presence of both eight cysteine and three methionine residues in the molecule argued against the use of a strategy which entails catalytic hydrogenation for the removal of protecting groups. Furthermore our investigations and those of Sarid and Patchornik[6] made unattractive the use of sodium–liquid ammonia to remove the frequently used S-benzyl blocking group for cysteine. For the protection of the sulfhydryls of the eight cysteines, we had developed[6] the acetamidomethyl blocking group which is stable, *e.g.*, to trifluoroacetic acid at 25°, to anhydrous HF at 0°, and to hydrazine and which may be selectively removed with Hg(II) under mild conditions. Moreover, use of the NCA's[7] of arginine and glutamic and aspartic acids and the NTA of histidine[8] in peptide bond formation permitted the development of a strategy[9] in which the third functionality needed to be protected only for the amino acids lysine and cysteine. Furthermore, the recent reports[10] that the benzyloxycarbonyl protecting group may be smoothly removed with liquid HF, in conjunction with fact that we had found S-protein to be stable in this solvent at 0°, led us to select this protecting group for the ε-amino function of lysine. This choice of the "permanent" protecting groups permitted the use of the butyloxycarbonyl group as the acid-labile, temporary blocking group of the growing peptide chains. In addition this combination of protecting groups enabled us to remove all of the N-blocking groups of the tetrahectapeptide while leaving the cysteines protected. The formation of the four disulfide bridges subsequent to the liberation of the ε-amino groups of the eight lysine residues was considered to be a desirable feature of our strategy. Finally the synthesis of this tetrahectapeptide—with only the sulfhydryl groups protected—from natural S-protein appeared feasible. Indeed the availability of the acetamidomethylated reduced natural S-protein enabled us to work out conditions for the final steps before committing any of our synthetic intermediates.

To synthesize S-protein we relied on the fragment condensation method. A total of 19 fragments were prepared. About 40% of the bonds in the peptide fragments were formed through the use of NCA's[11] and NTA's,[8] the remainder, with the Boc-hydroxysuccinimide esters of Anderson.[12] The latter were routinely employed for the incorporation of the amino-terminal amino acid of all fragments and also for the introductions of asparagine, serine, or threonine.

To permit the use of the unprotected ω-carboxy groups of aspartic and glutamic acids, we relied on the azide method to couple fragments. As a consequence, the carboxy-terminal amino acids of all fragments but one had to be converted to esters prior to the introdution of dibasic acids.[9] Those esters then served as precursors of the hydrazides. In this manner it was possible to synthesize fragments 65–124 and 21–64.

The preparation of these protected hexaconta- and tetratetracontapeptides and their coupling will be described in separate communications.

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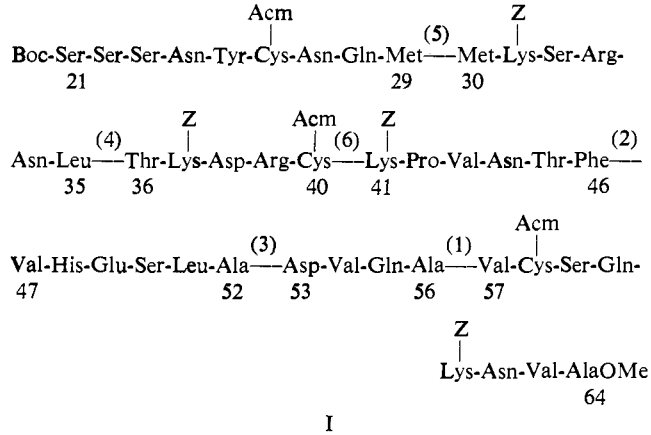
Studies on the Total Synthesis of an Enzyme. II. Synthesis of a Protected Tetratetracontapeptide Corresponding to the 21–64 Sequence of Ribonuclease A

Sir:

This communication describes the preparation of the protected tetratetracontapeptide fragment 21–64 (I) of RNase A. The fragments were prepared by the



I

general scheme outlined in the preceding communication.[1] The azide couplings of the fragments were performed in the order indicated by the numerals in parentheses above the appropriate bonds in I. The azide components, blocked at the amino-terminal end with the Boc protecting group, were prepared and allowed to react *in situ* under anhydrous conditions,[2] at temperatures ranging from −40 to +5°. Stabilities of the azide components and reactivities of the nucleophiles determined the choice of reaction temperatures. Removal of the Boc protecting groups was carried out either with anhydrous HCl in ethyl acetate at 0° or with anhydrous trifluoroacetic acid at room temperature.

