Notes

Design and Synthesis of New Linear and Cyclic Bradykinin Antagonists[1]

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We report here on the synthesis and pharmacological properties of a new series of small linear and cyclic peptides derived from the five C-terminal amino acid residues of second-generation bradykinin receptor antagonists. Variations of the two first residues of the pentapeptide (Thi-Ser-D-Tic-Oic-Arg) were shown to modulate the biological activities of the analogs on bradykinin-induced smooth muscle contractions in rabbit jugular vein (RJV), a tissue preparation specific of the B2 bradykinin receptor. Several analogs showed pA_2 values around 7 on this tissue preparation, and one cyclic compound, c[-Gly-Thi-D-Tic-Oic-Arg-], **24**, in which Thi-Ser was replaced by Gly-Thi, displayed a pA_2 of 7.4 on RJV. On the basis of these results, three cyclic molecules and their linear counterparts (compounds **22**−**24** and **4**−**6**, respectively) were tested on human umbilical vein, a tissue specific of the human B2 receptor. The pK_B values obtained for these compounds on these tissue preparations were equivalent to those obtained for the decapeptide NPC 567 (4.8 < pA_2 < 5.1). NMR and molecular modeling studies performed on compound **24** clearly demonstrated a type II′ β-turn structure. This analog may serve as a new lead for the design of nonpeptide ligands of the bradykinin B2 receptor subtype.

Introduction

Bradykinin, in addition to its algesic[2] and proinflammatory effects,[3] induces endothelium-dependent vasodilatation and vascular and bronchial smooth muscle contraction.[4] The actions of bradykinin are mediated through at least two distinct types of receptors, designated B1 and B2, that have been recently cloned and expressed in different species including human.[5,6] Although there is a dynamic upregulation of B1 receptor expression when a prolonged or chronic inflammatory condition is present,[7] most of the early inflammatory effects of bradykinin are mediated by the bradykinin B2 receptor subtype.[8] There is currently a considerable effort in the development of bradykinin receptor antagonists as potential human therapeutic agents. One of the most potent compounds is HOE 140 (D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9) which antagonizes the action of bradykinin on the B2 receptor at the nanomolar range[9,10] but which possesses agonistic properties on certain tissue preparations.[11,12] The clinical utility of these second-generation pseudopeptides might be limited by their relatively short duration of action, high costs of fabrication, and rare routes of administration, which justify the need for a nonpeptide bradykinin receptor antagonist. At the present time, the only nonpeptide bradykinin B2 receptor antagonist that has been reported is Win-64338.[13] This molecule, found by random screening of a large compound collection, appeared to be poorly selective, with submicromolar binding affinities to numerous receptors and agonistic properties on several isolated organ preparations.[14] In the absence of good nonpeptide leads, an alternative approach to bradykinin B2 receptor antagonist design is a targeted chemical modification of the original peptide lead. Extensive molecular modeling[15] has convincingly demonstrated the presence of a β-turn involving the four C-terminal residues of HOE 140 with a clear hydrogen bond between the NH of the last peptidic bond (Arg9) and the CO of the serine in position 6 of the molecule. However, N-terminally cyclized analogs[16,17] and a mimic of NPC 17731,[18] in which a stretch of the N-terminus was replaced by a 12-carbon chain, displayed low pA_2 values.

In contrast, we report here on the synthesis and pharmacological evaluation of small cyclic peptide bradykinin receptor antagonists. One of them demonstrated a good affinity for the rabbit jugular vein bradykinin B2 receptor (pA_2 value: 7.4) and activity on human bradykinin B2 receptor (human umbilical vein) equivalent to the decapeptide NPC 567 (pA_2 value: 5.13). This compound may represent an outstanding lead for the design of nonpeptide ligands of the bradykinin B2 receptor subtype.

Chemistry

All peptides were synthesized by the solid-phase method of Merrifield[19] on a *p*-alkoxybenzyl alcohol resin with the first amino acid routinely bound to the resin. N^{α}-Fluorenylmethoxycarbonyl (Fmoc)[20] protection was employed for peptide elongation, and groups of the *tert*-butyl type were used for side chain protection. Fmoc-Oic-OH was prepared according to a previously reported procedure.[21] Coupling of protected amino acids was carried out with DCC[22] or TBTU[23] in the presence of HOBt. Peptides were cleaved from the resin using standard TFA procedure, and the linear peptides were purified by preparative reverse-phase HPLC. Compounds **8**−**10** were prepared by conventional solid-phase synthesis. After specific deprotection of the Fmoc group from the amino terminal residue of the protected

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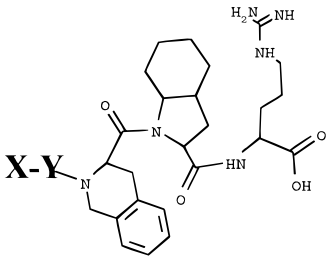
Table 1. Linear Peptide Bradykinin Receptor Antagonists



no.	X	Y	pA_2[a] or pK_B	slope[b]
1	Thi[c]	Ser	in[d]	
2	Tic	Ser	in[d]	
3	Phe	Ser	6.04 ± 0.37	0.24 ± 0.33
4	Gly	Phe	6.07± 0.10	
5	Abu[e]	Thi	6.6 ± 0.28	0.54 ± 0.58
6	Gly	Thi	in[d]	
7	Abu	Phe	6.44 ± 0.22	0.96 ± 0.49
8	GuaAbu[f]	Thi	in[d]	
9	GuaCapro[g]	Thi	5.47 ± 0.17	
10	GuaValeric[h]	Thi	5.52 ± 0.26	
11	Arg	Thi	6.10 ± 0.35	
12	aminodecanoic[i]	Thi	6.33 ± 0.23	

