# Total Synthesis of Bovine Pancreatic Ribonuclease A.† Part 1. Synthesis of the Protected Pentadecapeptide Ester (Positions 110—124) ‡

By **Nobutaka Fujii** and **Haruaki Yajima,*** Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku Kyoto, 606, Japan

The main strategy for the total synthesis of bovine pancreatic ribonuclease (RNase) A is outlined with respect to deprotecting procedures and the selection of methods for the construction of the entire amino-acid sequence of this enzyme. Amino-acid derivatives bearing protecting groups removable by methanesulphonic acid were employed in combination with the TFA-labile Z(OMe) $N^{\alpha}$-protecting group. Thirty relatively small peptide fragments were selected as the building blocks for construction of the peptide backbone. The C-terminal-protected pentadecapeptide ester, Z(OMe)-(RNase 110—124)-OBzl, was synthesized by successive condensation of three fragments, Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl (1), Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH (2), and Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH (3).

In this series of papers, we describe the total chemical synthesis of a protein having full and specific ribonuclease (RNase) A activity. The primary structure of bovine pancreatic ribonuclease A was disclosed by Hirs, Moore, and Stein [1] in 1960. Shortly afterwards, with the aid of improved methods for sequential analysis, the entire amino-acid sequence consisting of 124 amino-acids was confirmed [2] with minor revisions.[3] Thus, this protein became the first enzyme for which the entire structure was recorded in the literature. These authors emphasized that 'a formula derived by degradative experiments should be regarded as a working hypothesis until it is confirmed by other means, preferably by chemical synthesis.'

In 1969, two groups of investigators reported the chemical synthesis of materials possessing partial RNase enzyme activity.[4,5] Gutte and Merrifield [4] using automated solid-phase synthesis, reported a successful synthesis of 0.41 mg of a protein (in the supernatant solution of ammonium sulphate fractionation of the trypsin-resistant material) with a specific activity of 78%. Various side reactions that could occur during treatment of protected peptides with HF [6] were not clear at that time, for instance $\gamma$-anisylation of Glu(OBz) [7] and the intramolecular rearrangement of Tyr(Bzl) to Tyr(3-Bzl).[8,9] § Accumulation of errors or failure sequences are often observed in stepwise solid-phase syntheses, as reported by Tregear *et al.*[10] using the Edman procedure. As pointed out by Jones and Ridge,[11] if the synthesis is carried out without purification and characterization of the intermediates, the presence of active molecules closely related to, but not identical with, the natural enzyme cannot be excluded.

The other group, Denkewalter *et al.*,[5] reported the synthesis of an S-protein (tetrahectapeptide, RNase 21—124) [12] in a conventional manner using a modified Leuch's anhydride procedure [13] as the main strategy for the preparation of the necessary fragments with minimal protection. After Rudinger azide condensation [14] of two large fragments (positions 21—64 and 65—124), the Z group from lysine and the newly devised acetamidomethyl group [15] from cysteine were removed by HF and mercuric acetate respectively. They obtained a solution containing *ca.* 2$\gamma$ of S'-activity, upon combination with the S-peptide (RNase 1—20) derived from a natural source. Thus the synthetic material was estimated to have an activity of *ca.* 30—40%, but the minute amount made further purification and characterization of the end product impossible. Thus, we became aware that many problems remained to be solved and an unambiguous synthesis of RNase A or S-protein had yet to be accomplished.

Our synthesis of a protein with 124 amino-acids corresponding to the entire amino-acid sequence of RNase A was accomplished in a conventional manner by successive assembling of 30 peptide fragments, each of which was fully characterized by elemental and amino-acid analyses; after each condensation, all protected intermediates were characterized. In the final step of the synthesis, methanesulphonic acid (MSA) [16] was employed as a deprotecting reagent. After aerial oxidation,[17] the crude material (9—12% activity) was purified by affinity chromatography [18] to afford a compound of higher activity (74—82%) which was then subjected to ion-exchange chromatography to give essentially the fully active component. The synthetic enzyme exhibited identical behaviour to natural RNase A upon electrophoresis and specific activity against yeast RNA and 2',3'-cyclic cytidine phosphate.

† Preliminary communication, H. Yajima and N. Fujii, *J. Chem. Soc., Chem. Commun.*, 1980, 115.

‡ This paper is regarded as Part 88 in the series 'Studies on Peptides' (Part 87, H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano, and H. Adachi, *Int. J. Protein Res.*, 1980, **16**, 33).

§ All amino-acid residues have the L-configuration. Abbreviations used throughout this series are those recommended by the I.U.P.A.C.–I.U.B. Commission on Biochemical Nomenclature (*Biochem. J.*, 1967, **102**, 23; 1967, **104**, 17; 1972, **126**, 773) *Z* = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, MBzl = *p*-methoxybenzyl, Boc = t-butoxycarbonyl, Tri = trityl, MBS = *p*-methoxybenzenesulphonyl, Troc = βββ-trichloroethyloxycarbonyl, Picoc = 4-picolyloxycarbonyl, NP = *p*-nitrophenyl, PCP = pentachlorophenyl, DNP = 2,4-dinitrophenyl, DCC = dicyclohexylcarbodiimide, HOBT = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, DMF = *NN*-dimethylformamide, DMSO = *NN*-dimethyl sulphoxide, HMPA = hexamethylphosphoramide, NMP = *N*-methylpyrrolidone, THF = tetrahydrofuran, MSA = methanesulphonic acid, EDTA = ethylenediaminetetra-acetic acid disodium salt, PCP–O–TCA = pentachlorophenyl trichloroacetate, Su = *N*-hydroxysuccinimide.

Our synthetic strategy, outlined above, is shown schematically in the Figure.* In order to construct the peptide backbone, we selected a conventional procedure which would permit characterization of each intermediate at every step. In order to facilitate purification, relatively small peptide fragments were selected as building blocks and these were assembled for the most part using a modified azide procedure.[14] These building blocks were condensed stepwise from the C- towards the N-terminal end. This approach requires more coupling steps compared to the condensation of relatively large fragments, but is advantageous with respect to ease of purification of the protected intermediates. Smaller peptides couple efficiently by the azide procedure; however the efficiency decreased to a certain degree when the amino-component reached the S-protein stage. After reactions, the excess of azide component could in most cases be readily removed by repeated precipitation. Although chromatographic techniques are usually of limited value in purifying large protected peptides, we have found that, in certain instances, the Sephacryl S-200 5% $H_2O$—NMP (or DMSO) system is quite effective and preferable to Sephadex LH-20 or 60, and we accordingly used this technique at several stages of the synthesis.

The methanesulphonic acid (MSA) deprotecting procedure[16] employed in this synthesis was first introduced to this laboratory in 1975 and its scope and limitation have been examined through syntheses of model peptides, such as duck glucagon[19] and human β-endorphin.[20] For the removal, as the final step of the synthesis, of the many protecting groups necessary for the construction of the peptide chain, we considered the use of a strong acid, *e.g.* trifluoromethanesulphonic acid–TFA.[21a] However, this acid was found to decompose Cys(MBzl)[21b] and to give Lys(Bzl) from Lys(Z). The latter side reaction, reported by Mitchell and Merrifield,[22] did not occur in the MSA-deprotecting system,[23] and in this weaker acid system full recovery of cysteine from Cys(MBzl) was obtained. It was further found that this acid has no tendency to form a γ-anisyl-glutamic acid derivative from Glu(OBzl), a Friedel–Crafts-type reaction known as a side reaction of HF. Thus MSA was judged by us to be more attractive than either trifluoromethanesulphonic acid or HF.

