Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. VIII. Synthesis of [Ser^4,Orn^{10}]- and [Ser^5,Orn^{10}]-eicosapeptides[1,2]

Raniero Rocchi, Luigi Moroder, Fernando Marchiori, Ettore Ferrarese, and Ernesto Scoffone

Contribution from Istituto di Chimica Organica dell'Universitá, Sezione VIII del Centro Nazionale di Chimica delle Macromolecole del CNR, Padua, Italy.
Received June 3, 1968

Abstract: Syntheses are described of two analogs of the S-peptide in which the arginyl residue in position 10 is replaced by ornithine, and the alanyl residue in either position 4 or 5 is substituted by a seryl residue. The stereochemical homogeneity of these peptides, *i.e.*, lysylglutamylthreonylserylalanylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine and lysylglutamylthreonylalanylserylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. After recombination with S-protein the enzymic properties of the two synthetic eicosapeptides were checked against RNA. The activity data show that substitution of the alanyl residue either in position 4 or 5 with a seryl residue does not affect significantly the capacity of the [Orn^{10}]-S-peptide to activate S-protein.

During the course of the investigations of the relationship of the primary structure of bovine pancreatic ribonuclease S-peptide[3] to its potential enzymic activity it became apparent that conformational features are essential for the capacity of S-peptide to activate S-protein.

The ability of certain residues such as phenylalanine-8,[4,5] methionine-13,[6,7] and aspartic acid-14[8] to act as important "binding sites" has been demonstrated by several authors, either by modification of a functional group on the peptide or by a synthetic approach.

A fundamental advance in the interpretation of the influence of conformational features on the functions of the enzyme was the recent elucidation of the structure of RNase-S at 3.5-Å resolution by the Richards' group.[9] The electron density map can be interpretated in terms of the peptide backbone and many large side chains and shows that 50% of the S-peptide (residues 2–12) is in an α-helical conformation.

Moreover the S-peptide appears to be held in place largely by hydrophobic interactions involving phenylalanine-8, methionine-13, histidine-12, and alanine-4.

It may be noted that the helical model of the S-peptide, that we previously proposed[6b] as a working hypothesis, definitely appears to be substantially correct on the basis of the X-rays structure. In our model both of the alanyl residues in positions 4 and 5 lie in the hydrophobic face of the molecule directed toward the S-protein.

Thus it was reasonable to assume that such hydrophobic residues, or at least one of them, could be involved in the association process between S-peptide and S-protein which results in the formation of RNase S′. As a consequence substitution of the apolar side-chain residue of either Ala-4 or Ala-5 by the hydrophilic seryl residue was expected to affect differently the potential activity of the resulting eicosapeptide analog if the two alanyl residues differ in their capacity to contribute to the binding of the S-peptide.

Furthermore it is interesting to note that a hydrophilic–hydrophobic substitution at position 4 takes place in the rat pancreatic ribonuclease. By comparing the primary structure of bovine and rat ribonuclease according to the alignment proposed by Beintema[10] it is possible to see that the alanyl residue 4 in the beef enzyme sequence is replaced, in the rat enzyme, by a seryl residue, while in position 5, an alanyl residue is present in both the beef and the rat enzyme.

The present study describes synthetic routes to the eicosapeptide analogs [Ser^4,Orn^{10}]- and [Ser^5,Orn^{10}]-S-peptide (Chart I).

The ability of the synthetic materials to restore ribonuclease activity after recombination with S-protein was tested using yeast RNA as substrate. The [Ser^4,Orn^{10}]-RNase S′ is about 40% as active as RNase S′ while the [Ser^5,Orn^{10}]-RNase S′ is 50% as active.

(1) The peptides and peptide derivatives mentioned are of the L configuration. For a simpler description the customary L designation for individual amino acid residues is omitted. The following abbreviations (IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966)) are used: Z = benzyloxycarbonyl, Boc = *t*-butyloxycarbonyl, OMe = methyl ester, OEt = ethyl ester, OBu^t = *t*-butyl ester, ONp = *p*-nitrophenyl ester, ODNp = 2,4-dinitrophenyl ester, DMF = dimethylformamide, TFA = trifluoroacetic acid, TCA = trichloroacetic acid.

(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, Proceedings of the Symposium, in press.

(3) (a) 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-protein, the protein component obtained from RNase S; S-peptide, the eicosapeptide obtained from RNAse S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein. (b) According to 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.

