picrate and measuring the uptake of picrate into the organic phase spectrophotometrically. According to this constant (*i.e.*, the concentration of potassium picrate in the aqueous phase that caused half-saturation of the peptide with potassium picrate in the organic phase) the peptide ($K_{D_2} = 7 \times 10^{-6}$ *M*) has a sevenfold higher affinity for potassium picrate than valinomycin ($K_{D_2} = 5 \times 10^{-5}$ *M*).

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Studies on Polypeptides. LI. Application of *S*-Ethylcarbamoylcysteine to the Synthesis of a Protected Heptatetracontapeptide Related to the Primary Sequence of Ribonuclease T_1[1-4]

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Abstract: This paper describes the synthesis of a partially protected heptatetracontapeptide hydrazide (fragment ABCD) of molecular weight 5485 corresponding to positions 1–47 of the proposed primary structure of the enzyme ribonuclease T_1. *S*-Ethylcarbamoyl-L-cysteine was used to synthesize formylalanyl-*S*-ethylcarbamoylcysteinyl-aspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycylserylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine benzyloxycarbonylhydrazide (fragment A). This peptide derivative corresponds to positions 1–11 of the ribonuclease T_1 sequence and contains three of the four half-cystine residues of the enzyme. Fragment A was exposed to hydrogen bromide in trifluoroacetic acid, and the ensuing hydrazide *via* the azide was coupled to the previously described fragment B *tert*-butoxycarbonylhydrazide to give fragment AB, which spans positions 1–23 of the enzyme sequence. Fragment AB was converted to the hydrazide by exposure to trifluoroacetic acid, and the hydrazide, in the form of the corresponding azide, was coupled to fragment CD *tert*-butoxycarbonylhydrazide to give fragment ABCD. The sequential homogeneity of fragment ABCD is based on the fact that it was obtained by linking fragments, shown to be homogeneous, by azide couplings and on the observation that the ratios of the "diagnostic" amino acid residues agreed with those expected by theory. The insolubility of both fragments AB and ABCD eliminated thin layer chromatography as a useful tool for analytical evaluation. The stability of the *S*-ethylcarbamoyl group was investigated with simple model peptides and this sulfur protecting group was found to be stable under acid conditions (trifluoroacetic acid and hydrogen bromide in trifluoroacetic acid) but was cleaved in an alkaline milieu of pH 8.5 or higher. The *S*-ethylcarbamoyl group is readily cleaved by silver and mercuric acetate with formation of mercaptides. *S*-Ethylcarbamoylcysteine and peptides containing this protected amino acid fail to react with *p*-chloromercuribenzoate at pH 4.6 but are readily cleaved by this reagent at pH 7.0 or higher. This observation enabled us to determine the *S*-ethylcarbamoyl content of the various peptide intermediates in the synthesis of fragment A. Peptides containing *S*-ethylcarbamoylcysteine do not react with Ellman reagent on thin layer plates, but exposure of developed plates to ammonia vapor results in the formation of bright yellow spots. The *S*-ethylcarbamoyl group provides suitable protection of the thiol group of cysteine in peptide synthesis but care has to be exercised not to expose peptides containing this sulfhydryl protector to strongly alkaline conditions. Oxidation with performic acid converts peptides containing *S*-ethylcarbamoylcysteine into the corresponding cysteic acid derivatives.

In previous communications,[1,3,5] we have described syntheses of two protected peptide hydrazides, *i.e.*, fragments BCD spanning positions 12–47 and fragment EF corresponding to positions 48–80 (Figure 1,

(1) See R. Camble, G. Dupuis, K. Kawasaki, H. Romovacek, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **94**, 2091 (1972), for paper L in this series.

(2) Supported by grants from the U. S. Public Health Service and the Hoffmann-La Roche Foundation.

(3) A preliminary communication of some of the results presented in this paper has appeared: H. T. Storey and K. Hofmann, *Peptides, Proc. Eur. Peptide Symp., 11th, 1971*, in press.

(4) The amino acid residues except glycine are of the L configuration. The following abbreviations are used: AP-M, aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)]; Boc, *tert*-butoxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; EC, ethylcarbamoyl; EtOH, ethanol; F, formyl; MeOH, methanol; N_3, azide; NMM, *N*-methylmorpholine; OCP, 2,4,5-trichlorophenyl ester; ONHS, *N*-hydroxysuccinimido ester; *O-t*-Bu, *tert*-butyl ester; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; tlc, thin layer chromatography; X, *tert*-butoxycarbonylhydrazide; Y, benzyloxycarbonylhydrazide; Z, benzyloxycarbonyl. In order to simplify the designation of complex peptide derivatives, the following nomenclature is used: fragment A, the *N*-formyl benzyloxycarbonylhydrazide of the peptide corresponding to positions 1–11 of T_1; fragment B, the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 12–23 of T_1; fragment AB, the *N*-formyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 1–23 of T_1; fragment CD, the *N*-benzyloxycarbonyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 24–47 of T_1; fragment ABCD, the *N*-formyl *tert*-butoxycarbonylhydrazide of the peptide corresponding to positions 1–47 of T_1; fragments A, AB, CD, and ABCD hydrazides, the free hydrazides of the N-protected peptides; fragments B and CD *tert*-butoxycarbonylhydrazides, the amino-deprotected peptides.

(5) J. Beacham, G. Dupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Amer. Chem. Soc.*, **93**, 5526 (1971).

top) of the proposed primary sequence of the enzyme ribonuclease T_1 (ribonucleate guanine nucleotido-2′-transferase (cyclizing), 2.7.7.26).[6] This paper describes syntheses of fragments A, AB (Figure 1, center), and ABCD (Figure 1, bottom).

The key to the preparation of fragment ABCD was the development of a route to fragment A, a peptide derivative which embodies in its structure three of the four half-cystines of the enzyme. Hydrogenolysis which was used exclusively for deprotection of α-amino groups in the preparation of fragments BCD and EF was obviously not applicable in this case and a different approach had to be developed.

The synthesis of fragment BCD involved partial deprotection of fragment CD by hydrogenolysis and coupling of the ensuing fragment CD *tert*-butoxycarbonylhydrazide with fragment B azide. Fragment ABCD was synthesized by first condensing fragment A azide with fragment B *tert*-butoxycarbonylhydrazide to give fragment AB which was deprotected on the hydrazide side and coupled to fragment CD *tert*-butoxycarbonylhydrazide.[7]

Sulfhydryl Protection

The selection of a suitable sulfhydryl protecting group, cleavable under mild conditions, was of importance in the synthesis of fragment A. Since our approach[5] to the synthesis of the peptide chain of the T_1 enzyme involves generation of hydrazides by acid cleavage of protected hydrazides rather than exposure of esters to hydrazine, the utility of the ethylcarbamoyl group for cysteine sulfhydryl protection was explored. Guttmann[8] prepared *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteine and used this compound in syntheses of glutathione and oxytocin. He found the *S*-ethylcarbamoyl group stable to acid but labile to alkali. We prepared *N*-*tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteine from *S*-ethylcarbamoylcysteine[9] and *tert*-butoxycarbonyl azide[10] and converted the compound to the 2,4,5-trichlorophenyl ester. Zahn and Hammerström[11] generated *N*-*tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteine by another route. We were able to confirm the stability of the *S*-ethylcarbamoyl group toward strong acid, *i.e.*, trifluoroacetic acid and hydrogen bromide in trifluoroacetic acid, and its lability to alkali. In order to gain additional information regarding its behavior the reaction with metal ions of *S*-ethylcarbamoylcysteine and peptide derivatives containing this residue was explored.

The utility of metal ions for deprotection of S-substituted cysteine derivatives has been studied previously. For example, in their classical studies on sulfhydryl protection, Zervas and Photaki[12] observed that the *S*-trityl protecting group was smoothly removed from *S*-tritylcysteine containing peptides with silver nitrate in pyridine solution at 0° to yield the respective silver mercaptides. The free sulfhydryl group was liberated from the mercaptides by hydrogen halide or sulfide.

