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Synthesis of Granuliberin-R and Various Fragment Peptides and Their Histamine-Releasing Activities[1]

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Granuliberin-R and various fragment peptides were synthesized and their histamine-releasing activities were examined. The relative potencies of granuliberin-R, H–Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–NH_2, as liberators of histamine from mast cells are very similar to those of substance P and bradykinin. Studies on the histamine-releasing activity of various fragment peptides of granuliberin-R showed that a 4-amino acid central sequence, –Ile–Tyr–Arg–Arg–, is the minimum requirement for the releasing action of this frog skin peptide.

Keywords——granuliberin-R; mast cell-degranulating activity; histamine-releasing activity; substance P; bradykinin; neurotensin; fragment peptides of granuliberin-R

Nakajima and Yasuhara[3] recently isolated a dodecapeptide having mast cell-degranulating activity from a frog, *Rana rugosa*, and named it granuliberin-R (G-R). The entire amino acid sequence of this frog skin peptide is H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH_2. Its central partial sequence, -Pro-Ile-Tyr-Arg-Arg-Pro-, resembles the C-terminal part of neurotensin, -Pro-Arg-Arg-Pro-Tyr-Ile-, but G-R has neither hypotensive activity nor smooth muscle contraction activity. The synthetic material was chromatographically identical with natural G-R and had the same degranulating activity in rat peritoneal mast cells.[4] As mast cell-degranulating activity is accompanied by histamine release from the cell,[5] we synthesized this peptide and its various fragment peptides to test histamine-releasing activity and find the minimum sequence of the dodecapeptide required for the biological activity. We also compared the histamine-releasing activity of G-R with those of some known biologically active peptides, namely substance P, bradykinin and neurotensin, which are known to liberate histamine from mast cells or to have affinity with mast cell receptor sites.[5,6]

The dodecapeptide amide was synthesized by the conventional solution procedure as shown in Fig. 1. The only side chain-protecting group was the nitro group for arginine, which could be removed by catalytic hydrogenation at the end of the synthesis. The N-terminal fragment I was synthesized stepwise starting from proline *tert*-butyl ester by the

1) Amino acids and peptides mentioned here are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Boc=*t*-butyloxycarbonyl, Bzl=benzyl, Bu^t=*t*-butyl, Me=methyl, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, HOBt=N-hydroxy-benzotriazole, ONB=HONB ester, OSu=N-hydroxy-succinimide ester, DCC=N,N′-dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, TEA=triethylamine, DMF=dimethylformamide.

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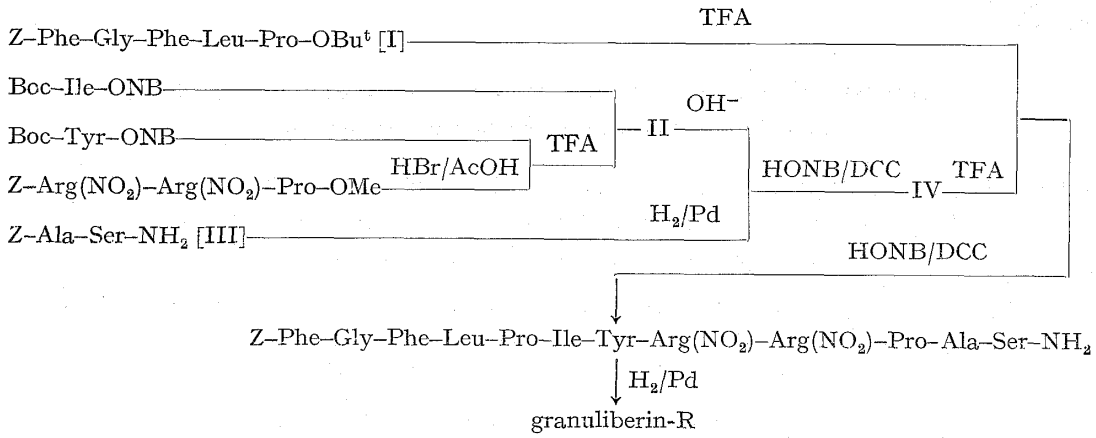

