemical modification of the "active" amino acid in a biologically active peptide may provide a rational approach to the discovery of competitive antagonists was tested with the S-peptide-S-protein system. A series of histidine modified analogs of S-peptide₁₋₁₄ and of N^δ-formylornithine¹⁰ and ornithine¹⁰ S-peptides₁₋₁₄ was prepared and tested for ability to compete with natural S-peptide for S-protein. β-(Pyrazolyl-3)-alanine¹², 3-carboxymethylhistidine¹², N^δ-formylornithine¹⁰ β-(pyrazolyl-3)-alanine¹², ornithine¹⁰ β-(pyrazolyl-3)-alanine¹², N^δ-formylornithine¹⁰ 3-carboxymethylhistidine¹², and ornithine¹⁰ 3-carboxymethylhistidine¹² S-peptides₁₋₁₄ proved to be potent competitive antagonists of S-peptide. The *d*-sulfoxides of β-(pyrazolyl-3)-alanine¹² and of 3-carboxymethylhistidine¹² S-peptides₁₋₁₄ were considerably less effective inhibitors than the corresponding peptides containing methionine. 1-Carboxymethylhistidine¹² S-peptide₁₋₁₄ was shown to possess the ability to activate S-protein with formation of active enzyme but only at high molar peptide to protein ratios. 1,3-Dicarboxymethylhistidine¹² S-peptide₁₋₁₄ was inactive. The replacement of arginine by ornithine in β-(pyrazolyl-3)-alanine¹² and 3-carboxymethylhistidine¹² S-peptide₁₋₁₄ significantly weakened the ability to antagonize S-peptide. The corresponding N^δ-formylornithine derivatives were as effective as the peptides containing ornithine. Unequivocal synthetic routes to the abovementioned peptides are described.

Structure-function studies with synthetic S-peptide⁵ analogs and fragments⁶ have led us to conclude that histidine is the catalytically active amino acid residue in

(1) See K. Hofmann, J. P. Visser, and F. M. Finn, *J. Amer. Chem. Soc.*, **91**, 4883 (1969), for paper XLIII in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation. Mr. Visser's participation in this investigation was made possible through support from the Pittsburgh Plate Glass Foundation program in International Education.

(3) Preliminary communications of some of the results presented in this study have appeared (a) F. M. Finn and K. Hofmann, *J. Amer. Chem. Soc.*, **89**, 5298 (1967); (b) F. M. Finn, J. P. Visser, and K. Hofmann in "Peptides 1968," E. Bricas, Ed., North Holland Publishing Company, Amsterdam, 1968, p 330.

(4) The amino acid residues are of the L configuration. Abbreviations used are: 1-CMHis = 1-carboxymethylhistidine; 3-CMHis = 3-carboxymethylhistidine; 1,3-DiCMHis = 1,3-dicarboxymethylhistidine; Pyr(3)ala = β-(pyrazolyl-3)-alanine; FOrn = N^δ-formylorni-

thine; NArg = nitroarginine; Met = methionine *d*-sulfoxide; B-M-W = 1-butanol-methanol-water, 1:1:1; B-M-1 N AcOH = 1-butanol-water-1 N acetic acid, 1:1:1, etc.; P-M-W = 2-propanol-methanol-water, 1:1:1; P-M-2% AcOH = 2-propanol-methanol-2% acetic acid, 1:1:1, etc; Boc = *t*-butoxycarbonyl; O-*t*-Bu = *t*-butyl ester; Z = benzyloxycarbonyl; TEA = triethylamine; TFA = trifluoroacetic acid; DMF = dimethylformamide; DCC = N,N'-dicyclohexylcarbodiimide; AG 1-X2 = anion-exchange resin (Bio-Rad); tlc = thin layer chromatography; AP-M = aminopeptidase M [G. Pfeleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determan, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

(5) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958); RNase S, subtilisin-modified beef ribonuclease A; S-peptide, the peptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S', the reconstituted enzyme obtained by mixing equimolar proportions of S-protein and S-peptide. According to M. S. Doscher and C. H. W. Hirs, *Biochemistry*, **6**, 304 (1967), natural S-peptide is a mixture of at least three components, very likely S-pep-

S-peptide and that the rest of the molecule functions as a vehicle to bring this histidine into the correct stereochemical position in the active site of the ribonuclease S molecule. In addition to its role in catalysis, the histidine residue may also contribute to binding. We are investigating analogs of S-peptide₁₋₁₄ since this compound is equivalent to natural S-peptide as concerns activation of S-protein.^{6c}

We reasoned that if these interpretations were correct and if histidine 12 did not contribute significantly to binding it should be possible to discover competitive inhibitors to S-peptide₁₋₂₀ via histidine substitutions in S-peptide₁₋₁₄.

To test this prediction experimentally we synthesized Pyr(3)ala¹² S-peptide₁₋₁₄ (XIV) (Scheme I) and explored its ability to compete with S-peptide for S-protein with RNA as the substrate. This analog was selected because the molecular dimensions of Pyr(3)ala are very similar if not identical with those of histidine. The two amino acids differ markedly as concerns the acid-base properties of the ring portions of their molecules.⁷

Carboxymethylation has provided significant information pertaining to the active site of pancreatic ribonuclease A.⁸ The enzyme is rapidly inactivated

tide₁₋₂₀, S-peptide₁₋₂₁, and S-peptide₁₋₂₂. For clarity natural S-peptide will be designated "S-peptide".

(6) (a) K. Hofmann, F. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaiharu, *J. Amer. Chem. Soc.*, **85**, 833 (1963); (b) F. M. Finn and K. Hofmann, *ibid.*, **87**, 645 (1965); (c) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *ibid.*, **88**, 3633 (1966).

(7) K. Hofmann and H. Bohn, *ibid.*, **88**, 5914 (1966).

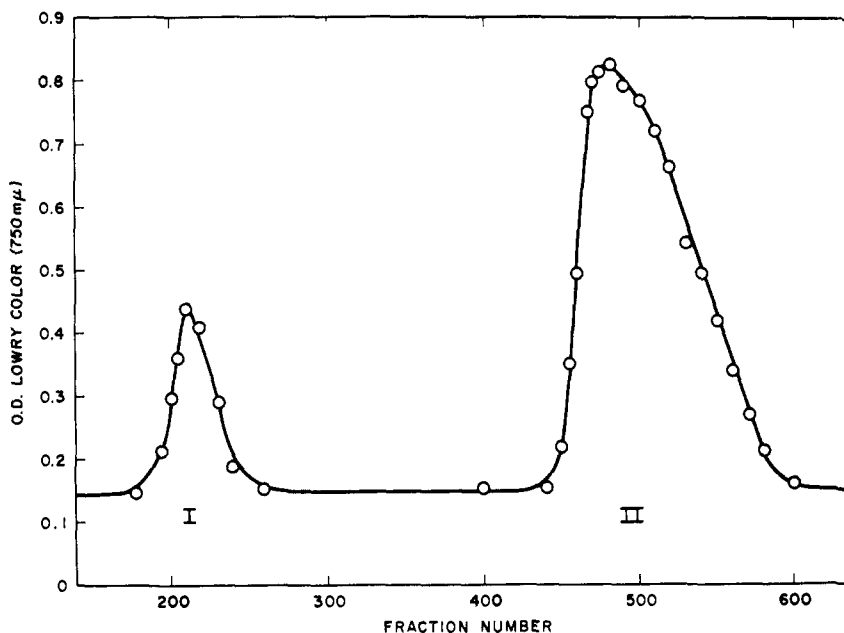


Figure 1. Separation of 1-CMHIS- (peak I) and 3-CM-histidylmethionylaspartic acid *d*-sulfoxide (peak II) on an AG 1-X2 column (1.9 × 60 cm); fraction size 2 ml.

when exposed to bromo- or iodoacetate at pH 5.5. Two products of this reaction, 1-CMHIS¹¹⁹ RNase A and 3-CMHIS¹² RNase A, each inactive enzymically, have been isolated and identified. In this context, it is difficult to rationalize a report⁹ that carboxymethylation of S-peptide sulfone does not alter the property of this peptide to activate S-protein.

In order to resolve this discrepancy we synthesized 1-CMHIS¹² (XI), 3-CMHIS¹² (XII), and 1,3-DiCMHIS¹² (XIII) S-peptides₁₋₁₄ by unequivocal routes and explored their interaction with S-protein and RNase S.

Scoffone and collaborators¹⁰ have carried out structure-function studies with derivatives of Orn¹⁰ S-peptide₁₋₂₀. Since their results, based on double substitutions, may not be comparable to those obtained with S-peptide₁₋₁₄ analogs which, as natural S-peptide, contain arginine we synthesized FOrn¹⁰ 3-CMHIS¹² (XV), Orn¹⁰ 3-CMHIS¹² (XVI), FOrn¹⁰ Pyr(3)ala¹² (XVII), and Orn¹⁰ Pyr(3)ala¹² (XVIII) S-peptides₁₋₁₄ and compared their interaction with S-protein and RNase S with that of corresponding peptides containing arginine.

Preparative Aspects

Carboxymethylated histidines are not well suited as starting materials for the unequivocal synthesis of peptides since they contain carboxyl groups whose differential protection is rather difficult. Carboxymethylation of suitably protected peptides containing histidine and separation of the carboxymethyl derivatives appeared to offer more promise. Since the carboxymethyl derivatives of histidine are known and are readily separable on the amino acid analyzer,^{8c,11}

(8) (a) E. A. Barnard and W. D. Stein, *J. Mol. Biol.*, **1**, 339, 350 (1959); (b) H. G. Grundlach, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **234**, 1754 (1959); (c) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 2413, 2421 (1963).

(9) P. J. Vithayathil and F. M. Richards, *ibid.*, **235**, 2343 (1960).

(10) (a) E. Scoffone, F. Marchiori, R. Rocchi, G. Vidali, A. Tamburro, A. Scatturin, and A. Marzotto, *Tetrahedron Lett.*, **9**, 943 (1966); (b) see R. Rocchi, F. Marchiori, L. Moroder, G. Borin, and E. Scoffone, *J. Amer. Chem. Soc.*, **91**, 3927 (1969), for additional references.

(11) K. D. Hapner, Ph.D. Thesis, Indiana University, 1966.

assignment of structure to carboxymethylated peptides is readily achieved.

The presence in S-peptide₁₋₁₄ of a methionine residue on the carboxyl side of histidine complicates the synthesis of carboxymethylated S-peptides₁₋₁₄ since methionine as well as histidine is subject to carboxymethylation. Thus, a method for reversible protection of methionine during carboxymethylation had to be found.

In previous syntheses of S-peptide₁₋₂₀ and related compounds^{1,6c,12} we employed methionine *d*-sulfoxide and reduced the final peptide sulfoxides to the corresponding methionine derivatives with thioglycolic acid. Since it was to be expected that the sulfoxide of methionine would not undergo S-alkylation we selected the *d*-sulfoxide of N^α-*t*-butoxycarbonylhistidylmethionylaspartic acid^{6c} for carboxymethylation studies.

