that the adenine and cytosine bases in these dinucleoside monophosphates are quite strongly stacked at room temperature, and that the intramolecular base-stacking tendencies are quite comparable for the two sequence isomers. A comparison of the results obtained in this work for ApC and CpA with those previously reported by Chan and Nelson[4] for ApA suggests that the intramolecular stacking interaction is probably somewhat stronger in ApA than in ApC and CpA. These conclusions are in general agreement with those reached by other workers,[18,20,21] based upon optical studies of these systems, at least with regard to the general classification of these dinucleoside monophosphates as being "stacked." The present pmr study also yielded information concerning the conformation of the intramolecular stacks and the conformational changes in the ribose–phosphate–ribose backbone accompanying the intramolecular base-stacking interaction. The chemical shift data were shown to be consistent with stacked conformations in which both bases of the dinucleoside monophosphates are preferentially oriented in the *anti* conformation, as in similar dApdC and dCpdA (dA ≡ deoxyadenosine; dC ≡ deoxycytidine) segments of double-helical DNA. The average ring conformation of the two ribose moieties has been found to become more 3′-*endo* as a result of the intramolecular base-stacking interaction. Finally, the concentration dependence of the pmr spectra of ApC and CpA indicates that these dinucleotides undergo extensive intermolecular self-association, pointing out the necessity of working at a low concentration of dinucleotide in order to obtain meaningful results regarding the intramolecular process.

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Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. XII. Synthesis of des-Lys^1-[Orn^{10}]-, des-Lys^1,Glu^2-[Orn^{10}]-, and des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptides[1,2]

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Abstract: Syntheses are described of three analogs of S-peptide in which the arginyl residue, in position 10, has been replaced by ornithine and one, two, or three of the residues of the 1–3 sequence are respectively removed. The stereochemical homogeneity of these peptides, *i.e.*, des-Lys^1-[Orn^{10}]-, des-Lys^1,Glu^2-[Orn^{10}]-, and des-Lys^1,Glu^2,-Thr^3-[Orn^{10}]-S-peptide, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. The enzymic properties of the three synthetic analogs were checked, with RNA, after recombination with S-protein. Both the des-Lys^1-[Orn^{10}]-S-peptide and the des-Lys^1,Glu^2-[Orn^{10}]-S-peptide form an about 50% active partially synthetic ribonuclease at a molar ratio of 1:1 with S-protein, while further removal of threonine in position 3 is accompanied by a significant decrease of the potential activity of the des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptide. The des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-RNase S′ shows 20% of the ability of RNase S′ to catalyze the depolymerization of RNA.

Much information is presently available about the importance of the different amino acid side chain residues in the S-peptide sequence in connection with the specific noncovalent binding responsible for the formation of the complex between S-peptide and S-protein.

Structure–function studies, carried out in our laboratory by the synthetic approach,[3] have shown that

(1) The peptides and peptide derivatives mentioned have the L configuration. For a simpler description the customary L designation for individual amino acid residues is omitted. The following abbreviations are used: (a) [IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966)]: Z = benzyloxycarbonyl, Boc = *t*-butyloxycarbonyl, OMe = methyl ester, OEt = ethyl ester, OBu^t = *t*-butyl ester, ONp = *p*-nitrophenyl ester, DMF = dimethylformamide, TFA = trifluoroacetic acid, TCA = trichloroacetic acid. (b) [F. M. Richards, *Proc. Natl. Acad. Sci. U. S.*, **44**, 162 (1958)]: RNase A, the principal chromatographic component of beef pancreatic ribonuclease; RNase S, subtilisin-modified RNase A; S-peptide, the eicosapeptide obtained from RNase S; S-protein, the protein component obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein. According to (c) M. S. Dosher and C. H. W. Hirs, *Federation Proc.*, **25**, 527 (1966), natural S-peptide is a mixture of at least (1–20)-S-peptide and (1–21)-S-peptide.

(2) Some of the results recorded in this paper have been presented at the IXth European Peptide Symposium, Orsay, France, April 15, 1968; E. Scoffone, F. Marchiori, L. Moroder, R. Rocchi, and A. Scatturin in "Peptides 1968," E. Bricas, Ed., North-Holland Publishing Co., Amsterdam, Netherlands, 1968, p 325.

(3) (a) E. Scoffone, R. Rocchi, F. Marchiori, L. Moroder, A. Marzotto, and A. M. Tamburro, *J. Am. Chem. Soc.*, **89**, 5450 (1967); (b) R. Rocchi, L. Moroder, F. Marchiori, E. Ferrarese, and E. Scoffone, *ibid.*, **90**, 5885 (1968); (c) F. Marchiori, R. Rocchi, L. Moroder, A. Fon-

Chart I. Amino Acid Sequence of S-Peptide and Its Synthetic Analogs

S-Peptide

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

Des-Lys^1-[Orn^{10}]-S-peptide

□ - Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

Des-Lys^1,Glu^2-[Orn^{10}]-S-peptide

□ - □ - Thr-Ala-Ala-Ala-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

Des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptide

□ - □ - □ - Ala-Ala-Ala-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

phenylalanine in position 8 is a very important hydrophobic binding site[3a,d] in the S-peptide but the alanyl residues in position 4, 5, or 6 can be respectively substituted for a seryl residue[3b,c] without drastically altering the S-protein activating characteristics.

