Studies on Polypeptides. XLIII. Synthesis of S-Peptide$_{1-20}$ by Two Routes

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Abstract: S-Peptide$_{1-20}$ was synthesized by two routes from a common intermediate. Within the limits of error the synthetic material was indistinguishable from natural S-peptide by ability to activate S-protein, optical rotation, thin layer chromatography, and amino acid ratios in AP-M digests. The refinements in synthetic methodology and purification procedures which are described have made *fully active* synthetic S-peptide readily available. The formation of ribonuclease S′ from the combination of synthetic S-peptide$_{1-20}$ with natural S-protein constitutes the first partial synthesis of this enzyme.

Richards[5] cleaved pancreatic ribonuclease A with subtilisin and from the digest isolated two fragments which he named S-peptide and S-protein. Neither fragment exhibited enzymic activity but an equimolar mixture of the two fragments (ribonuclease S′) possessed the full catalytic power of the original enzyme.

The correct amino acid sequence of S-peptide (Scheme I) was established in 1962.[6]

In 1966,[3] we reported a synthesis of S-peptide$_{1-20}$ and provided evidence to show that the synthetic compound combined with S-protein to form a ribonuclease S′ whose activity toward RNA was indistinguishable from that of the enzyme regenerated from natural S-peptide and S-protein. These experiments constituted a partial synthesis of ribonuclease S′.

In this paper we present the experimental details of the original synthesis and relate an alternate, more convenient, route to fully active S-peptide$_{1-20}$.

Preparative Aspects

The key intermediate for both routes (Scheme I) is the protected tetrapeptide hydrazide (I)[7] whose chain can be elongated from either the carboxyl or the amino end.[8] Hydrogenolysis converts I into the hydrazide II with simultaneous reduction of the nitroarginine residue to arginine. Using the Rudinger modification,[9] this hydrazide *via* the azide was coupled with the *d*-sulfoxide of histidylmethionylaspartylserylserylthreonylserylalanylalanine monoacetate trihydrate[10] (III). The ensuing partially protected tridecapeptide (IV) was readily separated from unreacted amino component (III) by countercurrent distribution in the solvent system 1-butanol–10% acetic acid. Trifluoroacetic acid deprotection followed by exchange of trifluoroacetate ions by acetate ions on Amberlite IRA-400 gave the free tridecapeptide (V) which was isolated in the form of the acetate hexahydrate.

The protected heptapeptide azide (VI)[11] served to introduce the N-terminal segment into the S-peptide molecule.

In earlier studies directed to the synthesis of S-peptide fragments[12] and S-peptide,[3] we employed the solid azide (VI) for this purpose, but the yields of the desired coupling products were unsatisfactory. Recently, we have realized improved yields in this step by the use of the Rudinger procedure.[9] The mixture of products resulting from coupling by this technique of the azide (VI) with V was distributed between 1-butanol and 2% acetic acid to remove unchanged amino component. Then, for removal of unreacted azide and azide rearrangement products, the butanol-soluble material was dissolved in a 1:1:1 mixture of 2-propanol–methanol–water and the desired partially protected S-peptide *d*-sulfoxide (VII) was adsorbed at 0° on Dowex 50W-X2 in a batch process. 2-Propanol–methanol–2% ammonium hydroxide eluted the desired product (VII) from the resin. The ensuing partially protected S-peptide *d*-sulfoxide (VII), without further purification, was deblocked with trifluoroacetic acid to give crude S-peptide *d*-sulfoxide which was purified by chromatography on CMC. The resulting material formed a single spot on thin layer chromatography (Figure 2) and its behavior on paper electrophoresis at various pH values was indistinguishable from that of S-peptide

(1) See N. Yanaihara, C. Yanaihara, G. Dupuis, T. Beacham, R. Camble, and K. Hofmann, *J. Amer. Chem. Soc.*, **91**, 2184 (1969), for paper XLII in this series.

(2) Supported by grants from the U. S. Public Health Service and the National Science Foundation.

(3) A preliminary communication of some of the results presented in this study has appeared: K. Hofmann, M. J. Smithers, and F. M. Finn, *J. Amer. Chem. Soc.*, **88**, 4107 (1966).

(4) The amino acid residues are of the L configuration. Abbreviations used are: Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; Narg = nitroarginine; O-*t*-Bu = *t*-butyl ester; TEA = triethylamine; TFA = trifluoroacetic acid; DMF = dimethylformamide; CMC = carboxymethylcellulose; AG-1X2 = anion-exchange resin (Bio-Rad); AP-M = aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

(5) F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958); RNase S, subtilisin modified beef ribonuclease A; S-peptide, the peptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar proportions of S-protein and S-peptide. According to M. S. Doscher and C. H. W. Hirs, *Biochemistry*, **6**, 304 (1967), natural S-peptide is a mixture of closely related compounds containing mainly S-peptide$_{1-20}$. For clarity natural S-peptide will be designated "S-peptide."

