predict that grayanotoxin II derivatives are inactive (nontoxic) because of a distortion of the relative positions of the two sites mentioned above or because there is a sterically restricted site near the C(10) position. Structural evidence does not as yet discriminate between these possibilities. These findings yield a model for the minimal features of a binding site for the lipid-soluble neutrotoxins. Further investigations must examine the differences between the fully active batrachotoxin compounds and the other partially active toxins. Attempts to model the cationic group interaction with the triangle of oxygen atoms are in progress.

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Registry No. 1, 38776-76-0; 3, 4678-45-9.

Supplementary Material Available: Fractional atomic coordinates for the hydrogen atoms, all temperature factors, bond distances and bond angles, observed and calculated structure factors for both structures, and calculated structure factors for the two enantiomers of α-H_2GTXII (40 pages). Ordering information is given on any current masthead page.

Synthesis of PHI (Peptide Histidine Isoleucine) and Related Peptides and Immunochemical Confirmation of Amino Acid Residue in Position 24 of PHI with use of the Synthetic Peptides†

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Contribution from the Laboratory of Bioorganic Chemistry, Shizuoka College of Pharmacy, Shizuoka 422, Japan, Department of Biochemistry II, Karolinska Institute, Stockholm, Sweden, and Laboratory of Cellular Metabolism, National Institute for Physiological Sciences, Okazaki, Aichi 444, Japan. *Received March 8, 1984*

Abstract: An immunochemical approach, using synthetic peptides, was employed to establish the nature of residue 24 in the amino acid sequence of PHI (peptide histidine isoleucine). PHI(20–27) and [Gln^{24}]-PHI(20–27) were synthesized by conventional solution methods and were used as haptenic immunogens for production of antisera. An antiserum raised against [Gln^{24}]-PHI(20–27), R8304, was shown to recognize specifically the glutaminyl residue in position 24. With use of this antiserum together with an anti-PHI(20–27) serum, R8201, the amino acid residue in position 24 of natural PHI preparation was radioimmunologically demonstrated to be a glutamyl and not a glutaminyl residue. Crossreactivity of a crude extract of porcine duodenum in radioimmunoassays with the two PHI antisera, R8201 and R8304, again confirmed the presence of a glutamyl residue in position 24 of the intact PHI molecule in the tissue. On the basis of these results, a heptacosapeptide amide corresponding to the proposed [Glu^{24}] sequence of PHI was synthesized by the azide fragment condensation method in solution. The synthetic preparation was identical with the natural preparation of PHI in high-performance liquid chromatography, radioimmunoassay, and bioassay. The present study not only provided an immunochemical method for confirmation of a single amino acid residue among the 27 constituent amino acids of the PHI molecule but also led to a synthetic PHI preparation identical with that of the natural peptide. This synthetic peptide provides a tool for the investigation of the physiological significance of PHI.

Porcine PHI (peptide histidine isoleucine) is a peptide of 27 amino acid residues having amino terminal histidine and carboxyl terminal isoleucine. The peptide was isolated by Tatemoto and Mutt[1] from porcine upper intestinal tissue. The peptide shows considerable sequence homology with porcine secretin and VIP (Figure 1). Tatemoto and Mutt directed their attention to the amino acid residues in position 24 in these three peptides. In secretin position 24 is occupied by a glutaminyl residue and in VIP by asparagine. These findings suggested the possibility that glutaminyl residue originally present in position 24 had been deamidated during the purification of the PHI.[1] In this study, we used a novel immonochemical approach to confirm the presence of a glutamyl residue in position 24 in the intact molecule of the PHI.

The first objective of this study was to develop a radioimmunoassay using specific antisera which could unequivocally identify the amino acid residue in position 24 of PHI. For the purpose of producing such antisera, PHI(20–27) and [Gln^{24}]-PHI(20–27) were chosen as haptenic immunogens and these two peptides were prepared by conventional solution methods (Figure 2). We employ the azide fragment condensation procedure with minimum side-chain protection for the synthesis of peptides. This strategy was used extensively in our previous studies on the syntheses of various peptides.[2a–e] The present synthesis of the two octapeptide amides proceeds along the same line and afforded homogeneous materials without resorting to complicated purification procedures.

† The amino acids except glycine are of the L configuration. The conventions and nomenclature used are those recommended by IUPAC: *Pure Appl. Chem.* **1974**, *40* 317–331.

‡ Shizuoka College of Pharmacy.

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§ National Institute for Physiological Sciences.

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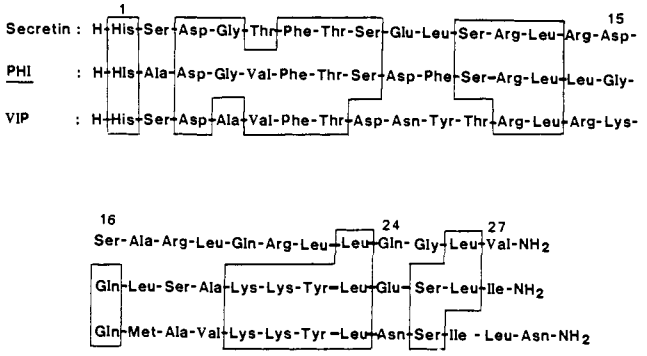

Figure 1. Comparsion of the amino acid sequences of secretin, PHI, and VIP.

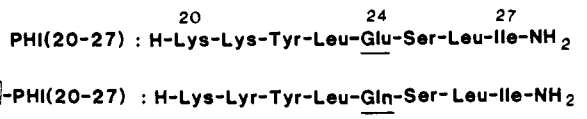

Figure 2. Two synthetic octapeptide amides used as haptenic immunogens.

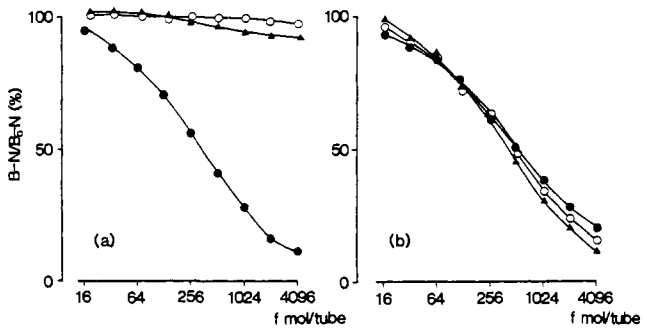

Figure 3. Inhibition curves of [Gln^{24}]-PHI(20–27) (●), PHI(20–27) (▲), and natural PHI (○) in radioimmunoassays with use of anti-[Gln^{24}]-PHI(20–27) serum R8304 (a) and anti-PHI(20–27) serum R8201 (b). Labeled antigen: ^{125}I-[Gln^{24}]-PHI(20–27) in part a and ^{125}I-PHI(20–27) in part b.

The two octapeptide amides, after having been conjugated with macromolecule carrier, were injected into rabbits. The theoretical prediction of Hopp and Woods[3] pertaining to the protein antigenic determinant does not necessarily apply to peptides, especially when macromolecule-conjugated peptides are used as immunogens. The successful production of an antiserum having the desired immunologic determinant may depend on chance. However, the use of a purposely designed synthetic peptide as the haptenic immunogen favors generation of an antiserum having the desired specificity.[4] In this context, it was fortunate that one of three rabbits which had received an injection with [Gln^{24}]-PHI(20–27)-protein conjugate generated an antiserum (R8304) that recognized specifically the glutaminyl residue in position 24. In a radioimmunoassay using this antiserum, and ^{125}I-[Gln^{24}]-PHI(20–27) as the labeled antigen, [Gln^{24}]-PHI(20–27) inhibited antibody binding of the labeled antigen in a dose-dependent manner, while the Glu^{24} analogue, PHI(20–27), showed little inhibiting activity in doses up to 4000 fmol (Figure 3a). Thus we succeeded in developing the desired radioimmunoassay that could discriminate the amino acid in position 24 of the PHI molecule. In this radioimmunoassay, purified natural PHI showed no inhibitory activity and thus behaved like the Glu^{24} analogue. This result confirmed that the amino acid residue in position 24 of natural PHI is not a glutaminyl residue.

Immunization of rabbits with the PHI(20–27)-protein conjugate also resulted in production of an antiserum (R8201). However,

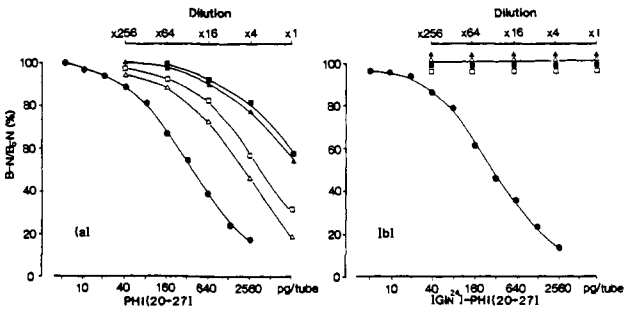

Figure 4. Dilution curves of porcine duodenal tissue crude extracts in radioimmunoassays with use of anti-PHI(20–27) serum R8201 (a) and anti-[Gln^{24}]-PHI(20–27) serum R8304 (b). The labeled antigens used in the radioimmunoassays were ^{125}I-PHI(20–27) (a) and ^{125}I-[Gln^{24}]-PHI(20–27) (b). The duodenal mucosa was extracted with boiling water (▲) or 0.1 M AcOH (△) and the duodenal muscle layer with boiling water (■) or 0.1 M AcOH (□).

the immunologic recognition site of the antiserum was not specifically restricted to the glutamyl residue in position 24. The peptide-protein conjugate against which antiserum R8201 was generated had been prepared by using a water-soluble carbodiimide as the coupling reagent. The γ-carboxyl group of the glutamyl residue may have been involved in this coupling reaction and became unavailable for immunoresponse during antiserum generation. Glutaraldehyde was alternatively used as the coupling reagent for conjugation of PHI(20–27) but the desired antiserum has not yet been obtained. In fact, antiserum R8201 crossreacted with PHI(20–27) and [Gln^{24}]-PHI(20–27) to the same extent (Figure 3b). Although the result indicated that the glutamyl residue in position 24 was not crucial for immunologic recognition of the antiserum, it was noteworthy that in a radioimmunoassay using antiserum R8201, purified natural PHI inhibited the antibody binding of ^{125}I-PHI(20–27) as did synthetic PHI(20–27).