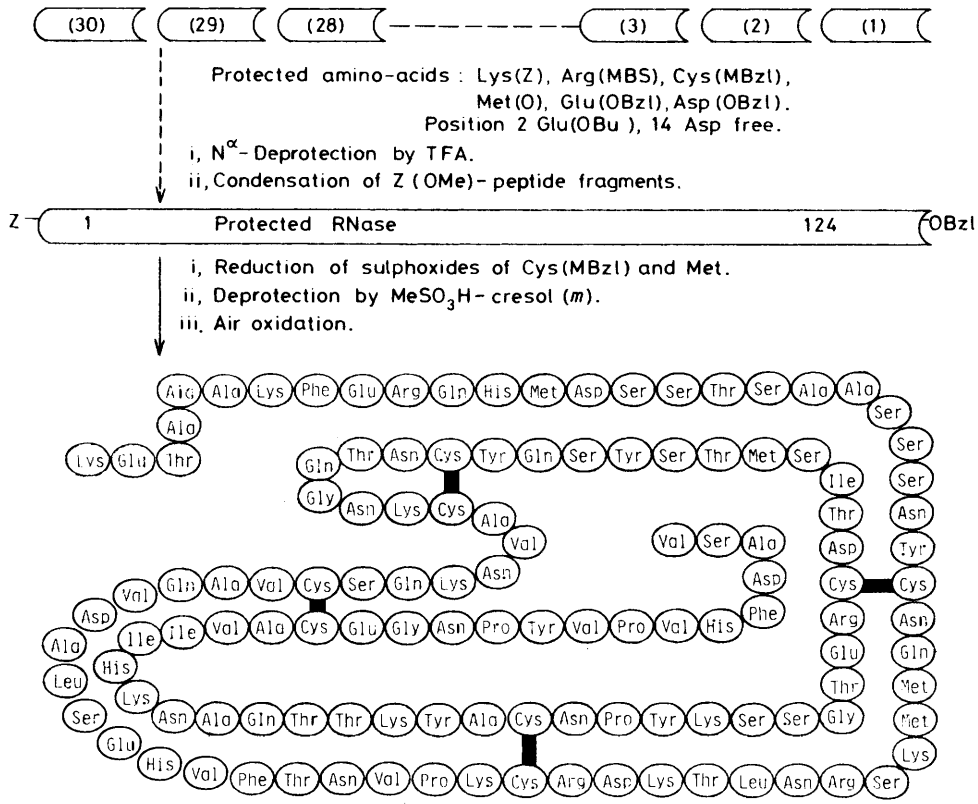



FIGURE Strategy for the total synthesis of RNase A

However, two additional problems needed to be solved before this reagent could be applied to a practical peptide synthesis, *viz.* choice of a suitable protecting group for arginine and prevention of *S*-alkylation of methionine. A new arginine derivative, Arg(MBS), introduced by Nishimura and Fujino,[24] satisfied our requirements, since MSA removed this protecting group within 60 min at 20 °C, whereas the nitro[25] and tosyl[26] groups employed currently in peptide synthesis resisted the action of MSA. Despite such ease of removal of the MBS group, we had to overcome an unexpected side

* Formulae are numbered consecutively throughout all six parts of this series.

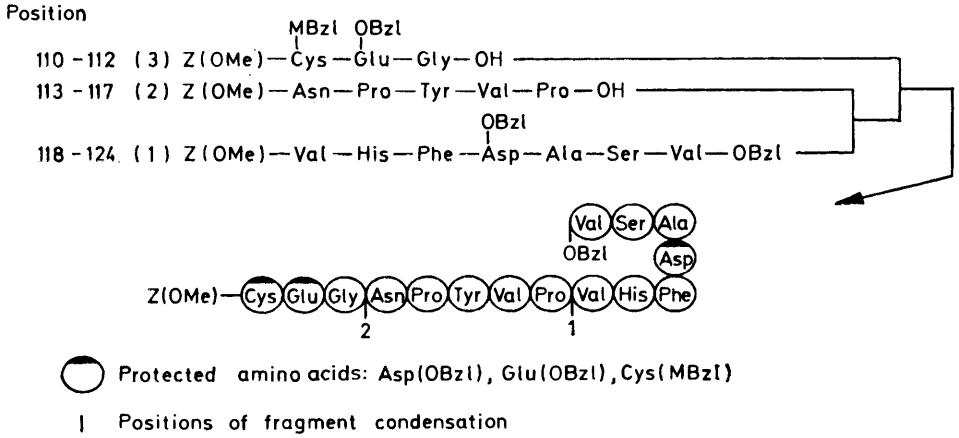
reaction at tyrosine. The MBS cation liberated by MSA (as well as by HF), attacked the phenolic group of tyrosine to form Tyr(MBS),[27] even in the presence of the cation scavenger anisole; this was overcome by the use of, as an alternative scavenger, *m*- or *o*-cresol. Recently we introduced a more acid-labile protecting group for arginine, the mesitylene-2-sulphonyl group.[28] However, this new derivative was not ready for use in the present synthesis.

The second problem, that of *S*-alkylation of methionine in the MSA–anisole system, was due to methyl transfer from anisole [29] and this side reaction could be totally prevented by the use of methionine sulphoxide, Met(O).[30] An easy procedure for the preparation of Met(O), by oxidation using sodium metaperiodate or sodium perborate, provided us with sufficient starting material.[31]

An unexpected problem arose with the formation of the sulphoxide of Cys(MBzl) during the peptide synthesis. The chemical nature of the Cys(MBzl) sulphoxide has hitherto not been examined in the field of peptide synthesis. We had an indication that partial oxidation of Cys(MBzl) [32] had indeed occurred during the long course of the synthesis, and when treated with MSA–anisole, as well as with HF, this compound was fully transformed into *p*-methoxyphenylcysteine,[33] instead of cysteine. Reduction of the sulphoxide of Cys(MBzl) with thiols thus became necessary prior to the MSA deprotection. This simultaneously reduced Met(O) to methionine. Thus, the protecting group for methionine needed to be removed prior to removal of the other protecting groups. This new situation prompted us to investigate an alternative scavenger, one that has no tendency to alkylate methionine. For this and for other reasons, we selected *m*-cresol. We believe that the use of Met(O) played an important role in preventing partial *S*-oxidation during the synthesis and partial *S*-alkylation during the $N^{\alpha}$-deprotection, as described by Iselin.[30]

With the above strategy and keeping the various side reactions in mind, we undertook the synthesis of RNase A with amino-acid derivatives bearing protecting groups removable by MSA, *i.e.* Asp(OBzl), Glu(OBzl), Lys(Z), Arg(MBS), and Cys(MBzl), in combination with the TFA-labile Z(OMe) group [34] as the $N^{\alpha}$-protecting group. Our reagent, *p*-methoxybenzyl-8-quinolyl carbonate,[35] gave sufficient quantities of Z(OMe)-amino-acids as starting materials. After performing a preliminary coupling on a small scale, two successive preparative-scale runs for each condensation step were performed to afford sufficient quantities of intermediates. Thus, we succeeded in synthesizing 4.9 g of the protected S-protein. We herein present a detailed account of the synthesis of RNase A, in which we have been engaged for the past $3\frac{1}{2}$ years.

