[Chem. Pharm. Bull. 32(11)4585—4592(1984)]

Amino Acids and Peptides. XII.[1,2] Synthesis of C-Terminal Decapeptide of Bovine Pancreatic Ribonuclease A (RNase A) and Its Analogs and Determination of Their Ability to Reactivate Des(121—124) RNase A

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(Received June 14, 1984)

A decapeptide corresponding to the C-terminal sequence (residues 115—124) of bovine pancreatic ribonuclease A (RNase A) and four analogs in which Asp-121 is replaced by Asn, Glu, Gln and Ala were synthesized by the fragment condensation procedure, and their abilities to reactivate RNase A from which the last 4 residues, Asp–Ala–Ser–Val, had been removed were examined. Asp-decapeptide (I) and Glu-decapeptide (III) bound with inactivated RNase 1—120 non-covalently and exhibited hydrolytic activity towards cyclic 2′,3′-cytidylic acid. In contrast, Asn-decapeptide (II), Gln-decapeptide (IV) and Ala-decapeptide (V) did not show any ability to restore the activity of RNase A 1—120. Our systematic syntheses of the C-terminal decapeptide of RNase A and its analogs indicate a very important role of the free carboxyl group at position 121 of RNase A for manifestation of RNase activity.

Keywords——bovine pancreatic RNase A; C-terminal decapeptide; Asp-121 replacement; four decapeptide analogs; chemical synthesis; reactivation ability; important carboxyl group; position 121; cyclic 2′,3′-cytidylic acid

Bovine pancreatic ribonuclease A (RNase A) catalyzes the hydrolysis of ribonucleic acid or nucleotide ester by a two-step reaction, transesterification and hydrolysis.[3-5] Although the exact mechanism of the enzyme has still not been fully resolved, it is generally recognized that the side-chains of Gln-11, His-12, Lys-41, Thr-45, His-119 and Asp-121 are closely involved in the enzyme action.[6,7] One interesting discovery with bovine pancreatic RNase A was the observation that the 20-residue S-peptide of the N-terminal section of the molecule containing His at position 12 could be mixed with the inactive 104-residue S-protein of the C-terminal section of the molecule containing His at position 119 to regenerate fully active RNase-S′.[8,9] Further, an active enzyme could be formed by the non-covalent interaction of the synthetic COOH-terminal tetradecapeptide of bovine pancreatic RNase A with an inactive derivative of the enzyme in which the COOH-terminal residue have been removed.[10] The latter finding has opened the way for a systematic study of the role in the enzyme action of individual amino acid residues near the carboxyl end of RNase A. The initial observations showed that inactive RNase 1—120, obtained by pepsin digestion[11,12] and the C-terminal 14-residue peptide, synthesized by the solid phase method[13] (in a ratio of 1 : 3) could combine to generate approximately 30% of the activity of native RNase A with ribonucleic acid (RNA) or cyclic 2′,3′-cytidylic acid as a substrate. It was found that the C-terminal decapeptide, H–Tyr–Val–Pro–Val–His–Phe–Asp–Ala–Ser–Val–OH, was sufficient to reactivate RNase from which the last 4 residues, Asp–Ala–Ser–Val, had been removed.[14]

This report deals with the synthesis of the C-terminal decapeptide and its analogs in which the Asp-121 residue is replaced by Asn, Glu, Gln or Ala, and their ability to restore the

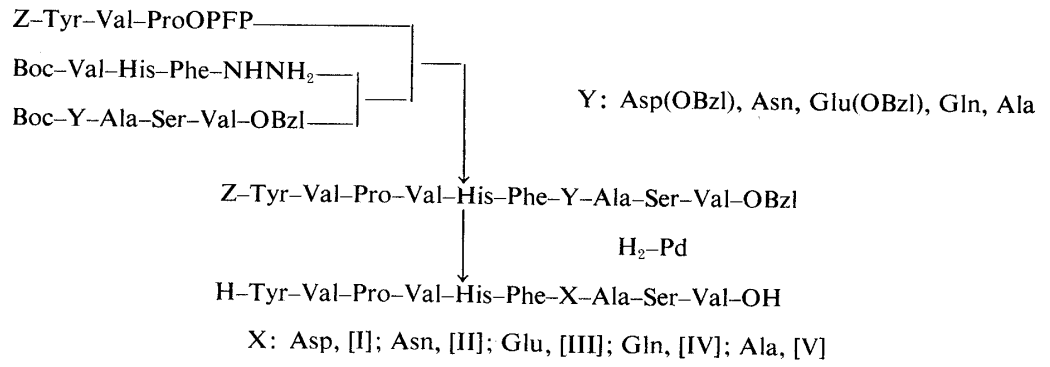

Fig. 1. Synthetic Scheme for the C-Terminal Decapeptide of RNase A [I] and Its Analogs [II—V]

enzymatic activity of RNase A 1—120 towards cyclic 2′,3′-cytidylic acid, in order to determine whether or not the carboxyl group of Asp-121 is essential for manifestation of the enzymatic activity and to determine the effect on the enzymatic activity of elongation of the side chain of the Asp residue by *ca.* 1.5 Å by introduction of a methylene group ($-CH_2-$). Previously, Yajima and Fujii reported the total synthesis of bovine pancreatic RNase A with full activity in a crystalline form.[15-17] With reference to their synthetic route for the C-terminal portion, the C-terminal decapeptide and four kinds of analogs (I—V) were prepared according to the scheme shown in Fig. 1.

