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New Bradykinin Analogues Modified in Position 6 and 7 with Naphthylalanine*

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We describe the synthesis and some pharmacological properties of eight new analogues of a previously synthesized bradykinin antagonist, D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg. Two peptides were designed by substitution of Ser^6 with L-1- and L-2-naphthylalanine. In two further analogues this modification was combined with placement in position 7 of D-naphthylalanine residue. We also obtained four analogues by acylation of N-terminus of the peptides mentioned above with 1-adamantaneacetic acid. The activity of analogues was assessed by their ability to inhibit vasodepressor response to exogenous bradykinin (rat blood pressure test). Our results indicate that the modifications proposed decreased significantly the B_2 antagonistic activity. Moreover, our earlier observation, suggesting that acylation of the N-terminus of many BK antagonists with bulky groups consistently improved the antagonistic potency, appears to be valid only for one pair of analogues.

Key words: bradykinin, B_2 antagonists, rat blood pressure assay

Bradykinin (BK), a nonapeptide naturally occurring in blood and urine, is known to possess pharmacological activities such as vasodilation and algesia [1]. Among the different research approaches employed for investigating BK activity and its interaction with other vasoactive systems *in vivo*, the most favoured one has been the synthesis and evaluation of various BK analogues that can bind specifically to BK receptor protein. The evidence published so far indicates that most of the clinically relevant effects of BK are functions of B_2 receptors. For this reason most attempts to synthesize effective BK antagonists have been directed towards analogues

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* Abbreviations: The symbols of the amino acids and peptides are in accordance with 1983 Recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [*Eur. J. Biochem.* **138**, 9 (1984)]. Other abbreviations: Aaa, 1-adamantaneacetic acid; L-1-Nal, L-3-(1-naphthyl)-alanine; L-2-Nal, L-3-(2-naphthyl)-alanine; DIPEA, N,N-diisopropylethylamine; DCM, dichloromethane; DMF, dimethylformamide; Hyp, hydroxyproline; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; TBTU, O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Thi, β-thienyl-L-alanine; TIS, triisopropylsilane; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl.

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commonly referred to as B_2 bradykinin antagonists [2]. It is believed that the critical change, conferring bradykinin antagonist activity upon analogues, is the replacement of Pro^7 with aromatic D-amino acids [2]. D-Phe has been most widely used and appears to be acceptable in analogues with a wide variety of additional modifications. It was later shown that only a narrow range of D-amino acid residues is acceptable for the production of antagonistic activity. Naphthylalanine (Nal) is one of the amino acids regarded as leading to less active compounds [2]. However, in 1997 we reported that two analogues synthesized in our laboratory, formed by replacement of D-Phe in position 7 of potent B_2 antagonist, Aaa(D-Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7)BK with D-1-Nal or D-2-Nal, showed strikingly different potencies in rat blood pressure assay: the peptide substituted with D-2-Nal exhibited a much greater antagonistic activity than its counterpart having D-1-Nal [3]. These two peptides differ only in that the naphthalene ring is connected to the backbone of the molecule through position 2 rather than 1. The hindering effect due to the bulky naphthalene fragment near the peptide bond in the case of D-3-(1-naphthyl)-alanine is much greater than in the case of D-3-(2-naphthyl)-alanine. We assumed that this factor has a significant impact on the bioactive conformations of molecules containing these amino acids, and can thus influence their interaction with receptors.

Recently we described the synthesis and some pharmacological properties of two analogues [4] designed by replacement of Thi^8 in the B_2 antagonist previously synthesized by Stewart and Vavrek [2], namely [D-Arg^0, Hyp^3,$Thi^{5,8}$, D-Phe^7]BK (model peptide) with L-3-(1-naphthyl)-alanine (L-1-Nal) and L-3-(2-naphthyl)-alanine (L-2-Nal). The next two peptides were designed by substitution of D-Phe^7 by D-2-Nal in the model peptide, combined with the introduction in position 8 of either L-1-Nal or L-2-Nal residues. Knowing that acylation of B_2 antagonists with various kinds of bulky acyl groups has improved their potency in rat blood pressure assay, four further analogues were designed by additional acylation of the N-terminal ends of the above-mentioned peptides with 1-adamantaneacetic acid. Our studies have demonstrated that the proposed modifications reduced B_2 antagonistic activity, although the range of this effect was different. We also observed that even minor changes in the structure of the C-terminal part of the antagonists could significantly influence their activity. Continuing in this direction, in this study we designed analogues of the model peptide, modified in a similar way as described above. However, while position 7 of new peptides was occupied, as before, by D-Phe or D-2-Nal, this time the Ser residue in position 6 was substituted by L-1-Nal or L-2-Nal. We assumed that this modification, located further from the C-terminal end of the antagonist, could significantly alter the pharmacological properties of resulting peptides. An additional stimulus for such an approach came from one of our recent papers, in which we demonstrated the importance to the potency of antagonistic activity of the appropriate location in the molecule of the relevant modification [5].