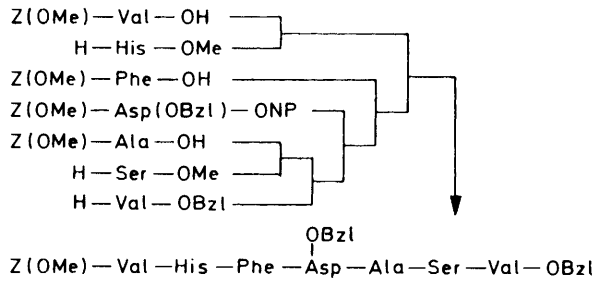
In the first paper, we report the synthesis of the protected pentadecapeptide ester corresponding to the C-terminal portion of RNase (positions 110—124). The pentadecapeptide ester, Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-




SCHEME 1 Synthetic route to the protected pentadecapeptide ester, Z(OMe)-(RNase 110—124)-OBzl

Ser-Val-OBzl, abbreviated as Z(OMe)-(RNase 110—124)-OBzl, was synthesized according to Scheme 1, where three peptide fragments, (1), (2) and (3), served as building blocks. The Merck group [5c] also selected these three units (two hydrazides and the C-terminal heptapeptide with the free carboxy-group), although no experimental details were given.

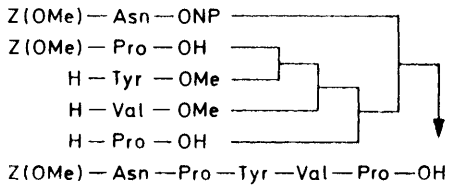
The first block (1), Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase 118—124)-OBzl, was synthesized according to Scheme 2. The C-terminal carboxy-group was, in our synthesis, protected as its benzyl ester. An attempt to prepare Z-Ala-Ser-Val-OH




SCHEME 2 Synthetic scheme for the protected heptapeptide ester, Z(OMe)-(RNase 118—124)-OBzl (1)

by azide coupling of Z-Ala-Ser-$NHNH_2$ [36] with valine gave a poor yield, because of the difficulty involved in bringing valine into aqueous solution, even in the presence of the theoretical amount of triethylamine. The coupling of Z(OMe)-Ala-Ser-$NHNH_2$ with H-Val-OBzl was performed to give Z(OMe)-Ala-Ser-Val-OBzl in satisfactory yield. Prior to each chain elongation, the Z(OMe) group was removed by TFA in the presence of anisole. To the resulting $N^{\alpha}$-deprotected peptide, H-Ala-Ser-Val-OBzl in this instance, Z(OMe)-Asp-(OBzl)-OH and Z(OMe)-Phe-OH were incorporated in a stepwise manner by the *p*-nitrophenyl ester procedure [37] and DCC condensation [38] respectively; subsequently Z(OMe)-Val-His-$NHNH_2$ was incorporated using the Rudinger azide procedure. The latter hydrazide was prepared by the mixed anhydride procedure [39] followed by the usual treatment of the resulting dipeptide ester, Z(OMe)-Val-His-OMe, with hydrazine. In this step, the DCC condensation is known to give the DCC adduct of histidine.[40]

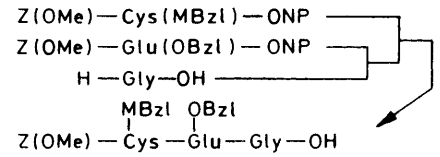
Fragment (2), Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH, Z(OMe)-(RNase 113—117)-OH, was synthesized according to Scheme 3. Construction of the peptide bonds, Val-Pro-Val (116—118), gave considerable difficulty due to the combination of sterically hindered amino-acids. Prior to synthesizing this fragment, the Val-Pro-Val-His unit was prepared as a model compound. The DCC condensation between proline and valine is known to proceed smoothly, but not that between valine and proline, because of the predominant formation of an acylurea, a side reaction of DCC.[41] Thus, we decided to terminate this fragment at proline (117) and form the Val–Pro bond by condensation of Z(OMe)-Pro-Tyr-Val-$NHNH_2$ and proline by the azide procedure. The former tripeptide hydrazide was the one derived from the azide condensation of the known hydrazide Z(OMe)-Pro-Tyr-$NHNH_2$ [42] with H-Val-OMe followed by the usual hydrazine treatment. The resulting tetrapeptide, Z-(OMe)-Pro-Tyr-Val-Pro-OH, after the treatment with TFA, was submitted to condensation with Z(OMe)-Asn-OH by the *p*-nitrophenyl ester method to give fragment (2), unaccompanied by any sizeable amount of the *O*-acyl component [43] at the tyrosine residue.




SCHEME 3 Synthetic scheme for the protected pentapeptide, Z(OMe)-(RNase 113—117)-OH (2)

Fragment (3), Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH, Z(OMe)-(RNase 110—112)-OH, was synthesized without particular difficulty by successive *p*-nitrophenyl ester condensations of Z(OMe)-Glu(OBzl)-OH and Z(OMe)-Cys(MBzl)-OH (Scheme 4).

The two fragments (2) and (3), having proline and glycine C-terminals, respectively, were successively condensed with fragment (1) by the pentachlorophenyl trichloroacetate (PCP-O-TCA) procedure [44] without risk of racemization. We noticed that each reaction was apparently accelerated by the addition of HOBT.[45] Each product was purified by washing and reprecipitation. The amino-acid ratios in 6N-HCl hydrolysates are listed in the Table, where recovery of phenylalanine was taken as the basis for the calculation, since this amino-acid occurs once in the C-terminal portion at position 120 and then at positions 46 and 8. Such diagnostic amino-acids should be selected from amino-acids present only in small amount and located in the C-terminal portion of the molecule. We believe that such a choice of amino-acid as the basis for the calculation throughout the synthesis is absolutely necessary for obtaining information on the purity of each condensation product, rather than varying the amino-acid for reasons of convenience. Various synthetic tactics arising in this synthesis of RNase will be reported.




SCHEME 4 Synthetic scheme for the protected tripeptide, Z(OMe)-(RNase 110—112)-OH (3)

Amino-acid ratios of Z(OMe)-(RNase 110—124)-OBzl and intermediates

| Position | 118—124 | 113—124 | 110—124 |
|---|---|---|---|
| Residue | (7) | (12) | (15) |
| Asp | 0.95(1) | 2.06(2) | 2.03(2) |
| Ser | 0.84(1) | 0.87(1) | 0.90(1) |
| Glu | | | 1.07(1) |
| Pro | | 2.12(2) | 2.19(2) |
| Gly | | | 0.99(1) |
| Ala | 0.94(1) | 0.98(1) | 1.02(1) |
| Val | 1.98(2) | 2.95(3) | 2.98(3) |
| Tyr | | 0.97(1) | 0.94(1) |
| Phe | 1.00(1) | 1.00(1) | 1.00(1) |
| His | 0.75(1) | 0.78(1) | 0.87(1) |
| Cys | | | (1) |
| Recovery (%) | 89 | 91 | 89 |

It should be mentioned that a number of C-terminal peptides have previously been synthesized for the purposes of structure–function studies on RNase. These include the fragment (118—124), prepared in a conventional manner,[46a] with different protecting groups from ours, the fragments (116—124), (113—124), and (111—124), synthesized by the solid method,[46b] and analogues of fragment (111—124), also obtained by the solid method.