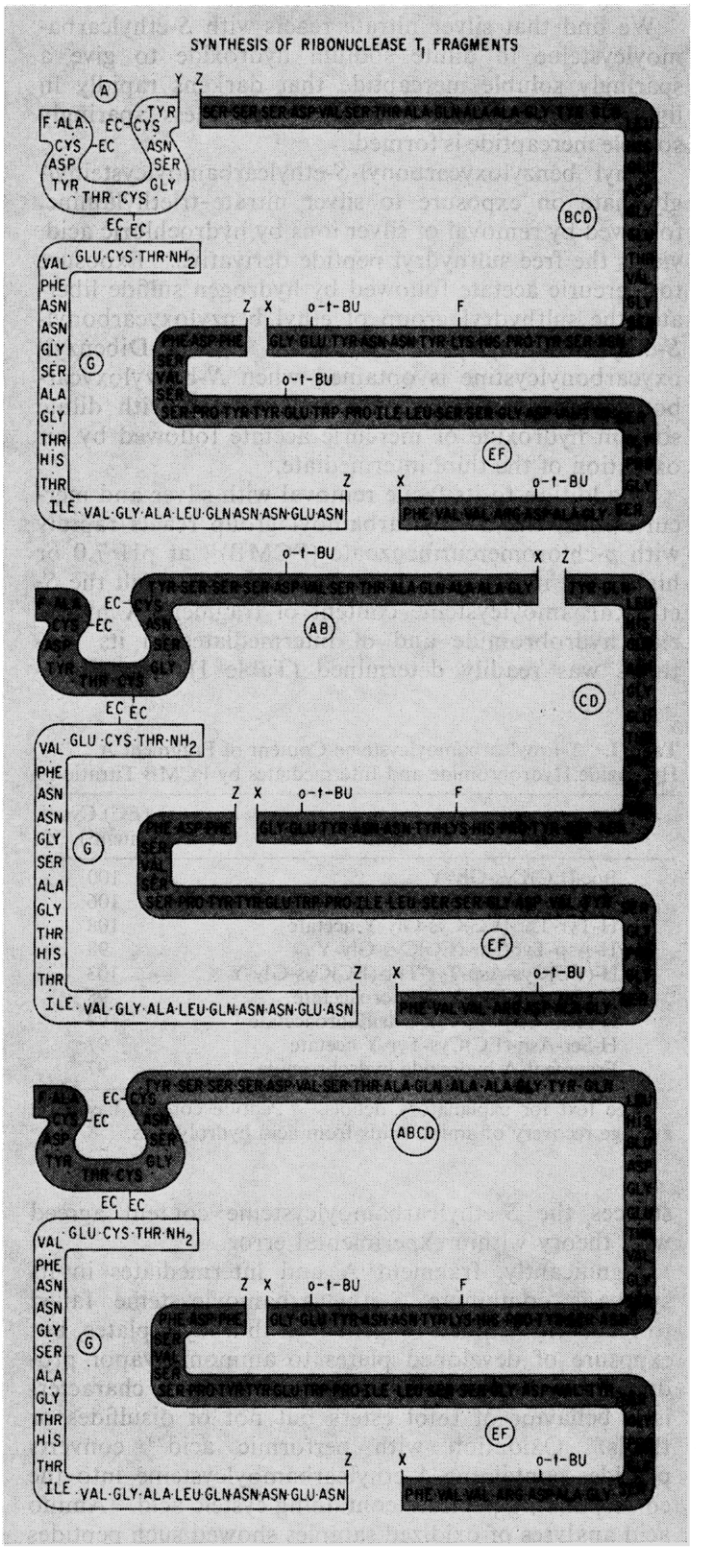

Figure 1. Location of completed sequences within the sequences of ribonuclease T_1.

Mercuric chloride similarly removed the trityl group and the free sulfhydryl was liberated from the ensuing mercaptides by hydrogen sulfide.[13] The *S*-benzylthiomethyl[14,15] and *S*-acetamidomethyl protecting groups[16] are readily removed with mercuric acetate.

(6) K. Takahashi, *J. Biol. Chem.*, **240**, 4117 (1965).
(7) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
(8) St. Guttmann, *Helv. Chim. Acta*, **49**, 83 (1966).
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(10) L. A. Carpino, *J. Amer. Chem. Soc.*, **79**, 98 (1957).
(11) H. Zahn and K. Hammerström, *Chem. Ber.*, **102**, 1048 (1969).
(12) L. Zervas and I. Photaki, *J. Amer. Chem. Soc.*, **84**, 3887 (1962).
(13) L. Zervas, I. Photaki, A. Cosmatos, and D. Borovas, *ibid.*, **87**, 4922 (1965).
(14) P. J. E. Brownlee, M. E. Cox, B. O. Handford, J. C. Marsden, and G. T. Young, *J. Chem. Soc.*, 3832 (1964).

We find that silver nitrate reacts with *S*-ethylcarbamoylcysteine in dilute sodium hydroxide to give a sparingly soluble mercaptide that darkens rapidly in light; with mercuric acetate a colorless sparingly soluble mercaptide is formed.

Ethyl benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate on exposure to silver nitrate–triethylamine, followed by removal of silver ions by hydrochloric acid, yields the free sulfhydryl peptide derivative. Exposure to mercuric acetate followed by hydrogen sulfide liberates the sulfhydryl group of ethyl benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate. *N,N'*-Dibenzyloxycarbonylcystine is obtained when *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteine is treated with dilute sodium hydroxide or mercuric acetate followed by air oxidation of the thiol intermediate.

In addition to its facile removal with silver and mercuric ions, the *S*-ethylcarbamoyl group reacts rapidly with *p*-chloromercuribenzoate (PCMB)[17] at pH 7.0 or higher but not at pH 4.6.[18] Based on this result the *S*-ethylcarbamoylcysteine content of fragment A hydrazide hydrobromide and of intermediates in its synthesis was readily determined (Table I). In all instances the *S*-ethylcarbamoylcysteine content agreed with theory within experimental error.

Table I. *S*-Ethylcarbamoylcysteine Content of Fragment A Hydrazide Hydrobromide and Intermediates by PCMB Titration[a]

Compound	(EC) Cys (content[b]), %
Boc-(EC)Cys-Gly-Y	100
Boc-Thr-(EC)Cys-Gly-Y	106
H-Tyr-Thr-(EC)Cys-Gly-Y acetate	108
H-Asp-Tyr-Thr-(EC)Cys-Gly-Y	98
H-(EC)Cys-Asp-Tyr-Thr-(EC)Cys-Gly-Y	103
H-(EC)Cys-Tyr-Y trifluoroacetate	98
H-Asn-(EC)Cys-Tyr-Y trifluoroacetate	99
H-Ser-Asn-(EC)Cys-Tyr-Y acetate	97
Fragment A hydrazide hydrobromide	97

[a] See text for explanatory details. [b] Peptide content based on average recovery of amino acids from acid hydrolysates.

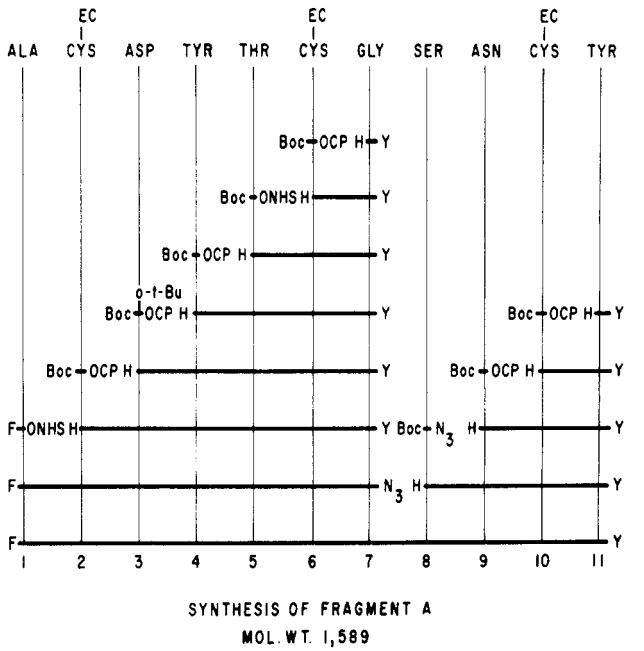
Significantly, fragment A and intermediates in its synthesis containing *S*-ethylcarbamoylcysteine failed to react with Ellman reagent[19] on thin layer plates, but exposure of developed plates to ammonia vapor produced sharp intense yellow spots. This is a characteristic behavior of thiol esters but not of disulfides or thiols. Oxidation with performic acid[20] converts peptides containing *S*-ethylcarbamoylcysteine into the corresponding peptides containing cysteic acid. Amino acid analyses of oxidized samples showed such peptides to contain the expected proportions of cysteic acid.

Based on our own experience we conclude that the *S*-ethylcarbamoyl group provides adequate protection of the thiol group of cysteine and that this group is readily removable under a variety of mild conditions. In view of the fact that this group is sensitive to alkali, basic conditions (pH 8 or higher) should be avoided in peptide syntheses involving its use.

Preparation of Fragment A

Fro the synthesis of fragment A two subfragments, an *N*-formyl heptapeptide benzyloxycarbonylhydrazide (positions 1–7) and a *tert*-butoxycarbonyl tetrapeptide benzyloxycarbonylhydrazide (positions 8–11), synthesized essentially by the stepwise active ester procedure[21] were selectively deblocked and linked *via* an azide coupling step. Intermediates in the synthesis of these subfragments were characterized by thin layer chromatography, optical rotation, amino acid composition of acid, and in many instances AP-M digests and elemental analyses. The synthetic route to these subfragments (Chart I) involved the use of *tert*-butoxycarbonyl amino acid derivatives and C-terminal amino acid benzyloxycarbonylhydrazides. Serine was introduced *via* *N-tert*-butoxycarbonylserine azide; other amino acids were coupled in the form of their *N-tert*-butoxycarbonyl *N*-hydroxysuccinimido[22] or 2,4,5-trichlorophenyl[23] esters. The β-carboxyl group of aspartic acid was protected by a *tert*-butyl ester group.[24] Treatment with aqueous 90% trifluoroacetic acid served to remove amino protecting groups. Although the *S*-ethylcarbamoyl group was found to be stable in DMF–TEA under the conditions used for coupling, only 90% of the required molar quantity of this amine was usually employed to minimize thiol deblocking during the stepwise elongation of the peptide chain. The two final active ester steps in the synthesis of the N-terminal heptapeptide derivative (positions 1–7) were performed at 40° in the absence of base.

Chart I



SYNTHESIS OF FRAGMENT A
MOL. WT. 1,589

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(19) C. B. Glaser, H. Maeda, and J. Meienhofer, *J. Chromatogr.*, **50**, 151 (1970).
(20) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).
(21) (a) M. Bodanszky and V. du Vigneaud, *J. Amer. Chem. Soc.*, **81**, 5688 (1959); (b) J. S. Morley, *J. Chem. Soc. C*, 2410 (1967).
(22) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).
(23) (a) J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963); (b) W. Broadbent, J. S. Morley, and B. E. Stone, *J. Chem. Soc. C*, 2632 (1967).
(24) R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, **44**, 1991 (1961).

As was pointed out in a previous communication,[5] tlc of protected peptide hydrazides has limited value as an index of purity for the reason that many of these intermediates migrate close to the solvent front. This was again observed during the present study.

Impurities were present at the tetrapeptide stage in both subfragments of fragment A, possibly because of O-acylation of the tyrosine residues. The C-terminal tetrapeptide benzyloxycarbonylhydrazide (positions 8–11) was readily purified by chromatography on a Dowex column with pyridinium acetate buffers[5,25] as the eluents. The purified peptide produced sharp spots on thin layer chromatograms and the amino acid composition of acid hydrolysates of the peptide and of performic acid oxidized specimens agreed within experimental error with theory. The average recovery of amino acids in AP-M digests of the hydrobromide salt of the free tetrapeptide hydrazide was 83% of theory.

