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Synthesis of Head Activator (HA)-Related Peptides and Development of HA-Radioimmunoassay

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Synthesis of peptides related to hydra head activator (HA; pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH) was performed by the solution method. The analogs synthesized were Tyr¹¹-HA, des-Phe¹¹-HA, Arg¹-HA and Phe⁵-HA. Antisera were prepared by immunizing three rabbits with the synthetic HA. Two anti-HA-antisera with sufficient titer were obtained. An HA-radioimmunoassay system, developed by using an antiserum (ASH-04) and ¹²⁵I-Tyr¹¹-HA as a tracer, was found to be sensitive and specific to HA.

Keywords—head activator; synthesis; antibody preparation; ¹²⁵I-Tyr¹¹-head activator; radioimmunoassay; specificity; droplet counter current chromatography; HPLC

The hydra head activator (HA) is an undecapeptide isolated from the fresh water hydra and the sea anemone, and sequenced as pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH¹⁾ (I). The peptide is a growth hormone-like factor in hydra stimulating head specific growth or bud formation, together with other morphogenous factors.²⁾ A peptide with complete sequence homology occurs not only in the coelenterates but also in mammals—human, bovine and rat hypothalami, and intestine.³⁾ Except for the induction of amylase release from rat pancreas *in vitro* but not *in vivo*, and a weak stimulatory effect on smooth muscle,⁴⁾ no biological activity or physiological function of HA has been reported in mammals.

In the present paper we describe: a) the synthesis of four HA-related peptides (Fig. 1), and b) the development of an HA-radioimmunoassay (RIA).

Synthesis of HA-related peptides was carried out by a conventional solution method. The protected peptides prepared as units for successive azide fragment condensation to build up the peptide backbones were as follow; Z-pGlu-Pro-Pro-Gly-NHNHBoc (VI), Z-Arg(NO₂)-Pro-Pro-Gly-NHNHBoc,⁵⁾ Boc-Gly-Ser-N₂H₃,⁶⁾ Boc-Phe-Ser-N₂H₃,⁷⁾ Boc-Lys(Z)-Val-Ile-Leu-Tyr-OH (VII), Boc-Lys(Z)-Val-Ile-Leu-OH (VIII) and Boc-Lys(Z)-Val-Ile-Leu-Phe-OH (IX). In Fig. 2, the synthetic route is shown for an analog, Tyr¹¹-HA (II), which

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HA (I)	pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH	
Tyr ¹¹ -HA (II)	pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Tyr-OH	
des-Phe ¹¹ -HA (III)	pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-OH	
Arg ¹ -HA (IV)	H-Arg-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH	
Phe ⁵ -HA (V)	pGlu-Pro-Pro-Gly-Phe-Ser-Lys-Val-Ile-Leu-Phe-OH	
bradykinin (BK)	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	

Fig. 1. Head Activator (HA) and Related Peptides

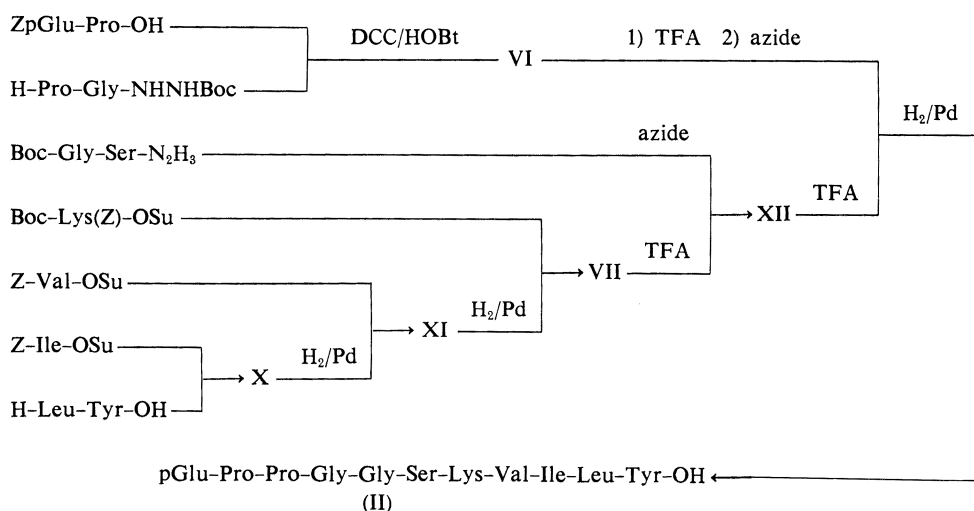


Fig. 2. Synthesis of Tyr¹¹-HA (II)

was designed as a labelled antigen preparation with minimum structural modification (replacing C-terminal Phe by Tyr). The C-terminal protected pentapeptide (VII) was prepared in a stepwise fashion by the active ester method. The starting material, H-Leu-Tyr-OH⁸⁾ was coupled with Z-Ile-OSu to yield Z-Ile-Leu-Tyr-OH (X). Debenzoyloxycarbonylation of X by catalytic hydrogenation followed by coupling with Z-Val-OSu gave Z-Val-Ile-Leu-Tyr-OH (XI), which was deprotected by the same procedure. Acylation of the resulting tetrapeptide with the active ester of Boc-Lys(Z)-OH gave VII. Removal of the N^z-Boc group of VII was carried out by treatment with trifluoroacetic acid (TFA) at 15 °C for 45 min to suppress N^ε-Z cleavage of Lys.⁹⁾ The resulting pentapeptide H-Lys(Z)-Val-Ile-Leu-Tyr-OH·TFA (VII') was condensed with Boc-Gly-Ser-N₂H₃⁶⁾ by the azide method¹⁰⁾ and the protected heptapeptide Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XII) obtained was further subjected to *tert*-butyloxycarbonylation under the same conditions as described above.

