Synthesis of Peptide Analogs of the N-Terminal Eicosapeptide Sequence of Ribonuclease A. XIII. Synthesis of des-Lys^{7}-[Orn^{10}]- and des-Phe^{8}-[Orn^{10}]-S-peptides[1,2]

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Abstract: Syntheses are described of two analogs of S-peptide in which the arginyl residue in position 10 has been replaced by ornithine and either the lysyl residue in position 7 or the phenylalanyl residue in position 8 has been removed. The stereochemical homogeneity of these peptides, *i.e.*, lysylglutamylthreonylalanylalanylalanyl-phenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine and lysyl-glutamylthreonylalanylalanylalanyllysylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine, was assessed by digestion with aminopeptidase M followed by quantitative amino acid analysis. The enzymic properties of the two synthetic nonadecapeptides were checked with RNA as well as with cytidine 2′,3′-phosphate, after recombination with S-protein. The des-Phe^{8}-[Orn^{10}]-S-peptide is practically inactive with both substrates, while the des-Lys^{7}-[Orn^{10}]-S-peptide, which shows a very low catalytic activity against RNA only at 100:1 molar ratio with S-protein, forms, with the synthetic substrate, a 10% active partially synthetic ribonuclease at 1:1 molar ratio with S-protein. Competitive inhibition studies with the S-peptide–S-protein system were also carried out and only the des-Lys^{7}-[Orn^{10}]-S-peptide proved to be quite a strong S-peptide antagonist exhibiting 50% inhibition at a molar ratio of approximately 20:1. In the case of the RNA substrate, some enzyme–substrate binding, involving the lysyl residue in position 7, is proposed.

Previous studies[3] in this laboratory have dealt with the importance of some amino acid side-chain residues in the binding process which allow the S-peptide to activate S-protein and with an evaluation of those conformational features which are responsible for the noncovalent binding of S-peptide with S-protein.

For these purposes the natural sequence of the S-peptide was modified by substitution or by omission of some amino acid residues.

The afforded substitution dealt with positions 4, 5, 6, 8, 10, 11, and 13 while the deleted amino acids were lysine-1, glutamic acid-2, and threonine-3.

The different ability of the S-peptide analogs to catalyze the depolymerization of RNA, after recombination with S-protein, showed that the side-chain residues of phenylalanine-8[3d,g] and methionine-13[3h,4-6] are very important for the capacity of S-peptide to bind S-protein while the contribution of other side-chain residues appears to be of little significance.

We describe here the synthesis of the nonadecapeptide in which the phenylalanyl residue in position 8 in the peptide sequence has been omitted (Chart I). As expected such a deletion gave a peptide unable to regenerate ribonuclease activity after recombination with S-protein when tested at molar ratios as high as 100:1, using both RNA and cytidine 2′,3′-phosphate as substrates.

The afforded modification does not deal with one of the residues which have been indicated to be part of the active site of the enzyme. In order to test if the lack of measurable S-protein activating characteristics in the des-Phe^{8}-[Orn^{10}]-S-peptide could be attributed to the inability of the synthetic nonadecapeptide to bind the partner S-protein, we explored the capacity of the modified peptide to compete with S-peptide for S-protein with RNA as the substrate.

The observation that des-Phe^{8}-[Orn^{10}]-S-peptide fails to compete with S-peptide at molar ratios as high as 100:1 further confirms the preeminent importance of phenylalanine-8 as a binding site in the peptide–protein system.

We already suggested[7] that a possible explanation of

(1) The peptides and peptide derivatives mentioned have the L configuration. For a simpler description the customary L designation for individual amino acid residue 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, OBu^{t} = *t*-butyl ester, ONp = *p*-nitrophenyl ester, DMF = dimethylformamide, TFA = trifluoroacetic 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-protein, the protein component obtained from RNase A; S-peptide, the eicosapeptide obtained from RNase S; RNase S′, the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein. (c) 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.