For the synthesis of the N-terminal fragment (positions 1–7), the stepwise procedure was carried through the pentapeptide stage without rigorous purification. The ensuing *tert*-butoxycarbonyl-β-*tert*-butylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide was then partially deblocked and the peptide benzyloxycarbonylhydrazide purified by Dowex chromatography. Following addition of the *N*-*tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteine residue (position 2), the doubly protected peptide hydrazide was isolated by chromatography of the crude reaction mixture on the ion exchange resin AG 1-X2. The free β-carbonyl group of the aspartic acid residue provided the negative charge for attachment to the resin. The doubly protected heptapeptide hydrazide (positions 1–7) was isolated by the same technique and was partially deblocked with trifluoroacetic acid freshly saturated with hydrogen bromide. Model experiments using formylalanine showed that this treatment fails to remove the *N*-formyl group. The ensuing free hydrazide was converted to the azide and the latter was coupled[7] with the C-terminal tetrapeptide benzyloxycarbonylhydrazide (positions 8–11) to give crude fragment A which was again chromatographed on AG 1-X2. Fragment A formed a sharp spot on thin layer chromatograms in two solvent systems and was Ellman negative but became positive when the developed plates were exposed to ammonia vapor; some samples exhibited slight tailing. Amino acid analyses of fragment A and of performic acid oxidized samples contained the constituent amino acids in the theoretically expected ratios. PCMB titration of fragment A hydrazide hydrobromide showed a thiol ester content of 97% of theory (Table I). Thin layer chromatograms of fragment A and the two subfragments from which it was derived have been presented.[3] These results support the sequential homogeneity of fragment A.

Preparation of Fragments AB and ABCD

Exposure to trifluoroacetic acid freshly saturated with hydrogen bromide removed the benzyloxycarbonyl group from fragment A to give fragment A hydrazide hydrobromide which was converted to the azide and coupled[7] with fragment B *tert*-butoxycarbonylhydrazide.[5] The ensuing fragment AB was purified by triturating the solid material first with ethyl acetate, then with water. The sparing solubility of this fragment in most common solvents precluded chromatographic purification and made difficult a critical assessment of its homogeneity by tlc, but the amino acid composition of acid hydrolysates and the ratio of the "diagnostic" amino acid residues[5] Val/Tyr in acid hydrolysates of several samples were in satisfactory agreement with theory.

Fragment AB was converted to the free hydrazide trifluoroacetate salt with 90% trifluoroacetic acid and the corresponding azide was coupled[7] to fragment CD *tert*-butoxycarbonylhydrazide[5] in a mixture of DMSO and DMF. After 48 hr at 4° the reaction mixture, which had set to a stiff gel, was stirred at 4° with a 2:3 mixture of DMF and 0.01 *M* acetic acid; insoluble material was removed by centrifugation and the clear supernatant was added to a column of the ion exchange resin AG 1-X2. The column was then eluted with 2:3 mixtures of DMF and dilute acetic acid of increasing concentration. Absorbancy measurements at 280 nm served to assess the progress of separation. The chromatogram showed the presence of three major peaks (I, II, and III) which were eluted, respectively, with DMF–0.03 *M* acetic acid, DMF–0.10 *M* acetic acid, and DMF–0.50 *M* acetic acid. Material corresponding to peak I was identified as unreacted fragment CD *tert*-butoxycarbonylhydrazide by tlc and amino acid analysis of an AP-M digest; the recovery yield was 55%. The material corresponding to peak II on amino acid analysis exhibited essentially the same amino acid composition as fragment CD *tert*-butoxycarbonylhydrazide but on tlc was shown to be a mixture. This fraction was not investigated further. The desired fragment ABCD (mol wt 5485) was isolated from the fractions corresponding to peak III in a yield of 17%. The low solubility of this material precluded extensive evaluation of its homogeneity by thin layer chromatography. However, since this peptide derivative was obtained by azide coupling of fragments of established sequential homogeneity we relied on the ratios of the "diagnostic" amino acid residues in acid hydrolysates of performic acid oxidized material for analytical characterization. Unfortunately, fragment B contains no diagnostic amino acid residues[5] but the ratio of cysteic acid to leucine, contributed by fragments A and C, and the ratio of cysteic acid to lysine, contributed by fragments A and D, are significant. These ratios were 3:1 as predicted by theory.

The amino acid composition of acid hydrolysates of the material not soluble in 0.01 *M* acetic acid–DMF was very similar to that of fragment ABCD suggesting the presence of polymers of this compound formed by partial sulfhydryl deblocking and disulfide formation. The high alanine and cysteic acid content suggests contamination with fragment AB.

Experimental Section[26]

Preparation of Amino Acid Derivatives. *S*-Ethylcarbamoylcysteine. This compound was prepared essentially as described:[8]

(25) W. A. Schroeder, R. T. Jones, J. Cormick, and K. McCalla, *Anal. Chem.*, 34, 1570 (1962).

(26) Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 nm and extrapolated to the 589-nm sodium line. Elemental analyses were by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. Analytical samples were dried *in vacuo* over P_2O_5 at 50–60°. The amino acid compositions of acid hydroly-

mp 190–191°; $[\alpha]^{30}D$ −87.9° (*c* 1.12, 95% acetic acid) (lit.[8] mp 219°; $[\alpha]^{22}D$ −91.1° (*c* 0.8, 95% acetic acid); lit.[9] mp 182°); R_f^{I} 0.4; R_f^{III} 0.5; thiol negative, thiol ester positive; elutes from the 160-cm column of the Beckman amino acid analyzer at 220 ml; from the 60-cm column at 85 ml of first buffer (position between Pro and Gly). Incubation with pH 8.5 0.25 *M* ammonium bicarbonate at room temperature for 66 hr showed 10% deblocking; 2.3% of thiol was formed under the same conditions at 0°.

***N*-*tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteine.** *tert*-Butoxycarbonyl azide[10] (36 g) and TEA (13.8 ml) were added dropwise at room temperature to a stirred solution of *S*-ethylcarbamoylcysteine (19.2 g) in a mixture of DMF (200 ml), water (150 ml), and dioxane (80 ml) and the mixture was stirred at room temperature for 24 hr. The precipitate was removed and the filtrate evaporated. The residue was dissolved in ethyl acetate and the solution was washed and dried. The solvent was removed and the residue solidified on addition of petroleum ether. Two recrystallizations from acetonitrile gave needles: 18.2 g (62%); mp 139–140°; $[\alpha]^{25}D$ −52.1° (*c* 2.33, DMF); $[\alpha]^{25}D$ −21.8° (*c* 1.0, EtOH) (lit.[11] mp 139°; $[\alpha]^{24}D$ −22.2° (*c* 1.0, EtOH); R_f^{I} 0.8; R_f^{III} 0.7; R_f^{IV} 0.8. *Anal.* Calcd for $C_{11}H_{20}N_2O_5S$: C, 45.2; H, 6.9; N, 9.6; S, 11.0. Found: C, 45.4; H, 7.0; N, 9.4; S, 10.8.

2,4,5-Trichlorophenyl *N*-*tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinate. DCC (6.19 g) was added at −10° to a solution of *N*-*tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteine (8.77 g) and 2,4,5-trichlorophenol (7.11 g) in THF (125 ml) and the mixture was stirred at 4° for 40 hr. The precipitate was removed, the filtrate was evaporated, and the residue washed with ether and dried. Recrystallization from ethyl acetate gave colorless needles: 9.84 g (69%); mp 152–154°; $[\alpha]^{28}D$ −35.1° (*c* 1.36, DMF); R_f^{I} 0.9; R_f^{III} 0.9; R_f^{IV} 0.9. *Anal.* Calcd for $C_{17}H_{21}N_2O_5SCl_3$: C, 43.3; H, 4.5; N, 5.9; S, 6.8; Cl, 22.6. Found: C, 43.0; H, 4.3; N, 5.7; S, 6.8; Cl, 22.4.

***N*-Hydroxysuccinimido Formylalaninate.** DCC (2.06 g) was added at 0° to a solution of formylalanine[27] (1.16 g) and *N*-hydroxysuccinimide (1.15 g) in acetonitrile (75 ml) and the mixture was stirred at room temperature for 18 hr. The precipitate was removed and the filtrate evaporated to dryness. The residue was recrystallized from EtOH: 1.59 g (75%); mp 155–157°; $[\alpha]^{25}D$ −81.9° (*c* 2.0, DMF); R_f^{I} 0.5; R_f^{III} 0.7. *Anal.* Calcd for $C_8H_{10}N_2O_5$: C, 44.9; H, 4.7; N, 13.1. Found: C, 44.9; H, 5.0; N, 13.0.

Glycine Benzyloxycarbonylhydrazide Trifluoroacetate. NMM (14.76 ml) was added at −18° to a solution of *tert*-butoxycarbonylglycine (21.02 g) in ethyl acetate (300 ml). To the stirred mixture was added isobutyl chloroformate (15.61 ml), followed, after 4 min, by benzyloxycarbonylhydrazide (21.94 g). The mixture was stirred at −18° for 15 min and then at room temperature for 45 min. The solvent was removed, the residue was dissolved in ethyl acetate, and the solution was washed and dried. The solvent was removed and the residue deblocked in 90% TFA (100 ml). The solvent was evaporated and the residue precipitated from ether with petroleum ether. The compound was crystallized from MeOH–ether: needles 35.13 g (87%); mp 176–177°; R_f^{I} 0.4. *Anal.* Calcd for $C_{12}H_{14}N_3O_5F_3$: C, 42.7; H, 4.2; N, 12.5. Found: C, 42.7; H, 4.1; N, 12.7.