The N-terminal tetrapeptide (VI) was prepared by the reaction between Z-pGlu-Pro-OH¹¹⁾ and H-Pro-Gly-NHNHBoc⁵⁾ with the dicyclohexylcarbodiimide (DCC)-*N*-hydroxybenzotriazole (HOBt) procedure.¹²⁾ The final coupling of Z-pGlu-Pro-Pro-Gly-N₃ derived from VI with H-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XII') yielded the protected undecapeptide Z-pGlu-Pro-Pro-Gly-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH, which was hydrogenated to give crude II. Purification of peptide II was performed by droplet counter current chromatography (DCCC),¹³⁾ with a solvent system of *n*-butanol-acetic acid-water (4 : 1 : 5). The desired peptide emerged after the solvent front of the lower phase used as the mobile phase. A highly purified product was obtained after gel filtration on Sephadex G-25 using 2 M acetic acid as the solvent. The homogeneity of this peptide was proved by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), elemental analysis and amino acid analysis of the acid hydrolysate.

The other three HA analogs, des-Phe¹¹-HA (III), Arg¹-HA (IV) and Phe⁵-HA (V), were synthesized in the same manner as described above, starting from VIII or IX as the C-terminal fragment. HA itself was also prepared and extensively purified by DCCC employing two different solvent systems. The homogeneity of the synthetic peptides was proved by the same means as used for II. The synthetic HA prepared in this study had the same biological activity as the native peptide. It also cross-reacted with the HA-RIA system developed by Bodenmüller *et al.*¹⁴⁾ Cross-reactivity and homogeneity data are summarized in Table I.

TABLE I. Characterization of Synthetic Peptides

Peptide	Rf^1	Rf^2	Retention time ^{a)} (min)	Cross-reactivity (%)
I	0.17	0.59	12.35	100
II	0.13	0.56	8.71	100
III	0.11	0.48	7.43	1
IV	0.02	0.53	9.53	0.03
V	0.25	0.64	16.06	0.3

a) Conditions of analytical HPLC: column, μ -Bondapak C_{18} (4×300 mm); flow rate, 1 ml/min; detection, 210 nm; eluent system, linear gradient from 25% to 45% CH_3CN (15 min) in 20 mM phosphate buffer (pH 3.0).

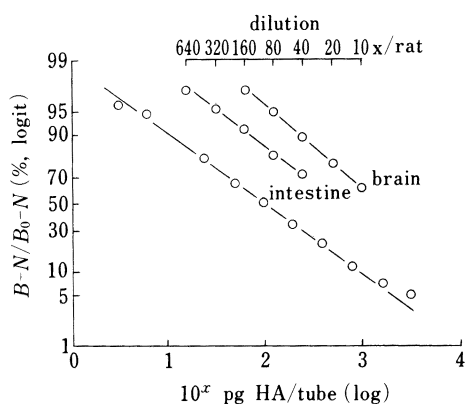


Fig. 3. Standard Dose-Response Curve and Dilution Plots of 2M AcOH Extract from Whole Intestine and Whole Brain of Rat Antiserum, ASH-04 (final 20000 \times); tracer, ^{125}I -Tyr¹¹-HA; F/B separation, double antibody method.

To obtain a specific antiserum, we used HA-ascaris protein conjugates,¹⁵⁾ prepared by using glutaraldehyde. Immunization of three rabbits with the conjugate gave antibodies with sufficient titer. ^{125}I -Tyr¹¹-HA was prepared by the Iodogen method.¹⁶⁾ Purified labelled antigen was separated from the unlabelled antigen and other compounds by reversed-phase HPLC.

The specificity of these antisera was examined, and the antiserum ASH-04 was found to be more specific in binding to the undecapeptide HA than the others. In the HA-RIA using ASH-04 at a final dilution of 20000 fold, the cross-reactivities of the three HA analogs and bradykinin (BK) were examined. The dose-response curves of III, IV and V, which resemble BK, revealed that the minor change of the HA molecule resulted in a drastic decrease of the binding activity with the antisera (Table I). The Gly⁵ residue seemed to be important for binding with the antibody ASH-04, while BK did not cross-react at all. These results clearly indicate that the assay system is specific for the undecapeptide. In HA-RIA by the double antibody method, less than 10 pg of HA per tube was detectable; 2 N acetic acid extracts of rat whole intestine and whole brain displaced the tracer in a manner parallel to that of synthetic HA (Fig. 3). The content of immunoreactive HA, however, was found to be extremely low. The results suggest that the RIA system established here could be useful for further immunological studies on the head activator.