(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, A. Marzotto, A. Scatturin, A. M. Tamburro, and G. Vidali, *J. Chem. Soc., C*, 606 (1967); (b) R. Rocchi, F. Marchiori, L. Moroder, A. Fontana, and E. Scoffone, *Gazz. Chim. Ital.*, **96**, 1537 (1966); (c) F. Marchiori, R. Rocchi, L. Moroder, and E. Scoffone, *ibid.*, **96**, 1549 (1966); (d) E. Scoffone, R. Rocchi, F. Marchiori, L. Moroder, A. Marzotto, and A. M. Tamburro, *J. Am. Chem. Soc.*, **89**, 5450 (1967); (e) R. Rocchi, L. Moroder, F. Marchiori, E. Ferrarese, and E. Scoffone, *ibid.*, **90**, 5885 (1968); (f) F. Marchiori, R. Rocchi, L. Moroder, A. Fontana, and E. Scoffone, *ibid.*, **90**, 5889 (1968); (g) R. Rocchi, F. Marchiori, A. Scatturin, L. Moroder, and E. Scoffone, *Gazz. Chim. Ital.*, **98**, 1270 (1968); (h) R. Rocchi, A. Scatturin, L. Moroder, F. Marchiori, A. M. Tamburro, and E. Scoffone, *J. Am. Chem. Soc.*, **91**, 492 (1969); (i) L. Moroder, F. Marchiori, R. Rocchi, A. Fontana, and E. Scoffone, *ibid.*, **91**, 3921 (1969).

(4) F. M. Finn and K. Hoffmann, *ibid.*, **87**, 645 (1965).

(5) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *ibid.*, **88**, 3633 (1966).

(6) (a) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 2343 (1960); (b) P. J. Vithayathil and F. M. Richards, *ibid.*, **236**, 1386 (1961).

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^{7}-[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-	□-	Phe-	Glu-	Orn-	Gln-	His-	Met-	Asp-	Ser-	Ser-	Thr-	Ser-	Ala-	Ala

Des-Phe^{8}-[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-	□-	Glu-	Orn-	Gln-	His-	Met-	Asp-	Ser-	Ser-	Thr-	Ser-	Ala-	Ala

the varied capacity of the synthetic S-peptide analogs to activate S-protein might be sought in their differing tendency to undergo a coil-to-helix conformational transition induced by strong hydrophobic interactions with S-protein.

The amino acids involved in the active site of the enzyme are then in the correct position. In the case of des-Phe^{8}-[Orn^{10}]-S-peptide the elimination of this important binding site could prevent the stabilization of the helical conformation and the synthetic nonadecapeptide is thus unable to bind S-protein.

A similar behavior is shown by the [Ile^{8},Orn^{10}]-[3d] and the [Gly^{8},Orn^{10}]-S-peptides[3g] which do not show any inhibitory capacity for S-protein when tested up to a 100:1 molar ratio with S-peptide.

In our opinion even the [Ile^{8},Orn^{10}]-[3d] and the [Gly^{8},Orn^{10}]-S-peptides[3g] remain in an unordered form also in presence of the protein and are therefore unable to bind as well to activate S-protein.

In addition, in order to test if a peptide lacking a residue which presumably is not involved in any hydrophobic interaction still possesses those conformational features responsible for the ability of S-peptide to bind S-protein, we synthesized the des-Lys^{7}-[Orn^{10}]-S-peptide (Chart I). The removal of the lysyl residue in position 7 has been suggested by the following considerations.

It has been already demonstrated[8] that, considering RNA as a substrate, the masking of the α- and ϵ-amino groups of the S-peptide as 1$^{\epsilon}$,7$^{\epsilon}$-diguanidino-, 1$^{\alpha}$,1$^{\epsilon}$,7$^{\epsilon}$-triguanidino-, and 1$^{\alpha}$-acetamido-1$^{\epsilon}$,7$^{\epsilon}$-diguanidino-S-peptide does not affect its potential ability to activate S-protein while the maximum activity obtained with a slight molar excess of 1$^{\alpha}$,1$^{\epsilon}$,7$^{\epsilon}$-triacetamido-S-peptide is only 40% of that observed with the unmodified peptide.

The observation that the activity is lowered by the acetylation of one or both the ϵ-amino groups but is not affected by the guanidination of the same groups seems to indicate that the presence of a basic function in the side chain of at least one of the residues in positions 1 and 7 is very important for the depolymerization of RNA. It is also interesting to note that, with uridine 2′,3′-phosphate as substrate, the triacetylated peptide derivative at 10:1 molar ratio with S-protein exhibits an activity which approaches that shown by the unmodified peptide, in contrast to the observations with the RNA substrate.