These structure–function studies, according to Richards'[4] and Hofmann's[5,6] findings, have identified methionine-13 as another significant binding site[3e] and have demonstrated that the apolar interaction involving such a residue is not specific for the methionyl side-chain thioether function, but more generally involve a hydrophobic group.[3e] Moreover, Hofmann has shown that aspartic acid-14[6] plays a significant role in the binding to the protein and that the removal of lysine-1[5] exerts little effect on the S-protein activating characteristics of the S-peptide but the elimination of both lysine-1 and glutamic acid-2 markedly decreases potency.[5]

These findings point to glutamic acid-2 and aspartic acid-14 as two significant binding sites in the S-peptide.

The Hofmann conclusions[5] about the importance of glutamic acid-2 were drawn from the activity data obtained for a peptide corresponding to the 3–13 sequence, lacking therefore aspartic acid in position 14. Such a residue on the basis of physicochemical evidence obtained by Li, *et al.*,[7] and of the interpretation of the X-ray structure of RNase S, elucidated by Wyckoff, *et al.*,[8] appears to be paired with tyrosine-25 and it is proposed[6] to play an important role in the formation of the peptide–protein complex.

In order to check the activity of a peptide lacking the glutamyl residue in position 2 but in which the aspartic acid-14 is present, we synthetized the des-Lys^1,Glu^2-[Orn^{10}]-S-peptide (Chart I).

Moreover we synthesized and we tested the catalytic activity, after recombination with S-protein, of the des-Lys^1-[Orn^{10}]- and des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptide (Chart I).

The activities of the partially synthetic RNase analogs, expressed as a percentage of RNase S′ and [Orn^{10}]-RNase S′[9] activity, are listed in Table I.

Removal of the lysyl residue in position 1 from the [Orn^{10}]-eicosapeptide sequence does not practically affect its capacity to activate the S-protein. This was expected from the maintenance of the catalytic activity in the des-Lys^1-RNase isolated by Eaker[10] with countercurrent distribution of a commercial ribonuclease sample and from the synthetic work of Hofmann on some fragments of the S-peptide sequence.[5]

Table I. S-Protein Activating Capacity of S-Peptide Analogs at 1:1 Molar Ratio, Substrate RNA

S-Peptide analogs	Activity %: RNase S′	Activity %: [Orn^{10}]-RNase S′
Des-Lys^1-[Orn^{10}]	50	80
Des-Lys^1,Glu^2-[Orn^{10}]	50	80
Des-Lys^1,Glu^2,Thr^3-[Orn^{10}]	20	32

The high potential catalytic activity of the des-Lys^1,-Glu^2-[Orn^{10}]-S-peptide which is practically equal to that of the des-Lys^1-[Orn^{10}]-S-peptide does not agree with the Hofmann results which point to glutamic acid-2 as a particularly effective binding site.[5]

A possible interpretation of the different potential catalytic activities shown by a peptide which lacks the first two amino acid residues in the N-terminal region is that glutamic acid-2 is important only when aspartic acid-14 is absent but it does not play a significant role in a peptide in which the C-terminal heptapeptide has not been removed.

Further removal of the threonyl residue in position 3 is accompanied by a decrease in the ability of the resulting partially synthetic modified RNase S′ to catalyze the depolymerization of the RNA.

The potential activity of the des-Lys^1,Glu^2,Thr^3-[Orn^{10}]-S-peptide is of the same order as that of the [Pro^6,Orn^{10}]-S-peptide.[3c] This result can be interpreted on the basis of the observation that a prolyl residue can be accommodated in the first three positions of an α helix.[11]

From these findings it is possible to assign to threonine-3 a certain role either directly in the S-peptide–S-protein association process, or in contributing to those structural features which allow the S-peptide to undergo the thermodynamically favorable coil-to-helix conformational transition when in the presence of S-protein.[12]

It is interesting to note that if one compares the primary structure of sheep,[13] bovine, and rat ribonuclease, according to the alignment proposed by Beintema,[14] position 3 is always occupied by a