Experimental

Melting points are uncorrected. Optical rotations were measured in a Nippon Bunkoh DIP-4 polarimeter. Amino acid analyses were performed with a Hitachi KLA-5 amino acid analyzer. HPLC was performed on a system

composed of two model 510 pumps, a model U6K injector, a model 680 gradient controller, a model 481 LC spectrophotometer and a data module, model 730 (Waters). Radioactivity was counted with an Auto Well Gamma System, ARC-360 (Aloka). Acid hydrolysis of samples for amino acid analysis was conducted with twice-distilled 5.8 N HCl at 110 °C for 24 h and for 48 h in evacuated sealed tubes, and phenol was added thereto when the peptide contained tyrosine. Evaporation of solvents was carried out *in vacuo* below 45 °C in a rotary evaporator. The solvent systems used for TLC on silica gel (Merck) were *n*-BuOH–AcOH–H₂O (4 : 1 : 5, the upper phase, *R_f¹*) and *n*-BuOH–pyridine–AcOH–H₂O (30 : 20 : 6 : 24, *R_f²*).

Z-Ile-Leu-Tyr-OH (X)—Z-Ile-OSu (2.21 g, 6.1 mmol) was allowed to react at room temperature with H-Leu-Tyr-OH⁸⁾ (1.38 g, 4.7 mmol) in dimethylformamide (DMF) (15 ml)–H₂O (6 ml) containing triethylamine (TEA) (1.32 ml, 9.4 mmol) for 16 h. After evaporation of the solvents, the residue in AcOEt was washed successively with 1 N HCl and saturated NaCl solution. The organic phase was dried over Na₂SO₄ and evaporated. The residue was triturated with ether–petr. ether and the solid was reprecipitated from the same solvents. Yield 2.47 g (96.9%). mp 123–125 °C. *R_f¹* 0.85, *R_f²* 0.82. $[\alpha]_D^{21} - 0.07^\circ$ (*c* = 1.0, DMF). Anal. Calcd for C₂₉H₃₉N₃O₇: C, 64.31; H, 7.26; N, 7.76. Found: C, 63.92; H, 7.30; N, 7.57.

Z-Val-Ile-Leu-Tyr-OH (XI)—X (2.33 g, 4.3 mmol) was hydrogenated in *n*-BuOH (40 ml)–MeOH (40 ml)–10% AcOH (80 ml) over Pd–black for 20 h. The catalyst was removed by filtration and the filtrate was concentrated to a small volume. The residue was precipitated from EtOH. Yield 1.75 g (87.0%). *R_f¹* 0.77, *R_f²* 0.80. Z-Val-OSu (1.42 g, 4.1 mmol) was coupled with H-Ile-Leu-Tyr-OH (1.59 g, 3.4 mmol) obtained above in H₂O (6 ml)–dimethylsulfoxide (DMSO) (2 ml)–DMF (20 ml) containing TEA (0.81 ml, 5.8 mmol) at room temperature for 20 h. The solvents were evaporated off and the residue was triturated with 10% AcOH. The solid was reprecipitated from MeOH–ether. Yield 1.73 g (79.4%). mp 232–233 °C. *R_f¹* 0.87, *R_f²* 0.75. $[\alpha]_D^{21} - 2.6^\circ$ (*c* = 1.0, DMF). Anal. Calcd for C₃₄H₄₈N₄O₈: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.37; H, 7.57; N, 8.46.

Boc-Lys(Z)-Val-Ile-Leu-Tyr-OH (VII)—XI (1.60 g, 2.5 mmol) was hydrogenated in *n*-BuOH (15 ml)–MeOH (60 ml)–50% AcOH (30 ml) in the same manner as described for XI. Yield 1.26 g (88.7%). *R_f¹* 0.75, *R_f²* 0.80. Boc-Lys(Z)-OSu [prepared from Boc-Lys(Z)-OH (0.989 g, 2.6 mmol), *N*-hydroxysuccinimide (HOSu) (0.314 g, 2.73 mmol), and DCC (0.563 g, 2.73 mmol) in DMF (1 ml)–THF (10 ml) as usual] was coupled with the tetrapeptide obtained above in H₂O (8 ml)–DMSO (15 ml)–DMF (40 ml) at room temperature for 20 h. The mixture was treated in the same manner as described for XI and reprecipitated from AcOEt–ether. Yield 0.58 g (39.1%). mp 275–277 °C (dec.). *R_f¹* 0.85, *R_f²* 0.81. $[\alpha]_D^{21} - 11.1^\circ$ (*c* = 0.7, DMF). Anal. Calcd for C₄₅H₆₈N₆O₁₁ · 3/2H₂O: C, 60.32; H, 7.99; N, 9.38. Found: C, 60.41; H, 7.85; N, 9.55.

