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New Conformationally Homogeneous β-Turn Antagonists of the Human B_2 Kinin Receptor

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Abstract: We have designed and synthesized a conformationally homogeneous series of cyclic pentapeptides of the general structure *c*[Pro-aa$_i$-D-Tic-Oic-aa$_{i+3}$] which adopt a type-II′ β-turn conformation believed important for high affinity antagonism of the bradykinin (BK) B_2 receptor. We incorporated D-Tic and octahydroindole-2-carboxylic acid (Oic) residues (present in known active antagonists) in a cyclic pentapeptide that would place the D-aa in the $i+1$ position of the β-turn and a proline as a bridge between the *C*- and *N*-termini sides of the turn. In positions i and $i+3$ alkyl, aromatic, polar or charged amino acids could be introduced without dramatically changing the overall structure. Ten analogues were studied using ^{1}H nuclear magnetic resonance (NMR) and evaluated for their binding affinity for the human B_2 receptor. The NMR data in dimethylsulfoxide (DMSO) confirmed the structural homogeneity within the class and, on the basis of this, one representative member of the series was chosen for a detailed structure determination using NMR data in sodium dodecylsulphate (SDS) micelles and molecular dynamics calculations. Despite the structural similarity, the binding affinity of the ten analogues was strongly influenced by the nature of the side-chains in positions i and $i+3$, with the doubly charged analogue **49** ($pK_i = 6.2$) proving best. This compound may serve as the starting point for the discovery of new non-peptide bradykinin B_2 receptor antagonists. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antagonists; biological activity; bradykinin; conformation; cyclic peptides; NMR studies; synthesis

INTRODUCTION

Bradykinin (BK: H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) is a linear nonapeptide hormone that plays a role in the regulation of major physiological processes as well as in a wide variety of pathological responses. It is one of the most potent vasodilators, increases vascular permeability and also elicits contraction of smooth muscles of the respiratory and gastrointestinal tract and the uterus. BK is active in the central nervous system, where it initiates pain *stimuli* and is responsible for the cardinal symptoms of inflammation [1]. BK is one of the kinin metabolites, together with kallidin (Lys-BK) [2], des-Arg^9-BK and des-Arg^9-Lys-BK [3], whose action is

Abbreviations: aa, amino acid; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Cbz, benzyloxycarbonyl; Dab, diaminobutyric acid; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; Dap, diaminopropionic acid; EtOAc, ethyl acetate; EtOH, ethanol; EDC, 1-ethyl-3-[(3′-dimethylamino)-propyl]-carbodiimide; HOBt, 1-hydroxybenzotriazole; HOAt, 1-hydroxy-7-azabenzotriazole; Oic, octahydroindole-2-carboxylic acid; Orn, ornithine; *i*PrOH, isopropanol; SDS, sodium dodecylsulphate; TFA, trifluoroacetic acid; Thi, β-thienylalanine; Tic, 1,2,3,4, tetrahydroisoquinoline-3-carboxylic acid; Standard three letter abbreviations are used for the amino acid residues. Unless otherwise specified, they are of the L-series.

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mediated by two *G*-protein coupled receptors, termed B_1 and B_2 [4] both of which have been cloned [5,6]. The B_1 kinin receptor has a low level of expression in normal tissues, but undergoes a marked up-regulation (*de novo* synthesis) during tissue trauma or inflammation [7]. On the other hand, the B_2 kinin receptor is constitutively expressed by a variety of cell types [4], and is ready to transduce the signals delivered by newly formed kinins in plasma and tissues.

Owing to the putative role of kinins in mediating pain and inflammation, there is remarkable interest in identifying potent and selective kinin receptor antagonists, in particular for the B_2 receptor due to its perceived role in the chronic disease asthma. To date, a number of peptide [8] and non-peptide antagonists [9] have been identified.

Different research groups support the hypothesis that high-affinity peptide antagonists of the B_2 receptor must adopt *C*-terminal β-turn conformations [10–16]. Moreover, there is the assumption that an *N*-terminal positive charge and a hydrophobic *C*-terminal β-turn are the minimum requirements for binding [17].

Previously, a series of linear and cyclic penta- and hexapeptides based upon the *C*-terminal sequence of HOE 140 [18] have been synthesized by Thurieau and coworkers [19]. Cyclization was accomplished *via* the introduction of a Gly residue between the *N*- and *C-termini*. NMR studies performed on their most active compound ($pK_b = 4.93$ on the human B_2 receptor) revealed the presence of a β-turn structure around the D-Tic-Oic moiety in dimethylsulfoxide (DMSO).

On the basis of the Thurieau results [19], indicating that stabilizing the conformation *via* cyclization is beneficial for the antagonistic activity and with the aim of designing new low molecular weight molecules, we focused our efforts on studying cyclic derivatives that would preserve the type-II′ β-turn conformation around the D-Tic-Oic sequence independently of the residues in position i and $i+3$ of the turn. This would allow the introduction of different amino acids (alkyl, aromatic, polar and charged) (Figure 1) without changing the overall ring structure [20,21], thereby permitting a systematic analysis of their effect upon binding to the human B_2 receptor. The availability of a conformationally homogeneous series, in which all the peptides share a common structure, is the information required for the design of a first generation peptidomimetic, in which a suitable non-peptidic scaffold will replace the backbone.

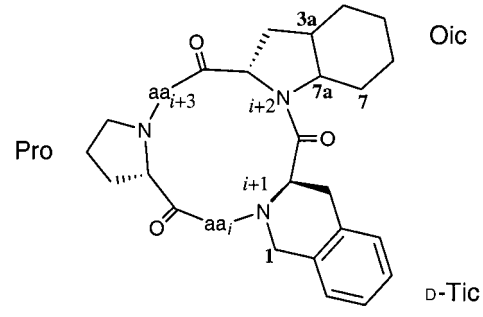


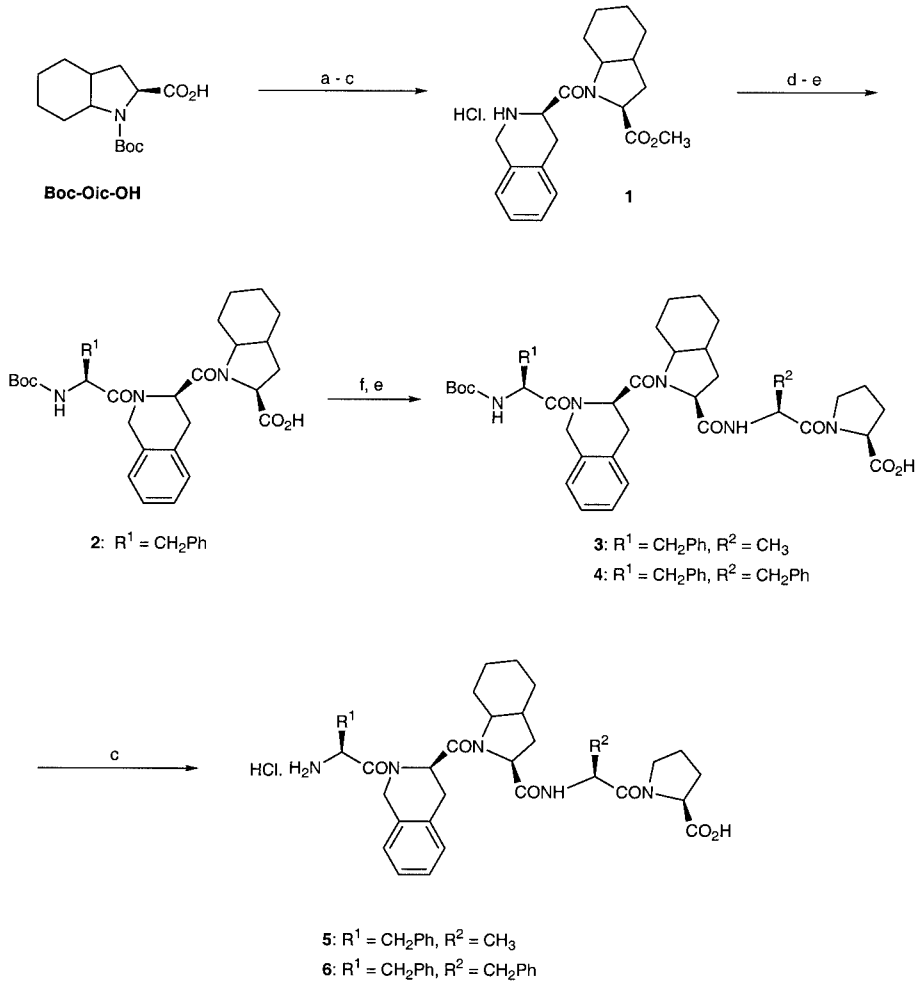
Figure 1 General structure of the cyclic pentapeptide family.