(6) (a) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 237, 1845 (1962); (b) J. T. Potts, A. Berger, J. Cooke, and C. B. Anfinsen, *ibid.*, 237, 1851 (1962); (c) E. Gross and B. Witkop, *ibid.*, 237, 1856 (1962).

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

(8) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *ibid.*, **74**, 470 (1952).

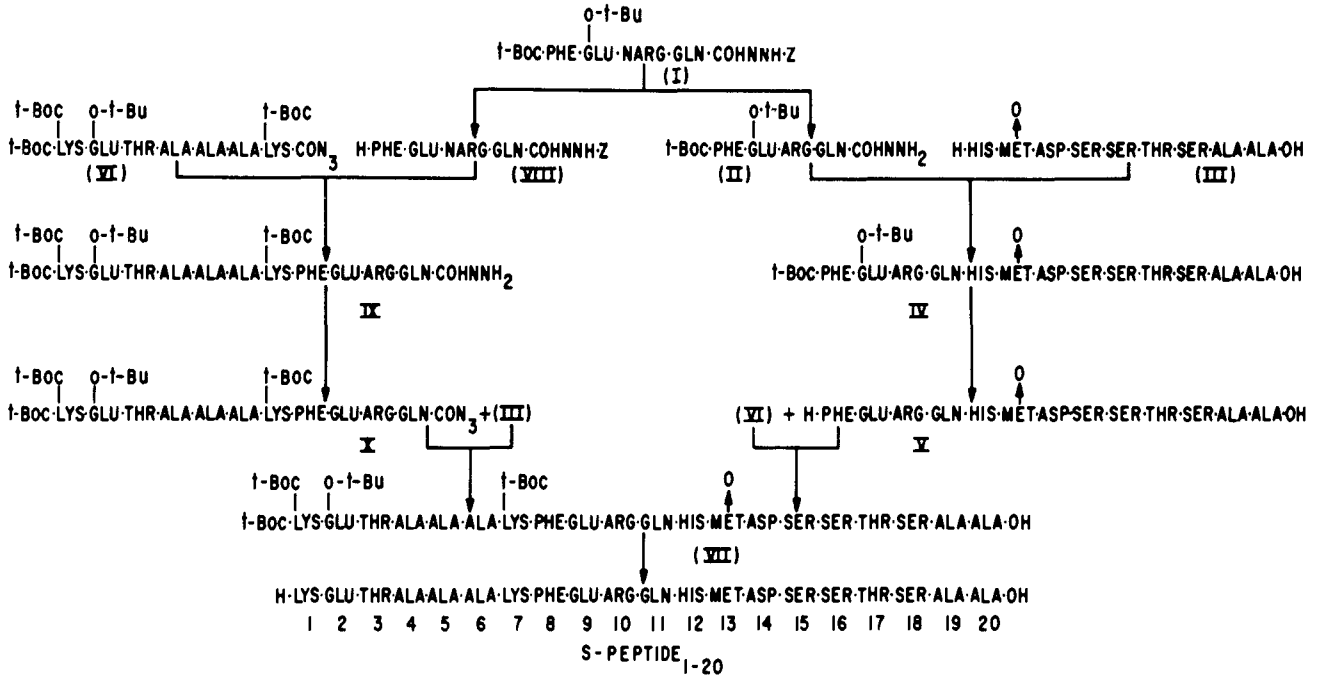
(9) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

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

(11) K. Hofmann, R. Schmiechen, R. D. Wells, Y. Wolman, and N. Yanaihara, *ibid.*, **87**, 611 (1965).

(12) K. Hofmann, R. Schmiechen, M. J. Smithers, R. D. Wells, Y. Wolman, and G. Zanetti, *ibid.*, **87**, 640 (1965).

Scheme I



sulfone, prepared from the natural material by oxidation with performic acid.[13]

An alternate more convenient route to S-peptide *d*-sulfoxide (Scheme I) also uses the protected tetrapeptide hydrazide (I) as the key intermediate. In this case exposure to trifluoroacetic acid removed the *t*-butoxycarbonyl and *t*-butyl ester groups from I with formation of the benzyloxycarbonylhydrazide (VIII). This compound was then coupled with VI and the resulting mixture was first washed with water and then exhaustively hydrogenated. The desired protected undecapeptide hydrazide (IX) was isolated from the mixture of hydrogenation products by adsorption on Dowex 50W-X2 and elution with 2-propanol–methanol–2% ammonium hydroxide. The azide (X) was then coupled with the nonapeptide (III). Chromatographically homogeneous partially protected S-peptide *d*-sulfoxide (VII) was isolated from the reaction mixture by a single chromatographic step on the ion-exchange resin AG-1X2 using a mixture of 1-butanol–methanol–aqueous acetic acid of increasing normality for elution.

