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Studies with the S-Peptide–S-Protein System: The Role of Glutamic Acid-2, Lysine-7, and Methionine-13 in S-Peptide$_{1-14}$ for Binding to and Activation of S-Protein[1-3]

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Syntheses are described of 12-serine S-peptide$_{1-14}$, 12-(β-pyrazolyl-3)-alanine S-peptide$_{2-14}$, 12-(β-pyrazolyl-3)-alanine S-peptide$_{3-14}$, 13-norleucine S-peptide$_{1-14}$, 13-norleucine S-peptide$_{2-14}$, 13-norleucine S-peptide$_{3-14}$, 7-norleucine S-peptide$_{1-14}$, and 7,13-dinorleucine S-peptide$_{1-14}$. The peptides were prepared by combination of the stepwise method and fragment condensation and were characterized by thin-layer chromatography and amino acid analyses of acid and enzymic hydrolysates. The interaction between these peptides and S-protein or ribonuclease S was determined with RNA as the substrate. The peptides in which 12-histidine is replaced by serine or β-(pyrazolyl-3)-alanine did not activate S-protein but had the ability to compete with S-peptide$_{1-20}$ for S-protein and thus inhibited RNase S. 12-(β-Pyrazolyl-3)-alanine S-peptide$_{2-14}$ was essentially as effective an inhibitor of RNase S as 12-(β-pyrazolyl-3)-alanine S-peptide$_{1-14}$; 12-(β-pyrazolyl-3)-alanine S-peptide$_{3-14}$ was about 30-fold less active. 13-Norleucine S-peptide$_{1-14}$ and 13-norleucine S-peptide$_{2-14}$ activated S-protein and were considerably more effective than 13-norleucine S-peptide$_{3-14}$. Together with previous observations, these results firmly establish the "binding" contribution of glutamic acid-2 in the S-peptide–S-protein system. A possible explanation for this phenomenon is presented. 12-Serine S-peptide$_{1-14}$ was approximately sevenfold less effective an inhibitor as 12-(β-pyrazolyl-3)-alanine S-peptide$_{1-14}$. This finding indicates that the aromatic azole ring of histidine and of β-(pyrazolyl-3)-alanine contributes to the stability of the respective S-peptide–S-protein complexes. The observation that both 7-norleucine S-peptide$_{1-14}$ and 7,13-dinorleucine S-peptide$_{1-14}$ activate S-protein does not support the suggestion that lysine-7 is an important substrate binding site in RNase S.

[1] This is Paper XLVIII in our series: Studies on Polypeptides. See K. Hofmann and C. Y. Bowers, *J. Med. Chem.* **13**, 1009 (1970) for paper XLVII.

[2] Supported by grants from the U.S. Public Health Service and the Hoffmann–La Roche Foundation.

[3] The amino acids 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 = *t*-butoxycarbonyl; DAPA = 3-dimethylaminopropylamine; DCC = *N*,*N*′-dicyclohexylcarbodiimide; DMF = dimethylformamide; EtOH = ethanol; MeOH = methanol; NMM = *N*-methylmorpholine; O-*t*-Bu = *t*-butyl ester; Pyr = β-(pyrazolyl-3)-alanine; TEA = triethylamine; TFA = trifluoroacetic acid; THF = tetrahydrofuran; tlc = thin-layer chromatography. RNase S, subtilisin modified beef RNase A, or an equimolar mixture of S-peptide and S-protein; S-peptide, the peptide obtained from RNase S; S-protein, the protein component obtained from RNase S. Natural S-peptide is a mixture of at least three components, very likely S-peptide$_{1-20}$, S-peptide$_{1-21}$, and S-peptide$_{1-22}$ [M. S. Doscher and C. H. W. Hirs, *Biochemistry* **6**, 304 (1967)].

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INTRODUCTION

From the point of view of fundamental knowledge, the S-peptide–S-protein system (*1*) is unique since it affords the opportunity to assess the structural features of S-peptide which are important for binding to S-protein and those which are responsible for the formation of the active site of ribonuclease S. Significantly, two biologically inert molecules combine noncovalently to form an active enzyme. A synthesis of S-peptide was developed which afforded a material which exhibited, within experimental error, the physical, chemical, and biological properties of S-peptide isolated from subtilisin digested ribonuclease A (*2*). It was also observed that S-peptide$_{1-14}$ possesses the same ability as natural S-peptide to activate S-protein, i.e., at a molar peptide to protein ratio of 1:1 both compounds formed a fully active ribonuclease S (*3*). From this result we concluded that amino acid residues 15–20 in S-peptide do not contribute either to binding or function of the ribonuclease S molecule. The X-ray diffraction studies of Wyckoff et al. (*4*) confirmed this conclusion.

Shortening of the peptide chain of S-peptide$_{1-14}$ from the carboxyl end provided us with S-peptide$_{1-13}$ and S-peptide$_{1-12}$, both possessing the ability to activate S-protein; but higher peptide-to-protein ratios were required for essentially full activation. S-Peptide$_{1-11}$, which lacks the histidine residue, failed to form active enzyme with S-protein at molar ratios as high as 8000:1 (*5*). This finding agreed with the results of carboxymethylation studies (*6*) which demonstrated that 12-(3-carboxymethylhistidine) ribonuclease A was enzymically inactive. These and other structure–function studies with analogs of S-peptide$_{1-13}$ and S-peptide$_{1-14}$ led us to conclude (*7*) that histidine-12 is the "active" residue in S-peptide and that the rest of the molecule acts as the vehicle which brings this histidine into the correct stereochemical position within the active site of the ribonuclease S molecule. This conclusion received experimental support from the observation that 12-β-(pyrazolyl-3)-alanine S-peptide$_{1-14}$ (III) is a powerful, competitive antagonist of S-peptide (*7*, *8*). Peptide (III) and S-peptide must exhibit identical affinity for S-protein since ribonuclease S is 50% inhibited when the ratio of (III) to S-peptide is unity. This discovery made it possible to compare the affinity for S-protein of S-peptide and a number of S-peptide analogs or fragments.

Evaluation of the affinity for S-protein of a peptide in which the azole ring of histidine or β-(pyrazolyl-3)-alanine was replaced by an aliphatic sidechain prompted the synthesis of 12-serine S-peptide$_{1-14}$ (II) (*9*).

During the early phases of our studies, we observed that S-peptide$_{2-13}$ exhibited essentially the same S-protein activating potency as S-peptide$_{1-13}$. This observation was not surprising since the naturally occurring 1-deslysine ribonuclease A had been shown to be as active as ribonuclease A in the hydrolysis of yeast RNA (*10*). S-Peptide$_{3-13}$ was a much weaker activator of S-protein. This result pointed to a binding function for glutamic acid-2 and we suggested that this residue was a "binding" site of S-peptide (*5*).

Since the inhibitor approach provides a more sensitive measure than S-protein activation for assessing the affinity of a peptide for S-protein, we synthesized 12-β-(pyrazolyl-3)-alanine S-peptide$_{2-14}$ (IV) and 12-β-(pyrazolyl-3)-alanine S-peptide$_{3-14}$ (V) and compared their ability to displace S-peptide from ribonuclease S with that of 12-β-(pyrazolyl-3)-alanine S-peptide$_{1-14}$ (III). In order to gain additional insight into the binding role of glutamic acid-2, we synthesized 13-norleucine S-peptide$_{1-14}$ (VI), 13-norleucine S-peptide$_{2-14}$ (VII), and 13-norleucine S-peptide$_{3-14}$ (VIII) and studied their interaction with S-protein.

In view of suggestions (*11*) that lysine-7 may provide a substrate binding site in

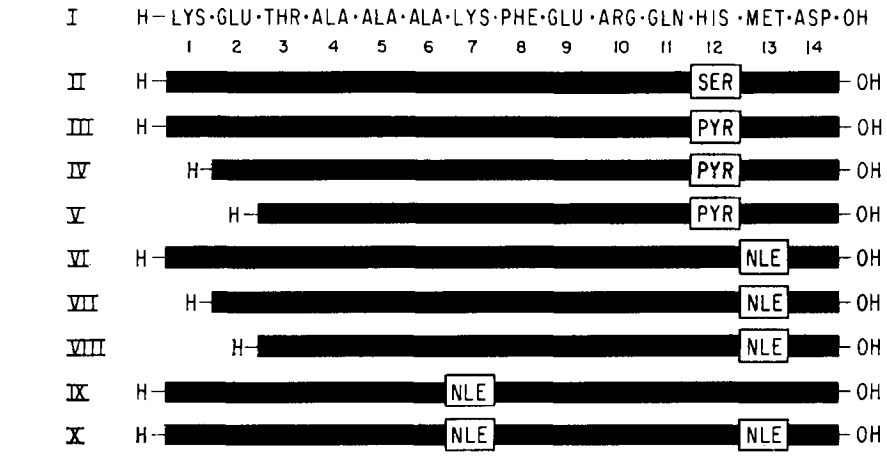
RNase A, the ability of 7-norleucine S-peptide$_{1-14}$ (IX) and 7,13-dinorleucine S-peptide$_{1-14}$ (X) to activate S-protein was also explored.