Fig. 1. Synthetic Route to Granuliberin-R

activated ester method. In the synthesis of fragment II, Z-Arg(NO_2)-Arg(NO_2)-Pro-OMe[7] was treated with 25% hydrogen bromide in acetic acid, further elongation being achieved by the HONB activated ester method.[8] Fragment III of the C-terminus was prepared from Z-Ala-ONB and H-Ser-NH_2. Each fragment was condensed from the C-terminus to the N-terminus by the HONB-DCC method,[8] after catalytic hydrogenation or acidolysis. Following the final condensation, the protecting groups were removed by catalytic hydrogenolysis and the product was purified by column chromatography on CM-cellulose and Sephadex LH-20. Instead of Z-Ala-Ser-NH_2, the corresponding benzyl ester Boc-Ala-Ser-OBzl was used to prepare des-amide G-R. The truncated peptides were prepared from the corresponding protected intermediates by catalytic hydrogenation and/or acidolysis then purified on CM-cellulose and/or Sephadex LH-20 columns. Other biologically active peptides used in this paper were synthesized in our laboratories by the conventional solution method.[9,10]

Table I. Histamine Release by Granuliberin-R and Its Fragment Peptides

Compound	Histamine % release[a]					
	1.0 μg/ml		10 μg/ml		100 μg/ml	
1 2 3 4 5 6 7 8 9 10 11 12	(a)[b]	(b)[b]	(a)	(b)	(a)	(b)
H–Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–NH_2	8	6	35	46	77	100
H–Phe–Gly–Phe–Leu–Pro–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–OH	6		20		87	
H–Phe–Gly–Phe–Leu–Pro–OH	7		7		13	
H–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–NH_2	7		38		80	
H–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–OH	7		13		73	
Boc–Ile–Tyr–Arg–Arg–Pro–Ala–Ser–OH	5		6		24	
H–Ile–Tyr–Arg–Arg–Pro–OH	6		15		76	
Boc–Ile–Tyr–Arg–Arg–Pro–OH	10		5		17	
H–Ile–Tyr–Arg–Arg–OH		1		12		84
H–Tyr–Arg–Arg–Pro–OH		3		3		21
H–Tyr–Arg–Arg–OH		1		1		27
H–Arg–OH		0		0		0
Bradykinin	1		13		54	

a) Spontaneous release (buffer)=4—5%. Compound 48/80=83% (1.0 μg/ml).
b) Different series of assays.

7) M. Fujino, O. Nishimura, and C. Hatanaka, *Chem. Pharm. Bull.*, **17**, 2135 (1969).
8) M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull.*, **22**, 1857 (1974).
9) M. Fujino and S. Shinagawa, *Chem. Pharm. Bull.*, **20**, 1021 (1972).
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Histamine-releasing activities of G-R and fragment peptides were determined (Table I) in rat peritoneal mast cells which were collected and purified by the method described by Sullivan *et al.*[11]

It is clear that des-amide G-R is as active as G-R, and the releasing activity of these peptides was found to increase dose-dependently. Even when the N-terminal pentapeptide was removed from the parent peptide, the biological activity of the heptapeptide amide or its des-amide peptide was equal to that of G-R. However, when the N-terminal amino function was blocked with a protecting group the activity decreased dramatically. Further removal of the amino acid residues from the C-terminus up to proline-10 caused no loss of the activity, while removal from the N-terminus up to isoleucine-6 reduced the activity to the level of the N-terminal-blocked peptides. Thus, the minimum sequence required for the histamine-releasing activity of G-R is assumed to be Ile-Tyr-Arg-Arg. This minimum sequence has two characteristic parts; Ile-Tyr part seems to produce a hydrophobic interaction and Arg-Arg an ionic interaction with the receptor of mast cells.

Fig. 2 shows the histamine release activities of G-R and its fragment peptide together with those of some other biologically active peptides which are known to be liberators of histamine from mast cells, *e.g.*, substance P, bradykinin and neurotensin. The potencies of substance P and bradykinin are almost equal to that of G-R. Johnson and Erdös[5] reported that peptides having this histamine-releasing activity contain two or more basic amino acid residues in their sequence as an essential functional moiety. However, our results that a fragment of G-R, Tyr-Arg-Arg-Pro, and the N-terminal fragment of substance P, Arg-Pro-Lys-Pro, were inactive or only slightly active in causing histamine release indicate that not only basic residues but also hydrophobic ones are necessary for histamine-releasing activity from mast cells.

