Articles

Design, Synthesis, and in Vitro Activity of Bis(succinimido)hexane Peptide Heterodimers with Combined B_1 and B_2 Antagonist Activity[1,2]

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We have developed a series of peptide heterodimers based on the B_2 antagonist D-Arg[0]-[Hyp[3],D-Phe[7],Leu[8]]-BK (**1**) and the B_1 antagonist Lys[0]-[Leu[8],des-Arg[9]]-BK (**7**) that are potent antagonists of both B_1 and B_2 receptors. From this series, compound **50** (alternatively, CP-0364), the 1,6-bis(succinimido)hexane heterodimer of D-Arg[0]-[Hyp[3],Cys[6],D-Phe[7],Leu[8]]-BK (**2**), and D-Arg[0]-[Cys[1],-Hyp[3],Leu[8],des-Arg[9]]-BK (**6**), was found to be the most active both in vitro and in vivo. Compound **50** has a pA_2 of 8.3 when measured against bradykinin (BK)-induced rat uterine smooth muscle contraction and an IC_{50} of approximately 10^{-8} M against [des-Arg[9]]-BK-induced rabbit aorta smooth muscle contraction in vitro. Compounds such as **50** may be useful in the treatment of both subacute and chronic inflammatory disorders wherein both B_2 and B_1 receptors appear to contribute to the clinical manifestations of the disease.

Introduction

There is increasing evidence in the literature that the response characteristics of a variety of biological systems to BK and related kinins change as a function of the underlying physiologic or pathophysiologic state. More specifically, while B_2 receptor responsiveness appears to be relatively constant in both normal and pathophysiologic conditions, there is a dynamic up-regulation of B_1 responsiveness when there is a more prolonged or chronic inflammatory condition present. For example, B_1 activity has been shown to be present in animal models of endotoxemia and sepsis, asthma, hyperalgesia, and arthritis, but absent in the same animals under normal conditions.[3-5] Furthermore, while there has been significant progress in the development of potent B_2 antagonists (CP-0127,[6] HOE-140,[7] and NPC-17731[8]), it is quite clear that none of these compounds have significant effects on B_1 activity. It would seem reasonable, therefore, that therapies that can be directed simultaneously to both of the major BK receptor populations may be superior to agents directed to a single receptor in a number of important clinical conditions.

Previously, we have reported on a series of B_2 antagonist dimers[6] which had significantly improved activities both in vitro and in vivo relative to their corresponding monomeric peptide precursors. While investigating the structure–activity relationships of these compounds, it became apparent that a significant component of the activities of these compounds at the B_2 receptor could be obtained by a portion of the dimer, and that additional activities could be incorporated into a single molecule by employing a heterodimeric structure. Described herein is a series of heterodimers based on the B_2 antagonist D-Arg[0]-[Hyp[3],D-Phe[7],Leu[8]]-BK (**1**)[9] and the B_1 antagonist Lys[0]-[Leu[8],des-Arg[9]]-BK (**7**);[10] heterodimers developed in order to explore the potential of incorporating combined B_1 and B_2 receptor antagonist activities in a single compound.

Initial studies using an alanine substituted "dummy" peptide, which was devoid of intrinsic B_1 or B_2 agonist or antagonist activity, indicated that a substantial proportion of the increased activities of the previously described homodimers[6] is a function of the linker moiety. Further, these studies allowed us to investigate the development of heterodimers in which one side of the dimer (B_2) was held constant while the geminal ligand (B_1) was varied so as to achieve optimal antagonist activity at both receptors.

We were able to demonstrate that the SAR for linker modification for B_2 antagonists was different than that for B_1 antagonists. These differences suggest that single-chain peptide-based antagonists capable of interacting with these two receptor populations may be difficult to develop. Despite these differences, however, compounds with potent *combined* B_1/B_2 antagonist activities were prepared. Compounds of this type may be useful in elucidating the roles these two interrelated receptor populations play in a variety of pathophysiologic processes.

Chemistry

Syntheses: Peptide Monomers. Syntheses of peptide monomers, listed in Table 1, were accomplished via standard solid-phase methodology[11] on a Pam resin, using protocols shown in Table 2. N^{α}-tert-Butyloxycarbonyl protection was employed for all peptide syntheses, and finished peptides were cleaved from the resin using standard HF procedures.[12] Anisole alone was found to be an efficient carbocation scavenger during HF cleavage; as a result, all peptides were cleaved from the resin without any additional scavengers. Free peptides were extracted with water and then purified by preparative reversed-phase HPLC. Occasionally, dithiothreitol treatment was required to reduce peptide disulfide dimers prior to HPLC purification. Peptide characterization data are given in Table 1. Overall isolated yields calculated from the starting Boc-amino acid-Pam resin were typically 50–60%.

Syntheses: *S*-(*N*-Hexylsuccinimido)-Modified Peptide Monomers. Syntheses of *S*-(*N*-hexylsuccinimido)-

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Table 1. Characterization of Cysteine-Containing Peptide Monomers[a]

