Design and Synthesis of Potent Bradykinin Agonists Containing a Benzothiazepine Moiety

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A bradykinin analogue (H-Arg-Pro-Pro-Gly-Phe-Ser-D-BT-Arg-OH, **3**) in which the Pro-Phe dipeptide was replaced by the (3*S*)[amino]-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety has been synthesized. The same modification was performed on the potent bradykinin B_2 receptor antagonist HOE 140 (H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH), in which the -D-Tic-Oic- moiety was replaced by D-BT to yield H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-Arg-OH, **1** (JMV1116). These compounds were examined in vitro for their binding affinity toward bradykinin B_1 and B_2 receptors as well as for their ability to interfere with bradykinin-induced contraction of both human umbilical vein and rat uterus. The two compounds **3** and **1** competed with [^{3}H]bradykinin binding to the human cloned B_2 receptor giving K_i values of 13 ± 2 and 0.7 ± 0.1 nM, respectively. Unexpectedly, both compounds were full bradykinin B_2 receptor agonists on the human umbilical vein ($pD_2 = 6.60 \pm 0.07$ for **3** and 6.80 ± 0.08 for **1**) and rat uterus ($pD_2 = 7.20 \pm 0.09$ for **3** and 7.50 ± 0.09 for **1**) preparations with the same efficacy as bradykinin. In addition **1** induced a concentration-dependent phosphoinositide production in CHO cells expressing the human cloned B_2 receptor. These data provide evidence for a bioactive conformation of bradykinin constrained at the dipeptide Pro-Phe.

Introduction

Bradykinin (BK), a linear nonapeptide hormone (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH), and related kinins are thought to be involved in a wide variety of physiological and pathophysiological responses through activation of two types of receptors named B_1 and B_2 that have been cloned.[1,2] Bradykinin induces vascular and bronchial smooth muscle contraction and causes vasodilation and microvascular leakage.[3,4] After local injection into the skin, bradykinin produces all of the classical signs of inflammation: pain, swelling, redness, and heat.[2,5,6] Due to the pathophysiological role of bradykinin, considerable effort toward the development of bradykinin receptor antagonists as potential therapeutic agents has been carried out for several years. Since the initial discovery of bradykinin, a large number of peptidic and pseudopeptidic antagonists of bradykinin receptors have been described.[7,8] One of the most potent and selective bradykinin B_2 receptor antagonists described so far is HOE 140,[9] a decapeptide (H-D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9-OH) containing several unusual amino acids. This antagonist has sufficient high receptor affinity and in vivo lifetime to be investigated as a potential drug. On the other hand, it has been suggested that the cardioprotective effects of angiotensin-converting enzyme (ACE) inhibitors are due, at least in part, to a metabolic protection of bradykinin[10−12] and are therefore resulting from bradykinin B_2 receptor activation. These findings along with the studies indicating that potent bradykinin pseudopeptide agonists were useful in the delivery of anticancer drugs into brain tumors by increasing the permeability of the blood−brain barrier[13] suggest that the development of agonists having improved metabolic stability and oral bioavailability might be of great interest. Among them, one of the most studied bradykinin agonist, RMP-7[13] (H-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-Pro^7-4-Me-Tyr$^8\Psi$(CH_2NH)-Arg^9-OH), was shown to enhance penetration of various anticancer drugs into human brain tumors[14,15] and of antiviral drugs through the blood−ocular barrier in the guinea pig.[16,17]

Extensive spectroscopic studies have shown that bradykinin possesses a high degree of conformational flexibility in solution, although evidence of a β-turn-like structure spanning the residues Ser^6-Pro^7-Phe^8-Arg^9 has been reported in DMSO and SDS micelles.[18,19] Based on ^{1}H NMR and computational studies performed on several bradykinin analogues, it has been suggested that the high affinity of bradykinin antagonists, including HOE 140, for the B_2 receptor was related to their propensity to adopt C-terminal β-turn conformations.[20−22] However, ACE, a major bradykinin-degrading enzyme,[23] cleaves its substrate at Pro^7-Phe^8 and Phe^5-Ser^6 amide bonds suggesting that ACE inhibitors may display features complementary to the bradykinin receptor.

Initially, our main goal was the design of bradykinin and HOE 140 analogues containing constrained non-

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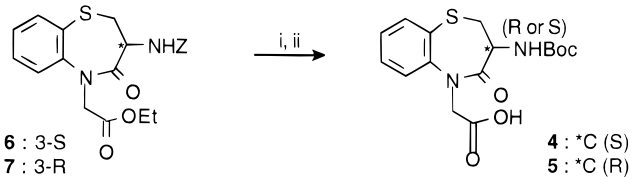
Scheme 1. *N-tert*-Butyloxycarbonylation of the 1,5-Benzothiazepine Derivatives[a]



[a] (i) 33% HBr in AcOH; (ii) Boc_2O, 1 N NaOH, dioxane.

peptidic moieties at positions 7 and 8 since these positions are thought to be important for the agonist/antagonist activity and represent the cleavage site for ACE. In the design of peptidomimetics, incorporation of nonpeptide scaffolds into bioactive molecules has been the focus of extensive research over the last 10 years.[24] Conformationally constrained surrogates have been used in the design and synthesis of enzyme inhibitors and antagonists of peptide hormones receptors.[25] Interestingly a nonpeptide β-turn mimetic was incorporated in the LHRH sequence by Freidinger et al.[26] to produce a potent LHRH agonist. Recently, investigators at Scios-Nova[27] reported that substitution of four amino acids (Pro-Hyp-Gly-Phe) in the N-terminal part of HOE 140 by a spirocyclic structure led to a bradykinin antagonist on the human B_2 receptor.