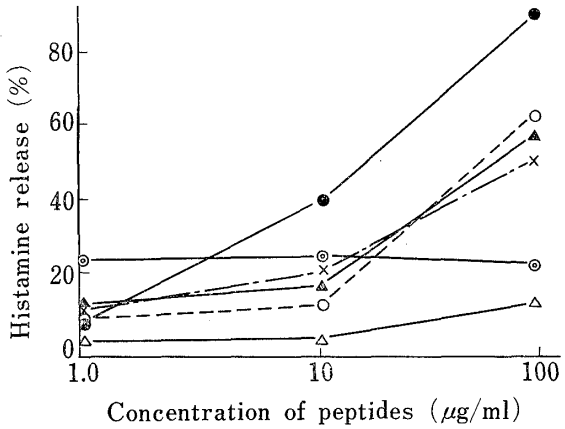

Fig. 2. Histamine-releasing Activities of Granuliberin-R and Some Other biologically Active Peptides

●; granuliberin-R, ○; granuliberin-R fragment (6—10), ▲; bradykinin, ×; substance P, ◎; neurotensin, △; substance P fragment (1—4).

Our preliminary experiments showed that the histamine release with G-R is associated with exclusion of trypan blue and retention of lactic dehydrogenase (unpublished data), as is the case with other kinins, and this indicates that G-R may stimulate exocytosis of mast cell granules rather than disrupting cell membranes.

Experimental

All melting points were taken in open capillaries and are uncorrected. Rotations were determined with a Perkin-Elmer model 141 polarimeter. Amino acid analyses were performed with a Hitachi KLA-3B amino acid analyzer. Acid (5.7 N HCl) hydrolysis was carried out at 110° for 24 hr. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 35—40°. Catalytic hydrogenations were performed at room temperature with palladium black as a catalyst. The purity of the products was tested by thin-layer chromatography (TLC) using Merck precoated silica gel $60F_{254}$ and/or cellulose plates (Avicel). Solvent systems used were: $CHCl_3$–MeOH (19: 1, v/v Rf^1), $CHCl_3$–MeOH–AcOH (9:1:0.5, v/v Rf^2), AcOEt–pyridine–AcOH–H_2O (60: 20: 6: 10, v/v Rf^3), *n*-BuOH–AcOEt–AcOH–H_2O (1: 1: 1: 1, v/v Rf^4), *n*-BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24, v/v Rf^5). *Rf* values given are those using silica gel plates unless otherwise stated.

11) T.J. Sullivan, K.L. Parker, W. Stenson, and C.W. Parker, *J. Immunol.*, **114**, 1473 (1975).

Z-Leu-Pro-OBu^t (Ia)——Z–Pro–OBu^t (15 g) was hydrogenolyzed in MeOH (150 ml) for 3 hr. After filtration and concentration, the resulting residue was dissolved in AcOEt (200 ml), then Z–Leu–OSu (18.1 g) was added. The mixture was stirred at room temperature for 16 hr, then diluted with AcOEt (200 ml). The mixture was washed with 4% aqueous $NaHCO_3$ and 0.2 N HCl, then dried over anhydr. Na_2SO_4. The solvent was removed to give the product as an oil: 17 g (81%), Rf^1 0.55.

Z-Phe-Leu-Pro-OBu^t (Ib)——Compound Ia (17 g) was hydrogenolyzed in MeOH (500 ml) for 4 hr. After filtration and concentration, the resulting residue was dissolved in AcOEt (300 ml) together with Z–Phe–OSu (15 g), and the mixture was stirred at room temperature for 16 hr. After the usual work-up, the product was crystallized from ether and then purified by recrystallization from AcOEt–ether: 6.3 g (34%), mp 129—131°, $[\alpha]_D^{20}$ −76.8° (c=0.98 in MeOH), Rf^2 0.86. *Anal.* Calcd for $C_{32}H_{43}N_3O_6$: C, 67.94; H, 7.66; N, 7.43. Found: C, 68.01; H, 7.69; N, 7.34.

Z-Gly-Phe-Leu-Pro-OBu^t (Ic)——Compound Ib (4.9 g) was hydrogenolyzed in MeOH (200 ml) for 4 hr. After filtration and concentration, the residue was coupled with Z–Gly–ONB (3.7 g) in a mixture of AcOEt (20 ml) and dioxane (20 ml) for 16 hr. After the usual work-up, the product was crystallized from pet. ether and then purified by recrystallization from AcOEt–pet. ether: 5.3 g (98.7%), mp 94—95°, $[\alpha]_D^{20}$ −67.6° (c=0.94 in MeOH), Rf^2 0.44. *Anal.* Calcd for $C_{34}H_{46}N_4O_7$: C, 65.57; H, 7.45; N, 9.00. Found: C, 64.74; H, 7.25; N, 8.89.

