Total Synthesis of Bovine Pancreatic Ribonuclease A. Part 3.[1] Synthesis of the Protected Hexapentacontapeptide Ester (Positions 69—124) †

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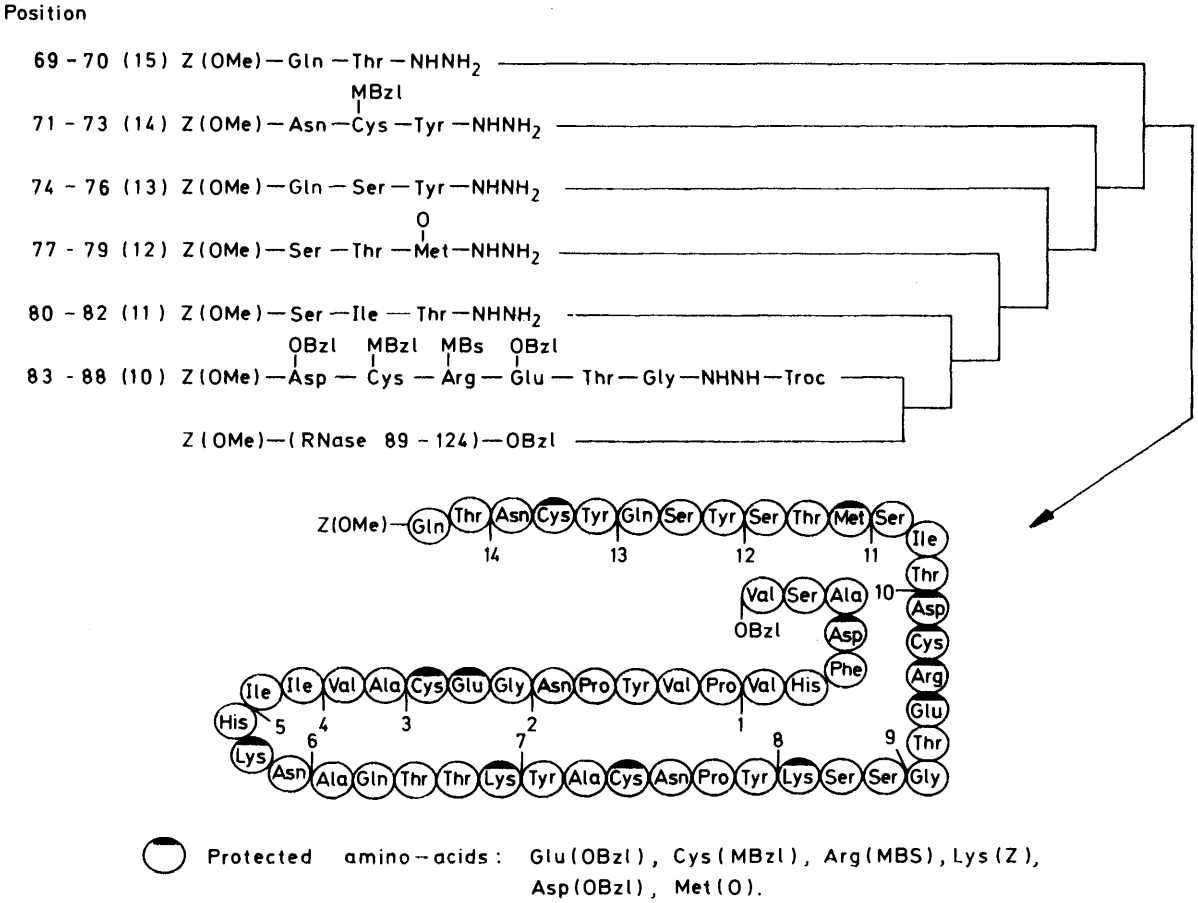
Commencing with the protected hexatriacontapeptide, corresponding to the sequence of bovine pancreatic RNase A, Z(OMe)-(RNase 89—124)-OBzl, chain elongation was carried out to the hexapentacontapeptide stage, Z(OMe)-(RNase 69—124)-OBzl, by six successive azide condensations of the peptide fragments Z(OMe)-Asp-(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc (10), Z(OMe)-Ser-Ile-Thr-$NHNH_2$ (11), Z(OMe)-Ser-Thr-Met(O)-$NHNH_2$ (12), Z(OMe)-Gln-Ser-Tyr-$NHNH_2$ (13), Z(OMe)-Asn-Cys(MBzl)-Tyr-$NHNH_2$ (14), and Z(OMe)-Gln-Thr-$NHNH_2$ (15). The Troc group was removed from fragment (10) by treatment with Zn prior to condensation. With *N*-methylpyrrolidone-5% H_2O as eluant, gel-filtration on Sephacryl S-200 was employed for purification of Z(OMe)-(RNase 69—124)-OBzl which was contaminated with the acyl component used in excess during the coupling.

THE synthesis of the protected hexatriacontapeptide ester corresponding to positions 89—124 of bovine pancreatic RNase A was described in the first two papers of this series;[1] we now report further chain elongation to the protected hexapentacontapeptide ester, Z(OMe)-(RNase 69—124)-OBzl, which was performed by successive condensations of six peptide fragments, from (10) to (15) (Scheme 1).



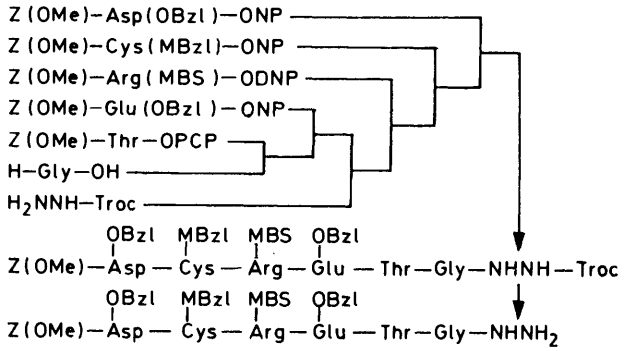
SCHEME 1 Synthetic route to the protected hexapentacontapeptide ester, Z(OMe)-(RNase 69—124)-OBzl

The protected hexapeptide hydrazide derivative Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc (10) (positions 83—88) was synthesized according to Scheme 2. The glycine residue at position 88 can be chosen as the racemization-free condensation point. Therefore, we initially planned to prepare the protected hexapeptide without protection of the carboxy-group and couple this unit by DCC in the presence of HOBT[2] to the relatively large amino-component, H-(89—124)-OBzl. However, consider-

† This paper is regarded as Part 90 in the series 'Studies on Peptides' [Part 89, ref. 1*b* (preceding paper)].

ation of the various preferable features of the Rudinger azide-coupling procedure [3] for our present synthesis led us to adopt a substituted hydrazine in the preparation of this hydrazide containing Asp(OBzl) and Glu(OBzl), since the usual hydrazinolysis of esters could not be applied. Troc-$NHNH_2$, introduced in our laboratory in 1971,[4] met our requirements, since the Troc group can be removed by treatment with Zn [5] without affecting the side-chain protecting groups; Z(OMe), Z, Bzl, MBzl, and MBS.[6] The known substituted hydrazines, Z-$NHNH_2$,[7] Boc-$NHNH_2$,[8] Tri-$NHNH_2$,[9] and Picoc-$NHNH_2$,[10] did not fulfil this requirement.