In our series of cyclic pentapeptides Pro was deemed a more appropriate bridging unit between the *N*- and *C-termini* of the type-II′ β-turn due to the known propensity of Gly to compete with the D-aa for the $i+1$ position in the turn [20]. In fact, a later independent molecular modelling study on the compounds from Thurieau *et al.* [19] revealed, among the low-energy conformers, turns centred upon both the Gly and the D-Tic moieties [22]. Proline has the added advantage that it could induce strongly favoured conformations in cyclic pentapeptides by means of a fused β,γ-turn system [20].

MATERIALS AND METHODS

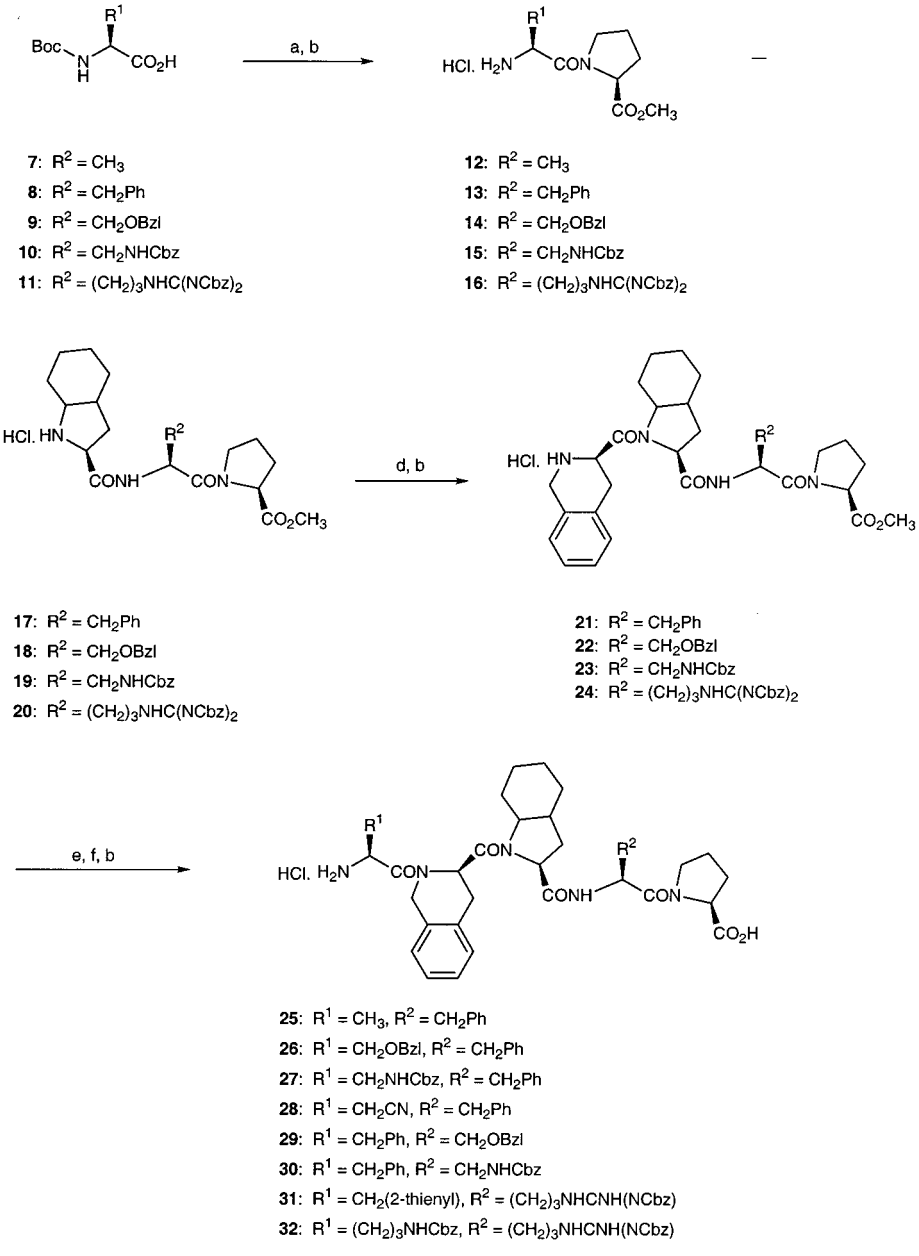
Synthesis of Cyclic Pentapeptides

The linear pentapeptides were synthesized in solution using either a convergent [3 + 2] approach (Scheme 1), or a stepwise approach (Scheme 2). The α-amino groups were *tert*-butoxycarbonyl (Boc) protected, while the side-chain amino groups of diaminopropionic acid (Dap) and ornithine (Orn) were benzyloxycarbonyl (Cbz) protected, the guanidinium group of Arg was *bis*-Cbz protected, the hydroxyl group of Ser was protected as its benzyl ether and methyl ester protection was used for the carboxylic acid group of the *C*-terminal proline. The coupling reactions were carried out using standard HOBt/EDC.HCl activation of the carboxylic acid moiety [23], and cleavage of the Boc group was performed with 4 N HCl/dioxane. Surprisingly, during the treatment with HCl/dioxane, partial removal of Cbz group on the side-chain of arginine was observed so



Scheme 1 Convergent [3 + 2] synthesis of the linear pentapeptides. Reagents and conditons: (a) $SOCl_2$, CH_3OH, -10–40°C; (b) i. Boc-D-Tic-OH, HOBt, EDC.HCl, CH_2Cl_2-DMF (4:1), 0°C, ii. HCl.H-Oic-OCH_3, DIEA, 0°C to rt; (c) 4 N HCl/dioxane, CH_2Cl_2, 0°C to rt; (d) i. Boc-Phe-OH, HOBt, EDC.HCl, CH_2Cl_2-DMF (4:1), 0°C, ii. 1, DIEA, 0°C to rt; (e) i. LiOH, THF-CH_3OH-H_2O (3:2:1), rt, ii. HCl (aq.); (f) i. HOBt, EDC.HCl, CH_2Cl_2-DMF (4:1), 0°C, ii. 12 or 13, DIEA 0°C to rt.

that the linear pentapeptides **31** and **32** were isolated as the *mono*-Cbz derivatives. All the intermediates, after workup and removal of the Boc group, were used in the next coupling reactions without chromatographic purification. After removal of the *N*- and *C*-termini protecting groups, macrocyclization of the pentapeptides was carried out under high dilution conditions (1 mM final concentration), using excess HOAt/EDC.HCl (Scheme 3) [24]. The benzyl-type side-chain protecting groups were removed by hydrogenation in the presence of 10% Pd/C. Problems were encountered in the removal of the Cbz protecting group from compound **46** due to the presence of the sulphur-containing moiety. The group was successfully removed by treatment with trifluoroacetic acid (TFA)/thioanisole [25]. The coupling reaction between Boc-Asn-OH and HCl.H-D-Tic-Oic-Phe-Pro-OMe (**21**) gave, after removal of the Boc group, compound **28** as the major product, dehydration of the primary amide occurring even in the presence of HOBt despite literature claims to the contrary [23]. The resulting nitrile was used to synthesize compound **40** (Scheme 3) and the nitrile group reduced to the amine using $NaBH_4$/$CoCl_2.6H_2O$ [26]. The final compounds were generally purified by flash chromatography [27] (purification by preparative reverse phase-high performance liquid chromatography (RP-HPLC) resulted in disappointing yields of recovered products) and their homogeneity confirmed by thin-layer