Z-Phe-Gly-Phe-Leu-Pro-OBu^t (I)——Compound Ic (3.1 g) was hydrogenolyzed in MeOH (150 ml) for 4 hr, and the residue was coupled with Z–Phe–OSu (2.4 g). The product was purified by reprecipitation from AcOEt–pet. ether: 3.2 g (83%), mp 98—102°, $[\alpha]_D^{20}$ −54.9° (c=1.03 in MeOH), Rf^2 0.72. *Anal.* Calcd for $C_{43}H_{55}N_5O_8$: C, 67.09; H, 7.20; N, 9.10. Found: C, 66.91; H, 7.28; N, 8.88.

Z-Phe-Gly-Phe-Leu-Pro-OH (Id)——Compound I (1.15 g) was treated with 90% aqueous TFA at room temperature for 35 min. After concentration, the residue was washed with dry ether to give a powder, which was purified by crystallization from AcOEt: 963 mg (90%), mp 195—196°, $[\alpha]_D^{20}$ −46.0° (c=1.05 in MeOH), Rf^2 0.68, Rf^3 0.86. *Anal.* Calcd for $C_{39}H_{47}N_5O_8$: C, 65.62; H, 6.64; N, 9.81. Found: C, 65.34; H, 6.70; N, 9.71.

Z-Tyr-Arg(NO$_2$)-Arg(NO$_2$)-Pro-OMe (IIa)——Z–Arg(NO$_2$)–Arg(NO$_2$)–Pro–OMe[7] (3.7 g) was treated with 25% HBr in AcOH (10 ml) at room temperature for 90 min. The resulting HBr salt was dissolved in DMF (6 ml) together with TEA (1.4 ml). Boc–Tyr–ONB (2.2 g) was added and the mixture was stirred at room temperature for 16 hr. The product was precipitated by the addition of ether and then purified by reprecipitation from MeOH–AcOEt: 3.43 g (86%), mp 144—146°, $[\alpha]_D^{20}$ −40.2° (c=0.40 in MeOH), Rf^2 0.09, Rf^3 0.61. *Anal.* Calcd for $C_{32}H_{50}N_{12}O_{12}\cdot H_2O$: C, 47.28; H, 6.45; N, 20.68. Found: C, 47.54; H, 6.33; N, 20.52.

Z-Ile-Tyr-Arg(NO$_2$)-Arg(NO$_2$)-Pro-OMe (II)——Compound IIa (2.0 g) was treated with TFA (10 ml) containing anisole (1.35 ml). After the usual work-up, the free base was coupled with Boc–Ile–ONB (1.2 g) in DMF at room temperature for 20 hr. The reaction mixture was diluted with AcOEt to give a precipitate which was collected by filtration and then purified by reprecipitation from aqueous MeOH: 1.9 g (84%), mp 120° (dec.), $[\alpha]_D^{20}$ −43.0° (c=0.50 in MeOH), Rf^3 0.20, Rf^4 0.60. *Anal.* Calcd for $C_{38}H_{61}N_{13}O_{13}\cdot H_2O$: C, 49.29; H, 6.86; N, 19.67. Found: C, 49.54; H, 6.87; N, 19.98.

Boc-Ile-Tyr-Arg(NO$_2$)-Arg(NO$_2$)-Pro-OH (IIb)——Compound II (1.8 g) was dissolved in 70% aqueous MeOH (6 ml) and 1 N NaOH (4.7 ml) was added at 0°. The mixture was stirred at 0° for 30 min and at room temperature for 2 hr. The reaction mixture was neutralized by the addition of 1 N HCl (4.7 ml). The precipitate formed was collected by filtration and then purified by column (4×4 cm) chromatography on silica gel using a mixture of AcOEt, pyridine, AcOH and H_2O (106: 30: 9: 15): 1.08 g (59%), mp 143° (dec.). $[\alpha]_D^{20}$ −35.8° (c=0.50 in MeOH), Rf^3 0.51. *Anal.* Calcd for $C_{37}H_{59}N_{13}O_{13}\cdot H_2O$: C, 48.73; H, 6.74; N, 19.97. Found: C, 48.80; H, 6.80; N, 19.04.