no.	structure[b]	amino acid analysis	peptide sequence analysis[c]
1[d]	*d*	Arg (2.88), Pro (1.04), Hyp (0.94), Gly (1.02), Phe (2.09), Ser (1.03), Leu (0.99)	1, Arg; 2, Arg; 3, Pro; 4, Hyp; 5, Gly; 6, Phe; 7, Ser; 8, Phe; 9, Leu; 10, Arg
2	[L-Cys[6]]-1	Arg (3.10), Pro (0.97), Hyp (0.92), Gly (0.99), Phe (2.04), Cys[e], Leu (0.97)	1, Arg; 2, Arg; 3, Pro; 4, Hyp; 5, Gly; 6, Phe; 7, Cys; 8, Phe; 9, Leu; 10, Arg
3	[L-Ala[5,8],D-Ala[7],-L-Cys[6]]-1	Arg (2.80), Pro (1.01), Hyp (0.94), Gly (1.08), Ala (3.17), Cys[e]	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Ala; 7, Cys(Meb); 8, Ala; 9, Ala; 10, Arg(Tos)
4	[L-Cys[6],des-L-Arg[9]]-1	Arg (2.18), Pro (0.99), Hyp (0.95), Gly (0.99), Phe (1.95), Cys[e], Leu (0.94)	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Cys(Meb); 8, Phe; 9, Leu
5	[des-L-Arg[9]]-1	Arg (2.18), Pro (1.01), Hyp (0.92), Gly (0.98), Phe (1.96), Ser (1.01), Leu (0.94)	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu
6	[L-Cys[1],L-Pro[7],-des-L-Arg[9]]-1	Arg (0.98), Cys[e], Pro (1.99), Hyp (0.95), Gly (1.12), Phe (1.05), Ser (1.00), Leu (0.92)	1, Arg; 2, Cys; 3, Pro; 4, Hyp; 5, Gly; 6, Phe; 7, Ser; 8, Pro; 9, Leu
7[f]	*f*	Lys (0.99), Arg (1.08), Pro (3.11), Gly (1.01), Phe (1.04), Ser (1.01), Leu (0.99)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Pro; 9, Leu
8	[L-Cys[0]]-7	Cys[e], Arg (1.06), Pro (2.96), Gly (0.97), Phe (0.98), Ser (0.97), Leu (0.95)	1, Cys(Meb); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Pro; 9, Leu
9	[L-Cys[1]]-7	Lys (0.98), Cys[e], Pro (3.03), Gly (0.97), Phe (0.98), Ser (0.98), Leu (0.96)	1, Lys; 2, Cys; 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Ser; 8, Pro; 9, Leu
10	[L-Cys[2]]-7	Lys (0.94), Arg (1.03), Cys[e], Pro (2.00), Gly (1.07), Phe (0.99), Ser (1.00), Leu (0.98)	1, Lys; 2, Arg; 3, cys; 4, Pro; 5, Gly; 6, Phe; 7, Ser; 8, Pro; 9, Leu
11	[L-Cys[3]]-7	Lys (0.97), Arg (1.07), Pro (1.95), Cys[e], Gly (0.98), Phe (0.98), Ser (0.99), Leu (0.96)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Cys(Meb); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Pro; 9, Leu
12	[L-Cys[4]]-7	Lys (0.96), Arg (1.07), Pro (2.94), Cys[e], Phe (0.97), Ser (0.97), Leu (0.95)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Cys(Meb); 6, Phe; 7, Ser(OBzl); 8, Pro; 9, Leu
13	[L-Cys[5]]-7	Lys (0.94), Arg (1.08), Pro (2.87), Gly (0.97), Cys[e], Ser (1.00), Leu (0.94)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Cys(Meb); 7, Ser(OBzl); 8, Pro; 9, Leu
14	[L-Cys[6]]-7	Lys (0.98), Arg (1.08), Pro (3.00), Gly (0.98), Phe (0.99), Cys[e], Leu (0.96)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Cys(Meb); 8, Pro; 9, Leu
15	[L-Cys[7]]-7	Lys (0.96), Arg (1.07), Pro (1.97), Gly (0.96), Phe (0.98), Ser (0.53)[g], Cys[e], Leu (0.94)	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Cys(Meb); 9, Leu
16	[L-Cys[8]]-7	Lys (0.98), Arg (1.07), Pro (2.95), Gly (0.97), Phe (0.98), Ser (0.97), Cys[e]	1, Lys(Cl-Z); 2, Arg(Tos); 3, Pro; 4, Pro; 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Pro; 9, Cys(Meb)

[a] All peptides ≥95% pure by analytical reversed-phase HPLC. [b] Peptides based on the B_2 bradykinin antagonist D-Arg[0]-[Hyp[3],D-Phe[7],Leu[8]]-bradykinin or the B_1 bradykinin antagonist Lys[0]-[Leu[8],des-Arg[9]]-bradykinin. [c] Peptide-Pam resin sequence data or purified peptide sequence data. [d] Included as the B_2 reference ligand; D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Phe[5]-Ser[6]-D-Phe[7]-Leu[8]-Arg[9]. [e] Cys not determined quantitatively. [f] Lys[0]-Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Leu[8]; the B_1 reference ligand. [g] Satisfactory Ser recovery could not be obtained from this peptide; laser desorption mass spectral analysis yielded correct molecular weight (1004 g/mol).

Table 2. Solid-Phase Synthesis: Schedule of Steps for 0.5-mmol Run Involving Preformed Symmetric Anhydrides[a]

step	reagent/solvent	volume (mL)	mix time (min)
1	CH_2Cl_2	4 × 30	2
2	50% TFA in CH_2Cl_2	1 × 30	5
3	50% TFA in CH_2Cl_2	1 × 30	20
4	CH_2Cl_2	4 × 30	2
5	DMF	3 × 30	2
6	10% DIPEA in DMF	2 × 30	2
7	DMF	4 × 30	2
8	Preformed symmetric anhydride[b] (from 4 equiv of Boc-amino acid and 2 equiv of DCC)	1 × 8	45
9	DMF	3 × 30	2
10	CH_2Cl_2	4 × 30	1

[a] Boc-D-Arg(Tos)-OH and Boc-Arg(Tos)-OH activated (DCC/HOBt) and coupled in DMF; Boc-Pro-OH activated/coupled in same fashion for Pro-Pro couplings. [b] Formed initially in CH_2Cl_2; DCU filtration followed by solvent exchange with DMF.

modified peptide monomers, listed in Table 3, were carried out in DMF/ammonium bicarbonate (0.1 M, pH 7.5) via a reaction[13] between a cysteine-containing peptide monomer and *N*-hexylmaleimide. Characterization data are given in Table 3. Overall isolated yields calculated from the starting peptide were 70–90%.

Syntheses: Bis(succinimido)hexane Peptide Homodimers. Syntheses of bis(succinimido)hexane peptide homodimers, listed in Table 4, were accomplished most efficiently when 2 equiv of cysteine-containing peptide monomer and 1 equiv of bis(maleimido)hexane (both dissolved in DMF) were allowed to react in ammonium bicarbonate (0.1 M) near pH 7.5. Such a reaction is but one example of the well-known tendency of thiol (SH) nucleophiles to react with α,β-unsaturated carbonyl systems in a 1,4-addition or conjugate addition.[13] Dimerization reactions were routinely allowed to proceed for 30 min at room temperature, and the resulting bis(succinimido)hexane peptide homodimers were then purified by preparative reversed-phase HPLC. Overall isolated yields calculated from the starting cysteine-containing peptide monomer were typically 60–80%.