(4) F. M. Richards and A. D. Logue, *J. Biol. Chem.*, **237**, 3693 (1962).

(5) (a) F. Marchiori, R. Rocchi, L. Moroder, and E. Scoffone, *Gazz. Chim. Ital.*, **96**, 1549 (1966); (b) E. Scoffone, R. Rocchi, F. Marchiori, L. Moroder, A. Marzotto, and A. M. Tamburro, *J. Am. Chem. Soc.*, **89**, 5450 (1967).

(6) F. M. Finn and K. Hofmann, *ibid.*, **87**, 645, 1965.

(7) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 2343 (1960).

(8) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

(9) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, **242**, 3984 (1967).

(10) J. J. Beintema and M. Gruber, *Biochim. Biophys. Acta*, **147**, 612 (1967).

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

[Orn[10]]-S-Peptide[11]

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-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

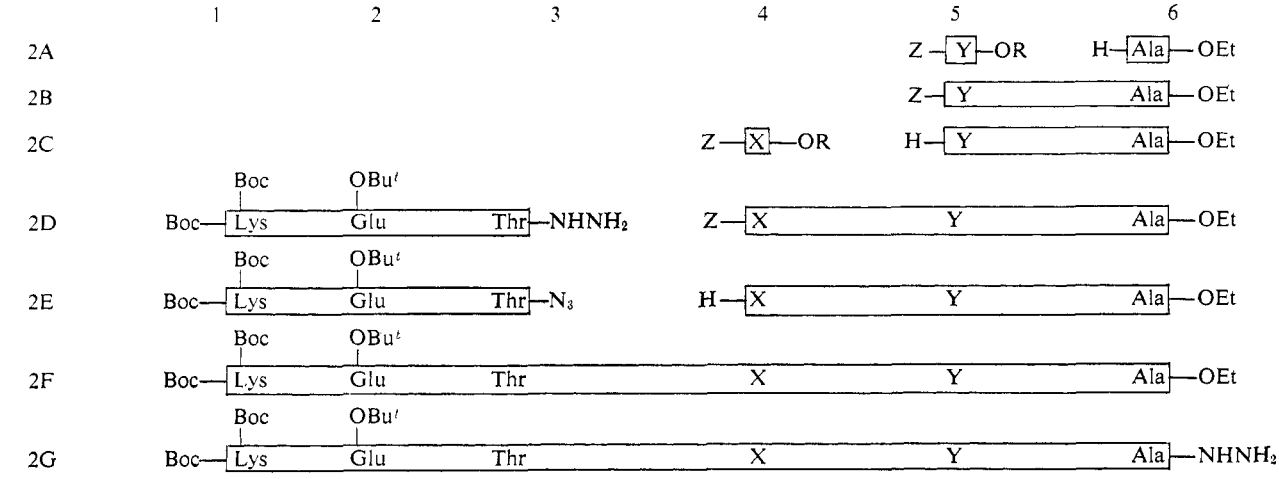
[Ser[4],Orn[10]]-S-Peptide

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Lys-Glu-Thr-Ser-Ala-Ala-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

[Ser[5],Orn[10]]-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-Ser-Ala-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

Chart II. Synthesis of the 1–6 Sequences



X—OR = Ser—ODNp or Ala—ONp
Y—OR = Ala—ONp or Ser—ODNp

The activity data show that both of the synthetic eicosapeptides generate high ribonuclease activity when combined with S-protein at 1:1 molar ratios.

Thus [Ser[5],Orn[10]]-S-peptide is substantially equal in activity to the [Orn[10]]-S-peptide,[11] whereas [Ser[4],-Orn[10]]-S-peptide is slightly less active.

However the accuracy of the procedure used to determine the enzymic activity does not allow us to exclude that such a difference may be within the limits of experimental errors.

At any rate it is evident that the contribution of both hydrophobic alanyl residues in positions 4 and 5 is not essential for the capacity of S-peptide to bind S-protein and that a hydrophilic group can be substituted for the methyl group without any marked effect on the peptide–protein association. Direct hydrophobic interactions between the side chain of the alanyl residue at position 4 or 5 and groups in the protein appear to be of little significance.

A more detailed examination of the enzymic properties of [Ser[4],Orn[10]]- and [Ser[5],Orn[10]]-RNase S′, including an evaluation of their conformational stability, will be reported in a forthcoming paper.