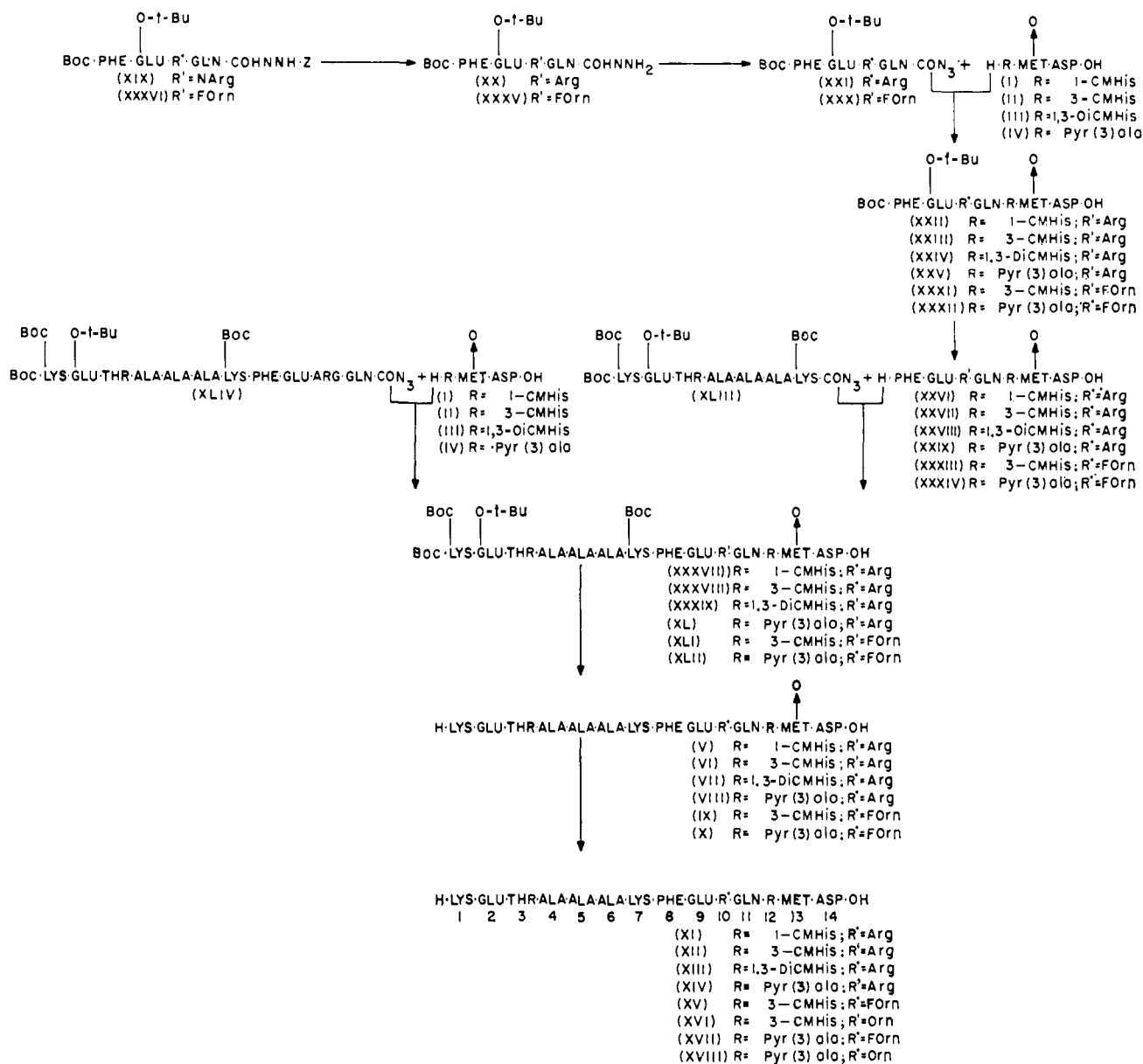
Incubation of N^α-*t*-butoxycarbonylhistidylmethionylaspartic acid *d*-sulfoxide with iodoacetate at 50° and pH 7.7-7.8 for 42 hr afforded a mixture of materials from which carboxymethylated products, because of their greater acidity, were readily separated by chromatography on the ion-exchange resin AG 1-X2. These materials, eluted with 1.7 *N* acetic acid, were deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions. The ensuing mixture was resolved by AG 1-X2 chromatography into the *d*-sulfoxides of 1-CM-, 3-CM-, and 1,3-di-CM-histidylmethionylaspartic acid (I), (II), and (III). Figure 1 illustrates the separation of the two monocarboxymethylated tripeptide *d*-sulfoxides. The synthesis of β-(pyrazolyl-3)-alanyl-methionylaspartic acid *d*-sulfoxide (IV) has been described.¹³

Routes (Scheme I), developed in connection with the synthesis of S-peptide₁₋₂₀,¹ were employed to convert the tripeptides I to IV into the tetradecapeptide *d*-sulfoxides V to X. Reduction with thioglycolic acid^{6c} served to convert these sulfoxides into peptides XI to

(12) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Amer. Chem. Soc.*, **87**, 631 (1965).

(13) K. Hofmann, R. Andreatta, and H. Bohn, *ibid.*, **90**, 6207 (1968)

Scheme I



XVIII. For the synthesis of the peptides containing arginine, *t*-butoxycarbonylphenylalanyl- γ -*t*-butylglutamylnitroarginylglutamine "benzyloxycarbonylhydrazide" (XIX) was converted to *t*-butoxycarbonylphenylalanyl- γ -*t*-butylglutamylarginylglutamine hydrazide (XX) by catalytic hydrogenation¹⁴ and the azide (XXI) corresponding to this hydrazide was coupled with the tripeptides I-IV to give the protected heptapeptides XXII-XXV which were deblocked with TFA to afford the heptapeptides XXVI-XXIX.

The formyl group was employed to protect selectively the δ -amino group of ornithine in the synthesis of the analogs containing this amino acid residue. Peptides II and IV were acylated with *t*-butoxycarbonylphenylalanyl- γ -*t*-butylglutamyl- N^{δ} -formylornithylglutamine azide (XXX) to give the protected heptapeptides XXXI and XXXII. Exposure of these materials to the action of TFA removed the *t*-butoxycarbonyl and *t*-butyl groups but left the formyl group intact.

(14) K. Hofmann, W. Haas, M. J. Smithers, R. D. Wells, Y. Wolman, N. Yanaihara, and G. Zanetti, *J. Amer. Chem. Soc.*, **87**, 620 (1965).

The preparation of the hydrazide XXXV was patterned according to the synthesis of XX. N^{δ} -Formylornithine, prepared from the copper complex of ornithine and ethyl formate,¹⁵ was converted into succinimido N^{α} -*t*-butoxycarbonyl- N^{δ} -formylornithinate. Acylation of glutamine "benzyloxycarbonylhydrazide"¹⁴ with this "active" ester gave N^{α} -*t*-butoxycarbonyl- N^{δ} -formylornithylglutamine "benzyloxycarbonylhydrazide" which was partially deblocked with TFA. The ensuing "benzyloxycarbonylhydrazide" of N^{δ} -formylornithylglutamine was then acylated with *t*-butoxycarbonylphenylalanyl- γ -*t*-butylglutamic acid azide¹⁴ to give XXXVI which was converted to XXXV by hydrogenation.

For the synthesis of the protected tetradecapeptides XXXVII-XLII the heptapeptides XXVI-XXXIV were coupled with the azide XLIII. An alternate route to the protected tetradecapeptides XXXVII-XL involved acylation of peptides I-IV with the azide XLIV.¹ The

(15) K. Hofmann, E. Stutz, G. Spühler, H. Yajima, and E. T. Schwartz, *ibid.*, **82**, 3727 (1960).

protected tetradecapeptides were deblocked with TFA to give the tetradecapeptide *d*-sulfoxides V–X. A minimum of side-chain protection was used in these syntheses since this approach allows the use of ion-exchange chromatography for purification of intermediates. Since all of the peptides contain an excess of carboxyl groups we relied heavily on the use of the ion-exchange resin AG 1-X2.

Inspection of the experimental results shows that coupling yields with peptides containing 1-CMHs are consistently lower than those observed with the other peptides. Unreacted amino component could not be isolated from the reaction mixtures suggesting that side reactions rather than poor acylation were responsible for the low yields.

Exposure to 50% thioglycolic acid at 37° was used to remove the formyl group with simultaneous reduction of the sulfoxide. This procedure converted peptide IX into a mixture of XV and XVI which was readily and cleanly separable by ion-exchange chromatography. The same procedure converted X into a separable mixture of XVII and XVIII. The composition of AP-M digests served to differentiate the formylated peptides from those containing unprotected ornithine. The digest of the former contains Lys and FORn, the digest of the latter Lys and Orn.

The 3-CMHs–Met *d*-sulfoxide bond is very resistant to AP-M hydrolysis. The rate of digestion is markedly increased in the corresponding methionine peptides; however, hydrolyses of both the 1-CMHs–Met and 3-CMHs–Met bonds proceed slowly. The 1,3-Di-CMHs–Met bond is resistant to the enzyme.

Experimental Section¹⁶

N^δ-Formylornithine. The preparation of this compound is patterned according to the procedure for the preparation of N^ε-formyllysine.¹⁵

(16) Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 m μ and extrapolated to the 589-m μ sodium line. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; oxygen values were actually determined and not computed by difference. The amino acid compositions of acid and AP-M hydrolysates were determined with a Beckman-Spinco Model 120 amino acid analyzer according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). The figures in parentheses are average recoveries of amino acids, based on formula weight when available or minimum residue weight. Nle and α -amino- β -guanidopropionic acid were used for internal standards. Acid hydrolyses were performed in constant boiling HCl at 110° for 24 hr in evacuated tubes; values are not corrected for amino acid destruction. AP-M digests were performed as described in ref 6c. Reference samples of 1-CM-, 3-CM-, and 1,3-DiCMHs (DL) were prepared as described in ref 8c. Designation of solvent systems for paper and tlc chromatograms are given in K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaiharu, *J. Amer. Chem. Soc.*, **87**, 611 (1965). The hydrazide reaction was performed according to H. Ertel and L. Horner, *J. Chromatogr.*, **7**, 268 (1962). AG 1-X2 (chloride form, 200–400 mesh, Bio-Rad Laboratories, Richmond, Cal.) was washed twice with 2 *N* KOH, then with water until neutral. This was followed by two washings with 10% acetic acid and water until neutral. Columns were equilibrated with the appropriate solvents before use. RNase S was prepared as described in ref 6b. Enzyme assays were performed as described in ref 1. Ribonuclease 100% activity corresponds to 130 Kunitz units/mg at 30°. Buffers for enzyme assays at different pH values were as follows: pH 2.0, 0.1 *M* sodium malonate or 0.1 *M* sodium citrate; pH 3.1, 0.1 *M* sodium citrate; pH 4.0, 0.1 *M* sodium formate; pH 5.0, 0.1 *M* sodium acetate; pH 6.1, 0.1 *M* sodium malonate. Yeast RNA was the substrate for all enzymatic measurements since strongly inhibited RNase S or weakly activated S-protein produced completely erratic kinetics with uridine or cytidine 2',3'-cyclic phosphates. The ability of S-protein to catalyze the polymerization of cyclic phosphates [M. R. Bernfield, *J. Biol. Chem.*, **241**, 2014 (1966)] appears to be responsible for this behavior. In the interest of space conservation much detail has been omitted from the Experimental

The copper chelate¹⁷ prepared from 30 g of ornithine monohydrochloride was dissolved in 1 *N* KOH (400 ml) and MeOH (400 ml) and the solution was cooled in an ice bath. Ethyl formate (200 ml) was added and the mixture was stirred vigorously with cooling for 20 hr, the pH of the reaction mixture being maintained between 8 and 9 by periodic addition of 5 *N* KOH.