7: $R^2 = CH_3$
8: $R^2 = CH_2Ph$
9: $R^2 = CH_2OBzl$
10: $R^2 = CH_2NHCbz$
11: $R^2 = (CH_2)_3NHC(NCbz)_2$

12: $R^2 = CH_3$
13: $R^2 = CH_2Ph$
14: $R^2 = CH_2OBzl$
15: $R^2 = CH_2NHCbz$
16: $R^2 = (CH_2)_3NHC(NCbz)_2$

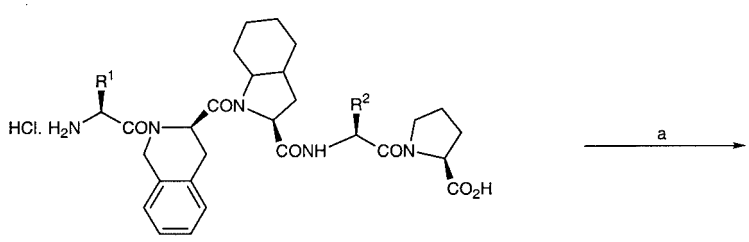
17: $R^2 = CH_2Ph$
18: $R^2 = CH_2OBzl$
19: $R^2 = CH_2NHCbz$
20: $R^2 = (CH_2)_3NHC(NCbz)_2$

21: $R^2 = CH_2Ph$
22: $R^2 = CH_2OBzl$
23: $R^2 = CH_2NHCbz$
24: $R^2 = (CH_2)_3NHC(NCbz)_2$

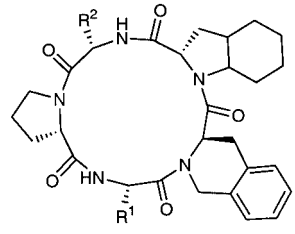
25: $R^1 = CH_3$, $R^2 = CH_2Ph$
26: $R^1 = CH_2OBzl$, $R^2 = CH_2Ph$
27: $R^1 = CH_2NHCbz$, $R^2 = CH_2Ph$
28: $R^1 = CH_2CN$, $R^2 = CH_2Ph$
29: $R^1 = CH_2Ph$, $R^2 = CH_2OBzl$
30: $R^1 = CH_2Ph$, $R^2 = CH_2NHCbz$
31: $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_3NHCNH(NCbz)$
32: $R^1 = (CH_2)_3NHCbz$, $R^2 = (CH_2)_3NHCNH(NCbz)$

Scheme 2 Stepwise synthesis of the linear pentapeptides. Reagents and conditions: (a) i. Boc-Ala-OH, Boc-Phe-OH, Boc-Ser(Bzl)-OH, Boc-Dpr(Cbz)-OH or Boc-Arg(Cbz)$_2$-OH, HOBt, EDC.HCl, CH_2Cl_2 or CH_2Cl_2-DMF (4:1), 0°C, ii. HCl.H-Pro-OCH_3, DIEA, 0°C to rt; (b) 4 N HCl/dioxane, CH_2Cl_2, 0°C to rt; (c) i. Boc-Oic-OH, HOBt, EDC.HCl, CH_2Cl_2 or CH_2Cl_2-DMF (4:1), 0°C, ii. 13–16, DIEA, 0°C to rt; (d) i. Boc-D-Tic-OH, HOBt, EDC.HCl, CH_2Cl_2 or CH_2Cl_2-DMF (4:1), 0°C, ii. 17–20, DIEA, 0°C to rt; (e) i. Boc-Ala-OH, Boc-Phe-OH, Boc-Ser(Bzl)-OH, Boc-Dpr(Cbz)-OH, Boc-Asn-OH, Boc-Thi-OH or Boc-Orn(Cbz)-OH, HOBt, EDC.HCl, CH_2Cl_2 or CH_2Cl_2-DMF (4:1), 0°C, ii. 21–24, DIEA, 0°C to rt; (f) i. LiOH, THF-CH_3OH-H_2O (3:2:1), rt, ii. HCl (aq.).

chromatography (TLC), HPLC, nuclear magnetic resonance (NMR), and mass spectroscopy prior to the testing of their biological activity. An exception was compound **39** whose HPLC-mass spectrum revealed the presence of *ca.* 6% of the *N*-methyl derivative arising from a side reaction during the hydrogenolysis of the Cbz protecting group: HPLC (Spherisorb®; isocratic 60% A: 40% B) $t_R = 16.28$ min; ESMS m/z 655 $(M + H)^+$.



5: $R^1 = CH_2Ph$, $R^2 = CH_3$
6: $R^1 = CH_2Ph$, $R^2 = CH_2Ph$
25: $R^1 = CH_3$, $R^2 = CH_2Ph$
26: $R^1 = CH_2OBzl$, $R^2 = CH_2Ph$
27: $R^1 = CH_2NHCbz$, $R^2 = CH_2Ph$
28: $R^1 = CH_2CN$, $R^2 = CH_2Ph$
29: $R^1 = CH_2Ph$, $R^2 = CH_2OBzl$
30: $R^1 = CH_2Ph$, $R^2 = CH_2NHCbz$
31: $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_3NHCNH(NCbz)$
32: $R^1 = (CH_2)_3NHCbz$, $R^2 = (CH_2)_3NHCNH(NCbz)$



33: $R^1 = CH_2Ph$, $R^2 = CH_3$
34: $R^1 = CH_2Ph$, $R^2 = CH_2Ph$
35: $R^1 = CH_3$, $R^2 = CH_2Ph$
b → **36**: $R^1 = CH_2OBzl$, $R^2 = CH_2Ph$
b → **37**: $R^1 = CH_2OH$, $R^2 = CH_2Ph$
b → **38**: $R^1 = CH_2NHCbz$, $R^2 = CH_2Ph$
b → **39**: $R^1 = CH_2NH_2$, $R^2 = CH_2Ph$
c → **40**: $R^1 = CH_2CN$, $R^2 = CH_2Ph$
c → **41**: $R^1 = (CH_2)_2NH_2.TFA$, $R^2 = CH_2Ph$
d → **42**: $R^1 = CH_2Ph$, $R^2 = CH_2OBzl$
d → **43**: $R^1 = CH_2Ph$, $R^2 = CH_2OH$
b → **44**: $R^1 = CH_2Ph$, $R^2 = CH_2NHCbz$
b → **45**: $R^1 = CH_2Ph$, $R^2 = CH_2NH_2.TFA$
e → **46**: $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_3NHCNH(NCbz)$
e → **47**: $R^1 = CH_2$(2-thienyl), $R^2 = (CH_2)_3NHC(NH)NH_2.TFA$
e → **48**: $R^1 = (CH_2)_3NHCbz$, $R^2 = (CH_2)_3NHCNH(NCbz)$
e → **49**: $R^1 = (CH_2)_3NH_2.TFA$, $R^2 = (CH_2)_3NHC(NH)NH_2.TFA$

Scheme 3 Macrocyclization and deprotection. Reagents and conditions: (a) HOAt/EDC.HCl, DIEA, CH_2Cl_2 or CH_2Cl_2-DMF (4:1), 0°C to rt; (b) H_2, 10% Pd/C, CH_3OH-H_2O, rt; (c) $CoCl_2.6H_2O$, $NaBH_4$, EtOH, rt; (d) H_2, 10% Pd/C, EtOH-H_2O, HCl/dioxane (cat.), rt; (e) TFA, thioanisole, rt.