Thus, Z(OMe)-Glu(OBzl)-Thr-Gly-OH, prepared in a stepwise manner, was converted quantitatively, by the DCC-plus-HOBT condensation [2] with Troc-$NHNH_2$, into the crystalline hydrazide derivative, Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc. This fragment, after N^{α}-deprotection with TFA,[11] was condensed with Z(OMe)-Arg(MBS)-OH,[6] a new arginine derivative bearing a protecting group removable by MSA,[12] by means of the DNP method.[13,14] The reaction gave a yield of 81%

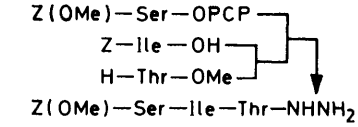


SCHEME 2 Synthetic scheme for the protected hexapeptide hydrazide derivative, Z(OMe)-(RNase 83—88)-NHNH-Troc (10)

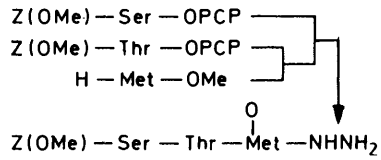
(by t.l.c.) without isolation of the active ester. This is the first time during the synthesis that an arginine residue has been incorporated into the chain. To the resulting protected tetrapeptide, Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc, two amino-acid derivatives, Z(OMe)-Cys(MBzl)-OH and Z(OMe)-Asp(OBzl)-OH, were successively introduced by the NP-active ester procedure [15] to give the protected hexapeptide hydrazide derivative (10), from which the Troc group was smoothly removed by Zn in acetic acid. The last trace of contaminative zinc acetate was removed by EDTA. The purity of the Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-$NHNH_2$ was confirmed by t.l.c., and elemental and amino-acid analyses.

The fragment Z(OMe)-Ser-Ile-Thr-$NHNH_2$ (11) (positions 80—82) was prepared using the known dipeptide Z-Ile-Thr-OMe [16] and an available active ester, Z(OMe)-Ser-OPCP (Scheme 3).

In the next fragment, Z(OMe)-Ser-Thr-Met(O)-NH-NH_2 (12) (positions 77—79), the methionine residue was protected as the sulphoxide [17] according to the strategy detailed earlier.[1a] In addition to the two hydrophilic amino-acid residues, serne and threonine, Met(O) derivatives are usually very water-soluble compounds. Therefore, we decided to oxidize methionine at the final stage of the synthesis, instead of starting with Z(OMe)-



SCHEME 3 Synthetic scheme for the protected tripeptide hydrazide, Z(OMe)-(RNase 80—82)-$NHNH_2$ (11)

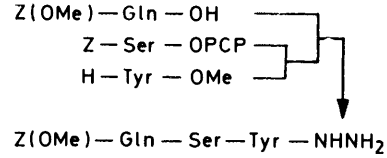


SCHEME 4 Synthetic scheme for the protected tripeptide hydrazide, Z(OMe)-(RNase 77—79)-$NHNH_2$ (12)

Met(O)-OMe. The tripeptide ester Z(OMe)-Ser-Thr-Met-OMe, prepared using available active esters, Z(OMe)-Thr-OPCP and Z(OMe)-Ser-OPCP, as shown in Scheme 4, was exposed to the action of tetrachloroauric(III) acid according to the method of Bordignon *et al.*[18] Oxidation with hydrogen peroxide [17] is known to yield Met(O) slightly contaminated with sulphone; the superior oxidant sodium perborate [19] was not available at this time. We were interested in this reagent, which stereospecifically oxidizes the sulphur atom of methionine. T.l.c. showed the reaction to be seemingly complete within 3 h at 40 °C. However, because of its high solubility in water, we were not able to isolate the desired compound in an analytically pure form. The crude product contaminated with some inorganic salt was converted into the corresponding analytically pure hydrazide (12).

The next fragment, Z(OMe)-Gln-Ser-Tyr-$NHNH_2$ (13) (positions 74—76), was prepared starting from the known dipeptide ester Z-Ser-Tyr-OMe,[20] which after hydrogenolysis was condensed, in an ice-bath, with Z(OMe)-Gln-OH by DCC in the presence of HOBT. The purified tripeptide, Z(OMe)-Gln-Ser-Tyr-OMe, free from any dehydro-derivative,[21] was converted into (13) in the usual manner (Scheme 5).

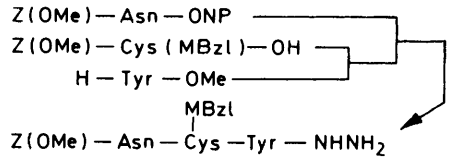
Next, we attempted to introduce as one unit the



SCHEME 5 Synthetic scheme for the protected tripeptide hydrazide, Z(OMe)-(RNase 74—76)-$NHNH_2$ (13)

sequence 69—73 by condensation of Z(OMe)-Gln-Thr-$NHNH_2$ (15) (positions 69—70) with H-Asn-Cys(MBzl)-Tyr-OMe. However the dipeptide hydrazide (15) was found to have low solubility in DMF. Thus, we decided to use two building blocks in a stepwise manner.

Z(OMe)-Asn-Cys(MBzl)-Tyr-$NHNH_2$ (14) (positions 71—73) was prepared according to Scheme 6. Z(OMe)-Cys(MBzl)-Tyr-OMe, prepared by the DCC condensation procedure as usual, was, after treatment with TFA, allowed to react with Z(OMe)-Asn-ONP. The resulting tripeptide ester was converted into (14) as usual.



