[Chem. Pharm. Bull. 21(3) 616—621 (1973)]

UDC 547.466.1.057 : 615.35.015.12

Synthesis of Bradykinyl-Val-Ala-Pro-Ala-Ser-OH and Its Biological Properties[1)]

Noboru Yanaihara, Chizuko Yanaihara, Masanori Sakagami,[2a)] Terumi Nakajima, Taeko Nakayama[2b)] and Kojiro Matsumoto[2c)]

Shizuoka College of Pharmacy,[2a)] Institute of Pharmaceutical Sciences, School of Medicine, Hiroshima University,[2b)] and Faculty of Pharmaceutical Sciences, University of Tokyo[2c)]

(Received October 7, 1972)

Synthesis is described of bradykinyl-Val-Ala-Pro-Ala-Ser-OH. All the fragment condensations were carried out by Rudinger's azide method in order to minimize the racemization during the coupling steps involving peptide fragments. The ensuing tetradecapeptide with a high degree of homogeneity was found to be identical with bradykinyl-Val-Ala-Pro-Ala-Ser-OH of natural origin with respect to chemical and biological properties.

Nakajima[3)] reported the isolation of a tetradecapeptide from the skin of *Rana nigromaculata* and the determination of the amino acid sequence as bradykinyl-Val-Ala-Pro-Ala-Ser-OH (I). We carried out the synthesis of I in order to confirm the proposed structure and to use the synthetic material for further biological and physiological studies.

The present communication describes the synthesis of I possessing the properties identical to those of the natural one.

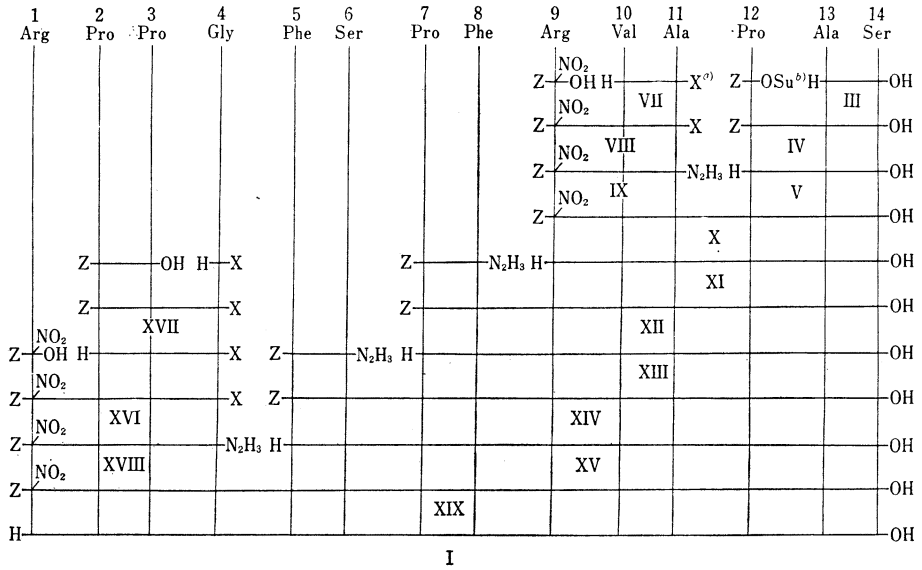

Chart 1. Route for the Preparation of Bradykinyl-Val-Ala-Pro-Ala-Ser-OH

a) X=NHNH-Boc *b*) OSu=N-hydroxysuccinimide ester

1) The amino acid residues except glycine are of the L-configuration. The abbreviations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967).

2) Location: *a*) *Oshika, Shizuoka*; *b*) *Kasumi-cho, Hiroshima*; *c*) *Hongo Bunkyo-ku, Tokyo*.

3) T. Nakajima, *Chem. Pharm. Bull.* (Tokyo), **16**, 2088 (1968).

Synthesis

The route for the preparation of I is shown in Chart 1.