General

TLC was performed on precoated silica gel 60 plates (Merck). The solvent systems are specified within the text. Spots were visualized by using ultraviolet light (UV) light, with iodine vapour, by spraying with ninhydrin dissolved in acetone (1:100), with cerium (IV) sulphatelammonium molybdenum (VI) oxide in 10% sulphuric acid, or with a basic solution of $KMnO_4$ [28]. HPLC analyses were carried out using a 600 E Waters pump coupled to a Jasco 875 UV detector, and a Merck-Hitachi D-2500 integrator, or on a system comprising a Jasco PU-980 pump, LG-980-02 gradient unit, and UV-975 UV/visible detector, and a Merck-Hitachi D-2500 integrator. Three analytical HPLC columns were used: a Symmetry™ C18, 5 μm, 4.6 × 150 mm, a Spherisorb® S5 ODS2, 5 μm, 4.6 × 250 mm and a Vydac Protein & Peptide C18, 5 μm, 4.6 × 250 mm. The eluants used were (A) water + 0.1% TFA; (B) acetonitrile + 0.1% TFA. The following gradient systems were routinely employed: (i) 20–80% B in 20 min, then 80% B for 5 min (Prg. 1); (ii) 20–92% B in 24 min, then 92% B for 10 min (Prg. 2). A flow rate of 1 mL/min was used, except in the case of the Symmetry™ column, where a flow rate of 0.6 mL/min was used in order to compensate for the shorter column length. Routine analyses were performed with the detector set at $\lambda = 230$ nm, while final compounds were also analysed under isocratic conditions with the detector set at $\lambda = 214$ nm. Retention times are reported in mm with the percentage purity in brackets. For preparative HPLC, a Waters 600 E apparatus with preparative heads was used with a Jasco

875 UV detector, a Kipp Zonen recorder and a 202 Gilson fraction collector. The columns used were a Symmetryprep™ C18, 7 μm, 19 × 300 mm column, a Hibar Lichrosorb RP-18, 7 μm, 25 × 250 mm column, or a Vydac C18, 10 μm, 22 × 250 mm column. The eluants used were the same as for the analytical runs. Silica gel column chromatography was performed on Silica gel 60 (40–63) using Still's procedure [27]. Proton NMR spectra were obtained at 300 MHz on a Varian Gemini (BB) or at 400 MHz on a Bruker Avance spectrometer. Sodium dodecylsulphate (SDS) was purchased from Cambridge Isotope Laboratories Inc., USA. Mass spectra were recorded on a Fisons VG 4 triple quadrupole equipped with a standard ionspray source scanning in the positive mode. The IS cone voltage was held at 23 V. Samples were prepared in water-CH_3CN (1:1) containing 2 mM ammonium acetate. Tandem mass spectra were obtained using argon ($P_{ar} \approx 0.8$ mbar) as the collision induced dissociation (CID) gas at a collision energy of 30 eV, and a cone voltage of 30 V. Hydrogen gas for hydrogenations was generated using a Packard 9200 hydrogen generator. The Boc protected D and L-amino acids were purchased from Synthetech (USA), Bachem (Switzerland) or Novabiochem (Switzerland), while the remaining reagents and solvents were purchased from Aldrich, Fluka or Sigma, and used without further purification.

Macrocyclic Peptides

c(Pro-Phe-D-Tic-Oic-Ala) (33). A solution of compound **5** (64 mg, 0.094 mmol) in CH_2Cl_2 (28 mL) was added dropwise at a rate of 10 mL/h to a chilled solution of 1-hydroxy-7-azabensotriazole (HOAt) (64 mg, 0.47 mmol), EDC.HCl (90 mg, 0.47 mmol) and diisopropylethylamine (DIEA) (82 μL, 0.47 mmol) in CH_2Cl_2 (47 mL)-dimethylformamide (DMF) (19 mL). After the addition was complete, HPLC analysis (Spherisorb®, Prg. 1) showed no starting material remained ($t_R = 20.40$ mm). The solvents were removed under reduced pressure and the residue was partitioned between ethyl acetate (EtOAc) (50 mL) and 1 N HCl (50 mL). The layers were separated and the organic phase was washed with 1 N HCl (50 mL). The combined acid washes were back-extracted with EtOAc (50 mL), and then the combined organic extracts were washed successively with brine (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The organic phase was dried (Na_2SO_4), filtered and the filtrate concentrated under reduced pressure. The crude product was purified by preparative RP-HPLC on a Hibar Lichrosorb column eluting with a gradient of 20–80% B in 1 h at a flow rate of 10 mL/mm to give **33** (8.4 mg, 19%): R_f 0.05–0.21 ($CHCl_3$-CH_3OH, 19:1); HPLC (Spherisorb®; Prg. 1) $t_R = 22.70$ mm (100%), (Spherisorb®; isocratic 35% A: 65% B) $t_R = 8.28$ min (100%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.44 (d, $J = 6.5$ Hz, 1H), 7.39–7.17 (m, 8H), 7.03 (d, $J = 9.60$ Hz, 1H), 6.45 (d, $J = 1.50$ Hz, 1H), 5.01 (m, 1H), 4.87 (d, $J = 14.6$ Hz, 1H), 4.61 (d, $J = 14.6$ Hz, 1H), 4.56 (m, 1H), 4.17 (m, 1H), 3.97 (m, 2H), 3.85 (m, 1H), 3.69 (m, 1H), 3.30 (m, 3H), 2.87 (m, 3H), 2.45–2.29 (m, 2H), 2.21–2.00 (m, 2H), 1.94–1.81 (m, 1H), 1.80–1.12 (m, 12H); ESMS m/z 626 $(M + H)^+$.

The following cyclic pentapeptides were synthesized using the procedure outlined for **33**.

c(Pro-Phe-D-Tic-Oic-Phe) (34). The crude peptide from the macrocyclization was purified by flash chromatography eluting with $CHCl_3$-CH_3OH (97:3) to give **34** (53 mg, 72%): R_f 0.21–0.31 ($CHCl_3$-CH_3OH, 97:3); HPLC (Spherisorb®; Prg. 1) $t_R = 23.94$ min (99.7%), (Spherisorb®; isocratic 35% A: 65% B) $t_R = 15.36$ min (98.8%); ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.41 (d, $J = 6.3$ Hz, 1H), 7.35–7.09 (m, 13H), 6.81 (d, $J = 9.5$ Hz, 1H), 6.51 (d, $J = 1.3$ Hz, 1H), 4.92 (dd, $J = 16.0$, 7.1 Hz, 1H), 4.85 (d, $J = 14.9$ Hz, 1H), 4.58 (d, $J = 14.8$Hz, 1H), 4.55 (m, 1H), 4.19 (m, 1H), 4.00 (m, 3H), 3.69 (m, 1H), 3.30 (m, 3H), 2.98–2.78 (m, 5H), 2.66–2.57 (m, 1H), 2.45–0.94 (m, 13H); ESMS m/z 702 $(M + H)^+$.

c(Pro-Ala-D-Tic-Oic-Phe) (35). Flash chromatography (eluting with $CHCl_3$-EtOAc-abs.ethanol (EtOH), 18:1:1) of the crude product from macrocyclization gave **35** (59 mg, 58%): R_f 0.10 ($CHCl_3$-EtOH-abs.EtOH, 18:1:1); HPLC (Spherisorb®; Prg. 1) $t_R =$ 20.34 mm (98.7%), (Spherisorb®; isocratic 35% A: 65% B) $t_R = 8.19$ min (98.8%); ^{1}H-NMR (400 MHZ, DMSO-d_6) δ 7.37–7.14 (m, 9H), 6.94 (d, $J = 9.5$ Hz, 1H), 6.58 (d, $J = 1.3$ Hz, 1H), 4.90–4.76 (m, 2H), 4.65–4.54 (m, 1H), 4.50 (m, 1H), 4.23 (m, 1H), 4.12–3.88 (m, 3H), 3.70 (m, 1H), 3.30 (m, 3H), 3.03–2.81 (m, 2H), 2.67–2.59 (m, 1H), 2.40–2.32 (m, 2H), 2.17–1.88 (m, 3H), 1.79–1.10 (m, 12H); ESMS m/z 626 $(M + H)^+$.

