Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 164–170
Published online 31 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.827

JOURNAL OF PEPTIDE SCIENCE

Bradykinin analogs containing the 4-amino-2-benzazepin-3-one scaffold at the *C*-terminus

S. BALLET,[a] R. DE WACHTER,[a] K. VAN ROMPAEY,[a] Cs. TÖMBÖLY,[b] D. FEYTENS,[a] G. TÖTH,[b] L. QUARTARA,[c] P. CUCCHI,[c] S. MEINI[c] and D. TOURWÉ[a]*

[a] Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium
[b] Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, Temesvàri krt. 62, H-6726 Szeged, Hungary
[c] Menarini Ricerce srl., Pharmacology Department, Via Rismondo 12A, I-50131 Florence, Italy

Received 9 October 2006; Revised 27 October 2006; Accepted 31 October 2006

Abstract: High affinity peptide ligands for the bradykinin (BK) B_2 subtype receptor have been shown to adopt a β-turn conformation of the *C*-terminal tetrapeptide (H-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH). We investigated the replacement of the Pro^7-Phe^8 dipeptide moiety in BK or the D-Tic^7-Oic^8 subunit in HOE140 (H-D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9-OH) by 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one templates (Aba). Binding studies to the human B_2 receptor showed a correlation between the affinities of the BK analogs and the propensity of the templates to adopt a β-turn conformation. The L-spiro-Aba-Gly containing HOE140 analog BK10 has the best affinity, which correlates with the known turn-inducing property of this template. All the compounds did not modify basal inositolphosphate (IP) output in B_2-expressing CHO cells up to 10 μM concentration. The antagonist properties were confirmed by the guinea pig ileum smooth muscle contractility assay. The new amino-benzazepinone (Aba) substituted BK analogs were found to be surmountable antagonists. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: bradykinin; HOE 140; 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-ones; β-turn conformation; B_2 antagonists

INTRODUCTION

Bradykinin (BK, H-Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9-OH) is implicated in a number of physiological and pathophysiological processes. It plays a key role in inflammatory diseases, vascular permeability and allergic reactions [1–3]. It is a potent elicitor of pain and is also suggested to be a growth factor for prostate and small cell lung carcinoma [4–6]. Its biological effects are mediated by two types of receptors, B_1 and B_2. Activation of B_2 receptors mediates nociception [7], therefore B_2 antagonists have antinociceptive effects [8]. The search for effective BK receptor antagonists has been going on since 1984 [9]. Key modifications in the first generation B_2 antagonists were the replacement of Pro^7 by D-aromatic amino acids (blocking ACE) and the addition of an extra D-Arg residue to the *N*-terminus, which blocks the enzymatic degradation by aminopeptidase P and consequently increases the lifetime *in vivo* [10]. The second and third generation antagonists contained conformationally constrained amino acids (D-Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and Oic = octahydroindole-2-carboxylic acid), as exemplified by HOE 140 (H-D-Arg^0-Arg^1-Pro^2-Hyp^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9-OH), which were capable of reducing the impact of carboxypeptidase N [11,12].

Since ACE cleaves BK at the Pro^7-Phe^8 position, Amblard proposed that ACE inhibitors might display features that are complementary to the BK receptor [13–16]. Therefore, the dipeptides Pro^7-Phe^8 and D-Tic^7-Oic^8 in BK and HOE 140, respectively, were replaced by the core of various ACE inhibitors. The introduction of the dipeptidomimetic D-BT **1** ((*S*)-[3-amino-4-oxo-2,3-dihydro-5*H*-benzo[b] [1,4] thiazepin-5-yl] acetic acid) in BK or in the HOE 140 sequence produced potent and selective B_2 receptor ligands, which unexpectedly turned out to be agonists [14,15].

Conformational analysis has revealed that potent peptide ligands for the B_2 receptor adopt a *C*-terminal type II′ β-turn at the level of the residues 6–9 in BK, i.e. Ser^6-Pro^7-Phe^8-Arg^9 [17–20]. The D-BT dipeptomimetic **1** adopts a type II′ β-turn in the solid state as well as in solution, as evidenced by IR, NMR and X-ray analysis [16]. This confirms the suggestion that high affinity for the B_2 receptor is related to a high propensity of the BK analogues to adopt a *C*-terminal β-turn conformation.

The structural resemblance between the D-BT **1** moiety and the 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) scaffolds (structures **2** to **6**) urged us to examine the effects of introducing this type of structure into the BK and HOE 140 sequence (Figure 1). Moreover, this dipeptide mimic has also been used to prepare ACE inhibitors [21]. Since we have recently demonstrated that Aba-containing dipeptide mimetics

*Correspondence to: D. Tourwé, Department of Organic Chemistry, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium; e-mail: datourwe@vub.ac.be

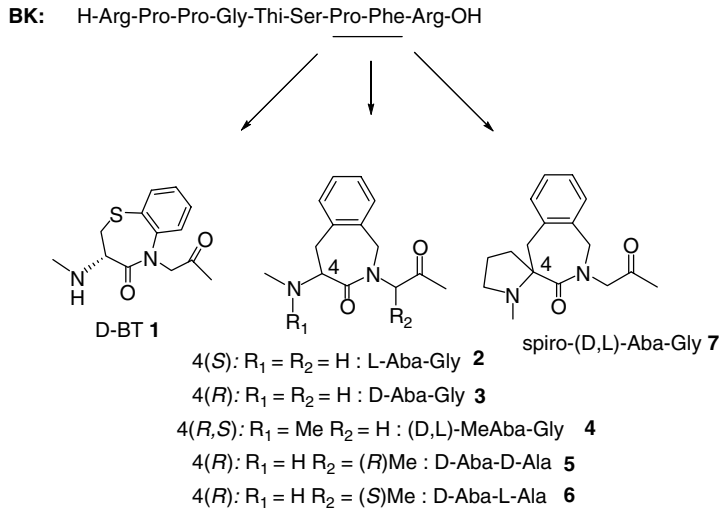

Figure 1 The structures of the D-BT motif **1** and Aba scaffolds **2–7**.

do not adopt turn conformations, and that the spiro-Aba dipeptidomimetic **7** strongly prefers a turn structure [22], the incorporation of these scaffolds into the BK and HOE 140 sequences provides a means to further probe the β-turn hypothesis. Very recently, the importance of the conformation of the *C*-terminus of BK was further demonstrated by incorporation of a dipeptide mimetic β-turn inducer [23].

