Bradykinin Receptor Antagonists Containing N-Substituted Amino Acids: *In Vitro* and *in Vivo* B_2 and B_1 Receptor Antagonist Activity[†]

Val S. Goodfellow,* Manoj V. Marathe, Karen G. Kuhlman, Timothy D. Fitzpatrick, David Cuadrado, Wendy Hanson, John S. Zuzack, Sherman E. Ross, Maciej Wieczorek, Michael Burkard, and Eric T. Whalley

Departments of New Leads Discovery, Biochemistry, and Pharmacology, Cortech Inc., 6850 North Broadway, Denver, Colorado 80221

We report a systematic probing of the structural requirements of the bradykinin (BK) type 2 (B_2) receptor for antagonist activity by incorporating *N*-alkyl-amino acid residues at positions 7 and 8 of a potent antagonist sequence. Compound **1** (D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Thi[5]-Ser[6]-D-Tic[7]-N-Chg[8]-Arg[9], CP-0597)[1,2] is a potent ($pA_2 = 9.3$, rat uterus; $pK_i = 9.62$, binding, human receptor clone) B_2 receptor antagonist devoid of *in vitro* B_1 antagonist activity (rabbit aorta). Compound **1** exhibits high potency ($ED_{50} = 29.2$ pmol/kg/min, iv, rabbit) and duration of action when tested in models for *in vivo* B_2 antagonist activity. Although devoid of activity in a classic B_1 isolated tissue assay, B_1 antagonist activity for **1** was demonstrated *in vivo*, in a LPS-treated, inducible BK_1 receptor rabbit blood pressure model ($ED_{50} = 1.7$ nmol/kg/min). D-Arg[0] of **1** can be formally replaced by an achiral arginine surrogate, without significant loss in antagonist potency on rat uterus (compound **11**, B_2 $pA_2 = 9.1$). Antagonist **13** (Hyp[2], NChg[8]), $pK_i = 10.2$, and agonist **4** (*N*-methylcyclohexyl-Gly[8]), $pK_i = 10.1$, also exhibited substantial binding to guinea pig ileum membrane receptors as well as a human B_2 receptor clone. Very minor structural changes in the *N*-alkyl amino acid residues in positions 7 and 8 can modify the activity of this class of compounds from being extremely potent antagonists to tight binding partial or full agonists. These studies have resulted in a series of compounds containing inexpensive amino acid residues but which produce broad spectrum BK receptor blocking potency and exceptional *in vivo* duration of action.

Bradykinin (BK) is a nonapeptide produced by proteolytic cleavage of high molecular weight kininogen by plasma kallikreins. Bradykinin and related kinins are believed to be significant mediators in generating many pathophysiological responses including hyperalgesia via stimulation of peripheral A- and C-fiber neurons.[2–9] There is evidence that BK plays an important role in the inflammatory response[10–13] and is a significant mediator in several disease states including hypotension associated with sepsis and bronchopulmonary disorders including asthma.[14–16] Bradykinin antagonists containing D-Phe at position 7 in the bradykinin sequence were developed by Stewart, and these have been extensively reviewed.[17] A number of much more potent second-generation antagonists of the B_2 receptor have been developed, including CP-0127,[18] NPC17731,[19] and HOE-140.[20] Although HOE-140 is an exceptionally potent compound on B_2 receptors ($pA_2 = 9.5$, rat uterus), it is also extremely selective and exhibits no activity on the majority of B_1 receptors tested to date. Recent data indicate that activation of the B_1 receptor is important in various models of chronic inflammation or persistent hyperalgesic conditions. HOE-140 is much less effective in these models than would be expected from the impressive B_2 antagonist activity and *in vivo* stability of the compound.[21] The more potent second-generation antagonists contain a large number of expensive amino acid residues such as L-2-thienylalanine, 4-substituted D-prolines, L-octahydroindole-3-carboxylic acid, and D-arginine. The high cost of peptides derived from these residues may preclude their practical use as therapeutic

agents for chronic inflammatory diseases. Recently small molecule nonpeptide bradykinin antagonists have been reported, but these competitive antagonists have exhibited activity at multiple receptors.[22,23]

We report a systematic study of positions 7 and 8 of the bradykinin sequence with N-substituted amino acids and describe the discovery of a highly potent B_2 receptor antagonist, **1** (CP-0597). A key finding which prompted the research reported here was the synthesis in our laboratory of a weak, small molecule B_2 antagonist (MW < 500), which contains nonproteinogenic amino acid residues, in which a hydrogen bond-stabilized type II′ β-turn conformation is irrelevant. In addition, during the early course of these studies, it was found in our laboratories that analogs of HOE-140 containing D-2-indanylglycine at position 7, may exhibit substantial antagonist activity at both B_1 and B_2 receptors.[24]

With a view toward developing compounds with combined B_2 and B_1 receptor antagonist activity, we undertook a study to produce analogs which could readily adopt a type II′ β-turn[25–29] at the C-terminal but would allow more conformational freedom to the residues in position 7 or 8 than is allowed in many second-generation bradykinin antagonists. Our assumption was that the bioactive conformation of potent antagonists in the B_2 receptor may be perturbed somewhat from the II′ β-turn proposed for the C-terminal region of potent antagonists and the orientation of the side chains of residues 7 and 8 would be even more greatly perturbed from an ideal II′ β-turn orientation in the B_1 receptor binding site. In addition, orientation of the side chain of the residue in position 8 may be critical in determining B_2 or B_1 receptor specificity. B_1
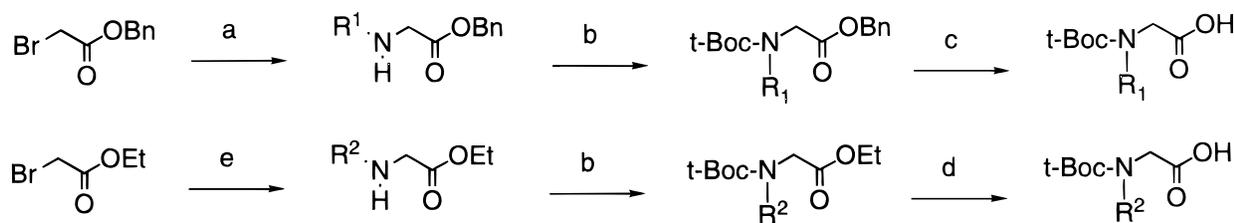
Scheme 1[a]

[a] (a) R^1NH_2, THF; (b) (t-BuOCO)$_2$O; (c) H_2, Pd/C; (d) NaOH, MeOH; (e) R^2NH_2. R^1 = C_5H_9, C_6H_{11}, $CH_2C_6H_{11}$, $CH_2CH_2C_6H_5$; R^2 = $CH_2C_6H_5$.

Scheme 2[a]
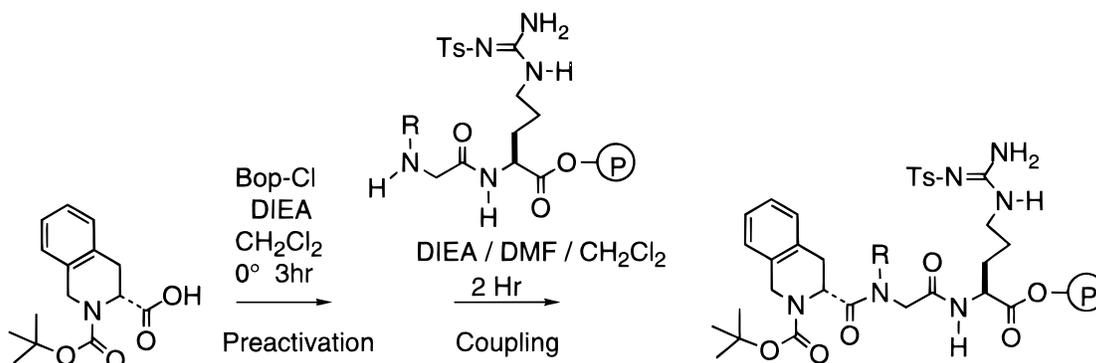
[a] R = C_5H_9, C_6H_{11}, $CH_2C_6H_{11}$, $CH_2CH_2C_6H_5$.

antagonist activity normally requires truncation of the Arg[9] residue.[30] Formal removal of the C-terminal arginine may alter the preferred conformation of the C-terminal region of the peptide, allowing greater conformational mobility to the penultimate residue. For example, HOE-140 possesses no measurable B$_1$ antagonist activity on rabbit aorta, while [des-Arg[9]]HOE-140 exhibits substantial B$_1$ activity ($-\log IC_{50}$ = 7.0, rabbit aorta). In early studies performed by Regoli, a flexible leucine side chain at position 8 was favored by the B$_1$ receptor for maximal activity in des-Arg[9] BK analogs.[30] Des-Arg[9] C-terminal amides exhibited only slightly attenuated B$_1$ activity with respect to corresponding C-terminal carboxylates. It appeared likely that proteolytic processing of B$_2$ ligands by removal of C-terminal arginine produces a free carboxylate and also more conformational freedom for residues at positions 7 and 8, which allows a conformation needed to bind to the B$_1$ receptor.

Numerous structure−activity studies of highly constrained residues at positions 7 and 8 have suggested that an aromatic residue of D-configuration in position 7 and a cyclic alkyl structure at position 8 produce potent B$_2$ antagonist activity. Stewart and co-workers have reported structures where D-cyclopentylglycine is an acceptable replacement for position 7 or L-cyclopentylglycine is a suitable replacement for position 8, but the antagonist activites of these analogs are not extremely high.[31,32] Young and co-workers have explored the use of *N*-benzylglycine analogs in bradykinin sequences, especially position 7, but they have not reported extremely potent agonists or antagonists when tested *in vitro*. Some of these compounds prepared by Young and collaborators are reported to exhibit impressive agonist activity *in vivo*.[33−35] The disparity between *in vitro* and *in vivo* agonist activity may be a manifestation of the stability of the *N*-benzylglycine analogs to proteolytic degradation when compared *in vivo* with rapidly degraded bradykinin. The use of N-substituted

glycine analogs in our studies, which were designed to probe the requirements for receptor binding and antagonism, allowed us to maintain a unique form of conformational constraint yet allowed a more relaxed population of conformers for the side chains for residues at positions 7 and 8.

Chemistry

N-Alkylglycine compounds which were not commercially available were prepared, in general, by alkylation of ethyl or benzyl 2-bromoacetate with excess monoalkylamines. The resulting N-substitued α-amino esters were protected as *N-t*-Boc derivatives, the ethyl or benzyl protecting groups were cleaved by base hydrolysis or hydrogenation, and the resulting N-protected amino acids were incorporated into peptides using solid-phase peptide synthesis. The synthesis of unusual amino acids by this technique is illustrated in Scheme 1. The coupling of sterically hindered amino acids, such as D-Tic to *N*-alkylglycine residues, required special techniques. (See Scheme 2.) Where couplings to *N*-alkyl residues were involved, the N-protected carboxylic acid component (2 equiv relative to peptidyl resin) was preactivated with Bop-Cl (2 equiv) in dichloromethane for 3 h, at 0 °C in the presence of 2 equiv of diisopropylethylamine (DIEA). The homogeneous mixture was then transferred to the reaction vessel containing peptidyl resin and treated with an additional 2 equiv of DIEA in DMF and allowed to react for 2 h. In some cases 4 equiv of preactivated amino acid component were used as noted in the Experimental Section. This method constitutes a novel approach to the solid-phase synthesis of peptides containing *N*-alkylglycine residues. The preactivation at 0 °C and the controlled exposure of the activated component to excess tertiary base suppress racemization in the coupling steps and are analogous to procedures and conditions developed for Bop-Cl couplings used in solution-phase synthesis.[36] The method of preactivation in dichloromethane followed by