The key intermediate in this approach was the protected C-terminal tetrapeptide with replacement of Asp-121 by Asn, Glu, Gln or Ala. These peptide derivatives were synthesized by the *p*-nitrophenyl ester method or DCC method. Boc–Asp(OBzl)–ONp, Boc–Asn–ONp, Boc–Glu(OBzl)–ONp and Boc–Gln–ONp were coupled with H–Ala–Ser–Val–OBzl, which was prepared from Boc–Ala–Ser–Val–OBzl by treating with TFA, to afford Boc–Asp(OBzl)–Ala–Ser–Val–OBzl, Boc–Asn–Ala–Ser–Val–OBzl, Boc–Glu(OBzl)–Ala–Ser–Val–OBzl and Boc–Gln–Ala–Ser–Val–OBzl, respectively. Although active esters were thought to be selective amine acylating agents, they were found to cause O-acylation if base were present.[18] In the *p*-nitrophenyl ester coupling reactions described above, Boc–Glu(OBzl)–ONp reacted with the hydroxyl group of the Ser residue most easily to provide Boc–Glu(OBzl)–Ala–Ser[Boc–Glu(OBzl)]–Val–OBzl in 20% yield. In the other active ester coupling reactions, we could not isolate the corresponding O-acylated peptides because the side reaction stated above was minimal. The descrepancy of reactivities during the active ester coupling reactions might be due to the differences in the electron density at the carbon atom of the activated carbonyl group and in the steric effect of the side chain. The formation of succinimide during the reaction of Boc–Asp(OBzl)–ONp[19] could not be suppressed completely even when the pH of the reaction mixture was maintained at 6.0, so the by-product was removed by silica gel coulmn chromatography. Boc–Ala–OH was coupled with H–Ala–Ser–Val–OBzl by the DCC–HOBt method[20] to give Boc–Ala–Ala–Ser–Val–OBzl. During this reaction, O-acylation could not be detected by TLC. Boc–Val–His–Phe–$NHNH_2$ was prepared as follows. Boc–Val–OH and H–His–Phe–OMe[21] were coupled by the mixed anhydride method to yield Boc–Val–His–Phe–OMe, which was converted to the corresponding hydrazide by hydrazine hydrate treatment. Finally, Z–Tyr–Val–Pro–OH was prepared in two different ways, one consisting of coupling of Z–Tyr–Val–$NHNH_2$ with H–Pro–OH and the other of Z–Tyr–$NHNH_2$ with H–Val–Pro–OH. The former method was more convenient than the latter, in which it was difficult to obtain H–Val–Pro–OH in a pure form.

Boc–Y–Ala–Ser–Val–OBzl [Y = Asp(OBzl), Asn, Glu(OBzl), Gln and Ala] was treated with HCl/dioxane or TFA/anisole to remove the Boc group, yielding the corresponding

TABLE I. *Rf* Values and Amino Acid Ratios in Acid Hydrolysates and AP-M Digests of H–Tyr–Val–Pro–Val–His–Phe–X–Ala–Ser–Val–OH

Compound (X)	TLC		Amino acid ratios in acid hydrolysates / Amino acid ratios in AP-M digests								Average recovery (%)
	Rf^3	Rf^4	Tyr[a]	Val[b]	Pro[b]	His	Phe	X	Ala	Ser	
Asp	0.16	0.88	0.32	3.00	0.99	1.21	1.07	1.04	1.05	0.89	77.3
			0.92	2.18	0.32	0.89	1.13	1.08	1.11	0.87	71.0
Asn	0.16	0.88	0.47	3.00	1.07	0.88	0.92	1.07	0.97	0.88	88.8
			0.89	2.25	0.55	0.91	1.08	1.08	1.03	1.01	80.5
Glu	0.16	0.88	0.25	3.00	1.11	1.01	1.01	1.01	0.96	0.82	86.7
			1.29	2.67	0.50	1.05	0.91	0.91	1.08	0.75	67.8
Gln[c]	0.21	0.88	0.38	3.00	0.93	0.92	0.94	1.02	0.87	0.74	80.3
			0.83	2.16	0.29	0.91	1.12	1.12	1.18	0.84	73.5
Ala	0.18	0.88	0.59	3.00	0.89	0.86	0.94	0.99	0.99	0.94	76.7
			0.88	2.38	0.58	0.90	0.98	1.22	1.22	0.78	79.7

a) Tyr was destroyed during acid hydrolysis.
b) The Val–Pro bond was not completely cleaved by AP-M.
c) Glu was recovered instead of Gln.
TLC: thin-layer chromatography.

