that of a methyl group, is the assumption that differences in substitution of a rotationally free methylene group, *i.e.*, $-CH_2H$ or $-CH_2R$, are not important in determining the magnitude of an nmr solvent shift. The excellent correlations obtained for this series of closely related quinones **2–7** suggest that the assumption is valid; however, the assumption is not necessarily valid for other systems.[20]

## Experimental Section

**Nmr Measurements.** All measurements were carried out using a Varian Associates HA-100 nmr spectrometer with 2–5% w/v solutions at ambient temperatures unless otherwise indicated. Chemical shifts are expressed in $\tau$ units relative to tetramethylsilane as an internal standard.

**Multiprenylquinones.** The multiprenylquinones used for this study have been described.[10]

**Acknowledgment.** We wish to express appreciation to Dr. R. F. Muraca and Mr. William R. Anderson, Jr., for the nmr spectra and for their helpful interest. We also thank Professor Harold W. Moore,[15] University of California, Irvine, Professor Dudley H. Williams, Cambridge University and their coworkers, for their manuscript prior to publication.

This research was partially supported by funds from the Merck Sharp and Dohme Research Laboratories and we express our appreciation to Dr. Max Tishler.

(20) Note (ref 15) the significant difference in the benzene solvent shift for the methyl group of epoxypropene (+0.30) as compared with that for the exocyclic methylene of 1,2-epoxybutene (+0.22).

# Synthesis of a Cyclic Disulfide-Linked Octapeptide Corresponding to Residues 65 to 72 of Bovine Pancreatic Ribonuclease A[1a]

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**Abstract:** This report describes the synthesis of the cyclic disulfide-linked octapeptide, cysteinyllysylasparaginylglycylglutaminylthreonylasparaginylcysteine, which corresponds to residues 65 to 72 in bovine pancreatic ribonuclease A. The protected tetrapeptides, tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycine and glutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester, were prepared by stepwise condensation of protected amino acids. These tetrapeptides were then coupled to give tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester. The *t*-BOC group was removed by treatment with trifluoroacetic acid, and the remaining protecting groups were removed by sodium in liquid ammonia to give the linear octapeptide. The disulfide bridge was then allowed to form in dilute solution at pH 6.5.

The optical rotatory dispersion of cyclic peptides composed of ten amino acid residues has been found to be similar to that of peptides with the $\alpha$-helix structure.[2,3] Since many proteins of known structure contain cyclic peptide moieties of approximately this size in the form of disulfide "loops," it is possible that such loops contribute to the over-all rotatory dispersion of the protein in such a way that the result is an overestimate of the per cent $\alpha$ helix in the molecule. For the purpose of estimating such a contribution in a protein of known structure, it seemed worthwhile to synthesize the loop peptide of bovine pancreatic ribonuclease A corresponding to residues 65 to 72.[4,5] This paper describes that synthesis.

The over-all scheme for the synthesis is outlined in Figures 1, 2, and 3. Carbobenzoxyasparaginylglycine ethyl ester (I)[6,7] was obtained in 78% yield from carbobenzoxyasparagine and glycine ethyl ester using the coupling reagent NEPIS[8a] according to the procedure of Woodward, *et al.*[7] No dehydration of the asparagine residue to $\beta$-cyanoalanine[8b,c] was detected by infrared spectroscopy. The carbobenzoxy group was removed by hydrogenolysis in the presence of palladium catalyst. The resultant dipeptide (II) was not isolated because it was found to be extremely hygroscopic but was treated directly with $\alpha$-carbobenzoxy-ε-*t*-BOC-lysine N-hydroxysuccinimide ester. Schwyzer and Rittel[9] have prepared $\alpha$-carbobenzoxy-ε-*t*-BOC-lysine in 25% over-all yield by acylation of the ε-amino group of lysine with *t*-BOC azide while protecting the $\alpha$-amino group by means of a complex with copper. The copper was then removed and a carbobenzoxy group added to the $\alpha$-amino group. In the present work, 48% over-all yield was obtained in a less tedious procedure by reversing the sequence of acylations. Thus, $\alpha$-carbobenzoxylysine was prepared by the method of Bezas and Zervas[10] and was allowed

(1) (a) This work was supported by U. S. Public Health Service Research Grant 1-F2-GM-29,584-01. A portion of this material was presented by this author at the Biochemical Section of the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966, Abstract C215. (b) Postdoctoral Research Fellow. Address correspondence to Mount Sinai School of Medicine, New York, N. Y. 10029.

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(8) (a) Abbreviations used: NEPIS, N-ethyl-5-phenylisoxazolium-3′-sulfonate; *t*-BOC, *t*-butyloxycarbonyl; DCCI, dicyclohexylcarbodiimide; (b) D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *J. Am. Chem., Soc.*, **78**, 5954 (1956); (c) P. G. Katsoyannis, D. T. Gish, G. P. Hess, and V. du Vigneaud, *ibid.*, **80**, 2558 (1958).

(9) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).

