Studies on the Secondary Structure of Bradykinin in Aqueous Solution. Syntheses, Circular Dichroism Spectra, and Biological Activities of Bradykinin Analogs Containing 5-Aminovaleric Acid Residue

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Eight bradykinin analogs containing 5-aminovaleric acid residue were synthesized by a solution method. Based on the consideration concerning their CD spectra, the presence of an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8, an intramolecular 4→1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5, and a salt bridge between the guanidino group of Arg^1 and the carboxyl group of Arg^9, was proposed for the secondary structure of bradykinin in aqueous solution. The biological activities of these analogs depend on the presence of both Phe residues at positions 5 and 8, and Arg residues at positions 1 and 9. No correlation between the biological activities and the secondary structure could be found.

Bradykinin (BK) was discovered by Rocha e Silva et al.,[1] isolated by Elliott et al.,[2] and synthesized by Boissonnas et al.[3] This peptide shows mainly activities on uterus and smooth muscle contraction, pain, and blood pressure etc. To date, a large number of BK analogs have been prepared in order to examine the relationship between structure and activity. The secondary structure of BK in solution has been studied by several research groups. Initial studies on the secondary structure of BK were made by Bodanszky et al.[4] and Brady et al.[5] They suggested that BK existed in an extended conformation, namely a random coil, from the results of the ORD and CD spectra. From a study on the CD spectra of BK, its fragments, and model compounds, a secondary structure model of BK was proposed by Cann et al.[6] It has an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser^6 and the amide proton of Phe^8, which was considered to cause the CD band at 234 nm of the peptide. On the basis of studies by electron spin resonance and fluorescence energy transfer, Ivanov et al.[7] suggested ionic bond formation between the guanidino group of Arg^1 and the carboxyl group of Arg^9. A ^{13}C NMR study by London et al.[8] provided evidence for the existence of the 3→1 hydrogen bond, but not for the formation of a salt bridge. Chipens et al.[9] implied a certain similarity in the secondary structure between the BK and *cyclo*-BK analogs from studies of their CD spectra. In the past, we reported on preliminary results of a study of BK analogs containing a 5-aminovaleric acid (5-Ava) residue.[10-12]

In this paper we wish to report on the syntheses, the CD spectra, and the biological activities of eight BK analogs containing the 5-Ava residue shown in Table 1. When each sequence of two adjacent amino acid residues is replaced by one residue of 5-Ava, each amide group is substituted by an ethylene group, and each side chain by a hydrogen atom, respectively. The contributions of each amide bond and each side chain to the secondary structure and the biological activity of BK is discussed here on the basis of the results of the CD spectra and biological activities.

Table 1. Primary Sequences of Bradykinin and Eight Bradykinin Analogs Containing 5-Ava Residue

	1	2	3	4	5	6	7	8	9	
Bradykinin (BK)	H–Arg–	Pro–	Pro–	Gly–	Phe–	Ser–	Pro–	Phe–	Arg–	OH
[5-Ava$^{1-2}$]-BK (**9**)	H–	5-Ava	–							–OH
[5-Ava$^{2-3}$]-BK (**16**)	H–	–	5-Ava	–						–OH
[5-Ava$^{3-4}$]-BK (**26**)	H–		–	5-Ava	–					–OH
[5-Ava$^{4-5}$]-BK (**32**)	H–			–	5-Ava	–				–OH
[5-Ava$^{5-6}$]-BK (**37**)	H–				–	5-Ava	–			–OH
[5-Ava$^{6-7}$]-BK (**46**)	H–					–	5-Ava	–		–OH
[5-Ava$^{7-8}$]-BK (**50**)	H–						–	5-Ava	–	–OH
[5-Ava$^{8-9}$]-BK (**56**)	H–							–	5-Ava	–OH

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Results and Discussion

Synthesis of BK Analogs Containing 5-Ava Residue. The synthetic methods for BK analogs containing the 5-Ava residue are shown in Figs. 1—8. All of them were synthesized by a solution method. Following protecting groups were used for synthesizing those compounds: Z[13] or Boc group for the α-amino groups; methyl, ethyl, or benzyl ester group for the carboxyl groups; and nitro group for the guanidino group of arginine. The hydroxyl group of serine was not protected. The above-mentioned protecting groups were deprotected by the following methods: HCl/THF, HCl/dioxane, or TFA for Boc group, HBr/AcOH or H_2/Pd for Z group, saponification for methyl and ethyl ester groups, and H_2/Pd for benzyl ester and nitro groups. For peptide bond formation, DCC,[14] DCC/HOBt,[15]

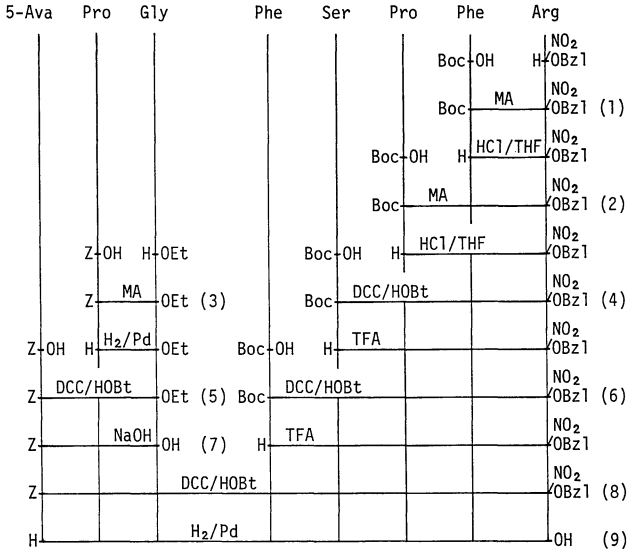

Fig. 1. Synthetic scheme of [5-Ava[1-2]]-BK.