Preparative Aspects

The peptides (Table 1) which were required for this study were prepared from the fragments listed in Table 2. Syntheses of a number of these compounds have been described; the remainder were prepared by methods previously developed in this

TABLE 1

SYNTHETIC S-PEPTIDE ANALOGS



laboratory (*2, 3, 8, 12*). The Rudinger modification of the azide procedure (*13*) was used exclusively for coupling of the various fragments. Fragment XXV was obtained by acylating fragment XXII with *N*-hydroxysuccinimido *t*-butoxycarbonylnorleucinate followed by deprotection. Peptides XXI and XXIV, which do not contain methionine, were prepared by the stepwise method starting with di-*t*-butyl aspartate (*14*). Histidine was incorporated via N^{α}-benzyloxycarbonylhistidine azide (*15*) and arginine as N^{α}-benzyloxycarbonylnitroarginine (*16*) by the Anderson modification (*17*) of the mixed anhydride procedure. Active esters of the respective benzyloxycarbonyl derivatives were used to incorporate the rest of the amino acid residues. The γ-carboxyl group of glutamic acid was protected by a *t*-butyl ester (*18*). The methionine residue was carried through the various synthetic steps in the form of the *d*-sulfoxide (*12c*) and was reduced with thioglycolic acid (*3*) just prior to the enzyme assays. Benzyloxycarbonyl groups and the nitro group were removed by hydrogenolysis. A number of intermediates and the final products were purified by chromatography on the ion exchanger AG 1-X2 using 1-butanol/MeOH/acetic acid mixtures for elution (*2b*). The final products were obtained in the form of fluffy, colorless powders by lyophilization. With few exceptions, protected intermediates were characterized by optical rotation, elemental analysis, tlc in at least two solvent systems, and amino acid analyses of acid hydrolysates. Optical rotation, tlc in several solvent systems, and amino acid analyses of AP-M digests served to characterize the free peptides.

TABLE 2

INTERMEDIATES FOR THE SYNTHESIS OF S-PEPTIDE ANALOGS

Peptide No.	Structure
XI	Boc·Phe·Glu(O-*t*-Bu)·Arg·Gln·$HNNH_2$ (*12b*)
XII	Z·Thr·Ala·Ala·Ala·Lys(Boc)·$HNNH_2$
XIII	Boc·Thr·Ala·Ala·Ala·Lys(Boc)·$HNNH_2$ (*12a*)
XIV	Z·Glu(O-*t*-Bu)·Thr·Ala·Ala·Ala·Lys(Boc)·$HNNH_2$
XV	Boc·Glu(O-*t*-Bu)·Thr·Ala·Ala·Ala·Lys(Boc)·$HNNH_2$
XVI	Boc·Lys(Boc)·Glu(O-*t*-Bu)·Thr·Ala·Ala·Ala·$HNNH_2$ (*19*)
XVII	Boc·Lys(Boc)·Glu(O-*t*-Bu)·Thr·Ala·Ala·Ala·Lys(Boc)·$HNNH_2$ (*12a*)
XVIII	Boc·Lys(Boc)·Glu(O-*t*-Bu)·Thr·Ala·Ala·Ala·Lys(Boc)·Phe·Glu(O-*t*-Bu)·Arg·Gln·$HNNH_2$ (*4b*)
XIX	H·Ser·Met(O)·Asp·OH
XX	H·His·Nle·Asp·OH
XXI	H·Phe·Glu·Arg·Gln·His·Nle·Asp·OH
XXII	H·Phe·Glu·Arg·Gln·His·Met(O)·Asp·OH (*3*)
XXIII	H·Phe·Glu·Arg·Gln·Pyr·Met(O)·Asp·OH (*8*)
XXIV	H·Nle·Phe·Glu·Arg·Gln·His·Nle·Asp·OH
XXV	H·Nle·Phe·Glu·Arg·Gln·His·Met(O)·Asp·OH

EXPERIMENTAL

General Procedures

Melting points are uncorrected. Except when noted otherwise, rotations were determined in 10% acetic acid 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.; oxygen values were actually determined and not computed by difference. Analytical samples were dried *in vacuo* over P_2O_5 at 50–60°C. The amino acid compositions of acid and AP-M hydrolysates were determined with a Beckman Spinco Model 120 amino acid analyzer according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.* **30**, 1185 (1958). The figures in parentheses are average recoveries of amino acids, based on formula weight. Nle and α-amino-β-guanidopropionic acid were used for internal standards. Acid hydrolyses were performed in constant boiling HCl at 110°C for 24 hr in evacuated tubes; values are not

corrected for amino acid destruction. AP-M digests were performed as described in (*3*). Azide couplings and purification of final products and intermediates were performed essentially as described (*2, 3, 8, 12*). Sparingly soluble intermediates were suspended in 1-butanol and the suspension washed in the usual manner. Protecting groups were removed with 90% TFA and trifluoroacetate ions were exchanged for acetate ions on AG 1-X2 or Amberlite IRA-400. Sulfoxides were reduced with thioglycolic acid (*3*). Details can be found in the references cited and in A. J. Quattrone, PhD Thesis, University of Pittsburgh, 1970. Designation of solvent systems for descending paper chromatograms 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. Designations 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 (1:19). AG 1-X2 (chloride form, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) was washed twice with 2 *N* KOH, then with water until neutral. This was followed by two washings with 10% acetic acid and water until neutral. Columns were equilibrated with the appropriate solvents before use. RNase S was prepared as described (*5*) and enzyme assays were performed according to the previously reported method (*2b*) using RNA as substrate. Ribonuclease 100% activity corresponds to 130 Kunitz units per mg at 30°C. Peptide concentrations are based on the average amino acid recovery in 24-hr acid hydrolysates.

Preparation of Peptides

α-N-*Hydroxysuccinimido* γ-t-*butyl* t-*butoxycarbonylglutamate.* To a THF solution, cooled at 0°C, of γ-*t*-butyl *t*-butoxycarbonylglutamate (270 mg) and *N*-hydroxysuccinimide (115 mg) was added a solution of DCC (205 mg) in THF (5 ml). The reaction mixture was kept at 4°C for 12 hr and was then filtered. The filtrate was evaporated and the residue recrystallized from 2-propanol; 290 mg (81%); mp 146–147°C; $[\alpha]_D^{28}$ −31.2° (*c* 1.77, DMF).

Anal. Calcd for $C_{18}H_{28}O_8N_2$: C, 54.0; H, 7.0; N, 7.0. Found: C, 54.1; H, 7.1; N, 7.0.

N-*Hydroxysuccinimido* t-*butoxycarbonylnorleucinate.* DCC (495 mg) in THF (10 ml) was added to a solution of *t*-butoxycarbonylnorleucine (*20*) (506 mg) and *N*-hydroxysuccinimide (277 mg) in THF (10 ml). The mixture was stirred for 2 hr at 0°C and for 24 hr at 25°C. The suspension was filtered; the filtrate was evaporated to yield an oil which soon crystallized. The compound was crystallized from a mixture of 2-propanol (1 ml) and petroleum ether (20 ml); 595 mg (83%); mp 107–108°C; $[\alpha]_D^{25}$ −30.7° (*c* 1.59, acetone); R_f^I 0.8; R_f^{III} 0.6; R_f^{IV} 0.8.

Anal. Calcd for $C_{15}H_{24}O_6N_2$: C, 54.9; H, 7.4; N, 8.5. Found: C, 54.6; H, 7.5; N, 8.3.

N-*Hydroxysuccinimido benzyloxycarbonylnorleucinate.* DCC (4.53 g) in THF (50 ml) at 0°C was added to a solution of benzyloxycarbonylnorleucine (21) (4.65 g) and *N*-hydroxysuccinimide (2.30 g) in THF (50 ml) and the solution was stirred at 0°C for 2 hr and at 25°C for 22 hr. The suspension was filtered and the filtrate evaporated yielding an oil which soon crystallized. Two recrystallizations from ethyl acetate/petroleum ether yielded prisms: 5.61 g (88%); mp 69–71°C; $[\alpha]_D^{26}$ −22.3° (*c* 3.94, acetone); R_f^I 0.8; R_f^{IV} 0.9.

Anal. Calcd for $C_{18}H_{22}O_6N_2$: C, 59.7; H, 6.1; N, 7.7. Found: C, 59.8; H, 6.0; N, 7.7.

N-*Benzyloxycarbonylserylmethionylaspartic acid* d-*sulfoxide.* A solution of methionylaspartic acid *d*-sulfoxide monohydrate (*3*) (2.40 g) in water (80 ml) and TEA (2.3 ml) was shaken for 60 hr at 4°C with a solution of benzyloxycarbonylserine azide

(prepared from 6.1 g of the hydrazide (*22*)) in ether (160 ml). The organic phase was separated and the aqueous layer extracted with three portions of ethyl acetate. The organic phases were discarded and the aqueous layer was passed through a column of Dowex-50-W-X2 (H^+) which was prepared with 60 g of resin. The column was eluted with water, the combined aqueous eluates were evaporated, and the residual oil was dried. Addition of ethyl acetate/light petroleum ether 1 : 1 (20 ml), cooling, and scratching brought about crystallization. Recrystallization from MeOH/ethyl acetate yielded colorless needles; 1.95 g (45%); mp 156–157°C; $[\alpha]_D^{25}$ +16.2° (*c* 5.59, MeOH); R_f^1 0.8; R_f^3 0.7 × His; R_f^I 0.5.

Anal. Calcd for $C_{20}H_{27}O_{10}N_3S$: C, 47.9; H, 5.4; N, 8.4. Found: C, 47.8; H, 5.7; N, 8.2.

Unreacted dipeptide was recovered by washing the column with 3% ammonium hydroxide. The eluates were evaporated and the dipeptide isolated as described (*3*). Recrystallization from aqueous EtOH afforded 1.1 g of dipeptide.