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Table I. Amino Acid Analyses of Fragments

Fragment[a]	Spinco amino acid analysis
Boc(57–64)OMe	$Val_{1.95}Ser_{1.00}Glu_{0.98}Lys_{0.98}Asp_{1.04}Ala_{1.02}$
Boc(53–56)$NHNH_2$	$Asp_{1.00}Val_{1.02}Glu_{0.98}Ala_{1.00}$
Boc(53–64)OMe	$Asp_{2.00}Val_{2.86}Glu_{2.11}Ala_{2.08}Ser_{0.95}Lys_{1.01}$
Boc(41–46)OMe	$Lys_{1.02}Pro_{1.00}Val_{1.00}Asp_{1.01}Thr_{0.98}Phe_{0.98}$
Boc(47–52)OMe[b]	$Val_{1.00}His_{1.00}Glu_{1.02}Ser_{1.00}Leu_{1.02}Ala_{1.00}$
Boc(41–52)OMe	$Lys_{1.00}Pro_{1.02}Val_{2.02}Asp_{1.02}Thr_{0.98}Phe_{0.98}His_{1.00}Glu_{1.02}Ser_{0.98}Leu_{1.00}Ala_{1.00}$
Boc(41–64)OMe	$Lys_{2.02}Pro_{0.96}Val_{4.91}Asp_{3.12}Thr_{1.00}Phe_{0.96}His_{0.91}Glu_{2.99}Ser_{2.09}Leu_{0.98}Ala_{3.12}$
H(36–40)OEt	$Thr_{1.00}Lys_{1.02}Asp_{1.00}Arg_{1.00}$
H(36–40)OEt[c,d]	$Thr_{0.99}Lys_{1.03}Asp_{2.01}$[e]$Arg_{0.99}$
Boc(30–35)$NHNH_2$	$Met_{0.63}Lys_{1.00}Ser_{1.00}Arg_{0.99}Asp_{1.03}Leu_{1.00}$
H(30–35)$NHNH_2$[c,d]	$Met_{1.02}$[f]$Lys_{1.02}Arg_{0.98}Leu_{0.98}$
H(30–40)OEt	$Met_{0.81}Lys_{2.03}Ser_{1.03}Arg_{2.00}Asp_{2.06}Leu_{1.00}Thr_{1.00}$
H(30–40)OEt[d,g]	$Met_{0.97}Lys_{2.03}$[h](Ser + Asn)[i]$_{1.82}Arg_{2.00}Leu_{1.00}Thr_{0.99}$
Boc(21–29)$NHNH_2$	$Ser_{2.87}Asp_{2.09}Tyr_{1.02}Glu_{1.02}Met_{0.99}$
Boc(21–40)$NHNH_2$	$Ser_{3.99}Asp_{3.98}Tyr_{0.88}Glu_{1.04}Met_{1.94}Lys_{1.98}Arg_{1.92}Leu_{1.04}Thr_{1.02}$
Boc(21–64)OMe[j]	$Ser_{6.30}Asp_{7.25}Tyr_{0.96}Glu_{3.90}Met_{1.99}Lys_{3.99}Arg_{1.99}Leu_{1.96}Thr_{1.84}Pro_{1.11}Val_{4.61}Phe_{0.94}$-$His_{0.52}Ala_{2.84}$

[a] Fragments refer to formula I and carry the substituents shown therein, unless otherwise indicated. Except as noted in the table, analyses were carried out after 20-hr acid hydrolysis. [b] 70-hr acid hydrolysis. [c] Blocking groups removed with anhydrous HF. [d] Submitted to enzymatic degradation with aminopeptidase M. [e] Acetamidomethylcysteine emerges with aspartic acid on the amino acid analyzer. [f] 0.05 of total as methionine oxide. [g] Butyloxycarbonyl blocking group removed with HCl in ethyl acetate. [h] About 6% of this total is ε-unblocked lysine. [i] Asparagine and serine are not separated. The reported value is calculated as serine. The theoretical value is 1.88, since the height–width constant of asparagine is only 0.88 times that of serine. This column was developed essentially according to the Beckmann accelerated protocol A-TB-305 which only partially separates aspartic acid from acetamidomethylcysteine and thus prevents calculation of these amino acids. [j] Low values for Val and His presumably because of incomplete cleavage of the Val-His bond.