[a] pA_2 values are means ± SEM of $n \geq 3$ and were determined in rabbit jugular vein as reported elsewhere.[10] A pK_B value was determined when activity was only detected at the highest concentration tested (10^{-5} M). [b] Slope is indicated only when pA_2 could be calculated. [c] Thi: thienylalanine. [d] in: inactive at 10^{-5} M. [e] Abu: 4-aminobutyric acid. [f] GuaAbu: 4-guanidinobutyric acid. [g] GuaCapro: 5-guanidinocaproic acid. [h] Guavaleric: 6-guanidinovaleric acid. [i] Aminodecanoic: 10-aminodecanoic acid.

peptide, the peptidyl resin was guanylated using an excess of 1*H*-pyrazole-1-carboxamide hydrochloride and DIEA in DMF as described by Bernatowicz et al.[24] The final pure peptides **8**−**10** were then obtained after cleavage from the resin and preparative HPLC.

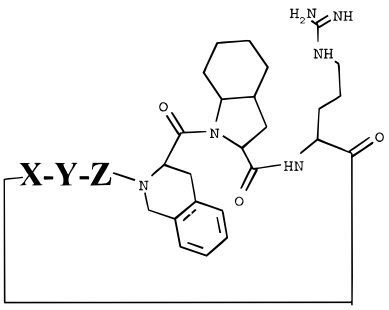
Cyclization reactions were achieved in DMF using TBTU as the condensation agent. The final cyclic peptides (Table 1) were obtained after a two-step purification procedure including desalting on Sephadex gel and preparative HPLC purification. For the synthesis of compounds **26** and **27**, allyloxycarbonyl protection of the side chains of Lys and Arg was used. After the cyclization reaction, the allyl group was removed according to previously described procedures.[25]

Results and Discussion

The bradykinin C-terminal-related analogs were evaluated for their ability to antagonize kinin-induced rabbit jugular vein and rabbit aorta smooth muscle contraction (pA_2). The results are shown in Tables 1 and 2. All the compounds were tested at three different concentrations except for compounds **25**− **27** that were evaluated only at 10^{-5} M. No detectable activities were observed in the rabbit aorta which only expresses the bradykinin B1 receptor subtype.[8] In the rabbit jugular vein some of the compounds induced a rightward shift of the bradykinin response−concentration curve. The maximal contractile response was not affected by the various compounds tested, and in addition, the effects obtained with active compounds were fully washable so they appeared to be selective and reversible antagonists for the bradykinin B2 subtype receptor.

Results of the linear peptide bradykinin receptor antagonists are summarized in Table 1. Analog **1** which corresponds to the five C-terminal amino acids of the HOE 140 molecule had no detectable activity on the B2

Table 2. Cyclic Peptide Bradykinin Receptor Antagonists



no.	X	Y	Z	pA_2[a] or pK_B	slope[b]
13	Abu[c]	Thi[d]	Ser	7.34 ± 0.25	0.67± 0.23
14	Bal[e]	Thi	Ser	7.12 ± 0.21	0.91± 0.20
15	Abu	Tic	Ser	6.36 ± 0.10	0.47± 0.21
16	Abu	Phg[f]	Ser	6.27 ± 0.36	0.52 ± 0.37
17	Abu	Thi	Ala	7.20 ± 0.24	0.74 ± 0.35
18	Abu	Ala	Ser	6.51 ± 0.29	1.22 ± 0.23
19	Abu	Trp	Ala	6.31 ± 0.17	
20	Abu	phe	Ala	in[g]	
21		Gly	Ala	6.62 ± 0.46	1.62 ± 1.05
22		Gly	Phe	6.93 ± 0.22	0.59 ± 0.44
23		Abu	Thi	6.49 ± 0.15	0.89 ± 0.32
24		Gly	Thi	7.39 ± 0.24	1.11 ± 0.54
25		Sar	Thi	6.66 ± 0.39	
26		Lys	Thi	6.84 ± 0.20	
27		Arg	Thi	7.12 ± 0.51	

[a] pA_2 values are means ± SEM of $n \geq 3$ and were determined in rabbit jugular vein as reported elsewhere.[10] A pK_B value was determined when activity was only detected at the highest concentration tested (10^{-5} M). [b] Slope is indicated only when pA_2 could be calculated. [c] Abu: 4-aminobutyric acid. [d] Thi: thienylalanine. [e] Bal: β-alanine. [f] Phg: phenylglycine. [g] in: inactive at 10^{-5} M.

receptor (rabbit jugular vein). Replacement of the thienylalanine residue in compound **1** by phenylalanine gave compound **3** with a pA_2 of 6.04 in the rabbit jugular vein, while, by contrast, a substitution by a more constrained amino acid, like Tic, gave compound **2** with no activity. An important finding was that serine could be replaced by phenylalanine (compound **4**) with no detectable loss of activity. Surprisingly, when thienylalanine was used for this substitution, it led to a totally inactive analog (compound **6**). An increase in the distance between the N-terminus of the peptide and the four last amino acids seemed to be well correlated with an increase in activity (analogs **5, 7**, and **12** compared to analog **6**). Introduction of a guanidino function in the N-terminal abolished activity (analog **8)** or induced a 10-fold decrease in activity (compounds **9** and **10**).