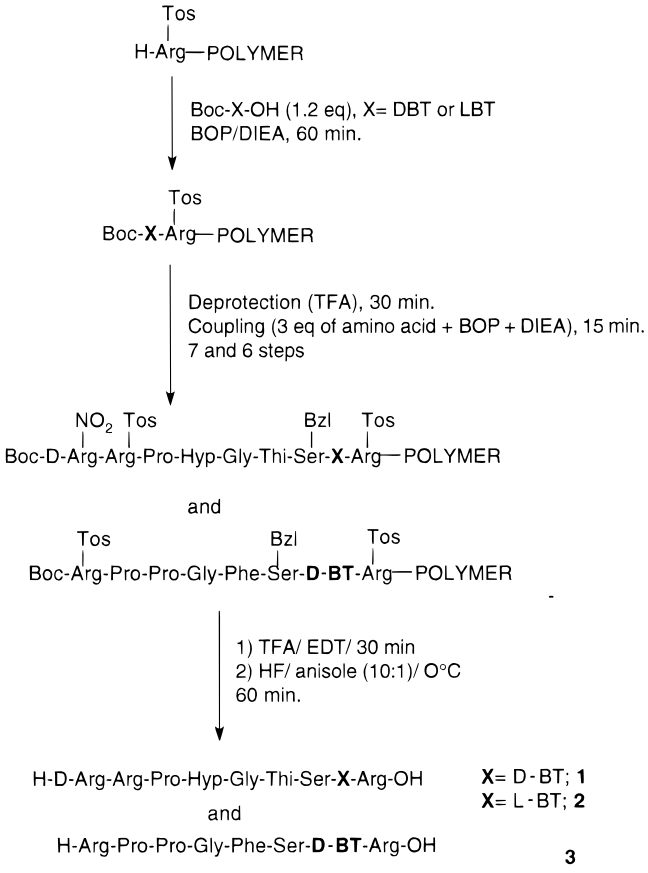
We report here the synthesis and pharmacological evaluation of analogues of both bradykinin and HOE 140 in which amino acids in positions 7 and 8 (Pro-Phe in bradykinin and D-Tic-Oic in HOE 140) were replaced by the ACE inhibitor-containing structure 3-[amino]-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one moiety.[28]

Chemistry

The constrained dipeptide mimetics Boc-D-BT-OH (**4**) and Boc-L-BT-OH (**5**) are shown in Scheme 1. The benzothiazepine intermediates **6** and **7** were prepared according to Slade et al.[28] starting from aromatic nucleophilic substitution of *o*-fluoronitrobenzene with respectively L- and D-*N*-acetylcysteine. Cleavage of both the Z protecting group and the ethyl ester of intermediates **6** and **7** by HBr in acetic acid followed by introduction of the *N-tert*-butyloxycarbonyl group afforded compounds **4** and **5** (Scheme 1). These moieties were introduced in bradykinin and in HOE 140 analogues replacing respectively the dipeptide Pro-Phe to afford the bradykinin analogue **3** and the dipeptide D-Tic-Oic to afford HOE 140 analogues **1** and **2** according to Scheme 2.

All peptides were synthesized on a chloromethylated resin by the solid-phase method with the first amino acid [Boc-Arg(Tos)-] routinely bound to the resin.[29] N^{α}-*tert*-butyloxycarbonyl (Boc) protection was used as temporary protection of the N-terminal amino groups, and tosyl, nitro, and benzyl groups were used for side chain protections. Couplings of protected amino acids or dipeptide mimetics were carried out with BOP reagent.[30] After deprotection of the Boc group by TFA, HOE 140 and bradykinin analogues were deprotected and cleaved from the resin using standard liquid HF procedure, and compounds **1**−**3** were purified by preparative reverse-phase HPLC. The synthetic routes used for their preparation are described in Scheme 2.