SCHEME 6 Synthetic scheme for the protected tripeptide hydrazide, Z(OMe)-(RNase 71—73)-$NHNH_2$ (14)

In order to synthesize Z(OMe)-(RNase 69—124)-OBzl, the six fragments thus obtained were assembled according to Scheme 1. Each azide condensation was performed with a 2.5—3.5-fold excess of the azide component in, most instances, the solvent system DMSO–DMF. Compared with the condensations described in the preceding paper,[1] the amount of the azide was increased to bring every condensation to completion. After repeated precipitation from DMSO with methanol, the desired pure (t.l.c.) products were isolated; their homogeneities were assessed at each step by amino-acid analysis, using the recovery of phenylalanine as standard (Table 1).

TABLE 1

Amino-acid ratios of Z(OMe)-(RNase 69—124)-OBzl and intermediates

Position	83—124	80—124	77—124	74—124	71—124	69—124
Residue	(42) *	(45)	(48)	(51)	(54)	(56)
Asp	5.21(5)	5.18(5)	5.14(5)	5.09(5)	6.11(6)	6.14(6)
Thr	2.76(3)	3.90(4)	4.87(5)	4.86(5)	4.83(5)	5.76(6)
Ser	2.47(3)	3.01(4)	3.92(5)	4.50(6)	4.48(6)	4.51(6)
Glu	3.12(3)	3.01(3)	3.06(3)	4.10(4)	4.09(4)	5.13(5)
Pro	2.90(3)	2.79(3)	2.81(3)	2.77(3)	2.79(3)	2.76(3)
Gly	2.22(2)	2.14(2)	2.16(2)	2.11(2)	2.13(2)	2.17(2)
Ala	4.14(4)	3.99(4)	3.94(4)	3.91(4)	3.96(4)	3.98(4)
Val	3.94(4)	3.88(4)	3.88(4)	3.89(4)	3.90(4)	3.91(4)
Met†			0.67(1)	0.64(1)	0.69(1)	0.67(1)
Ile	2.08(2)	2.41(3)	2.39(3)	2.47(3)	2.42(3)	2.44(3)
Tyr	2.72(3)	3.21(3)	3.25(3)	4.34(4)	5.31(5)	5.33(5)
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)
Lys	2.93(3)	3.18(3)	3.20(3)	3.19(3)	3.22(3)	3.24(3)
His	2.12(2)	1.71(2)	1.69(2)	1.73(2)	1.72(2)	1.74(2)
Arg	1.01(1)	0.90(1)	0.92(1)	0.91(1)	0.90(1)	0.89(1)
Cys	(3)	(3)	(3)	(3)	(4)	(4)
Recovery (%)	89	91	87	87	89	88

* Hydrolysis for 72 h. † Met(O) was not calculated.

We must mention that some problems were encountered in removing the excess of unchanged azide component. Estimation of purity by amino-acid analysis is often difficult, as was found in the case of fragments (11) and (12). Serine and threonine decomposed to some extent during the acid hydrolysis and Met(O) was mostly converted into methionine. Isoleucine (3 mol) gave a low recovery, because of the presence of the Ile–Ile bond (position 106–107), which resisted even hydrolysis for 72 h.[22,23] Thus, after these two coupling steps, the ratios of these newly incorporated amino-acids to phenylalanine were uncertain. When our preliminary run was carried out, we noticed that after the two successive condensations of (13) and (14), the recoveries of aspartic acid, tyrosine, and glutamic acid were higher than those predicted by theory, as was that of threonine after condensation of (15). Each product exhibited some tailing on t.l.c. Simple precipitation procedures seemed hopeless for yielding pure compounds. Gel-filtration on Sephadex LH-20 or 60 had been previously shown to be a useful tool for purification of protected peptides, but, as the molecular weight of our peptides increased, as was the case for Z(OMe)-(RNase 69—124)-OBzl, LH-20 or 60 proved ineffective. Galpin *et al.*[24] have recommended the use of Enzacryl K-2 (DMF or NMP) and Sephadex G-50 (HMPA–5% H_2O) for larger peptides. In the present studies, we found that partial resolution of a contaminant could be achieved by the use of Sepharose CL-6B (DMSO–5% H_2O), or, even better, Sephacryl S-200 (NMP–5% H_2O) (Figure). Acid hydrolysis of the main product obtained, using the latter system, gave amino-acid ratios identical to those predicted by theory; the material obtained from the side-peak revealed the presence of amino-acids consisting predominantly of positions 69 to 82 (Table 2).

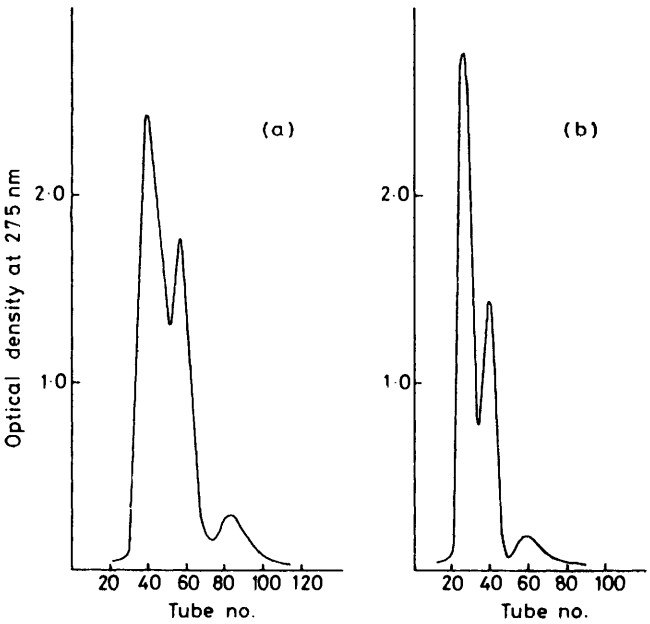


FIGURE Gel-filtration of the crude sample of Z(OMe)-(RNase 69—124)-OBzl on (a) Sepharose CL-6B and (b) Sephacryl S-200

The result indicated that such contamination was initiated by successive chain elongation of the Ser-Ile-Thr unit (11), which was not removed completely during the purification of Z(OMe)-(RNase 80—124)-OBzl. It showed that even a small azide can occasionally give a rearrangement product which is difficult to remove by a single precipitation procedure.