In addition, our synthetic PHI(20–27), but not [Gln^{24}]-PHI-(20–27), crossreacted with two C-terminal specific PHI antisera raised against natural PHI. The dose–response curve of PHI-(20–27) was superimposable on that of a preparation of natural PHI in two independent radioimmunoassay systems (G. Dockray, 1982, and S. R. Bloom, 1982, personal communications).

These results demonstrated that natural PHI possesses a glutamyl residue in position 24. However, the question regarding deamidation during the purification procedure of the PHI still remained unanswered. To answer this question, crude extracts of porcine duodenal tissue were examined with the available two radioimmunoassay systems. The extraction procedures were chosen so as to minimize the possibility of deamidation of the glutaminyl residue. The tissues were extracted with hot water or dilute acetic acid. As can be seen in Figure 4, parts a and b, all the porcine duodenal crude extracts displaced the labeled antigen, ^{125}I-PHI(20–27), dose dependently in the radioimmunoassay using anti-PHI(20–27) serum R8201, while the same extracts showed no immunoreactivity in the radioimmunoassay with anti-[Gln^{24}]-PHI(20–27) serum R8304 in the concentrations examined. The parallelism between the dilution curves of the extracts and the standard curve supported the conclusion that the immunoreactive component(s) in the extracts were indistinguishable immunologically from PHI(20–27) in this assay system. It seems unlikely that the glutaminyl residue in position 24 was deaminated during the extraction processes under such mild conditions. Thus, these results provide additional support for the presence of a glutamyl, and not a glutaminyl, residue in position 24 in the native PHI molecule.

Since the physiological significance of PHI has not yet been established, it was important to have available synthetic material for this purpose. Consequently, we carried out the synthesis of PHI possessing a glutamyl residue in position 24. Moroder et al. reported[5] a synthesis of PHI by solution methodology using

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Z-Leu-Glu-Ser-Leu-Ile-NH_2	(IV)	(positions 23--27)
Z-Lys(Tos)-Lys(Tos)-Tyr-N_2H_3	(VII)	(positions 20--22)
Z-Leu-Ser-Ala-N_2H_3	(VIII)	(positions 17--19)
Z-Leu-Gly-Gln-N_2H_2-Boc	(IX)	(positions 14--16)
Z-Ser-Arg-Leu-N_2H_2-Boc	(X)	(positions 11--13)
Z-Thr-Ser-Asp-Phe-N_2H_2-Boc	(XI)	(positions 7--10)
Z-His-Ala-Asp-Gly-Val-Phe-N_2H_2-Boc	(XII)	(positions 1-- 6)

Figure 5. Protected peptide fragments used for azide fragment condensation.

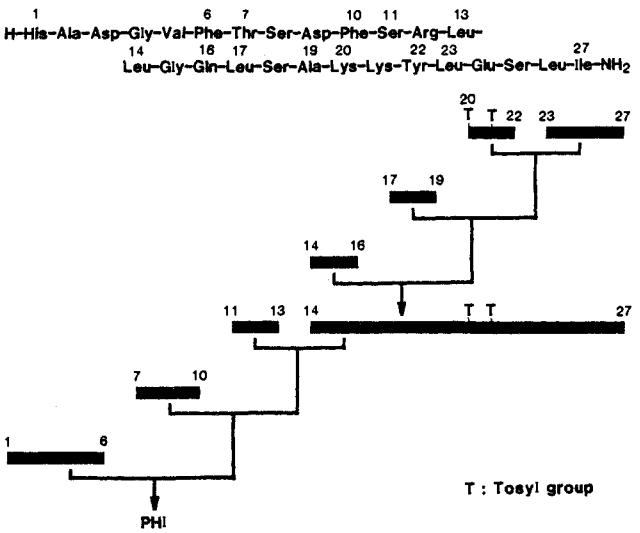

Figure 6. Schematic representation of chain elongation by azide fragment condensation in PHI synthesis.

maximum side-chain protection. In the present study, only the ϵ-amino group of the lysine residues in positions 20 and 21 was protected with the tosyl (Tos) group throughout the synthesis. The tosyl group was chosen because of its expected stability during repeated chain elongation reactions. The absence of a prolyl residue in PHI allowed the use of sodium in liquid ammonia reduction for removal of tosyl groups in the final step of the synthesis. We prefer basic deblocking which, under well-controlled conditions, proceeds without marked side reactions as was demonstrated in some of our previous synthetic works.[2b,6a,b]

The seven protected peptide fragments used for azide condensations are listed in Figure 5. Each of these protected fragments was prepared in a stepwise manner using the *N*-hydroxysuccinimide, 1-hydroxybenztriazole active ester, mixed anhydride, or azide methods. The N^{α}-amino function was protected with the benzyloxycarbonyl (Z) group which was removed by catalytic hydrogenation prior to each peptide chain elongation reaction. The ω-carboxyl function of the glutamyl or aspartyl residues was protected as the benzyl ester (OBzl) and the guanido function of the arginyl residue with the nitro (NO_2) group. These protecting groups were removed by hydrogenolysis together with the N^{α}-Z group prior to further chain elongation. Intermediates were purified mainly by crystallization, precipitation, or/and washing with a suitable aqueous or organic solvent.

Starting from the carboxyl terminal fragment, Z-Leu-Glu-Ser-Leu-Ile-NH_2 (IV) (positions 23–27), the six protected peptide fragments were consecutively coupled by the azide method (Figure 6). The *tert*-butyloxycarbonyl (Boc) group on the hydrazide function of the peptide fragments IX, X, XI, and XII was removed by acidolysis with use of trifluoroacetic acid. In the cases of less soluble peptide intermediates, the azide coupling reactions were carried out in solvent mixtures consisting of hexamethylphosphoric triamide, dimethylformamide, dimethyl sulfoxide, and water. In each coupling reaction, a 1.2–5-fold (10-fold in the final coupling) excess of the acyl component was used to assure complete acylation of the amino component. Gel chromatography on Sephadex LH-20 with 5% aqueous dimethylformamide as eluent was used effectively for purification of each coupling product. After the final coupling reaction, Z-His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (positions 1–27) was isolated by gel chromatography on Sephadex LH-20 and was, without further purification, treated with sodium in liquid ammonia. The product was easily purified by one-step semipreparative reverse-phase high-performance liquid chromatography (HPLC). The use of dilute hydrochloric acid as the aqueous phase simplified the final isolation by lyophilization. Ion-exchange column chromatography on carboxymethyl cellulose or droplet countercurrent distribution could also be used for purification. However, the recoveries of the applied material were extremely low and these procedures proved inpractical for production of larger quantities of peptide.

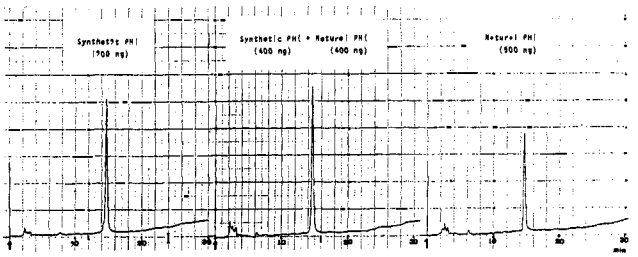

Figure 7. Elution profiles of synthetic and natural preparations of PHI in HPLC. The conditions are described in the Experimental Section.

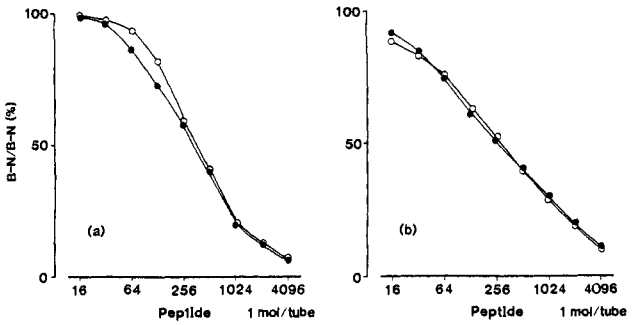

Figure 8. Dose–response curves of synthetic (●) and natural (○) preparations of PHI in radioimmunoassays with use of anti-natural PHI serum T33 (a) and anti-PHI(20–27) serum R8201 (b). Labeled antigen: ^{125}I-natural PHI.

The purified heptacosapeptide amide was compared with natural PHI in HPLC, radioimmunoassay, and bioassay. The synthetic heptacosapeptide amide coeluted with the natural PHI in HPLC in the elution systems used (Figure 7). It also behaved in the same manner as the natural PHI in two different radioimmunoassay systems with N-terminal specific anti-natural PHI serum T33 and with C-terminal specific PHI antiserum R8201 using ^{125}I-natural PHI as tracer (Figure 8). The vasodilating effects of the natural and synthetic preparations of PHI on the cat submandibular gland blood flow were almost identical (Figure 9). The synthetic heptacosapeptide amide was thus identified with the natural PHI as far as it was examined.

^{125}I-synthetic PHI did not bind to anti-[Gln^{24}]-PHI(20–27) serum R8304 under such a condition that the antiserum could bind an appropriate amount of ^{125}I-[Gln^{24}]-PHI(20–27). On the other hand, ^{125}I-synthetic PHI exhibited a binding activity against anti-PHI(20–27) serum R8201 as potent as that of ^{125}I-PHI-(20–27) and the antibody-bound labeled PHI could be displaced with synthetic PHI dose dependently (Figure 10). Porcine colon crude extract also inhibited the antibody binding of ^{125}I-synthetic PHI, and the dilution curve of the extract was parallel to the dose–response curve of synthetic PHI (Figure 10). These results

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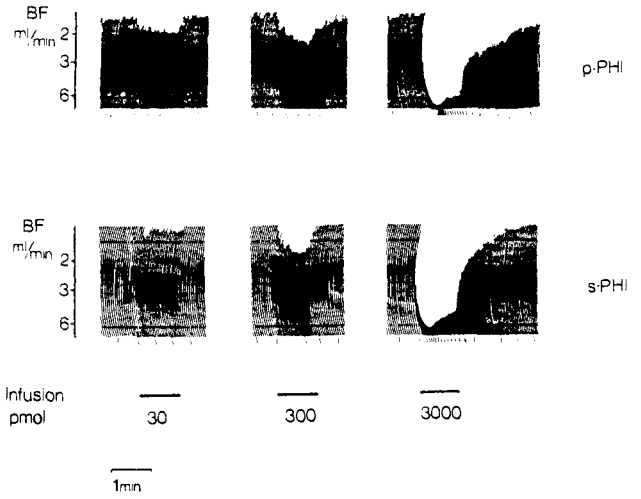

Figure 9. Effects of natural (p-PHI) and synthetic (s-PHI) preparations of PHI on cat submandibular gland blood flow. The details are in the Experimental Section.