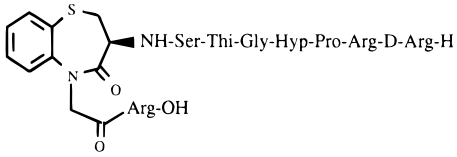
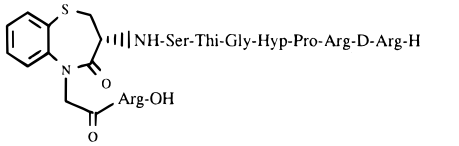
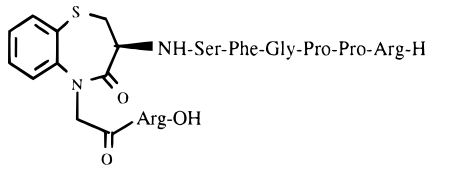
Scheme 2. Synthesis of HOE 140 and Bradykinin Analogues



Results and Discussion

Bradykinin analogues were evaluated for their ability to recognize cloned human and rat B_2 receptors expressed in CHO cells[31] as well as human cloned B_1 receptors expressed in HEK 293 cells.[32] The most potent analogues were tested for their ability to interfere with bradykinin-induced response in human umbilical vein and rat uterus preparations. All compounds inhibited in a concentration-dependent manner the binding of radiolabeled bradykinin to both human and rat cloned B_2 bradykinin receptors with potency varying from subnanomolar to micromolar affinity (Table 1). The most potent compound **1** (containing the *S* isomer of the benzothiazepinone derivative) had an affinity (K_i) of 0.70 $\pm$ 0.1 and 6 $\pm$ 2 nM for the human and rat B_2 receptor, respectively. In the same experiments, the affinity of bradykinin was 0.65 $\pm$ 0.08 and 0.46 $\pm$ 0.09 nM for the human and rat B_2 receptor, respectively. Compound **2** having in its sequence the 3(*R*)-[amino]-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one moiety exhibited a 240-fold lower affinity for the human B_2 receptor (K_i = 162 $\pm$ 18 nM) than **1** pointing out the major role of the orientation of the peptide chain from the benzothiazepine moiety. Compared to bradykinin and HOE 140, **1** was approximately 10 times more potent at the human than at the rat B_2 receptor. Substitution of the dipeptide Pro-Phe at positions 7 and 8 in bradykinin by D-BT led to compound **3** which also bound to human bradykinin B_2 receptors with a K_i value of 13.0 $\pm$ 4.5 nM. Both **1** and **3** bound as potently as

Table 1. Binding Affinities of Bradykinin and HOE 140 Analogues to B_2 and B_1 Human and Rat Receptors[a]

	Compounds	Ki (nM) Human receptor B_2	Ki (nM) Human receptor B_1	Ki (nM) Rat receptor B_2
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	0.65 ± 0.08	n. d.	0.46 ± 0.09
RMP 7	H-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-Me-Tyrψ(CH_2NH)-Arg-OH	19 ± 0.2	n.d.	n.d.
HOE140	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH	0.064 ± 0.019	n.d.	0.077 ± 0.020
1 JMV1116	 NH-Ser-Thi-Gly-Hyp-Pro-Arg-D-Arg-H; Arg-OH	0.7 ± 0.1	>10000	6.0 ± 2.0
2	 NH-Ser-Thi-Gly-Hyp-Pro-Arg-D-Arg-H; Arg-OH	162 ± 18	>10000	n.d.
3	 NH-Ser-Phe-Gly-Pro-Pro-Arg-H; Arg-OH	13.0 ± 4.5	>10,000	n.d.

[a] Binding studies were performed on membranes of transfected CHO or 293 cells using 400 pM [^{3}H]bradykinin for the bradykinin B_2 receptor or 1 nM [^{3}H]des-Arg^{10}-[Leu^9]-kallidin for the bradykinin B_1 receptor. Nonspecific binding was determined in the presence of 10 μM bradykinin or des-Arg^{10}-[Leu^9]-kallidin. Results are given as means ± SEM (n = 3 or 4); n.d., not determined.

RMP-7 to bradykinin B_2 receptors. Compounds **1**−**3** had no affinity for the human cloned B_1 receptor (K_i > 10 000 nM).

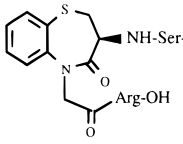
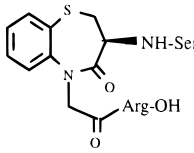
We have evaluated the effect of the two most potent compounds (**1** and **3**) on both isolated human umbilical vein and rat uterus preparations. Unexpectedly, both compounds behaved as full agonists (Table 2). Although pD_2 values for bradykinin were 5−13-fold higher than those for **1**, both compounds developed a similar maximal contraction (Figure 1) indicating that **1** behaved as a full agonist. In addition, the contractile effects of **1** were similar in the presence or absence of protease inhibitors (pD_2 6.80 ± 0.08 vs 6.70 ± 0.09), while the sensitivity to bradykinin was increased in the presence of protein degradation inhibitors (pD_2 7.90 ± 0.09 vs 7.40 ± 0.16) (Figure 1) (Table 2). Compound **1** also behaved as a contractile agent on rat uterus with the same efficacy as bradykinin (Figure 2). As observed in the human umbilical vein, pD_2 values of **1** were not significantly different in the presence and absence of protease inhibitors (7.50 ± 0.09 vs 7.20 ± 0.05). In contrast, the potency of bradykinin was changed by approximately 1 log unit (pD_2, 8.3 ± 0.2 vs 7.33 ± 0.09) (Figure 2, Table 2). These results suggest that **1** is more resistant to protease-mediated degradation than bradykinin.