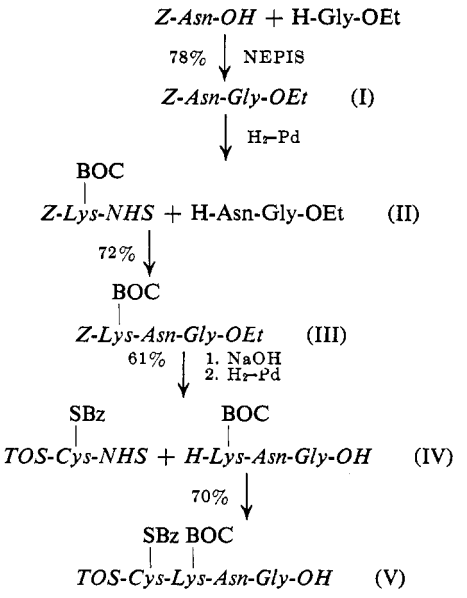


Figure 1. Synthetic scheme for the tetrapeptide V: Z, carbobenzoxy; Et, ethyl; NHS, N-hydroxysuccinimide; TOS, *p*-toluenesulfonyl; Bz, benzyl.

to react with *t*-BOC azide to give α-carbobenzoxy-ε-*t*-BOC-lysine, obtained as an oil. This material was converted to the N-hydroxysuccinimide ester with DCCI and N-hydroxysuccinimide by the method of Anderson, *et al.*,[11] to give the crystalline active ester. The coupling of II to this lysine active ester gave α-carbobenzoxy-ε-*t*-BOC-lysylasparaginylglycine ethyl ester (III). At this point, the ethyl ester was saponified with 1.2 equiv of NaOH, and the resultant free acid of III was subjected to hydrogenolysis in the presence of palladium catalyst to give crystalline ε-*t*-BOC-lysylasparaginylglycine (IV) in 61% yield. A sample of this tripeptide was digested with leucine aminopeptidase and the 24-hr digest was applied to the Spinco amino acid analyzer. A 98% recovery of asparagine and glycine was obtained (ε-*t*-BOC-lysine was not determined). This indicated that neither racemization nor α-to-β peptide bond shift at asparagine had occurred during the saponification of the ethyl ester.

The compound tosyl-S-benzylcysteine was prepared by the method of du Vigneaud, *et al.*[12] This was coupled to N-hydroxysuccinimide with DCCI to give crystalline tosyl-S-benzylcysteine N-hydroxysuccinimide ester. This compound was combined with the triethylamine salt of the tripeptide IV to give in 70% yield the crystalline tetrapeptide tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycine (V).

The synthesis of the tetrapeptide XI was begun by condensing carbobenzoxyasparagine with S-benzylcysteine benzyl ester using NEPIS to give carbobenzoxyasparaginyl-S-benzylcysteine benzyl ester (VI) in 82% yield. As with carbobenzoxyasparaginylglycine ethyl ester, no dehydration to β-cyanoalanine was detected by infrared spectroscopy. The carbobenzoxy group was removed by brief treatment with HBr in glacial acetic acid[13] and the peptide hydrobromide was distributed

(10) B. Bezas and L. Zervas, *J. Am. Chem. Soc.*, **83**, 719 (1961).
(11) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *ibid.*, **86**, 1839 (1964).
(12) V. du Vigneaud, M. F. Bartlett, and A. Jöhl, *ibid.*, **79**, 5572 (1957).
(13) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

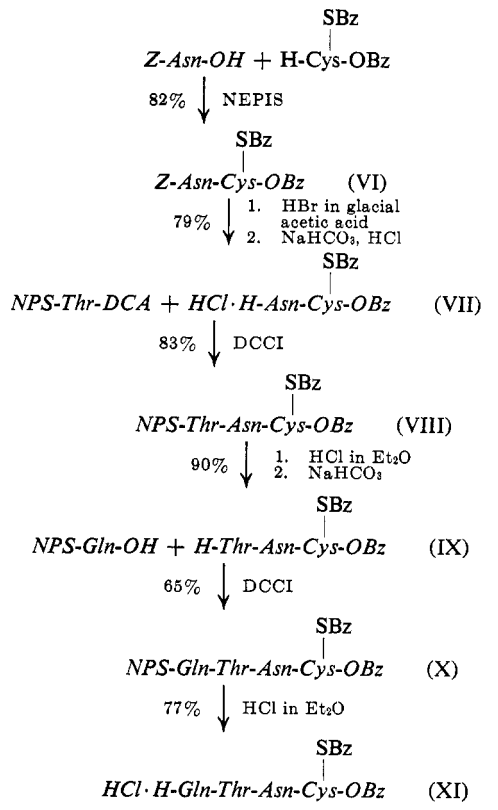


Figure 2. Synthetic scheme for the tetrapeptide XI: NPS, *o*-nitrophenylsulfenyl; DCA, dicyclohexylammonium.

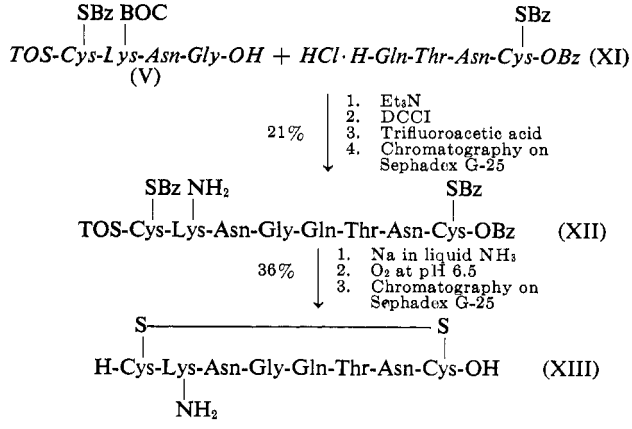


Figure 3. Synthetic scheme for the cyclic octapeptide XIII.

between water and ethyl acetate and washed with $NaHCO_3$ to remove any free acid of the dipeptide. The peptide free base was then converted to the crystalline hydrochloride of asparaginyl-S-benzylcysteine benzyl ester in an over-all yield of 79%. This dipeptide hydrochloride was then neutralized with 1 equiv of the dicyclohexylammonium salt of *o*-nitrophenylsulfenylthreonine (prepared by the method of Zervas, *et al.*[14]) and then coupled by means of DCCI. The crystalline tripeptide *o*-nitrophenylsulfenylthreonylasparaginyl-S-benzylcysteine benzyl ester (VIII) was obtained in 83% yield. The *o*-nitrophenylsulfenyl group was removed by treatment of VIII with HCl in ether (Zervas, *et al.*[14]), and the tripeptide hydrochloride was neutralized in water to give crystalline threonylasparaginyl-S-benzyl-

(14) L. Zervas, D. Borovas, and E. Gazis, *J. Am. Chem. Soc.*, **85**, 3660 (1963).

