Synthesis and Activity of Dimeric Bradykinin Antagonists Containing Diaminodicarboxylic Acid Bridge Residues

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Abstract: Enhancement of a ligand's interaction with a receptor through presenting the ligand in multimeric form is a topic of general interest. Thus dimerization of single-chain bradykinin antagonist peptides has previously been shown to be beneficial in terms of potency and duration of action. While crosslinking polypeptides at terminal positions using suitable dicarboxylic acids and diamines is comparatively straightforward synthetically, internal dimerizations are usually achieved through oxidation or double S-alkylations of cysteine residues, resulting in metabolically unfavourable disulphide and thioether cross-links. Using suitably modified standard solid-phase peptide synthesis protocols, dimeric bradykinin antagonist peptides [H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe]$_2$-X-[(D-Phe)-Leu-Arg-OH]$_2$ were synthesized where X corresponds to a L,L-2,7-diaminosuberic or L,L-2,9-diaminosebacic acid residue, respectively. The biological activity of these peptides was comparable to that of conventional dimeric bradykinin antagonists cross-linked through cystine or bis(succinimido)alkyl bridges. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Bradykinin antagonist; dimer; diaminodicarboxylic acid; bridge residue

INTRODUCTION

Bradykinin (H-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH) acts on smooth muscle, dilates peripheral vessels, increases vascular permeability and is a potent pain-producing agent. Because of the latter property and because of bradykinin's involvement in many pathophysiological processes, there exists an interest in developing potent bradykinin B$_2$ receptor antagonists as potential therapeutic agents for the treatment of inflammation and pain.
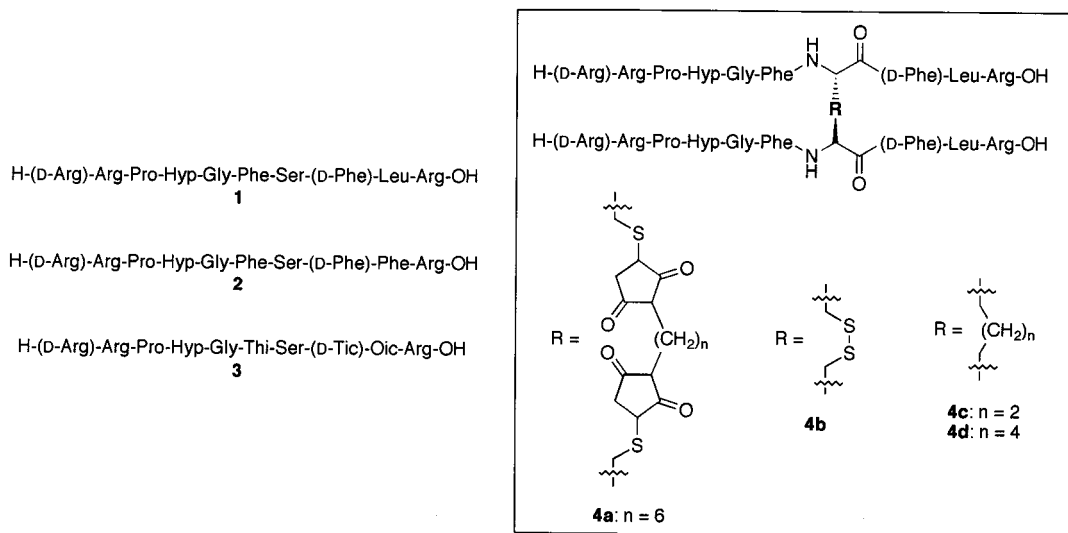
Replacement of Pro^7 in bradykinin with D-Phe confers antagonism properties on the resulting analogue [1]. A marked and selective increase in affinity for the B$_2$ receptor is achieved by replacing Pro^3 with Hyp and by extension of the peptide chain at the amino terminus with D-Arg [2]. Antagonist affinity for both the B$_1$ and B$_2$ receptors is increased by the replacement of Phe^5 or Phe^8 with Thi [3]. Similar effects are achieved with either D-Phe in position 7 or with Leu in position 8, provided D-Arg^0 and Hyp^3 are present (compounds **1** and **2**, Scheme 1). Combination of the D-Phe^7 and Leu^8, and particularly D-Tic^7 and Oic^8, replacements enhances affinity and confers metabolic stability [4]. These findings have led to the most potent bradykinin antagonist known to date (HOE-140, peptide **3**) [5].