The observation that the des-Lys^{1}-RNase A, isolated by Eaker,[9] shows full catalytic activity, the results of Hofmann[4] on the 2–13 synthetic fragment of S-peptide, and the demonstration that the des-Lys^{1}-[Orn^{10}]-S-peptide[3i] is practically as active as the [Orn^{10}]-S-peptide rule out lysine-1 as essential for the enzymic activity. Moreover, lysine-7 is one of the residues which are cross-linked when ribonuclease is treated with small bifunctional reagents such as 1,5-difluoro-2,4-dinitrobenzene[10] or dimethyl adipimidate.[11]

In the case of 1,5-difluoro-2,4-dinitrobenzene, the cross-link is between the ϵ-amino groups of the lysyl residues 7 and 41. This result is consistent with the data of Hirs[12] concerning the arylation of these residues with 1-fluoro-2,4-dinitrobenzene, from which he inferred that the two lysyl residues are in the same region of the molecule and that the ϵ-amino group of lysine-41 is closely related to the catalytic function of the protein.

The capacity of the des-Lys^{7}-[Orn^{10}]-S-peptide to activate S-protein using RNA as well as cytidine 2′,3′-phosphate as the substrate has been tested following the procedures reported by Kunitz[13] and by Crook, *et al.*[14] This analog is unable to regenerate any catalytic potency against RNA when tested in 10:1 molar ratio with the protein. The activity, when tested against cytidine 2′,3′-phosphate in 1:1 peptide–protein molar ratio, rises up to 10% if compared with the RNase S′ activity.

The binding capacity of the des-Lys^{7}-[Orn^{10}]-S-peptide was evaluated by competitive inhibition studies in the S-peptide–S-protein system. The preparation of inhibitor to S-peptide has been already reported. The [β-pyrazolyl-3[12]]-S-peptide 1–14,[15] the [3-carboxymethyl-His[12]]-S-peptide 1–14,[16] and the [Ser[12]]-S-peptide 1–14[16] in which only the active site is modified fail to activate S-protein but are capable of antagonizing S-peptide.

In our system the modification deals with a residue which, presumably, is not directly involved either in the active site of the enzyme or in the hydrophobic interaction with S-protein. Nevertheless, the des-Lys^{7}-[Orn^{10}]-S-peptide lacks any potential catalytic activity against RNA but is able to catalyze the hydrolysis of cytidine 2′,3′-phosphate after recombination with S-protein.

Moreover, it is a rather strong S-peptide antagonist exhibiting 50% inhibition at a molar ratio of approximately 20:1 (Figure 1). One could tentatively explain both the different potential catalytic activity against RNA and cytidine 2′,3′-phosphate and the inhibitory capacity by admitting that lysine-7 is involved in some

(7) A. Scatturin, A. M. Tamburro, R. Rocchi, and E. Scoffone, *Chem. Commun.*, 1273 (1967).
(8) P. J. Vithayathil and F. M. Richards, *J. Biol. Chem.*, **235**, 1029 (1960).
(9) D. Eaker, *J. Polymer Sci.*, **49**, 45 (1961).
(10) P. S. Marfey, M. Uziel, and J. Little, *J. Biol. Chem.*, **240**, 3270 (1965).
(11) F. C. Hartman and F. Wold, *Biochemistry*, **6**, 2439 (1967).
(12) (a) H. W. Hirs, M. Halmann, and J. H. Kycia, "Biological Structure and Function," Vol. 1, T. W. Goodwin and O. Lindberg, Ed., Academic Press, New York, N. Y., 1961, p 41; (b) C. H. W. Hirs, *Brookhaven Symp. Biol.*, **15**, 154 (1962).
(13) M. Kunitz, *J. Biol. Chem.*, **164**, 583 (1946).
(14) E. M. Crook, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **74**, 234 (1960).
(15) F. M. Finn and K. Hofmann, *J. Am. Chem. Soc.*, **89**, 5298 (1967).
(16) K. Hofmann, Communication to the IX European Peptide Symposium, Orsay, France, April 15, 1968.

binding between the enzyme and the natural substrate. The removal of such a residue does not alter remarkably the association between S-peptide and S-protein but strongly affects the formation of the complex with the RNA substrate.