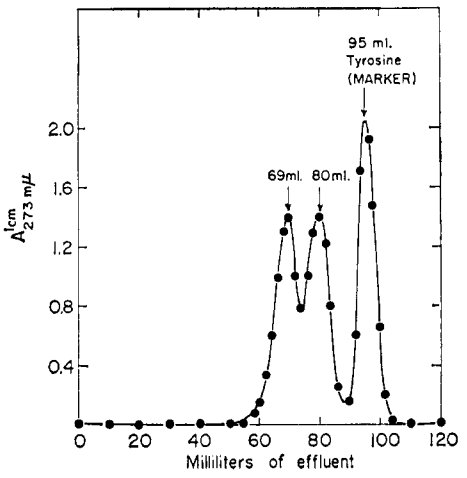


Figure 4. Chromatography on a 0.9 × 150 cm column of Sephadex G-25 in 50% acetic acid of the product from condensation of the tetrapeptide V and XI following the subsequent removal of the ε-*t*-BOC group from lysine. Fractions (2 ml) were collected at 25° at a flow rate of 8 ml/hr.

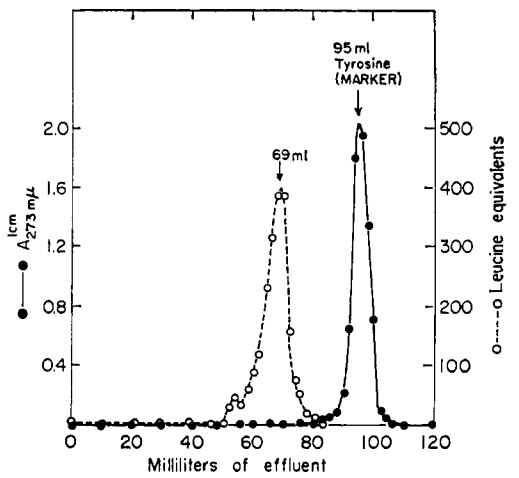


Figure 5. Chromatography on a 0.9 × 150 cm column of Sephadex G-25 in 50% acetic acid of the cyclic octapeptide XIII. Fractions (2 ml) were collected at 25° at a flow rate of 8 ml/hr.

cysteine benzyl ester (IX) in 90% yield. Peptide IX was then coupled to *o*-nitrophenylsulfenylglutamine with DCCI to give a 65% yield of *o*-nitrophenylsulfenylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester (X). The *o*-nitrophenylsulfenyl group was removed from X with HCl in ether to give in 77% yield the tetrapeptide hydrochloride glutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester. When this peptide was stored at room temperature it was found that a large portion of it underwent cyclization at the amino-terminal end to form pyrrolidone. This occurred despite the fact that the amino group was protected as the hydrochloride. In order to eliminate the cyclization, the peptide was stored and used at temperatures below 5°.

It should be mentioned that the use of the *o*-nitrophenylsulfenyl protecting group in the synthesis of this tetrapeptide had several advantages. The protected intermediates were nicely crystalline and had high melting points. In addition, the yellow color of the protected peptides made it easy to follow the partition of the peptide during various extraction procedures.

For the synthesis of the octapeptide XIII, the tetrapeptides V and XI were condensed by the use of DCCI in dimethylformamide after the addition of 1 equiv of triethylamine. This gave the fully protected linear octapeptide. The *t*-BOC group was removed with trifluoroacetic acid to give tosyl-S-benzylcysteinyllysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester (XII) which was contaminated with tetrapeptides and with dicyclohexylurea. The removal of the *t*-BOC group from the lysine with trifluoroacetic acid rendered the octapeptide soluble in 50% acetic acid. The material was chromatographed on a column of Sephadex G-25 in this solvent to give the pattern shown in Figure 4. The first peak was found to contain the desired octapeptide and the second peak contained tetrapeptide fragments. The octapeptide was isolated by lyophilization. It was found to be homogeneous in paper chromatography in three solvent systems and had the correct amino acid composition as shown in Table I. The yield based on the tetrapeptides was 21%. This octapeptide was dried at high vacuum over NaOH and $P_2O_5$ and then treated with sodium in liquid ammonia as described by du Vigneaud, *et al.*,[15] in order to remove the remaining protecting groups. The disulfide bridge was then allowed to form in dilute solution at pH 6.5 as described by du Vigneaud, *et al.*,[15] for oxytocin. This product was desalted on Sephadex (Figure 5), and its elution position was found to be the same as that for the linear octapeptide. This indicated that neither dimerization nor polymerization had taken place during the oxidation of the sulfhydryl groups. This cyclic disulfide octapeptide XIII was obtained in 36% yield based on the linear protected octapeptide. It was reduced with mercaptoethanol as described by Crestfield, *et al.*,[16] to the linear sulfhydryl peptide and again chromatographed on Sephadex. Its elution position