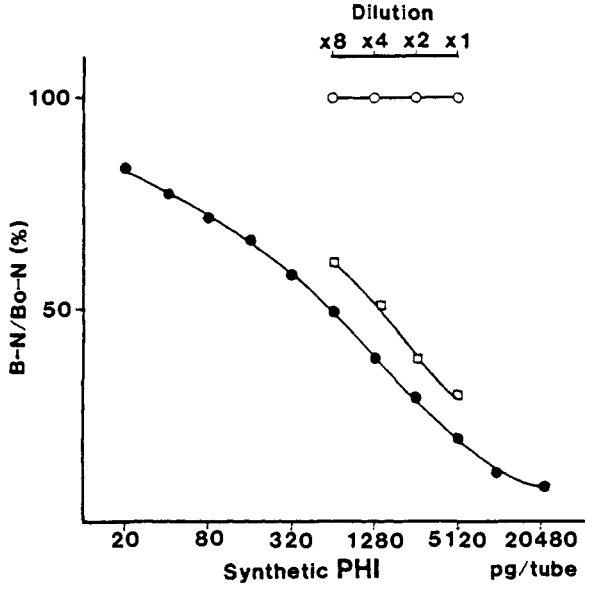

Figure 10. Inhibition curves of synthetic preparation of PHI (●), porcine colon crude extract (□), and human colon crude extract (○) in a radioimmunoassay with use of anti-PHI(20–27) serum R8201 and ^{125}I-synthetic PHI as labeled antigen.

again confirmed glutamyl residue in position 24 of the intact PHI molecule.

As can be seen in Figure 10, human colon crude extract did not displace the labeled synthetic PHI which bound to antiserum R8201. It was recently disclosed[7] by nucleotide sequence analysis that a VIP precursor protein molecule in human neuroblastoma cells contained a PHI-like peptide (PHM) sequence. The amino acid sequences of PHI and PHM differ in only two residues in positions 12 and 27. Assuming that PHM is human PHI, the result shown in Figure 10 can be considered to reflect the amino acid difference in position 27. Antiserum R8201 is supposed to recognize the extremely C-terminal portion of PHI including at least the sequence from the glutamyl residue in position 24 to isoleucyl amide in position 27, and the lack of crossreactivity of this antiserum with human colon extract strongly supports the substitution of the C-terminal residue in the proposed structure of human PHI (PHM).

We have been focusing our attention on the application of synthetic peptides to immunochemical studies not only on biologically active peptides but also on their biosynthetic precursors.[8]

In this paper, we describe an application of the immunochemical approach to the structural confirmation of a particular amino acid residue in a peptide. The synthetic preparation of PHI which is identical with that of the natural PHI will be useful in investigations designed to clarify the physiological significant of this peptide.

Experimental Section

Abbreviations used are as follows: BSA, bovine serum albumin; DCC, *N,N'*-dicyclohexylcarbodiimide; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; HMPT, hexamethylphosphoric triamide; HPLC, high-performance liquid chromatography; -OSu, *N*-hydroxysuccinimide ester; TFA, trifluoroacetic acid; TLC, thin-layer chromatography. The previously established procedures for preparation of peptide fragments are briefly described. Details of the procedures are given in our previous papers.[2a-e] The moles of peptides used as starting materials and the percent yields were calculated on the basis of elementary analysis or amino acid analysis. Melting points are uncorrected. R_f^{I}, R_f^{II}, and R_f^{III} values on TLC (Kieselgel G Type 60) refer to solvent systems 1-BuOH–AcOH–H_2O (4:1:5, v/v) (upper layer), 1-BuOH–pyridine–HOAc–H_2O (30:20:6:24, v/v), and $CHCl_3$–MeOH–HOAc (95:5:3, v/v), respectively. Optical rotations were measured on a Jasco automatic polarimeter Model DIP-4. Amino acid analyses were performed with a Hitachi amino acid analyzer Model 835. HPLC was performed on a Toyo-Soda Model HLC-803D-GE-4, and the eluates were monitored with a Toyo-Soda spectrophotometer Model UV-8-II at wavelength 210 nm. Acid hydrolysis of samples for amino acid analysis was conducted with twice-distilled 6 N HCl at 110 °C for 24 h [48 h for peptides containing Lys(Tos)] in evacuated sealed tubes. When tyrosine-containing peptides were hydrolyzed, phenol was added to the hydrolysis tubes. Amino acid ratios in acid hydrolysates were calculated on the basis of the mean moles of all the amino acids, except Ser, recovered on amino acid analysis and figures in parentheses stand for theoretical values. Aminopeptidase M digestion was carried out according to the method described by Hofmann et al.[9] All solvents were of reagent grade and were distilled before use. Evaporations were carried out in vacuo at 40–45 °C in rotary evaporators. On gel chromatography, the fractions eluted were examined by UV absorption at 278 nm and by chlorine–tolidine reagent on TLC. 1-Hydroxybenztriazole, *N*-hydroxysuccinimide, DCC, and TFA were purchased from Protein Research Foundation (Osaka). The solvents used for HPLC (CH_3CN and H_2O) were obtained from E. Merck (Darmstadt), and all other reagents and solvents were from Wako Chemicals (Tokyo). Complete Freund's adjuvant was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Synthetic Experiments. Synthesis of PHI(20–27) and [Gln^{24}]-PHI-(20–27). Z-Leu-Glu-Ser-Leu-Ile-NH$_2$ (IV) (Positions 23–27 in PHI). Z-Ile-OSu (31.1 g, 85.8 mmol) was converted to the amide in tetrahydrofuran (600 mL) by treatment with 28% NH_4OH (52.2 mL). The precipitated product was purified by washing with cold MeOH and then with Et_2O: yield 20.1 g (89%); mp 213–214 °C; R_f^{I} 0.82, R_f^{II} 0.84; $[\alpha]^{28}_D$ −3.4° (*c* 1.21, HOAc). Anal. ($C_{14}H_{20}N_2O_3$): C, H, N. Z-Ile-NH_2 (10.6 g, 40 mmol) was debenzyloxycarbonylated by treatment with 25% HBr in HOAc (20 mL). The deblocked amide hydrobromide in a mixture of tetrahydrofuran (40 mL) and H_2O (5 mL) containing triethylamine (5.6 mL) was coupled with Z-Leu-OSu (18.1 g, 50 mmol) in tetrahydrofuran (40 mL). The product was precipitated by adding H_2O and was purified by reprecipitation from MeOH–EtOAc to give Z-Leu-Ile-NH_2: yield 13.8 g (91%); mp 227–228 °C; R_f^{I} 0.86, R_f^{II} 0.88; $[\alpha]^{28}_D$ −28.9° (*c* 1.22, HOAc). Anal. ($C_{20}H_{31}N_3O_3$); C, H, N. Z-Leu-Ile-NH_2 (3.57 g, 9.46 mmol) was debenzyloxycarbonylated by treatment with 25% HBr in HOAc (13 mL): R_f^{I} 0.63, R_f^{II} 0.76. The deblocked dipeptide amide hydrobromide in DMF (50 mL) containing H_2O (5 mL) and triethylamine (1.32 mL) was coupled with the azide prepared from Z-Ser-N_2H_3 (4.78 g, 19 mmol) in DMF (30 mL) with 6 N HCl in dioxane (9.44 mL) and isoamyl nitrite (2.53 mL). The product was precipitated by adding H_2O and purified by reprecipitation from DMF–Et_2O to give Z-Ser-Leu-Ile-NH_2: yield 3.50 g (80%); mp 222–223 °C; R_f^{I} 0.84, R_f^{II} 0.85; $[\alpha]^{27}_D$ −35.7° (*c* 1.21, HOAc). Anal. ($C_{23}H_{36}N_4O_6$): C, H, N. Z-Ser-Leu-Ile-NH_2 (3.10 g, 6.67 mmol) was hydrogenated to H-Ser-Leu-Ile-NH_2·HOAc over Pd in a mixture of MeOH (50 mL), 1-BuOH (50 mL), H_2O (50 mL), and HOAc (10 mL): R_f^{I} 0.55, R_f^{II} 0.77. The deblocked tripeptide amide acetate in DMF (20 mL) containing triethylamine (0.93 mL) was coupled, in the presence of *N*-hydroxysuccinimide (1.73 g, 15 mmol), with the mixed anhydride prepared from Z-Glu(OBzl)-OH (3.49 g, 9.40 mmol) with *N*-methyl-

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morpholine (1.03 mL) and isobutyl chloroformate (1.24 mL). The product was precipitated by adding 1 N citric acid and purified by reprecipitation from DMF–Et_2O to afford Z-Glu(OBzl)-Ser-Leu-Ile-NH_2: yield 4.32 g (93%); mp 246 °C dec; R_f^I 0.83, R_f^{II} 0.84; $[\alpha]^{27}_D$ −29.8° (*c* 1.05, HOAc). Anal. ($C_{35}H_{49}N_5O_9 \cdot 0.5H_2O$): C, H, N. Z-Glu-(OBzl)-Ser-Leu-Ile-NH_2 (2.63 g, 3.80 mmol) was hydrogenated over Pd in a mixture of MeOH (30 mL), 1-BuOH (50 mL), H_2O (50 mL), and HOAc (20 mL) to give H-Glu-Ser-Leu-Ile-NH_2·HOAc: R_f^I 0.61, R_f^{II} 0.73. The deblocked tetrapeptide amide acetate in a mixture of DMF (100 mL), HMPT (5 mL), and H_2O (50 mL) containing triethylamine (1.08 mL) was coupled with Z-Leu-OSu (3.89 g, 10.7 mmol) in DMF (15 mL). The product was precipitated by adding H_2O and purified by reprecipitation from DMF–Et_2O to give IV: yield 2.54 g (92%); mp 240–243 °C; R_f^I 0.81, R_f^{II} 0.82; $[\alpha]^{28}_D$ −17.4° (*c* 1.15, DMF). Anal. ($C_{34}H_{54}N_6O_{10} \cdot H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Ser(1) 0.82, Glu(1) 1.00, Ile(1) 0.97, Leu(2) 2.03.