Peptide Syntheses

The synthetic route to [Ser[4],Orn[10]]-, and [Ser[5],-Orn[10]]-S peptide, illustrated in Charts II and III, is similar to that which we used for the preparation of other analogs.[6,11,12]

The condensation by an azide coupling step of the tripeptide N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonine hydrazide[13] (2D, 1–3) with serylalanylalanine ethyl ester (2E, Ser[4] 4–6) or alanylserylalanine ethyl ester (2E, Ser[5] 4–6), respectively, gave the two protected hexapeptides (2F, Ser[4] 1–6, and 2F, Ser[5] 1–6), which were converted into hydrazides (2G or 3A, Ser[4] 1–6, and 2G or 3A, Ser[5] 1–6) in the usual manner.

The corresponding azides were coupled with N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester (3B, 7–12) obtained by catalytic hydrogenolysis from the corresponding N^{α}-benzyloxycarbonyl derivative[14] (3A, 7–12) to give the two protected dodecapeptide esters (3C, Ser[4] 1–12, and 3C, Ser[5] 1–12) which were converted into hydrazides (3D, Ser[4] 1–12, and 3D, Ser[5] 1–12).

The corresponding azides were then treated with the octapeptide methionylaspartylserylserylthreonylseryl-alanylalanine[15] (3E, 13–20) to give the partially protected eicosapeptides 3F, 1–20.

(11) (a) E. Scoffone, F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, and A. Marzotto, *Tetrahedron Letters*, 943 (1966); (b) E. Scoffone, R. Rocchi, F. Marchiori, A. Marzotto, A. Scatturin, A. M. Tamburro, and G. Vidali, *J. Chem. Soc., C*, 606 (1967).

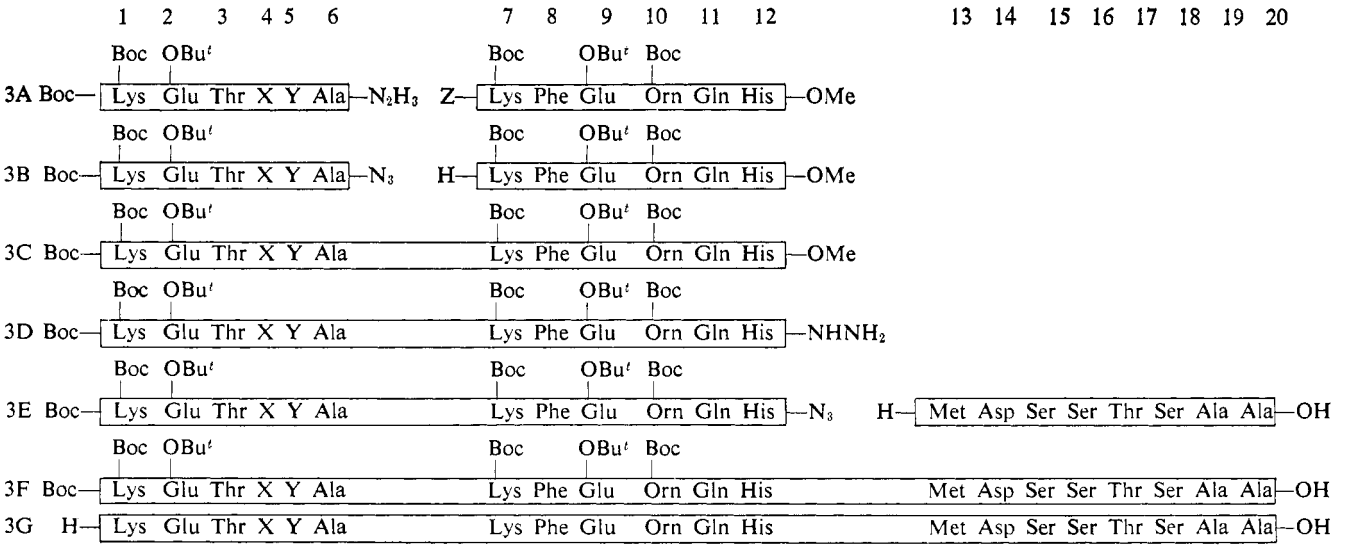
(12) R. Rocchi, F. Marchiori, L. Moroder, A. Fontana, and E. Scoffone, *Gazz. Chim. Ital.*, **96**, 1537 (1966).

