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Synthesis, Conformation and Biological Activity of Linear and Cyclic Thr^6-bradykinin Analogues Containing *N*-benzylglycine in Place of Phenylalanine

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Abstract: Three linear Thr^6-bradykinin analogues in which either one or both the two phenylalanine residues in the peptide sequence have been substituted by *N*-benzylglycine (BzlGly) and their head-to-tail cyclic analogues were synthesized and tested on an isolated rat duodenum preparation. The linear (BzlGly^5,Thr^6-BK, BzlGly^8,Thr^6-BK and BzlGly^{5,8},Thr^6-BK) and the cyclic (*cyclo* BzlGly^5,Thr^6-BK, *cyclo* BzlGly^8,Thr^6-BK and *cyclo* BzlGly^{5,8},Thr^6-BK) peptoid-like analogues were characterized by amino acid analysis, optical rotation, analytical HPLC and MALDI-TOF mass spectroscopy. The conformational features of both the linear and cyclic derivatives were investigated by FT-IR and CD measurements. Preliminary molecular mechanics calculations were also performed on some synthetic peptides. Pharmacological screening using the relaxation of the isolated rat duodenum preparation showed that incorporation of *N*-benzylglycine at positions 5 and/or 8 in the linear Thr^6-BK causes a substantial decrease in potency. Comparable incorporation in *cyclo* Thr^6-BK, at position 8, or 5 and 8, resulted in nearly inactive analogues. However, *cyclo* BzlGly^5,Thr^6-BK showed a potency which is of the same order of magnitude as for *cyclo*-BK and *cyclo* Thr^6-BK.

Keywords: bradykinin; *N*-alkylglycine; peptoids; synthesis; kinins

INTRODUCTION

Bradykinin, a nonapeptide isolated from mammalian blood serum, affects a number of tissues in the mammalian body and blocks neurotransmission in the insect CNS [1,2]. In the mammalian body, this peptide as well as its linear analogues are susceptible to enzymatic degradation. Extensive attempts to develop potent and stable bradykinin (BK) agonists have been carried out by several groups including

Abbreviations: The amino acid residues are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are according to the suggestions of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem. 1984;* **138**: 9–37). Abbreviations listed in the guide recently published in *J. Peptide Sci.* 1999; **5**: 465–471 are used without explanation. Other abbreviations are: 2-Cl-Trt resin, 2-chloro trityl chloride resin; HMP resin, 4-(hydroxymethyl)-phenoxymethyl resin; NMP, *N*-methylpyrrolidone; BzlGly, *N*-benzylglycine; Sasrin resin, 2-methoxy-4-alkoxybenzyl alcohol resin; TCP resin, trityl chloride polystyrene resin; TDM, *N*,*N*,*N*′,*N*′-tetramethyl-4-4-diamino-diphenylmethane.

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the authors' laboratory. Cyclic bradykinin analogues have also been synthesized in order to study the influence of conformational restrictions on biological activity. Recent results [3] showed that the three linear kinins Lys-Lys-BK, Nle-Lys-BK and Lys-Nle-BK are more active than BK according to the rat duodenum relaxation test. The most potent analogue (Lys-Nle-BK) is about 40 times more active. In agreement with previous observations ([3] and references therein cited) the head-to-tail cyclic analogues are less active than the linear ones, but the most potent derivative (*cyclo* Nle-Lys-BK) is still about 6 times more active than BK itself. An interesting idea for modifying the structure of a biologically active peptide was the proposal to shift the amino acid side chain from the α-carbon to the nitrogen atom of the peptide bond. The resulting *N*-substituted glycine derivatives, called peptoids, are achiral and possess proteolytic stability and high flexibility [4]. The first incorporation of *N*-benzylglycine as an achiral structural isomer of phenylalanine into a biologically active peptide was with bradykinin: BzlGly-containing BK analogues behaved as potent agonists or antagonists [5,6]

In continuation of our investigations on the structure–activity relationship of BK and BK analogues ([3] and references therein cited) we focused our attention on Thr^6-BK, a native kinin discovered in the venom of a solitary wasp and present also in ant venom and frog skin. The wasp's sting results in an irreversible paralysis of the prey, an effect which has been explained as an immediate and permanent block of transmission at the synaptic level [1]. Thr^6-BK proved to be about 10 times more potent than BK in the insect central nervous system [1] as well as in smooth muscle [7]. The structural analysis of Thr^6-BK analogues is made particularly interesting by the fact that despite the high sequence homology with native bradykinin (only one conservative substitution: Ser^6/Thr^6) there is a marked and significant difference in the biological profiles of the two peptides. The conformation of the natural Thr^6-BK has been already investigated by NMR spectroscopy and computer simulations [8,9]. The structural characterization supports the hypothesis of the significant role of the residue in position 6 on both conformation as well as biological activities, and indicates the presence of partially populated turns at the *C*-terminus (residues Thr^6-Arg^9) and at the *N*-terminus (residues Pro^2-Phe^5), suggesting also that the *N*-terminal turn might contribute to the bioactive conformation. We report in this communication the synthesis and some preliminary pharmacological experiments and structural investigations of new linear and cyclic Thr^6-BK analogues, in which either one or two amino acid residues in the peptide sequence have been substituted by *N*-benzylglycine. Looking at the Thr^6-BK sequence, it appears that the corresponding *N*-substituted glycine isomer can only be prepared for the threonine, arginine and phenylalanine residues. We decided to replace first the two phenylalanine residues in position 5 and 8, without modifying the threonine residue or the *N*- and *C*-terminal arginines (Table 1). Solid phase syntheses of linear peptoid-like analogues **I–III** were based on Fmoc chemistry, starting with Fmoc–Arg(Pmc)-HMP resin. The threonine hydroxyl function was protected as its *tert*-butyl ether, and the Pmc group was used for protecting arginine side chains. The solid-phase procedure was also used for preparing peptides **Ia–IIIa,** starting with Fmoc-Gly-Sasrin-, H-Gly-2-Cl-Trt- or Fmoc-Gly-TCP resin. The nonapeptides **Ia, IIa** and **IIIa**, covering the entire sequence of BzlGly^5, Thr^6-BK, BzlGly^8, Thr^6-BK and BzlGly^{5,8}, Thr^6-BK, respectively, were cyclized at room temperature in the presence of HATU and HOAt, yielding the protected *cyclo*-BzlGly^5, Thr^6-BK

Table 1 Linear and Cyclic Thr^6-BK Peptoid-like Analogues

I	H-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-Phe-Arg-OH	**BzlGly^5,Thr^6-BK**
II	H-Arg-Pro-Pro-Gly-Phe-Thr-Pro-BzlGly-Arg-OH	**BzlGly^8,Thr^6-BK**
III	H-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-BzlGly-Arg-OH	**BzlGly^{5,8},Thr^6-BK**
Ia	H-BzlGly-Thr(tBu)-Pro-Phe-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH	
IIa	H-Phe-Thr(tBu)-Pro-BzGly-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH	
IIIa	H-BzlGly-Thr(tBu)-Pro-BzlGly-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH	
Ib	-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-Phe-Arg-	***cyclo*-BzlGly^5,Thr^6-BK**
IIb	-Arg-Pro-Pro-Gly-Phe-Thr-Pro-BzlGly-Arg-	***cyclo*-BzlGly^8,Thr^6-BK**
IIIb	-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-BzlGly-Arg-	***cyclo*-BzlGly^{5,8},Thr^6-BK**

(**Ib**), *cyclo*-BzlGly^{8}, Thr^{6}-BK (**IIb**) and *cyclo*-BzlGly^{5,8}, Thr^{6}-BK (**IIIb**) which were fully deblocked, purified by semipreparative HPLC and characterized by amino acid analysis, optical rotation, analytical HPLC and mass spectrometry. The conformational features of both the linear and cyclic Thr^{6}-BK peptoid-like analogues were investigated by FT-IR and CD measurements, and their pharmacological properties were tested using the isolated rat duodenum preparation.