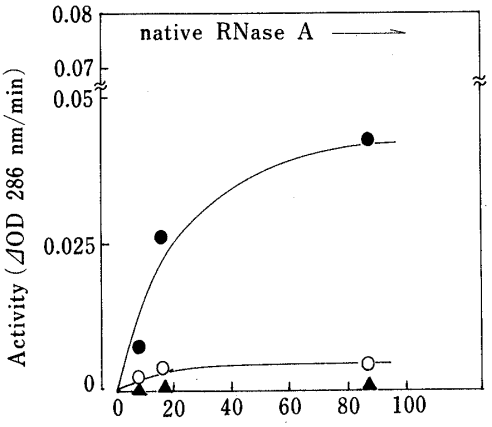

Fig. 2. Reactivation of Des(121—124) RNase A
● X: Asp(I); ○ X: Glu(III);
▲ X: Asn, Gln, Ala(II, IV, V).

amine, which was coupled with Boc–Val–His–Phe–N_3 to afford five kinds of protected heptapeptides, Boc–Val–His–Phe–Y–Ala–Ser–Val–OBzl [Y = Asp(OBzl), Asn, Glu(OBzl), Gln and Ala]. The Boc group of these heptapeptides was removed by TFA/anisole treatment to give the heptapeptide amines. These amines were coupled with Z–Tyr–Val–Pro–OPFP to yield protected decapeptides Z–Tyr–Val–Pro–Val–His–Phe–Y–Ala–Ser–Val–OBzl [Y = Asp(OBzl), Asn, Glu(OBzl), Gln and Ala]. Pentafluorophenyl ester is more reactive than *p*-nitrophenyl ester,[22] but *O*-acylation was not observed during the above coupling reactions. After purification of these five kinds of protected decapeptides by column chromatography on Sephadex LH-20 using EtOH as an eluant and/or by recrystallization, the Z and Bzl groups were removed by catalytic hydrogenation to afford H–Tyr–Val–Pro–Val–His–Phe–X–Ala–Ser–Val–OH [X = Asp, I; Asn, II; Glu, III; Gln, IV; and Ala, V] in a pure form. Each peptide exhibited a sharp single spot positive to ninhydrin and Pauly reagents on TLC. Amino acid analysis of acid hydrolysates gave molar ratios in good agreement with the theoretically expected values except for Tyr residue, which was destroyed during acid hydrolysis. Amino acid analysis of amino peptidase (AP-M)[23] digests was also performed and gave molar ratios in good agreement with the expected values (except for Val and Pro residues in all peptides

and Gln residue in peptide IV) as summarized in Table I. AP-M employed in this experiment (purchased from Seikagaku Kogyo Co., Ltd.) does not cleave the Val–Pro bond completely,[24] so the values of Val and Pro residues were lower than the theoretically expected values. With regard to the value of Gln in peptide (IV) one mol of Glu residue instead of Gln residue was recovered. However, from the TLC data, amino acid analysis of the acid hydrolysate and elemental analysis, it can be deduced that the AP-M employed converted Gln residue to Glu during the digestion. Partial conversion of the Gln residue to Glu by AP-M was reported previously.[25] In peptide I, the molar ratio of Asp was 1.08, indicating that succinimide formation followed by $\alpha \rightarrow \beta$ shift did not occur and the benzyl group did not remain on the β-carboxyl group. In peptide II, the molar ratio of the Asn was 1.08 without any trace of Asp residue, indicating that AP-M did not convert the Asn residue to Asp, and moreover, succinimide formation did not occur. The homogeneity of the peptides (I—V) was thus ascertained.

The ability of the peptides to regenerate hydrolytic activity of RNase A 1—120 towards cyclic 2′,3′-cytidylic acid was examined, and the results are shown in Fig. 2. Asp-decapeptide (I) regenerated 56% of the activity of native RNase A (in a ratio 1 : 87), but Asn-decapeptide (II) did not reactivate RNase A 1—120. Glu-decapeptide (III) gave a 10% recovery of RNase activity; when the concentration of the peptide was increased, however, the recovery % of activity did not increase. Gln-decapeptide (IV) did not show any ability to restore the activity of inactivated RNase 1—120. Ala-decapeptide (V) also showed no reactivation activity. From the results described above, it can be concluded that the free carboxyl group at position 121 of peptide I or III plays a very important role in the reactivation of RNase A 1—120, while the amide group of the side chain of Asn or Gln in peptide II or IV can not participate in the interaction with His-119 to manifest RNase A activity. It is also interesting that on incorporation of a methylene group (*ca.* 1.5 Å) into the side chain of the Asp residue to change the position of the free carboxyl group of Asp-121, reactivation activity still remained (10%) but was decreased significantly. The carboxyl group of Asp might be more favorable for formation of hydrogen bonding with His-119 than that of Glu. The results obtained here are compatible with the previous reports that Asp-121 interacts with His-119 *via* hydrogen bonding,[4,6,7,26–28] controlling the p*K* of His-119.[10,29] We have established a system suitable for studies of the exact role of Asp-121 and the interaction of Asp-121 with His-119 of RNase A.