The bulk of the organic solvent was removed *in vacuo* and the copper chelate which had precipitated was collected and washed with water. The chelate was suspended in water (approximately 200 ml) at 70° and hydrogen sulfide was passed through the solution for 2 hr. The copper sulfide was removed by filtration with the aid of Norit A and Filter-cel and was washed with water. The combined filtrate and washings were concentrated to a volume of approximately 100 ml and Dowex 50W-X2 (ammonium form) (approximately 2 ml settled in water) was added to remove small amounts of ornithine. The resin was removed by filtration, washed with water, and the combined filtrate and washings were evaporated to a small volume. Addition of EtOH gave crystals which were collected and washed with EtOH, 13.3 g (47%): mp 215–216°; $[\alpha]^{25}_D + 2.1^\circ$ (*c* 2.4, water); R_f^{VI} 0.1; R_f^{VII} 0.4; R_f^I 0.3; R_f^S 1.3 \times His; single ninhydrin- and chlorine-positive spot.

Anal. Calcd for C₈H₁₂O₃N₂: C, 45.0; H, 7.6; N, 17.5; O, 30.0. Found: C, 45.1; H, 7.8; N, 17.5; O, 30.1.

N^α-*t*-Butoxycarbonyl-N^δ-formylornithine. *t*-Butylazidoformate (5 ml) was added to a solution of FORn (2.8 g) in water (45 ml), dioxane (60 ml), and TEA (2.5 ml). The mixture was stirred at 45–50° for 48 hr, TEA (2.5 ml) being added slowly during the first 6 hr. The bulk of the organic solvents was removed, and the residue was extracted with three 100-ml portions of ethyl acetate. The ethyl acetate phases were washed with two 50-ml portions of water and the combined aqueous phases were concentrated to a volume of approximately 100 ml. The solution was cooled in an ice bath, acidified with solid citric acid, and extracted with 100 ml of ether which was discarded. The solution was then extracted with three 150-ml portions of 1-butanol-ethyl acetate (1:2) and the organic phases were washed with four 50-ml portions of saturated NaCl, three 50-ml portions of 5% acetic acid, and ten 50-ml portions of water. The combined organic phases were evaporated and the resulting oil was dried *in vacuo*, 3.5 g (77%): $[\alpha]^{25}_D - 2.2^\circ$ (*c* 8.8, MeOH); $[\alpha]^{25}_D + 5.1^\circ$ (*c* 1.44, 3% NH₄OH); R_f^I 0.7; R_f^{VI} 0.8; R_f^{VII} 0.7; R_f^I 0.9; R_f^S 3.0 \times His, single chlorine-positive spot.

Succinimido N^α-*t*-Butoxycarbonyl-N^δ-formylornithinate. DCC (1.74 g) was added to an ice-cold solution of N^α-*t*-butoxycarbonyl-N^δ-formylornithine (2.09 g) and N-hydroxysuccinimide (0.92 g) in dichloromethane (150 ml). The mixture was kept at 0° for 30 min and at room temperature for 2 hr when the N,N'-dicyclohexylurea was removed by filtration. Evaporation of the filtrate gave a solid which was crystallized first from 150 ml of ethyl acetate, then from 40 ml of 2-propanol, 2.12 g (74%): mp 141–142°; $[\alpha]^{25}_D - 28.9^\circ$ (*c* 1.0, DMF); $[\alpha]^{25}_D - 34.7^\circ$ (*c* 1.13, MeOH); R_f^I 0.9.

Anal. Calcd for C₁₅H₂₃O₅N₃: C, 50.4; H, 6.5; N, 11.8; O, 31.3. Found: C, 50.7; H, 6.5; N, 11.8; O, 31.0.

N^α-*t*-Butoxycarbonyl-N^δ-formylornithylglutamine "Benzylloxycarbonylhydrazide." Succinimido N^α-*t*-butoxycarbonyl-N^δ-formylornithinate (1.79 g) was added to a solution of glutamine "benzylloxycarbonylhydrazide" acetate¹⁴ (1.77 g) in DMF (50 ml). The reaction mixture was kept at room temperature for 16 hr, then the solvent was removed. The residue was dissolved in 1-butanol-ethyl acetate, 1:1 (300 ml) and the extract was washed with five 30-ml portions of 2% acetic acid and two 30-ml portions of water and the solvent was evaporated. Crystallization occurred when ethyl acetate (150 ml) was added to a solution of the residue in 30 ml of MeOH. The crystals were washed with ethyl acetate and recrystallized from MeOH-ethyl acetate, 1.51 g (56%); mp 186–188°; $[\alpha]^{25}_D - 38.9^\circ$ (*c* 1.09, MeOH); R_f^I 0.9; R_f^{VI} 0.7; R_f^{VII} 0.7; R_f^I 0.9; R_f^S 3.5 \times His; single chlorine- and hydrazide-positive spot; amino acid ratios in acid hydrolysate: Orn_{1.1}Glu_{0.9} (100%).

Anal. Calcd for C₂₄H₃₈O₈N₆: C, 53.7; H, 6.8; N, 15.7; O, 23.9. Found: C, 53.8; H, 6.5; N, 15.9; O, 23.9.

Carboxymethylation of N^α-*t*-Butoxycarbonylhistidylmethionyl-aspartic Acid *d*-Sulfoxide. A solution of N^α-*t*-butoxycarbonyl-histidylmethionylaspartic acid *d*-sulfoxide monohydrate^{6c} (2.07 g), iodoacetic acid (1.12 g), and sodium bicarbonate (0.45 g) in 1 *N*

Section. In general, only the synthesis of the first of a series of analogs is given in detail. Those that follow are described only in terms of the most essential information. Details can be found in J. P. Visser, Ph.D. Thesis, University of Pittsburgh, Pittsburgh, Pa., 1969.

(17) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

NaOH (14 ml) was incubated at 50° for 42 hr. The pH of the reaction mixture was 7.7–7.8. The solution was concentrated to a small volume *in vacuo* and water (50 ml) was added. This solution was concentrated to a small volume *in vacuo* and water (50 ml) was added. This solution was added to a column (3 × 35 cm) of acetate cycle AG 1-X2 which was eluted with 0.05 *N* acetic acid (300 ml), 0.1 *N* acetic acid (700 ml), 0.8 *N* acetic acid (300 ml), and 1.7 *N* acetic acid (1000 ml). Crystalline starting material (310 mg) was isolated from the 0.1 *N* acetic acid eluates. Evaporation of pooled chlorine positive fractions from the 1.7 *N* acetic acid eluates gave 840 mg of a mixture of carboxymethylation products. This material was dissolved in TFA (10 ml) and the solution was kept at room temperature for 30 min. The bulk of the TFA was evaporated, the residue was dissolved in water (20 ml), and the pH of the solution was adjusted to approximately 7 by addition of pyridine. For separation of the carboxymethylation products, this solution was added to a column of acetate cycle AG 1-X2 (1.9 × 60 cm) equilibrated with 0.1 *M* pyridinium acetate. The column was eluted with 0.2 *M* pyridinium acetate (1300 ml) and 1.7 *N* acetic acid (600 ml). Fractions (2 ml each) were collected. The mono-carboxymethylated peptides I and II were located in the pyridinium acetate eluates as widely separated peaks (Figure 1). The dicarboxymethyl derivative (III) was located in the acetic acid eluates.

1-Carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide Hemihydrate (I). Pooled fractions containing the less retarded ninhydrin-positive material from the pyridinium acetate eluates (Peak I, Figure 1) were evaporated to a small volume and the residue was lyophilized to constant weight from 10% acetic acid; 60 mg (3%) of 1-CM-histidylmethionylaspartic acid *d*-sulfoxide hemihydrate was obtained in the form of an amorphous hygroscopic solid: $[\alpha]^{25D} +53.5^\circ$ (*c* 1.05, water); R_f^{VII} 0.1; R_f^S 0.4 × His; single Pauly-negative, ninhydrin-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: 1-CMHist_{1.0}Met_{0.8}Asp_{1.0} (90%).

Anal. Calcd for C₁₇H₂₅O₉N₅S·0.5H₂O: C, 42.1; H, 5.4; N, 14.5; O, 31.4. Found: C, 41.5; H, 6.0; N, 14.0; O, 30.6.

3-Carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (II). Evaporation and lyophilization of pooled fractions containing the more retarded ninhydrin-positive material from the pyridinium acetate eluates (Peak II, Figure 1) gave 490 mg (25%) of 3-CM-histidylmethionylaspartic acid *d*-sulfoxide in the form of an amorphous hygroscopic powder: $[\alpha]^{25D} +29.1^\circ$ (*c* 1.02, water); R_f^{VII} 0.1; R_f^S 0.4 × His; single Pauly-negative, ninhydrin-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: 3-CMHist_{1.0}Met_{0.8}Asp_{1.0} (92%).

Anal. Calcd for C₁₇H₂₅O₉N₅S: C, 42.9; H, 5.3; N, 14.7; O, 30.3; S, 6.7. Found: C, 42.5; H, 5.4; N, 14.5; O, 30.6; S, 6.8.

1,3-Dicarboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide Monoacetate Sesquihydrate (III). Evaporation and lyophilization of the pooled ninhydrin-positive 1.7 *N* acetic acid eluates gave 110 mg (5%) of 1,3-di-CM-histidylmethionylaspartic acid *d*-sulfoxide monoacetate sesquihydrate in the form of an amorphous hygroscopic powder; $[\alpha]^{25D} +35.4^\circ$ (*c* 1.01, water); R_f^{VII} 0.1; R_f^S 0.1 × His; amino acid ratios in acid hydrolysate: 1,3-DiCMHist_{1.0}Met_{0.8}Asp_{1.0} (98%).

Anal. Calcd for C₁₉H₂₇O₁₁N₅S·CH₃COOH·1.5H₂O: C, 40.6; H, 5.5; N, 11.3; O, 37.4. Found: C, 40.5; H, 5.5; N, 11.3; O, 37.3.