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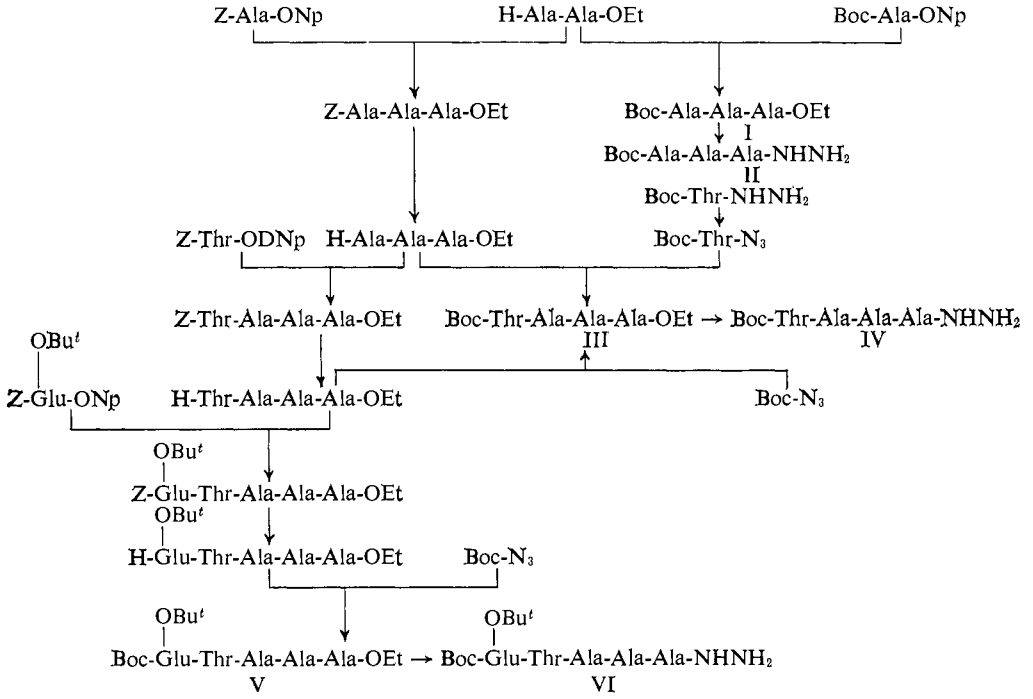
Chart II. Synthesis of 2–6, 3–6, and 4–6 Sequences



hydroxylated amino acid residue such as threonine or serine.

Peptide Syntheses

The synthetic route to des-Lys[1]-[Orn[10]]-, des-Lys[1],-Glu[2]-[Orn[10]]-, and des-Lys[1],Glu[2],Thr[3]-[Orn[10]]-S-peptide, illustrated in Charts II and III, is similar to that we used for the preparation of other analogs.[8,9,15,16]

The peptide esters corresponding to the sequences 2–6, 3–6, and 4–6, in the form of N^{α}-*t*-butyloxycarbonyl derivatives, were prepared by stepwise elongation starting from the C-terminal dipeptide alanylalanine ethyl ester[17] (Chart II). The introduction of the *t*-butyloxycarbonyl group as N^{α}-protecting agent was achieved either by using in the last step of the synthesis an activated *t*-butyloxycarbonyl amino acid or by reaction of the N^{α}-free peptide with *t*-butyl azidoformate.[18]

The protected peptide esters (2, I; 2, III; and 2, V) were transformed into the corresponding hydrazides (2, II or 3A, I 4–6; 2, IV or 3A, II 3–6; and 2, VI or 3A, III 2–6) in the usual manner and coupled by an azide coupling step with the hexapeptide 3B, 7–12[3c] to give respectively the peptide esters 3C, I 4–12; 3C, II 3–12; and 3C, III 2–12, which were converted into hydrazides (3D, I 4–12; 3D, II 3–12; and 3D, III 2–12) (Chart III).

The corresponding azides were then treated with the octapeptide methionylaspartylserylserylthreonylseryl-alanylalanine[19] (3E, 13–20) to give the partially protected eicosapeptides 3F, I 4–20; 3F, II 3–20; and 3F, III 2–20. The protecting groups were removed with TFA and the crude products 3G, I 4–20, 3G, II 3–20, and 3G, III 2–20 were purified by chromatography through an Amberlite CG 50 column eluted with 0.2 *M* sodium phosphate, desalted by gel filtration on Sephadex G 25 using 5% acetic acid as the eluent, and lyophilized.

The chemical homogeneity of the three S-peptide analogs was evaluated by paper electrophoresis at different pH values and quantitative amino acid analyses of the acid hydrolysates. Aminopeptidase M (AP-M) was employed for the evaluation of the stereochemical homogeneity of synthetic materials according to the procedure described by Hofmann, *et al.*[6]

Experimental Section[20]

RNase A was prepared from bovine pancreatic ribonuclease (Fluka AG four times crystallized) by the procedure of Crestfield, *et al.*[21] RNase S, S-protein, and S-peptide were prepared from RNase A, by using the proteolytic enzyme designated subtilopeptidase A, which was a gift from Novo Industri A/S, Copenhagen, Denmark, essentially by the method of Richards[22] modified by Dosher and Hirs.[1c] Aminopeptidase M (AP-M) was obtained from Rohm and Haas GmbH, Darmstadt, West Germany. Commercial yeast RNA was obtained from Schwarz Laboratories and purified by exhaustive dialysis, first against 0.1 *M* sodium chloride and then against water.[23]

Aminopeptidase M digests were prepared in the manner described by Hofmann, *et al.*[6] The amino acid composition of acid and enzymic hydrolysates was determined by quantitative analysis using either a Technicon or a Carlo Erba amino acid analyzer. Ascending thin layer chromatography was performed on silica gel G