Such an explanation agrees also with the findings of Richards[8] that the potential activity of the triacetylated S-peptide derivative is lowered much more against RNA than against uridine 2′,3′-phosphate.

The hydrolysis of the synthetic substrate, which probably involves the formation of a different protein–substrate complex, is much less affected by the modification induced by the removal of the residue in position 7.

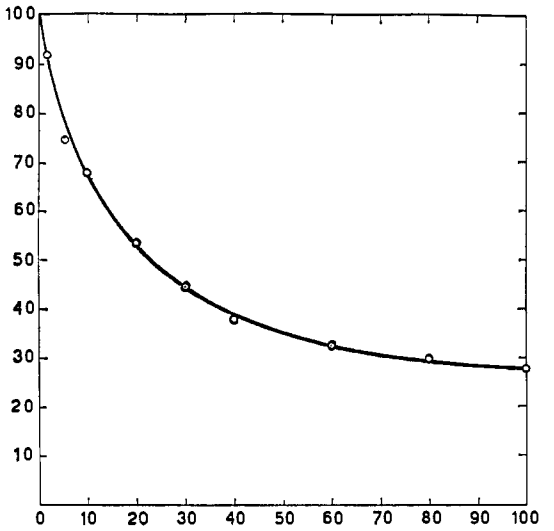
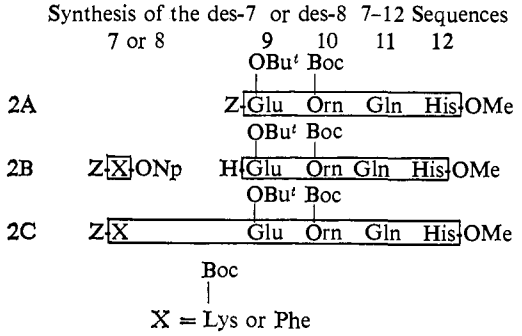


Figure 1. Inhibition of RNase S′ by des-Lys[7]-[Orn[10]]-S-peptide with yeast RNA as the substrate. Molar ratio S-peptide:S-protein 1:1. Abscissa: molar ratio of inhibitor to S-peptide. Ordinate: per cent activity remaining.

Peptide Syntheses

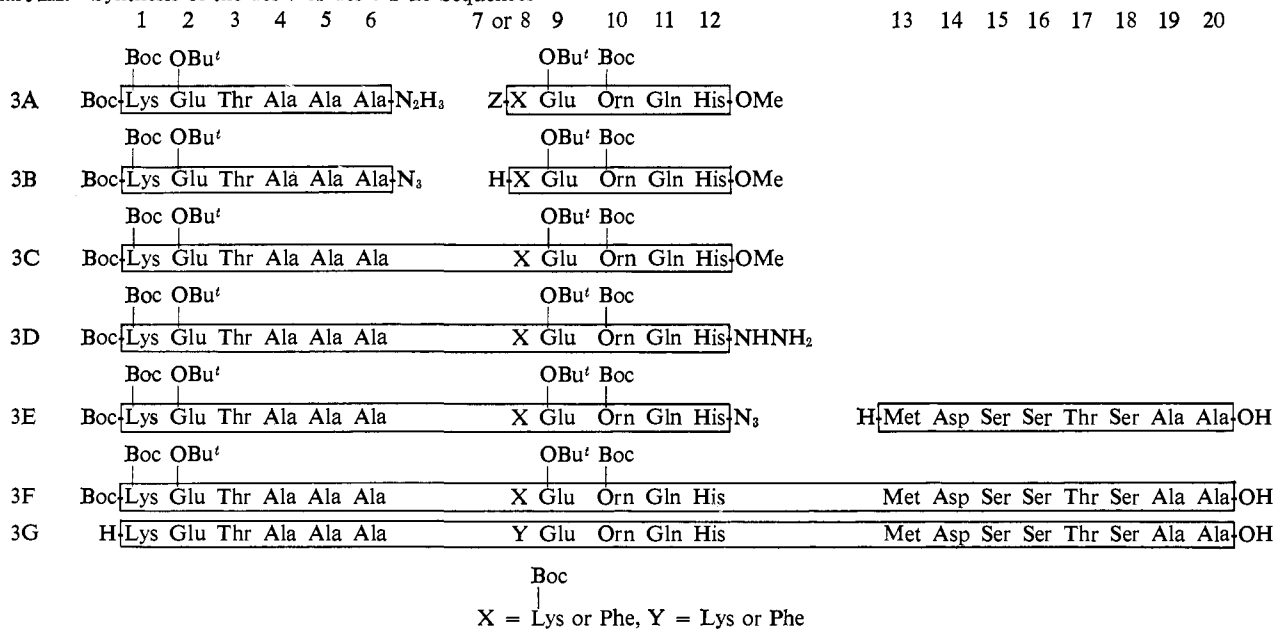
The method of synthesis of des-Lys[7]-[Orn[10]]- and des-Phe[8]-[Orn[10]]-S-peptide, illustrated on Charts II and III, is based on our previous studies with other analogs.[8]