To further evaluate the agonist property of **1**, we studied its effect on the phosphoinositide cascade which is coupled to the B_2 receptor. Compound **1** increased in a concentration-dependent manner the level of IP_1, IP_2, and IP_3 in CHO cells stably transfected with the human B_2 receptor (Figure 3). The calculated EC_{50} values for **1** were 3.27 ± 0.75, 5.49 ± 1.1, and 4.38 ± 0.73 nM for IP_1, IP_2, and IP_3 production, respectively. EC_{50} values for bradykinin for IP_1, IP_2, and IP_3 production were 2.25 ± 0.98, 2.99 ± 0.19, and 5.03 ± 0.74 nM, respectively. **1** and bradykinin produced a similar maximum increase in IPs levels confirming that **1** behaved as a full agonist (Figure 3).

Figure 4 shows the effect of various doses of HOE 140 on the **1**-induced contraction of rat uterus. HOE 140 behaved as a competitive antagonist giving a pA_2 value of 9.03 ± 0.49. The slope of the Schild plot (1.38 ± 0.30) was not significantly different from unity. We previously reported a pA_2 value of 8.94 ± 0.38 for HOE 140 against bradykinin in the same model.[31] This indicates that there was no difference in the antagonist potency of HOE 140 against **1** or bradykinin. Similar findings were obtained in the human umbilical vein in which HOE 140 had a pK_B value of 8.28 ± 0.33 against **1** (n = 5; data not shown) and a pA_2 value of 8.18 ± 0.28 against bradykinin.[31]

Taken together these results show that **1** is a full, potent, and selective agonist of bradykinin B_2 receptors. They also indicate that replacement of Pro-Phe in bradykinin or D-Tic-Oic in HOE 140 by D-BT yielded selective ligands of the B_2 bradykinin receptor which

Table 2. Agonist Activity of HOE 140 and Bradykinin Analogues on Human Umbilical Vein (HUV) and Rat Uterus (RUT)

	Compounds	pD2 HUV		pD2 RUT	
		Presence of inhibitors*	Absence of inhibitors*	Presence of inhibitors*	Absence of inhibitors*
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	7.90 ± 0.09	7.40 ± 0.16	8.3 ± 0.2	7.33 ± 0.09
RMP 7	H-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-Me-Tyrψ(CH_2NH)-Arg-OH	7.10 ± 0.8	6.90 ± 1.10	n. d.	n. d.
HOE140	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg-OH antagonist	pA_2 = 8.18 ± 0.28		pA_2 = 8.94 ± 0.38	
1	 NH-Ser-Thi-Gly-Hyp-Pro-Arg-D-Arg-H	6.80 ± 0.08	6.70 ± 0.09	7.50 ± 0.09	7.20 ± 0.05
3	 NH-Ser-Phe-Gly-Pro-Pro-Arg-H	6.6 ± 0.17	n. d.	7.2 ± 0.09	n. d.

[a] See Experimental Section; n.d., not determined.

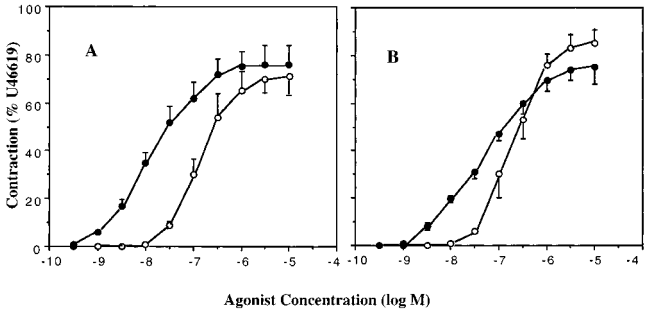


Figure 1. Concentration-related contractile response to bradykinin (●) and **1** (○) in human umbilical vein. Experiments were carried out in the presence (A) or absence (B) of protease inhibitors, and values are means ± SEM of 6 separate experiments.

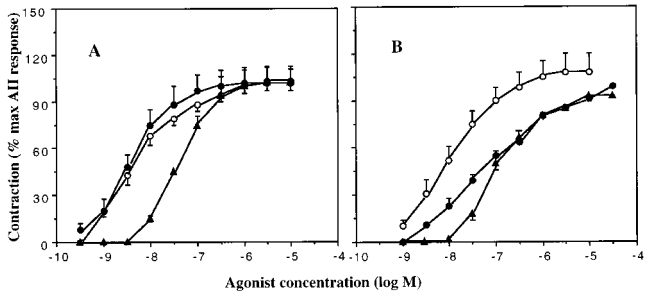


Figure 2. Concentration-related contractile response to oxytocin (○), bradykinin (●), and **1** (▲) in rat uterus. Experiments were carried out in the presence (A) or absence (B) of protease inhibitors, and values are means ± SEM of 5 separate experiments.

acted as full agonists. They suggest that the agonist active conformation of bradykinin at the B_2 receptor can be approached through introduction of constrained dipeptide mimetics such as D-BT at positions 7 and 8. This successful demonstration of the application of D-BT as a new type of conformational constraint of Pro-Phe in bradykinin provides inference of bioactive conformation and should facilitate the study of the interactions between the ligand and the receptor at the active site as well as the design of analogues with improved biological activity and longer duration of action.