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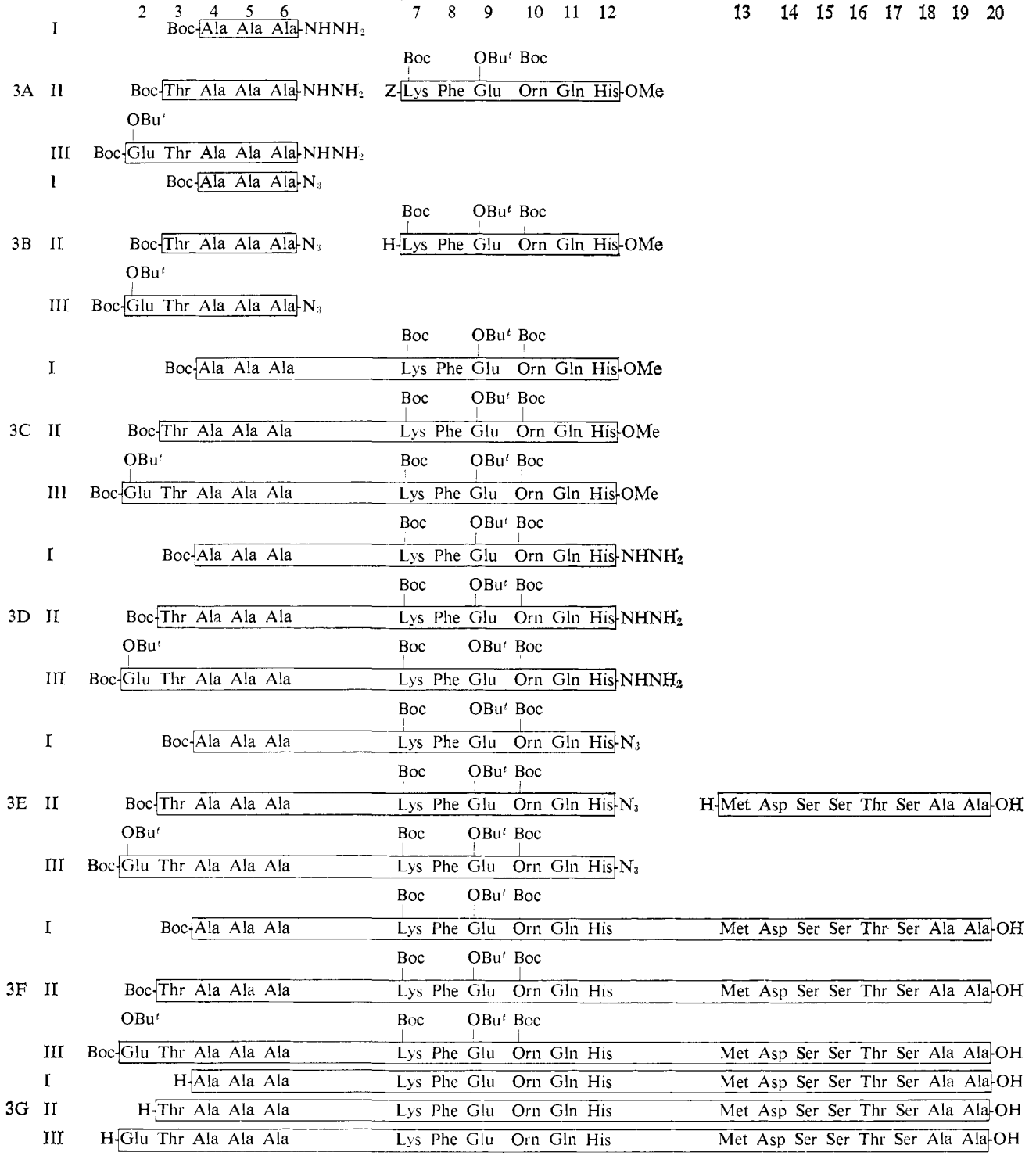
(20) The melting points were determined by the Tottoli's capillary melting point apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. The acid hydrolyses were carried out with 6 *N* hydrochloric acid, in sealed evacuated ampoules, for 22 hr at 110°.
(21) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 618 (1963).
(22) (a) F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.*, **234**, 1459 (1959); (b) G. Gordillo, P. J. Vithayathil, and F. M. Richards, *Yale J. Biol. Med.*, **34**, 582 (1962); (c) A. Marzotto, A. Scatturin, G. Vidali, and E. Scoffone, *Gazz. Chim. Ital.*, **94**, 760 (1964).
(23) D. Wellner, H. J. Silman, and M. Sela, *J. Biol. Chem.*, **238**, 1324 (1963).

Chart III. Synthesis of the 4–20, 3–20, and 2–20 Sequences



(Merck) with the following solvent systems: R_{f_1}, 1-butanol–glacial acetic acid–water (3:1:1); R_{f_2}, ethyl acetate–pyridine–glacial acetic acid–water (60:20:6:14); R_{f_3}, chloroform–methanol–32% ammonium hydrate (2:2:1). Electrophoreses were carried out on Whatman No. 1 filter paper, at a gradient of about 20 V/cm, for 3 hr, at pH 1.9 (25% acetic acid), 3.5, and 6.4 [pyridine–acetic acid–water (1:10:89 and 5:0.2:95)], and 9.4 (Na_2CO_3–$NaHCO_3$).[24] The chlorine[25] and the Pauly[26] tests were carried out according to the literature. The hydrazides were also revealed by spraying the chromatograms with a 1% picryl chloride solution in 95% ethanol, followed by exposure to ammonia vapors.