Syntheses: Bis(succinimido)hexane Peptide Heterodimers. Syntheses of bis(succinimido)hexane peptide heterodimers, listed in Table 4, were carried out via initial reaction[13] of 1 equiv of cysteine-containing peptide monomer with 1.5 equiv of bis(maleimido)hexane (both dissolved in DMF) in ammonium bicarbonate (0.1 M) near pH 7.5. Following a 30-min reaction, the peptide–bis(maleimido)-hexane monoadduct was purified by preparative reversed-phase HPLC, lyophilized, and then condensed with 1.5 equiv of a different cysteine-containing peptide monomer in ammonium bicarbonate (0.1 M) near pH 7.5. This reaction[13] was allowed to proceed for 30 min at room temperature, after which time the resulting bis(succinimido)hexane peptide heterodimer was purified by pre-

Table 3. Characterization of *S*-(*N*-Hexylsuccinimido)-Modified Peptide Monomers[a]

no.	structure[b]	amino acid analysis	formula	calcd FW	*m/e* value[c] (M + H)
17	[L-Cys^0(HS)]-1	Succ-Cys[d], Arg (2.05), Pro (0.97), Hyp (0.90), Gly (1.01), Phe (2.05), Ser (1.04), Leu (0.97)	$C_{64}H_{97}N_{17}O_{15}S$	1376.	1375.
18	[L-Cys^1(HS)]-1	Arg (2.03), Succ-Cys[d], Pro (0.98), Hyp (0.91), Gly (1.01), Phe (2.05), Ser (1.04), Leu (0.97)	$C_{64}H_{97}N_{17}O_{15}S$	1376.	1376.
19	[L-Cys^2(HS)]-1	Arg (2.86), Succ-Cys[d], Hyp (0.92), Gly (1.05), Phe (2.09), Ser (1.09), Leu (0.99)	$C_{65}H_{102}N_{20}O_{15}S$	1436.	1435.
20	[L-Cys^3(HS)]-1	Arg (3.02), Pro (0.99), Succ-Cys[d], Gly (1.00), Phe (2.02), Ser (1.03), Leu (0.94)	$C_{65}H_{102}N_{20}O_{14}S$	1420.	1419.
21	[L-Cys^4(HS)]-1	Arg (3.10), Pro (1.09), Hyp (1.05), Succ-Cys[d], Phe (1.94), Ser (0.97), Leu (0.85)	$C_{68}H_{106}N_{20}O_{15}S$	1476.	1477.
22	[L-Cys^5(HS)]-1	Arg (3.01), Pro (0.98), Hyp (0.93), Gly (1.03), Succ-Cys[d], Ser (1.06), Phe (1.01), Leu (0.97)	$C_{61}H_{100}N_{20}O_{15}S$	1386.	1385.
23	[L-Cys^6(HS)]-1	Arg (3.31), Pro (0.96), Hyp (0.95), Gly (0.92), Phe (1.87), Succ-Cys[d], Leu (0.91)	$C_{67}H_{104}N_{20}O_{14}S$	1446.	1446.
24	[L-Cys^7(HS)]-1	Arg (2.93), Pro (0.97), Hyp (0.94), Gly (1.05), Phe (1.04), Ser (1.07), Succ-Cys[d], Leu (0.99)	$C_{61}H_{100}N_{20}O_{15}S$	1386.	1385.
25	[L-Cys^8(HS)]-1	Arg (2.93), Pro (1.00), Hyp (0.93), Gly (1.04), Phe (2.05), Ser (1.04), Succ-Cys[d]	$C_{64}H_{98}N_{20}O_{15}S$	1419.	1418.
26	[L-Cys^9(HS)]-1	Arg (1.98), Pro (0.97), Hyp (0.96), Gly (1.01), Phe (2.06), Ser (1.06), Leu (0.96), Succ-Cys[d]	$C_{64}H_{97}N_{17}O_{15}S$	1376.	1376.
27	[L-Cys^0(HS)]-7	Succ-Cys[d], Arg (1.03), Pro (3.01), Gly (1.07), Phe (1.01), Ser (0.97), Leu (0.91)	$C_{54}H_{83}N_{13}O_{13}S$	1154.	1155.
28	[L-Cys^1(HS)]-7	Lys (1.03), Succ-Cys[d], Pro (2.92), Gly (1.09), Phe (0.97), Ser (1.05), Leu (0.93)	$C_{54}H_{83}N_{11}O_{13}S$	1126.	1127.
29	[L-Cys^2(HS)]-7	Lys (0.92), Arg (1.04), Succ-Cys[d], Pro (2.02), Gly (1.06), Phe (1.00), Ser (1.02), Leu (0.93)	$C_{55}H_{88}N_{14}O_{13}S$	1185.	1186.
30	[L-Cys^3(HS)]-7	Lys (1.07), Arg (1.04), Pro (2.01), Succ-Cys[d], Gly (1.03), Phe (1.02), Ser (0.94), Leu (0.91)	$C_{55}H_{88}N_{14}O_{13}S$	1185.	1186.
31	[L-Cys^4(HS)]-7	Lys (0.95), Arg (1.07), Pro (3.05), Succ-Cys[d], Phe (1.02), Ser (0.97), Leu (0.94)	$C_{58}H_{92}N_{14}O_{13}S$	1225.	1226.
32	[L-Cys^5(HS)]-7	Lys (0.98), Arg (1.04), Pro (3.02), Gly (1.03), Succ-Cys[d], Ser (1.00), Leu (0.94)	$C_{51}H_{86}N_{14}O_{13}S$	1135.	1135.
33	[L-Cys^6(HS)]-7	Lys (0.98), Arg (1.05), Pro (2.96), Gly (1.01), Phe (1.03), Succ-Cys[d], Leu (0.97)	$C_{57}H_{90}N_{14}O_{12}S$	1195.	1195.
34	[L-Cys^7(HS)]-7	Lys (0.97), Arg (1.03), Pro (1.99), Gly (1.05), Phe (1.02), Ser (0.99), Succ-Cys[d], Leu (0.94)	$C_{55}H_{88}N_{14}O_{13}S$	1185.	1186.
35	[L-Cys^8(HS)]-7	Lys (1.06), Arg (1.03), Pro (2.96), Gly (1.02), Phe (1.00), Ser (0.92), Succ-Cys[d]	$C_{54}H_{84}N_{14}O_{13}S$	1169.	1170.

[a] All peptides ≥95% pure by analytical reversed-phase HPLC. [b] See Table 1 for sequence; HS = *S*-(*N*-hexylsuccinimido). [c] Electrospray or laser desorption mass spectra obtained. [d] Succ-Cys not determined quantitatively.

parative reversed-phase HPLC. Overall isolated yields calculated from the initially added cysteine-containing peptide monomer ranged from 10–50%.

Results and Discussion

Analysis of the Importance of the Dimer Structure on the in Vitro Activity of Homodimeric Compounds. The activities of the original series of cysteine-containing monomeric homologues of reference B_2 antagonist **1** and their corresponding bis(succinimido)hexane dimers[6] on bradykinin-induced rat uterine smooth muscle contraction are graphically represented in Figure 1. As can be appreciated from these data, there is a significant improvement in the potency (increased pA_2) when dimers are formed at several of the positions, with the most potent being the dimer formed at position 6 (compound **36**; alternatively, CP-0127[6] is currently in human clinical trials for sepsis). Similar data were observed in other assay systems of in vitro B_2 antagonist activity (guinea pig ileum and rabbit jugular vein—data not shown).

