Synthesis and Characterization of Bradykinin B_2 Receptor Agonists Containing Constrained Dipeptide Mimics

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We have previously shown that substitution of the D-Tic-Oic dipeptide by a (3*S*)-[amino]-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety in the bradykinin B_2 receptor antagonist HOE 140 resulted in a full potent and selective bradykinin B_2 receptor agonist (H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-D-BT-Arg-OH, JMV1116) exhibiting a high affinity for the human receptor (K_i 0.7 nM). In the present study, we have investigated the effects of replacement of the D-Tic-Oic moiety by various constrained dipeptide mimetics. The resulting compounds were tested for their binding affinity toward the cloned human B_2 receptor and for their functional interaction with the bradykinin-induced contraction of isolated human umbilical vein. Subsequently, we have designed novel bradykinin B_2 receptor agonists which are likely to be resistant to enzymatic cleavage by endopeptidases and which might represent interesting new pharmacological tools. In an attempt to increase the potency of compound JMV1116, both its N-terminal part and the D-BT moiety were modified. Substitution of the D-arginine residue by a L-lysine residue led to a 10-fold more potent bradykinin B_2 ligand [compound **22** (JMV1465) (K_i 0.07 nM)], retaining full agonist activity on human umbilical vein. Substitution of the D-BT moiety by a (3*S*)-[amino]-5-(carbonylmethyl)-2,3-dihydro-8-methyl-1,5-benzothiazepin-4(5*H*)-one [D-BT(Me)] moiety led to compound **23** (JMV1609) which exhibited a higher agonist activity ($pD_2 = 7.4$) than JMV1116 ($pD_2 = 6.8$).

Introduction

In the design of peptidomimetics, incorporation of nonpeptidic scaffolds into bioactive molecules has been the focus of extensive research over the last years.[1] Conformationally restrained surrogates have been used in the design and synthesis of enzyme inhibitors and antagonists of peptide hormone receptors.[2] Backbone conformational constraints are of interest in limiting the number of conformations available to a peptide. Potential advantages resulting from such constrained compounds include (i) increased potency by stabilizing a biologically active conformation; (ii) enhanced stability toward degradation by enzymes; (iii) improved biological selectivity through elimination of bioactive conformers that give unwanted biological responses. In addition, some information can be obtained about the molecular interactions between the ligand and the receptor through the introduction of conformational constraints.

We have recently shown[3] that replacement of the dipeptide Pro[7]-Phe[8] by the (3*S*)-[amino]-5-(carbonylmethyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one (D-BT) moiety in the bradykinin sequence resulted in a full bradykinin agonist. The same modification applied to the bradykinin antagonist HOE 140[4] (H-D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Thi[5]-Ser[6]-D-Tic[7]-Oic[8]-Arg[9]-OH), in which replacement of the -D-Tic[7]-Oic[8]- dipeptide resulted in potent bradykinin agonists, resulted in binding with a 10 times higher affinity to the human than to rat bradykinin B_2 receptors.[3] HOE 140 analogues containing various constrained and nonpeptide moieties replacing the dipeptide -D-Tic-Oic- were synthesized to further investigate the effect of this modification.

Since ACE is a major kinin-degrading enzyme[5] which cleaves bradykinin at the Pro[7]-Phe[8] (and Phe[5]-Ser[6]) amide bond, ACE inhibitors might display features complementary to the bradykinin receptor. Therefore, we used the core of ACE inhibitors as templates to design HOE 140 analogues. In fact, the D-Tic-Oic dipeptide was replaced by the core of various ACE inhibitors including benzothiazepine and benzodiazepine derivatives as well as five-, six-, and seven-membered ring lactams.[6,7] This strategy was successfully approached by Hoyer et al.[8] for the design of nonpeptide bradykinin B_2 receptor antagonists.

Spectroscopic studies have shown that the high affinity of bradykinin analogues (including HOE 140) for B_2 receptors is related to their high propensity to adopt a C-terminal β-turn conformation[9−11] spanning the residues Ser[6]-Pro[7]-Phe[8]-Arg[9]. To follow this hypothesis the D-Tic-Oic moiety was substituted by several β-turn mimetics as well as by other constrained dipeptide mimetics such as a benzodiazepine moiety[12] and a pyrrolidine-2,4-dione moiety.[13]

Chemistry

All suitably protected constrained dipeptide mimetics **1**−**10** used in this study are reported in Table 1. The

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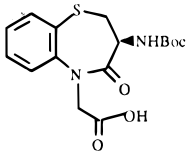
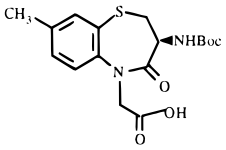
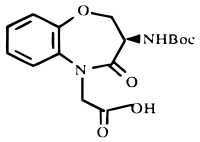
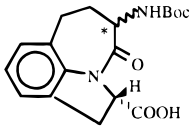
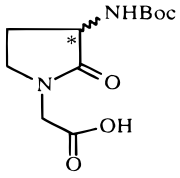
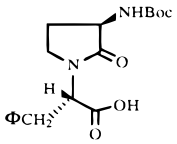
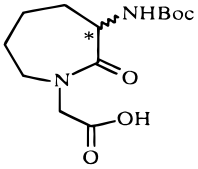
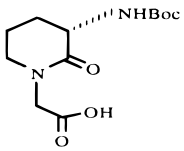
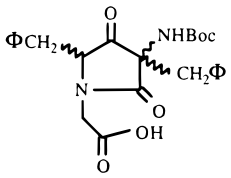
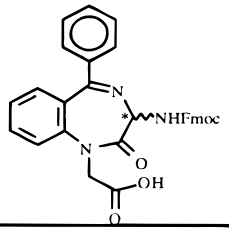
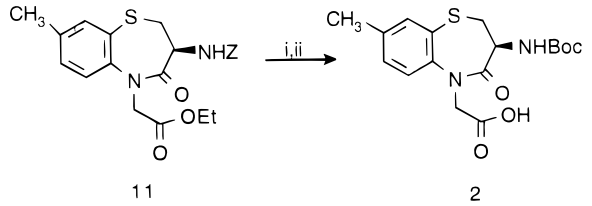
Table 1. Structures of Protected Constrained Dipeptide Mimetics

