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: $Lys_{2.00}Orn_{1.10}Glu_{2.05}Thr_{2.00}Ala_{3.90}Phe_{0.97}His_{0.97}Met_{0.98}Asp_{0.98}Ser_{3.95}$; 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.

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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]

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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.

Chart I. Amino Acid Sequences 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

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

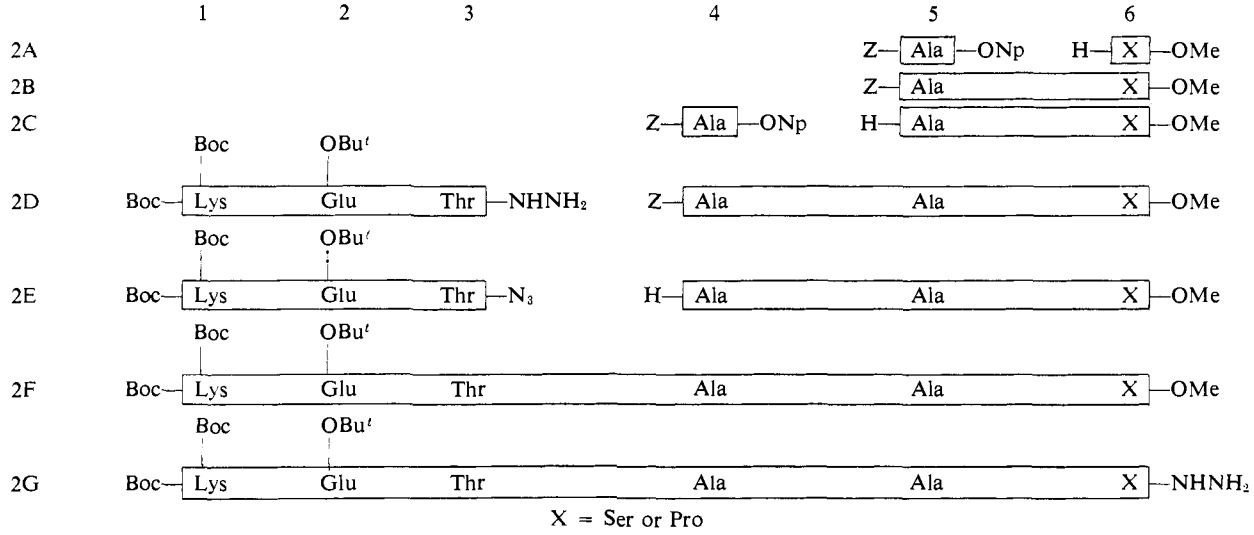
[Ser^{6},Orn^{10}]-S-Peptide

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

[Pro^{6},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-Ala-Pro-Lys-Phe-Glu-Orn-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

Chart II. Synthesis of the 1–6 Sequences



The X-ray structure of RNase-S[5] shows that the section of the S-peptide molecule which contains the sequence 2–12 is in an α-helical conformation and that the alanyl residue in position 4 appears to be involved in the hydrophobic interaction responsible for the correct S-peptide–S-protein association. The results of our substitution at positions 4 and 5 showed that direct hydrophobic interactions between the alanyl-4 or -5 side-chain residue and groups in the protein appears to be of little significance.

In order to see how the alanyl residue in position 6 affects the ability of the S-peptide to assume its correct conformation when it is bound to S-protein, we synthesized the [Ser^{6},Orn^{10}]-S-peptide (Chart I).

The findings of Beintema,[6] that an aspartyl residue replaces alanine-6 in the rat ribonuclease sequence, makes probable that the substitution of the apolar residue in position 6 by an amino acid with a polar side chain does not significantly affect the catalytic function.

In order to explore the influence of the dimensions of the helical portion on the S-peptide's ability to bind and to activate the S-protein, we carried out the synthesis of the [Pro^{6},Orn^{10}]-S-peptide (Chart I). The steric restrictions of proline prevent such a residue from taking part in an α-helical conformation. The expected consequence of the replacement of alanine-6 with proline is then a shortening or the disappearance of the helical section of the S-peptide, which could affect its capacity to regenerate ribonuclease activity with the S-protein.