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH (XII)—VII (0.56 g, 0.64 mmol) was dissolved in cold TFA (5 ml) containing anisole (0.5 ml) and the solution kept at 15 °C for 45 min. Excess of TFA was evaporated off and anhydrous Et₂O was added to the residue. The precipitate VII' was washed with Et₂O and dried over KOH/P₂O₅. *R_f¹* 0.78, *R_f²* 0.76. Isoamyl nitrite (0.18 ml, 1.34 mmol) was added to a solution of Boc-Gly-Ser-N₂H₃ (0.35 g, 1.28 mmol) in DMF (8 ml) containing 6 N HCl in dioxane (0.640 ml, 3.84 mmol) at –15 °C. This mixture was stirred at –10 °C for 2 min and neutralized with an ice-cold solution of TEA (0.538 ml, 3.84 mmol) in DMF (3 ml), and then was combined with an ice-cold solution of VII' in DMSO (7 ml)–DMF (4 ml) containing TEA (0.179 ml, 1.28 mmol). The reaction mixture was kept for 1 h at –5 °C and for 24 h at 4 °C and then concentrated. The product was precipitated in the same manner as described for XI and reprecipitated with MeOH–AcOEt–ether. Yield 0.48 g (73.8%). mp 284–285 °C (dec.). *R_f¹* 0.92, *R_f²* 0.89. $[\alpha]_D^{13} - 9.1^\circ$ (*c* = 1, DMF). Anal. Calcd for C₅₀H₇₆N₈O₁₄ · 1/2H₂O: C, 58.75; H, 7.59; N, 10.96. Found: C, 58.69; H, 7.41; N, 10.95.

Z-pGlu-Pro-Pro-Gly-NHNH-Boc (VI)—DCC (1.14 g, 5.5 mmol) was added to an ice-cold solution of Z-pGlu-Pro-OH¹¹⁾ (1.80 g, 5 mmol), H-Pro-Gly-NHNH-Boc⁵⁾ (5 mmol) [prepared from Z-Pro-Gly-NHNH-Boc⁵⁾ (2.10 g) by catalytic hydrogenation] and HOBt (0.74 g, 5.5 mmol) in DMF (5 ml)–tetrahydrofuran (THF) (25 ml). The mixture was stirred for 40 h at 4 °C and the resulting solid was filtered off. The filtrate was concentrated to a small volume and the residue was distributed between five portions of *n*-BuOH and ten portions of H₂O. The combined upper phase was concentrated to give a residue, which was passed through a column of Sephadex LH-20 (3.2 × 90 cm) with MeOH–1% AcOH (9 : 1) as the eluent. The fractions (8 g each, No. 35–37) were concentrated. The product was triturated with ether. Yield 2.09 g (66.6%). mp 151–155 °C (dec.). *R_f¹* 0.57, *R_f²* 0.79. $[\alpha]_D^{15} - 99.0^\circ$ (*c* = 1.0, DMF). Anal. Calcd for C₃₀H₄₀N₆O₉: C, 57.32; H, 6.41; N, 13.37. Found: C, 57.20; H, 6.44; N, 13.19. Amino acid ratios in an acid hydrolysate (24 h): Glu 1.00, Pro 2.00, Gly 1.00.

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Tyr-OH (II)—XII (101 mg, 0.1 mmol) was partially deprotected with TFA–anisole at 15 °C in the same manner as described for XII to give H-Gly-Ser-Lys(Z)-Val-Ile-Leu-Tyr-OH · TFA (XII'). *R_f¹* 0.55, *R_f²* 0.71. VI (107 mg, 0.17 mmol) was treated with TFA (5 ml) at 20 °C for 45 min in the same manner as described for XII to give Z-pGlu-Pro-Pro-Gly-N₂H₃ · TFA (VI'). *R_f¹* 0.41, *R_f²* 0.66. The product VI' was converted to the azide in DMF (3 ml) by using 6 N HCl in dioxane (0.283 ml, 1.7 mmol) and isoamyl nitrite (23 μl, 0.17 mmol) in the same manner as described for XII. The azide was coupled with XII' (0.1 mmol) in DMSO (5 ml)–DMF (5 ml) at 4 °C for 15 h. The product was precipitated from 20% AcOH (80 ml), washed with MeOH (1 ml)–AcOEt (10 ml) (yield 85 mg; *R_f¹* 0.43, *R_f²* 0.79), and hydrogenated over Pd in *n*-BuOH (5 ml)–MeOH (25 ml)–50% AcOH (5 ml). The product was gel-filtered on a Sephadex G-25 column with 2 M AcOH as the eluent and

lyophilized. Yield 65 mg. The crude material (20 mg) was dissolved in a mixture (2 ml) of the upper and lower phases of *n*-BuOH–AcOEt–H₂O (4 : 1 : 5) and subjected to DCCC which employed 200 tubes (0.2 × 30 cm each) filled with the upper phase. The lower phase (350 ml) was pumped through the tubes from top to bottom at a flow rate of 3 ml/h, followed by the upper phase (300 ml). The fractions (3 g each, No. 66–120) were gel-filtered and lyophilized. Yield 17 mg (48.4%). $[\alpha]_D^{21} - 115.6^\circ$ ($c = 1$, 3 M AcOH). *Anal.* Calcd for C₅₄H₈₄N₁₂O₁₀ · 2AcOH · 2H₂O: C, 53.69; H, 7.46; N, 12.95. Found: C, 53.44; H, 6.90; N, 12.99. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.94 (1.01), Ser 0.80 (0.85), Glu 0.97 (1.05), Pro 2.34 (2.04), Gly 2.00 (2.06), Val 0.58 (0.78), Ile 0.54 (0.77), Leu 1.01 (0.97), Tyr 0.95 (1.02), NH₃ trace (0.30).