In contrast to the linear analogs, the pA_2 values of the cyclic compounds for the B2 receptor displayed in Table 2 varied between 6.3 and 7.4, with compound **20** (D-Phe residue, inactive) being the sole notable exception. Selectivity for B2 with respect to B1 receptor was also observed, since no detectable affinity for the B1 receptor preparation was noted. The results obtained with compounds **13**−**20** stressed the importance of the aromatic Thi residue and the possibility to replace serine by a neutral side chain residue like alanine. On the basis of these results and those obtained on the linear analogs, a series of more constrained molecules was synthesized (compounds **21**−**27**). Comparison between compounds **23** and **24** showed the importance of the cycle size, since a difference of two atoms in the ring induced a difference of nearly 1 log unit in activity. Replacement of glycine by either lysine or arginine in

Table 3. Bradykinin Antagonist Pseudopeptide on Human Umbilical Vein

no.	structure					human umbilical vein pK_b[a]
NPC 567[b]						5.16 ± 0.75
4	Gly	Phe	Tic	Oic	Arg	in[c]
5	Abu[d]	Thi[e]	Tic	Oic	Arg	in[c]
6	Gly	Thi	Tic	Oic	Arg	4.79 ± 0.68
22	c(Gly	Phe	Tic	Oic	Arg)	5.13 ± 0.57
23	c(Abu	Thi	Tic	Oic	Arg)	in[c]
24	c(Gly	Thi	Tic	Oic	Arg)	4.93 ± 0.65

[a] pK_b values are means ± SEM of $n \geq 3$ and were determined as reported elsewhere.[26] [b] NPC 567: D-Arg-[Hyp^3,D-Phe^7]bradykinin. [c] in: inactive at 10^{-5} M. [d] Abu: 4-aminobutyric acid. [e] Thi: thienylalanine.

order to mimic the bipolar structure of the HOE 140 molecule gave compounds **26** and **27** with no improvement in activity (pA_2 of 6.8 and 7.1, respectively). The results obtained on these analogs clearly demonstrate that stabilizing the conformation via cyclization is beneficial for the antagonistic activity.

In order to assess for tissue specificity, three of the more potent cyclic compounds (**22**−**24**) and their three linear counterparts (**4**−**6**) were tested on the human umbilical vein, a tissue specific of the human B2 receptor[26] (Table 3). The pK_B values obtained for compounds **6**, **22**, and **24** were in the same range as the one obtained for NPC 567, a B2 receptor decapeptide antagonist. However, they are less potent to the pA_2 values measured on rabbit jugular vein pointing out the existence of large species differences within this type of compounds. Based on these results, NMR experiments were performed on the most active compound of the series (**24**) to determine if the molecule adopts the designed β-turn structure obtained with the tetrapeptide Ser-D-Tic-Oic-Arg[15] and which has been confirmed on the entire molecule by a combined approach of NMR and molecular dynamics within a biphasic membrane mimetic.[27] For structural analysis we used three different kinds of parameters: coupling constant, hydrogen bonds, and dipolar interactions. $^3J_{NH\text{-}H\alpha}$ coupling constants were determined from the 1D ^{1}H NMR spectrum and are given in Table 1 of Supporting Information. Hydrogen bonds were studied by temperature dependence. The short mixing time excluded all spin-diffusion phenomena; 62 cross-peaks (17 inter-residues) were obtained within this experiment. The Roesy map revealed some characteristic dipolar interactions (Figures 1 and 2 and Table 1 provided in Supporting Information) which reflected the unique conformation of the molecule. Dipolar coupling observed between the Arg and Gly NH's and between the Gly and Thi NH's are in favor of an orientation of the NH of Thi and Arg toward the center of the molecule. In addition, temperature coefficients calculated for Arg and Thi (Table 1, Supporting Information) may be related to the presence of hydrogen bonds between NHThi and COArg and between NHArg and COThi, respectively. NHGly showed a greater sensitivity to temperature variations, thus reflecting the absence of a hydrogen bond.

Using the NMR data, it was possible to find one major conformation for the peptidic backbone of the molecule **24**. The temperature coefficient showed that the NH of arginine participates in an internal hydrogen bond. The 3D modeling representation displayed in Figure 1 shows that this hydrogen bond could be made with the C=O of the thienyl residue, thus realizing a type II′ β-turn with values for the corresponding Φ and Ψ angles of $\Phi_2 = 53°$, $\Psi_2 = -142°$ and $\Phi_3 = -51°$, $\Psi_3 = -40°$, for Tic and Oic, respectively, comparable to those of an ideal type II′ β-turn: $\Phi_2 = 60°$, $\Psi_2 = -120°$ and $\Phi_3 = -80°$, $\Psi_3 = 0°$.[28] In order to define a minimal pharmacophore, intramolecular distances have been calculated as follows: d(Arg NH−CO Thi) = 3.41 Å, d(Tic CO−CH Gly) = 5.63 Å, d(Thi CO−CO Oic) = 5.19 Å. Whenever possible, the conformation of the side chains were determined from the coupling constant value between the protons, adjusted manually, and then minimized. For instance the preferred side chain disposition of the Tic residue in the molecule appeared to be the trans gauche, g(+), conformation (exo).