**Table I.** Amino Acid Composition of Octapeptides[a]

| Amino acid | Peptide XII[b] Theor | Peptide XII[b] Found | Peptide XIII[d] Theor | Peptide XIII[d] Found |
|---|---|---|---|---|
| S-Benzylcysteine | 2 | 1.26[c] | 0 | 0 |
| Lysine | 1 | 1.02 | 1 | 1.03 |
| Aspartic acid | 2 | 2.00 | 2 | 2.00 |
| Glycine | 1 | 0.93 | 1 | 0.94 |
| Glutamic acid | 1 | 0.98 | 1 | 0.97 |
| Threonine | 1 | 0.96 | 1 | 0.94 |
| Cysteic acid | 0 | 0 | 2 | 2.02 |
| Ammonia | 2 | 2.08 | 2 | 2.10 |

[a] Values given refer to relative number of residues. [b] The amount of peptide used for the analysis was 0.46 mg. The recovery of micromoles of amino acid accounted for 93% of the weight of the sample. [c] The low recovery of S-benzylcysteine was due to the incomplete cleavage of the N-tosyl group by 6 *N* HCl. A value of 2.00 was assumed for the purpose of calculating the over-all recovery. [d] The amount of peptide used for the analysis was 0.31 mg. The material was oxidized with performic acid according to the procedure of Schram, *et al.* [E. Schram, S. Moore, and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954)], before the hydrolysis in order to convert cystine to cysteic acid. The recovery of micromoles of amino acid accounted for 91% of the weight of the sample.

(15) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).

(16) A. M. Crestfield, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **238**, 622 (1963).

was found to be the same as the cyclic peptide. The material was then reoxidized and rechromatographed. It was isolated by lyophilization.

The cyclic octapeptide was found to be homogeneous in paper chromatography and paper electrophoresis. Digestion with leucine aminopeptidase showed that lysine and asparagine were liberated first, followed by the subsequent amino acids and lastly by the appearance of cystine (Table II). This indicated that the octapeptide had been cyclized through a disulfide bridge.

**Table II.** Results of Leucine Aminopeptidase Digestion of Peptide XIII[a,c]

| Amino acid | Digestion time 30 min | Digestion time 12 hr |
|---|---|---|
| Half-cystine | 0.11 | 1.89 |
| Lysine | 1.00 | 1.05 |
| Asparagine and glutamine[b] | 1.44 | 3.04 |
| Glycine | 0.68 | 1.02 |
| Threonine | 0.18 | 1.00 |

[a] Values given refer to relative number of residues. [b] These amino acids did not separate on the amino acid analyzer. [c] The amount of amino acid residues obtained accounted for 92% of the weight of the sample.

## Experimental Section

All amino acids employed in the synthesis were of the L configuration, except glycine. Melting points were performed on a Fisher-Johns melting point block and are uncorrected. Reported yields are those obtained after crystallization or purification. Analytical samples were dried *in vacuo* over phosphorus pentoxide at 60°. Paper chromatography was carried out on Whatman No. 1 filter paper in the following systems: (BAW) 1-butanol–acetic acid–water, 4:1:1; (SBA) 2-butanol–10% ammonia, 85:15; (BPAW) 1-butanol–pyridine–acetic acid–water, 30:20:6:24. The chromatography was run at room temperature, and the peptide spots were located by ninhydrin and by the chlorine method.[17] Samples of peptide for amino acid analysis were hydrolyzed for 20 hr in 6 *N* HCl in evacuated, sealed Pyrex tubes. The analyses were performed on a Spinco Model 120B amino acid analyzer as described by Spackman, *et al.*[18] Digestion of peptides with leucine aminopeptidase was carried out at 37° with an enzyme:substrate ratio of 1:20 in 0.01 *M* Tris buffer containing 10 μmol/ml of peptide. Portions of the digest were removed after various time intervals and applied directly to the Spinco amino acid analyzer.

**α-Carbobenzoxy-ε-*t*-BOC-lysine N-Hydroxysuccinimide Ester.** α-Carbobenzoxylysine was obtained in 60% yield from lysine monohydrochloride by the method of Bezas and Zervas.[10] Ten grams of this material (36 mmol) was ground in a mortar and pestle with MgO (2 g, 49 mmol). The mixture was then stirred for 1 hr at 50° in 150 ml of 50% aqueous dioxane. Six grams (42 mmol) of *t*-BOC azide[19–21] was added, and the mixture was stirred for 48 hr. The small amount of undissolved material was removed by filtration, and the filtrate was diluted with water and brought to pH 4 at 5° with 10% citric acid. The solution was extracted with ethyl acetate, and the organic phase was washed with water and dried over $Na_2SO_4$. The solvent was removed in a rotary evaporator to give α-carbobenzoxy-ε-*t*-BOC-lysine as an oil. The product, obtained in 79% yield, was ninhydrin negative and was homogeneous in paper chromatography: $R_f$(BAW) 0.81, $R_f$(SBA) 0.59, $R_f$(BPAW) 0.88.

The oil (10.7 g, 28 mmol) was dissolved in 50 ml of a mixture of ethyl acetate and dioxane (1:4) together with 3.2 g (28 mmol) of N-hydroxysuccinimide (prepared by the method of Anderson, *et al.*[11]). The solution was cooled in an ice bath, 5.73 g (18 mmol) of DCCI[22,23] was added, and the mixture was stirred overnight at 5°. The dicyclohexylurea which was removed at the end of the reaction by filtration weighed 6.1 g (95% of the theoretical). The filtrate was acidified with a few drops of glacial acetic acid (in order to consume any unreacted DCCI) and brought to dryness in a rotary evaporator. The residue was crystallized from isopropyl alcohol–petroleum ether (bp 30–60°) to give 11 g (82%) of soft needles of α-carbobenzoxy-ε-*t*-BOC-lysine N-hydroxysuccinimide ester, mp 96–98°, $[\alpha]^{27}D$ −15.9° (*c* 3.5, dioxane). The over-all yield of the ester based on α-carbobenzoxylysine was 66%.