Z-Ala-Ser-NH$_2$ (III)——Z–Ser–NH$_2$ (2.4 g) was hydrogenolyzed in MeOH (200 ml) for 3 hr. After filtration and concentration, the residue was coupled with Z–Ala–ONB (3.8 g) in a mixture of DMF (20 ml) and tetrahydrofuran (20 ml) at room temperature for 15 hr. After concentration, the product was crystallized from H_2O: 1.22 g (41%), mp 212—214°, $[\alpha]_D^{23}$ −10.5° (c=1.1 in MeOH), Rf^2 0.20, Rf^3 0.75. *Anal.* Calcd for $C_{14}H_{19}N_3O_5$: C, 54.36; H, 6.19; N, 13.59. Found: C, 54.26; H, 6.23; N, 13.55.

Boc-Ala-Ser-OBzl (IIIa)——This compound was prepared from H–Ser–OBzl p-toluenesulfonate (17.7 g) and Boc–Ala–ONB (7.5 g) in the usual manner and purified by recrystallization from AcOEt-pet. ether: 15.3 g (86%), mp 50—52°, $[\alpha]_D^{20}$ −27.3° (c=1.05 in MeOH), Rf^2 0.71. *Anal.* Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65. Found: C, 58.96; H, 7.03; N, 7.60.

Boc-Ile-Tyr-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Ala-Ser-NH$_2$ (IV)——Compound III (130 mg) was hydrogenolyzed, and the resulting free base and compound IIb (313 mg) were coupled in DMF (7 ml) using DCC in the presence of HONB at room temperature for 20 hr. After the usual work-up, the product was precipitated from ether and purified by reprecipitation from MeOH–AcOEt: 196 mg (53%), mp 157—160° (dec.), $[\alpha]_D^{25}$ −48.3° (c=0.48 in MeOH), Rf^3 0.36, Rf^4 0.73. *Anal.* Calcd for $C_{43}H_{70}N_{16}O_{15}\cdot CH_3OH\cdot H_2O$: C, 47.99; H, 6.96; N, 20.35. Found: C, 47.53; H, 6.68; N, 19.93.

Boc-Ile-Tyr-Arg(NO$_2$)-Arg(NO$_2$) Pro-Ala-Ser-OBzl (IVa)——Compound IIIa (1.8 g) was treated with 6.5 N HCl in dioxane (3 ml) at room temperature for 15 min, and the product was isolated as a crystalline material by the addition of dry ether (1.5 g, 100%). The product was dissolved in DMF (5 ml) together

with TEA (0.24 ml) and then coupled with compound IIb (1.07 g) using DCC in the presence of HONB. After concentration, the residue was washed well with 2% aqueous $NaHCO_3$ and 1% aqueous AcOH. The product was then purified by reprecipitation from DMF–AcOEt–ether: 1.2 g (88%), mp 140° (dec.), $[\alpha]_D^{20}$ −35.7° (c=0.75 in AcOH), Rf^3 0.82. *Anal.* Calcd for $C_{50}H_{75}N_{16}O_{15}\cdot H_2O$: C, 51.76; H, 6.69; N, 18.11. Found: C, 51.70; H, 6.95; N, 17.87.

H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH_2 (G-R)——Compound IV (158 mg) was treated with TFA (1 ml) containing anisole (0.1 ml), and the product and compound Id (120 mg) were coupled in DMF (6 ml) using DCC in the presence of HONB. After the usual work-up, the resulting crude product was hydrogenolyzed to remove the protecting groups and then purified by column chromatography on CM-cellulose (2.2×23 cm, gradient elution: 0.005 M ammonium acetate/0.2 M ammonium acetate=400ml/400 ml). The fractions (549—608 ml) containing the product were collected and lyophilized, and the powder obtained was further purified on a column (1.0×85 cm) of Sephadex LH-20 (1 N AcOH). The fractions (34—43.5 ml) containing the pure product were collected and lyophilized: 60 mg (29.8%), $[\alpha]_D^{23}$ −87.6°(c=0.55 in 1 N AcOH), Rf^4 0.57, Rf^5 0.57, Rf^4 (cellulose) 0.82, Rf^5 (cellulose) 0.70, paper electrophoresis (pH 6.5 pyridine–acetate buffer, 600 volt)=0.82×Arg. Amino acid analysis: Arg 2.1, Ser 0.94, Pro 2.0, Gly 1.0, Ala 0.96, Ile 0.88, Leu 1.0, Tyr 0.84, Phe 1.96 (average recovery, 76.2%).