c(Pro-Ser-D-Tic-Oic-Phe) (37). The linear peptide **26** was cyclized to give the protected peptide **36**. To remove the O-benzyl ether protecting group the crude product (210 mg, 0.29 mmol) was dissolved in 95% EtOH (17 mL), 4 N HCl/dioxane (0.14 mL, 0.58 mmol) was added, followed by a suspension of 10% Pd/C (105 mg) in 95% EtOH (2 mL). The mixture was

degassed with N_2 for 10 min, then H_2 was bubbled through the mixture until HPLC showed complete consumption of the starting material. The mixture was degassed again with N_2, filtered through Celite® to remove the catalyst, and the filtrate concentrated under reduced pressure. The crude product was purified by flash chromatography eluting first with $CHCl_3$, then with $CHCl_3$-abs.EtOH (9:1), to give **37** (99 mg, 44%): R_f 0.23 ($CHCl_3$-EtOAc-abs.EtOH, 8:1:1); HPLC (Spherisorb®; Prg. 1) $t_R = 16.43$ min (98.7%), (Spherisorb®; isocratic 50% A: 50% B) $t_R =$ 9.48 mm (100%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.70–7.67 (m, 1H), 7.30–7.14 (m, 9H), 6.81 (d, $J = 9.2$ Hz, 1H), 6.55 (d, $J = 1.3$ Hz, 1H), 5.07 (t, $J = 12.0$, 6.1 Hz, 1H), 4.93–4.83 (m, 2H), 4.51–4.43 (m, 2H), 4.22–4.19 (m, 1H), 4.12 (m, 1H), 4.05–3.98 (m, 2H), 3.62–3.54 (m, 2H), 3.45–3.30 (m, 3H), 3.01–2.94 (m, 1H), 2.85–2.78 (m, 1H), 2.60 (t, $J =$ 22.3, 10.8 Hz, 1H), 2.37–2.11 (m, 3H), 1.97–1.90 (m, 1H), 1.79–1.17 (m, 10H); ESMS m/z 642 $(M + H)^+$.

c(Pro-Dap-D-Tic-Oic-Phe) (39). Macrocyclization of the linear pentapeptide **27** gave the cyclic product **38** which was purified by flash chromatography eluting with $CHCl_3$-EtOAc-abs.EtOH (18:1:1) (51 mg, 61%): HPLC (Spherisorb®; Prg. 2) $t_R = 21.76$ min (99.2%); ESMS m/z 775 $(M + H)^+$. A portion of this product (20 mg, 0.026 mmol) was Cbz side-chain deprotected by hydrogenation using the procedure outlined above for **36** (the solvent system used in this case was CH_3OH-H_2O, and the 4 N HCl/dioxane was omitted), to give **39** (16 mg, 96%): HPLC (Spherisorb®; isocratic 60% A: 40% B) $t_R = 11.95$ min (93.2%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.29–7.15 (m, 9H), 6.85 (d, $J = 9.0$ Hz, 1H), 6.50 (d, $J = 1.4$ Hz, 1H), 5.05–4.84 (m, 2H), 4.55–4.50 (m, 2H), 4.20–4.01 (m, 4H), 3.71 (m, 1H), 3.54–3.27 (m, 6H), 3.11–2.89 (m, 4H), 2.36–2.11 (m, 3H), 1.92–1.22 (m, 11H); ESMS m/z 641 $(M + H)^+$.

c(Pro-Dab(TFA)-D-Tic-Oic-Phe) (41). The linear peptide **28** (78 mg, 0.11 mmol) was cyclized using the procedure described above, to give, after workup, the crude cyclic compound **40**. To a solution of crude product **40** (50 mg, 0.077 mmol) in EtOH (3 mL), was added $CoCl_2.6H_2O$ (37 mg, 0.16 mmol), followed by portionwise addition of $NaBH_4$ (12 mg, 0.31 mmol), until the mixture remained grey in colour. Further $CoCl_2.6H_2O$ (36 mg, 0.15 mmol), was added followed by $NaBH_4$ (11 mg, 0.29 mmol). The mixture was stirred at room temperature for 2 h, then filtered through Celite® and the filtrate concentrated under reduced pressure. The crude product was purified by preparative RP-HPLC on a SymmetryPrep™ column eluting with a gradient of 20% B to 80% B in 50 min at a flow rate of 17 mL/min. The fractions containing the product were combined, concentrated under reduced pressure, and the residue freeze-dried from water to give **41** (3 mg, 6%): HPLC (Vydac; gradient 25–50% B in 20 min) $t_R = 11.83$ min (95.8%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.65 (brs, 3H), 7.36–7.15 (m, 9H), 7.01 (d, $J = 9.5$ Hz, 1H), 6.52 (d, $J = 1.7$ Hz, 1H), 4.82–4.74 (m, 2H), 4.56–4.49 (m, 2H), 4.20–4.17 (m, 1H), 4.05–3.99 (m, 3H), 3.66 (m, 1H), 3.50 (m, 1H), 3.30 (m, 4H), 2.97–2.89 (m, 3H), 2.39–2.11 (m, 3H), 1.93 (m, 1H), 1.80–1.14 (m, 12H); ESMS m/z 671 $(M + H)^+$.

c(Pro-Phe-D-Tic-Oic-Ser) (43). The linear pentapeptide **29** was cyclized, and the crude product purified on a short column of silica gel, eluting with $CHCl_3$-isopropanol (*i*PrOH) (10:1), to give compound **42** (30 mg, 32%): R_f 0.41 ($CHCl_3$-*i*PrOH, 10:1); HPLC (Spherisorb®; isocratic 20% A: 80% B) $t_R = 8.78$ min (100%); ESMS m/z 732 $(M + H)^+$. The benzyl ether protecting group was removed (see compound **37**), and the crude product purified on a short column of silica gel, eluting with $CHCl_3$-*i*PrOH (10:1), to give compound **43** (6 mg, 60%): R_f 0.30 ($CHCl_3$-*i*PrOH, 10:1); HPLC (Spherisorb®; isocratic 40% A: 60% B) $t_R = 7.90$ min (95%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.45 (m, 1H), 5.22 (m, 1H), 5.02 (m, 1H), 5.89 (m, 1H), 4.62 (m, 1H), 4.56 (m, 1H), 4.34 (m, 1H), 4.24 (m, 1H), 4.03 (m, 2H), 3.74 (m, 1H), 3.72 (m, 1H), 3.48 (m, 1H), 3.35 (m, 2H), 2.92 (m, 3H), 2.37 (m, 2H), 2.10 (m, 2H), 1.86 (m, 1H), 2.40 (m, 2H), 1.75 (m, 3H), 1.63 (m, 3H), 1.52–1.11 (m, 4H); ESMS m/z 642 $(M + H)^+$.

c(Pro-Phe-D-Tic-Oic-Dap(TFA)) (45). Cyclization of the linear peptide **30** gave the crude protected pentapeptide **44**, which was purified by flash chromatography, eluting first with $CHCl_3$-EtOAc (9:1) then with $CHCl_3$-EtOAc-abs.EtOH (18:1:1); R_f 0.06–0.10 ($CHCl_3$-EtOAc-abs.EtOH, 18:1:1); HPLC (Spherisorb®; Prg. 1) $t_R = 23.68$ mm (87%). Hydrogenolysis of the Cbz protecting group (see **37**), and purification of the resulting product by preparative HPLC on a SymmetryPrep™ column, eluting with a gradient of 30–40% B in 1 h at a flow rate of 10 mL/min, gave compound **45** (11 mg, 8%) as its TFA salt; HPLC (Symmetry™; Prg. 2) $t_R = 14.02$ mm (99%), (Symmetry™; isocratic 60% A: 40% B) $t_R =$ 9.62 min (99.7%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.95 (brs, 3H), 7.47 (d, $J = 6.6$ Hz, 1H), 7.38 (d, $J = 7.1$ Hz, 2H), 7.35–7.20 (m, 6H), 7.07 (d, $J = 9.6$ Hz, 1H), 6.80 (d, $J = 1.3$ Hz, 1H), 5.02 (m, 1H), 4.92 (d, $J = 14.5$ Hz, 1H), 4.62 (d, $J = 15.2$Hz, 1H), 4.57

(dd, $J = 10.1$, 3.6 Hz, 1H), 4.54 (dd, $J = 8.4$, 1.6Hz, 1H), 4.15 (dd, $J = 10.1$, 8.2 Hz, 1H), 4.06 (m, 1H), 4.02–3.90 (m, 3H), 3.30 (m, 3H), 3.13 (m, 1H), 2.89 (m, 3H), 2.40 (m, 2H), 2.20–1.98 (m, 3H), 1.87–1.59 (m, 5H), 1.50 (m, 1H), 1.44–1.14 (m, 3H); ESMS m/z 641 $(M + H)^+$.