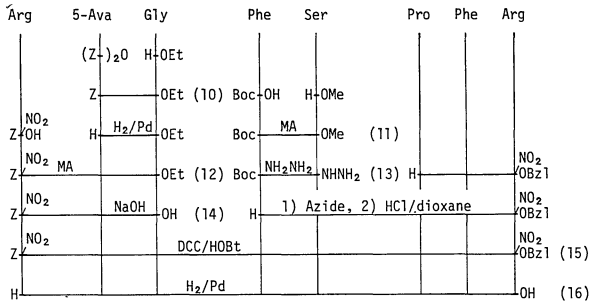

Fig. 2. Synthetic scheme of [5-Ava[2-3]]-BK.

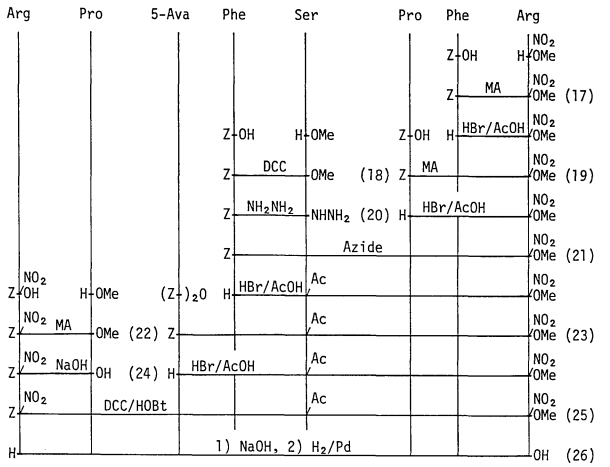

Fig. 3. Synthetic scheme of [5-Ava[3-4]]-BK.

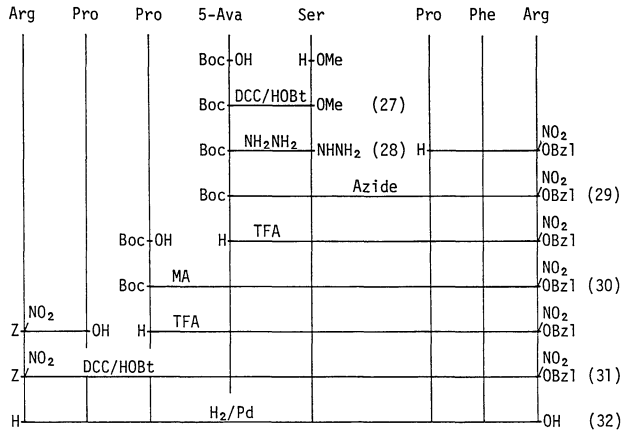

Fig. 4. Synthetic scheme of [5-Ava[4-5]]-BK.

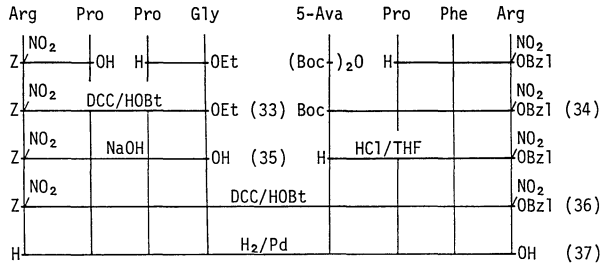

Fig. 5. Synthetic scheme of [5-Ava[5-6]]-BK.

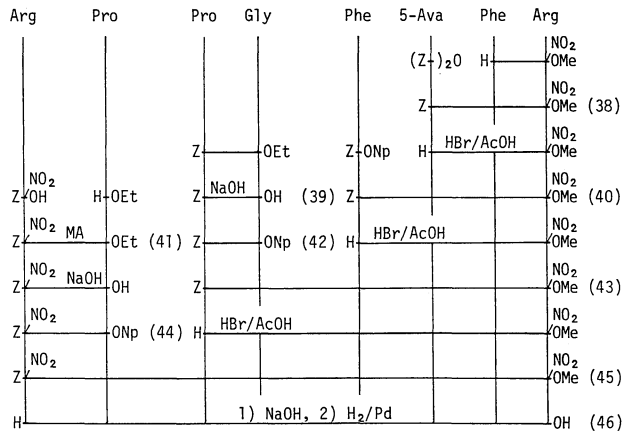

Fig. 6. Synthetic scheme of [5-Ava[6-7]]-BK.

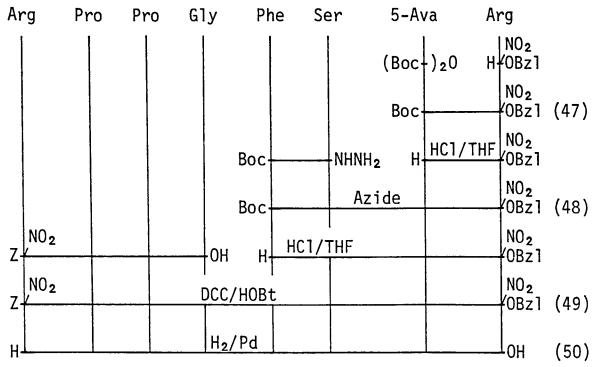

Fig. 7. Synthetic scheme of [5-Ava$^{7-8}$]-BK.

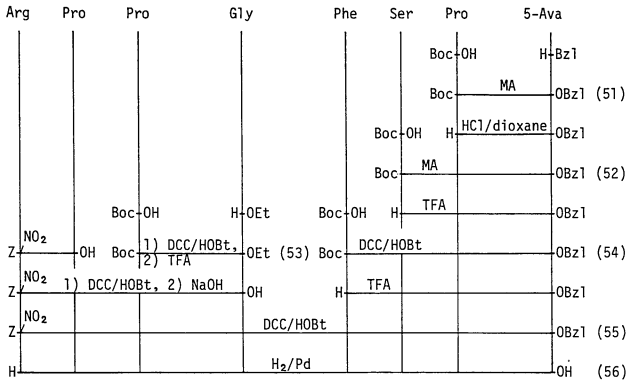

Fig. 8. Synthetic scheme of [5-Ava$^{8-9}$]-BK.

