24-48 h before the experiments. Cumulative dose-response curves were constructed. The antagonists were applied to the bath 1 min before the construction of the oxytocin dose-response curve was started. The galactogogic test was performed on ethanol-anesthetized lactating rats 5-15 days after delivery.[21,22] Synthetic oxytocin was used as standard in these assays. Pressor activity was determined on pithed rat preparation[23] using synthetic arginine-vasopressin as a standard. Detailed descriptions of the tests are given in ref 14.

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A New Class of Bradykinin Antagonists: Synthesis and in Vitro Activity of Bissuccinimidoalkane Peptide Dimers[1,2]

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A systematic study on the dimerization of the bradykinin (BK) antagonist D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Leu^8-Arg^9 has been performed. The first part of this study involved compounds wherein dimerization was carried out by sequentially replacing each amino acid with cysteine and cross-linking with bismaleimidohexane. The second part of this study utilized a series of bissuccinimidoalkane dimers wherein the intervening methylene chain was varied systematically from $n = 2$ to $n = 12$ while the point of dimerization was held constant at position 6. The biological activities of these dimers were then evaluated on BK-induced smooth muscle contraction in two different isolated tissue preparations: guinea pig ileum (GPI) and rat uterus (RU). Several of the dimeric BK antagonists displayed remarkable activities and long durations of action. In addition, dimerization at position 4, 7, 8, or 9 produced dimeric analogues with markedly reduced potency. Rank order of antagonist potency as a function of dimerization position is as follows: rat uterus, $6 > 5 > 0 > 2 > 1 > 3 \gg 4, 7, 8, 9$; guinea pig ileum, $6 > 5 > 3 > 2 > 1 > 0 \gg 4, 7, 8, 9$. Evaluation of the linker length as represented by the number of methylene units indicated an optimal distance between the two monomeric peptides of six to eight methylene moieties. These studies also revealed that the carbon-chain length significantly affected the duration of action in vitro and resulted in partial agonism effects when $n > 8$. The optimum activity in vitro was achieved with dimerization at position 6 and $n = 6$ (designated herein as compound **25**; alternatively, CP-0127). Similar effects in potency were also seen when the monomeric antagonist D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Phe^8-Arg^9 (NPC-567) was dimerized using similar chemistry. These results suggest that the development of BK antagonists of significant therapeutic potential may be possible using a dimerization strategy that can overcome the heretofore limiting problems of potency and in vivo duration of action found with many of the BK antagonists in the literature.

Introduction

The design and synthesis of potent, stable, and specific bradykinin antagonists has long been considered a desirable goal in medicinal chemistry. Compounds with these characteristics could be important in the treatment of such diverse disorders as septic shock, asthma, and rhinitis.[3,4] Up until now, however, most antagonists have been plagued with the dual problem of relatively low potency and poor in vivo stability. These problems have been solved recently by an approach involving the introduction of the conformationally constrained amino acid analogues 1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (D-Tic) and/or (7*S*,8*S*)-*endo-cis*-octahydroindole-2-carboxylic acid (Oic) in the carboxy-terminal region of decapeptide inhibitors, which improves potency and confers metabolic stability on these compounds.[5-7] This study describes an alternative approach which appears to have produced similar results. Described herein are two series of compounds that were produced using a standardized and

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(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: Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DIPEA, diisopropylethylamine; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; 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 antagonist; Pam, (phenylacetamido)methyl; PBS, phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate); Succ-Cys, SuccinylCysteine; TFA, trifluoroacetic acid; THF, tetrahydrofuran.
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Table I. Characterization of Cysteine-Containing Peptide Monomers[a]

no.	structure[b]	amino acid analysis[c]	peptide sequence analysis[d]
1[e]	*e*	Arg, Pro, Hyp, Gly, Phe, Ser, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
2	[L-Cys[0]]-1	Cys,[f] Arg, Pro, Hyp, Gly, Phe, Ser, Leu	1, Cys(Meb); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
3	[D-Cys[0]]-1	Cys,[f] Arg, Pro, Hyp, Gly, Phe, Ser, Leu	1, Cys(Meb); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
4	[L-Cys[1]]-1	Arg, Cys,[f] Pro, Hyp, Gly, Phe, Ser, Leu	1, Arg(Tos); 2, Cys(Meb); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
5	[L-Cys[2]]-1	Arg, Cys,[f] Hyp, Gly, Phe, Ser, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Cys(Meb); 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
6	[L-Cys[3]]-1	Arg, Pro, Cys,[f] Gly, Phe, Ser, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Cys(Meb); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
7	[L-Cys[4]]-1	Arg, Pro, Hyp, Cys,[f] Phe, Ser, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Cys(Meb); 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
8	[D-Cys[4]]-1	Arg, Pro, Hyp, Cys,[f] Phe, Ser, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Cys(Meb); 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
9	[L-Cys[5]]-1	Arg, Pro, Hyp, Gly, Cys,[f] Ser, Phe, Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Cys(Meb); 7, Ser(OBzl); 8, Phe; 9, Leu; 10, Arg(Tos)
10	[L-Cys[6]]-1	Arg, Pro, Hyp, Gly, Phe, Cys,[f] Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Cys(Meb); 8, Phe; 9, Leu; 10, Arg(Tos)
11	[L-Cys[7]]-1	Arg, Pro, Hyp, Gly, Phe, Ser, Cys,[f] Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly, 6, Phe, 7, Ser(OBzl); 8, Cys(Meb); 9, Leu; 10, Arg(Tos)
12	[D-Cys[7]]-1	Arg, Pro, Hyp, Gly, Phe, Ser, Cys,[f] Leu	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Cys(Meb); 9, Leu; 10, Arg(Tos)
13	[L-Cys[8]]-1	Arg, Pro, Hyp, Gly, Phe, Ser, Cys[f]	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Cys(Meb); 10, Arg(Tos)
14	[L-Cys[9]]-1	Arg, Pro, Hyp, Gly, Phe, Ser, Leu, Cys[f]	1, Arg(Tos); 2, Arg(Tos);, 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe, 9, Leu; 10, Cys(Meb)
15[g]	*g*	Arg, Pro, Hyp, Gly, Phe, Ser	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Ser(OBzl); 8, Phe; 9, Phe; 10, Arg(Tos)
16	[L-Cys[6]]-15	Arg, Pro, Hyp, Gly, Phe, Cys[f]	1, Arg(Tos); 2, Arg(Tos); 3, Pro; 4, Hyp(OBzl); 5, Gly; 6, Phe; 7, Cys(Meb); 8, Phe; 9, Phe; 10, Arg(Tos)

[a] Peptides based on the bradykinin antagonist D-Arg[0]-[Hyp[3],D-Phe[7],Leu[8]]-bradykinin. [b] All peptides ≥95% pure by analytical HPLC. [c] Values ±5%. [d] Peptide-Pam resin sequence data. [e] Included as the reference ligand; D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Phe[5]-Ser[6]-D-Phe[7]-Leu[8]-Arg[9]. [f] Cys not determined quantitatively. [g] NPC-567, D-Arg[0]-Arg[1]-Pro[2]-Hyp[3]-Gly[4]-Phe[5]-Ser[6]-D-Phe[7]-Phe[8]-Arg[9]; included as an alternative reference ligand.

systematic design strategy involving the synthesis of peptide antagonist dimers based upon the introduction of cysteine residues in the parent peptide at certain defined positions followed by dimerization utilizing various bismaleimidoalkane linkers.