The structures of the new analogues are as follows:

X-D-Arg-Arg-Pro-Hyp-Gly-Thi-Y-M-Thi-Arg

Where:

X = H	Y = L-1-Nal	M = D-Phe	(I)
X = Aaa	Y = L-1-Nal	M = D-Phe	(II)
X = H	Y = L-2-Nal	M = D-Phe	(III)
X = Aaa	Y = L-2-Nal	M = D-Phe	(IV)
X = H	Y = L-1-Nal	M = D-2-Nal	(V)
X = Aaa	Y = L-1-Nal	M = D-2-Nal	(VI)
X = H	Y = L-2-Nal	M = D-2-Nal	(VII)
X = Aaa	Y = L-2-Nal	M = D-2-Nal	(VIII)

EXPERIMENTAL

Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Microtechna type AAA881 analyzer. For analysis, the peptides (0.5 mg) were hydrolyzed by azeotropic hydrochloric acid (400 μl), containing phenol (20 μl), in evacuated sealed ampoules at 110°C for 18 h. TLC was carried out on silica plates (Merck), and the spots were visualized using iodine or ninhydrin. The following solvent systems were used: A: butan-1-ol – acetic acid – water 4:1:5 (v/v), upper phase; B: butan-1-ol – acetic acid – water – ethyl acetate 1:1:1:1 (v/v/v/v).

The purity of the peptides was also ascertained by HPLC. Analyses of the analogues were performed on a Gold System Beckman chromatograph with an Ultrasphere ODS column (5 μm, 4.6×200 mm) and with an ODS precolumn (5 μm, 4.6×50 mm). Solvent system: (A) 0.1% trifluoroacetic acid (TFA), (B) acetonitrile – 0.1% TFA (80:20 v/v), linear gradient 10–70% of (B) for 25 min, λ = 226 nm, flow rate 1 ml/min. Mass spectra were determined on Finningan Mat 95 spectrometer with FAB ionization technique.

Peptide synthesis: Peptides I–IV were synthesized manually, peptides V–VIII were synthesized by the solid-phase method on a Symphony/Multiplex$_{TM}$ Multiple Peptide Synthesizer (Protein Technologies Inc., USA) using the Fmoc-strategy and starting from Fmoc-Arg(Pbf)-Wang resin [10] (loading 0.33 mmol/g, 50 μmol). Fmoc was removed by 20% piperidine in DMF. A five-fold excess of the respective Fmoc-amino acids was activated *in situ* using TBTU (1 eq.)/HOBt (1 eq.) in DMF and coupling reactions were base catalyzed with DIPEA (4 equivalents). Amino acid side chain protecting groups were tBu for Hyp and Pbf for Arg and D-Arg. All Fmoc-protected amino acids and Fmoc-Arg(Pbf)-Wang resin were commercially available (NovaBiochem, Bad Soden, Germany). 1-Adamantaneacetic acid was coupled in the final coupling step (peptide II, IV, VI, VIII) using the same procedure as for Fmoc-amino acids. Each cycle of solid phase synthesis consisted of the following steps: 1. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) three times for 30 seconds; 2. Equilibrating the resin with 2.5 ml of a 20% solution of piperidine (Pip) in DMF, twice for 5 minutes each time; 3. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) six times for 30 seconds; 4. Equilibration with a solution of five-fold excess of Fmoc-amino acid followed by the addition of five-fold excess of a solution of: TBTU (1 eq.)/HOBt (1 eq.)/DIPEA (4 equivalents) in DMF and mixing for 45 minutes. Coupling reactions were performed twice without monitoring. 5. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) three times for 30 seconds.