*Anal.* Calcd for $C_{23}H_{31}O_8N_3$: C, 57.86; H, 6.54; N, 8.80. Found: C, 58.0; H, 6.59; N, 8.76.

**α-Carbobenzoxy-ε-*t*-BOC-lysylasparaginylglycine Ethyl Ester (III).** Carbobenzoxyasparaginylglycine ethyl ester was obtained in 78% yield by the method of Woodward, *et al.*[7] Nine grams (25.6 mmol) of the compound was dissolved in 250 ml of methanol containing 13 ml of 2 *N* HCl (26 mmol). One gram of palladium oxide catalyst was added and the suspension was stirred vigorously while hydrogen gas was bubbled through it at 60°. After 12 hr, the flask was flushed with nitrogen, and the catalyst was removed by gravity filtration. The solvent was distilled off, and the residue was repeatedly dried in a rotary evaporator after the addition of 50-ml portions of benzene in order to remove the last traces of water. The product (6 g, 98%) was an oil which was homogeneous by paper chromatography: $R_f$(BAW) 0.04, $R_f$(SBA) 0.48, $R_f$(BPAW) 0.61. The entire material was shaken at 0° for 1 hr with 3.6 ml (25.6 mmol) of triethylamine in 30 ml of ethyl acetate. The mixture was filtered and 11 g (23.1 mmol) of α-carbobenzoxy-ε-*t*-BOC-lysine N-hydroxysuccinimide ester was added. The reaction was allowed to proceed for 40 min at room temperature, after which the solution was placed in the refrigerator for 24 hr. The solution was then washed with dilute $NaHCO_3$, water, cold 0.02 *N* $H_2SO_4$, and water and dried over $Na_2SO_4$. The ethyl acetate was removed in a rotary evaporator, the residue taken up in a small volume of methanol, and the product precipitated by dropwise addition of the methanol solution into a large volume of rapidly stirred water. The material was crystallized from methanol–ether to give 10 g (72%) of α-carbobenzoxy-ε-*t*-BOC-lysylasparaginylglycine ethyl ester, mp 165–166°, $[\alpha]^{27}D$ −12.6° (*c* 2, acetic acid). The compound was found to be homogeneous by paper chromatography: $R_f$(BAW) 0.79, $R_f$(SBA) 0.83, $R_f$(BPAW) 0.90.

*Anal.* Calcd for $C_{27}H_{41}N_5O_9$: C, 55.94; H, 7.13; N, 12.08. Found: C, 55.7; H, 7.19; N, 12.01.

**ε-*t*-BOC-lysylasparaginylglycine (IV).** α-Carbobenzoxy-ε-*t*-BOC-lysylasparaginylglycine ethyl ester (5.8 g, 10 mmol) was dissolved in 80 ml of ethanol. To this was added 7.5 ml of 2 *N* NaOH with stirring at room temperature. After 15 min the solution was diluted with water and extracted with 1-butanol. It was then cooled to 0° and carefully acidified with 2 *N* $H_2SO_4$. The aqueous solution was again extracted with 1-butanol, and the organic phase was thoroughly washed with water and then brought to dryness in a rotary evaporator at 35° using a vacuum pump and Dry Ice trap. The residue was homogeneous in paper chromatography: $R_f$(BAW) 0.67, $R_f$(SBA) 0.00, $R_f$(BPAW) 0.72. The entire material was dissolved in a mixture of methanol and water and hydrogenated in the presence of 0.5 g of palladium oxide catalyst for 24 hr at room temperature. The catalyst was removed by filtration, and the product, obtained by evaporation of the solvent, was crystallized from methanol–ether to give 2.5 g of material, mp 198° dec, $[\alpha]^{27}D$ +7.5° (*c* 1.5, acetic acid). The yield of ε-*t*-BOC-lysylasparaginylglycine based on α-carbobenzoxy-ε-*t*-BOC-lysylasparaginylglycine ethyl ester was 61%.

*Anal.* Calcd for $C_{17}H_{31}N_5O_7$: C, 48.91; H, 7.49; N, 16.78. Found: C, 48.7; H, 7.56; N, 16.69.

**Tosyl-S-benzylcysteine N-Hydroxysuccinimide Ester.** S-Benzylcysteine was prepared by the method of Wood and du Vigneaud.[24] The N-tosyl derivative was prepared by the method of du Vigneaud, *et al.*[12] For the synthesis of the active ester, 10 g (27 mmol) of tosyl-S-benzylcysteine was dissolved in a mixture of dioxane and ethyl acetate (4:1) together with 3.2 g (27 mmol) of N-hydroxysuccinimide. The solution was stirred at 5°, and 5.65 g (27 mmol) of DCCI was added. After 24 hr at 5° the dicyclohexylurea was removed by filtration (yield, 5.90 g, 96%), and the filtrate was acidified with a few drops of glacial acetic acid and brought to dryness in a rotary evaporator. The residue was crystallized from isopropyl

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(18) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958),
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(20) L. A. Carpino, *ibid.*, **82**, 2725 (1960).
(21) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).
(22) H. G. Khorana, *J. Chem. Soc.*, 2081 (1952).
(23) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
(24) J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 110 (1939).

alcohol to give 10 g (81%) of long needles, mp 138–140°, $[\alpha]^{27}D$ −13.5° (*c* 2, dioxane).