Compounds		Mimetic numbers	Final derived compounds
	S	**1**	**22** (JMV1465)
	S	**2**	**23** (JMV1609)
	R	**3**	**24** (JMV1442)
	S	**4**	**25**
	R	**4'**	**26**
	S	**5**	**27**
	R	**5'**	**28**
	R	**6**	**29**
	S	**7**	**30**
	R	**7'**	**31**
	S	**8**	**32**
	mixture of diastereoisomers	**9**	**33**
	R or S	**10**	**34**
	R or S		**35**

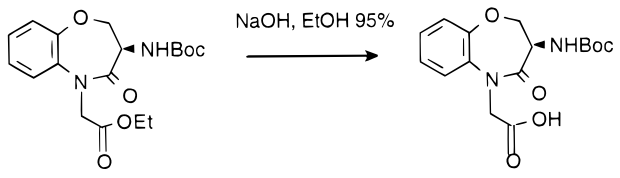
Scheme 1. Preparation of Constrained Dipeptide Mimetic **2**[a]



11 2

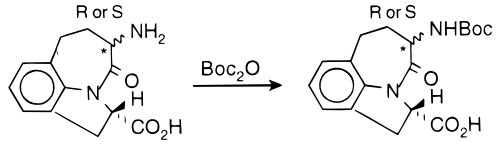
[a] (i) 33% HBr in AcOH; (ii) Boc_2O, 1 N NaOH, dioxane.

Scheme 2. Preparation of Constrained Dipeptide Mimetic **3**



12 3

Scheme 3. *N*-*tert*-Butyloxycarbonylation of 5(*S* and *R*)-Amino-1,2,4,5,6,7-hexahydroazepino[3,2,1-*hi*]indo-4-one (*S*)-Carboxylic Acid Compounds



13: 5-S
14: 5-R

4: 5-S
4': 5-R

synthesis of Boc-D-BT-OH (mimetic **1**) has been described previously.[3] Compound **11** was prepared according to Slade et al.[14] starting from D-acetylcysteine. Acidolysis of **11** by HBr in acetic acid followed by *N*-*tert*-butyloxycarbonylation afforded compound **2** (Scheme 1). Saponification of compound **12** which was prepared according to Nagel et al.[15] yielded compound **3** (Scheme 2).

Compounds **4** and **4′** were obtained according to Scheme 3. The intermediates **13** and **14** were prepared following the synthetic route described by De Lombaert et al.[16] *N*-*tert*-Butyloxycarbonylation of **13** and **14** led respectively to N^{α}-Boc dipeptide mimetics **4** and **4′**. The four dipeptide mimetics **5**, **5′**, **6**, and **8** were prepared according to Freidinger et al.[17]

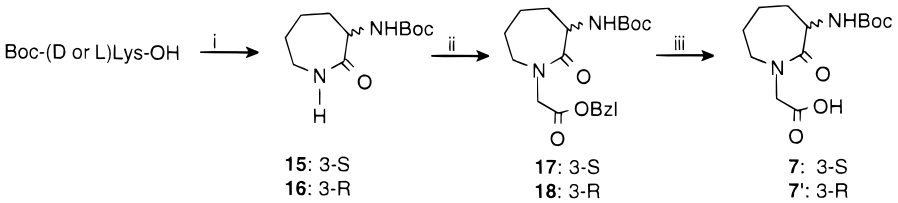
Compounds **7** and **7′** were synthesized following Scheme 4. The seven-membered lactam intermediates **15** and **16** were prepared by cyclization of Boc-(D or L)-Lys-OH in DMF at a 10 mM concentration, with BOP as coupling reagent in the presence of sodium bicarbonate as "insoluble base". This methodology, derived from the procedure described by Brady et al.,[18] led to the lactams in fairly good yields. Alkylation of the amide with benzyl bromoacetate afforded compounds **17** and **18**. Despite the presence of a urethane function, only alkylation of the lactam nitrogen occurred. Deprotection of the benzyl group by hydrogenolysis produced compounds **7** and **7′**.

The pyrrolidine-2,4-dione **19** was prepared according to Pothion et al.[13] from the *N*-(*tert*-butyloxycarbonyl)-*N*-carboxyanhydride derivative of phenylalanine. Acidolysis of compound **19** followed by *N*-*tert*-butyloxycarbonylation led to compound **20**. Alkylation of the pyrrolidine nitrogen with benzyl bromoacetate yielded compound **21** which was deprotected by hydrogenolysis to yield compound **9** (Scheme 5). Compound **10** was purchased from Neosystem Laboratoire (Strasbourg, France).

The above-mentioned dipeptide mimetics **1**−**10** were used for the synthesis of the corresponding HOE 140 analogues **22**−**35** (Table 2). These compounds were synthesized on a chloromethylated resin by the solid-phase method with the first amino acid (Boc-Arg(Tos)-) routinely bound to the resin.[19] N^{α}-*tert*-Butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc) protections were used as temporary protection of 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 using BOP[20] reagent. Boc and Fmoc groups were removed by TFA and piperidine, respectively. **22**−**35** were obtained according to Scheme 6. The two diastereoisomers **34** and **35** were separated by HPLC, but the determination of the absolute configuration was not performed. Compound **33** was tested as a mixture of diastereoisomers.