The activity data show that both synthetic analogs are able to catalyze the depolymerization of RNA, after recombination with S-protein in a 1:1 molar ratio. The [Ser^{6},Orn^{10}]- and the [Pro^{6},Orn^{10}]-RNase S′ show about 65 and 25%, respectively, of the enzymic activity of [Orn^{10}]-RNase S′[7] (40 and 15% in comparison with RNase S′).

Since the alanyl residues in positions 4, 5, or 6 can be replaced by the seryl residue without significantly affecting the potential catalytic properties of the resulting S-peptide analogs, it is possible to conclude that the hydrophobic character of these residues is not an important feature for the correct peptide–protein association.

Moreover, the potential catalytic activity of the synthetic analog containing, in position 6, an imino acid (proline) in place of an amino acid (alanine) shows that such a modified eicosapeptide still posseses those basic structural features which are responsible for the activation of the S-protein in spite of the considerable difference in conformation which undoubtedly is found in the polypeptide backbone.

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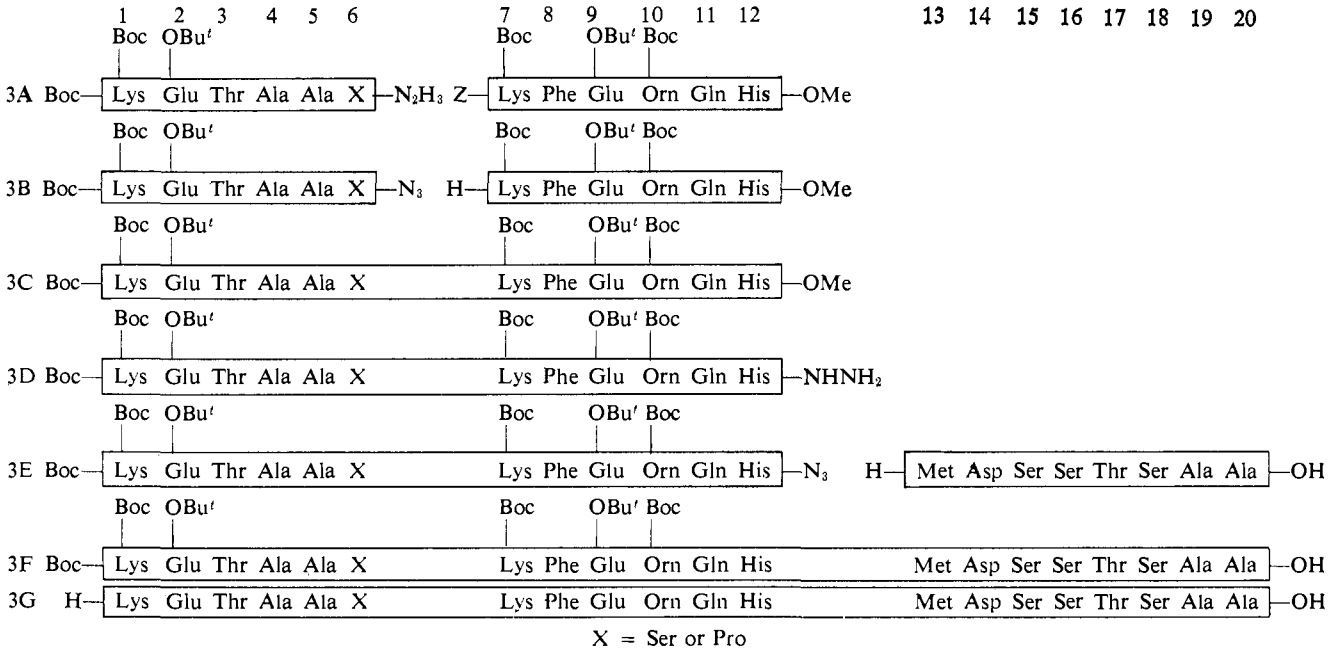
Chart III. Synthesis of the 1–20 Sequences



These findings agree with Hofmann's[8] observation that the heptapeptide N^{ϵ}-formyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine still possesses the ability to activate the S-protein, with RNA as substrate (although at high peptide:protein molar ratios).