MATERIALS AND METHODS

All chemicals were commercial products of the best grade available. Fmoc-Thr(tBu)-OH and Fmoc-Arg(Pmc)-HMP-resin were Novabiochem products (Läufelfingen, Switzerland), Fmoc-Arg(Pmc)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH and H-Gly-2-Cl-Trt resin were supplied by Advanced ChemTech (Machelen, Bruxelles, Belgium). Fmoc-Phe-OH and Fmoc-Gly-Sasrin-resin were obtained from Bachem Feinchemikalen A.G. (Bubendorf, Switzerland). Fmoc-Gly-TCP-resin was supplied by PepChem (Tübingen, Germany) and Fmoc-BzlGly-OH was prepared essentially according to the literature [10]. HOAt and HATU were obtained from PerSeptive Biosystems (Hamburg, Germany). All other chemicals for the solid phase peptide synthesis were supplied by Applied Biosystems/Perkin Elmer (Forster City, CA USA). Optical rotations were determined, at 20 °C, with a Perkin Elmer model 241 polarimeter. Amino acid analyses were performed with a Carlo Erba model 3A 30 amino acid analyser interfaced with a Shimadzu C-R4A chromatopac, following hydrolysis for 22 h at 110 °C in sealed, evacuated vials with constant boiling hydrochloric acid. Ascending TLC was performed on Merck F_{254} silica plates (Darmstad, Germany) (solvent system: butan-1-ol/acetic acid/water: 3/1/1, by vol.). Peptides were visualized by one or more of the following procedures: ninhydrin, TDM reagent [11], and UV light.

Analytical HPLC separations were performed either on an Aquapore RP-300 column (220 × 4.6 mm, 7 μm, Brownlee Labs. Santa Clara, CA, USA, flow rate 1.5 ml/min) or on a Vydac C_{18} 218 TP104 column (250 × 4.6 mm, 10 μm, Hesperia, CA, USA, flow rate 1.5 ml/min) using a Perkin Elmer series 3B liquid chromatograph equipped with an LC-90 UV detector (230 nm) and LC 100 integrator. Semipreparative HPLC separations (Vydac C_{18}218 TP1022 column, 250 × 22 mm, 10 μm, flow rate 15 ml/min) were performed on a Shimadzu series LC-6A chromatograph equipped with two independent LC-8A pump units, an SPD-6A detector and a Perkin Elmer 561 recorder. Eluants A (0.1% TFA in 90% aqueous acetonitrile) and B (0.1% aqueous TFA) were used for preparing binary gradients. See text for elution conditions. Solvents were dried and freshly distilled and evaporations were carried out under reduced pressure at 40°–45 °C using a rotary evaporator. Yields are based on the weights of vacuum-dried products. Sodium sulphate or magnesium sulphate were used for drying purposes.

The pharmacological activities of the linear and cyclic analogues were tested using the isolated preparation of rat duodenum in a 10 ml bath filled with aerated Krebs solution. Kinins were dissolved in the physiological salt solution and added to the bath at doses of 100 μl, resulting in different final concentrations. The duodenal relaxations were transduced isotonically using a HSE-type B (368) lever transducer, connected to a HSE-2-channel-bridge amplifier (type 301). Amplitudes were plotted as the percentages of maximal relaxation on a probit scale against log doses. EC_{50} (50% effective concentration values) were found by interpolation (±SEM, $n = 4$).

Mass Spectra

Molecular weight determinations were made by MALDI-TOF MS carried out on a Maldi I Kratos Shimadzu instrument operating in a linear mode at a nominal accelerating potential of +10 KeV (matrix α-cyano-4-hydroxycinnamic acid).

Infrared Absorption

Solid-state IR absorption spectra (KBr disk technique) and solution spectra (TFE, sample concentration about 10 mM) were recorded at room temperature using a nitrogen flushed Perkin Elmer Model 1720X FT-IR spectrophotometer connected to a PC IBM PS/2 model 50Z. Elaboration of the spectra by baseline subtraction and second derivative formation was achieved using the Spectra Calc program (Galactic, Salem USA).

Circular Dichroism

CD measurements were performed, in the solvents indicated, at 298 K, over 250–185 nm, using a Jasco 715 spectropolarimeter connected with a PC IBM PS/2 Model 40 SIC for the spectra elaboration

(J700 Windows program). A Suprasil quartz cell of 1 mm path length was used, and six scans were accumulated for all spectra. The sample concentration was about 2×10^{-5} M for the linear compounds and about 10^{-4} M for the cyclic compounds. Solutions of the desired concentration were prepared by dissolving samples of the different peptides in the minimum amount of water; peptide concentrations were determined by quantitative amino acid analysis. Aliquots of the mother solutions were diluted with the appropriate volumes of water, aqueous SDS or TFE. Final aqueous solutions were 30 mM SDS and 97% TFE (v/v). The spectra reported are original computer-drawn CD curves; $[\Theta]_R$ represents the mean residue molar ellipticity (deg cm^2 dmol^{-1}).

Solid-phase Peptide Synthesis

Assemblies of linear peptides on the Applied Biosystems Model 431A Peptide Synthesizer were performed on a 0.25 mmol scale starting with Fmoc-Arg(Pmc)-HMP resin (substitution 0.45 mmol/g) for peptides **I**, **II** and **III**, and with Fmoc-Gly-Sasrin, H-Gly-2-Cl-Trt or Fmoc-Gly-TCP resin for peptides **Ia, IIa** and **IIIa**. The FastMoc methodology (HBTU/HOBt/DIEA in DMF) and a single acylation protocol (NMP as the solvent) were used through all syntheses for the acylation of the L-amino acid residues. For the acylation of the BzlGly residues, HATU was added as a solid into the cartridges containing either Fmoc-Gly-OH or Fmoc-Pro-OH; the mixture was dissolved with DMF, without adding HOBt, and the time of coupling was doubled. Coupling yields were determined by ninhydrin analysis [12] of small aliquots of peptide resin removed automatically after each coupling reaction. The final peptide-resins were N^{α}-deprotected with 20% piperidine in NMP, thoroughly washed and dried. Cleavage from the HMP resin and removal of the side chain protecting groups were simultaneously achieved by treatment with a TFA : triethylsilane : H_2O mixture (95 : 2.5 : 2.5, v/v/v) (≈10 ml/g peptide-resin, 3.5 h at room temperature). The acid solution was evaporated *in vacuo* and the residue was dissolved in a small volume of TFA, precipitated twice with excess ether (or *tert*-butyl methyl ether), collected and dried in the presence of P_2O_5 and KOH pellets.

In the amino acid sequence of the linear precursors (**Ia, IIa** and **IIIa**) of the cyclic peptides, proline is the second *C*-terminal aminoacid residue. The possible intrachain aminolysis at the dipeptide stage [12] was prevented either by using the dipeptide Fmoc-Pro-Pro-OH in the first acylation step of the H-Gly-Sasrin resin [13], or by growing the peptide on the TCP or 2-Cl-Trt resin [14,15].

In particular, peptide **Ia** was assembled starting with the Fmoc-Gly-TCP resin (substitution 0.51 mmol/g) and the synthesis of peptide **IIa** was performed on the H-Gly-2-Cl-Trt resin (substitution 1.1 mmol/g), either by stepwise elongation or by using Fmoc-Pro-Pro-OH in the first acylation step. The procedure based on the use of Fmoc-Gly-Sasrin resin (substitution 0.69 mmol/g) and of Fmoc-Pro-Pro-OH as the first acylating reagent was utilized for preparing peptide **IIIa** and for an alternative synthesis of **IIa**.