Experimental

General experimental methods employed here were essentially the same as those described in the previous paper[1] of this series. Thin layer chromatography was performed on silica gel (Kieselgel G, Merck). Rf^1, Rf^2, Rf^3 and Rf^4 values refer to the systems of $CHCl_3$, MeOH and AcOH (90 : 8 : 2), $CHCl_3$, MeOH and H_2O (8 : 3 : 1, lower phase), *n*-BuOH, AcOH and H_2O (4 : 1 : 5, upper phase) and *n*-BuOH, pyridine, AcOH and H_2O (4 : 1 : 1 : 2), respectively.

Boc–Ala–Ser–NHNH$_2$——Boc–Ala–OH (5.7 g), H–Ser–OMe (prepared from 4.7 g of H–Ser–OMe·HCl and 4.2 ml of Et_3N) and HOBt (4.9 g) were dissolved in DMF (30 ml) and cooled with ice-salt. DCC (7.4 g) was added to the cold solution, and the reaction mixture was stirred at 4 °C overnight. After removal of the urea derivative and the solvent, the residue was dissolved in AcOEt and the AcOEt solution was washed with 5% Na_2CO_3, 10% citric acid and water, then dried over Na_2SO_4 and evaporated down. Hydrazine hydrate (80%, 4.5 ml) was added to a solution of the resulting oily product (Boc–Ala–Ser–OMe) in MeOH (14 ml). The reaction mixture was kept at room temperature overnight. The precipitate formed was collected by filtration and washed with MeOH, yield 4.37 g (50.2%), mp 170—175 °C, $[\alpha]_D^{24}$ −40.9° (c = 0.9, AcOH), Rf^4 0.67. *Anal.* Calcd for $C_{11}H_{22}N_4O_5$: C, 45.5; H, 7.58; N, 19.3. Found: C, 45.6; H, 7.66; N, 19.3.

Boc–Ala–Ser–Val–OBzl——Boc–Ala–Ser–N_3 (prepared from 3.3 g of Boc–Ala–Ser–NHNH$_2$ and 1.4 ml of isopentyl nitrite as usual) in DMF (45 ml) was added to an ice-cold solution of H–Val–OBzl (prepared from 6.0 g of *H–Val–OBzl·TosOH* and 2.1 ml of Et_3N). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid and water, dried over

Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford a precipitate, which was collected by filtration and recrystallized from AcOEt, yield 3.65 g (78.5%), mp 74—77 °C, $[\alpha]_D^{24}$ −51.9° (c=1.0, MeOH), Rf^1 0.51, Rf^2 0.81. *Anal.* Calcd for $C_{23}H_{35}N_3O_7$: C, 59.3; H, 7.58; N, 9.0. Found: C, 59.5; H, 7.70; N, 9.1.

Synthesis of Boc–Y–Ala–Ser–Val–OBzl——a) Y=Asp(OBzl): Boc–Asp(OBzl)–ONp (3.81 g) and H–Ala–Ser–Val–OBzl·TFA (prepared from 2.9 g of Boc–Ala–Ser–Val–OBzl and 4 ml of TFA containing 1.5 ml of anisole) were dissolved in DMF (20 ml) containing Et_3N (1.1 ml). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated down. The oily product in $CHCl_3$ (4 ml) was applied to a column of silica gel (2×32.5 cm), which was eluted with the following solvents: $CHCl_3$ (1000 ml) and 1% MeOH in $CHCl_3$ (1450 ml). The solvent of the latter eluate was removed by evaporation. Petroleum ether was added to the residue to afford crystals, yield 0.42 g (11.4%), mp 68—72 °C, $[\alpha]_D^{24}$ −39.3° (c=0.4, MeOH), Rf^1 0.38, Rf^2 0.71. *Anal.* Calcd for $C_{34}H_{46}N_4O_{10}$: C, 61.0; H, 6.72; N, 8.4. Found: C, 61.3; H, 6.41; N, 8.4.

b) Y=Asn: The title compound was prepared from Boc–Asn–ONp (1.08 g) and H–Ala–Ser–Val–OBzl·TFA (prepared from 1.43 g of Boc–Ala–Ser–Val–OBzl and 3.5 ml of TFA containing 0.75 ml of anisole). Yield 1.06 g (68.2%), mp 180—182 °C, $[\alpha]_D^{24}$ −50.5° (c=0.7, MeOH), Rf^1 0.22, Rf^2 0.50. *Anal.* Calcd for $C_{27}H_{41}N_5O_9$: C, 56.1; H, 6.91; N, 12.1. Found: C, 56.1; H, 7.21; N, 12.0.