Rudinger's azide modification[4] was employed entirely for the fragment condensations. Interaction of Z-Ala-OSu[5] with triethylammonium salt of H-Ser-OH gave Z-Ala-Ser-OH[6] (II), which was hydrogenated to yield H-Ala-Ser-OH[6,7] (III). Coupling of Z-Pro-OSu[5] with III produced Z-Pro-Ala-Ser-OH (IV), which was deblocked by hydrogenolysis to give H-Pro-Ala-Ser-OH (V). This tripeptide served as the amino component in the fragment condensation. Z-Val-OSu[5] was coupled with H-Ala-NHNH-Boc derived from Z-Ala-NHNH-Boc to yield Z-Val-Ala-NHNH-Boc (VI). Hydrogenation of VI gave H-Val-Ala-NHNH-Boc (VII), which was coupled with a mixed anhydride of Z-Arg(NO_2)-OH.[8] The resulting tripeptide Boc hydrazide (VIII) was treated with TFA to give the corresponding hydrazide (IX). The azide derived from IX was coupled with V to give protected hexapeptide (X).

Hydrogenation of X, followed by column chromatography on carboxy methyl cellulose (CMC), gave homogeneous H-Arg-Val-Ala-Pro-Ala-Ser-OH acetate tetrahydrate (XI), which was coupled with the azide derived from Z-Pro-Phe-$NHNH_2$.[9] The resulting protected octapeptide (XII) was hydrogenated, and the material XIII thus obtained was coupled with the azide derived from Z-Phe-Ser-$NHNH_2$[9-11] to give protected decapeptide acetate pentahydrate (XIV). Hydrogenation of XIV gave decapeptide acetate (XV).

Z-Arg(NO_2)-Pro-Pro-Gly-NHNH-Boc(XVI) was prepared by the following route. A mixed anhydride of Z-Pro-Pro-OH[12] was coupled with H-Gly-NHNH-Boc which was derived by the hydrogenation of Z-Gly-NHNH-Boc to give Z-Pro-Pro-Gly-NHNH-Boc (XVII). The material obtained by hydrogenation of XVII was coupled with a mixed anhydride of Z-Arg (NO_2)-OH[8] to give XVI in the form of monohydrate. Boissonnas, *et al.*[11] had reported the synthesis of XVI by a different route. However, the melting point and optical rotation of the highly purified protected tetrapeptide XVI obtained in this experiment differed from those reported.[11]

Treatment of XVI with TFA gave the corresponding hydrazide (XVIII). Coupling of the azide derived from XVIII with XV gave crude protected tetradecapeptide (XIX), which was hydrogenated over Pd to give crude I. This material was purified by column chromatography on CMC and gelfiltration on Sephadex G-25. The purified tetradecapeptide I behaved as a single component on thin-layer chromatography (TLC) in two solvent systems and its acid hydrolysate contained the constituent amino acids in the theoretical ratios.

Identification and Biological Activity

Because of an extremely limited amount of I of natural origin which is available for use, the identification of synthetic product with natural one was performed in the following manner using dansyl method.[13] Dansylation of synthetic I produced dansyl derivative of bradykinyl-Val-Ala-Pro-Ala-Ser-OH, I, which gave a single spot having identical *Rf* value with

4) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
5) G.W. Anderson, J. E. Zimmerman, and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
6) J.I. Harris and J.S. Fruton, *J. Biol. Chem.*, **191**, 143 (1951).
7) R. Hirschmann, R.G. Strachan, H. Schwam, E.F. Schoenewaldt, H. Joshua, B. Barkemeyer, D.F. Veber, W.J. Paleveda, Jr., T.A. Jacob, T.E. Beesley, and R.G. Denkewalter, *J. Org. Chem.*, **32**, 3415 (1967).
8) K. Hofmann, W.D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **78**, 238 (1956).
9) E. Schroder and H. Gibian, *Ann.*, **673**, 176 (1964).
10) E.D. Nicolaides and H.A. DeWald, *J. Org. Chem.*, **26**, 3872 (1961).
11) R.A. Boissonnas, St. Guttmann, and P.-A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).
12) S. Lande, *J. Org. Chem.*, **27**, 4558 (1962).
13) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).