The final peptide resins were N^{α}-deprotected and the side chain protected peptides were removed from the resin. Removal from the TCP- and 2-Cl-Trt resins was achieved by treatment with 20% 1,1,1,3,3,3-hexafluoro-2-propanol/DCM (3–4 times, 10–20 ml/g peptide-resin, 20 min at room temperature) with monitoring of the reaction by TLC. The resin was filtered off and the filtrates were pooled and evaporated to dryness. Removal of **IIa** and **IIIa** from the Sasrin resin was achieved by treatment with 1% TFA/DCM (10–20 ml/g peptide-resin for 2 min at room temperature). The resin was collected by filtration, and the acid filtrate was neutralized by adding 10% pyridine in CH_3OH (1 ml). The acid treatment was repeated several times, the reaction was monitored by TLC and the neutralized solutions were pooled and concentrated to small volume. Addition of excess H_2O gave a precipitate which was collected, thoroughly washed with H_2O and dried. Comparable results were obtained by the different procedures. Peptides **Ia**, **IIa** and **IIIa**, practically homogeneous by analytical HPLC, were directly used in the next cyclization reaction.

Synthesis of Cyclic Derivatives

Typically, solid HATU (3 eq) and HOAt (3 eq) were added to a DMF solution of the linear peptide **Ia**, **IIa** or **IIIa** (peptide concentration 1.0×10^{-3} or 2.0×10^{-3} M), the pH value was adjusted to 8–9 by adding DIEA, and the reaction was allowed to proceed under vigorous stirring. When HPLC monitoring indicated that the reaction was over, the solvent was removed and the residue was taken up in DCM, washed with saturated NaCl

aqueous solution, 0.1 M citric acid, 5% $NaHCO_3$, saturated NaCl aqueous solution, dried and evaporated to dryness. Removal of the side chain protecting groups was achieved by dissolving the cyclic peptide in a mixture (10 ml/100 mg) of TFA/H_2O/triethylsilane (95 : 2.5 : 2.5 by vol). After 1.5 h stirring at room temperature most of the solvent was removed, ether was added and the resulting precipitate was collected, washed with ether (3 × 10 ml) and dried *in vacuo* in the presence of KOH pellets. Purification by semipreparative HPLC yielded the desired cyclic analogues. As observed with other cyclic BK-analogues [16–18], the analytical HPLC elution profiles of **Ib**, **IIb** and **IIIb** were temperature-dependent and showed evidence of the existence of a conformational equilibrium between two or more slowly interchanging conformers.

H-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-Phe-Arg-OH.3TFA (BzlGly^5, Thr^6-BK, I)

The synthesis started from Fmoc-Arg(Pmc)-HMP resin (556 mg, 0.25 mmol) and the final peptide-resin weighed 745 mg. Cleavage from the resin and side chain deprotection were carried out on a portion (455 mg) of peptide-resin. The crude product was precipitated twice from TFA with *tert*-butyl methyl ether and further purified by semipreparative HPLC (elution: isocratic 22% A for 2 min, linear gradient 22–52% A in 30 min and 52–90% A in 5 min). Yield 123 mg (57%); amino acid ratios: Thr 1.03, Pro 3.05, Gly 0.99, Phe 0.92, BzlGly 0.93, Arg 2.06; $[\alpha]_D$ 94.5° (c 0.99, H_2O); $[\alpha]_{365\ nm}$ 315.5° (c 0.99, H_2O); MALDI-TOF 1073.76 [M + H], (calculated average mass 1074.20); single peak by analytical HPLC (Vydac C18 column, elution conditions as those used for the semipreparative HPLC).

H-Arg-Pro-Pro-Gly-Phe-Thr-Pro-BzlGly-Arg-OH. 3TFA (BzlGly^8, Thr^6-BK, II)

The synthesis was performed as described for **I**. The final peptide-resin weighed 738.5 mg. Cleavage from the resin, side chain deprotection and purification by semipreparative HPLC were carried out on 453 mg of peptide-resin. Yield 172 mg (78%); amino acid ratios: Thr 0.99, Pro 3.03, Gly 1.02, Phe 0.93, BzlGly 0.97, Arg 2.03; $[\alpha]_D$ 73.4° (c 1.00, H_2O), $[\alpha]_{365\ nm}$ 244.9° (c 1.00, H_2O); MALDI-TOF 1073.76 [M + H], (calculated average mass 1074.20); single peak by analytical HPLC (Vydac C18 column, elution conditions as those used for **I**).

H-Arg-Pro-Pro-Gly-BzlGly-Thr-Pro-BzlGly-Arg-OH. 3TFA. (BzlGly^{5,8}, Thr^6-BK, III)

The synthesis was performed as described for **I**. The final peptide-resin weighed 743 mg. Cleavage from the resin, deprotection and purification were carried out on 453 mg of peptide-resin. Yield 180 mg (83%); amino acid ratios: Thr 1.05, Pro 2.90, Gly 0.98, BzlGly 2.01, Arg 2.05; $[\alpha]_D$ 86.9° (c 1.01, H_2O), $[\alpha]_{365\ nm}$ 290.8° (c 1.01, H_2O); MALDI-TOF 1073.76 [M + H], (calculated average mass 1074.2); single peak by analytical HPLC (Vydac C18 column, elution conditions as those used for **I**).

H-BzlGly-Thr(tBu)-Pro-Phe-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH, Ia

The synthesis was performed starting with Fmoc-Gly-TCP resin (490 mg, 0.25 mmol). The final peptide-resin weighed 670 mg. Cleavage from the resin was separately carried out on portions (100–200 mg) of peptide-resin as already described. Total yield 244 mg (59%); amino acid ratios: Thr 0.97, Pro 3.12, Gly 1.01, Phe 0.94, BzlGly 0.97, Arg 1.98; practically homogeneous by analytical HPLC (Aquapore RP-300 column, elution: isocratic 20% A for 2 min, gradient 20–90% A in 20 min).

H-Phe-Thr(tBu)-BzlGly-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH, IIa

Procedure a. The synthesis was performed by the standard procedure starting from H-Gly-2-Cl-Trt resin (227 mg, 0.25 mmol). The final peptide-resin weighed 355 mg. After cleavage from the resin, the product (126 mg) was dissolved in CH_2Cl_2 (20 ml), extracted with H_2O (3 × 20 ml), dried and evaporated to dryness. Yield 110 mg (27%); amino acid ratios: Thr 0.90, Pro 2.99, Gly 1.10, Phe 0.88, BzlGly 1.00, Arg 2.12; homogeneous by analytical HPLC (column and elution conditions as described for **Ia**). The molecular weight determination was carried out on a peptide sample after removal of the side chain protecting groups by treatment with 95% aqueous TFA. MALDI-TOF 1072.9, [M + H] (calculated average mass 1074.2).

Procedure b. The title compound was prepared essentially as described in *procedure a* but Fmoc-Pro-Pro-OH was used in the first acylation step. The final peptide-resin weighed 355 mg. After cleavage from the resin, the product (137 mg) was worked up as already described yielding the side chain

protected nonapeptide (120 mg, 30%) identical to that obtained by *procedure a*.

Procedure c. The synthesis started from Fmoc-Gly-Sasrin resin (362 mg, 0.25 mmol) and Fmoc-Pro-Pro-OH was used in the first acylation step. The final peptide-resin weighed 638 mg. Cleavage from the resin yielded 230 mg (55%) of the title compound identical to those obtained by *procedures a* and *b*.