MATERIALS AND METHODS

General Information

Boc-Arg(Tos)-Merrifield resin, 2–1*H*(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and Boc-amino acids were purchased from Senn Chemicals (Dielsdorf, Switzerland). D-α-(*o*-cyanobenzyl)proline was donated by Bioquadrant (Laval, QC, Canada).

[^{3}H]-BK (specific activity 90 Ci mmol^{-1}) and myo-[1,2-^{3}H] inositol (specific activity 74.7 Ci · mmol^{-1}) were provided by PerkinElmer New England Nuclear (Boston, MA, USA). BK and bestatin were obtained from Peninsula (St. Helens, UK). Leupeptin was obtained from Boehringer Mannheim (Germany) and Thiorphan from Bachem (Essex, UK). MERGEPTA was from Calbiochem (La Jolla, CA, USA). All salts used were purchased from Merck (Darmstadt, Germany). All other materials were obtained from Sigma (St. Louis, LA, USA). HOE 140 was synthesized in Menarini Ricerche (Florence, Italy). All compounds were stored at −25 °C.

Thin layer chromatography (TLC) was performed on a plastic sheet precoated with silica gel 60F$_{254}$ (Merck, Darmstadt, Germany) using specified solvent systems. Melting points were determined on a Büchi B$_{540}$ melting point apparatus (Flawil1, Switzerland) with a temperature gradient of 1 °C min^{-1}. Mass spectrometry (MS) was recorded on a VG Quattro II spectrometer using electrospray (ESP) ionization (positive or negative ion mode). Data collection was done with Masslynx software. Analytical RP-HPLC was performed using an Agilent 1100 Series system (Waldbronn, Germany) with a Supelco Discovery BIO Wide Pore (Bellefonte, PA, USA) RP C-18 column (25 cm × 4.6 mm, 5 μm) using UV detection at 215 nm. The mobile phase (water/acetonitrile) contained 0.1% TFA. The standard gradient consisted of a 30 min run from 3 to 97% acetonitrile at a flow rate of 1 ml min^{-1}. Preparative RP-HPLC was performed on a Gilson apparatus, and controlled with the software package Unipoint. The Reverse Phase C-18 column (Discovery BIO Wide Pore 25 cm × 21.2 mm, 10 μm) was used under the same conditions as the analytical RP-HPLC, but with a flow rate of 20 ml min^{-1}. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 250 MHz and 63 MHz, respectively, on a Bruker AC 250 spectrometer. Calibration was done with TMS (tetramethylsilane) or residual solvent signals as the internal standard. The solvent used is mentioned in all cases and the abbreviations used are as follows: s (singlet), d (doublet), dd (double doublet), ps t (pseudo triplet), t (triplet), q (quadruplet), p (pentuplet), br s (broad singlet), m (multiplet), M (massive).

Peptide Synthesis

All peptides in this study were synthesized manually by the Boc solid-phase methodology using TBTU and HOBt as the coupling reagents. Boc-Arg(Tos)-Merrifield resin (loading 0.57 mmol g^{-1}) was placed into a 50 ml glass vial with fritted disc and swollen in CH_2Cl_2 (20 ml) for 1 h. The Boc protecting group on the resin was removed by a 49% TFA/49% DCM/2% anisole mixture (5 min+15 min). The resin was washed with DMF (3 × 15 ml) and then with DCM (3 × 15 ml). Boc-Aba-Xxx-OH (3 equiv.) and TBTU/HOBt (3 equiv.) were dissolved in 10 ml of DMF and then NMM (9 equiv.) was added. The coupling mixture was transferred into the glass vial with the resin and shaken for 2.5 h. The resin was washed three times with DMF (15 ml) and three times with DCM (15 ml). Completion of the coupling was tested by means of the Kaiser or NF-31 test. In case of a positive color test, the coupling was repeated until a negative test was obtained. The next amino acid was consecutively coupled using the procedure described above. The removal of the orthogonal protecting groups as well as final cleavage of the peptide from the resin were induced by HF$_{(l)}$ treatment (Asti, France). After lyofilization, final purification was accomplished by preparative RP-HPLC. The purified peptides (>95% purity) were isolated and their structures were confirmed by electrospray ionization (ESI)