Unless stated otherwise, solvents were evaporated at a bath temperature of 40–50° in a rotatory evaporator. The enzymic activity of the partially synthetic, modified ribonucleases was determined, with RNA substrate, essentially as described by Kunitz,[27] as well as by following the release of acid-soluble nucleotides by the uracyl acetate–TCA procedure.[28] For reasons outlined in a previous communication,[3a] prior to performing the ribonuclease assays, the synthetic eicosapeptides were incubated under nitrogen at 45°, in

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(26) K. Randerath, "Thin Layer Chromatography," Academic Press, New York, N. Y., 1963, p 176.
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1% aqueous thioglycolic acid, according to the procedure described by Hofmann, *et al.*[6]

The crude S-peptide analogs (100–200 mg) obtained by treatment of the partially protected eicosapeptides with TFA were dissolved in 0.2 *M* sodium phosphate buffer (pH 6.47) and purified by chromatography through an Amberlite CG 50 column (1.8 × 90 cm) with the same phosphate buffer as the eluent.

Individual fractions (2.5 ml) were collected (rate *ca.* 16 ml/hr), and the products detected by the ninhydrin test and the Pauly reaction.

The ninhydrin-, Pauly-positive fractions were pooled, concentrated under reduced pressure, lyophilized, and desalted by passing through a Sephadex G-25 column (1.8 × 140 cm) with 5% acetic acid as the eluent (rate *ca.* 20 ml/hr, individual fractions of 2.5 ml). The peptide was detected as described above and the peptide-containing fractions were pooled, concentrated to a syrup, and lyophilized from water to constant weight.

***t*-Butyloxycarbonylalanylalanylalanine Ethyl Ester (2, I).** *t*-Butyloxycarbonylalanine *p*-nitrophenyl ester[29] (2.48 g, 8.0 mmol) and triethylamine (1.4 ml) were added to a pyridine solution (25 ml) of alanylalanine ethyl ester hydrobromide, which was obtained by treatment of benzyloxycarbonylalanylalanine ethyl ester (3.22 g, 10.0 mmol) with 2 *N* hydrobromic acid in glacial acetic acid as already described.[17]

After 24 hr the solvent was removed under reduced pressure and the residue, taken up in chloroform, was washed with 5% sodium carbonate, 5% citric acid, and water and dried over sodium sulfate. The solution was evaporated to dryness *in vacuo* and the residue crystallized twice from ethyl acetate–petroleum ether (bp 30–60°); yield 1.85 g (65%), mp 174–175°, $[\alpha]^{20}D$ −33.0 ± 0.5° (*c* 1.0, DMF), R_{f_1} 0.80, R_{f_2} 0.90, single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{16}H_{29}N_3O_6$ (359.4): C, 53.5; H, 8.1; N, 11.7. Found: C, 53.4; H, 8.2; N, 11.6.

***t*-Butyloxycarbonylalanylalanylalanine Hydrazide (2, II or 3A, I 4–6).** The above protected tripeptide ester (2, I) (1.8 g, 4.8 mmol) was dissolved in ethanol (150 ml), and hydrazine hydrate (5 ml) was added. The mixture was kept 1 hr at 50° and a further 24 hr at room temperature. After cooling at 0°, the crystalline precipitate was collected, washed with ice-cold ethanol and ether, and dried over concentrated sulfuric acid; yield 1.4 g (80%), mp 249–250°, $[\alpha]^{20}D$ −17.0 ± 0.3° (*c* 1.0, DMF), R_{f_1} 0.60, R_{f_2} 0.80, single chlorine- and picryl chloride-positive spot.

Anal. Calcd for $C_{14}H_{27}N_5O_5$ (345.4): C, 48.7; H, 7.9; N, 20.3. Found: C, 48.4; H, 7.8; N, 20.2.

***t*-Butyloxycarbonylthreonylalanylalanylalanine Ethyl Ester (2, III). a. Azide Procedure.** Sodium nitrite (0.83 g, 12 mmol) in water (2.5 ml) was added to a suspension of *t*-butyloxycarbonylthreonine hydrazide[30] (2.33 g, 10 mmol) in 1 *N* hydrochloric acid (15 ml) containing sodium chloride (1.5 g) to lower the freezing point, cooled to −10°. After stirring at −10° for 10 min, the *t*-butyloxycarbonylthreonine azide was extracted with three 10-ml portions of ethyl acetate cooled to −10°. The combined organic layers were quickly washed with ice-cold, saturated solutions of potassium carbonate and sodium chloride and dried for 15 min, at −10°, over sodium sulfate. Simultaneously, alanylalanylalanine ethyl ester hydrobromide[17] (3.75 g, 11 mmol) was suspended in DMF (15 ml) and cooled to 0°, and triethylamine (1.55 ml) was added. After stirring at 0° for 30 min, the precipitated triethylammonium hydrobromide was filtered off, and the filtrate was added to the ethyl acetate solution of the *t*-butyloxycarbonylthreonine azide prepared above. DMF (60 ml) was then added, and the resulting solution was concentrated under reduced pressure, at 0°, to remove most of the ethyl acetate, and allowed to react at 5° for 3 days. The solution was then concentrated to small volume (*ca.* 20 ml) under reduced pressure, whereupon ether was added. After 12 hr at 0° the precipitate was collected, washed with ether, and crystallized twice from ethanol–ether; yield 2.8 g (61%), mp 210–211°, $[\alpha]^{20}D$ −19.0 ± 0.5° (*c* 1.0, DMF), R_{f_1} 0.70, R_{f_3} 0.90, single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{20}H_{36}N_4O_8$ (460.5): C, 52.1; H, 7.8; N, 12.2. Found: C, 51.6; H, 7.7; N, 12.1.