H-BzlGly-Thr(tBu)-Pro-BzlGly-Arg(Pmc)-Arg(Pmc)-Pro-Pro-Gly-OH, IIIa

The synthesis was performed as described for **IIa** (*procedure c*). The final peptide-resin weighed 872 mg. Cleavage from the resin yielded 395 mg (95%) of peptide; amino acid ratios: Thr 0.95, Pro 2.92, Gly 1.05, BzlGly 2.00, Arg 2.08; practically homogeneous by analytical HPLC (column and conditions as described for **IIa**).

Cyclo BzlGly^5, Thr^6-Bradykinin di-trifluoroacetate,*cyclo* BzlGly^5, Thr^6-BK, Ib

Cyclization of **Ia** (152 mg) was carried out as already described (peptide concentration 2.0×10^{-3}M; reaction time 90 min.; yield 137 mg, 91%). The analytical HPLC elution profile (Aquapore RP-300 column, elution: isocratic 55% A for 2 min, linear gradient 55–100% A in 15 min) indicated the presence of a minor contaminant. Removal of the side chain protecting groups from a sample (88 mg) of the protected cyclic peptide yielded a crude product (51 mg, 90%) which was purified by semipreparative HPLC (elution: isocratic 20% A for 2 min, linear gradient 20–60% A in 25 min) Yield 46 mg (89%) of pure **Ib**; amino acid ratios: Thr 1.00, Pro 2.84, Gly 1.03, Phe 0.99, BzlGly 0.93, Arg 2.13; $[\alpha]_D$ 74.0° (c 1.03, H_2O); MALDI-TOF 1056.63, [M + H] (calculated average mass 1056.2); homogeneous by analytical HPLC (Aquapore RP-300 column; elution: isocratic 20% A for 2 min, linear gradient 20–60% A in 25 min).

Cyclo BzlGly^8, Thr^6-Bradykinin di-trifluoroacetate,*cyclo* BzlGly^8, Thr^6-BK, IIb

The crude title compound prepared by cyclization of **IIa** (88 mg, peptide concentration 1.0×10^{-3} M, reaction time 120 min, yield 84 mg, 97%) followed by removal of the side chain protecting groups, behaved as an inhomogeneous product by analytical HPLC (Aquapore RP-300 column, elution: isocratic 45% A for 2 min, linear gradient 45–100% A in 20 min). Purification of the side chain protected cyclization product by semipreparative HPLC column (elution: isocratic 70% A for 2 min, linear gradient 70–100% A in 25 min) yielded two main components which were separately collected and side chain deprotected. The faster moving component (30 mg) yielded a product (15 mg) which was identified as the title compound {amino acid ratios: Thr 0.98, Pro 2.82, Gly 1.07, Phe 0.99, BzlGly 1.07, Arg 2.07; $[\alpha]_D$ 76.3° (c 1.01, H_2O); MALDI-TOF 1054.92, [M + H] (calculated average mass 1056.2); homogeneous by analytical HPLC (Aquapore RP-300 column; elution: isocratic 25% A for 2 min, linear gradient 25–55% A in 25 min)} and the slower moving component (7 mg) gave the corresponding cyclodimer (4 mg). MALDI-TOF 2111.04, [M + H] (calculated average mass 2112.4). Cyclodimer formation was slightly increased when the cyclization reaction was carried out at a higher (2×10^{-3} M) peptide concentration.

Cyclo BzlGly^{5,8}, Thr^6-Bradykinin di-trifluoroacetate,*cyclo* BzlGly^{5,8}, Thr^6-BK, IIIb

Cyclization of **IIIa** (100 mg) was carried out as already described (peptide concentration 2.0×10^{-3} M; reaction time 60 min, yield 97 mg, 98%). The crude product, inhomogeneous by analytical HPLC (column and elution conditions as for **IIb**) was side chain deprotected and the resulting cyclic peptide (62 mg) was purified by semipreparative HPLC (elution: isocratic 25% A for 2 min, linear gradient 25–60% A in 15 min). Yield 28 mg (46%); amino acid ratios: Thr 1.04, Pro 2.88, Gly 1.00, BzlGly 2.08, Arg 2.09. $[\alpha]_D$ 77.4° (c 1.01, H_2O); MALDI-TOF 1054.92 [M + H] (calculated average mass 1056.2); homogeneous by analytical HPLC (elution conditions as those used for the semipreparative separation).

Conformational Studies

Infrared absorption. The conformational preferences of the linear peptides were examined by FT-IR absorption in TFE solution as well as in the solid state. The poor solubility of cyclic peptides **Ib, IIb** and **IIIb** prevented measurements in solution. The spectra of the linear and cyclic peptides in the solid state are quite similar, and exhibit broad absorption bands in the NH and OH stretching region (3200–3600 cm^{-1}). The higher wave-number band, at 3450–3480 cm^{-1}, is associated with the free NH oscillators while those

Table 2 Infrared Absorption Frequencies (cm^{-1}) in the Solid State (KBr pellets) of Linear and Cyclic Thr^6-BK Peptoid-like Analogues

Thr^6-BK	1741 b	1666 m	1656 sh	1639 s	1618 s	1603 w	1584 w	1498 s
I	1737 b	1668 m	1656 m	1639 s	1618 s	1603 w	1584 w	1497 s
II	1741 b	1666 m	1656 w	1639 s	1618 s	1603 w	1584 w	1498 s
III	1741 b	1670 m		1639 m	1618 s	1603 w	1584 w	1498 s
c-Thr^6-BK		1670 m	1656 w	1639 s	1618 s	1603 w	1584 w	1498 s
Ib		1666 m	1655 w	1639 s	1618 s	1603 w	1584 w	1498 s
IIb		1668 m	1655 w	1639 s	1618 s	1603 w	1584 w	1498 s
IIIb		1667 m	1655 w	1639 s	1618 s	1603 w	1584 w	1498 s

b, broad; w, weak; m, medium; s, strong; sh, shoulder.

near 3340 and 3250 cm^{-1} can be assigned to intramolecular and intermolecular hydrogen bonds, respectively [19]. The sharp band at 3415 cm^{-1} may be ascribed to weak Thr NH...O^{γ} intramolecular H bonding. This bond was first observed in linear serine-containing model peptides [20] and in proline- and serine-containing cyclic peptides [19]. The infrared absorption frequencies of the linear and cyclic peptides in the 1800–1500 cm^{-1} region (C=O stretching mode) are shown in Table 2. Only linear peptides show a broad band at about 1740 cm^{-1} which can be ascribed to the presence of the *C*-terminal carboxyl function. The band at 1497–1498 cm^{-1} arises from the aromatic side chains, and the bands at 1584 and 1603 cm^{-1} may be assigned to symmetrical and asymmetrical vibrations of the C–N bonds in the guanidinium groups [21]. The band at 1639 cm^{-1} is consistent with the existence of β-turn structures stabilized by $1 \leftarrow 4$ intramolecular H bonds. Mantsch *et al.* [22], on the basis of detailed FT-IR studies on a series of proline-containing cyclic peptides, have in fact associated an amide I component band near 1640 cm^{-1} with the acceptor amide C=O of the $1 \leftarrow 4$ intramolecular H bonded β-turn structure. The bands at 1655–1656 cm^{-1} and 1666–1670 cm^{-1} can be assigned to weakly H bonded carbonyl groups. The band at 1618 cm^{-1} could be assigned to the stretching of a H bonded C=O of a γ-turn with a proline residue in position $(i+1)$. In a γ-turn, the H bond involving a proline residue may be stronger than that enclosing residues other than proline [23]. Proline has an high probability to be in β-, or γ-turns in solution as well as in the solid state [24], and in *cyclo* BK a reverse γ-turn involving Pro^3 has been indicated in solution by NMR investigations [25]. In the amide II region, the spectra of both the linear and cyclic peptides show a weak and broad absorption band which did not resolve into definite components even after derivatization.

