but washing out the serum or bovine serum albumin removed the inhibition of insulin binding. Therefore, the inhibitory effect of serum on insulin binding is not due to the degradation of insulin receptors but to some other unknown factor (s).

In summary, erythrocyte membranes serve as receptor sites for the radioreceptor assay; a more sensitive assay should be possible simply by using solubilized erythrocyte membranes or larger amounts of membranes.

[Chem. Pharm. Bull. 28(1) 310—313 (1980)]

Synthesis of Bradykinin Fragments and Their Analogs modified at a Phenylalanine Residue[1,2]

Yoshio Okada, Yuko Tsuda, and Masami Yagyu

Faculty of Pharmaceutical Sciences, Kobe-Gakuin University[3]

(Received May 25, 1979)

Bradykinin fragments and their analogs, Gly–X–Ser–Pro (X: L-Phe I, D-Phe II, L-Ala III, D-Ala IV, β-Ala V and Gly VI) and X–Ser–Pro (X: L-Phe VII and D-Phe VIII) were synthesized. Compounds I, II, V, VII and VIII prolonged the pentobarbital-induced sleeping time in mice at the dosage of 2.5 nmol per mouse.

Keywords——bradykinin fragments; analogs; chemical synthesis; AP–M digest; effects on central nervous systems

Bradykinin injected intracerebrally into experimental animals produces a short-lasting phase of behavioral excitation, followed by sedation.[4] Iwata *et al.*, suggested that the excited state might be elicited by bradykinin, and that its metabolites might cause the depression.[5]

Synthesis of bradykinin fragments and their analogs is under way in our laboratory to investigate their action on the central nervous system by assessing their effects on pentobarbital-induced sleeping time in mice as an index. In a preliminary report,[1] we stated that some synthetic bradykinin fragments, Gly–Phe–Ser–Pro (I), Phe–Ser–Pro (VII) and Ser–Pro (IX) (Fig. 1) prolonged the pentobarbital-induced sleeping time in mice, and it was shown that the Ser–Pro moiety at the C-terminus of the peptides was required for effectiveness in this action. In this paper, we describe the synthesis of Gly–X–Ser–Pro (X: L-Phe I, D-Phe II, L-Ala III, D-Ala IV, β-Ala V, and Gly VI) and X–Ser–Pro (X: L-Phe VII, D-Phe VIII), as shown in Fig. 2, to study their effects on pentobarbital-induced sleeping time in mice.

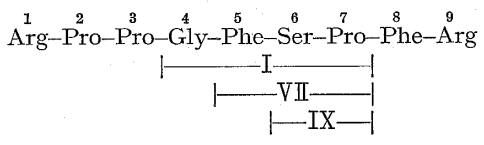
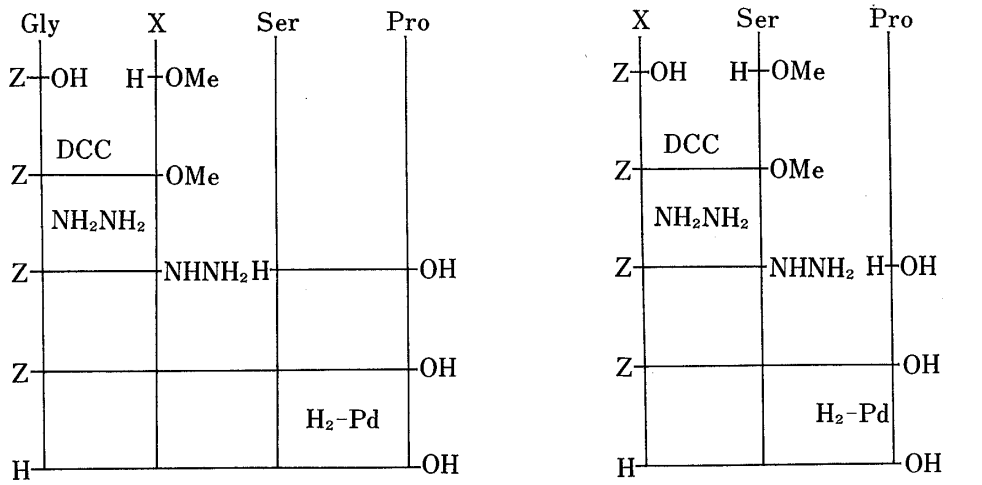