HOE 140 analogues were obtained after cleavage of the precursors from the resin using standard HF

Scheme 4. Syntheses of Constrained Dipeptide Mimetics **7** and **7′** [a]



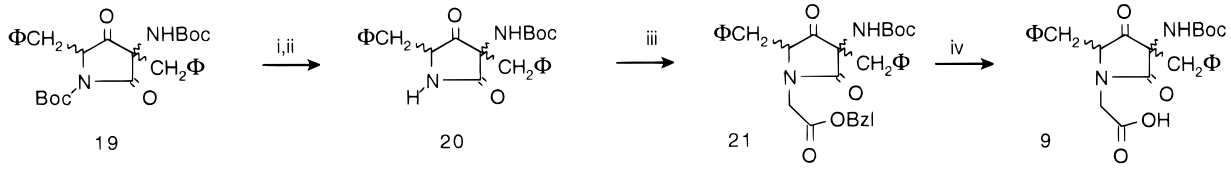
15: 3-S
16: 3-R

17: 3-S
18: 3-R

7: 3-S
7': 3-R

[a] (i) BOP, $NaHCO_3$, NMM; (ii) Br-CH_2-COOBzl, NaH; (iii) H_2/Pd/C.

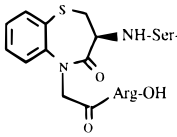
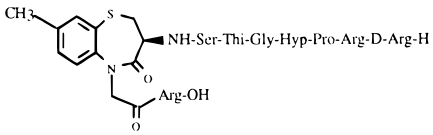
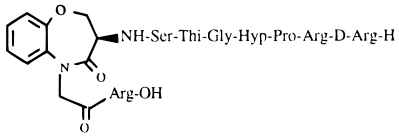
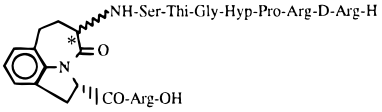
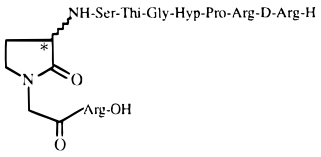
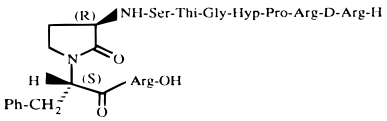
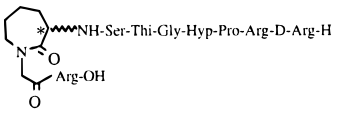
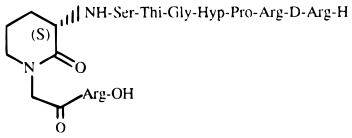
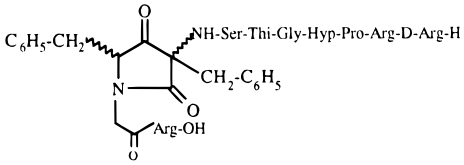
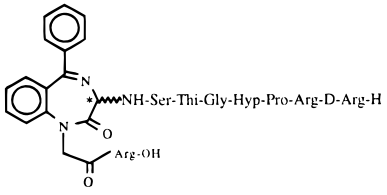
Scheme 5. Syntheses of Constrained Dipeptide Mimetic **9**[a]



19 20 21 9

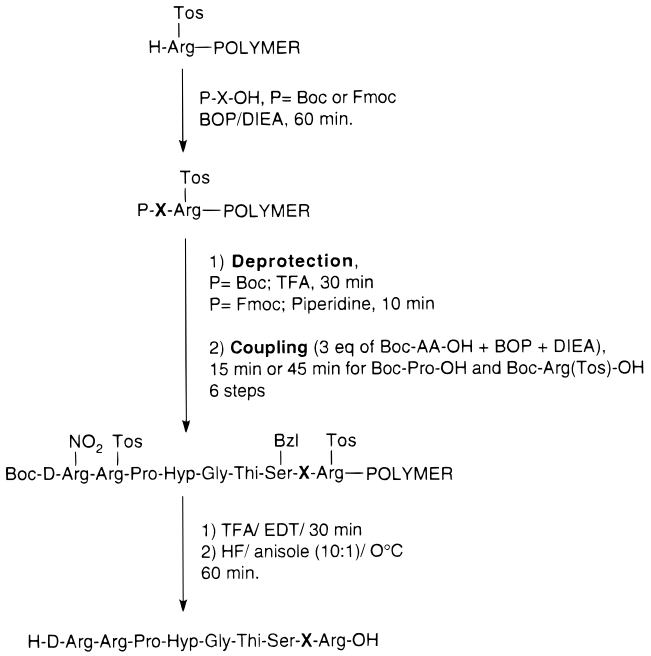
[a] (i) TFA; (ii) Boc_2O; (iii) Br-CH_2-COOBzl, NaH; (iv) H_2/Pd/C.

Table 2. Binding Affinities of Bradykinin and HOE 140 Analogues to B_2 Human Receptors[a]

	Compounds		Ki (nM),B2
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH		0.65
RMP 7	H-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-Me-Tyrψ(CH_2NH)-Arg-OH		19
HOE140	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH		0.06
JMV1116	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-**D-BT**-Arg-OH	S	0.7
22		S	0.07
23		S	6
24		R	2.5
25		S	160
26		R	140
27		S	1030
28		R	4900
29		R	2070
30		S	520
31		R	770
32		S	18600
33		mixture of diastereois omers	14
34		R or S	86
35		R or S	1040

[a] Results are means of at least three separate experiments in duplicate.