In order to assess the contribution of various components of the total structure to the activity of the final dimeric compound, a series of control monomers and dimers was designed and evaluated for B_2 antagonist activity. The data listed for the first three compounds in Table 5 are the original data for the reference monomer, **1**, its Cys^6 homologue, **2**, and the bis(succinimido)hexane dimer, **36**. These data are listed for comparison purposes. Compound **3** is the Ala^5, D-Ala^7, Ala^8, Cys^6 homologue of reference monomer **1**. This compound was synthesized in order to provide an alternative geminal ligand for monomer **2** which possessed the same general physical properties, but was devoid of any intrinsic B_1 or B_2 agonist or antagonist activity.

As can be seen from these data, both monomer **3** and its bis(succinimido)hexane dimer, compound **37**, were without B_2 antagonist activity, but the bis(succinimido)-hexane heterodimer made up of monomer **2** and monomer **3**, namely compound **38**, displayed an inhibitory activity that was superior to reference monomer **1**, but slightly inferior to that of the corresponding homodimer, compound **36**. These data suggest that alternative ligands can be paired with monomer **2** without a significant loss of potency at the B_2 receptor. Furthermore, these data also imply that a substantial component of the activity of the original series of homodimers is attributable to the linking elements of the dimers and not to the dimer structure per se. Such differences in potency between monomers, homodimeric, and heterodimeric compounds have also been noticed in some in vivo systems (unpublished observations). Further experiments are being performed to investigate this phenomenon.

In order to explore the various contributions of the different components of the dimer, the effect of the "linker" (as represented by the *S*-(*N*-hexylsuccinimido) moiety) *as a function of position* was assessed. Illustrated in Figure 2 are the data comparing the pA_2 values of a series of *S*-(*N*-hexylsuccinimido)-modified monomers and their

Table 4. Characterization of Bis(succinimido)hexane Peptide Dimers[a]

no.	structure[b]	dimer type	amino acid analysis	formula	calcd FW	*m/e* value[c] (M + H)
36	2-[BSH]-2	homodimer	Arg (5.98), Pro (1.87), Hyp (1.91), Gly (2.12), Phe (4.07), Succ-Cys[d], Leu (2.04)	$C_{128}H_{194}N_{40}O_{28}S_2$	2805.	2806.
37	3-[BSH]-3	homodimer	Arg (5.53), Pro (2.00), Hyp (1.85), Gly (2.19), Ala (6.44), Succ-Cys[d]	$C_{98}H_{166}N_{40}O_{28}S_2$	2417.	2417.
38	2-[BSH]-3	heterodimer	Arg (6.16), Pro (1.98), Hyp (1.82), Gly (2.07), Ala (3.06), Phe (1.98), Succ-Cys[d], Leu (0.93)	$C_{113}H_{180}N_{40}O_{28}S_2$	2611.	2611.
39	2-[BSH]-4	heterodimer	Arg (5.27), Pro (1.97), Hyp (1.83), Gly (2.06), Phe (4.02), Succ-Cys[d], Leu (1.85)	$C_{122}H_{182}N_{36}O_{27}S_2$	2649.	2649.
40	4-[BSH]-4	homodimer	Arg (4.15), Pro (2.00), Hyp (1.83), Gly (2.15), Phe (4.00), Succ-Cys[d], Leu (1.87)	$C_{116}H_{170}N_{32}O_{28}S_2$	2493.	2494.
41	2-[BSH]-8	heterodimer	Arg (3.95), Pro (4.20), Hyp (0.83), Gly (2.06), Phe (2.94), Ser (1.09), Succ-Cys[d], Leu (1.92)	$C_{115}H_{173}N_{33}O_{27}S_2$	2514.	2516.
42	2-[BSH]-9	heterodimer	Lys (1.11), Arg (3.01), Pro (3.94), Hyp (0.90), Gly (2.02), Phe (3.04), Ser (1.02), Succ-Cys[d], Leu (1.95)	$C_{115}H_{173}N_{31}O_{27}S_2$	2486.	2487.
43	2-[BSH]-10	heterodimer	Lys (1.09), Arg (4.10), Pro (2.96), Hyp (0.87), Gly (2.00), Phe (3.03), Ser (1.01), Succ-Cys[d], Leu (1.96)	$C_{116}H_{178}N_{34}O_{27}S_2$	2545.	2545.
44	2-[BSH]-11	heterodimer	Lys (1.01), Arg (4.06), Pro (2.98), Hyp (0.90), Gly (2.00), Phe (3.07), Ser (1.01), Succ-Cys[d], Leu (1.98)	$C_{116}H_{178}N_{34}O_{27}S_2$	2545.	2547.
45	2-[BSH]-12	heterodimer	Lys (1.03), Arg (4.02), Pro (3.94), Hyp (0.88), Gly (1.03), Phe (3.06), Ser (1.03), Succ-Cys[d], Leu (2.00)	$C_{119}H_{182}N_{34}O_{27}S_2$	2585.	2585.
46	2-[BSH]-13	heterodimer	Lys (1.02), Arg (4.14), Pro (3.96), Hyp (0.90), Gly (2.01), Phe (2.02), Ser (0.99), Succ-Cys[d], Leu (1.95)	$C_{112}H_{176}N_{34}O_{27}S_2$	2495.	2498.
47	2-[BSH]-14	heterodimer	Lys (1.01), Arg (4.01), Pro (3.93), Hyp (0.96), Gly (2.05), Phe (3.08), Succ-Cys[d], Leu (1.97)	$C_{116}H_{180}N_{34}O_{28}S_2$	2555.	2555.
48	2-[BSH]-15	heterodimer	Lys (1.04), Arg (4.13), Pro (2.97), Hyp (0.92), Gly (2.03), Phe (3.01), Ser (0.96), Succ-Cys[d], Leu (1.94)	$C_{116}H_{178}N_{34}O_{27}S_2$	2545.	2548.
49	2-[BSH]-16	heterodimer	Lys (1.08), Arg (4.11), Pro (3.92), Hyp (0.89), Gly (1.99), Phe (3.01), Ser (0.98), Succ-Cys[d], Leu (1.03)	$C_{115}H_{174}N_{34}O_{27}S_2$	2529.	2530.
50	2-[BSH]-6	heterodimer	Arg (4.28), Pro (3.00), Hyp (1.83), Gly (2.04), Phe (3.04), Ser (0.96), Succ-Cys[d], Leu (1.84)	$C_{115}H_{173}N_{33}O_{28}S_2$	2530.	2530.

[a] All peptide dimers ≥95% pure by analytical reversed-phase HPLC. [b] See Table 1 for sequence; BSH = bis(succinimido)hexane. [c] Electrospray, laser desorption or plasma desorption mass spectra obtained. [d] Succ-Cys not determined quantitatively.