Table I. Relative Activity of Bradykinyl-Val-Ala-Pro-Ala-Ser-OH(I)

	Synthetic Bradykinyl-Val-Ala-Pro-Ala-Ser-OH			
	Non-treated	Trypsin-treated		
	40ng	2.0ng	2.5ng	5.0ng
Weight(ng) of bradykinin exhibiting an equivalent oxytocic activity	2.4 2.2	1.4 1.6 1.7	2.3 1.9	3.5 3.2 3.5
Relative activity to bradykinin (molar basis)	6.9%	94%	100% average 92%	82%

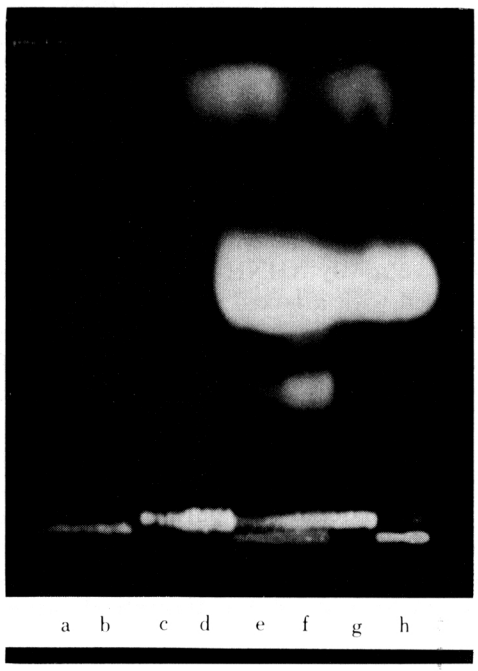

Fig. 1. Comparison of Natural and Synthetic I on TLC

solvent system: isopropanol–methyl acetate–28% ammonia (7:9:4); detection: UV 366 nm. a: dansylated natural I; b: dansylated synthetic I; c: trypsin-treated product of dansylated natural I; d: trypsin-treated product of dansylated synthetic I; e: dansylated product of trypsin-treated natural I; f: dansylated product of trypsin-treated synthetic I; g: dansylated bradykinin; h: blank (dansylated trypsin).

that of dansyl derivative of I of natural origin on TLC. The *Rf* value was slightly lower than that of dansylated bradykinin in a solvent system of isopropanol–methyl acetate–28% ammonia (7: 9: 4) as described.[3]

Treatments of synthetic and natural products with trypsin, followed by dansylation, gave spots identical with those of dansylated bradykinin and dansylated H-Val-Ala-Pro-Ala-Ser-OH (XX) in both cases.

Preparation of XX as standard was performed in the following manner. Coupling of the azide derived from Z-Val-Ala-$NHNH_2$[14] with V, followed by hydrogenation, gave crude H-Val-Ala-Pro-Ala-Ser-OH (XXI), which was purified by column chromatography on CM-Sephadex and then dansylated to yield XX.

The activity of I was examined by the response on the rat uterus. Approximately 7% activity was observed when compared with that of bradykinin on molar basis. After treatment of synthetic I with trypsin, the activity was raised to 92% of bradykinin activity on the average. The assay results of the intact and trypsin-treated synthetic I are shown in Table I.

Result

All the fragment condensations were carried out by Rudinger's azide method in order to minimize the racemization during the coupling steps involving peptide fragments. The synthesis of I according to the route shown in Chart 1 was repeated twice. The ensuing tetradecapeptide I with a high degree of homogeneity was found to be identical with bradykinyl-Val-Ala-Pro-Ala-Ser-OH (I) of natural origin with respect to chemical and biological properties. Since our synthetic route excludes any possibility of the formation of bradykinin molecule as a by-product, bradykinyl-Val-Ala-Pro-Ala-Ser-OH, I, seems to have little if any

14) J.H. Seu, R.R. Semby, and F.M. Bumpus, *J. Amer. Chem. Soc.*, **84**, 4948 (1962).

intrinsic bradykinin-like activity. Any evidence was not obtained for partial enzymic degradation of I to bradykinin in the assay system.

Further investigation on biological properties of I is now under way. In addition, synthetic I and the peptide fragments prepared in the present study may be employed as substrates for study on kinin-kininogen system.