***N*-*tert*-Butoxycarbonyltyrosine Benzyloxycarbonylhydrazide.** *N*-*tert*-Butoxycarbonyltyrosine dicyclohexylammonium salt (23.13 g) was partitioned between aqueous citric acid and ethyl acetate. The ethyl acetate layer was washed with water, dried, and evaporated. The residue was dissolved in ethyl acetate (200 ml), *tert*-butoxycarbonylhydrazide (9.9 g) and DCC (10.32 g) were added at −10°, and the mixture was stirred in the cold for 1 hr and then at room temperature for 18 hr. The precipitate was removed, the filtrate was diluted with ethyl acetate, and the solution was washed and dried. The solvent was removed and the solid residue which was obtained by trituration with petroleum ether was precipitated from ethyl acetate with petroleum ether, collected, and dried: 21.25 g (99%); mp 136–139°; $[\alpha]^{26}D$ −6.6° (*c* 4.52, MeOH); R_f^{I} 0.9; R_f^{III} 0.8. *Anal.* Calcd for $C_{22}H_{27}N_3O_6$: C, 61.5; H, 6.3; N, 9.8. Found: C, 61.7; H, 6.7; N, 9.6.

Tyrosine Benzyloxycarbonylhydrazide Monoacetate. *N*-*tert*-Butoxycarbonyltyrosine benzyloxycarbonylhydrazide (8.59 g) was deblocked in cold aqueous 90% TFA (100 ml). The solvent was removed and the residual oil triturated first under ether and then under petroleum ether to give a solid which was collected and dried. The crude product was dissolved in aqueous 10% acetic acid and applied to a column (2 × 35 cm) of AG1-X2 ion-exchange resin which was eluted with 10% acetic acid. The eluates were combined, concentrated, and lyophilized to constant weight to give the acetate salt: 5.5 g (70%); $[\alpha]^{25}D$ +36.9° (*c* 1.14, H_2O); R_f^{I} 0.6; R_f^{III} 0.6; R_f^{IV} 0.7; R_f^{1} 0.7; R_f^{3} 3.2 × His.

***N*-*tert*-Butoxycarbonylserine Hydrazide.** Hydrazine hydrate (22.8 ml) was added to a solution of methyl *N*-*tert*-butoxycarbonylserinate (30.2 g) in MeOH (150 ml) and the mixture was kept at room temperature for 24 hr. The solvent was removed and the residue dried over concentrated sulfuric acid *in vacuo*. Trituration of the residual oil under ethyl acetate gave a solid which was collected, washed with ether, and dried. The compound was recrystallized from ethyl acetate–ether: 20.0 g (66%); mp 106–108°; $[\alpha]^{30}D$ −9.0° (*c* 3.95, MeOH); R_f^{I} 0.8; R_f^{III} 0.7. *Anal.* Calcd for $C_8H_{17}N_3O_4$: C, 43.8; H, 7.8; N, 19.2. Found: C, 43.9; H, 7.7; N, 19.5.

***N*-Hydroxysuccinimido *N*-*tert*-Butoxycarbonylthreoninate.** NMM (3.1 ml) was added at −18° to a solution of *tert*-butoxycarbonylthreonine (5.48 g) in ethyl acetate (50 ml). To the stirred solution was added isobutyl chloroformate (3.3 ml) followed, after 3 min, by a precooled solution of *N*-hydroxysuccinimide (3.18 g) in THF (15 ml). The mixture was stirred at −18° for 10 min, then at room temperature for 15 min, and finally at 40° for 20 min. The precipitate was removed and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate and the solution cooled to 0° and washed twice with ice-cold water and dried over magnesium sulfate. The solvent was evaporated, the residue was dissolved

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sates and AP-M digests 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. Nle and α-amino-β-guanidopropionic acid served as internal standards. Acid hydrolyses were performed in constant boiling HCl at 110° for 24 hr in evacuated tubes except with peptides containing valine or isoleucine which were hydrolyzed for 48 hr; values are not corrected for amino acid destruction. AP-M digests were performed as described by K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966), except when noted otherwise. Designations of solvent systems for descending paper chromatography on Whatman No. 1 filter paper are: R_f^{1} 1-butanol–glacial acetic acid–water (4:1:5) top layer; R_f^{3} 1-butanol–pyridine–water–glacial acetic acid (45:30:36:9); with this system R_f values are expressed as multiples of the distance traveled by a histidine marker. Designation of solvent systems for ascending tlc on silica gel G. (E. Merck and Co., Darmstadt, West Germany) are: R_f^{I} 1-butanol–glacial acetic acid–water (60:20:20); R_f^{III} 1-butanol–pyridine–glacial acetic acid–water (30:20:6:24); R_f^{IV} MeOH–chloroform (1:1); R_f^{V}, a freshly prepared mixture of 1-butanol–50% formic acid (1:1). Sparingly soluble compounds were prepared for spotting by dissolving them in a few drops of glacial acetic acid with slight warming and adding a few drops of water. Solvents were freshly distilled and, except when noted otherwise, evaporations were carried out *in vacuo* at a temperature of 40–45° in rotary evaporators. Petroleum ether (bp 30–60°) was employed. Except when noted otherwise the ethyl acetate solutions were washed three times with 1 *N* citric acid, once with saturated sodium chloride, three times with saturated sodium bicarbonate, and three times with saturated sodium chloride. For removal of *tert*-butoxycarbonyl groups the protected peptides were kept in 90% aqueous TFA for 30 min at room temperature. Yields are based on weight of vacuum dried (over P_2O_5 and KOH) substance. The anion exchanger AG 1-X2 was purchased from Bio-Rad Laboratories, Richmond, Calif.

For determination of *S*-ethylcarbamoylcysteine in peptides the PCMB determination of Boyer[17] was modified. Stock solutions were prepared by dissolving the peptides in a minimum of DMF (0.1–0.2 ml) and diluting to a concentration of 2 × 10^{-3} *M* with 0.05 *M* sodium borate buffer, pH 10. Aliquots (0.5–1.0 ml) of the stock solutions were mixed with varying amounts of a PCMB solution (3 × 10^{-3} *M*) prepared with the same borate buffer. The solutions were diluted to a final volume of 2 ml with borate buffer and allowed to stand at room temperature for 30 min. Aliquots (0.2 ml) were withdrawn and mixed with 2.8 ml of 0.33 *M* sodium acetate buffer, pH 4.6. Absorbance was measured at 255 nm against a buffer blank. Absorbance due to PCMB alone was determined by preparing a similar set of solutions without peptide.

Performic acid oxidations were performed as follows: a performic acid solution was prepared by adding 30% v/v hydrogen peroxide (1.0 ml) to redistilled formic acid (9.0 ml). The solution was kept at room temperature for 2 hr and then cooled to 0°. The peptide (*ca.* 3–5 mg) was dissolved in formic acid (1.0 ml) and the solution was cooled to 0°. Performic acid solution (0.2 ml) was added and the mixture was kept at 0° for 4 hr. Water (15 ml) was added and the solvents were removed *in vacuo*. The residue was dissolved in water (1 ml) and lyophilized prior to acid hydrolysis.

(27) G. C. Sheehan and D.-DH Yang, *J. Amer. Chem. Soc.*, **80**, 1154 (1958).

in ether and the solution kept in a refrigerator for 2 days. The ensuing crystals were collected, washed with ether, and dried: 5.93 g (75%); mp 134–135°; $[\alpha]^{25}D$ −33.3° (*c* 1.94, ethyl acetate). *Anal.* Calcd for $C_{13}H_{20}N_2O_7$: C, 49.4; H, 6.4; N, 8.9. Found: C, 49.6; H, 6.4; N, 8.8.

Model Sulfhydryl Deblocking Studies. *N,N'*-Dibenzyloxycarbonylcystine Bisdicyclohexylammonium Salt. (a) From *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteine with Alkali. *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteine (2.00 g) was dissolved in 1 *N* aqueous NaOH (5 ml). After 2 hr at room temperature aqueous 1 *N* HCl (5 ml) was added and the pH adjusted to 8 by the addition of aqueous sodium bicarbonate. The solution was aerated for 18 hr, the pH was adjusted to 2 by the addition of HCl, and the solution was extracted with ethyl acetate. The extracts were washed with water and dried. The solvent was evaporated; DCHA was added to the residual oil to give a solid which was crystallized from MeOH–ether. Recrystallization from the same solvents gave: 2.08 g (78%); mp 189–190°; $[\alpha]^{27}D$ −71.4° (*c* 1.01, MeOH); R_f^I 0.8 (chlorine-positive spot) and 0.6 (DCHA spot). *Anal.* Calcd for $C_{46}H_{70}N_4O_8S_2$: C, 63.4; H, 8.1; N, 6.4; S, 7.4. Found: C, 63.3; H, 8.2; N, 6.3; S, 7.6.

(b) From *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteine with Mercuric Acetate. A solution of mercuric acetate (4.00 g) in water (5 ml) was added with stirring to a solution of *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteine[9] (2.00 g) in MeOH (100 ml). The resultant suspension was stirred for 30 min, acetic acid (5 ml) was added, and H_2S was passed through the solution for 4 hr with vigorous stirring. The black precipitate was removed by filtration through Celite and the filtrate reduced to dryness *in vacuo*. The residue was dissolved in aqueous sodium bicarbonate and aerated for 18 hr. The solution was treated as described in the previous experiment to yield: 1.70 g (64%); mp 189–190°; $[\alpha]^{27}D$ −74.0° (*c* 1.01, MeOH); R_f^I 0.8 (chlorine-positive spot) and 0.6 (DCHA spot).

(c) From Cystine. Cystine (1.00 g) was carbobenzoxylated in the usual fashion in 1 *N* aqueous NaOH, and DCHA was added to a solution of the resultant oil in ether. The ensuring crystalline solid was recrystallized from MeOH–ether: 2.51 g (60%); mp 189–190°; $[\alpha]^{27}D$ −73.1° (*c* 1.02, MeOH); R_f^I 0.8 (chlorine-positive spot) and 0.6 (DCHA spot). *Anal.* Calcd for $C_{46}H_{70}N_4O_8S_2$: C, 63.4; H, 8.1; N, 6.4; S, 7.4. Found: C, 63.6; H, 8.3; N, 6.5; S, 7.5.