DOI: 10.1002/psc

Table 1 Physicochemical Properties of the BK Hybrid Peptides

No.	Sequence	*m/z* (M + 1)		HPLC[a]		TLC[b]	
		Calcd	Obsd	System 1	System 2	System 1	System 2
BK1	H-Arg-Pro-Pro-Gly-Phe-Ser-D-Aba-Gly-OH	1031.5	1032	13.8	16.8	0.33	0.63
BK2	H-Arg-Pro-Pro-Gly-Phe-Ser-(D,L)-MeAba-Gly-OH	1045.6	1046	12.7	16.3	0.37	0.61
BK3	H-Arg-Pro-Pro-Gly-Phe-Ser-D-Aba-L-Ala-OH	1045.6	1046	11.0	16.0	0.33	0.59
BK4	H-Arg-Pro-Pro-Gly-Phe-Ser-D-Aba-D-Ala-OH	1045.6	1046	11.3	16.6	0.37	0.61
BK5	H-Arg-Pro-Pro-Gly-Phe-Ser-spiro-(D or L)-Aba-Gly-OH	1071.6	1072	12.3	17.2	0.33	0.69
BK6	H-Arg-Pro-Pro-Gly-Phe-Ser-spiro-(D or L)-Aba-Gly-OH	1071.6	1072	12.5	17.3	0.35	0.67
BK7	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Aba-Gly-OH	1209.6	1210	9.7	15.0	0.37	0.63
BK8	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-L-Aba-Gly-OH	1209.6	1210	8.0[c]	15.3	0.35	0.70
BK9	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-spiro-Aba-Gly-OH	1249.6	1250	11.0	16.3	0.37	0.67
BK10	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-L-spiro-Aba-Gly-OH	1249.6	1250	11.1	16.4	0.39	0.55

[a] HPLC: RP C-18 column: Supelco Discovery BIO Wide Pore, 25 cm × 5 4.6 mm, 5 μm. System 1: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 3–97% B in A over 30 min, flow rate 1.2 ml min^{-1}.
[b] TLC System 1: EtOAc : nBuOH : AcOH : H_2O 1 : 1 : 1 : 1. TLC System 2: nBuOH : AcOH : H_2O 4 : 2 : 1.
[c] Run over 20 min. System 2: solvent A, 0.1% TFA in water, solvent B, 0.1% TFA in MeOH; gradient, 3–97% B in A over 20 min, flow rate 1.2 ml min^{-1}.

mass spectrometry. The physicochemical properties of the BK hybrid peptides are shown in Table 1.

Radioligand Binding Studies

Binding experiments were performed in *N*-tris[hydroxymethyl] methyl-2-aminoethanesulphonic acid (10 mM, pH 7.4) containing 1,10-phenanthroline (1 mM), bacitracin (140 μg ml^{-1}) and bovine serum albumin (1 g l^{-1}) on membranes from a pool of CHO cell clones stably expressing the human B_2 receptor as previously described [24]. Experiments were performed at room temperature for an incubation time of 60 min and in a final volume of 0.5 ml. Competition-binding experiments were carried out at [^{3}H]BK radioligand concentration comparable with the calculated K_d value (0.1–0.2 nM). Competing ligands were tested in a wide range of concentrations (1 pM–10 μM). Nonspecific binding was defined as the amount of radioligand bound in the presence of 1 μM of unlabelled BK, and represented less than 10% of the total bound [^{3}H]BK. Each experiment was performed in duplicate. All incubations were terminated by rapid filtration through UniFilter-96 plates (Packard Instrument Company) presoaked for at least 2 h in polyethylenimine 0.6%, and using a MicroMate 96 Cell Harvester (Packard Instrument Company). After washing 5 times with 0.5 ml aliquots of Tris buffer (50 mM, pH 7.4, 4 °C), the filters were dried and soaked in 50 μl per well of Microscint 40 (Packard Instrument Company), and the bound radioactivity was counted by a TopCount Microplate Scintillation Counter (Packard Instrument Company).

Inositol Phosphate (IP) Determination

Cells grown in the 24 wells were labelled for 24 h with *myo*-[1,2-^{3}H]inositol (0.5 ml per well, 1 μ Ci/ml) in Iscove' modified Dulbecco's medium (IMDM) and Ham's F12 Medium (F12) (1 : 1) containing fetal bovine serum dialyzed 1% and L-glutamine (2 mM). After a 15-min preincubation period at 37 °C in a buffer consisting of Dulbecco's phosphate-buffered saline (PBS), Ca^{2+}/Mg^{2+} free (135 mM) HEPES (20 mM), $CaCl_2$ (2 mM), $MgSO_4$ (1.2 mM), ethylene glycol tetraacetic acid (EGTA) (1 mM), glucose (11.1 mM), bovine serum albumin 0.05% and LiCl (25 mM) (IP buffer) cells were stimulated for 30 min at 37 °C in 0.5 ml of IP buffer added with different concentrations of the agonist. Antagonists were added 15 min prior to stimulation with the agonist. IP were then extracted and isolated with anion exchange chromatography as previously described [25].

Smooth Muscle Contractility Assay

Guinea pig ileum longitudinal smooth muscle-myenteric plexus smooth muscle preparation was undertaken as previously described [26]. Smooth muscle strips were placed in organ baths (5 ml capacity) containing oxygenated and gassed (95% O_2, 5% CO_2) Krebs'solution. Mechanical activity was isotonically recorded (load: 5 mN; Basile 7050 pen recorder). After a 1-h equilibration period, BK (1 μM) was administered 3–4 times to the preparations at 20-min intervals to assay sensibility and reproducibility of the contractile response. Afterwards, a cumulative concentration-response curve to BK (1 nM–1 μM) was constructed. At the end of each curve, the maximal contractile response of the preparation was evaluated by administration of KCl (80 mM). After washout and recovery of basal tone, the concentration-response curve to BK was repeated in the presence of the receptor antagonist. Peptidase inhibitors (thiorphan, bestatin, and captopril, 1 μM) were added 15 min prior to determination of the bradykinin-induced concentration-response curve, and the antagonists' contact time was 15 min.

