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New Bradykinin Analogues Substituted in Positions 6 and 7 with Enantiomers of N-Methylphenylalanine*

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Four new analogues of a previously designed bradykinin antagonist, D-Arg-Arg-Pro--Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg, containing replacements in positions 6 and 7 with all possible combinations of enantiomers of N-methylphenylalanine (MePhe) were designed, synthesized and bioassayed. The presence of two consecutive MePhe residues in the sequence of the analogues caused great difficulties in the synthesis. The best results for the CO–N(CH_3) bond formation were obtained using O-(7-azabenzotriazol-1-yl)-1,1,3,3--tetramethyluronium hexafluorophosphate/7-azabenzotriazol-1-ol (HATU/HOAt) as coupling reagent (Fmoc strategy). The antagonistic potency of these peptides was assessed by their ability to inhibit vasodepressor response to exogenous bradykinin in conscious rats. Our results showed that the modifications proposed resulted in a decrease in antagonistic activity. However, we demonstrated once again that the D-amino acid in position 7 of BK antagonists may be replaced by a suitable L-amino acid residue. Our results may be of value in the design of new B_2-antagonists.

Key words: bradykinin, N-methylphenylalanine substitution, B_2-antagonists

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg; BK) is a peptide produced by proteolytic cleavage of high-molecular-weight kininogen by plasma kallikreins. Nearly all cells in most species express kinin receptors, which mediate the diverse physiological and pathophysiological activities of BK. Kinin receptors are G-protein-coupled, and their activation leads to slow, sustained contraction of smooth muscles, increased vascular permeability, increased mucous gland secretion, stimulation of sensory neurons, production of nitric oxide, alteration of ion secretion of epithelial cells, release of cytokines from leukocytes and production of eicosanoids from various cell types [1]. Because of their broad spectrum of activities, kinins have been

* 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; DCM, dichloromethane; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; Hyp, hydroxyproline; HOAt, 7-azabenzotriazol-1-ol; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MePhe, N-methyl-L-phenylalanine; NMM; N-methylmorpholine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid; Thi, β-thienyl-L-alanine; TIS, triisopropylsilane.

implicated in many disorders including pain, sepsis, asthma rheumatoid arthritis, pancreatitis and in a wide range of other inflammatory diseases.

Two different receptors B_1 and B_2 mediate the biological actions of BK. B_2 receptors require the entire BK sequence for recognition, whereas B_1 receptors recognize and bind only des-Arg^9-bradykinin. From clinical studies it is clear that most relevant effects caused by BK are functions of the B_2 receptors. For this reason, most attempts to synthesize effective BK antagonists have been directed to analogues commonly called B_2 bradykinin antagonists [2]. B_1 antagonists did not attract much interest until the demonstration that B_1 receptors, normally not present in most tissues, are expressed in chronic inflammation [3,4].

Although almost four decades have passed since the discovery of BK, stable, potent and specific bradykinin antagonists, which may be used as therapeutic agents in the treatment of BK-mediated disorders continue to be unavailable. Recent studies on the structure and conformation of bioactive endogenous peptides suggested that their flexibility constitutes one of their characteristics associated with their biological activity [5]. In this field, N-methylation is an attractive modification currently used as a local and subtle mode of conformational constraint, because of the facility of its introduction and its occurrence in many families of peptide congeners isolated from microorganisms and plants. There is considerable information outlining structural perturbations induced by N-methylation: steric constraints, suppression of a proton-donating ability of the NH group capable of hydrogen bonding, reduction of predominance of the *trans vs cis* peptide bond, and increased basicity of the carbonyl group. Detailed conformational studies indicated that the influence of N-methylation on conformation depends to a large extent on the chirality of the residues surrounding the modified peptide bond [6].

Having all this in mind and knowing that the conformation of the C-terminal part of BK analogues is crucial for biological activity [7], we decided to replace residues 6 and 7 of the B_2 antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]bradykinin, previously synthesized in Stewart's group [8], by all possible combinations of enantiomers of N-methylphenylalanine. The synthesized new analogues (I–IV) have the following structures:

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

Where:	X = MePhe	Y = MePhe	(I)
	X = MePhe	Y = D-MePhe	(II)
	X = D-MePhe	Y = MePhe	(III)
	X = D-MePhe	Y = D-MePhe	(IV)

A further stimulus investigating the proposed peptides came from our recent finding [9] that the MePhe^{7,8} modification of the Stewart's analogue, although disadvantageous for B_2 antagonism, allowed us to demonstrate that D-amino acid residue in position 7 of BK antagonists can be replaced by a suitable L-amino acid residue (analogue VII, Table 2). Until recently the presence of a D-amino acid residue in position 7 was considered to be essential for B_2 antagonism.