Cleavage of the peptides from the resin with side-chain deprotection was performed by treatment with TFA:H_2O:TIS (95.5:2.5:2.5 v/v/v) for 4 h. The total volume of the TFA filtrate was reduced to about 1 ml and the peptides were precipitated with cold diethyl ether. The crude material was desalted by gel filtration on a Sephadex G-15 column (10×1.5 cm) eluted with aqueous acetic acid (50%) at a flow rate of 4 ml/h. Fractions comprising the major peak were pooled and lyophilized, and the residue was subjected

further to reversed-phase HPLC on an Ultrasphere ODS column (5 μm, 10×200 mm). Purification was performed on a Gold System Beckman chromatograph. Solvent system: (A) 0.1% trifluoroacetic acid (TFA), (B) acetonitrile – 0.1% TFA (80:20 v/v); isocratic system – 40% of (B), λ = 226 nm, flow rate 2 ml/min. Lyophilization of the pertinent fractions gave the bradykinin analogues. Physicochemical properties of the new analogues I–VIII are presented in Table 1.

Table 1. Physicochemical characteristics of bradykinin analogues.

Analogue	FAB-MS M+H$^+$		RP-HPLC T_R [min]	R_F [a,b]
	calculated	found		
I	1403.8	1405	27.53	0.44
II	1579.9	–	30.93	0.47
III	1403.8	–	34.44	0.44
IV	1579.9	1581	34.47	0.48
V	1454	1455	32.08	0.43
VI	1630.1	1631	35.29	0.48
VII	1454	1455	32.04	0.43
VIII	1630.1	1631	36.03	0.48

[a] Butan-1-ol – acetic acid – water – ethyl acetate 1:1:1:1 (v/v/v/v).
[b] All peptides gave expected amino acid analysis ratios after hydrolysis (±0.05).
The purity of analogues determined on HPLC was between 95 and 97%.

Bioassay methods

Effect of bradykinin analogues on rat blood pressure: The antagonistic potency of the analogues was assessed by their ability to inhibit the vasodepressor response to exogenous bradykinin in conscious rats. Male, intact Wistar albino rats (350–400 g) were maintained on a regular chow diet and tap water at a constant room temperature (23±l°C). One day before the experiment polyethylene tubing (PE5O) was inserted into the right carotid and the iliac artery under pentobarbital (pentobarbital sodium – 20 mg/kg i.p.) anaesthesia. We provided 24 hours for recovery from the surgical catheterization to reduce the effect of neurohumoral activation related to trauma. In particular, we tried to minimize incoherent reactions related to an increase in endogenous catecholamines and bradykinin, which might interfere with our assay.

A Y-type connection was attached to the carotid artery for injection of bradykinin and infusion of the bradykinin analogues. All catheters were exteriorized subcutaneously at the back of the neck. On the day of the experiment, the rats were put into plastic cages. Mean arterial pressure (MAP) and heart rate (HR) were monitored through a Gould-Statham P23-ID pressure transducer (Gould, Cleveland, OH, USA) connected to the iliac catheter and recorded on a paper chart recorder (TZ 4200, Laboratorni Pristroje, Prague, Czech Republic). Thirty minutes prior to the experiment, pentobarbital (pentobarbital sodium – 10 mg/kg) was injected i.p. to sedate the rats. The next dose of pentobarbital (5 mg/kg) was repeated 60–70 min later. Following the injection of the first dose, a 30-min stabilization period was allowed prior to the start of the experiment. Since we observed in our previous studies performed on conscious rats, that some analogues of bradykinin evoked agitation of animals related to direct sensory effect, we prevented rats from anxiety in the present assay with the use of a moderate dose of an anesthetic, pentobarbital sodium. The measures implied resulted in a relatively small deviation of the mean.

An angiotensin-converting enzyme inhibitor, enalapril (Merck Sharp and Dohme Research Lab., Rahway, NJ, USA; l mg/kg) was injected into the iliac catheter. Thirty to sixty minutes later, after stabilization of the blood pressure, bradykinin acetate salt (Sigma) (62.5 and 125 ng), dissolved in 5% D-glucose solution at a concentration of 2.5 μg/ml, was injected every 4 to 5 min. into one branch of the carotid catheter. Each dose was repeated twice or three times until the vasodepressor responses to exogenous bradykinin were stable. *(Prior to the administration of the doses of BK, MAP averaged*

101±1.2 mm Hg and HR was 344±2.7 beats/min. BK evoked significant dose-dependent vasodepressive response (–29.9±0.9 and –39.2±1.2 mm Hg following 62.5 and 125 ng BK, respectively), accompanied by a transient increase in HR (21.7±5.8 and 49.5±8.5 beats/min, following 62.5 and 125 ng BK, respectively).