A different approach towards increased potency and resistance to metabolic inactivation is provided by dimerization of single-chain bradykinin peptide

H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe-Ser-(D-Phe)-Leu-Arg-OH
1

H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe-Ser-(D-Phe)-Phe-Arg-OH
2

H-(D-Arg)-Arg-Pro-Hyp-Gly-Thi-Ser-(D-Tic)-Oic-Arg-OH
3

Scheme 1

antagonists. Additionally, this approach permits combination of two single-chain antagonists with different receptor subtype specificities [6]. Enhancement of a ligand's interaction with a receptor through presenting the ligand in multimeric form has been shown to be feasible in several systems [7–13]. It is thought that such enhancement operates through an increase of the effective local ligand concentration at the receptor and, in some cases, through actual multivalent interactions of the ligand with the receptor. In a systematic study of dimerizations based on the single-chain bradykinin antagonist **1**, Cheronis *et al.* [14] showed that dimerization at position 6 through a bis(succinimido)alkane linker containing a six-carbon alkyl spacer (**4a**) resulted in optimal activity *in vitro*. We were interested in replacing such disulphide and thioether bridges with non-reducible and stable alkane moieties. This can be achieved with the aid of appropriate diaminodicarboxylic acids [15,16] through solid-phase peptide synthesis without the need for post-assembly dimerization.

MATERIALS AND METHODS

General

Bis(Fmoc)-L,L-2,7-diaminosuberic acid and bis-(Fmoc)-L,L-2,9-diaminosebacic acid [16,17] were synthesized using Schöllkopf asymmetric alkylation technology [18]. Peptides **1–3** were obtained from Bachem AG, Switzerland.

Amino acid derivatives and general peptide synthesis reagents were obtained from Novabiochem AG and Bachem AG (Switzerland), as well as from Applied Biosystems Inc. (USA). DMF and TFA were from Rathburn Ltd (UK). Other reagents and solvents used were of the highest commercially available grades.

Amino acid compositions were determined by the Amino Acid Analysis Laboratory, University of Uppsala, Sweden. MALDI-TOF MS was performed using a Lasermat 2000 instrument (Thermo Bioanalysis Ltd, Hemel Hempstead, UK). The matrix used was α-cyano-4-hydroxycinnamic acid. RP-HPLC was carried out using Beckman System Gold equipment and Vydac columns (218TP1022 for preparative, and 218TP54 for analytical work). Gradient elution with MeCN/H_2O) (0.1% TFA) was applied.

Cystine Dimer 4a and Bis(succinimido)hexane Dimer 4b

Both these peptides were prepared, essentially as described [14], from the common precursor H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe-Cys-(D-Phe)-Leu-Arg-OH, the former by air oxidation (1 mg/ml; pH 7.4), the latter by selective *S*-alkylation with bis(-maleimido)hexane (2:1 molar ratio of peptide/crosslinker; 1:10 DMF/H_2O pH 7.4). Reactions were followed by analytical RP-HPLC (oxidation complete after 13 days, alkylation complete after 1 h). The reaction mixtures were concentrated and chromatographed by preparative RP-HPLC to afford the pure title peptides. Cystine dimer **4a**: analytical

RP-HPLC $- t_R = 22.7$ min, purity 98% (5–50% MeCN over 30 min, 1 ml/min, $\lambda = 215$ nm); MALDI-TOF MS $- [M + H]^+ = 2526$; $C_{114}H_{177}N_{38}O_{24}S_2$ requires 2526.3. Bis(succinimido)hexane dimer **4b**: analytical RP-HPLC $- t_R = 13.6$ min, purity 98% (5–75% MeCN over 20 min, 1 ml/min, $\lambda = 215$ nm); MALDI-TOF MS $- [M + H]^+ = 2805$; $C_{128}H_{195}N_{40}O_{28}S_2$ requires 2804.4.