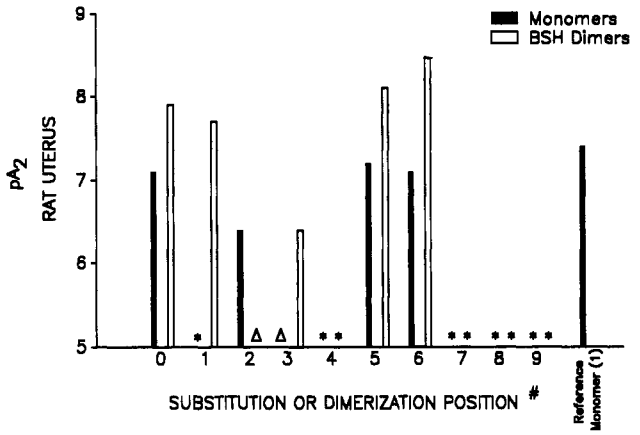

Figure 1. Effect of monomeric and bis(succinimido)hexane dimeric cysteine-substituted homologues of reference monomer 1 on bradykinin-induced contraction of rat uterus in vitro.[6] 1 = D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Phe[5]-Ser[6]-D-Phe[7]-Leu[8]-Arg[9]. pA_2 values are means of $n \geq 3$. * = inactive; Δ = partial agonist; BSH = bis(succinimido)hexane; # = all compounds characterized/assessed in ref 6.

corresponding bis(succinimido)hexane homodimers. As in the previous series, the reference sample is compound 1 (pA_2 = 7.4). As can be seen from these data, there is a general concurrence of activities of *S*-(*N*-hexylsuccinimido)-modified monomer and bis(succinimido)hexane homodimer with positions 5 and 6 (compounds **22** and **23**) again being the preferred positions for "linking". The unexpected activities of the monomers modified at positions 2, 7, 8, and 9 (compounds **19**, **24**, **25**, and **26**, respectively) are currently under investigation, and a more complete analysis of the in vitro and in vivo pharmacology of these compounds as well as those described in Table 5 will be published elsewhere.

Table 5. Effect of Monomeric and Dimeric Cysteine-Substituted Analogues of Reference Monomer 1 on Bradykinin-Induced Contraction of Rat Uterus in Vitro

no.	structure[a]	pA_2[b]	% recovery
1[c]	DR^0-R^1-P^2-J^3-G^4-F^5-S^6-DF^7-L^8-R^9	7.4 ± 0.2	100
2	DR^0-R^1-P^2-J^3-G^4-F^5-C^6-DF^7-L^8-R^9	7.1 ± 0.1	100
36	DR^0-R^1-P^2-J^3-G^4-F^5-C^6-DF^7-L^8-R^9 (BSH) DR^0-R^1-P^2-J^3-G^4-F^5-C^6-DF^7-L^8-R^9	8.5 ± 0.3	50
3	DR^0-R^1-P^2-J^3-G^4-A^5-C^6-DA^7-A^8-R^9	inactive	–
37	DR^0-R^1-P^2-J^3-G^4-A^5-C^6-DA^7-A^8-R^9 (BSH) DR^0-R^1-P^2-J^3-G^4-A^5-C^6-DA^7-A^8-R^9	inactive	–
38	DR^0-R^1-P^2-J^3-G^4-F^5-C^6-DF^7-L^8-R^9 (BSH) DR^0-R^1-P^2-J^3-G^4-A^5-C^6-DA^7-A^8-R^9	8.3 ± 0.2	57

[a] BSH = bis(succinimido)hexane. [b] pA_2 values are means ± SEM of $n \geq 3$. [c] Included as the reference ligand.

Design, Synthesis, and Analysis of Compounds Containing Combined B_1 and B_2 Antagonist Activities. While compound **36** appears to be significantly resistant to a variety of kininases, it is a reasonably good substrate for kininase I or carboxypeptidase (data not shown). Since the des-Arg[9] derivatives of a number of B_2 antagonists are known to possess B_1 antagonist properties, the mono- and bis-des-Arg[9] derivatives of compound **36** were evaluated for their B_1 and B_2 antagonist activities. As can be seen from the data listed in Table 6, compound **36** is strictly a B_2 antagonist while its mono- and bis-des-Arg[9] metabolites, compounds **39** and **40**, respectively, possess moderate antagonist activity at B_1 receptors. Interestingly, unlike the situation with the B_2 antagonists, there is a *loss* of potency at this receptor when the

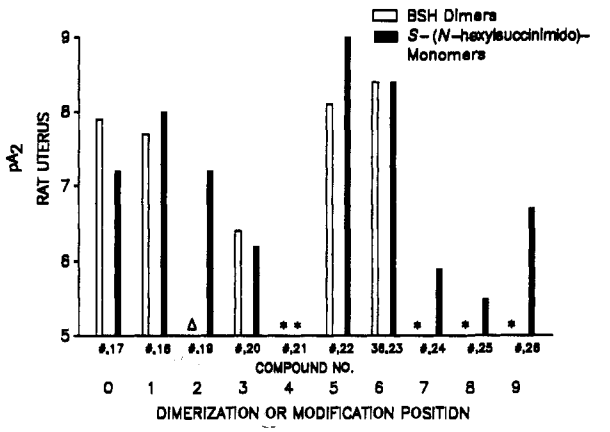

Figure 2. Effect of cysteine-substituted, *S*-(*N*-hexylsuccinimido)-modified monomeric and cysteine-substituted, bis(succinimido)hexane dimeric homologues of reference monomer 1[6] on bradykinin-induced contraction of rat uterus in vitro. 1 = D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Leu^8-Arg^9. pA_2 values are means of $n \geq 3$. * = inactive; Δ = partial agonist; BSH = bis(succinimido)hexane; # = compounds characterized/assessed in ref 6.

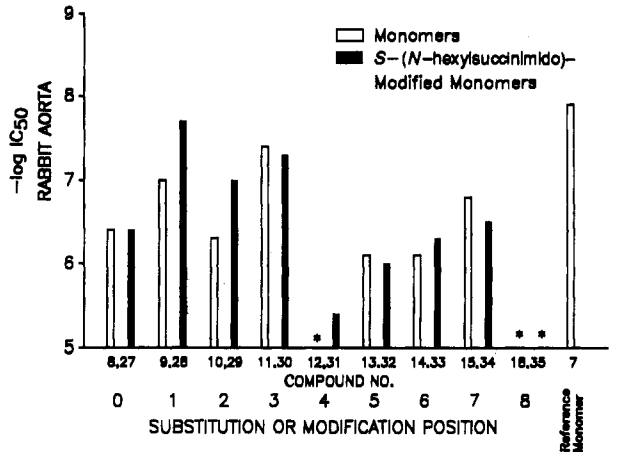

Figure 3. Effect of cysteine-substituted, *S*-(*N*-hexylsuccinimido)-modified and cysteine-substituted, unmodified monomeric homologues of reference monomer 7 on [des-Arg^9]-bradykinin-induced contraction of rabbit aorta in vitro. 7 = Lys^0-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Leu^8. $-\log_{10} IC_{50}$ values are means of $n \geq 3$. * = inactive.