1-Carboxymethylhistidylmethionylaspartic Acid. A sample of the *d*-sulfoxide (I) was reduced with thioglycolic acid⁶⁶ and the product was isolated by lyophilization: $[\alpha]^{25D} +18.7^\circ$ (*c* 0.96, water); R_f^{VII} 0.3; amino acid ratios in 96 hr AP-M digest (410 mU of enzyme per μM of peptide: 1-CMHist_{1.0}Met_{1.0}Asp_{1.1}) (69%).

3-Carboxymethylhistidylmethionylaspartic Acid. A sample of the *d*-sulfoxide (II) was reduced with thioglycolic acid⁶⁶ and the product was isolated by lyophilization; $[\alpha]^{25D} -5.8^\circ$ (*c* 1.16, water); R_f^{VII} 0.3; amino acid ratios in acid hydrolysate: 3-CMHist_{0.9}Met_{1.0}Asp_{1.0} (88%); amino acid ratios in 96-hr AP-M digest (330 mU of enzyme per μM of peptide: 3-CMHist_{1.0}Met_{1.0}Asp_{1.0}) (86%).

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamyl-N^δ-formylornithylglutamine "Benzylloxycarbonylhydrazide" (XXXVI).** *N*^ε-*t*-Butoxycarbonyl-N^δ-formylornithylglutamine "benzyloxycarbonylhydrazide" (1.07 g) was dissolved in TFA (10 ml) and the solution was kept at room temperature for 20 min. Ice-cold ether was added and the precipitate was collected and washed with ether. The solid was dissolved in water (50 ml) and acetate cycle Amberlite IRA-400 (10 ml settled in water) was added. The suspension was stirred for 10 min, the resin was removed by filtration, and was washed with water until the filtrate was ninhydrin negative. Filtrate and washings were combined, concentrated to a small volume, and lyophilized.

The ensuing N^δ-formylornithylglutamine "benzyloxycarbonylhydrazide" acetate (0.99 g) was dissolved in DMF (10 ml) and TEA (0.28 ml) and this solution was added at -60° to a solution containing the azide of *t*-butoxycarbonylphenylalanyl-γ-*t*-butylglutamic acid prepared from 1.16 g of the hydrazide.¹⁴ The mixture was kept at 4° for 16 hr, the pH being maintained at approximately 8 by addition of TEA. The bulk of the solvents was removed, the residue was dissolved in 95% EtOH (approximately 50 ml), and water was added to precipitate the product. The compound was recrystallized from 95% EtOH, 1.62 g (93%); mp 228–229°; $[\alpha]^{25D} -18.0^\circ$ (*c* 1.12, DMF); R_f^{VI} 0.8; R_f^{VII} 0.8; R_f^I 0.9; R_f^S 3.5 × His; single chlorine- and hydrazide-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Orn_{1.0} (93%).

Anal. Calcd for C₄₂H₈₀O₁₂N₈: C, 58.0; H, 7.0; N, 12.9; O, 22.1. Found: C, 58.4; H, 7.3; N, 12.8; O, 22.0.

For enzymic digestion the *t*-butoxycarbonyl derivative (200 mg) was partially deblocked with TFA and TFA ions were exchanged by acetate ions on acetate cycle Amberlite IRA-400. The product was lyophilized from water, 154 mg (94%); $[\alpha]^{25D} -32.9^\circ$ (*c* 0.93, 10% acetic acid); R_f^{VI} 0.5; R_f^{VII} 0.6; R_f^I 0.7; R_f^S 2.9 × His; amino acid ratios in AP-M digest: Phe_{1.0}Glu_{1.0}FORn_{1.0}Gln_{1.0} (96%).

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamyl-N^δ-formylornithylglutamine Hydrazide (XXXV).** The protected hydrazide XXXVI (400 mg) was hydrogenated over palladium in 100 ml of a mixture of 1-butanol-methanol-water (2:2:1). The catalyst was removed by filtration and the filtrate was concentrated to a small volume. Addition of water precipitated the product, 305 mg (90%); mp 231–233°; R_f^{VI} 0.6; R_f^{VII} 0.7; R_f^I 0.9; R_f^S 3.5 × His; single chlorine- and hydrazide-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Orn_{1.0} (95%).

Anal. Calcd for C₃₄H₅₄O₁₀N₈: C, 55.6; H, 7.4; N, 15.2; O, 21.8. Found: C, 55.9; H, 7.3; N, 15.5; O, 22.1.

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamyl-1-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide Monoacetate Trihydrate (XXII).** A solution of 1-CM-histidylmethionylaspartic acid *d*-sulfoxide (I) (95 mg) in 50% aqueous DMF (2 ml) and TEA (0.81 ml of a 10% solution in DMF) was added at -60° to a DMF solution (4 ml) containing the azide XXI derived from 370 mg of the hydrazide XX. See ref 1 for standard method to prepare this azide. The mixture was stirred for 20 hr at 4° and was then diluted with water (100 ml). This solution was added to a column (2 × 10 cm) of acetate cycle AG 1-X2 which was eluted with water (300 ml) and 0.03 *N* acetic acid (300 ml). The desired product was located in the acetic acid eluates by the chlorine and Sakaguchi reactions. The desired fractions were combined, the bulk of the solvent was removed, and the residue was lyophilized, 50 mg (20%); $[\alpha]^{25D} -19.9^\circ$ (*c* 0.89, water); R_f^{VI} 0.2; R_f^{VII} 0.5; R_f^I 0.7; R_f^S 2.6 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Arg_{1.0}1-CMHist_{1.0}Met_{0.8}Asp_{1.0} (92%).

Anal. Calcd for C₅₁H₇₇O₁₈N₁₃S·CH₃COOH·3H₂O: C, 48.7; H, 6.7; N, 13.9; O, 28.2. Found: C, 48.9; H, 6.9; N, 14.3; O, 27.9.

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide Monoacetate Monohydrate (XXIII).** A solution of 3-CM-histidylmethionylaspartic acid *d*-sulfoxide (II) (285 mg) in 50% aqueous DMF (4 ml) and TEA (0.25 ml) was added at -60° to a DMF solution (6 ml) containing the azide XXI¹ derived from 555 mg of the hydrazide XX. After 20 hr at 4° the reaction mixture was diluted with water (100 ml) and the solution was added to an acetate cycle AG 1-X2 column (2 × 15 cm) which was eluted with water (200 ml), 0.05 *N* acetic acid (600 ml), and 0.1 *N* acetic acid (100 ml). The desired product was isolated from the 0.05 *N* acetic acid eluates and lyophilized: 430 mg (56%); $[\alpha]^{25D} -17.2^\circ$ (*c* 1.2, water); R_f^{VI} 0.2; R_f^{VII} 0.5; R_f^I 0.7; R_f^S 2.6 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Arg_{1.0}3-CMHist_{1.0}Met_{0.8}Asp_{1.0} (97%).

Anal. Calcd for C₅₁H₇₇O₁₈N₁₃S·CH₃COOH·H₂O: C, 50.1; H, 6.6; N, 14.3; O, 26.4. Found: C, 49.9; H, 6.6; N, 14.3; O, 26.5. Unreacted amino component II (75 mg) was isolated from the 0.1 *N* acetic acid eluates.

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutamyl-1,3-dicarboxymethylhistidylmethionylaspartic Acid Monoacetate Tetrahydrate (XXIV).** A solution of 1,3-di-CM-histidylmethionylaspartic acid *d*-sulfoxide (III) (106 mg) in 50% aqueous DMF (4 ml) and TEA (0.12 ml) was added at -60° to a DMF solution (5 ml) containing the azide XXI¹ derived from 370 mg of the hydrazide XX. After 20 hr at 4° the reaction mixture was diluted with water

(100 ml) and the solution was added to a (2 × 15 cm) column of acetate cycle AG 1-X2 resin which was eluted with water (150 ml 0.7 N acetic acid (200 ml), and 3 N acetic acid (100 ml). The desired product was isolated from the 0.7 N eluates and was lyophilized from 10% acetic acid, 130 mg (55%): $[\alpha]^{25D} - 16.3^\circ$ (c 1.12, water); R_f^{VI} 0.2; R_f^{VII} 0.5; R_f^I 0.6; R_f^S 2.2 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Arg_{1.0}1,3-DiCMHis_{1.0}Met_{0.8}Asp_{1.0} (93%).

Anal. Calcd for C₅₃H₇₅O₂₀N₁₃S·CH₃COOH·4H₂O: C, 47.8; H, 6.6; N, 13.2; O, 30.1. Found: C, 47.7; H, 6.4; N, 13.3; O, 30.4. Unreacted amino component (III) (40 mg) was isolated from the 3 N acetic acid eluates.

t-Butoxycarbonylphenylalanyl- γ -*t*-butylglutamylarginylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide Tetrahydrate (XXV). A solution of β -(pyrazolyl-3)-alanylmethionylaspartic acid *d*-sulfoxide IV¹³ (835 mg) in DMF (10 ml), water (4 ml), and TEA (0.83 ml) was added at -60° to a DMF solution (20 ml) containing the azide XXI derived from 1.85 g of the hydrazide XX. After 19 hr at 4° and 5 hr at room temperature, the solvent was evaporated, and the residue distributed between 1-butanol and 2% acetic acid.^{18,19} The butanol phases were evaporated and the residue dissolved in P-M-W (150 ml) was added to an AG 1-X2 column (4 × 10 cm) which was eluted with 150 ml of the same solvent followed by P-M-5% AcOH (300 ml). Chlorine-positive 5% acetic acid eluates were pooled, concentrated to a small volume *in vacuo*, and the residue was lyophilized from 10% acetic acid, 508 mg (21%): $[\alpha]^{25D} - 16.8^\circ$ (c 1.31, 10% acetic acid); R_f^{VI} 0.6; R_f^{VII} 0.6; single chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{0.9}Glu_{2.2}Arg_{0.9}Pyr(3)ala_{0.9}Met_{0.8}Asp_{1.1} (100%).