Z-Leu-Gln-Ser-Leu-Ile-NH_2 (V) [Positions 23–27 in [Gln^{24}]-PHI(20–27)]. Z-Leu-OSu (7.25 g, 20 mmol) in tetrahydrofuran (200 mL) was coupled with H-Gln-OH (3.51 g, 24 mmol) in H_2O (100 mL). The product was extracted with AcOEt and purified by recrystallization from AcOEt–Et_2O to afford Z-Leu-Gln-OH: yield 6.09 g (77%); mp 126–128 °C; R_f^I 0.72, R_f^{II} 0.74; $[\alpha]^{29}_D$ −8.0° (*c* 1.07, DMF). Anal. ($C_{19}H_{27}N_3O_6$): C, H, N. Z-Leu-Gln-OH (1.97 g, 5 mmol) was converted to the mixed anhydride in DMF (10 mL) with *N*-methylmorpholine (0.51 mL) and isobutyl chloroformate (0.65 mL) and coupled with H-Ser-Leu-Ile-NH_2·HOAc in DMF (30 mL) prepared from the previously obtained Z-Ser-Leu-Ile-NH_2 (2.44 g, 5.25 mmol) by catalytic hydrogenation. The product was precipitated by adding 1 N citric acid and purified by reprecipitation from DMF–MeOH to give V: yield 2.90 g (76%); mp 251 °C dec; R_f^I 0.74, R_f^{II} 0.82; $[\alpha]^{27}_D$ −16.6° (*c* 1.13, DMF). Anal. ($C_{34}H_{55}N_7O_9 \cdot H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Ser(1) 0.93, Glu(1) 0.97, Ile(1) 1.01, Leu(2) 2.02.

Z-Lys(Z)-Lys(Z)-Tyr-N_2H_3 (VI) Positions 20–22). H-Tyr-OMe·HCl (3.48 g, 15 mmol) in DMF (10 mL) containing triethylamine (2.10 mL) was coupled with the mixed anhydride prepared from Boc-Lys(Z)-OH (5.71 g, 15 mmol) in tetrahydrofuran (15 mL) with *N*-methylmorpholine (1.53 mL) and isobutyl chloroformate (1.58 mL). The product was extracted with AcOEt and purified by reprecipitation from AcOEt–petroleum ether to give Boc-Lys(Z)-Tyr-OMe: yield 6.67 g (80%); mp 60–61 °C; R_f^I 0.77, R_f^{II} 0.74; $[\alpha]^{27}_D$ −4.4° (*c* 1.34, MeOH). Anal. ($C_{29}H_{39}N_3O_8$): C, H, N. Boc-Lys(Z)-Tyr-OMe (2.23 g, 4 mmol) was treated with TFA (5 mL) to give H-Lys(Z)-Tyr-OMe·TFA—R_f^I 0.70, R_f^{II} 0.77—which, in DMF (10 mL) containing *N*-methylmorpholine (0.44 mL) was coupled with the mixed anhydride prepared from Z-Lys(Z)-OH (1.66 g, 4 mmol) in tetrahydrofuran (10 mL) with *N*-methylmorpholine (0.44 mL) and isobutyl chloroformate (0.52 mL). The product was extracted with AcOEt and purified by reprecipitation with MeOH–Et_2O to yield Z-Lys(Z)-Lys(Z)-Tyr-OMe: yield 2.86 g (84%); mp 97–98 °C; R_f^I 0.91, R_f^{II} 0.92; $[\alpha]^{27}_D$ −2.4° (*c* 1.28, DMF). Anal. ($C_{46}H_{55}N_5O_{11}$): C, H, N. Z-Lys(Z)-Lys(Z)-Tyr-OMe (1.50 g, 1.76 mmol) was treated with hydrazine hydrate (0.90 mL) in a mixture of MeOH (5 mL) and DMF (5 mL). The product precipitated was purified by reprecipitation from DMF–Et_2O to give VI: yield 1.50 g (theoretical yield); mp 153–154 °C; R_f^I 0.90, R_f^{II} 0.92; $[\alpha]^{24}_D$ −15.5° (*c* 0.96, DMF). Anal. ($C_{45}H_{55}N_7O_{10}$): C, H, N.

PHI(20–27): H-Lys-Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (II). Compound IV (705 mg, 0.97 mmol) was hydrogenated over Pd in 30% aqueous HOAc (40 mL) and 1-BuOH (10 mL) to give H-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc—R_f^I 0.63, R_f^{II} 0.76—which, in a mixture of DMF (10 mL) and H_2O (2 mL) containing triethylamine (0.14 mL), was coupled with the azide prepared from compound VI (1.02 g, 1.20 mmol) in DMF (10 mL) with 6 N HCl in dioxane (0.6 mL) and isoamyl nitrite (0.16 mL). The product was precipitated by adding H_2O and purified by washing with hot MeOH to give Z-Lys(Z)-Lys(Z)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2: yield 1.16 g (84%); mp 251 °C dec; R_f^I 0.80, R_f^{II} 0.81; $[\alpha]^{30}_D$ −20.3° (*c* 1.18, DMF). Anal. ($C_{71}H_{99}N_{11}O_{18} \cdot H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 2.10, Ser(1) 0.80, Glu(1) 0.97, Ile(1) 0.95, Leu(2) 1.99, Tyr(1) 0.99. The protected octapeptide amide (200 mg, 0.14 mmol) was hydrogenated over Pd in 20% aqueous HOAc (20 mL) to give II·3HOAc: yield 170 mg (theoretical yield); R_f^I 0.27, R_f^{II} 0.59; $[\alpha]^{29}_D$ −39.5° (*c* 1.05, 1 M HOAc). Amino acid ratios in acid hydrolysate: Lys(2) 2.02, Ser(1) 0.82, Glu(1) 0.97, Ile(1) 0.98, Leu(2) 2.03, Tyr(1) 1.00. The product showed a single sharp peak at a retention time of 4.1 min in reverse-phase HPLC on a C_{18} TSK-GEL LS-410K column (0.4 × 30 cm) in 0.01 N HCl/CH_3CN (75:25, v/v) at a flow rate of 1.0 mL/min.

[Gln^{24}]-PHI(20–27): H-Lys-Lys-Tyr-Leu-Gln-Ser-Leu-Ile-NH_2 (III). Compound V (706 mg, 0.98 mmol) was hydrogenated over Pd in a mixture of MeOH (20 mL), 1-BuOH (20 mL), H_2O (20 mL), and HOAc (15 mL) to give H-Leu-Gln-Ser-Leu-Ile-NH_2·HOAc—R_f^I 0.49, R_f^{II} 0.74—which, in DMF (30 mL) containing H_2O (2 mL) and triethylamine (0.41 mL), was coupled with the azide prepared from compound VI (1.02 g, 1.20 mmol) in DMF (10 mL) with 6 N HCl in dioxane (0.60 mL) and isoamyl nitrite (0.16 mL). The product was precipitated by adding 1 N citric acid and purified by reprecipitation from DMF–MeOH to give Z-Lys(Z)-Lys(Z)-Tyr-Leu-Gln-Ser-Leu-Ile-NH_2: yield 1.03 g (75%); mp 256 °C dec; R_f^I 0.81, R_f^{II} 0.90; $[\alpha]^{27}_D$ −18.9° (*c* 1.10, DMF). Anal. ($C_{71}H_{100}N_{12}O_{17} \cdot H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 2.11, Ser(1) 0.89, Glu(1) 0.99, Ile(1) 0.96, Leu(2) 1.94, Tyr(1) 1.00. The protected octapeptide amide (320 mg, 0.23 mmol) was hydrogenated over Pd in a mixture of DMF (50 mL) and 20% aqueous HOAc (10 mL). The product was chromatographed on a Sephadex G10 column (2.5 × 140 cm) with 1 M HOAc as eluent to give III·3HOAc: yield 215 mg (80%); R_f^I 0.20, R_f^{II} 0.64; $[\alpha]^{24}_D$ −40.0° (*c* 1.02, 1 M HOAc). Amino acid ratios in acid hydrolysate: Lys(2) 2.03, Ser(1) 0.87, Glu(1) 0.98 Ile(1) 0.98, Leu(2) 1.99, Tyr(1) 1.02. The preparation was eluted as a single sharp peak at a retention time of 3.8 min in reverse-phase HPLC on a C_{18} TSK-GEL LS-410K (0.4 × 30 cm) column in 0.01 N HCl/CH_3CN (75:25, v/v) at a flow rate of 1.0 mL/min.

Synthesis of PHI. Preparation of Protected Peptide Fragments. Z-Lys(Tos)-Lys(Tos)-Tyr-N_2H_3 (VII) (Positions 20–22). Z-Lys(Tos)-OH (17.4 g, 40 mmol) in tetrahydrofuran (20 mL) was coupled with H-Tyr-OMe·HCl (9.27 g, 40 mmol) in DMF (25 mL) containing triethylamine (5.6 mL, 40 mmol) in the presence of 1-hydroxybenztriazole (6.49 g, 48 mmol) and DCC (9.08 g, 44 mmol). The product was purified by recrystallization from AcOEt–petroleum ether to give Z-Lys(Tos)-Tyr-OMe: yield 21.0 g (86%); mp 141–143 °C; R_f^I 0.90, R_f^{II} 0.89; $[\alpha]^{28}_D$ −3.0° (*c* 1.29, MeOH). Anal. ($C_{31}H_{37}N_3O_8S$): C, H, N. Z-Lys(Tos)-Tyr-OMe (6.11 g, 10 mmol) was hydrogenated to H-Lys-(Tos)-Tyr-OMe·HCl over Pd in MeOH (100 mL) containing 1 N HCl (10 mL)—R_f^I 0.69, R_f^{II} 0.80—which, in DMF (20 mL) containing triethylamine (1.4 mL), was coupled with Z-Lys(Tos)-OH (4.34 g, 10 mmol) in DMF (20 mL) in the presence of 1-hydroxybenztriazole (1.62 g, 12 mmol) and DCC (2.25 g, 11 mmol). The product was purified by reprecipitation with AcOEt–petroleum ether to yield Z-Lys(Tos)-Lys-(Tos)-Tyr-OMe: yield 7.83 g (88%); mp 106–107 °C; R_f^I 0.95, R_f^{II} 0.95, R_f^{III} 0.43; $[\alpha]^{29}_D$ −6.8° (*c* 1.12, MeOH). Anal. ($C_{44}H_{55}N_5O_{11}S_2$): C, H, N. The protected tripeptide methyl ester (4.47 g, 5 mmol) was converted to the hydrazide VII with hydrazine hydrate (2.5 mL) in MeOH (30 mL) followed by reprecipitation from DMF–MeOH–Et_2O: yield 4.38 g (98%); mp 188–189 °C; R_f^I 0.91, R_f^{II} 0.90, R_f^{III} 0.12; $[\alpha]^{28}_D$ −9.7° (*c* 1.07, DMF). Anal. ($C_{43}H_{55}N_7O_{10}S_2$): C, H, N.