MA,[16] azide,[17] active ester,[18] and symmetrical anhydride methods were employed. The final deprotection of protected [5-Ava$^{1-2}$]-BK (**8**), [5-Ava$^{2-3}$]-BK (**15**), [5-Ava$^{4-5}$]-BK (**31**), [5-Ava$^{5-6}$]-BK (**36**), [5-Ava$^{7-8}$]-BK (**49**), and [5-Ava$^{8-9}$]-BK (**55**) was carried out by catalytic hydrogenation. On the other hand, deprotection of protected [5-Ava$^{3-4}$]-BK (**25**) and [5-Ava$^{6-7}$]-BK (**45**) was performed by saponification followed by catalytic hydrogenation.

In coupling of the carboxyl group of 5-Ava, a mixed anhydride method gave some by-products; a DCC method did not afford the expected product. A symmetrical anhydride and DCC/HOBt methods gave satisfactory results. Purification of protected octapeptides was performed by gel filtration on a Sephadex LH-20 column using DMF as the eluent. Free peptides were purified by ion-exchange chromatography on a carboxymethyl cellulose column, gel filtration on a Sephadex G-10 column, and partition chromatography on a Sephadex G-25 column.[19] The purity of all products was confirmed by TLC, elemental analysis, and amino acid analysis. Table 2 shows physical data concerning other compounds, except for those described in the experimental section.

CD Spectra of BK Analogs Containing 5-Ava Residue. The CD spectra were measured in the region of about 190 to 270 nm in aqueous solution. The data are reported as molar ellipticity, [θ], which is expressed in degree square centimeter per decimole.

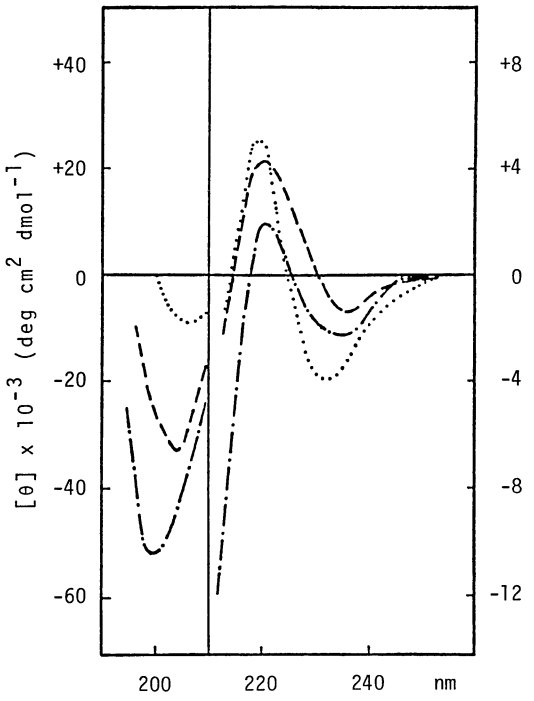

Fig. 9. CD spectra of [5-Ava$^{3-4}$]-BK (**26**) (·····), [5-Ava$^{5-6}$]-BK (**37**) (————), and [5-Ava$^{8-9}$]-BK (**56**) (—·—·—) in water.

The CD spectrum of BK shows a negative band at 234 nm, a positive band at 221 nm, and a deep negative band at about 200 nm (Fig. 11). The CD spectra of the BK analogs containing the 5-Ava residue are classified into three types in the region 210—260 nm. The CD spectra of [5-Ava$^{3-4}$]-BK (**26**), [5-Ava$^{5-6}$]-BK (**37**), and [5-Ava$^{8-9}$]-BK (**56**) (Fig. 9) show a negative band near 234 nm and a positive band near 221 nm, resembling that of BK. In the region near 200 nm, [5-Ava$^{3-4}$]-BK shows a considerably shallower trough, as compared with that of BK. The CD spectra of [5-Ava$^{1-2}$]-BK (**9**), [5-Ava$^{2-3}$]-BK (**16**), and [5-Ava$^{4-5}$]-BK (**32**) (Fig. 10) near 234 nm are similar to that of BK, although their peak near 221 nm is lower. Concerning the negative band near 200 nm, [5-Ava$^{1-2}$]-BK and [5-Ava$^{2-3}$]-BK show a much shallower trough. The CD spectra of [5-Ava$^{6-7}$]-BK (**46**) and [5-Ava$^{7-8}$]-BK (**50**) (Fig. 11) do not show a negative band at 234 nm and exhibit a simple peak near 221 nm. The ellipticity of [5-Ava$^{6-7}$]-BK is very large. Regarding the negative band near 200 nm, the ellipticity of [5-Ava$^{7-8}$]-BK is similar to that of BK, although that of [5-Ava$^{6-7}$]-BK is small.

In [5-Ava$^{6-7}$]-BK and [5-Ava$^{7-8}$]-BK, the absence of the negative band at 234 nm suggests that both the amide bonds of Ser^{6}–Pro^{7} and Pro^{7}–Phe^{8} of BK relate to this negative band. That is, these two analogs can

Table 2. Yields, Physical Properties, and Analytical Data of Intermediate and Final Peptides[a)]