Experimental

Melting points were determined on a Mitamura Riken capillary melting point apparatus and are uncorrected. Optical rotations were measured on a Yanaco automatic polarimeter OR-50. Microanalyses were performed by the Analytical Center of Shizuoka College of Pharmacy. Analytical samples were dried *in vacuo* over P_2O_5 at 60—70°. Amino acid analyses were performed with a Hitachi Model KLA-3B amino acid analyzer. Acid hydrolysis of a sample for amino acid analysis was conducted with 6N HCl at 110° for 24 hr in a sealed tube. Designations of solvent systems for TLC on Silica gel G (Merck) are: Rf^{I} 1-BuOH–AcOH–H_2O (4:1:5) upper layer; Rf^{II} 1-BuOH–pyridine–AcOH–H_2O (30:20:6:24). All solvents were of reagent grade and were distilled before use. Evaporations were carried out *in vacuo* at 40—45° in rotary evaporators.

Z-Ala-NHNH-Boc——DCC (5.15 g) was added to a solution of Z-Ala-OH (5.60 g) and Boc-$NHNH_2$ (3.30 g) in THF (50 ml) cooled at 4°. The mixture was stirred at 4° for 30 min and then at room temperature for 3 hr. The dicyclohexylurea was removed by filtration, and the solvent was evaporated. The residue was dissolved in EtOAc, and the solution was washed successively with 1N citric acid, saturated $NaHCO_3$ and saturated NaCl, and dried over Na_2SO_4. The solvent was evaporated. The residue was solidified by addition of petroleum ether. Recrystallization from EtOAc with petroleum ether gave pure product, yield 8.10 g (97%), mp 92—93°, $[\alpha]_D^{27}$ −45.3° (c=2.0, MeOH). *Anal.* Calcd. for $C_{16}H_{23}O_5N_3$: C, 56.96; H, 6.87; N, 12.46. Found: C, 57.45; H, 6.65; N, 12.40.

Z-Gly-NHNH-Boc——Isobutylchloroformate (1.37 ml) was added to a solution of Z-Gly-OH (1.92 g) and N-methylmorpholine (1.06 ml) in THF (20 ml) at −15°. After 1 min, Boc-$NHNH_2$ (1.37 g) dissolved in THF (6 ml) was added. The mixture was stirred at 0° for 5 min and at room temperature for further 30 min. The solvent was evaporated. The residue was dissolved in EtOAc, and the solution was washed successively with 1N citric acid, saturated $NaHCO_3$ and saturated NaCl and dried over Na_2SO_4. The solvent was evaporated and the residue was solidified from H_2O. Recrystallization from MeOH with H_2O gave pure product, yield 2.50 g (77%), mp 37—41°. *Anal.* Calcd. for $C_{15}H_{21}O_5N_3$: C, 55.72; H, 6.55; N, 13.00. Found: C, 55.37; H, 6.36; N, 13.13.

Z-Ala-Ser-OH (II)——A solution of Z-Ala-OSu[5] (4.80 g) in THF (15 ml) was added to a solution of H-Ser-OH (1.56 g) and triethylamine (2.10 ml) in H_2O (15 ml). The mixture was kept at room temperature for 20 hr and the bulk of the solvents was evaporated. To the residue was added 3N citric acid (30 ml). The crystalline material thus formed was collected by filtration and washed with ether and H_2O; needles, yield 3.55 g (76%), mp 205—206°, lit.[6] mp 204—205°, $[\alpha]_D^{25}$ +28.2° (c=2.1, DMF). *Anal.* Calcd. for $C_{14}H_{18}O_6N_2$: C, 54.18; H, 5.84; N, 9.03. Found: C, 54.09; H, 5.88; N, 9.17.

H-Ala-Ser-OH (III)——The above compound II (3.10 g) was hydrogenated over Pd in 50% aqueous MeOH (100 ml) in the usual manner. The catalyst was removed by filtration and the solution was evaporated. The residue was washed with MeOH and collected by filtration; yield 1.62 g (92%), mp 208—209° (decomp.); $[\alpha]_D^{25}$ +10.9° (c=2.0, H_2O) (lit.[7] mp 208—210°, $[\alpha]_D^{24}$ +11.2° (c=1.97, H_2O): lit.[6] $[\alpha]_D^{23}$ +11.5° (c=2.0, H_2O)); Rf^{I} 0.22, Rf^{II} 0.52. *Anal.* Calcd. for $C_6H_{12}O_4N_2$: C, 40.90; H, 6.86; N, 15.90. Found: C, 40.84; H, 6.96; N, 15.74.