In conclusion, we have reported in this paper new potent full bradykinin B_2 receptor agonists containing in their sequence the D-BT constrained structure replacing the dipeptide Pro-Phe. These preliminary results on potent pseudopeptide agonists are significant in our ongoing efforts to understand the structural characteristics required in the C-terminal tetrapeptide of bradykinin analogues for agonist or antagonist activity. Comparison of the conformational profiles of different agonists and antagonists are in progress in our laboratory to determine the characteristics and the spatial arrangement of the moieties involved in recognition and activation of the bradykinin B_2 receptor.

Experimental Section

All pseudopeptides were prepared by solid-phase synthesis according to Merrifield[29] with a manual apparatus, using a Boc strategy. Boc-amino acids, with the side chain protecting groups tosyl for Arg, nitro for D-Arg, and benzyl for Ser, were obtained from Bachem. 3(*R* and *S*)-[(benzyloxycarbonyl)amino]-5-(carbethoxymethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one moieties were synthesized in our laboratory. The Merrifield chloromethylated resin was purchased from Pierce. The first amino acid was bound to the resin according to Gisin.[33] Peptides were cleaved from the resin by a mixture of HF: anisole (10:1) at 0 °C for 60 min. HF was removed under reduced pressure at 0 °C, the product was washed with ether, and the desired goods were extracted with a mixture of CH_3CN:H_2O:TFA (50:50:1) and lyophilized. The crude products were purified by preparative reverse-phase HPLC on a Waters DeltaPrep 4000 system equipped with a PrePak cartridge (40 × 100 μm) filled with a C18 DeltaPrep silica gel (15 mm, 100 Å) phase. Separation was performed with a flow rate of 50 mL/min and UV detection at 220 nm using gradient condition in a solvent system of A (0.1% TFA in H_2O) and B (0.1% TFA in

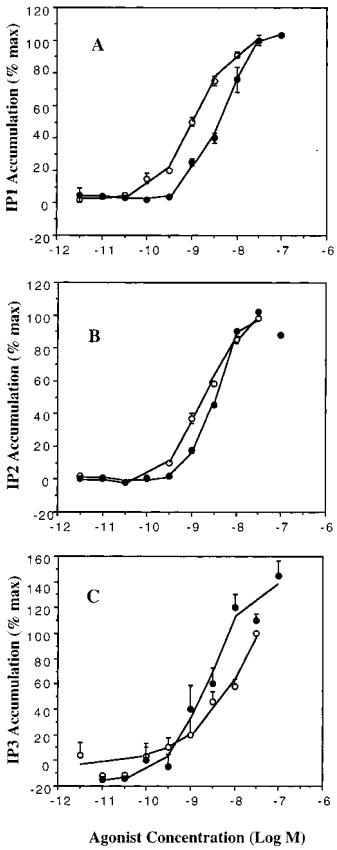


Figure 3. Inositol phosphate accumulation induced by bradykinin (○) and **1** (●) in CHO cells transfected with the human B_2 receptor. Values are means ± SEM of at least 3 separate experiments.

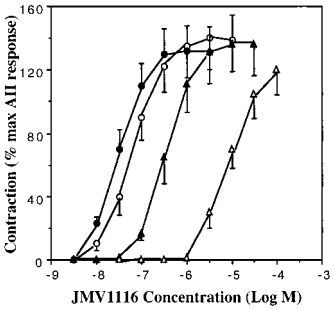


Figure 4. Concentration−response curves of **1** in the presence of increasing concentrations of HOE 140 (● vehicle; ○ 3 nM; ▲ 10 nM; △ 100 nM). Values are means ± SEM of 6 separate experiments.

CH_3CN). HPLC controls were run on a Merck/Hitachi instrument on a DeltaPak C18 (5 μm), 150 × 3.9 mm, 100 Å column, with a UV detection at 220 nm and a flow rate of 1 mL/min. Capillary zone electophoresis (CZE) was run on a PACE 5000 Beckman using an uncoated fused silica capillary (75 μm × 50 cm × 800 μm aperture), pressure injection, run conditions 15 min, 20 °C, 15 kV, 65 mM tetraborate buffer (pH 8.4). Molecular weights of peptides were determined by FAB mass spectrometry on a JEOL JMS-DX-300 apparatus. ^{1}H NMR spectra were performed on a Brüker AC 250 instrument. ^{1}H NMR experiments on compound **1** were run on a Brüker DRX spectrometer operating at a proton frequency of 500.13 MHz and were performed by Dr G. Metz (Fournier Pharma, Research Center Heidelberg). Melting points were taken on a Büchi apparatus in open capillary tubes. Ascending TLC was performed on precoated plates of silica gel 60 F 254 (Merck). Compounds were located with UV light (254 nm), charring reagent, or ninhydrin. All reagents and solvents were of analytical grade. Abbreviations: DMF, dimethylformamide; NMM, *N*-methylmorpholine; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid. Other abbreviations used were those recommended by the IUPAC−IUB Commission (*Eur. J. Biochem.* **1984**, *138*, 9−37).