The FT-IR spectra of Thr^6-BK and of the linear peptides **I, II** and **III**, in TFE solution, in the 1800–1500 cm^{-1} region, are shown in Figure 1. The substitution of the phenylalanine residue with *N*-benzylglycine induces a gradual increase of the band at about 1640 cm^{-1} in the order $BzlGly^{5,8}$, Thr^6-BK > $BzlGly^8$, Thr^6-BK > $BzlGly^5$, Thr^6-BK > Thr^6-BK. The absorption frequencies are indicated in Table 3.

The absorption bands of the *C*-terminal carboxyl functions, of the aromatic residues and the C–N bonds of the guanidino groups are similar to those observed in the solid state. The amide I bands appear at 1637–1634, 1660–1661 and 1678–1679 cm^{-1}. The lower frequency band can be assigned to the stretching of H bonded C=O in β-turn structures. Comparative CD and FT-IR spectroscopic data on linear peptides with the β-turn motif–Pro-Gly-,

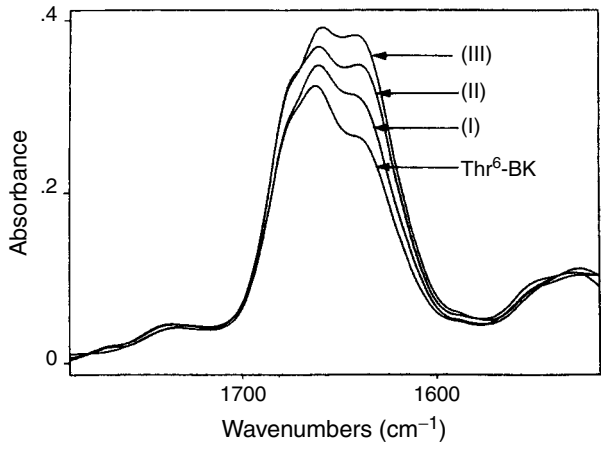

Figure 1 FT-IR absorption spectra of Thr^6-BK and the linear Thr^6-BK peptoid-like analogues **I**, **II** and **III** in TFE solution (concentration 1.0×10^{-2} M).

Table 3 Infrared Absorption Frequencies (cm^{-1}) in TFE Solution of Linear Thr^6-BK Peptoid-like Analogues

Thr^6-BK	1754 b	1678 s	1661 s	1636 s	1607 ww	1586 w	1500 s
I	1754 b	1679 s	1661 s	1637 s	1607 ww	1586 w	1499 s
II	1745 b	1678 s	1660 s	1634 s	1607 ww	1586 w	1499 s
III	1745 b	1679 s	1660 s	1635 s	1607 ww	1586 w	1498 s

ww, very weak; w, weak; b, broad; s, strong.

showed that, in TFE, the β-turn band can shift as low as about 1633 cm^{-1} [26]. The bands at 1660 and 1678 cm^{-1} can be attributed to amide C=O groups exposed to the solvent. These spectral differences suggest different features of the major conformers in the solid state and in TFE solution. The organic solvent appears to have a β-turn stabilizing effect, and increases the H-bonded β-turn conformer population. Moreover the relative increase of the intensity of the band at about 1640 cm^{-1} (Figure 1) suggests that the *N*-benzylglycine residue promotes the formation of β-turn structures.

Circular dichroism. The CD spectra of Thr^6-BK and of the linear peptides **I, II** and **III**, in water are shown in Figure 2A. The spectrum of Thr^6-BK, which is very similar to those already reported for BK [27–30], is characterized by a negative band with a maximum at 195 nm, and a minimum at 202 nm ($\pi-\pi^*$ transition), a small shoulder at 212 nm and two weak bands centred at 221 nm (positive) and at 234 (negative), respectively. Previous systematic CD investigations [29] showed that, in aqueous solution, the secondary structure of BK should be viewed as a time average of interconverting unordered and partially ordered structures with $3 \rightarrow 1$ type (γ turn) and $4 \rightarrow 1$ type (β turn) hydrogen bonds bridging the Pro^7 residue. The spectrum of Thr^6-BK is indicative of the existence of a similar situation. In peptides **I, II** and **III,** the negative band is centred at 203 nm and its intensity increases regularly in the order Thr^6-BK < **I** < **II** < **III**. This feature could indicate the presence of more ordered or more rigid structures. In the spectrum of **I** the positive band at 220 nm is missing. The CD spectrum of **II** lacks the weak negative band at 230 nm which has been attributed to a $3 \leftarrow 1$ type hydrogen bond [29] and cannot form owing to the lack of the amide proton on the BzlGly^8 residue. Substitution of both Phe residues prevent

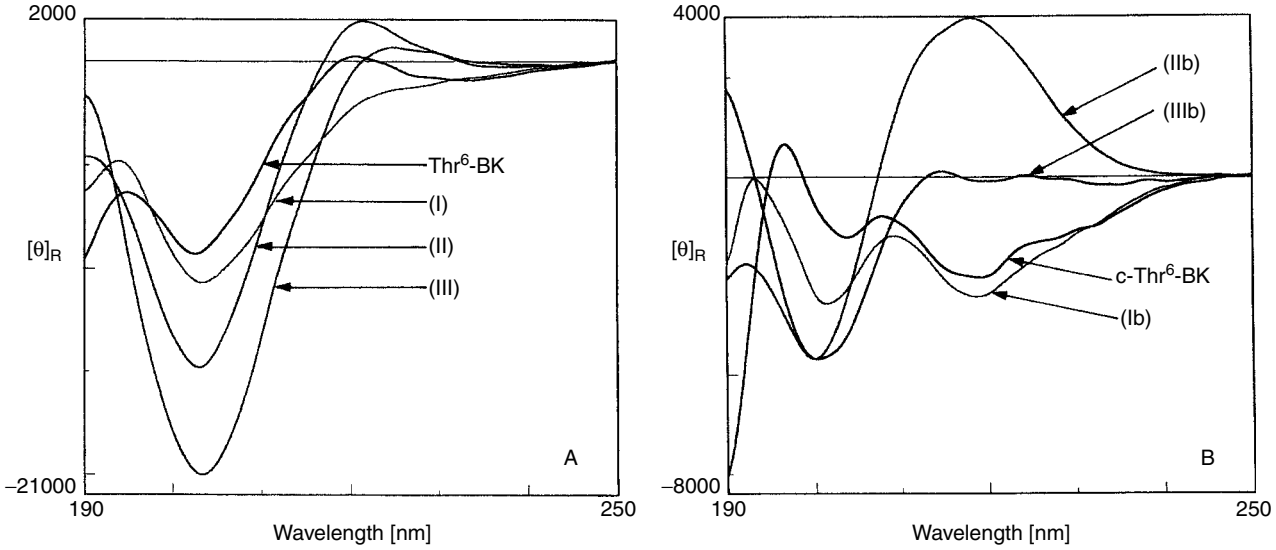

Figure 2 Far ultraviolet CD spectra of Thr^6-BK and Thr^6-BK peptoid-like analogues in water solution: **A**, linear peptides (Thr^6-BK, **I**, **II** and **III**, concentration 2.0×10^{-5} M); **B**, cyclic peptides (*cyclo* Thr^6-BK, **Ib**, **IIb** and **IIIb**, concentration 1.0×10^{-4} M).