Scheme 6. Syntheses of **22**−**35**[a]



[a] **X** = constrained dipeptide mimetics **1**−**10**. In compound **22**, the N-terminal residue is lysine.

procedures. Compounds **22**−**35** were purified by preparative reverse-phase HPLC on a C18 column.

Results and Discussion

Analogues were evaluated for their binding properties to cloned human B_2 and B_1 receptors expressed in CHO and 293 cells, respectively. Compounds **22**−**35** inhibited the binding of [^{3}H]bradykinin to the cloned B_2 bradykinin receptor with subnanomolar to micromolar affinity (Table 2). The most potent compound (**22**) had a 10-fold higher affinity for the B_2 receptor than bradykinin (K_i 0.07 nM). Its methyl analogue (**23**) having a N-terminal D-Arg bound to the B_2 receptor with a lower affinity (K_i 6 nM). Compound **24** (JMV1442), having in its sequence a (*S*)-benzoxazepine moiety, exhibited a fairly high affinity for the B_2 receptor (K_i 2.5 nM) as well as interestingly the diastereoisomeric mixture of HOE analogues (compounds **33**) containing the pyrrolidine-2,4-dione moiety (K_i 14 nM). Among the analogues containing the benzodiazepine moiety, one isomer (e.g. **35**, K_i 86 nM) was more potent at the B_2 receptor than the other (**34**, K_i 1040 nM). In general, the (*S*)-containing isomer analogues were more potent in recognizing the B_2 receptor than their (*R*)-counterparts with the exception of **25** and **26** (K_i 160 and 140 nM, respectively) for which both diastereoisomers showed low potencies. A dramatic decrease in affinity for the human B_2 receptor was observed with the five- and six-membered lactam-containing analogues (**27**, **28**, **29**, **32**, K_i varying from 1030 to 18600 nM), while the seven-membered ring lactams **30** and **31** had a slightly improved affinity. The structure−activity relationship in the bradykinin series pointed out the crucial role of an aromatic moiety at position 8. We have synthesized the compound **29** (an analogue of compounds **27** and **28**) bearing a benzyl group (Table 1). This analogue exhibited weak affinity for the B_2 human receptor (K_i 2070 nM) although it was about 2.5 times more potent than its corresponding analogue without the benzyl group (**28**). None of these HOE 140 analogues in the micromolar range were able to recognize the human cloned B_1 bradykinin receptor.

The most potent compounds, i.e. **22**−**24**, were tested for their ability to contract isolated human umbilical vein in comparison with bradykinin, the bradykinin B_2 receptor agonist RMP-7,[21] and HOE 140. Results are reported in Table 3.

As in the case of JMV1116, these three new derivatives behaved as agonists. Effectively they contracted human umbilical vein rings in a concentration-dependent manner giving calculated pD_2 values ranging from 5.1 to 7.4. Addition of a methyl group onto the benzothiazepinone moiety of JMV1116 led to **23**, which contracted HUV with a higher potency (pD_2 7.4) than JMV1116, and **22**, although its affinity for the B_2 receptor remained lower. Replacement of the benzothiazepinone in JMV1116 by a benzoxazepinone led to a less potent derivative, i.e. **24**.

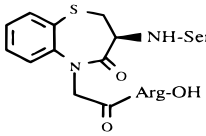
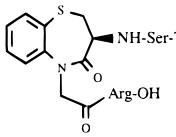
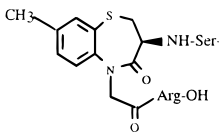
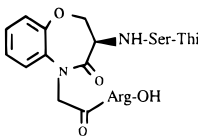
Modification of the peptide moiety was approached by replacing the N-terminal Arg in JMV1116 by a L-lysine residue. The resulting compound (**22**) behaved as a potent agonist (pD_2 7.1). Although the pD_2 value for bradykinin in the presence of a cocktail of enzyme inhibitors was about 5−8 times higher than that of **22** and **23** (7.9 vs 7.1 and 7.4, respectively), both compounds developed a similar maximal contraction of the human umbilical vein. The activity of compounds **22** and **23** was not affected in the absence of protease inhibitors. HOE 140 did not stimulate contraction of the human umbilical vein but was able to potently antagonize the bradykinin-, **22**-, and **23**-induced HUV contraction with the same potency (pA_2 of 8.2 nM) (Table 3).

In conclusion, we have synthesized bradykinin analogues in which the dipeptide Pro-Phe (positions 7 and 8 in the bradykinin sequence) was replaced by β-turn mimetics or lactams. Most of these compounds bound to the human cloned B_2 receptor. Introduction of ACE inhibitor cores produced potent and selective agonists of the B_2 bradykinin receptor. These analogues are apparently resistant to enzymatic (ACE) degradation. Among the constrained structures that were studied, the best mimetic of the Pro-Phe dipeptide was the D-BT moiety. N-terminal modification (replacement of D-Arg in position 1 by Lys) of D-BT-containing compounds yielded even more potent agonists, i.e. **22**. Introduction of a methyl group in position 8 of the benzothiazepine of JMV1116 produced **23** which was more potent than the parent compound.