Scheme 3
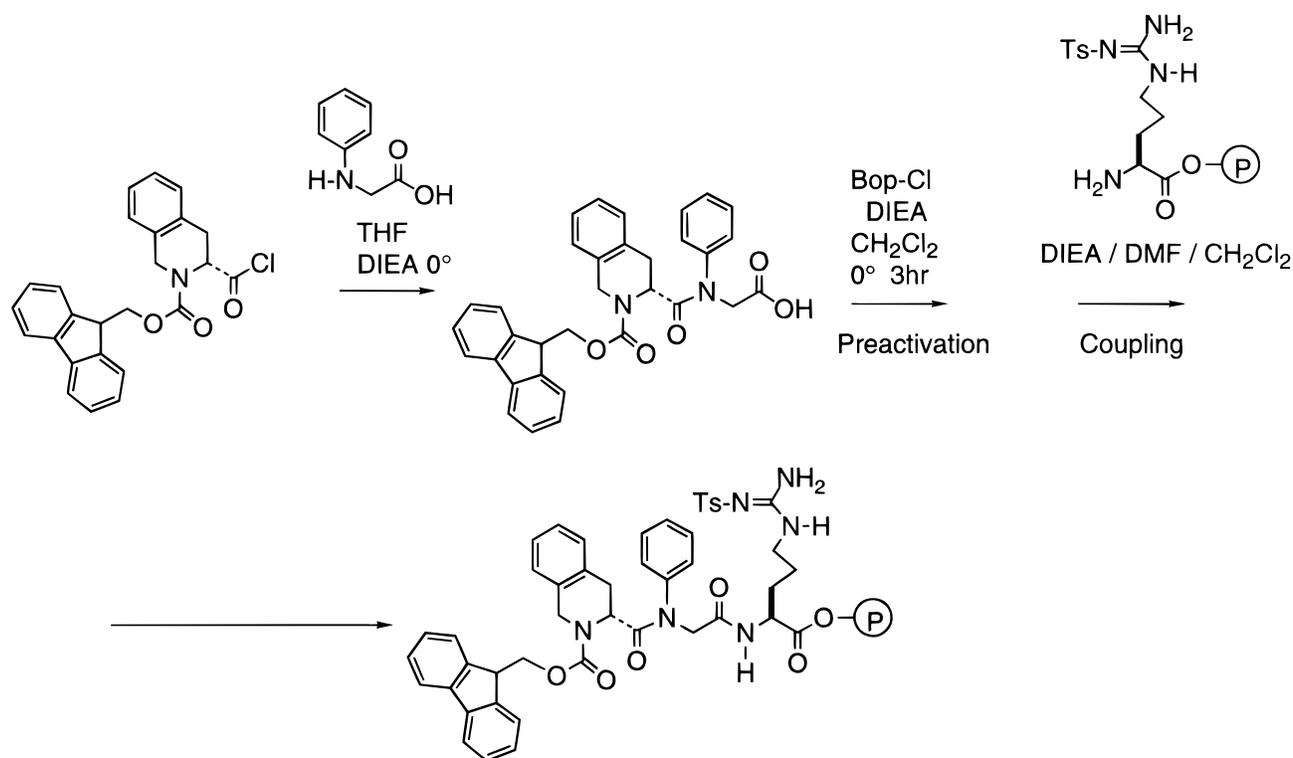
dilution with DMF is necessary to prevent racemization during preactivation but facilitates rapid reaction with the amine component bound in the solid phase. A detailed study of this method will be reported elsewhere.

To test the coupling efficiency of the Bop-Cl method, the dipeptide NChg-Phe was synthesized on Wang resin using a submonomer synthetic route. Boc-D-Tic-OH or Boc-L-Tic-OH was then coupled to the dipeptide on the Wang resin, using the previously described Bop-Cl technique with 4 equiv of preactivated amino acid, 3 h preactivation at 0 °C, and 2 h of reaction time at room temperature. Each peptide was "double-coupled". Resin samples were taken after each coupling, the unreacted dipeptide and product tripeptides were released from the resin by TFA cleavage, and the resulting mixture was analyzed for reaction completion by HPLC analysis. After one coupling, the ratio of coupled L-Tic-NChg-Phe to NChg-Phe was 93.5:6.5. After the second coupling, the ratio was 98.3:1.7. After one coupling, the ratio of coupled D-Tic-NChg-Phe to NChg-Phe was 94.9:5.1. After the second coupling, the ratio was 98.8:1.2.

The tripeptides were reacted with GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate) to give thiourea derivatives which were easily separable by HPLC for L-Tic-NChg-Phe and D-Tic-NChg-Phe. The maximum percentage of the minor epimers arising from racemization during coupling, determined by GITC/HPLC analysis and corrected for minor amounts of the opposite enantiomer of the amino acid found in the D- or L-Tic starting materials, was 1.65% in the L-Tic-NChg-Phe tripeptide and 1.50% in the D-Tic-NChg-Phe tripeptide. Similar results were obtained using couplings of Fmoc-2-indanylglycine.

The coupling of Fmoc-D-Tic-OH or Fmoc-D-Phe-OH to the poorly nucleophilic aniline nitrogen of *N*-phenylglycine was sluggish using a variety of coupling methods including the Bop-Cl method. In this instance, Fmoc-

D-Tic-OH was converted to the acid chloride and coupled to *N*-phenylglycine, in a solution-phase reaction, using conditions previously reported for Fmoc-protected acid chlorides.[37] The resulting dipeptide was coupled to Arg-Hmp resin manually, using the Bop-Cl approach. This process is illustrated in Scheme 3. Epimerization of the achiral *N*-alkylglycine residue is not a concern in this fragment-coupling step. Attempts to detect epimerization occurring during couplings with Fmoc acid chloride derivatives were not undertaken, but we expect that minor amounts of racemization during such couplings can occur as reported by Carpino.[37]

The N-terminal 5-guanidinopentanoyl residues were synthesized on the resin by coupling *N*-*t*-Boc-5-aminopentanoic acid to the peptide by conventional solid-phase technology. The *t*-Boc group was then removed by treatment with TFA, and the amine was converted to guanidine by the action of of 1*H*-pyrazole-1-carboxamidine in the presence of DIEA in DMF at 47 °C using a modification of the method of Bernatowicz[38] as illustrated in Scheme 4.

Results and Discussion

In Vitro **B$_2$ Activity.** We undertook a systematic study of N-substituted glycine replacements for A and B in the sequence D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-A^7-B^8-Arg^9. Initial choices for synthesis were based on the results of multiple conformational searches for the truncated structures Ac-Ser- D-Tic-B-Arg, which suggested that when B was *N*-cyclohexylglycine or *N*-cyclopentylglycine, families of conformations that resembled a type II' β-turn for the sequence Ser-D-Tic-Oic-Arg were energetically favorable. Indeed the combination A = D-Tic and B = NChg (compound **1**) proved to be a potent antagonist of the B$_2$ receptor (pA_2 = 9.3, rat uterus), where the *N*-cyclohexyl moiety apparently provides sufficient affinity to produce a very

Scheme 4
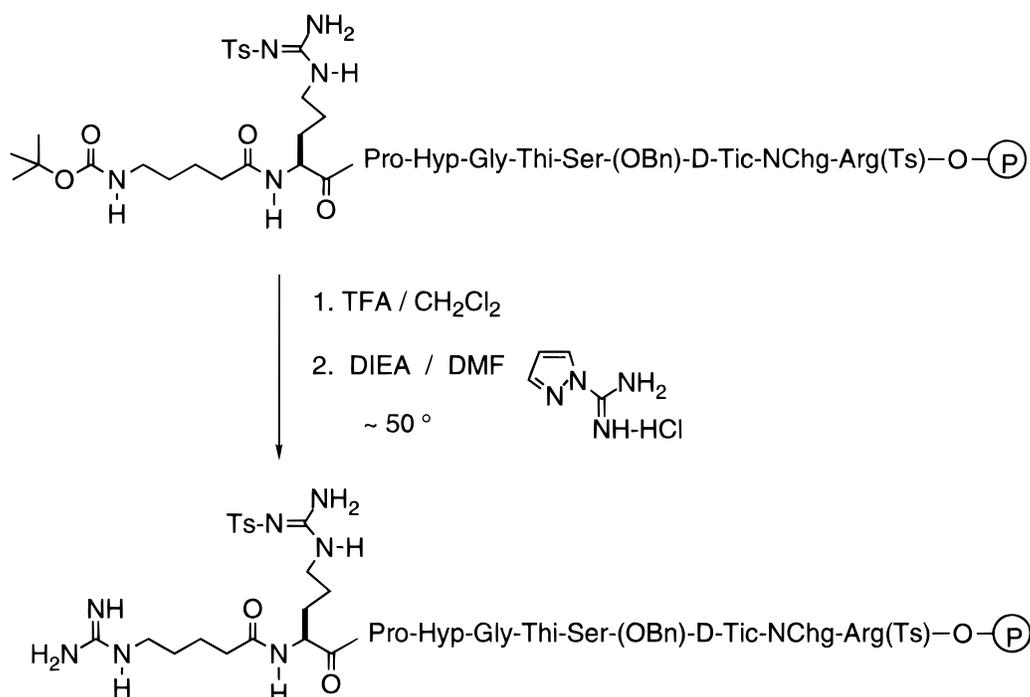
potent compound, although the position 8 residue side chain is not rigidly constrained. Formal contraction of the cyclohexyl ring by one carbon produced a cyclopentyl analog (**2**) with significantly attenuated B_2 antagonist activity, in the rat uterus isolated tissue assay; however, the compound exhibited good affinity ($pK_i = 9.96$) for the guinea pig ileum (GPI) receptor. Formal substitution of phenyl for cyclohexyl, represented by compound **3**, resulted in only a slight attenuation of B_2 antagonist activity in isolated rat uterus tissue assays and resulted in a compound with good affinity for the GPI or human receptor. The formal extension of the cyclohexyl moeity by one methylene unit, however, produced a compound (**4**) with agonist activity on rat uterus. Subsequent binding studies using membranes prepared from GPI showed that compound **4** binds competively to the receptor with a K_i versus radiolabeled bradykinin of 84 pM. The apparent paradox between extremely good binding and moderate potency of antagonists or agonists for bradykinin receptors has been discussed elsewhere.[39] Compound **1**, in preliminary assays, was shown to be a potent, competitive antagonist on human ileum. Compounds **1**–**3** were competitive antagonists on rat uterus. Compound **1** and HOE-140 were non-competitive antagonists on rabbit jugular vein.

Systematic variation of aromatic N-substituents at the 7 position was not as profitable in the search for novel potent antagonists. The *N*-benzylglycine[7] analog **5** produced a much less potent antagonist ($pA_2 = 7.3$). Interestingly formal extension of the benzyl substituent by one methylene unit again produced a compound (**6**) with agonist activity. Dual substitution of *N*-benzylglycine in the 7 position and *N*-cyclohexylglycine in the 8 position produced a compound (**7**) with weak agonist activity.

Using the optimal residue *N*-cyclohexylglycine in position 8, several analogs were prepared with variations in the residue at position 7. Three representative examples are reported here. The order of B_2 antagonist potency for substitutions at position 7 was as follows:
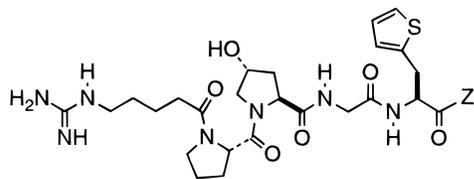
Chart 1[a]
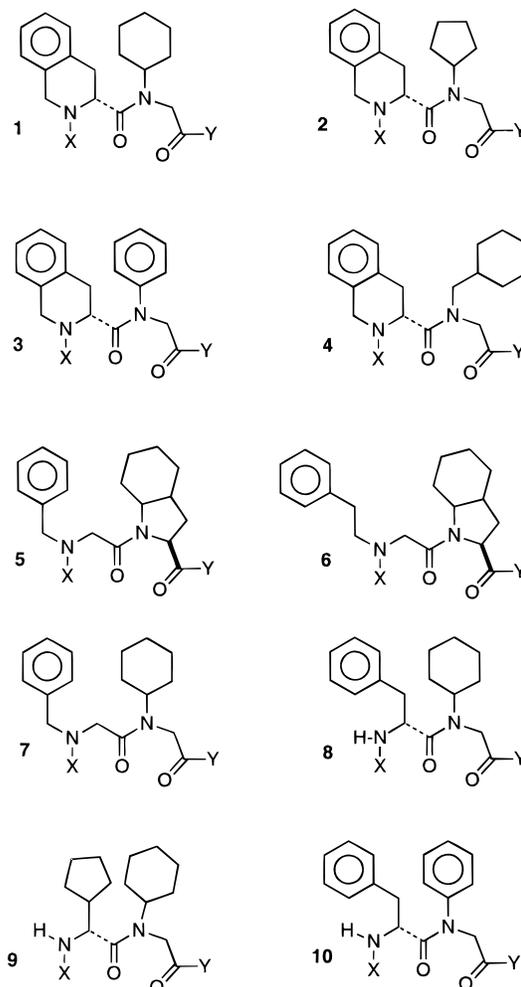
[a] X = D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-. Y = Arg.

D-Tic > D-Phe > D-Cpg (analogs **1**, **8**, and **9**, respectively), with the weakest analog, D-Cpg[7], exibiting low potency ($pA_2 = 5.85$). The combination D-Phe[7]-NChg[8]

Chart 2[a]
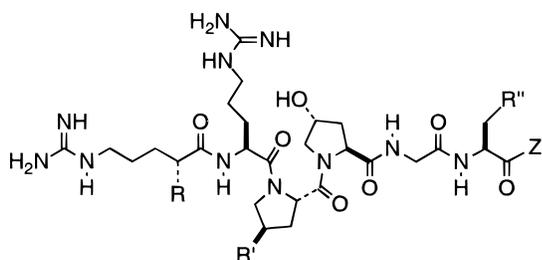
11 R = H,   R' = H,   R'' = 2- Thienyl

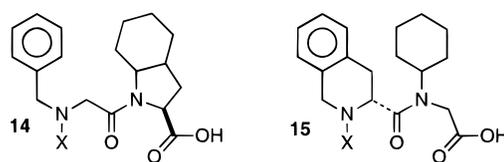13 R = NH$_2$,   R' = OH,   R''= 2- Thienyl

16 R = NH$_2$,   R' = H,   R''= Phenyl

12

[a] Z = -Ser-D-Tic-NChg-Arg.