Compound	Yield/%	Mp θ_m/°C	$[\alpha]_D^{24}$/° (*c* 1.0, DMF)	Molecular formula	Elemental analysis/% Found (F:), Calcd (C:)	C	H	N	R_f[b)]
3	95	Oil	—	—			—		R^1 0.77, R^2 0.52
5	68	Oil	—	—			—		R^1 0.69, R^4 0.74, R^5 0.71
7	88	Oil	—	—			—		R^4 0.48, R^5 0.58
9	52	—	−64.7[f)] (*c* 0.1, H_2O)	$C_{44}H_{63}O_{10}N_{11}\cdot CH_3COOH\cdot 2.5H_2O$	F: C:	54.64 54.64	6.75 7.18	15.07 15.24	R^9 0.61 (Cellulose)
12	66	97—102	+1.7 (*c* 1.24)	$C_{23}H_{35}O_8N_7\cdot H_2O$	F: C:	49.60 49.72	6.39 6.71	18.09 17.65	R^1 0.40, R^4 0.53, R^8 0.71
13	54	165	−3.6 (*c* 0.84)	$C_{17}H_{26}O_5N_4\cdot 1/2H_2O$	F: C:	54.22 54.38	7.73 7.24	15.70 14.92	R^4 0.36
14	63	139—142	−3.5 (*c* 0.90, MeOH)	$C_{21}H_{31}O_8N_7\cdot 1/2H_2O$	F: C:	48.84 48.64	6.24 6.22	18.88 18.91	R^5 0.49
15	41	135—140	−35.4 (*c* 0.88)	$C_{60}H_{78}O_{16}N_{16}\cdot 6H_2O$	F: C:	52.69 51.94	5.77 6.53	15.68 16.15	R^1 0.11, R^4 0.24, R^8 0.79
16	15	—	−36.7 (*c* 0.1, H_2O)	$C_{45}H_{68}O_{10}N_{14}\cdot 2CH_3COOH\cdot 3H_2O$	F: C:	51.78 51.65	6.98 7.25	17.85 17.21	R^6 0.87, R^7 0.28, R^8 0.64 (Cellulose)
23	81	204—208	−45.2[g)]	$C_{48}H_{62}O_{13}N_{10}$	F: C:	57.74 58.40	6.28 6.33	13.93 14.19	R^1 0.53, R^2 0.50
25	37	168—171	−53[d)] (*c* 0.5)	$C_{59}H_{80}O_{17}N_{16}$	F: C:	56.02 55.13	6.21 6.27	17.73 17.43	R^2 0.23, R^5 0.73
26	63	—	−77.2[e)] (*c* 0.1, H_2O)	$C_{48}H_{72}O_{10}N_{14}\cdot 2CH_3COOH\cdot 5H_2O$	F: C:	51.71 51.39	7.04 7.46	16.09 16.13	R^8 0.43, R^{10} 0.66
27	66	Oil	—	—			—		R^1 0.44
31	71	123—125.5 (Decomp)	−53.1 (*c* 0.167)	$C_{59}H_{80}O_{16}N_{16}\cdot 2H_2O$	F: C:	54.45 54.29	6.32 6.49	17.49 17.17	R^1 0.29, R^4 0.36, R^5 0.63
32	44	—	−93.7[f)] (*c* 0.1, H_2O)	$C_{44}H_{70}O_{10}N_{14}\cdot 2CH_3COOH\cdot 3H_2O$	F: C:	51.09 51.05	7.01 7.50	17.54 17.36	R^8 0.29, R^5 0.45, R^6 0.65, R^8 0.56 (Cellulose)
34	80	Oil	—	—			—		R^2 0.47
36	80	Oil	—	—			—		R^1 0.41
37	5	—	−64.1[e)] (*c* 0.1, H_2O)	$C_{43}H_{68}O_9N_{14}\cdot 2CH_3COOH\cdot 7H_2O$	F: C:	47.98 48.19	6.67 7.74	16.58 16.74	R^8 0.35
38	60	110—111	−9.6	$C_{29}H_{39}O_8N_7$	F: C:	56.01 56.76	6.73 6.41	15.33 15.98	R^2 0.40
40	70	127—129	−11.7[c)]	$C_{38}H_{48}O_9N_8$	F: C:	60.12 59.99	6.31 6.36	14.60 14.73	R^2 0.55
41	49	146—147	−39.4	$C_{21}H_{30}O_7N_6$	F: C:	52.90 52.71	6.47 6.32	17.25 17.56	R^2 0.63
44	33	78—94	−61.5[d)]	$C_{25}H_{29}O_9N_7$	F: C:	53.57 52.53	5.66 5.11	17.01 17.15	R^2 0.60
45	35	124—126	−31.5[d)] (*c* 0.1)	$C_{56}H_{76}O_{15}N_{16}$	F: C:	55.34 55.44	6.21 6.31	18.26 18.47	R^3 0.65
46	56	—	−27.0[e)] (*c* 0.1, H_2O)	$C_{47}H_{70}O_9N_{14}\cdot 2CH_3COOH\cdot 6H_2O$	F: C:	50.42 50.90	7.06 7.54	16.19 16.30	R^{10} 0.41, R^{10} 0.78 (Cellulose)

Table 2. (Continued)

Compound	Yield/%	Mp θ_m/°C	$[\alpha]_D^{24}$/°	Molecular formula	Elemental analysis/% Found (F:), Calcd (C:) C	H	N	R_f[b]
50	9	—	−40.5[e] (*c* 0.12, H_2O)	$C_{41}H_{66}O_{10}N_{14}\cdot 2CH_3COOH\cdot 4H_2O$	F: 48.81	7.47	17.71	R^9 0.44 (Cellulose)
					C: 48.82	7.46	17.71	
51	73	Oil	—	—		—		R^1 0.83, R^4 0.90
52	52	134—135.5	−29.6	$C_{25}H_{37}O_7N_3$	F: 61.42	7.75	8.51	R^1 0.65, R^4 0.80, R^5 0.86
					C: 61.08	7.59	8.55	
53	100	Oil	—	—		—		R^1 0.79, R^4 0.81
54	97	Oil	—	—		—		R^1 0.60, R^4 0.76, R^5 0.87
55	97	108—113	−53.8	$C_{55}H_{72}O_{14}N_{12}\cdot H_2O$	F: 57.89	6.56	14.68	R^1 0.33, R^4 0.37, R^5 0.61
					C: 57.78	6.52	14.70	

a) Compound **1**[24] has mp 155—157°C; **2**[24] mp 85—92°C; **4**[24] mp 155.5—159°C; **6**[24] mp 110—113°C; **11**[25] mp 67—69°C; **17**[26] mp 152—155°C; **18**[3] mp 126—129°C; **19**[26] mp 135—138°C; **20**[3] mp 189°C; **22**[27] mp 162—164°C; **24**[28] mp 114—117°C; **33**[29] mp 87—90°C; **39**[26] oil; **42**[26] mp 143.5—144°C. Compound **21** produced in 62% yield; **47** 40% yield; **48** 66% yield; **49** 45% yield: other data of these compounds are unlisted. b) The solvent systems were described in experimental section. Silica-gel plate was used for TLC except when noted herein. c) At 19°C. d) At 20°C. e) At 21°C. f) At 22°C. g) At 25.5°C.