Amino acid composition of acid hydrolysates and behavior on thin layer chromatography of the partially protected S-peptide *d*-sulfoxide (VII) prepared by this route agreed well with the same properties of VII prepared by the earlier method.

The partially protected S-peptide *d*-sulfoxide was deblocked in the usual manner. Optical rotation and thin layer chromatograms of the ensuing product showed good agreement with those of S-peptide *d*-sulfoxide prepared by the first route. Reduction with thioglycolic acid of the *d*-sulfoxide prepared by either procedure gave an S-peptide which was indistinguishable from the natural compound by its ability to fully activate S-protein (Figure 1), optical rotation, amino acid ratios in AP-M digest (Table I), and thin layer chromatography (Figure 2).

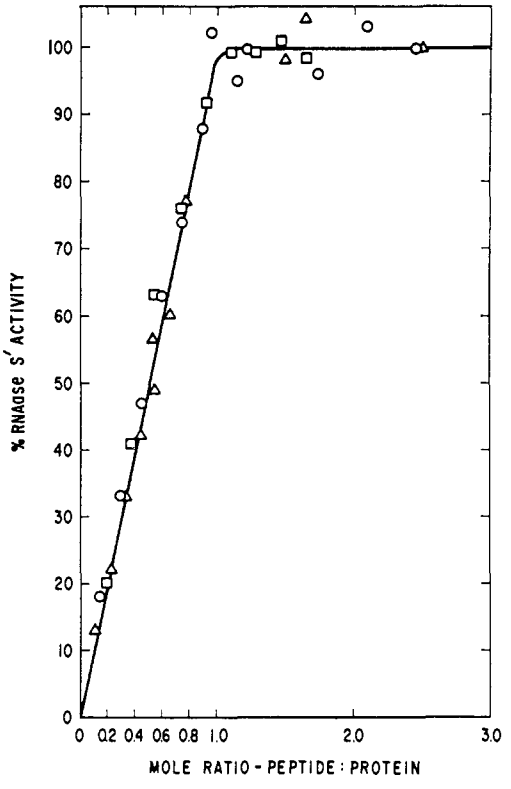


Figure 1. Activity of partially synthetic and natural ribonucleases S′ using yeast RNA as substrate: ○, natural enzyme; △, partially synthetic enzyme, method a; □, partially synthetic enzyme, method b. Each point is based on triplicate assays. For assay conditions see text.

Experimental Section[14]

A. Peptide Syntheses. Phenylalanylglutamylnitroarginylglutamine "Benzyloxycarbonylhydrazide" Monoacetate (VIII).[15] The

(13) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).

(14) Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 mμ and extrapolated to the 589-mμ sodium line. Elemental analyses were performed by Schwarzkopf

Table I. Amino Acid Ratios in AP-M Digests and Optical Rotation in 10% Acetic Acid of Natural and Synthetic S-Peptide

	Lys	Glu	Thr	Ala	Phe	Arg	Gln	His	Met	Asp	Ser	Rec,[a] %	$[\alpha]^{28}D$,[b] deg
Natural	2.40	2.16	1.95	4.81	0.99	0.96	0.89	1.08	0.85	1.06	2.86	75	−74.9 (0.78)
Syn a	2.36	2.16	2.12	5.25	0.86	0.96	0.90	0.86	0.77	0.86	2.70	75	−75.5 (0.46)
Syn b	2.24	2.13	2.11	5.18	0.96	1.04	0.89	0.89	0.85	0.89	2.67	85	−74.5 (0.86)

[a] Recoveries based on Lys, Glu, Phe, and Arg. [b] The numbers in parentheses are concentrations based on peptide content.

protected hydrazide (I)[7] (5.0 g) was dissolved in TFA (20 ml), and the solution was kept at room temperature for 30 min. Ice-cold ether (approximately 200 ml) was added and the mixture was kept in a refrigerator at −10° for 2 hr. The precipitate was collected, washed with ice-cold ether, and dried at room temperature over KOH pellets. The material was then dissolved in water (100 ml) and the solution was passed through a column of acetate cycle Amberlite IRA-400 (2 × 40 cm). The column was washed with water and ninhydrin-positive eluates were combined and evaporated to dryness. The residue was suspended in water, glacial acetic acid was added to dissolve the material, and the solution was lyophilized: 3.9 g (87%); $[\alpha]^{29}D$ −26.2° (*c* 1.02, 10% acetic acid); R_f^I 0.6; amino acid ratios in AP-M digest: $Phe_{0.99}Glu_{1.04}Narg_{1.00}Gln_{0.98}$ (recovered 87%).