Both vasodepressor responses to 62.5 and 125 ng were taken as the control responses. The BK analogue dissolved in 5% D-glucose solution was infused to a branch of the carotid catheter other than the BK. A constant rate of infusion – 125 μl/min was provided using an infusion pump (F5z Dialyse 15; Dascon BV, Uden, Netherlands). The BK analogue administration was initiated with its 8-min infusion at a concentration of 0.4 μg/ml (giving a dose of 50 ng/min), during the infusion, 125 ng BK was injected into the carotid artery. This procedure was repeated twice or three times until the vasodepressor responses were stable. The dose of bradykinin antagonist infused was afterwards increased (1, 4, 16, 64, 150 or 256 and, if necessary, 500 and 1000 μg/ml) and the same procedure was repeated until the vasodepressor response to 250 ng of exogenous bradykinin decreased to less than 10% of the control response.

The inhibition of the vasodepressor response to 125 ng BK by each BK-antagonist tested, was plotted against the logarithm of its dose. This dose-effect plot was used for quantitative estimation of the antagonistic potencies of the tested compounds. As indices of these potencies we used the effective doses: ED_{20}, ED_{50} and ED_{90}, representing the respective doses of bradykinin antagonist (μg/kg/min) that inhibit the vasodepressor response to its agonist (125 ng of BK) by 20, 50 and 90%. We also calculated pA_2 from the dose-effect plot as a supplementary index. In our assay pA_2 represents a negative logarithm of the molar concentration of an antagonist (concentration injected divided by the estimated volume of distribution – 67 ml/kg), which reduces the response to the double dose of agonist (125 ng BK) to a value obtained as a response to its single dose (62.5 ng BK) [6]. The results are reported as mean values of ± S.E. A comparison of the two analogues was accomplished by Student`s non-paired t-test [7]. Differences were considered to be significant for $P < 0.05$.

RESULTS AND DISCUSSION

The synthesis of our peptides was based on Fmoc chemistry and started with Fmoc-Arg(Pbf)-Wang resin. Peptides were cleaved from the resin with simultaneous side-chain deprotection by treatment with a mixture of liquid trifluoroacetic acid, H_2O and TIS. The crude compounds were desalted by gel filtration on a Sephadex G-15 column and purified on reversed-phase HPLC. The purity and identity of new analogues was ascertained by analytical HPLC and mass spectrometry.

The antagonistic potencies of our new analogues I–VIII, as well as those of [Arg^0, Hyp^3, $Thi^{5,8}$, D-Phe^7] BK (model peptide) and some related peptides (IX and X), are presented in Table 2. The antagonistic potency of the analogues was assessed by their ability to inhibit vasodepressor response to exogenous BK in conscious rats [8,9]. In this assay only peptides I, III and V are moderately potent antagonists, especially in low doses, whereas analogues II, IV, VI and VIII exhibit a very low potency. Compound VII shows only negligible antagonistic activity. It is characteristic for all new analogues that even if their activity in lower doses (ED_{20}) is not significantly lower than that of the model peptide, in higher doses (ED_{50} and ED_{90}) this difference increases.

Table 2. Pharmacological properties of bradykinin analogues.