c) Y=Glu(OBzl): The title compound was prepared from Boc–Glu(OBzl)–ONp (3.94 g) and H–Ala–Ser–Val–OBzl·TFA (prepared from 2.77 g of Boc–Ala–Ser–Val–OBzl as described above). The crude product in $CHCl_3$ (5 ml) was applied to a column of silica gel (3×23 cm), and eluted with $CHCl_3$ (350 ml), followed by 1% MeOH in $CHCl_3$. Individual fractions (50 ml each) were collected. The solvent of the effluent in tube Nos. 11—14 (Rf^1 0.52) was removed by evaporation. Petroleum ether was added to the residue to afford Boc–Glu(OBzl)–Ala–Ser–[Boc–Glu(OBzl)]–Val–OBzl, yield 0.10 g (2.0%), mp 96—97 °C, $[\alpha]_D^{24}$ −34.0° (c=1.0, MeOH), Rf^1 0.50. Amino acid ratios in an acid hydrolysate: Glu 1.91; Ala 0.86; Ser 0.80; Val 1.00 (average recovery 85.3%). *Anal.* Calcd for $C_{52}H_{69}N_5O_{14}$: C, 62.2; H, 6.87; N, 7.0. Found: C, 62.1; H, 6.89; N, 7.0. In tube Nos. 15—19, two components (Rf^1 0.52 and 0.36) were contained. After removal of the solvent from these fractions by evaporation, petroleum ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt to give the purified desired peptide, Boc–Glu(OBzl)–Ala–Ser–Val–OBzl, yield 0.42 g (11.7%), mp 135—136 °C, $[\alpha]_D^{24}$ −39.8° (c=0.7, MeOH), Rf^1 0.35, Rf^2 0.68. Amino acid ratios in an acid hydrolysate: Glu 1.04; Ala 0.96; Ser 0.84; Val 1.00 (average recovery 95.0%). *Anal.* Calcd for $C_{35}H_{48}N_4O_{10}$: C, 61.4; H, 7.01; N, 8.2. Found: C, 61.4; H, 7.09; N, 8.3.

d) Y=Gln: The title compound was prepared from Boc–Gln–ONp (3.18 g) and H–Ala–Ser–Val–OBzl·TFA (prepared from 4.0 g of Boc–Ala–Ser–Val–OBzl as described above). Yield 1.28 g (26.2%), mp 155—157 °C, $[\alpha]_D^{24}$ −37.5° (c=1.4, MeOH), Rf^1 0.16, Rf^2 0.48. *Anal.* Calcd for $C_{28}H_{43}N_5O_9 \cdot 3H_2O$: C, 52.0; H, 7.57; N, 10.8. Found: C, 51.4; H, 7.15; N, 10.2.

e) Y=Ala: The title compound was prepared from Boc–Ala–OH (1.87 g) and H–Ala–Ser–Val–OBzl·HCl (prepared from 3.09 g of Boc–Ala–Ser–Val–OBzl and 7.9 ml of 7.35 N HCl/dioxane as usual) by the DCC–HOBt method. Yield 1.16 g (37.2%), mp 183—184 °C, $[\alpha]_D^{24}$ −49.6° (c=1.5, MeOH), Rf^1 0.19, Rf^2 0.52. *Anal.* Calcd for $C_{26}H_{40}N_4O_8$: C, 58.2; H, 7.46; N, 10.4. Found: C, 57.2; H, 7.78; N, 10.3.

Boc–Val–His–OMe——A mixed anhydride (prepared from 10.0 g of Boc–Val–OH and 4.8 ml of ethyl chloroformate as usual) in THF (100 ml) was added to an ice-cold solution of H–His–OMe (prepared from 12.1 g of H–His–OMe·2HCl and 7 ml of Et_3N) in DMF (100 ml). The reaction mixture was stirred at 4 °C for 2 d. After removal of the solvent, the residue was dissolved in AcOEt and 5% Na_2CO_3. This solution was stored in a cold room for 2 d. The gelatinous powder that formed was collected by filtration and recrystallized from AcOEt, yield 7.73 g (42%), mp 145 °C, $[\alpha]_D^{24}$ −19.2° (c=1.0, MeOH), Rf^2 0.68. *Anal.* Calcd for $C_{17}H_{28}N_4O_5$: C, 55.4; H, 7.66; N, 15.2. Found: C, 55.2; H, 7.66; N, 15.0.

Boc–Val–His–NHNH$_2$——Hydrazine hydrate (80%, 0.23 ml) was added to a solution of Boc–Val–His–OMe (1.6 g) in MeOH (10 ml). The mixture was stored at room temperature overnight. After concentration of the solution to half the initial volume, ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from MeOH and ether, yield 0.86 g (62.3%), mp 114—116 °C, $[\alpha]_D^{24}$ −11.5° (c=0.9, AcOH), Rf^2 0.46. *Anal.* Calcd for $C_{16}H_{28}N_6O_4$: C, 52.2; H, 7.66; N, 22.8. Found: C, 52.0; H, 7.79; N, 22.5.