Serylmethionylaspartic acid d-*sulfoxide* (*XIX*). Benzyloxycarbonylserylmethionylaspartic acid *d*-sulfoxide (3.90 g) was dissolved in sodium-dried liquid ammonia (approximately 300 ml) and sodium was added in small portions with stirring until a permanent blue color remained. After 5 min Dowex-50W-X2 (NH_4^+) (25 g) was added in small portions and the ammonia was allowed to evaporate at room temperature. A slow stream of nitrogen was passed over the residue to remove the last traces of ammonia. The residue was dissolved in water and the solution was applied to a column of AG 1-X2. The column was eluted first with water (200 ml), then with 0.25 *N* acetic acid. Evaporation of the acetic acid eluate yielded a colorless oil which soon crystallized. The compound was recrystallized from aqueous EtOH; 2.46 g (86%); mp 242°C (dec); $[\alpha]_D^{25}$ +42.5° (*c* 2.91, water); R_f^1 0.2; R_f^{III} 0.2; amino acid ratios in AP-M digest: $Ser_{0.9}Met(O)_{1.0}Asp_{1.1}$ (89%).

Anal. Calcd for $C_{12}H_{21}O_8N_3S$: C, 39.2; H, 5.8; N, 11.4; O, 34.8; S, 8.7. Found: C, 39.4; H, 5.9; N, 10.9; O, 34.2; S, 9.3.

N-*Benzyloxycarbonylnorleucylaspartic acid.* An ice-cold solution of aspartic acid (4.0 g) and TEA (12.5 ml) in water (50 ml) was added to an ice-cold solution of *N*-hydroxysuccinimido benzyloxycarbonylnorleucinate (10.0 g) in THF (50 ml). The mixture was kept at 0°C for 2 hr and then at room temperature for 24 hr. The solution was concentrated and the residue extracted with three portions of ethyl acetate which were discarded. The aqueous layer was acidified to Congo red with 2 *N* HCl and extracted with ethyl acetate. The organic layers were washed in the usual manner, dried, and evaporated. The ensuing oil soon crystallized and the material was recrystallized from ethyl acetate/petroleum ether; colorless needles; 8.25 g (72%); mp 142–144°C; $[\alpha]_D^{26}$ −9.8° (*c* 4.35, MeOH); R_f^1 0.8; R_f^I 0.7; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Nle_{1.0}Asp_{1.1}$ (84%).

Anal. Calcd for $C_{18}H_{24}O_7N_2$: C, 56.8; H, 6.4; N, 7.4; O, 29.4. Found: C, 57.0; H, 6.2; N, 7.5; O, 29.6.

Norleucylaspartic acid. The above benzyloxycarbonyldipeptide (7.25 g) was hydrogenated in 50% aqueous MeOH (100 ml). The catalyst was removed by filtration, the filtrate was evaporated to dryness, and the residue was crystallized from aqueous EtOH. Colorless needles; 4.46 g (95%); mp 239–240°C $[\alpha]_D^{26}$ +24.7° (*c* 4.57, water); R_f^I 0.5; R_f^{III} 0.4; single chlorine and ninhydrin positive spot; amino acid ratios in AP-M digest: $Nle_{1.0}Asp_{1.0}$ (92%).

Anal. Calcd for $C_{10}H_{18}O_5N_2$: C, 48.8; H, 7.4; N, 11.4; O, 32.5. Found: C, 48.3; H, 8.0; N, 11.2; O, 33.2.

Benzyloxycarbonylthreonylalanylalanylalanyl-N^{ϵ}*-t-butoxycarbonyllysine hydrazide* (*XII*). Hydrazine hydrate (3 ml) was added to a solution of the required methyl ester (*12a*) (980 mg) in MeOH (100 ml) and the mixture was kept at room temperature for 48 hr. The gel which had precipitated was collected, washed with ice-cold MeOH and was dried over KOH and concd sulfuric acid; 800 mg (81%); $[\alpha]_D^{25}$ −6.5° (*c* 1.04, DMF).

Anal. Calcd for $C_{32}H_{52}O_{10}N_8$: C, 54.2; H, 7.4; N, 15.8. Found: C, 54.1; H, 7.4; N, 16.1.

Benzyloxycarbonyl - γ - t - butylglutamylthreonylalanylalanylalanyl - N^{ϵ} *- t - butoxy - carbonyllysine hydrazide* (*XIV*). Hydrazine hydrate (2 ml) was added to a solution of the required methyl ester (*12a*) (1.02 g) in MeOH (100 ml) and the mixture was kept at room temperature for 48 hr. The gel which had precipitated was collected, washed with ice-cold MeOH, and dried over KOH and concd sulfuric acid; 860 mg (84%); mp 229°C (dec); $[\alpha]_D^{27}$ −11.6° (*c* 1.73, DMF).

Anal. Calcd for $C_{41}H_{67}O_{13}N_9$: C, 55.1; H, 7.6; N, 14.1. Found: C, 55.2; H, 7.7; N, 14.4.

t - *Butoxycarbonyl - γ - t - butylglutamylthreonylalanylalanylalanyl -* N^{ϵ} *- t - butoxy - carbonyllysine hydrazide* (*XV*). α-*N*-Hydroxysuccinimido-γ-*t*-butyl *t*-butoxycarbonylglutamate (365 mg) was added to a DMF solution (8 ml) containing methyl threonylalanylalanylalanyl-N^{ϵ}-*t*-butoxycarbonyllysinate acetate (*12a*) (485 mg) and TEA (0.11 ml). The mixture was kept at room temperature for 16 hr, water (100 ml) was added, and the precipitate was collected and washed with water, 1 *N* citric acid, and water. For purification the product was twice precipitated from DMF with water and dried; 580 mg (92%); mp 227–228°C (dec); $[\alpha]_D^{28}$ −15.2° (*c* 1.14, DMF); R_f^{I} 0.7; R_f^{III} 0.8. Hydrazine hydrate (0.18 ml) was added to a MeOH solution (5 ml) containing the above methyl ester (300 mg) and the mixture was kept at room temperature for 6 hr. The gelatinous precipitate was collected, washed with ether, and dried over KOH and concd sulfuric acid; 280 mg (93%); mp 240–241°C (dec); $[\alpha]_D^{26}$ −45.2° (*c* 0.92, acetic acid); R_f^{I} 0.5; R_f^{III} 0.7.

Anal. Calcd for $C_{38}H_{69}O_{13}N_9$: C, 53.1; H, 8.1; N, 14.7. Found: C, 53.1; H, 8.3; N, 14.4.

α,β-*Di*-t-*butyl norleucylaspartate.* *N*-Hydroxysuccinimido benzyloxycarbonylnorleucinate (10.2 g) in THF (75 ml) was added to a solution of α,β-di-*t*-butyl aspartate (*14*) (8.0 g) and TEA (4.8 ml) in THF (75 ml). The mixture was stirred for 2 hr at 0°C and for 22 hr at room temperature and filtered. The filtrate was evaporated, the residual oil dissolved in ethyl acetate, and the solution was washed in the usual manner. Evaporation of the ethyl acetate gave an oil; 13.6 (86%). This material (9.9 g) was hydrogenated in MeOH (50 ml) and the resulting oily product dried; 6.45 g (90%); R_f^{I} 0.6; R_f^{III} 0.7. A sample was deblocked with TFA, TFA ions were exchanged for acetate ions on AG 1-X2, and the material was isolated by lyophilization from 0.1 *N* acetic acid. Colorless powder; $[\alpha]_D^{23}$ +25.4° (*c* 2.59, H_2O); R_f^{I} 0.4; R_f^{III} 0.4; amino acid ratios in AP-M digest: $Nle_{1.0}Asp_{1.0}$ (84%).

α,β-*Di*-t-*butyl* N^{α}*-benzyloxycarbonylhistidylnorleucylaspartate.* *t*-Butyl nitrite (1.8 ml) was added at −30°C with stirring to a DMF solution (35 ml) of N^{α}-benzyloxycarbonylhistidine hydrazide (*15*) (4.4 g) and 5.7 *N* HCl in dioxane (8.8 ml). After 30 min, the solution was cooled at −60°C and TEA (6.9 ml) was added, followed by α,β-di-*t*-butyl norleucylaspartate (2.6 g) in dioxane (50 ml) and TEA (2.2 ml). The mixture was stirred for 20 hr at 4°C, then the solvents were evaporated. The residue was dissolved in ethyl acetate and water and the solution washed in the usual manner and dried. Evaporation gave an amorphous material which was purified by two

precipitations from ethyl acetate with ether; 2.69 g (80%); mp 155–157°C (dec); $[\alpha]_D^{25}$ −29.5° (*c* 3.74, MeOH); R_f^{I} 0.8; R_f^{III} 0.9.

Anal. Calcd for $C_{32}H_{47}O_8N_5$: C, 61.0; H, 7.5; N, 11.1. Found: C, 61.1; H, 7.5; N, 11.3.

α,β-Di-t-butyl histidylnorleucylaspartate monoacetate. The benzyloxycarbonyl derivative (876 mg) was hydrogenated in MeOH containing acetic acid and the product was isolated by lyophilization from water; 769 mg (99%); $[\alpha]_D^{30}$ −20.7° (*c* 2.2); R_f^{I} 0.4; R_f^{III} 0.7.

Anal. Calcd for $C_{26}H_{44}O_8N_5$: C, 56.3; H, 8.0; N, 12.6; O, 23.1. Found: C, 55.9; H, 8.2; N, 12.5; O, 23.1.