Several studies have previously reported that dimerization can result in an increase in potency and/or resistance to inactivation for peptide agonists[8-15] and antagonists.[16] To our knowledge, however, this approach has not been employed in the design and synthesis of bradykinin antagonists. To explore this possibility, we chose to use a well-characterized and potent peptide antagonist, D-Arg[0]-[Hyp[3],D-Phe[7],Leu[8]]-bradykinin,[17] as the reference

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Table II. 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. [b] Formed initially in CH_2Cl_2; DCU filtration followed by solvent exchange with DMF.

ligand (referred to herein as compound 1).

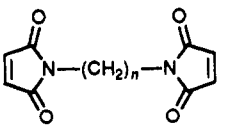
Initial attempts at dimerization using the free α amino or carboxyl groups at the respective termini of this decapeptide and a variety of linking chemistries yielded variable but uniformly unimpressive results in vitro (unpublished observations). On the basis of these findings, a different strategy was employed, wherein a D- or L-cysteine residue was systematically substituted along the peptide chain, and the resulting sulfhydryl-containing monomer dimerized by reaction with the readily available homobifunctional cross-linking reagent bismaleimidohexane. Unlike dimers formed using the free terminal amino or carboxyl moieties, dimers formed at several different positions using the cysteine/bismaleimidohexane dimerization strategy were found to have dramatically improved potency in a number of standard in vitro assay systems. The most impressive change in measured pA_2 from monomer to dimer resulted from dimers formed using L-cysteine in place of serine in position 6 in the reference ligand.

Once an optimal dimerization position was found, the effect of alkyl chain length on in vitro activity was explored. Significant differences were observed that could only be attributed to chain length, with the bissuccinimidohexane dimer providing optimal pharmacologic behavior in these screening assays. Alternative linking chemistries were also explored, with the most desirable outcome being provided by simple alkane chains of moderate (C_6 or C_7) chain length. These alkyl chain lengths were found to have the optimal constellation of increased potency, prolonged duration of action, and absence of high-dose partial agonism which was observed for dimers containing linkers of greater than eight methylene groups in length. No further improvement in activity was found when higher oligomers (trimers) were tested. Finally, an alternative reference antagonist ligand was chosen and similar results were obtained.

Chemistry

Syntheses: Peptide Monomers. Syntheses of peptide monomers, listed in Table I, were accomplished via standard solid-phase methodology[18] on a Pam resin, using protocols shown in Table II. N^{α}-*tert*-Butyloxycarbonyl protection was employed for all peptide syntheses, and finished peptides were cleaved from the resin using standard HF procedures.[19] Anisole alone was found to be an efficient carbocation scavenger during HF cleavage; as a result, all cysteine-containing peptides were cleaved from the resin without any additional scavengers (thiol or otherwise). Free peptides were extracted with 10% aqueous acetic acid, concentrated in vacuo, and then purified by preparative reverse-phase HPLC. Occasionally, dithiothreitol treatment was required to reduce peptide disulfide dimers prior to HPLC purification. Peptide characterization data are given in Table I. Overall isolated yields calculated from the starting Boc-amino acid-Pam resin were typically 50–60%.

Table III. Characterization of Bismaleimidoalkane Linkers



n[a]	isolated % yield	TLC, R_f[b]	^{1}H NMR spectral data, δ
2 (ethane)	64	0.73 (A), 0.65 (B)	3.74 (s, 4 H), 6.69 (s, 4 H)[c]
3 (propane)	53	0.74 (A), 0.67 (B)	1.61 (p, 2 H), 3.51 (t, 4 H, J = 7.0 Hz), 6.70 (s, 4 H)[c]
4 (butane)	51	0.76 (A), 0.67 (B)	1.50 (p, 4 H), 3.54 (t, 4 H, J = 7.0 Hz), 6.70 (s, 4 H)[c]
6 (hexane)	60	0.80 (A), 0.75 (B)	1.30 (p, 4 H), 1.57 (p, 4 H), 3.50 (t, 4 H, J = 7.2 Hz), 6.68 (s, 4 H)[c]
8 (octane)	58	0.85 (A), 0.74 (B)	1.28 (m, 8 H), 1.56 (p, 4 H), 3.50 (t, 4 H, J = 7.3 Hz), 6.69 (s, 4 H)[c]
9 (nonane)	90	0.86 (A), 0.77 (B)	1.26 (m, 10 H), 1.56 (p, 4 H), 3.50 (t, 4 H, J = 7.2 Hz), 6.69 (s, 4 H)[c]
10 (decane)	90	0.86 (A), 0.77 (B)	1.27 (m, 12 H), 1.57 (p, 4 H), 3.51 (t, 4 H, J = 7.2 Hz), 6.69 (s, 4 H)[c]
12 (dodecane)	82	0.84 (A), 0.74 (B)	1.22 (m, 16 H), 1.47 (p, 4 H), 3.38 (t, 4 H, J = 7.0 Hz), 7.02 (s, 4 H)[d]

[a] Starting N,N'-diaminoalkanes: 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,6-diaminohexane, 1,8-diaminooctane, 1,9-diaminononane, 1,10-diaminodecane, 1,12-diaminododecane. [b] For the solvent systems (A and B), see Experimental Section. [c] $CDCl_3$ data. [d] DMSO-d_6 data.

Syntheses: Bismaleimidoalkane Linkers. Syntheses of the variable bismaleimidoalkane linkers, listed in Table III, were carried out via a (modified) one-step maleimidation of the appropriate N,N'-diaminoalkane with N-(methoxycarbonyl)maleimide.[20] Bismaleimidoalkanes so prepared were at least 95% pure on the basis of NMR spectroscopy and analytical HPLC. Characterization data are given in Table III. Overall isolated yields calculated

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Table IV. Characterization of Bis/Trissuccinimido–Peptide Oligomers[a]