b. By *t*-Butyloxycarboxylation of the Tetrapeptide Ethyl Ester. *t*-Butyl azidoformate[18] (0.86 g, 6.0 mmol) and triethylamine (0.85 ml) were added to a solution of threonylalanylalanylalanine ethyl ester[17] (1.08 g, 3 mmol) in DMF (100 ml). After 24 hr at 40°, a second portion of *t*-butyl azidoformate[18] (0.86 g, 6.0 mmol) was added, and the mixture was allowed to react a further 30 hr at 40°. The solvent was then evaporated to dryness under reduced pressure and the residue was dissolved, by heating, in ethanol (100 ml), filtered, and precipitated by addition of ether (200 ml). After 12 hr at 0°, the precipitate was collected, washed with ether, and dried; yield 1.15 g (84%), mp 210–211°, $[\alpha]^{20}D$ −19 ± 0.5° (*c* 1.0, DMF), R_{f_1} 0.70, R_{f_3} 0.90, single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{20}H_{36}N_4O_8$ (460.5): C, 52.1; H, 7.8; N, 12.2. Found: C, 51.7; H, 7.7; N, 12.2.

***t*-Butyloxycarbonylthreonylalanylalanylalanine Hydrazide (2, IV or 3A, II 3–6).** Hydrazine hydrate (2.5 ml) was added to a solution of *t*-butyloxycarbonylthreonylalanylalanylalanine ethyl ester (1.1 g, 2.4 mmol) in a mixture of DMF (20 ml) and ethanol (20 ml). After heating for 1 hr at 50°, the solution was kept at room temperature for 5 days when ether was added. After 12 hr at 0°, the precipitation was completed by addition of ether; the precipitate was collected, washed with ether, and dried over concentrated sulfuric acid; yield 0.963 g (90%), mp 237–238°, $[\alpha]^{20}D$ −6.4 ± 0.3° (*c* 1.0, DMF), R_{f_1} 0.60, R_{f_2} 0.95, single chlorine- and picryl chloride-positive spot.

Anal. Calcd for $C_{18}H_{34}N_6O_7$ (446.5): C, 48.4; H, 7.7; N, 18.8. Found: C, 47.8; H, 7.5; N, 18.6.

***t*-Butyloxycarbonyl-γ-*t*-butylglutamylthreonylalanylalanylalanine Ethyl Ester (2, V).** *t*-Butyl azidoformate[18] (0.5 g, 3.5 mmol) and triethylamine (0.5 ml) were added to a solution of γ-*t*-butylglutamylthreonylalanylalanylalanine ethyl ester[31] (0.92 g, 1.68 mmol) in 40 ml of 50% methanolic DMF. After 24 hr at 40°, further *t*-butyl azidoformate[18] (0.5 g, 3.5 mmol) was added and the mixture was then kept 48 hr at 40°. Methanol was eliminated from the solution by evaporation under reduced pressure and the product was precipitated by addition of ether, collected after 12 hr at 0°, and recrystallized from DMF–ether; yield 0.69 g (63%), mp 209–210°, $[\alpha]^{20}D$ −18.5 ± 0.5° (*c* 1.0, DMF), R_{f_1} 0.70, R_{f_2} 0.90, single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{29}H_{51}N_5O_{11}$ (645.8): C, 53.9; H, 7.9; N, 10.8. Found: C, 53.2; H, 7.6; N, 10.7.

Alternatively in another experiment the solvent was completely removed from the reaction mixture and the residue, taken up in chloroform, was washed with 0.5 *M* citric acid and water, dried over sodium sulfate, and evaporated to dryness.

Crystallization of the residue from DMF–ether gave 65% yield of pure product.

***t*-Butyloxycarbonyl-γ-*t*-butylglutamylthreonylalanylalanylalanine Hydrazide (2, VI or 3A, III 2–6).** Hydrazine hydrate (1.2 ml) was added to a solution of 2, V (0.775 g, 1.2 mmol) in ethanol (30 ml). After 1 hr at 50°, the mixture was allowed to react 5 days, at room temperature. The little amorphous gel was dissolved by addition of the minimum amount of DMF; the solution was filtered to remove some insoluble product and poured into an excess of stirred, ice-cold ether. The precipitated product was collected, washed with ether, and dried over concentrated sulfuric acid; yield 0.61 g (80%), mp 222–223° dec, $[\alpha]^{25}D$ −9.5 ± 0.3° (*c* 0.67, DMF), R_{f_1} 0.60, R_{f_2} 0.80, single chlorine- and picryl chloride-positive spot.

Anal. Calcd for $C_{27}H_{49}N_7O_{10}$ (631.8): C, 51.3; H, 7.8; N, 15.5. Found: C, 50.8; H, 7.8; N, 15.6.