This result was deduced from the coupling constants $J_{H\alpha/H\beta}$ of the Tic residue which are different (i.e., 6.1 and 9.8, Table 3) in the exo conformation and similar in the endo conformation.[29] The conformations of the side chains of Thi and Arg were not well determined due to their high flexibilities. Finally, the conformation obtained from NMR results has been fully optimized without any constraints using the semiempirical AM1 method with the Gaussian92 package.[30] This calculation gave a molecule (Figure 1) with a nearly unchanged backbone conformation. In addition the positions of the side chains were mostly conserved, thus showing the high rigidity of the molecule. All these experiments strongly support the hypothesis that high-affinity peptide bradykinin receptor antagonists, like bradykinin itself, adopt C-terminal β-turn conformations when bound to a receptor.[31]

In conclusion, we are reporting here a series of small peptides derived from the C-terminal fragment of the second-generation bradykinin receptor antagonist HOE 140. Five compounds which exhibit pA_2's higher than 7 in rabbit jugular vein represent examples of small, cyclic, and selective bradykinin receptor antagonists. Since their potencies are moderate when compared to that of HOE 140, an important structural feature should also be present in the N-terminal part of this decapeptide antagonist. However, because of their small size and their constrained conformation, these cyclized peptides can be considered as leads for the design of nonpeptide antagonists of the bradykinin receptor, a strategy currently pursued in our laboratory.

Experimental Section

General Methods. All peptides were synthesized by the solid-phase method of Merrifield[19] using standard procedures on a Milligen 9050 or a Labortec SP650 peptide synthesizer. The *p*-alkoxybenzyl alcohol resin and N^{α}-protected (Fmoc) amino acids[20] were purchased from Bachem except for Oic (L-3as,7as-octahydroindole-2-carboxylic acid) which was synthesized in house by procedures previously described.[21] Single diisopropylcarbodiimide-mediated coupling reaction was carried out on the automatic synthesizer with the first amino acid routinely bound to the resin. Peptides were cleaved from the resin by TFA (10 mL/g of the resin) containing 30% of a dichloromethane/anisole/ethanedithiol (2/1/1, in mL/g of the resin) mixture. Reverse-phase HPLC was performed on a Waters 625 LC system equipped with a photodiode array UV detector, utilizing a DeltaPak C18 (spherical 5 μm) column (3.6 × 150 mm). Retention times are given for gradient elution at 1 mL/min in the binary solvent system 0.1% TFA in water/0.1% TFA in acetonitrile. Preparative HPLC was routinely performed on a Waters Prep LC 3000 system equipped with a Waters 490E multiwavelength detector on a PrePak cartridge

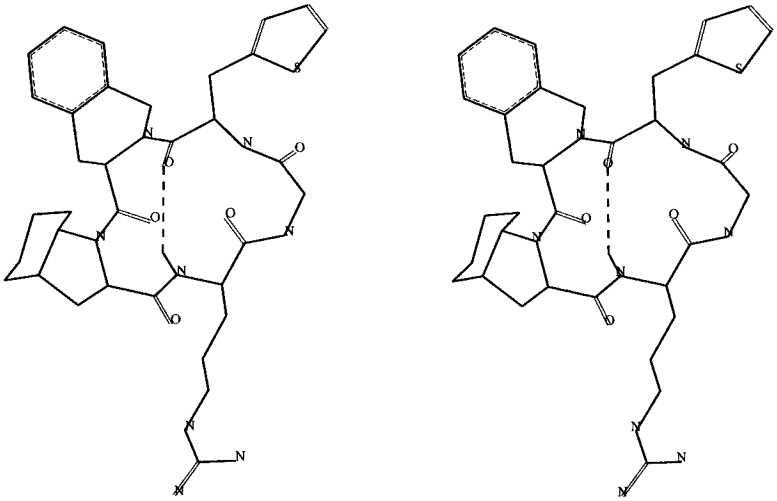


Figure 1. Stereoview of the NMR-derived conformation of the cyclic peptide **24**. The hydrogen bond within the β-turn is indicated by a dashed line.

(47 × 300 mm) filled with a C18 silica gel (300 Å, 15 μm) phase. The operating flow rate was 60 mL/min.

For amino acid analyses, peptides were hydrolyzed in 6 N HCl (0.3 mL) for 20 h at 110 °C in sealed tubes. Hydrolysates were analyzed with a Varian LC90 Star system. The whole procedure including liquid transfer, mixing, Fmoc-derivatization, pentane extraction, and separation on an Aminotag C18 (5 mm) column (4.6 × 150 mm) was completed within 40 min.

Molecular weights of peptides were determined by FAB mass spectrometry on a Normag R10-10C apparatus. The samples were dissolved in a glycerol/thioglycerol matrix (1/1), and ionization was effected by a beam of krypton atoms accelerated through 6−8 keV. ^{1}H NMR spectra were recorded on Bruker spectrophotometers at 200 or 400 mHz as indicated, with Me_4Si as external standard.