## EXPERIMENTAL

Rotations were determined with a Union digital polarimeter PM-101. Hydrolyses of protected peptides containing Tyr with 6N-HCl were performed in the presence of phenol (*ca.* 20 mol equiv.).[47] The amino-acid compositions of acid hydrolysates were determined with a Hitachi model KLA-5 amino-acid analyser and values are un-

corrected for amino-acid destruction. Solvents were freshly distilled and evaporations were carried out *in vacuo* at 40—50 °C (bath temperature). The $N^{\alpha}$-protecting group, Z(OMe), was cleaved by TFA in the presence of anisole (⩾2 mol equiv.) in an ice-bath for 45—60 min. The DCC and the active ester condensations were performed at room temperature (17—25 °C).

The azide condensation was performed according to Honzl and Rudinger.[14] To an ice-chilled solution of a hydrazide in DMF, HCl–DMF (2 mol equiv.) was added followed by isopentyl nitrite (1.1 mol equiv.). After stirring for 15—20 min, when the hydrazine test [48] became negative, the solution was neutralized with $Et_3N$ (2 mol equiv.) and added to an ice-chilled solution of an amino-component, together with additional $Et_3N$ (1 mol equiv.). In the azide reaction derived from His-hydrazides, *N*-methylmorpholine, instead of $Et_3N$, was employed for suppression of possible racemization.[49] After stirring at 4 °C for 48 h (in most instances), a few drops of AcOH were added and the solution was concentrated.

A mixed anhydride was prepared according to Vaughan and Osato.[39] Isobutyl chloroformate (1.1 mol equiv.) was added to an ice-chilled solution of a carboxy-component and $Et_3N$ (1 mol equiv.) in dry THF. After stirring for 20 min, the solution was added to an ice-chilled solution of an amino-component in DMF.

Unless otherwise mentioned, products obtained in Parts 1—5 of this series were purified by one of the following procedures.

*Procedure A.* For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$, and $H_2O$–NaCl, dried over $Na_2SO_4$, and concentrated. The residue was recrystallized or precipitated from appropriate solvents.

*Procedure B.* For purification of protected peptide esters less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$, and $H_2O$ and recrystallized or precipitated from appropriate solvents. For purification of His-containing peptides, 3% AcOH, instead of 5% citric acid, was used for washing.

*Procedure C.* For purification of protected peptides with free carboxy-groups, the crude product was dissolved in 3% ammonia and the aqueous phase, after washing with AcOEt, was acidified with citric acid. The resulting powder (if an oil was obtained, it was extracted with AcOEt) was washed with 5% citric acid and $H_2O$ and recrystallized or precipitated from appropriate solvents.

T.l.c. of products obtained in this series was performed on silica gel (Kieselgel G, Merck). $R_F$ Values refer to the following v/v solvent systems: $R_{F_1}$ $CHCl_3$–MeOH–$H_2O$ (8 : 3 : 1), $R_{F_2}$ $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), $R_{F_3}$ $Bu^nOH$–AcOH–AcOEt–$H_2O$ (1 : 1 : 1 : 1), $R_{F_4}$ $Bu^nOH$–AcOH–pyridine–$H_2O$ (4 : 1 : 1 : 2), $R_{F_5}$ $Bu^nOH$–AcOH–$H_2O$ (4 : 1 : 5), $R_{F_6}$ $CHCl_3$–$CF_3CH_2OH$ (3 : 1).

*Z(OMe)-Ala-Ser-OMe.*—DCC (41.20 g, 0.2 mol) was added to a stirred mixture of Z(OMe)-Ala-OH (43.05 g, 0.17 mol) and H-Ser-OMe [from the hydrochloride (26.45 g, 0.17 mol) with $Et_3N$ (23.5 ml, 0.17 mol)] in DMF (300 ml). After 48 h, the solution was filtered, the filtrate was concentrated, and the residue was purified by procedure A. Recrystallization from AcOEt gave the *ester* (40.01 g, 66%), m.p. 143—145 °C, $[\alpha]_D^{22}$ +6.3° (*c*, 0.6 in DMF), $R_{F_1}$ 0.81 (Found: C, 54.45; H, 6.3; N, 7.95. $C_{16}H_{22}N_2O_7$ requires C, 54.23; H, 6.26; N, 7.91%).

*Z(OMe)-Ala-Ser-NHNH₂.*—To a solution of Z(OMe)-Ala-Ser-OMe (25.0 g, 71 mmol) in MeOH (250 ml) was added 80% hydrazine hydrate (25 ml, 0.4 mol). The precipitate formed on standing at room temperature overnight was collected by filtration and recrystallized from MeOH to give crystalline *hydrazide* (24.07 g, 96%), m.p. 210—211 °C, $[\alpha]_D^{22}$ +15.5° (*c*, 0.6 in DMSO), $R_{F_1}$ 0.72 (Found: C, 51.05; H, 6.3; N, 15.55. $C_{15}H_{22}N_4O_6$ requires C, 50.84; H, 6.26; N, 15.81%).

*Z(OMe)-Ala-Ser-Val-OBzl.*—The azide [from Z(OMe)-Ala-Ser-$NHNH_2$ (17.70 g, 50 mmol)] in DMF (85 ml) and $Et_3N$ (6.9 ml, 50 mmol) were added to a stirred solution of H-Val-OBzl [from the hydrobromide (14.41 g, 50 mmol) with $Et_3N$ (6.9 ml, 50 mmol)] in DMF (85 ml) and after the reaction, the solution was concentrated. The residue was purified by procedure A followed by recrystallization from MeOH to afford the crystalline *tripeptide ester* (16.33 g, 62%), m.p. 146—151 °C, $[\alpha]_D^{22}$ −29.3° (*c*, 0.6 in MeOH), $R_{F_1}$ 0.68 (Found: C, 61.2; H, 6.7; N, 8.1. $C_{27}H_{35}N_3O_8$ requires C, 61.23; H, 6.66; N, 7.94%).

*Z(OMe)-Asp(OBzl)-Ala-Ser-Val-OBzl.*—Z(OMe)-Ala-Ser-Val-OBzl (23.71 g, 45 mmol) was treated with TFA–anisole (42 ml; 5 : 2 v/v) as usual and n-hexane was added to form an oily precipitate, which was washed with ether, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (200 ml), together with $Et_3N$ (12.4 ml, 90 mmol) and Z(OMe)-Asp(OBzl)-ONP (29.49 g, 58 mmol). After stirring for 48 h, the solution was concentrated. The product was purified by procedure A and further by column chromatography on silica (3 × 35 cm) with $CHCl_3$–MeOH (8 : 2 v/v) as eluant. Fractions containing the substance of $R_{F_1}$ 0.98 were combined and the solvent was evaporated off. The residue was recrystallized from AcOEt to give the *protected tetrapeptide ester* (26.79 g, 81%), m.p. 129—131 °C, $[\alpha]_D^{22}$ −12.1° (*c*, 1.2 in DMF) (Found: C, 62.15; H, 6.35; N, 7.6. $C_{38}H_{46}N_4O_{11}$ requires C, 62.11; H, 6.31; N, 7.62%).