EXPERIMENTAL

The 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 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 tehnique.

Peptide synthesis. Peptides 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 HATU (1 eq.) / HOAt (1 eq.) in DMF and coupling reactions were base catalyzed with NMM (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 (Nova-Biochem, Bad Soden, Germany). 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 second. 2. Equilibrating the resin with 2.5 ml of a 20% solution of piperidine (Pip) in DMF, two times for 5 minutes. 3. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) six times for 30 second. 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: HATU (1 eq.) / HOAt (1 eq.) / NMM (4 equivalents) in DMF and mixing for 45 minutes. Coupling reactions were performed two times without monitoring. 5. Washing the resin with a 2.5 ml portion of dimethylformamide (DMF) three times for 30 second.

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 a 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–IV are presented in Table l.

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) were 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 Pristnje, Prague, Czech Republic). Thirty minutes prior to the experiment, pentobarbital (pentobarbital sodium – 10 mg/kg) was injected i.p. to make the rats sedate. 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.

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, 125, 250 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 102 ± 2 mm Hg and HR was 342 ± 6 beats/min. BK evoked significant dose-dependent vasodepressive response (–23.7 ± 1.1, –31.7 ± 1.3 and –39.4 ± 1.7 mm HG following 62.5, 125 and 250 ng BK, respectively), accompanied by a transient increase in HR (23 ± 5; 45 ± 7 and 56 ± 8 beats/min, following 62.5, 125 and 250 ng BK, respectively).*

The vasodepressor response to BK was plotted against the logarithm of the bradykinin dose. The average values of the responses to 125 ng and 250 ng were calculated from the regression line obtained from the log dose-effect plot. Both vasodepressor responses to 125 ng and 250 ng were taken as the control responses. The BK analogue dissolved in 5% D-glucose solution was infused to another branch of the carotid catheter 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, 250 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 250 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 (250 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 (250 ng BK) to a value obtained as a response to its single dose (125 ng BK) [11]. 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 [12]. Differences were considered to be significant for $P < 0.05$.

Table 1. Physicochemical characteristics of bradykinin analogues.

Analogue	FAB-MS $M + H^+$ *calculated*	*found*	RP-HPLC T_R [min.]	R_F [a,b]
I	**$C_{64}H_{91}N_{19}O_{12}S_2$ + H**	1382.1	18.0	0.39 (A)
	1382.7			
II	**$C_{64}H_{91}N_{19}O_{12}S_2$ + H**	1382.2	20.4	0.38 (A)
	1382.7			
III	**$C_{64}H_{91}N_{19}O_{12}S_2$ + H**	1382.1	21.9	0.38 (A)
	1382.7			
IV	**$C_{64}H_{91}N_{19}O_{12}S_2$ + H**	1382.3	21.2	0.36 (A)
	1382.7			

[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%.

Table 2. Pharmacological properties of bradykinin analogues.

Analogue	Antagonistic potency				
	N[a]	ED_{20}[b] (μg/min)	ED_{50}[b] (μg/min)	ED_{90}[b] (μg/min)	pA_2[c]
[D-Arg^0, Hyp^3, Thi^{5,8}, MePhe^{6,7}]BK (I)	6	10 ± 1.5	521 ± 55	>>1000[d]	6.96 ± 0.12
[D-Arg^0, Hyp^3, Thi^{5,8}, MePhe^6, D-MePhe^7]BK (II)	8	1.5 ± 0.2	23 ± 3.9	808 ± 174	7.81 ± 0.13
[D-Arg^0, Hyp^3, Thi^{5,8}, D-MePhe^6, MePhe ^{7}]BK (III)	6	0.8 ± 0.1	12.9 ± 0.8	577 ± 70	7.91 ± 0.13
[D-Arg^0, Hyp^3, Thi^{5,8}, D-MePhe^{6,7}]BK (IV)	7	8.8 ± 0.3	276 ± 13	>>1000[d]	6.74 ± 0.08
[D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK[e] (V)	10	0.7 ± 0.2	5.4 ± 0.4	97 ± 8	7.95 ± 0.07
[D-Arg^0, Hyp^3, Thi^5, MePhe^{7,8}]BK[f] (VI)	3	weak agonist			
Aaa[D-Arg^0, Hyp^3, Thi^5, MePhe^{7,8}]BK[f] (VII)	4	4.6 ± 2.8	49 ± 41	>1000	–

[a] Number of rats tested.
[b] 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 (250 ng of BK) by 20, 50 and 90%.
[c] 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, [11]), which reduces the response to the double dose of agonist (250 ng BK) to a value obtained as a response to its single dose (125 ng BK).
[d] Evaluated by extrapolation from ED_{20} and ED_{50} values.
[e] This peptide was previously designed by Stewart's group [8]. As we used a different assay for the evaluation of antagonistic properties of our peptides, we tested this analogue in our system as a reference.
[f] Data from [9].