Histidylnorleucylaspartic acid trihydrate (XX). The di-*t*-butyl ester (2.43 g) was deblocked with TFA and the product purified by chromatography on AG 1-X2. The title peptide was eluted with 0.033 *M* acetic acid. For final purification, the compound was precipitated from water with MeOH; 1.53 g (80%); $[\alpha]_D^{29}$ −29.7° (*c* 1.1); R_f^{I} 0.3; R_f^{III} 0.4; amino acid ratios in AP-M digest: $His_{1.0}Nle_{1.0}Asp_{1.0}$ (80%).

Anal. Calcd for $C_{16}H_{25}O_6N_5 \cdot 3\,H_2O$: C, 43.9; H, 7.1; N, 16.0; O, 32.9. Found: C, 44.3; H, 6.7; N, 16.5; O, 33.0.

α,β-Di-t-butyl benzyloxycarbonylglutaminylhistidylnorleucylaspartate. 2,4,5-Trichlorophenyl benzyloxycarbonylglutaminate (*23*) (10.5 g) in DMF (100 ml) was added to a solution of α,β-di-*t*-butyl histidylnorleucylaspartate monoacetate (13.0 g) and TEA (5.85 ml) in DMF (100 ml). The mixture was stirred at 0°C for 2 hr and at room temperature for 22 hr, then the solvents were evaporated. The resulting gel was distributed between ethyl acetate and water, collected, and dried; 13.6 g (76%); $[\alpha]_D^{28}$ −24.3° (*c* 1.455, DMF); mp 205°C (dec); R_f^{I} 0.6; R_f^{III} 0.8; amino acid ratios in acid hydrolysate: $Glu_{1.0}His_{1.0}Nle_{1.0}Asp_{1.0}$ (97%).

Anal. Calcd for $C_{37}H_{55}O_{10}N_7$: C, 58.6; H, 7.3; N, 12.9. Found: C, 58.5; H, 7.5; N, 13.0.

α,β-Di-t-butyl benzyloxycarbonylnitroarginylglutaminylhistidylnorleucylaspartate monoacetate. The protected tetrapeptide di-*t*-butyl ester (2.58 g) was hydrogenated in DMF (50 ml) and EtOH (50 ml) and the filtrate from the catalyst was concentrated to a volume of approximately 40 ml. To this solution was added a solution of a mixed anhydride prepared from benzyloxycarbonylnitroarginine (2.33 g), NMM (0.74 ml), isobutyl chloroformate (0.78 ml) in THF (50 ml) (*17*). The solution was stirred at −10°C for 1 min, then NMM (0.74 ml) was added and stirring was continued for 30 min at 0°C and for 2 hr at room temperature. The solvents were evaporated, the residue was distributed between ether and water and was collected. The material was then suspended in 1-butanol and the suspension washed in the usual manner. The butanol phases were evaporated and the residue lyophilized from 20% acetic acid; 2.59 g (75%); $[\alpha]_D^{25}$ −21.6° (*c* 0.928, DMF); R_f^{I} 0.6; R_f^{III} 0.8.

Anal. Calcd for $C_{45}H_{70}O_{15}N_{12}$: C, 53.0; H, 6.9; N, 16.5; O, 23.6. Found: C, 52.8; H, 7.1; N, 16.5; O, 23.9.

α,β-Di-t-butyl arginylglutaminylhistidylnorleucylaspartate diacetate. The protected pentapeptide ester (5.03 g) was hydrogenated for 20 hr at 30°C in 50% EtOH (200 ml) and 10% acetic acid (20 ml) and the product was isolated by lyophilization from 5% acetic acid; 4.7 g (99%); mp 150°C (dec); $[\alpha]_D^{25}$ −23.0° (*c* 0.89, DMF); R_f^{I} 0.3; R_f^{III} 0.7. A sample for analytical evaluation was deblocked with TFA; amino acid ratios in AP-M digest: $Arg_{1.1}Gln_{0.9}His_{1.1}Nle_{0.9}Asp_{0.9}$ (104%).

α,β-Di-t-butyl benzyloxycarbonyl-γ-t-butylglutamylarginylglutaminylhistidylnorleucylaspartate diacetate. α-*N*-Hydroxysuccinimido γ-*t*-butyl benzyloxycarbonylglutamate (*24*) (1.47 g) in DMF (10 ml) was added at 0°C with stirring to a solution of

α,β-di-*t*-butyl arginylglutaminylhistidylnorleucylaspartate diacetate (2.51 g) in DMF (15 ml). After 5 min, TEA (0.75 ml) was added and the mixture was stirred for 2 hr at 0°C and for 22 hr at room temperature. DAPA (0.09 ml) was added and the solvents were evaporated. The residue was distributed between ethyl acetate and water; the solid was collected, suspended in 1-butanol, and the suspension washed in the usual manner. Evaporation of the butanol solution gave a colorless powder; 2.8 g (98%); $[\alpha]_D^{29}$ −36.1° (*c* 2.00, 1-butanol/MeOH/H_2O, 1:1:1); R_f^{I} 0.6; R_f^{III} 0.8; amino acid ratios in acid hydrolysate: $Glu_{2.0}Arg_{0.9}His_{1.1}Nle_{1.0}Asp_{1.0}$ (82%).

Anal. Calcd for $C_{56}H_{89}O_{18}N_{12}$: C, 55.2; H, 7.4; N, 13.8; O, 23.6. Found: C, 55.0; H, 7.4; N, 13.9; O, 23.4.

α,β-Di-t-butyl γ-t-butylglutamylarginylglutaminylhistidylnorleucylaspartate diacetate. The protected hexapeptide di-*t*-butyl ester (6.7 g) was hydrogenated in 50% EtOH (200 ml) and 20% acetic acid (10 ml). Lyophilization of the filtrate from the catalyst gave a colorless powder; 5.8 g (97%); $[\alpha]_D^{28}$ −20.2° (*c* 1.05, DMF); R_f^{I} 0.4; R_f^{III} 0.7. A sample was deblocked with TFA and the ether precipitated material was washed with ether and dried; amino acid ratios in AP-M digest: $Glu_{0.9}Arg_{0.8}Gln_{1.1}His_{1.1}Nle_{1.1}Asp_{1.0}$ (68%).

α,β - Di - t - butyl benzyloxycarbonylphenylalanyl - γ - t - butylglutamylarginyl - glutaminylhistidylnorleucylaspartate triacetate. *N*-Hydroxysuccinimido benzyloxycarbonylphenylalaninate (*25*) (2.62 g) in DMF (10 ml) was added at 0°C to a stirred solution of the hexapeptide di-*t*-butyl ester diacetate (6.3 g) in DMF (15 ml). After 5 min, TEA (1.52 ml) was added and the solution was stirred for 2 hr at 0°C and for 22 hr at room temperature. DAPA (1.58 ml) was added and the solvents were evaporated. The residue was distributed between ethyl acetate and water (50 ml each) and the resulting gel was collected, suspended in 1-butanol and the suspension washed in the usual manner. Evaporation of the butanol gave a colorless powder; 6.1 g (82%); mp 206–207°C (dec); $[\alpha]_D^{29}$ −33.3° (*c* 2.1, 1-butanol/MeOH/H_2O, 1:1:1); R_f^{I} 0.6; R_f^{III} 0.8; amino acid ratios in acid hydrolysate: $Phe_{1.0}Glu_{2.1}Arg_{0.9}His_{1.1}Nle_{1.0}Asp_{1.0}$ (86%).

Anal. Calcd for $C_{67}H_{102}O_{21}N_{13}$: C, 56.4; H, 7.2; N, 12.8; O, 23.6. Found: C, 56.6; H, 7.4; N, 12.6; O, 23.9.

Phenylalanylglutamylarginylglutaminylhistidylnorleucylaspartic acid (XXI). The protected heptapeptide di-*t*-butyl ester (5.7 g) was hydrogenated in 2-propanol (200 ml) and 10% acetic acid (10 ml) and the product was isolated from the catalyst filtrates by lyophilization; 5.0 g (98%); $[\alpha]_D^{28}$ −23.7° (*c* 1.01, DMF); R_f^{I} 0.3; R_f^{III} 0.8. This material (2.73 g) was deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions on AG 1-X2. Chlorine positive fractions were pooled, evaporated to a small volume, and lyophilized; 1.90 g (100%); $[\alpha]_D^{28}$ −32.9° (*c* 1.016); R_f^{1} 0.1; R_f^{3} 1.4 × His; R_f^{III} 0.5; amino acid ratios in AP-M digest: $Phe_{1.0}Glu_{1.0}Arg_{0.9}Gln_{1.1}His_{1.1}Nle_{0.9}Asp_{1.0}$ (71%).

α,β - Di - t - butyl benzyloxycarbonylnorleucylphenylalanyl - γ - t - butylglutamyl - arginylglutaminylhistidylnorleucylaspartate diacetate. *N*-Hydroxysuccinimido benzyloxycarbonylnorleucinate (272 mg) in DMF (5 ml) was added at 0°C to a stirred solution of the heptapeptide di-*t*-butyl ester (646 mg) in water (0.5 ml), DMF (4.5 ml), and TEA (0.104 ml). The solution was stirred at room temperature for 22 hr; DAPA (0.02 ml) was added, and the solution was evaporated. The residue was suspended in 1-butanol and the suspension was washed in the usual manner. The butanol was removed, the residue was suspended in ethyl acetate (50 ml) and water (5 ml), and the gelatinous product was collected, washed with ethyl acetate, and dried; 539 mg (61%) $[\alpha]_D^{28}$ −20.4° (*c* 1.41, DMF); R_f^{I} 0.8; R_f^{III} 0.9; amino acid ratios in acid hydrolysate: $Nle_{1.9}Phe_{0.9}Glu_{2.1}Arg_{1.0}His_{1.1}Asp_{1.0}$ (95%).