We demonstrated[9] that the S-peptide is essentially randomly coiled in the absence of the partner S-protein while it undergoes a coil-to-helix transition in the presence of the protein.

If one assumes that the existence of a helical portion in the S-peptide, stabilized by a cooperative effect of S-protein, is essential to facilitate the hydrophobic interactions which allow the association process, it is possible to deduce, from the above-mentioned findings, that only the helical section including residue 7–12 is essential for the correct peptide–protein binding, while residue 2–6 could be in a different conformation without a remarkable effect on the affinity of synthetic peptides for S-protein. A more detailed examination of the enzymic properties of [Ser[6],Orn[10]]- and [Pro[6],Orn[10]]-S-peptide including the evaluation of their conformational stability will be reported in a forthcoming paper.

Peptide Syntheses

The synthetic route to [Ser[6],Orn[10]]- and [Pro[6],-Orn[10]]-S-peptide, illustrated in Charts II and III, is similar to that which was used for the preparation of other analogs.[3,7,10] The sequence 1–6 was prepared by condensing the azide corresponding to the N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonine hydrazide[11] (2D, 1–3) with alanylalanylserine methyl ester (2E, Ser[6] 4–6) or alanylalanylproline methyl ester (2E, Pro[6] 4–6), respectively, to give the two protected hexapeptides 2F, Ser[6] or 2F, Pro[6] 1–6.

The above-mentioned hexapeptides were converted into hydrazides (2G or 3A, Ser[6] 1–6, and 2G or 3A, Pro[6] 1–6) in the usual manner.

The corresponding azides (3B, Ser[6] or Pro[6] 1–6) 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[12] (3A, 7–12) to give the two protected dodecapeptides esters (3C, Ser[6] or Pro[6] 1–12).

The esters were converted into hydrazides (3D, Ser[6] or Pro[6] 1–12) and azides (3E, Ser[6] or Pro[6] 1–12) which were coupled with the octapeptide methionylaspartylserylserylthreonylserylalanylalanine[13] (3E, 13–20) to give the partially protected eicosapeptides 3F, Ser[6] or Pro[6] 1–20. The protecting groups were removed by exposure to TFA, and the crude eicosapeptides 3G, Ser[6] or Pro[6] 1–20, 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 both 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 the evaluation of the stereochemical homogeneity of synthetic materials according to the procedure suggested by Hofmann.[14]

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Experimental Section[15]

RNase A was prepared by the procedure of Crestfield, *et al.*,[16] starting with bovine pancreatic ribonuclease (Fluka AG four times crystallized). RNase S, S-protein, and S-peptide were prepared from RNase A, essentially by the method of Richards.[4b,17]

Aminopeptidase M (AP-M) was obtained from Rohm and Haas HmbH, Darmstadt, West Germany. Yeast RNA, obtained from Schwarz Laboratories, was purified according to the procedure of Wellner, *et al.*[18]

The crude S-peptide analogs (100–200 mg) obtained by treatment of the partially protected eicosapeptide with TFA were dissolved in 0.2 *M* sodium phosphate buffer (pH 6.47) and purified by passing the solutions 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- and 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. 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.

Benzyloxycarbonylalanylserine Methyl Ester (2B, Ser^6 5–6). Benzyloxycarbonylalanine *p*-nitrophenyl ester[19] (20.66 g, 60.0 mmol) and triethylamine (8.4 ml) were added to a pyridine solution (150 ml) containing the serine methyl ester[20] (prepared from 10.23 g, 66.0 mmol, of the hydrochloride with 2 *N* ammonia in chloroform in the usual manner), and the reaction mixture was kept overnight at room temperature. The solvent was then removed *in vacuo* and the residue crystallized twice from ethyl acetate and once from ethanol–petroleum ether (bp 30–60°); yield 15.18 g (78%); mp 134–135° (lit.[21] mp 134–135°); $[\alpha]^{20}D$ −20.5 ± 0.2° (*c* 1.0, methanol). Alternatively in another experiment the residue obtained by removing the solvent from the reaction mixture was taken up in ethyl acetate and washed successively with 5% sodium carbonate, 1 *N* hydrochloric acid, and water. The solution was then dried over sodium sulfate and evaporated to dryness. Crystallization of the residue from ethanol–petroleum ether gave only 44% yield of the pure product, probably owing to its partial solubility in water.