Dimeric Peptides 4c and 4d

Using a 'bubbler' apparatus for manual peptide synthesis [19], Fmoc-Arg(Pmc)-Tentagel S PHB resin (2 g, 0.42 mmol; from Rapp Polymere, Tübingen, Germany) was Fmoc-deprotected with 20% piperidine/DMF during 20 min and was then washed extensively with DFM. The drained resin was acylated with Fmoc-Leu-OH, PyBOP and HOBt (2 mmol each) and NMM (3 mmol) in DMF (4 ml) during 2 h and was again washed with DMF. After repeated deprotection, a further acylation was carried out similarly using Fmoc-(D-Phe)-OH. Aliquots of the drained resin (0.2 mmol each) were again deprotected, washed and reacted with bis(Fmoc)-2,7-diaminosuberic acid or bis(Fmoc)-2,9-diaminosebacic acid (0.08 mmol), PyBOP (0.16 mmol), HOBt (0.16 mmol) and NNM (0.24 mmol) in DMF (1 ml) for 18 h. Unreacted resin-bound amino groups were then capped by reaction with excess acetic anhydride in DMF (2×10 min). The resin samples were washed and transferred to reaction cartridges of an ABI 433A peptide synthesizer. The remaining residues were assembled employing the standard single-couple (1 mmol scale) chemistry programme, successively using the following amino acid derivatives: Fmoc-Phe-Oh, Fmoc-Gly-OH, Fmoc-Hyp(Bu^t)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pmc)-OH and Fmoc-D-Arg(Pmc)-OH. After completed assembly and terminal Fmoc-deprotection, the resin aliquots were washed successively with DMF, CH_2Cl_2 and Et_2O) and were dried. Cleavage and side-chain deprotection was performed for 2 h with TFA containing phenol (5%) and H_2O (5%). Resin residue was removed by filtration and was washed with neat TFA. The combined filtrate and washings from each cleavage were evaporated under reduced pressure and the crude peptides were precipitated with Et_2O. They were collected by centrifugation and were dried under high vacuum.

The crude peptides were purified in aliquots of *ca.* 20 mg by preparative RP-HPLC (5–40% MeCN over 50 min at 10 ml/min). Semi pure material from two runs was pooled and rechromatographed (same conditions) to afford the pure peptides (purification yield *ca.* 20%) [H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe]$_2$-(2,7-diaminosuberyl)-[(D-Phe)-Leu-Arg-OH]$_2$ **(4c)**: analytical RP-HPLC $- t_R = 17.6$ min, purity 98% (5–50% MeCN over 20 min, 1 ml/min, $\lambda = 215$ nm); MALDI-TOF MS $- [M + H]^+ = 2490$; $C_{116}H_{181}N_{38}O_{24}$ requires 2490.4; amino acid analysis–Arg, 5.86 (6); Pro, 2.03 (2); Hyp, 2.10 (2); Gly, 1.98 (2); Phe, 3.87 (4); Leu, 2.00 (2). [H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe]$_2$-(2,9-diaminosebacyl)-[(D-Phe)-Leu-Arg-OH]$_2$ **(4d)**: analytical RP-HPLC $- t_R = 16.8$ min, purity 98% (5–50% MeCN over 20 min, 1 ml/min, $\lambda = 215$ nm); MALDI-TOF MS $- [M + H]^+ = 2519$; $C_{118}H_{185}N_{38}O_{24}$ requires 2518.4; amino acid analysis $-$ Arg, 6.15 (6); Pro, 1.92 (2); Hyp, 2.11 (2); Gly, 1.78 (2); Phe, 4.06 (4); Leu, 2.02 (2).

RESULTS AND DISCUSSION

The lead structures for these studies were the bis(-succinimido)alkane peptide dimer **4a** (BRADYCOR) and the cystine peptide dimer **4b**, which were prepared from H-(D-Arg)-Arg-Pro-Hyp-Gly-Phe-Cys-(D-Phe)-Leu-Arg-OH by selective *S*-alkylation with bis(maleimido)hexane and by oxidation, respectively, as described [14].

The novel peptides **4c** and **4d**, on the other hand, were prepared using *N*-Fmoc-protected [17] derivatives of L,L-2,7-diaminosuberic acid and L,L-2,9-diaminosebacic acid, respectively [15,16]. Standard peptide synthesis methods [20] with appropriate modifications for obtaining the resin-bound dimeric intermediates, were employed. It was found that acylation of H-(D-Phe)-Leu-Arg-Resin with somewhat less than one half molar equivalent of Fmoc-protected diaminodicarboxylic acid favoured formation of the desired dimeric resin-bound intermediate while preventing formation of the intermediate with only one of the carboxyl groups of the diaminodicarboxylic acid residue amidated to the tripeptidyl resin. Unreacted peptidyl resin amino groups were then capped by acetylation prior to Fmoc-deprotection and completion of peptide assembly. The protected dimeric decapeptidyl resins were cleaved and deprotected, affording the desired peptides as the only major products (Figure 1).