Anal. Calcd for C₄₉H₇₅O₁₆N₁₃S·4H₂O: C, 48.8; H, 6.9; N, 15.1; O, 26.5. Found: C, 48.5; H, 6.7; N, 15.0; O, 27.0.

t-Butoxycarbonylphenylalanyl- γ -*t*-butylglutamyl-N⁶-formylornithylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide Tetrahydrate (XXXI). A solution of 3-CM-histidylmethionylaspartic acid *d*-sulfoxide II (142 mg) in 50% aqueous DMF (2 ml) containing TEA (0.12 ml) was added at -60° to a DMF solution (6 ml) containing the azide XXX derived from 220 mg of the hydrazide XXXV. After 20 hr at 4° and 1 hr at room temperature the reaction mixture was diluted with 50 ml of B-M-W and the solution was added to a (2 × 10 cm) column of acetate cycle AG 1-X2 equilibrated with the same solvent. The column was eluted with B-M-W (50 ml) and B-M-2 N AcOH (300 ml). Evaluation by tlc of the B-M-2 N AcOH eluates showed them to contain a mixture of the desired product and unreacted amino component. These fractions were pooled, the solvent was removed, and the residue was distributed between 1-butanol and 2% acetic acid.¹⁸ The butanol phases were evaporated to a small volume, 10% acetic acid was added to the residue, and the solution was lyophilized, 278 mg (74%): $[\alpha]^{30D} - 7.7^\circ$ (c 1.75, DMF); R_f^{VI} 0.3; R_f^{VII} 0.5; R_f^I 0.8; R_f^S 3.0 × His; single Pauly-negative, chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Orn_{1.1}-3-CMHis_{0.9}Met_{0.8}Asp_{1.0} (92%).

Anal. Calcd for C₅₁H₇₅O₁₉N₁₁S·4H₂O: C, 49.0; H, 6.7; N, 12.3; O, 29.4. Found: C, 48.8; H, 6.6; N, 12.0; O, 30.1. Unreacted amino component (16 mg) was isolated from the aqueous phases.

t-Butoxycarbonylphenylalanyl- γ -*t*-butylglutamyl-N⁶-formylornithylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide Sesquihydrate (XXXII). A solution of β -(pyrazolyl-3)-alanylmethionylaspartic acid *d*-sulfoxide (IV) (104 mg) in water (2 ml) and TEA (0.07 ml) was added at -60° to a DMF solution (6 ml) containing the azide XXX derived from 184 mg of the hydrazide XXXV. After 20 hr at 4° and 1 hr at room temperature B-M-W (50 ml) was added and the solution was applied to a column (2 × 10 cm) of AG 1-X2 which was eluted with 50 ml of B-M-W and 300 ml of B-M-1.5 N AcOH. The desired material plus unreacted amino component was located in the B-M-1.5 N AcOH eluates by the chlorine reaction. These fractions were pooled, evaporated to a small volume, and the residue was distributed between 1-butanol and 2% acetic acid.¹⁸ The product was isolated from the butanol phases by evaporation and lyophilization, 205

mg (72%): $[\alpha]^{25D} - 3.9^\circ$ (c 0.75, DMF); R_f^{VI} 0.3; R_f^{VII} 0.5; R_f^I 0.9; R_f^S 3.1 × His; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Orn_{1.1}Pyr(3)ala_{1.0}Met_{0.8}Asp_{1.0} (96%).

Anal. Calcd for C₄₉H₇₅O₁₇N₁₁S·1.5H₂O: C, 51.3; H, 6.7; N, 13.4; O, 25.8. Found: C, 51.6; H, 6.9; N, 12.9; O, 26.1. Unreacted amino component (20 mg) was isolated from the aqueous phases.

Phenylalanylglutamylarginylglutamyl-1-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXVI). The protected peptide XXII (48 mg) was dissolved in TFA (2 ml) and the solution was kept at room temperature for 45 min. The bulk of the TFA was removed and the residue dissolved in water (20 ml). The pH of the solution was adjusted to approximately 7 by addition of TEA and the solution was added to an acetate cycle AG 1-X2 column (2 × 10 cm) which was eluted with water (100 ml) and 0.05 N acetic acid (150 ml). The desired product was isolated from the 0.05 N acetic acid eluates and lyophilized from water, 39 mg: $[\alpha]^{25D} - 2.6^\circ$ (c 0.66, water); R_f^{VII} 0.2; R_f^I 0.2; R_f^S 0.7 × His; single ninhydrin, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Arg_{1.0}1-CMHis_{1.0}Met_{0.8}Asp_{1.0} (77%).

Phenylalanylglutamylarginylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXVII). The protected peptide XXIII (125 mg) was deblocked with TFA and the product was lyophilized, 125 mg: $[\alpha]^{27D} - 13.3^\circ$ (c 0.9, water); R_f^{VII} 0.2; R_f^S 0.7 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Arg_{1.0}-3-CMHis_{1.0}Met_{0.8}Asp_{1.0} (92%).

Phenylalanylglutamylarginylglutamyl-1,3-dicarboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXVIII). The protected peptide XXIV (98 mg) was deblocked with TFA and the product was lyophilized from water; 78 mg: $[\alpha]^{25D} - 11.7^\circ$ (c 1.15, water); R_f^{VII} 0.1; R_f^I 0.1; R_f^S 0.6 × His; single ninhydrin, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.1}Arg_{1.0}1,3-DiCMHis_{1.0}Met_{0.8}Asp_{1.0} (87%).

Phenylalanylglutamylarginylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide (XXIX). The protected peptide XXV (463 mg) was deblocked with TFA and the product was lyophilized, 409 mg: $[\alpha]^{25D} - 10.7^\circ$ (c 1.06, 10% acetic acid); R_f^{VI} 0.1; R_f^{VII} 0.2; ninhydrin- and chlorine-positive spot; amino acid

ratios in AP-M digest: Phe_{1.0}Glu_{1.1}Arg_{1.1}Gln_{1.0}Pyr(3)ala_{0.8}Met_{1.0}Asp_{0.9} (79%).

Phenylalanylglutamyl-N⁶-formylornithylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXXIII). The protected peptide XXXI (220 mg) was deblocked with TFA and the product was lyophilized from water, 192 mg: $[\alpha]^{25D} - 13.0^\circ$ (c 1.05, water); R_f^{VII} 0.2; R_f^I 0.3; R_f^S 0.4 × His; single ninhydrin, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Phe_{1.0}Glu_{2.0}Orn_{1.1}-3-CMHis_{0.9}Met_{0.8}Asp_{1.0} (82%).

Phenylalanylglutamyl-N⁶-formylornithylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide (XXXIV). The protected peptide XXXII (250 mg) was deblocked with TFA and the product was lyophilized 215 mg: $[\alpha]^{27D} - 10.4^\circ$ (c 0.75, water); R_f^{VI} 0.2; R_f^{VII} 0.3; R_f^I 0.4; R_f^S 0.5 × His; single chlorine- and ninhydrin-positive spot; amino acid ratios in AP-M digest: Phe_{1.2}

Glu_{1.1}FOrn_{1.0}Gln_{0.8}Pyr(3)ala_{1.1}Met_{0.9}Asp_{1.0} (78%).

N⁶,N⁶-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N⁶-*t*-butoxycarbonyllslylphenylalanylglutamylarginylglutamyl-1-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXXVII). a. From the Azide XLIV and the Tripeptide I. A solution of I (48 mg) in 50% aqueous DMF (2 ml) and TEA (0.41 ml) of a 10% solution in DMF was added at -60° to a DMF solution (5 ml) containing the azide XLIV derived from 348 mg of the corresponding hydrazide. See ref 1 for standard method to prepare this azide. After 20 hr at 4° and 2 hr at room temperature, the reaction mixture was diluted with 100 ml of B-M-W and the solution was added to an acetate cycle AG 1-X2 column (2 × 8 cm) which was eluted with 100 ml of the same solvent, then with 150 ml of B-M-0.2 N AcOH. The desired compound which was located in the acetic acid eluates by the Sakaguchi and chlorine reactions was isolated and lyophilized from 10% acetic acid, 55 mg (20%): R_f^{VI} 0.3; R_f^{VII} 0.5; R_f^I 0.8; R_f^S 3.0 × His; amino acid ratios in acid hydrolysate: Lys_{2.2}Glu_{3.0}Thr_{0.9}Ala_{2.9}Phe_{0.9}Arg_{1.1}1-CMHis_{1.0}Met_{0.8}Asp_{1.0} (85%).

b. From the Azide XLIII and the Heptapeptide XXVI. A solution of XXVI (39 mg) in 50% aqueous DMF (1 ml) and TEA (0.2 ml) of a 10% solution in DMF was added at -60° to a DMF

(18) K. Hofmann, R. Schmiechen, M. J. Smithers, R. D. Wells, Y. Wolman, and G. Zanetti, *J. Amer. Chem. Soc.*, **87**, 640 (1965).

(19) In the description of the preparation of *t*-butoxycarbonylphenylalanyl- γ -*t*-butylglutamylarginylglutamylhistidylmethionylaspartic acid *d*-sulfoxide⁶ it was erroneously stated that the desired product was present in the aqueous phase of the distribution mixture.

solution (7 ml) containing the azide XLIII derived from 109 mg of the corresponding hydrazide.¹ After 48 hr at 4° the product was isolated and lyophilized from 10% acetic acid; 16 mg (20%); chromatographically identical with the material prepared according to method a.

N^ε,N^ε-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N^ε-*t*-butoxycarbonyllysylphenylalanylglutamylarginylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXXVIII). a. From the Azide XLIV and the Tripeptide II. A solution of II (95 mg) in 50% aqueous DMF (2 ml) and TEA (0.83 ml of a 10% solution in DMF) was added at -60° to a DMF solution (6 ml) containing the azide XLIV derived from 698 mg of the corresponding hydrazide.¹ After 20 hr at 4° and 2 hr at room temperature, the reaction mixture was evaporated and the residue distributed between 1-butanol and 2% acetic acid. The butanol phases were pooled and evaporated and the residue dissolved in 50 ml of B-M-W was added to an acetate cycle AG 1-X2 column (2 × 10 cm). The column was eluted with 100 ml of the same solvent followed by B-M-0.3 *N* AcOH (400 ml). The product was isolated from the latter eluates and lyophilized from 10% acetic acid, 225 mg (54%); R_f^{VI} 0.3; R_f^{VII} 0.5; R_f^I 0.8; R_f^3 3.0 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.1}Glu_{3.0}Thr_{1.0}Ala_{3.0}Phe_{1.0}Arg_{1.0}3-CMHis_{0.9}Met_{0.8}Asp_{1.0} (97%).

b. From the Azide XLIII and the Heptapeptide XXVII. A solution of XXVII (207 mg) in water (2 ml) and TEA (0.11 ml) was added at -60° to a DMF solution (20 ml) containing the azide XLIII derived from 325 mg of the corresponding hydrazide.¹ After 96 hr at 4° the reaction mixture was diluted with 100 ml of B-M-W and this solution was added to an acetate cycle AG 1-X2 column (2 × 15 cm) which was eluted with B-M-W (100 ml), B-M-0.15 *N* AcOH (500 ml), and aqueous 0.05 *N* acetic acid (150 ml). The desired product was isolated from the B-M-0.15 *N* AcOH eluates in the usual manner and was lyophilized from 10% acetic acid; 330 mg (79%); chromatographically identical with the material prepared according to method a. Unreacted amino component XXVII (24 mg) was isolated from the 0.05 *N* aqueous acetic acid eluates. A preparation, performed in the same manner, except that XXVII was dissolved in 10 ml rather than 2 ml of water, afforded XXXVIII in a 26% yield.