H-Phe-Gly-Phe-Leu-Pro-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-OH (des-amide G-R)——Compound IVa (171 mg) was treated with TFA (1 ml) containing anisole (0.1 ml) and the product was coupled with compound Id (107 mg) using DCC in the presence of HONB to give the protected des-amide G-R, which was deblocked and purified as described for G–R: 66 mg (27.6%), $[\alpha]_D^{20}$ −88.1° (c=0.47 in 1 N AcOH), Rf^4 0.62, Rf^5 0.44, Rf^4 (cellulose) 0.83, Rf^5 (cellulose) 0.81, paper electrophoresis (pH 6.5 pyridine–acetate buffer, 600 volt)=0.74×Arg. Amino acid analysis: Arg 2.0, Ser 0.86, Pro 2.03, Gly 1.0, Ala 1.05, Ile 1.05, Leu 1.14, Tyr 1.0, Phe 1.91 (average recovery, 80.7%).

Boc-Ile-Tyr-Arg-Arg-Pro-OH (IIc)——Compound IIb (450 mg) was hydrogenolyzed and the product was purified as described for G-R: 126 mg (34.2%), $[\alpha]_D^{20}$ −54.3° (c=0.18 in 3 N AcOH), Rf^4 0.63. Amino acid analysis: Arg 2.05, Pro 1.0, Ile 0.86, Tyr 0.86 (average recovery, 73.6%).

H-Ile-Tyr-Arg-Arg-Pro-OH (IId)——Compound IIc (50 mg) was treated with TFA (2 ml) and the resulting material was purified on a Sephadex LH-20 column (1.0×91.5 cm, 3 N AcOH). The fractions (32—40 ml) containing the pure product were collected and lyophilized: 28 mg (64%), $[\alpha]_D^{20}$ −18.1° (c=0.32 in 1 N AcOH), Rf^5 (cellulose) 0.57. Amino acid analysis: Arg 2.18, Pro 1.00, Ile 1.00, Tyr 0.87 (average recovery, 79%).

H-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-NH_2 (IVb)——Compound IV (89 mg) was treated with TFA (1 ml) containing anisole (0.06 ml) followed by hydrogenolysis in AcOH. The resulting material was purified on a Sephadex LH-20 column (1.0×85 cm, 1 N AcOH) and the fractions (31—38 ml) were collected and lyophilized: 24 mg (32.9%), $[\alpha]_D^{23}$ −50.5° (c=0.46 in 1 N AcOH), Rf^4 0.10, Rf^4 (cellulose) 0.57, Rf^5 (cellulose) 0.52. Amino acid analysis: Arg 2.08, Ser 0.96, Pro 1.12, Ala 0.94, Ile 1.00, Tyr 1.04 (average recovery, 84%).

Boc-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-OH (IVc)——Compound IVa (1.0 g) was hydrogenolyzed in AcOH and the product was purified as described for G-R: 511 mg (60.7%), $[\alpha]_D^{20}$ −76.4° (c=0.22 in 3 N AcOH), Rf^4 0.60. Amino acid analysis: Arg 1.89, Ser 1.07, Pro 1.0, Ala 1.07, Ile 1.0, Tyr 0.89 (average recovery, 67.5%).

H-Ile-Tyr-Arg-Arg-Pro-Ala-Ser-OH (IVd)——Compound IVc (17 mg) was treated with TFA (1 ml) and the resulting material was purified as described for IId: 9 mg (52.2%), $[\alpha]_D^{20}$ −50.5° (c=0.2 in 1 N AcOH), Rf^5 (cellulose) 0.50. Amino acid analysis: Arg 1.96, Ser 1.0, Pro 1.05, Ala 1.10, Ile 0.99, Tyr 0.89 (average recovery, 86.9%).