Because of these observations in our preliminary run, the next preparative runs were performed with particular

TABLE 2

Amino-acid analysis of Z(OMe)-(RNase 69—124)-OBzl purified by gel-filtration and crude intermediates

Position	80—124	77—124	74—124	71—124	69—124	Sepharose CL-6B		Sephacryl S-200	
Residue	(45)	(48)	(51)	(54)	(56)	Peak 1	Peak 2	Peak 1	Peak 2
Asp	5.20(5)	5.10(5)	4.89(5)	6.24(6)	6.47(6)	6.18	11.52	6.21	12.64
Thr	3.55(4)	5.02(5)	5.00(5)	5.00(5)	6.42(6)	5.62	18.31	5.79	19.25
Ser	3.12(4)	4.69(5)	5.76(6)	5.27(6)	5.36(6)	4.42	10.76	4.46	13.93
Glu	3.31(3)	3.26(3)	4.96(4)	4.77(4)	6.09(5)	5.06	17.35	5.02	19.30
Pro	2.96(3)	2.98(3)	2.80(3)	2.86(3)	2.69(3)	2.69	2.37	2.71	2.47
Gly	2.32(2)	2.22(2)	2.17(2)	2.16(2)	2.11(2)	2.13	1.78	2.11	1.96
Ala	4.26(4)	4.07(4)	3.97(4)	3.90(4)	3.76(4)	4.05	3.65	4.06	3.76
Val	4.02(4)	3.69(4)	3.49(4)	3.47(4)	3.57(4)	3.91	3.49	3.88	3.74
Met		1.08(1)	0.94(1)	0.94(1)	0.92(1)	0.72	4.79	0.69	4.98
Ile	2.85(3)	2.68(3)	2.62(3)	2.68(3)	2.53(3)	2.51	2.52	2.49	2.32
Tyr	2.90(3)	2.73(3)	4.04(4)	5.17(5)	5.41(5)	5.23	17.09	5.21	18.00
Phe	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00(1)	1.00	1.00	1.00	1.00
Lys	2.86(3)	3.21(3)	2.75(3)	2.85(3)	2.82(3)	2.99	2.76	3.17	2.97
His	1.85(2)	1.89(2)	1.62(2)	1.70(2)	1.68(2)	1.72	1.49	1.69	1.54
Arg	1.03(1)	1.06(1)	1.07(1)	1.07(1)	0.97(1)	0.97	1.07	0.91	1.02
Cys	(3)	(3)	(3)	(4)	(4)				
Recovery (%)	83	85	89	88	88	89		89	

care being taken when purifying Z(OMe)-(RNase 80—124)-OBzl, as well as Z(OMe)-(RNase 77—124)-OBzl, taking the isoleucine recovery of the purified sample of Z(OMe)-(RNase 69—124)-OBzl (Ile/Phe = 2.4, after hydrolysis for 48 h) as a marker. Although a sharp single spot on t.l.c. in the solvent system Bu^nOH–AcOH–AcOEt–H_2O (1 : 1 : 1 : 1, v/v) is still one of the guides for routine assessment of the purity of protected intermediates, we decided to examine the purity by, in addition to amino-acid analysis, column chromatography on Sephacryl S-200 at the stage of Z(OMe)-(RNase 69—124)-OBzl. This is a good check-point for establishment of the purity of the synthetic peptides being used in successive condensations at the C-terminal end. A tool for the purification of relatively large protected peptides was thus now available, for use when discrepancies between the observed and theoretical values of amino-acid compositions were noted. Thus, we were able with confidence to prepare 19 g of Z(OMe)-(RNase 69—124)-OBzl having a high degree of homogeneity.

EXPERIMENTAL

General experimental procedures were described in Part 1.[1a] The N^{α}-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 by the method of Honzl and Rudinger.[3]

Z(OMe)-Thr-Gly-OH.—Z(OMe)-Thr-OPCP (53.16 g, 0.1 mol) dissolved in THF (250 ml) was added to a stirred solution of Gly (22.52 g, 0.3 mol) in H_2O (150 ml) containing Et_3N (48 ml, 0.35 mol). After 24 h, the solvent was evaporated off and the product was isolated by procedure C. Recrystallization from AcOEt–ether gave the *protected dipeptide* (14.91 g, 44%), m.p. 113—115 °C, $[\alpha]_D^{23}$ +7.6° (*c*, 0.8 in DMF), R_{F_1} 0.29 (Found: C, 52.65; H, 5.9; N, 8.1. $C_{15}H_{20}N_2O_7$ requires C, 52.93; H, 5.92; N, 8.23%).

Z(OMe)-Glu(OBzl)-Thr-Gly-OH.—Z(OMe)-Thr-Gly-OH (28.80 g, 85 mmol) in MeOH (100 ml) containing a few drops of AcOH was hydrogenated over Pd for 8 h. The filtered solution was concentrated and the residue was recrystallized from H_2O–MeOH to give H-Thr-Gly-OH (14.02 g, 94%). This dipeptide (6.34 g, 36 mmol) was dissolved in H_2O (50 ml) containing Et_3N (9.9 ml, 72 mmol), and a solution of Z(OMe)-Glu(OBzl)-ONP (18.81 g, 36 mmol) in THF (150 ml) was added. After stirring for 48 h, the mixture was concentrated and the residue was washed with 5% citric acid and H_2O and then recrystallized from MeOH–AcOEt to yield the *protected tripeptide* (17.91 g, 89%), m.p. 122—124 °C, $[\alpha]_D^{22}$ +1.5° (*c*, 0.7 in DMF), R_{F_1} 0.25 (Found: C, 57.9; H, 5.9; N, 7.65. $C_{27}H_{33}N_3O_{10}$ requires C, 57.95; H, 5.94; N, 7.51%).

Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc.—DCC (2.47 g, 12 mmol) was added to a stirred mixture of Z(OMe)-Glu(OBzl)-Thr-Gly-OH (5.60 g, 10 mmol), Troc-$NHNH_2$ (2.49 g, 12 mmol), and HOBT (1.62 g, 12 mmol) in DMF (50 ml). After 48 h, the solution was filtered, the filtrate was concentrated, and the residue was purified by procedure A. Trituration with ether followed by recrystallization from AcOEt–ether gave the *substituted hydrazide* (7.30 g, 97%), m.p. 87—89 °C, $[\alpha]_D^{23}$ −2.4° (*c*, 0.4 in DMF), R_{F_1} 0.62 (Found: C, 48.15; H, 5.0; N, 9.3. $C_{30}H_{36}Cl_3N_5O_{11}$ requires C, 48.10; H, 4.84; N, 9.35%).

Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc.—Z(OMe)-Glu(OBzl)-Thr-Gly-NHNH-Troc (10.74 g, 14 mmol) was treated with a mixture of TFA (21 ml) and anisole (5.2 ml) as usual, then dry ether was added. The resulting powder was dissolved in 4.2N-HCl–dioxan (3.3 ml, 14 mmol), the solvent was evaporated off, and the residue was again treated with ether to afford HCl·H-Glu(OBzl)-Thr-Gly-NHNH-Troc as a powder, which was dissolved in DMF (40 ml) containing Et_3N (3.9 ml, 28 mmol). DCC (3.17 g, 15 mmol) was added to a stirred solution of Z(OMe)-Arg(MBS)-OH (7.83 g, 15 mmol) and DNP-OH (2.84 g, 15 mmol), in AcOEt (20 ml). After 5 h, the solution was filtered, the filtrate was added to the above solution containing the tripeptide, and the mixture, after stirring for 48 h, was concentrated. The product was isolated by procedure B. Recrystallization from MeOH–ether afforded the *protected tetrapeptide* (12.14 g, 81%), m.p. 108—110 °C, $[\alpha]_D^{22}$ −2.2° (*c*, 0.5 in DMF), R_{F_1} 0.61 (Found: C, 47.9; H, 4.9; N, 11.7. $C_{43}H_{54}Cl_3N_9O_{15}S$ requires C, 48.02; H, 5.06; N, 11.72%).

Z(OMe)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc.—Z(OMe)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc (5.38 g, 5 mmol) was treated with a mixture of TFA (11 ml) and anisole (2.7 ml) as usual and dry ether was added. The resulting powder was collected by filtration and dissolved in DMF (50 ml) together with Et_3N (1.4 ml,

10 mmol) and Z(OMe)-Cys(MBzl)-ONP (2.63 g, 5 mmol). After stirring for 48 h, the solution was concentrated and the product was isolated by procedure B. Recrystallization from MeOH–ether afforded the *protected pentapeptide* (5.76 g, 89%), m.p. 103—105 °C, $[\alpha]_D^{22}$ −9.2° (*c*, 0.4 in DMF), R_{F_1} 0.60 (Found: C, 49.9; H, 5.1; N, 10.85. $C_{54}H_{67}Cl_3N_{10}O_{17}S_2$ requires C, 49.94; H, 5.20; N, 10.79%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH-Troc (10).—The above protected pentapeptide (6.49 g, 5 mmol) was treated with a mixture of TFA (13 ml) and anisole (3.2 ml) as usual, then dry ether was added. The resulting powder was dissolved in DMF (35 ml) together with Et_3N (1.4 ml, 10 mmol) and Z(OMe)-Asp(OBzl)-ONP (2.80 g, 5.5 mmol). After stirring for 48 h, the solution was concentrated, and the product was isolated by procedure B. Recrystallization from MeOH–ether afforded the *protected hexapeptide* (6.25 g, 83%), m.p. 140—142 °C, $[\alpha]_D^{22}$ −10.4° (*c*, 0.5 in DMF), R_{F_1} 0.64 (Found: C, 51.65; H, 5.3; N, 10.05. $C_{65}H_{78}Cl_3N_{11}O_{20}S_2$ requires C, 51.91; H, 5.23; N, 10.25%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH$_2$, *Z(OMe)-(RNase* 83—88)-*NHNH$_2$*.—The above protected hexapeptide (9.02 g, 6 mmol) in AcOH (90 ml) was treated with Zn powder (4.8 g, 12 mol equiv.) for 48 h. The filtered solution was concentrated and the residue was treated with a saturated solution of EDTA and 5% Na_2CO_3. The resulting gelatinous mass was washed with H_2O and recrystallized from MeOH to give the *protected hexapeptide hydrazide* (5.40 g, 68%), m.p. 130—133 °C, $[\alpha]_D^{22}$ −8.0° (*c*, 0.4 in DMSO), R_{F_1} 0.55. Amino-acid analysis: Asp 1.04, Arg 1.09, Glu 1.05, Thr 1.01, Gly 1.00 (average recovery 90%) (Found: C, 55.55; H, 5.85; N, 11.35. $C_{62}H_{77}N_{13}O_{18}S_2 \cdot H_2O$ requires C, 55.30; H, 5.91; N, 11.44%).

Z(OMe)-Ser-Ile-Thr-OMe.—Z-Ile-Thr-OMe [16] (30.40 g, 80 mmol) in THF (200 ml) was hydrogenated over Pd in the presence of 1N-HCl (80 ml, 1 mol equiv.) for 8 h. The catalyst was removed by filtration, the filtrate was concentrated and the residue was dissolved in DMF (300 ml) together with Et_3N (22.1 ml, 0.16 mol) and Z(OMe)-Ser-OPCP (45.55 g, 88 mmol). After stirring for 48 h, the solution was concentrated and the product was isolated by procedure B. Recrystallization from MeOH–AcOEt afforded the *protected tripeptide ester* (28.76 g, 72%), m.p. 189—191 °C, $[\alpha]_D^{23}$ +3.6° (*c*, 0.8 in DMF), R_{F_1} 0.43 (Found: C, 55.55; H, 7.15; N, 8.45. $C_{23}H_{35}N_3O_9$ requires C, 55.52; H, 7.09; N, 8.45%).

Z(OMe)-Ser-Ile-Thr-NHNH$_2$, *Z(OMe)-(RNase* 80—82)-*NHNH$_2$* (11).—The above protected tripeptide ester (10.40 g, 21 mmol) in DMF–MeOH (100 ml; 4 : 1 v/v) was treated with 80% hydrazine hydrate (11 ml, 13 mol equiv.) overnight. Precipitation of the resulting mass from DMF with MeOH afforded the *hydrazide* (8.11 g, 78%), m.p. 240—242 °C, $[\alpha]_D^{22}$ −1.3° (*c*, 0.8 in DMSO), R_{F_1}, 0.52. Amino-acid analysis: Ser 0.74, Ile 1.00, Thr 0.91 (average recovery 77%) (Found: C, 52.9; H, 6.9; N, 13.8. $C_{22}H_{35}N_5O_8$ requires C, 53.11; H, 7.09; N, 14.08%).

Z(OMe)-Thr-Met-OMe.—Z(OMe)-Thr-OPCP (79.65 g, 0.15 mol) was added to a stirred solution of H-Met-OMe [from the hydrochloride (30.0 g, 0.15 mol) with Et_3N (20.7 ml, 0.15 mol)] in DMF (300 ml). After 48 h, the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH–AcOEt to give the *protected dieptide ester* (37.23 g, 58%), m.p. 111—112 °C, $[\alpha]_D^{22}$ −5.2° (*c*, 1.0 in DMF), R_{F_1} 0.77 (Found: C, 53.0; H, 6.3; N, 6.3. $C_{19}H_{28}N_2O_7S$ requires C, 53.25; H, 6.59; N, 6.54%).