Chart II. Synthesis of the des-7 or des-8 7–12 Sequences



The resulting pentapeptides 2 C (or 3A), des-Phe[8] 7–12 and 2C (or 3A), des-Lys[7] 7–12 were hydrogenated and coupled with N^{α},N^{ϵ}-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanine azide[19] (3 B, 1–6) to give the protected undecapeptide esters (3 C, des-Lys[7] 1–12 and 3 C, des-Phe[8] 1–12) which were converted into hydrazides (3 D, des-Lys[7] 1–12 and 3 D, des-Phe[8] 1–12) in the usual manner.

Chart III. Synthesis of the des-7 or des-8 1–20 Sequences



N^{α}-Benzyloxycarbonyl-N^{ϵ}-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[17] or benzyloxycarbonylphenylalanine *p*-nitrophenyl ester[18] were coupled with the tetrapeptide γ-*t*-butylglutamyl-N^{δ}-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester[17] (2 B, 9–12).

The corresponding azides (3 E, des-Lys[7] 1–12 and 3 E, des-Phe[8] 1–12) were then treated with the octapeptide methionylaspartylserylserylthreonylserylalanylalanine[20] (3 E, 13–20) to give the partially protected nonadecapeptides 3 F, des-Lys[7] 1–20 and 3 F, des-Phe[8] 1–20. After treatment with TFA, the crude free nonadeca-

(17) F. Marchiori, R. Rocchi, G. Vidali, A. M. Tamburro, and E. Scoffone, *J. Chem. Soc., C*, 81 (1967).
(18) F. Marchiori, R. Rocchi, and E. Scoffone, *Ric. Sci. Rend.*, **A32**, 647 (1962).
(19) R. Rocchi, F. Marchiori, A. Scatturin, and E. Scoffone, *J. Chem. Soc., C*, 86 (1967).
(20) F. Marchiori, R. Rocchi, L. Moroder, G. Vidali, and E. Scoffone, *ibid., C*, 89 (1967).

peptides 3 G, des-Lys[7] 1–20 and 3 G, des-Phe[8] 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 an eluent, and lyophilized.

The free peptides behaved as a single component in paper electrophoresis at different pH values and yielded the expected ratio of amino acid residues on acid hydrolysis.

Complete enzymic digestion of the purified nonadecapeptides by aminopeptidase M[5] (AP-M) indicated that the amino acids have the L configuration.

Experimental Section[21]

RNase A was prepared by the procedure of Crestfield, *et al.*,[22] starting with bovine pancreatic ribonuclease (Fluka AG four times crystallized). 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[23] modified by Dosher and Hirs.[1c] Aminopeptidase M (AP-M) was obtained from Rohm and Haas GmbH, Darmstadt, West Germany. Commercial yeast RNA, obtained from Schwarz Laboratories, was purified by exhaustive dialysis, first against 0.1 *M* sodium chloride and then against water.[24]

The crude S-peptide analogs (100–200 mg), obtained by treatment of the partially protected nonadecapeptides with TFA, were dissolved in 0.2 *M* sodium phosphate buffer (pH 6.4) and purified by passing the solutions through an Amberlite GC 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.

Benzyloxycarbonylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester [2 C (or 3 A), des-Lys[7] 7–12]. Benzyloxycarbonyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine methyl ester[17] (2 A, 9–12) (1.08 g, 1.3 mmol) was dissolved in a mixture of glacial acetic acid (30 ml) and methanol (10 ml) and hydrogenated for 2 hr in the presence of palladium (10%) on charcoal catalyst. The catalyst was filtered off, the filtrate was evaporated to dryness under reduced pressure, and the residue was dissolved in pyridine (60 ml) containing triethylamine (0.40 ml).