Table 3. Analytical Characteristics of HOE 140 and Bradykinin Analogues

Compounds	t_R, min[a]	CZE Rt[b]	FAB-MS (M + H$^+$) Calcd	Found
1	18.52	11.92	1228	1228
2	27.04	9.84	1228	1228
3	27.16	10.66	1050	1050

[a] HPLC controls were run on a Merck/Hitachi instrument on a DeltaPak C18 (5 mm), 150 × 3.9 mm, 100 Å column using gradient conditions in a solvent system of A (0.1% TFA) and B (0.1% TFA in CH_3CN); gradient 0−100% B in 100 min. [b]CZE were performed on a PACE 5000 Beckman using an uncoated fused silica capillary (75 μm × 50 cm × 800 μm aperture), pressure injection, run conditions 15 min, 20 °C, 15 kV, 65 mM tetraborate buffer (pH 8.4).

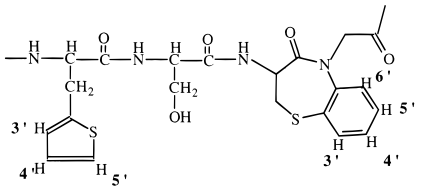
General Procedure for the Preparation of HOE 140 and Bradykinin Analogues. Boc-Arg(Tos)-Merrifield-resin (0.5 g, 0.24 mmol) was used, and the following protected amino acids were coupled to the growing peptide chain in stepwise fashion: 1.2 equiv of Boc-D-BT-OH (or 1.2 equiv of Boc-L-BT-OH), 3 equiv of Boc-Ser(Bzl)-OH, 3 equiv of Boc-Thi-OH for HOE 140 analogues (or 3 equiv of Boc-Phe-OH for BK analogues), 3 equiv of Boc-Gly-OH, 3 equiv of Boc-Hyp-OH for HOE 140 analogues (or 3 equiv of Boc-Pro-OH for BK analogues), 6 equiv of Boc-Pro-OH, 6 equiv of Boc-Arg(Tos)-OH, and 3 equiv of Boc-D-Arg(NO_2)-OH for HOE 140 analogues. All amino acids and mimetics were coupled with BOP. Reaction times for complete couplings were 15 min for Boc-Ser(Bzl)-OH, Boc-Thi-OH, Boc-Gly-OH, Boc-Hyp-OH, 45 min for Boc-Pro-OH and Boc-Arg(Tos)-OH, and 60 min for Boc-D-BT-OH and Boc-L-BT-OH. Completion of the reaction was checked by the ninhydrin test of Kaiser.[34] N^{α}-Boc deprotection was achieved with a mixture of TFA:CH_2Cl_2:ethanedithiol (40:60:2). Washings of the substituted resin with isopropyl alcohol, methylene chloride, and DMF were applied. After coupling of the last amino acid, the Boc protecting group was removed, and final deprotections and cleavage from the resin were performed with HF:anisole. Analytical data of the synthesized compounds are reported in Table 3. ^{1}H NMR values of compound **1** are reported in Table 4.

3(*S* and *R*)-[(Benzyloxycarbonyl)amino]-5-(carbethoxymethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (6 and 7). The synthesis of chiral 1,5-L- or -D-benzothiazepine was achieved according to the literature from L- or D-acetylcysteine.[28]

Boc-D-BT-OH (4). Compound **6** (2.24 g, 5.4 mmol) was deprotected with a 33% solution of HBr in acetic acid (20 mL). After standing at room temperature for 2 h, the solution of HBr/AcOH was concentrated under reduced pressure and the deprotected compound was precipitated in ether and hexane. It was collected and dried in vacuo over KOH pellets. To a solution of the deprotected material (1.6 g, 4.8 mmol) in a mixture of 1 N aqueous solution hydroxide (9.6 mL) and dioxane (10 mL) was added di-*tert*-butyl dicarbonate (1.4 g, 6.5 mmol), and the pH was continuously adjusted to 10 by addition of 1 N aqueous solution hydroxide. When no starting material could be detected by TLC the reaction mixture was diluted with water (50 mL) and extracted with ether (2 × 30 mL). The aqueous phase was acidified to pH 3 with 1 M potassium bisulfate and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (2 × 30 mL) and brine, dried over magnesium sulfate, filtered,

Table 4. 500-MHz ^{1}H NMR of **1** (JMV1116)[a]