Analysis of Data

Each value in the text is the mean value ±S.D. Competition-binding data were fitted by nonlinear regression using

DOI: 10.1002/psc

GraphPad Prism 4.0 (GraphPad, San Diego, CA) in order to determine the ligand concentration inhibiting the radioligand binding of the 50% (IC_{50}) competition experiments. K_i values were calculated from IC_{50} using the Cheng–Prusoff equation ($K_i = IC_{50}/(1 + [\text{radioligand}]/K_d)$). Functional data were fitted by sigmoidal nonlinear regression (GraphPad Prism 4.0) to determine the agonist concentration producing 50% (EC_{50}) of the maximal response (Emax) from the concentration-response curves. The nature of antagonists' interactions with the B_2 receptor was studied by performing the Schild analysis, and the apparent affinities of the antagonists were expressed in terms of pA_2 values.

Molecular Modeling

The global minimum and an ensemble of low-energy conformations of Ac-L-spiro-Aba-Gly-NHMe were obtained by molecular modeling [27] using Macromodel 5.0 [28] and the GB/SA solvation model [27]. Duplicate structures and those greater than 50 kJ mol^{-1} above the global minimum were discarded. The ensembles of generated structures were clustered into families using Xcluster 1.7. A RMDS value of 0.2 Å was used.

Ac-D-Pro-L-Phe-NHMe was built using the torsional angles for an ideal II′ β-turn: $(\varphi, \psi)_{i+1} = (60, -120)$; $(\varphi, \psi)i_{+2} = (-80, 0)$ and with $X_1(\text{Phe}) = -60°$ [16]. The MM3* force field [29] was used *in vacuo* for the energy minimization on this structure. The minimization with constraints was carried out with the Polak–Ribière conjugate gradient method as implemented in Macromodel 5.0, using a gradient convergence criterion of 0.02 kJ mol^{-1} Å.

All the atom pairs of the backbone were used for the superimpositions of the structures (shown later in Figure 3).

Synthesis of Benzazepinones

(4(R,S)-(Boc-methylamino)-1,2,4,5-tetrahydro-2-benzazepin-3-one-2-yl) acetic acid (Boc-(D,L)-MeAba-Gly-OH) 4. Boc-(D,L)-MeAba-Gly-OBn (600 mg, 1.37 mmol, 1 equiv.) [30] was dissolved in dioxane/water (3 : 2, 60 ml). 10% Pd/C (10 wt%, 60 mg) was added and the suspension was hydrogenated in a Parr apparatus (50 psi, r.t., 2 h), after which the mixture was filtered over dicalite. The filtrate was evaporated and lyofilized from AcN : H_2O (1 : 1). A white powder was obtained.

Yield: 464 mg, 97%. Formula, $C_{18}H_{24}N_2O_5$; MW, 348.40; m.p., 110–127 °C; MS (ES^-): 247 (-Boc), 291 (-*t*Bu), 347 ($M-H^-$); HPLC: $t_{ret} = 20.5$ min, R_f (EtOAc/MeOH 2 : 1) 0.53. ^{1}H NMR ($CDCl_3$, 250 MHz) δ_H 1.43 (9H, s, *t*Bu), 2.97 (4H, broad s, N–Me + H_β), 3.43 (1H, m, $H_{\beta'}$), 4.26 (3H, M, H_α Gly + 2 H_ε), 4.83 (1H, m, $H_{\alpha'}$ Gly), 5.15 (1H, m, H_α), 7.10–7.41 (4H, M, H arom) ^{13}C NMR ($CDCl_3$, 63 MHz) δ_C 28.37 (CH_3 Boc), 31.75 (N-Me), 34.35 (CH_2 β), 44.68 (CH_2 ε), 53.33 (CH_2 Gly), 55.64 (CH α), 80.38 (C_q Boc), 126.64, 128.13, 128.66, 130.13 (CH arom), 134.55, 135.68 (C_q arom), 156.46 (C=O Boc), 172.92 (2 C=O).

(2R)-2-{(4R)-4-((tert-butoxycarbonyl)amino)-3-oxo-1,2,4,5-tetrahydro-2H-2-benzazepin-2-yl} propanoic acid (Boc-D-Aba-D-Ala-OH) 5. The product was prepared by standard Boc-protection using Boc_2O as the protecting agent and Et_3N as a base in a dioxane : water (9 : 1) mixture [30].

Yield: 446 mg, 90%. Formula: $C_{18}H_{24}N_2O_5$. MW: 348.4, m.p., 115.9–118.1 °C; MS (ES^+), 349 ($M^+ + 1$); HPLC: $t_{ret} =$ 19.6 min, R_f (EtOAc/MeOH 3 : 7) 0.67. ^{1}H NMR (DMSO-d_6, 250 MHz): δ_H 1.18 (3H, d ($J = 7$ Hz), CH_3 Ala), 1.41 (9H, s, *t*Bu), 2.88–3.10 (2H, m, $H_\beta + H_{\beta'}$), 4.15 (1H, d($^2J =$ 17 Hz), H_ε), 4.83 (1H, d($^2J = 17$ Hz), $H_{\varepsilon'}$), 5.0–5.13 (2H, m, H_α Aba + H_α Ala), 7.13–7.29 (4H, M, H arom.) ^{13}C NMR (DMSO-d_6, 63 MHz)- δ_C 15.7 (CH_3 Ala), 28.6 (CH_3 Boc), 36.2 (CH_2 β), 48.0 (CH_2 ε), 49.5 (CH α Ala), 52.9 (CH α benzazepine), 78.6 (C_q Boc), 126.4, 127.7, 128.8, 130.7 (4 CH arom), 135.4, 135.8 (2 C_q arom), 155.1 (C=O Boc), 171.9 (C=O COOH), 173.2 (C=O $CONR_2$).