Benzyloxycarbonylalanylproline Methyl Ester (2B, Pro^6 5–6). Benzyloxycarbonylalanine *p*-nitrophenyl ester[19] (5.86 g, 17 mmol) and triethylamine (5.2 ml) were added to a pyridine solution (100 ml) containing proline methyl ester hydrochloride[22] (3.31 g, 20 mmol), and the reaction mixture was kept 20 hr at room temperature. The solvent was then removed *in vacuo*, and the residue, taken up in ethyl acetate, was thoroughly washed with 5% sodium carbonate, 1 *N* hydrochloric acid, and water. The solution, dried over sodium sulfate and taken to dryness *in vacuo*, gave the title compound as an oil; yield 4.34 g (76%); R_{f_1} 0.73, R_{f_2} 0.95; single ninhydrin-negative and chlorine-positive spot.

Since our attempts to crystallize the oily methyl ester failed, the characterization of 2B, Pro^6 5–6, was achieved as follows. Benzyloxycarbonylalanylproline methyl ester (0.2 g, 0.6 mmol) was dissolved in acetone (2 ml); 1 *N* sodium hydroxide (0.66 ml) was added, and the solution was stirred for 45 min at room temperature. The acetone was then removed under reduced pressure. The residue was dissolved in water (30 ml), and the solution was extracted twice with ether (two 30-ml portions), cooled to 2°, and acidified to pH 2 with 1 *N* hydrochloric acid. The acid solution was extracted three times with ethyl acetate (three 30-ml portions), and the combined organic layers were thoroughly washed with water, dried over sodium sulfate, and concentrated to small volume *in vacuo*. Dicyclohexylamine (0.14 ml) was added, and the resulting dicyclohexylammonium salt was precipitated by addition of ether, collected, and dried *in vacuo* over concentrated sulfuric acid; yield 0.225 g (71%); mp 204–205°; $[\alpha]^{20}D$ −58.4 ± 0.2° (*c* 1.0, glacial acetic acid).

Anal. Calcd for $C_{28}H_{43}N_3O_5$ (501.66): C, 67.0; H, 8.7; N, 8.4. Found: C, 67.2; H, 8.6; N, 8.5.

Benzyloxycarbonylalanylalanylserine Methyl Ester (2D, Ser^6 4–6). Benzyloxycarbonylalanine *p*-nitrophenyl ester[19] (14.63 g, 42.5 mmol) and triethylamine (12 ml) were added to a pyridine solution (300 ml) of alanylserine methyl ester acetate obtained by hydrogenolysis of 2B, Ser^6 5–6 (14.9 g, 46.0 mmol), in 50% methanolic acetic acid (300 ml). The reaction mixture was kept 24 hr at room temperature, the solvent was evaporated to dryness *in vacuo*, and the residue was crystallized twice from ethyl acetate and once from ethanol–ether; yield 11.6 g (69%); mp 192–193°; $[\alpha]^{20}D$ −52.0 ± 0.5 (*c* 1.0, methanol); R_{f_1} 0.70, R_{f_2} 0.85; single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{18}H_{25}N_3O_7$ (395.4): C, 54.7; H, 6.4; N, 10.6. Found: C, 54.6; H, 6.2; N, 10.6.

As in the case of 2B, Ser^6 5–6, the yield of pure product dropped to 22% when the purification of the residue, obtained by evaporation of the solvent from the reaction mixture, was carried out by taking up this residue in ethyl acetate and washing with 5% sodium carbonate, 1 *N* hydrochloric acid, and water.