*Z(OMe)-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl.*— Z(OMe)-Asp(OBzl)-Ala-Ser-Val-OBzl (7.34 g, 10 mmol) was treated with TFA–anisole (10 ml; 7 : 3 v/v) and n-hexane was added. An oily residue was washed with n-hexane and dissolved in 3.15N-HCl–dioxan (3.1 ml, 10 mmol). After evaporation of the solvent, the residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 h, and then dissolved in DMF (70 ml) together with $Et_3N$ (1.4 ml, 10 mmol), Z(OMe)-Phe-OH (3.96 g, 12 mmol), and DCC (2.47 g, 12 mmol). After stirring for 48 h, the solution was filtered, the filtrate was concentrated, and the residue was purified by procedure B followed by recrystallization from THF–AcOEt to give a white *powder* (8.55 g, 97%), m.p. 203—206 °C, $[\alpha]_D^{22}$ −13.9° (*c*, 0.7 in DMF), $R_{F_5}$ 0.80 (Found: C, 63.2; H, 6.3; N, 7.9. $C_{47}H_{55}N_5O_{12}\cdot 1/2H_2O$ requires C, 63.35; H, 6.34; N, 7.86%).

*Z(OMe)-Val-His-OMe.*—A mixed anhydride from Z-(OMe)-Val-OH (45.01 g, 0.16 mol) in THF (250 ml) was added to an ice-chilled solution of H-His-OMe [from the dihydrochloride (38.56 g, 0.16 mol) with $Et_3N$ (44.2 ml, 0.32 mol) in DMF (200 ml). After stirring in an ice-bath for 5 h, the solution was concentrated and the residue was triturated with ether. Batchwise washing with 5% $Na_2CO_3$ and $H_2O$ followed by recrystallization from MeOH–ether afforded the crystalline *ester* (40.03 g, 58%), m.p. 166—167 °C, $[\alpha]_D^{22}$ +4.1° (*c*, 0.7 in DMF), $R_{F_1}$ 0.54 (Found: C, 58.2; H, 6.7; N, 12.85. $C_{21}H_{28}N_4O_6$ requires C, 58.32; H, 6.53; N, 12.96%).

*Z(OMe)-Val-His-NHNH₂.*—Z(OMe)-Val-His-OMe (40.02

g, 92 mmol) in MeOH (200 ml) was treated with 80% hydrazine hydrate (25.7 ml, 0.41 mol) at room temperature overnight. The resulting solid was collected by filtration and washed with MeOH to give a *powder* (27.50 g, 69%), m.p. 160—161 °C, $[\alpha]_D^{25}$ −10.8° (*c*, 0.8 in DMSO), $R_{F_1}$ 0.47 (Found: C, 54.8; H, 6.65; N, 19.3. $C_{20}H_{28}N_6O_5 \cdot 1/2H_2O$ requires C, 54.41; H, 6.62; N, 19.04%).

*Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase* 118—124)*-OBzl*(1).—Z(OMe)-Phe-Asp-(OBzl)-Ala-Ser-Val-OBzl (8.82 g, 10 mmol) was treated with TFA–anisole (16 ml; 3 : 1 v/v) as usual and n-hexane was added. An oily residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 h, and then dissolved in DMF (20 ml) containing $Et_3N$ (1.4 ml, 10 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Val-His-$NHNH_2$ (5.56 g, 13 mmol)] in DMF (40 ml) and *N*-methylmorpholine (1.4 ml, 13 mmol). After the reaction, the solution was concentrated and the residue was treated with ether and 5% $NaHCO_3$. The resulting powder was purified by column chromatography on silica (3 × 35 cm), which was eluted with $CHCl_3$–MeOH–$H_2O$ (8 : 3 : 1 v/v). Fractions containing the substance of $R_{F_1}$ 0.72 were combined and the solvent was evaporated off. The residue was triturated with $H_2O$ and then recrystallized from THF–ether to give the *protected heptapeptide ester* as a powder (8.72 g, 78%), m.p. 190—193 °C, $[\alpha]_D^{22}$ −18.9° (*c*, 1.0 in DMF), $R_{F_1}$ 0.72 (Found: C, 62.4; H, 6.4; N, 11.05. $C_{58}H_{71}N_9O_{14}$ requires C, 62.29; H, 6.40; N, 11.27%).

*Z(OMe)-Pro-Tyr-Val-OMe.*—The azide [from Z(OMe)-Pro-Tyr-$NHNH_2$[42] (25.30 g, 55 mmol)] in DMF (100 ml) and $Et_3N$ (7.6 ml, 55 mmol) was added to an ice-chilled solution of H-Val-OMe [from the hydrochloride (11.20 g, 67 mmol) with $Et_3N$ (9.2 ml, 67 mmol)] in DMF (50 ml). After the reaction, the solution was concentrated and the residue was purified by procedure A. Trituration with ether and recrystallization twice from AcOEt afforded the *tripeptide ester* as a powder (29.42 g, 96%), m.p. 166—169 °C, $[\alpha]_D^{16}$ −49.7° (*c*, 0.9 in DMF), $R_{F_2}$ 0.63 (Found: C, 62.55; H, 6.7; N, 7.5. $C_{29}H_{37}N_3O_8$ requires C, 62.69; H, 6.71; N, 7.56%).

*Z(OMe)-Pro-Tyr-Val-*$NHNH_2$.—Z(OMe)-Pro-Tyr-Val-OMe (29.40 g, 53 mmol) in MeOH (200 ml) was treated with 80% hydrazine hydrate (6 ml, 1.0 mol) at 60 °C for 8 h. After cooling with ice, the resulting solid was collected by filtration and precipitated twice from DMF with MeOH to afford the *tripeptide hydrazide* (22.52 g, 73%), m.p. 253—255 °C, $[\alpha]_D^{16}$ −27.7° (*c*, 0.8 in DMSO), $R_{F_2}$ 0.40 (Found: C, 60.75; H, 6.8; N, 12.5. $C_{28}H_{37}N_5O_7$ requires C, 60.52; H, 6.71; N, 12.61%).

*Z(OMe)-Pro-Tyr-Val-Pro-OH.*—The azide [from Z(OMe)-Pro-Tyr-Val-$NHNH_2$ (22.50 g, 41 mmol)] in DMF (100 ml) and $Et_3N$ (5.6 ml, 41 mmol) were added to an ice-chilled solution of Pro (7.01 g, 61 mmol) in $H_2O$ (30 ml) containing $Et_3N$ (8.4 ml, 61 mmol). After stirring for 24 h, the mixture was concentrated and the residue purified by procedure C. Trituration with ether and recrystallization twice from AcOEt–ether afforded a *powder* (20.66 g, 80%), m.p. 137—141 °C, $[\alpha]_D^{25}$ −57.0° (*c*, 0.8 in DMF), $R_{F_2}$ 0.26 (Found: C, 61.55; H, 6.95; N, 8.5. $C_{33}H_{42}N_4O_9 \cdot 1/2\ H_2O$ requires C, 61.19; H, 6.69; N, 8.65%).

*Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH, Z(OMe)-(RNase* 113—117)*-OH*(2).—Z(OMe)-Pro-Tyr-Val-Pro-OH (17.30 g, 26 mmol) was treated with TFA–anisole (45 ml; 7 : 2 v/v) as usual and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (100 ml) together with $Et_3N$ (8.0 ml, 58 mmol), HOBT (6.10 g, 29 mmol), and Z(OMe)-Asn-ONP (12.10 g, 29 mmol). After stirring for 24 h, the mixture was concentrated and the residue was purified by procedure C followed by recrystallization twice from MeOH–AcOEt to give the *protected pentapeptide* (14.54 g, 72%), m.p. 144—148 °C, $[\alpha]_D^{25}$ − 74.5° (*c*, 0.8 in DMF), $R_{F_1}$ 0.38, $R_{F_2}$ 0.20. Amino-acid analysis (numbers in parentheses are theoretical values): Asp 1.00 (1), Pro 2.11 (2), Tyr 0.96 (1), Val 0.93 (1) (average recovery 90%) (Found: C, 57.9; H, 6.4; N, 10.7. $C_{37}H_{48}N_6O_{11} \cdot H_2O$ requires C, 57.65; H, 6.54; N, 10.90%).

*Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH, Z(OMe)-(RNase* 110—112)*-OH*(3).—Z(OMe)-Glu(OBzl)-Gly-OH [42] (22.60 g, 50 mmol) was treated with TFA–anisole (36 ml; 2 : 1 v/v) as usual and dry ether was added. The oily precipitate was dried over KOH pellets *in vacuo* for 3 h and then dissolved in DMF (200 ml) containing $Et_3N$ (14 ml, 0.1 mol) and Z-(OMe)-Cys(MBzl)-ONP (31.57 g, 60 mmol). After stirring for 48 h, the mixture was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was recrystallized twice from AcOEt–ether to give the *protected tripeptide* (23.80 g, 70%), m.p. 124—126 °C, $[\alpha]_D^{22}$ −50.3° (*c*, 0.8 in DMF), $R_{F_1}$ 0.42. Amino-acid analysis: Glu 1.07 (1), Gly 1.00 (1) (average recovery 91%) (Found: C, 59.7; H, 5.8; N, 6.0. $C_{34}H_{39}N_3O_{10}S$ requires C, 59.90; H, 5.77; N, 6.16%).

*Z(OMe)-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl. Z(OMe)-(RNase* 113—124)*-OBzl.*—To a solution of Z(OMe)-Asn-Pro-Tyr-Val-Pro-OH (25.0 g, 32 mmol) in DMF (150 ml) was added $Et_3N$ (4.4 ml, 32 mmol) followed by PCP-O-TCA (14.41 g, 35 mmol). While stirring for 2 h, a new spot of $R_{F_2}$ 0.32 appeared on t.l.c. and the starting material of $R_{F_1}$ 0.20 disappeared. The solvent was evaporated and the residue was triturated with $H_2O$ and the resulting active ester was recrystallized from THF and ether as a powder (27.55 g, 86%), m.p. 136—141 °C. Z(OMe)-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl (23.52 g, 21 mmol) was treated with TFA–anisole (85 ml; 14 : 3 v/v) as usual and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and then dissolved in DMF (150 ml) together with $Et_3N$ (9.6 ml, 69 mmol), HOBT (4.83 g, 23 mmol), and the active ester obtained above (23.20 g, 23 mmol). After stirring for 48 h, the solution was concentrated and the residue was purified by procedure B followed by precipitation from MeOH–AcOEt to give a *powder* (31.47 g, 89%), m.p. 148—151 °C, $[\alpha]_D^{22}$ −50.3° (*c*, 0.8 in DMF), $R_{F_1}$ 0.60, $R_{F_3}$ 0.76 (Found: C, 60.0; H, 6.55; N, 12.25. $C_{86}H_{109}N_{15}O_{21} \cdot 2H_2O$ requires C, 59.88; H, 6.60; N, 12.18%).

*Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl, Z(OMe)-(RNase* 110—124)*-OBzl.*—PCP-O-TCA (9.49 g, 23 mmol) was added to a solution of Z(OMe)-Cys(MBzl)-Glu(OBzl)-Gly-OH (14.20 g, 21 mmol) in DMF (70 ml) containing $Et_3N$ (3.15 ml, 23 mmol). While stirring for 2.5 h, the starting material ($R_{F_1}$ 0.42) disappeared and a new spot ($R_{F_1}$ 0.92) was detected on t.l.c. The solvent was evaporated off and the residue was treated with $H_2O$ and the resulting powder was precipitated from THF with ether to afford the active ester (15.96 g, 82%). Z(OMe)-(RNase 113—124)-OBzl (20.30 g, 12 mmol) was treated with TFA–anisole (75 ml; 4 : 1 v/v) and the deprotected peptide, isolated as above, was dissolved in DMF (100 ml) together with $Et_3N$ (5.46 ml, 40 mmol), HOBT (2.71 g, 13 mmol), and the active ester obtained above (12.10 g, 13 mmol). After stirring

for 48 h, the mixture was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH to give the *protected pentadecapeptide ester* as a powder (18.02 g, 70%), m.p. 226—231 °C, $[\alpha]_D^{22}$ −51.2° (*c*, 0.9 in DMF), $R_{F_1}$ 0.58 (Found: C, 60.05; H, 6.45; N, 11.35; S, 1.65. $C_{111}H_{138}N_{18}O_{27}S\cdot 2H_2O$ requires C, 59.93; H, 6.43; N, 11.34; S, 1.44%).

*Trial Experiments.—Z-Ala-Ser-Val-OH.* The azide [from Z-Ala-Ser-$NHNH_2$ [36] (19.20 g, 60 mmol)] in DMF (150 ml) and $Et_3N$ (8.3 ml, 60 mmol) were added to a solution of Val (13.92 g, 0.12 mol) in $H_2O$ (130 ml) and pyridine (70 ml) containing $Et_3N$ (16.6 ml, 0.12 mol). While stirring for 48 h, a certain amount of Val separated from the solution. The solution was filtered and the filtrate concentrated. The residue was purified by procedure C followed by recrystallization from AcOEt to give the *protected tripeptide* (7.85 g, 32%), m.p. 165—166 °C, $[\alpha]_D^{22}$ +5.2° (*c*, 1.0 in DMF), $R_{F_1}$ 0.15 (Found: C, 56.0; H, 6.9; N, 10.25. $C_{19}H_{27}N_3O_7$ requires C, 55.73; H, 6.65; N, 10.26%).

*H-Ala-Ser-Val-OH.* Z-Ala-Ser-Val-OH (13.10 g, 32 mmol) in MeOH (250 ml) containing a few drops of AcOH was hydrogenated over Pd for 8 h. The solution was filtered, the filtrate was concentrated, and the residue, after trituration with EtOH, was recrystallized from $H_2O$–EtOH to give *crystals* (8.60 g, 98%), m.p. 210 °C (decomp.), $[\alpha]_D^{22}$ −39.1° (*c*, 1.0 in 3% AcOH), $R_{F_4}$ 0.41 (Found: C, 48.1; H, 7.8; N, 15.05. $C_{11}H_{21}N_3O_5$ requires C, 47.99; H, 7.69; N, 15.27%).