Ethyl *N*-Benzyloxycarbonylcysteinylglycinate. (a) From Ethyl *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate with Silver Nitrate. A solution of silver nitrate (186 mg) in water (5 ml) and MeOH (10 ml) was added to ethyl *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate (412 mg) in MeOH (10 ml). TEA (0.14 ml) was added dropwise and the resultant precipitate was collected after the solution had been heated to its boiling point and then cooled. This precipitate (425 mg) was slurried in DMF (5 ml) and concentrated HCl (0.3 ml) and the suspension was shaken for 3 hr. Chloroform was added to the residue and the solution was washed with water and dried. Evaporation of the solvent gave a solid which was crystallized from ethyl acetate–petroleum ether: 212 mg (62%); mp 122–123°; $[\alpha]^{28}D$ −16.1° (*c* 1.2, EtOH) (lit.[12] mp 123–124°; $[\alpha]^{28}D$ −16.8° (*c* 3, EtOH). *Anal.* Calcd for $C_{15}H_{20}N_2O_5S$: C, 52.9; H, 5.9; N, 8.2; S, 9.4. Found: C, 52.7; H, 6.0; N, 8.1; S, 9.7.

(b) From Ethyl *N*-Benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate with Mercuric Acetate. Mercuric acetate (1.55 g) was added with stirring to a solution of ethyl *N*-benzyloxycarbonyl-*S*-ethylcarbamoylcysteinylglycinate (1.00 g) in 80% aqueous acetic acid (50 ml) at room temperature. After 1 hr 80% aqueous acetic acid (75 ml) was added and H_2S was bubbled through the solution for 4 hr with vigorous stirring. The precipitate was removed by filtration through a bed of Celite and the filtrate was evaporated. The solid residue was crystallized from ethyl acetate–petroleum ether: 650 mg (79%); mp 121–122°; $[\alpha]^{28}D$ −17.5° (*c* 1.02, EtOH). *Anal.* Calcd for $C_{15}H_{20}N_2O_5S$: C, 52.9; H, 5.9; N, 8.2; S, 9.4. Found: C, 53.2; H, 6.0; N, 8.3; S, 9.7.

Preparation of Fragment A (Positions 1–11). (Positions 10 and 11) *N-tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinyltyrosine Benzyloxycarbonylhydrazide. 2,4,5-Trichlorophenyl *N-tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteinate (26.4 g) was added to a stirred solution of tyrosine benzyloxycarbonylhydrazide monoacetate (21.8 g) in THF (450 ml) at −10°. The mixture was kept at 4° for 44 hr and the solvent was evaporated. The residue was dissolved in ethyl acetate and the solution was washed and dried. The solution was concentrated, and the product precipitated with petroleum ether. Reprecipitation with petroleum ether from a solution in ethyl acetate gave a white solid: 21.37 g (60%); mp 142–144°; $[\alpha]^{29}D$ −37.5° (*c* 3.79, MeOH); R_f^I 0.8; R_f^{III} 0.8; amino acid ratios in acid hydrolysate, $Cys_{1.1}Tyr_{0.9}$ (70%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Cys(SO_3H)_{1.1}Tyr_{0.9}$. *Anal.* Calcd for $C_{28}H_{37}N_5O_8S$: C, 55.7; H, 6.2; N, 11.6; S, 5.3. Found: C, 56.0; H, 6.3; N, 11.5; S, 5.6.

(Positions 9–11) *N-tert*-Butoxycarbonylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine Benzyloxycarbonylhydrazide. The protected dipeptide hydrazide (21.37 g) was deblocked in aqueous 90% TFA (100 ml). The solvent was evaporated, a mixture of ether and petroleum ether (1:1) was added, and the resultant precipitate was collected, washed with ether, and dried. Reprecipitation from EtOH with a mixture of ether and petroleum ether (1:1) gave a white solid: 17.47 g (80%); mp 122–124° dec; $[\alpha]^{29}D$ +2.3° (*c* 1.97, H_2O); $[\alpha]^{29}D$ −7.6° (*c* 2.14, MeOH); R_f^I 0.7; R_f^{III} 0.8; R_f^{IV} 0.8; R_f^1 0.8; R_f^3 3.2 × His.

To a solution of *S*-ethylcarbamoylcysteinyltyrosine benzyloxycarbonylhydrazide trifluoroacetate (5.49 g) in THF (150 ml) at −10° was added a solution of 2,4,5-trichlorophenyl *N-tert*-butoxycarbonylasparaginate[23] (3.66 g) in THF (150 ml) followed by TEA (1.16 ml). After stirring the mixture at −10° for 30 min and then at room temperature for 24 hr, the solvent was evaporated and ethyl acetate was added to the residue. The mixture was kept in a refrigerator for 48 hr and the precipitate was collected, washed with ethyl acetate, and dried: 3.52 g (55%); mp 186–188°; $[\alpha]^{32}D$ −54.4° (*c* 3.72, MeOH); R_f^I 0.8; R_f^{III} 0.7. *Anal.* Calcd for $C_{32}H_{43}N_7O_{10}S$: C, 53.6; H, 6.1; N, 13.7; S, 4.5. Found: C, 53.6; H, 6.2; N, 13.5; S, 4.7.

(Positions 9–11) Asparaginyl-*S*-ethylcarbamoylcysteinyltyrosine Benzyloxycarbonylhydrazide Trifluoroacetate. The protected tripeptide hydrazide (10.52 g) was deblocked in cold aqueous 90% TFA (100 ml). The solvent was evaporated, ether was added, and the resultant precipitate was collected, washed with ether, and dried. Reprecipitation from EtOH with ether gave a white solid: 10.15 g (94%); $[\alpha]^{27}D$ −25.7° (*c* 1.70, DMF); R_f^I 0.6 with minor impurity, R_f^I 0.5; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Asp_{1.0}Cys_{0.8}Tyr_{1.0}$ (85%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Asp_{1.0}Cys(SO_3H)_{1.1}Tyr_{0.9}$.

(Positions 8–11) Serylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine Benzyloxycarbonylhydrazide Acetate. *tert*-Butyl nitrite (0.61 ml) was added to a solution cooled at −20° of *N-tert*-butoxycarbonylserine hydrazide (1.02 g) in DMF (10 ml) containing 6.91 *N* hydrogen chloride in dioxane (3.4 ml). The mixture was stirred at −20° for 20 min and then cooled to −40° and TEA (3.86 ml) was added. To this solution containing the azide of Boc-Ser was added a solution of asparaginyl-*S*-ethylcarbamoylcysteinyltyrosine benzyloxycarbonylhydrazide trifluoroacetate (3.40 g) in DMF (25 ml). The pH of the reaction mixture was adjusted to 7.5–8.0 by dropwise addition of TEA, as necessary. The mixture was stirred for 72 hr at 4° and diluted with ethyl acetate and the solution was washed and dried. The solvent was evaporated, a mixture of ether and petroleum ether (1:1) was added, and the resultant precipitate was collected and dried. Reprecipitation from MeOH with petroleum ether gave a solid: 3.06 g (82%); $[\alpha]^{29}D$ −51.2° (*c* 1.29, MeOH); R_f^I 0.7; R_f^{III} 0.8; amino acid ratios in acid hydrolysate, $Ser_{1.0}Asp_{1.1}Cys_{0.6}Tyr_{0.9}$ (82%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Ser_{1.0}Asp_{1.1}Cys(SO_3H)_{1.1}Tyr_{0.8}$.

The impure protected tetrapeptide hydrazide (1.00 g) was deblocked in cold aqueous 90% TFA (3 ml). The solvent was removed, ether (80 ml) was added, and the resultant precipitate was collected, washed with ether, and dried: 1.09 g (107%); R_f^I 0.3 with impurities at 0.2, 0.5, and 0.7; R_f^{III} 0.7 with streaking. This impure material (250 mg) was dissolved in 0.1 *M* pH 3.1 pyridinium acetate buffer (5 ml) and the solution was applied to a column (1.9 × 15 cm) of Beckman ion-exchange resin type 15A. The column was eluted at approximately 15 ml/hr with a nonlinear buffer gradient obtained by mixing 0.1 *M* pyridinium acetate, pH 3.1 (125 ml), with 2.0 *M* pyridinium acetate, pH 5.0 (two 125-ml portions), in a Buchler gradient mixer, and fractions (30 min) were collected. Samples (10 μl) were withdrawn from each fraction and evaluated by tlc using the chlorine test for visualization. Fractions containing the desired homogeneous material were pooled, concentrated to a small volume, and lyophilized to constant weight: 139 mg (59% recovery as acetate salt); $[\alpha]^{25}D$ −48.6° (*c* 1.15, MeOH); R_f^I 0.3; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Ser_{1.0}Asp_{1.1}Cys_{1.0}Tyr_{1.0}$ (93%); amino acid ratios in performic acid oxidized sample, $Ser_{1.0}Asp_{1.2}Cys(SO_3H)_{1.0}Tyr_{0.9}$; amino acid ratios in AP-M digest of HBr–TFA deblocked material, $(Ser + Asn)_{2.1}Cys_{0.9}Tyr_{1.0}$ (83%). *Anal.* Calcd for $C_{32}H_{44}N_8O_{12}S$: C, 50.3; H, 5.8; N, 14.7; S, 4.2. Found: C, 50.1; H, 5.9; N, 14.9; S, 4.4.

(Positions 6–7) *N-tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of glycine benzyloxycarbonylhydrazide trifluoroacetate (1.69 g) in DMF (5 ml) was added to a solution of 2,4,5-trichlorophenyl *N-tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteinate (2.48 g) in DMF (25 ml) at −10°. TEA (0.61 ml) was then added and the mixture was stirred at 0° for 3 hr and then at room temperature for 72 hr. The bulk of the solvents was removed, the residue was dissolved in ethyl acetate, and the solution was washed and dried. Evaporation of the solvent gave a solid which was recrystallized from ethyl acetate: 2.30 g (92%); mp 164–166°; $[\alpha]^{29}D$ −22.1° (*c* 1.73, DMF); $[\alpha]^{26}D$ −9.2° (*c* 1.22, MeOH); R_f^{I} 0.7; R_f^{III} 0.8; iodine, chlorine, and thio ester positive ninhydrin negative spot; amino acid ratios in acid hydrolysate, $Cys_{0.9}Gly_{1.1}$ (91%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Cys(SO_3H)_{1.0}Gly_{1.0}$. *Anal.* Calcd for $C_{21}H_{31}N_5O_7S$: C, 50.7; H, 6.3; N, 14.1; S, 6.4. Found: C, 50.9; H, 6.5; N, 14.2; S, 6.6.