Benzyloxycarbonylalanylalanylproline Methyl Ester (2D, Pro^6 4–6). Benzyloxycarbonylalanylproline methyl ester (2B, Pro^6 5–6) (4.14 g, 12.37 mmol) was dissolved in 2.5 *N* hydrobromic acid in glacial acetic acid and kept at 50° for 45 min and at room temperature for an additional hour. Ether was added to precipitate the dipeptide hydrobromide, and the oily residue was collected, dissolved in methanol, and reprecipitated with ether (three times). The resulting oil (2.78 g, 80%) was dried *in vacuo* over potassium hydroxide pellets and proved to be chromatographically homogeneous; R_{f_1} 0.35, R_{f_2} 0.30. The alanylproline methyl ester hydrobromide was dissolved in pyridine, and benzyloxycarbonylalanine *p*-nitrophenyl ester[19] (3.1 g, 9 mmol) and triethylamine (2.65 ml) were added. The reaction mixture was kept 40 hr at room temperature, then the solvent was evaporated to dryness *in vacuo*, and the residue, taken up in ethyl acetate, was washed with 5% sodium carbonate, 1 *N* hydrochloric acid, and water and dried over sodium sulfate. The solvent was removed under reduced pressure, and the product was crystallized twice from methanol–ether; yield 1.6 g (44%); mp 130–131°; $[\alpha]^{20}D$ −116.9 ± 0.5° (*c* 1.0, glacial acetic acid); R_{f_1} 0.70, R_{f_2} 0.95; single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{20}H_{27}N_3O_6$ (405.44): C, 59.2; H, 6.7; N, 10.4. Found: C, 59.1; H, 6.7; N, 10.4.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylserine Methyl Ester (2F, Ser^6 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[11] (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 (4 ml) cooled to −10°. After stirring at −10° for 15 min precooled saturated sodium chloride (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, benzyloxycarbonylalanylalanylserine methyl ester (2D, Ser^6 4–6) (1.78 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, and the residue was dissolved in methanol. Precipitation occurred on addition of ether. 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 2.80 g (80%); mp 182–184°; $[\alpha]^{20}D$ −15.3 ± 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 $C_{39}H_{69}N_7O_{15}$ (876.0): C, 53.5; H, 8.0; N, 11.2. Found: C, 53.2; H, 7.9; N, 11.4.

(15) General experimental and analytical procedures used were those described in paper VIII of this series.[3]

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N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylproline Methyl Ester (2F, Pro^6 1–6). The condensation of 2E, 1–3 (obtained from 2.52 g, 3.9 mmol of 2D, 1–3[11]), with 2E, Pro^6 4–6 (R_{f_1} 0.30; obtained by hydrogenolysis in methanol from 1.58 g, 3.9 mmol, of 2D, Pro^6 4–6), was carried out by the same procedure described above for 2F, Ser^6 1–6. After 6 days at 5°, the solvent was removed *in vacuo* from the reaction mixture, and the residue, taken up in chloroform, was extracted with 5% sodium carbonate, 5% citric acid, and water. The solution was dried over sodium sulfate and evaporated to dryness *in vacuo*. The residue was crystallized from methanol–ethyl acetate and recrystallized from methanol–ether; yield 2.62 g (76%); mp 140–141°; $[\alpha]^{20}D$ −41 ± 0.5° (*c* 1.0, DMF); R_{f_1} 0.70, R_{f_2} 0.80; single ninhydrin-negative and chlorine-positive spot.

Anal. Calcd for $C_{41}H_{71}N_7O_{14}$ (886.09): C, 55.5; H, 8.1; N, 11.1. Found: C, 54.8; H, 8.1; N, 10.9.