*Z-Pro-Val-His-OMe.* Z-Val-His-OMe [50] (40.20 g, 0.1 mol) dissolved in MeOH (300 ml) and 1N-HCl (200 ml, 0.2 mol) was hydrogenated over Pd for 8 h. The catalyst was removed by filtration, the filtrate was concentrated, and the residue ($R_{F_3}$ 0.48) was dissolved in acetonitrile (400 ml) together with $Et_3N$ (28 ml, 0.2 mol) and Z-Pro-OH (24.90 g, 0.1 mol). DCC (24.80 g, 0.12 mol) was added and the mixture was stirred overnight. The solution was filtered, the filtrate was concentrated, and the residue was dissolved in AcOEt. The organic phase was washed with 5% $Na_2CO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$, and then concentrated. The residue was recrystallized from MeOH–ether to give the *protected tripeptide ester* (28.11 g, 56%), m.p. 131—135 °C, $[\alpha]_D^{22}$ −28.7° (*c*, 0.9 in DMF), $R_{F_1}$ 0.66 (Found: C, 58.35; H, 6.95; N, 13.65. $C_{25}H_{33}N_5O_6\cdot H_2O$ requires C, 58.01; H, 6.82; N, 13.52%).

*Z(OMe)-Val-Pro-Val-His-OMe.* Z-Pro-Val-His-OMe (26.0 g, 50 mmol) in MeOH (300 ml) and 1N-HCl (110 ml, 0.11 mol) was hydrogenated over Pd for 8 h. After filtration, the solution was concentrated and the residue was dissolved in DMF (100 ml) together with $Et_3N$ (13.8 ml, 0.1 mol) and Z(OMe)-Val-OH (14.60 g, 50 mmol). After addition of DCC (12.40 g, 60 mmol), the mixture was stirred for 48 h, filtered, and the filtrate was concentrated. The residue was purified as above to give a *powder* (11.90 g, 38%), m.p. 122—124 °C, $[\alpha]_D^{22}$ −37.6° (*c*, 1.0 in DMF), $R_{F_1}$ 0.22 (Found: C, 59.45; H, 7.3; N, 13.5. $C_{31}H_{44}N_6O_8$ requires C, 59.22; H, 7.05; N, 13.37%).

*Z(OMe)-Val-Pro-Val-His-$NHNH_2$.* Z(OMe)-Val-Pro-Val-His-OMe (19.11 g, 30 mmol) in MeOH (60 ml) was treated with 80% hydrazine hydrate (11.3 ml, 6 mol equiv.) overnight. The solution was concentrated and the residue was treated with ether. Recrystallization from MeOH–$Et_2O$ afforded the *hydrazide* as a powder (18.10 g, 95%), m.p. 138—140 °C, $[\alpha]_D^{22}$ −47.1° (*c*, 0.9 in DMSO), $R_{F_1}$ 0.11 (Found: C, 56.1; H, 7.05; N, 17.35. $C_{30}H_{44}N_8O_7\cdot 1/2H_2O$ requires C, 56.49; H, 7.11; N, 17.57%).

*Z(OMe)-Asn-Pro-Tyr-OMe.* Z-Pro-Tyr-OMe [51] (4.26 g, 10 mmol) in MeOH (30 ml) and 1N-HCl (10 ml, 1 mol equiv.) was hydrogenated as usual. The solution was filtered, the filtrate was concentrated, and the residue ($R_{F_1}$ 0.29) was dissolved in DMF (30 ml) together with $Et_3N$ (1.4 ml, 10 mmol) and Z(OMe)-Asn-ONP (4.17 g, 10 mmol). After stirring for 24 h, the solution was concentrated and the residue was purified by procedure A followed by precipitation from AcOEt with ether to afford an *amorphous powder* (2.50 g, 44%), $[\alpha]_D^{20}$ −41.9° (*c*, 0.9 in DMF), $R_{F_1}$ 0.61 (Found: C, 57.85; H, 6.2; N, 10.0. $C_{28}H_{34}N_4O_9\cdot 1/2H_2O$ requires C, 58.02; H, 6.08; N, 9.66%). Use of an excess of $Et_3N$ in the reaction afforded a heterogeneous oily product.

*Z(OMe)-Asn-Pro-Tyr-$NHNH_2$.* Z(OMe)-Asn-Pro-Tyr-OMe (7.0 g, 12 mmol) in MeOH (30 ml) was treated with 80% hydrazine hydrate (4.5 ml, 6 mol equiv.) overnight. The gelatinous mass formed was recrystallized from MeOH to give the *tripeptide hydrazide* (3.71 g, 53%), m.p. 177—180 °C, $[\alpha]_D^{22}$ −45.3° (*c*, 1.0 in DMF), $R_{F_1}$ 0.33 (Found: C, 55.8; H, 6.4; N, 14.55. $C_{27}H_{34}N_6O_8\cdot 1/2H_2O$ requires C, 55.95; H, 6.09; N, 14.50%).

This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan, and by the Yamada Science Foundation (Osaka, Japan). The authors thank Dr. Teh-yung Liu, Bureau of Biologics, Food and Drug Administration, Rockville, Maryland, U.S.A., for his valuable discussions and encouragement throughout this investigation.

[9/1715 *Received*, 26*th October*, 1979]

REFERENCES

[1] C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1960, **235**, 633; D. H. Spackman, W. H. Stein, and S. Moore, *ibid.*, p. 648.
[2] D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 1963, **238**, 227.
[3] D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.* 1962, **237**, 1845; J. T. Potts, A. Berger, J. Cooke, and C. B. Anfinsen, *ibid.*, p. 1851; E. Gross and B. Witkop, *ibid.*, p. 1856.
[4] B. Gutte and R. B. Merrifield, *J. Am. Chem. Soc.*, 1969, **91**, 501; *J. Biol. Chem.*, 1971, **246**, 1922.
[5] (*a*) R. G. Denkewalter, D. F. Veber, F. W. Holly, and R. Hirschmann, *J. Am. Chem. Soc.*, 1969, **91**, 502; (*b*) R. G. Strachan, W. J. Paleveda, jun., R. F. Nutt, R. A. Vitali, D. F. Veber, M. J. Dickinson, V. Garsky, J. E. Deak, E. Walton, S. R. Jenkins, F. W. Holly, and R. Hirschmann, *ibid.*, p. 503; (*c*) S. R. Jenkins, R. F. Nutt, R. S. Dewey, D. F. Veber, F. W. Holly, W. J. Paleveda, jun., T. Lanza, jun., R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, M. J. Dickinson, J. Sondey, R. Hirschmann, and E. Walton, *ibid.*, p. 505; (*d*) D. F. Veber, S. L. Varga, J. D. Milkowski, H. Joshua, J. B. Conn, R. Hirschmann, and R. G. Denkewalter, *ibid.*, p. 506; (*e*) R. Hirschmann, R. F. Nutt D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *ibid.*, p. 507.
[6] S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, 1967, **40**, 2164.
[7] S. Sano and S. Kawanishi, *J. Am. Chem. Soc.*, 1975, **97**, 3480; R. S. Feinberg, and R. B. Merrifield, *ibid.*, p. 3485.
[8] B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, 1973, **95**, 3750; D. Yamashiro, and C. H. Li, *Int. J. Pept. Protein Res.*, 1972, **4**, 181; *J. Org. Chem.*, 1973, **38**, 591; Y. Kiso, H. Isawa, K. Kitagawa, and T. Akita, *Chem. Pharm. Bull.*, 1978, **26**, 2562.
[9] M. Engelhard and R. B. Merrifield, *J. Am. Chem. Soc.*, 1978, **100**, 3559.
[10] G. W. Tregear, R. Sauer, H. D. Niall, H. Y. Keutmann, and J. T. Potts, jun., *Biochem.*, 1977, **16**, 2817.
[11] J. H. Jones and B. Ridge in 'Amino acids, Peptides, and Proteins,' ed. G. T. Young, Specialist Periodical Reports, The Chemical Society, London, 1972, vol. 4, p. 379.