Table 6. Effect of the Primary Metabolites of Compound 36 on Bradykinin-Induced Contraction of Rat Uterus in Vitro and on des-Arg^9-Bradykinin-Induced Contraction of Rabbit Aorta in Vitro

no.	structure[a]	rat uterus pA_2[b]	rabbit aorta $-\log_{10} IC_{50}$[c]
36	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8\text{-}R^9$ (BSH) $DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8\text{-}R^9$	8.5 ± 0.3	inactive (<5)
39	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8$ (BSH) $DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8\text{-}R^9$	8.2 ± 0.2	5.7 ± 0.1
40	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8$ (BSH) $DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8$	6.7 ± 0.2	5.7 ± 0.3
2	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8\text{-}R^9$	7.1 ± 0.1	inactive (<5)
4	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}C^6\text{-}DF^7\text{-}L^8$	inactive	6.4 ± 0.1
5	$DR^0\text{-}R^1\text{-}P^2\text{-}J^3\text{-}G^4\text{-}F^5\text{-}S^6\text{-}DF^7\text{-}L^8$	inactive	6.9 ± 0.1

[a] BSH = bis(succinimido)hexane. [b] pA_2 valus are means ± SEM of $n \geq 3$. [c] IC_{50} values are means ± SEM of $n \geq 3$.

monomers are compared with the relevant homo- and heterodimers (compounds **4** and **5** versus **39** and **40**). These data suggest that the SAR for modifications of a B_1 antagonist are substantially different than those of a B_2 antagonist.

In order to explore this point more fully, a series of cysteine- and *S*-(*N*-hexylsuccinimido)-cysteine-containing homologues of the well characterized B_1 antagonist Lys^0-[Leu^8,des-Arg^9]-BK (**7**) were synthesized and tested for B_1 antagonist activity. Illustrated in Figure 3 are the potencies of these modified monomers as a function of modification position. Unlike several of the compounds from the series of *S*-(*N*-hexylsuccinimido)-modified B_2 antagonists, no compound was found to have *increased* activity over the B_1 reference monomer (**7**). However, modifications at position 1 (compound **28**) appeared to be well tolerated. These data suggested that, while the differences between B_1 and B_2 receptors appear to be even greater than originally appreciated, the development of compounds with combined B_1 and B_2 antagonist activity should be possible if one was judicious in the choice of linkage positions within the two respective ligands.

This conclusion was verified by the synthesis and in vitro analysis of a series of heterodimeric compounds designed to have *combined* B_2 and B_1 antagonist activities. In these compounds, the B_2 antagonist ligand was maintained as the prototype Cys^6 peptide, monomer **2**, while the dimerization position of the B_1 antagonist ligand was systematically moved through the reference ligand, **7**, using our standard cysteine–bis(succinimido)hexane dimerization chemistry.

As can be seen from the data presented in Table 7, each of the ligands can have profound effects on the activity of the geminal ligand at its specific receptor. Interestingly, the resulting activity of the B_1 antagonist is maximal when it is dimerized from position 1, a result consistent with the data obtained from the *S*-(*N*-hexylsuccinimido)-modified monomer series (Figure 3). Furthermore, the optimal activity at *both* receptors was found when the B_2 antagonist was linked from position 6 and the B_1 antagonist from position 1 to yield compound **42**.

Finally, compound **50**, the bis(succinimido)hexane heterodimer consisting of monomer **2** and a further optimized B_1 antagonist, monomer **6**, is the optimal compound reported here for overall combined B_2/B_1 antagonist activity. This compound and others are being evaluated in a number of animal models of acute and chronic inflammatory conditions in which combined B_2 and B_1 kinin activities have been implicated. Preliminary data on the in vivo activity of this compound have been published elsewhere.[14]

General Discussion

In inflammatory conditions, a variety of both humoral (kinins, complement, and other contact system derived peptides) and cellularly-derived mediators and effectors (lipid metabolites, cytokines, nitric oxide, histamine, neuropeptides, leukocyte proteases, etc.) interact in a complex fashion. This interaction is dependent on a number of factors including the inciting cause of the inflammatory response, its location, the underlying physiologic state of the responding organism, and the temporal duration of the inflammatory condition. This dynamic interplay of responses is well illustrated in the change in

Table 7. Effect of B_1 Antagonist Dimerization Position in B_2 (= Compound 2)/B_1 Antagonist Heterodimers on Bradykinin-Induced Contraction of Rat Uterus in Vitro and on des-Arg^9-Bradykinin-Induced Contraction of Rabbit Aorta in Vitro

		B_1 dimerization	rat uterus B_2		rabbit aorta B_1
no.	structure[a]	position	pA_2[b]	% recovery	$-\log_{10} IC_{50}$[c]
41	2-[BSH]-8	0	8.0 ± 0.6	92	7.0 ± 0.1
42	2-[BSH]-9	1	7.6 ± 0.2	66	7.9 ± 0.2
43	2-[BSH]-10	2	8.1 ± 0.4	90	6.4 ± 0.2
44	2-[BSH]-11	3	8.3 ± 0.3	95	6.6 ± 0.2
45	2-[BSH]-12	4	8.0 ± 0.4	84	5.1 ± 0.1
46	2-[BSH]-13	5	7.6 ± 0.3	100	6.6 ± 0.1
47	2-[BSH]-14	6	7.5 ± 0.2	83	5.8 ± 0.1
48	2-[BSH]-15	7	8.3 ± 0.3	63	5.5 ± 0.1
49	2-[BSH]-16	8	8.7 ± 0.3	79	inactive
50	DR^0-C^1-P^2-J^3-G^4-F^5-S^6-P^7-L^8 (BSH) DR^0-R^1-P^2-J^3-G^4-F^5-C^6-DF^7-L^8-R^9		8.3 ± 0.2	66	7.5 ± 0.1

[a] See Table 1 for sequence; BSH = bis(succinimido)hexane. [b] pA_2 values are means ± SEM of $n \geq 3$. [c] IC_{50} values are means ± SEM of $n \geq 3$.

the response characteristics to the kinins in a number of different animal models of both acute and chronic inflammatory conditions.[3-5]

The B_1/B_2 receptor system is unique, however, in that the agonists to the upregulated (B_1) receptor ([des-Arg^9]-BK and Lys^0-[des-Arg^9]-BK) are the primary metabolites of the agonists to the constitutively present (B_2) receptor. While there are a number of enzymes capable of degrading BK and Lys^0-BK, in peripheral tissues and in the circulation the primary metabolism of these potent inflammatory mediators is via circulating and tissue-bound carboxypeptidases.[15] As a result, the des-Arg^9 derivatives of these peptides will be produced in direct proportion to the concentrations of their parent peptides. Perhaps even more importantly is the observation that fibrin degradation products, which are also products of an activated contact system, will inhibit kininase II (angiotensin converting enzyme or ACE).[16] However, unlike the situation with the carboxypeptidase-derived products of the kinins, which possess significant biological activities, the ACE-derived metabolites are biologically and pharmacologically silent. If this inhibition is of physiologic significance, then, in the setting of massive contact system activation (sepsis, trauma and burns), the biological activities of both BK and Lys^0-BK and their des-Arg^9 derivatives would likely be enhanced, further augmenting the significance of the upregulated B_1 system.

