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ORIGINAL ARTICLE

2-Alkyl-2-carboxyazetidines as γ-turn inducers: incorporation into neurotrophin fragments

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Abstract Conveniently substituted 2-alkyl-2-carboxyazetidine amino acids have been incorporated into NGF and NT3 tetrapeptide sequences to investigate their utility as reverse turn inducers (γ- vs. β-turns). Despite the presence of an Asp residue at *i* position, highly preferred in β-turns, molecular modeling and NMR studies indicated that the azetidine-containing peptides mainly stabilized γ-turn conformations.

Keywords 2-Alkylazetidines · Reverse γ-turn inducers · Conformationally restricted amino acids · Peptides

Introduction

The development of new drugs based on the protein recognition motifs, such as α-helix, β-sheets or reverse turns has become an area of interest (Perez de Vega et al. 2007; Yin and Hamilton 2005). A strategy to stabilize these common structural motifs has been the search of compounds able to mimic or induce particular elements of the peptide secondary structure. In these sense, reverse turns have deserved attention due to their frequent localization on the surface of proteins and peptides, and thus, their broad involvement in biomolecular recognition events. β-Turns are the most common type and comprise four residues, being frequently stabilized by an intramolecular hydrogen bond between the carbonyl oxygen of residue *i* and the amide proton of residue $i + 3$. Among the proteinogenic amino acids, Pro is conformationally unique because its ϕ torsion angle is restricted ($-65° \pm 10°$) and it has marked preference for participating in β-turns (Wilmot and Thornton 1988). Conformational studies on the Pro lower homolog, 2-carboxyazetidine, suggested that this natural amino acid is also able to induce β-turns (Toniolo et al. 1996). Taking into consideration the importance of the side chains as key elements in molecular recognition events, we have developed a synthetic methodology to 2-substituted-2-carboxyazetidines able to carry the side chain of amino acids (Gerona-Navarro et al. 2004). However, conformational studies on the model dipeptide sequences, RCO-Azx-Ala-NHMe and RCO-Ala-Azx-NHMe,[1] have established that these α,α-disubstituted azetidines preferentially induce γ-turn conformations (Baeza et al. 2008, 2009). γ-Turns comprised three amino acids with a hydrogen bond between the oxygen of the CO moiety of the *i* residue and the amide proton of the $i + 2$ residue (Toniolo 1980; Guruprasad and Rajkumar 2000). In addition to γ-turns, molecular dynamic and NMR studies on the azetidine containing models indicated the presence of β-turn-like structures although in lower percentage (Baeza et al. 2008). Therefore, it is of interest to investigate to which extent these restricted amino acids fix γ- versus β-turn conformations when inserted into longer peptides and such a study could be more significant if we select a real peptide system with an amino acid composition having a certain tendency to be in β-turns.

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[1] For a new nomenclature of azetidine containing amino acids (see Bonache 2006). Azx indicates any azetidine, Aze, Azk and Azo refer to Glu, Lys and Nle-derived azetidines, respectively.

Owing to our interest in neuroprotective agents (Alonso de Diego et al. 2005, 2006) we have focused our attention on neurotrophins (NT), in particular on nerve growth factor (NGF) and NT3. Extensive site-directed mutagenesis experiments and 3D crystal structures of neurotrophins and their receptors have contributed to define important regions for binding (Longo et al. 2005; McDonald et al. 1991; Ultsch et al. 1999; Wehrman et al. 2007; Wiesmann et al. 1999). In particular, three surface β-turn regions (loops 1, 2 and 4) are likely candidates for TrK receptors interaction sites. The sequences of loops 1 of NGF (Asp-Ile-Lys-Gly) and NT3 (Asp-Ile-Arg-Gly), and NGF loop 4 (Asp-Gly-Lys-Gln, human, Asp-Glu-Lys-Gln, mouse) contain an Asp residue at i position. This residue has a high tendency to be at this position in β-turns, but the lowest overall positional preference for i position of γ-turns (Guruprasad and Rajkumar 2000). Thus, if the Asp^i favors the adoption of β-turn conformations, these sequences are a good choice to study whether our 2-alkyl-2-carboxyazetidines are able to induce γ-turns, even in non-favorable conditions, or on the contrary, to stabilize β-turns.

To determine the general applicability of azetidines derivatives as γ-turns inducers, this paper describes the synthesis and conformational characteristic of a series of tetrapeptides derived from NGF and NT3 loops, incorporating the corresponding 2-alkyl-2-carboxyazetidine derivative at $i + 1$ or $i + 2$ position, and Pro or α-MePro at $i + 1$ position for comparative purposes.