no.	structure[b]	linker	formula	calcd FW	m/e value[c]	amino acid analysis[d]
17	[BSH(L-Cys^0)]-1	$-(CH_2)_6-$	$C_{122}H_{180}N_{34}O_{30}S_2$	2665	2664	Succ-Cys,[e] Arg, Pro, Hyp, Gly, Phe, Ser, Leu
18	[BSH(D-Cys^0)]-1	$-(CH_2)_6-$	$C_{122}H_{180}N_{34}O_{30}S_2$	2665	2664	Succ-Cys,[e] Arg, Pro, Hyp, Gly, Phe, Ser, Leu
19	[BSH(L-Cys^1)]-1	$-(CH_2)_6-$	$C_{122}H_{180}N_{34}O_{30}S_2$	2665	2664	Arg, Succ-Cys,[e] Pro, Hyp, Gly, Phe, Ser, Leu
20	[BSH(L-Cys^2)]-1	$-(CH_2)_6-$	$C_{124}H_{190}N_{40}O_{30}S_2$	2784	2784	Arg, Succ-Cys,[e] Pro, Hyp, Gly, Phe, Ser, Leu
21	[BSH(L-Cys^3)]-1	$-(CH_2)_6-$	$C_{124}H_{190}N_{40}O_{28}S_2$	2751	2751	Arg, Pro, Succ-Cys,[e] Gly, Phe, Ser, Leu
22	[BSH(L-Cys^4)]-1	$-(CH_2)_6-$	$C_{130}H_{198}N_{40}O_{30}S_2$	2865	2865	Arg, Pro, Hyp, Succ-Cys,[e] Phe, Ser, Leu
23	[BSH(D-Cys^4)]-1	$-(CH_2)_6-$	$C_{130}H_{198}N_{40}O_{30}S_2$	2865	2865	Arg, Pro, Hyp, Succ-Cys,[e] Phe, Ser, Leu
24	[BSH(L-Cys^5)]-1	$-(CH_2)_6-$	$C_{116}H_{186}N_{40}O_{30}S_2$	2684	2685	Arg, Pro, Hyp, Gly, Succ-Cys,[e] Ser, Phe, Leu
25	[BSH(L-Cys^6)]-1	$-(CH_2)_6-$	$C_{128}H_{194}N_{40}O_{28}S_2$	2805	2805	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
26[f]	[disulfide (L-Cys^6)]-1		$C_{114}H_{176}N_{38}O_{24}S_2$	2526	2527	Arg, Pro, Hyp, Gly, Phe, cystine,[g] Leu
27	[BSH(L-Cys^7)]-1	$-(CH_2)_6-$	$C_{116}H_{186}N_{40}O_{30}S_2$	2684	2685	Arg, Pro, Hyp, Gly, Phe, Ser, Succ-Cys,[e] Leu
28	[BSH(D-Cys^7)]-1	$-(CH_2)_6-$	$C_{116}H_{186}N_{40}O_{30}S_2$	2684	2685	Arg, Pro, Hyp, Gly, Phe, Ser, Succ-Cys,[e] Leu
29	[BSH(L-Cys^8)]-1	$-(CH_2)_6-$	$C_{122}H_{182}N_{40}O_{30}S_2$	2751	2751	Arg, Pro, Hyp, Gly, Phe, Ser, Succ-Cys[e]
30	[BSH(L-Cys^9)]-1	$-(CH_2)_6-$	$C_{122}H_{180}N_{34}O_{30}S_2$	2665	2664	Arg, Pro, Hyp, Gly, Phe, Ser, Leu, Succ-Cys[e]
31	[BSE(L-Cys^6)]-1	$-(CH_2)_2-$	$C_{174}H_{186}N_{40}O_{28}S_2$	2749	2750	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
32	[BSP(L-Cys^6)]-1	$-(CH_2)_3-$	$C_{125}H_{188}N_{40}O_{28}S_2$	2763	2764	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
33	[BSB(L-Cys^6)]-1	$-(CH_2)_4-$	$C_{126}H_{190}N_{40}O_{28}S_2$	2777	2778	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
34	[BSO(L-Cys^6)]-1	$-(CH_2)_8-$	$C_{130}H_{198}N_{40}O_{28}S_2$	2833	2834	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
35	[BSN(L-Cys^6)]-1	$-(CH_2)_9-$	$C_{131}H_{200}N_{40}O_{28}S_2$	2847	2847	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
36	[BSD(L-Cys^6)]-1	$-(CH_2)_{10}-$	$C_{132}H_{202}N_{40}O_{28}S_2$	2861	2861	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
37	[BSDD(L-Cys^6)]-1	$-(CH_2)_{12}-$	$C_{134}H_{206}N_{40}O_{28}S_2$	2889	2889	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
38	[BS amide (L-Cys^6)]-1	$-(CH_2)_3C(O)NH(CH_2)_2NHC(O)(CH_2)_3-$	$C_{132}H_{200}N_{42}O_{30}S_2$	2916	2916	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
39	[TS amide(L-Cys^6)]-1	$-[(CH_2)_3C(O)NH(CH_2)_2]_3N$	$C_{201}H_{306}N_{64}O_{45}S_3$	4434	4434	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
40	[BS aryl(L-Cys^6)]-1	$-C_6H_4-$	$C_{128}H_{186}N_{40}O_{28}S_2$	2797	2796	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
41	[TS alkane(L-Cys^6)]-1	$-[(CH_2)_2]_3N$	$C_{189}H_{285}N_{61}O_{42}S_3$	4175	4175	Arg, Pro, Hyp, Gly, Phe, Succ-Cys,[e] Leu
42	[BSH(L-Cys^6)]-15	$-(CH_2)_6-$	$C_{134}H_{190}N_{40}O_{28}S_2$	2872	2871	Arg, Pro, Hyp, Gly, Phe, Succ-Cys[e]

[a] All compounds ≥98% pure by analytical HPLC. [b] See Table I for sequence; BSH = bissuccinimidohexane, BSE = bissuccinimidoethane, BSP = bissuccinimidopropane, BSB = bissuccinimidobutane, BSO = bissuccinimidooctane, BSN = bissuccinimidononane, BSD = bissuccinimidodecane, BSDD = bissuccinimidododecane, BS = bissuccinimido, TS = trissuccinimido. [c] Electrospray mass spectra obtained. [d] Values ±5%. [e] Succ-Cys not determined quantitatively. [f] Non-succinimido–peptide dimer. [g] Cystine not determined quantitatively.

from the starting N,N'-diaminoalkane were 50–90%.

Syntheses: Bissuccinimidoalkane Peptide Dimers. Syntheses of bissuccinimidoalkane peptide dimers, listed in Table IV, were accomplished most efficiently when 2 equiv of cysteine-containing peptide monomer and 1 equiv of the appropriate bismaleimidoalkane linker (both dissolved in DMF) were allowed to react in PBS 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.[21] Dimerization reactions were routinely allowed to proceed overnight at room temperature and the resulting bissuccinimidoalkane peptide dimers were then purified by preparative reverse-phase HPLC. Characterization data are given in Table IV. Overall isolated yields calculated from the starting cysteine-containing peptide monomer were typically 60–80%.

Syntheses: Bissuccinimidoamide, Bissuccinimidoaryl, Trissuccinimidoamide, and Trissuccinimidoalkane Peptide Oligomers. These types of succinimido peptide oligomers, also listed in Table IV, were synthesized using the same procedure as that employed in the production of bissuccinimidoalkane peptide dimers. Four to five equivalents of cysteine-containing peptide and 1 equiv of trismaleimide-containing linker were utilized in the preparation of peptide trimers. Characterization data regarding peptide dimers and trimers are given in Table IV. Overall isolated yields calculated from the starting cysteine-containing peptide monomer were typically 40–60%. Characterization data pertaining to novel

(21) Bakuzis, P.; Bakuzis, M. L. F. Oxidative Functionalization of the β-Carbon in α,β-Unsaturated Systems, Preparation of 3-Phenylthio Enones, Acrylates, and Other Vinyl Derivatives. *J. Org. Chem.* 1981, *46*, 235–239.