Boc–Val–His–Phe–OMe——a) Boc–Val–His–N_3 (prepared from 0.45 g of Boc–Val–His–NHNH$_2$ and 0.13 ml of isopentyl nitrite) in DMF (10 ml) was added to a solution of H–Phe–OMe (prepared from 0.18 g of H–Phe–OMe·HCl and 0.14 ml of Et_3N) in THF (40 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 0.31 g (60.8%), mp 170—173 °C, $[\alpha]_D^{24}$ −22.5° (c=1.0, MeOH), Rf^1 0.43, Rf^2 0.76. *Anal.* Calcd for $C_{26}H_{37}N_5O_6$: C, 60.6; H, 7.23; N, 13.6. Found: C, 60.0; H, 7.15; N, 13.5.

b) A mixed anhydride (prepared from 1.1 g of Boc–Val–OH and 0.52 ml of ethyl chloroformate as usual) in THF (50 ml) was added to an ice-cold solution of H–His–Phe–OMe (prepared from 2.17 g of Z–His–Phe–OMe by catalytic hydrogenation) in DMF (50 ml). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was dissolved in AcOEt and 5% Na_2CO_3. After storage of the solution in a refrigerator overnight, the crystals

Table II. Yield, mp, $[\alpha]_D$ Values, Elemental Analysis and *Rf* Values of Boc–Val–His–Phe–Y–Ala–Ser–Val–OBzl

Compound (Y)	Yield (%)	mp (°C)	$[\alpha]_D$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^2	Rf^3
Asp(OBzl)[a]	47.7	189—192	−14.7 (c=0.3)	$C_{54}H_{71}N_9O_{13}$	61.6 (61.6)	6.65 6.59	12.0 11.9)	0.55	0.67
Asn[b]	54.9	232—233	−19.3 (c=1.0)	$C_{47}H_{66}N_{10}O_{12}\cdot 3H_2O$	55.5 (55.6)	7.08 6.79	13.8 13.8)	0.60	0.58
Glu(OBzl)[a]	78.4	204—207	−14.6 (c=0.6)	$C_{55}N_{73}N_9O_{13}\cdot 3H_2O$	58.9 (58.6	7.04 7.02	11.2 11.1)	0.60	0.50
Gln[b]	39.4	233—234	−18.2 (c=1.4)	$C_{48}H_{68}N_{10}O_{12}\cdot 2H_2O$	56.9 (56.7	7.11 6.81	13.8 13.8)	0.50	0.58
Ala[b]	54.2	226—227	−15.3 (c=1.3)	$C_{46}H_{65}N_9O_{11}\cdot 3H_2O$	56.7 (56.5	7.29 7.06	12.9 12.7)	0.48	0.57

a) Recrystallized from AcOEt. *b*) From EtOH.

that had formed were collected by filtration and recrystallized from AcOEt, yield 2.29 g (75.3%), mp 165—168 °C, $[\alpha]_D^{24}$ −22.8° (c=1.0, MeOH), Rf^1 0.45, Rf^2 0.75. *Anal.* Calcd for $C_{26}H_{37}N_5O_6$: C, 60.6; H, 7.23; N, 13.6. Found: C, 60.7; H, 7.25; N, 13.3.

Boc–Val–His–Phe–NHNH$_2$——Hydrazine hydrate (80%, 0.3 ml) was added to a solution of Boc–Val–His–Phe–OMe (1.0 g) in MeOH (5 ml). The solution was kept at room temperature overnight. The crystals that formed were collected by filtration and washed with MeOH, yield 0.95 g (92.3%), mp 241—243 °C, $[\alpha]_D^{22}$ −10.3° (c=0.3, AcOH), Rf^2 0.31, Rf^3 0.63. *Anal.* Calcd for $C_{25}H_{37}N_7O_5$: C, 58.3; H, 7.18; N, 19.0. Found: C, 58.2; H, 7.18; N, 18.8.

General Procedure for the Synthesis of Boc–Val–His–Phe–Y–Ala–Ser–Val–OBzl (Y=Asp(OBzl), Asn, Glu(OBzl), Gln and Ala)——Boc–Val–His–Phe–N_3 (prepared from 0.78 mmol of Boc–Val–His–Phe–NHNH$_2$ and 0.78 mmol of isopentyl nitrite) was added to a solution of H–Y–Ala–Ser–Val–OBzl·TFA (prepared from 0.78 mmol of Boc–Y–Ala–Ser–Val–OBzl and 1.3 ml of TFA containing 0.25 ml of anisole as usual). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and water were added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt or EtOH. Yield, mp, $[\alpha]_D$ values, elemental analysis, *Rf* values are summarized in Table II.