Benzyloxycarbonylphenylalanine *p*-nitrophenyl ester[18] (0.588 g, 1.4 mmol) was added and the mixture was allowed to react for 24 hr at room temperature. The solvent was then evaporated *in vacuo*, and the residue was crystallized first from methanol–ether and then from methanol–water yielding 0.70 g (55%), mp 190–191°, $[\alpha]^{20}D$ −22.7 ± 0.2° (*c* 1.0, methanol), R_{f_1} 0.60, R_{f_2} 0.70, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{48}H_{67}N_9O_{13}$ (987.14): C, 58.9; H, 6.9; N, 12.9. Found: C, 59.3; H, 6.9; N, 13.0.

N$^{\alpha}$-Benzyloxycarbonyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester [2 C (or 3 A), des-Phe[8] 7–12]. The title compound was prepared from 2 A, 9–12[17] (1.08 g, 1.3 mmol) and N$^{\alpha}$-benzyloxycarbonyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysine *p*-nitrophenyl ester[17] (0.702 g, 1.4 mmol) by the above reported procedure used for the preparation of 2 C, des-Lys[7] 7–12; yield 0.59 g (43%), mp 182–183°, $[\alpha]^{20}D$ −23.6 ± 0.2° (*c* 1.0, methanol); R_{f_1} 0.65, R_{f_2} 0.75, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{80}H_{78}N_{10}O_{15}$ (1059.25): C, 56.6; H, 7.4; N, 13.2. Found: C, 56.1; H, 7.4; N, 13.1.

N$^{\alpha}$,N$^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3 C, des-Lys[7] 1—12). The protected pentapeptide ester (3 A, des-Lys[7] 7–12) (0.684 g, 0.7 mmol) was dissolved in a mixture of glacial acetic acid (30 ml) and methanol (20 ml) and hydrogenated for 2 hr in the presence of palladium (10%) on charcoal catalyst. The catalyst was removed by filtration and the solvent was evaporated *in vacuo*. The residue (R_{f_1} 0.45, R_{f_2} 0.55, single ninhydrin-, chlorine-, and Pauly-positive spot) was dissolved in DMF (30 ml) and N$^{\alpha}$,N$^{\epsilon}$-di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanine azide[19] (3 B, 1–6) (0.645 g, 0.74 mmol) and triethylamine (0.2 ml) were added.

After stirring for 6 days at 5° water was added, and the resulting precipitate was collected, washed with water and ether, and dried; yield 0.468 g (40%), mp 228–229°, $[\alpha]^{20}D$ −16.0 ± 0.2° (*c* 1.0, DMF), R_{f_1} 0.65, R_{f_2} 0.95, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{76}H_{126}N_{16}O_{24}$ (1672.04): C, 56.0; H, 7.6; N, 13.4. Found: C, 55.6; H, 7.6; N, 13.2.

N$^{\alpha}$,N$^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Methyl Ester (3 C, des-Phe[8] 1–12). The condensation of 3 B, 1–6[19] (0.488 g, 0.56 mmol) with 3 B, des-Phe[8] 7–12 (R_{f_1} 0.45, R_{f_2} 0.55, single ninhydrin-, chlorine-, and Pauly-positive spot) obtained by hydrogenolysis in methanol from 3 A, des-Phe[7] 7–12 (0.572 g, 0.54 mmol) was carried out as described above for 3 C, des-Lys[7] 1–12, yielding 0.34 g (36%), mp 227–228°, $[\alpha]^{20}D$ −17 ± 1° (*c* 1.0, DMF), R_{f_1} 0.65, R_{f_2} 0.95, single ninhydrin-negative, chlorine- and Pauly-positive spot.

Anal. Calcd for $C_{80}H_{137}N_{17}O_{26}$ (1753.14): C, 54.8; H, 7.9; N, 13.6. Found: C, 54.3; H, 7.8; N, 13.3.