c(Pro-Thi-D-Tic-Oic-Arg(TFA)) (47). Macrocyclization of the *mono*-Cbz protected pentapeptide **31** gave the cyclic compound **46** which was used without further purification. Thioanisole (380 mL, 3.25 mmol) was added to a solution of **46** (55 mg, 0.065 mmol) in TFA (1.5 mL, 19.5 mmol). The solution was stirred at room temperature for 20 h at which point HPLC analysis (Symmetry™; Prg. 2) showed complete consumption of the starting material ($t_R = 14.60$ min). Diethyl ether was added and the precipitate was filtered and washed with diethyl ether. The crude product was purified on a Vydac C18 preparative column eluting with a gradient of 33–43% B in lh at a flow rate of 20 mL/min to give **47** (13 mg, 25%); HPLC (Vydac; isocratic 64% A: 36% B) $t_R = 8.46$ min (100%); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 7.45–6.40 (m, 13H), 4.96 (m, 1H), 4.82 (m, 1H), 4.60 (m, 2H), 4.47 (m, 1H), 4.08–3.79 (m, 4H), 3.67–3.42 (m, 2H), 2.36 (m, 2H), 2.29–1.11 (m, 18H); ESMS m/z 717 $(M + H)^+$.

c(Pro-Orn(TFA)-D-Tic-Oic-Arg(TFA)) (49). Cyclization of the linear pentapeptide **32** gave the crude product **48** [HPLC (Symmetry™; Prg. 2) $t_R = 14.24$ min (30%); ESMS m/z 946 $(M + H)^+$], which was *bis*-deprotected using thioanisole/TFA (see **47**). The crude product was purified by preparative HPLC on a Vydac column, eluting with a gradient of 15–35% B in 60 min, at a flow rate of 20 mL/min, to give compound **49** (5 mg, 10%); HPLC (Vydac; isocratic 79% A: 21% B) $t_R = 10.08$ min (98.6%); ESMS m/z 678 $(M + H)^+$, 339.5 $(M + 2H)^{++}$.

NMR Spectroscopy

NMR experiments were recorded on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm inverse probe and processed using Xwin-NMR version 2.1. ^{1}H-NMR experiments on all the analogues were carried out on a 2.5 mM sample in DMSO-d_6 at 300 K. The exchange experiment was performed by adding 50 μL of D_2O to 450 μL of a 2.5 mM solution of **33** in DMSO-d_6 at 300 K.

^{1}H-NMR experiments of compound **33** were performed on a 3.8 mM sample in aqueous solution (90% H_2O:10% D_2O) containing 320 mM SDS-d_{25} (98%). Chemical shifts were calibrated with respect to internal tetramethylsilane. For assignment of the spin systems, TOCSY (Total Correlation Spectroscopy) [29] and NOESY (Nuclear Overhauser Enhanced Spectroscopy) [30] spectra were recorded in the phase-sensitive mode using TPPI (Time Proportional Phase Increments Method). NOESY spectra were collected at 300 K with mixing times varying from 100 to 200 ms. No evidence of spin diffusion was observed up to a mixing time of 200 ms. Suppression of the solvent signal was achieved using excitation sculpting [31]. The spectral width was 6400 Hz in both dimensions, with 4 K points in t_2 and 256 data points in t_1 and 96 scans at each increment. Forward linear prediction to 512 points and zero filling to 1024 were applied to the incremented dimension. Sinusoidal apodization was used in both t_2 and t_1. Coupling constants were measured from monodimensional experiments. Cross-peak intensities from the 200 ms spectrum were classified in three different categories: s (strong), m (medium), w (weak). For the temperature coefficient measurements one-dimensional spectra were recorded at 300 and 330 K.

Molecular Dynamics of Compound 33

Molecule sketch, molecular dynamics and analysis of the structures were carried out using the Builder, Discover and Analysis modules of Insight II v.98.0 [32]. Calculations were performed with the CVFF force field as implemented in Discover v.2.98. A dielectric constant of 10.0 was used to mimic a membrane-like environment [33]. For all parameters affecting molecular dynamics, Discover default values were used.

Based on NMR data, obtained in SDS micelles, the following constraints were defined for molecular dynamics. Five constraints (ω constraints) were defined to maintain all the amide bonds in the *trans* conformation; ω dihedral angle values were constrained to $(180° \pm 30°)$. Two constraints were provided from the observed CH_α-NH coupling constants for Ala and Phe; the H-N-Cα-Hα dihedral angle was constrained to $(180° \pm 20°)$ for Ala and to $(180° \pm 25°)$ for Phe. A constraint for the NH-HN distance, with lower and upper bounds of 1.8 and 3.3 Å, respectively, was defined according to the NH Phe-NH Ala NOE interaction. Constraints were defined for the D-Tic and Oic side-chains according to the observed Hα-Hβ coupling constants for the two amino acids. The dihedral angle Hα-Cα-Cβ-H$\beta_{pro\text{-}R}$ was constrained to (35°,85°) for D-Tic and to $(-160°, -120°)$ for Oic; Hα-Cα-Cβ-H$\beta_{pro\text{-}S}$ was

constrained to (180° ± 25°) for D-Tic and to (−40°,0°) for Oic. All constraints were applied adding an extra energy biharmonic function to the general energy term, using the constraint command of Discover. Force constants of 10 kcal/mol/Å^2 and 10 kcal/mol/radian^2 have been used for distance and dihedral angle constraints, respectively.

An unrestrained molecular dynamics of 1500 ps was performed at 1200 K, with a snapshot taken every 5 ps, to generate 300 structures. Each structure was used as starting point for a three step molecular dynamics with constraints. In the first step, applying only ω constraints, molecules were cooled from 1200 to 600 K in 15 ps. In the second step, molecules were cooled from 600 to 300 K in 10 ps using all defined constraints. Finally, ω constraints were removed and dynamics were continued at 300 K for 20 ps. The average of the potential energy over the last 15 ps of dynamics was evaluated for each of the 300 trajectories. The final structures of the 150 trajectories with lowest energy were used for analysis.

RESULTS AND DISCUSSION

NMR and Molecular Dynamics

The conformation of 10 analogues of the general structure presented in Figure 1 were studied using NMR. The proton spectra of the compounds were completely assigned by analysing the TOCSY and NOESY data in DMSO-d_6. The ^{1}H spectra in this solvent revealed the existence of two isomers showing some signals in slow exchange on the NMR time scale. The major isomer (varying from 80–100%) showed strong/medium NOE contacts between: Hα of D-Tic and H-7a of Oic, the NH of $i+3$ aa and Hα of Oic, both the H − 1 protons of D-Tic and Hα of the i aa, Hα of Pro and NH of the i aa and Hα of the $i+3$ aa and Hα of proline. These data indicated a *trans* conformation for all the amide bonds except for the Pro-$i+3$ amide bond that presented a *cis* conformation. Table 1 lists the NH (i and $i+3$ residues) and Hα ($i+1$ and $i+2$ residues) chemical shifts as well as the ^3J NH-α coupling constants (i and $i+3$ residues) for the 10 analogues. All these parameters were very similar within this series indicating a close structural relationship. Moreover, the temperature coefficients were higher than 4 ppb/K for the NHs in position i and lower than 1.5 ppb/K for the NHs in position $i+3$ for all the compounds studied herein, pointing to a hydrogen bonding of the NH of the amino acid in the $i+3$ position. In view of these results, indicating structural homogeneity within the class [34], one representative member of the series could be indifferently chosen for a detailed structure determination. This made possible to perform the conformational analysis of the backbone

Table 1 ^{1}H-NMR Chemical Shifts[a], Coupling Constants[b] and NH Temperature Coefficients[c] of All the Synthesized Analogues in DMSO-d_6[d]