***t*-Butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, I 4–12).** *t*-Butyl nitrite (0.89 ml, 0.75 mmol) in DMF (3.5 ml) and 2.16 *N* hydrochloric acid in tetrahydrofuran (0.65 ml) were added to a stirred solution, cooled to −10°, of *t*-butyloxycarbonylalanylalanylalanine hydrazide (0.242 g, 0.7 mmol) in DMF (6 ml). After 10 min of stirring, a solution of N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester acetate[3c] (0.792 g, 0.7 mmol) and triethylamine (0.4 ml) in DMF (9 ml) was added. The combined solutions were allowed to react for 7 days at 4°, the solution was concentrated under reduced pressure, and precipitation occurred by addition of water. The precipitate was collected and the crude product (0.67 g, 70%), which proved to be inhomogeneous in thin layer chromatography, was purified by preparative thin layer chromatography on silica gel. The substance (0.15 g) was applied on a plate (20 × 20 cm, 2 mm thickness) and eluted with ethyl acetate–pyridine–glacial acetic acid–water (60:20:6:14). The portion of the gel where the product has been localized was removed and extracted with

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DMF, the silica gel was filtered off, and the combined solutions were concentrated (5 ml) under reduced pressure.

The product was purified by gel filtration on a Sephadex L H/20 column (3 × 21 cm) with DMF as the eluent.

Individual fractions (3 ml) were collected (rate *ca.* 30 ml/hr), and the peptide was detected by the chlorine and the Pauly tests. The peptide-containing fractions were pooled and concentrated (5 ml), and precipitation occurred by addition of ether; yield 0.342 g (35%), mp 223–225°, $[\alpha]^{20}D$ −16.7 ± 0.2° (*c* 1.0, DMF), R_{f_1} 0.75, R_{f_2} 0.90, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{65}H_{104}N_{14}O_{19}$ (1385.65): C, 56.3; H, 7.6; N, 14.1. Found: C, 55.6; H, 7.6; N, 13.7.

***t*-Butyloxycarbonylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, II 3–12).** The condensation of 3A, II 3–6 (0.337 g, 0.755 mmol) with 3B, 7–12[3c] (0.855 g, 0.755 mmol) and the following purification by preparative thin layer chromatography and gel filtration on a Sephadex L H/20 column were carried out as described above for 3C, I 4–12, yielding 0.33 g (30%), mp 238–40°, $[\alpha]^{20}D$ −10.7 ± 0.2° (*c* 1.0, DMF), R_{f_1} 0.65, R_{f_2} 0.80, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{69}H_{111}N_{15}O_{21}$ (1486.75): C, 55.7; H, 7.5; N, 14.1. Found: C, 55.0; H, 7.3; N, 13.8.

***t*-Butyloxycarbonyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, III 2–12).** The title compound was obtained by condensation of 3A, III 2–6 (0.425 g, 0.673 mmol) with 3B, 7–12[3c] (0.76 g, 0.673 mmol).

The azide procedure and the purification of the crude product were carried out as described above for 3C, I 4–12, yield 0.429 g (38%), mp 239–241°, $[\alpha]^{20}D$ −15.5 ± 0.2° (*c* 1.0, DMF), R_{f_1} 0.80, R_{f_2} 0.95, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{75}H_{126}N_{16}O_{24}$ (1672.04): C, 56.1; H, 7.6; N, 13.4. Found: C, 55.7; H, 7.7; N, 13.4.

***t*-Butyloxycarbonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, I 4–12).** The nonapeptide methyl ester (3C, I 4–12) (0.340 g, 0.245 mmol) was dissolved in DMF (15 ml), and hydrazine hydrate (0.40 ml) was added. The solution was heated for 1 hr at 50° and then kept 7 days at room temperature. The addition of ether gave a precipitate which was collected, washed with ether, and dried *in vacuo* first over concentrated sulfuric acid and then at 110°. The product (0.312 g, 92%) had mp 224–226° dec, $[\alpha]^{20}D$ −24.3 ± 0.2° (*c* 1.01, 90% acetic acid), R_{f_1} 0.65, R_{f_2} 0.65, single chlorine-, picryl chloride-, and Pauly-positive spot.

Anal. Calcd for $C_{64}H_{104}N_{16}O_{18}$ (1385.66): C, 55.4; H, 7.6; N, 16.2. Found: C, 54.8; H, 7.4; N, 16.4.

***t*-Butyloxycarbonylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, II 3–12).** This compound was obtained from 3C, II 3–12 (0.322 g, 0.216 mmol) by the same procedure reported above for the preparation of 3D, I 4–12; yield 0.305 g (95%), mp 237–239° dec, $[\alpha]^{20}D$ −22.2 ± 0.4° (*c* 1.01, 90% acetic acid), R_{f_1} 0.65, R_{f_2} 0.70, single chlorine-, picryl chloride-, and Pauly-positive spot.

Anal. Calcd for $C_{66}H_{111}N_{17}O_{20}$ (1486.76): C, 54.9; H, 7.5; N, 16.0. Found: C, 54.2; H, 7.7; N, 15.8.