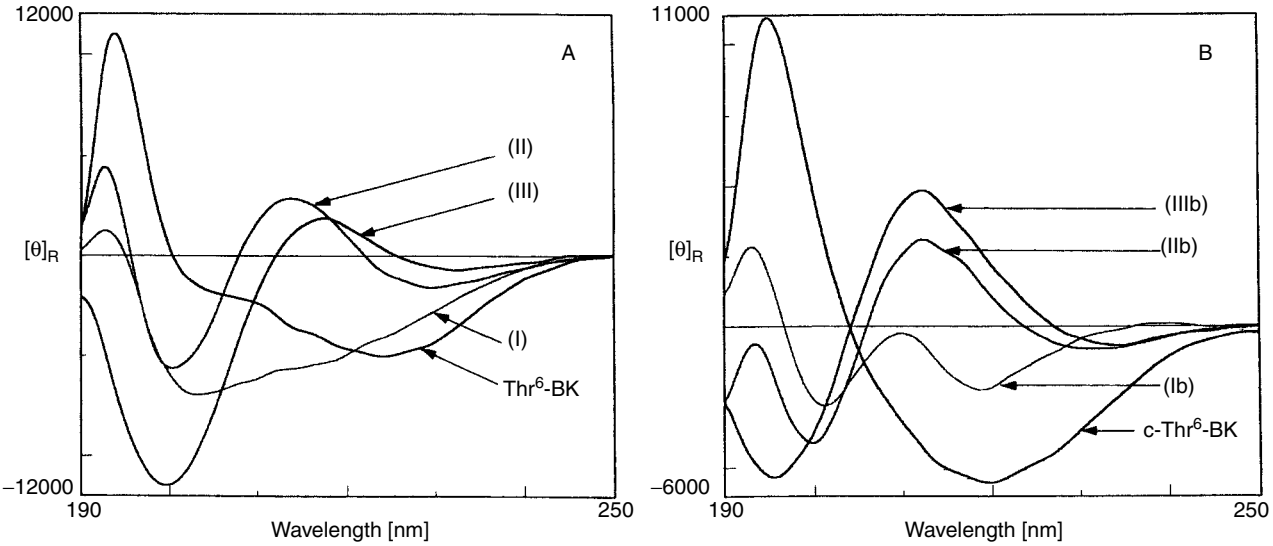

Figure 3 Far ultraviolet CD spectra of Thr^6-BK and Thr^6-BK peptoid-like analogues in TFE solution: **A**, linear peptides (Thr^6-BK, **I**, **II** and **III**, concentration 2.0×10^{-5} M); **B**, cyclic peptides (*cyclo* Thr^6-BK, **Ib**, **IIb** and **IIIb**, concentration 1.0×10^{-4} M).

the possibility of formation of some intramolecular H bonds and peptide **III** assumes a polyproline II type structure characterized by an intense negative band at 203 nm and a weak, broad positive band at 225 nm [31].

The spectra of Thr^6-BK and of the linear peptides **I, II** and **III**, in aqueous TFE are shown in Figure 3A. Previous CD and NMR investigations [30] showed that at a TFE concentration $\geq$95%, BK is present in two predominant conformations: one is all *trans* and extended, and in the other the Pro^2-Pro^3 bond is *cis* with a VIa β turn present between Arg^1 and Gly^4. The spectrum of Thr^6-BK is very similar, showing a n-π^* transition around 224 nm and an exciton couplet ($\pi-\pi^*$) with a shoulder at 205 nm and a positive band at 195 nm. It can be classified as a class C-like spectrum [32] and is indicative of the presence of a significant number of type I β turns in the conformational mixture. Also, the spectrum of peptide **I** can be considered as a class C-like spectrum, but the increased amplitude of the negative band below 210 nm reflects a change of the dominant conformation or, most likely, a shift of the conformational equilibrium. The shape of the CD spectrum of **II**, even if the bands do not present typical intensities, is similar to a type B spectrum which, according to the definition proposed by Woody [33], is indicative of type II β turn structures. Similar structures could also be present in peptide **III**.

The spectra of Thr^6-BK, **I**, **II** and **III**, in 30 mM SDS are shown in Figure 4A. The micellar systems have been often used to mimic a membrane-like environment. Previous conformational investigations carried out on the Thr^6-BK by NMR spectroscopy and distance geometry [9] showed that the micellar environment induced the formation of a secondary structure in the flexible peptide. The ensemble calculations allowed for the identifications of three different families of conformers differing in the conformation adopted at the *C*-terminus: type I β-turn, type II β-turn and a third conformation intermediate between the two β-turns. The CD spectrum of Thr^6-BK in SDS solution is indicative of a conformation more ordered than in water (Figure 2A), with a contribution of type I β-turns, but the negative exciton splitting of the $\pi-\pi^*$ transition suggests the presence of unordered structures. The CD curves of **I** in water and SDS solution are similar, but the differences in their intensities at 195 nm and 204 nm indicate that the micellar system enhances the ordered structure. In SDS solution the spectrum of **I** is compatible with proline-rich β-turn helices which consist predominantly of *trans*-proline [34]. The structures of peptide **II** in SDS solution and in aqueous TFE seem to be quite similar. The micellar environment induces a limited red-shift of the maxima and a decrease in the intensity of the positive peaks. The CD spectrum of **III** in SDS solution suggests a perturbation of

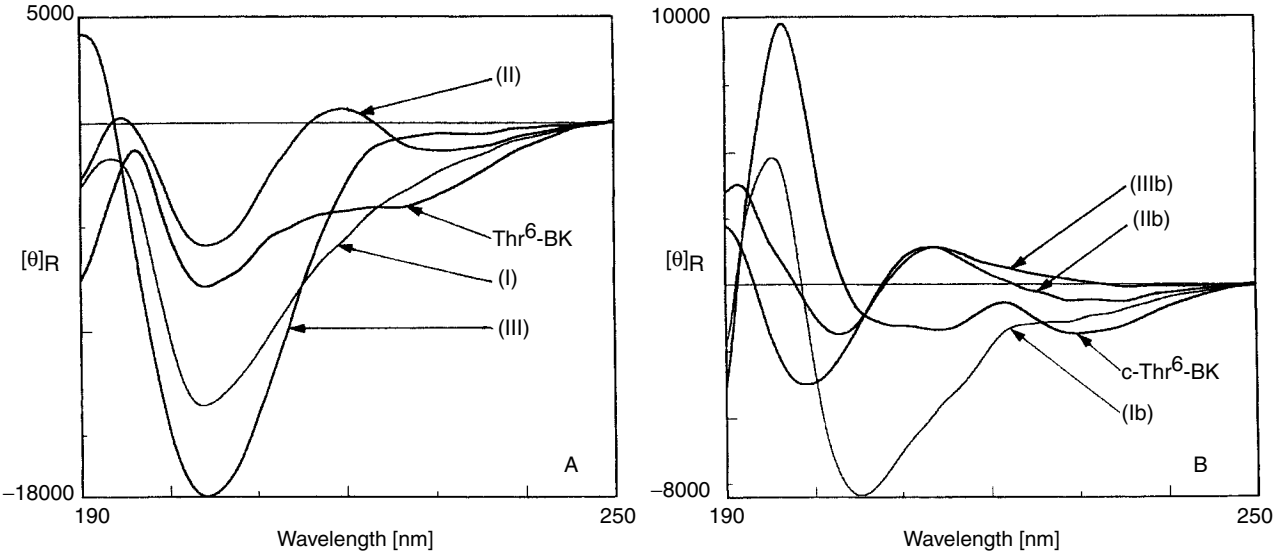

Figure 4 Far ultraviolet CD spectra of Thr[6]-BK and Thr[6]-BK peptoid-like analogues in aqueous 30 mM SDS solution: **A**, linear peptides (Thr[6]-BK, **I**, **II** and **III**, concentration 2.0×10^{-5} M); **B**, cyclic peptides (*cyclo* Thr[6]-BK, **Ib**, **IIb** and **IIIb**, concentration 1.0×10^{-4} M).

the type II polyproline helical conformation present in water. The negative band is shifted to higher wavelength with its intensity diminished and the positive band has disappeared. A possible steric interaction [35] involving Pro[7] and BzlGly[8] could favour a conformer population with a *cis* amide bond and the consequent destabilization of the polyproline II conformation.