(2S)-2-{(4R)-4-((tert-butoxycarbonyl)amino)-3-oxo-1,2,4,5-tetrahydro-2H-2-benzazepin-2-yl} propanoic acid (Boc-D-Aba-L-Ala-OH) 6. The product was prepared by standard Boc-protection using Boc_2O as the protecting agent and Et_3N as a base in a dioxane : water (9 : 1) mixture [30].

Yield, 459 mg; quantitative Formula, $C_{18}H_{24}N_2O_5$; MW, 348.4; m.p., 116.2–118.4 °C; MS (ES^+), 349 ($M^+ + 1$). HPLC: $t_{ret} = 19.4$ min; R_f (EtOAc/MeOH 3 : 7) 0.65. ^{1}H NMR (DMSO-d_6, 250 MHz): δ_H 1.24 (3H, d ($J = 7$ Hz), CH_3 Ala), 1.39 (9H, s, *t*Bu), 2.93 (1H, dd ($^2J = 17$ Hz, $^3J = 13$ Hz), H_β), 3.11 (1H, dd($^2J = 17$ Hz, $^3J = 4$ Hz), $H_{\beta'}$), 4.13 (1H, d($^2J = 17$ Hz), H_ε), 4.81 (1H, q($^3J = 7$ Hz), H_α Ala), 5.02 (1H, m, H_α Aba), 5.07 (1H, d($^2J = 17$ Hz), $H_{\varepsilon'}$), 6.76 (1H, d ($^3J = 7$ Hz), 4-NH), 7.11 (4H, M, H arom.) ^{13}C NMR (DMSO-d_6, 63 MHz)- δ_C 16.8 (CH_3 Ala), 28.6 (CH_3 Boc), 37.0 (CH_2 β), 49.0 (CH_2 ε), 50. 7 (CH α Ala), 52.0 (CH α benzazepine), 79.3 (C_q Boc), 125.4, 127.7, 128.9, 131.0 (4 CH arom), 136.0, 137.2 (2 C_q arom), 155.0 (C=O Boc), 171.9 (C=O COOH), 172.2 (C=O $CONR_2$).

RESULTS AND DISCUSSION

Chemistry

The synthesis of Boc-protected L- and D-Aba-Gly **2** and **3**, rac-MeAba-Gly **4**, D-Aba-Ala **6** and D-Aba-D-Ala **5** was performed using previously published methods [30–32]. The racemic spiro-dipeptomimetic **7** was prepared as recently described, starting from racemic Boc-α-(*o*-cyanobenzyl)proline [22].

The dipeptidomimetics **2** to **7** were incorporated into the BK and HOE 140 peptide sequences using Boc solid-phase peptide synthesis on a Merrifield resin. The peptides were purified to homogeneity by preparative HPLC. When the racemic building blocks **4** and **7** were used, two epimeric peptides were obtained. The MeAba-Gly epimeric peptides could not be separated. The spiro-Aba-Gly analogues of BK (BK7 and BK8, see Table 2) and of HOE 140 (BK9 and BK10, see Table 2) were separated. In order to assign the configuration of the chiral center in these epimeric peptides, an asymmetric synthesis was performed starting from D-α-(*o*-cyanobenzyl)proline (having (*S*) absolute configuration) using the protocol described for the racemic compound [22] and the resulting L-spiro-Aba-Gly was incorporated into the HOE 140 sequence. A comparison of the HPLC retention times allowed us to assign the L-configuration to BK10 ((S) absolute configuration).

DOI: 10.1002/psc

Binding Affinity and Activity for Human B_2 Receptor

The binding affinity was evaluated by inhibiting the [^{3}H]BK binding to the human B_2 receptor, performed with membranes of CHO cells expressing the human B_2 receptor. The results of these experiments are shown in Figure 2.

The data are summarized in Table 2:

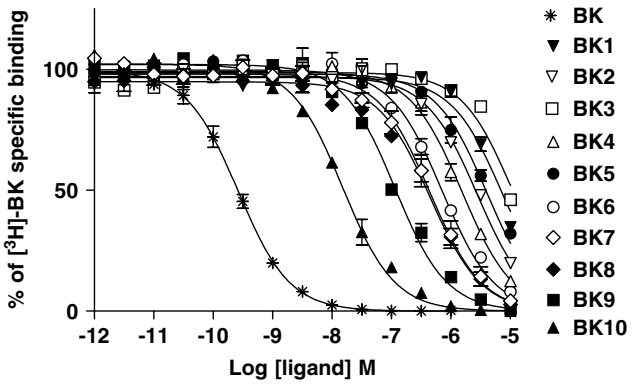

Figure 2 Inhibition binding experiments of [^{3}H]BK binding to the human B_2 receptor.

The substitution of the Pro^7-Phe^8 dipeptide in the BK sequence by the dipeptidomimetics **2–7** resulted in a large drop in B_2 receptor affinity. Only the D-Aba-D-Ala containing BK4 and the spiro analogue BK6 showed moderate affinity. When introducing L- or D-Aba-Gly into the HOE 140 sequence, slightly more potent compounds are obtained; however, they still show considerably less affinity than the parent compound. The most potent ligands result from the incorporation of the spiro-Aba-Gly scaffold (BK9 and BK10). The asymmetric synthesis indicated that the most potent epimer, BK10, has the L-stereochemistry of the asymmetric carbon.

The HOE 140 analogues BK7, BK9 and BK10 were tested in B_2-expressing CHO cells for their activity in the IP accumulation assay, as index for phospholipase C activation [25]. All the compounds did not modify the basal IP output up to 10 μM concentration, thus indicating that they are not inverse agonists or agonists. Compounds (10 μM) were incubated for 15 min before agonist incubation (30 min). However, no shifts of the BK concentration-response curves were observed.