***t*-Butyloxycarbonyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, III 2–12).** The title compound was obtained from 3C, III 2–12 (0.275 g, 0.164 mmol) by the same procedure reported above for the preparation of 3D, I 4–12; yield 0.214 g (78%), mp 239–240° dec, $[\alpha]^{20}D$ −25.4 ± 0.5° (*c* 0.515, 90% acetic acid), R_{f_1} 0.55, R_{f_2} 0.65, single chlorine-, picryl chloride-, and Pauly-positive spot.

Anal. Calcd for $C_{77}H_{126}N_{18}O_{23}$ (1672.05): C, 55.3; H, 7.6; N, 15.1. Found: C, 54.8; H, 7.5; N, 15.1.

Alanylalanylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, I 4–20). Sodium nitrite (1 *M*, 0.44 ml) was added to a solution of 3D, I 4–12 (0.304 g, 0.219 mmol) in a mixture of glacial acetic acid (9 ml), 1 *N* hydrochloric acid (1.0 ml), and 20% sodium chloride (2 ml) at −10°.

After stirring for 15 min at −10°, precooled 20% sodium chloride (100 ml) was added, and the resulting precipitate was collected and washed with ice-cold water. The still-wet material was dissolved in DMF (25 ml) at −10° and dried over sodium sulfate. The drying agent was filtered off, and a solution of methionylaspartylserylserylthreonylserylalanylalanine[19] (0.388 g, 0.44 mmol as monoacetate trihydrate) and triethylamine (0.15 ml) in DMF (25 ml) was added. The reaction mixture was stirred for 7 days at 5° and for 1 day at room temperature, concentrated to 10 ml, and diluted with water (150 ml). The resulting precipitate was centrifuged, washed with water and ether, and dried over phosphorus pentoxide yielding 0.246 g (53%).

The crude material (3F, I 4–20) was dissolved in anhydrous TFA (2.5 ml) and the solution was kept for 150 min at room temperature. Ice-cold ether (60 ml) was added, and after 30 min at 0° the peptide was collected by centrifugation, washed with ether, and dried *in vacuo* over potassium hydroxide pellets.

The residue, dissolved in 0.2 *M* sodium phosphate buffer, was purified by passing through an Amberlite CG 50 column, desalted by gel filtration on a Sephadex G-25 column, and lyophilized as described previously.

The product (3G, I 4–20) (0.057 g, 28% of the crude product) had $[\alpha]^{20}D$ −93 ± 2° (*c* 0.101, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{1.10}Orn_{0.95}Glu_{2.00}Thr_{1.00}Ala_{4.80}Phe_{1.10}His_{1.00}Met_{0.97}Asp_{0.95}Ser_{2.80}$; amino acid ratios in AP-M digest: $Lys_{1.05}Orn_{1.00}Glu_{1.00}Thr_{0.95}Ala_{4.85}Phe_{1.10}(Gln + Ser)_{3.80}His_{0.95}Met_{0.95}Asp_{1.00}$.

Threonylalanylalanylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, II 3–20). The condensation of 3D, II 3–12 (0.311 g, 0.209 mmol) with 3E, 13–20[19] (0.370 g, 0.42 mmol as monoacetate trihydrate) by the azide procedure was carried out as described above for 3G, I 4–20 and gave the partially protected des-Lys[1],-Glu[2]-[Orn[10]]-S-peptide (3F, II 3–20, 0.27 g, 58%).

Treatment with anhydrous TFA, purification on Amberlite CG 50 and on Sephadex G-25, followed by lyophilization gave the pure des-Lys[1],Glu[2]-[Orn[10]]-S-peptide (3G, II 3–20) (0.067 g, 30% of the crude product), $[\alpha]^{20}D$ −90 ± 2° (*c* 0.1, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{1.05}Glu_{2.00}Thr_{1.95}Ala_{4.70}Phe_{0.93}Orn_{1.00}His_{1.00}Met_{0.92}Asp_{1.00}Ser_{2.80}$; amino acid ratios in AP-M digest: $Lys_{1.00}Glu_{1.05}Thr_{1.95}Ala_{4.90}Phe_{1.05}Orn_{1.05}(Gln + Ser)_{3.85}His_{1.00}Met_{0.98}Asp_{1.00}$.

Glutamylthreonylalanylalanylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, III 2–20). The title compound was prepared by coupling 3D, III 2–12 (0.21 g, 0.126 mmol) with 3E, 13–20[19] (0.21 g, 0.25 mmol as monoacetate trihydrate) by the same procedure described above for 3G, I 4–20 and 3G, II 3–20. The crude product (3F, III 2–20, 0.191 g, 63%) was deblocked and purified as previously described and gave 0.092 g (58% of the crude product) of des-Lys[1]-[Orn[10]]-S-peptide (3 G, III 2–20), $[\alpha]^{20}D$ −82 ± 2° (*c* 0.129, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{1.02}Glu_{2.95}Thr_{1.95}Ala_{4.80}Phe_{1.00}Orn_{0.95}His_{1.00}Met_{0.95}Asp_{1.00}Ser_{2.85}$; amino acid ratios in AP-M digest: $Lys_{1.00}Glu_{2.05}Thr_{2.00}Ala_{4.85}Phe_{1.05}Orn_{0.95}(Gln+Ser)_{3.85}His_{0.95}Met_{1.00}Asp_{1.05}$.

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