N$^{\alpha}$-N$^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanylphenylalanyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3D, des-Lys[7] 1–12). The undecapeptide methyl ester (3 C, des-Lys[7] 1–12) (0.464 g, 0.277 mmol) was dissolved in DMF (15 ml), and hydrazine hydrate (0.54 ml) was added. The solution was heated for 12 hr at 60°, hydrazine hydrate (0.27 ml) was then added, and the reaction mixture was kept 4 days at room temperature.

The solution was concentrated under reduced pressure and ether was added. The resulting precipitate was collected, crystallized from DMF–water, and dried *in vacuo* first over concentrated sulfuric acid and then at 110°.

The product (0.186 g, 40%) had mp 237–238°, $[\alpha]^{20}D$ −23 ± 0.5° (*c* 0.985, 90% acetic acid), R_{f_1} 0.70, R_{f_2} 0.95, single chlorine-, picryl chloride-, and Pauly-positive spot.

Anal. Calcd for $C_{77}H_{126}N_{18}O_{23}$ (1672.05): C, 55.4; H, 7.6; N, 15.0. Found: C, 54.8; H, 7.5; N, 14.8.

N$^{\alpha}$,N$^{\epsilon}$-Di-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamylthreonylalanylalanylalanyl-N$^{\epsilon}$-*t*-butyloxycarbonyllysyl-γ-*t*-butylglutamyl-N$^{\delta}$-*t*-butyloxycarbonylornithylglutaminylhistidine Hydrazide (3 D, des-Phe[8] 1–12). This compound was obtained from 3 C, des-Phe[8] 1–12 (0.33 g, 0.188 mmol) by the same procedure reported above for the preparation of 3 D, des-Lys[7] 1–12; yield 0.152 g (46%), mp 236–237°, $[\alpha]^{20}D$ −28.5 ± 0.5° (*c* 0.986, 90% acetic acid), R_{f1} 0.70, R_{f2} 0.95, single chlorine-, picryl chloride-, and Pauly-positive spot.

Anal. Calcd for $C_{79}H_{137}N_{19}O_{25}$ (1753.15): C, 54.0; H, 7.9; N, 15.2. Found: C, 53.6; H, 7.7; N, 14.8.

Lysylglutamylthreonylalanylalanylalanylphenylalanylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylseryl-alanylalanine (3 G, des-Lys[7] 1–20). Sodium nitrite (1 *M*, 0.2 ml) was added to a solution of 3 D, des-Lys[7] 1–12 (0.17 g, 0.102 mmol) in a mixture of 90% acetic acid (8 ml), 1 *N* hydrochloric acid (0.4 ml), and 20% sodium chloride (1.6 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 (15 ml) at −10° and dried over sodium sulfate. The drying agent was filtered off and a solution of methionylaspartylserylserylthreonylserylalanylalanine[20] (3 E, 13–20) (0.18 g, 0.204 mmol as monoacetate trihydrate) in DMF (30 ml) and triethylamine (0.05 ml) were added.

The reaction mixture was stirred for 7 days at 5°, filtered, concentrated to small volume under reduced pressure, and diluted with water (80 ml). The resultant precipitate was centrifuged,

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

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washed with water and ether, and dried over phosphorus pentoxide yielding 0.206 g (84%).

The crude material (3 F, des-Lys[7] 1–20) was dissolved in anhydrous TFA (1.5 ml), and the solution was kept for 150 min at room temperature. Ice-cold ether was added, and after 30 min at −10° the precipitate was collected by centrifugation, washed with ether, and dried 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 (3 G, des-Lys[7] 1–20) (0.085 g, 50% of the crude product) had $[\alpha]_D$ −75.0 ± 1° (*c* 0.104, 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: $Lys_{1.04}Orn_{1.05}His_{1.00}Glu_{2.96}Thr_{1.92}Ala_{4.85}Phe_{1.10}Met_{0.90}Asp_{0.92}Ser_{2.85}$; amino acid ratios in AP-M digest: $Lys_{1.05}Orn_{1.00}His_{0.95}Glu_{2.10}(Gln + Ser)_{3.78}Thr_{2.10}Ala_{4.95}Phe_{0.98}Met_{1.03}Asp_{1.00}$.