Z-Val-Ile-Leu-OH (XIII)—Z-Val-OSu (2.68 g, 7.7 mmol) was allowed to react with H-Ile-Leu-OH · HCl¹⁷ (1.97 g, 7.0 mmol) in DMF (20 ml) containing TEA (2.66 ml, 19 mmol) at room temperature for 18 h. The product was worked up in the same manner as described for X and precipitated from AcOEt–ether. The solid was reprecipitated from the same solvents. Yield 3.13 g, (93.7%). mp 214–216°C (dec.). R_f^1 0.93, R_f^2 0.86. $[\alpha]_D^{25} - 10.0^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₂₅H₃₉N₃O₆: C, 62.87; H, 8.23; N, 8.80. Found: C, 63.00; H, 8.25; N, 8.73.

Boc-Lys(Z)-Val-Ile-Leu-OH (VIII)—XIII (995 mg, 2 mmol) was hydrogenated in MeOH (100 ml)–5% AcOH (50 ml) in the same manner as described for XI. The product (R_f^1 0.61, R_f^2 0.73) was reacted with Boc-Lys(Z)-OSu (2.6 mmol) in DMF (20 ml)–H₂O (5 ml) containing TEA (0.433 ml, 3.1 mmol) at room temperature for 20 h. The product was worked up in the same manner as described for XI and reprecipitated from AcOEt–ether. Yield 1.10 g (78.0%). mp 164–167°C. R_f^1 0.91, R_f^2 0.86. $[\alpha]_D^{25} - 22.4^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₃₅H₅₉N₅O₉: C, 61.26; H, 8.42; N, 9.92. Found: C, 60.97; H, 8.53; N, 10.02. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.03 (0.90), Val 0.80 (1.02), Ile 0.74 (0.97), Leu 0.97 (1.10), NH₃ 0.33 (0.26).

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-OH (XIV)—VIII (1.19 g, 1.4 mmol) was partially deblocked with ice-chilled TFA (5 ml)–anisole (0.5 ml) in the same manner as described for XII to give H-Lys(Z)-Val-Ile-Leu-OH · TFA (VIII'). R_f^1 0.75, R_f^2 0.74. The product was allowed to react with the azide [prepared from Boc-Gly-Ser-N₂H₃⁶¹ (0.58 g, 2.1 mmol), 6 N HCl in dioxane (1.12 ml, 8.0 mmol) and isoamyl nitrite (0.28 ml, 2.1 mmol)] in DMSO (30 ml)–DMF (20 ml) at 4°C for 18 h. The desired product was isolated in the same manner as described for XI and reprecipitated from MeOH (40 ml)–AcOEt (80 ml). Yield 1.04 g (87.5%). mp 226–227°C (dec.). R_f^1 0.85, R_f^2 0.85. $[\alpha]_D^{25} - 14.5^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₄₁H₆₇N₇O₁₂ · H₂O: C, 56.73; H, 8.01; N, 11.30. Found: C, 57.00; H, 8.14; N, 11.07. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 0.93 (1.08), Ser 0.95 (0.85), Gly 1.06 (1.07), Val 0.69 (1.01), Ile 0.80 (0.92), Leu 1.06 (1.00), NH₃ 0.49 (0.45).

pGlu-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-OH (III)—XIV (128 mg, 0.15 mmol) was partially deblocked in the same manner as described for XII to give H-Gly-Ser-Lys(Z)-Val-Ile-Leu-OH · TFA (XIV'). R_f^1 0.53, R_f^2 0.74. The azide [prepared from VI' (0.22 mmol), 6 N HCl in dioxane (178 μl, 1.10 mmol) and isoamyl nitrite (30 μl, 0.22 mmol) in DMF (4 ml)] was allowed to react with XIV' in DMSO (4 ml)–DMF (6 ml) containing TEA (55 μl, 0.39 mmol) at 4°C for 20 h, then the mixture was concentrated. The resulting residue was triturated with 5% AcOH, reprecipitated from MeOH–AcOEt (115 mg, R_f^1 0.47, R_f^2 0.77 as main spot), and hydrogenated in *n*-BuOH (15 ml)–MeOH (15 ml)–10% AcOH (15 ml). The product in 2 M AcOH was applied to a column (3.0 × 103 cm) of Sephadex G-25 (fine). The fractions (8 g each, No. 51–59) were lyophilized. Yield 41 mg (45.0%). R_f^1 0.11, R_f^2 0.48. $[\alpha]_D^{25} - 117.6^\circ$ ($c = 1.0$, 3 M AcOH). *Anal.* Calcd for C₄₅H₇₅N₁₁O₁₃ · AcOH: C, 54.37; H, 7.67; N, 14.84. Found: C, 54.35; H, 7.40; N, 14.59. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Lys 1.01 (1.08), Ser 0.91 (0.82), Glu 1.05 (1.05), Pro 2.02 (2.02), Gly 2.06 (2.07), Val 0.79 (0.98), Ile 0.74 (0.90), Leu 0.95 (0.95), NH₃ 0.54 (0.65).