Given the potential importance of the B_1 as well as the B_2 receptor systems in severe and/or chronic inflammatory conditions, we felt that having a compound capable of interacting with these two related, but clearly independent, receptors could be of importance. Initially, however, it was not at all clear that these types of compounds would be successful because peptide–receptor interactions involving two different receptor types might not be tolerant of the presence of the geminal ligand. The studies involving the modified monomers, wherein the SAR of the two antagonist series were found to be different, emphasize how different these two receptors really are. From the data presented, however, it is apparent that heterodimeric compounds that interact effectively and specifically with their targeted receptors can be made. Furthermore, although the geminal ligand does influence activity negatively, it is reasonable to ask whether other receptor systems can be targeted using a similar heterodimeric compound approach. Additional studies exploring the potential of the heterodimer concept will be presented in a subsequent publication.

Another point that needs to be made concerning the comparison of the homodimers, heterodimers, and modified monomers is the fact that a significant component of the activities of the original B_2 antagonist series of compounds is being conferred by the "linker" moiety itself and is not a function of the dimer structure, per se. This observation implies that there are additional "peri-receptor" sites that can be utilized to develop other types of monomeric antagonists (perhaps nonpeptides) for the B_2 receptor.

Summary and Conclusions

Based on the data derived from these studies, two important points can be made regarding B_2 or B_1/B_2 homo- and heterodimers. These are as follows:

1. Compounds with combined B_2 and B_1 antagonist activity can be made using a heterodimer strategy.

2. The differences between the structure–activity relationships of the B_2 and B_1 receptor populations for their respective antagonists appear to be significant. This suggests that monomeric ligands with combined and approximately equivalent B_2/B_1 antagonist activities will be difficult to develop.

Compounds with combined B_2/B_1 receptor antagonist activities may be useful in elucidating the role of the kinins in a variety of pathophysiologic states and, eventually, may become useful agents for the treatment of a number of both acute and chronic diseases.

Experimental Section

Peptides were prepared using an automated peptide synthesizer (Applied Biosystems (ABI), Model 430A) according to protocols shown in Table 2 for a 0.5-mmol solid-phase synthesis. Peptide characterization data are given in Tables 1, 3, and 4. Unless stated otherwise, amino acids were of the L-configuration. Boc-protected amino acids, solvents, and other reagents for automated peptide synthesis were purchased from Applied Biosystems (ABI). Trifunctional amino acids were protected as follows: Boc-D-Arg(Tos)-OH, Boc-Arg(Tos)-OH, Boc-Ser(OBzl)-OH, Boc-Lys(Cl-Z)-OH, Boc-Hyp(OBzl)-OH, and Boc-Cys(Meb)-OH. Tetrahydrofuran and dimethylformamide were reagent grade and were used without further purification.

Synthetic peptides were purified by reversed-phase HPLC on a Waters Delta-Prep 3000 preparative chromatography system equipped with a variable-wavelength detector, using either a 47-mm × 30-cm Waters Delta-Pak radial compression cartridge

(column 1:300 Å, 15 μm C_{18}) or a 10-mm × 25-cm Vydac column (column 2: 300 Å, 5 μm C_{18}). Typically, peptides were eluted over a 35–45-min period with a linear acetonitrile gradient (column 1: 0–80%, 100 mL/min; column 2: 15–70%, 10 mL/min) containing a constant concentration of TFA (0.1%, v/v). The effluent was monitored at 215 nm, and the homogeneity of purified material was established by analytical HPLC on a 4.6-mm × 15-cm Vydac reversed-phase column (300 Å, 5 μm C_{18}) using a 20-min linear acetonitrile gradient (15–40%, 1 mL/min) containing a constant concentration of TFA (0.1%, v/v). NMR spectra (^1H and ^{13}C) were recorded on a Varian Gemini-300 spectrometer operating at 300 MHz. Chemical shift values are expressed in ppm downfield from tetramethylsilane (TMS) as the internal standard. Amino acid analyses were carried out via the Waters PICO·TAG chemistry[17] following 22–24-h vapor-phase hydrolysis with constant boiling 6 M HCl. Microsequencing analyses of resin-bound or free peptides were performed on an automated liquid-phase sequencer (Applied Biosystems (ABI), Model 473A) coupled to an on-line Macintosh workstation. Electrospray mass spectrometry was performed on a R30–10 Nermag/Delsi Instruments mass spectrometer at Texas Analytical Services (Houston, TX). Plasma desorption mass spectrometry was carried out at Multiple Peptide Systems (San Diego, CA) on an Applied Biosystems, Inc. Bio/Ion 20 mass spectrometer. Laser desorption mass spectrometry was performed at Cortech on a Finnigan LaserMat mass spectrometer. Thin-layer chromatography (TLC) was done on silica gel plates (J. P. Baker, Flex) and components were visualized by fluorescence quench or by Cl_2/starch–KI spray.[18] Solvent systems used in TLC were as follows: 1-butanol/acetic acid/water, 4:1:1, and 95% CH_3CH_2OH.

General Procedure for the Removal of Peptide from Resin: HF Cleavage. Briefly, 0.5 mmol of completed (side chain protected) peptidyl-resin was dried in vacuo and placed in one of the reaction vessels associated with a Peninsula Laboratories Type I HF apparatus. The resin was treated with anisole (1 mL) and placed back in vacuo, and 10 mL of liquid HF was allowed to distill into the reaction vessel (cooled to −78 °C in dry ice/acetone). The peptidyl-resin/anisole/HF reaction mixture was then allowed to stir at 0 °C for 1 h. After removal of the HF in vacuo, the resin was washed successively with ethyl ether, ethyl acetate, and ethyl ether to remove organic byproducts, and the peptide was extracted from the resin with water (3 × 10-mL volumes). The crude peptide was then purified by preparative reversed-phase HPLC as described above. Lyophilization afforded the pure peptide as a fluffy, white powder.