Anal. Calcd for $C_{71}H_{110}O_{20}N_{14}$: C, 57.6; H, 7.5; N, 13.3; O, 21.6. Found: C, 57.6; H, 7.3; N, 13.6; O, 21.9.

Norleucylphenylalanylglutamylarginylglutaminylhistidylnorleucylaspartic acid trifluoroacetate (XXIV). The protected octapeptide di-*t*-butyl ester diacetate (263 mg) was hydrogenated in MeOH (25 ml) and 10% acetic acid (25 ml) and the product was isolated in the usual manner; 227 mg (94%); $[\alpha]_D^{28}$ −19.3° (*c* 1.27, DMF); R_f^{I} 0.5; R_f^{III} 0.7. This material (98 mg) was deblocked with TFA and precipitated with ether; 96 mg (90%); $[\alpha]_D^{28}$ −26.3° (*c* 0.858, H_2O); R_f^{1} 0.7; R_f^{3} 2.7 × His; R_f^{I} 0.2; R_f^{III} 0.5; amino acid ratios in AP-M digest: $Nle_{2.0}Phe_{0.9}Glu_{1.1}Arg_{0.9}Gln_{1.1}His_{1.0}Arg_{1.1}$ (47%).

t - *Butoxycarbonylnorleucylphenylalanylglutamylarginylglutaminylhistidylmethionyl - aspartic acid* d-*sulfoxide monoacetate dihydrate.* *N*-Hydroxysuccinimido *t*-butoxycarbonylnorleucinate (98 mg) in DMF (2 ml) was added to a solution of (XXII) tri-trifluoroacetate (*3*) (330 mg) in water (0.5 ml), DMF (4.5 ml), and TEA (0.14 ml). The mixture was stirred for 1 hr at 0°C and for 16 hr at room temperature and the solvent was evaporated. The product was dissolved in 1-butanol/MeOH/water (1:1:1) and was chromatographed on a column (1.8 × 14 cm) of AG 1-X2. Elution with 1-butanol/MeOH/acetic acid mixtures was performed essentially in the manner described (*2b*); the desired product was eluted with 1-butanol/MeOH/0.02 *M* acetic acid. Fractions containing homogeneous material (tlc) were pooled, evaporated to a small volume and lyophilized; 246 mg (74%); $[\alpha]_D^{28}$ −27.5° (*c* 0.54); R_f^{I} 0.3; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Nle_{1.0}Phe_{1.0}Glu_{2.1}Arg_{1.0}His_{0.9}Met_{0.7}Asp_{1.0}$ (79%).

Anal. Calcd for $C_{53}H_{86}O_{21}N_{14}S$: C, 49.4; H, 6.7; N, 15.2; O, 26.1; S, 2.5. Found: C, 49.8; H, 6.8; N, 15.3; O, 26.0; S, 2.7.

Norleucylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid d-*sulfoxide (XXV).* The protected octapeptide di-*t*-butyl ester (62 mg) was deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions on AG 1-X2. The peptide was isolated by lyophilization of the desired column effluents; 45 mg (85%); $[\alpha]_D^{28}$ −20.5° (*c* 1.02, H_2O); R_f^{1} 0.1; R_f^{3} 1.23 × His; R_f^{III} 0.4; amino acid ratios in AP-M digest: $Nle_{1.0}Phe_{1.0}Glu_{1.1}Arg_{1.0}Gln_{0.9}His_{0.9}\overset{O}{\overset{\|}{M}}et_{1.0}Asp_{1.0}$ (63%).

N^{α},N^{ϵ} - *Di* - t - *butoxycarbonyllysyl* - *γ* - t - *butylglutamylthreonylalanylalanylalanyl* - N^{ϵ} - t - *butoxycarbonyllysylphenylalanylglutamylarginylglutaminylserylmethionyl - aspartic acid* d-*sulfoxide.* From 330 mg of (XVIII) and 73.4 mg of (XIX); 146 mg (35%); R_f^{I} 0.3; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Lys_{2.5}Glu_{3.0}Thr_{0.9}Ala_{3.1}Phe_{0.9}Arg_{1.1}Ser_{0.8}Met_{0.6}Asp_{1.0}$ (95%).

12-Serine S-peptide$_{1-14}$ *(II).* The protected peptide *d*-sulfoxide (146 mg) was deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions; 94 mg (78%); $[\alpha]_D^{25}$ −49.8° (*c* 1.47); R_f^{I} origin; R_f^{III} 0.1; amino acid ratios in acid hydrolysate: $Lys_{2.0}Glu_{3.1}Thr_{1.0}Arg_{3.0}Phe_{1.0}Arg_{1.0}Ser_{0.9}Met_{0.5}Asp_{1.0}$ (78%). A sample of the *d*-sulfoxide was reduced with thioglycolic acid; $[\alpha]_D^{29}$ −54.3° (*c* 1.19); R_f^{III} 0.2; amino acid ratios in AP-M digest: $Lys_{2.1}Glu_{2.2}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{1.1}Gln_{0.8}Ser_{0.8}\overset{O}{\overset{\|}{M}}et_{0.9}Asp_{1.0}$ (78%).

12-β-(Pyrazolyl-3)-alanine S-peptide$_{2-14}$ *(IV).* The protected tridecapeptide (100 mg) which was prepared from 427 mg of (XV) and 130 mg of (XXIII) (*8*) was deblocked with TFA, and trifluoroacetate ions were exchanged for acetate ions to give 60 mg of the *d*-sulfoxide of (IV); $[\alpha]_D^{27}$ −48.4° (*c* 0.97, H_2O); R_f^{III} 0.2; amino acid ratios in acid hydrolysate: $Glu_{3.2}Thr_{1.0}Ala_{3.1}Lys_{1.0}Phe_{1.0}Arg_{1.0}Pyr_{0.7}Met_{0.7}Asp_{1.1}$ (101%); amino

acid ratios in AP-M digest: $Glu_{2.3}Thr_{1.0}Ala_{3.3}Lys_{0.9}Phe_{0.9}Gln_{0.9}Arg_{0.9}Pyr_{0.7}Met_{1.0}Asp_{1.1}$ (94%). A sample of this *d*-sulfoxide (55 mg) was reduced with thioglycolic acid to give 53 mg of (IV); $[\alpha]_D^{28}$ −54.0° (*c* 1.13); R_f^{III} 0.2; amino acid ratios in acid hydrolysate: $Glu_{3.3}Thr_{0.9}Ala_{3.0}Lys_{1.0}Phe_{1.0}Arg_{1.0}Pyr_{1.0}Met_{1.0}Asp_{1.0}$ (99%).

12-β-(Pyrazolyl-3)-alanine S-peptide$_{3-14}$ (*V*). The protected dodecapeptide (160 mg) which was prepared from 723 mg of (XIII) and 330 mg of (XXIII) (*8*) was deblocked with TFA, and trifluoroacetate ions were exchanged for acetate ions to give 137 mg of the *d*-sulfoxide of (V) (137 mg); $[\alpha]_D^{27}$ −47.2° (*c* 0.92, H_2O); R_f^{III} 0.2; amino acid ratios in acid hydrolysate: $Thr_{1.1}Ala_{3.1}Lys_{1.1}Phe_{1.0}Arg_{1.1}Glu_{2.2}Pyr_{0.6}Met_{0.8}Asp_{1.1}$ (87%); amino acid ratios in AP-M digest: $Thr_{1.1}Ala_{3.4}Lys_{1.2}Phe_{1.0}Arg_{1.0}Glu_{1.2}Gln_{0.6}Pyr_{0.7}$ O $Met_{0.9}Asp_{1.0}$ (72%). A sample of this *d*-sulfoxide (75 mg) was reduced with thioglycolic acid to afford 73 mg of (V); $[\alpha]_D^{28}$ −56.8° (*c* 1.05, H_2O); R_f^{I} 0.1; R_f^{III} 0.3; amino acid ratios in acid hydrolysate: $Thr_{1.0}Ala_{3.1}Lys_{1.0}Phe_{1.0}Glu_{2.2}Arg_{0.9}Pyr_{1.0}Met_{0.9}Asp_{1.0}$ (72%).

N^{α},N^{ϵ} - *Di* - t - *butoxycarbonyllysyl* - γ - t - *butylglutamylthreonylalanylalanylalanyl* - N^{ϵ} - t - *butoxycarbonyllysylphenylalanylglutamylarginylglutaminylhistidylnorleucyl* - *aspartic acid*. From 109 mg of (XVII) and 65.7 mg of (XXI); 47 mg (37%); R_f^{I} 0.5; R_f^{III} 0.7; amino acid ratios in acid hydrolysate: $Lys_{2.1}Glu_{3.1}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{0.8}His_{0.9}Nle_{1.0}Asp_{1.0}$ (80%).

13-Norleucine S-peptide$_{1-14}$ (*VI*). The protected peptide (25 mg) was deblocked with TFA. Yield 19 mg (84%); $[\alpha]_D^{27}$ −53.1° (*c* 2.10); R_f^{1} 0.1; R_f^{3} 0.8 × His; R_f^{I} origin; R_f^{III} 0.2; amino acid ratios in AP-M digest: $Lys_{2.1}Glu_{2.1}Thr_{1.0}Ala_{3.1}Phe_{0.9}Arg_{0.9}Gln_{1.0}His_{1.0}Nle_{1.0}Asp_{1.0}$ (74%).