Z-Ile-Leu-Phe-OH (XV)—Z-Ile-OSu (6.12 g, 16.9 mmol) was allowed to react at room temperature for 20 h with H-Leu-Phe-OH¹⁸ (3.62 g, 13 mmol) in DMF (25 ml)–H₂O (10 ml) containing TEA (3.09 ml, 22.1 mmol). The mixture was treated in the same manner as described for X. The residue was triturated with ether–petr. ether. The solid was reprecipitated from AcOEt–ether. Yield 5.34 g (76.8%). mp 156–158°C. R_f^1 0.88, R_f^2 0.78. $[\alpha]_D^{25} - 5.7^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₂₉H₃₉N₃O₆ · 1/2H₂O: C, 65.15; H, 7.54; N, 7.86. Found: C, 65.15; H, 7.50; N, 7.73.

Z-Val-Ile-Leu-Phe-OH (XVI)—XV (3.48 g, 6.5 mmol) was hydrogenated in MeOH (40 ml)–50% AcOH (20 ml) in the same manner as described for XI. The product (R_f^1 0.66, R_f^2 0.73) was precipitated from EtOH and allowed to react with Z-Val-OSu (2.72 g, 7.8 mmol) at room temperature for 20 h in H₂O (5 ml)–DMSO (5 ml)–DMF (40 ml) containing TEA (1.54 ml, 11 mmol). XVI was isolated in the same manner as described for XI and reprecipitated from MeOH–ether. Yield 3.17 g (76.9%). mp 227–228°C. R_f^1 0.86, R_f^2 0.78. $[\alpha]_D^{25} - 9.5^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₃₄H₄₈N₄O₇ · 1/2H₂O: C, 64.43; H, 7.79; N, 8.84. Found: C, 64.77; H, 7.89; N, 8.94.

Boc-Lys(Z)-Val-Ile-Leu-Phe-OH (IX)—XVI (1.90 g, 3 mmol) was hydrogenated in MeOH (40 ml)–*n*-BuOH (20 ml)–50% AcOH (20 ml) in the same manner as described for XI. The product (R_f^1 0.66, R_f^2 0.78) in DMSO (20 ml)–DMF (20 ml) containing TEA (0.56 ml, 6 mmol) was coupled with Boc-Lys(Z)-OSu (6 mmol) at room temperature. After 18 h, the mixture was treated in the same manner as described for XI and the product was reprecipitated from MeOH–ether. Yield 2.03 g (79.4%). mp 295–297°C (dec.). R_f^1 0.89, R_f^2 0.74. $[\alpha]_D^{25} - 15.7^\circ$ ($c = 1.0$, DMF). *Anal.* Calcd for C₄₅H₆₈N₆O₁₀: C, 63.36; H, 8.03; N, 9.85. Found: C, 63.00; H, 8.06; N, 9.88.

Boc-Gly-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH (XVII)—IX (1.11 g, 1.3 mmol) was partially deblocked with ice-chilled TFA (10 ml)–anisole (1.3 ml) in the same manner as described for XII to give H-Lys(Z)-Val-Ile-Leu-Phe-OH · TFA (IX'). R_f^1 0.75, R_f^2 0.73. IX' in DMSO (30 ml)–DMF (15 ml) was allowed to react with azide [prepared

from Boc-Gly-Ser-N₂H₃ (0.72 g, 2.6 mmol), 6 N HCl in dioxane (1.73 ml, 10.4 mmol) and isoamyl nitrite (0.35 ml, 2.6 mmol) at 4 °C for 20 h. The mixture was treated in the same manner as described for XI and the product was washed with MeOH. Yield 1.00 g (76.8%). mp 275–279 °C (dec.). *Rf*¹ 0.86, *Rf*² 0.80. $[\alpha]_D^{25} - 13.9^\circ$ (*c* = 1.0, DMF). *Anal.* Calcd for C₅₀H₇₆N₈O₁₃: C, 60.22; H, 7.68; N, 11.24. Found: C, 59.77; H, 7.70; N, 11.35. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.99 (0.82), Gly 1.06 (0.98), Val 0.61 (0.73), Ile 0.63 (0.78), Leu 1.03 (0.97), Phe 0.98 (0.91), Lys 0.93 (1.15), NH₃ 0.25 (0.43).