(Positions 6 and 7) *S*-Ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide Trifluoroacetate. *N-tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (2.00 g) was deblocked in aqueous 90% TFA (10 ml). The solvent was evaporated, ether (150 ml) was added, and the resulting precipitate collected, washed with ether, and dried: 2.02 g (98%). A sample of the trifluoroacetate was dissolved in 5% aqueous acetic acid and TFA ions were exchanged for acetate ions on a column of Amberlite IRA 400 ion-exchange resin (20–50 mesh, acetate form). The acetate salt of the dipeptide was isolated by lyophilization: $[\alpha]^{28}D$ +9.5° (*c* 2.30, MeOH); R_f^{I} 0.4; R_f^{III} 0.6; ninhydrin, chlorine, hydrazide, and thio ester positive, thiol negative spot; amino acid ratios in acid hydrolysate, $Cys_{1.0}Gly_{1.0}$ (78%).

(Positions 5–7) *N-tert*-Butoxycarbonylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of *N*-succinimido *N-tert*-butoxycarbonylthreoninate (1.30 g) in DMF (10 ml) was added to a solution of *S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide trifluoroacetate (1.99 g) in DMF (15 ml) at −10°. TEA (0.48 ml) was then added and the mixture was stirred for 3 hr at 0° followed by 40 hr at room temperature, when the bulk of the solvents was removed. The residue was dissolved in ethyl acetate and the solution was washed and dried. The solvents were evaporated and the residue precipitated from ethyl acetate with petroleum ether and dried: 2.26 g (97%); mp 100–105°; $[\alpha]^{27}D$ −29.6° (*c* 2.43, MeOH); $[\alpha]^{26}D$ −23.2° (*c* 1.96, DMF); R_f^{I} 0.8; R_f^{III} 0.8; iodine, hydrazide, and thio ester positive, thiol and ninhydrin negative spot; amino acid ratios in acid hydrolysate, $Thr_{0.9}Cys_{1.1}Gly_{1.0}$ (95%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Thr_{1.1}Cys(SO_3H)_{1.0}Gly_{0.9}$. *Anal.* Calcd for $C_{25}H_{38}N_6O_9S$: C, 50.2; H, 6.4; N, 14.0; S, 5.4. Found: C, 50.3; H, 6.4; N, 13.6; S, 4.9.

(Positions 5–7) Threonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide Trifluoroacetate. *N-tert*-Butoxycarbonylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (2.26 g) was deblocked in aqueous 90% TFA (10 ml). The solvent was evaporated, ether (150 ml) was added, and the resulting precipitate was collected, washed with ether, and dried: 2.19 g (95%); white amorphous solid; $[\alpha]^{27}D$ −4.4° (*c* 2.18, MeOH); R_f^{I} 0.5; R_f^{III} 0.6; ninhydrin, chlorine, hydrazide, and thio ester positive, thiol negative spot; amino acid ratios in acid hydrolysate, $Thr_{1.0}Cys_{0.9}Gly_{1.1}$ (76%); amino acid ratios in AP-M digest, $Thr_{1.1}Cys_{1.1}Gly_{0.9}$ (73%).

(Positions 4–7) *N-tert*-Butoxycarbonyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of 2,4,5-trichlorophenyl *N-tert*-butoxycarbonyltyrosinate[23] (1.45 g) in DMF (15 ml) was added to a solution of threonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide trifluoroacetate (1.84 g) in DMF (50 ml) at −10°. After stirring for 5 min, TEA (0.37 ml) was added. The mixture was stirred 3 hr at 0° followed by 72 hr at room temperature when the bulk of the solvents was removed. The residue was dissolved in ethyl acetate and the solution was washed and dried. Evaporation of the solvent gave a solid which was recrystallized from ethyl acetate–ether: 1.58 g (69%); $[\alpha]^{26}D$ −18.1° (*c* 1.06, MeOH); R_f^{I} 0.8; R_f^{III} 0.8; iodine, hydrazide, and thio ester positive, thiol and ninhydrin negative spot; amino acid ratios in acid hydrolysate, $Tyr_{1.1}Thr_{0.8}Cys_{1.1}Gly_{1.0}$ (92%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Tyr_{0.9}Thr_{1.0}Cys(SO_3H)_{1.1}Gly_{1.1}$. *Anal.* Calcd for $C_{34}H_{47}N_7O_{11}S$: C, 53.6; H, 6.2; N, 12.9; S, 4.2. Found: C, 53.4; H, 6.3; N, 12.5; S, 4.2.

(Positions 4–7) Tyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide Acetate. *N-tert*-Butoxycarbonyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (1.22 g) was deblocked in 90% TFA (10 ml). The solvent was evaporated, ether (200 ml) was added, and the resulting precipitate collected, washed with ether and dried: 1.20 g (97%), white amorphous solid; R_f^{I} 0.6 with trace impurity at 0.4; R_f^{III} 0.7. This material was dissolved in 5% aqueous acetic acid and the solution was applied to a column (1.9 × 20 cm) of Amberlite IRA 400 ion-exchange resin (20–50 mesh, acetate form) which was eluted with 5% aqueous acetic acid. The eluates were combined, concentrated, and lyophilized to give the acetate salt as a white fluffy powder: 1.05 g (91%); R_f^{I} 0.6 with trace impurity at 0.4; R_f^{III} 0.7; $[\alpha]^{26}D$ −15.0° (*c* 1.08, MeOH); amino acid ratios in acid hydrolysate, $Tyr_{1.0}Thr_{0.9}Cys_{1.1}Gly_{1.0}$ (75%); amino acid ratios in AP-M digest, $Tyr_{1.1}Thr_{1.1}Cys_{0.8}Gly_{1.0}$ (75%).

(Positions 3–7) *N-tert*-Butoxycarbonyl-β-*tert*-butylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of 2,4,5-trichlorophenyl *N-tert*-butoxycarbonyl-β-*tert*-butylaspartate[23] (342 mg) in DMF (5 ml) was added to a solution of tyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine acetate (500 mg) in DMF (20 ml) at −10°. After stirring for 5 min, TEA (0.85 ml) was added and the mixture was stirred for 3 hr at 0° followed by 40 hr at room temperature, when the bulk of the solvents was removed. The residue was dissolved in ethyl acetate and the solution was washed and dried. Evaporation of the solvent gave a solid which was precipitated from MeOH with ether: 524 mg (81%); $[\alpha]^{26}D$ −21.3° (*c* 2.74, MeOH); R_f^{I} 0.8; R_f^{III} 0.9; amino acid ratios in acid hydrolysate, $Asp_{1.0}Tyr_{1.0}Thr_{0.8}Cys_{1.1}Gly_{1.0}$ (100%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Asp_{1.1}Tyr_{0.8}Thr_{1.0}Cys(SO_3H)_{1.1}Gly_{1.0}$. *Anal.* Calcd for $C_{42}H_{60}N_8O_{14}S$: C, 54.1; H, 6.5; N, 12.0; S, 3.4. Found: C, 53.5; H, 6.6; N, 12.1; S, 3.3.

(Positions 3–7) Aspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. *N-tert*-Butoxycarbonyl-β-*tert*-butylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (410 mg) was deblocked for 40 min in aqueous 90% TFA (8 ml). The solvent was evaporated, ether (60 ml) was added, and the resulting precipitate collected, washed with ether, and dried: 390 mg of white amorphous solid; R_f^{I} 0.4 with impurity at 0.2 and trace impurities at 0.5, 0.6, and 0.7; R_f^{III} 0.7 with impurities at 0.4, 0.6, and 0.8.

A sample of the crude deblocked pentapeptide (196 mg) was dissolved in 0.1 *M*, pH 3.1 pyridinium acetate buffer (7 ml) and the solution applied to a column (1.9 × 15 cm) of Beckman ion-exchange resin type 15A. The column was eluted at approximately 15 ml/hr with a nonlinear gradient obtained by mixing 0.1 *M* pyridinium acetate, pH 3.1 (two 125-ml portions), with 2.0 *M* pyridinium acetate, pH 5.0 (125 ml), in a Buchler gradient mixer and fractions (30 min) were collected. Samples (10 μl) were withdrawn from each fraction and evaluated by tlc using the chlorine test for visualization. Fractions containing the desired homogeneous material were pooled, concentrated to a small volume, and lyophilized to constant weight: 144 mg; $[\alpha]^{25}D$ −25.1° (*c* 1.06, DMSO); $[\alpha]^{25}D$ −27.6° (*c* 0.99, DMF); R_f^{I} 0.4; R_f^{III} 0.7; ninhydrin, chlorine, hydrazide, and thio ester positive, thiol negative spot; amino acid ratios in acid hydrolysate, $Asp_{1.1}Tyr_{0.9}Thr_{0.9}Cys_{1.1}Gly_{1.1}$ (83%); amino acid ratios in performic acid oxidized sample, $Asp_{1.0}Tyr_{0.9}Thr_{1.0}Cys(SO_3H)_{1.0}Gly_{1.0}$; amino acid ratios in AP-M digest, $Asp_{1.1}Tyr_{1.0}Thr_{1.0}Cys_{0.7}Gly_{1.0}$ (68%).