Z(OMe)-Ser-Thr-Met-OMe.—Z(OMe)-Thr-Met-OMe (38.02 g, 89 mmol) was treated with TFA–anisole (68 ml; 25 : 9 v/v) as usual, then the excess of TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (300 ml) containing Et_3N (24.8 ml, 0.18 mol) and Z(OMe)-Ser-OPCP (46.03 g, 89 mmol). After stirring for 48 h the solution was concentrated and the residue was purified by procedure B followed by recrystallization from MeOH–AcOEt to give the *protected tripeptide ester* (37.71 g, 82%), m.p. 148—150 °C, $[\alpha]_D^{23}$ −6.6° (*c*, 1.1 in DMF), R_{F_1} 0.77 (Found: C, 49.3; H, 6.5; N, 7.7. $C_{22}H_{33}N_3O_9S \cdot H_2O$ requires C, 49.52; H, 6.61; N, 7.88%).

Z(OMe)-Ser-Thr-Met(O)-NHNH$_2$, *Z(OMe)-(RNase* 77—79)-*NHNH$_2$* (12).—Z(OMe)-Ser-Thr-Met-OMe (15.04 g, 29 mmol) in DMF–MeOH (72 ml; 1 : 5 v/v) was treated with $HAuCl_4 \cdot 4H_2O$ (13.22 g, 32 mmol) in H_2O in the presence of $NaHCO_3$ (7.36 g, 3 mol equiv.) at 40 °C for 3 h. After filtration, the filtrate was concentrated and the residue was treated with AcOEt. Recrystallization of the resulting powder from MeOH–EtOH (×3) afforded the sulphoxide contaminated with a small amount of inorganic salts (11.49 g, 74%), m.p. 161—163 °C, R_{F_1} 0.57. This sulphoxide (22 mmol) in MeOH (110 ml) was treated with 80% hydrazine hydrate (6.9 ml, 5 mol equiv.) overnight. Precipitation of the resulting mass from DMF with MeOH (×2) gave the *protected tripeptide hydrazide* (10.89 g, 95%), m.p. 183—189 °C, $[\alpha]_D^{23}$ +3.5° (*c*, 0.9 in DMSO), R_{F_1} 0.47. Amino-acid analysis: Ser 0.91, Thr 1.00, Met 0.80 (average recovery 80%) (Found: C, 46.9; H, 6.5; N, 13.05. $C_{21}H_{33}N_5O_9S \cdot 1/2H_2O$ requires C, 46.65; H, 6.34; N, 12.96%).

Z(OMe)-Gln-Ser-Tyr-OMe.—Z-Ser-Tyr-OMe [20] (29.24 g, 70 mmol) in THF (240 ml) was hydrogenated over Pd in the presence of 1N-HCl (70 ml, 1 mol equiv.) for 8 h. The catalyst was filtered off, the filtrate was concentrated, and the residue was dissolved in DMF (200 ml). To this ice-chilled solution, Et_3N (9.7 ml, 70 mmol), Z(OMe)-Gln-OH (21.72 g, 70 mmol), HOBT (9.45 g, 70 mmol), and DCC (15.86 g, 77 mmol) were successively added. The mixture was stirred in an ice-bath for 48 h and the filtered solution was concentrated. Treatment of the residue with AcOEt afforded a powder, which was purified by procedure B followed by recrystallization from MeOH to afford the *protected tripeptide ester* (27.44 g, 68%), m.p. 206—209 °C, $[\alpha]_D^{23}$ +8.8° (*c*, 0.9 in DMF), R_{F_1} 0.64. No CN bond vibration was observed in the i.r. spectrum (Found: C, 56.5; H, 5.95; N, 9.7. $C_{27}H_{34}N_4O_{10}$ requires C, 56.44; H, 5.96; N, 9.75%).

Z(OMe)-Gln-Ser-Tyr-NHNH$_2$, *Z(OMe)-(RNase* 74—76)-*NHNH$_2$* (13).—Z(OMe)-Gln-Ser-Tyr-OMe (20.20 g, 35 mmol) in DMF–MeOH (120 ml; 1 : 2 v/v) was treated with 80% hydrazine hydrate (14 ml, 6.4 mol equiv.) overnight. The resulting mass was precipitated from DMF with MeOH to yield the *hydrazide* (17.72 g, 88%), m.p. 231—234 °C, $[\alpha]_D^{22}$ +5.5° (*c*, 0.9 in .DMSO), R_{F_1} 0.21. Amino-acid analysis: Glu 1.00, Ser 0.86, Tyr 0.95 (average recovery 99%) (Found: C, 54.35; H, 5.8; N, 14.4. $C_{36}H_{34}N_6O_9$ requires C, 54.35; H, 5.96; N, 14.63%).

Z(OMe)-Cys(MBzl)-Tyr-OMe.—DCC (22.66 g, 0.11 mol) was added to a mixture of Z(OMe)-Cys(MBzl)-OH (40.54 g, 0.1 mol) and H-Tyr-OMe [from the hydrochloride (23.17 g, 0.1 mol) with Et_3N (13.8 ml, 0.1 mol)] in DMF (400 ml) and the mixture was stirred for 48 h. The solution was filtered,

the filtrate was concentrated, and the residue was purified by procedure A followed by recrystallization from MeOH to afford the *protected tripeptide ester* (45.51 g, 78%), m.p. 141—143 °C, $[\alpha]_D^{23}$ −26.8° (*c*, 1.0 in DMF), R_{F_1} 0.80 (Found: C, 61.8; H, 6.05; N, 4.75. $C_{30}H_{34}N_2O_8S$ requires C, 61.84; H, 5.88; N, 4.80%).

Z(OMe)-Asn-Cys(MBzl)-Tyr-OMe.—Z(OMe)-Cys(MBzl)-Tyr-OMe (58.26 g, 0.1 mol) was treated with TFA–anisole (110 ml; 8 : 3 v/v) as usual, then the excess of TFA was removed by evaporation. The oily residue was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMF (400 ml) together with Et_3N (13.8 ml, 0.1 mol) and Z(OMe)-Asn-ONP (41.73 g, 0.1 mol). After stirring for 48 h, the solution was concentrated. Treatment of the residue with AcOEt afforded a fine powder, which was purified by procedure B followed by recrystallization from dioxan–MeOH to give the *protected tripeptide ester* (45.79 g, 66%), m.p. 178—181 °C, $[\alpha]_D^{23}$ −27.5° (*c*, 0.7 in DMF), R_{F_1} 0.65 (Found: C, 57.95; H, 5.85; N, 7.9. $C_{34}H_{40}N_4O_{10}S\cdot 1/2H_2O$ requires C, 57.86; H, 5.86; N, 7.94%).