Chart 3[a]

[a] X = D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-.

Table 1. *In Vitro* Bradykinin Antagonist Activity in Functional Tissue Assays

compd	B$_2$ pA_2 (rat uterus)	n	recovery (%)	B$_1$ $-$log(IC$_{50}$) (rabbit aorta)	n
1	9.31 ± 0.11	26	73	<5	3
2	8.50 ± 0.09	3	56	<5	4
3	8.88 ± 0.12	6	54	inactive	
4	agonist			<5	4
5	7.30 ± 0.3	3	93	<5	2
6	agonist			<5	4
7	agonist			<5	2
8	7.73 ± 0.09	3	87	5.52	4
9	5.85 ± 0.09	3	75	5.34	4
10	7.68 ± 0.09	6	60		
11	9.09 ± 0.25	10	66		
12	7.65 ± 0.25	6	81		
13	8.48 ± 0.38	5	60		
14	5.67 ± 0.17	3	32	6.41 ± 0.25	3
15	6.76 ± 0.71	3	0	5.79 ± 0.06	5
16	8.79 ± 0.15	12	96		
HOE-140	9.50 ± 0.30	7	41	inactive	
NPC-567	7.29 ± 0.29	3	100		

(**8**) or D-Phe-NPhg[8] (**10**) exhibited essentially identical potency (pA_2 ~ 7.7) on rat uterus tissue.

Our interest in producing compounds with optimal pharmacological parameters led us to explore further modifications of compound **1**. Substitution of D-Arg[0] with the 5-guanidinopentanoyl moiety produced a compound (**11**, pA_2 = 9.1) with potency similar to that of compound **1** in the rat uterus functional tissue assay. Truncation at the N-terminal and replacement of Arg[1] with the 5-guanidinopentanoyl moiety resulted in a compound (**12**) with a ca. 10-fold reduction in potency. Replacement of Pro[3] with hydroxyproline resulted in a slight attentuation in rat uterus antagonist activity for compound **13** but excellent affinity in the GPI binding assay (pK_i = 10.22). Compound **11** is of interest due to the replacement of D-Arg[0] with a relative inexpensive residue which still provides stability to the action of aminopeptidases. The desamino modification can also alter important pharmacokinetic and pharmacodynamic properties of the drug. Compounds **1** and **11** exhibited higher reversibility or washoff recovery than HOE-140 during *in vitro* experiments, a property that may be of interest in toxicological comparisons of the three compounds.

***In Vitro* B$_1$ Activity.** Only two decapeptides disclosed here, exhibited measureable *in vitro* B$_1$ activity in the rabbit aorta functional tissue assay. Both of these compounds contained nonconstrained residues at positions 7 and 8: D-Phe[7]-NChg[8] ($-$log IC$_{50}$ = 5.52) and D-Cpg-NChg ($-$log IC$_{50}$ = 5.34). Two des-Arg[9] analogs are reported here, **14** and **15**. These compounds exhibited only modest activity on both B$_2$ and B$_1$ receptors. The *in vitro* B$_1$ antagonist activity of des-Arg analog **14** ($-$log IC$_{50}$ = 6.41) is measureably lower than that observed for HOE-140 (des-Arg[9]) ($-$log IC$_{50}$ = 7.0). This B$_1$ antagonist activity of **14** is in the range observerd for des-Arg[9] NPC-567 ($-$log IC$_{50}$ = 6.25). It is probable that some *in vivo* antiinflammatory effect of NPC-567 arises from antagonism of B$_1$ receptors by the action of the des-Arg[9] analog produced by the activity of carboxypeptidase. It is unlikely that compounds related to **1**
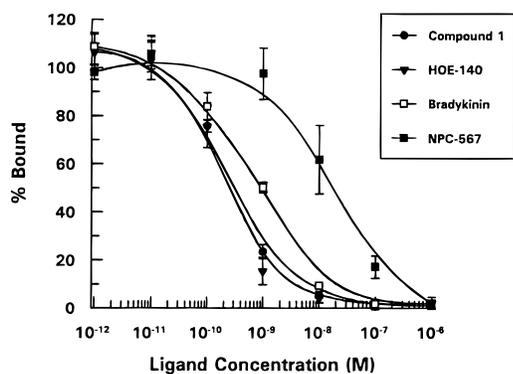
could serve as prodrugs with B$_1$ activity unmasked by the removal of the C-terminal arginine residue by carboxypeptidases, due to their exceptional stability to C-terminal proteolysis.

***In Vitro* Binding Studies (Guinea pig ileum and human receptor clones).** Competitive binding studies were performed using radiolabeled bradykinin and receptor membrane preparations derived from guinea pig ilea. Such studies are a useful tool in primary identification of compounds with high affinity for B$_2$ receptors; however, there is much evidence of variability in the affinity of various antagonist ligands for receptors from different species (guinea pig, mouse, rat, human) and between tissue types within species. Differences in individual compounds functioning as either agonists or antagonists on guinea pig ileum or rat uterus tissues are a well-known phenomenon.[17] Four antagonists reported here (**1**–**3** and **13**) exhibited K_i values in the picomolar range (61–121 pM) on the GPI receptor. These compounds contain closely topographically related substitutions: NCpg, NChg, or NPhg at position 8. In subsequent studies antagonists **1**–**3** and **13** also exhibited excellent binding to the human receptor, with K_i values of 0.24, 0.40, 0.40, and 0.20 nM, respectively. The agonist compound **4** also exhibited significant binding to the human and GPI receptors with K_i values of 169 and 84 pM, respectively. Compound **11**, the desamino analog of compound **1**, showed much lower affinity in the human binding assay than was anticipated from preliminary functional studies with rat uterus.

These data broaden our understanding of the structural requiments for agonist and antagonist activity, but they also illustrate the variability between receptors obtained from different species and the added insight provided by functional tissue assays.

Table 2. Receptor Binding Data ($n = 4$, $\pm$SD)

	pK_i	
compd	guinea pig ileum	human S34 clone
1	10.11 ± 0.11	9.62 ± 0.06
2	9.96 ± 0.11	9.41 ± 0.08
3	9.92 ± 0.06	9.40 ± 0.04
4	10.09 ± 0.09	9.78 ± 0.09
5	8.32 ± 0.08	8.05 ± 0.11
8	8.98 ± 0.07	8.57 ± 0.09
9	7.56 ± 0.02	7.64 ± 0.10
10	8.12 ± 0.10	7.84 ± 0.12
11	8.85 ± 0.05	7.92 ± 0.11
13	10.22 ± 0.06	9.69 ± 0.05
14	5.96 ± 0.09	6.24 ± 0.07
bradykinin	9.58 ± 0.06	9.17 ± 0.07
HOE-140	10.18 ± 0.12	9.74 ± 0.18
NPC-567	7.45 ± 0.08	7.90 ± 0.25

Table 3. Stability of Selected Compounds (Half-life, h) Measured by *in Vitro* Tissue Preparations

	1	**11**	HOE-140	BK	NPC17761	NPC17731
human plasma	>6	>6	>6	0.45	ND	ND
rat kidney	>6	ND	>6	0.13	>6	>6
porcine kidney	>6	3.9	>6	0.05	1.17	4.34
porcine lung	>6	>6	>6	0.05	ND	ND

Figure 1. Inhibition of [³H]BK binding to cloned human receptors. Results are expressed as mean $\pm$ SD; $n = 4$.
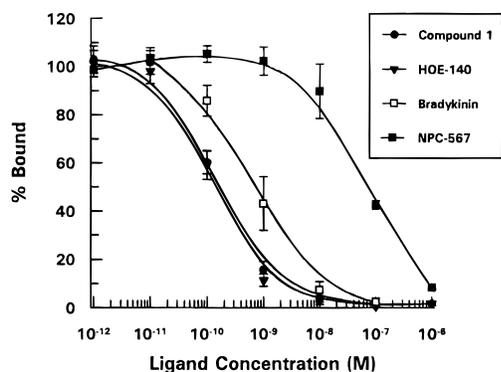
Figure 2. Inhibition of [³H]BK binding to guinea pig ileum membranes. Results are expressed as mean $\pm$ SD; $n = 4$.

***In Vitro* Stability Studies.** Selected compounds were studied for stability using HPLC assays and *in vitro* preparations of human plasma, rat kidney, rat lung, and porcine lung membranes.[40] Peptide drugs are rapidly degraded by the action of proteases. In particular, molecules related to bradykinin are rapidly degraded by aminopeptidases, carboxypeptidases (CP), and endopeptidases such as neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE). Human plasma is rich in peptidase activity arising from the actions of carboxypeptidases and aminopeptidases. Pig or rat kidney possesses significant activity from NEP, ACE, and meprin. Pig and rat lung possess significant protease activity arising from ACE, carboxypeptidase-M, and NEP.[40] Compound **1** exhibited excellent stability ($t_{1/2} > 6$ h) toward proteolysis or metabolic degradation in all of these *in vitro* tissue preparation assays as measured by HPLC. As expected, compound **11**, lacking an N-terminal amino group

required for recognition by aminopeptidases, exhibited excellent stability in the *in vitro* human plasma assay ($t_{1/2} > 6$ h). In contrast to **1**, compound **11** exhibited lower stability in the *in vitro* porcine kidney preparation $t_{1/2} = 3.9$ h). Moderate stability in limited sets of organs which metabolize peptide drugs is not a fatal flaw for bradykinin antagonists. Compounds which exhibit exceptional stability in all tissues may be problematic from a toxicological standpoint; therefore, it may be desirable to produce compounds with various levels of stability in different target organs to achieve maximal potency and minimal toxicity in chronic applications.

***In Vivo* Activity.** Compound **1** was tested in rats and rabbits to establish efficacy of antagonism at B₂ and B₁ receptors, duration of action, and receptor selectivity. Compound **1** demonstrated excellent efficacy at B₂ receptors in rabbit blood pressure models with an ED₅₀ of 29.2 pmol/kg/min, iv. In a lipopolysacharide challenge blood pressure model (B₁ assay), **1** exhibited an ED₅₀ of 1.7 nmol/kg/min. HOE-140 was inactive in this assay at a dose of 5 nmol/kg/min. The specificity of **1** for bradykinin receptors in rats and rabbits was demonstrated by the lack of effect of **1** on blood pressure responses induced by the following vasoactive agents: acetylcholine, norepinephrine, substance P, angiotensin, and angiotensin II. Duration of action experiments were conducted with bolus subcutaneous doses, bolus iv, and continuous iv infusion of **1**. In all cases **1** exhibited substantial duration of action. For example, the hypotensive effect of bradykinin in rabbits was 100% inhibited over 60 min, after iv infusion. In addition complete blockade of agonist effect up to 3 h after a subcutaneous dose (1 mg/kg, rat) was demonstrated.

Conclusions

The most potent, specific, B₂ antagonists reported in the literature possess rigid, constrained, cyclic amino acids at positions 7 and 8. Examples of these are D-Tic⁷-Oic⁸ as in HOE-140[20] or 4-(thioaryl)- or 4-(oxyalkyl)-substituted D-Pro⁷ followed by similar residues of L-configuration at position 8 or Oic, as reported by investigators at Scios-Nova.[19] We have demonstrated that it is unnecessary to include a constrained heterocyclic residue at position 8 to maintain excellent B₂ potency.

Minor modifications of *N*-alkylglycine residues at positions 7 and 8 (extension of the substituent by one methylene unit) in two antagonist structures disclosed here led to homologs with agonist activity. One such agonist, **4**, showed significant affinity for the receptors from either guinea pig ilea or a human receptor clone. Agonists **4**, **6**, and **7** share identical structural backbones and closely related side chain substitution. Agonists **4** and **6** differ in structure from parent antagonists **1** and **5**, respectively, only by the extension of a methylene group in one side chain, at either position 7 or 8, yet both homologs behave as agonists on rat uterus B₂ receptors. Although it is possible that there may be many modes of binding for synthetic peptide agonists

Table 4. *In Vivo* Biological Data Summary for Compound **1** (CP-0597)

experiment	species	dose	n	results
B_2 ED_{50}	rabbit	0.01, 0.03, 0.10 μg/kg/min	3	ED_{50} = 0.051 μg/kg/min (29.2 pmol/kg/min)
B_1 ED_{50}	rabbit	1, 3, 10 μg/kg/min	4	ED_{50} = 2.9 μg/kg/min (1.7 nmol/kg/min)
duration of action (iv infusion)	rabbit	0.1 μg/kg/min	3	100% inhibited at 60 min
selectivity (iv infusion)	rabbit	0.1 μg/kg/min	3	B_2 block only
duration of action (sc)	rat	1 mg/kg	2	100% inhibited at 3 h

and antagonists, the interesting structure−activity relationships illustrated here for such closely related peptides invite speculation into what structural features may lead to the observed effect. It is possible that the three novel synthetic compounds reported to be agonists on rat uterus tissue, in this work, derive their agonist activity from an increase in conformational freedom (increased side chain and backbone mobility), relative to more constrained parent antagonist structures, and bind in a mode more closely related to the binding of the natural agonist ligand bradykinin. An alternative model utilizes backbone conformations related to a II′ β-turn as the presumed mode of binding for both antagonists and the synthetic agonists reported here. This model suggests that agonism arises from increased steric extension of side chains for position 7 or 8. By superimposing several structures for agonists and antagonists, a common "trigger surface" can be constructed from the extraneous steric bulk presented by the agonists near positions 7 and 8. This extraneous steric bulk provided by the synthetic agonists may play a role in stabilizing the active form of the receptor leading to signal transduction. Both the conformational and steric models are consistent with the data we have reported here. Conclusive models await the availability of high-resolution structures of the the bradykinin receptor.