H-Tyr-Arg-Arg-Pro-OH (IIe)——Compound IIa (190 mg) was treated with 1 N NaOH (2 ml) in MeOH (4 ml) to remove the methyl ester, followed by treatment with TFA (4 ml), and the product was hydrogenolyzed in AcOH (20 ml). After concentration, the product was purified as described for IId: 28 mg (20.4%), $[\alpha]_D^{22}$ −21.6° (c=0.26 in 1 N AcOH), Rf^5 (cellulose) 0.45. Amino acid analysis: Arg 2.0, Pro 1.0, Tyr 0.92 (average recovery, 84%).

H-Phe-Gly-Phe-Leu-Pro-OH (Ie)——Compound Id (350 mg) was hydrogenolyzed in AcOH and the resulting material was purified as described for IId: 170 mg (59.9%), $[\alpha]_D^{20}$ −28.8° (c=0.22 in 3 N AcOH), Rf^3 0.38, Rf^4 0.82. Amino acid analysis: Pro 0.94, Gly 1.0, Leu 1.0, Phe 1.94 (average recovery, 79.1%).

Boc-Tyr-Arg(NO_2)-Arg(NO_2)-OBzl (Va)——Boc–Arg(NO_2)–Arg(NO_2)–OBzl[12] (915 mg) was treated with TFA (3 ml) and the resulting material was coupled with Boc–Tyr–ONB (791 mg) in DMF (10 ml). After the usual work-up, the product was purified by column chromatography on silica gel (2.5×4.5 cm) with $CHCl_3$–MeOH–AcOH (9:1:0.5): 450 mg (38.8%), mp 95—100° (dec.), $[\alpha]_D^{28}$ −13.0° (c=0.33 in DMF), Rf^2 0.28, Rf^3 0.72. *Anal.* Calcd for $C_{33}H_{47}N_{11}O_{11}$: $CH_3COOH\cdot H_2O$: C, 49.34; H, 6.36; N, 18.34. Found: C, 49.64; H, 6.62; N, 17.65.

Z-Ile-Tyr-Arg(NO_2)-Arg(NO_2)-OBzl (Vb)——Compound Va (250 mg) was treated with TFA (2 ml) and then coupled with Z–Ile–ONB (165 mg) in DMF (12 ml). After the usual work-up, the product was purified by reprecipitation from AcOEt–ether: 226 mg (76.1%), mp 212—215° (dec.), $[\alpha]_D^{28}$ −19.9° (c=0.12 in DMF), Rf^3 0.86. *Anal.* Calcd for $C_{42}H_{56}N_{12}O_{12}\cdot H_2O$: C, 53.72; H, 6.23; N, 17.90. Found: C, 53.44; H, 5.91; N, 17.72.

12) H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Japan*, **39**, 882 (1966).

H-Tyr-Arg-Arg-OH (Vc)——Compound Va (200 mg) was treated with TFA (2 ml) and the product was then hydrogenolyzed in AcOH to remove all the protecting groups. The resulting material was purified as described for IVb: 30 mg (23.6%), $[\alpha]_D^{22}$ +6.3° (c=0.41 in 1 N AcOH), Rf^5 (cellulose) 0.41. Amino acid analysis: Arg 2.1, Tyr 1.0 (average recovery, 53%).

H-Ile-Tyr-Arg-Arg-OH (Vd)——Compound Vb (150 mg) was hydrogenolyzed in 70% aqueous AcOH, and the product was purified as described for IVb: 27 mg (27.3%), $[\alpha]_D^{22}$ +8.3° (c=0.51 in 1 N AcOH), Rf^5 (cellulose) 0.55. Amino acid analysis: Arg 2.05, Ile 1.0, Tyr 1.03 (average recovery, 68.8%).

Biological Assay for Histamine Release——Rat peritoneal mast cells were harvested and purified by the method described by Sullivan *et al.*[11] A mast cell suspension (0.9 ml, total cells: 1×10^5) was treated with 0.1 ml of peptide or compound 48/80 (10 μg—1 mg/ml) and incubation was continued for 10 min at 37°. After cooling at 0°, the cell suspension was separated into cells and supernatant by centrifugation at $780\times g$ for 10 min. The amounts of histamine in the supernatant were determined by the *o*-phthalaldehyde spectrofluorometric procedure of Shore *et al.*[13]

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13) P.A. Shore, A. Burkehalt, and V.H. Cohn, *J. Pharmacol. Exptl. Therap.*, **127**, 182 (1959).