To further investigate the antagonist properties of our analogues (BK7, BK9, BK10), these compounds were

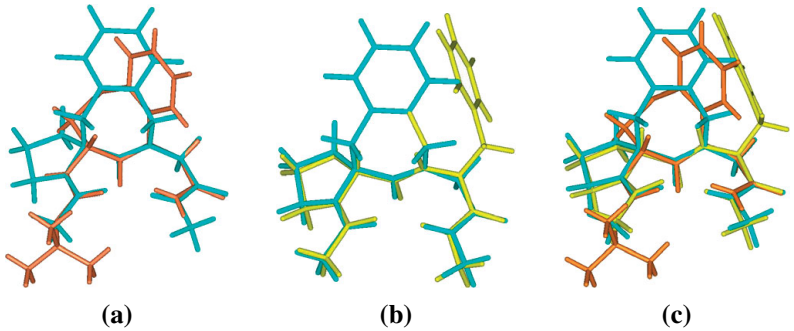

Figure 3 Superimposition of Ac-L-spiro-Aba-Gly-NHMe (cyan) with (a) β II′ folded conformation of Boc-D-BT-NH_2 (orange), (b) β II′ folded Ac-D-Pro-L-Phe-NHMe model dipeptide (yellow), (c) β II′ folded conformation of Boc-D-BT-NH_2 (orange) and β II′ folded Ac-D-Pro-L-Phe-NHMe model dipeptide (yellow).

Table 2 Sequences and Numbering of the Peptides and Affinity Values Towards the B_2 Receptor

Name/no.	Peptide sequence	pKi (95% C.I.)
BK	H-Arg-Pro-Pro-Gly-Phe-Ser-**Pro-Phe**-Arg-OH	10 ± 0.02
HOE 140	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-**D-Tic-Oic**-Arg-OH	10.1 ± 00.7
BK1	H-Arg-Pro-Pro-Gly-Phe-Ser-**D-Aba-Gly**-Arg-OH	5.6 ± 0.05
BK2	H-Arg-Pro-Pro-Gly-Phe-Ser-**(D, L)-MeAba-Gly**-Arg-OH	6.1 ± 0.03
BK3	H-Arg-Pro-Pro-Gly-Phe-Ser-**D-Aba-L-Ala**-Arg-OH	53.9% ± 0.3 inhibition at 10 mM
BK4	H-Arg-Pro-Pro-Gly-Phe-Ser-**D-Aba-D-Ala**-Arg-OH	6.3 ± 0.03
BK5	H-Arg-Pro-Pro-Gly-Phe-Ser-**(Dor L)-spiro-Aba-Gly**-Arg-OH	5.9 ± 0.04
BK6	H-Arg-Pro-Pro-Gly-Phe-Ser-**(Lor D)-spiro-Aba-Gly**-Arg-OH	6.6 ± 0.05
BK7	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-**D-Aba-Gly**-Arg-OH	6.5 ± 0.04
BK8	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-**L-Aba-Gly**-Arg-OH	6.7 ± 0.04
BK9	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-**D-spiro-Aba-Gly-**Arg-OH	7.6 ± 0.03
BK10	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-**L-spiro-Aba-Gly**-Arg-OH	8.5 ± 0.03

DOI: 10.1002/psc

Table 3 Peptide Numbering, Sequences and pA_2 Values of Peptides Tested in Guinea Pig Smooth Muscle Contraction Assay

Peptide.	Sequence	pA_2 values
HOE140	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg.OH	9.5 ± 0.1[a]
BK 7	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Aba-Gly-Arg-OH	5.6 ± 0.06
BK 9	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-spiro-Aba-Gly-Arg-OH	5.5 ± 0.12
BK 10	H-D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-L-spiro-Aba-Gly-Arg-OH	5.7 ± 0.05

[a] HOE140 value from Ref. 17.

investigated in the guinea pig ileum smooth muscle contractility assay [17].

The measured antagonist potencies (pA_2 values) in blocking the concentration-dependent contractile responses induced by BK are shown in Table 3. Contrary to what was previously reported with Icatibant (HOE 140), its analog compounds bearing amino-benzazepinone (Aba) substitutions were surmountable antagonists.

These data further support the hypothesis that high affinity of the BK analogues is related to their ability to adopt a *C*-terminal β-turn conformation. Indeed, we have previously shown that the Aba-Gly dipeptidomimetics do not adopt a turn conformation, but rather prefer extended conformations. In contrast, the spiro-Aba scaffold strongly prefers a turn conformation [22]. We have compared the low-energy conformation of the Ac-L-spiro-Aba-Gly-NHMe tetrapeptide model with the preferred β-turn conformation of the Boc-D-BT-NH_2 scaffold (Figure 3(a)), which, when incorporated into the HOE140 sequence, resulted in a potent B_2 agonist [16]. An almost perfect overlap (RMSD = 0.1360 Å) of the backbone is observed, the major difference being the positioning of the aromatic ring. Figure 3(b) shows an overlap of Ac-L-spiro-Aba-Gly-NHMe with Ac-D-Pro-Phe-NHMe in a type II′ β-turn conformation. Again, an excellent overlap (RMSD = 0.2055 Å) of the backbone and of the 5-membered Pro and spiro-ring is observed, whereas the orientation of the aromatic rings is different. Finally, a superimposition of Ac-L-spiro-Aba-Gly-NHMe, Boc-D-BT-NH_2 and Ac-D-Pro-Phe-NHMe in a type II′ β-turn conformation is illustrated in Figure 3(c). From these models, one can conclude that the different positioning of the aromatic ring is probably the cause of the differences in potency, and also influences the agonist/antagonist character of these peptide analogs.