Anal. Calcd for $C_{33}H_{45}O_{11}N_{11}\cdot CH_3COOH$: C, 50.5; H, 5.9; N, 18.5; O, 25.0. Found: C, 50.3; H, 6.2; N, 18.2; O, 24.7.

N^{α},N^{ϵ}-Di-*t*-butoxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-*t*-butoxycarbonyllysylphenylalanylglutamylarginylglutamine Hydrazide Monoacetate Dihydrate (IX).[15] The hydrazide VI[11] (2.176 g; 2 mmol) was dissolved in freshly distilled DMF (80 ml). The solution was cooled at −25 to −30° and 6.9 *N* hydrogen chloride in dioxane (1.16 ml; 8 mmol) was added, followed by 10% *t*-butyl nitrite in DMF (2.6 ml; 2.2 mmol). The very viscous DMF solution liquified on addition of the acid. The mixture was stirred at −25 to −30° for 30 min when a sample spotted on filter paper was hydrazide negative. The mixture was cooled at −60° and TEA (0.10 ml; 8 mmol) was added. The solution at this point should turn wet Alkacid paper green. Additional TEA (10% in DMF) was added if this was not the case. A solution of VIII (1.66 g; 2 mmol) in DMF (20 ml) and TEA (0.28 ml; 2 mmol) was added dropwise with stirring to the azide solution and the mixture was stirred at 4° for 20 hr and at room temperature for 20 hr. The solvents were removed, the residue was triturated with water (80 ml), and the suspension was filtered. The solid was washed twice more with water (80 ml) in a similar manner and was hydrogenated for 24 hr over palladium in 200 ml of 2-propanol–methanol–2.5 *N* acetic acid (1:1:1). The catalyst was removed by filtration, the filtrate was cooled at 0°, and Dowex 50W-X2 (40 ml settled in 2-propanol–methanol–water) was added with stirring. The suspension was stirred at 0° for 1 hr and was then filtered. The resin was resuspended in 200 ml of 2-propanol–methanol–water (1:1:1) and the suspension was stirred for 30 min at 0° and filtered. This procedure was repeated until the filtrate was chlorine negative, then the resin was exhaustively eluted with 2-propanol–methanol–2% ammonium hydroxide in a batch process. The combined eluates were evaporated to a small volume and lyophilized: 2.42 g (69%); R_f^I 0.6; amino acid ratios in acid hydrolysate: $Lys_{2.00}Glu_{2.95}Thr_{0.92}Ala_{3.20}Phe_{1.02}Arg_{0.91}$.

Anal. Calcd for $C_{76}H_{133}O_{27}N_{19}\cdot CH_3COOH\cdot 2H_2O$: C, 52.3; H, 7.7; N, 15.2; O, 24.8. Found: C, 52.9; H, 8.0; N, 14.8; O, 24.3.

Microanalytical Laboratory, Woodside, N. Y. Oxygen values were determined and not computed by difference. The amino acid composition of acid and enzymic hydrolysates was 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). Paper chromatograms were performed on Whatman No. 1 filter paper by the descending technique with the following solvent systems: R_f^1, 1-butanol–acetic acid–water, 4:1:5 (upper phase); R_f^3, 1-butanol–pyridine–acetic acid–water, 45:30:9:36. Thin layer chromatograms were performed with the following solvent systems: R_f^I, 1-butanol–acetic acid–water, 60:20:20; R_f^{III}, 1-butanol–pyridine–acetic acid–water, 30:20:6:24. The hydrazide reaction was performed according to H. Ertel and L. Horner, *J. Chromatogr.*, **7**, 268 (1962); AP-M digests were performed as described [K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966)]. Paper electropherograms were carried out in pyridinium acetate buffers with a Wieland-Pfleiderer Pherograph using Whatman No. 3 paper; 1600 V, 1.5 hr. Papers were developed with ninhydrin and Pauly reagents.

(15) F. M. Finn and K. Hofmann, *ibid.*, **89**, 5298 (1967).

***t*-Butoxycarbonylphenylalanyl-γ-*t*-butylglutamylarginylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine *d*-Sulfoxide Hexahydrate (IV).**[3] An azide solution was prepared from II[7] (923 mg; 1 mmol) in DMF (10 ml) with 6.9 *N* HCl in dioxane (0.72 ml; 5 mmol) and *t*-butyl nitrite (0.13 ml; 1.1 mmol) at −25 to −30°. The solution was stirred for 30 min when the hydrazide test was negative. The solution was cooled at −60° and