N,$^{\alpha}N^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylserine Hydrazide (2G, Ser^6 1–6, or 3A, Ser^6 1–6). The protected hexapeptide methyl ester 2F, Ser^6 1–6 (2.63 g, 3.0 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 at room temperature for 4 days. 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 2.26 g (86%); mp 217–218°; $[\alpha]^{22}D$ −34.0 ± 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, 52.0; H, 8.0; N, 14.5.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylproline hydrazide (2G, Pro^6 1–6, or 3A, Pro^6 1–6) was obtained by addition of hydrazine hydrate (2.5 ml) to a solution of 2F, Pro^6 1–6 (2.5 g, 2.82 mmol), in DMF (50 ml). After heating for 1 hr at 70° the reaction mixture was kept 3 days at room temperature. The solution was concentrated under reduced pressure, and the product was precipitated by addition of water. The precipitate was collected, washed with water, dried *in vacuo* over concentrated sulfuric acid, and crystallized twice from DMF–ether, yielding 1.44 g (57%); mp 144–145°; $[\alpha]^{20}D$ −69.6 ± 0.2° (*c* 1.0, 90% acetic acid); R_{f_1} 0.55, R_{f_2} 0.80; single chlorine- and picryl chloride positive spot.

Anal. Calcd for $C_{40}H_{71}N_9O_{13}$ (886.1): C, 54.2; H, 8.1; N, 14.2. Found: C, 53.7; H, 7.8; N, 14.1.

N^{ϵ}-*t*-Butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester Acetate (3B, 7–12). We have previously reported[3,10] the hydrogenolysis of 3A, 7–12, to prepare some dodecapeptide analogs corresponding to the 1–12 sequence of the S-peptide. We have now isolated and characterized the N^{α}-free hexapeptide 3B, 7–12, as the acetate salt. The N^{α}-benzyloxycarbonyl group was removed from 3A, 7–12, by hydrogenating for 2 hr over 10% palladized charcoal, the protected hexapeptide (1.325 g, 1.1 mmol) dissolved in a mixture of glacial acetic acid (60 ml) and methanol (20 ml). The catalyst was filtered off, the solvent evaporated under reduced pressure, and the residue crystallized twice from methanol–ether; yield 0.97 g (78%); mp 173–175°; $[\alpha]^{20}D$ −12.0 ± 0.2° (*c* 1.0, DMF); R_{f_1} 0.55, R_{f_2} 0.20; single ninhydrin-, chlorine-, and Pauly-positive spot.

Anal. Calcd for $C_{53}H_{85}N_{11}O_{16}$ (1132.36): C, 56.2; H, 7.5; N, 13.6. Found: C, 56.1; H, 7.6; N, 13.8.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylseryl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3C, Ser^6 1–12). Sodium nitrite (1 *M*, 0.6 ml) was added to a solution of 3A, Ser^6 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 (50 ml) at −10°, dried over sodium sulfate, and added to a solution of N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester acetate (3B, 7–12) (0.57 g, 0.504 mmol) in DMF (20 ml) containing triethylamine (0.15 ml). 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 the crude material from DMF–ether gave the pure product; yield 0.242 g (25%); mp 232–233°; $[\alpha]^{20}D$ −19.6 ± 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: $Lys_{1.97}Orn_{1.05}Glu_{3.00}Thr_{0.95}Ser_{0.95}Ala_{2.00}Phe_{1.05}His_{0.95}$.

Anal. Calcd for $C_{89}H_{146}N_{18}O_{28}$ (1916.3): C, 55.8; H, 7.7; N, 13.2. Found: C, 55.6; H, 7.6; N, 13.0.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylprolyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester (3C, Pro^6 1–12) was prepared by condensation of 3B, Pro^6 1–6 (obtained from 0.532 g, 0.6 mmol, of 3A, Pro^6 1–6), with 3B, 7–12 (0.68 g, 0.6 mmol), by an azide coupling step as described above for the preparation of 3C, Ser^6 1–12; yield 0.8 g (69%); mp 184–185°; $[\alpha]^{20}D$ −27.5 ± 0.5° (*c* 1.0, DMF); R_{f_1} 0.80, R_{f_2} 0.95; single ninhydrin-negative and chlorine- and Pauly-positive spot; amino acid ratios in acid hydrolysate: $Lys_{1.90}Orn_{1.10}His_{0.95}Glu_{3.10}Thr_{0.92}Ala_{1.90}Pro_{0.95}$.