General Procedure for the Preparation of Compounds 1−7 and 11−12. The protected peptide was assembled starting with Fmoc-Arg(Pmc)-*p*-alkoxybenzyl alcohol-resin (2 g, 1 mmol) and using 20% piperidine in DMF (2 × 15 min) for N^{α}-deprotection and DCC/HOBt for coupling (90 min) except for D-Tic and Ser(tBu), where TBTU and HOBt were used as coupling reagents. The usual sequence of 1 min washings of the substituted resin with isopropyl alcohol, methylene chloride and DMF was applied.

H-Thi-Ser-D-Tic-Oic-Arg-OH (1). For the synthesis of peptide **1** the protected amino acids (3 mmol, 3 equiv) were introduced in the following order: Fmoc-Oic-OH, Fmoc-D-Tic-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thi-OH. Cleavage from the resin and removal of the Pmc and the *tert*-butyl-type protecting groups were achieved in the mixture of TFA (24 mL), CH_2Cl_2 (6 mL), anisole (1 mL), and ethanedithiol (1 mL) for 90 min at room temperature. The oily residue obtained from the filtrate after evaporation in vacuum was then triturated in ether and gathered as a powder: 746 mg (70% yield, trifluoroacetate). The crude product was then purified by preparative HPLC which gave 440 mg of the linear pure compound **1** as a tris-(trifluoroacetate) (0.41 mmol, 41.2% yield). The final product was >99% pure according to analytical HPLC: AAA Arg 1.07 (1), Ser 0.93 (1), Tic 1.03 (1), Oic 1.00 (1), Thi nd; HPLC t_R 17 min, gradient 10−50% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 725.

H-Tic-Ser-D-Tic-Oic-Arg-OH (2): yield 469 mg (49%); AAA Arg 1.02 (1), Ser 1.03 (1), Tic 1.97 (1), Oic 0.97 (1); HPLC t_R 20 min, gradient 15−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 731.

H-Phe-Ser-D-Tic-Oic-Arg-OH (3): yield 561 mg (53%); AAA Arg 1.04 (1), Ser 1.01 (1), Tic 0.99 (1), Phe 1.01 (1), Oic 0.97 (1); HPLC t_R 22 min, gradient 15−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 719.

H-Gly-Phe-D-Tic-Oic-Arg-OH (4): yield 357 mg (39%); AAA Arg 0.90 (1), Gly 0.98 (1), Tic 1.04 (1), Phe 1.05 (1), Oic 1.02 (1); HPLC t_R 9.5 min, gradient 25−50% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 689.

H-Abu-Thi-D-Tic-Oic-Arg-OH (5): yield 446 mg (42%); AAA Arg 0.96 (1), Abu 1.04 (1), Tic 1.04 (1), Oic 1.02 (1), Thi nd; HPLC t_R 16 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 723.

H-Gly-Thi-D-Tic-Oic-Arg-OH (6): yield 497 mg (48%); AAA Arg 1.04 (1), Gly 0.97 (1), Tic 1.08 (1), Oic 0.91 (1), Thi nd; HPLC t_R 14 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 695.

H-Abu-Phe-D-Tic-Oic-Arg-OH (7): yield 497 mg (47%); AAA Arg 1.01 (1), Abu 0.97 (1), Phe 1.05 (1), Tic 0.96 (1), Oic 1.01 (1), Thi nd; HPLC t_R 17 min, gradient 15−50% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 717.

3-Guanidinobutyryl-Thi-D-Tic-Oic-Arg-OH (8). The protected peptide Fmoc-Abu-Thi-Ser(Bu)-D-Tic-Oic-Arg(Pmc)-resin was synthesized using the general procedure. After N^{α}-deprotection using 20% piperidine, the guanidinylation reaction was performed using a procedure similar to that described by Bernatowicz et al.[24] Briefly, to the peptide-resin (0.7 mmol) were added 1*H*-pyrazole-1-carboxamide hydrochloride (1.02 g, 7 mmol) and DIEA (1.34 mL, 7.7 mmol) in 15 mL of DMF, and the reaction was performed during 18 h at room temperature. After washes with CH_2Cl_2 and DMF, the peptide was cleaved from the resin and purified as described in the general procedure: yield 120 mg (18%); AAA Arg 1.13 (1), Tic 0.90 (1), Oic 1.03 (1), Thi nd; HPLC t_R 23 min, gradient 20−45% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 765.

5-Guanidinocaproyl-Thi-D-Tic-Oic-Arg-OH (9). This compound was obtained using a similar procedure as described for the synthesis of compound **8:** yield 110 mg (10.8%); AAA Arg 1.06 (1), Tic 0.94 (1), Oic 0.97 (1), Thi nd; HPLC t_R 22.7 min, gradient 15−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 793.

4-Guanidinovaleroyl-Thi-D-Tic-Oic-Arg-OH (10). This compound was obtained using a similar procedure as described for the synthesis of compound **8:** yield 100 mg (9.9%); AAA Arg 1.10 (1), Tic 0.95 (1), Oic 1.02 (1), Thi nd; HPLC t_R 22 min, gradient 15−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 779.

H-Arg-Thi-D-Tic-Oic-Arg-OH (11). The compound was obtained by the general procedure: yield 30 mg (55%); AAA Arg 2.11 (2), Tic 0.95 (1), Oic 0.98 (1), Thi nd; HPLC t_R 20 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 794.