Smaller intermediates encountered in the synthesis of the fragments were usually purified by conventional procedures, including dry column chromatography on silica gel,[3] and gave amino acid analyses comparable to those reported for the larger fragments reported in Table I. All reactions were monitored by tlc in several solvent systems.

The synthesis of the tetratetracontapeptide was accomplished *via* the eicosapeptide 21–40. To this end about a 10% excess of the protected azide of fragment 30–35 was allowed to react with fragment 36–40 to afford the undecapeptide in >90% yield by precipitation with ethyl acetate. In the preparation of the hydrazide of fragment 30–35, replacement of the ester group was found to be complete, and hydrazinolysis of the guanidino group of arginine-33 prevented, when the reaction was carried out in methanolic solution with a large excess of hydrazine for a short period of time. We have found this procedure (using methanol or dimethylformamide as solvent and reaction times as brief as 2 min) satisfactory for the preparation of hydrazides of arginine-containing peptides. In the coupling of fragment 30–40 with the azide of the protected fragment 21–29 about twice the theoretical amount of the latter was employed. The yield was 45–55% (based on nucleophile) after gel filtration on Sephadex G-50 in 50% acetic acid. Our peptide fragments were found to have adequate solubility and stability[4] in this solvent pair.

The intermediates used to prepare the other component, fragment 41–64, are shown in formula I. Hexapeptide 47–52, which was impure as shown by tlc, was nevertheless suitable for condensation with 1 equiv of the azide of fragment 41–46, because satisfactory purification of the resulting dodecapeptide (60% yield) was conveniently accomplished by dry column chromatography on silica gel. More extensive purification of the hexapeptide 47–52 provided the specimen which is described in Table I. Although the nucleophile 57–64 proved to be extraordinarily insoluble and had to be introduced as a suspension in dimethylformamide for coupling with a 10% excess of the azide of fragment 53–56, the desired dodecapeptide 53–64 was obtained in about 60% yield by precipitation with aqueous methanol. Attempts to condense the azide of fragment 41–52 with fragment 53–64 failed to give a pure product in dimethylformamide but proceeded smoothly in a mixture of that solvent and hexamethylphosphoramide[5] at 5°.

The combination of 1.5 equiv of the azide of the eicosapeptide 21–40 with fragment 41–64 was carried out in the same mixed solvent system at 5°. Purification of Sephadex G-50 separated the desired product from the expected low molecular weight contaminants as well as from a small amount of a by-product which appeared to be the result of the condensation of two parts of fragment 21–40 with one of fragment 41–64. The desired product, which could be distinguished from the high molecular weight impurity by tlc, was isolated in about 25% yield based on the nucleophile. Treatment of this ester with a large excess of hydrazine in dimethylformamide for 3 min led to a hydrazide which could be distinguished from the ester precursor in the chloroform–methanol–60% aqueous ammonium hydroxide tlc system (60:32:10). The view that no unwanted bonds involving the hydroxyls of serine, threonine,

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and tyrosine or the imidazole ring of histidine are present in the purified tetratetracontapeptide ester is supported by the results of the hydrazinolysis step.

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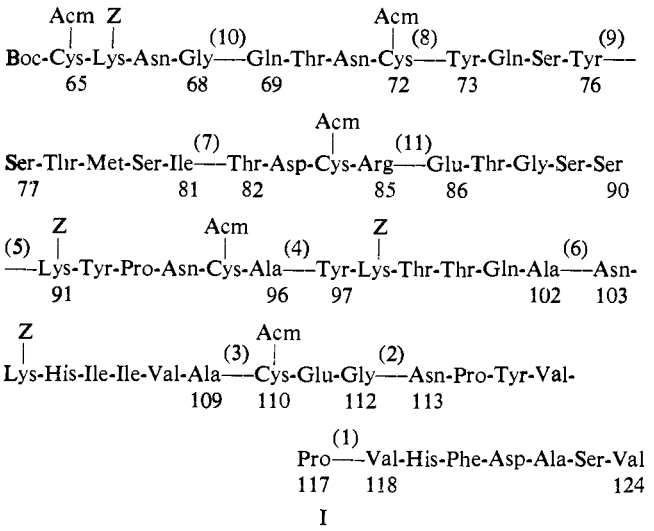
Studies on the Total Synthesis of an Enzyme. III. Synthesis of a Protected Hexacontapeptide Corresponding to the 65–124 Sequence of Ribonuclease A