Materials and methods

Experimental part

General methods

^{1}H-NMR spectra of peptide derivatives were recorded at 400 or 500 MHz, and their ^{13}C-NMR spectra at 100 or 125 MHz. The NMR spectra assignment was based on COSY, HSQC and HMBC spectra. Electrospray mass spectra (ES-MS) were performed, in positive mode. Analytical RP-HPLCs, except for **(*RS*)-8,** were performed on a Novapak C18 (3.9 × 150 mm, 4 μm) column, with a flow rate of 1 mL min^{-1}, using a tunable UV detector set at 214 nm and mixtures of CH_3CN (solvent A) and 0.05% TFA in H_2O (solvent B) as mobile phase, using a 30 min gradient (0–100% of A in B). RP-HPLC of derivative **(*RS*)-8** was performed on an ACE 5 C18-300 (4.6 × 250 mm, analytic; 10 × 250 mm, semi-preparative) column, using a gradient from 0 to 20% of A in B, with a flow rate of 1.3 mL min^{-1} and a gradient of 30 min (analytic) or 6 mL min^{-1} and a 75 min gradient (semi-preparative), using a continuous UV detector set at 214 and 256 nm. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. All compounds were synthesized manually in parallel on resin, following the Fmoc/*t*Bu strategy.

Solid-phase synthesis of tetrapeptides **1–9**

General coupling procedure

The corresponding Fmoc-Xaa-protected resin (268 mg, 0.82 mmol of Wang resin), previously swollen, was treated with 20% piperidine in DMF (1 × 1 min) and (3 × 10 min) and washed with DCM/DMF/DCM/DMF (4 × 0.5 min each). Then, a solution of the corresponding amino acid Fmoc-Yaa-OH (1.5 eq. for coupling to primary amines, or 3 eq. to secondary amines) in anhydrous DMF, HOBt (1.5 eq. for coupling to primary amines, or 3 eq. to secondary amines) and DIC (3 eq. for coupling to primary amines, or 6 eq. to secondary amines) were added. Couplings were allowed to proceed at room temperature overnight. When necessary, the coupling was repeated with a fresh portion of the Fmoc-amino acid and the indicated coupling reagents. After complete coupling, the resins were washed with DMF/DCM/DMF/DCM (4 × 0.5 min each) and drained. Coupling reactions to primary amines were monitored by the Kaiser test and to secondary amines by the chloranil test.

General procedure for the cleavage from Wang resin

After formation of the tetrapeptides, the N-terminal Fmoc group was removed by treatment of the previously swollen resin with 20% of piperidine in DMF (1 × 1 min) and (3 × 10 min). The resulting resin-bounded derivative was treated with a mixture of TFA:H_2O, 95:5 at r.t. for 3 h [for peptide sequences without Arg(Pbf) or Gln(Trt) residues] or with a mixture of TFA/EDT/TA/H_2O/TIS (75:10:10:4:1, v/v/v/v/v) for 3 h [for peptides that contain Arg(Pbf) or Gln(Trt) residues]. After adding ether, the solution was centrifuged, the sobrenadant was eliminated by decantation and the crude was lyophilized.

H-Asp-Pro-Lys-Gln-OH · 2 TFA (1) Syrup, 64% yield. HPLC: t_R = 5.57 min. $[\alpha]_D = -50.11$ (c = 0.91, H_2O). ^{1}H-NMR (400 MHz, DMSO-d_6): rotamers ratio 4.5:1. Major rotamer: δ 8.05 (d, 1H, J = 7.1, α-NH-Lys), 8.01 (d, 1H, J = 8.3, α-NH-Gln), 7.78 (bs, 2H, NH_2), 7.30 (s, 1H, $CONH_2$), 6.81 (s, 1H, $CONH_2$), 4.39 (m, 2H, α-H, Pro and Asp), 4.16 (m, 2H, α-H, Lys and Gln), 3.63 (m, 1H, δ-H, Pro), 3.55 (m, 1H, δ-H, Pro), 2.82 (dd, 1H, J = 17.7, 4.3, β-H, Asp), 2.76 (m, 2H, ε-H, Lys), 2.60 (dd, 1H, J = 17.7, 8.1, β-H, Asp), 2.11 (m, 3H, β-H Pro, γ-H, Gln), 1.95 (m, 1H, β-H, Gln), 1.86 (m, 3H, β-H and γ-H, Pro), 1.77 (m, 1H, β-H, Gln), 1.65 (m, 1H, δ-H, Lys), 1.52 (m, 3H, β-H,

Lys, δ-H, Lys), 1.35 (m, 2H, γ-H, Lys), minor rotamer: δ 8.37 (d, 1H, $J = 7.6$, α-NH-Lys), 8.30 (d, 1H, $J = 7.8$, α-NH-Gln), 7.78 (bs, 2H, NH_2), 7.30 (s, 1H, $CONH_2$), 6.81 (s, 1H, $CONH_2$), 4.57 (m, 1H, α-H, Pro), 4.31 (m, 1H, α-H, Lys), 4.03 (m, 1H, α-H, Gln), 3.82 (m, 1H, α-H, Asp), 3.63 (m, 1H, δ-H, Pro), 3.55 (m, 1H, δ-H, Pro), 2.82 (m, 1H, β-H, Asp), 2.76 (m, 2H, ε-H, Lys), 2.60 (m, 1H, β-H, Asp), 2.25-1.70 (m, 8H, β-H and γ-H, Pro and Gln), 1.65 (m, 1H, δ-H, Lys), 1.52 (m, 3H, β-H and δ-H Lys), 1.35 (m, 2H, γ-H, Lys). ES-MS: 487.3 $[M + 1]^+$.