Entry	Compound	NH		Hα		^3J NH-α (Hz)	
	i *i*+3	*i*	*i*+3	*i*+1	*i*+2	*i*	*i*+3
33	*c*[Pro-*Phe*-D-Tic-Oic-*Ala*]	7.03	6.45	4.55	4.00	9.60	1.50
34	*c*[Pro-*Phe*-D-Tic-Oic-*Phe*]	6.80	6.50	4.55	4.00	9.50	1.30
35	*c*[Pro-*Ala*-D-Tic-Oic-*Phe*]	6.94	6.58	4.50	4.03	9.50	1.30
37	*c*[Pro-*Ser*-D-Tic-Oic-*Phe*]	6.81	6.55	4.50	4.04	9.22	1.30
39	*c*[Pro-*Dap*-D-Tic-Oic-*Phe*]	6.85	6.50	4.50	4.00	9.00	1.37
41	*c*[Pro-*Dab*(ThA)-D-Tic-Oic-*Phe*]	7.01	6.52	4.53	4.05	9.50	1.71
43	*c*[Pro-*Phe*-D-Tic-Oic-*Ser*]	7.13	6.60	4.57	4.03	9.90	1.30
45	*c*[Pro-*Phe*-D-Tic-Oic-*Dap*(TFA)]	7.07	6.80	4.52	4.00	9.56	1.30
47	*c*[Pro-*Thi*-D-Tic-Oic-*Arg*(TFA)]	7.12	6.47	4.55	4.00	9.60	1.70
49	*c*[Pro-*Orn*(TFA)-D-Tic-Oic-*Arg*(TFA)]	7.00	6.45	4.50	4.02	9.23	1.30

[a] Chemical shifts are reported in ppm using the methyl group of DMSO-d_6 at 2.50 ppm as an internal reference.
[b] Coupling constants were measured from the one-dimensional spectra.
[c] Temperature coefficients were >4 ppb/K for NH in position i and <1.5 ppb/K for NH in position $i+3$. Data collected at 300 and 330 K from the one-dimensional spectra.
[d] The data were recorded at 400 MHz in DMSO-d_6 at a concentration of 2.5 mM and a temperature of 300 K.

using the inactive compound **33** (see binding activities below), that was available in good quantity after biological tests.

The NMR study of compound **33** was performed in SDS micelles to mimic a membrane-like environment [16].

The ^{1}H-NMR spectra in micelles showed, in contrast to the spectra in DMSO, only one set of resonances precluding the possibility of *cis*/*trans* isomerism around the amide bonds. ^{1}H-NMR chemical shifts and HN-Hα coupling constants of compound **33** in SDS micelles are presented in Table 2. Experiments were carried out in H_2O and D_2O. The Ala NH exchanged very slowly with D_2O (complete after 48 h), indicating that this proton was not exposed to the solvent and/or hydrogen bonded. In our case, if the desired β-turn was present, there should be a hydrogen bond between Ala NH and Phe CO.

All the amide bond conformations were determined to be *trans* using NOE data. Table 3 lists the most significant NOE contacts observed. These data indicated the flipping of the amide bond of the proline when changing from a DMSO to a SDS environment [35].

The extreme values for the coupling constants CHα-NH of Ala (9.4 Hz) and for CHα-NH of Phe (9.0 Hz), the unique amide bond conformation for all the amino acids, and the Ala NH-Phe NH NOE interaction were used to perform a restrained molecular dynamics.

Due to the cyclic structure of the compounds and the presence of D-Tic and Oic residues, the aforementioned experimental constraints were enough to determine the presence of a type-II′ β-turn about the D-Tic and Oic residues. The superimposition of the backbone atoms of 30 low-energy conformations obtained using restraint molecular dynamics of compound **33** is presented in Figure 2. The average backbone dihedral angles, in agreement with a type II′ β-turn conformation [20] at the $i+1$ and $i+2$ were: $\phi_i = -114 \pm 17°$, $\psi_i = 128 \pm 10°$; $\phi_{i+1} = 62 \pm 10°$, $\psi_{i+1} = -120 \pm 8°$; $\phi_{i+2} = -65 \pm 7°$, $\psi_{i+2} = -25 \pm 14°$; $\phi_{i+3} = -120 \pm 16°$, $\psi_{i+3} = -89 \pm 16°$; $\phi_{i+4} = -67 \pm 9°$, $\psi_{i+4} = -35 \pm 11°$. The comparison between the experimental NOE data and the calculated distances was in agreement with the conformation defined by the above dihedral angles (Table 3).

Only the side-chain conformations of the amino acids of the β-turn motif that show a clear conformational preference, as D-Tic and Oic, were analysed. The remaining side-chain conformations were not studied due to their high flexibilities and because they would probably suffer a pronounced induced fit upon interaction with the receptor. The preferred side-chain disposition of the D-Tic residue was analysed, deducing from the different coupling constants JHα/Hβ an *exo* conformation [36]. The Oic residue presented two similar JHα/Hβ of 8.5 Hz and two different NOE interactions between the α and β protons (Hα-$H\beta_{pro\text{-}S}$ strong NOE interaction, Hα-$H\beta_{pro\text{-}R}$ medium NOE interaction). These data indicated a χ_1 angle of (-15 ± 5) for this amino acid.

Table 2 ^{1}H-NMR Chemical Shifts[a] and HN-Hα Coupling Constants[b] of Compound 33 in the Presence of SDS Micelles

aa	HN	Hα	Hβ	Hγ	Others		J (Hz)
Phe	6.97	5.33	3.13				9.00
			2.70				
D-Tic	–	4.47	3.50		$H1_{pro\text{-}S}$	5.02	
			3.18		$H1_{pro\text{-}R}$	4.81	
Oic	–	4.75	2.57		H3a	2.77	
			2.07		H7a	4.08	
					$H7_{pro\text{-}R}$	2.32	
					$H7_{pro\text{-}S}$	1.48	
Ala	7.90	4.96	1.70				9.40
Pro	–	4.36	2.43	1.71	Hδ	4.17	
			2.16	1.66		3.75	

[a] Chemical shifts are reported in ppm using TMS as an internal reference.
[b] Coupling constants were measured from one-dimensional spectra.
[c] The data were recorded at 400 MHz in SDS micelles on a 3.8 mM sample at 300 K.

Table 3 The Most Significant NOE Intensities for **33** in Comparison with Distances for the Calculated Structure

Protons	Exp[a]	Calc[b]	Protons	Exp[a]	Calc[b]
Hα D-Tic-H7a Oic	s	2.3	NH Ala-Hα D-Tic	w	4.3
Hβ D-Tic$_{pro-S}$-H7a Oic	w	4.0	Hα D-Tic-H7 Oic$_{pro-R}$	s	2.5
Hβ D-Tic$_{pro-R}$-H7a Oic	m	2.9	NH Phe-Hα D-Tic	w	5.1
Hβ Ala-δ Pro$_{pro-R}$	m	2.9	NH Phe-Hδ Pro$_{pro-S}$	m	2.9
H1 D-Tic$_{pro-S}$-Hβ$_c$ Phe	m	3.7	NH Phe-Hγ Pro$_c$	m	3.8
H1 D-Tic$_{pro-S}$-Hα Phe	s	2.1	NH Phe-Hδ Pro$_{pro-R}$	w	4.0
H1 D-Tic$_{pro-R}$-Hα Phe	s	2.8	NH Phe-Hα Pro	m	3.5
NH Ala-H7a Oic	w	4.5	NH Ala-H7 Oic$_{pro-R}$	m	3.7
NH Ala-H7 Oic$_{pro-S}$	s	2.7	NH Phe-NH Ala	m	3.4
Hβ$_c$ Ala-δ Pro$_{pro-S}$	m	3.0			

[a] NOE intensities determined from NOESY experiment. w = weak, m = medium, s = strong, c = centroid.
[b] Average distances obtained from restraint molecular dynamics in Å.

Receptor Binding Affinity

The cyclic pentapeptides were evaluated at 10 μM for their ability to inhibit the binding of [^{3}H]-BK to the human B_2 receptor and the results are summarized in Table 4 [37]. Tests were performed directly on the human receptor due to the known non-transferability of binding results from animal versions of the B_2 receptor, e.g. rabbit or rat, to that of humans [19].