(Positions 2–7) *N-tert*-Butoxycarbonyl-*S*-ethylcarbamoylcysteinylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of 2,4,5-trichlorophenyl *N-tert*-butoxycarbonyl-*S*-ethylcarbamoylcysteinate (190 mg) in DMF (5 ml) was added to a solution of aspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (285 mg) in DMF (10 ml) at 0°. The mixture was kept at room temperature for 18 hr and then at 40° for 24 hr, when the bulk of the solvents was removed. The residue was dissolved in a 1:1:1 mixture of 1-butanol–MeOH–water (100 ml) and the solution was applied to a column (1.9 × 10 cm) of AG 1-X2 ion-exchange resin which was eluted with 50 ml of the same solvent followed by 1-butanol–MeOH–0.03 *M* acetic acid (150 ml), 1-butanol–MeOH–0.06 *M* acetic acid (250 ml), and 1-butanol–MeOH–0.09 *M* acetic acid (300 ml). Fractions of 10 ml each were collected. The desired material was located in the 0.09 *M* eluates by absorbancy measurements at 280 nm and tlc. Fractions containing homogeneous material were pooled and concentrated to a small volume, and the residue was lyophilized to constant weight: 289 mg (74%); white fluffy powder; $[\alpha]^{28}D$ −27.1° (*c* 1.08, MeOH); R_f^{I} 0.7; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Asp_{1.1}Tyr_{1.0}Thr_{0.0}Cys_{2.0}Gly_{1.1}$ (83%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Asp_{1.1}Tyr_{0.8}Thr_{1.0}Cys(SO_3H)_{2.0}Gly_{1.1}$. *Anal.* Calcd for

$C_{44}H_{62}N_{10}O_{16}S_2$: C, 50.3; H, 5.9; N, 13.3; S, 6.1. Found: C, 50.2; H, 5.9; N, 13.1; S, 6.3.

(Positions 2–7) *S*-Ethylcarbamoylcysteinylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. The protected hexapeptide hydrazide (347 mg) was deblocked in aqueous 90% TFA (10 ml). The solvent was removed, ether was added, and the precipitate was collected, washed with ether, and dried. The material was dissolved in aqueous 5% acetic acid (200 ml) and applied to a column (1.9 × 15 cm) of Amberlite IRA 400 ion-exchange resin. The column was eluted with 5% acetic acid, the eluates were combined, and the solution was concentrated and then lyophilized to constant weight: 318 mg (101%), white fluffy powder; $[\alpha]^{27}D$ −14.3° (*c* 1.16, MeOH); R_f^{I} 0.5; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Asp_{1.0}Tyr_{1.0}Thr_{0.9}Cys_{2.0}Gly_{1.1}$ (93%); amino acid ratios in AP-M digest, $Asp_{0.9}Tyr_{0.8}Thr_{0.9}Gly_{0.9}$ (84%).

(Positions 1–7) Formylalanyl-*S*-ethylcarbamoylcysteinylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine Benzyloxycarbonylhydrazide. A solution of *N*-hydroxysuccinimido formylalaninate (65 mg) in DMF (2 ml) was added to a solution of *S*-ethylcarbamoylcysteinylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycine benzyloxycarbonylhydrazide (256 mg) in DMF (10 ml) at −10°. The mixture was kept at 0° for 4 hr and then at room temperature for 40 hr. The bulk of the solvents was removed and the residue dissolved in a 1:1:1 mixture of 1-butanol–MeOH–water (100 ml) and the solution was applied to a column (1.9 × 10 cm) of AG 1-X2 ion-exchange resin. The column was eluted with 50 ml of the same solvent followed by 1-butanol–MeOH–0.06 *M* acetic acid (150 ml), 1-butanol–MeOH–0.09 *M* acetic acid (150 ml), and 1-butanol–MeOH–0.12 *M* acetic acid (600 ml). Fractions of 10 ml each were collected. The desired material was located in the 0.12 *M* eluates by absorbancy measurements at 280 nm and tlc. Fractions containing homogeneous material were pooled, concentrated to a small volume, and lyophilized to constant weight: 187 mg (66%); white fluffy solid; $[\alpha]^{29}D$ −42.9° (*c* 1.08, DMF); R_f^{I} 0.6; R_f^{III} 0.6; amino acid ratios in acid hydrolysate, $Ala_{1.1}Asp_{1.1}Tyr_{0.9}Thr_{0.9}Cys_{2.1}Gly_{1.1}$ (97%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Ala_{1.1}Asp_{1.2}Tyr_{0.9}Thr_{1.0}Cys(SO_3H)_{1.8}Gly_{1.1}$. *Anal.* Calcd for $C_{45}H_{59}N_{11}O_{16}S_2$: C, 49.2; H, 5.7; N, 14.7; S, 6.1. Found: C, 49.1; H, 6.0; N, 14.0; S, 6.1.

(Positions 1–11)-Formylalanyl-*S*-ethylcarbamoylcysteinylaspartyltyrosylthreonyl-*S*-ethylcarbamoylcysteinylglycylserylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine Benzyloxycarbonylhydrazide (Fragment A). The protected heptapeptide hydrazide (230 mg) was dissolved in TFA, freshly saturated with hydrogen bromide (15 ml) at 0°. Hydrogen bromide was passed through the solution which was kept at 0° for 35 min and then at room temperature for 35 min. The solvent was removed, ether (100 ml) was added, and the precipitate was collected, washed with ether, and dried; 210 mg (96%) of a colorless, nonhygroscopic powder was obtained.

A solution of the hydrobromide in DMF (1 ml) was cooled at −10° and 6.91 *N* hydrogen chloride in dioxane diluted 1:10 with DMF (1.53 ml) was added followed by 10% *tert*-butyl nitrite in DMF (0.28 ml). The solution was stirred at −10° for 15 min and was then cooled at −25 to −30°. A 10% solution of TEA in DMF (1.76 ml) was then added and the mixture was stirred at −25 to −30° for 5 min. A solution of serylasparaginyl-*S*-ethylcarbamoylcysteinyltyrosine benzyloxycarbonylhydrazide acetate (161 mg) in DMF (1 ml) containing 10% TEA in DMF (0.29 ml) was then added and the mixture was stirred at 4° for 84 hr. TEA (10% in DMF) was added dropwise from time to time to adjust the pH of the mixture to 7.5–8.0. The reaction mixture was diluted with 100 ml of 1-butanol–MeOH–water and the solution was applied to a column (1.9 × 7 cm) of AG 1-X2 ion-exchange resin. The column was eluted with 50 ml of the same solvent followed by 1-butanol–MeOH–0.03 *M* acetic acid (200 ml) and 1-butanol–MeOH–0.09 *M* acetic acid (900 ml). Fractions of 20 ml each were collected and the desired material was located in the 0.09 *M* eluates by absorbancy measurements at 280 nm and tlc, and these fractions were pooled.[28] The solvent was removed, ethyl acetate (30 ml) was added, and after trituration the product was collected by centrifugation, washed with ethyl acetate, and dried: 224 mg (64%); $[\alpha]^{29}D$ −43.9° (*c* 1.08, DMF); R_f^{I} 0.5 with minor impurity at 0.4; R_f^{III} 0.7; amino acid ratios in acid hydrolysate, $Ala_{1.2}Asp_{2.0}Tyr_{1.8}Thr_{1.0}Cys_{3.0}Gly_{1.1}Ser_{1.0}$ (98%); amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Ala_{1.1}Asp_{2.1}Tyr_{1.7}Thr_{1.2}Cys(SO_3H)_{2.9}Gly_{1.1}Ser_{1.0}$. *Anal.* Calcd for $C_{65}H_{89}N_{17}O_{24}S_3$: C, 49.1; H, 5.7; N, 15.0; S, 6.1. Found: C, 48.9; H, 5.8; N, 15.0; S, 6.4

Fragment A Hydrazide Hydrobromide. Fragment A (135 mg) was dissolved at 0° in TFA freshly saturated with hydrogen bromide (15 ml). Hydrogen bromide was passed through this solution which was kept at 0° for 30 min and then at room temperature for 30 min. The solvent was removed, ether (50 ml) was added, and the resultant precipitate was collected, washed with ether, and dried: 125 mg (96%) of a colorless, nonhygroscopic powder; amino acid ratios in acid hydrolysate, $Ala_{1.2}Asp_{2.0}Tyr_{2.0}Thr_{1.0}Cys_{2.7}Gly_{1.2}Ser_{0.9}$ (85%). See Table I for PCMB titration.

(Positions 1–23) Preparation of Fragment AB. Fragment A hydrazide hydrobromide (120 mg) was dissolved in freshly distilled DMF (1 ml) and the clear solution was cooled at −10°. To this solution was added 6.91 *N* hydrogen chloride in dioxane diluted 1:10 with DMF (0.56 ml) followed by *tert*-butyl nitrite (1:10 in DMF) (0.10 ml) and the solution was stirred at −10° for 15 min. The solution was cooled at −25° and TEA (1:10 in DMF) (0.76 ml) was added. To this azide solution was added a solution of fragment B *tert*-butoxycarbonyl hydrazide[5] (69 mg) in DMSO (1 ml) and TEA (1:10 in DMF) (0.074 ml). The mixture was stirred at 4° for 72 hr and was then added to 50 ml of ethyl acetate. The precipitate was collected by centrifugation and washed with three 10-ml portions of ethyl acetate and dried. The dry residue was then triturated with four 10-ml portions of water and redried: 94 mg (61%); R_f^{III} 0.7; single chlorine, tyrosine, thio ester positive, ninhydrin, and thiol negative tailing spot; practically insoluble in DMF, 1-butanol–MeOH–water (1:1:1), 50% aqueous formic acid, and 50% acetic acid; soluble in DMSO; amino acid ratios in acid hydrolysate, $Asp_{3.2}Thr_{1.9}Ser_{4.3}Glu_{1.1}Gly_{2.2}Ala_{4.1}Cys_{2.8}Val_{0.94}Tyr_{1.96}$ (98%); ratios of diagnostic amino acids Val/Tyr 0.48; amino acid ratios in acid hydrolysate of performic acid oxidized sample, $Asp_{3.1}Thr_{2.1}Ser_{4.3}Glu_{1.2}Gly_{2.1}Ala_{3.9}Cys(SO_3H)_{3.5}Val_{0.98}Tyr_{1.84}$; ratio Val/Tyr 0.53.