The constraints present in the cyclic nonapeptides containing three prolines and one or two BzlGly residues, would reduce the conformational space available. The CD spectra, in water, of *cyclo* Thr[6]-BK and of **Ib** (Figure 2B) reflect either their conformational flexibility or, more likely, the existence of several stable isomers, differing in the configurations of the X-Pro and X-BzlGly bonds. Previous NMR investigations on *cyclo* BK in DMSO solution showed the presence of three families of conformers containing VIb type β-turns between Arg[1] and Gly[4] and between Phe[5] and Phe[8], and inverse γ-turn bridging Pro[3] as the most pronounced structural features [25]. In TFE solution the spectra of *cyclo* Thr[6]-BK and linear Thr[6]-BK are very similar, and have a class C character but the organic solvent does not affect significantly the structure of **Ib**. In SDS solution the spectrum of *cyclo* Thr[6]-BK is similar to that observed in water and the spectrum of **Ib** assumes a class C character.

According to the Woody notation [33] the CD curve of **IIb**, in water, corresponds to β-turn of type II (class B). A similar type of spectrum was registered for **IIb** in TFE and in SDS solution (Figure 3B and Figure 4B) even if, particularly in SDS solution, the bands do not have the intensities typical of the class B spectra. The CD spectrum of **IIIb** in water is compatible with a proline-rich β-turn structure but in TFE and in SDS the spectra are similar to those of **IIb**.

Pharmacological activities. The EC_{50} values of the synthetic BK analogues, in comparison with the values published earlier for BK [7] and *cyclo* BK [13], are shown in Table 4. The EC_{50} value for the isotonic relaxation of the rat duodenum caused by Thr[6]-BK is somewhat lower than that of BK, which confirms the higher potency of Thr[6]-BK [7]. Substitution of Phe residues by *N*-benzylglycine either at position 8 (**II**), or 5 and 8 (**III**), resulted in a reduction of potency by at least four orders of magnitude, whereas substitution at position 5 alone (**I**) resulted in an inactive peptide. Although some end-to-end cyclic BK analogues with a backbone atom number of 33 are about equipotent with BK, *cyclo* BK itself (27 atoms) is less potent [13]. It is worth noting that **Ib** shows an EC_{50} value of the same order of magnitude as the value for *cyclo* BK (Table 4). However, **IIb** only caused a small relaxation at a very

Table 4 EC_{50} Values (±SEM, $n = 4$) for the Relaxation of the Rat Duodenum by Linear and Cyclic Thr^6-BK Peptoid-like Analogues in Comparison with BK and Cyclo BK

Peptide	EC_{50}
BK	$8.3\ (\pm 1.2) \times 10^{-10}$ M (7)
Thr^6-BK	$1.8\ (\pm 0.2) \times 10^{-10}$ M
$BzlGly^5$,Thr^6-BK (**I**)	inactive at 3.0×10^{-5} M
$BzlGly^8$,Thr^6-BK (**II**)	$2.1\ (\pm 1.0) \times 10^{-6}$ M
$BzlGly^{5,8}$,Thr^6-BK (**III**)	$9.1\ (\pm 3.2) \times 10^{-6}$ M
cyclo BK	$2.5\ (\pm 0.3) \times 10^{-8}$ M (13)
cyclo $BzlGly^5$,Thr^6-BK (**Ib**)	$9.1\ (\pm 1.3) \times 10^{-8}$ M
cyclo $BzlGly^8$,Thr^6-BK (**IIb**)	$>3.0 \times 10^{-5}$ M
cyclo $BzlGly^{5,8}$,Thr^6-BK (**IIIb**)	$>5.0 \times 10^{-5}$ M*

* IIIb at a very high concentration causes an enhanced spontaneous activity with a concurrent contraction. See Figure 5.

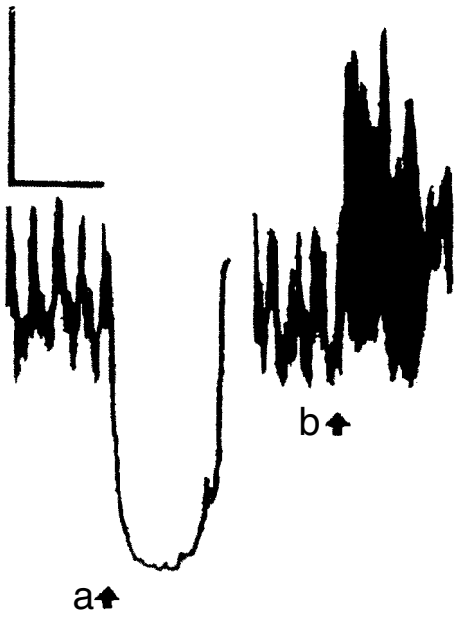

Figure 5 Isotonic registration of the relaxation or contraction of the isolated rat duodenum. The relaxation by BK (1.0×10^{-8} M, **a**) is always accompanied by a transient loss of spontaneous activity. The contraction by **IIIb** (5.0×10^{-5} M, **b**) is accompanied (or caused) by an increase in spontaneous activity. Calibration: 1 min and 1 mm (= 5%).

high concentration (3.0×10^{-5} M) suggesting that the possible EC_{50} must be present at even higher values. **IIIb** was inactive as a relaxation factor, but caused enhanced spontaneous activity and a concurrent small contraction (Figure 5).

Preliminary experiments in order to evaluate the stability to enzymatic degradation showed that BK and Thr^6-BK (10^{-8} M) are inactivated within 5 min in the presence of the duodenum preparation. *Cyclo* Nle,Lys-BK (10^{-8} M) [3] is not destroyed measurably in 15 min. However, both in the presence and absence of the duodenum, **Ib** (10^{-8} M) is broken down slowly by about 10% in 10 min and by about 50% in 10 min, at 10^{-7} M. Further experiments are needed to clarify this point.

Conformational analysis. Molecular mechanics calculations on $BzlGly^5$, Thr^6-BK (**I**), *cyclo* $BzlGly^5$,Thr^6-BK (**Ib**), *cyclo* $BzlGly^8$,Thr^6-BK (**IIb**) and *cyclo* Thr^6-BK were performed using the implementation of Amber all-atom force field (AMBER*) [36] within the framework of Macromodel version 5.5 [37]. The AMBER* force field in MMOD 5.5 contains a new set of parameters for proline containing peptides, recently developed on the basis of high-level *ab initio* calculations [38]. The torsional space of each molecule was randomly varied with the usage-directed Monte Carlo conformational search of Chang-Guida-Still [39]. For each search, at least 1000 starting structures for each variable torsion angle were generated and minimized until the gradient was less than 0.002 Kcal/Å mol. Duplicate conformations, and conformations with an energy excess of 5 Kcal/mol above the global minimum were discarded. All conformations above 3 Kcal/mol were fully analysed. First, 44 conformations were found for compound **I**, 75 for **Ib**, 24 for **IIb** and 20 for *cyclo* Thr^6-BK, but the number of conformers was dramatically reduced by considering only the pharmacophoric conformations ($\Delta E = 0.5$ Kcal/mol). The energies, and ϕ and ψ values of the most significant conformations are shown in Table 5.