H-10-Aminodecanoyl-Thi-D-Tic-Oic-Arg-OH (12). The compound was obtained by the general procedure: yield 100 mg (38%); AAA Arg 1.0 (1), Tic 1.0 (1), Oic 0.96 (1), Thi nd; HPLC t_R 17.5 min, gradient 25−45% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 821.

cyclo[-Abu-Thi-Ser-D-Tic-Oic-Arg-] (13). For the cyclization the pure product H-Abu-Thi-Ser-D-Tic-Oic-Arg-OH (400 mg, 0.38 mmol) was dissolved in DMF to a concentration of 2.5×10^{-4} mol/L, treated with 792 mL of diisopropylamine,

488 mg of TBTU, and 247 mg of HOBt, and kept for 12 h at room temperature under stirring. Chromatography of the residue obtained by evaporation on Sephadex LH20 in DMF allowed the pure fractions to be isolated as an oily residue. Preparative HPLC of the cyclic analog afforded 98 mg of **1** (0.098 mmol, overall yield from Fmoc-Arg(Pmc)-resin 9.8%). The final product was >99% pure according to analytical HPLC: AAA Arg 1.05 (1), Ser 0.95 (1), Abu 0.96 (1), Tic 1.03 (1), Oic 1.00 (1), Thi nd; HPLC t_R 11 min, gradient 25−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 792.

cyclo[-Bal-Thi-Ser-D-Tic-Oic-Arg-] (14). This compound was obtained by the procedure described for **13**: yield 16 mg (15%); AAA Arg 0.96 (1), Ser 0.99 (1), Tic 1.00 (1), Oic 1.04 (1), Bal nd, Thi nd; HPLC t_R 8 min, gradient 25−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 778.

cyclo[-Abu-Tic-Ser-D-Tic-Oic-Arg-] (15). This compound was obtained by the procedure described for **13**: yield 63.6 mg (6.2%); AAA Arg 1.01 (1), Ser 1.00 (1), Abu 0.98 (1), Tic 2.04 (2), Oic 0.98 (1); HPLC t_R 21.5 min, gradient 20−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 798.

cyclo[-Abu-Phg-Ser-D-Tic-Oic-Arg-] (16). This compound was obtained by the procedure described for **13**: yield 44.9 mg (4.5%); AAA Arg 1.01 (1), Ser 1.06 (1), Abu 0.96 (1), Phg 0.96 (1), Tic 1.00 (1), Oic 1.01 (1); HPLC t_R 17 min, gradient 20−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 772.

cyclo[-Abu-Thi-Ala-D-Tic-Oic-Arg-] (17). This compound was obtained by the procedure described for **13**: yield 51.6 mg (5.1%); AAA Arg 0.97 (1), Ala 1.00 (1), Abu 1.01 (1), Tic 1.07 (1), Oic 0.96 (1), Thi nd; HPLC t_R 18 min, gradient 25−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 776.

cyclo[-Abu-Ala-Ser-D-Tic-Oic-Arg-] (18). This compound was obtained by the procedure described for **13**: yield 56 mg (6%); AAA Arg 0.96 (1), Ala 1.05 (1), Ser 0.96 (1), Abu 1.02 (1), Tic 1.07 (1), Oic 0.96 (1); HPLC t_R 18.5 min, gradient 17−25% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 710.

cyclo[-Abu-Trp-Ala-D-Tic-Oic-Arg-] (19). This compound was obtained by the procedure described for **13**: yield 53.8 mg (5.2%); AAA Arg 0.92 (1), Ala 0.97 (1), Abu 1.1 (1), Tic 1.06 (1), Oic 0.94 (1), Trp nd; HPLC t_R 17.5 min, gradient 20−50% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 809.

cyclo[-Abu-D-Phe-Ala-D-Tic-Oic-Arg-] (20). This compound was obtained by the procedure described for **13**: yield 41 mg (4.6%); AAA Arg 0.95 (1), Ala 1.08 (1), Abu 1.01 (1), Phe 1.05 (1), Tic 0.97 (1), Oic 0.97 (1); HPLC t_R 19 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 770.

cyclo[-Gly-Ala-D-Tic-Oic-Arg-] (21). This compound was obtained by the procedure described for **13**: yield 33 mg (4%); AAA Arg 1.04 (1), Ala 0.94 (1), Gly 1.00 (1), Tic 1.01 (1), Oic 0.99 (1); HPLC t_R 18 min, gradient 15−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 595.

cyclo[-Gly-Phe-D-Tic-Oic-Arg-] (22). This compound was obtained by the procedure described for **13**: yield 30 mg (3.3%); AAA Arg 0.95 (1), Phe 1.09 (1), Gly 0.98 (1), Tic 1.07 (1), Oic 0.91 (1); HPLC t_R 15 min, gradient 25−50% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 671.

cyclo[-Abu-Thi-D-Tic-Oic-Arg-] (23). This compound was obtained by the procedure described for **13**: yield 53 mg (5.6%); AAA Arg 0.99 (1), Abu 0.91 (1), Tic 1.04 (1), Oic 1.06 (1), Thi nd; HPLC t_R 19 min, gradient 25−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 705.

cyclo[-Gly-Thi-D-Tic-Oic-Arg-] (24). This compound was obtained by the procedure described for **13**: yield 43 mg (4.7%); AAA Arg 0.91 (1), Gly 0.94 (1), Tic 1.09 (1), Oic 1.05 (1), Thi nd; HPLC t_R 24 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 677.