Fig. 1. Structure of Bradykinin and Its Fragments

1) Previous paper of this series, Y. Okada, Y. Tsuchiya, M. Yagyu, S. Kozawa, and K. Kariya, *Neuropharmacology*, **16**, 381 (1977).
2) Abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 3485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, DCC=N,N′-dicyclohexylcarbodiimide.
3) Location: *Ikawadani-machi, Tarumi-ku, Kobe, 673, Japan.*
4) F.G. Graeff, R.I. Pela, and M. Rocha E. Silva, *J. Pharmacol.*, **37**, 723 (1969).
5) H. Iwata, T. Shikimi, M. Iida, and H. Miichi, *Jap. J. Pharmacol.*, **20**, 80 (1970).



I: X=L-Phe; II: X=D-Phe; III: X=L-Ala; IV: X=D-Ala; V: X=β-Ala; VI: X=Gly

VII: X=L-Phe; VIII: X=D-Phe

Fig. 2. Synthetic Scheme for Bradykinin Fragments and Their Analogs

The synthesis of N-protected tetrapeptides, Z–Gly–X–Ser–Pro–OH, and tripeptides, Z–X–Ser–Pro–OH, was performed as shown in Fig. 2. The azide coupling of the N-terminal dipeptide fragment, Z–Gly–X–$NHNH_2$ with the C-terminal moiety, H–Ser–Pro–OH[6] and of Z–X–Ser–$NHNH_2$ with proline afforded Z–Gly–X–Ser–Pro–OH and Z–X–Ser–Pro–OH, respectively. The resulting N-protected peptides were purified by silica gel column chromatography (Table I). The Z group was removed by hydrogenation over a palladium catalyst, and the resulting peptides were purified by column chromatography on Dowex 50wx2 (H^+ form) using pyridine acetate buffer to give the purified peptides, I—VIII (Tables II and III).

Table I. N-Protected Peptides

Compound	mp (°C)	Yield (%)	$[\alpha]_D^{25}$ (MeOH)	Formula	Analysis (%) Calcd (Found.) C	H	N
Z–Gly–L-Phe–Ser–Pro–OH[a]	94—97	65.8	−41.2 (c =1.1)	$C_{27}H_{32}N_4O_8 \cdot H_2O$	58.1 (58.2	6.13 5.92	10.0 10.1)
Z–Gly–D-Phe–Ser–Pro–OH	Amorphous	77.9	−27.6 (c =1.0)	$C_{27}H_{32}N_4O_8$	60.0 (59.6	5.96 5.97	10.4 10.2)
Z–Gly–L-Ala–Ser–Pro–OH	80—86	49.5	−72.3 (c =0.9)	$C_{21}H_{28}N_4O_8 \cdot 1/2H_2O$	53.3 (53.4	6.17 6.19	11.8 11.8)
Z–Gly–D-Ala–Ser–Pro–OH	65—89	40.0	−25.1 (c =1.1)	$C_{21}H_{28}N_4O_8 \cdot 1/2H_2O$	53.3 (53.1	6.17 6.42	11.8 11.5)
Z–Gly–β-Ala–Ser–Pro–OH	Amorphous	39.1	−44.2 (c =1.1)	$C_{21}H_{28}N_4O_8 \cdot H_2O$	52.3 (52.7	6.26 6.10	11.6 11.1)
Z–Gly–Gly–Ser–Pro–OH	Amorphous	56.9	−48.5 (c =0.8)	$C_{20}H_{26}N_4O_8 \cdot 1/2H_2O$	51.3 (51.4	6.02 6.12	12.0 11.6)
Z–L-Phe–Ser–Pro–OH[b]	74—95	75.0	−48.7 (c =1.0)	$C_{25}H_{29}N_3O_7$	62.1 (62.0	6.05 6.23	8.7 8.4)
Z–D-Phe–Ser–Pro–OH	Amorphous	58.0	−31.5 (c =0.8)	$C_{25}H_{29}N_3O_7$	62.1 (61.8	6.05 6.12	8.7 8.5)

a) $[\alpha]_D^{25}$ −26.4° (DMF), Lit.[6] mp 50—60°, $[\alpha]_D^{18}$ −20.8° (DMF).
b) $[\alpha]_D^{25}$ −37.5° (DMF), Lit.[6] mp 75—86°, $[\alpha]_D^{21}$ −50.7° (DMF).