Sir:

We report herewith the preparation of the protected hexacontapeptide fragment 65–124 (I) of RNase A. This polypeptide was prepared by the addition of the



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N-protected azide of the heneicosapeptide 65–85 to the nonatriacontapeptide 86–124. Coupling reactions involving peptidal azides in which the carboxy-terminal amino acid is unprotected arginine[1] had not, to our knowledge, been previously employed, but we had successfully carried out such reactions in a synthesis of porcine calcitonin[2] and in other instances.[2] The fragments were prepared and the azide coupling reactions were carried out by the procedures described in the two preceding communications.[3,4] The azide couplings of the fragments were performed in the order indicated by the numerals in parentheses above the appropriate bonds in I.

The heneicosapeptide 65–85 was synthesized *via* two routes. In one of these the protected octapeptide azide of fragment 69–76 was allowed to react with 1 equiv of fragment 77–85. The resulting heptadecapeptide, obtained in about 60% yield, was treated with trifluoroacetic acid to remove the butyloxycarbonyl blocking group, and the resulting salt was dissolved in hexamethylphosphoramide and treated with 2 equiv of a solution of the azide of the blocked tetrapeptide[5] 65–68 in dimethylformamide at 5° for 2 days. The product (fragment 65–85) was obtained in about 70% yield.

An alternate route to the heneicosapeptide 65–85 involved the addition of the above blocked tetrapeptide azide in 20% excess to the octapeptide 69–76 to give the desired dodecapeptide in >80% yield. The coupling of the protected azide of fragment 65–76 with the nonapeptide fragment 77–85 proceeded in 15–30% yield, and isolation of pure product generally required purification on Sephadex G-50.

In the preparation of the nonapeptide it is noteworthy that the methyl ester of fragment 77–81, though carboxy terminal in isoleucine, could nevertheless be converted quantitatively to the hydrazide on treatment at 25° with a 25% solution of hydrazine in dimethylformamide overnight and that the azide derived therefrom smoothly reacted with fragment 82–85 at −20° in 60% yield.

The tetrapeptide fragment 65–68 which was required for this synthesis contains an asparaginylglycine sequence whose rearrangement to β-aspartylglycine, presumably *via* a succinimide intermediate, has been described.[6] Our protected tetrapeptide ester and hydrazide were shown to be single components by tlc in two systems, and the absence of a carboxylate anion in the hydrazide was shown by electrophoresis at pH 9. Infrared spectroscopy showed no evidence for the presence of a succinimido moiety[6] in the tetrapeptide ester. The integrity of the asparaginylglycine sequence is also supported by enzymic degradation studies on a sample of the tetrapeptide hydrazide which had been deblocked with anhydrous HF.[3] After incubation with aminopeptidase M at 36° the resulting hydrolysate was indistinguishable by tlc from a synthetic mixture of the component amino acids, and only 2% of β-aspartylglycine was detected in the hydrolysate by Spinco amino acid analysis. Finally, the protected tetrapeptide ester was stable in 50% acetic acid for 2 days. It may be concluded, therefore, that the Asp-Gly bond is reasonably stable to the reaction conditions to which it was to be exposed in our scheme[3] for the synthesis of S-protein.

The synthesis of the nonatriacontapeptide 86–124

(1) Treatment of the carbobenzyloxylated, acetamidomethylated reduced S-protein with trypsin should afford fragment 86–124 in which the sulfhydryl groups and the ε-amino functions of lysine are protected. This material could serve as a relay. Work along these lines is in progress. For this reason an azide coupling with carboxy-terminal arginine-85 became attractive.

(2) Unpublished results from these laboratories.

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