cyclo[-Sar-Thi-D-Tic-Oic-Arg-] (25). This compound was obtained by the procedure described for **13**: yield 18 mg (2.2%); AAA Arg 0.95 (1), Tic 1.02 (1), Oic 1.01 (1), Sar nd, Thi nd; HPLC t_R 24 min, gradient 20−40% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 691.

cyclo[-Lys-Thi-D-Tic-Oic-Arg-] (26). The protected peptide Boc-Lys(Alloc)-Thi-D-Tic-Oic-Arg-resin was obtained by the procedure described for **1**. After the cyclization reaction, the allyl side chain protection was removed using a procedure similar to that described by Loffet et al.[25] Briefly, the peptide (300 mg, 0.36 mmol) was suspended in 2 mL of CH_2Cl_2, and $PdCl_2(PPh_3)_2$ (10 mg, 0.0144 mmol), Bu_3Sn (0.14 mL, 0.72 mmol), and CH_3COOH (0.05 mL) were added to the reaction medium under nitrogen. After 1.5 h at room temperature, the reaction was stopped by evaporation and the residue obtained was purified by preparative HPLC on reverse phase and lyophilized: yield 50 mg (5.1%, total yield); AAA Arg 1.06 (1), Lys 0.94 (1), Tic 0.99 (1), Oic 1.01 (1), Thi nd; HPLC t_R 12 min, gradient 30−60% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 747.

cyclo[-Arg-Thi-D-Tic-Oic-Arg-] (27). This compound was obtained by the procedure described for **26**: yield 40 mg (3.9%); AAA Arg 2.02 (2), Tic 0.99 (1), Oic 1.01 (1), Thi nd; HPLC t_R 18.5 min, gradient 24−30% CH_3CN in 0.1% TFA aqueous buffer per 30 min; MS *m/e* MH^+ 776.

NMR Studies and Signal Assignments. NMR experiments were performed on a Bruker AMX 500 spectrometer equipped with a 5 mm inverse probe; 5 mg of each sample was dissolved in DMSO-d_6 (SDS, 99.96%). 2D HOHAHA and ROESY were performed in the phase sensitive mode (TPPI) with 8 and 64 scans and a spectral width in w2 of 5000 Hz. Temperature-dependence chemical shifts were determined with a series of five spectra from 300 to 320 K.

The cross-peak assignments were calculated on 2D NMR experiments (TOCSY-ROESY) by identifying spin systems of each amino acid. The different cross-peaks were classified in three different categories: S (strong), M (medium), W (weak). Sequential assigments using dαN and dβN was done to confirm resonance assignments. Single sets of resonance were found for all the amino acids, thus excluding conformationnal equilibrium.

Computational Methodology. Molecules were modeled using standard bond lengths and angles, with the SYBYL program versions 6.04 and 6.1a running on an Indigo R4400 Extreme Silicon Graphics workstation. The conformation of the molecule **24** was minimized by molecular mechanics using the NMR constraints as described in the next paragraph. The optimized geometry was adjusted and minimized in vacuo, using 200 steps of simplex and then the conjugate gradient of the Powell[32] algorithm, and the energy calculations were based on the Tripos force field.[33] Globally neutral molecules were considered, omitting the electrostatic term. The Kollman force field[34] was not used because of the presence of the nonnatural amino acids Thi, Tic, and Oic. The $^3J_{NH/H\alpha}$ coupling constants and the NOE between HαTIC and HδOIC and between HαTHI and HγTIC gave information on the geometry of the cyclic backbone, showing that the peptide bonds were all trans. Two other types of NMR-derived constraints, mostly the H-bonds and only a few NOE-derived interatomic distances, were introduced as distance-range constraints within SYBYL, using a *k* range force constant varying between 50 or 100 kcal/mol·A^2 for the H-bonds, depending on the temperature coefficients, and between 1 and 20 kcal/mol·A^2, depending on the intensities of the NOE. These NOE are listed in Table 1 (Supporting Information).

An optimized NMR conformation of compound **24**, without any constraints, was used as the starting point of the molecular dynamics simulation. After a 5 ps equilibrium period in vacuo at 300 K with constant temperature and volume (using the Berendson algorithm), molecular dynamics simulation was processed during 295 ps, at 300 K, with configurations saved every picosecond. For all the MD simulations a time step of 1 fs was employed, the nonbonded interactions were updated every 25 fs within a radius of 8 Å, and a temperature coupling of 10 fs was used. The 295 structures generated were energy-minimized as described before with a convergence criteria of 0.01 kcal/Å·mol, and then the Φ and Ψ torsion angles were measured on each conformer.

For the full optimization of **24** by the semiempirical method AM1 using Gaussian92, all the atoms of the molecule **24** were considered, using the following options in the route of the input file, in *Z*-matrix: #P RAM1(s1978) opt=(grad,noeigentest,-newton). The convergence criterions used were the defaults of Gaussian92, i.e., 0.000 450 for the maximum force.