N^ε,N^ε-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N^ε-*t*-butoxycarbonyllysylphenylalanylglutamylarginylglutamyl-1,3-dicarboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XXXIX). a. From the Azide XLIV and the Tripeptide III. A solution of III (54 mg) in water (1 ml) and TEA (0.6 ml of a 10% solution in DMF) was added at -60° to a DMF solution (6 ml) containing the azide XLIV derived from 261 mg of the corresponding hydrazide.¹ After 20 hr at 4° and 2 hr at room temperature, the reaction mixture was diluted with 50 ml of B-M-W and the solution was added to an acetate cycle AG 1-X2 column (2 × 10 cm) which was eluted with B-M-W (150 ml), B-M-0.6 *N* AcOH (150 ml), B-M-1.5 *N* AcOH (200 ml), and finally B-M-1.7 *N* AcOH (100 ml). The desired compound was isolated from the B-M-1.5 *N* AcOH eluates in the usual manner and was lyophilized from 10% acetic acid, 84 mg (45%); R_f^{VI} 0.3; R_f^{VII} 0.5; R_f^I 0.7; R_f^3 2.8 × His; single Pauly-negative, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.1}Glu_{3.1}Thr_{1.0}Ala_{3.0}Phe_{1.0}Arg_{0.9}1,3-DiCMHis_{1.0}Met_{0.8}Asp_{1.0} (96%). Unreacted amino component (22 mg) was isolated from the B-M-1.7 *N* AcOH eluates.

b. From the Azide XLIII and the Heptapeptide XXVIII. A solution of the heptapeptide XXVIII (78 mg) in water (1 ml) and TEA (0.49 ml of a 10% solution in DMF) was added at -60° to a DMF solution (7 ml) containing the azide XLIII derived from 152 mg of the corresponding hydrazide.¹ After 72 hr at 4° the reaction mixture was diluted with 50 ml of B-M-W and this solution was added to an acetate cycle AG 1-X2 column (2 × 10 cm) which was eluted with 50 ml of B-M-W, 150 ml of B-M-0.6 *N* AcOH, 150 ml of B-M-1.5 *N* AcOH, and finally with 300 ml of B-M-2.0 *N* AcOH. The desired product was isolated from the B-M-1.5 *N* AcOH eluates in the usual manner and lyophilized from 10% acetic acid; 30 mg (20%); chromatographically identical with the material prepared according to a. Unchanged amino component (50 mg) was isolated from the B-M-2.0 *N* AcOH eluates.

N^ε,N^ε-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N^ε-*t*-butoxycarbonyllysylphenylalanylglutamylarginylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide (XL). a. From the Azide XLIV and the Tripeptide IV. A solution of the tripeptide IV¹³ (84 mg) in water (0.4 ml), TEA (0.84 ml of a 10% solution in DMF), and DMF (2 ml) was added at -60°

to a DMF solution (4 ml) containing the azide XLIV derived from 330 mg of the corresponding hydrazide.¹ After 40 hr at 4° and 2 hr at room temperature, the solution was evaporated and the residue distributed between 2% acetic acid and 1-butanol.¹⁸ The butanol phases were pooled, the solvent was evaporated, and the residue was dissolved in P-M-W (50 ml). This solution was added to a column of AG 1-X2 (2 × 10 cm) which was eluted with the same solvent (150 ml) followed by P-M-2% AcOH (100 ml) and P-M-5% AcOH (120 ml). The chlorine test served to detect the desired peptide in the P-M-5% AcOH eluates which were pooled, evaporated to a small volume, and lyophilized from dilute acetic acid, 143 mg (35%); R_f^{VI} 0.3; R_f^{VII} 0.6; amino acid ratios in acid hydrolysate: Lys_{2.0}Glu_{3.1}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{1.0}Pyr(3)Ala_{1.0}Met_{0.8}Asp_{1.0} (97%).

b. From the Azide XLIII and the Heptapeptide XXIX. A solution of XXIX (272 mg) in water (2 ml), TEA (1.04 ml of a 10% solution in DMF), and DMF (5 ml) was added at -60° to a DMF solution (20 ml) containing the azide XLIII derived from 544 mg of the corresponding hydrazide.¹ After 24 hr at 4° and 24 hr at room temperature, the crude product was isolated in the manner described above and lyophilized from dilute acetic acid, 608 mg (107%); R_f^{VI} 0.4; R_f^{VII} 0.7 chlorine-positive spot, contaminated with a material of R_f^{VII} 0.1.

N^ε,N^ε-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N^ε-*t*-butoxycarbonyllysylphenylalanylglutamyl-N⁶-formylornithylglutamyl-3-carboxymethylhistidylmethionylaspartic Acid *d*-Sulfoxide (XLI). A solution of the heptapeptide XXXIII (164 mg) in water (2 ml) and TEA (0.09 ml) was added at -60° to a DMF solution (20 ml) containing the azide XLIII derived from 349 mg of the corresponding hydrazide.¹ After 44 hr at 4° and 1 hr at room temperature, the reaction mixture was diluted with 100 ml of B-M-W and this solution was added to an acetate cycle AG 1-X2 column (2 × 10 cm) which was eluted with 50 ml of B-M-W and 400 ml of B-M-1.5 *N* AcOH. The desired product was isolated from the 1.5 *N* acetic acid eluates and was lyophilized from 10% acetic acid, 260 mg (78%); R_f^{VI} 0.4; R_f^{VII} 0.6; R_f^I 0.8; R_f^3 3.0 × His; single Pauly-negative, chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{2.0}Glu_{3.1}Thr_{1.0}Ala_{3.0}Phe_{1.0}3-CMHis_{1.0}Met_{0.8}Asp_{1.0} (95%).

N^ε,N^ε-Di-*t*-butoxycarbonyllysyl- γ -*t*-butylglutamylthreonylalanylalanylalanyl-N^ε-*t*-butoxycarbonyllysylphenylalanylglutamyl-N⁶-formylornithylglutamyl- β -(pyrazolyl-3)-alanylmethionylaspartic Acid *d*-Sulfoxide (XLII). A solution of XXXIV (193 mg) in water (1 ml), DMF (3 ml), and TEA (0.08 ml) was added at -60° to a DMF solution (20 ml) containing the azide XLIII derived from 435 mg of the corresponding hydrazide.¹ After 44 hr at 4° and 1 hr at room temperature, the reaction mixture was diluted with B-M-W (100 ml) and the solution was added to an AG 1-X2 column (2 × 10 cm) which was eluted with B-M-W (100 ml) and B-M-0.9 *N* AcOH (300 ml). Chlorine-positive B-M-0.9 *N* AcOH eluates were pooled, evaporated, and the residue was distributed between 1-butanol and 2% acetic acid.¹⁸ The butanol phases were concentrated to a small volume and the residue was lyophilized from 10% acetic acid, 335 mg (83%); R_f^{VI} 0.4; R_f^{VII} 0.6; R_f^I 0.9; R_f^3 3.2 × His; single chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{2.2}Glu_{3.0}Thr_{1.0}Ala_{3.0}Phe_{1.0}Pyr(3)-ala_{1.0}Met_{0.8}Asp_{1.0} (84%). Unreacted amino component XXIV (16 mg) was isolated from the aqueous phases.

1-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (V). The protected peptide XXXVII (50 mg) was deblocked with TFA, TFA ions were exchanged for acetate ions on AG 1-X2, and the product was lyophilized from water, 30 mg; $[\alpha]_D^{25}$ -45.5° (*c* 1.05, water); R_f^{VII} 0.1; R_f^3 0.3 × His; single Pauly-negative, ninhydrin-, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.2}Glu_{3.0}Thr_{1.0}Ala_{2.8}Phe_{0.9}Arg_{1.0}1-CMHis_{1.0}Met_{0.7}Asp_{1.0} (91%).

3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (VI). The protected peptide XXXVIII (170 mg) was deblocked with TFA, 126 mg; $[\alpha]_D^{25}$ -50.8° (*c* 0.80, water); R_f^{VII} 0.1; R_f^3 0.3 × His; single Pauly-negative, ninhydrin-, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.0}Glu_{3.1}Thr_{1.0}Ala_{3.0}Phe_{1.0}Arg_{1.0}3-CMHis_{0.9}Met_{0.8}Asp_{1.0} (92%).

1,3-Dicarboxymethylhistidine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (VII). The protected peptide XXXIX (105 mg) was deblocked with TFA, 76 mg; $[\alpha]_D^{25}$ -44.3° (*c* 1.07, water); R_f^{VII} 0.1; R_f^3 0.3 × His; single Pauly-negative, ninhydrin-, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.1}Glu_{3.1}Thr_{1.0}Ala_{3.0}Phe_{0.9}Arg_{0.9}1,3-DiCMHis_{1.0}Met_{0.7}Asp_{1.0} (90%).

β -(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (VIII). The protected peptide XL (140 mg) prepared by method a was deblocked

with TFA; 116 mg. This material was chromatographed on a CMC column (0.9 × 13 cm) using a gradient produced by mixing 150 ml of 0.01 M ammonium acetate with 150 ml of 0.05 M ammonium acetate. Lowry²⁰-positive fractions were evaporated and the residue was lyophilized from water, 90 mg; $[\alpha]^{25D} -47.1^\circ$ (*c* 1.31, 10% acetic acid); R_f^{VI} origin; R_f^{VII} 0.2; single chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.0}Glu_{3.1}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{1.0}Pyr(3)ala_{0.9}Met_{0.9}Asp_{1.0} (78%).

The protected peptide XL (600 mg) prepared by method b was deblocked with TFA and the product was lyophilized from water, 216 mg; $[\alpha]^{25D} -47.4^\circ$ (*c* 1.09, 10% acetic acid); R_f^{VI} origin; R_f^{VII} 0.2; amino acid ratios in acid hydrolysate: Lys_{2.0}Glu_{3.2}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{0.9}Pyr(3)ala_{1.0}Met_{0.9}Asp_{1.0} (78%).

N⁶-Formylornithine¹⁰ 3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (IX). The protected peptide XLI (213 mg) was deblocked with TFA, 165 mg; $[\alpha]^{25D} -45.1^\circ$ (*c* 1.1, water); R_f^{VI} 0.1; R_f^3 0.5 × His; single Pauly-negative, ninhydrin-, Sakaguchi-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.0}Glu_{3.1}Thr_{1.0}Ala_{3.0}Phe_{1.0}3-CMHIS_{1.0}Met_{0.7}Asp_{1.0} (99%).

N⁶-Formylornithine¹⁰ β-(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (X). The protected tetradecapeptide XLII (310 mg) was deblocked with TFA, 221 mg; $[\alpha]^{25D} -48.6^\circ$ (*c* 1.05, water); R_f^{VII} 0.1; R_f^3 0.6 × His; ninhydrin-, and chlorine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.2}Glu_{3.0}Thr_{1.0}Ala_{3.0}Phe_{1.0}Pyr(3)ala_{1.0}Met_{0.6}Asp_{1.0} (82%); amino acid ratios in AP-M digest: Lys_{2.2}Glu_{1.9}Thr_{1.0}Ala_{3.0}Phe_{0.9}Forn_{1.1}Gln_{1.0}Pyr(3)ala_{1.0}Met_{1.0}Asp_{1.0} (87%).