Added flexibility, relative to the D-Tic-Oic motif, obtained by utilizing a NChg residue at position 8 substantially increases B_1 blocking activity for compound **1** in an *in vivo* model where susceptability to B_1 type agonists is induced by LPS challenge. Conformational mobility at positions 7 and 8 allows for modest B_1 blocking potency in *in vitro* assays (rabbit aorta) by related decapeptides containing C-terminal arginine. Additional studies dealing with this observation are underway. The relevance of animal models and classical bradykinin B_1 or B_2 receptor subtypes defined by traditional pharmacology, to human pathophysiological conditions, is yet to be clearly established.

The development of the compounds related to compound **11** which possess NChg[8] and replace D-Arg[0] with a 5-guanidinopentanoyl substituent or alternative mimetics may have important economic advantage. Impressive decapeptide bradykinin antagonists such as HOE-140, NPC17776, and NPC17731 contain several very expensive or nonnaturally occurring amino acids such as complicated 4-substituted D-prolines, L-Oic, Thi, and D-Arg. In contrast *N*-cyclohexylglycine is achiral and can be synthesized in high yield using very economical chemistry; the same remarks apply to 5-guanidinopentanoyl residues. Phenylalanine or related inexpensive aromatics at the 5 position may be viable alternatives to 2-thienylalanine in antagonists targeted for human applications. Thienylalanine at the 5 position was originally found to greatly increase potency of agonists on GPI.[17] Compound **1** was shown to be highly

potent, was stable to proteolysis in *in vitro* tests, and exhibited significant duration of action in animal assays. The structures reported here have provided us with detailed structural insights into the requirements for antagonism and agonism from receptors derived from different species and tissues and have led to the design of nonpeptide bradykinin antagonists[50] and antagonists with potent blocking activity on multiple receptor targets.

Experimental Section

General Methods. Peptides were prepared by solid-phase methodology. A combination of manual and automated methods was utilized. Automated synthesis utilizing "Boc"-protected amino acids was accomplished with a CS-Bio Model 536 peptide synthesizer, using protocols which were identical with those described for manual solid-phase synthesis. Automated "Fmoc" synthesis was performed using an ABI 431A peptide synthesizer and the ABI "Fast-Moc" protocols.[42] Common Boc-amino acids were purchased from Bachem; Fmoc-amino acids were purchased from Bachem or ABI. All solvents were analytical grade and used without further purification. Analytical HPLC was performed on Vydac C_{18} 5 μm columns eluting with acetonitrile/water mixtures containing 0.1% TFA, 1 mL/min flow rate, and detection at 215 nm. All final compounds tested in biological assays showed purity, by HPLC, of >97%. Preparative C_{18} chromatography was performed using a Rainin chromatographic system, utilizing Vydac 300 × 25 mm C_{18} columns and eluting with gradients composed of acetonitrile/water (0.1% TFA), 10−20 mL/min flow rate. Peptide sequence analysis was obtained using an ABI Model 437A automated liquid-phase microsequencer. FAB-MS were obtained from M-Scan Inc., West Chester, PA. Laser desorption-mass spectra were obtained on a Finnigan Laser-Mat mass spectrometer. NMR spectra were obtained with a Varian Gemini spectrometer; ¹H NMR spectra were acquired at 300 MHz, and ¹³C NMR spectra were obtained at 75.6 MHz. Unless stated otherwise, all spectra were obtained in $CDCl_3$. Amino acid analysis was carried out following 24 h vapor-phase hydrolysis in 6 N HCl, utilizing Waters PICO-TAG chemistry.[43] Methods of amino acid analysis provided quantification of commonly occurring amino acids. Quantitation of unusual amino acids often required extensive methods development. Unusual amino acids such as D-Tic, D-Cpg, Igl, Oic, Thi, N-substituted glycines, and Gpa were all qualitatively identified by retention time on the amino acid analyzer but were not routinely quantified by our amino acid analysis service facility. Similar remarks apply to peptide sequencing as well. Correct sequence analysis was found for all compounds containing a free N-terminal amino group. Our laser desorption-mass spectrometer allows determination of molecular ions with an accuracy of ca. 0.1%. This minor expected limitation in accuracy accounts for the ∼1 amu. difference between calculated and observed molecular weights which are reported in two cases. High-resolution mass spectra and high-field NMR data are given for compound **1**, the first compound in this series reported here.

The general method of synthesis of *t*-Boc-protected amino acid derivatives is given for Boc-*N*-cyclohexylglycine. Analogous procedures were followed for the additional N-substituted glycine derivatives.

***N*-Cyclohexylglycine Benzyl Ester.** Cyclohexylamine (14.3 mL, 125 mmol) was dissolved in 50 mL of THF and

chilled to 0 °C under nitrogen; to this solution was added, by dropwise addition, a solution of benzyl 2-bromoacetate (7.93 mL, 50 mmol) in 50 mL of THF. The reaction mixture was allowed to warm to room temperature and then stirred for ca. 15 h. The solvent was removed *in vacuo*, and the residue was taken up in 200 mL of CH_2Cl_2 and washed with 100 mL of 10% Na_2CO_3 solution. The Na_2CO_3 solution was extracted twice with 50 mL of CH_2Cl_2. All CH_2Cl_2 layers were combined, dried over anhydrous $MgSO_4$, and concentrated *in vacuo.* The residue was treated with 26 mL of 4 N HCl in dioxane, volatiles were removed *in vacuo*, and the residue was triturated with cold, anhydrous diethyl ether. The solid was collected and dried for several hours under reduced pressure to yield 15 g of the hydrochloride salt contaminated with traces of cyclohexylamine hydrochloride. This mixture was dissolved in 300 mL of CH_2Cl_2, and the solution was washed with 100 mL of 10% Na_2CO_3 solution. The aqueous solution was back-extracted with CH_2Cl_2. All CH_2Cl_2 layers were combined, dried over $MgSO_4$, and dried under high vacuum (~1 Torr) to provide 11.63 g (91%) of the title compound as an oil: ¹H NMR (CDCl₃) δ 1.0–1.32 (m, 4H), 1.55–1.78 (m, 4H), 1.83 (m, 2H), 2.41 (tt, J = 10, 4 Hz, 1H), 3.48 (s, 2H), 5.17 (s, 2H), 7.36 (s, 5H); ¹³C NMR (DMSO) δ 24.62, 25.82, 33.08, 48.02, 56.12, 66.28, 128.15, 128.36, 135.47, 142.53.

Boc-*N*-cyclohexylglycine Benzyl Ester. *N*-Cyclohexylglycine benzyl ester (11.0 g, 44.4 mmol) was dissolved in 44.4 mL of dioxane, and 44.4 mL of 1 N NaOH solution was added followed by di-*tert*-butyl dicarbonate (10.68 g, 48.8 mmol). Stirring was continued for ca. 15 h, after which volatiles were removed by rotary evaporation. The resulting residue was partitioned between 100 mL of water and 100 mL EtOAc. The layers were separated, and the aqueous layer was acidified to pH 3 with 5% $KHSO_4$ solution. The aqueous layer was extracted with EtOAc, and all EtOAc solutions were combined, washed with saturated $NaHCO_3$ solution and saturated NaCl solution, and dried over $MgSO_4$. Removal of the solvent by rotary evaporation and further drying under high vacuum provided 14.50 g (94%) of the title compound as an oil: ¹H NMR (CDCl₃) δ 0.95–1.55 (m, 14H), 1.63 (m, 1H), 1.77 (m, 4H), 3.70–4.1 (m, 3H), 5.16 (s, 2H), 7.36 (s, 5H).

Boc-*N*-cyclohexylglycine. Boc-*N*-cyclohexylglycine benzyl ester (14.0 g, 40 mmol) was dissolved in 240 mL of anhydrous EtOH, flushed with dry nitrogen, and combined carefully under inert atmosphere with 1.5 g of 10% palladium on carbon. Using a Parr apparatus, the nitrogen atmosphere was replaced with hydrogen (43 psi), and the mixture was shaken over 24 h at room temperature. The mixture was purged with nitrogen, and the catalyst and solids were removed by filtration through a pad of Celite. The filtrate was concentrated by rotary evaporation, and the residue was taken up in 300 mL of EtOAc. The EtOAc layer was extracted with 150 mL of 1 N NaOH solution. The basic solution was acidified in an ice bath to pH 3 with 1 N HCl. The acid solution was extracted with EtOAc (3 × 100 mL). This solution was washed with saturated NaCl solution and concentrated *in vacuo* to a colorless oil which was further dried under high vacuum. The resulting glass/solid was triturated with hexane, which after filtration and drying provided the title compound (8.89 g, 87%) as a colorless powder: mp 103–104 °C (uncorrected); ¹H NMR (CDCl₃) δ 1.07 (m, 1H), 1.10–1.55 (m, 4H), 1.43 (s, 9H), 1.55–1.9 (m, 5H), 3.67–4.13 (m, 3H), 11.33 (br s, 1H); ¹³C NMR (CDCl₃) δ 25.43, 25.68, 28.20, 30.91, 44.03, 44.26, 44.36, 44.42, 54.03, 54.18, 56.13, 80.36, 80.47, 154.81, 176.83. Anal. ($C_{13}H_{23}NO_4$) C, H, N.

N^α-Boc-N-cyclopentylglycine. The title compound was prepared as described for Boc-*N*-cyclohexylglycine. The resulting compound was recrystallized from EtOAc/hexane to yield 1.09 g (47.0%) of the title compound as a colorless solid: ¹H NMR (CDCl₃) δ 1.2–1.72 (m, 15H), 1.76–1.96 (m, 2H), 3.64–4.0 (br s, 2H), 4.1–4.6 (m, 1H), 10.36 (br s, 1H). Anal. ($C_{12}H_{21}NO_4$) C, H, N.

N^α-Boc-N-phenethylglycine. The title compound was prepared as described for Boc-*N*-cyclohexylglycine: ¹H NMR (CDCl₃) δ 1.45 (s, 9H), 2.8–2.92 (m, 2H), 3.44–3.6 (m, 2H), 3.8, 3.92 (rotamers, 2H), 7.12–7.36 (m, 5H), 11.32 (br s, 1H); ¹³C NMR (CDCl₃) δ 34.65, 35.01, 49.15, 49.69, 50.37, 50.55,

80.64, 80.77, 126.32, 126.42, 128.5, 128.56, 128.80, 138.79, 138.93, 155.97, 175.32, 176.02. Anal. ($C_{15}H_{21}NO_4$) C, H, N.

N^α-Boc-N-Cyclohexylmethylglycine. The title compound was prepared as described for Boc-*N*-cyclohexylglycine: ¹H NMR (CDCl₃) δ 0.84–1.8 (m, 20H), 3.04–3.2 (m, 2H), 3.9–4.04 (rotamers, 2H), 11.44 (br s, 1H); ¹³C NMR (CDCl₃) δ 25.67, 25.78, 26.25, 26.36, 28.13, 28.19, 28.24, 30.59, 30.63, 30.72, 36.85, 37.08, 37.69, 45.53, 49.08, 49.69, 54.47, 54.62, 80.17, 80.37, 155.46, 156.37, 174.68, 175.06. Anal. ($C_{14}H_{25}NO_4$) C, H, N.