Experimental Section

All analogues were prepared by solid-phase synthesis on a chloromethylated resin according to Barany and Merrifield[19] with a manual apparatus. Boc-amino acids were obtained from Bachem (Switzerland), and the chloromethylated resin of Merrifield was from Pierce. The first amino acid was bound to the resin according to the Gisin[22] method. Peptides were cleaved from the resin by HF:anisole (10:1) at 0 °C, 60 min. HF was removed under reduced pressure at 0 °C, the product was washed with ether, and the peptides 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 Prep 4000 Waters system equipped with a DeltaPrep cartridge (40 × 100 mm) filled with a C_{18} DeltaPrep silica gel (15 μm, 100 Å) phase. Separation was performed with a flow rate of 50 mL/min and UV detection at 220 nm using

Table 3. Biological Activity of HOE 140 Analogues and HOE 140 on Human Umbilical Vein

	Compounds	pD_2 Absence of inhibitors*	pD_2 Presence of inhibitors*
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	7.4	7.9
RMP 7	H-Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-Me-Tyrψ(CH_2NH)-Arg-OH	6.9	7.1
HOE140	H-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg-OH (antagonist)	N.D.	8.2 (pA_2)
JMV1116		6.7	6.8
22		7.0	7.1
23		7.3	7.4
24		5.1	N.D

[a] See Experimental Section. Results are the means of at least three separate experiments in duplicate.

gradient condition in a solvent system of A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). HPLC controls were run on a Merck/Hitachi instrument on a DeltaPak C_{18} (5 μm), 150 × 3.9 mm, 100 Å column, with UV detection at 220 nm and a flow rate of 1 mL/min. ^1H NMR spectra were recorded on a Bruker AC 250 spectrometer. The HOE 140 analogues were identified by FAB mass spectrometry on a JEOL JMS-DX-300 apparatus. Capillary zone electrophoresis (CZE) was performed on a PACE 5000 Beckman instrument, using an uncoated fused silica capillary. Melting points were taken on a Büchi apparatus in open capillary tubes. TLC was performed on precoated plates of silica gel 60F254 (Merck) using the following solvent systems (by volume): A, AcOEt/hexane, 1:5; B, AcOEt/hexane, 3:7; C, AcOEt/hexane, 5:5; D, AcOEt/hexane, 7:3; E, chloroform/methanol/acetic acid, 180:10:5; F, chloroform/methanol/acetic acid, 120:10:5; G, chloroform/methanol/acetic acid, 60:10:5. Derivatives were located with UV light (254 nm), charring reagent, or ninhydrin. All reagents and solvents were of analytical grade. Abbreviations: DMF, dimethylformamide; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid. Other abbreviations used were those recommended by the IUPAC−IUB Commission (*Eur. J. Biochem.* **1984**, *138*, 9−37).

General Procedure for the Preparation of Compounds 22−35. The protected peptides were assembled starting with the Boc-Arg(Tos)-Merrifield-resin (0.5 g, 0.24 mmol). The dipeptide mimetics **1**−**10** (1.2 equiv) were first coupled to H-Arg(Tos)-Merrifield-resin. The reaction time for complete couplings was 60 min. The protected amino acids (3 equiv) were then introduced in the following order: Boc-Ser(Bzl)-OH, Boc-Thi-OH, Boc-Gly-OH, Boc-Hyp-OH, Boc-Pro-OH, Boc-Arg(Tos)-OH, Boc-D-Arg(NO_2)-OH, or Boc-Lys(Boc)-OH. The reaction times for complete couplings were 15 min for Boc-Ser(Bzl)-OH, Boc-Thi-OH, and Boc-Gly-OH and and Boc-Hyp-OH and 45 min for Boc-Pro-OH and Boc-Arg(Tos)-OH. All amino acids and constraint structures were coupled with BOP. Completion of the reaction was checked by the ninhydrin test of Kaiser.[23] N^{α}-Boc deprotection was achieved with 40% TFA in CH_2Cl_2 for 5 min and then for 25 min, and N^{α}-Fmoc deprotection was achieved with a mixture of piperidine:DMF (20:80) for 10 min. After incorporation of the constrained dipeptide mimetics **1**, **2**, **3**, **4**, **4′**, and **10**, deprotection was performed with a mixture of TFA:CH_2Cl_2:ethanedithiol (40:60:2) and washings with isopropyl alcohol, methylene chloride, and DMF were applied. After coupling of the last amino acid, the Boc protecting group was removed, and the cleavage and deprotection of the compound linked to the resin were performed with HF:anisole (10:1) at 0 °C. Analytical data are reported in Table 4.

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-5-(carboxymethyl)-2,3-dihydro-8-methyl-1,5-benzothiazepin-4(5*H*)-one, Boc-D-BT(Me)-OH (2). Compound **11** (850 mg, 1.98 mmol) was deprotected with a 33% solution of HBr in acetic acid (15 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 (684 mg, 1.98 mmol) in a mixture of 1 N sodium hydroxide (4 mL) and dioxane (10 mL) was added di-*tert*-butyl dicarbonate (518 g, 2.38 mmol). The pH was continuously adjusted to 10 by addition of 1 N sodium hydroxide. When no starting material could be detected by TLC, the reaction mixture was diluted with water (30 mL) and extracted with ether (2 × 20 mL). The aqueous phase was acidified to pH 3 with 1 M potassium bisulfate and extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with water (2 × 20 mL) and brine, dried over magnesium sulfate, filtered, 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

Table 4. Analytical Characteristics of Bradykinin and HOE 140 Analogues

Compounds	HPLC t_R, min[a]	CZE[b] t_R, min	FAB-MS (M + H$^+$)
22	23.9	6.80	1200
23	28.5	6.91	1242
24	24.4	7.44	1212
25	26.7	6.10	1222
26	26.3	6.53	1222
27	19.6	6.93	1134
28	19.9	6.76	1134
29	27.4	6.66	1224
30	22.4	6.57	1162
31	16.5	6.90	1162
32	20.5	6.60	1148
33	-	-	1328
34	29.8	6.88	1285
35	29.1	7.16	1285

[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, 30 kV, 25 mM phosphate buffer (pH 2.75).

in vacuo over phosphorus pentoxide: yield 700 mg (97%); mp 98−100 °C; *Rf* (F) 0.63; HPLC[24] t_R = 23.5 min; FAB-MS *m/z* 367 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.39 (9 H, s), 2.34 (3 H, s), 2.83 (1H, t, $J_1 = J_2 = 11.1$ Hz), 3.72 (1H, dd, $J_1 = 6.8$ Hz, $J_2 = 11.1$ Hz), 4.14 (1H, d, $J = 17.4$ Hz), 4.44 (1H, m), 4.82 (1H, d, $J = 17.4$ Hz), 5.60 (1H, d, $J = 7.7$ Hz), 7.23 (2H, d), 7.45 (1H, s).