1-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ (XI). The *d*-sulfoxide V (30 mg) was reduced with thioglycolic acid^{6c} and the product was lyophilized, 30 mg; $[\alpha]^{27D} -49.2^\circ$ (*c* 1.03, water); R_f^{VII} 0.1; R_f^3 0.5 × His; single Pauly-negative, ninhydrin, Sakaguchi-, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.2}Glu_{3.0}Thr_{0.9}Ala_{3.0}Phe_{1.0}Arg_{0.9}1-CMHIS_{1.0}Met_{1.0}Asp_{1.0} (85%).

3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ (XII). The *d*-sulfoxide VI (80 mg) was reduced^{6c} and the product was lyophilized, 80 mg; $[\alpha]^{27D} -57.9^\circ$ (*c* 1.01, water); R_f^{VII} 0.1; R_f^3 0.5 × His; single Pauly-negative, ninhydrin-, Sakaguchi-, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate: Lys_{2.1}Glu_{3.0}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{0.9}3-CMHIS_{1.0}Met_{1.0}Asp_{1.0} (80%).

1,3-Dicarboxymethylhistidine¹² S-Peptide₁₋₁₄ (XIII). The *d*-sulfoxide VII (50 mg) was reduced^{6c} and the product was lyophilized, 50 mg; $[\alpha]^{25D} -43.1^\circ$ (*c* 1.01, water); R_f^{VII} 0.1; R_f^3 0.5 × His; Pauly-negative-, Sakaguchi-, chlorine-, methionine-, and ninhydrin-positive spot containing an impurity with R_f^3 0.6 × His; amino acid ratios in acid hydrolysate: Lys_{2.1}Glu_{3.1}Thr_{0.9}Ala_{3.1}Phe_{1.0}Arg_{0.9}1,3-diCMHIS_{1.0}Met_{1.0}Asp_{1.0} (64%).

β-(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ (XIV). The *d*-sulfoxide VIII (48 mg) was reduced^{6c} and the product was lyophilized; $[\alpha]^{25D} -51.4^\circ$ (*c* 0.96, 10% acetic acid); R_f^{VI} origin; R_f^{VII} 0.2; amino acid ratios in acid hydrolysate: Lys_{2.0}Glu_{3.2}Thr_{1.0}Ala_{3.0}Phe_{1.0}Arg_{0.9}Pyr(3)ala_{1.0}Met_{1.0}Asp_{0.9} (80%); amino acid ratios in AP-M digest: Lys_{2.2}Glu_{2.2}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{1.0}Gln_{0.9}Pyr(3)ala_{0.8}Met_{0.9}Asp_{0.9} (80%).

Reduction and Deformylation of N⁶-Formylornithine¹⁰ 3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (IX). The *d*-sulfoxide IX (61 mg) was incubated for 44 hr at 37° in 4 ml of 50% aqueous thioglycolic acid. The solution was added to an acetate cycle IRA-400 column (2 × 15 cm) which was eluted with 2% acetic acid. Chlorine positive eluates were pooled and lyophilized. The residue was dissolved in water (50 ml), the pH adjusted to approximately 7 by addition of TEA, and the solution was added to an AG 1-X2 column (2 × 5 cm) equilibrated with 10 ml of 1% thioglycolic acid and water. The column was then eluted with water (100 ml), 0.005 N acetic acid (150 ml), and 0.05 N acetic acid (100 ml).

Ornithine¹⁰ 3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ (XVI). Chlorine positive 0.005 N acetic acid eluates from the above column were pooled, evaporated to a small volume, and lyophilized, 26 mg; $[\alpha]^{25D} -61.4^\circ$ (*c* 1.06, water); R_f^{VII} 0.1; R_f^3 0.6 × His; single Pauly-negative, ninhydrin, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.1}Glu_{3.1}Thr_{0.9}Ala_{3.0}Phe_{1.0}3-CMHIS_{1.0}Met_{1.0}Asp_{1.0} (92%); amino acid ratios in AP-M digest: Lys + Orn_{3.2}Glu_{3.3}Thr_{1.1}Ala_{3.3}Phe_{0.9}Gln_{0.8}3-CMHIS_{0.8}Met_{0.9}Asp_{0.9}.

N⁶-Formylornithine¹⁰ 3-Carboxymethylhistidine¹² S-Peptide₁₋₁₄ (XV). Chlorine-positive 0.05 N acetic acid eluates from the above

column were pooled, evaporated to a small volume, and lyophilized, 23 mg; $[\alpha]^{25D} -55.9^\circ$ (*c* 0.91, water); R_f^{VII} 0.1; R_f^3 0.8 × His; single Pauly-negative, chlorine-, methionine-, and ninhydrin-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.0}Glu_{3.1}Thr_{0.9}Ala_{3.0}Phe_{1.0}3-CMHIS_{1.1}Met_{0.9}Asp_{1.0} (92%); amino acid ratios in AP-M digest: Lys_{2.1}Glu_{2.2}Thr_{1.1}Ala_{3.3}Phe_{1.0}Forn_{1.0}Gln_{0.7}3-CMHIS_{0.8}Met_{0.9}Asp_{0.9}.

Reduction and Deformylation of N⁶-Formylornithine¹⁰ β-(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ *d*-Sulfoxide (X). The *d*-sulfoxide X (48 mg) was incubated for 22 hr at 37° in 50% aqueous thioglycolic acid (4 ml) and the products were isolated in the manner described above. Water (50 ml) and 0.005 N acetic acid were used to elute the (2 × 5 cm) AG 1-X2 column.

Ornithine¹⁰ β-(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ (XVIII). The chlorine-positive water eluates from the AG 1-X2 column were pooled, evaporated to a small volume, and lyophilized; 16 mg; $[\alpha]^{27D} -62.3^\circ$ (*c* 1.12, water); R_f^{VII} 0.2; R_f^3 0.7 × His; single ninhydrin-, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.0}Glu_{3.1}Thr_{1.0}Ala_{3.1}Phe_{1.0}Pyr(3)ala_{1.0}Met_{0.9}Asp_{0.9} (87%); amino acid ratios in AP-M digest: Lys + Orn_{2.9}Glu_{2.2}Thr_{1.1}Ala_{3.3}Phe_{1.0}Gln_{0.7}Pyr(3)ala_{0.9}Met_{0.9}Asp_{1.0}.

N⁶-Formylornithine¹⁰ β-(Pyrazolyl-3)-alanine¹² S-Peptide₁₋₁₄ (XVII). The chlorine-positive 0.005 N acetic acid eluates were lyophilized, 30 mg; $[\alpha]^{27D} -57.1^\circ$ (*c* 0.62, water); R_f^{VII} 0.2; R_f^3 1.0 × His; single ninhydrin, chlorine-, and methionine-positive spot; amino acid ratios in acid hydrolysate: Lys + Orn_{3.1}Glu_{3.1}Thr_{0.9}Ala_{3.0}Phe_{1.0}Pyr(3)ala_{0.9}Met_{0.9}Asp_{1.0} (83%); amino acid ratios in AP-M digest: Lys_{2.0}Glu_{2.1}Thr_{1.1}Ala_{3.3}Phe_{1.0}Forn_{1.0}Gln_{0.7}Pyr(3)ala_{0.8}Met_{0.9}Asp_{1.0}.

Results and Discussion

At molar ratios as high as 1000:1, Pyr(3)ala¹² S-peptide₁₋₁₄ fails to activate S-protein over the pH range of 2–6, but, as has been shown previously,^{3a} this peptide is a potent S-peptide antagonist. Figure 2 shows that Pyr(3)ala¹² S-peptide₁₋₁₄ competes equally well with S-peptide₁₋₁₄ or S-peptide with a 50% inhibition ratio of approximately 1:1. This finding further supports the concept^{6c} that S-peptide₁₋₁₄ is equivalent to natural S-peptide as concerns activation of and binding to S-protein. The conclusion that amino acid residues 15–20 are not concerned with the association between S-peptide and S-protein has received support from the X-ray investigations of Wyckoff, *et al.*²¹ Their results indicate that this portion of the S-peptide molecule is not in contact with the S-protein portion in ribonuclease-S crystals. Thus, a dynamic structure-function study has been correlated with a molecular model derived from X-ray diffraction findings with a crystalline enzyme.

The pK of the imidazole ring of histidine, which is critical for catalytic function of ribonuclease S, does not appear to be involved in the association between S-peptide and S-protein. However, the five-membered aromatic azole ring seems to be important for binding, since Ser¹² S-peptide₁₋₁₄,^{3b} another competitive antagonist of S-peptide, exhibits a 50% inhibition ratio of only 8:1.

In their early investigations on the relation between structure and ability to activate S-protein, Vithayathil and Richards⁹ concluded that methionine is an important binding site in S-peptide. This conclusion has been confirmed by our own studies with the *d*-sulfoxides of a number of synthetic S-peptide fragments.^{6b,c} The observation that the 50% inhibition ratio (approximately unity with Pyr(3)ala¹² S-peptide₁₋₁₄)^{3a} is increased to about 600:1 when the methionine sulfur is converted to the *d*-sulfoxide, provides convincing con-

(20) O. H. Lowry, N. J. Rosebough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(21) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).

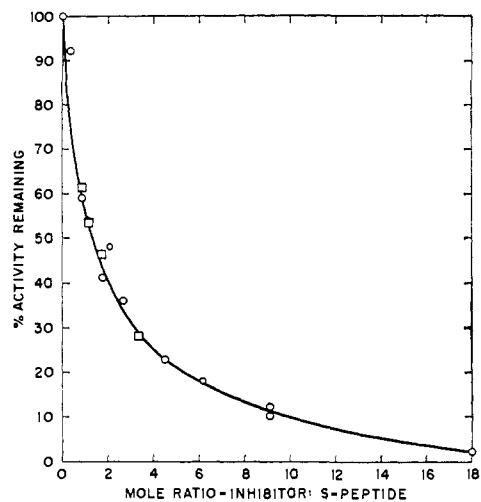


Figure 2. Competition of Pyr(3)ala¹² S-peptide₁₋₁₄ with natural S-peptide (O) and S-peptide₁₋₁₄ (o) for S-protein; substrate, yeast RNA. Molar ratio of either natural S-peptide or S-peptide₁₋₁₄ to S-protein, 1:1. Abscissa: molar ratio of inhibitor to S-peptide. Ordinate: per cent activity remaining. Reversal of inhibition by addition of increasing amounts of natural S-peptide to inhibited enzyme (□).

firmation of this point *via* a different experimental approach.