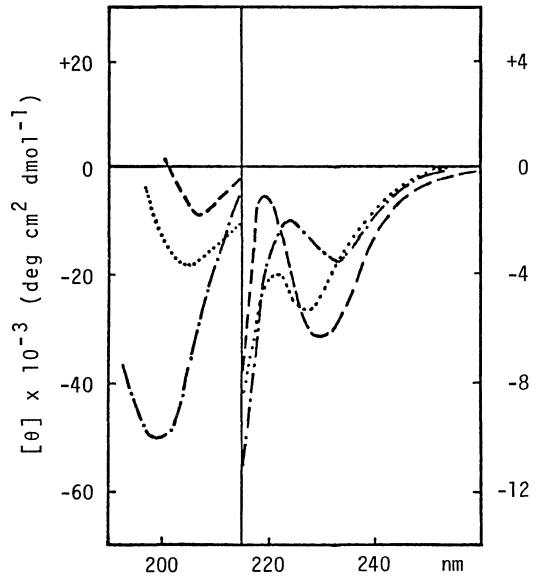

Fig. 10. CD spectra of [5-Ava[1-2]]-BK (**9**) (·····), [5-Ava[2-3]]-BK (**16**) (————), and [5-Ava[4-5]]-BK (**32**) (—·—·—) in water.

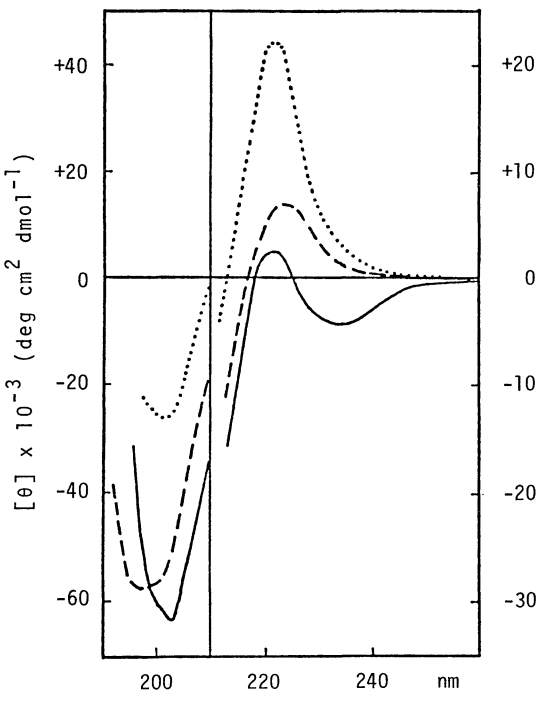

Fig. 11. CD spectra of BK (————), [5-Ava[6-7]]-BK (**46**) (·····), and [5-Ava[7-8]]-BK (**50**) (————) in water.

not form an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Ser[6] and the amide proton of Phe[8] as the result of replacement with the 5-Ava residue. The existence of the hydrogen bond in the BK molecule was pointed out by Cann et al.[6] Our result provides additional evidence for the formation of the intramolecular 3→1 hydrogen bond.

Cann et al. indicated the importance of an N-terminal sequence and Phe residues as a contribution for the positive band at 221 nm, suggesting an intramolecular 3→1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Gly^4.[6] Ivanov et al. proposed four energetically preferable BK-conformers which contain the salt bridge.[7] One of them forms an intramolecular 4→1 hydrogen bond between the carbonyl oxygen of Pro^2 and the amide proton of Phe^5 in the molecule. Although the positive band near 221 nm is generally referred to as arising from a Phe residue, the present result suggests that both amide bonds of Pro^2–Pro^3 and Gly^4–Phe^5 also contribute to this band. This result may be explained by supposing the formation of a partial structure stabilized with the intramolecular 4→1 hydrogen bond. Also, the similarity of the positive bands of [5-Ava^{3-4}]-BK and BK appears to make the 3→1 hydrogen bond in N-terminal sequence unlikely. In addition, the peak of [5-Ava^{1-2}]-BK, which lacks the strongly basic group on the side chain of Arg^1, is lower than those of [5-Ava^{2-3}]-BK and [5-Ava^{4-5}]-BK. This may be due to a change of secondary structure, owing to an impossibility to form the salt bridge between the guanidino group of Arg^1 and the C-terminal carboxyl group, proposed by Ivanov et al.[7] The similarity of the positive bands near 220 nm of BK-amide and [5-Ava^{1-2}]-BK implies the presence of the salt bridge in BK.[12] That is, the stability of the intramolecular 4→1 hydrogen bond is lowered by the incapability of salt-bridge formation because of a blocking of the C-terminal carboxyl group. This impossibility may bring about a decrease of ellipticity near 221 nm. The similarity between the peaks of BK and [5-Ava^{8-9}]-BK, in which the formation of salt bridge is possible, supports this supposition.

The analogs, (in which each sequence of Arg^1–Pro^2, Pro^2–Pro^3, Pro^3–Gly^4, Phe^5–Ser^6, and Ser^6–Pro^7 was replaced with a 5-Ava residue), show a shallower trough near 200 nm, as compared with that of BK; that is, this band probably arises mainly from the both sequences of Arg^1–Pro^2–Pro^3–Gly^4 and Phe^5–Ser^6–Pro^7 in BK.

The contributions for each CD band of BK are suggested as follows: the intramolecular 3→1 hydrogen bond for 234 nm band, Phe residues and the intramolecular 4→1 hydrogen bond for 221 nm band, and the both sequences of Arg^1–Pro^2–Pro^3–Gly^4 and Phe^5–Ser^6–Pro^7 for about the 200 nm band. Much smaller ellipticities of BK, compared with other peptides carrying a rigid conformation, such as gramicidin S, may reflect a higher flexibility in the backbone structure of BK.