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-5-(carboxymethyl)-2,3-dihydro-1,5-benzoxazepin-4(5*H*)-one, Boc-D-BO-OH (3). 1 M sodium hydroxide (3.3 mL, 3.3 mmol) was added to a solution of Boc-D-BO-OEt (**12**) (1 g, 2.75 mmol) in 95% EtOH (10 mL). After stirring at room temperature for 2 h, the solvent was partially concentrated under reduced pressure and the resulting solution was diluted with water (20 mL) and extracted with ether (2 × 10 mL). The aqueous phase was acidified to pH 3 with 1 M potassium bisulfate and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with water (2 × 10 mL) and brine, dried over magnesium sulfate, filtered, and concentrated in vacuo to give 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 830 mg (90%); mp 60−65 °C; *Rf* (E) 0.44; HPLC[24] t_R = 19.7 min; FAB-MS *m/z* 337 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 400 MHz) d 1.33 (9 H, s), 4.13 (1H, t, $J_1 = J_2 = 10$ Hz), 4.22 (1H, d, $J = 17.6$), 4.54 (1H, t, $J = 10$ Hz), 4.66 (1H, m), 4.68 (1H, d, $J = 17.6$), 5.46 (1H, d, $J = 7.1$), 7.13 (4H, m).

5(*S*)-[(*tert*-Butyloxycarbonyl)amino]-1,2,4,5,6,7-hexahydroazepino[3,2,1-*hi*]indol-4-one 2-(*S*)-Carboxylic Acid (4). To a solution of compound **13** (150 mg, 0.6 mmol) in a mixture of 0.1 M aqueous solution hydroxide (6 mL) and dioxane (6 mL) was added di-*tert*-butyl dicarbonate (157 mg, 7.2 mmol), and the pH was continuously adjusted to 10 by addition of 1 M sodium hydroxide. When no starting material could be detected by TLC, the reaction mixture was diluted with water (40 mL) and extracted with ether (2 × 20 mL). The expected compound precipitated upon addition of the aqueous phase into 1 M potassium hydrogenosulfate (40 mL). It was collected, thoroughly washed with water, and dried in vacuo over phosphorus pentoxide: yield 180 mg (84%); mp 170−172 °C; *Rf* (E) 0.41; HPLC[24] t_R = 22.5 min; FAB-MS *m/z* 347 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.40 (9 H, s), 2.06 (1 H, m), 2.41 (1 H, m), 3.02 (1 H, m), 3.11 (1 H, m), 3.27 (1 H, dd, $J_1 = 5.6$ Hz, $J_2 = 16.0$ Hz), 3.49 (1 H, dd, $J_1 = 11.1$ Hz, $J_2 = 16.0$ Hz), 4.32 (1 H, m), 5.12 (1 H, dd, $J_1 = 6.1$ Hz, $J_2 = 11.1$ Hz), 5.88 (1 H, d, $J = 5.0$ Hz), 7.02 (3H, m, H).

5(*R*)-[(*tert*-Butyloxycarbonyl)amino]-1,2,4,5,6,7-hexahydroazepino[3,2,1-*hi*]indol-4-one 2-(*S*)-carboxylic acid (4′) was obtained as described for compound **4**: yield 150 mg (70%); mp 144−146 °C; *Rf* (E) 0.48; HPLC[24] t_R = 22.9 min; FAB-MS *m/z* 347 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.40 (9 H, s), 2.05 (1 H, m), 2.30 (1 H, m), 3.00 (1 H, m), 3.26 (1 H, m), 3.24 (1 H, dd, $J_1 = 2.9$ Hz, $J_2 = 16.7$ Hz), 3.41 (1 H, dd, $J_1 = 10.5$ Hz, $J_2 = 16.7$ Hz), 4.23 (1 H, m), 5.22 (1 H, dd, $J_1 = 2.9$ Hz, $J_2 = 10.5$ Hz), 5.86 (1 H, d, $J = 5.6$ Hz), 7.00 (3H, m, H).

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-pyrrolidineacetic acid (5) was synthesized according to Freidinger et al.:[17] yield (60%); mp 168−170 °C; *Rf* (G) 0.30; HPLC[24] t_R = 20.80 min; FAB-MS *m/z* 259 [M + H$^+$]; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.38 (9 H, s), 1.81 (1 H, m), 2.22 (1 H, m), 3.30 (2 H, m), 3.81 (1H, d, $J = 17.5$ Hz), 4.01 (1H, d, $J = 17.5$ Hz), 4.09 (1 H, m), 7.14 (1 H, d, $J = 8.8$ Hz).

3(*R*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-pyrrolidineacetic acid (5′) was obtained by the synthetic route described by Freidinger et al.[17] starting from Boc-D-Met-Gly-OMe: yield (57%); mp 170−172 °C; *Rf* (G) 0.30; HPLC[24] t_R = 20.80 min; FAB-MS *m/z* 259 [M + H$^+$]; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.38 (9 H, s), 1.81 (1 H, m), 2.22 (1 H, m), 3.30 (2 H, m), 3.81 (1H, d, $J = 17.5$ Hz), 4.01 (1H, d, $J = 17.5$ Hz), 4.09 (1 H, m), 7.14 (1 H, d, $J = 8.8$ Hz).