Carboxymethylation of N-*t*-butoxycarbonylhistidyl-methionylaspartic acid *d*-sulfoxide produces predominantly the less-hindered 3-CMHis derivative, but the expected 1-CMHis and 1,3-diCMHis derivatives were also obtained. All three carboxymethyl derivatives were converted into the corresponding S-peptides₁₋₁₄. From the results of the carboxymethylation of RNase A,^{8c} it could be predicted that 3-CMHis¹² RNase S would be inactive. Indeed 3-CMHis¹² S-peptide₁₋₁₄ at molar ratios as high as 1400:1 failed to produce an active enzyme with S-protein. However, introduction of the bulky carboxymethyl group into the 3 position of the imidazole ring does not interfere with, but rather favors association between the peptide and S-protein.

3-CMHis¹² S-peptide₁₋₁₄ is the most powerful S-peptide antagonist discovered thus far. Its 50% inhibition ratio^{3b} of approximately 0.8 with RNA as substrate indicates that this peptide has a higher affinity for S-protein than either S-peptide, S-peptide₁₋₁₄, or Pyr(3)ala¹² S-peptide₁₋₁₄. The *d*-sulfoxide of 3-CMHis¹² S-peptide₁₋₁₄ is a 3000-times less effective inhibitor. Inhibition is of the competitive type since addition of S-peptide to the enzymically inactive 3-CMHis¹² S-peptide₁₋₁₄-S-protein complex brings about reactivation.

Surprisingly 1-CMHis¹² S-peptide₁₋₁₄ activates S-protein with RNA as the substrate (Figure 3) but large proportions of peptide are required to produce an effect.²² 1,3-DiCMHis¹² S-peptide₁₋₁₄ failed to activate S-protein or to inhibit RNase S.

The difference in the behavior of these carboxymethylated S-peptide fragments is remarkable indeed. As concerns 1-CMHis¹² S-peptide₁₋₁₄ the presence of the carboxymethyl group appears to interfere with the binding of the peptide to S-protein; hence, the large

(22) The carboxymethylated peptide could not be tested at molar ratios higher than 1200:1 since the peptide causes precipitation of the RNA in the assay solution.

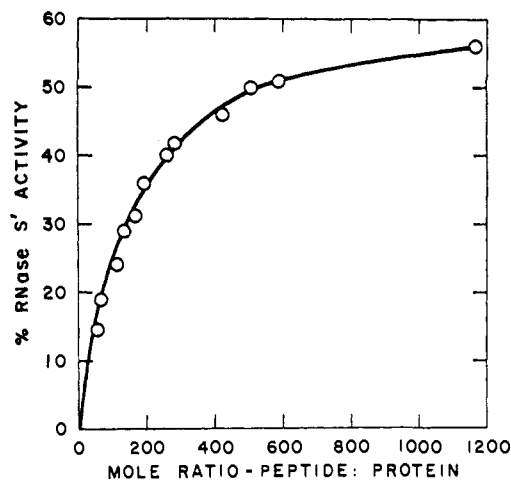
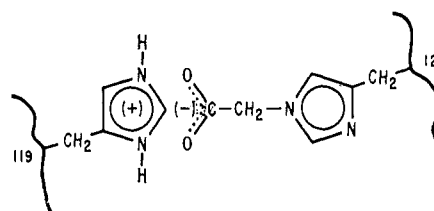


Figure 3. Activation of S-protein by 1-CMHis¹² S-peptide₁₋₁₄; substrate, yeast RNA.

proportions required for activation. The *pK* of the imidazole ring of 1-CMHis (6.33)¹¹ is very similar to that of His (6.13), thus carboxymethylation is not likely to interfere with the reversible protonation of the unsubstituted nitrogen.

The situation with 3-CMHis¹² S-peptide₁₋₁₄ is quite different. Here again the difference between the *pK* of 3-CMHis (5.74)¹¹ and histidine is probably not great enough to account for the inability of this peptide to function catalytically. It is tempting to postulate that the CM-carboxyl group of this peptide, *via* a proton exchange, forms a salt bridge with the imidazole ring of histidine 119 in the manner illustrated in Scheme II.

Scheme II



Such interaction would be expected to prevent His¹¹⁹ from undergoing deprotonation, strengthen the association between peptide and S-protein and interfere with substrate binding. Yang and Hummel²³ using ultraviolet spectral changes as the indicator, have reported that 3-CMHis¹² RNase A fails to bind 2'-cytidine monophosphate. They have also observed that 3-CMHis¹² and 1-CMHis¹⁹ RNase A exhibit higher stability toward urea below pH 6.5 than RNase A and they attribute this stability to an interaction between the carboxymethyl group and a cationic site on the enzyme. The model proposed in Scheme II could explain these results.

Scoffone and collaborators¹⁰ have prepared a number of analogs of Orn¹⁰ S-peptide₁₋₂₀ and have evaluated the ability of these peptides to activate S-protein. In these analogs the strongly basic guanidinium group of arginine is replaced by the less basic δ -amino group of ornithine with a concomitant shortening of the side chain. Although Orn¹⁰ S-peptide activates S-protein approximately 65% at a 1:1 molar ratio this peptide

(23) S-T. Yang and J. P. Hummel, *J. Biol. Chem.*, **239**, 3775 (1964).

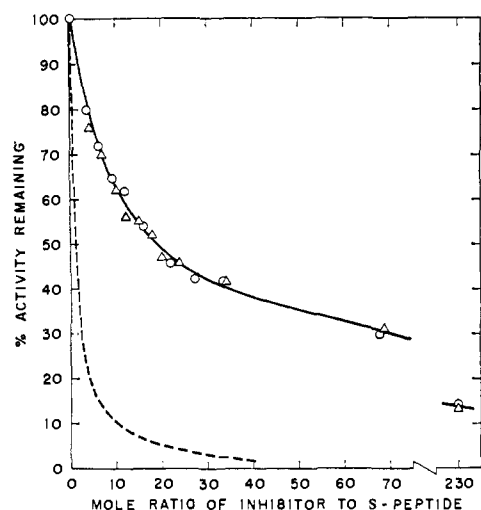


Figure 4. Inhibition of RNase S by FOrn¹⁰ Pyr(3)ala¹² S-peptide₁₋₁₄ (Δ) and Orn¹⁰ Pyr(3)ala¹² S-peptide₁₋₁₄ (○) with yeast RNA as substrate. Molar ratio of S-peptide to S-protein, 1:1. For comparison inhibition by Pyr(3)ala¹² S-peptide₁₋₁₄ (---) is given.

fails to produce a fully active enzyme even at high peptide to protein ratios. The possibility exists that the arginine-ornithine replacement may alter the conformation of S-peptide. Since the inhibitor approach provides a much more sensitive index of binding than do activation measurements we replaced arginine by N^δ-formylornithine and ornithine in our most potent S-peptide antagonists, Pyr(3)ala¹² S-peptide₁₋₁₄ and 3-CMHis¹² S-peptide₁₋₁₄, and measured the ability of these analogs to inhibit RNase S. From the results presented in Figures 4 and 5, it is clearly apparent that the Orn substitution lowers markedly the ability of the analogs to compete with S-peptide for S-protein. Here again the 3-CMHis peptides (Figure 5) bind more firmly than the corresponding peptides containing Pyr(3)ala (Figure 4). Substitution of arginine by ornithine decreases the binding to S-protein of Pyr(3)ala¹² S-peptide₁₋₁₄ 20-fold (Figure 4) and that of 3-CMHis¹² S-peptide₁₋₁₄ 8-fold (Figure 5). It is particularly significant to note that formylation of the δ-amino group of ornithine exerts no measurable effect on the affinity of the ornithine analogs for S-protein. It follows from these results that arginine makes a unique contribution to the strong association between S-peptide and S-protein which is not duplicated by ornithine.

From S-protein activation studies with de-Lys¹ and de-Lys¹, de-Glu² S-peptides₁₋₁₃ we discovered Glu² as a "binding" site in S-peptide.^{6b} Moroder, *et al.*,²⁴ contend that the removal of Lys¹ and Glu² exerts little effect on the S-peptide activating properties of Orn¹⁰ S-peptide. These authors questioned the significance of Glu² for binding and suggested that the absence of Asp¹⁴ in our peptides was responsible for the marked differences we observed.

(24) L. Moroder, F. Marchiori, R. Rocchi, A. Fontana, and E. Scoffone, *J. Amer. Chem. Soc.*, **91**, 3921 (1969).

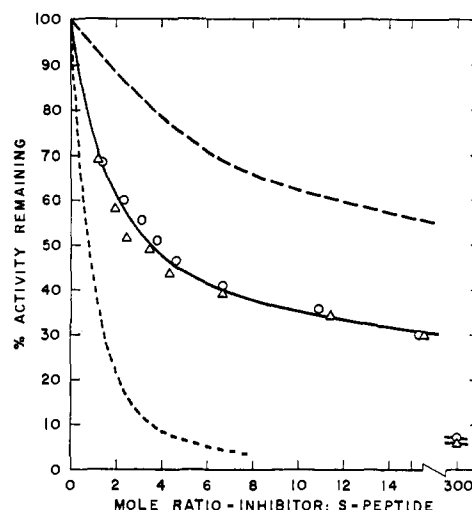


Figure 5. Inhibition of RNase S by FOrn¹⁰ 3-CMHis¹² S-peptide₁₋₁₄ (Δ) and Orn¹⁰ 3-CMHis¹² S-peptide₁₋₁₄ (○) with yeast RNA as substrate. Molar ratio of S-peptide to S-protein, 1:1. For comparison the inhibition curves for Orn¹⁰ Pyr(3)ala¹² S-peptide₁₋₁₄ (— — —) and 3-CMHis¹² S-peptide₁₋₁₄ (---) are given.

We submit that the apparent discrepancies between our results and those of Moroder, *et al.*, are a reflection of the presence or absence of arginine¹⁰ in the peptides under discussion rather than the presence or absence of aspartic acid.¹⁴

The functional groups of both Glu² and Arg¹⁰ in RNase A are relatively unreactive^{25,26} even though models derived from X-ray crystallographic studies of RNases A and S^{21,27} indicate that these amino acids are exposed to the solvent. In addition pyroglutamic acid² RNase₂₋₁₂₄ exhibits markedly lower enzymic activity than either RNase A or RNase₂₋₁₂₄.²⁸

The X-ray structure of RNase A shows that the γ-carboxyl of Glu² and the guanidinium group of Arg¹⁰ are within hydrogen bonding distance of each other.²⁹ An interaction between these two residues could contribute to the particular S-peptide conformation required for optimal binding to S-protein. The effect would be lost in Orn¹⁰ S-peptide because the greater distance between the γ-carboxyl of Glu² and the δ-amino group of ornithine precludes interaction. This interpretation is supported by the observation that formylation of the δ-amino group of the ornithine peptides does not alter their affinity for S-protein. Hence, disruption of this delicate Glu-Arg interaction by substitution of Orn in position 10 could very well destroy the significant binding contribution of Glu².

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