*Anal.* Calcd for $C_{21}H_{22}N_2O_6S_2$: C, 54.53; H, 4.80; N, 6.06; S, 13.87. Found: C, 54.5; H, 4.80; N, 6.04; S, 13.81.

**Tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycine (V).** ε-*t*-BOC-lysylasparaginylglycine (1.66 g, 4 mmol) and exactly 1 equiv of triethylamine were dissolved in 20 ml of tetrahydrofuran and water (2:1). To this was added 1.85 g (4 mmol) of tosyl-S-benzylcysteine N-hydroxysuccinimide ester dissolved in 2 ml of tetrahydrofuran. After 1 hr at room temperature, the reaction mixture was placed in the refrigerator overnight. The mixture was diluted with cold 0.01 *N* $H_2SO_4$, and the precipitate was filtered, washed with water, and dried. The peptide was crystallized from methanol to give 2.1 g (70%) of tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycine, mp 168° dec, $[\alpha]^{27}D$ +20.6° (*c* 1, water–dimethylformamide 1:10). The tetrapeptide was found to be homogeneous in paper chromatography in three solvents: $R_f$(BAW) 0.88, $R_f$(SBA) 0.03, $R_f$(BPAW) 0.92.

*Anal.* Calcd for $C_{34}H_{48}O_{10}N_6S_2$: N, 10.95; S, 8.38. Found: N, 11.03; S, 8.30.

**S-Benzylcysteine Benzyl Ester Hydrochloride.** This ester was prepared from the N-carboxy anhydride of S-benzylcysteine according to the procedure of Sokolovsky, *et al.*,[25] for S-carbobenzoxycysteine benzyl ester hydrochloride. A suspension of 21.1 g (0.1 mmol) of S-benzylcysteine (Wood and du Vigneaud[24]) was made in 300 ml of anhydrous dioxane, and dry phosgene was bubbled through it at 40° for 1 hr. The slightly turbid solution was filtered and the solvent removed in a rotary evaporator at 45°. Benzyl alcohol (60 ml) and dry ether (300 ml) saturated with dry HCl were added, and the solution was allowed to stand overnight at room temperature. The ester hydrochloride which separated was filtered, washed with ether, and recrystallized from methanol–ether. The yield was 23 g (70%), mp 128–129°, $[\alpha]^{27}D$ −19.5° (*c* 2, 95% ethanol).

*Anal.* Calcd for $C_{17}H_{19}O_2NS \cdot HCl$: C, 60.4; H, 5.97; N, 4.15; S, 9.50. Found: C, 60.2; H, 6.08; N, 4.15; S, 9.47.

**Carbobenzoxyasparaginyl-S-benzylcysteine Benzyl Ester (VI).** Carbobenzoxyasparagine (7.86 g, 30 mmol) in 20 ml of acetonitrile was stirred for 1 hr at 0° with 7.5 g (30 mmol) of Woodward's reagent K[26] (N-ethyl-5-phenylisoxazolium-3′-sulfonate, NEPIS) and 1 equiv of triethylamine. S-Benzylcysteine benzyl ester hydrochloride (10 g, 30 mmol) was added to the mixture together with 1 equiv of triethylamine. The reaction mixture was stirred at room temperature for 48 hr, after which the solvent was removed *in vacuo* and the residue washed successively with dilute $NaHCO_3$, water, dilute HCl, and water. The dipeptide was crystallized from ethyl acetate to give 13.5 g (82%) of small needles of carbobenzoxyasparaginyl-S-benzylcysteine benzyl ester, mp 184–185°, $[\alpha]^{27}D$ −33.3° (*c* 1, acetic acid). The material was found to be homogeneous by paper chromatography in three solvent systems: $R_f$(BAW) 0.86, $R_f$(SBA) 0.77, $R_f$(BPAW) 0.90.

*Anal.* Calcd for $C_{29}H_{31}N_3O_6S$: C, 63.37; H, 5.69; N, 7.65; S, 5.83. Found: C, 63.3; H, 5.73; N, 7.66; S, 5.80.

**Asparaginyl-S-benzylcysteine Benzyl Ester Hydrochloride (VII).** Carbobenzoxyasparaginyl-S-benzylcysteine benzyl ester (6.1 g, 11 mmol) was dissolved in 30 ml of warm glacial acetic acid. The solution was cooled to room temperature and 90 ml of 40% HBr in glacial acetic acid was added. After 15 min at 25°, the solution was poured into a volume of dry ether. The mixture was cooled in an ice bath and scratched with a glass rod to give a granular precipitate of the dipeptide ester hydrobromide. This precipitate was filtered, washed with ether, and dissolved in a mixture of ethyl acetate and 1 *M* $NaHCO_3$. The ethyl acetate phase was washed with water and dried over $Na_2SO_4$. Addition of dry ether saturated with HCl gas gave 3.9 g (79%) of beautiful white needles of asparaginyl-S-benzylcysteine benzyl ester hydrochloride, mp 134–135°, $[\alpha]^{27}D$ −21.4° (*c* 1, acetic acid). The peptide was found to be homogeneous by paper chromatography in three solvent systems: $R_f$(BAW) 0.15, $R_f$(SBA) 0.56, $R_f$(BPAW) 0.71.

*Anal.* Calcd for $C_{21}H_{26}O_4N_3SCl$: N, 9.30; S, 7.09. Found: N, 9.33; S, 7.05.