Z–Tyr–Val–NHNH$_2$——Z–Tyr–OH (7.45 g), H–Val–OBzl (prepared from 10.0 g of H–Val–OBzl·TosOH) and HOBt (4.05 g) were dissolved in DMF (20 ml) and cooled with ice-salt. DCC (6.18 g) was added to the cold solution prepared above and the reaction mixture was stirred at 4 °C overnight. After removal of dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3, 1 N HCl and water, dried over Na_2SO_4 and evaporated down. Hydrazine hydrate (80%, 3.75 ml) was added to a solution of the oily product obtained above in MeOH (8 ml) and the solution was stored at room temperature overnight. Crystals that formed were collected by filtration and recrystallized from EtOH, yield 10.4 g (97.1%), mp 258—260 °C, $[\alpha]_D^{24}$ −8.1° (c=0.9, DMF), Rf^2 0.38, Rf^3 0.61. *Anal.* Calcd for $C_{22}H_{28}N_4O_5$: C, 61.7; H, 6.59; N, 13.1. Found: C, 61.4; H, 6.48; N, 12.9.

Z–Tyr–Val–Pro–OH——a) Z–Tyr–N_3 (prepared from 0.79 g of Z–Tyr–NHNH$_2$ and 0.31 ml of isopentyl nitrite as usual) in DMF (15 ml) was added to a solution of H–Val–Pro–OH (prepared from 1.0 g of Z–Val–Pro–OH[30] by catalytic hydrogenation) in DMF (10 ml) containing Et_3N (0.32 ml) under cooling with ice. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, AcOEt and 5% $NaHCO_3$ were added. The $NaHCO_3$ layer was acidified with conc. HCl to pH 3. The oily precipitate was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to give crystals, which were collected by filtration and washed with ether, yield 0.61 g (52.4%), mp 105—108 °C, $[\alpha]_D^{24}$ −51.3° (c=0.9, MeOH), Rf^2 0.33, Rf^3 0.86, Rf^4 0.72. *Anal.* Calcd for $C_{27}H_{33}N_3O_7\cdot 1/2H_2O$: C, 62.4; H, 6.54; N, 8.1. Found: C, 62.8; H, 6.42; N, 7.9.

b) Z–Tyr–Val–N_3 (prepared from 0.96 g of Z–Tyr–Val–NHNH$_2$ and 0.32 ml of isopentyl nitrite as usual) in DMF (50 ml) was added to a solution of H–Pro–OH (0.32 g) in water (10 ml) and DMF (10 ml) containing Et_3N (0.39 ml). The reaction mixture was stirred in a cold room overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 1 N HCl and water, dried over Na_2SO_4 and evaporated down. Ether was added to the residue to afford crystals, which were collected by filtration and recrystallized from AcOEt, yield 0.39 g (32.3%), mp 108—110 °C, $[\alpha]_D^{24}$ −51.3° (c=1.1, MeOH), Rf^2 0.34, Rf^4 0.75. *Anal.* Calcd for $C_{27}H_{33}N_3O_7\cdot 1/2H_2O$: C, 62.4; H, 6.54; N, 8.1. Found: C, 62.9; H, 6.52; N, 8.0.

Z–Tyr–Val–Pro–OPFP——DCC–PFP complex (0.54 g)[22] was added to a solution of Z–Tyr–Val–Pro–OH

TABLE III. Yield, mp, $[\alpha]_D$ Values, Elemental Analysis and *Rf* Values of Z–Tyr–Val–Pro–Val–His–Phe–Y–Ala–Ser–Val–OBzl

Compound (Y)	Yield (%)	mp (°C)	$[\alpha]_D$ (DMF)	Formula	Elemental analysis Calcd (Found)			TLC	
					C	H	N	Rf^2	Rf^3
Asp (OBzl)[a]	62.5	199—202	−33.6 (c=1.0)	$C_{76}H_{94}N_{12}O_{17}\cdot 2H_2O$	63.1 (63.2	6.49 6.29	11.6 11.6)	0.74	0.74
Asn	41.6	247—250	−36.5 (c=1.0)	$C_{69}H_{89}N_{13}O_{16}\cdot 3H_2O$	58.8 (58.6	6.73 6.45	12.9 12.8)	0.68	0.70
Glu (OBzl)[a]	41.6	184—185	−37.5 (c=0.3)	$C_{77}H_{96}N_{12}O_{17}\cdot 6H_2O$	59.0 (58.7	6.88 6.55	10.7 11.0)	0.70	0.71
Gln	75.8	245—247	−32.4 (c=1.0)	$C_{70}H_{91}N_{13}O_{16}\cdot 3H_2O$	59.1 (59.1	6.81 6.94	12.8 13.0)	0.65	0.68
Ala	58.3	188—191	−2.6 (c=0.3)	$C_{68}H_{88}N_{12}O_{15}\cdot 3H_2O$	59.8 (59.8	6.87 6.94	12.3 12.6)	0.75	0.78

a) Purified by column chromatography on Sephadex LH-20 using EtOH as an eluate.