Anal. Calcd for $C_{91}H_{148}O_{27}N_{18}$ (1926.35): C, 56.7; H, 7.8; N, 13.1. Found: C, 56.0; H, 7.8; N, 12.8.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylseryl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, Ser^6 1–12). The dodecapeptide methyl ester 3C, Ser^6 1–12 (0.230 g, 0.12 mmol), was dissolved in DMF (10 ml), and hydrazine hydrate (0.28 ml) was added. The solution was heated for 1 hr at 70°, the hydrazine hydrate (0.14 ml) was then added, and the reaction mixture was kept overnight at 50° and then 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 0.198 g (86%); mp 240–242° dec; $[\alpha]^{20}D$ −25.5 ± 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, 54.8; H, 7.6; N, 14.5.

N^{α},N^{ϵ}-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylprolyl-N^{ϵ}-*t*-butyloxycarbonyllysylphenylalanyl-γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine hydrazide (3D, Pro^6 1–12) was prepared from the corresponding methyl ester (3C, Pro^6 1–12; 0.79 g, 0.41 mmol) by the procedure described above for 3D, Ser^6 1–12. The product (0.557 g, 70%) had mp 203–205°; $[\alpha]^{20}D$ −43.4 ± 0.5° (*c* 0.96, 90% acetic acid); R_{f_1} 0.75, R_{f_2} 0.90; single Pauly-, chlorine-, and picryl chloride positive spot.

Anal. Calcd for $C_{90}H_{148}O_{26}N_{20}$ (1926.36): C, 56.1; H, 7.7; N, 14.5. Found: C, 55.6; H, 7.7; N, 14.2.

Lysylglutamylthreonylalanylalanylseryllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, Ser^6 1–20). Sodium nitrite (1 *M*, 0.3 ml) was added to a solution of 3D, Ser^6 1–12 (0.198 g, 0.103 mmol), in a mixture of 90% acetic acid (10 ml), 1 *N* hydrochloric acid (0.6 ml), and saturated sodium chloride (0.8 ml) at −10°. After stirring for 15 min at −10°, precooled 20% sodium chloride (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[13] (3E, 13–20; 0.182 g, 0.206 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 0.153 g (58%). The crude material (3F, Ser^6 1–20) was dissolved in anhydrous TFA (1.5 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^6 1–20) (0.03 g, 24.4%) had $[\alpha]^{22}D$ −65.1 ± 2° (*c* 0.11, water) and a 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: $Lys_{1.99}Orn_{1.05}Glu_{2.90}Thr_{2.00}Ala_{3.90}Phe_{0.97}His_{0.96}Met_{0.97}Asp_{0.98}Ser_{3.95}$; amino acid ratios in AP-M di-

gest: $Lys_{2.05}Orn_{1.05}Glu_{1.99}Thr_{1.90}Ala_{4.05}Phe_{1.00}His_{0.95}Met_{1.02}Asp_{0.96}$-$(Ser + Gln)_{4.90}$. The [Ser[6],Orn[10]]-S-peptide gave, after recombination in a 1:1 molar ratio with S-protein, a 40% active partially synthetic ribonuclease.

Lysylglutamylthreonylalanylalanylprolyllysylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3G, Pro[6] 1–20). The condensation of 3D, Pro[6] 1–12 (0.335 g, 0.174 mmol), with 3E, 13–20[13] (0.306 g, 0.348 mmol, as monoacetate, trihydrate), by the azide procedure was carried out as described above for 3G, Ser[6] 1–20, and gave the partially protected [Pro[6],Orn[10]]-S-peptide (3F, Ser[6] 1–20; 0.264 g, 57%). Treatment of the crude product with anhydrous TFA (2.5 ml), purification on Amberlite CG 50 and on Sephadex G-25, followed by lyophilization gave the pure [Pro[6],Orn[11]]-S-peptide (3G, Pro[6] 1–20; 0.053 g, 25%), $[\alpha]^{23}D$ $-97.5 \pm 1°$ (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: $Lys_{2.00}Glu_{2.95}Thr_{1.95}Ala_{3.80}Orn_{1.00}Phe_{1.05}His_{1.05}Pro_{0.95}$-$Met_{0.97}Asp_{1.05}Ser_{2.95}$; amino acid ratios in AP-M digest: $Lys_{1.97}$-$Glu_{1.95}Thr_{2.00}Ala_{3.90}Orn_{0.95}Phe_{0.97}His_{0.97}Pro_{0.97}Met_{1.00}Asp_{0.95}(Ser + Gln)_{4.05}$. The [Pro[6],Orn[10]]-S-peptide gave, after recombination in a 1:1 molar ratio with S-protein, a 15% active partially synthetic ribonuclease.