Biological Activities of BK Analogs Containing 5-Ava Residue. The biological activities of these analogs were examined for uterus contraction of rat and blood flow of dog. The results shown in Table 3 are expressed as relative activity compared with the threshold dose of BK. [5-Ava^{1-2}]-BK, [5-Ava^{4-5}]-BK, [5-Ava^{5-6}]-BK, [5-Ava^{7-8}]-BK, and [5-Ava^{8-9}]-BK have no activity, while [5-Ava^{2-3}]-BK, [5-Ava^{3-4}]-BK, and [5-Ava^{6-7}]-BK indicate some of the activity, although very weak compared with that of BK. It has been known that the biological activity of BK analogs are drastically reduced by substitution of both or one of the Arg residue at positions 1 and 9 with a neutral amino acid or acidic amino acid residue, whereas the substitution of the Arg residue with other basic amino acids preserves considerable biological activities. [5-Ava^{1-2}]-BK and [5-Ava^{8-9}]-BK lack in Arg residue at positions 1 and 9, respectively. The present results also emphasize the importance of these two basic amino acid residues regarding biological activity. One or two of the Phe residues at positions 5 and 8 in BK can be substituted with an amino acid containing a restricted side chain, such as tyrosine methyl ether and 3-cyclohexylalanine, without any striking change regarding the biological activities.[20,21] However, replacement of the Phe residue by an aliphatic amino acid residue, such as the Ala, Leu, or Ile residue, results in a loss of activity.[21-23] [5-Ava^{4-5}]-BK, [5-Ava^{5-6}]-BK, [5-Ava^{7-8}]-BK, and [5-Ava^{8-9}]-BK did not show any biological activity. These results indicate similarly the importance for biological activity of the Phe residues at positions 5 and 8.

It is an interesting problem to elucidate the relationship between the structure in an aqueous solution and the activities of BK. [5-Ava^{3-4}]-BK, in which the peptide bond substituted with 5-Ava residue does not take part in the intramolecular 4→1 hydrogen bond, showed biological activity. Also, [5-Ava^{2-3}]-BK and [5-Ava^{6-7}]-

Table 3. Biological Activities of Eight Bradykinin Analogs Containing 5-Ava Residue

Compound	Blood flow[a] (dog, femoral artery)	Uterus contraction[b] (rat, isolated)
Bradykinin (BK)	1	1
[5-Ava^{1-2}]-BK (**9**)	Inactive	Inactive
[5-Ava^{2-3}]-BK (**16**)	4×10^{-5}	1×10^{-2}
[5-Ava^{3-4}]-BK (**26**)	1×10^{-3} [c]	1×10^{-3}
[5-Ava^{4-5}]-BK (**32**)	Inactive	Inactive
[5-Ava^{5-6}]-BK (**37**)	Inactive	Inactive
[5-Ava^{6-7}]-BK (**46**)	1×10^{-2}	1×10^{-3}
[5-Ava^{7-8}]-BK (**50**)	Inactive	Inactive
[5-Ava^{8-9}]-BK (**56**)	Inactive	Inactive

a) The threshold dose of BK: 1×10^{-9} g (=1). b) The threshold dose of BK: 1×10^{-9} g ml^{-1} (=1). c) Rat.

BK have biological activity, although not so strong as that of BK, in spite of an absence of the carbonyl oxygen of Pro^2 or Ser^6, which participates in the intramolecular 4→1 hydrogen bond or the intramolecular 3→1 hydrogen bond, respectively. These results imply that the biological activities of BK are affected by the absence of Arg and Phe residues, rather than the one of the intramolecular hydrogen bonds. On account of the structural flexibility of these linear peptides, a number of conformers may exist in equilibrium in solution. Therefore, the contribution of secondary structure for biological activity is ambiguous. We could not find any appreciable relationship between the CD spectra and the biological activities.

Experimental

Melting points were measured by a micro melting-point apparatus (Yanagimoto MFG Co.) and are given as uncorrected values. Optical rotations were determined with a polarimeter III (Shimadzu Co.). Amino acid analyses were performed on a JLC-6AS automatic analyzer (JEOL) after acid hydrolysis with 6 M (1 M=1 mol dm^{-3}) HCl in a sealed tube at 110 °C for 16—24 h. The theoretical values of the amino acid ratios are shown in parentheses after each result.

Thin-layer chromatography was performed on a TLC plate of silica gel 60 F_{254} (Merck and Co.) or cellulose (Avicel), using the following solvent systems (volume ratios): R_f^1, chloroform–methanol (9:1); R_f^2, chloroform–methanol–acetic acid (95:5:3); R_f^3, chloroform–methanol–acetic acid (95:15:3); R_f^4, chloroform–methanol–acetic acid (9:1:1); R_f^5, 1-butanol–acetic acid–water (4:1:1); R_f^6, 1-butanol–pyridine–acetic acid–water (15:10:3:12); R_f^7, 1-butanol–pyridine–acetic acid–water (16:3:1:4); R_f^8, 1-butanol–pyridine–acetic acid–water (4:1:1:2); R_f^9, 1-butanol–pyridine–acetic acid–water (16:10:3:12); R_f^{10}, 1-butanol–pyridine–water–ethanol (4:1:5:4). Silica-gel plate was used for TLC if not mentioned otherwise.

CD measurements were performed using a JASCO J-20 CD, ORD spectropolarimeter at 21—23 °C. A 0.50-mm thick cell was used. The measurements were carried out at concentrations of (3.2—4.2)×10^{-4} M and at pH 5.49—7.30.

Female rats weighing 180—250 g were used in order to assay the ability for the contraction of isolated rat uterus. A uterus horn applied to the load-tension of 0.5 g was suspended in an organ-bath of de Jalon's solution (10 ml) at 26 °C. The uterus contractions caused by the addition of BK analogs were recorded as isometric data using a JD-111S physiological displacement transducer (Nihonkoden Kogyo Co.). A dose-response curve was obtained in the concentration range of 3×10^{-6}—1×10^{-5} g ml^{-1} by using a cumulative method.