(13) E. Scoffone, R. Rocchi, G. Vidali, A. Scatturin, and F. Marchiori, *ibid.*, **94**, 743 (1964).

(14) F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, and E. Scoffone, *J. Chem. Soc., C*, 81 (1967).

(15) F. Marchiori, R. Rocchi, L. Moroder, G. Vidali, and E. Scoffone, *ibid.*, 89 (1967).

Chart III. Synthesis of the 1–20 Sequences



The protecting groups were removed with TFA and the crude eicosapeptides 3G, Ser[4] 1–20, and 3G, Ser[5] 1–20, so obtained were purified by chromatography through an Amberlite CG 50 column with 0.2 *M* sodium phosphate, desalted by gel filtration on Sephadex G-25 using 5% acetic acid as an eluent, and lyophilized.

The chemical homogeneity of the two S-peptide analogs was evaluated by electrophoresis at different pH values and quantitative amino acid analysis of the acid hydrolysates.

Aminopeptidase M (AP-M) was employed for evaluation of the stereochemical homogeneity of synthetic materials according to the procedure described by Hofmann.[8]

Experimental Section[16]

RNase A was prepared from bovine pancreatic ribonuclease (Fluka AG four times crystallized) by the procedure of Crestfield, *et al.*;[17] 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[18] modified by Dosher and Hirs.[3b] 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.[19]

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, 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.

Benzyloxycarbonylserylalanine Ethyl Ester (2B, Ser[5] 5–6). Benzyloxycarbonylserine 2,4-dinitrophenyl ester[20] (11.34 g, 28 mmol) was added to a solution of alanine ethyl ester hydrochloride[21] (4.6 g, 30 mmol) in chloroform (300 ml) containing triethylamine (8.4 ml). After 12 hr at room temperature the solution was extracted with 5% sodium carbonate, 1 *N* hydrochloric acid, and water, dried over sodium sulfate, and taken to dryness *in vacuo*. The residue was crystallized from ethanol–petroleum ether (bp 30–60°); yield 7.98 g (83%), mp 100–101°, $[\alpha]^{20}D$ −29.8 ± 0.3° (*c* 1.0, methanol); R_{f_1} 0.80; single ninhydrin-negative, chlorine-positive spot.

Anal. Calcd for $C_{16}H_{22}N_2O_6$ (338.35): C, 56.8; H, 6.6; N, 8.3. Found: C, 56.6; H, 6.5; N, 8.3.

Benzyloxycarbonylalanylserylalanine Ethyl Ester (2D, Ser[5] 4–6). Benzyloxycarbonylalanine *p*-nitrophenyl ester[22] (7.44 g, 21.6 mmol)

(16) The melting points were determined by a Tottoli 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°.

Aminopeptidase M digests were prepared in the manner described by Hofmann, *et al.*[8] The amino acid composition of acid and enzymic hydrolysates was determined by quantitative analysis using a Technicon amino acid analyzer. Ascending thin layer chromatography was performed on silica gel G (Merck) with the following solvent systems: R_{f_1}, *n*-butyl alcohol–glacial acetic acid–water (3:1:1); R_{f_2}, ethyl acetate–pyridine–glacial acetic acid–water (60:20:6:14).

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–$NaHCO_3$; G. E. Delory and E. J. King, *Biochem. J.*, **39**, 245 (1945)).

The chlorine (H. N. Rydon and P. Smith, *Nature*, **169**, 922 (1952)) and the Pauly (K. Randerath, "Thin Layer Chromatography," Academic Press, New York, N. Y., 1963, p 176) 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. Catalytic hydrogenolysis was carried out in the indicated solvent, containing a few drops of glacial acetic acid, over 10% palladized charcoal, for the indicated time. 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 M. Kunitz (*J. Biol. Chem.*, **164**, 563 (1946)), as well as by following the release of acid-soluble nucleotides by the uranyl acetate–TCA procedure (W. A. Klee and F. M. Richards, *ibid.*, **229**, 489 (1957)). For reasons outlined in a previous communication,[6b] prior to performing the ribonuclease assays, the synthetic eicosapeptides were incubated under nitrogen at 45°, in 1% aqueous thioglycolic acid, according to the procedure described by Hofmann, *et al.*[8]

(17) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 618 (1963).

(18) (a) F. M. Richards and P. J. Vithayathil, *ibid.*, **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).

(19) D. Wellner, H. J. Silman, and M. Sela, *J. Biol. Chem.*, **238**, 1324 (1963).