Z-Leu-Ser-Ala-N_2H_3 (VIII) (Positions 17–19). Z-Ser-Ala-OMe[10] (3.96 g, 12.2 mmol) was hydrogenated over Pd in MeOH (100 mL) containing 1 N HCl (12.2 mL)—R_f^I 0.43, R_f^{II} 0.69—which, in H_2O (30 mL) containing triethylamine (1.7i mL), was coupled with the *N*-hydroxysuccinimide ester of Z-Leu-OH (5.68 g, 15.7 mmol) in tetrahydrofuran (50 mL). The product was purified by washing with AcOEt followed by precipitation from MeOH–Et_2O to yield Z-Leu-Ser-Ala-OMe: yield 4.02 g (75%); mp 180–181 °C; R_f^I 0.79, R_f^{II} 0.85; $[\alpha]^{28}_D$ −41.2° (*c* 1.05, MeOH). Anal. ($C_{21}H_{31}N_3O_7$): C, H, N. Z-Leu-Ser-Ala-OMe (3.33 g, 7.61 mmol) was converted to the hydrazide, VIII, with hydrazine hydrate (3.7 mL) in MeOH (50 mL) followed by reprecipitation from DMF–Et_2O: yield 3.03 g (91%); mp 228–229 °C; R_f^I 0.68, R_f^{II} 0.82; $[\alpha]^{28}_D$ −32.9° (*c* 1.00, HOAc). Anal. ($C_{20}H_{31}N_5O_6$): C, H, N. Amino acid ratios in acid hydrolysate: Ser(1) 0.82, Ala(1) 1.00, Leu(1) 1.00.

Z-Leu-Gly-Gln-N_2H_2-Boc (IX) (Positions 14–16). Z-Gln-N_2H_2-Boc (7.89 g, 20 mmol) was hydrogenated to H-Gln-N_2H_2-Boc over Pd in MeOH (100 mL): R_f^I 0.47, R_f^{II} 0.65. Z-Leu-Gly-OH[11] (5.04 g, 19 mmol) was converted to the mixed anhydride with *N*-methylmorpholine (1.94 mL) and isobutyl chloroformate (2.50 mL) in DMF (20 mL) and coupled with the above H-Gln-N_2H_2-Boc in DMF (20 mL). The product was purified by reprecipitation from MeOH–Et_2O to give IX: yield 7.41 g (69%); mp 145–147 °C; R_f^I 0.82, R_f^{II} 0.81; $[\alpha]^{27}_D$ −23.5° (*c* 1.03, MeOH). Anal. ($C_{26}H_{40}N_6O_8$): C, H, N. Amino acid ratios in acid hydrolysate: Glu(1) 0.98, Gly(1) 1.02, Leu(1) 1.00.

Z-Ser-Arg-Leu-N_2H_2-Boc (X) (Positions 11–13). Z-Leu-OH (dicyclohexylamine salt: 14.7 g, 33 mmol) was converted to the mixed anhydride with *N*-methylmorpholine (3.06 mL) and isobutyl chloroformate (3.96 mL) in tetrahydrofuran (45 mL) and coupled with NH_2NH-Boc (4.35 g, 33 mmol) in tetrahydrofuran (30 mL). The product was obtained in oily form: yield 10.2 g (89%); R_f^I 0.76, R_f^{II} 0.80. The oily preparation of Z-Leu-N_2H_2-Boc was hydrogenated to H-Leu-

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N_2H_2-Boc over Pd in MeOH (100 mL)—R_f^I 0.58, R_f^{II} 0.78—which, in DMF (20 mL), was coupled with the mixed anhydride prepared from Z-Arg(NO_2)-OH (7.56 g, 21.4 mmol) with *N*-methylmorpholine (2.18 mL) and isobutyl chloroformate (2.82 mL) in DMF (40 mL). The product was purified by reprecipitation from MeOH–Et_2O to afford Z-Arg(NO_2)-Leu-N_2H_2-Boc: yield 9.60 g (77%); mp 139–141 °C; R_f^I 0.83, R_f^{II} 0.81; $[\alpha]^{27}_D$ −39.3° (*c* 1.01, MeOH). Anal. ($C_{25}H_{40}N_8O_8$): C, H, N. Z-Arg(NO_2)-Leu-N_2H_2-Boc (3.89 g, 6.70 mmol) was hydrogenated to H-Arg-Leu-N_2H_2-Boc·2HOAc over Pd in a mixture of MeOH (15 mL) and 25% aqueous HOAc (15 mL): R_f^I 0.61, R_f^{II} 0.35. The resulting dipeptide Boc-hydrazide diacetate in DMF (20 mL) containing triethylamine (0.94 mL) was coupled with the azide prepared from Z-Ser-N_2H_3 (2.04 g, 8.04 mmol) in DMF (15 mL) with 6 N HCl in dioxane (4.02 mL) and isoamyl nitrite (1.08 mL). The product was purified by reprecipitation from MeOH–Et_2O to give X·2HOAc and converted to the hydrochloride form by lyophilization from diluted HCl: yield 3.16 g (71%); mp 135–137 °C; R_f^I 0.67, R_f^{II} 0.75; $[\alpha]^{28}_D$ −31.8° (*c* 1.07, MeOH). Anal. ($C_{28}H_{46}N_8O_8$·HCl·0.5H_2O): C, H, N. Amino acid ratios in acid hydrolysate: Arg(1) 0.97, Ser(1) 1.00, Leu(1) 1.03.

Z-Thr-Ser-Asp-Phe-N_2H_2-Boc (XI) (Positions 7–10). Z-Phe-N_2H_2-Boc (5.01 g, 12 mmol) was hydrogenated to H-Phe-N_2H_2-Boc over Pd in MeOH (50 mL)—R_f^I 0.70, R_f^{II} 0.80—which, in tetrahydrofuran (20 mL), was then coupled with the mixed anhydride prepared from Z-Asp(OBzl)-OH (3.90 g, 11 mmol) with *N*-methylmorpholine (1.20 mL) and isobutyl chloroformate (1.43 mL) in tetrahydrofuran (20 mL). The product was purified by recrystallization from AcOEt–Et_2O to give Z-Asp(OBzl)-Phe-N_2H_2-Boc: yield 5.70 g (84%); mp 124–125 °C; R_f^I 0.81, R_f^{II} 0.86; $[\alpha]^{27}_D$ −34.8° (*c* 1.79, MeOH). Anal. ($C_{33}H_{38}N_4O_8$): C, H, N. Z-Thr-Ser-OMe[12] (4.00 g, 11 mmol) was converted to the hydrazide with hydrazine hydrate (6 mL) in EtOH (20 mL): yield 3.49 g (87%); mp 233–234 °C; R_f^I 0.80, R_f^{II} 0.71; $[\alpha]^{28}_D$ −13.5° (*c* 1.30, DMF). Anal. ($C_{15}H_{22}N_4O_6$): C, H, N. The above Z-Asp(OBzl)-Phe-N_2H_2-Boc (3.07 g, 5 mmol) was hydrogenated to H-Asp-Phe-N_2H_2-Boc over Pd in MeOH (50 mL)—R_f^I 0.54, R_f^{II} 0.68—which, in DMF (10 mL) containing triethylamine (2.08 mL), was coupled with the azide prepared from Z-Thr-Ser-N_2H_3 (1.76 g, 5 mmol) in DMF (10 mL) with 6 N HCl in dioxane (2.48 mL) and isoamyl nitrite (0.67 mL). The product was purified by reprecipitation from MeOH–Et_2O to give XI: yield 2.47 g (69%); mp 139–141 °C; R_f^I 0.69, R_f^{II} 0.69; $[\alpha]^{28}_D$ −38.3° (*c* 1.03, MeOH). Anal. ($C_{33}H_{44}N_6O_{12}$·0.5H_2O): C, H, N. Amino acid ratios in acid hydrolysate: Asp(1) 1.00, Ser(1) 0.91, Thr(1) 0.99, Phe(1) 1.01.