H-Arg-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH (IV)—Z-Arg(NO₂)-Pro-Pro-Gly-NHNHBoc⁵ (144 mg, 0.2 mmol) was treated with TFA (3 ml) in the usual manner. The product was converted to the corresponding azide with 6 N HCl in dioxane (0.167 ml, 1 mmol) and isoamyl nitrite (0.027 ml, 0.2 mmol) in DMF (4 ml) and coupled at 4 °C for 22 h with H-Gly-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH·TFA (XVII'), which was derived from XVII (100 mg, 0.1 mmol) in the same manner as described for XII. The mixture was concentrated. The residue was precipitated from 20% AcOH and reprecipitated from MeOH-AcOEt. Yield 106 mg, *Rf*¹ 0.55, *Rf*² 0.73. The solid was hydrogenated over Pd and purified by DCCC using a solvent system of *n*-BuOH-AcOH-H₂O (4 : 1 : 5) in the same manner as described for II. The lower phase fractions (2 g each, No. 11–31) were gel-filtered on a Sephadex G-25 column. Yield 57 mg (49.0%). $[\alpha]_D^{13} - 99.3^\circ$ (*c* = 1, 1 M AcOH). *Anal.* Calcd for C₅₅H₉₉N₁₅O₁₃·3AcOH·3H₂O: C, 51.80; H, 7.82; N, 14.96. Found: C, 51.80; H, 7.41; N, 15.16. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.69 (0.75), Pro 1.85 (1.92), Gly 1.86 (2.19), Val 0.67 (0.96), Ile 0.64 (0.95), Leu 0.86 (1.06), Phe 0.89 (1.04), Lys 1.12 (0.98), Arg 1.14 (1.13), NH₃ 0.34 (0.58).

Boc-Phe-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH (XVIII)—The azide [prepared from Boc-Phe-Ser-N₂H₃⁷ (257 mg, 0.70 mmol), 6 N HCl in dioxane (0.55 ml, 3.5 mmol), and isoamyl nitrite (0.094 ml, 0.70 mmol) in DMF (30 ml) in the usual manner] was coupled with IX' [prepared from IX (0.299 g, 0.35 mmol) in the same manner as described for XII] at 4 °C for 20 h. The product was isolated in the same manner as described for XI and reprecipitated from DMF-MeOH. Yield 0.32 g (83.0%). *Rf*¹ 0.85, *Rf*² 0.78. mp 292–294 °C (dec.). $[\alpha]_D^{15} - 11.2^\circ$ (*c* = 1, DMF). *Anal.* Calcd for C₅₇H₈₂N₈O₁₃: C, 62.96; H, 7.60; N, 10.31. Found: C, 62.63; H, 7.70; N, 10.41. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.92 (0.80), Val 0.52 (0.74), Ile 0.57 (0.75), Leu 0.97 (1.02), Phe 1.94 (1.88), Lys 1.09 (1.08), NH₃ 0.20 (0.17).

pGlu-Pro-Pro-Gly-Phe-Ser-Lys-Val-Ile-Leu-Phe-OH (V)—XVIII (218 mg, 0.2 mmol) was treated with TFA (5 ml)-anisole (0.5 ml) in the usual manner to give H-Phe-Ser-Lys(Z)-Val-Ile-Leu-Phe-OH·TFA (XVIII'). *Rf*¹ 0.72, *Rf*² 0.70. The azide [prepared from VI' (0.34 mmol) as described for II] was coupled with XVIII' in DMSO (20 ml)-DMF (20 ml) at 4 °C for 48 h. The product was isolated in the same manner as described for XI, washed with MeOH-AcOEt [yield 190 mg; *Rf*¹ 0.72, *Rf*² 0.67 (main spot)], hydrogenated over Pd in *n*-BuOH-MeOH-50% AcOH (20 ml : 20 ml : 10 ml) and gel-filtered on Sephadex G-25 to give crude V (41 mg), which was subjected to DCCC using *n*-BuOH-AcOH-H₂O (4 : 1 : 5) in the same manner as described for II. After elution with the lower phase (300 ml), fractions (4 g each, No. 112–121) of the upper phase eluate were gel-filtered on Sephadex G-25. Yield 30 mg (15.0%). $[\alpha]_D^{15} - 113.0^\circ$ (*c* = 0.5, 3 M AcOH). *Anal.* Calcd for C₆₁H₉₉N₁₂O₁₄·AcOH·2H₂O: C, 57.70; H, 7.53; N, 12.82. Found: C, 57.47; H, 7.26; N, 13.03. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 1.17 (1.08), Glu 0.89 (0.97), Pro 1.95 (1.99), Gly 0.93 (1.06), Val 0.56 (0.87), Ile 0.59 (0.85), Leu 0.98 (1.03), Phe 2.02 (1.95), Lys 0.85 (1.01), NH₃ 0.15 (0.27).