(20) F. Marchiori, R. Rocchi, and E. Scoffone, *Gazz. Chim. Ital.*, **93**, 834 (1963).

(21) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud, and E. Sandrin, *Helv. Chim. Acta*, **38**, 1491 (1955).

and triethylamine (6.5 ml) were added to a chloroform solution (200 ml) of serylalanine ethyl ester acetate obtained by hydrogenolysis of 2B, Ser[5] 5–6 (7.95 g, 23.5 mmol), in 50% methanolic acetic acid (300 ml). The solution was kept for 24 hr at room temperature and then washed with 5% sodium carbonate, 1 *N* hydrochloric acid, and water, dried over sodium sulfate, and evaporated to dryness *in vacuo*. The residue was dissolved in ethanol, and petroleum ether was added to precipitate the product; yield 6.2 g (70%); mp 156–157°; $[\alpha]^{20}D$ −47.7 ± 0.2° (*c* 1.0 methanol); R_{f_1} 0.75, R_{f_2} 0.95; single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{19}H_{27}N_3O_7$ (409.4): C, 55.7; H, 6.6; N, 10.3. Found: C, 55.3; H, 6.6; N, 10.1.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylseryl-alanylalanine Ethyl Ester (2F, Ser[4] 1–6). Sodium nitrite, 1 *M* (4.2 ml), was added to a solution of N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonine hydrazide[13] (2D, 1–3; 2.59 g, 4.0 mmol) in a mixture of glacial acetic acid (20 ml), 1 *N* hydrochloric acid (8 ml), and saturated sodium chloride solution (4 ml) cooled to −10°. After stirring at −10° for 15 min precooled, saturated sodium chloride solution (15 ml) and ice-cold water (15 ml) were added, and the N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonine azide (2E, 1–3) was extracted with three 20-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 10 min at −10°, over sodium sulfate. Simultaneously, benzyloxycarbonylserylalanylalanine ethyl ester[23] (2D, Ser[4] 4–6) (1.84 g, 4.5 mmol) was dissolved in methanol (100 ml) and hydrogenated for 4 hr and the catalyst filtered off. The filtrate was evaporated to dryness under reduced pressure; the residue was dissolved in methanol, and precipitation occurred by addition of ether; yield 1.1 g (80%); mp 144–145°; R_{f_1} 0.50; single ninhydrin- and chlorine-positive spot. The product was dissolved in DMF (25 ml) containing triethylamine (1.1 ml) and added to the ethyl acetate solution of the protected tripeptide azide (2E, 1–3) prepared above. The resulting solution was concentrated under reduced pressure, at 0°, to remove most of the ethyl acetate and then allowed to react at 5° for 5 days whereupon ether was added. The precipitate was collected, washed with ether, and crystallized twice from DMF–ethyl acetate; yield 2.16 g (70%); mp 194–196°; $[\alpha]^{25}D$ −19.0 ± 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 $C_{40}H_{71}N_7O_{15}$ (890.0): C, 54.0; H, 8.0; N, 11.0. Found: C, 53.4; H, 8.0; N, 11.2.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylala-nylserylalanine Ethyl Ester (2F, Ser[5] 1–6). The condensation of 2E, 1–3 (obtained from 2.59 g, 4.0 mmol of 2D, 1–3),[13] with 2E, Ser[5] 4–6 (R_{f_1} 0.50; obtained by hydrogenolysis in methanol from 1.84 g, 4.5 mmol, of 2D, Ser[5] 4–6), was carried out by the same procedure described above for 2F, Ser[4] 1–6; yield 2.6 g (81%); mp 182–184°; $[\alpha]^{20}D$ −18.5 ± 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 $C_{40}H_{71}N_7O_{15}$ (890.0): C, 54.0; H, 8.0; N, 11.0. Found: C, 53.6; H, 7.9; N, 11.1.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonyl-serylalanylalanine Hydrazide (2G, Ser[4] 1–6, or 3A, Ser[4] 1–6). The protected hexapeptide ethyl ester 2F, Ser[4] 1–6 (2.08 g, 2.34 mmol), was dissolved in DMF (70 ml), and hydrazine hydrate (2.5 ml) was added. The reaction mixture was kept overnight at 60° and then a further 4 days at room temperature. The slightly turbid solution was filtered and concentrated under reduced pressure, and the product was precipitated by addition of ethanol. The precipitate was collected by centrifugation, washed with ethanol and ether, recrystallized twice from DMF–ether, and dried *in vacuo* over concentrated sulfuric acid; yield 1.8 g (88%); mp 217–218°; $[\alpha]^{22}D$ −32.0 ± 0.5° (*c* 1.0, glacial acetic acid); R_{f_1} 0.65, R_{f_2} 0.95; single chlorine- and picryl chloride positive spot.