The bradykinin B$_2$ receptor antagonistic potency of compounds **1–4** was tested; the results are summarized in Table 1. The pA_2 values obtained for the single-chain antagonists **1–3** are in good agreement with those published previously using a similar bioassay. A statistically significant potency differ-

ence between the bis(succinimido)alkane dimer **4a** and the simple cystine dimer **4b** was not found, and the potency of peptide **4a** was lower than previously reported. Replacement of cystine in **4b** with its isosteric dicarba analogue 2,7-diaminosuberic acid surprisingly resulted in a dimer peptide **4c** which was distinctly less potent. Increasing the chain length of the bridge amino acid improved activity, resulting in a dimeric bradykinin antagonist **4d** which was equipotent with the cystine dimer **4b** and was marginally more potent than the parent single-chain compound **1**, as well as the related compound **2**.
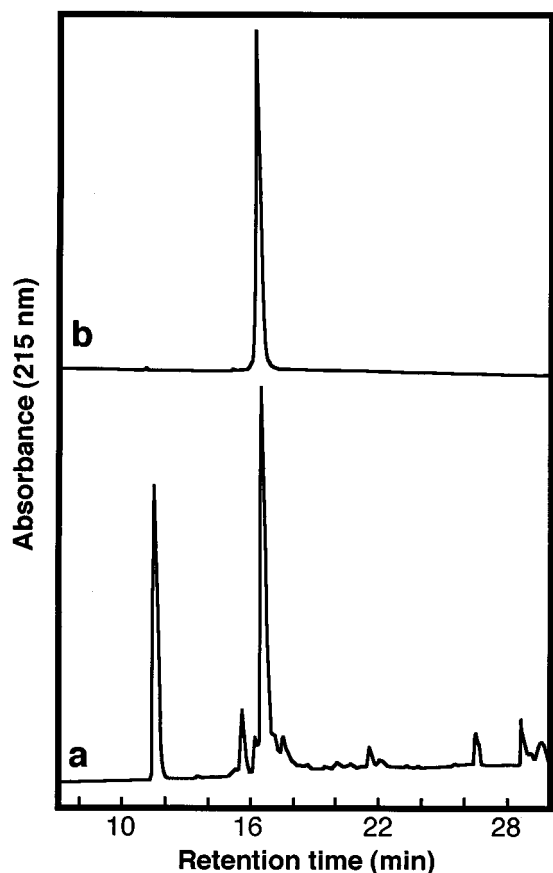

Figure 1 HPLC analysis pf peptide **4d**. Crude product after peptidyl resin cleavage and deprotection (a) and purified peptide (b). HPLC conditions: Vydac 218TP54 column (octadecylsilane; 4.6 × 250 mm), 1 ml/min flow rate, gradient elution from 5 to 50% acetonitrile in water (containing 0.1% TFA) over 20 min. The eluent corresponding to the peak at 11.5 min in (a) does not contain peptidic material but is due to cleavage artefacts.

Table 1 Antagonistic Effect of Peptides on Bradykinin-induced Guinea Pig Ileum Contraction[a]

Compound	Biological activity	
	pA_2 measured[b]	pA_2 published
1	6.3 ± 0.3	6.5 ± 0.2 [14]
2	6.0 ± 0.1	5.9 [23]
3	8.4 ± 0.2	8.5 [5]
4a	6.4 ± 0.4	7.7 ± 0.2 [14]
4b	6.9 ± 0.2	7.2 ± 0.1 [14]
4c	5.9 ± 0.4	
4d	6.8 ± 0.2	

[a] An assay system was used essentially as described [14].
[b] Average and standard deviation derived from three experiments.

CONCLUSIONS

These results show that in principle it is possible to dimerize single-chain bradykinin antagonist **1** through the introduction of diaminodicarboxylic acid residues in place of Cys[6], without loss of potency. From Table 1 it can be seen that none of the dimeric bradykinin antagonists **4** approach the potency of the single-chain antagonist **3**. Recent results with conventionally dimerized third generation bradykinin antagonists [21,22] would indicate that the dimerization methods introduced here will be applicable to such peptides and should provide highly potent, stable antagonists.

Acknowledgements

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