Compounds **33**, **34**, **35**, **37** and **43** showed no B_2 binding affinity at 10 μM despite adopting a type-II′ β-turn conformation. This lack of affinity is not caused by the introduction of the Pro residue since compound **47** is equipotent with the Gly containing analogue of Thurieau *et al.* [19], indicating that the *extra* steric hindrance of the Pro residue does not impair the interaction with the receptor, further suggesting that this part of the molecule is not in direct contact with the surface of its biological target. Thus, the absence of activity in the aforementioned analogues is attributable to the unfavourable interactions of the side-chains of the

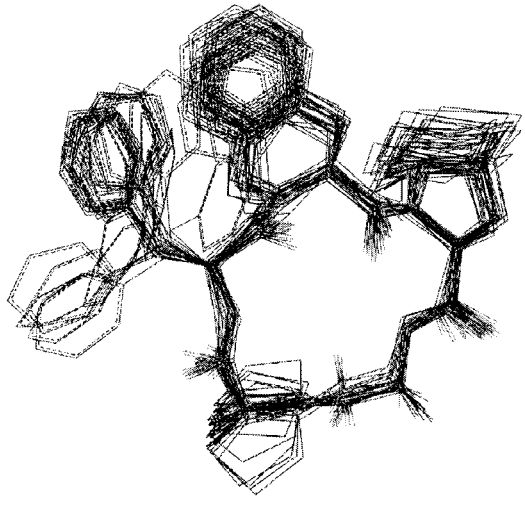


Figure 2 Superimposition of the structures obtained from restrained molecular dynamics of **33**. Superimposition was perfomed using all the backbone atoms.

Table 4 Relative Potencies[a] of the Compounds at the Human Kinin B_2 Receptor

Entry	Compound	hB$_2$R/WI38
33	*c*[Pro-*Phe*-D-Tic-Oic-*Ala*]	IN
34	*c*[Pro-*Phe*-D-Tic-Oic-*Phe*]	IN
35	*c*[Pro-*Ala*-D-Tic-Oic-*Phe*]	IN
37	*c*[Pro-*Ser*-D-Tic-Oic-*Phe*]	IN
39	*c*[Pro-*Dap*-D-Tic-Oic-*Phe*]	18%
41	*c*[Pro-*Dab*-(TFA)-D-Tic-Oic-*Phe*]	36%
43	*c*[Pro-*Phe*-D-Tic-Oic-*Ser*]	IN
45	*c*[Pro-*Phe*-D-Tic-Oic-*Dap*(TFA)]	IN
47	*c*[Pro-*Thi*-D-Tic-Oic-*Arg*(TFA)]	40%
49	*c*[Pro-*Orn*(TFA)-D-Tic-Oic-*Arg*(TFA)]	86% (6.2)

[a] Binding studies at the human B_2 receptor were performed using W138 fibroblast cell membranes [27]. Results are indicated as: IN, inactive at 10 μM concentration; % of binding inhibition at 10 μM concentration; pK_i as −log of the constant of binding inhibition calculated as follows $K_i = IC_{50}/(1 + [L^*]/K_D)$, where K_D and $[L^*]$ are the affinity constant of [^{3}H]-BK (measured in saturation studies) and its concentration used in the displacement studies (0.25–0.30 nM), respectively.

residues present in positions *i* and $i+3$. The introduction of a Dap residue in position *i*, compound **39**, results in a weak binding affinity, which increases upon elongation of the alkyl chain, resulting in compound **41** which has a value comparable with that of the reference compound **47** (the cyclized *C*-terminal sequence of HOE-140). These data are in conflict with the hypothesis put forward by Thurieau *et al.* [19] regarding the importance of an aromatic residue in position *i*. The possibility that the activity of compound **39** arises from the interchangeability of positions *i* and $i+3$ at the receptor level is excluded by the inactivity of compound **45**, which has the amino group in position $i+3$. At this point, it was decided to investigate the effect of the introduction of a residue bearing a positive charge in position *i* of compound 47. The resulting compound **49** showed a 10-fold increase in binding affinity in comparison with the reference compound, thus confirming the beneficial effect of a positive charge in position *i* in our family of cyclic pentapeptides.

CONCLUSION

A series of cyclic pentapeptides, based upon the *C*-terminal fragment of the known BK antagonist HOE-140, has been designed with the aim at obtaining a type-II′ β-turn around the D-Tic and Oic residues. NMR studies on all 10 analogues synthesized revealed the presence of this structure irrespective of the nature of the side-chains of the residues in positions *i* and $i+3$ of the turn. Our binding data suggest that this structural motif is recognized by the B_2 receptor, but alone is not sufficient to guarantee the binding affinity of the small peptides. In fact, it is the nature of the side-chains in positions *i* and $i+3$ which is vital for the moderate affinity demonstrated by certain analogues. The fundamental difference between our most active compound, **49**, and those previously synthesized [19] can be rationalized *via* two hypotheses. Firstly, that compound **49** mimics the *C*-terminal fragment of the high affinity peptide antagonists, such as HOE-140, and that in this case the positively charged residue in position *i* interacts with a region of the receptor usually occupied by the *N*-terminal portion of these larger peptides. This would lend support to the hypothesis that these large peptide antagonists adopt a *quasi* cyclic structure with the *N*- and *C*-termini near to one another [11]. If this is the case, the rigid nature of our cyclic pentapeptides will allow us to investigate the optimal distance between the positive charges and the lipophilic β-turn core. An alternative hypothesis is that this doubly positively charged cyclic pentapeptide is mimicking the *N*-terminal fragment of the larger antagonists with the Orn and Arg in **49** occupying the position of the Arg and Phe or Thi residues found in BK (Arg-Pro-Pro-Gly-Phe) and in HOE-140 (Arg-Pro-Hyp-Gly-Thi), respectively. NMR studies in SDS micelles on large peptide antagonists have revealed the presence of a β-turn in the *N*-terminus of these molecules [38]. This second hypothesis would require that these aromatic residues are involved in a β-interaction with a residue on the receptor and that the guanidinium group in **49** is able to mimic this interaction. Such a situation might explain the difference in activity between compounds **39** and **45**. In the former, the Dap residue in position *i* and the Phe residue in position $i+3$ are able to play these roles, while in the latter, where the positions are reversed, an aromatic residue is present in the region of the molecule requiring a positive charge. Further studies are necessary to discriminate between these two hypotheses.

Materials Suppliers

HOBt (Fluka, Sigma-Aldrich, Milan, Italy); EDC.HCl (Aldrich, Sigma-Aldrich, Milan, Italy); HCl/Dioxane (Aldrich); HOAt (PerSeptive Biosystems, Hamburg, Germany); Pd/C (Fluka); TFA (Merck, Darmstadt, Germany); Thioanisole (Aldrich); Boc-Asn-OH (Bachem, Bubendorf; Switzerland); $NaBH_4$ (Aldrich); $CoCl_2.6H_2O$ (Aldrich); Spherisorb (Waters, Milan, Italy). Ninhydrin (Aldrich); Acetone (Carlo Erba, Milan, Italy); Cerium (Carlo Erba); Sulphuric acid (Carlo Erba); $KMnO_4$ (Carlo Erba); Jasco (L.I. Services, Rome, Italy); Symmetry (Waters); Vydac (Vydac, California, USA); Water (MilliQ, Waters); Acetonitrile (Baker, Mallinckrodt-Baker, Milan, Italy); Kipp Zonen recorder (Gilson, Milan, Italy); Gilson fraction collector (Gilson); Hibar-Lichrosorb (Merck); Silica gel (Carlo Erba); Varian Gemini (Varian, California, USA); Bruker Avance (Bruker, Karlsruhe, Germany); Fisons VG 4 (Fisons, Manchester, England); Ammonium acetate (Fluka); Argon (Rivoira, Turin, Italy); Packard (Alltech, Illinois, USA); CH_2Cl_2 (Aldrich); DIEA (Aldrich); DMF (Fluka); EtOAc (Prolabo, Bois, France); $NaHCO_3$ (Carlo Erba); Na_2SO_4 (Carlo Erba); DMSO d_6, (Cortech, Paris, France); $CHCl_3$ (Prolabo); CH_3OH (Carlo Erba); EtOH (Carlo Erba); N_2 (Rivoira); Celite (Aldrich); i-PrOH (Carlo Erba); Diethyl ether (Carlo Erba); D_2O (Cortech).

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