Z-Pro-Ala-Ser-OH (IV)——A solution of Z-Pro-OSu[5] (3.80 g) in THF (30 ml) was added to a solution of III (1.76 g) and triethylamine (2.80 ml) in H_2O (25 ml). The mixture was allowed to stand at room temperature for 3 hr and the solution was evaporated. The residue was extracted with five portions of 1-BuOH which were washed with six portions of 2% AcOH. Evaporation of the 1-BuOH gave an oil which was solidified from EtOAc with petroleum ether, yield 4.01 g (98%), mp 182—183°, $[\alpha]_D^{27}$ −74.0° (c=1.0, MeOH). *Anal.* Calcd. for $C_{19}H_{25}O_7N_3$: C, 56.01; H, 6.18; N, 10.31. Found: C, 56.10; H, 6.54; N, 10.18.

H-Pro-Ala-Ser-OH hemihydrate (V)——The above compound IV (0.81 g) was hydrogenated over Pd in 50% aqueous MeOH (50 ml) in the usual manner. Isolation of the product was carried out in the manner as described for the preparation of III, yield 0.52 g (93%), mp 214—216° (decomp.), $[\alpha]_D^{27}$ −74.8° (c=1.1, 10% AcOH); Rf^{I} 0.13, Rf^{II} 0.50. *Anal.* Calcd. for $C_{11}H_{19}O_5N_3 \cdot 0.5H_2O$: C, 46.78; H, 7.14; N, 14.88. Found: C, 46.63; H, 7.26; N, 14.85.

Z-Val-Ala-NHNH-Boc (VI)——Z-Ala-NHNH-Boc (1.69 g) was hydrogenated over Pd in MeOH (30 ml) in the usual manner. The resulting partially deblocked material was dissolved in THF (10 ml) containing triethylamine (0.70 ml), and Z-Val-OSu[5] (1.74 g) was added. The mixture was allowed to stand at room temperature for 20 hr, and H_2O (100 ml) was added. The resulting mass was collected by filtration and dried. Recrystallization from THF gave pure VI, yield 1.58 g (73%), mp 155—158°, $[\alpha]_D^{25}$ −22.1° (c=2.1,

DMF); Rf^{I} 0.85. *Anal.* Calcd. for $C_{21}H_{32}O_6N_4$: C, 57.80; H, 7.39; N, 12.83. Found: C, 58.11; H, 7.61; N, 12.47.

Z-Arg(NO_2)-Val-Ala-NHNH-Boc (VIII)——Hydrogenation of VI (1.31 g) was carried out over Pd in MeOH (30 ml) in the usual manner. The catalyst was removed by filtration, the solvent was evaporated and the residue was dried *in vacuo* over P_2O_5.

A solution of Z-Arg(NO_2)-OH[8] (1.01 g) and N-methylmorpholine (0.31 ml) in THF (20 ml) was cooled to −15° and isobutylchloroformate (0.40 ml) was added. After 1 min, the resulting mixed anhydride was combined with an ice-cooled solution of the above hydrogenated material (VII) and triethylamine (0.42 ml) in THF (10 ml). The mixture was kept at 4° for 5 min and at 15° for further 30 min. The solution was evaporated and the residue was dissolved in EtOAc, which was washed successively with 1N citric acid, saturated $NaHCO_3$ and H_2O, and dried. The solvent was evaporated and the residue was crystallized from ether Recrystallization from MeOH with EtOAc gave pure VIII, yield 1.32 g (69%), mp 203—204° (decomp.), $[\alpha]_D^{25}$ −18.9° (c=2.0, DMF); Rf^{I} 0.74. *Anal.* Calcd. for $C_{27}H_{43}O_9N_9$: C, 50.85; H, 6.80; N, 19.77. Found: C, 51.04; H, 6.87; N, 19.65.

Z-Pro-Pro-Gly-NHNH-Boc (XVII)——Z-Gly-NHNH-Boc (1.85 g) was hydrogenated over Pd in MeOH (50 ml) in the usual manner. Isolation was performed in the manner as described for the hydrogenation of VI.