Rabbit Jugular Vein in Vitro: pA_2 Measurements. Experiments were performed in male New Zealand rabbits (12 weeks old; Charles River, France). Rabbits were anesthetized with an overdose of pentobarbital (40 mg kg^{-1} in the ear vein). The rabbit aorta and jugular veins were excised, immersed in cold (4 °C) physiological salt solution, and cleaned of adherent connective tissue. Arteries and veins were cut into rings (4 mm long) and mounted in an organ bath for isometric tension recording. In rabbit aortic rings the endothelium was carefully removed by inserting a pair of watchmaker forceps into the lumen and rolling them back and forth on saline-wetted paper. Endothelium removal was demonstrated by the disappearance of the relaxation to acetylcholine (10^{-6} M). The tissues were bathed in modified Krebs-Ringer solution (37 °C, bubbled with a 95% O_2, 5% CO_2 gas mixture, pH 7.4) of the following composition (mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; calcium-disodium EDTA, 0.026; and glucose, 11.1. The tissues were connected to a UC_2 force transducer (Gould, France), and changes in tension were recorded on a polygraph (Gould, France).

Rings were stretched step by step (1 g of passive tension for the rabbit jugular vein, 8−10 g for the rabbit aorta) until optimal and reproducible contraction to KCl (40 mM added into the bath) was achieved. Then a reference contraction was produced with a concentration of KCl (60 mM added into the bath) which gave the maximum contraction to the depolarizing solution in those tissues. After repeated rinses, rabbit jugular venous rings were subjected to a resting period of 45 min. Rabbit aortic rings were allowed to equilibrate for 6 h in order to express the B1 bradykinin receptor.[8] A 45 min incubation period with the antagonists was observed before the cumulative addition of agonist (e.g., B2 receptor, bradykinin; B1 receptor, des-Arg^9-bradykinin). Experiments were performed in parallel in the rings from the same tissue. Only one agonist concentration−response curve was performed on a single ring. For most of the compounds three concentrations (10^{-7}, 10^{-6}, and 10^{-5} M) were tested.

Human Umbilical Vein in Vitro: pK_B Measurements. Umbilical cords were collected just after delivery and transported to the laboratory in an ice-cold Krebs solution previously aerated with a mixture of 95% O_2, 5% CO_2. Umbilical veins were dissected free of connective tissue, cut into rings, and suspended in organ chambers filled with Krebs solution (37 °C gassed with 95% O_2, 5% CO_2, pH 7.40) of the following composition: NaCl, 118.3 mM; KCl 4.7 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; and glucose, 11.0 mM. The isolated rings were connected to a force transducer, and isometric changes in tension were recorded. The endothelium was carefully removed by inserting a pair of watchmaker forceps into the lumen and rolling the rings back and forth on saline-wetted paper. Umbilical veinous rings were submitted to an initial load of 2−4 g and pretreated with indomethacin (5×10^{-6} M) and L-nitroarginine (10^{-4} M). Umbilical rings were contracted several times with KCl (40 mM), and after a reference contractile, response to KCl (60 mM) was obtained. Experiments were done in parallel, and a single cumulative concentration−response curve for bradykinin was made. As in the rabbit jugular vein assay, a 45 min incubation period with the antagonists was observed before the cumulative addition of agonist. Umbilical vessel contractions are expressed as a percentage of the reference contraction to KCl (60 mM).

Calculation and Statistical Evaluation. Statistical evaluation was performed with a three-way analysis of variance (treatment × concentration × tissue). When a significant interaction was observed ($p < 0.05$), a complementary analysis was undertaken (Newman-Keul's test) to identify differences among groups. EC_{50} calculation was performed with a linear regression within the two half-log concentrations surrounding the 50% value. pA_2 values were calculated according to Tallarida's method; the slope of the Schild plot was constrained to unity (Tallarida et al.).[35] When activity was only detected at the highest concentration tested (10^{-5} M), a pK_B value was determined; the apparent antagonist dissociation constants were determined according to the equation: K_B = [Ant]/(concentration ratio − 1), where [Ant] is the concentration of the antagonist and the concentration ratio is the EC_{50} in the presence of the antagonist divided by the EC_{50} of the agonist in the absence of the antagonist. These results were then expressed as the negative logarithm of the K_B (i.e., $-\log(K_B) = pK_B$).

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Supporting Information Available: ^{1}H NMR chemical shift assignments and ^{1}H ROESY map of the peptide **24** (Table 1 and Figure 1) and sequential and medium range NOE figure and table of NOE data for peptide **24** (Figure 2 and Table 2) (4 pages). Ordering information can be found on any current masthead page.

References

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission on Biomedical Nomenclature (Nomenclature and Symbolism for Amino Acids and Peptides. *Biochem. J.* **1984**, *219*, 345−373): AAA, amino acid analysis; B1, bradykinin receptor subtype 1; B2, bradykinin receptor subtype 2; Abu, 4-aminobutyric acid; aminodecanoic, 10-aminodecanoic acid; Bal, *β*-alanine; tBu, *tert*-butyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, dimethylformamide; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; GuaAbu, 4-guanidinobutyric acid; GuaCapro, 5-guanidinocaproic acid; Guavaleric, 6-guanidinovaleric acid; Hyp, hydroxyproline; NMM, *N*-methylmorpholine; Oic, L-3as,7as-octahydroindole-2-carboxylic acid; Tic, 1,2,3,4-tetraisoquinoline-3-carboxylic acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Thi, tetrahydro-1,4-thiazine-3-carboxylic acid.

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