3(*R*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-pyrrolidine-3(*S*)-phenyl-2-propionic acid (6) was obtained starting from Boc-D-Met-Phe-OMe according to Freidinger et al.:[17] yield (52%); foam; *Rf* (E) 0.32; HPLC[24] t_R = 21.3 min; FAB-MS *m/z* 349 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 400 MHz) d 1.36 (9 H, s), 1.77 (1 H, m), 2.38 (1 H, br), 2.96 (1 H, dd, $J_1 = 11.8$ Hz, $J_2 = 14.6$ Hz), 3.16 (1 H, m), 3.31 (1 H, m), 3.38 (1 H, dd, $J_1 = 4.3$ Hz, $J_2 = 14.9$ Hz), 3.88 (1H, m), 4.96 (1 H, dd, $J_1 = 4.6$ Hz, $J_2 = 11.4$ Hz), 5.23 (1 H, br), 7.17 (5 H, m).

***N*-α-Boc-L-α-amino-ε-caprolactam (15).** To a solution of Boc-Lys-OH (1 g, 4 mmol) in DMF (400 mL) were added BOP (1.8 g, 4 mmol) and sodium bicarbonate (1.7 g, 20 mmol). After 12 h stirring at room temperature, the mixture was concentrated to a small volume (5 mL). Ethyl acetate (100 mL) was then added, and the solution was washed with a saturated sodium bicarbonate solution (3 × 100 mL), water, 1 M potassium hydrogenosulfate solution (3 × 100 mL), and brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure to afford a residue that crystallized upon trituration in hexane: yield (85%); mp 110 °C dec; *Rf* (C) 0.27; *Rf* (D) 0.44.

***N*-α-Boc-D-α-amino-ε-caprolactam (16)** was obtained as described for compound **15** from Boc-D-Lys-OH: yield (90%); mp 105 °C dec; *Rf* (C) 0.27; *Rf* (D) 0.44.

1-*N*-[(Benzyloxycarbonyl)methyl]-3(*S*)-[(*tert*-butyloxycarbonyl)amino]azepan-2-one (17). A solution of *N*-α-Boc-L-α-amino-ε-caprolactam (**15**) (740 mg, 3.2 mmol) in 5 mL of THF was added to a suspension of sodium hydride (147 mg, 6.4 mmol) in 5 mL of THF. The reaction was stirred at room temperature for 15 min, and benzyl bromoacetate was added (613 mL, 3.2 mmol). After 5 h stirring at room temperature, ethyl acetate (100 mL) was added followed by water (100 mL). The organic phase was washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure to afford an oily compound: yield 1.1 g (95%); *Rf* (B) 0.40; *Rf* (C) 0.62.

***N*-[(Benzyloxycarbonyl)methyl]-3(*R*)-[(*tert*-butyloxycarbonyl)amino]azepan-2-one (18)** was obtained as described for compound **17** from the corresponding D-caprolactam **16**: yield (82%); oil; *Rf* (B) 0.40; *Rf* (C) 0.0.62.

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-azepineacetic Acid (7). Compound **17** (1.1 g, 3.2 mmol) was hydrogenated overnight at room temperature in 95% EtOH (100 mL) in the presence of a 10% Pd/C catalyst. The catalyst was then removed by filtration, and the filtrate was concentrated in vacuo to give a residue which crystallized upon trituration in

hexane: yield 780 mg (96%); mp 125 °C dec; *Rf* (E) 0.40; HPLC[24] t_R = 16.8 min; FAB-MS *m/z* 287 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.46 (9 H, s), 1.71 (4 H, m), 2.01 (2 H, m), 3.21 (1 H, dd, J_1 = 4.6 Hz, J_2 = 15.4 Hz), 3.70 (1 H, dd, J_1 = 11.4 Hz, J_2 = 15.4 Hz), 4.20 (2 H, AB system, J = 7.5 Hz), 4.45 (1 H, m), 5.91 (1H, d, J = 6.2 Hz).

3(*R*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-azepineacetic acid (7′) was obtained as described for compound **7**: yield (95%); mp 85 °C dec; *Rf* (E) 0.40; HPLC[24] t_R = 16.8 min; FAB-MS *m/z* 287 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.46 (9 H, s), 1.71 (4 H, m), 2.01 (2 H, m), 3.21 (1 H, dd, J_1 = 4.5 Hz, J_2 = 15.4 Hz), 3.70 (1 H, dd, J_1 = 11.3 Hz, J_2 = 15.4 Hz), 4.20 (2 H, AB system, J = 7.5 Hz), 4.45 (1 H, m), 5.91 (1H, d, J = 6.2 Hz).

3(*S*)-[(*tert*-Butyloxycarbonyl)amino]-2-oxo-1-piperidineacetic acid (8) was obtained according to Freidinger et al.:[17] yield 800 mg (98%); mp 110−113 °C; *Rf* (E) 0.70; HPLC[24] t_R = 24.65 min; FAB-MS *m/z* 273 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 1.46 (9 H, s), 1.77 (1 H, m), 2.02 (2 H, m), 2.45 (1 H, m), 3.40−3.60 (2 H, m), 3.92 (1 H, d, J = 15 Hz), 4.18 (1 H, m), 4.37 (1 H, d, J = 15 Hz), 5.70 (1H, m).