Z(OMe)-Asn-Cys(MBzl)-Tyr-NHNH$_2$, Z(OMe)-(RNase 71—73)-NHNH$_2$(14).—Z(OMe)-Asn-Cys(MBzl)-Tyr-OMe (6.97 g, 10 mmol) dissolved in DMF (60 ml) was treated with 80% hydrazine hydrate (3.2 ml, 5 mol equiv.) overnight. The resulting gelatinous mass was precipitated from DMF with MeOH to yield the *hydrazide* (5.77 g, 83%), m.p. 243—247 °C, $[\alpha]_D^{23}$ −19.8° (*c*, 1.1 in DMSO), R_{F_1} 0.42. Amino-acid analysis: Asp 1.00, Tyr 0.98 (average recovery 81%) (Found: C, 56.65; H, 6.05; N, 11.95. $C_{33}H_{40}N_6O_9S$ requires C, 56.88; H, 5.70; N, 12.06%).

Z(OMe)-Gln-Thr-OMe.—DCC (20.60 g, 0.1 mol) was added to an ice-chilled solution of Z(OMe)-Gln-OH (31.03 g, 0.1 mol), HOBT (13.40 g, 0.1 mol), and H-Thr-OMe [from the hydrochloride (16.81 g, 0.1 mol) with Et_3N (13.8 ml, 0.1 mol)] in DMF (300 ml). After stirring in an ice-bath for 48 h, the solution was filtered, the filtrate was concentrated, and the residue was treated with ether. The resulting powder was purified by procedure B followed by recrystallization from MeOH to yield the *dipeptide ester* (17.44 g, 41%), m.p. 175—177 °C, $[\alpha]_D^{22}$ +4.0° (*c*, 0.5 in DMF), R_{F_1} 0.76 (Found: C, 53.7; H, 6.35; N, 10.0. $C_{19}H_{27}N_3O_8$ requires C, 53.64; H, 6.40; N, 9.88%).

Z(OMe)-Gln-Thr-NHNH$_2$, Z(OMe)-(RNase 69—70)-NHNH$_2$ (15).—Z(OMe)-Gln-Thr-OMe (16.0 g, 38 mmol) in DMF–MeOH (200 ml; 1 : 1 v/v) was treated with 80% hydrazine hydrate (12.2 ml, 5 mol equiv.). The gelatinous mass formed on standing overnight was precipitated from DMF with MeOH to yield the *hydrazide* (13.20 g, 83%), m.p. 234—237 °C, $[\alpha]_D^{22}$ +5.2° (*c*, 0.6 in DMSO), R_{F_1} 0.44. Amino-acid analysis: Glu 1.00, Thr 0.86 (average recovery 82%) (Found: C, 50.7; H, 6.3; N, 16.55. $C_{18}H_{27}N_5O_7$ requires C, 50.81; H, 6.40; N, 16.46%).

Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-Ser-Ser-Lys(Z)-Tyr-Pro-Asn-Cys(MBzl)-Ala-Tyr-Lys(Z)-Thr-Thr-Gln-Ala-Asn-Lys(Z)-His-Ile-Ile-Val-Ala-Cys(MBzl)-Glu(OBzl)-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe-Asp(OBzl)-Ala-Ser-Val-OBzl [Abbreviated as Z(OMe)-(RNase 83—124)-OBzl or Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-(89—124)-OBzl].—Z(OMe)-(RNase 89—124)-OBzl (14.38 g, 2.79 mmol) was treated with TFA–anisole (75 ml; 4 : 1 v/v) as usual, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h, and dissolved in DMSO–DMF (100 ml; 1 : 1 v/v) containing Et_3N (1.16 ml, 8.37 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Asp(OBzl)-Cys(MBzl)-Arg(MBS)-Glu(OBzl)-Thr-Gly-NHNH$_2$ (9.37 g, 6.98 mmol)] in DMF (50 ml) and Et_3N (0.96 ml, 6.96 mmol) and the mixture was stirred at 4 °C for 48 h. The solvent was evaporated off and the product was purified by procedure B followed by precipitation from DMSO with MeOH (×4) to give the *protected dotetracontapeptide ester* (14.35 g, 81%), m.p. 273 °C (decomp.), $[\alpha]_D^{23}$ −29.5° (*c*, 1.0 in DMSO), R_{F_3} 0.69 (Found: C, 56.6; H, 6.4; N, 12.65. $C_{300}H_{394}N_{56}O_{79}S_4\cdot 9H_2O$ requires C, 56.84; H, 6.55; N, 12.37%).

Z(OMe)-(RNase 80—124)-OBzl [Z(OMe)-Ser-Ile-Thr-(83—124)-OBzl].—Z(OMe)-(RNase 83—124)-OBzl (11.16 g, 1.76 mmol) was treated with TFA–anisole (60 ml; 5 : 1 v/v) and the N$^{\alpha}$-deprotected peptide isolated as mentioned above was dissolved in DMSO–DMF (1 : 1 v/v; 100 ml) containing Et_3N (0.73 ml, 5.28 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Ser-Ile-Thr-NHNH$_2$ (2.19 g, 4.40 mmol)] in DMF (20 ml) and Et_3N (0.61 ml, 4.40 mmol). After 48 h, the solvent was evaporated off and the product was purified by procedure B followed by precipitation from DMSO with MeOH (×3) to yield the *protected pentatetracontapeptide ester* (10.23 g, 87%), m.p. 252 °C (decomp.), $[\alpha]_D^{23}$ − 32.6° (*c*, − 0.9 in DMSO), R_{F_3} 0.84 (Found: C, 55.8; H, 6.55; N, 12.7. $C_{313}H_{417}N_{59}O_{84}S_4\cdot 13H_2O$ requires C, 56.00; H, 6.55; N, 12.31%).

Z(OMe)-(RNase 77—124)-OBzl [Z(OMe)-Ser-Thr-Met(O)-(80—124)-OBzl].—Z(OMe)-(RNase 80—124)-OBzl (9.41 g, 1.40 mmol) was treated with TFA–anisole (60 ml; 5 : 1 v/v) and the N$^{\alpha}$-deprotected peptide, isolated as above, was dissolved in DMSO–DMF (2 : 1 v/v; 100 ml) containing Et_3N (0.58 ml, 4.20 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Ser-Thr-Met(O)-NHNH$_2$ (1.89 g, 3.50 mmol)] in DMF (15 ml) and Et_3N (0.48 ml, 3.50 mmol). After 48 h, the solvent was evaporated off and the residue was purified by procedure B followed by precipitation from DMSO with MeOH (×2) to yield the *protected octatetracontapeptide ester* (9.44 g, 96%), m.p. 257 °C (decomp.), $[\alpha]_D^{23}$ −23.8° (*c*, 0.7 in DMSO), R_{F_3} 0.86 (Found: C, 55.0; H, 6.45; N, 12.9. $C_{325}H_{438}N_{62}O_{90}S_5\cdot 13H_2O$ requires C, 55.38; H, 6.45; N, 12.32%).