A solution of Z-Pro-Pro-OH[12] (2.18 g) and N-methylmorpholine (0.63 ml) in THF (20 ml) and DMF (15 ml) was cooled to −15° and isobutylchloroformate (0.83 ml) was added. After 1 min, the resulting mixed anhydride was combined with an ice-cooled solution of the above hydrogenated material and triethylamine (0.80 ml) in DMF (20 ml). The mixture was kept at 0° for 5 min and at 15° for additional 30 min. The solvent was evaporated. The residue was solidified by addition of H_2O and washed with MeOH, yield 1.96 g (66%), mp 205—207° (decomp.), $[\alpha]_D^{28}$ −51.6° (c=1.1, DMF); Rf^{I} 0.85. *Anal.* Calcd. for $C_{25}H_{35}O_7N_5$: C, 58.01; H, 6.82; N, 13.53. Found: C, 57.66; H, 7.07; N, 13.18.

Z-Arg(NO_2)-Pro-Pro-Gly-NHNH-Boc Hydrate (XVI)——Compound XVII (1.55 g) was hydrogenated over Pd in MeOH (50 ml) containing 10% AcOH (10 ml). Isolation was performed in the manner as described for the hydrogenation of VI, Rf^{I} 0.30, Rf^{II} 0.71.

A solution of Z-Arg(NO_2)-OH[8] (1.06 g) and N-methylmorpholine (0.31 ml) in THF (50 ml) was cooled to −15° and isobutylchloroformate (0.40 ml) was added. After 1 min, the resulting mixed anhydride was combined with an ice-cooled solution of the above hydrogenated material and triethylamine (0.42 ml) in THF (50 ml). The mixture was kept at 0° for 5 min and at 15° for further 30 min and the solvent was evaporated. The residue was dissolved in EtOAc, which was washed 1N cirtic acid, saturated $NaHCO_3$ and NaCl solution, and dried over Na_2SO_4. The solvent was evaporated and the residue was precipitated from EtOAc with ether, yield 1.30 g (59%), mp 115—121°, $[\alpha]_D^{27}$ −86.4° (c=1.1, MeOH), $[\alpha]_D^{27}$ −56.8° (c=1.0, DMF), [lit.[11] Z-Arg(NO_2)-Pro-Pro-Gly-NHNH-Boc: mp 115°, $[\alpha]_D^{26}$ −66.6° ±0.5 (c=1.2, MeOH), −39.9° ±0.5 (c=1.6, DMF)]; Rf^{I} 0.65, Rf^{II} 0.80. *Anal.* Calcd. for $C_{31}H_{46}O_{10}N_{10}\cdot H_2O$ C, 50.53; H, 6.56; N, 19.01. Found: C, 50.27; H, 6.70; N, 18.25.

H-Arg-Val-Ala-Pro-Ala-Ser-OH Acetate Tetrahydrate (XI)——Z-Arg(NO_2)-Val-Ala-NHNH-Boc (VIII) (2.29 g) was treated with TFA (6 ml) in the usual manner and ether was added. The resulting Z-tripeptide hydrazide (IX) was collected by filtration and dried over KOH. This material IX was dissolved in DMF (12 ml) and cooled to −15°. To the solution were added 6N HCl in dioxane (1.80 ml) and isoamyl nitrite (0.48 ml). The mixture was left at −10° for 5 min and neutralized with triethylamine. An ice-cold solution of V (0.77 g) and triethylamine (0.42 ml) in H_2O (6 ml) was added. The mixture was stirred at 4° for 15 hr and the solution was evaporated. The residue was extracted with five portions of 1-BuOH which were washed with six portions of 2% AcOH. Evaporation of 1-BuOH gave an oil which was solidified from MeOH with EtOAc.

The resulting crude Z-Arg(NO_2)-Val-Ala-Pro-Ala-Ser-OH (X) (2.24 g) was hydrogenated over Pd in MeOH (50 ml) and 50% AcOH (50 ml). The catalyst was removed and the solvent were evaporated. The residue was dissolved in H_2O (250 ml).

The solution was applied to a column of CMC (2.5×25 cm), which was eluted successively with H_2O (600 ml) and NH_4OAc buffer: 0.001M (250 ml), 0.002M (500 ml), 0.0075M (650 ml) and finally 0.01M (400 ml). The 0.0075M NH_4OAc eluates containing the desired material were pooled and the solvent was evaporated. The product was lyophilized three times with H_2O, yield 0.90 g (45%), $[\alpha]_D^{27}$ −110.1° (c=1.0, 10% AcOH); Rf^{I} 0.08; amino acid ratios in acid hydrolysate $Arg_{0.97}$ $Ser_{0.95}$ $Pro_{1.04}$ $Ala_{1.98}$ $Val_{1.07}$. *Anal.* Calcd. for $C_{27}H_{49}O_{10}N_9\cdot 4H_2O$: C, 44.31; H, 7.86; N, 17.22. Found: C, 44.19; H, 8.16; N, 17.60.