Z-His-Ala-Asp-Gly-Val-Phe-N_2H_2-Boc (XII) (Positions 1–6). Z-Val-Phe-N_2H_2-Boc[13] (4.88 g, 9.52 mmol) was hydrogenated over Pd in MeOH (80 mL) to give H-Val-Phe-N_2H_2-Boc—R_f^I 0.58, R_f^{II} 0.76—and coupled with the mixed anhydride prepared from Z-Gly-OH (1.99 g, 9.52 mmol) in tetrahydrofuran (10 mL) with *N*-methylmorpholine (0.97 mL) and isobutyl chloroformate (1.26 mL). The product was purified by reprecipitation from AcOEt–petroleum ether to afford Z-Gly-Val-Phe-N_2H_2-Boc: yield 4.42 g (82%); mp 128–130 °C; R_f^I 0.90, R_f^{II} 0.88; $[\alpha]^{28}_D$ −37.8° (*c* 1.27, MeOH). Anal. ($C_{29}H_{39}N_5O_7$): C, H, N. Z-Gly-Val-Phe-N_2H_2-Boc (4.33 g, 7.60 mmol) was hydrogenated to H-Gly-Val-Phe-N_2H_2-Boc over Pd in MeOH (50 mL)—R_f^I 0.62, R_f^{II} 0.74—which, in a mixture of DMF (10 mL), tetrahydrofuran (20 mL), and *N*-methylmorpholine (0.84 mL), was coupled with the mixed anhydride prepared from Z-Asp(OBzl)-OH (2.72 g, 7.60 mmol) in tetrahydrofuran (30 mL) with *N*-methylmorpholine (0.84 mL) and isobutyl chloroformate (1.00 mL). The product was purified by reprecipitation from DMF–Et_2O to give Z-Asp(OBzl)-Gly-Val-Phe-N_2H_2-Boc: yield 5.29 g (90%); mp 154–156 °C; R_f^I 0.88, R_f^{II} 0.89; $[\alpha]^{28}_D$ −19.8° (*c* 1.25, DMF). Anal. ($C_{40}H_{50}N_6O_{10}$): C, H, N. Z-His-N_2H_3 (5.26 g, 17.3 mmol) was coupled with H-Ala-OMe·HCl (2.66 g, 19.1 mmol) in DMF (10 mL) containing triethylamine (2.67 mL) via the azide prepared with 6 N HCl in dioxane (5.78 mL) and isoamyl nitrite (2.31 mL) in DMF (20 mL). The product was purified by recrystallization from MeOH to give Z-His-Ala-OMe: yield 4.31 g (65%); mp 137–140 °C; R_f^I 0.55, R_f^{II} 0.55; $[\alpha]^{28}_D$ −15.0° (*c* 1.01, DMF). Anal. ($C_{18}H_{22}N_4O_5$·0.5H_2O): C, H, N. Z-His-Ala-OMe (2.00 g, 5.22 mmol) was converted to the hydrazide with hydrazine hydrate (2.7 mL) in DMF (5 mL) and MeOH (1 mL): yield 1.68 g (86%); mp 190–192 °C; R_f^I 0.40, R_f^{II} 0.71; $[\alpha]^{28}_D$ −3.4° (*c* 1.03, DMF). Anal. ($C_{17}H_{22}N_6O_4$): C, H, N. Z-Asp(OBzl)-Gly-Val-Phe-N_2H_2-Boc (2.00 g, 2.58 mmol) was hydrogenated to H-Asp-Val-Phe-N_2H_2-Boc over Pd in DMF (70 mL) and MeOH (30 mL)—R_f^I 0.52, R_f^{II} 0.65—which, in DMF (20 mL) and H_2O (5 mL), was coupled with the azide prepared from Z-His-Ala-N_2H_3 (1.16 g, 3.10 mmol) in DMF (15 mL) with 6 N HCl in dioxane (1.55 mL) and isoamyl nitrite (0.41 mL). The product was purified by reprecipitation from DMF–Et_2O to yield XII: yield 1.70 g (72%); mp 198–200 °C; R_f^I 0.68, R_f^{II} 0.79; $[\alpha]^{28}_D$ −23.5° (*c* 1.10, DMF). Anal. ($C_{42}H_{56}N_{10}O_{12}$·1.5H_2O): C, H, N. Amino acid ratios in acid hydrolysate: His(1) 0.99, Asp(1) 1.03, Gly(1) 1.01, Ala(1) 1.02, Val(1) 0.95, Phe(1) 1.00.

Fragment Condensation. Z-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (XIII) (Positions 20–27). Compound IV (2.25 g, 3.10 mmol) was hydrogenated to H-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc in the same manner as described in the preparation of PHI(20–27). Compound VII (2.84 g, 3.18 mmol) was converted to the azide in DMF (30 mL) with 6 N HCl in dioxane (1.59 mL) and isoamyl nitrite (0.42 mL) at −18 °C, which, after being neutralized with triethylamine, was combined at −15 °C with a stirred solution of the above deprotected pentapeptide amide acetate in a mixture of DMF (70 mL) and H_2O (30 mL) containing triethylamine (0.45 mL). The mixture was stirred at −15 °C for 2 h and at 4 °C for 20 h. The solvents were evaporated and the residue was solidified by adding 1 N citric acid. The precipitate was collected by filtration and washed with H_2O. The product was purified by precipitation from DMF–AcOEt–Et_2O to give XIII: yield 4.36 g (97%); mp 240–241 °C dec; R_f^I 0.83, R_f^{II} 0.79; $[\alpha]^{29}_D$ −17.6° (*c* 1.06, DMF). Anal. ($C_{69}H_{99}N_{11}O_{18}S_2$·$H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 2.08, Ser(1) 0.72, Glu(1) 1.00, Ile(1) 0.95, Leu(2) 1.99, Tyr(1) 0.98.

Z-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (XIV) (Positions 17–27). Compound XIII (2.05 g, 1.41 mmol) was hydrogenated over Pd in a mixture of 1-BuOH (30 mL), MeOH (40 mL), HOAc (15 mL), and H_2O (50 mL) to give H-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc: R_f^I 0.79, R_f^{II} 0.79. Compound VIII (750 mg, 1.71 mmol) was converted to the azide in DMF (10 mL) with 6 N HCl in dioxane (0.86 mL) and isoamyl nitrite (0.23 mL) at −18 °C, which, after being neutralized with triethylamine, was combined at −15 °C with a stirred solution of the above hydrogenated octapeptide amide acetate in a mixture of DMF (20 mL) and H_2O (2 mL) containing triethylamine (0.20 mL). The mixture was treated in the same manner as described in the preparation of XIII. The product was purified by precipitation from DMF–AcOEt to give XIV: yield 2.10 g (86%); mp 232–233 °C dec; R_f^I 0.83, R_f^{II} 0.85; $[\alpha]^{29}_D$ −5.9° (*c* 0.82, DMF), $[\alpha]^{24}_D$ −21.7° (*c* 1.15, HOAc). Anal. ($C_{81}H_{120}N_{14}O_{22}S_2$·$H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 1.95, Ser(2) 1.64, Glu(1) 1.02, Ala(1) 1.02, Ile(1) 0.93, Leu(3) 3.06, Tyr(1) 1.00.

Z-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (XV) (Positions 14–27). Compound XIV (460 mg, 0.27 mmol) was hydrogenated over Pd in a mixture of DMF (10 mL), 1-BuOH (20 mL), HOAc (10 mL), and H_2O (30 mL) to give H-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc: R_f^I 0.67, R_f^{II} 0.79. Compound IX (760 mg, 1.35 mmol) was treated with TFA (1 mL) to give Z-Leu-Gly-Gln-N_2H_3·TFA—R_f^I 0.65, R_f^{II} 0.79—and converted to the azide in DMF (10 mL) at −18 °C with 6 N HCl in dioxane (0.67 mL) and isoamyl nitrite (0.18 mL). The solution, after being neutralized with triethylamine, was combined at −15 °C with a stirred solution of the above hydrogenated undecapeptide amide acetate in DMF (10 mL) containing triethylamine (0.18 mL). The mixture was treated in the same manner as described in the preparation of XIII. The product was purified by precipitation from DMF–MeOH followed by gel chromatography on Sephadex LH-20 using 5% aqueous DMF to give XV: yield 480 mg (88%); mp 210 °C dec; R_f^I 0.76, R_f^{II} 0.83; $[\alpha]^{29}_D$ −1.2° (*c* 1.00, DMF), $[\alpha]^{24}_D$ −19.7° (*c* 0.70, 65% aqueous HOAc). Anal. ($C_{94}H_{142}N_{18}O_{26}S_2$·$2H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 1.94, Ser(2) 1.54, Glu(2) 2.01, Gly(1) 1.08, Ala(1) 1.05, Ile(1) 0.94, Leu(4) 4.03, Tyr(1) 0.95.

Z-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (XVI) (Positions 11–27). Compound XV (444 mg, 218 μmol) was hydrogenated over Pd in a mixture of 1-BuOH (15 mL), MeOH (20 mL), HOAc (3 mL), and H_2O (7 mL) to give H-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc: R_f^I 0.57, R_f^{II} 0.79. Compound X (hydrochloride 280 mg, 420 μmol) was converted to the azide in DMF (10 mL) with 6 N HCl in dioxane (0.25 mL) and isoamyl nitrite (66 μL) at −18 °C, which, after being neutralized with triethylamine, was combined with the above hydrogenated tetradecapeptide amide in a mixture of DMF (5 mL) and H_2O (2 mL) containing triethylamine (62 μL) at −15 °C. The mixture was treated in the same manner as described in the preparation of XIII. The product was purified by precipitation from DMF–AcOEt to give XVI·HCl: yield 531 mg (theoretical yield); mp 216 °C dec; R_f^I 0.65, R_f^{II} 0.79; $[\alpha]^{19}_D$ −19.5° (*c* 1.19, 50% aqueous HOAc). Anal. ($C_{109}H_{170}N_{24}O_{30}S_2$·HCl·$2H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 1.89, Arg(1) 1.15, Ser(3) 2.63, Glu(2) 1.89, Gly(1) 1.04, Ala(1) 0.99, Ile(1) 0.98, Leu(5) 5.08, Tyr(1) 0.98.

Z-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (XVII) (Positions 7–27).

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Compound XVI·HCl (463 mg, 190 μmol) was hydrogenated over Pd in 50% aqueous HOAc to give H-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HCl·HOAc: R_f^I 0.44, R_f^{II} 0.69. Compound XI (717 mg, 1.00 mmol) was treated with TFA (2 mL) to give Z-Thr-Ser-Asp-Phe-N_2H_3·TFA—R_f^I 0.68, R_f^{II} 0.69—and converted to the azide in DMF (10 mL) at −18 °C with 6 N HCl in dioxane (0.50 mL) and isoamyl nitrite (0.13 mL). The solution, after being neutralized with triethylamine, was combined at −15 °C with the above hydrogenated heptadecapeptide amide acetate hydrochloride in a mixture of DMF (10 mL), Me_2SO (1 mL), and H_2O (1 mL) containing triethylamine (81 μL). The mixture was treated in the same manner as described in the preparation of XIII, and the product was purified by gel chromatography on a Sephadex LH-20 column (3.5 × 140 cm) with 5% aqueous DMF to yield XVII·HCl: yield 440 mg (79%); mp 176–178 °C; R_f^I 0.54, R_f^{II} 0.74; $[\alpha]^{24}_D$ −8.3° (*c* 0.56, 50% aqueous HOAc). Anal. ($C_{129}H_{196}N_{28}O_{38}S_2$·HCl·4$H_2O$): C, H, N. Amino acid ratios in acid hydrolysate: Lys(2) 1.89, Arg(1) 0.90, Asp(1) 1.17, Thr(1) 1.10, Ser(4) 3.38, Glu(2) 1.94, Gly(1) 1.02, Ala(1) 0.96, Ile(1) 0.93, Leu(5) 5.00, Tyr(1) 0.92, Phe(1) 1.17.