[12] F. M. Richards, *Proc. Natl. Acad. Sci. USA*, 1958, **44**, 162; F. M. Richard and P. J. Vithayathil, *J. Biol. Chem.*, 1959, **234**, 1459.
[13] J. L. Bailey, *J. Chem. Soc.*, 1950, 3461; R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, B. Barkemeyer, D. F. Veber, W. J. Paleveda, jun., T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.*, 1967, **32**, 3415.
[14] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, 1961, **26**, 2333.
[15] D. F. Veber, J. D. Milkowski, R. G. Denkewalter, and R. Hirschmann, *Tetrahedron Lett.*, 1968, 3057; D. F. Veber, J. D.Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, 1972, **94**, 5456.
[16] H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, 1975, **23**, 1164.
[17] C. B. Anfinsen and E. Haber, *J. Biol. Chem.*, 1961, **236**, 1361.
[18] M. Wilchek and M. Gorecki, *European J. Biochem.*, 1969, **11**, 491; in ' Methods in Enzymology,' eds. W. B. Jakoby and M. Wilcheck, Academic Press, New York and London, 1974, vol. 34, p. 492; R. K. Wierenga, J. D. Huizinga, W. Gaastra, G. W, Welling, and J. J. Beintema, *FEBS Letters*, 1973, **31**, 181; I. M. Chaiken and H. C. Taylor, *J. Biol. Chem.*, 1976, **251**, 2044.
[19] H. Yajima, H. Ogawa, and H. Sakurai, *J. Chem. Soc., Chem. Commun.*, 1977, 909; H. Ogawa and H. Yajima, *Chem. Pharm Bull.*, 1978, **26**, 1540; H. Ogawa, M. Sugihara, H. Yajima, H. Sakurai, and K. Tusda, *ibid.*, p. 1549.
[20] M. Kubota, T. Hirayama, O. Nagase, and H. Yajima, *Chem. Pharm. Bull.*, 1978, **26**, 2132, 2139.
[21] (*a*) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, 1974, 107; (*b*) N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, 1975, **23**, 1596.
[22] R. A. Mitchell and R. B. Merrifield, *J. Org. Chem.*, 1976, **41**, 2015.
[23] N. Fujii, S. Funakoshi, T. Sasaki, and H. Yajima, *Chem. Pharm. Bull.*, 1977, **25**, 3096.
[24] O. Nishimura and M. Fujino, *Chem. Pharm. Bull.*, 1976, **24**, 1568.
[25] M. Bergmann, L. Zervas, and H. Rinke, *Z. Physiol. Chem.*, 1934, **224**, 40.
[26] E. Schnabel and C. H. Li, *J. Am. Chem. Soc.*, 1960, **82**, 4576.
[27] H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, 1978, **26**, 3752.
[28] H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, 1978, 482.
[29] H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *J. Chem. Soc., Chem. Commun.*, 1976, 922; *Chem. Pharm. Bull.*, 1977, **25**, 2929.
[30] B. Iselin, *Helv. Chim. Acta*, 1961, **44**, 61.
[31] N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, 1978, **26**, 650.
[32] S. Akabori, S. Sakakibara, Y. Shimonishi, and Y. Nobuhara, *Bull. Chem. Soc. Jpn.*, 1964, **37**, 433.
[33] H. Yajima, S. Funakoshi, N. Fujii, K. Akaji, and H. Irie, *Chem. Pharm. Bull.*, 1979, **27**, 1060.
[34] F. Weygand and K. Hunger, *Chem. Ber.*, 1962, **95**, 1.
[35] H. Yajima, F. Tamura, and Y. Kiso, *Chem. Pharm. Bull.*, 1970, **18**, 2574; H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *ibid.*, 1973, **21**, 1380.
[36] E. Schröder, *Justus Liebigs Ann. Chem.*, 1964, **673**, 186.
[37] M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, 1959, **81**, 5688.
[38] J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, 1955, **77**, 1067.
[39] Th. Wieland and H. Bernhard, *Justus Liebigs Ann. Chem.*, 1951, **572**, 190; J. R. Vaughan, jun., and R. L. Osato, *J. Am. Chem. Soc.*, 1951, **73**, 5553; R. A. Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874.
[40] H. Rink and B. Riniker, *Helv. Chim. Acta*, 1974, **57**, 831.
[41] J. C. Sheehan, M. Goodman, and G. P. Hess, *J. Am. Chem. Soc.*, 1956, **78**, 1367; H. Schüssler and H. Zahn, *Chem. Ber.*, 1962, **95**, 1076.
[42] H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.*, 1975, **23**, 375.
[43] J. Ramachandran and C. H. Li, *J. Org. Chem.*, 1963, **28**, 173; H. Zahn, H. Bremer, and R. Zabel, *Z. Naturforsch., Teil B*, 1965, **20**, 653; R. Geiger, W. König, J. Sandow, and A. V. Schalley, *Z. Physiol. Chem.*, 1974, **355**, 1526; M. Bodanszky, M. L. Fink, Y. S. Klausner, S. Natarajan, K. Tatemoto, A. E. Yiotakis, and A. Bodanszky, *J. Org. Chem.*, 1977, **42**, 149; J. Martinez, J. C. Tolle, and M. Bodanszky, *Int. J. Pept. Protein Res.*, 1979, **13**, 22.
[44] M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.*, 1968, **16**, 929.
[45] W. König and R. Geiger, *Chem. Ber.*, 1973, **106**, 3626.
[46] (*a*) J. E. Shields and H. Renner, *J. Am. Chem. Soc.*, 1970, **88**, 2304; (*b*) M. C. Lin, B. Gutte, S. Moore, and R. B. Merrifield, *J. Biol. Chem.*, 1970, **245**, 5169; R. Hayashi, S. Moore, and R. B. Merrifield, *ibid.*, 1973, **248**, 3889; (*c*) R. S. Hodge and R. B. Merrifield, *Int. J. Pept. Protein Res.*, 1974, **6**, 379; B. Gutte, *J. Biol. Chem.*, 1975, **250**, 889; R. S. Hodge and R. B. Merrifield, *ibid.*, p. 1231.
[47] B. Iselin, *Helv. Chim. Acta*, 1962, **45**, 1510.
[48] H. E. Ertel and L. Horner, *J. Chromatogr.*, 1962, **7**, 268.
[49] M. Shin and K. Inouye in ' Peptide Chemistry 1977,' ed. T. Shiba, Protein Research Foundation, Osaka, 1977, p. 67.
[50] R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, 1958, **41**, 1273.
[51] H. Yajima, Y. Kinomura, T. Oshima, and Y. Okada, *Chem. Pharm. Bull.*, 1967, **15**, 1922.