Benzyloxycarbonyl - γ - t - *butylglutamylthreonylalanylalanylalanyl* - N^{ϵ} - t - *butoxy* - *carbonyllysylphenylalanylglutamylarginylglutaminylhistidylnorleucylaspartic acid*. From 90 mg of (XIV) and 56 mg of (XXI); 37 mg (46%); R_f^{I} 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Glu_{3.0}Thr_{1.0}Ala_{3.1}Lys_{1.1}Phe_{0.9}Arg_{0.9}His_{1.0}Nle_{1.0}Asp_{1.1}$.

13-Norleucine S-peptide$_{2-14}$ *hydrochloride* (*VII*). The protected peptide (22 mg) was deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions on AG 1-X2. This material was hydrogenated in 0.01 *N* HCl to remove the benzyloxycarbonyl group; 18 mg (70%); $[\alpha]_D^{24}$ −52.3° (*c* 1.06); R_f^{3} 0.9 × His; R_f^{III} 0.3; amino acid ratios in AP-M digest: $Glu_{2.0}Thr_{1.0}Ala_{3.0}Lys_{1.0}Phe_{0.8}Arg_{0.9}Gln_{1.1}His_{1.0}Nle_{1.0}Asp_{1.1}$ (78%).

Benzyloxycarbonylthreonylalanylalanylalanyl - N^{ϵ} - t - *butoxycarbonyllysylphenyl* - *alanylglutamylarginylglutaminylhistidylnorleucylaspartic acid*. From 72 mg of (XII) and 56 mg of (XXI); 21 mg (30%); R_f^{I} 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Thr_{1.0}Ala_{3.1}Lys_{1.0}Phe_{1.0}Glu_{2.1}Arg_{0.9}His_{1.0}Nle_{1.0}Asp_{1.0}$ (95%).

13-Norleucine S-peptide$_{3-14}$ (*VIII*). The protected peptide (17 mg) was first subjected to hydrogenolysis, then the product was further deblocked with TFA, and trifluoroacetate ions were exchanged for acetate ions; 14 mg (77%); $[\alpha]_D^{24}$ −35.1° (*c* 0.77); R_f^{3} 0.9 × His; R_f^{III} 0.4; amino acid ratios in AP-M digest: $Thr_{1.0}Ala_{3.1}Lys_{1.0}Phe_{1.0}Glu_{1.0}Arg_{0.9}Gln_{1.1}His_{0.9}Nle_{1.0}Asp_{1.0}$ (78%).

N^{α},N^{ϵ} - *Di* - t - *butoxycarbonyllysyl* - γ - t - *butylglutamylthreonylalanylalanylalanyl* - *norleucylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartic acid* d-*sulfoxide*. From 84 mg of (XVI) and 145 mg of (XXV); 59 mg (32%); R_f^{I} 0.4; R_f^{III} 0.6; amino acid ratios in acid hydrolysate: $Lys_{1.1}Glu_{3.2}Thr_{1.1}Ala_{3.2}Nle_{1.0}Phe_{1.0}Arg_{0.9}His_{0.9}Met_{0.8}Asp_{1.0}$ (86%).

7-Norleucine S-peptide$_{1-14}$ (*IX*). The protected peptide (36 mg) was deblocked with

TFA and trifluoroacetate ions were exchanged for acetate ions; 38 mg (88%); $[\alpha]_D^{24}$ −35.7° (*c* 0.755); R_f^3 0.6 × His; R_f^{III} 0.3; amino acid ratios in AP-M digest: $Lys_{1.0}$ $Glu_{2.2}Thr_{1.0}Ala_{3.3}Nle_{1.0}Phe_{1.0}Arg_{0.9}Gln_{0.8}His_{0.9}\overset{O}{\overset{\downarrow}{M}}et_{1.1}Asp_{1.0}$ (62%). The sulfoxide was reduced with thioglycolic acid; $[\alpha]_D^{24}$ −30.3° (*c* 0.80); R_f^3 1.0 × His; R_f^{III} 0.3; amino acid ratios in AP-M digest: $Lys_{1.4}$[5]$Glu_{2.1}Thr_{1.1}Ala_{3.4}Nle_{0.9}Phe_{1.0}Arg_{1.0}Gln_{1.0}His_{0.8}$ $Met_{0.8}Asp_{1.0}$ (60%).

N^{α},N^{ϵ} - *Di* - t - *butoxycarbonyllysyl* - γ - t - *butylglutamylthreonylalanylalanylalanyl* - *norleucylphenylalanylglutamylarginylglutaminylhistidylnorleucylaspartic acid.* From 88 mg of (XVI) and 95 mg of (XXIV); 56 mg (53%); R_f^1 0.5; R_f^{III} 0.7; amino acid ratios in acid hydrolysate: $Lys_{1.1}Glu_{3.2}Thr_{1.0}Ala_{3.1}Phe_{1.0}Arg_{0.9}His_{0.9}Nle_{1.9}Asp_{1.0}$ (93%).

7,13-Di-norleucine S-peptide$_{1-14}$ (*X*). The protected peptide (25 mg) was deblocked with TFA and trifluoroacetate ions were exchanged for acetate ions. Yield 25 mg (90%); $[\alpha]_D^{25}$ −47.4° (*c* 1.07); R_f^{III} 0.4; amino acid ratios in AP-M digest: $Lys_{1.0}Glu_{2.2}$ $Thr_{1.1}Ala_{3.2}Nle_{1.9}Phe_{0.9}Arg_{1.0}Gln_{0.9}His_{1.1}Asp_{1.0}$ (78%).

RESULTS AND DISCUSSION

Our original observation (*26*) that S-peptide$_{1-13}$ produced an active enzyme when added to S-protein demonstrated that a fragment of this peptide contained the structural features which are necessary for activation. The results of Potts et al. (*27*) showing that removal of the five C-terminal amino acid residues from S-peptide by digestion with carboxypeptidase A afforded a material which formed active enzyme with S-protein, confirmed our result.

By synthesizing analogs of S-peptide$_{1-14}$ (I), the smallest fully active fragment of natural S-peptide, and testing their ability to bind to and activate S-protein, it was possible to delineate binding sites within the peptide molecule and judge the relative importance of their contribution to the association between peptide and protein. It was shown that the hydrophobic sidechains of phenylalanine and methionine are involved in the formation of the ribonuclease S complex. The importance of the aromatic sidechain of phenylalanine is based on the observation that replacement of this residue by tyrosine is not detrimental to reformation of ribonuclease S, but that its substitution by aliphatic amino acid residues such as isoleucine or alanine reduce sharply the capacity of the resulting peptide to regenerate active enzyme (*28*).

The hydrophobic sidechain of methionine is a particularly strong binding site. Oxidation of its thioether sulfur to the sulfoxide or sulfone has a twofold effect on reformation of active enzyme (*3*). By inhibition experiments, it was possible to demonstrate that oxidation of the methionine alters drastically the binding ability of the peptide. For example, 12-Pyr S-peptide$_{1-14}$ (III) inhibits ribonuclease S 50% at a molar ratio of 1:1. The corresponding *d*-sulfoxide exhibits a 50% inhibition ratio of 600:1. The same behavior is observed with 12-(3-carboxymethylhistidine) S-peptide$_{1-14}$ (*8*). Furthermore, although the weakened binding is not evident from the early points in the activation curve, full activation is never reached using RNA as substrate when either S-peptide sulfone or S-peptide$_{1-14}$ *d*-sulfoxide are added to S-protein. We have demonstrated conclusively that the sulfur atom of methionine cannot be involved in ribonuclease catalysis since 13-(α-amino-*n*-butyric acid) S-peptide$_{1-13}$ combines with S-protein to afford active enzyme (*5*). Since it is generally assumed that the sidechain of norleucine exhibits the same degree of hydrophobicity as that of methionine, 13-Nle

[5] The Tris buffer peak overlapped with the lysine peak.

S-peptide$_{1-14}$ (VI) was tested for its ability to activate S-protein. This peptide brings about a 75% activation at a molar ratio of 1:1 (Fig. 1); however, considerably higher ratios resulted in only slightly higher activation. Full activation could not be achieved with this peptide. Competition experiments in which 12-Pyr S-peptide$_{1-14}$ was compared with 13-Nle S-peptide$_{1-14}$ (Fig. 1) indicated that the two compounds have essentially the same affinity for S-protein. These results agree with those of Rocchi et al. (*20*) who compared the S-protein activation of 10-Orn S-peptide$_{1-20}$ with that of 10-Orn 13-Nle S-peptide$_{1-20}$. It may be reasoned that the active site which is formed by the combination of S-protein with 13-Nle S-peptide$_{1-14}$ or with the oxidized forms of S-peptide or

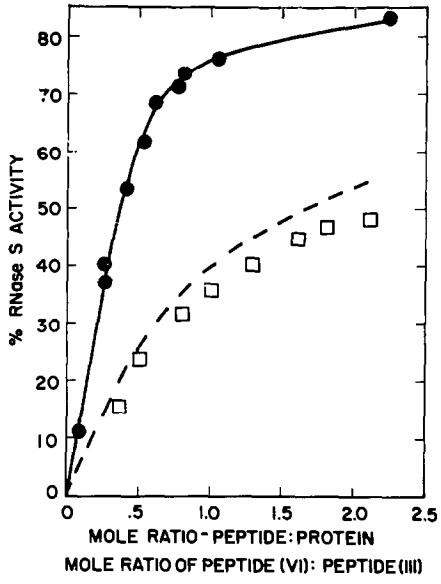


FIG. 1. Activation of S-protein by 13-Nle S-peptide$_{1-14}$ (VI) —●— and reactivation of a 1:1 mixture of 12-Pyr S-peptide$_{1-14}$ (III) and S-protein by (VI) —□—. The dashed line is the calculated curve which would be obtained if both peptides bind equally well to S-protein and if 80% activity represents full reactivation.