PHI: H-His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2 (I). Compound XVII·HCl (101 mg, 34.6 μmol) was hydrogenated over Pd in 40% aqueous HOAc to give H-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HCl·HOAc: R_f^I 0.28, R_f^{II} 0.76. Compound XII (158 mg, 172 μmol) was treated with TFA (1.5 mL) to give Z-His-Ala-Asp-Gly-Val-Phe-N_2H_3·TFA—R_f^I 0.44, R_f^{II} 0.78—and converted to the azide in DMF (5 mL) at −18 °C with 6 N HCl in dioxane (88 μL) and 10% isoamyl nitrite in DMF (234 μL). The solution, after being neutralized with triethylamine, was combined at −18 °C with the above hydrogenated heneicosapeptide amide acetate hydrochloride in a mixture of DMF (5 mL) and HMPT (1.5 mL) containing triethylamine (20 μL). The mixture was stirred at −18 °C for 2 h and at 4 °C for 20 h. To the mixture was added at −18 °C another aliquot of the azide solution prepared from the same amount of compound XII and the mixture was stirred at −18 °C for 2 h and at 4 °C for 20 h. The mixture was concentrated and chromatographed on a Sephadex LH-20 column (3.5 × 140 cm) with 5% aqueous DMF as eluent. The eluates corresponding to the main peak which was detected by UV absorption and TLC were pooled and concentrated, to which AcOEt was added. The precipitate was collected by filtration to give N^{α},N^{ϵ},N^{ϵ}-protected PHI: yield 90 mg (73%); R_f^I 0.40, R_f^{II} 0.79. Amino acid ratios in acid hydrolysate: Lys(2) 1.84, His(1) 1.08, Arg(1) 0.84, Asp(2) 2.24, Thr(1) 1.01, Ser(4) 3.25, Glu(2) 1.87, Gly(2) 2.13, Ala(2) 2.08, Val(1) 1.12, Ile(1) 0.93, Leu(5) 4.80, Tyr(1) 0.90, Phe(2) 2.16. The N^{α},N^{ϵ},N^{ϵ}-protected PHI (88 mg, 25.3 μmol) was dissolved in liquid NH_3 (50 mL) at −50 °C and small pieces of metal sodium were added to blue end point over a period of 30 s. After the additiion of NH_4Cl (1.0 g) at −50 °C, NH_3 was evaporated and 1 N HCl was added to pH 4.5 at 0 °C. The precipitate was collected and washed three times with 1 M aqueous HOAc by centrifugation. The solid was suspended in H_2O and lyophilized (product A, 45 mg): R_f^I 0.13, R_f^{II} 0.64. Amino acid ratios in acid hydrolysate: Lys(2) 2.04, His(1) 0.90, Arg(1) 0.90, Asp(2) 2.14, Thr(1) 0.83, Ser(4) 3.45, Glu(2) 1.90, Gly(2) 2.03, Ala(2) 2.10, Val(1) 1.08, Ile(1) 1.09, Leu(5) 4.89, Tyr(1) 1.01, Phe(2) 2.09. The supernatant and washings were pooled and desalted by gel chromatography on Sephadex G-25 with 1 M aqueous HOAc as eluent and the peak fractions were lyophilized (product B, 24 mg). Products A and B showed similar elution profiles in HPLC, but the main component in product A was approximately 60% and that in product B about 20%. Each of the products was submitted to purification by semipreparative reverse-phase HPLC on a C_{18} TSK-GEL LS-410AK column (0.75 × 30 cm) with 22.5% CH_3CN in 0.01 N HCl as eluant to give I·2HCl: total yield 25 mg (19.1%); R_f^I 0.13, R_f^II 0.64; $[\alpha]^{25}_D$ −46.0° (*c* 0.72, 20% aqueous HOAc) [lit.[14] $[\alpha]^{17}_D$ −62.4° (*c* 0.2, 0.2 N HOAc), not identified with natural PHI]. Amino acid ratios in acid hydrolysate: Lys(2) 1.98, His(1) 0.96, Arg(1) 1.03, Asp(2) 1.98, Thr(1) 0.93, Ser(4) 3.62, Glu(2) 2.02, Gly(2) 2.12, Ala(2) 2.02, Val(1) 1.04, Ile(1) 0.99, Leu(5) 5.01, Tyr(1) 0.98, Phe(2) 1.94 (recovery 89%). Amino acid ratios (His as 1.00) in aminopeptidase M digest: Lys(2) 1.99, His(1) 1.00, Arg(1) 1.02, Asp(2) 1.89, Thr(1) 1.02, Ser(4) 4.44, Gln(1) nd, Glu(1) 1.04, Gly(2) 2.00, Ala(2) 1.97, Val(1) 0.86, Ile(1) 0.98, Leu(5) 5.28, Tyr(1) 1.03, Phe(2) 2.02 (recovery 87%). The synthetic preparation showed a single sharp peak at a retention time of 14.7 min and was coeluted exactly with the natural preparation of PHI in analytical reverse-phase HPLC on a C_{18} TSK-GEL LS-410AK column (0.4 × 30 cm) in 0.01 N HCl/CH_3CN (70:30–60:40, v/v) over a period of 30 min at a flow rate of 1 mL/min (Figure 7). It was also eluted as a single sharp peak at a retention time of 46.2 min in reverse-phase HPLC on a μBondapak C_{18} column (0.4 × 30 cm) in a linear gradient solvent system from 0.12% TFA to 0.1% TFA/CH_3CN (40/60, v/v) over a period of 60 min at a flow rate of 1 mL/min. In two radioimmunoassay systems using antisera R8201 and T33 and ^{125}I-natural PHI as tracer, the synthetic preparation showed dose–response curves superimposable on that of natural PHI in the range between 16 and 4096 fmol (Figure 8).

Immunochemical Experiments. Preparation of Immunogen. (1) To a solution of PHI(20–27) (4.5 mg) in H_2O (1.5 mL) were added at 0 °C crude protein extract of Ascaris suilla (20 mg) and 1-ethyl-3-(3-dimethylaminopropyl)-1-carbodiimide hydrochloride (130 mg). The mixture was adjusted to pH 6–6.5 with 0.1 N HCl and allowed to stand at 4 °C for 15 h, when unreacted molecules of the peptide were detected no more in the reaction mixture by TLC. After adding a few drops of HOAc, the mixture was submitted to gel chromatography on a Sephadex G-10 column (2.5 × 120 cm) with 1 M aqueous HOAc as eluent. The excluded peak fractions containing the conjugate were pooled and lyophilized (24 mg, theoretical yield). [Gln^{24}]-PHI(20–27) was also conjugated with the protein extract in the same manner.

(2) To a solution of PHI(20–27) (5 mg) and BSA (15 mg) in 0.1 M NH_4OAc (pH 7.0) (2 mL) was added dropwise 0.02 M glutaraldehyde (1.3 mL) at 0 °C. The mixture was stirred at room temperature for 5 h, when unreacted molecules of the peptide were detected no more in the reaction mixture by TLC. The solution was dialyzed against H_2O at 4 °C for 24 h while H_2O (2 L each) was changed four times. The dialysate was lyophilized (19.2 mg, theoretical yield). [Gln^{24}]-PHI(20–27) was also conjugated with the macromolecules in the same manner.

Immunization. Each of the four kinds of the above-obtained protein conjugates of PHI(20–27) or [Gln^{24}]-PHI(20–27) (1 mg/rabbit for the first injection and 0.5 mg/rabbit thereafter) was dissolved in saline (0.5 mL/rabbit) and the solution was emulsified with complete Freund's adjuvant (0.5 mL/rabbit) in the conventional manner. The emulsion was injected subcutaneously at multiple sites of mixed-bred female rabbits at 2-week intervals. Three rabbits were used for each conjugate. Blood was taken 10 days after injection for examination of titer. All rabbits produced more or less antibodies against the immunogens. Among them one of the rabbits received the PHI(20–27)-conjugate (carbodiimide method) to give anti-PHI(20–27) serum R8201 after the 12th injection and another received the [Gln^{24}]-PHI(20–27)-conjugate (carbodiimide method) to yield anti-[Gln^{24}]-PHI(20–27) serum R8304 after the 8th injection, both of which exhibited titers sufficiently high enough for radioimmunoassay.

Radioimmunoassay. Preparation of Labeled Antigens: Iodination of PHI(20–27), [Gln^{24}]-PHI(20–27) or synthetic or natural PHI was carried out by the method of Hunter and Greenwood.[15] To a solution of a peptide (3–5 μg) in 1 M phosphate buffer, pH 7.5, (20 μL) were added ^{125}INa (500 μCi) and chloramine T (20 μg) in H_2O (10 μL). The mixture was shaken for 30 s, and sodium metabisulfite (150 μg) in H_2O (50 μL) and 10% KI (10 μL) were successively added. The mixture was applied to a column (1.0 × 30 cm) of Sephadex G-10, which was eluted with a mixture of 1 M HOAc containing 0.1% BSA. The excluded peak fractions were collected and used for radioimmunoassay. Specific activities of the labeled antigens were in the range of 180–310 μCi/μg.

Radioimmunoassay. The standard diluent used was 0.01 M phosphate buffer, pH 7.4, containing 0.025 M EDTA, 0.5% BSA, and 0.14 M NaCl. A mixture in each assay tube comprising standard diluent (0.4 mL), appropriate peptide solution (0.1 mL), diluted antiserum (R8304 × 1000, R8201 × 1000, and T33 ×600 diluted) (0.1 mL), and labeled antigen (0.1 mL) was incubated at 4 °C for 48 h, when normal rabbit serum (×100 diluted) (0.1 mL), goat anti-rabbit γ-globulin serum (×20 diluted) (0.1 mL), an d 5% polyethylene glycol in 0.01 M phosphate buffered saline, pH 7.4 (0.9 mL), were added. The mixture was kept at 4 °C for 3 h and then centrifuged at 3000 rpm for 30 min at 4 °C. The supernatant was decanted and the precipitate counted with an automatic γ counter.