After a dog was anesthetized with sodium pentobarbital (30 mg kg^{-1}), hepaline was injected into a vein. The blood flow in the femoral artery of the dog was measured by using an electromagnetic blood flowmeter. The injection-volume into the artery of the BK analogs was (1—3)×10^{-5} l, and the administration range of these was 2×(10^{-6}—10^{-4}) g.

N^{α}-Boc groups were removed by TFA, HCl/THF, or HCl/dioxane with or without anisole, respectively. The reaction was run in an ice-bath or at room temperature according to the requisite. The concentration of HCl in the solution was kept above 2.5 M and at 10—30 equivalents for each peptide. N^{α}-Z groups were removed by H_2/Pd in an acidic solution at room temperature or the treatment with 25% HBr/AcOH for 1.0—2.5 h at room temperature. The symmetrical anhydrides described in the schemes were prepared by DCC. The protected products were purified by the usual methods. Only the typical procedures are herein described.

Z-5-Ava-Pro-Gly-Phe-Ser-Pro-Phe-Arg(NO_2)-OBzl (8) by a DCC/HOBt Method. The deprotection of **6** (0.719 g) with TFA (7.0 ml) and anisole (0.50 ml) in the usual manner gave an oily residue. DCC (0.239 g) in DMF (2.0 ml), and an ice-chilled solution of the above residue and NMM (0.349 ml) in DMF (8.0 ml) were added to an ice-cooled solution of **7** (0.427 g) and HOBt (0.285 g) in DMF (7.0 ml). The mixture was stirred for 1 h in an ice-bath and then for 41 h at room temperature. After the precipitates were removed, the solvent was evaporated. The residue was dissolved in ethyl acetate. The ethyl acetate solution was washed by the routine method. After removing the solvent, the residue was crystallized from diethyl ether. The crude product was further recrystallized from ethyl acetate–diethyl ether: Yield 0.640 g (67%); mp 103—106.5 °C; $[\alpha]_D^{24}$ −31.5° (*c* 0.5, DMF); R_f^1 0.71, R_f^4 0.70, R_f^5 0.80, R_f^8 0.77. Amino acid ratios in acid hydrolyzate: Ser 0.83 (1), Pro 2.21 (2), Gly 1.03 (1), Phe 1.81 (2), 5-Ava 1.19 (1), Arg+Orn 0.93 (1). Found: C, 59.85; H, 6.75; N, 14.01%. Calcd for $C_{59}H_{74}O_{14}N_{12}$: C, 60.29; H, 6.35; N, 14.30%.

Z-5-Ava-Gly-OEt (10) by a Symmetrical Anhydride Method. $(Z\text{-}5\text{-}Ava)_2O$ (3.03 g) was added to a solution of HCl·H-Gly-OEt (1.12 g) and Et_3N (2.50 ml) in chloroform (30 ml). After stirring for 2 d, the solvent was evaporated. The residue was dissolved in ethyl acetate. The organic layer was washed using the usual method. After evaporation of the solvent, the residue was recrystallized from ethyl acetate: Yield 0.81 g (35%); mp 62—64.2 °C; $[\alpha]_D^{24}$ −12.8° (*c* 0.265, DMF); R_f^1 0.59. Found: C, 60.22; H, 7.28; N, 8.86%. Calcd for $C_{17}H_{24}O_5N_7$: C, 60.70; H, 7.19; N, 8.33%.

Boc-5-Ava-Ser-$NHNH_2$ (28). Compound **27** (4.210 g) was dissolved in methanol (12.0 ml), and to this solution was added 80% $NH_2NH_2 \cdot H_2O$ (8.05 ml). The mixture was stirred for 45 h at room temperature. The resulting crystals were collected, then washed onto a filter funnel with methanol–diethyl ether (1:1), and further triturated with ethanol: Yield 3.483 g (83%); mp 171—173.5 °C; $[\alpha]_D^{24}$ −1.0° (*c* 1.0, DMF); R_f^8 0.65. Amino acid ratios in acid hydrolyzate: Ser 0.92 (1), 5-Ava 1.08 (1). Found: C, 49.01; H, 8.81; N, 17.65%. Calcd for $C_{13}H_{26}O_5N_4$: C, 49.04; H, 8.23; N, 17.60%.

Boc-5-Ava-Ser-Pro-Phe-Arg(NO_2)-OBzl (29) by an Azide Method. The treatment of **2** (1.961 g) in dioxane (11.1 ml) with 5.04 M HCl/dioxane (11.90 ml) in the presence of anisole (1.0 ml) for 1 h in an ice-bath gave a solid. A solution of **28** (0.952 g) in DMF (12 ml) was cooled below −30 °C. To the solution were added 5.04 M HCl/dioxane (1.79 ml) and isopentyl nitrite (0.402 ml) with stirring. The mixture was stirred for 15 min at −22 °C and then chilled below −30 °C. To the mixture were added Et_3N (1.25 ml) and an ice-chilled solution of the above solid and Et_3N (0.42 ml) in DMF (11 ml). After stirring for ca. 3 d, the solvent was evaporated. The residue was dissolved in ethyl acetate. The organic layer was washed using the usual procedure. After concentration of the solution, the residue was crystallized from diethyl ether. The product was further recrystallized from ethanol–diethyl ether:

Yield 2.156 g (86%); mp 110.5—114.5°C; $[\alpha]_D^{24}$ −36.7° (*c* 0.5, DMF); R_f^1 0.38, R_f^5 0.90. Amino acid ratios in acid hydrolyzate: Ser 0.93 (1), Pro 1.05 (1), Phe 0.99 (1), 5-Ava 1.12 (1), Arg+Orn 0.91 (1). Found: C, 56.60; H, 6.84; N, 15.19%. Calcd for $C_{40}H_{57}O_{11}N_9 \cdot 1/2H_2O$: C, 56.59; H, 6.89; N, 14.85%.