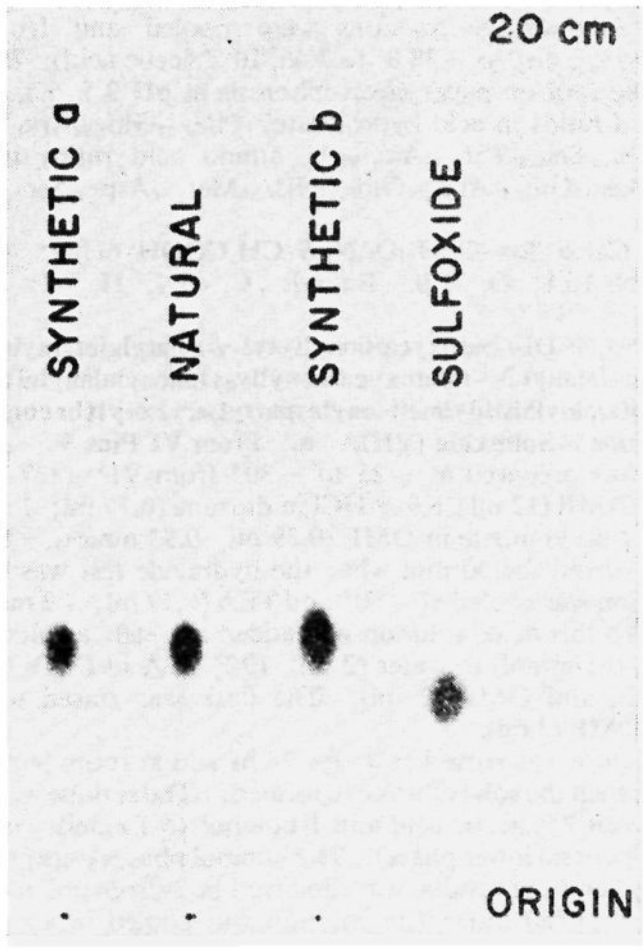


Figure 2. Photograph of a 20 × 20 cm thin layer chromatogram developed for 8 hr in system III. Compounds (three S-peptide preparations and S-peptide *d*-sulfoxide) were spotted 2 cm from the lower edge of the plate. Materials were visualized by the chlorine test and immediately photographed. The slight tailing noticeable in all three S-peptide preparations can be attributed to the presence of trace amounts of S-peptide sulfoxide.

TEA (0.69 ml; 5 mmol) was added. To this azide solution was added at −60° a solution of III[10] (518 mg; 0.5 mmol) in water (10 ml), TEA (0.21 ml; 1.5 mmol), and DMF (10 ml). The flask was rinsed with two 2-ml portions of 50% aqueous DMF. The mixture was stirred at 4° for 27 hr and at room temperature for 72 hr; then the solvent was evaporated. The residue was distributed between 1-butanol and 2% acetic acid (6 funnels, 50 ml of upper and 50 ml of lower phase each) and the pooled aqueous phases were evaporated. The residue dissolved in 10% acetic acid (80 ml) was added to the first eight tubes of a countercurrent machine and distributed in the solvent system 1-butanol–10% acetic acid (187 transfers). The contents of tubes 74–99, which contained the desired product, were pooled, the solvent was evaporated, and the residue was lyophilized: 351 mg (41%); $[\alpha]^{23}D$ −37.4° (*c* 2.07, 10% acetic acid); R_f^1 0.5; R_f^3 2.3 × His; single ninhydrin-negative, chlorine-, Pauly-, and Sakaguchi-positive spot; amino acid ratios in acid hydrolysate: $Phe_{0.98}Glu_{1.97}Arg_{1.03}His_{1.03}Met_{0.76}Asp_{1.02}Ser_{2.99}Thr_{0.93}Ala_{2.05}$.[16]

Anal. Calcd for $C_{68}H_{107}O_{26}N_{19}S\cdot 6H_2O$: C, 46.8; H, 6.9; N, 15.2; O, 29.3; S, 1.8. Found: C, 46.5; H, 7.3; N, 15.6; O, 29.2; S, 2.3.

(16) Values are not corrected for destruction.

For isolation of unreacted III the contents of tubes 4–44 were pooled, and the solvent was evaporated. The residue was twice evaporated with water (50 ml each) and dried over KOH pellets *in vacuo*. This material dissolved in water (500 ml) was applied to a column of AG-1X2 (3 × 20 cm) which was eluted with water (500 ml), then with 0.01 *N* acetic acid. Fractions (20 ml each) were collected. Pauly-positive fractions were pooled, evaporated to a small volume, and lyophilized: 200 mg; $[\alpha]^{24}D$ −41.5° (*c* 2.05, 10% acetic acid); lit.[10] $[\alpha]^{27}D$ −39.5° (*c* 0.40, 10% acetic acid); chromatographically identical with starting material; amino acid ratios in acid hydrolysate: $His_{1.11}Met_{0.80}Asp_{1.09}Ser_{2.86}Thr_{1.05}Ala_{2.15}$.[16]

Phenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine *d*-Sulfoxide Acetate Hexahydrate (V).[3] The partially protected tridecapeptide hexahydrate (IV) (308 mg) was dissolved in TFA (10 ml) and the solution was kept at room temperature for 30 min. The TFA was evaporated; the residue was lyophilized twice from water, and TFA ions were exchanged for acetate ions on a column (1.5 × 20 cm) of acetate cycle Amberlite IRA-400 (prepared from 30 ml of water-settled resin). Pauly-positive fractions were pooled and lyophilized: 279 mg (96%); $[\alpha]^{24}D$ −38.8° (*c* 2.46, 10% acetic acid); R_f[3] 0.5 × His; single spot on paper electrophoresis at pH 3.5, 6.5, and 8.0; amino acid ratios in acid hydrolysate: $Phe_{0.96}Glu_{2.24}Arg_{1.06}His_{1.00}Met_{0.78}Asp_{0.98}Ser_{2.89}Thr_{0.90}Ala_{1.98}$;[16] amino acid ratios in AP-M digest: $Phe_{1.15}Glu_{1.20}Arg_{0.91}Gln_{0.84}His_{0.80}Met_{0.56}Asp_{0.82}Ser_{3.26}Thr_{1.07}Ala_{2.09}$.

Anal. Calcd for $C_{59}H_{91}O_{24}N_{19}S \cdot CH_3COOH \cdot 6H_2O$: C, 44.4; H, 6.5; N, 16.1; O, 31.0. Found: C, 44.4; H, 7.2; N, 15.6; O, 29.8.

N^{α},N^{ϵ}-Di-*t*-butoxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N^{ϵ}-*t*-butoxycarbonyllysylphenylalanylglutamylarginylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine *d*-Sulfoxide (VII).[3] **a. From VI Plus V.** An azide solution was prepared at −25 to −30° from VI[11] (327 mg; 0.3 mmol) in DMF (12 ml), 6.9 *N* HCl in dioxane (0.17 ml; 1.2 mmol), and 10% *t*-butyl nitrite in DMF (0.39 ml; 0.33 mmol). The solution was stirred for 30 min when the hydrazide test was negative. The solution was cooled at −60° and TEA (0.17 ml; 1.2 mmol) was added. To this azide solution was added at −60° a solution of V (165 mg; 0.1 mmol) in water (2 ml), 10% TEA in DMF (0.42 ml; 0.3 mmol), and DMF (2 ml). The flask was rinsed with 50% aqueous DMF (3 ml).

The mixture was stirred at 4° for 24 hr and at room temperature for 24 hr; then the solvent was evaporated. The residue was distributed between 2% acetic acid and 1-butanol (6 funnels with 20 ml each of upper and lower phase). The butanol phases were pooled and evaporated and the residue was dissolved in 2-propanol–methanol–water (1:1:1) (50 ml). The solution was cooled in an ice bath, Dowex 50W-X2 (25 ml, settled in the same solvent) was added, and the suspension was stirred with cooling for 30 min. The solvent was discarded and the resin was washed (by suspension) with the same solvent until the washings were chlorine negative. The resin was then eluted with 2-propanol–methanol–2% ammonia (1:1:1) until the washings were Pauly negative. These eluates were pooled, the solvent was evaporated, and the residue lyophilized from dilute acetic acid: 239 mg (94%); R_f^I 0.2 single Pauly-positive spot; chlorine test showed a major spot at R_f^I 0.2 with small impurities at origin and R_f^I 0.8; amino acid ratios in acid hydrolysate: $Lys_{2.09}Glu_{3.11}Thr_{1.94}Ala_{5.10}Phe_{0.77}Arg_{0.97}His_{0.92}Met_{0.72}Asp_{0.97}Ser_{2.91}$.[16]

b. From X Plus III. *t*-Butyl nitrite 10% in DMF (0.59 ml; 0.50 mmol) was added to a stirred solution cooled at −30° of X (792 mg; 0.45 mmol) in DMF (15 ml) containing 6.7 *N* HCl in dioxane (0.34 ml; 2.3 mmol). The solution was stirred for 30 min at −25°, then cooled at −60°, and TEA (0.31 ml; 2.3 mmol) was added. To this solution of the azide was added a solution of III[10] (235 mg; 0.23 mmol) in water (3 ml), DMF (7 ml), and TEA 10% in DMF (0.94 ml; 0.68 mmol). The reaction mixture was stirred for 20 hr at 4° and for 1 hr at room temperature, then the gelatinous mixture was diluted with 150 ml of 1-butanol–methanol–water (1:1:1). The solution was added to an acetate cycle AG-1X2 column (2 × 10 cm) which was eluted with 1-butanol–methanol–water (1:1:1) (50 ml); 1-butanol–methanol–0.03 *N* acetic acid (1:1:1) (125 ml); 1-butanol–methanol–0.06 *N* acetic acid (1:1:1) (500 ml); and finally 1-butanol–methanol–0.25 *N* acetic acid (1:1:1) (200 ml). The partially protected peptide (VII) was located in the 1-butanol–methanol–0.06 *N* acetic acid eluates by the chlorine reaction on tlc. Fractions containing homogeneous material were pooled, concentrated to a small volume, and lyophilized from 10% acetic acid: 275 mg (48%); R_f^I 0.2; R_f^{III} 0.5; single chlorine-, Pauly-, and Sakaguchi-positive spot; amino acid ratios in acid hydrolysate: $Lys_{2.01}Glu_{3.13}Thr_{1.93}Ala_{5.17}Phe_{1.02}Arg_{0.96}His_{0.97}Met_{0.88}Asp_{1.02}Ser_{2.63}$.[16]