pGlu-Pro-Pro-Gly-Gly-Ser-Lys-Val-Ile-Leu-Phe-OH (I)—The azide [prepared from VI' (1.0 mmol)] was coupled with XVII' (0.5 mmol) in DMSO (15 ml)-DMF (10 ml) at 4 °C for 20 h. The product was isolated in the same manner as described for XI, and washed with MeOH-AcOEt. Yield 632 mg (90.7%). *Rf*¹ 0.48, *Rf*² 0.78 (main spot). The solid (500 mg) was hydrogenated in *n*-BuOH-MeOH-10% AcOH (1 : 1 : 1) (60 ml) over Pd and lyophilized from 1 M AcOH. Yield 401 mg. Crude I (100 mg) was subjected to DCCC using a solvent system of *n*-BuOH-AcOH-H₂O (4 : 1 : 5) in the same manner as described for II. The fractions (4 g each, No. 92–121) were concentrated to a small volume and passed through a column (3.2 × 90 cm) of Sephadex G-25 (fine) using 2 M AcOH as the eluent. The fractions (8 g each, No. 45–51) were lyophilized. Yield 68 mg. The product was then subjected to DCCC again using a solvent system of *n*-BuOH-pyridine-0.1% AcOH (5 : 3 : 11). The lower phase (300 ml) was used as the mobile phase. The fractions (4 g each, No. 65–85) were concentrated, lyophilized, and passed through a Sephadex G-25 column. The product was lyophilized from 2 M AcOH. Yield 56 mg (36.0%). $[\alpha]_D^{15} - 127.5^\circ$ (*c* = 1, 3 M AcOH). *Anal.* Calcd for C₅₄H₈N₁₂O₁₄·AcOH·3H₂O: C, 54.27; H, 7.64; N, 13.56. Found: C, 54.52; H, 7.30; N, 13.95. Amino acid ratios in an acid hydrolysate [24 h (48 h)]: Ser 0.93 (0.81), Glu 1.05 (1.05), Pro 1.98 (1.94), Gly 2.06 (1.95), Val 0.61 (0.82), Ile 0.58 (0.75), Leu 1.02 (0.99), Phe 0.99 (0.98), Lys 1.02 (1.05), NH₃ 0.14 (0.38).

Immunochemical Experiments

Preparation of Conjugate—A 2% aqueous solution of glutaraldehyde (1 ml) was added dropwise to an ice-cold solution of HA (7.5 mg) and crude protein extract of *Ascaris suilla* (15 mg) in H₂O (7.5 ml) during 30 min. The mixture was stirred overnight at 4 °C and taken into dialysis tubing (Spectrapor, MW cut-off 1000). The dialysis was continued at 4 °C for 20 h with two changes of H₂O (1 l each) and the dialysate was lyophilized. Yield 13 mg.

Immunization—The conjugate (3 mg) was dissolved in saline (1.5 ml) and the solution was emulsified with complete Freund's adjuvant (1.5 ml, Calbiochem-Behring) in a Sorval Omni-Mixer at 50000 rpm. The emulsion was injected subcutaneously at multiple sites on three mixed-bred male rabbits. The rabbits were boosted three times at

biweekly intervals and monthly thereafter using a half amount of the conjugate. Blood was taken at 10 d after the injection. All rabbits produced antibodies after the 5th injection.

Labeled Antigen Preparation—A solution of II (11 μg , 9.2 nmol) in H_2O (22 μl) was added to a solution of $^{125}\text{I}\text{Na}$ (250 μCi , 0.115 nmol) in 0.25 M phosphate buffer (50 μl , pH 7.40) in a glass tube, which had been filmed with 1,3,4,6-tetrachloro-3 α ,6 α -diphenyl-glycouril (0.99 μg , 2.3 nmol, Iodogen, Pierce) at the bottom.¹⁶⁾ The mixture was stirred on a test tube mixer and allowed to react for 5 min. The product was purified on a column (3.9 \times 30 mm) of Chemcosorb 7-ODS-H with a linear gradient of acetonitrile from 10% to 30% over a period of 15 min in 20 mM phosphate buffer (pH 3.0), at flow rate of 0.8 ml/min. Fractions of the highest radioactivity (retention time: 13.5 min) were used as the labelled antigen in HA-RIA.

Radioimmunoassay (RIA)—The standard diluent (SD) used was 10 mM phosphate buffer (pH 7.40), containing 0.5% bovine serum albumin (BSA), 0.025 M ethylenediaminetetraacetic acid (EDTA) and 0.15 M NaCl. The mixture in each assay tube consisted of SD (0.5 ml), peptide or tissue extract (0.1 ml), diluted antiserum (0.1 ml) and labelled antigen (0.1 ml, approximately 10000 cpm). The mixture was incubated at 4 °C overnight, and 0.5% normal rabbit serum (0.1 ml), 10% goat anti-rabbit γ -globulin serum (0.1 ml) and 30% polyethyleneglycol¹⁹⁾ in BSA free SD were added. After an additional incubation for 1 h at 4 °C, the mixture was centrifuged at 3000 rpm at 4 °C. The supernatant was removed and the radioactivity in the tube was counted.

Tissue Extraction—Rat whole intestine and whole brain were diced, immersed in a small amount of 2 M AcOH and then plunged into a boiling water bath for 5 min. The mixture was cooled and homogenized in a ten fold excess of ice-cold 2 M AcOH. A supernatant was obtained by centrifugation at 30000 $\times g$ for 20 min, and the precipitate was re-extracted in the same manner. The combined supernatant was washed with three portions of CHCl_3 and lyophilized.

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