Z(OMe)-(RNase 74—124)-OBzl [Z(OMe)-Gln-Ser-Tyr-(77—124)-OBzl].—Z(OMe)-(RNase 77—124)-OBzl (9.44 g, 1.34 mmol) was treated with TFA–anisole (60 ml; 5 : 1 v/v) and the N$^{\alpha}$-deprotected peptide isolated as above was dissolved in DMSO–DMF (1 : 1 v/v; 100 ml) containing Et_3N (0.55 ml, 4.02 mmol). To this ice-chilled solution the azide [from Z(OMe)-Gln-Ser-Tyr-NHNH$_2$ (2.31 g, 4.02 mmol)] in DMF (20 ml) and Et_3N (0.55 ml, 4.02 mmol) were added. After 48 h, the solvent was evaporated off and the residue was purified by procedure B followed by precipitation from DMSO with MeOH (×5) to yield the *protected henpentacontapeptide ester* (9.25 g, 92%), m.p. 246 °C (decomp.), $[\alpha]_D^{23}$ −28.5° (*c*, 0.8 in DMSO), R_{F_3} 0.81, R_{F_5} 0.43 (Found: C, 54.5; H, 6.35; N, 12.5. $C_{325}H_{438}N_{66}O_{96}S_5\cdot 18H_2O$ requires C, 54.64; H, 6.41; N, 12.30%).

Z(OMe)-(RNase 71—124)-OBzl [Z(OMe)-Asn-Cys-(MBzl)-Tyr-(74—124)-OBzl].—Z(OMe)-(RNase 74—124)-OBzl (9.25 g, 1.23 mmol) was treated with TFA–anisole (60 ml; 5 : 1 v/v) and the N$^{\alpha}$-deprotected peptide, isolated as above, was dissolved in DMF–NMP (1 : 2 v/v 100 ml) containing Et_3N (0.41 ml, 3.69 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Asn-Cys-(MBzl)-Tyr-NHNH$_2$ (2.15 g, 3.08 mmol)] in DMF (15 ml) and Et_3N (0.43 ml, 3.08 mmol). After stirring for 48 h,

the solution was concentrated and the residue was purified by procedure B followed by precipitation from DMSO–MeOH (×3) to yield the *protected tetrapentacontapeptide ester* (9.04 g, 92%), m.p. 253 °C (decomp.), $[\alpha]_D^{23}$ −26.8° (*c*, 0.9 in DMSO), R_{F_3} 0.78 (Found: C, 54.7; H, 6.35; N, 12.65. $C_{366}H_{488}N_{70}O_{102}S_6 \cdot 17H_2O$ requires C, 54.95; H, 6.36; N, 12.26%).

Z(OMe)-(RNase 69—124)*-OBzl* [*Z(OMe)-Gln-Thr-*(71—124)*-OBzl*].—Z(OMe)-(RNase 71—124)-OBzl (9.04 g, 1.18 mmol) was treated with TFA–anisole (54 ml; 9 : 1 v/v) and the N^{α}-deprotected peptide, isolated as above, was dissolved in DMSO–DMF (2 : 1 v/v, 90 ml) containing Et_3N (0.49 ml, 3.54 mmol). To this ice-chilled solution were added the azide [from Z(OMe)-Gln-Thr-$NHNH_2$ (1.26 g, 2.95 mmol)] in DMF (10 ml) and Et_3N (0.41 ml, 2.95 mmol). After 48 h, the solvent was removed by evaporation and the residue was purified by procedure B followed by precipitation from DMSO–MeOH (×3) to yield the *protected hexapentacontapeptide ester* (8.74 g, 94%), m.p. 238 °C (decomp.), $[\alpha]_D^{23}$ −28.4° (*c*, 1.1 in DMSO), R_{F_3} 0.72 (Found: C, 52.75; H, 6.25; N, 12.2. $C_{375}H_{503}N_{73}O_{106}S_6 \cdot 32H_2O$ requires C, 53.00; H, 6.73; N, 12.03%).

Purification of the Crude Sample of Z(OMe)-(RNase 69—124)*-OBzl by Gel-filtration on Sephacryl* S-200.—The first batch of Z(OMe)-(RNase 69—124)-OBzl prepared as above, gave somewhat higher recoveries of Asp, Thr, and Glu than those predicted by theory (Table 2). Such a tendency was also noted in the intermediates Z(OMe)-(RNase 71—124)-OBzl and Z(OMe)-(RNase 74—124)-OBzl, where newly incorporated amino-acid ratios were somewhat higher than expected. The impure samples of Z(OMe)-(RNase 69—124)-OBzl (100 mg each) were submitted to gel-filtration on various supports (2.8 × 130 cm) with DMSO, 5% H_2O–DMSO, or NMP as eluants. The supports used were 1, LH-20 (DMSO); 2, Sephadex G-50 (5% H_2O–DMSO); 3, Sephadex G-100 (5% H_2O–DMSO); 4, Sepharose CL-6B (5% H_2O–DMSO); 5, Sephacryl S-200 (5% H_2O–NMP); 6, Biogel P-2 (for the N^{α}-deprotected peptide with 66% AcOH as eluant). Individual fractions (9.5 ml each) were collected and absorptions at 275 nm were measured. No separation was obtained in experiments 1, 2, 3, and 6. Gel-filtration on Sepharose CL-6B gave some separation (peak 1, tube nos. 35—50, 46 mg; peak 2, tube nos. 56—70, 14 mg) and on Sephacryl S-200 a better separation was achieved (peak 1, tube nos. 24—31, 58 mg; peak 2, tube nos. 36—45, 7 mg) as shown in the Figure. The purity of other batches of Z(OMe)-(RNase 69—124)-OBzl was confirmed by gel-filtration on Sephacryl S-200.

This investigation was supported in part by a grant from the Ministry of Education, Science and Culture, Japan, and by the Yamada Science Foundation (Osaka, Japan).

[9/1717 *Received*, 26*th October*, 1979]

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