The conformational restriction in the Aba analogs was apparently less optimal than the one imposed by D-BT (cfr. JMV1429, $K_i = 13 \pm 4.5$ nM, [13]). Affinity was improved by using the 'second generation' antagonist sequence of HOE140. D-Tic-Oic was replaced by the spiro-Aba derivative **7**, leading to two low nanomolar ligands for the human B_2 receptor.

Differences obtained with the two functional tests, i.e. the BK induced IP accumulation in CHO cells expressing the hB_2R and the guinea pig smooth muscle contractility assay, indicate that despite the nM binding affinity, these compounds dissociate quickly from the receptor. In fact they become inactive in the assay in which their interaction is unfavored by the long agonist exposure (in the IP accumulation assay each agonist concentration is left in contact with cells for 30 min), whereas their interaction is favored in the isolated smooth muscle contractility assay in which the agonist cumulative concentration-response curves are produced in less than 10 min.

CONCLUSIONS

The replacement of the Pro^7-Phe^8 dipeptide in BK by various Aba-constrained dipeptidomimetics resulted in a loss of B_2 receptor affinity. Incorporation of these dipeptidomimetics into the HOE 140 sequence indicated that the mimetics which adopt extended conformations were less potent than the spiro-Aba mimetics that adopt a turn conformation. These replacements in HOE 140 provided two new potent analogues with a $K_i = 25$ nM (BK9) and a $K_i = 3.2$ nM (BK10), and maintained the B_2 antagonist character of the constrained peptides. This is in contrast to the results obtained for the D-BT replacement, which resulted in a potent agonist (JMV1116, $K_i = 0.7 \pm 0.1$ nM) [13,14.] Molecular modeling indicated excellent overlap of the backbone of the spiro-L-Aba-Gly mimetic with that of the D-BT mimetic, and with a type II′ β-turn conformation of Ac-D-Pro-L-Phe-NHMe. The orientations of the aromatic rings were, however, different, suggesting that this difference in orientation might be responsible for the difference in affinity and for the changes in the agonist/antagonist character.

Acknowledgements

The authors thank the I.W.T. (Institution for the Promotion of Innovation by Science and Technology in Flanders) for financial support. D. Feytens is

DOI: 10.1002/psc

a Research Assistant with the Fund for Scientific Research-Flanders (Belgium). We also thank Claude Didierjean from the UMR CNRS-UHP 7036, Faculté des Sciences, Vandoeuvre, France, for the crystallographic data of Boc-D-BT-NH_2.