Table V. Effect of Monomeric and Dimeric Cysteine-Substituted Analogues of 1 on Bradykinin-Induced Contractions of Guinea Pig Ileum in Vitro

no.	structure[a]	description reference	pA_2[b]
1	c	monomer	6.5 ● 0.2
2	[L-Cys^0]-1	monomer	6.7 ± 0.1
17	[BSH(L-Cys^0)]-1	dimer	<6
3	[D-Cys^0]-1	monomer	6.7 ± 0.5
18	[BSH(D-Cys^0)]-1	dimer	6.5 ± 0.1
4	[L-Cys^1]-1	monomer	6.1 ● 0.1
19	[BSH(L-Cys^1)]-1	dimer	7.6 ± 0.6
5	[L-Cys^2]-1	monomer	<6
20	[BSH(L-Cys^2)]-1	dimer	6.9 ± 0.3
6	[L-Cys^3]-1	monomer	6.7 ± 0.4
21	[BSH(L-Cys^3)]-1	dimer	7.7 ± 0.2
7	[L-Cys^4]-1	monomer	inactive
22	[BSH(L-Cys^4)]-1	dimer	<6
8	[D-Cys^4]-1	monomer	inactive
23	[BSH(D-Cys^4)]-1	dimer	inactive
9	[L-Cys^5]-1	monomer	6.4 ● 0.2
24	[BSH(L-Cys^5)]-1	dimer	7.9 ± 0.1
10	[L-Cys^6]-1	monomer	6.6 ● 0.2
25	[BSH(L-Cys^6)]-1	dimer	7.7 ± 0.2
11	[L-Cys^7]-1	monomer	inactive
27	[BSH(L-Cys^7)]-1	dimer	inactive
12	[D-Cys^7]-1	monomer	inactive
28	[BSH(D-Cys^7)]-1	dimer	inactive
13	[L-Cys^8]-1	monomer	inactive
29	[BSH(L-Cys^8)]-1	dimer	inactive
14	[L-Cys^9]-1	monomer	inactive
30	[BSH(L-Cys^9)]-1	dimer	inactive

[a] All dimers are the bissuccinimidohexane type. [b] pA_2 values are means ±SEM of $n \geq 3$. [c] D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Leu^8-Arg^9.

maleimide-containing linkers are given in the Experimental Section.

Results and Discussion

Effect of Dimerization Position on the Inhibition of Bradykinin-Induced Smooth Muscle Contraction in Vitro. The initial studies investigating the effects of dimerization based on position were conducted in a standard guinea pig ileum assay of bradykinin-induced smooth muscle contraction, a preparation known to contain a BK_2-type receptor. In addition to estimating the pA_2 values for these compounds, we also assessed the ability of these inhibitors to sustain their interaction with the receptor. This was measured by assessing the degree to which the preparation returned to baseline sensitivity and responsiveness after exposure to 10^{-5} M inhibitor, followed by a 40-min recovery period wherein the tissue was washed every 10 min with fresh Krebs buffer. Table V is a summary of the data comparing the reference ligand (1) to the series of cysteine-substituted monomers and their corresponding bissuccinimidohexane dimers.

A number of points can be made from these data. First, for the majority of positions, the replacement of the reference residue with cysteine does not dramatically alter the activity of the resulting monomeric inhibitor. This is not true, however, for positions 4, 7, 8, and 9, wherein cysteine substitution completely eliminates activity. These data are consistent with previous studies in which alanine was substituted for the corresponding amino acids in positions 1–9 of bradykinin.[22] Second, except for position "O", dimers were consistently more potent than their corresponding monomer when activity remained after cysteine substitution. Maximal activity was seen with dimers formed from a monomer containing cysteine in position 3, 5, or 6 (dimers **21**, **24**, and **25**, respectively). Third, dimers formed from the "O" position, regardless of the chirality of the cysteine residue, are equipotent to the reference ligand, consistent with data obtained from dimers formed using the terminal α amino group (data not shown). Lastly, following a 40-min wash-out period of all compounds which possessed antagonist activity, the dose–response curve for bradykinin was equivalent to that seen before exposure to the antagonist. This demonstrates that the effects of the antagonists in this tissue are fully reversible.

We then expanded our investigation to an assay system employing a second BK_2 receptor subtype, bradykinin-induced smooth muscle contraction in the rat uterus. Table VI is a summary of the data concerning monomers and dimers formed by the introduction of L-cysteine at various positions within the ligand. Data from D-cysteine substitutions are qualitatively similar to that seen in the guinea pig ileum assay system.

As the data indicate, we again see a dramatic enhancement of activity with dimerization except for dimers formed from positions 4, 7, 8, and 9. Interestingly, dimers formed from the "O" position now show enhanced activity in this system, which is in sharp contrast to the lack of enhancement found using the guinea pig ileum assay. Finally, dimer **25** (the bissuccinimidohexane dimer containing cysteine in position 6) was again found to be substantially more potent than any other dimer, including dimer **24**. These distinctions may be useful in further differentiating the receptor subtype populations found in these and other tissues.

Another important difference between monomeric and dimeric inhibitors in rat uterine tissue is the reduced recovery seen with a number of the dimers. In particular, exposure to 10^{-5} M **25** was found to reduce recovery by approximately 50%. Recovery improved another 50% (to 75% of preexposure responsiveness) with an additional 40-min recovery period and reached control levels after a third period. These data suggest that reduced recovery may be a function of a sustained interaction with the receptor as opposed to a "down regulation" or inactivation of receptors in this tissue. (This is in contrast to the guinea pig ileum, whereby full recovery of bradykinin responsiveness and sensitivity was seen after a 40 min wash-out period and may reflect differences in BK_2-receptor subtypes.) The nature of this "sustained interaction" on rat uterus is currently being investigated in our laboratories, but may reflect a difference in receptor "off-rate". From these data we determined that the 6 position was optimal for enhancing the activity of these types of antagonist dimers. We recognize that dimers formed using this same type of chemistry but with cysteine residues substituted in other positions could be better suited for other bradykinin receptor types. Despite this potentially confounding variable, however, further studies concerning the effects of linker chemistry on activity utilized a monomer containing cysteine in position 6 as the "base" ligand; this compound is referred to herein as monomer **10**.

Effect of Alkyl Chain Length on the Inhibition of Bradykinin-Induced Uterine Smooth Muscle Contraction in Vitro. As was mentioned in the introduction, bismaleimidohexane was chosen for the initial studies simply on the basis of its availability and ease of use. To assess the effects of alkyl chain length on both potency and inhibition of recovery, a series of bismaleimidoalkane linkers were synthesized and then used to dimerize the cysteine-containing "base" ligand (**10**). The resulting compounds were then tested in the rat uterine smooth muscle assay system for inhibitory potency as well as their ability to inhibit recovery. The data from these studies are summarized in Table VII.

Two important points can be made from these data. First, enhanced potency can be demonstrated for the entire series of dimers, independent of the alkyl chain length. However, potency appears to decline for alkyl chains less than six or greater than 10 methylene groups in length. Second, inhibition of recovery appears to increase as a function of chain length, to the point where the bissuccinimidododecane dimer (**37**) exhibits an essentially irreversible blockade of bradykinin-induced smooth muscle contraction after exposure to 10^{-5} M inhibitor, despite an extended (120-min) recovery or "wash-off" period. These data suggest that this sustained interaction with the tissue may be due to hydrophobic interactions of the alkyl chain with the receptor itself or with "peri-receptor" membrane components, a mechanism which has been proposed previously to explain the long-acting selective β_2-adrenoceptor agonism of salmeterol.[23]

(22) Regoli, D.; Barabé, J. Pharmacology of Bradykinin and Related Kinins. *Pharmacol. Rev.* **1980**, *32*, 1–46.