Anal. Calcd for $C_{38}H_{69}N_9O_{14}$ (876.0): C, 52.1; H, 7.9; N, 14.4. Found: C, 51.7; H, 7.9; N, 14.4.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylala-nylserylalanine hydrazide (2G, Ser[5] 1–6, or 3A, Ser[5] 1–6) was obtained from 2F, Ser[5] 1–6 (2.22 g, 2.5 mmol), by the same procedure reported above for the preparation of 2G, Ser[4] 1–6; yield 1.8 g (82%); mp 221–222°; $[\alpha]^{22}D$ −31.3 ± 0.5° (*c* 1.0, glacial acetic acid); R_{f_1} 0.65, R_{f_2} 0.95; single chlorine- and picryl chloride positive spot.

Anal. Calcd for $C_{38}H_{69}N_9O_{14}$ (876.0): C, 52.1; H, 7.9; N, 14.4. Found: C, 51.6; H, 7.9; N, 14.3.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylseryl-alanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butyl-glutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, Ser[4] 1–12). Sodium nitrite, 1 *M* (0.6 ml), was added to a solution of 3A, Ser[4] 1–6 (0.442 g, 0.504 mmol), in a mixture of glacial acetic acid (6 ml), 1 *N* hydrochloric acid (1.2 ml), and 20% sodium chloride (2 ml) at −10°. After stirring for 20 min at −10°, precooled 20% sodium chloride (60 ml) was added, and the resulting precipitate was collected by centrifugation and washed with ice-cold water. The moist azide was then dissolved in DMF (20 ml) at −10° and dried over sodium sulfate. Simultaneously N^{α}-benzyloxycarbonyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester[14] (3A, 7–12) (0.61 g, 0.504 mmol) was dissolved in a mixture of DMF–methanol (1:1 by volume) and hydrogenated for 3 hr and the catalyst filtered off. The filtrate was evaporated to dryness under reduced pressure, and the residue was dissolved in DMF (50 ml) containing triethylamine (0.14 ml) and added to the DMF solution of the protected hexapeptide azide (3B, Ser[4] 1–6). The reaction mixture was kept 6 days at 5° and a further 24 hr at room temperature; the solution was concentrated under reduced pressure, and precipitation occurred on addition of water. The precipitate was collected by centrifugation, washed with water, and dried *in vacuo* over phosphorus pentoxide. Crystallization of this crude material from DMF–ether gave the pure product; yield 0.290 g (30%); mp 232–233°; $[\alpha]^{20}D$ −15.5 ± 0.5° (*c* 1.0, DMF); R_{f_1} 0.85, R_{f_2} 0.95; single ninhydrin-negative and chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{1.97}Orn_{1.05}Glu_{3.00}Thr_{1.00}Ser_{1.00}Ala_{2.00}Phe_{1.05}His_{0.95}$.

Anal. Calcd for $C_{89}H_{146}N_{18}O_{28}$ (1916.3): C, 55.8; H, 7.7; N, 13.2. Found: C, 54.7; H, 7.5; N, 12.9.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonyl-alanylserylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butyl-glutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester (3C, Ser[5] 1–12) was prepared by condensation of 3B, Ser[5] 1–6 (obtained from 0.442 g, 0.504 mmol, of 3A, Ser[5] 1–6), with 3B, 7–12 (obtained by hydrogenolysis in 50% methanolic DMF from 0.61 g, 0.504 mmol, of 3A, 7–12),[14] by an azide coupling step as described above for the preparation of 3C, Ser[4] 1–12; yield 0.454 g (47%); mp 234–236°; $[\alpha]^{20}D$ −25.0 ± 1° (*c* 0.5, DMF); R_{f_1} 0.85, R_{f_2} 0.95; single ninhydrin-negative and chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{2.00}Orn_{1.05}Glu_{3.00}Thr_{0.98}Ser_{0.98}Ala_{2.00}Phe_{0.90}His_{0.90}$.