RESULTS AND DISCUSSION

The synthesis of our peptides was based on Fmoc chemistry and started with Fmoc-Arg(Pbf)-Wang resin. The presence of two consecutive MePhe residues in the sequence of the analogues caused great difficulties, due to anticipated problems known for coupling reactions with sterically hindered amino acids. For acylation of MePhe residues the best results were obtained with the coupling reagent HATU/HOAt. Nevertheless, peptides were obtained in rather low yields and with considerable amounts of side products. Crude peptides were purified by RP-HPLC.

It should be mentioned that we first tried to use a chloromethylated Merrifield resin and Boc-methodology for the synthesis of our peptides. However, we found out that apart from all other difficulties during splitting off peptides from the resin by HF treatment, a side reaction occurs, which seems to consist in breaking the products between the two MePhe residues. A similar effect was described previously [13].

The antagonistic potencies of our new analogues I–IV compared to that of the antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]BK (peptide V) synthesized by Stewart's group [8] and our previously obtained peptides VI, VII [9] are summarized in Table 2. The antagonistic potency of the analogues was assessed by their ability to inhibit vasodepressor response to exogenous BK in conscious rats [14,15]. In this assay, peptide III is a moderately potent antagonist, whereas compound II exhibits a lower potency. Analogues I and IV show only negligible antagonistic activity. Whereas in lower doses (ED_{20}) our most active analogue III is equipotent to Stewart's peptide, in higher doses (ED_{50}) its activity is approximately twice lower to that compound. However, our peptide is about six times less active than peptide V comparing the ED_{90} of both analogues.

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. Noteworthy, the effective doses (ED) have been obtained from the responses to only one dose of bradykinin (250 ng), whereas pA_2's were calculated from the responses to two doses of this hormone (125 ng, 250 ng). Values of pA_2 are not necessarily parallel to the ED, since the response to a single dose, (125 ng BK) is not virtually the same fraction of the response to its doubled (250 ng) ranging from 69 up to 81% in our previous studies. Here, the response fraction ranged from 76.1 ± 1.8% to 79.5 ± 1.7%, depending on the groups of rats (78.9 ± 1.5%, 79.0 ± 1.4%, 79.5 ± 1.6%, 79.5 ± 1.7% and 76.1 ± 1.8%; in groups tested with BK analogues: I, II, III, IV and V, respectively). Since in the present study, the response fractions are not substantially different from 80%, the obtained values of pA_2 are closely related to ED_{20} and in fact represent only the initial part of the dose-effect plot of an antagonist. Having in mind that pA_2 not only depends on the activity of a given antagonist, but also on the response fraction of the two doses of agonist, that is somehow unpredictable before the experiment, we emphasize that values of ED, provide a more relevant measure than pA_2.

As mentioned in the introduction, we previously synthesized and evaluated pharmacological properties of two analogues (VI and VII) having N-methyl-L-phenylalanine residues in positions 7 and 8 [9]. We demonstrated that these modifications resulted in a dramatic decrease in antagonistic activity {Aaa[D-Arg^0, Hyp^3, Thi^5, Me-Phe^{7,8}]BK (VII)}, or even in conversion into a weak agonist, [D-Arg^0, Hyp^3, Thi^5, Me-Phe^{7,8}]BK (VI). On the other hand, peptide VII is one of the first examples of B_2 antagonists having a L-amino acid residue in position 7. Until recently, the presence of a D-amino acid residue in position 7 was considered to be essential for B_2 antagonism [2,5].

From the data presented, it is clear that also the replacement of the amino acid residues in positions 6 and 7 of Stewart's peptide by each of the combinations of MePhe enantiomers results in a decrease in antagonistic activity. The range of this effect is dramatic for analogues I and VI. Both peptides contain only one enantiomer of MePhe in their structure. However, it should be pointed out that analogue I, which differs from the previously described compound VI by localization of the inserted modification only, possesses antagonistic activity in contrast to the latter. It is also interesting, that the best antagonist from the series described here contains L-MePhe in position 7.

Summing up, we report a continuation of our earlier studies aimed at clarifying the structural requirements for B_2 antagonistic activity of bradykinin analogues. We demonstrated again, that a D-amino acid residue in position 7 of BK antagonists may be replaced by an appropriate L-amino acid residue. Our results may be of certain value in the design of new B_2 antagonists.

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