	NH	Hα	Hβ	Hγ	Hδ	other H	^{3}J	ΔT
D-Arg^0	*	4.02	1.92	1.72	3.19	ε: 7.18	*	*
Arg^1	8.62	4.35	1.82	1.64	3.15	ε: 7.13	>6	4.4
Pro^2	-	4.77	1.92,2.45	1.96,2.05	3.41,3.90	-	-	-
Hyp^3	-	4.49	2.09,2.34	4.61	3.9	- -	-	
Gly^4	8.45	3.89,3.97	-	-	-	-	5.5	7.0
Thi^5	7.83	(4.75)	3.14,3.36	-	-	3': 6.96 4': 6.87 5': 7.04	7.5	3.6
Ser^6	8.19	4.36	3.78,3.85	-	- -		8.0	5.3
DBT^7	7.94	4.35	3.23,3.42	-	-		*	4.6
DBT'^{8}						3',6' : 7.6 5' or 6' : 7.29,7.66	-	-
Arg9	7.92	4.2	1.76,1.89	1.54	3.14	ε: 7.10	*	4.6



-Thi-Ser-DBT-

[a] Chemical shifts are in ppm (δ). *Not determined. The guanidium NH_2 resonances resulted in a broad peak near 6.7 ppm and are not included in the table.

and concentrated in vacuo to yield the expected compound which precipitated upon addition of a mixture of ether/hexane. It was collected by filtration and dried in vacuo over phosphorus pentoxide: yield 1.6 g (95%); mp 88−90 °C; R_f (chloroform/methanol/acetic acid, 120:10:5) 0.50; HPLC t_R = 21.7;[35] MS *m/e* MH$^+$ 353; ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.39 (9 H, s), 2.90 (1 H, t, $J_1 = J_2 = 11.1$ Hz), 3.78 (1H, dd, $J_1 = 6.7$ Hz, $J_2 = 11.1$ Hz), 4.15 (1H, d, $J = 17.5$ Hz), 4.47 (1H, m), 4.90 (1H, d, $J = 17.5$ Hz), 5.60 (1H, d, $J = 7.7$ Hz), 7.28 (1H, m, H arom), 7.43 (1H, m, H arom), 7.65 (1H, m, H arom).

Boc-L-BT-OH (5). It was obtained as described for compound **4**: yield 96%; mp 88−90 °C; R_f (chloroform/methanol/acetic acid, 120:10:5) 0.50; HPLC, t_R = 21.7;[35] MS *m/e* MH$^+$ 353; ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.39 (9 H, s), 2.89 (1 H, t, $J_1 = J_2 = 11.1$ Hz), 3.78 (1H, dd, $J_1 = 6.7$ Hz, $J_2 = 11.1$ Hz), 4.15 (1H, d, $J = 17.5$ Hz), 4.47 (1H, m), 4.90 (1H, d, $J = 17.5$ Hz), 5.60 (1H, d, $J = 7.7$ Hz), 7.28 (1H, m, H arom), 7.42 (1H, m, H arom), 7.65 (1H, m, H arom).

Pharmacological Studies. 1. Materials. HOE 140 (D-Arg^0-[Hyp^3,Thi^5, D-Tic^7,Oic^8]bradykinin) was synthesized in our laboratory. MERGETPA (DL-2-(mercaptomethyl)-3-(guanidinoethylthio)propanoic acid) was obtained from Calbiochem (La Jolla, CA). All molecular biology and cell culture reagents were purchased from Life Technologies (Cergy-Pontoise, France). Other chemicals were from Sigma Chemical Co. (St Louis, MO). The cDNA of the rat B_2 receptor subcloned in pRC/CMV was kindly provided by Prof. J. Navarro (University of Texas Medical Branch, Galveston, TX). [^{3}H]Bradykinin and [^{3}H]des-Arg^{10}-[Leu^9]-kallidin (80−120 Ci/mmol; 1 Ci = 37 GBq) were purchased from New England Nuclear.

2. Cloning of Human and Rat Bradykinin Receptors. As previously described[31] the coding region of the human B_2 receptor was isolated by PCR from genomic DNA of HepG2 cells using specific primers. The PCR product was subcloned into the *Eco*RI and *Xba*I sites of the vector pBlueScript SK$^-$ (Stratagene). The recombinant plasmid was digested with *Eco*RI and *Xba*I, and the insert was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen). The human B_1 receptor was cloned as previously described.[32] Briefly, total RNA was obtained from IMR-90 cells and poly(A$^+$) RNA was subsequently prepared by oligo(dT) cellulose chromatography. cDNA was obtained using Superscipt II RT kit (Life Technologies). The 1.1-kb cDNA encoding the human B_1 receptor was amplified by PCR and subcloned into the *Bam*HI and *Kpn*I restriction sites of the vector pGEM7zf(+) (Promega). The insert obtained following digestion of the plasmid with *Bam*HI and *Kpn*I was subcloned into pcDNA3 (Invitrogen). The cDNA of the rat B_2 receptor subcloned into pRC/CMV was kindly provided by Prof. J. Navarro (University of Texas Medical Branch, Galveston, TX).

3. Cell Culture and Transfection. CHO cells were maintained in HAM F12 containing 10% fetal calf serum, 4.5 g/L glucose, 100 mg/L streptomycin, and 10^5 units/L penicillin. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagles medium containing 10% fetal calf serum, 4.5 g/L glucose, 1% Glutamax (v/v), 1% nonessential amino acids (v/v), 1 mM sodium pyruvate, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were transfected with the different cDNA containing vectors (10 μg/plate of 150 mm in diameter) using the calcium phosphate precipitation method. After 48−

72 h of recovery, the selection of transfectants was performed using 500 μg/mL Geneticin. Cell clones were isolated by dilution plating, screened for receptor expression, and then propagated.