TABLE IV. Yield, mp, $[\alpha]_D$ Values, Elemental Analysis of H–Tyr–Val–Pro–Val–His–Phe–X–Ala–Ser–Val–OH

Compound (X)	Yield (%)	mp (°C)	$[\alpha]_D$	Formula	Elemental analysis Calcd (Found)		
					C	H	N
Asp[a] (I)	82.6	—	−62.8 (c=0.9)[c]	$C_{54}H_{76}N_{12}O_{15}\cdot 2HCl\cdot 5H_2O$	50.1 (49.8	6.79 6.25	13.0 13.2)
Asn (II)	76.5	195—200	−33.2 (c=1.0)[b]	$C_{54}H_{77}N_{13}O_{14}\cdot 3H_2O$	54.7 (54.6	7.00 6.84	15.4 14.8)
Glu (III)	81.4	180—184	−30.0 (c=0.2)[b]	$C_{55}H_{78}N_{12}O_{15}\cdot 2H_2O$	55.9 (55.5	7.44 7.10	14.2 14.1)
Gln (IV)	69.1	218—222	−26.2 (c=0.5)[b]	$C_{55}H_{79}N_{13}O_{14}\cdot 2H_2O$	55.9 (55.8	7.02 7.24	15.4 15.0)
Ala (V)	76.4	184—188	−183.5 (c=0.2)[c]	$C_{53}H_{76}N_{12}O_{13}\cdot 2H_2O$	56.6 (56.7	7.11 7.19	14.9 14.4)

a) Peptide (I) was isolated by lyophilization from water.
b) AcOH. *c*) H_2O.

(0.29 g) in AcOEt (5 ml). The mixture was stirred at room temperature for 10 min and at 0 °C for 10 min, then the urea derivative was removed by filtration and the solvent of the filtrate was removed by evaporation. Petroleum ether was added to the residue to give an amorphous product, yield 0.33 g (82.5%) Rf^2 0.94, Rf^4 0.82.

General Procedure for the Synthesis of Z–Tyr–Val–Pro–Val–His–Phe–Y–Ala–Ser–Val–OBzl (Y = Asp(OBzl), Asn, Glu(OBzl), Gln and Ala)——Z–Tyr–Val–Pro–OPFP (0.25 g) and H–Val–His–Phe–Y–Ala–Ser–Val–OBzl·TFA (prepared from 0.41 g of Boc–Val–His–Phe–Y–Ala–Ser–Val–OBzl and 1.2 ml of TFA containing 0.5 ml of anisole) were dissolved in DMF (10 ml) containing Et_3N (0.07 ml). The reaction mixture was stirred at room temperature for 3 h. After removal of the solvent, AcOEt and water were added to the residue to give crystals, which were collected by filtration and recrystallized from EtOH. Yield, mp, $[\alpha]_D$ values, elemental analysis and *Rf* values are summarized in Table III.

General Procedure for the Synthesis of H–Tyr–Val–Pro–Val–His–Phe–X–Ala–Ser–Val–OH (X = Asp, I; Asn, II; Glu, III; Gln, IV; Ala, V)——Z–Tyr–Val–Pro–Val–His–Phe–Y–Ala–Ser–Val–OBzl (0.093 mmol) in MeOH (4 ml) and DMF (8 ml) was hydrogenated over a Pd catalyst. After removal of Pd and the solvent, EtOH was added to the residue to give crystals, which were collected by filtration and washed with EtOH. Yield, mp, $[\alpha]_D$ values and elemental analysis are summarized in Table IV.

Enzyme Assay——The activity with 2′,3′-cyclic CMP as a substrate was determined by the spectrophotometric

method described by Crook *et al.*[31] The absorbancy difference between the substrate and product was followed at 286 nm with a Shimadzu UV 200S spectrophotometer with a thermostated cell holder. The substrate concentration used was 1 mM in 0.1 M Tris-HCl buffer (pH 6.0) containing 0.1 M NaCl. The final enzyme concentration was 1 μM. The reaction rate was expressed as $\Delta OD_{286\,nm}$/min.

Complex Formation between Des(121—124) RNase A and C-Terminal Decapeptide and Its Analogs—Des(121—124) RNase A solution (55 μM; 100 μl) was mixed with decapeptide (8.7—87 eq) in 150 μl of 0.1 M Tris-HCl-acetate buffer (pH 6.0) containing 0.1 M NaCl, and the pH of the mixture was adjusted to 6.0 by the addition of 10% trimethylamine or 10% acetic acid. After a 10 min incubation, 50 μl of the aliquot was used for the enzyme assay.

Added in Proof (July 13, 1984) After the present paper had been submitted, we became aware of a paper describing the synthesis of the tetradecapeptide composed of residues 111—124 of RNase A with Asp-121 replaced by Asn (Asn-121 tetradecapeptide) by the solid phase method and showing that its ability to restore the RNase activity of RNase 1—118 towards cyclic 2′,3′-cytidylic acid was significantly reduced (4.5%).[32]

References and Notes

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