6) K. Suzuki, T. Abiko, and N. Endo, *Chem. Pharm. Bull.*, **17**, 1671 (1969).

Table II. Yields, $[\alpha]_D$ Values and *Rf* Values of Peptides I—VIII

Compound No.		Yield (%)	$[\alpha]_D^{25}$ (H_2O)	TLC *Rf*¹	TLC *Rf*²
I	Gly–L-Phe–Ser–Pro[a]	48.6	−66.5 (c =0.4)	0.26	0.28
II	Gly–D-Phe–Ser–Pro	51.8	−67.9 (c =0.5)	0.30	0.31
III	Gly–L-Ala–Ser–Pro	49.5	−26.9 (c =0.5)	0.10	0.31
IV	Gly–D-Ala–Ser–Pro	55.0	−48.7 (c =0.3)	0.12	0.31
V	Gly–β-Ala–Ser–Pro	63.5	−70.7 (c =0.9)		0.31
VI	Gly–Gly–Ser–Pro	20.0	−87.9 (c =1.0)	0.17	0.30
VII	L-Phe–Ser–Pro[b]	83.3	−43.6 (c =0.5)	0.41	0.42
VIII	D-Phe–Ser–Pro	90.0	−135.8 (c =0.1)	0.41	0.43

a) Lit[6] $[\alpha]_D^{18}$ −42.5° (H_2O).
b) Lit[6] $[\alpha]_D^{24}$ −24.7° (H_2O).

Table III. Results of Amino Acid Analysis of the Peptides I—VIII

Compound No.		Acid hydrolysate (Treated with D-amino acid oxidase) Gly	X	Ser	Pro	APM digest (Recovery %) Gly	X	Ser	Pro
I	Gly–L-Phe–Ser–Pro	1.00	1.00	0.86	0.98	1.1 (82.6)	1.0 (79.1)	1.0 (74.8)	1.0 (77.4)
II	Gly–D-Phe–Ser–Pro	1.00 (0.96)	1.18 (<0.01)	0.89 (1.00)	0.85 (1.08)	1.4 (22.3)	1.0 (15.9)	0.9 (13.9)	1.0 (15.7)
III	Gly–L-Ala–Ser–Pro	1.00	1.00	0.89	1.00	1.1 (79.4)	1.0 (73.8)	0.9 (68.0)	1.0 (73.0)
IV	Gly–D-Ala–Ser–Pro	1.00 (1.00)	1.00 (<0.01)	0.84 (0.94)	1.10 (1.06)	2.3 (56.1)	1.0 (26.1)	0.9 (22.8)	1.0 (24.0)
V	Gly–β-Ala–Ser–Pro	1.00	0.99	0.91	1.15	3.0 (82.0)	1.0 (27.8)	1.0 (27.0)	1.0 (26.7)
VI	Gly–Gly–Ser–Pro	1.96	—	0.91	1.00	2.2 (81.7)	—	1.0 (73.6)	1.0 (71.7)
VII	L-Phe–Ser–Pro	—	1.00	0.97	1.17	—	0.9 (84.5)	1.0 (89.7)	1.0 (88.1)
VIII	D-Phe–Ser–Pro	—	0.93 (<0.01)	0.92 (0.92)	1.00 (1.00)	—	1.0 (19.6)	1.0 (18.7)	0.9 (16.0)

All peptides thus obtained were homogeneous upon thin layer chromatography on silica gel G; furthermore, each peptide gave a single symmetrical peak on an amino acid analyzer at a concentration of 10 μM. Table III shows the results of amino acid analysis of the peptides. Amino acid ratios in the acid hydrolysates are in good agreement with the theoretically expected values. Treatment of acid hydrolysates of II, IV and VIII with D-amino acid oxidase (Sigma Chemical Co., Lot. 23C–0390)[7] destroyed the D-amino acid completely. Amino acid ratios in aminopeptidase M (AP–M) digests[8] of these peptides are also shown in Table III. All L-peptides (I, III, VII) and VI were digested completely. In addition, it was observed that the AP–M employed (Pierce Chemical Co., Lot. 08307.33) cleaved the Gly–D-Phe bond to the extent of about 20%, the Gly–D-Ala bond by more than 50%, the D-Phe–Ser bond by about 20% and even the β-Ala–Ser bond by 30%.