***o*-Nitrophenylsulfenylthreonylasparaginyl-S-benzylcysteine Benzyl Ester (VIII).** Asparaginyl-S-benzylcysteine benzyl ester hydrochloride (3.5 g, 7.8 mmol) was combined in 100 ml of chloroform with 3.5 g (7.8 mmol) of the dicyclohexylamine salt of *o*-nitrophenylsulfenylthreonine (prepared by the method of Zervas, *et al.*[14]). The mixture was cooled to 5°, 1.6 g (7.8 mmol) of DCCI was added, and the reaction mixture was shaken for 15 hr. The chloroform was removed in a rotary evaporator and the residue was shaken with warm ethyl acetate and filtered. The yellow filtrate was washed with cold 0.01 *N* $H_2SO_4$, water, dilute $NaHCO_3$, and water, and then dried over $Na_2SO_4$. The *o*-nitrophenylsulfenylthreonylasparaginyl-S-benzylcysteine benzyl ester crystallized from the ethyl acetate on standing, in 83% yield, mp 215–216°, $[\alpha]^{27}D$ −57.5° (*c* 1, water–dimethylformamide 1:10). The peptide was found to be homogeneous in paper chromatography in three solvent systems: $R_f$(BAW) 0.90, $R_f$(SBA) 0.88, $R_f$(BPAW) 0.95.

*Anal.* Calcd for $C_{31}H_{35}O_8N_5S_2$: N, 10.46; S, 9.57. Found: N, 10.41; S, 9.52.

**Threonylasparaginyl-S-benzylcysteine Benzyl Ester (IX).** Five grams (7.5 mmol) of *o*-nitrophenylsulfenylthreonylasparaginyl-S-benzylcysteine benzyl ester was dissolved in 30 ml of ethyl acetate. Dry ether (3 ml) saturated with HCl gas was added, and the mixture was then diluted with water and extracted with ethyl acetate. The aqueous phase was neutralized with 1 *N* $NaHCO_3$ to give a flocculent precipitate of the tripeptide free base. Crystallization from ethyl acetate gave 3.5 g (90%) of threonylasparaginyl-S-benzylcysteine benzyl ester, mp 162–163°, $[\alpha]^{27}D$ −35.4° (*c* 1.5, acetic acid). The peptide was homogeneous in paper chromatography: $R_f$(BAW) 0.51, $R_f$(SBA) 0.62, $R_f$(BPAW) 0.82.

*Anal.* Calcd for $C_{25}H_{32}O_6N_4S$: N, 10.85; S, 6.20. Found: N, 10.88; S, 6.18.

***o*-Nitrophenylsulfenylglutaminylthreonylasparaginyl-S-benzylcysteine Benzyl Ester (X).** *o*-Nitrophenylsulfenylglutamine (1.2 g, 4 mmol), prepared by the method of Zervas, *et al.*,[14] was combined with 2.1 g (4 mmol) of threonylasparaginyl-S-benzylcysteine and stirred at 5° in 20 ml of dimethylformamide. DCCI (0.83 g, 4 mmol) was added, and the mixture was stirred for 2 days. The mixture was filtered, and the filtrate was added with stirring to a large volume of cold 0.01 *N* $H_2SO_4$. The yellow precipitate which formed was filtered, washed with dilute $NaHCO_3$ and water, and crystallized from ethyl acetate to give 2 g (65%) of *o*-nitrophenylsulfenylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester, mp 210° dec, $[\alpha]^{27}D$ −11.8° (*c* 2, water–dimethylformamide 1:10). With paper chromatography, the material was found to be homogeneous in three solvent systems: $R_f$(BAW) 0.86, $R_f$(SBA) 0.87, $R_f$(BPAW) 0.88.

*Anal.* Calcd for $C_{36}H_{43}O_{20}N_7S_2$: N, 12.29; S, 8.03. Found: N, 12.20; S, 8.00.

**Glutaminylthreonylasparaginyl-S-benzylcysteine Benzyl Ester Hydrochloride (XI).** *o*-Nitrophenylsulfenylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester (1.8 g, 2.2 mmol) was dissolved in 3 ml of dimethylformamide. The solution was cooled to 5°, and 1.5 ml of ether saturated with HCl was added. The mixture was diluted with ether, filtered, and washed with ether. Crystallization from dimethylformamide–ether gave 1.1 g (77%) of glutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester hydrochloride, mp 195–196°, $[\alpha]^{27}D$ −51.1° (*c* 1.5, acetic acid). When prepared and stored at temperatures below 5°, the peptide was found to be homogeneous in paper chromatography in two solvent systems (in SBA, the peptide precipitated at the origin): $R_f$(BAW) 0.30, $R_f$(BPAW) 0.70.

*Anal.* Calcd for $C_{30}H_{41}O_8N_6SCl$: N, 12.34; S, 4.70; Cl, 5.21. Found: N, 12.32; S, 4.66; Cl, 5.28.

**Tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine Benzyl Ester (XII).** Tosyl-S-benzylcysteinyl-ε-*t*-BOC-lysylasparaginylglycine (1.20 g, 1.56 mmol) was dissolved in 10 ml of dimethylformamide at 5° together with 1.06 g (1.56 mmol) of glutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester hydrochloride and 1 equiv of triethylamine. DCCI (0.32 g, 1.56 mmol) was added and the reaction mixture was stirred for 72 hr at 5°. At the end of this time, the solvent was removed in a rotary evaporator using a vacuum pump and Dry Ice trap. The residue was dissolved in 5 ml of trifluoroacetic acid and allowed to stand for 2 hr at room temperature. The trifluoroacetic acid was then removed *in vacuo* and the residue was dissolved in 2 ml of 50% acetic acid and chromatographed on a 0.9 × 150 cm column of Sephadex G-25 using 50% acetic acid as eluent. The pattern obtained is shown in Figure 4. The material which emerged at 69 ml was the octapeptide. This was removed and rechromatographed on the same column. The peak at 80 ml consisted of tetrapeptide material. The octapeptide from the rechromatography was isolated by lyophilization, and a portion was hydrolyzed with 6 *N* HCl and subjected to amino acid analysis. The results are given in Table I. The lyophilized peptide was found

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to be homogeneous in paper chromatography: $R_f$(BAW) 0.05, $R_f$(SBA) 0.61; $R_f$(BPAW) 0.86, $[\alpha]^{27}D$ −40.0° (*c* 0.5, acetic acid). The yield of tosyl-S-benzylcysteinyllysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester was 21% (0.45 g).