Boc-Pro-5-Ava-Ser-Pro-Phe-Arg(NO_2)-OBzl (30) by an MA Method. Compound **29** (0.400 g) was treated with TFA (4.0 ml) for 50 min to yield a solid in the usual manner. Boc-Pro-OH (0.103 g) was dissolved in THF (2.0 ml) and the solution was chilled below −40 °C. NMM (0.053 ml) and ethyl chloroformate (0.046 ml) were added to this solution. The mixture was stirred for 5 min at −13—−15 °C, and then chilled below −40°C. To the mixture was added an ice-chilled solution of the above solid and NMM (0.083 ml) in DMF (3.5 ml). The mixture was stirred in an ice-bath and then at room temperature for 22 h. After evaporation of the solvent, the residue was dissolved in ethyl acetate. The organic layer was washed in the usual method. After the solution was concentrated, the residue was recrystallized from ethyl acetate–diethyl ether: Yield 0.309 g (69%); mp 103.5—107.5 °C; $[\alpha]_D^{24}$ −45.5° (*c* 0.5, DMF); R_f^1 0.49, R_f^5 0.80. Amino acid ratios in acid hydrolyzate: Ser 0.86 (1), Pro 1.97 (2), Phe 1.04 (1), 5-Ava 1.15 (1), Arg+Orn 0.98 (1). Found: C, 56.13; H, 6.87; N, 14.39%. Calcd for $C_{45}H_{64}O_{12}N_{10} \cdot H_2O$: C, 56.59; H, 6.97; N, 14.67%.

Z-Arg(NO_2)-Pro-Pro-Gly-OH (35). To an ice-chilled solution of **33** (0.506 g) in ethanol (3.0 ml) was added 1.90 M NaOH (0.85 ml). The mixture was stirred for 30 min at room temperature and then 1 M HCl (1.70 ml) was added to this solution in an ice-bath. After evaporation of ethanol, to the residual solution were added ethyl acetate and 5% Na_2CO_3. The aqueous layer was acidified with concd HCl in an ice-bath, and then saturated with NaCl. It was extracted with ethyl acetate and the organic layer was washed with water saturated with NaCl. The aqueous layer was further extracted with ethyl acetate in the same manner. The organic layers were combined and the solvent was evaporated. The residue was crystallized from diethyl ether: Yield 0.463 g (96%); mp 112.5—118 °C; $[\alpha]_D^{24}$ −54.8° (*c* 0.5, DMF); R_f^4 0.17, R_f^5 0.47. Amino acid ratios in acid hydrolyzate: Pro 2.21 (2), Gly 0.79 (1), Arg+Orn 0.60 (1). Found: C, 47.96; H, 5.69; N, 16.69%. Calcd for $C_{26}H_{36}O_9N_8 \cdot 3H_2O$: C, 47.41; H, 6.42; N, 17.01%.

Z-Pro-Gly-Phe-5-Ava-Phe-Arg(NO_2)-OMe (43) by an Active Ester Method. Compound **40** (1.52 g) was treated with 25% HBr/AcOH (3.0 ml) for 1 h at room temperature to afford a solid in the usual way. Compound **42** (0.890 g) was added to a solution of the above solid, Et_3N (0.30 ml), and NMM (0.60 ml) in DMF (5.0 ml). After stirring for 7 d at room temperature, water (200 ml) was added to this mixture. The resulting precipitates were washed onto a filter funnel as usual. The crude product was washed with ethyl acetate, and then recrystallized from ethanol. The product was washed with diethyl ether: Yield 0.960 g (53%); mp 140—143 °C; $[\alpha]_D^{19.5}$ −25.0° (*c* 1.0, DMF); R_f^2 0.32. Found: C, 58.90; H, 6.67; N, 15.35%. Calcd for $C_{45}H_{58}O_{11}N_{10}$: C, 59.07; H, 6.39; N, 15.31%.

H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-5-Ava-OH (56). Compound **55** (201 mg) in a solution of DMF–water–acetic acid (10 – 5 – 1 ml) was hydrogenated over palladium black for 7 d. Water (14 ml) was added to the mixture during the hydrogenation. After the catalyst was removed, the solution was concentrated. The residue was dissolved in ammonium acetate buffer (0.04 M, pH 6.0) (3.0 ml) and subjected to a carboxymethyl cellulose column (1.7×22.3 cm; Whatman CM-52). It was eluted with ammonium acetate buffers (0.04—0.40 M, pH 6.0) by a linear gradient method. Fractions of 3.0 g each were collected and checked by UV absorption at 254 nm, TLC, and amino acid analysis. Fractions 37—42 were combined and concentrated. The residue was dissolved in 4% acetic acid (2.0 ml) and applied to a Sephadex G-10 column (1.7×41 cm) using the same solvent. Fractions 11—17 (2.5 g each) were combined and concentrated. For further purification, the residue was dissolved in 1-butanol–pyridine–acetic acid–water (16 : 10 : 3 : 12) (1.0 ml) and applied to a column (1.7×26.0 cm) of Sephadex G-25. Fractions 12—25 (3.0 g each), including a pure peptide, were combined and concentrated. The residue was lyophilized from water: Yield 72.7 mg (42%); $[\alpha]_D^{22}$ −97.7° (*c* 0.1, H_2O); R_f^9 0.62 (cellulose). Amino acid ratios in acid hydrolyzate: Ser 0.89 (1), Pro 3.07 (3), Gly 0.98 (1), Phe 1.00 (1), 5-Ava 1.14 (1), Arg 0.92 (1). Found: C, 52.60; H, 6.76; N, 16.10%. Calcd for $C_{40}H_{61}O_{10}N_{11} \cdot CH_3COOH \cdot 2H_2O$: C, 52.98; H, 7.30; N, 16.18%.

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13) The amino acids except glycine used are of L-configuration. The abbreviations used are those recommended by IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): *Eur. J. Biochem.*, **138**, 9 (1984). The following additional abbreviations are used: 5-Ava, 5-aminovaleric acid; Z, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; THF, tetrahydrofuran; AcOH, acetic acid; DCC, dicyclohexylcarbodiimide; HOBt, 1-benzotriazolol; MA, mixed anhydride; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; NMM, *N*-methylmorpholine; Et_3N, triethylamine; Ac, acetyl.

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