As described previously,[1] bradykinin fragments, Gly–Phe–Ser–Pro (I) (132.0±7.6 in comparison to saline=100) and Phe–Ser–Pro (VII) (131.4±9.2), prolonged the pentobarbital-induced sleeping time at a dosage of 2.5 nmol per mouse. The β-Ala analog (V) (123.7±

7) T.Y. Liu and E.C. Gotshlich, *J. Biol. Chem.*, **238**, 1928 (1963).
8) K. Hoffman, F.M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

12.8) and D-Phe analog (VIII) (124.0±9.5) showed activities which were significant, but lower than those of the parent molecules, (I) and (VII), and the D-Phe analog (II) (114.1 ±7.2) also showed a low activity at the same dosage. The L-Ala analog (III) (103.6±5.6), D-Ala analog (IV) (99.6±6.2) and Gly analog (VI) (113.7±13.4) did not exhibit any marked effects on sleeping time at the dosage of 2.5 nmol per mouse. The biological activity will be reported in detail elsewhere (K. Kariya *et al.*, in preparation).

Experimental

General experimental methods employed here were essentially the same as those described in the previous paper.[9] Thin layer chromatography was performed on silica gel (Kieselgel G, Merck). Rf^1 and Rf^2 values refer to the systems of *n*-butanol, AcOH and H_2O (4:1:5), and *n*-butanol, pyridine, AcOH and H_2O (4:1:1:2), respectively.

Z-Gly-X-Ser-Pro-OH (X: L-Phe, D-Phe, L-Ala, D-Ala, β-Ala, Gly)——Z–Gly–X–$NHNH_2$ (10 mmol) was dissolved in DMF (30 ml) containing 5.8 N HCl in dioxane (3.4 ml). The solution was cooled to −20°, and isoamylnitrite (1.4 ml, 10 mmol) was added. After 5 min, the pH of the solution was adjusted to 8 with triethylamine. This solution was mixed with a solution of H–Ser–Pro–OH[6] (2.0 g, 10 mmol) in H_2O (30 ml) containing triethylamine (1.4 ml, 10 mmol). The reaction mixture was stirred at 4° for 48 hr. After removal of the solvents, the residue was dissolved in 5% $NaHCO_3$ (50 ml), which was washed with AcOEt. The water layer was acidified with conc. HCl and the oily material was extracted with *n*-butanol, which was washed with water and concentrated. Ether was added to the residue to afford a solid material, which was purified by silica gel column chromatography. The yield, physical constants and analytical data are presented in Table I.

Z-X-Ser-Pro-OH (X: L-Phe, D-Phe)——Z–X–Ser–$NHNH_2$ (2 g, 5 mmol) was converted to the corresponding azide by the method described above. This azide in DMF (20 ml) was mixed with a solution of proline (1.15 g, 10 mmol) in H_2O (10 ml) containing triethylamine (1.4 ml, 10 mmol). The reaction mixture was stirred at 4° for 48 hr. After removal of the solvents, the residue was dissolved in 5% $NaHCO_3$ (50 ml), and the solution was washed with AcOEt. The water layer was acidified with conc. HCl and the oily precipitate was extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4 and concentrated. Petroleum ether was added to the residue to give a white powder (Table I).

General Procedure for the Debenzyloxycarbonylation of N-Protected Peptides——N-Protected peptide (0.9 mmol) in MeOH (15 ml) was hydrogenated over a palladium catalyst. After removal of palladium and of the solvent, the residue was dissolved in H_2O (5 ml) and lyophilized. This amorphous material in H_2O (2 ml) was applied to a column of Dowex 50 wx2 (2×15 cm) equilibrated with 0.05 M pyridine acetate buffer (pH 3.8). The peptide was eluted with a gradient to 0.05 M pyridine acetate buffer (pH 5.8). Fractions of 15 ml were collected. After reaction with ninhydrin, the absorbancy at 570 nm was used to monitor the chromatographic pattern. The yield, *Rf* values and physical constants are presented in Table II.

9) Y. Okada, C. Kawasaki, M. Okinaka, and K. Kawasaki, *Chem. Pharm. Bull.*, **27**, 1360 (1979).