N^α-Boc-N-benzylglycine. NaOH (0.3 g, 7.57 mmol) dissolved in a minimum amount of water was added to a stirred solution of N^α-Boc-N-benzylglycine ethyl ester (0.74 g, 2.52 mmol) in MeOH (5.0 mL). The reaction mixture was stirred at room temperature for 18 h. MeOH was evaporated *in vacuo,* and the resulting residue was dissolved in water. The aqueous layer was extracted with CHCl₃ (2 × 50 mL) and chilled to 0 °C; then the pH was adjusted to 2.0 with 1 N HCl, and the solution was then extracted with EtOAc. The EtOAc layer was then washed with brine, dried ($MgSO_4$), and evaporated *in vacuo* to yield 1.18 g (88.0%) of the title compound: ¹H NMR (CDCl₃) δ 1.49 (s, 9H), 3.82 (s, 1H), 3.98 (s, 1H), 4.52 (s, 1H), 4.56 (s, 1H), 7.2–7.4 (m, 5H), 11.5 (br s, 1H); ¹³C NMR (CDCl₃) δ 28.19, 28.26, 47.43, 47.57, 50.85, 51.49, 80.92, 81.10, 127.46, 127.52, 128.08, 128.6, 136.98, 137.16, 155.58, 155.99, 175.40, 175.70. Anal. ($C_{14}H_{19}NO_4$) C, H, N.

N^α-Boc-D-cyclopentylglycine. D-Cyclopentylglycine was synthesized by the method of Dunn.[44] D-Cyclopentylglycine (3.93 g, 27.5 mmol) and NaOH (27.5 mL, 1 M solution) were added to a mixture of 150 mL of dioxane and 75 mL of H_2O. The solution was chilled to 0 °C, and di-*tert*-butyl dicarbonate (6.60 g, 30.2 mmol) was added. The reaction mixture was warmed to room temperature and stirred for ca. 15 h. Volatiles were removed *in vacuo*, and the residue was taken up in water and basified, using 5% NaOH, to pH 9. The aqueous layer was extracted three times with EtOAc, and the combined extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to yield 5.44 g (81.5%) of N^α-Boc-cyclopentylglycine as a glass: ¹H NMR (CDCl₃) δ 1.15–1.9 (m, 8H), 1.45 (s, 9H), 2.25 (m, 1H), 3.99 (m, 0.32H), 4.25 (m, 0.68H), 5.01 (d, J = 7.0 Hz, 0.68H), 6.27 (d, J = 7.0 Hz, 0.32H), NH and α-H yield two signals arising from rotamers.

General Procedure for Solid-Phase Synthesis of Peptides: Peptides Ending in C-Terminal Arginine. All amino acids were protected at the α-nitrogen with the *tert*-butoxycarbonyl group. Serine was protected as the benzyl ether. The guanidine of arginine was protected with the tosyl group. No side chain protection was employed for hydroxyproline.

Resin Preparation. PAM resin (generally 0.20–2 g; Bachem) prederivatized with N^α-Boc-N^g-p-tosyl-L-arginine (~0.25–0.75 equiv/g) was charged in a vessel designed for manual solid-phase peptide synthesis.[45] The resin was treated with 25 mL of dry CH_2Cl_2, with agitation provided by nitrogen bubbling for 1 min. The solution was drained away, and washing with CH_2Cl_2 was repeated (2 × 25 mL). A similar wash was repeated with dimethylformamide (3 × 25 mL) followed by a wash with CH_2Cl_2 (3 × 25 mL).

Deprotection. The washed resin was treated with 25 mL of a 1:1 mixture of TFA in CH_2Cl_2. Agitation was maintained for 5 min by nitrogen bubbling; then the solvent was filtered away. The resin was again treated with 25 mL of a 1:1 mixture of TFA in CH_2Cl_2, agitation was maintained for 25 min by nitrogen bubbling, and then the solvent was filtered away.

Neutralization. The resin was washed sequentially with CH_2Cl_2 (3 × 25 mL), DMF (2 × 25 mL), and again with CH_2Cl_2. The resin was washed with a solution of 10% (v/v) diisopropylethylamine in CH_2Cl_2 (3 × 25 mL). To remove traces of base, the resin was washed with CH_2Cl_2 (3 × 25 mL).

Procedure for Coupling with *N*-Boc-amino Acid HOBt Esters. Four equivalents of the Boc-protected amino acids were dissolved in a minimum amount of DMF followed by 4 equiv of HOBt (1-hydroxybenzotriazole monohydrate). To this was added 4 equiv of DCC (dicyclohexylcarbodiimide). The reaction mixture was stirred at room temperature for 1 h. The

dicyclohexylurea was removed by filtration, and the resulting filtrate was added to the peptide synthesis vessel containing the N-deprotected peptidyl resin. Agitation was continued for 1 h.

Procedure for Coupling Boc-Protected Amino Acids to the Peptidyl Resin Containing an N-Terminal-Hindered Secondary Amine Such as D-Tic, Oic, or an N-Substituted Glycine Residue. Two equivalents of the Boc-protected amino acid and 2 equiv of diisopropylethylamine were dissolved in a minimum amount of CH_2Cl_2. The solution was chilled to 0 °C under nitrogen and treated with 2 equiv of Bop-Cl. The reaction mixture was stirred under nitrogen at 0 °C for 3 h. The homogeneous solution was added to the peptide synthesis vessel containing the N-deprotected peptidyl resin followed by an equivalent volume of DMF containing an additional 2 equiv of diisopropylethylamine. Agitation was continued for 2 h. In specific cases identified within specific examples, 4 equiv of the activated amino acid was employed by doubling the amount of reagents and solvents used in this procedure.

Postcoupling Washes. The resin was washed with dimethylformamide (3 × 25 mL) followed by CH_2Cl_2 (3 × 25 mL).

Evaluation of Coupling Completion. A few particles of the resin were reacted with ninhydrin using a modification of the method developed by Kaiser[46] to qualitatively determine if the reaction had gone to completion. If the reaction was complete, the resin was washed again with CH_2Cl_2 and the deprotection and coupling steps were continued as above. If unreacted amine appeared to be present, the resin was again submitted to the neutralization and coupling procedure. *N*-Alkyl-amino acid residues may give erratic results when subjected to the Kaiser test; in such cases, the use of quantitative amino acid analysis performed after hydrolysis of a small sample of the peptidyl resin may be of value in determining the completion of coupling reactions.

HF Deprotection. The peptide resin was carefully dried and transferred to a vessel especially prepared for HF reactions (Peninsula Laboratories). The peptide was treated with 1 mL of anisole followed by the condensation of ca. 9 mL of HF at low temperature. The reaction was allowed to continue for 45–60 min at 0 °C, and the HF was removed carefully under vacuum. The resin/scavenger mixture was dried under vacuum for 1 h; then the residue was carefully washed with anhydrous diethyl ether and the peptide extracted into 10% acetic acid solution. The acetic acid solution was lyophilized to a solid which was purified by reverse-phase preparative C_{18} HPLC chromatography (Dynamax or Vydac column, 30 × 2.5 cm, 10 μm C_{18}) to give the desired peptides.

Peptides Ending in C-Terminal Oic or N-Substituted Glycines. The C-terminal Boc-amino acid (1.0 mmol) was dissolved in a mixture of 10 mL of 95% EtOH and 3 mL of H_2O. Cesium bicarbonate (1.0 mmol) was added and the reaction mixture stirred for 1 h. The solvent was removed *in vacuo* on a rotary evaporator. Small portions of benzene were added and removed *in vacuo* to remove traces of water, until a white free-flowing powder was obtained. Merrifield resin (Bachem; 1% cross-linked, 100–200 mesh, ~1 mequiv/g, 1.0 mequiv) and the cesium salt were suspended in dry, nitrogen-purged DMF (6–8 mL/g of resin), and the reaction mixture was stirred under nitrogen at 50 °C for 24–36 h. The solution was filtered, and the resin was washed three times, with 50 mL of each of the following solvents in sequential manner: DMF, 50% DMF in water, DMF, and ethanol. The resin was dried overnight. An approximation of the Boc-amino acid substitution density of the resin was made by the mass gained by the resin during derivitization. The Boc-amino acid-derivatized resin was prepared for peptide synthesis and deprotected as described above for PAM resins.

Synthesis of Unusual Peptides: D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-NPhg-Arg (3). Fmoc-D-Tic Acid Chloride. One equivalent of the Fmoc-D-Tic was dissolved in CH_2Cl_2 (0.3 M) and placed in a flask equipped with a stir bar and reflux condenser. Thionyl chloride (10 equiv) was added with stirring and the mixture refluxed, under nitrogen, for 2 h. The reaction mixture was concentrated by rotary evaporation, and the residue was diluted with CH_2Cl_2 and concen-

trated again to remove excess thionyl chloride. This was repeated twice, and the resulting acid chloride was triturated with hexane (or alternatively recrystallized from CH_2Cl_2/hexane) until a fine powder was obtained. This was used without further purification.

Fmoc-D-Tic-*N*-phenylglycine. *N*-Phenylglycine (Aldrich; 1.2 equiv) was suspended in dry THF (0.5 M) in a flask, and 3 equiv of diisopropylethylamine was added. The flask was placed in an ice–water bath and stirred for 15 min under nitrogen; 1 equiv of Fmoc-D-Tic acid chloride was dissolved in dry THF (0.15 M) and added slowly to the cooled flask. A precipitate began to form immediately, and the reaction mixture was allowed to warm to room temperature and stirred for 2 h. Once the reaction was completed, the solvent was removed by rotary evaporation and the residue taken up in EtOAc and washed with 5% $KHSO_4$, H_2O, and brine. Drying with Na_2SO_4, rotary evaporation, and placement under high vacuum yielded a white foam. The dipeptide was analyzed by HPLC (C_{18} RP column, 4.6 × 250 mm, 1 mL/min flow rate, 30 min gradient of 30–100% CH_3CN/H_2O containing 0.1% TFA, detection at 254 nm) for purity and was utilized without further cleanup.

Attachment of Fmoc-D-Tic-NPhg-OH to Arg-OHMP Resin. The coupling of the Fmoc-D-Tic-NPhg-OH dipeptide (~0.785 mmol) to the peptidyl resin (0.25 mmol) was carried out using Bop-Cl under conditions as described above for the couplings of N-protected amino acids to hindered N-substituted amino acids.

Peptide Synthesis. The Fmoc-D-Tic-NPhg-Arg-OHMP resin was N-deprotected by reaction with 20% piperidine in DMF (2 × 30 min). The resin was then washed, sequentially, three times with DMF, twice with CH_2Cl_2, twice with MeOH, and twice with CH_2Cl_2. Fmoc-serine(O-*t*-Bu)-OH was coupled using the Bop-Cl procedure as described above. The resin was then transferred to an ABI Model 431 automated peptide synthesizer, and the additional residues were added using standard Fmoc/HBTU coupling procedures.[42] The resin was then washed several times with CH_2Cl_2 and dried in a stream of anhydrous nitrogen. The resin was then treated with 10 mL of TFA containing 0.5 mL of thioanisole and 0.25 mL of ethanedithiol. The reaction mixture bubbled for 3 h at room temperature. The mixture was filtered, and the resin was washed with ~1 mL of TFA. The combined filtrates were concentrated *in vacuo*, and the residue was treated with anhydrous diethyl ether and allowed to stand at ice bath temperature for 15 min. The precipitate was then collected by filtration, washed well with cold, anhydrous diethyl ether, and dried *in vacuo*, resulting in 205 mg of colorless powder. HPLC purification of a small portion of this material (0–35% CH_3CN, 0.1% TFA, gradient over 50 min, 10 mL/min) provided 10 mg of **3** as a colorless lyophilate.

D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-NPhg-Arg (10). The dipeptide Fmoc-D-Phe-NPhg-OH was prepared according to the method used to prepare Fmoc-D-Tic-NPhg-OH. The dipeptide Fmoc-D-Phe-NPhg-OH (~0.5 mmol) was coupled to Arg-HMP resin (0.25 mmol) using the procedures as described for compound **3**. The peptide was synthesized and cleaved from the resin as described for compound **3** to provide 260 mg of crude peptide. HPLC purification of 75 mg of this material (5–50% CH_3CN, 0.1% TFA, gradient over 60 min, 10 mL/min) provided 18.9 mg of **10** as a colorless lyophilate.