REFERENCES

1. Stewart JM. Bradykinin antagonists: discovery and development. *Peptides* 2004; **25**: 527–532.
2. Stewart JM, Gera L, York EJ, Chan DC, Bunn P. Bradykinin antagonists: Present progress and future prospects. *Immunopharmacology* 1999; **43**: 155–161.
3. Bock MG, Longmore J. Bradykinin antagonists: new opportunities. *Curr. Opin. Chem. Biol.* 2000; **4**: 401–406.
4. Chan D, Gera L, Helfrich B, Helm B, Stewart K, Whalley E, Bunn P. Novel bradykinin antagonist dimers for the treatment of human lung cancers. *Immunopharmacology* 1996; **33**: 201–204.
5. Maeda H, Akaike JW, Noguchi Y, Sakata Y. Bradykinin and nitric oxide in infectious disease and cancer. *Immunopharmacology* 1996; **33**: 222–230.
6. Stewart JM, Gera L, Chan DC, York EJ, Simkeviciene V, Bunn PA Jr, Taraseviciene-Stewart L. Combination cancer chemotherapy with one compound: pluripotent bradykinin antagonists. *Peptides* 2005; **26**: 1288–1291.
7. Ueda H. Molecular mechanisms of neuropathic pain-phenotypic switch and initiation mechanisms. *Pharmacol. Ther.* 2006; **109**: 57–77.
8. Sufka KJ, Roach JY. Stimulus properties and antinociceptive effects of selective bradykinin B1 and B2 receptor antagonists in rats. *Pain* 1996; **66**: 99–103.
9. Vavrek RJ, Stewart JM. Competitive antagonists of bradykinin. *Peptides* 1985; **6**: 161–164.
10. Stewart JM, Vavrek RJ. Chemistry of peptide B2 bradykinin antagonists. In *Bradykinin Antagonists. Basic and Clinical Research*, Burch RM (ed.). Marcel Dekker: New York, 1991; 51–96.
11. Hock FJ, Wirth K, Albus U, Linz W, Gerhards HJ, Wiemer G, Henke G, Breipohl ST, König W, Schölkens BA. HOE-140, a new, long acting bradykinin antagonist: in vitro studies. *Br. J. Pharmacol.* 1991; **102**: 769–773.
12. Wirth K, Hock FJ, Albus U, Linz W, Alpermann HG, Anagnostopoulos H, Henke S, Breipohl G, Koenig W. HOE 140 a new potent and long acting bradykinin-antagonist: in vivo studies. *Br. J. Pharmacol.* 1991; **102**: 774–777.
13. Amblard M, Daffix I, Bedos P, Bergé G, Dodey P, Paquet J-L, Luccarini J-M, Bélichard P, Pruneau D, Bellamy F, Martinez J. Synthesis and pharmacological evaluation of bradykinin analogs containing dipeptide mimics. In: *Proceedings of the 25th European Peptide Symposium*, Bajusz S, Hudecz F (eds). Akadémiai Kiadó: Budapest, Hungary, 1998; 20–23.
14. Amblard M, Daffix I, Bedos P, Bergé G, Pruneau D, Paquet J-L, Luccarini J-M, Bélichard P, Dodey P, Martinez J. Design and synthesis of potent bradykinin agonist Containing a Benzothiazepine moiety. *J. Med. Chem.* 1999; **42**: 4185–4192.
15. Amblard M, Daffix I, Bergé G, Calmès M, Dodey P, Pruneau D, Paquet J-L, Luccarini J-M, Bélichard P, Martinez J. Synthesis and characterization of bradykinin B2 receptor agonists containing constrained dipeptide mimics. *J. Med. Chem.* 1999; **42**: 4193–4201.
16. Amblard M, Raynal N, Averlant-Petit M-C, Didierjean C, Calmès M, Fabre O, Aubry A, Marraud M, Martinez J. Structural elucidation of the β-turn inducing (*S*)[3-amino-4-oxo-2,3-dihydro-5*H*-benzo[b][1,4]thiazepin-5-yl] acetic acid (DBT) motif. *Tetrahedron Lett.* 2005; **46**: 3733–3735.
17. Kyle DJ, Blake PR, Smithwick D, Green LM, Martin JA, Sinsko JA, Summers MF. NMR and computational evidence that high-affinity bradykinin receptor antagonists adopt C-terminal β-Turns. *J. Med. Chem.* 1993; **36**: 1450–1460.
18. Kyle DJ, Martin JA, Burch RM, Carter JP, Lu S, Meeker S, Prosser JC, Sullivan JP, Togo J, Noronha-Blob L, Sinsko JA, Walters RF, Whaley LW, Hiner RN. Probing the bradykinin receptor: mapping the geometric topography using ethers of hydroxyproline in novel peptides. *J. Med. Chem.* 1991; **34**: 2649–2653.
19. Kyle DJ, Martin JA, Farmer SG, Burch RM. Design and conformational analysis of several highly potent bradykinin receptor antagonists. *J Med. Chem.* 1991; **34**: 1230–1233.
20. Chacravarty S, Wilkins D, Kyle DJ. Design of potent cyclic peptide bradykinin receptor antagonists from conformationally constrained linear peptides. *J. Med. Chem.* 1993; **36**: 2569–2571.
21. Robl JA, Simpkins LM, Sulsky R, Sieber-McMaster E, Stevenson J, Kelly YF, Sun C-Q, Misra RN, Ryono DE, Asaad MM, Bird JE, Trippodo NC, Karanewsky DS. Dual metalloprotease inhibitors. II. Effect of substitution and stereochemistry on benzazepinone based mercaptoacetyls. *Bioorg. Med. Chem. Lett.* 1994; **4**: 1794–1800.
22. Van Rompaey K, Ballet S, Tömböly CS, De Wachter R, Vannomeslaeghe K, Biesemans M, Willem R, Tourwé D. Synthesis and evaluation of the β-turn properties of 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-ones and their spirocyclic derivative. *Eur. J. Org. Chem.* 2006; 2899–2911.
23. Alcaro MC, Vinci V, D'Ursi AM, Scrima M, Chelli M, Giuliani S, Meini S, Di Giacomo M, Colombo L, Papini AM. Bradykinin antagonists modified with dipeptidic mimetic β-turn inducers. *Bioorg. Med. Chem. Lett.* 2006; **16**: 2387–2390.
24. Meini S, Cucchi P, Bellucci F, Catalani C, Faiella A, Rotondaro L, Quartara L, Giolitti A, Maggi CA. Site-directed mutagenesis at the human B_2 receptor and molecular modelling to define the pharmacophore of non-peptide bradykinin receptor antagonists. *Biochem. Pharmacol.* 2004; **67**: 601–609.
25. Bellucci F, Meini S, Cucchi P, Catalani C, Reichert W, Zappitelli S, Rotondaro L, Quartara L, Giolitti A, Maggi CA. A different molecular interaction of bradykinin and the synthetic agonist FR190997 with the human B_2 receptor: evidence from mutational analysis. *Br. J. Pharmacol.* 2003; **140**: 500–506.
26. Meini S, Patacchini R, Lecci A, Quartara L, Maggi CA. Peptide and non-peptide bradykinin B_2 receptor agonists and antagonists: a reappraisal of their pharmacology in the guinea pig ileum. *Eur. J. Pharmacol.* 2000; **409**: 185–194.
27. Still WC, Tempczyk A, Hawley RC, Hendrickson T. Semianalytical treatment of solvation for molecular mechanics and dynamis. *J. Am. Chem. Soc.* 1990; **112**: 6127–6129.
28. Mohamadi F, Richards NGJ, Guida WC, Liskamp R, Lipton M, Caufield C, Chang G, Hendrickson T, Still WC. Macromodel–an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. *J. Comput. Chem.* 1990; **11**: 440–467.
29. Allinger NL, Yuh YH, Lii J-H. Molecular mechanics. The MM3 force field for hydrocarbons. *J. Am. Chem. Soc.* 1989; **111**: 8551–8577.
30. Ballet S, Frycia A, Piron J, Chung NN, Schiller PW, Kosson P, Lipkowski AW, Tourwé D. Synthesis and biological evaluation of constrained analogues of the opioid peptide H-Tyr-D-Ala-Phe-Gly-NH_2 using the 4-amino-2-benzazepin-3-one scaffold. *J. Pept. Res.* 2005; **66**: 222–230.
31. Tourwé D, Verschueren K, Frycia A, Davis P, Porreca F, Hruby VJ, Toth G, Jaspers H, Verheyden P, Van Binst G. Conformational restriction of Tyr and Phe side chains in opioid peptides: information about preferred and bioactive side-chain topology. *Biopolymers* 1995; **38**: 1–12.
32. Van Rompaey K, Van den Eynde I, De Kimpe N, Tourwé D. A new versatile synthesis of 2-substituted 2-benzazepin-3-ones. *Tetrahedron* 2003; **59**: 4421–4432.