S-peptide$_{1-14}$ differs slightly from that obtained with reduced S-peptide or S-peptide$_{1-14}$ and that this deformation may explain the differences in catalytic efficiency. Such a hypothesis appears reasonable since methionine is situated in close proximity to histidine-12, which is essential for catalysis. In addition to its essential function in ribonuclease catalysis, histidine-12 contributes to binding through the aromatic azole ring. The characteristic ionization behavior of the imidazole ring has already been excluded as a factor in binding by the demonstration that 12-Pyr S-peptide$_{1-14}$ (III) associates as firmly with S-protein as natural S-peptide (*8*). We have now replaced His-12 by serine in S-peptide$_{1-14}$. As may have been anticipated, the resulting 12-Ser S-peptide$_{1-14}$ (II) fails to activate S-protein, but rather inhibits RNase S competitively (Fig. 2). The substitution of the azole ring by an aliphatic hydroxymethyl group lowers the binding capacity approximately sevenfold.

Hydrophobic binding sites are not alone responsible for the strong noncovalent interaction of peptide and protein. The marked increase in binding observed when the chain of S-peptide$_{1-13}$ is lengthened by the addition of aspartic acid-14 clearly demon-

strates the role of a charged group in binding (*3*). Furthermore, in a previous study (*5*) we compared the 50% S-protein activation ratio of S-peptide$_{1-13}$ with that of S-peptide$_{2-13}$ and found the latter peptide to be approximately 50 times less active than

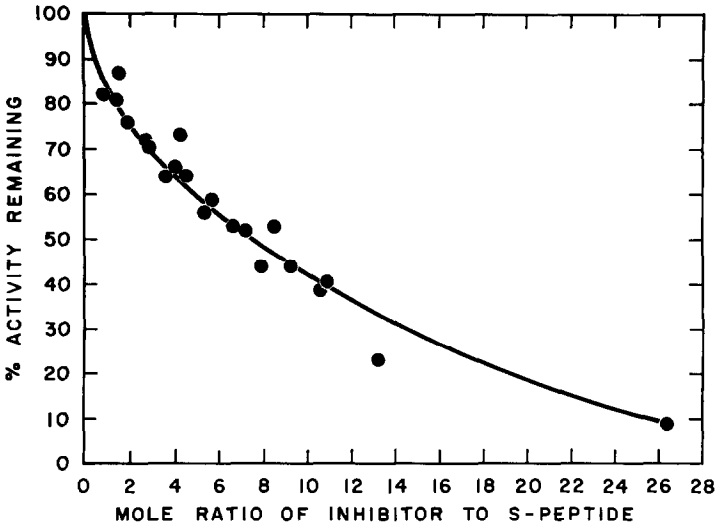


FIG. 2. Inhibition of RNase S with 12-Ser S-peptide$_{1-14}$ (II).

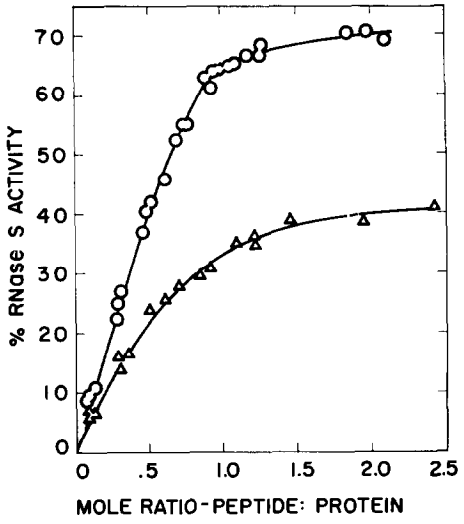


FIG. 3. Activation of S-protein by 13-Nle S-peptide$_{2-14}$ (VII) —o— and 13-Nle S-peptide$_{3-14}$ VIII) —△—.

the former. We concluded from this experiment that Glu-2 was a binding site of S-peptide. We have now compared the S-protein activating potential of 13-Nle S-peptide$_{1-14}$ (VI), 13-Nle S-peptide$_{2-14}$ (VII), and 13-Nle S-peptide$_{3-14}$ (VIII) and observed essentially the same behavior (Figs. 1 and 3). The desLys peptide is somewhat less active than the 1–14 fragment, but the peptide which lacks both Lys-1 and Glu-2 is considerably less potent.

Since the inhibitor approach provides a more sensitive criterion for binding, we have also compared the inhibition of ribonuclease S by 12-Pyr S-peptide$_{1-14}$ (III) with that of 12-Pyr S-peptide$_{2-14}$ (IV) and 12-Pyr S-peptide$_{3-14}$ (V) (Figs. 4 and 5). Like 12-Pyr S-peptide$_{1-14}$, the 1-desLys member of this series exhibits a 50% inhibition ratio of approximately 1 and thus binds as firmly to S-protein as natural S-peptide. Once again, removal of both Lys-1 and Glu-2 significantly lowers the inhibitory capacity. Interestingly, Moroder et al. (29) observed little difference in the S-protein activating properties of 10-Orn S-peptide$_{1-20}$, 10-Orn S-peptide$_{2-20}$, and 10-Orn

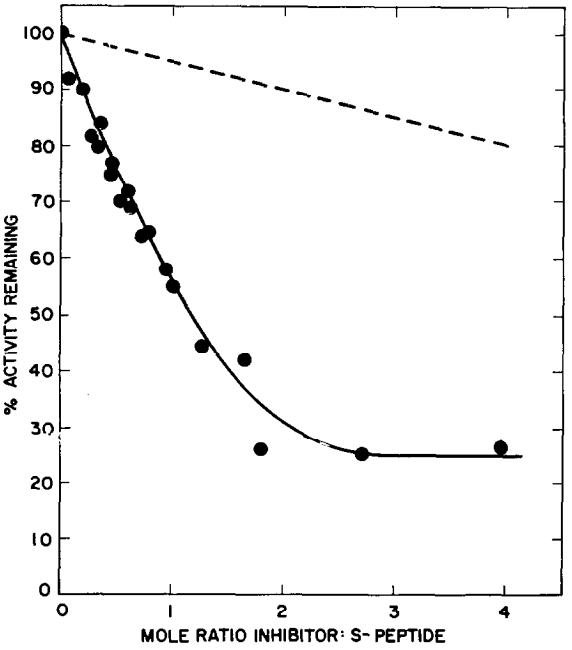


FIG. 4. Inhibition of RNase S by 12-Pyr S-peptide$_{2-14}$ (IV). The dashed line illustrates the inhibition by 12-Pyr S-peptide$_{3-14}$ (V) on the same scale.

S-peptide$_{3-20}$ in the same system. Recently, we have shown (*8*) that the replacement of Arg by Orn or N^{δ}-formylOrn in 12-Pyr S-peptide$_{1-14}$ diminishes significantly its ability to antagonize RNase S. Based on these results, we concluded that Arg and Orn are not equivalent as concerns the S-peptide–S-protein system and that ionic interactions between the sidechains of Arg-10 and Glu-2 may contribute to the stability of RNase S. This electrostatic attraction is not possible when Arg-10 is replaced by Orn since the Orn sidechain is shorter than that of Arg and thus may not be capable of interacting with the γ-carboxyl group of Glu-2. The observation that blocking of the δ-amino group of Orn does not alter the binding appears to support these conclusions. In this connection, it is of interest to note that Kerling (*30*) has shown that lengthening the sidechain of Arg-10 as in 10-homoArg 13-Leu S-peptide$_{1-13}$ similarly diminishes peptide-S-protein binding.

The arginine sidechain appears to be critical for maximum interaction with the γ-carboxyl group of glutamic acid-2 in the S-peptide–S-protein complex. Kartha (*31*) has indicated that the R groups of Glu-2 and Arg-10 are within hydrogen bonding

distance in the RNase A crystal. Unfortunately, neither residue sidechain is clearly resolved in the RNase S-2-Å electron-density map (*4b*).

Rocchi et al. (*11*) prepared 7-desLys 10-Orn S-peptide$_{1-20}$ and found that this peptide behaved both as an activator of S-protein with cytidine 2′, 3′ phosphate as

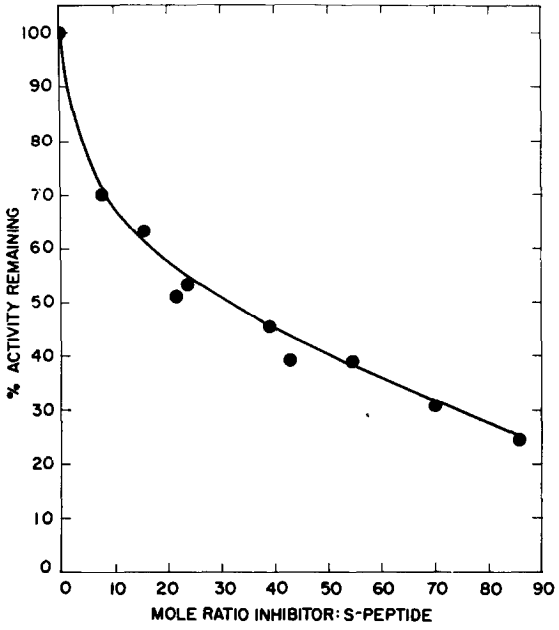


FIG. 5. Inhibition of RNase S by 12-Pyr S-peptide$_{3-14}$ (V).