(23) Jack, D. A Way of Looking at Agonism and Antagonism: Lessons from Salbutamol, Salmeterol and Other β-adrenoceptor Agonists. *Br. J. Clin. Pharmacol.* **1991**, *31*, 501–514.

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

monomer	structure	bissuccinimidohexane dimer	pA_2[a] monomer	pA_2[a] dimer	% recovery monomer	% recovery dimer
1	[b]		7.4 ± 0.2		100	
2	[L-Cys^0]-1	17	7.1 ± 0.4	7.9 ± 0.1	50	50
4	[L-Cys^1]-1	19	<6	7.5 ± 0.2	100	100
5	[L-Cys^2]-1	20	6.4 ± 0.2	PA[c]	80	90
6	[L-Cys^3]-1	21	PA[c]	6.2 ± 0		100
7	[L-Cys^4]-1	22	inactive	inactive		
9	[L-Cys^5]-1	24	7.2 ± 0.2	8.1 ± 0.1	95	75
10	[L-Cys^6]-1	25	7.1 ± 0.1	8.5 ± 0.3	100	50
11	[L-Cys^7]-1	27	inactive	inactive		
13	[L-Cys^8]-1	29	inactive	inactive		
14	[L-Cys^9]-1	30	inactive	inactive		

[a] pA_2 values are means ± SEM of $n = 3$. [b] D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Leu^8-Arg^9. [c] PA = partial agonist.

Table VII. Effect of Linker Length in Dimeric Analogues of 10 on Bradykinin-Induced Contraction of Rat Uterus in Vitro

no. carbon atoms in linker	no.[a]	pA_2[b]	% recovert (40 min)
2	31	8.4 ± 0.2	90
3	32	8.6 ± 0.2	90
4	33	8.3 ± 0.2	50
6	25	8.5 ± 0.3	50
8	34	8.4[c]	25
9	35	9.3 ± 0.4	10
10	36	8.6 ± 0.2	0
12	37	8.2 ± 0.3	0[d]

[a] See Tables I and IV for sequence. [b] pA_2 values are the means ± SEM of $n \geq 3$. [c] $n < 3$. [d] Irreversible at 80 min.

Effect of Other Maleimide-Based Linkers on the in Vitro Activity of Various Peptide Dimers. In order to assess further the importance of the linker element with respect to enhancing potency of these antagonists, we investigated a number of alternative linkers and modified monomers. Illustrated in Table VIII are a number of modifications of monomer **10** and/or alternative linkers used to produce compounds with which we evaluated several different aspects of the dimers described above. Except for compound **43**, which was used to assess the contribution of the succinimide moiety to the improved activity found with our previously described compounds, all of the compounds screened were either dimers or trimers. As can be seen from the data summarized in Table VIII, there does not seem to be an improvement in activity when the inhibitor is changed from a dimer to a trimer. Nor do other types of "spacing" elements besides alkyl chains appear to improve potency as well as the latter type of chemistry. Finally, while there does appear to be a small effect of the succinimide ring on measured inhibitory activity (**10** vs **43**), the "spacer" element or the geminal ligand must also play a role in the observed effect of dimerization (**43** vs **25**). The contribution of each of these components is currently being investigated more thoroughly in our laboratories.

It is important to note that when a dimer is formed by the reaction of a cysteine-containing peptide monomer with a bismaleimide linker (regardless of the spacer), three stereoisomers (i.e., diastereomers) are formed. Current separation and analytical techniques do not allow us to assess the importance of these stereochemical differences or whether there is preferential production of one of these diastereomers under our current reaction conditions. Preliminary data suggest that the two diastereomers formed by the reaction of cysteine-containing monomers with various monomaleimide-based compounds (yielding succinimide-modified monomers) are produced in equal molar amounts and have essentially equivalent activity in these simple in vitro systems (data not shown). Again, the importance of the chirality of the sulfur–succinimide ring junction is currently under investigation, as is the importance of the succinimide ring itself. The data from these investigations are still being developed and are beyond the scope of this paper.

Effect of Dimerization on Another Well-Characterized, Peptide-Based Bradykinin Antagonist. Finally, we decided to investigate whether dimerization could improve the efficacy of another well-characterized bradykinin antagonist besides compound **1**. Summarized in Table IX are the data comparing the improvement of activity found when the reference ligands **1** and **15** (NPC-567) are modified to contain cysteine in position 6 and then dimerized with bismaleimidohexane. Clearly, dimerization improves the potency of this alternative ligand (i.e., **15**) equally well as the original ligand chosen. We are currently expanding these investigations to include antagonists containing the conformationally constrained amino acid analogues D-Tic and Oic.

General Discussion

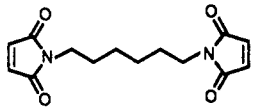
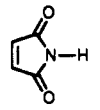
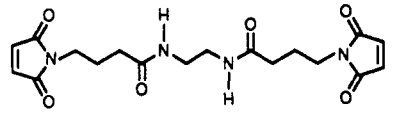
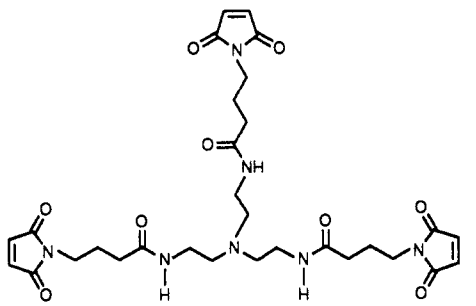
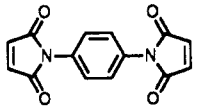
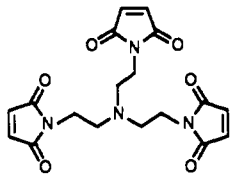
Synthetic Strategy. As mentioned in the introduction, a variety of peptide dimers have been shown to have increased potencies and durations of action when compared to their monomeric counterparts.[8-16] Most of these studies utilized the terminal amino or carboxyl groups as their points of dimerization, which in our case proved to be unsuccessful. As a result, we chose to investigate dimers utilizing an "internal" dimerization position since there was no a priori reason to assume that dimers formed from positions other than the two termini would be uniformly without activity. In order to effect this strategy, however, we had to choose some type of "orthogonal" chemistry so that dimers of unambiguous structure and composition could be easily synthesized.

Two types of alternative synthetic strategies present themselves for this task. The first would be to use an amino acid with a primary amine- (or carboxylic acid) containing side chain that could be differentially deprotected as the residue used systematically to explore the various internal dimerization positions. The second strategy would be to use an amino acid with a side-chain functionality that was inherently "orthogonal" in nature. Either approach has its advantages and disadvantages, but the wealth of alternative sulfhydryl-based conjugation chemistries made the choice of the cysteine substitution series the preferred one for these initial studies. We are currently exploring the alternative approach in a different series of dimers in order to compare and contrast these two dimerization strategies.