***N*-Hexylmaleimide** was prepared from *N*-(methoxycarbonyl)-maleimide and *N*-hexylamine as follows: To a stirred solution of *N*-hexylamine (3.06 g, 29.98 mmol) in saturated $NaHCO_3$ (150 mL) at 0 °C was added portionwise *N*-(methoxycarbonyl)-maleimide (5.60 g, 36.10 mmol).[19] The reaction mixture was stirred at 0 °C for 5 min, saturated $NaHCO_3$ and THF were added (100 mL of each), and the reaction mixture was then allowed to stir at room temperature for 1 h. Additional saturated $NaHCO_3$ and THF (100 mL of each) were added every hour over the next 3-h period. The resulting reaction mixture was transferred in toto to a separatory funnel (2 L) and the product isolated by extraction into ethyl acetate (3 × 250-mL volumes). The combined ethyl acetate extracts were washed with water (2 × 250-mL volumes) and brine (2 × 250-mL volumes), dried over Na_2SO_4, filtered, and concentrated in vacuo to afford *N*-hexylmaleimide as a light yellow oil. Yield: 6.23 g (34.41 mmol, 115%). TLC: R_f = 0.80 (1-butanol/acetic acid/water, 4:1:1); R_f = 0.73 (95% CH_3CH_2OH). ^1H-NMR (300 MHz, $CDCl_3$): δ 0.88 (t, 3 H, J = 6.6 Hz), 1.22–1.35 (m, 6 H), 1.57 (p, 2 H, J = 7.0 Hz), 3.51 (t, 2 H, J = 7.4 Hz), 6.69 (s, 2 H). (Approximately 5% residual *N*-(methoxycarbonyl)maleimide was evident in the ^1H-NMR spectrum.) ^{13}C-NMR (300 MHz, $CDCl_3$): δ 13.96, 22.47, 26.37, 28.47, 31.26, 37.89, 133.99, 170.89.

General Procedure for the Synthesis of *S*-(*N*-Hexylsuccinimido)-Modified Peptide Monomers. To a mixture of peptide monomer (1 equiv) and *N*-hexylmaleimide (1.5 equiv) in DMF (ca. 3.3 mL/mmol of peptide) was added 30 volumes of 0.1 M ammonium bicarbonate (pH 7.5). The reaction mixture was stirred at room temperature and monitored periodically by analytical reversed-phase HPLC. Modification (*S*-alkylation) reactions carried out in this manner were complete within 30 min. The resulting *S*-(*N*-hexylsuccinimido)-modified peptide monomer was purified by preparative reversed-phase HPLC as described above. Lyophilization then afforded the product as a fluffy, white powder.

General Procedure for the Synthesis of Bis(succinimido)-hexane Peptide Homodimers. To a mixture of peptide monomer (2 equiv) and bis(maleimido)hexane (1 equiv) in DMF (ca. 3.3 mL/mmol of peptide) was added 30 volumes of 0.1 M ammonium bicarbonate (pH 7.5). The reaction mixture was stirred at room temperature and monitored periodically by analytical reversed-phase HPLC. Dimerization reactions carried out in this manner were complete within 30 min. The resulting bis(succinimido)hexane peptide homodimer was purified by preparative reversed-phase HPLC as described above. Lyophilization then afforded the product as a fluffy, white powder.

General Procedure for the Synthesis of Bis(succinimido)-hexane Peptide Heterodimers. To a mixture of peptide monomer (1 equiv) and bis(maleimido)hexane (1.5 equiv) in DMF (ca. 3.3 mL/mmol of peptide) was added 30 volumes of 0.1 M ammonium bicarbonate (pH 7.5). The reaction mixture was stirred at room temperature and monitored periodically by analytical reversed-phase HPLC. Modification (*S*-alkylation) reactions carried out in this manner were complete within 30 min. The resulting peptide–bis(maleimido)hexane monoadduct was purified by preparative reversed-phase HPLC as described above, lyophilized, and then condensed with a different cysteine-containing peptide monomer (1.5 equiv) in 0.1 M ammonium bicarbonate (pH 7.5, ca. 100 mL/mmol of peptide). The reaction mixture was stirred at room temperature and monitored periodically by analytical reversed-phase HPLC. Dimerization reactions carried out in this manner were complete within 30 min. The resulting bis(succinimido)hexane peptide heterodimer was purified by preparative reversed-phase HPLC as described above and then lyophilized to afford a fluffy, white powder.

Rat Uterus in Vitro pA_2 Measurement. Female Sprague–Dawley rats (200–250 g) in natural estrus were euthanized by a blow on the head and exsanguinated. Uterine horns were removed, placed under a 1-g resting tension in 4-mL tissue baths containing De Jalon's solution at 31 °C, and aerated with air. The De Jalon's solution had the following composition (mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 0.55; $MgCl_2$, 0.053; $NaHCO_3$, 5.92; glucose, 2.77. Concentration effect curves were constructed to BK in the absence and presence of antagonist (preincubated for 15 min) via isometric tension recordings. Antagonist potency (pA_2 value) was calculated according to the method of Arunlakshana and Schild.[20] Following exposure to the highest concentration of antagonist (in each case 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 BK. The pD_2 (−log molar concentration of agonist producing 50% of the original maximum response) for BK at this time was calculated and compared to the pD_2 of the initial control concentration–effect curve for BK. The difference in pD_2 values compared to concurrent control reflected the percentage recovery of agonist response.

Rabbit Aorta in Vitro IC_{50} Measurement. Female NZW rabbits were euthanized by overdose of pentobarbital (80 mg/kg i.v.) and the thoracic aortas removed. Spiral strips were mounted under 2-g resting isometric tension in 5-mL tissue baths containing Krebs solution aerated with 95% O_2–5% CO_2. The Krebs solution had 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; glucose, 11; and contained indomethacin (2.8 μM). Following a 5-h period (during which time two concentration–effect (C–E) curves to [des-Arg^9]-BK were performed at 1 and 3 h), [des-Arg^9]-BK was added to the bath to a final concentration of 10^{-7} M. This produced a stable, sustained, prolonged (up to 45 min) contraction. The selected antagonist was then added in a cumulative fashion to the bath on top of this contraction. The IC_{50} (molar antagonist concentration producing 50% reversal of the contraction) was then calculated from the resulting tracings.

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McLeod for preparation of additional quantities of *S*-(*N*-hexylsuccinimido)-modified peptide monomers.

References

(1) Abbreviations follow the IUPAC–IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides: *Eur. J. Biochem.* **1984**, *158*, 9–31. Additional abbreviations used are as follows: BK, bradykinin; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIPEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, hydroxybenzotriazole; J, hydroxyproline; pA_2, –log molar concentration of antagonist in the presence of which twice the concentration of agonist is required to produce the same response as in the absence of the antagonist; PAM, (phenylacetamido)methyl; SAR, structure–activity relationship; Succ-Cys, succinylcysteine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

(2) A preliminary account of this work was presented at the Sixth International Conference of the Inflammation Research Association in White Haven, PA, September 20–24, 1992, Abstract No. P88 and P89.

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