δ-Gpa-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-NChg-Arg (11). Boc-N^g-*p*-tosyl-L-arginine Merrifield resin (4.20 g, Bachem RBoc20, 0.48 mequiv/g) was charged in a vessel designed for manual solid-phase peptide synthesis. The peptide was coupled sequentially to form a tetrapeptide (Ser(OBn) coupling); then the process was transferred to a CS-BIO automated peptide synthesizer for the addition of Gly, Hyp, Pro, and Arg. A portion of this peptidyl resin (0.53 g) was removed from the reaction vessel. The resin sample was deprotected with TFA as described above and N-Boc-δ-aminovaleric acid was coupled using the procedure described above for HOBt/diimide activation. The N-Boc-δ-aminovaleric acid residue was deprotected with TFA as described previously and neutralized by washing sequentially with CH_2Cl_2, 10% diisopropylethylamine in CH_2Cl_2, CH_2Cl_2, and finally DMF. The resin was

Table 5. Analytical Data for Synthetic Peptides

	amino acid analysis[a]						HPLC analysis		MS
no.	Arg	Pro	Hyp	Gly	Phe	Ser	gradient[b]	t_R (min)	(M + H)
1	3.13	0.95	0.90	0.98		1.04	B	30.3	1293
2	2.93	1.01	0.96	1.04		0.90	B	28.7	1279.6
3	3.01	0.97	0.89	1.02		0.91	A	20.9	1287.5
4	3.16	0.94	0.91	1.00		0.99	B	33.7	1308
5	2.98	1.08	0.97	1.10		0.87	C	14.0	1293
6	3.16	0.94	0.91	1.00		1.00	B	32.8	1305[c]
7	3.03	0.99	1.06	1.02		0.90	B	30.5	1279.6
8	3.11	0.95	0.91	1.00	0.97	1.06	B	30.5	1281.5
9	3.13	0.96	0.92	0.98		1.02	B	29.2	1259
10	3.02	0.95	0.97	0.99	1.03	0.88	A	21.9	1274[c]
11	2.09	1.03	1.05	0.99		0.90	A	22.6	1278
12	1.06	1.00	1.05	1.03		0.90	A	22.3	1123
13	3.10		2.08	1.00		0.89	B	25.3	1309
14	2.06	0.99	0.93	0.99		1.12	B	27.6	1136
15	2.01	0.94	0.91	0.98		1.27	D	28.8	1137
16	3.14	0.91	1.08	0.98	0.93	0.96	A	26.3	1287

[a] See comments in general experimental. [b] All gradients CH₃CN/ H₂O mixtures (percentage of CH₃CN specified) containing 0.1% TFA: A, 5−55%, 50 min; B, 5−70%, 65 min; C, 20−60%, 30 min; D, 10−60%, 50 min. [c] Observed molecular ion differs by 1 amu from expected value but is within 0.1% instrument accuracy.

heated with a mixture of 0.566 g of 1*H*-pyrazole-1-carboxamidine[38] and 0.76 mL of diisopropylethylamine in 20 mL of DMF at 47 °C for 2.25 h. The resin was washed with CH₂Cl₂ and DMF, and the reaction with 1H-pyrazole-1-carboxamidine was repeated two additional times. The resin was washed sequentially with CH₂Cl₂, MeOH, and then CH₂Cl₂ and dried *in vacuo*. HF deprotection provided 80 mg of example **11** as a colorless lyophilate which was purified by preparative HPLC (10−65% CH₃CN, 0.1% TFA, over 55 min).

δ-Gpa-Pro-Hyp-Gly-Thi-Ser-D-Tic-NChg-Arg (12). Boc-*N*ᵍ-*p*-tosyl-L-arginine Merrifield resin (4.20 g, 0.48 mequiv/g) was charged in a vessel designed for manual solid-phase peptide synthesis. The peptide was coupled sequentially to form a tetrapeptide (Ser(OBn) coupling); then the process was transferred to a CS-BIO automated peptide synthesizer for the addition of Gly, Hyp, and Pro. A portion of this peptidyl resin (0.260 g) was removed from the reaction vessel. The resin was deprotected with TFA as described above, and N-Boc-δ-aminovaleric acid was coupled using the procedure described above for HOBt/diimide activation. This residue was deprotected with trifluoracetic acid as described previously and neutralized by washing sequentially with CH₂Cl₂, 10% diisopropylethylamine in CH₂Cl₂, CH₂Cl₂, and finally DMF. The resin was heated with a mixture of 0.405 g of 1*H*-pyrazole-1-carboxamidine[42] and 0.545 mL of diisopropylethylamine in 14.5 mL of DMF at 47 0 °C for 2.25 h. The resin was washed with CH₂Cl₂ and DMF, and the reaction with 1*H*-pyrazole-1-carboxamidine was repeated. The resin was washed sequentially with CH₂Cl₂, MeOH, and then CH₂Cl₂ and dried *in vacuo*. HF deprotection provided 30 mg of crude **12** as a colorless lyophilate, which was purified by preparative HPLC.

D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-NBng-Oic (14). *N*ᵅ-Boc-Oic resin (0.60 g, 0.83 mequiv/g) was prepared as described above; the peptide was coupled sequentially and cleaved from the resin using the procedures previously described, to provide 91.3 mg of crude material. HPLC purification (10−65% CH₃-CN, 0.1% TFA, gradient over 55 min, 20 mL/min) provided 46.1 mg of **14** as a colorless lyophilate.

D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-NChg (15). *N*ᵅ-Boc-NChg resin (0.63 g, 0.79 mequiv/g) was prepared as described above. The peptide was coupled sequentially and cleaved from the resin using the procedures previously described, to provide 71 mg of crude material, which was purified by preparative HPLC.

Proton NMR and high-resolution mass spectral data[51] for compound 1: (300 MHz, DMSO-d_6) δ 0.93−1.5 (m, 5H, Chg⁸-CH), 1.50−1.70 (m, 6H, Arg⁰ β, γ, Arg⁹ β, Chg⁸-CH), 1.70−2.10 (m, 19H, Arg⁰ β, Arg¹ β, γ, Pro² β, γ, Hyp³ β, Chg⁸ CH, Arg⁹ β, γ), 2.80−3.21 (m, 7H, Tic⁷ β, Thi⁵ β, Arg⁰ δ, Arg⁹

δ), 3.21−3.65 (m, 5H, Tic⁷ NCH, Thi⁸ β, Arg¹ δ, Pro² δ), 3.65− 3.91 (m, 9H, Hyp³ δ, Pro² δ, Ser⁶ β, Chg⁸ α, Gly⁴ α), 4.05−4.21 (m, 3H, Arg⁰ α, Arg⁹ α, Tic⁷ NCH), 4.31−4.45 (m, 2H, Tic⁷ α, Arg¹ α), 4.45−4.61 (m, 2H, Hyp³ γ, α), 4.61−5.07 (m, 3H, Pro² α, Thi⁵ α, Ser⁶ α), 5.08 (br s, 1H, OH), 5.51 (br s, 1H, OH), 6.66−6.90 (m, 3H, Thi⁵ Ar-H), 6.95−7.40 (m, 13H, Arg⁰,¹,⁹ C=NH(NH₂), Tic⁷ ArH), 7.51 (1H, Arg⁰ NH), 7.63 (1H, Arg⁹ NH), 7.80 (1H, Thi⁵ NH), 8.05 (br s, 3H, Arg⁰,¹,⁹ -NHC=N), 8.31 (1H, Arg¹ NH), 8.09 (br t, 1H, Gly⁴ NH), 8.85 (1H, Ser⁶ NH).

Coupling Efficiency and Racemization Study. NChg-Phe Dipeptide Formation. Fmoc-L-Phe-Wang resin (615 mg, 0.448 mmole) was placed in a solid-phase peptide synthesis vessel and washed seven times with DMF and then allowed to swell for 30 min in 10 mL of DMF. The DMF was filtered away, and the resin was reacted with 10 mL of 20% piperidine in DMF, two times, for 30 min with nitrogen bubbling. The piperidine/DMF solution was filtered away, and the resin was washed seven times with DMF. In a separate flask, bromoacetic acid (717 mg, 5.16 mmol) was dissolved in 10 mL of DMF and treated with 0.97 mL of diisopropylcarbodiimide (6.19 mmol). The solution was added to the resin and agitated with nitrogen bubbling for 1 h. The solution was filtered away, and the bromoacetylation reaction was repeated two additional times, using 30 min reaction times. The DMF solution was filtered away, and the resin was washed seven times with DMF and then seven times with CH₂Cl₂. Cyclohexylamine (1.00 g) was dissolved in 10 mL of anhydrous DMSO and added to the resin. The reaction mixture was agitated with nitrogen bubbling for 15 h. The DMSO was drained away, and the resin was washed sequentially with DMSO, DMF. and then CH₂-Cl₂. The resin was dried under vacuum, and the change in resin weight was consistent with quantitative conversion to the NChg-Phe dipeptide. A 15 mg sample was deprotected with TFA (2 h, room temperature). HPLC analysis (5−60% CH₃CN, 0.1% TFA, over 30 min, t_R = 17.7 min) showed the expected NChg-Phe dipeptide formed with 93% purity as the crude product. MALDI-TOF mass spectrometry confirmed the identify of the dipeptide (M + H, 305). The resin was divided into two equal portions for subsequent reactions.

Bop-Cl-Mediated Solid-Phase Couplings of Boc-D-Tic and Boc-L-Tic to NChg-Phe Dipeptidyl Resin. In two separate flasks either Boc-L-Tic-OH or Boc-D-Tic-OH (247 mg, 0.896 mmol, 4 equiv) was dissolved in 5 mL of dry CH₂Cl₂ and treated with 0.155 mL of DIEA. The solution was chilled to 0 °C, under nitrogen, and treated with Bop-Cl (227 mg). The reaction mixture was stirred for 3 h at 0 °C. DMF 95 mL) was added to the reaction flask, and the mixture was transferred to a round bottom flask containing the dipeptidyl resin. An additional 0.155 mL of DIEA was added, and the reaction mixture was allowed to stir for 2 h at ambient temperature under nitrogen. After 2 h of reaction time, the solution was removed by a filterstick-mediated filtration, a 15 mg resin sample was removed, and a fresh, cold, preactivated solution of Boc-Tic-OH/Bop-Cl in DMF/CH₂Cl₂ was added followed by an additional 0.155 mL of DIEA. After 2 h of stirring at ambient temperature, the resin was washed well with DMF and CH₂Cl₂. The resin samples were washed well with DMF and then CH₂Cl₂ and deprotected with 1 mL of TFA at room temperature for 2 h. The solutions were evaporated, and the residue was treated with 1 mL of CH₂Cl₂ and evaporated. The residues were taken up in 1 mL of MeOH and analyzed by HPLC (5−60% CH₃CN, 0.1% TFA, over 30 min) for the ratio of unreacted dipeptide (t_R = 17.7 min) and resultant Tic-NChg-Phe tripeptide (24.1 min for both L-L- and D-L-diastereomers). The L-L-diastereomer showed 93.5% completion after one coupling and 98.3% completion after two couplings. The D-L-diastereomer showed 94.9% completion after one coupling and 98.8% completion after two couplings.

Determination of Epimer Formation during Coupling. A 0.200 mL aliquot of either the L-L- or D-L-tripeptide solution in methanol was evaporated to dryness. The residue was taken up in 0.100 mL of DMF and treated with 0.010 mL of DIEA followed by 3 mg of GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate). The mixture was sonicated for 5 min. Then the reaction was quenched by the addition of

0.400 mL of methanol and the mixture analyzed by HPLC (5–90% CH_3CN, 0.1% TFA, over 30 min). The L-L-diastereomer yielded a thiourea derivative with a retention time of 24.5 min, with 1.65% D-L-diastereomeric epimer (t_R = 25.3 min). The D-L-diastereomer yielded a thiourea derivative with a retention time of 25.3 min, with 1.5% L-L-diastereomeric epimer (t_R = 24.5 min). The percentages of epimer were corrected for 1.1% D-isomer found in the Boc-L-Tic-OH (Advanced Chemtech) and 0.5% L-isomer found in the Boc-D-Tic-OH (Synthetech) by similar GITC analysis.

***In Vitro* B_2 Antagonist Activity Measurements.** The standard rat uterus functional assay was conducted as follows: Female Sprague–Dawley rats (200–250 g) were pretreated with stilbesterol (100 μg/kg), killed 18 h later by a blow on the head, and exsanguinated. Uterine horns were removed, placed under a 1 g resting tension in 5 mL tissue baths containing De Jalon's solution at 31 °C, and aerated. Concentration–effect curves were constructed for bradykinin in the absence and presence of antagonist (preincubated for 15 min). Antagonist potency was calculated according to the method of Arunlakshana and Schild.[47] Following exposure to the highest concentration of antagonist (usually 10^{-5} M), each tissue was washed at 10 min intervals for 40 min, after which time a concentration–effect curve was again constructed for bradykinin. The pD_2 (−log(molar concentration producing 50% of the maximum original response to bradykinin)) for bradykinin at this time was calculated and compared to the pD_2 of the initial control concentration–effect curve for bradykinin. The difference in pD_2 values compared to concurrent control reflected the "percentage recovery" of agonist response.

***In Vitro* B_1 Antagonist Activity Measurements.** Female New Zealand white rabbits were killed by overdose of pentobarbital (80 mg/kg iv) and the thoracic aortas removed. Spiral strips were mounted under 2 g resting tension in 5 mL tissue baths containing Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 25 mM glucose, and 2.8 μM indomethacin) and aerated with 95% O_2/5% CO_2. Two concentration–effect curves for des-Arg⁹-bradykinin were constructed at 1 and 3 h. At 5 h des-Arg⁹-bradykinin was added to the bath to a final concentration of 10^{-7} M. This produced stable, sustained, and prolonged contractions of up to 45 min. The compounds of interest were added to the bath in a cumulative fashion, and the IC_{50} (concentration producing 50% reversal of the contraction) was calculated from the resulting tracings.