Mechanism(s) Responsible for Enhanced Activity. The enhanced activity of these dimers may be a function of one or more of a number of different molecular mechanisms. The most obvious (but least likely) of these is that the dimer is interacting with two receptors simultaneously and the increase in potency and duration of action is the result of bivalent binding cooperativity. This type of activity enhancement would be mechanistically similar to the enhanced binding of bivalent immunoglobulins to multivalent versus monovalent antigens.[24]

(24) Hornick, C. L.; Karush, F. Antibody affinity-III. The role of multivalence. *Biochemistry* **1972**, *9*, 325–340.

Table VIII. Effect of Different Maleimide-Based Modifiers or Linkers on Bradykinin-Induced Contractions of Guinea Pig Ileum and Rat Uterus in Vitro

no.	modifier or linker	pA_2[a] guinea pig ileum	pA_2[a] rat uterus	rat uterus % recovery
10[b]	D-Arg-Arg-Pro-Hyp-Gly-Phe-Cys(SH)-D-Phe-Leu-Arg	6.6 ± 0.2	7.1 ± 0.1	100
25[c]		7.7 ± 0.2	8.5 ● 0.3	50
26	disulfide dimer	7.2 ● 0.1	8.0 ± 0.1	100
43		6.2 ± 0.2	7.3 ± 0.2	100
38[c]		6.2 ● 0.2	7.6 ● 0.3	100
39[c]		7.5[d]	7.6[d]	50
40		6.3[d]	7.1 ± 0.4	100
41[b]		7.0[d]	8.6[d]	50

[a] pA_2 values are the means ± SEM of $n \geq 3$. [b] Reference monomer. [c] See Experimental Section for synthesis of corresponding linker; **25**, bismaleimidohexane; **38**, bismaleimidoamide (**45**); **39**, trismaleimidoamide (**46**); **41**, trismaleimidoalkane (**47**). [d] $n < 3$.

Table IX. Effect of Dimerized 1 and 15 on Bradykinin-Induced Contractions of Rat Uterus in Vitro

compound	rat uterus pA_2[a]	% recovery
1, D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Leu^8-Arg^9	7.4 ± 0.2	100
25, [BSH(L-Cys^6)]-1 dimer	8.5 ± 0.3	50
15, D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Phe^5-Ser^6-D-Phe^7-Phe^8-Arg^9	7.1 ± 0.3	100
42, [BSH(L-Cys^6)]-15 dimer	8.2 ● 0.3	40

[a] pA_2 values are the means ± SEM of $n \geq 3$.

There are three ways that bivalent interactions can be accomplished with such small dimeric structures. The first is that the receptor population is fluid with respect to membrane localization and that a dimeric compound can bring two receptors together on the surface of the responding cell population (again, in an analogous fashion to what is believed to occur on the surface of mast cells when surface bound IgE encounters a multivalent antigen).[25] Unlike these immunoglobulin receptors, whose three-dimensional structure is designed to optimize receptor cross-linking by multivalent antigens, the bradykinin receptor has evolved to bind a monovalent ligand and is not believed to require receptor cross-linking for signal transduction. As a result, it is difficult to visualize how two macromolecular, membrane-bound receptors can be spanned by dimers formed with alkane chain linkers of such short lengths. In addition, even if receptor cross-linking were possible, it is hard to explain why there is such a difference in potency between dimers formed with alkane linkers of 9 and 12 methylene groups in length (pA_2's of 9.3 and 8.2, respectively) with the *shorter* of the two being more potent.

Alternatively, the receptor population could be highly organized in the responding cell membrane (similar to the high density of nicotinic acetylcholine receptors at the neuromuscular junction).[26,27] In this case, the receptor

(25) Metzger, H.; Alcarez, G.; Holman, R.; Kinet, J-P.; Pribluda, V.; Quarto, R. The receptor with high affinity for immunoglobulin E. *Ann. Rev. Immunol.* **1986**, *4*, 419–470.

population is already at maximum spacial density and the bivalent ligand need only span the distance between two receptor binding sites. However, there are no data to suggest that the bradykinin receptor is displayed on the surface of the responding cells in such a fashion and the arguments based on the differences observed between the C_9- and C_{12}-based dimers discussed above still hold in this case.

Finally, bradykinin receptors might be displayed on the surfaces of responding cells in dimeric, trimeric, or some other oligomeric type of clusters with their ligand binding sites being arranged such that they are opposite each other (as if they were being reflected in a mirror). This type of receptor expression may be the only case that can both explain our data based on bivalent interactions and still account for the structure–activity relationships we have observed. Definitive binding studies which would help to resolve this question require the use of high specific activity radiolabeled antagonists. These compounds are currently being developed.

If bivalent binding is not the basis for the observed results, there are other explanations for the enhanced potency observed for this series of compounds. As discussed above, one possibility includes the existence of secondary binding sites in the vicinity of the primary ligand binding site that can interact with various components of the linker and/or geminal ligand. Alternatively, the existence of the linker and geminal ligand by virtue of steric and/or electrostatic interactions may impart a certain degree of "preorganization" to the primary ligand, so as to improve binding by decreasing the entropic nature of the free peptide in solution. To investigate these possibilities, we are developing a series of "heterodimers" incorporating several of the bissuccinimido linkers, one of the primary ligands (10), and various "nonsense" or "dummy" geminal ligands ranging from cysteine to glutathione to nonsense decapeptides with alanine substitutions at positions 4, 5, 7, and/or 8, which should be devoid of intrinsic antagonist or agonist activity yet retain the majority of the steric and electrostatic characteristics of the reference ligand.

In addition, dimers formed using more conformationally constrained ligands containing the modified amino acids D-Tic and/or Oic (analogues of HOE-140 for example) may also help to elucidate the mechanism of potency enhancement seen with the current series of dimers. If "preorganization" of the primary ligand by the linker and/or geminal ligand is responsible for improving the activity of the final compound, these constrained analogues should be less likely to show similar enhancements of activity.

Finally, exploration of the linker's contribution to the enhanced potency and duration of action is far from complete. Simple alkane chains appear to be highly desirable but the potential for further improvements in activity using more hydrophilic linkers, linkers with more constrained geometries (cis or trans double bonds, saturated or unsaturated ring systems, etc.), and linkers based on different conjugation chemistries (other than maleimide/succinimide conjugates) will also need to be incorporated into future compounds for a complete understanding of the role these elements play in the enhancement of activity to emerge. All of these possibilities are being investigated concurrently in our laboratories and will be the subject of future reports.

Conclusions

It appears that dimerization of peptide-based bradykinin antagonists can improve the potency of the parent ligand by as much as 100-fold, as measured in simple in vitro systems. Preliminary data indicate that this improvement in activity will, in fact, transfer into improved potency and duration of activity in a number of in vivo model systems of both bradykinin-specific and nonspecific pathophysiologic conditions. These types of compounds may be useful in the treatment of a number of disease states.