DISCUSSION

The aim of the present study was to continue earlier work to prepare stable and potent BK analogues. We prepared first some cyclic BK analogues which showed the same specific effect as linear bradykinin, albeit at a much higher concentration [7,13,16,18]. Then we investigated the potency of a number of cyclic BK analogues with a backbone atom number

Table 5 Significant Conformations of BzlGly^5,Thr^6-BK (**I**), *cyclo* BzlGly^5,Thr^6-BK (**Ib**), *cyclo* BzlGly^8, Thr^6-BK (**IIb**) and *cyclo* Thr^6-BK with Relative Energies, ϕ and ψ values

	BzlGly^5, Thr^6-BK (**I**)				*cyclo* BzlGly^5, Thr^6-BK (**Ib**)			*cyclo* BzlGly^8, Thr^6-BK (**IIb**)			*cyclo* Thr^6-BK	
	Conf. 1	Conf. 2	Conf. 4	Conf. 6	Conf. 1	Conf. 2	Conf. 5	Conf. 1	Conf. 2	Conf. 9	Conf. 1	Conf. 2
E(Kcal/mol)	−156.53	−156.46	−155.97	−155.53	−135.57	−135.21	−134.89	−133.43	−133.26	−132.38	−147.71	−147.40
ϕ_1	36.4	32.7	29.5	−33.5	−66.8	−67.0	−72.7	161.2	163.3	163.4	−52.2	−61.5
Ψ_1	145.2	143.7	156.6	174.4	126.4	125.0	109.6	−147.7	−161.4	−161.7	118.1	123.8
ϕ_2	−57.4	−51.9	−63.4	−55.9	−66.4	−69.1	−66.6	−56.4	−55.9	−55.7	−54.3	−63.3
Ψ_2	149.7	151.2	144.8	152.5	−47.6	−39.1	−51.9	158.0	162.0	162.3	−53.9	−44.6
ϕ_3	−61.7	−55.1	−82.3	−53.2	−78.8	−79.4	−77.5	−95.0	−97.6	−97.4	−79.5	−83.0
Ψ_3	−32.3	−39.2	66.8	−52.5	77.8	73.2	91.2	−175.8	−172.1	172.3	68.3	75.8
ϕ_4	174.6	175.6	145.2	172.9	−172.2	−175.0	−163.5	30.2	42.0	40.8	149.7	144.7
Ψ_4	−157.2	−154.8	−135.1	−155.0	156.4	160.4	147.4	−116.2	−116.8	−115.4	−154.3	−142.3
ϕ_5	82.2	81.7	74.9	80.3	64.1	65.0	62.0	−114.8	−110.9	−116.9	−148.0	−136.6
Ψ_5	−3.3	−3.1	5.3	−1.1	22.0	22.4	16.1	118.5	18.0	26.3	39.0	21.7
ϕ_6	−117.1	−121.8	−38.1	−117.4	−159.2	−158.8	168.1	−149.0	−65.9	−67.2	−145.0	−135.8
Ψ_6	99.3	98.8	108.6	98.8	131.5	131.2	139.3	142.0	137.7	138.2	104.5	98.1
ϕ_7	−86.1	−86.2	−92.2	−86.2	−71.2	−71.1	−76.7	−75.8	−80.3	−80.1	−55.7	−60.7
Ψ_7	60.7	60.7	53.1	59.9	−41.4	−41.6	−51.0	166.5	168.8	168.7	157.9	154.7
ϕ_8	−39.1	−38.8	−43.7	−39.6	−73.4	−73.5	−67.9	126.2	129.5	129.4	−64.7	−68.0
Ψ_8	128.2	128.1	132.0	128.4	121.5	120.8	147.7	−95.0	−93.9	−94.1	97.5	96.9
ϕ_9	−106.3	−106.3	−99.6	−106.2	−84.2	−84.0	−81.9	147.7	145.7	145.8	154.8	163.1
Ψ_9	45.0	45.1	−46.5	45.0	67.6	67.6	69.4	−118.4	−113.8	−113.6	−46.0	−56.5

higher than 27, showing that the optimal ring size seems to be 33 atoms [13] and that *cyclo* Nle,Lys-BK has a potency of the same order of magnitude as BK itself [3]. The present results show that the partial modification of the peptide structure by incorporation of *N*-benzylglycine as an achiral structural isomer of Phe changes the potency of both the linear and cyclic kinins. In the linear BK analogues, substitution BzlGly/Phe at position 5 (**I**) causes a complete loss in activity. This is not surprising if the NH of Phe^5 is involved in stabilizing the bioactive structure. The decrease in potency is less dramatic, but still significant, for the substitution at position 8 (**II**). However, substitution at both positions 5 and 8 (**III**) causes a loss in potency comparable to that of **II** suggesting that the variation induced by the substitution in position 8 minimizes the role of Phe^5. The situation observed for the cyclic peptides is quite different. The EC_{50} values of *cyclo* BK and **Ib** are comparable suggesting that the $BzlGly^5/Phe^5$ substitution does not significantly affect the bioactive conformation of the conformationally constrained cyclopeptide. On the contrary, substitution either at position 8 (**IIb**) or 5 and 8 (**IIIb**) causes a dramatic decrease in potency, indicating the importance of this residue in cyclokinins.

Comparison of the conformer distribution for compounds **I** and **Ib** obtained from molecular mechanics calculations showed that peptide **I** preferentially adopts extended conformations in which H bonding plays an important role. Conformational analysis of compounds **Ib**, **IIb** and *cyclo* Thr^6-BK was then carried out to explore how the conformer distribution is affected by the BzlGly/Phe substitution either in position 5 or in position 8. An inverse γ-turn at Pro^7 was found in all 44 conformers for $BzlGly^5$,Thr^6-BK (**I**), whereas in only four conformers another γ-turn was present at Pro^3. All the stable conformers of *cyclo* $BzlGly^5$,Thr^6-BK, (**Ib**), showed two inverse γ-turns involving both Pro^3 and Arg^9. A β II′ turn at Gly^4 and Phe^5 was also observed in some conformers of *cyclo* $BzlGly^8$,Thr^6-BK, (**IIb**). All the conformations of *cyclo* Thr^6-BK under 3 Kcal/mol showed an inverse γ-turn at Pro^3. In one conformation ($\Delta E = 2.23$ Kcal/mol) a γ-turn at the *C*-terminus Arg^9 was also present.

The conformer distribution was very similar for *cyclo* Thr^6-BK and **Ib**. In the low energy conformations of both peptides, an inverse γ-turn, involving Pro^3, was present while another inverse γ-turn involving Arg^9, was present in some conformations.

As already pointed out, the substitution BzlGly/Phe at position 5 does not affect significantly the biological activity and a strong correlation between activity and conformer distributions can be thus suggested for *cyclo* Thr^6-BK and **Ib**. On the contrary, the substitution BzlGly/Phe at position 8, which is accompanied by a dramatic decrease in potency, drastically changes the conformer distribution with respect to compounds **Ib** and *cyclo* Thr^6-BK, confirming the close correlation between conformations and biological activity.

The present results do not allow a precise description of the role of Phe^5 and Phe^8 in the linear and cyclic kinins or of the effect of the *N*-benzylglycine residues on bioactive conformation, but in view of the previous demonstration [40] that linear and cyclic analogues show comparable neurotoxic activity on the insect CNS, and the stability of cyclic peptoid-like kinins, this work can be considered a step forward in the development of a potent, stable Thr^6-BK mimic.

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