**Cysteinyllysylasparaginylglycylglutaminylthreonylasparaginylcysteine (XIII).** Tosyl-S-benzylcysteinyllysylasparaginylglycylglutaminylthreonylasparaginyl-S-benzylcysteine benzyl ester (0.42 g, 0.32 mmol) was dried in a vacuum desiccator for 24 hr over $P_2O_5$ and NaOH. The material was dissolved in 50 ml of liquid ammonia in a 250-ml, round-bottomed flask with rigid exclusion of moisture. Stirring was accomplished by means of a glass-coated magnetic bar. Small pieces of sodium were added to the solution until a blue color persisted. Ammonium chloride (20 mg) was then added, and the liquid ammonia was allowed to evaporate. The residue was dissolved at room temperature in 2 l. of 0.001 *M* acetic acid which had been boiled and flushed with nitrogen. The pH was carefully adjusted to 6.5 with 0.1 *M* NaOH, and $CO_2$-free oxygen was bubbled through the solution for 3 hr. At the end of this time, the solution was acidified with 1 ml of glacial acetic acid, and the solvent was removed in a rotary evaporator. The residue was dissolved in 2 ml of 50% acetic acid and chromatographed on a 0.9 × 150 cm column of Sephadex G-25 with 50% acetic acid as eluent. The pattern obtained is shown in Figure 5. The cyclic octapeptide was found to be homogeneous in paper chromatography in three solvent systems: $R_f$(BAW) 0.00, $R_f$(SBA) 0.80, $R_f$(BPAW) 0.47. Quantitative amino acid analysis of an acid hydrolysate of the peptide is given in Table I. The yield of the cyclic octapeptide after chromatography was 36% (0.1 g), $[\alpha]^{27}D$ −82.0° (*c* 0.5, water). Molecular weight by titration was found to be 871 (theoretical value, 866). Electrophoresis on Whatman No. 1 filter paper for 3 hr at 20 V/cm gave a single ninhydrin-positive spot at pH 5.6 with pyridine–acetate buffer and at pH 7.9 with *N*-ethylmorpholine–acetate. Digestion with leucine aminopeptidase gave quantitative recovery of free amino acids, indicating that no racemized amino acid residues were in the peptide (Table II).

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# The pH Dependence of the Pepsin-Catalyzed Hydrolysis of Neutral Dipeptides[1a]

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**Abstract:** The pH dependences of the kinetic constants for the pepsin-catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester were determined in the pH range 1.05–5.05 at 25° using twice-crystallized pepsin. The pH *vs.* $k_{cat}$ and $K_m$ figures were bell-shaped curves. The pH *vs.* $k_{cat}$ curves depend on a pair of catalytic groups with p$K_a$'s in water and deuterium oxide of 1.62, 3.48, and 1.92, 4.01, respectively. For this reaction, there is no deuterium oxide solvent isotope effect with $k_{H_2O\ lim}/k_{D_2O\ lim} = 1.05 \pm 0.30$. The competitive inhibition constant, $K_I$, for N-acetyl-D-phenylalanyl-D-tyrosine methyl ester was found to be equal to the Michaelis constant, $K_m$, for the L–L substrate at various pH's between 1.10 and 4.05. This indicates that the $K_m$ measured under turnover conditions is a true equilibrium constant not modified by additional rate constants. The pH dependences of the kinetic constants for the pepsin- (prepared according to Rajagopalan, Moore, and Stein) catalyzed hydrolyses of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester, N-acetyl-L-phenylalanyl-L-phenylalanine methyl ester, and N-acetyl-L-tyrosyl-L-phenylalanine methyl ester were also determined in this pH range. The pH *vs.* $k_{cat}$ and $K_m$ profiles were all bell-shaped curves. Evidence is presented that $k_{cat} = k_2$, the first catalytic step of the reaction. Based on these results a mechanism is proposed for these reactions which accounts for the prototropic equilibria and a rate-determining step which does not involve a proton transfer. Finally the inhibition constants for some of the products and dioxane were also determined in this pH range.

Previous work has established that pepsin catalyzes the hydrolysis of N-acyl-L-dipeptides[1c,2–6] and N-acyl-L-tripeptide esters.[7,8] All but one of the N-acyl-L-dipeptides, which exhibit maximum hydrolysis near pH 2, contained a free C-terminal carboxyl group which ionizes in this low pH region of pepsin activity. The N-acyl-L-tripeptide esters all contained a positively charged histidine moiety which was included to help increase the substrate solubility. These positively charged compounds (*e.g.*, Z-His-Phe-Trp-OET)[9] are the most catalytically active peptide substrates which have been prepared for pepsin. Unfortunately the electric charge of these substrates makes them less

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(9) Abbreviations used: His, L-histidyl; Phe, L-phenylalanyl; Tyr, L-tyrosine; $I_2$Tyr, L-diiodotyrosine; $Br_2$Tyr, L-dibromotyrosine; Trp, L-tryptophyl; Ac, acetyl; Z, benzyloxycarbonyl; OEt, ethyl ester; OMe, methyl ester; Glu, L-glutamyl.