Lysylglutamylthreonylalanylalanylalanyllysylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3 G, des-Phe[8] 1–20). The condensation of 3 D, des-Phe[8] 1–12 (0.13 g, 0.074 mmol) with 3 E, 13–20[20] (0.132 g, 0.15 mmol as monoacetate trihydrate) by the azide procedure was carried out as described above for 3 G, des-Lys[7] 1–20 and gave the partially protected des-Phe[8]-[Orn[10]]-S-peptide (3 F, des-Phe[8] 1–20, 0.113 g, 61%).

Treatment with anhydrous TFA, purification on Amberlite CG-50 and on Sephadex G-25, followed by lyophilization, gave the pure nonadecapeptide 3 G, des-Phe[8] 1–20 (0.03 g, 33%), $[\alpha]^{20}_D$ −79.0 ± 1° (*c* 0.0994, 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: $Lys_{2.00}Orn_{1.02}His_{1.04}Glu_{2.93}Thr_{2.00}Ala_{4.80}Met_{1.05}Asp_{1.00}Ser_{3.07}$; amino acid ratios in AP-M digest: $Lys_{2.10}Orn_{0.95}His_{1.00}Glu_{2.10}Thr_{2.05}(Gln + Ser)_{3.70}Ala_{5.02}Met_{0.94}Asp_{1.00}$.

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The Structure of Batrachotoxin, a Steroidal Alkaloid from the Colombian Arrow Poison Frog, *Phyllobates aurotaenia*, and Partial Synthesis of Batrachotoxin and Its Analogs and Homologs

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Abstract: Four major toxic steroidal alkaloids are contained in skin extracts of the Colombian arrow poison frog *Phyllobates aurotaenia:* (i) batrachotoxinin A (LD_{50} 1 mg/kg mice), $C_{24}H_{35}NO_5$, 3α,9α-epoxy-14β,18β-(epoxyethano-N-methylimino)-5β-pregna-7,16-diene-3β,11α,20α-triol, the structure of which was elucidated by X-ray crystallography of the 20α-*p*-bromobenzoate; (ii) pseudobatrachotoxin, an extremely labile alkaloid of unknown composition which on standing spontaneously forms batrachotoxinin A; (iii) batrachotoxin, $C_{31}H_{42}N_2O_6$, the most toxic principle (LD_{50} 2 μg/kg mice), which has now been recognized as the 20α ester of batrachotoxinin A with 2,4-dimethylpyrrole-3-carboxylic acid, and (iv) homobatrachotoxin, the former "isobatrachotoxin," $C_{32}H_{44}N_2O_6$ (LD_{50} 3 μg/kg mice), now formulated as the 20α ester of batrachotoxinin A with 2-ethyl-4-methylpyrrole-3-carboxylic acid. A partial synthesis of batrachotoxin was achieved by the reaction of the anhydride of ethyl chloroformate and 2,4-dimethylpyrrole-3-carboxylic acid with batrachotoxinin A. The analogous esterification with the anhydride of the fully substituted 2,4,5-trimethylpyrrole-3-carboxylic acid gave a homolog of batrachotoxin which was *more stable* and *twice as active* (LD_{50} 1 μg/kg). The 20α ester of batrachotoxinin A with 1,2,4,5-tetramethylpyrrole-3-carboxylic acid was much less active than batrachotoxinin A itself. Reductive opening of the 3,9-hemiketal oxygen bridge of batrachotoxin with sodium borohydride leads to an acid-sensitive dihydrobatrachotoxin with 1/250 of the activity of batrachotoxin.

After the elucidation of tetrodotoxin the many times more lethal venom of the Colombian arrow poison frog remained as one of the major challenges in the chemistry of natural products. Compared with the classical studies of Wieland on toad venoms[2] the difficulties were considerable. During the past 8 years four expeditions[3] had to be sent into the impervious jungle of the Choco region of western Colombia. The lability of the venom was a problem with which we learned to cope only after numerous failures. The paucity of the material forced us to carry out most operations on the level of micrograms. The advent of mass spectrometry, nuclear magnetic resonance, and, most important of all, the method of X-ray analysis of small asymmetric molecules without markers by heavy atoms[4] secured the final solution.

Four major steroidal alkaloids, batrachotoxin, "isobatrachotoxin," pseudobatrachotoxin, and batrachotoxinin A, have been isolated from extracts of the skin of the Colombian arrow poison frog.[5–7] Batrachotoxin and isobatrachotoxin[7] are extremely active

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