Experimental Section

Peptides were prepared using an automated peptide synthesizer (Applied Biosystems (ABI), Model 430A) according to protocols shown in Table II for a 0.5-mmol solid-phase synthesis. Peptide characterization data are given in Tables I and IV. 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-Hyp(OBzl)-OH, and Boc-Cys(Meb)-OH. *N*-(Methoxycarbonyl)maleimide and γ-maleimidobutyric acid were purchased from Sigma Chemical Co. (St. Louis, MO). Tris(2-aminoethyl)amine, 1,4-phenylenedimaleimide, maleimide, and the various *N,N'*-diaminoalkanes were obtained from Aldrich Chemical Co. (Milwaukee, WI). Isobutyl chloroformate and *N*-methylmorpholine were purchased from Fisher Scientific. Tetrahydrofuran and dimethylformamide were reagent grade and were used without further purification.

Synthetic peptides were purified by reverse-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 40–45-min period with a linear acetonitrile gradient (column 1, 0%–80%, 100 mL/min; column 2, 15%–50%, 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 reverse-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) 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[28] following 22–24-h vapor-phase hydrolysis with constantly boiling 6 M HCl. Microsequencing analyses of resin-bound peptides were performed on an automated liquid-phase sequencer (Applied Biosystems (ABI), Model 473A) coupled to an on-line Macintosh workstation. Electrospray mass spectrometry (Electrospray-MS) was performed on a R30-10 Nermag/Delsi Instruments mass spectrometer by Mr. Kevin McManus of Texas Analytical Services (Houston, TX). Thin-layer chromatography (TLC) was done on silica gel plates (E. Merck #5775) and components were visualized by fluorescence quench and/or by Cl_2/starch–KI spray.[29] Solvent systems used in TLC were as follows: 1-butanol/acetic acid/H_2O, 4:1:1 (A), and 95% CH_3CH_2OH (B).

General Procedure for the Removal of Peptide from Resin: HF Cleavage. Removal of the crude peptide as its C-terminal acid from the Pam resin was achieved by cleavage with HF.[19] 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.

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The resin was treated with anisole (1 mL) and placed back in vacuo and 10 mL of liquid HF 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 three times with dry ethyl ether to remove organic byproducts and the peptide extracted from the resin with 10% aqueous acetic acid (3 × 10 mL volumes). The combined acetic acid was evaporated and the crude peptide purified by preparative reverse-phase HPLC as described above. Lyophilization then afforded the pure peptide as a fluffy, white solid.

General Procedure for the Synthesis of Bismaleimidoalkane Linkers. Bismaleimidoalkane linkers were prepared from *N*-(methoxycarbonyl)maleimide and the appropriate *N*,*N*′-diaminoalkane as follows:[20] To a stirred solution of *N*,*N*′-diaminoalkane (1 equiv) in saturated $NaHCO_3$/THF (1:1 v/v, ca. 9 mL of total solvent per millimole of *N*,*N*′-diaminoalkane) at 0 °C was added portionwise *N*-(methoxycarbonyl)maleimide (2.4 equiv). The reaction mixture was stirred at 0 °C for 10 min, additional saturated $NaHCO_3$ and THF were added (same ratio and total volume per millimole), and the reaction mixture was then allowed to stir at room temperature for 3 h. During this time, the reaction was kept basic and clear by the addition of saturated $NaHCO_3$ and THF (same ratio and total volume per millimole) every hour. The (bismaleimidoalkane) product was then isolated by extraction into ethyl acetate. The combined ethyl acetate extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford the bismaleimidoalkane as a white to off-white solid.

Disulfide Dimer of D-Arg-Arg-Pro-Hyp-Gly-Phe-Cys-D-Phe-Leu-Arg (26). The corresponding thiol-containing monomer (**10**; 20 mg, 14.3 μmol) was dissolved in 20 mL of 0.1 M NH_4HCO_3 (pH 8) and allowed to stir at room temperature for 2 days. Reverse-phase HPLC purification (as described above) then afforded 12 mg (4.28 μmol, 60%) of **26** as a fluffy, white solid. Characterization data are given in Table IV.

***S*-Succinimidocysteine Derivative of D-Arg-Arg-Pro-Hyp-Gly-Phe-Cys-D-Phe-Leu-Arg (43).** The corresponding thiol-containing monomer (**10**; 20 mg, 14.3 μmol) was dissolved in 20 mL of 0.1 M NH_4HCO_3 (pH = 8), maleimide (5 equiv, 7 mg, 72 μmol) was added, and the resulting reaction mixture was allowed to stir at room temperature overnight. Reverse-phase HPLC purification (as described above) then afforded 18.3 mg (10.07 μmol, 70%) of **43** as a fluffy, white solid. Electrospray mass spectrum: *m*/*e* value, 1361; calcd formula weight, 1361. Amino acid analysis: Arg, Pro, Hyp, Gly, Phe, Leu (±5%), Succ-Cys (not determined quantitatively).

Conversion of γ-Maleimidobutyric Acid to Mixed Anhydride 44 with Isobutyl Chloroformate. To a stirring solution (under N_2) of γ-maleimidobutyric acid (220 mg, 1.20 mmol) and *N*-methylmorpholine (0.121 g, 1.20 mmol) in 5 mL of THF at −15 °C was added dropwise isobutyl chloroformate (0.164 g, 1.20 mmol) to generate the mixed anhydride. Following complete addition, the reaction mixture was allowed to warm slowly to room temperature over a 30-min period. It was then poured in toto into a separatory funnel containing 50 mL of water and extracted with ethyl acetate (3 × 100 mL volumes). The combined ethyl acetate was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to afford 313 mg (1.1 mmol, 92%) of product (**44**) as a viscous oil. NMR (300 MHz, $CDCl_3$): δ 0.97 (d, 6 H, *J* = 6.9 Hz), 1.90–2.10 (m, 3 H), 2.51 (t, 2 H, *J* = 7.5 Hz), 3.63 (t, 2 H, *J* = 6.9 Hz), 4.05 (d, 2 H, *J* = 6.6 Hz), 6.73 (s, 2 H). This material was used without further purification in the preparation of the bis/trismaleimidoamide linkers described below. It is a stable compound and was found to be superior to γ-maleimidobutyric acid *N*-hydroxysuccinimide ester (Sigma Chemical Co.) for carrying out amine acylation reactions.

Preparation of Bis/Trismaleimidoamide Linkers. 1,2-Diaminoethane and tris(2-aminoethyl)amine were converted into their respective bismaleimidoamide and trismaleimidoamide linkers as follows: To a stirring solution (under N_2) of mixed anhydride (**44**, 2 or 3 equiv, respectively) in THF at 0 °C was added 1 equiv of the appropriate amine. Following complete addition, the reaction mixture was allowed to warm slowly to room temperature and it was then stirred overnight. The solvent was evaporated under reduced pressure and the resulting semisolid residue dried well in vacuo. The two linkers prepared by this procedure are described below.

1,2-Bis(γ-maleimidobutyramido)ethane (45) was prepared in 98% yield (80 mg) from **44** (0.117 g, 0.413 mmol) and 1,2-diaminoethane (0.0126 g, 0.209 mmol) in 2 mL of THF. NMR (300 MHz, DMSO-d_6): δ 1.63–1.86 (m, 4 H), 2.03 (t, 4 H, *J* = 7.5 Hz), 3.29–3.55 (m, 8 H), 7.02 (s, 4 H), 7.81 (br t, 2 CO*NH*). Traces of mixed anhydride (**44**) were also visible in the NMR spectrum. This material was used without further purification.