H-Pro-Phe-Arg-Val-Ala-Pro-Ala-Ser-OH Acetate Trihydrate (XIII)——Z-Pro-Phe-$NHNH_2$[9] (0.55 g) was dissolved in DMF (10 ml) and 6N HCl in dioxane (0.90 ml), and the solution was cooled to −15° and isoamyl nitrite (0.20 ml) was added. The mixture was left at −10° for 5 min and neutralized with triethylamine. An ice-cold solution of XI (0.44 g) and triethylamine (0.19 ml) in DMF (10 ml) was added thereto. The mixture was stirred at 4° for 15 hr. Isolation of the product was carried out in the manner as described for X; Rf^{I} 0.40, Rf^{II} 0.68. The resulting protected octapeptide XII (0.66 g) was hydrogenated over Pd in MeOH (50 ml) and 10% AcOH (10 ml). The catalyst was removed and the solvent was evaporated. The product was precipitated from MeOH with ether, yield 0.56 g (97%), $[\alpha]_D^{27}$ −101.3° (c=1.0, 10% AcOH); Rf^{I} 0.22, Rf^{II}

0.63; amino acid ratios in acid hydrolysate $Arg_{0.94}$ $Ser_{0.89}$ $Pro_{2.06}$ $Ala_{1.94}$ $Val_{1.04}$ $Phe_{1.02}$. *Anal.* Calcd. for $C_{41}H_{65}O_{12}N_{11}\cdot 3H_2O$: C, 51.39; H, 7.47; N, 16.08. Found: C, 51.40; H, 7.43; N, 15.98.

Z-Phe-Ser-Pro-Phe-Arg-Val-Ala-Pro-Ala-Ser-OH Acetate Pentahydrate (XIV)——Z-Phe-Ser-NH-NH_2[9-11] (0.32 g) was dissolved in DMF (10 ml) containing 6N HCl in dioxane (0.53 ml), and the solution was cooled to −15° and isoamyl nitrite (0.12 ml) was added. The mixture was left at −10° for 5 min and neutralized with triethylamine. An ice-cold solution of XIII (0.38 g) and triethylamine (0.12 ml) in DMF (10 ml) was added thereto. The mixture was stirred at 4° for 15 hr. Isolation of the product was carried out in the manner as described for X, yield 0.44 g (79%), mp 188—191° (sintering at 165°), $[\alpha]_D^{27}$ −74.4° (c=1.0, MeOH); Rf^{I} 0.49, Rf^{II} 0.65. *Anal.* Calcd. for $C_{61}H_{85}O_{17}N_{13}\cdot 5H_2O$: C, 53.76; H, 7.03; N, 13.36. Found: C, 53.53; H, 7.31; N, 13.63.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Val-Ala-Pro-Ala-Ser-OH (Bradykinyl-Val-Ala-Pro-Ala-Ser-OH) (I)——Protected decapeptide XIV (50 mg) was hydrogenated over Pd in MeOH (30 ml) and 10% AcOH (10 ml). The catalyst was removed, the solvents were evaporated, and the resulting product XV was dried *in vacuo*; $[\alpha]_D^{27}$ −107.1° (c=1.0, 10% AcOH), Rf^{I} 0.39, Rf^{II} 0.75; amino acid ratios in acid hydrolysate $Arg_{1.01}$ $Ser_{1.39}$ $Pro_{2.21}$ $Ala_{1.94}$ $Val_{0.93}$ $Phe_{2.04}$.