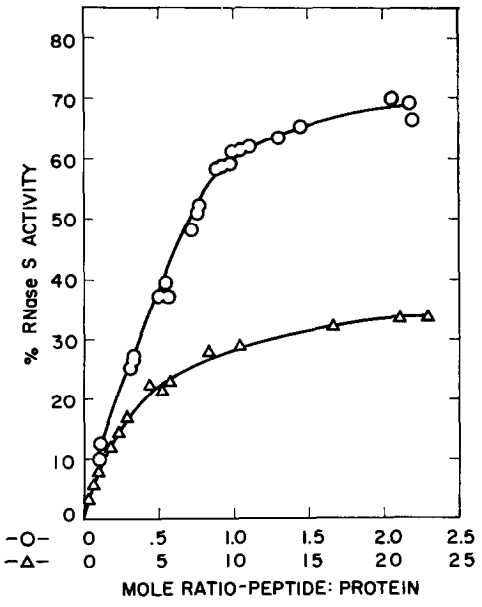


FIG. 6. Activation of S-protein by 7-Nle S-peptide$_{1-14}$ (IX) —o— and its *d*-sulfoxide —△—.

substrate and an inhibitor of RNase S using RNA hydrolysis as a measure of activity. These authors postulated that the peculiar properties of this peptide can be explained in terms of its inability to form an enzyme–substrate complex with RNA. They also suggested that Lys-7 was involved in substrate binding. The elimination of an amino acid residue from the middle of the peptide chain alters the relative positions of the remainder of the amino acid residues and complicates interpretation of biological results. The interesting question of the role of Lys-7 in substrate binding prompted us

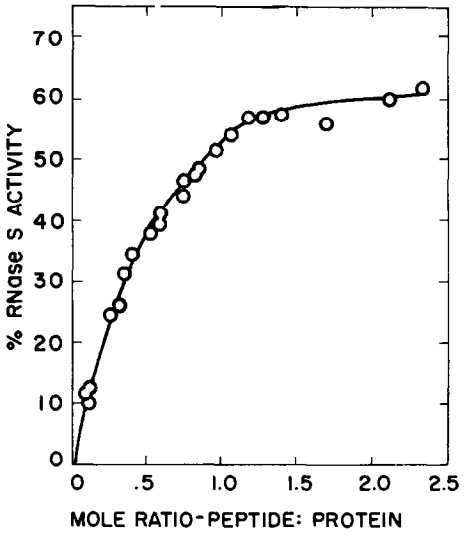


FIG. 7. Activation of S-protein by 7,13-di-Nle S-peptide$_{1-14}$ (X).

to synthesize analogs of S-peptide$_{1-14}$ in which Lys-7 is not eliminated but replaced by the hydrophobic norleucine residue. 7-Nle S-peptide$_{1-14}$ (IX) is not an inhibitor of RNase S. This peptide and the analog (X) in which both Lys-7 and Met-13 are replaced by Nle activate S-protein equally well, but neither is as effective as S-peptide$_{1-14}$ (Figs. 6 and 7). The *d*-sulfoxide of peptide (IX) exhibits weak S-protein activating activity. Based on these results, Lys-7 cannot be regarded as an essential substrate binding site.

Enzyme studies with synthetic fragments or analogs of S-peptide have provided convincing evidence that the strong and specific noncovalent interaction between S-peptide and S-protein depends on the subtle interplay between hydrophobic and ionic interactions of as yet not completely identified amino acid residues of S-protein and residues comprising positions 2–14 in S-peptide.

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REFERENCES

1. F. M. RICHARDS, *Proc. Nat. Acad. Sci. U.S.* **44**, 162 (1958).
2. (a) K. HOFMANN, M. J. SMITHERS, AND F. M. FINN, *J. Amer. Chem. Soc.* **88**, 4107 (1966). (b) K. HOFMANN, J. P. VISSER, AND F. M. FINN, *J. Amer. Chem. Soc.* **91**, 4883 (1969).

3. K. HOFMANN, F. M. FINN, M. LIMETTI, J. MONTIBELLER, AND G. ZANETTI, *J. Amer. Chem. Soc.* **88**, 3633 (1966).
4. (a) H. W. WYCKOFF, K. D. HARDMAN, N. M. ALLEWELL, T. INAGAMI, L. N. JOHNSON, AND F. M. RICHARDS, *J. Biol. Chem.* **242**, 3984 (1967). (b) H. W. WYCKOFF, D. TSERNOGLOU, A. W. HANSON, J. R. KNOX, B. LEE, AND F. M. RICHARDS, *J. Biol. Chem.* **245**, 305 (1970).
5. F. M. FINN AND K. HOFMANN, *J. Amer. Chem. Soc.* **87**, 645 (1965).
6. A. M. CRESTFIELD, W. H. STEIN, AND S. MOORE, *J. Biol. Chem.* **238**, 2413, 2421 (1963).
7. F. M. FINN AND K. HOFMANN, *J. Amer. Chem. Soc.* **89**, 5298 (1967).
8. K. HOFMANN, J. P. VISSER, AND F. M. FINN, *J. Amer. Chem. Soc.* **92**, 2900 (1970).
9. F. M. FINN, J. P. VISSER, AND K. HOFMANN, *in* "Peptides" (E. Bricas, Ed.), p. 330. North-Holland Publishing Company, Amsterdam, 1968.
10. D. L. EAKER, T. P. KING, AND L. C. CRAIN, *Biochemistry* **4**, 1486 (1965).
11. R. ROCCHI, F. MARCHIORI, L. MORODER, G. BORIN, AND E. SCOFFONE, *J. Amer. Chem. Soc.* **91**, 3927 (1969).
12. (a) K. HOFMANN, R. SCHMIECHEN, R. D. WELLS, Y. WOLMAN, AND N. YANAIHARA, *J. Amer. Chem. Soc.* **87**, 611 (1965). (b) K. HOFMANN, W. HAAS, M. J. SMITHERS, R. D. WELLS, Y. WOLMAN, N. YANAIHARA, AND G. ZANETTI, *J. Amer. Chem. Soc.* **87**, 620 (1965). (c) K. HOFMANN, W. HAAS, M. J. SMITHERS, AND G. ZANETTI, *J. Amer. Chem. Soc.* **87**, 631 (1965). (d) K. HOFMANN, R. SCHMIECHEN, M. J. SMITHERS, R. D. WELLS, Y. WOLMAN, AND G. ZANETTI, *J. Amer. Chem. Soc.* **87**, 640 (1965). (e) K. HOFMANN AND H. BOHN, *J. Amer. Chem. Soc.* **88**, 5914 (1966).
13. J. HONZL AND J. RUDINGER, *Czech. Chem. Commun.* **26**, 2333 (1961).
14. R. SCHWYZER, B. ISELIN, H. KAPPELER, B. RINIKER, W. RITTEL, AND H. ZUBER, *Helv. Chim. Acta* **46**, 1975 (1963).
15. R. HOLLEY AND E. SONDHEIMER, *J. Amer. Chem. Soc.* **76**, 1326 (1954).
16. K. HOFMANN, W. D. PECKHAM, AND A. RHEINER, *J. Amer. Chem. Soc.* **78**, 238 (1956).
17. G. W. ANDERSON, J. E. ZIMMERMAN, AND F. M. CALLAHAN, *J. Amer. Chem. Soc.* **88**, 1338 (1966).
18. R. SCHWYZER AND H. KAPPELER, *Helv. Chim. Acta* **44**, 1991 (1961).
19. R. ROCCHI, F. MARCHIORI, A. SCATTURIN, AND E. SCOFFONE, *J. Chem. Soc. C*, 86 (1967).
20. R. ROCCHI, A. SCATTURIN, L. MORODER, F. MARCHIORI, A. M. TAMBURRO, AND E. SCOFFONE, *J. Amer. Chem. Soc.* **91**, 492 (1969).
21. N. IZUMIYA, H. UCHINO, AND T. YAMASHITA, *Nippon Kaguku Zasshi* **79**, 420 (1958); *Chem. Abstr.* **54**, 4408i (1960).
22. J. S. FRUTON, *J. Biol. Chem.* **146**, 463 (1942).
23. J. PLESS AND R. A. BOISSONNAS, *Helv. Chim. Acta* **46**, 1609 (1963).
24. R. ZABEL AND H. ZAHN, *Z. Naturforsch. B* **20**, 650 (1965).
25. G. W. ANDERSON, J. E. ZIMMERMAN, AND F. M. CALLAHAN, *J. Amer. Chem. Soc.* **86**, 1839 (1964).
26. K. HOFMANN, F. FINN, W. HAAS, M. J. SMITHERS, Y. WOLMAN, AND N. YANAIHARA, *J. Amer. Chem. Soc.* **85**, 833 (1963).
27. J. T. POTTS, JR., M. YOUNG, AND C. B. ANFINSEN, *J. Biol. Chem.* **238**, PC 2593 (1963).
28. E. SCOFFONE, R. ROCHHI, F. MARCHIORI, L. MORODER, A. MARZOTTO, AND A. M. TAMBURRO, *J. Amer. Chem. Soc.* **89**, 5450 (1967).
29. L. MORODER, F. MARCHIORI, R. ROCCHI, A. FONTANA, AND E. SCOFFONE, *J. Amer. Chem. Soc.* **91**, 3921 (1969).
30. K. E. TH. KERLING, personal communication.
31. G. KARTHA, personal communication.