Stability Studies. Plasma samples were prepared by collection of whole blood from healthy male and female human volunteers or guinea pigs. Samples were collected into culture tubes containing sodium heparin and then spun at 4 °C at 2000 rpm for 10 min. Supernatant fractions were removed by aspiration and stored in vials at −20 °C. Rat or porcine lung and kidney cortical membranes were prepared using differential centrifugation as described by Skidgel[48] for lung or by Booth[49] for kidney. Membrane preparations were stored at −20 °C. Bradykinin antagonists were diluted to 1 mM concentration in PBS (0.0132 M phosphate, 0.1454 M NaCl, pH 7.2). Ten microliters of this working solution was delivered into a series of Eppendorf tubes followed by human or guinea pig plasma (90 μL) or PBS as a control blank and incubated for various time periods. At each time point, the reaction was quenched with the addition of 100 μL of 1 N HCl in either acetonitrile or ethanol. Samples were allowed to stand for ca. 15 min and then spun at 14 000 rpm for 10 min. Supernatant fractions were removed, filtered through 0.22 μM filters (Millipore), and analyzed by HPLC (C_{18}, 12–80% acetonitrile in water, both containing 0.1% TFA, monitoring at 214 nm).

Porcine kidney preparations were diluted 1:10 with PBS; rat kidney preparations were diluted 1:100 with PBS; porcine lung preparations were diluted 1:1 in PBS. The bradykinin antagonist solutions (10 μL) were added to a series of Eppendorf tubes followed by the respective diluted membrane preparations (90 μL). At various time points the reactions were quenched by the addition of 100 μL of ethanol, and the mixture was centrifuged and analyzed by HPLC as described

above. The half-life for the disappearance of the HPLC peak was determined using the computer program ENZFIT (Elsevier).

***In Vivo* Biological Data: 1. Rabbit Blood Pressure.** Male New Zealand white rabbits were anesthetized with pentobarbital, and the femoral arteries were cannulated for the recording of blood pressure. Cannulae were placed in the femoral veins for the bolus injection or continuous infusion of compounds. The femoral arterial catheters were connected to a Gould pressure transducer, and blood pressure was recorded and displayed on a Grass polygraph recorder. Following an equilibration period, the specific experimental procedure was started.

A. B_2 ED_{50}. Following the establishment of a stable baseline blood pressure, the animals were administered bolus injections of bradykinin that produced ca. a 15–25% decrease in blood pressure (0.2 and 0.4 nmol, iv). Bradykinin was tested in the absence and then in the presence of various doses of compound **1** (0.01, 0.03, and 0.10 μg/kg/min iv) for the determination of the ED_{50} (i.e., the dose of **1** reducing the response to bradykinin by 50%). In this system the ED_{50} was found to be 0.051 ± 0.006 μg/kg/min (29.2 pmol/kg/min).

B. B_1 ED_{50}. Rabbits were injected with lipopolysaccharide (LPS) from *Escherichia coli* (10 μg/animal) intravenously 12–18 h before the experiment. Preadministration of endotoxin results in the upregulation of B_1 receptors in the vascular system and allows the evaluation of B_1 antagonist activity. Stimulation of both preexisting B_2 and induced B_1 receptors produces hypotension. After blood pressure equilibration, the animals were administered bolus injections of bradykinin (0.2 and 0.4 nmol) and des-Arg⁹-bradykinin (4.0 and 8.0 nmol). Both agonists were tested in the absence and then in the presence of increasing doses of **1** (1, 3, and 10 μg/kg/min). The ED_{50} for B_1 activity in this system was found to be 2.9 ± 0.92 μg/kg/min (1.7 nmol/kg/min). HOE-140 was inactive at doses up to 10 μg/kg/min.

C. Selectivity of Intravenous Administration of Compound 1. Following equilibration of blood pressure responses produced to acetylcholine (20 nmol), norepinephrine (20 nmol), substance P (20 pmol), angiotensin (100 pmol), angiotensin II (2 pmol), and bradykinin (200 pmol), each vasoactive agent was then retested after the administration of **1** (0.1 μg/kg/min iv) and compared to preantagonist responses. In this study the response to bradykinin was antagonized, whereas those to the other vasoactive agents were unaffected compared to controls.

D. Duration of Action of Intravenously Infused Compound 1. Compound **1** (0.1 μg/kg/min) was intravenously infused for the above-mentioned selectivity experiments. After the selectivity experiment was complete, the infusion of **1** was stopped and the response to bradykinin (0.2 and 0.4 nmol) was tested at 5 min intervals for the first 30 min and at 15 min intervals for up to 1 h. At 1 h after the infusion had been stopped, there was still 100% inhibition of the response to bradykinin.

2. Rat Blood Pressure. Male Sprague–Dawley rats were anesthetized with pentobarbital, and the femoral arteries were cannulated for the recording of blood pressure. Both femoral veins in each animal were cannulated for the administration of test compounds. The femoral arterial catheters were connected to a Gould pressure transducer, and blood pressure was recorded and displayed on a Grass polygraph recorder. Following an equilibration period, the specific experimental procedure was started.

A. Selectivity of Subcutaneous Administered Compound 1. Following equilibration of blood pressure, responses were produced to acetylcholine (20 nmol), norepinephrine (1 nmol), substance P (2 pmol), angiotensin I (20 pmol), angiotensin II (2 pmol), and bradykinin (20 pmol i.a.). Animals were then treated with **1** (1.0 mg/kg sc). Thirty minutes after the sc injection, each vasoactive agent was retested and compared to preantagonist responses. In this study the response to bradykinin was antagonized, whereas those to the other vasoactive agents were unaffected compared to controls.

B. Duration of Action of iv Bolus Injections of Compound 1. Following equilibration of blood pressure, responses

to bradykinin (10 and 20 pmol i.a.) were produced as control responses. Compound **1** was then administered as an iv bolus (either 3, 10, or 30 μg/kg) and the response to bradykinin retested at 5 min intervals until the response returned to control levels. At 3 μg/kg, the response was ca. 50% inhibited and had returned to control by 30 min. At 10 and 30 μg/kg, the response was 100% inhibited with a 50% return to control level by 60 min and full recovery of response by 90 min.

C. Duration of Action of Subcutaneous Compound 1. After equilibration of mean arterial blood pressure, animals were challenged with bradykinin (10 and 20 pmol i.a.) to establish control responses. Animals were then given subcutaneous injections of **1** (1 and 3 mg/kg sc). Thirty minutes after injection of **1** and at 30 min intervals thereafter, animals were retested with bradykinin until responses had returned to control levels. At 1 mg/kg sc, the responses were still 100% inhibited at 3 h postinjection. At 3 mg/kg sc, the responses were still 100% inhibited at 5 h postinjection.

Binding to Guinea Pig Ileum. Guinea pig ileum membranes were obtained commercially or prepared from ilea collected from CO_2-asphyxiated, Duncan–Hartley strain guinea pigs. The ilea (35 g) were flushed with ice-cold saline and added to 350 mL of homogenation buffer containing 25 mM TES, pH 6.8, 1 mM 1,10-phenanthroline, 5 μg/mL soybean trypsin inhibitor, 100 μg/mL bacitracin, 1 mM benzamidine, and 100 μM phenylmethanesulfonyl fluoride. The membranes were then homogenized in a Waring blender. The membranes were isolated by centrifugation at 4 °C as follows: 1000g for 10 min, then supernatant centrifuged at 43000g for 15 min. The resulting pellet was resuspended in homogenation buffer and centrifuged at 43000g. The resulting pellet was resuspended in 25 mM TES, pH 6.8, and centrifuged at 43000g for 15 min. The resulting pellet was suspended in 175 mL of 25 mM TES, pH 6.8, dispensed into 4 mL aliquots, quick frozen, and stored at −70 °C. Assays were carried out at room temperature in 25 mM TES, pH 6.8, containing 1 mM 1,10-phenanthroline, 1 mM DTT, 1 μM captopril, 140 μg/mL bacitracin, 100 μM thiorphan, 0.1% bovine serum albumin (BSA), 0.3 nM [³H]bradykinin, 0.2 mg/mL prepared membranes, and appropriate concentrations of test compounds. The samples were rapidly filtered through Whatman GF/B filters (pretreated with 0.1% aqueous poly(ethylenimine) and air-dried). The filters were washed (8 × 1 mL) with an ice-cold solution of 10 mM Tris, pH 7.5, containing 100 mM NaCl and 0.02% BSA. The radioactivity retained by the filters was determined with a Wallac 1450 microbeta scintillation counter.

Antagonist Binding to Human Receptor. Human bradykinin B₂ receptor was expressed in CHO-K1 (ATCC) cells. Preparation of membranes for binding assay was carried out by scraping cells from roller bottles in ice-cold PBS and centrifuging at 1000g, at 4 °C for 15 min. The supernatant was discarded and the pellet resuspended in buffer A consisting of 25 mM TES (pH 6.8) with 2 μM 1,10-phenanthroline and centrifuged at 27000g for 15 min. The pellet was washed once using the same buffer and centrifugation parameters. The final pellet was resuspended in buffer B (buffer A with 2 μM captopril, 140 μg/mL bacitracin, 0.1% BSA), stored in 1 mL aliquots, and frozen at −70 °C until needed.

Binding assays were performed by incubating human clone membrane solution with [³H]bradykinin (final concentration 0.3 nM) with or without test compounds in assay buffer (buffer B with 1 mM dithiothreitol) at room temperature for 45 min at a final volume of 315 μL. All test compound dilutions were done in triplicate. Assays were harvested by quick filtration in a Tomtec harvester 96, with ice-cold wash buffer consisting of 10 mM Tris/HCl, pH 7.5, 100 mM NaCl, and 0.2% BSA, onto Wallac printed glass fiber filtermat "B", which had been presoaked with 0.1% PEI and previously air-dried. Filtermats were counted in 9.5 mL of Wallac Beta-Plate Scint, in a Wallac 1450 microbeta counter.

For both guinea pig ileum membranes and human cloned receptors, K_d values were obtained from saturation experiments. Briefly, assays were run under the same conditions as for competition studies except that [³H]bradykinin was used at a concentration range of 0.02–15 nM in the presence of buffer or 1 μM bradykinin (nonspecific binding). The results

were analyzed to calculate K_d using nonlinear regression. (Human S34f clone $K_d = 0.723 \pm 0.23$ nM, GPI $K_d = 0.23 \pm 0.09$ nM, $n = 3$.) Representative binding curves are illustrated in Figures 1 and 2.