The unreacted amino component was located in the 1-butanol–methanol–0.25 *N* acetic acid eluates, by the ninhydrin reaction. These fractions were evaporated and the residue was lyophilized from water to give 85 mg (37%) of III.

S-Peptide$_{1-20}$ *d*-Sulfoxide.[3,17] **a.** The partially protected peptide VII (256 mg) prepared according to method a above was dissolved in 90% TFA (2 ml); the solution was kept at room temperature for 25 min, then the TFA was evaporated. The residue, lyophilized twice from 10 of water, was dried over KOH pellets *in vacuo* at room temperature. This material, dissolved in 10% acetic acid, was passed through a column of acetate cycle Amberlite IRA-400 (prepared from 10 ml of 10% acetic acid settled resin) and Pauly-positive fractions were pooled and evaporated to dryness. The residue was lyophilized from water (214 mg). This material (106 mg) dissolved in water (5 ml) was applied to a CMC column (1 × 11 cm) which was eluted first with 0.01 *M* ammonium acetate (50 ml), then with 0.025 *M* ammonium acetate (200 ml). Fractions (3 ml each) were collected at a flow rate of 60 ml/hr. Individual fractions were evaluated by tlc using the chlorine test. Fractions containing a single component, R_f^{III} 0.1, were pooled, evaporated to a small volume, and lyophilized to constant weight from water: 46 mg; $[\alpha]^{25}D$ −57.1° (*c* 0.99, 10% acetic acid); R_f[3] 0.3 × His; single Pauly-, ninhydrin-, chlorine-, and Sakaguchi-positive spot on paper electrophoresis at pH 3.5, 6.5, and 8.0 indistinguishable from an authentic sample of performic acid oxidized natural S-peptide;[13] amino acid ratios in acid hydrolysate: $Lys_{1.99}Glu_{3.22}Thr_{2.01}Ala_{5.12}Phe_{0.99}Arg_{0.85}His_{0.93}Met_{1.01}Asp_{1.03}Ser_{2.76}$.[16]

b. The partially protected peptide VII (255 mg) prepared according to method b above was dissolved in 90% aqueous TFA (3 ml) and the solution was kept at room temperature for 45 min. The solvent was evaporated and the residue lyophilized from water. Trifluoroacetate ions were exchanged for acetate ions on acetate cycle Amberlite IRA-400 in the usual manner and the solution was lyophilized: 233 mg; $[\alpha]^{21}D$ −58.4° (*c* 1.16, 10% acetic acid); R_f^{III} 0.1; single chlorine-, ninhydrin-, Pauly-, and Sakaguchi-positive spot; amino acid ratios in acid hydrolysate: $Lys_{2.12}Glu_{3.00}Thr_{2.07}Ala_{5.31}Phe_{0.96}Arg_{0.89}His_{0.83}Met_{0.92}Asp_{0.94}Ser_{3.00}$.[16]

S-Peptide$_{1-20}$.[3] For conversion to S-peptide$_{1-20}$ the *d*-sulfoxides were reduced with thioglycolic acid as described.[18] Comparisons of some properties of the synthetic and natural S-peptides are presented in Figures 1 and 2 and Table I.

B. Enzymology. The Kunitz assay[19] based on measurement of the rate of decrease in absorption of RNA at 300 mμ as a function of enzyme concentration has been adopted for measurement of RNase S′ activity with the following modifications. Enzyme solutions (1 ml each) were prepared by mixing S-protein (1.5 to 2.0×10^{-6} *M*) with varying concentrations of the S-peptide preparations. Aliquots (0.2 ml) of these solutions were added to substrate solutions precooled at 20° (3.8 ml) containing RNA (a 0.1% solution in 0.1 *M* sodium acetate buffer, 2.0 ml) and water (1.8 ml). Yeast RNA (Sigma commercial grade, 25 g) was dissolved in 0.1 *M* sodium acetate buffer, pH 5.0 (1 l.). The RNA was precipitated with four volumes of absolute ethanol and centrifuged at 1000 rpm. The precipitate was washed four times with 400 ml each of ethanol and dried *in vacuo* over P_2O_5. Optical density changes at 300 mμ were recorded with a Cary 15 recording spectrophotometer using the 0–0.1 optical density scale. Silica cuvettes (1-cm light path) containing the complete assay solution were placed in a thermostated chamber at 20° within 15 sec after the addition of enzyme. Slopes obtained under these assay conditions are linear for highly active enzyme solutions for at least 40 sec. Equipment used for assay was prewashed with 0.1% thioglycolic acid followed by a wash with glass-distilled water. Cuvettes were washed between assays with 0.1 *N* sodium hydroxide. Ribonuclease 100% activity (Figure 1) corresponds to 130 Kunitz units at 30°.