Tris[2-(γ-maleimidobutyramido)ethyl]amine (46) was prepared in 89% yield (50 mg) from **44** (0.078 g, 0.275 mmol) and tris(2-aminoethyl)amine (0.0127 g, 0.087 mmol) in 2 mL of THF. NMR (300 MHz, DMSO-d_6): δ 1.62–1.85 (m, 6 H), 2.05 (t, 6 H, *J* = 7.5 Hz), 2.39–2.52 (m, 6 H), 3.27–3.55 (m, 12 H), 7.00 (s, 6 H), 7.69 (br t, 3 CO*NH*). Traces of mixed anhydride (**44**) were also visible in the NMR spectrum. This material was used without further purification.

Conversion of Tris(2-aminoethyl)amine to a Trismaleimidoalkane Linker. To a stirred solution of tris(2-aminoethyl)amine (0.073 g, 0.50 mmol) in saturated $NaHCO_3$/THF (V_t = 5 mL, 1:1 v/v) at 0 °C was added, portionwise, *N*-(methoxycarbonyl)maleimide (0.468 g, 3.02 mmol).[20] The reaction mixture was stirred at 0 °C for 4 h with additional saturated $NaHCO_3$/THF (10 mL/10 mL) being added every hour. The product was then isolated by extraction into ethyl acetate (3 × 100 mL volumes). The combined ethyl acetate extracts were washed with water and brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to afford 141 mg (0.365 mmol, 73%) of tris(2-maleimidoethyl)amine (**47**) as a light yellow solid. NMR (300 MHz, $CDCl_3$): δ 2.71 (t, 6 H, *J* = 6.6 Hz), 3.53 (t, 6 H, *J* = 6.6 Hz), 6.68 (s, 6 H).

General Procedure for the Synthesis of Bissuccinimido Peptide Dimers. Bissuccinimidoalkane, bissuccinimidoaryl, and bissuccinimidoamide peptide dimers were prepared from a cysteine-containing peptide monomer and the appropriate bismaleimide-containing linker as follows: To a mixture of peptide monomer (2 equiv) and bismaleimide-containing linker (1 equiv) in DMF (ca. 21 mL/mmol of peptide) was added 10 volumes of PBS (pH 7.5). The reaction mixture was stirred overnight at room temperature (monitored periodically by analytical HPLC) and the resulting bissuccinimido–peptide dimer purified by preparative reverse-phase HPLC as described above. Lyophilization then afforded the pure dimer as a fluffy, white solid.

General Procedure for the Synthesis of Trissuccinimido Peptide Trimers. Trissuccinimidoamide and trissuccinimidoalkane peptide trimers were prepared from a cysteine-containing peptide monomer (**10**) and the appropriate trismaleimide-containing linker as follows: To a mixture of peptide monomer (4–5 equiv) and trismaleimide-containing linker (1 equiv) in DMF (ca. 21 mL/mmol of peptide) was added 10 volumes of PBS (pH 7.5). The reaction mixture was stirred overnight at room temperature (monitored periodically by analytical HPLC) and the resulting trissuccinimido–peptide trimer purified by preparative reverse-phase HPLC as described above. Lyophilization then afforded the pure trimer as a fluffy, white solid.

Rat Uterus in Vitro pA_2 Measurement. Female Sprague–Dawley rats (200–250 g) were pretreated with stilbesterol (100 μg/kg sc) and killed 18 h later 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. Concentration effect curves were constructed to bradykinin in the absence and presence of antagonist (preincubated for 5 min). Antagonist potency was calculated according to the method of Arunlakshana and Schild.[30] 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 bradykinin. The pD_2 (−log molar concentration of agonist producing 50% of the original maximum response to bradykinin) for bradykinin at this time was calculated and compared to the pD_2 of the initial control concentration–effect curve

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for bradykinin. The difference in pD_2 values compared to concurrent control reflected the percentage recovery of agonist response.

Guinea Pig Ileum in Vitro pA_2 Measurement. Male Dunkin Hartley guinea pigs (350–450 g) were killed by cervical dislocation, and the ilea removed. Segments of ileum 2.5 cm in length were prepared and mounted under 2 g of resting tension in 4-mL tissue baths containing Tyrode's solution at 37 °C and bubbled with O_2/CO_2 (95%/5%). Concentration–effect curves were constructed for bradykinin in the absence and presence of the antagonist (preincubated for 5 min). Antagonist potency and recovery from antagonism were calculated as described above.

Rationally Designed "Dipeptoid" Analogues of CCK. A Free–Wilson/Fujita–Ban Analysis of Some α-Methyltryptophan Derivatives as CCK-B Antagonists

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A Free–Wilson/Fujita–Ban (FW/FB) analysis is reported on 36 "dipeptoid" antagonists of the CCK-B receptor. This series of compounds includes [*R*-(*R**,*R**)]-4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxobutanoic acid (CI-988, 1, Figure 1), the first rationally designed non-peptide antagonist of a neuropeptide receptor. The analysis treats the compounds in three parts: the N-terminus, variants on the tryptophan moiety, and the C-terminus. A highly significant correlation was found ($n = 36$, $r^2 = 0.97$, $s = 0.22$, $F = 57$, $p = 2 \times 10^{-8}$), suggesting that these three domains of these compounds contribute to binding affinity independently of each other, and are therefore additive in their effects on receptor affinity. The relative free-energies of binding of the individual substituents are calculated from the coefficients of the regression equation. The substitution of D-α-methyltryptophan for L-tryptophan increases the free-energy of binding by 3.5 kcal mol^{-1}. This increase in binding energy is explained by a 300-fold difference in conformational entropy between the methylated and desmethyl analogues.

Introduction

The synthesis of potent, highly selective non-peptide antagonists for the central cholecystokinin (CCK-B) receptor has been described previously.[1-5] The strategy involved the independent optimization of the N and C terminal structure–activity relationships (SAR) of compound 2[3] in Figure 1. Such a strategy assumes that the binding energies of the N and C terminus groups are additive when the ligand binds to the receptor. This is thought to be a reasonable approach with these semirigid molecules.

This paper describes a justification of this assumption using Free–Wilson/Fujita–Ban (FW/FB) analysis[6,7] for the binding of these "dipeptoids" to the CCK-B receptor. This quantitative structure–activity relationship (QSAR) method assumes that the individual substituents act independently of each other, and that they act in an additive fashion. Thus any deviation from this assumption should be evident in the differences between predicted and actual binding constants (K_i). The coefficients of the regression equation gained from this analysis are directly proportional to the free-energy change (ΔG) when replacing one group with another.[8] Therefore, in addition to testing the additivity hypothesis for this series a quantitative measure of the binding contribution of each group is also gained. The relative binding of the substituents are interpreted in terms of the additivity concept of the binding constants of the relevant functional groups put forward previously by Andrews et al.[9]

The choice of substituents (and hence compounds) used in any QSAR analysis is of considerable importance.[10] In

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