A solution of XVI (86 mg) in TFA (0.6 ml) was allowed to stand at room temperature for 40 min and anhydrous ether was added. The resulting Z-Arg(NO_2)-Pro-Pro-Gly-$NHNH_2$ (XVIII) was dissolved in DMF (6 ml) containing 6N HCl in dioxane (0.06 ml), and the solution was cooled to −15° and 10% isoamyl nitrite in DMF (0.16 ml) was added. The solution was left at −10° for 5 min and neutralized with triethylamine. An ice-cald solution of XV derived from XIV (50 mg) and triethylamine (0.015 ml) in DMF (5 ml) was added thereto. The mixture was stirred at 4° for 20 hr. Isolation of the product was performed in the manner as described for X. The resulting crude product was hydrogenated over Pd in MeOH (20 ml) and 50% AcOH (20 ml) for 40 hr. The catalyst was removed and the solvent were evaporated. The residue was lyophilized twice with a small amount of H_2O and then dissolved in H_2O (200 ml). The solution was applied to a column of CMC (3×7 cm), which was eluted successively with H_2O (200 ml) and NH_4OAc buffer: 0.005M (150 ml), 0.015M (250 ml), 0.025M (100 ml), and 0.05M (400 ml). The 0.05M NH_4OAc eluates containing the desired material were pooled and the solvent was evaporated. The product was lyophilized three times with H_2O and completely desalted by gelfiltration on Sephadex G-25 using 1M AcOH as eluent, yield 45 mg, $[\alpha]_D^{27}$ −113.4° (c=0.5, 10% AcOH), Rf^{I} 0.12, Rf^{II} 0.64, amino acid ratio in acid hydrolysate $Arg_{2.03}$ $Pro_{4.17}$ $Gly_{1.01}$ $Phe_{1.94}$ $Ser_{1.82}$ $Val_{1.01}$ $Ala_{1.86}$.

H-Val-Ala-Pro-Ala-Ser-OH Hydrate (XXI)——Z-Val-Ala-$NHNH_2$[14] (0.34 g) was dissolved in DMF (10 ml) and 6N HCl in dioxane (0.50 ml), and the solution was cooled to −15° and isoamyl nitrite (0.14 ml) was added. The solution was left at −10° for 5 min and neutralized with triethylamine. An ice-cold solution of V (0.14 g) and triethylamine (0.07 ml) in H_2O (6 ml) was added. The mixture was stirred at 4° for 15 hr and the solvent was evaporated. The crude product was isolated in the manner as described X and it was hydrogenated over Pd in MeOH (30 ml) and H_2O (20 ml). The resulting hydrogenated product was dissolved in H_2O (30 ml). The solution was applied to a CM-Sephadex C-25 (free form) column (1.3×20 cm), which was eluted successively with H_2O (100 ml), 0.1% AcOH (200 ml) and 0.5% AcOH (100 ml). The 0.1% AcOH eluates containing the desired material were pooled and the solvent was evaporated. The product was lyophilized with H_2O, yield 0.12 g (52%), $[\alpha]_D^{27}$ −131.1° (c=1.0, 10% AcOH), Rf^{I} 0.14, Rf^{II} 0.39; amino acid ratios in acid hydrolysate $Ser_{0.96}$ $Pro_{1.05}$ $Ala_{1.92}$ $Val_{1.07}$. *Anal.* Calcd. for $C_{19}H_{33}O_7N_5\cdot H_2O$: C, 49.44; H, 7.64; N, 15.17. Found: C, 48.75; H, 7.65; N, 15.46.

Enzymic Experiment——A peptide (2 μg) in 0.1M triethylammonium carbonate buffer (pH 8.0) (0.2 μl) was mixed with a solution of trypsin (TPCK-treated,[15] Worthington 3× crystallized) (2 μg) in the same buffer (2 μl). Incubation was performed at 25° for 2 hr and the reaction was terminated by addition of soy bean trypsin inhibitor (Sigma) (2 μg) in the same buffer (2 μl). The solution was evaporated to dryness in a vacuum desiccator over silica gel. The residue was subjected to biological assay[16] and dansylation.

Dansylation——Dansylation was carried out according to the method of Gray, *et al.*[13] Dansylated peptides were developed on Silica gel H plates in a solvent system of isopropanol–methyl acetate –28% ammonia (7 : 9 : 4).

15) V. Kostka and F.H. Carpenter, *J. Biol. Chem.*, 239, 1799 (1964).
16) I. Trautschols, "Handbook of Experimental Pharmacology," Bradykinin, Kallidin and Kallikrein, Vol. XXV, ed. by E.G. Erdos, Springer-Verlag, New York, 1970, p. 55.