(Positions 1–47) Preparation of Fragment ABCD. Fragment AB (180 mg) was deblocked in 90% TFA (4 ml) for 40 min, the solvent was evaporated, and the residue was precipitated with ether, washed with ether, and dried; yield 160 mg. This fragment AB hydrazide trifluoroacetate (120 mg) was dissolved at room temperature in DMSO (1 ml) and DMF (1 ml) was added to the clear solution. The solution was cooled at −10°, 6.91 *N* hydrogen chloride in dioxane diluted 1:10 with DMF (0.33 ml) was added with stirring and this was followed by *tert*-butyl nitrite (1:10 in DMF) (0.058 ml). The mixture was stirred at −10° for 15 min and then cooled at −25° and TEA (1:10 in DMF) (0.507 ml) was added. This was followed by a solution of fragment CD *tert*-butoxycarbonylhydrazide[5] (132 mg) in DMF (1 ml) and DMSO (1 ml) and TEA (1:10 in DMF) (0.122 ml). The mixture was stirred at 4° for 48 hr, the resulting stiff gel was suspended in 100 ml of DMF–0.01 *M* acetic acid 2:3, and the mixture was stirred for 5 hr at 4° and was then centrifuged. The residue (polymers + fragment AB) was washed by suspension and centrifugation with water and was dried: yield 44 mg; amino acid ratios in acid hydrolysate, $Lys_{0.8}His_{1.4}Asp_{6.9}Thr_{3.5}Ser_{7.5}Glu_{4.4}Pro_{1.0}Gly_{4.9}Ala_{5.5}Cys_{3.2}Val_{2.1}Leu_{0.9}Tyr_{5.1}$; amino acid ratios in performic acid oxidized sample, $Lys_{0.9}His_{1.7}Asp_{7.4}Thr_{3.5}Ser_{7.1}Glu_{4.5}Pro_{0.9}Gly_{4.9}Ala_{5.1}Cys(SO_3H)_{4.1}Val_{2.1}Leu_{0.9}Tyr_{4.1}$. The clear supernatant from the above centrifugation was added to an AG 1-X2 column (1.9 × 10 cm) equilibrated with DMF–0.01 *M* acetic acid (2:3). The column was then eluted with the following solvent mixtures: DMF–0.01 *M* acetic acid (2:3) (150 ml); DMF–0.03 *M* acetic acid (2:3) (150 ml); DMF–0.10 *M* acetic acid (2:3) (100 ml); DMF–0.20 *M* acetic acid (2:3) (10 ml); and DMF–0.50 *M* acetic acid (2:3) (300 ml). Fractions of 10 ml each were collected and peptides were located in the various fractions by absorbancy measurements at 280 nm. Major peptide peaks were located in tubes 19–30 (peak I), 35–42 (peak II), and in the 0.5 *M* acetic acid containing eluent tubes, 56–69 (peak III). The contents of tubes corresponding to peak I (regenerated fragment CD *tert*-butoxycarbonyl hydrazide) were pooled, the solvent was evaporated, and the residue lyophilized from 5% acetic acid: yield 73 mg (55%); R_f^{I} 0.1; R_f^{III} 0.6; amino acid ratios in AP-M digest, $Tyr_{4.3}(Gln + Asn + Ser)_{5.7}Leu_{1.2}His_{2.1}Glu_{2.1}Asp_{1.0}Gly_{2.8}Thr_{0.8}Val_{0.9}Pro_{1.0}Formlys_{1.1}$ (65%). The material corresponding to peak II was isolated as described above and the residue was washed with ethanol and dried: yield 12 mg; tlc in solvent system I showed the presence of several spots; amino acid ratios in acid hydrolysate, $Lys_{1.2}His_{2.0}Asp_{4.1}Thr_{1.2}Ser_{2.7}Glu_{3.9}Pro_{1.0}Gly_{3.0}Val_{1.0}Leu_{0.9}Tyr_{3.1}$. The material corresponding to peak III (fragment ABCD) was isolated as described above and the residue was lyophilized from 50% acetic acid: yield 37 mg (17%); R_f^{I} remains at origin; R_f^{III} 0.5;

(28) Since fragment A has a tendency to precipitate on standing in the eluent tubes, the tubes were rinsed with DMF.

R_f^V 0.5; both contain material remaining at origin; amino acid ratios in acid hydrolysate, $Lys_{1.1}His_{2.0}Asp_{7.0}Thr_{3.1}Ser_{7.3}{}^{29}Glu_{5.1}Pro_{1.0}Gly_{5.0}Ala_{4.3}Cys_{2.5}Val_{1.9}Leu_{1.1}Tyr_{5.7}{}^{29}$ (80%); amino acid ratios in performic acid oxidized sample, $Lys_{1.0}His_{1.7}Asp_{7.0}Thr_{3.2}Ser_{6.8}{}^{29}Glu_{5.0}Pro_{1.0}Gly_{5.0}Ala_{4.2}Cys(SO_3H)_{3.0}Val_{2.0}Leu_{1.0}Tyr_{5.6}$;[29]

(29) Corrected for destruction during acid hydrolysis.

ratios of diagnostic amino acids, fragment A/fragment C (Cys-(SO_3H)/Leu) = 3.0; fragment A/fragment D (Cys(SO_3H)/Lys) = 3.0; fragment B contains no diagnostic residues.

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Structure of the Borohydride Reduction Product of Photolinked 4-Thiouracil and Cytosine. Fluorescent Probe of Transfer Ribonucleic Acid Tertiary Structure

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Abstract: 5-(4-Pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (**2**), a photoproduct which can be isolated from the irradiation (335 nm) of certain *E. coli* transfer RNAs and irradiation (254 nm) of polycytidylic acid, deoxycytidine, and cytidine, is reduced by sodium borohydride to a fluorescent compound, 5-(4-pyrimidin-2-one)-3,6-dihydrocytosine, Pyo(4–5)hCyt (**3**). Catalytic oxygenation (Pt, O_2) converted Pyo(4–5)hCyt back to Pyo(4–5)Cyt. Treatment of Pyo(4–5)hCyt with aqueous acid gave 5-(4-pyrimidin-2-one)-3,6-dihydrouracil, Pyo(4–5)hUra (**8**), which could also be obtained by the treatment of 5-(4-pyrimidin-2-one)uracil, Pyo(4–5)Ura (**7**), with sodium borohydride. 5-(4-Pyrimidin-2-one)-4-thiouracil, Pyo(4–5)Sur (**9**), a photoproduct from irradiation (335 nm) of 4-thiouracil in aqueous solution, is reduced by sodium borohydride to 5-(4-pyrimidin-2-one)-4-thio-3,6-dihydrouracil, Pyo(4–5)hSur (**10**). The fluorescent nature of Pyo(4–5)hCyt provides a useful monitor of the photoreaction of tRNAs containing proximate 4-thiouridine and cytidine moieties.

A specific intramolecular photoreaction has been shown to occur between 4-thiouridine and a cytidine in *E. coli* $tRNA_1^{Val}$ on irradiation at 335 nm.[1] Only those *E. coli* tRNAs known to possess a 4-thiouridine moiety in nucleoside position 8 and a cytidine in position 13 from the 5′-terminal end yield a photoproduct under these conditions. Evidence for the covalent cross-linking between the two nucleosides after photolysis of the intact *E. coli* $tRNA_1^{Val}$ at 335 nm was provided by enzymic fragmentation sequence studies.[1] Subsequently the photochemically cross-linked binucleotide unit was isolated by the complete enzymic digestion of the irradiated tRNA.[1b]. The structure of the corresponding binucleoside photoproduct has recently been determined as **1**.[2a] Compound **1**, 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine, was obtained in fair yield by irradiating 4-thiouridine and cytidine in aqueous solution at 4° at 335 nm. The corresponding bipyrimidine product, 5-(4-pyrimidin-2-one)cytosine, Pyo(4–5)Cyt (**2**),[3] resulted on photolysis (335 nm) of 4-thiouracil in the presence of cytosine.[2]

The nature of the photoreaction and the structure of the photoproduct may lead to the acquisition of important structural and functional information about tRNA. It has been shown[4] that the photolytically cross-linked *E. coli* $tRNA_1^{Val}$ can be charged with valine in the presence of its corresponding aminoacyl synthetase, although the affinity for the synthetase is decreased. The Val-$tRNA_1^{Val}$ functions normally in a reconstructed *in vitro* protein-synthesizing system. Qualitatively similar results have been obtained in experiments with *E. coli* $tRNA^{Arg}$ and $tRNA_1^{Phe}$.[5]

The susceptibility of 4-thiouridine in tRNA to borohydride reduction[6] apparently led to an attempted borohydride reduction of the photolytically cross-linked tRNA. When the photoproduct was treated with sodium borohydride it was converted to a new, highly fluorescent compound with emission maximum 440 nm and excitation maximum 386 nm.[7] The degree and

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(3) Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations (*J. Mol. Biol.*, **55**, 299 (1971)) are used throughout. The photochemistry symbolism employed in ref 2 has been modified and improved after discussions with Dr. Waldo E. Cohn, Director of the Office of Biochemical Nomenclature. For example, the earlier abbreviation, Cyt-Sur, for the photoproduct **2**, which indicated the *source* of the two fragments, has been replaced by Pyo(4–5)Cyt, which represents the *actual* structure, now that it is known.[2] Pyo stands for pyrimidin-2-one and 4–5 indicates that it is attached by covalent linkage from the 4 position to the 5 position of cytosine. Based on the bipyrimidine system of nomenclature, which is less indicative of the biochemical connotation and interest, 5-(4-pyrimidin-2-one)cytosine is 4-amino-4′,5-bipyrimidine-2,2′(1*H*,1′*H*)-dione. Other abbreviations follow the new photochemistry symbolism, *e.g.*, Pdo(4–5)Cyd (in place of Cyd-Srd[2a]) for 5-(1-β-D-ribofuranosyl-4-pyrimidin-2-one)cytidine (**1**); Pyo(4–5)Ura for 5-(4-pyrimidin-2-one)-uracil (**7**); Pyo(4–5)Sur for 5-(4-pyrimidin-2-one)-4-thiouracil (**9**). The corresponding dihydro products obtained by treatment of **2**, **7**, and **9** with sodium borohydride are designated, respectively, as Pyo(4–5)-hCyt (**3**) (rather than Cyt-Sur_{red}), Pyo(4–5)hUra (**8**), and Pyo(4–5)hSur (**10**).

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