Anal. Calcd for $C_{89}H_{146}N_{18}O_{28}$ (1916.3): C, 55.8; H, 7.7; N, 13.2. Found: C, 55.1; H, 7.7; N, 13.2.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonyl-serylalanylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butyl-glutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, Ser[4] 1–12). The dodecapeptide methyl ester 3C, Ser[4] 1–12 (0.272 g, 0.142 mmol), was dissolved in DMF (10 ml), and hydrazine hydrate (0.28 ml) was added. The solution was heated for 1 hr at 70°, hydrazine hydrate (0.14 ml) was then added, and the reaction mixture was kept overnight at 50° and a further 3 days at room temperature. The solution was concentrated under reduced pressure, ether was added, and the precipitate was collected, washed with ether, and dried *in vacuo* over concentrated sulfuric acid. The crude product was recrystallized from DMF–water; yield 0.190 g (70%); mp 238–240° dec; $[\alpha]^{20}D$ −24.8 ± 0.5° (*c* 1.0, 90% acetic acid); R_{f_1} 0.70, R_{f_2} 0.95; single Pauly-, chlorine-, and picryl chloride positive spot.

Anal. Calcd for $C_{88}H_{146}N_{20}O_{27}$ (1916.3): C, 55.1; H, 7.7; N, 14.6. Found: C, 54.4; H, 7.5; N, 14.3.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylala-nylserylalanyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butyl-glutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine hydra-zide (3D, Ser[5] 1–12) was prepared from the corresponding methyl ester (3C, Ser[5] 1–12; 0.450 g, 0.233 mmol) by the procedure described above for 3D, Ser[4] 1–12. The product (0.326 g, 73%) had mp 238–240° dec; $[\alpha]^{20}D$ −27.2 ± 0.5° (*c* 1.0, 90% acetic acid); R_{f_1} 0.70, R_{f_2} 0.95; single Pauly-, chlorine-, and picryl chloride positive spot.

Anal. Calcd for $C_{88}H_{146}N_{20}O_{27}$ (1916.3): C, 55.1; H, 7.7; N, 14.6. Found: C, 54.9; H, 7.7; N, 14.4.

Lysylglutamylthreonylserylalanylalanyllysylphenylalanylglutam-ylornithylglutaminylhistidylmethionylaspartylserylserylthreonylser-ylalanylalanine (3G, Ser[4] 1–20). Sodium nitrite (1 *M*, 0.2 ml)

(22) F. Marchiori, R. Rocchi, and E. Scoffone, *Ric. Sci. Rend.*, **A2**, 647 (1962).

(23) F. Marchiori, R. Rocchi, and E. Scoffone, *Gazz. Chim. Ital.*, **94**, 834 (1963).

was added to a solution of 3D, Ser[4] 1–12 (0.186 g, 0.096 mmol), in a mixture of 90% acetic acid (8 ml), 1 *N* hydrochloric acid (0.4 ml), and saturated sodium chloride solution (0.8 ml) at −10°. After stirring for 15 min at −10°, precooled 20% sodium chloride solution (80 ml) was added, and the resulting precipitate was collected and washed with ice-cold water. The still-wet material was dissolved in DMF (20 ml) at −10° and dried over sodium sulfate. The drying agent was filtered off, and a solution of methionylaspartylserylserylthreonylserylalanylalanine[15] (3E, 13–20; 0.17 g, 0.192 mmol, as monoacetate, trihydrate) in DMF (5 ml) and triethylamine (0.06 ml) was added. The reaction mixture was stirred for 7 days at 5° and for 1 day at room temperature, filtered, and concentrated to 10 ml under reduced pressure and water was added (150 ml). The resultant precipitate was centrifuged, washed with water and ether, and dried, yielding 0.14 g (58%). The crude material (3F, Ser[4] 1–20) (0.132 g) was dissolved in anhydrous TFA (1.3 ml), and the solution was kept for 150 min at room temperature. An excess of ice-cold ether was added and after 30 min at −10° the peptide was collected by centrifugation, washed with ether, and dried. The residue, dissolved in 0.2 *M* sodium phosphate buffer (pH 6.4), 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, Ser[4] 1–20) (0.02 g, 19%) had $[\alpha]^{22}D$ −70.6 ± 1° (*c* 0.117, 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: $Lys_{2.00}Orn_{1.10}Glu_{2.93}Thr_{2.00}Ala_{3.90}Phe_{0.97}His_{0.97}Met_{0.98}Asp_{0.98}Ser_{3.93}$; amino acid ratios in AP-M digest: $Lys_{1.97}Orn_{1.01}Glu_{1.97}Thr_{1.98}Ala_{3.92}Phe_{1.02}His_{0.96}Met_{0.95}Asp_{0.95}(Ser + Gln)_{4.90}$. The [Ser[4],Orn[10]]-S-peptide gave, after recombination in 1:1 molar ratio with S-protein, a 40% active partially synthetic ribonuclease.

Lysylglutamylthreonylalanylserylalanyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, Ser[5] 1–20). The condensation of 3D, Ser[5] 1–12 (0.315 g, 0.164 mmol), with 3E, 13–20[15] (0.29 g, 0.328 mmol, as monoacetate, trihydrate), by the azide procedure was carried out as described above for 3G, Ser[4] 1–20, and gave the partially protected [Ser[5],Orn[10]]-S-peptide (3F, Ser[5] 1–20; 0.357 g, 82%). Treatment of 0.15 g of crude product with anhydrous TFA (1.5 ml), purification on Amberlite CG 50 and on Sephadex G-25, followed by lyophilization gave the pure [Ser[5],Orn[10]]-S-peptide (3G, Ser[5] 1–20; 0.04 g, 33%); $[\alpha]^{22}D$ −71.4 ± 1° (*c* 0.115, 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: $Lys_{2.04}Glu_{2.96}Thr_{1.97}Ala_{4.02}Orn_{1.01}Phe_{1.03}His_{0.99}Met_{0.95}Asp_{0.96}Ser_{3.97}$; amino acid ratios in AP-M digest: $Lys_{2.05}Glu_{1.99}Thr_{1.95}Ala_{4.02}Orn_{1.05}Phe_{1.02}His_{0.96}Met_{1.02}Asp_{0.96}(Ser + Gln)_{5.00}$. The [Ser[5],Orn[10]]-S-peptide gave, after recombination in 1:1 molar ratio with S-protein, a 50% active partially synthetic ribonuclease.

Acknowledgment. The authors wish to thank Dr. E. Celon for carrying out the microanalyses, Dr. L. Carpino for reading the manuscript, and Mr. U. Anselmi and Mr. D. Stivanello for the skillful technical assistance.

Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. IX. Synthesis of [Ser[6],Orn[10]]- and [Pro[6],Orn[10]]-eicosapeptides[1,2]

Fernando Marchiori, Raniero Rocchi, Luigi Moroder, Angelo Fontana, and Ernesto Scoffone

Contribution from Istituto di Chimica Organica dell'Universitá, Sezione VIII del Centro Nazionale di Chimica delle Macromolecole del CNR, Padua, Italy.
Received June 3, 1968

Abstract: Syntheses are described of two analogs of the S-peptide in which the arginyl residue in position 10 is replaced by ornithine and the alanyl residue in position 6 by either serine or proline. The stereochemical homogeneity of these peptides, *i.e.*, lysylglutamylthreonylalanylalanylseryllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine and lysylglutamylthreonylalanylalanylprolyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. The enzymic properties of the two synthetic eicosapeptides were checked, with RNA, after recombination with S-protein. The [Ser[6],Orn[10]]-S-peptide and the [Pro[6],Orn[10]]-S-peptide form respectively a 40 and 15% active partially synthetic ribonuclease at a molar ratio of 1:1 with S-protein.

In a previous communication[3] we described the syntheses of [Ser[4],Orn[10]]- and [Ser[5],Orn[10]]-eicosapeptide analogs of the N-terminal sequence of bovine pancreatic ribonuclease A[4] and we reported that these synthetic modified S-peptides show high ribonuclease activity after recombination with S-protein in a 1:1 molar ratio when tested with RNA as substrate.

The present study deals with the synthesis of two new eicosapeptide analogs where the arginyl residue in position 10 has been replaced by ornithine and the alanyl residue in position 6 either by serine or proline.

(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 (IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **241**, 2491 (1966)) are used: 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.

(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, Proceedings of the Symposium, in press.

(3) Part VIII: R. Rocchi, L. Moroder, F. Marchiori, E. Ferrarese, and E. Scoffone, *J. Am. Chem. Soc.*, **90**, 5885 (1968).

(4) (a) 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-protein, the protein component obtained from RNase S; S-peptide, the eicosapeptide obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein. (b) According to 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.