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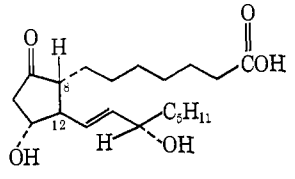
Communications to the Editor

Isolation and Characterization of a New Prostaglandin Isomer

Sir:

Prostaglandins are biosynthesized from C-20 polyunsaturated fatty acids when the latter are incubated in a variety of mammalian tissue homogenates;[1] for example, prostaglandin E_1 (PGE_1) (**1**) has been obtained from all-*cis*-8,11,14-eicosatrienoic acid. In addition to PGE_1, the corresponding 9α-hydroxy compound, prostaglandin $F_1\alpha$ ($PGF_1\alpha$), has also been obtained from the same C-20:3 acid,[2] and recently an isomer of PGE_1, 11-dehydro-$PGF_1\alpha$, has been isolated as the main product when a purified microsomal enzyme fraction from sheep seminal vesicular glands was employed for the conversion.[3] We wish to report the isolation and structure determination of a new isomer of PGE_1, namely 8-isoprostaglandin E_1 (**2**). This acid is of special interest in view of its chemical instability and significant biological properties. Relative to PGE_1 this new isomer has only very low activity in stimulating smooth muscle contraction *in vitro* or in



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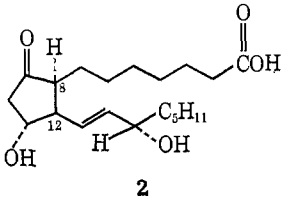

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causing an acute vasodepression in rats,[4] but is of the same order of potency as PGE_1 in its antilipolytic effects and in its inhibition of platelet aggregation.[4] Conditions for the large-scale bioconversion of all-*cis*-8,11,14-eicosatrienoic acid and extraction and purification of PGE_1 have been described earlier.[5] Mother liquors (20.9 g) from the crystallization of PGE_1 obtained biosynthetically were chromatographed on an Amberlyst-15 (silver form) ion-exchange column.[5] The fraction (11.0 g) containing both PGE_1 and 8-iso-PGE_1 was further fractionated on a column containing acid-washed silica gel (E. Merck AG., 0.05–2 mm). Solvents containing an increasing concentration of ethyl acetate in benzene were used for elution. Crystallization of a fraction eluted just prior to PGE_1 from an ethyl acetate–hexane mixture yielded 955 mg of material[6] which had a slightly faster mobility than PGE_1 on silica gel thin layers using system A IX[7] for development. The infrared spectrum (Nujol) of 8-iso-PGE_1 showed evidence for the presence of the same functional groups as in PGE_1[8] but was clearly different from the spectrum of PGE_1 in the fingerprint region. The mass spectra of the 8-iso-PGE_1 and its methyl ester were identical with the corresponding spectra of PGE_1 and PGE_1 methyl ester.[8] When 8-iso-PGE_1 was treated at 37° for 1 hr with 1 N sodium hydroxide a material was obtained that was identical with prostaglandin B_1 by thin layer chromatography and mass spectrometry (as the methyl ester) and in the sign and shape of its optical rotatory dispersion curve between 310 and 600 mμ.

The ORD spectrum of 8-iso-PGE_1 is nearly the mirror image of that of PGE_1 which has a negative Cotton effect curve.[8] As the most important determinant of the sign of the Cotton effect in cyclopentanones seems to be the stereochemistry of substituents α to the ketone, this suggests that a change has occurred at the C-8 position to give a β-oriented carboxy side chain (*i.e.*,

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