Discussion

The work reported in this paper constitutes a total synthesis of S-peptide$_{1-20}$ according to generally accepted standards of organic chemistry. The synthetic

(17) The optical rotation of S-peptide$_{1-20}$ *d*-sulfoxide, −57.1 and −58.4°, differs significantly from the value −43.1° reported earlier.[3] A difference in the degree of hydration appears to be responsible for this discrepancy.

(18) See Hofmann, *et al.*, ref 14.

(19) M. Kunitz, *J. Biol. Chem.*, **164**, 563 (1946).

material exhibits, within the limits of error of measurement, the same physical, chemical, and biological properties as the natural counterpart (Figures 1 and 2, Table I).

We selected S-peptide$_{1-20}$ as the object for synthetic studies since its biological activity, *i.e.*, its ability to activate S-protein to form enzymically fully active ribonuclease S′ at a molar ratio of 1:1, can be measured with a degree of accuracy not usually achieved with biological assays. In addition, the availability of a practical route to S-peptide$_{1-20}$ has paved the way for preparation of fragments and analogs whose biological properties have contributed to an understanding of the forces which bring about the interaction of S-peptide and S-protein and to a better comprehension of some aspects of ribonuclease catalysis.[15,18,20-22] Moreover, the nature and distribution of the amino acid residues which constitute S-peptide$_{1-20}$ offer a challenge from the point of view of peptide synthesis.

For reasons outlined in a previous communication[10] we employed the *d*-sulfoxide of methionine and converted the S-peptide$_{1-20}$ *d*-sulfoxide into S-peptide$_{1-20}$ in the final step. Our routes to S-peptide$_{1-20}$ are based on fragment condensation. For purification of intermediates and final product we relied on the use of ion-exchange resins and for this reason selected synthetic schemes which involve a minimum of side-chain protection. For example, the partially protected S-peptide$_{1-20}$ *d*-sulfoxide (VII) was cleanly separated from excess azide (VI) and its rearrangement products by adsorption at 0° on Dowex 50W-X2 in a batch process. This separation is based on the fact that the desired product (VII) contains unprotected arginine and histidine while the acylating component (VI) is devoid of basic residues. The *t*-butoxycarbonyl groups which protect the lysine residues of the azide (VI) are resistant to the acidic resin at the low temperature used in the separation. Unreacted amino component (V) was removed prior to the Dowex step by distribution of the reaction mixture between 1-butanol and 2% acetic acid. The same principles were employed in the preparation of the partially protected undecapeptide hydrazide (IX).

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Extensive experience with the ion-exchange resin AG-1X2[3,10,12,18,23-26] has shown that this material is very useful for the isolation and separation of peptides which differ in negative charge. Such peptides are selectively displaced from the resin by elution with acetic acid of increasing normality. Columns operate well with both aqueous and organic solvents. The high degree of selectivity of AG-1X2 is well documented in the Experimental Section.

The amino acid ratios in acid hydrolysates of synthetic S-peptide *d*-sulfoxide are, within the error of the amino acid determinations, those expected by theory. We attribute the somewhat low recoveries of glutamine, histidine, methionine, and aspartic acid in AP-M digests of both synthetic and natural S-peptide (Table I) to pyrrolidonecarboxylic acid formation of the glutamine residue and resistance of the aspartyl–serine bond to carboxypeptidase.[27] The AP-M preparation used in our experiments contained carboxypeptidase activity.

In the earlier phase of our studies[3] we were unable to obtain fully active S-peptide$_{1-20}$ by CMC chromatography of the final thioglycolic acid reduced peptide. Purification to full activity was achieved, however, by combination of the synthetic material with natural S-protein and chromatography of the ensuing ribonuclease S′ on Amberlite CG-50.[28] The enzyme thus purified was then dissociated with trichloroacetic acid,[29] and the regenerated S-peptide separated from protein contaminants by CMC chromatography.

The refinements in synthetic methodology and purification procedures which are described in this communication have made *fully active* synthetic S-peptide readily available without resorting to adsorption on S-protein.

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