Tissue Extraction. Fresh pig intestinal tissues were obtained from the local slaughter house. The mucosa was separated by scrapping from the muscle layer with a glass slide. The tissue samples were immediately frozen on dry ice and homogenized in 5–10-fold excess of hot water or hot 0.1 M HOAc. The homogenates were heated in a boiling water bath for 5–10 min, cooled, and centrifuged. The supernatants were separated and lyophilized and were used for radioimmunoassay.

Bioassay. Biological effects of synthetic and natural preparations of PHI were comparatively examined on the cat submandibular gland blood flow.[16] The peptides were initially dissolved in a small amount of 0.01

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M HOAc and the solutions were diluted in 0.01 M phosphate buffered saline (pH 7.4) and used for bioassay. Local intraarterial infusion of the synthetic preparation of PHI induced a dose-dependent vasodilation as the natural PHI. The vasodilating potencies of both preparations were almost identical ($n = 4$) with respect to threshold doses (ca. 30–100 pmol min^{-1}) as well as maximal effects (ca. 10 nmol min^{-1}) (Figure 9).

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Registry No. I, 92398-82-8; II, 91379-55-4; III, 92398-83-9; IV, 92398-84-0; V, 92398-85-1; VI, 92398-86-2; VII, 92398-87-3; VIII, 92398-88-4; IX, 92398-89-5; X·HCl, 92398-90-8; XI, 92398-91-9; XII, 92086-78-7; XIII, 92398-92-0; XIV, 92398-93-1; XV, 92420-10-5; XVI·HCl, 92398-94-2; XVII·HCl, 92420-22-9; Z-Ile-OSu, 3391-99-9; Z-Ile-NH_2, 86161-49-1; H-Ile-NH_2·HBr, 92398-95-3; Z-Leu-OSu, 3397-35-1; Z-Leu-Ile-NH_2, 82985-39-5; H-Leu-Ile-NH_2·HBr, 92398-96-4; Z-Ser-N_2H_3, 26582-86-5; Z-Ser-Leu-Ile-NH_2, 92398-97-5; H-Ser-Leu-Ile-NH_2·HOAc, 92398-98-6; Z-Glu(OBzl)-OH, 5680-86-4; Z-Glu(OBzl)-Ser-Leu-Ile-NH_2, 92398-99-7; H-Glu-Ser-Leu-Ile-NH_2·HOAc, 92399-01-4; H-Gln-OH, 56-85-9; Z-Leu-Gln-OH, 39802-26-1; H-Tyr-OMe·HCl, 3417-91-2; Boc-Lys(Z)-OH, 2389-45-9; Boc-Lys(Z)-Tyr-OMe, 92399-02-5; H-Lys(Z)-Tyr-OMe·TFA, 92399-03-6; Z-Lys(Z)-OH, 405-39-0; Z-Lys(Z)-Lys(Z)-Tyr-OMe, 92399-05-8; H-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc, 92399-07-0; Z-Lys(Z)-Lys(Z)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2, 92399-08-1; H-Leu-Gln-Ser-Leu-Ile-NH_2·HOAc, 92399-10-5; Z-Lys(Z)-Lys(Z)-Tyr-Leu-Gln-Ser-Leu-Ile-NH_2, 92399-11-6; Z-Lys(Tos)-OH, 2362-45-0; Z-Lys(Tos)-Tyr-OMe, 92399-12-7; H-Lys(Tos)-Tyr-OMe·HCl, 92399-13-8; Z-Lys(Tos)-Lys(Tos)-Tyr-OMe, 92399-14-9; Z-Ser-Ala-OMe, 38428-20-5; Z-Leu-Ser-Ala-OMe, 92399-15-0; Z-Gln-N_2H_2-Boc, 2899-14-1; H-Gln-N_2H_2-Boc, 14485-97-3; Z-Leu-Gly-OH, 2706-38-9; Z-Leu-OH·dicyclohexylamine salt, 53363-87-4; H_2NNH-Boc, 870-46-2; Z-Leu-N_2H_2-Boc, 20898-09-3; H-Leu-N_2H_2-Boc, 2419-37-6; Z-Arg(NO_2)-OH, 2304-98-5; Z-Arg(NO_2)-Leu-N_2H_2-Boc, 92399-16-1; H-Arg-Leu-N_2H_2-Boc·2HOAc, 92399-18-3; Z-Phe-N_2H_2-Boc, 36374-63-7; H-Phe-N_2H_2-Boc, 36261-38-8; Z-Asp(OBzl)-OH, 3479-47-8; Z-Asp(OBzl)-Phe-N_2H_2-Boc, 68801-61-6; Z-Thr-Ser-OMe, 2488-24-6; Z-Thr-Ser-N_2H_3, 2488-25-7; H-Asp-Phe-N_2H_2-Boc, 68801-62-7; Z-Val-Phe-N_2H_2-Boc, 36254-67-8; H-Val-Phe-N_2H_2-Boc, 47555-32-8; Z-Gly-OH, 1138-80-3; Z-Gly-Val-Phe-N_2H_2-Boc, 92399-..-4; H-Gly-Val-Phe-N_2H_2-Boc, 92399-20-7; Z-Asp(OBzl)-Gly-Val-Phe-N_2H_2-Boc, 92399-21-8; Z-His-N_2H_3, 49706-31-2; H-Ala-OMe·HCl, 2491-20-5; Z-His-Ala-OMe, 28944-91-4; Z-His-Ala-N_2H_3, 61486-56-4; H-Asp-Gly-Val-Phe-N_2H_2-Boc, 92399-22-9; H-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc, 92399-24-1; H-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc, 92420-24-1; Z-Leu-Gly-Gln-N_2H_3·TFA, 92399-26-3; H-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HOAc, 92399-28-5; H-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HCl·HOAc, 92420-26-3; Z-Thr-Ser-Asp-Phe-N_2H_3·TFA, 92420-28-5; H-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys(Tos)-Lys(Tos)-Tyr-Leu-Glu-Ser-Leu-Ile-NH_2·HCl·HOAc, 92399-30-9; Z-His-Ala-Asp-Gly-Val-Phe-N_2H_3·TFA, 92399-31-0; N^{α},N^{ϵ},N^{ϵ}-protected-PHI, 92399-32-1.

Supplementary Material Available: Listing of elemental analysis (4 pages). Ordering information is given on any current masthead page.

The Interconversion of the 5,6,7,8-Tetrahydro-, 7,8-Dihydro-, and Radical Forms of 6,6,7,7-Tetramethyldihydropterin. A Model for the Biopterin Center of Aromatic Amino Acid Mixed Function Oxidases

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Abstract: The 6,7-blocked pterins 6,6,7,7-tetramethyl-5,6,7,8-tetrahydropterin ($\mathbf{1}_{red}$), 6,6,7,7-tetramethyl-7,8-dihydropterin ($\mathbf{1}_{ox}$), and 5,6,6,7,7-pentamethyl-5,6,7,8-tetrahydropterin ($\mathbf{2}_{red}$) have been synthesized. $\mathbf{1}_{ox}$ represents the quinonoid product obtained upon $2e^-$ oxidation (electrochemical, Br_2) of $\mathbf{1}_{red}$. Two-electron oxidation of $\mathbf{2}_{red}$ yields $\mathbf{1}_{ox}$ rather than $\mathbf{2}_{ox}$ due to the rapid demethylation of the latter (the pseudo-first-order rate constant determined from elelectrochemical measurements at pH 5.9 is 1.4×10^{-2} s^{-1}). Solvolysis of $\mathbf{1}_{ox}$ yields the ring contracted imidazolone $\mathbf{3}_{ox}$ (Scheme III). The acid–base and spectral properties of $\mathbf{1}_{red}$ (Scheme I), $\mathbf{1}_{ox}$ (Scheme II), and $\mathbf{2}_{red}$ (Scheme IV) are described. The comproportionation equilibrium constant for the formation of the pterin radical $\mathbf{1}_{sem}$ has been determined by spectral and EPR measurements. The comproportionation constant for formation of $\mathbf{1}_{sem}$ from $\mathbf{1}_{red}$ and $\mathbf{1}_{ox}$ is much smaller ($\sim 10^7$-fold at pH 1.0 and $\sim 10^2$-fold at pH 7.0) than the like constants for flavin radical formation. The pH dependence of the redox potentials associated with $2e^-$ interconversion of $\mathbf{1}_{red}$ and $\mathbf{1}_{ox}$ has been determined and the like dependence of the $1e^-$ redox potentials for the interconversion of $\mathbf{1}_{red}$, $\mathbf{1}_{sem}$, and $\mathbf{1}_{ox}$ have been approximated. At scan speeds of 50 mV/s and at pH 5.9, the oxidation of $\mathbf{2}_{red}$ to $\mathbf{2}_{ox}$ can be shown to represent two $1e^-$ transfer steps in both anodic and cathodic sweeps. Nernst–Clark plots of potential vs. pH for **1** and **2** are provided (Figure 6). The mechanism of reaction of $\mathbf{1}_{red}$ and $\mathbf{2}_{red}$ with O_2 has been explored by comparing $\Delta G^{\ddagger}$ (from initial rates) to ΔG° values (electrochemical calculations) for $1e^-$ transfer from the tetrahydropterins to O_2. Since the difference $\Delta G^{\ddagger} - \Delta G^{\circ}$ is very small (13 kJ M^{-1}) and protons are not involved in the critical transition state, it is concluded that the transition state closely resembles the radical pairs $\{\mathbf{1}_{sem}O_2^{-}\cdot\}$ and $\{\mathbf{2}_{sem}O_2^{-}\cdot\}$ which must couple to provide 4a-hydroperoxypterins. The hydroperoxide moiety is consumed in the overall autocatalytic oxidation of $\mathbf{1}_{red}$ and $\mathbf{2}_{red}$.

The pterin-dependent aromatic hydroxylases phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophane hydroxylase are involved in the synthesis of tyrosine and the neurotransmitters epinephrine and norepinephrine and the central nervous system transmitter serotonin. In the recycling of the cofactor (biopterin), the pterin ring structure is interconverted from its tetrahydro to a $2e^-$ oxidized quinonoid form shown as the para isomer in eq 1. It has been hypothesized that, in the cycle of eq 1, phenyl-

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