4. Binding Studies. Stably transfected CHO cells were scrapped from dishes in 5 mL of binding buffer containing 20 mM TES (pH 6.8), 1 mM 1,10-phenanthroline, 140 μg/mL bacitracine, and 0.1% bovine serum albumin; 293 cells stably transfected with the B_1 receptor were treated as described above except that TES was used at 25 mM and pH 7.4. Cell membranes were obtained by centrifugation (40 000*g* for 15 min). Competition binding experiments were carried out by incubating membranes with the competitor ligands and 400 pM [^{3}H]bradykinin for the B_2 receptor (0.5 mL final volume for 90 min) or 1 nM [^{3}H]des-Arg^{10}-[Leu^9]-kallidin for the B_1 receptor (0.5 mL final volume for 60 min). Nonspecific binding was determined in the presence of 10 mM bradykinin or des-Arg^{10}-[Leu^9]-kallidin. Reactions were terminated by filtration with a Brandel cell harvester through Whatman GF/B filters presoaked for 2 h in poly(ethylenimide) 0.1% (w/v). Filters were washed three times with ice-cold 50 mM TES or Tris, and the radioactivity retained onto the filters was counted with a Beckman liquid scintillation counter. Protein concentration was measured by the method of Bradford.[36]

5. Measurements of Inositol Phosphates (IPs). CHO cells stably transfected with the human B_2 receptor were grown in 12-well plates and were then labeled for 18−24 h with 1 μCi/well *myo*-[^{3}H]inositol in serum-free 199 medium. Cells were washed with PBS and then incubated 15 min at 37 °C in 500 μL of IPs assay buffer (NaCl 116 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 5 mM, glucose 11 mM, HEPES 20 mM, captopril 10 μM, and 140 mg/mL bacitracine) supplemented with 10 mM LiCl. Cells were incubated for 15 min with bradykinin or **1**. After removal of the IPs buffer, the reaction was stopped by adding 500 μL of an ice-cold 5% $HClO_4$ solution containing 50 μg/mL phytic acid. After 15 min on ice, the mixture was neutralized by 2 M K_2CO_3 solution. Samples were then applied on anion-exchange columns (Dowex AG1-X8) to obtain different IPs components. Values are expressed as percent of the maximal value (4 times over basal value) obtained with 30 nM bradykinin.

6. Human Umbilical Vein (HUV) Contraction. Human umbilical cords were collected postdelivery and immediately placed in a Krebs solution of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylenediaminetetraacetic acid (EDTA) 0.026, glucose 5.5, bubbled with 95% O_2 plus 5% CO_2 and maintained at 4 °C. Vein rings (3−4 mm in length) without endothelium were set up in 8-mL jacketed organ baths containing Krebs solution and maintained at 37 °C. The resting tension was 1 g. After a maximal contraction was obtained with a high-potassium-containing Krebs solution (KPSS) in which NaCl was replaced by KCl and return to the baseline following repetitive washings, the following compounds were added into the organ bath: mepyramine (1 μM), atropine (1 μM), indomethacin (3 μM), N^G-nitro-L-arginine (L-NOARG, 30 μM), captotril (10 μM), thiorphan (1 μM), DL-2-(mercaptomethyl)-3-(guanidinoethylthio)propanoic acid (MERGEPTA, 5 mM), and nifedipine (0.1 μM). Thirty minutes later the concentration−response curve to bradykinin or **1** was obtained. At the end of the experiments, after washing and return to the baseline level, the maximal contraction of each vein segment was obtained by adding the thromboxane A_2 mimetic, U46619 (1 μM).

7. Rat Uterus (RUT) Contraction. Female Sprague−Dawley rats weighing 250−300 g (Iffa Credo, L′Arbresles, France) were pretreated with diethylstilbestrol at 0.1 mg/kg subcutaneously; 18 h later, rats were sacrified by CO_2 intoxication and the uterus was dissected out and immediately placed in a Jalon solution of the following composition in (mM): NaCl 154, KCl 5.6, $NaHCO_3$ 1.7, $MgCl_2$ 1.4, glucose 5.5, and $CaCl_2$ 0.3. Four segments (approximatively 10 mm in length) were prepared and suspended in jacketed organ baths containing 8 mL of Jalon's solution maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2. The resting tension was 1 g. After a 90-min resting period, captopril (1 μM), atropine (1 μM), indomethacin (3 μM), mepyramine (1 μM), and DL-thiorphan (1 μM) were added to the bath. The B_2 receptor antagonist HOE 140 was added at various concentrations 15 min before cumulative addition of bradykinin or **1**. A single concentration−response curve to bradykinin, **1**, or oxytocin was obtained for each uterus segment. At the end of the experiment, after washing and return to the baseline, angiotensin II (3 μM) was added in order to obtain the maximal contractile response of each segment.

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