Peptide	N	Antagonistic potency			
		ED_{20} [a]	ED_{50} [a]	ED_{90} [a]	pA_2 [b]
[D-Arg^0,Hyp^3,Thi^{5,8},1-Nal^6,D-Phe^7]BK I	5	1.46±0.3	18.26±3.5	>1000	7.78±0.1
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},1-Nal^6,D-Phe^7]BK II	4	16.88 ±2.17	659.5±138.5	>>1000	6.61±0.07
[D-Arg^0,Hyp^3,Thi^{5,8},2-Nal^6,D-Phe^7]BK III	4	3.41±0.55	30.36±4.04	575±81.3	7.53±0.15
Aaa [D-Arg^0,Hyp^3,Thi^{5,8},2-Nal^6,D-Phe^7]BK IV	4	11.7±2.77	49.4±6.28	375.5±8.26	6.83±0.1
[D-Arg^0,Hyp^3,Thi^{5,8},1-Nal^6,D-2-Nal^7]BK V	5	5.07±1.49	339.5±66.6	>>1000	7.22±0.13
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},1-Nal^6,D-2-Nal^7]BK VI	5	16.86±3.13	302.17±94.9	>>1000	6.75±0.11
[D-Arg^0,Hyp^3,Thi^{5,8},2-Nal^6,D-2-Nal^7]BK VII	6	471.1±104.7	>>1000	inactive	5.09±0.17
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},2-Nal^6,D-2-Nal^7]BK VIII	5	12.73±1.51	190.33±22.14	>1000	6.79±0.03
[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK [c] (Stewart's peptide)		0.68±0.04	5.04±0.28	75.90±7.51	7.98±0.05
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-2-Nal^7]BK [d] IX		0.37±0.09	–	18.57±3.27	–
Aaa[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK [e] X		0.84±0.09	–	13.94±1.69	–

N – number of rats tested.
[a] ED_{20}, ED_{50} and ED_{90} represent the respective doses of bradykinin antagonist (μg/kg/min) that inhibit the vasodepressor response to its agonist (125 ng of BK) by 20, 50 and 90%.
[b] pA_2 represents a negative logarithm of the molar concentration of an antagonist (concentration injected divided by the estimated volume of distribution – 67 ml/kg [6], which reduces the response to the double dose of agonist (125 ng BK) to a value obtained as a response to its single dose (62.5 ng BK).
[c] This peptide was previously designed by Stewart's group [10]. As we used a different assay for evaluation of our peptides, we tested this analogue in our system as a reference.
[d] Data from [3].
[e] Data from [8].

In the present studies we provide ED and pA_2 values for pharmacological characteristics of our analogues, since both indices have been widely employed in other, related reports. It is noteworthy that the effective doses (ED) were obtained from the responses to only one dose of bradykinin (125 ng), whereas the pA_2 values were calculated from the responses to two doses of this hormone (62.5 ng, 125 ng). The values of pA_2 are not necessarily parallel to the ED, since the response to a single dose, (62.5 ng BK) is not virtually the same fraction of the response to its doubled one (125 ng), ranging from 69 up to 81% in our previous studies. Here, the response fraction ranged from 72.2±2.2% to 77.9±1.2%, depending on the groups of rats (77.3±1.5%, 76.7±1.2%, 76.2±2.5%, 72.2±1.9%, 77.8±1.5%; 78.0±1.9%; 76.1±0.8 and 75.5±0.8%; in groups tested with BK analogues: I, II, III, IV, V, VI, VII and VIII respectively). Since, in the present study, the response fractions do not differ substantially from 80%, the pA_2 values obtained are closely related to ED_{20} and in fact represent only the initial part of the dose-effect plot of an antagonist. Bearing in mind that pA_2 depends not only on the activity of a given antagonist, but also on the response fraction of the two doses of the agonist, which cannot really be predicted before the experiment, we emphasize that values of EDs provide a more relevant measure than pA_2.

As mentioned in the introduction, we previously synthesized and evaluated the pharmacological properties of eight analogues with L-1-Nal or L-2-Nal in position 8 and D-Phe or D-2-Nal in position 7 [4]. We demonstrated that those modifications resulted in a decrease in antagonistic activity. From the data presented here, it is clear that the modifications applied in the present study led to even a more significant drop in the antagonistic properties of the new analogues. Although the range of this effect is different, the decrease in activity is significant, even when compared with peptides previously modified in similar way in positions 7 and 8 [4]. Moreover, our earlier observation suggesting that acylation of the N-terminus of many bradykinin antagonists with bulky groups consistently improves antagonistic potency, appears to be valid only for one pair of analogues, namely, VII and VIII. As regards other pairs, the acylated compounds are less potent than the nonacylated ones. Summing up, we report a continuation of our earlier studies aimed at clarifying the structural requirements for B_2 antagonistic activity of bradykinin analogues. The results clearly show that our modification has significantly decreased antagonistic activity. It appears that the future substitutions in the structure of antagonists should be inserted closer to the C-terminal end of the molecule.

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