References

(1) The original residue numbering of the parent bradykinin structure (Arg¹- Pro²-Pro³-Gly⁴-Phe⁵-Ser⁶-Pro⁷-Phe⁸-Arg⁹) is retained.
(2) Abbreviations of nonstandard amino acids or mimetics: Cpg = cyclopentylglycine, δ-Gpa = 5-guanidinopentanoic acid, Igl = 2-indanylglycine, NBng = N-benzylglycine, NChg = N-cyclohexylglycine, NCpg = N-cyclopentylglycine, NMch N-(methylcyclohexyl)glycine, NPeg = N-phenethylglycine, NPhg = N-phenylglycine. Abbreviations for standard bradykinin antagonists: NPC-567= [D-Arg⁰,Hyp³,D-Phe⁷]-bradykinin, NPC17761 = [D-Arg⁰,Hyp³,D-(4S)-(thiophenyl)-Pro⁷,Oic⁸]-bradykinin, NPC17731 = [D-Arg⁰,Hyp³,D-(4S)-(oxypropyl)-Pro⁷,Oic⁸]-bradykinin, HOE-140 = [D-Arg⁰,Hyp³,Thi⁵,D-Tic⁷,Oic⁸]-bradykinin. CP-0127 is the bis(succinimidohexane) cross-linked homodimer formed from [D-Arg⁰,Hyp³,Cys⁶,D-Phe⁷,Leu⁸]-bradykinin.
(3) Farmer, S. G.; Burch, R. M. The Pharmacology of Bradykinin Receptors. In *Bradykinin Antagonists*; Burch, R. M., Ed.; Marcel Dekker, Inc.: New York, 1991; pp 1–31.
(4) Griesbacher, T.; Lembeck, F. Effect of Bradykinin Antagonists on Bradykinin Induced Plasma Extravasation, Venoconstriction, Prostaglandin E₂ Release, and Nociceptor Stimulation and Contraction of the Iris Sphincter Muscle in the Rabbit. *Br. J. Pharmacol.* **1987**, *92*, 333–340.
(5) Taiwo, Y. O.; Levine, J. D. Characterization of the Arachadonic Acid Metabolites Mediating Bradykinin and Noradrenaline Hyperalgesia. *Brain Res.* **1988**, *458*, 402–406.
(6) Steranka, L. R.; Manning, D. C.; De Haas, C. J.; Ferkany, J. W.; Borosky, S. A.; Connor, J. R.; Vavrek, R. J.; Stewart, J. M.; Snyder, S. H. Bradykinin as a Pain Mediator: Receptors Are Localized to Sensory Neurons, and Antagonists Have Analgesic Actions. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, 85, 3245–3249.
(7) Dray, A.; Bettaney, J.; Forster, P.; Perkins, M. N. Bradykinin-Induced Stimulation of Afferent Fibres is Mediated Through Protein Kinase C. *Neurosci. Lett.* **1988**, *91*, 301–307.
(8) Sterankan, L. R.; Farmer, S. G.; Burch, R. M. Antagonists of B₂ Bradykinin Receptors. *FASEB J.* **1989**, 2019–2025.
(9) Haley, J.; Dickenson, A. H.; Schacter, M. Electrophysiological Evidence for a Role of Bradykinin in Chemical Nociception in the Rat. *Neurosci. Lett.* **1989**, *97*, 198–202.
(10) Marceau, F.; Lussier, A.; Regoli, D.; Giroud, J. P. Pharmacology of Kinins: Their Relevance to Tissue Injury and Inflammation. *Gen. Pharmacol.* **1983**, *14*, 209–229.
(11) Proud, D.; Kaplan, A. P. Kinin Formation: Mechanism and Role in Inflammatory Disorders. *Annu. Rev. Immunol.* **1988**, *6*, 49–84.
(12) Colman, R. W.; Wong, P. Y. Kallikrein-Kinin System in Pathologic Conditions. In *Bradykinin, Kallidin and Kallikrein, Handbook of Experimental Pharmacology*; Erdos, E. G., Ed.; Springer Verlag: New York, 1979; Vol. 25, pp 569–607.
(13) Greaves, M. W. Inflammation and Mediators. *Br. J. Dermatol.* **1988**, *119*, 419–426.
(14) Martinez-Brotons, F.; Oncins, J. R.; Mestres, J.; Amargos, V.; Reynaldo, C. Plasma kallikrein-kinin System in Patients with Uncomplicated Sepsis and Septic Shock - Comparison with Cardiogenic Shock. *Thrombosis Haemostasis* **1978**, *58*, 709–719.
(15) Whalley, E. T.; Solomon, J. A.; Modafferi, D. M.; Bonham, K. A.; Cheronis, J. C. CP-0127, A Novel Potent Bradykinin Antagonist, Increases Survival In Rat and Rabbit Models of Endotoxin Shock. *Agents Actions* **1992**, *38*, 413–420.
(16) Farmer, S. G. Airway Pharmacology of Bradykinin. In *Bradykinin Antagonists*; Burch, R. M., Ed.; Marcel Dekker, Inc.: New York, 1991; pp 213–236. Abraham, W. M. Bradykinin Antagonists in a Sheep model of allergic Asthma. In *Bradykinin Antagonists*; Burch, R. M., Ed.; Marcel Dekker, Inc.: New York, 1991; pp 261–276.
(17) Stewart, J. M.; Vavrek, R. J. Chemistry of Bradykinin B2 Antagonists. In *Bradykinin Antagonists*; Burch, R. M., Ed.; Marcel Dekker, Inc.: New York, 1991; pp 51–96.
(18) Cheronis, J. C.; Whalley, E. T.; Nguyen, K. T.; Eubanks, S. R.; Allen, L. G.; Duggan, M. J.; Loy, S. D.; Bonham, K. A.; Blodgett, J. K. A New Class of Bradykinin Antagonists: Synthesis and In Vitro Activity of Bissuccinimidoalkane Peptide Dimers. *J. Med. Chem.* **1992**, *35*, 1563–1572.
(19) Kyle, D. J.; Burch, R. M. A Survey of Bradykinin Receptors and Their Antagonists. *Curr. Opin. Invest. Drugs* **1993**, *2*, 5–20.

(20) Hock, F. J.; Wirth, K.; Albus, U.; Linz, W.; Gerhards, H. J.; Wiemer, G.; Henke, S.; Breipohl, G.; Knoig, W.; Knolle, J.; Scholkens, B. A. HOE 140 A New Potent and Long Acting Bradykinin Antagonist: In Vitro Studies. *Br. J. Pharmacol.* **1991**, *102*, 769−773. Hock, F. J.; Wirth, K.; Albus, L. W.; Alperman, H. G.; Anagnostopoulous, H.; Henke, S.; Breipohl, G.; Knoig, W.; Knolle, J.; Scholkens, B. A. U. HOE 140 A New Potent and Long Acting Bradykinin Antagonist: In Vivo Studies. *Br. J. Pharmacol.* **1991**, *102*, 774−777.

(21) Dray, A.; Bevan, S. Inflammation and Hyperalgesia: Highlighting the Team Effort. *TIPS* **1993**, *14*, 287.

(22) Mobilio, D.; Musser, J. H. N-Substituted Aminoquinolines As Analgesic Agents. U.S. Patent 5,216,165, June 1, 1993. Musser, J. H.; Molinari, A. J.; Mobilio, D. Substituted Quinolinyl- and Naphthalenylbenzamides or Benzylamines and Related Compounds Useful as Analgesics. U.S. Patent 5,212,182, May 18, 1993. Witherup, K. M.; Ransom, R. W.; Varga, S. L.; Pitzenberger, S. M.; Lotti, V. J.; Lumma, W. C. Pyrroloquinoline Bradykinin Antagonists. International Patent Application WO 94/09001, Apr. 28, 1994.

(23) Salvino, J. M.; Seoane, P. R.; Douty, B. D.; Awad, M. M. A.; Dolle, R. E.; Houck, W. T.; Faunce, D. M.; Sawutz, D. G. Design of Potent Non-Peptide Competitive Antagonists of the Human Bradykinin B$_2$ Receptor. *J. Med. Chem.* **1993**, *36*, 2583−2584.

(24) Stewart, J. M.; Gera, L.; Whalley, E. T.; Hanson, W.; McCulloogh, R. Remarkable New Bradykinin Antagonists Having High Potency at Both the B$_1$ and B$_2$ receptors. In *Proceedings of the Fourteenth American Peptide Symposium*; in press.

(25) Guba, G.; Haessner, R.; Breipohl, G.; Henke, S.; Knolle, J.; Santagada, V.; Kessler, H. Combined Approach of NMR and Molecular Dynamics within a Biphasic Membrane Mimetic: Conformation and Orientation of the Bradykinin Antagonist HOE-140. *J. Am. Chem. Soc.* **1994**, *116*, 7532−7540.

(26) Kyle, D. J.; Martin, J. A.; Farmer, S. G.; Burch, R. M. Design and Conformational Analysis of Several Highly Potent Bradykinin Receptor Antagonists. *J. Med. Chem.* **1991**, *34*, 1230−1233.

(27) Kyle, D. J.; Green, L. M.; Blake, P. R.; Smithwick, D.; Summers, M. F. A Novel β-Turn Mimetic Useful for Mapping the Unknown Topology of Peptide Receptors. *Pept. Res.* **1992**, *5*, 206−209.

(28) Kyle, D. J.; Blake, P. R.; Smithwick, D.; Green, L. M.; Martin, J. A.; Sinsko, J. A.; Summers, M. F. NMR and Computational Evidence That High Affinity Bradykinin Receptor Antagonists Adopt C-Terminal β-Turns. *J. Med. Chem.* **1993**, *36*, 1450−1460.

(29) Sawutz, D. G.; Salvino, J. M.; Seoane, P. R.; Douty, B. D.; Houck, W. T.; Bobko, M. A.; Doleman, M. S.; Dolle, R. E.; Wolfe, H. R. Synthesis, Characterization, and Conformational Analysis of the D/L Tic^7 Stereoisomers of Bradykinin Receptor Antagonist D-Arg^0 [Hyp^3, Thi^5, D-Tic^7, Oic^8]- Bradykinin. *Biochemistry* **1994**, *33*, 2373−2379.

(30) Marceu, F.; Regoli, D. C. Kinin Receptors of the B$_1$ Type and Their Antagonists. In *Bradykinin Antagonists*; Burch, R. M., Ed.; Marcel Dekker, Inc.: New York, 1991; pp 33−50.

(31) Stewart, J. M.; Gera, L.; Srivastava, V. P. Bradykinin Antagonists Without Aromatic Amino Acids. In *Peptides 1992*; Scheider, C. H., Eberle, A. N., Eds.; Escom: Leiden, 1992; pp 691−692.

(32) Gera, L.; Srivastava, V. P.; Dunn, F. W.; Stewart, J. M. Bradykinin Antagonists Containing Unusual Amino Acids Show Long Lasting Action In Vivo. In *Peptides Structure, Function, Biology: Procedings of the 13th American Peptide Symposium*; Hodges, R. S., Ed.; Escom: Leiden, 1994; pp 449−451.

(33) Mitchel, A. R.; Young, J. D.; Ghofrani, F.; Stewart, J. M. Synthesis of Bradykinin Analogs Containing N-Benzyglycine. In *Peptides Structure, Function, Biology: Procedings of the 13th American Peptide Symposium*; Hodges, R. S., Ed.; Escom: Leiden, 1994; pp 427−429.

(34) Ghofrani, F.; Mitchel, A. R.; Young, J. D. Use of N-Benzylglycine For the Preparation of Peptide-Peptoid Analogs of Biologically Active Peptides, P323. Thirteenth American Peptide Symposium, Edmonton, Alberta, Canada, June 20, 1993.

(35) Young, D.; Mitchel, A. R. Use of N-benzylglycine as a Replacement for Aromatic Amino Acid Residues. In *Peptides: Chemistry, Structure and Biology, Proc. 11th American Peptide Symposium*; Rivier, J. E., Marshal, G. R., Eds.; Escom: Leiden, 1990; pp 155−156.

(36) Tung, R. D.; Rich, D. H.; Dhaon, M. K. BOP-Cl Mediated Synthesis of the Clyclosporine A 8−11 Tetrapeptide Fragment. *J. Am. Chem. Soc.* **1985**, *107*, 4342.

(37) Beyermann, M.; Bienert, M.; Niedrich, H.; Carpino, L. A.; Sadat-Aalaee, D. Rapid Continuous Peptide Synthesis via FMOC Amino Acid Chloride Coupling and 4-(Aminomethyl)piperidine Deblocking. *J. Org. Chem.* **1990**, *55*, 721−728.

(38) Bernatowicz, M. S.; Matsueda, G.; Wu, Y. 1H-Pyrazole-1-carboxamidine Hydrochloride: An Attractive Reagent for Guanylation of Amines and Its Application to Peptide Synthesis. *J. Org. Chem.* **1992**, *57*, 2497−2502.

(39) Burch, R. M.; Kyle, D. J.; Stormann, T. M. *Molecular Biology And Pharmacology Of Bradykinin Receptors*; R. G. Landes Co.: Austin, TX, 1993; pp 13−15.

(40) Fauchere, J. L.; Thurieau, C. Evaluation of the Stability of Peptides and Pseudopeptides as a Tool in Peptide Drug Design. *Adv. Drug Res.* **1992**, *23*, 128−156.

(41) Field, J. L.; Hall, J. M.; Morton, I. K. M. Bradykinin Receptors in the Guinea-Pig Taenia Caeci Are Similar to Proposed BK$_3$ Receptors in the Guinea-Pig Trachea, and Are Blocked by HOE 140. *Br. J. Pharmacol.* **1992**, *105*, 293−296.

(42) ABI 431 Fmoc Procedure. *Applied Biosystems Model 431A Peptide Synthesizer User's Manual*, Version 2.0; January 1992.

(43) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid Analysis of Amino Acids Using Pre-Column Derivatization. *J. Chromatogr.* **1984**, *336*, 93−104.

(44) Hill, J. T.; Dunn, F. W. Preparation and Resolution of Cyclopentaneglycine. *J. Org. Chem.* **1965**, *30*, 1321−1331.

(45) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984.

(46) Reference 45, pp 105−107.

(47) Arunlakshana, O.; Schild, H. O. Some Quantitative Uses of Drug Antagonists. *Br. J. Pharmacol.* **1959**, *14*, 48−58.

(48) Skidgel, R. A.; Erdos, E. G.; Johnson, A. R. Hydrolysis of Opioid Hexapeptides by Carboxypeptidase N. *Biochem. Pharmacol.* **1984**, *33*, 3471−3478.

(49) Booth, A. G.; Kenny, A. J. A Rapid Method for the Preparation of Microvilli from Rabbit Kidney. *Biochem. J.* **1974**, *142*, 575−581.

(50) Goodfellow, V. S.; Laudeman, C. P.; Gerrity, J. I.; Burkard, M.; Strobel, E.; McLeod, D. A. Rationally Designed Diverse Non-Peptides: Variously Substituted Piperazine and Piperazinedione Libraries Targeted for the Discovery of Bradykinin and Other G-Protein Coupled Receptor Antagonists. *Molecular Diversity* **1996**, submitted.

(51) Note Added in Proof. High-resolution FAB-MS data for compound **1** were obtained by M-Scan (West Chester, PA) using 3-nitrobenzyl alcohol matrix with PEG1000 as internal standard. The sample signal was measured as 1292.6732, which agrees with the calculated protonated molecular ion of 1292.6686 at 3.5 ppm deviation.

JM950716I