3-[(*tert*-Butyloxycarbonyl)amino]-3,5-dibenzyl-2,4-dioxopyrrolidine (20). 1-(*tert*-Butyloxycarbonyl)-3-[(*tert*-butyloxycarbonyl)amino]-3,5-dibenzyl-2,4-dioxopyrrolidine[13] (**19**) (1 g, 2.1 mmol) was deprotected with TFA (7 mL). After standing 30 min at room temperature, the expected compound precipitated upon addition of ether (50 mL). It was collected, thoroughly washed with ether, and dried in vacuo over KOH. To a solution of this deprotected compound (700 mg, 1.7 mmol) in a mixture of 0.1 M sodium hydroxide (17 mL) and dioxane (34 mL) was added di-*tert*-butyl dicarbonate (370 mg, 1.7 mmol), and the pH was continuously adjusted to 10 by addition of 1 M aqueous sodium hydroxide solution. When no starting material could be detected by TLC, the reaction mixture was diluted with water (40 mL) and extracted with ether (2 × 20 mL). The expected compound precipitated upon addition of the aqueous phase into 1 M potassium hydrogenosulfate (40 mL). It was collected, thoroughly washed with water, and dried in vacuo over phosphorus pentoxide: yield 600 mg (90%); yield 1.2 g (85%); mp 235−237 °C; *Rf* (B) 0.20; *Rf* (C) 0.60.

1-[(Benzyloxycarbonyl)methyl]-3-[(*tert*-butyloxycarbonyl)amino]-3,5-dibenzyl-2,4-dioxopyrrolidine (21). A solution of compound **20** (190 mg, 0.5 mmol) in 5 mL of THF was added to a suspension of sodium hydride (24 mg, 1 mmol) in 5 mL of THF. The reaction was stirred at room temperature fo 15 min, and benzyl bromoacetate was added (80 mL, 0.5 mmol). After 5 h stirring at room temperature, ethyl acetate (20 mL) was added followed by water (20 mL). The organic phase was washed with brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure to afford an oily compound: yield 260 mg (90%); mp 125−127 °C; *Rf* (A) 0.12; *Rf* (B) 0.60.

3-[(*tert*-Butyloxycarbonyl)amino]-3,5-dibenzyl-2,4-dioxo-1-pyrrolidineacetic Acid (9). Compound **21** (260 mg, 0.47 mmol) was hydrogenated overnight at room temperature in 95% EtOH (100 mL) in the presence of a 10% Pd/C catalyst. The catalyst was then removed by filtration, and the filtrate was concentrated in vacuo to leave a residue which crystallized upon trituration in hexane: yield 200 mg (95%); mp 90−92 °C; *Rf* (E) 0.62; HPLC[24] t_R = 30.28 min; FAB-MS *m/z* 453 [M + H$^+$]; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.30 (4.5 H, s), 1.34 (4.5 H, s), 1.41 (0.5 H, dd, J_1 = 9.2 Hz, J_2 = 14.5 Hz), 2.59 (0.5 H, dd, J_1 = 3.8 Hz, J_2 = 14.7 Hz), 2.69 (0.5 H, d, J = 12.7 Hz), 2.90 (0.5 H, dd, J_1 = 8.4 Hz, J_2 = 15 Hz), 2.92 (0.5 H, d, J = 13.1 Hz), 2.98 (0.5 H, d, J = 12.8 Hz), 3.05 (0.5 H, d, J = 12.8 Hz), 3.36 (0.5 H, dd, J_1 = 4 Hz, J_2 = 15.2 Hz), 3.58 (0.5 H, d, J = 17.7 Hz), 3.60 (0.5 H, d, J = 18.02 Hz), 3.67 (0.5 H, dd, J_1 = 4 Hz, J_2 = 8.9 Hz), 3.98 (0.5 H, d, J = 18.9 Hz), 4.03 (0.5 H, d, J = 18.5 Hz), 4.28 (0.5 H, dd, J_1 = 3.8 Hz, J_2 = 9.2 Hz), 5.22 (0.5 H, s), 5.33 (0.5 H, s), 6.88 (1 H, d), 7.15 (3 H, m), 7.22 (5 H, m), 7.28 (1 H, m).

Pharmacological Studies. 1. Materials. HOE 140 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). [^{3}H]Bradykinin (80−120 mCi/mmol, 1 Ci = 37 GBq) and [^{3}H]des-Arg^{10}-[Leu^9]-kallidin (74−77 Ci/mmol) were purchased from New England Nuclear.

2. Cloning of Human and Rat Bradykinin Receptors. As previously described[25] 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[26] and expressed in 293 cells.

3. Cell Culture and Transfection. CHO and 293 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 mg/mL penicillin, and 100 mg/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 and 293 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 (40000*g* for 15 min). Competition binding experiments were carried out by incubating membranes with the competitor ligands (bradykinin and analogues) 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 μM 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 on the filters was counted with a Beckman liquid scintillation counter. Protein concentration was measured by the method of Bradford.[27]

5. Functional Experiments. 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 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 in the organ bath: mepyramine (1 μM), atropine (1 μM), indomethacin (3 μM), N^G-nitro-L-arginine (L-NOARG, 30 μM), captopril (10 μM), thiorphan (1 μM), DL-2 -(mercaptomethyl)-3-(guanidinoethylthio)-propanoic acid (MERGETPA, 5 mM), and nifedipine (0.1 μM). Thirty minutes later the concentration−response curve to bradykinin or bradykinin analogues 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).

Female Sprague−Dawley rats weighing 250−300 g (Iffa Credo, L'Arbresles, France) were pretrated with diethylstilbestrol at 0.1 mg/kg subcutaneously; 18 h later, rats were sacrified by a 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 (approximately 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 analogues. A single concentration−response curve to bradykinin or the tested analogues 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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