H-Asp-α-MePro-Lys-Gln-OH · 2 TFA (2) Syrup, 67% yield. HPLC: $t_R = 7.68$ min. $[\alpha]_D = -24.07$ ($c = 0.78$, H_2O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 7.84 (bs, 2H, NH_2), 7.78 (d, 1H, $J = 7.7$, α-NH-Gln), 7.44 (s, 1H, $CONH_2$), 7.42 (d, 1H, $J = 7.3$, α-NH-Lys), 6.90 (s, 1H, $CONH_2$), 4.36 (t, 1H, $J = 6.3$, α-H, Asp), 4.19 (m, 1H, α-H, Lys), 4.12 (m, 1H, α-H, Gln), 3.71 (m, 2H, δ-H, Pro), 3.00 (dd, 1H, $J = 17.7$, 5.0, β-H, Asp), 2.77 (m, 2H, ε-H, Lys), 2.64 (dd, 1H, $J = 17.7$, 7.6, β-H, Asp), 2.11 (m, 2H, γ-H, Gln), 1.93 (m, 2H, γ-H, Pro), 1.88 (m, 2H, β-H, Gln), 1.84 (m, 2H, β-H, Pro), 1.75 (m, 1H, β-H, Lys), 1.56 (m, 1H, β-H, Lys), 1.50 (s, 2H, δ-H, Lys), 1.49 (m, 3H, α-CH_3, Pro), 1.34 (m, 2H, γ-H, Lys). ES-MS: 501.3 $[M + 1]^+$.

H-Asp-Azg-Lys-Gln-OH · 2 TFA (3) Syrup, 62% yield. HPLC: $t_R = 3.17$ min. $[\alpha]_D = -61.08$ ($c = 0.86$, H_2O). ^{1}H-NMR (400 MHz, DMSO-d_6): rotamers ratio 1.5:1. Major rotamer: δ 8.10 (d, 1H, $J = 7.3$, α-NH-Gln), 8.06 (d, 1H, $J = 7.8$, α-NH-Lys), 7.90 (m, 2H, NH_2), 7.29 (s, 1H, $CONH_2$), 6.79 (s, 1H, $CONH_2$), 4.70 (m, 1H, H-2, Azg), 4.25 (m, 1H, α-H, Lys), 4.12 (m, 2H, H-4, Azg), 4.10 (m, 2H, α-H, Asp and Gln), 2.73 (m, 3H, β-H, Asp, ε-H, Lys), 2.56 (m, 1H, β-H, Asp), 2.46 (m, 1H, H-3, Azg), 2.09 (m, 3H, γ-H, Gln, H-3, Azg), 1.91 (m, 1H, β-H, Gln), 1.75 (m, 1H, β-H, Gln), 1.63 (m, 1H, β-H, Lys), 1.50 (m, 3H, β-H and δ-H, Lys), 1.31 (m, 2H, γ-H, Lys), minor rotamer: δ 8.34 (d, 1H, $J = 7.5$, α-NH-Gln), 8.30 (d, 1H, $J = 7.3$, α-NH-Lys), 7.90 (m, 2H, NH_2), 7.33 (s, 1H, NH-$CONH_2$), 6.79 (s, 1H, NH-$CONH_2$), 5.06 (m, 1H, H-2, Azg), 4.25 (m, 1H, α-H, Lys), 4.10 (m, 2H, α-H, Asp, α-H, Gln), 3.80 (m, 2H, H-4, Azg), 2.73 (m, 2H, ε-H, Lys), 2.56 (m, 2H, β-H, Asp, H-3, Azg), 2.46 (m, 1H, β-H, Asp), 2.09 (m, 3H, γ-H, Gln, H-3, Azg), 1.91 (m, 1H, β-H, Gln), 1.75 (m, 1H, β-H, Gln), 1.63 (m, 1H, β-H, Lys), 1.50 (m, 3H, β-H and δ-H, Lys), 1.31 (m, 2H, γ-H, Lys). ES-MS: 473.3 $[M + 1]^+$.

H-Asp-(*RS*)-Aze-Lys-Gln-OH · 2 TFA [(*RS*)-4] Syrup, 54% yield. HPLC: $t_R = 4.00$ min (minor diastereoisomer) and 6.56 min (major diastereoisomer). ^{1}H-NMR (400 MHz, DMSO-d_6): diastereoisomers ratio 1.1:1. Major diastereoisomer: δ 8.15 (d, 1H, $J = 8.1$, α-NH-Lys), 8.12 (d, 1H, $J = 8.1$, α-NH-Gln), 7.84 (m, 2H, NH_2), 7.46 (s, 1H, $CONH_2$), 6.82 (s, 1H, $CONH_2$), 4.27 (m, 1H, α-H, Lys), 4.17 (m, 3H, α-H, Gln, H-4, Aze), 4.02 (m, 1H, α-H, Asp), 2.76 (m, 3H, ε-H, Lys, β-H, Asp), 2.61 (dd, 1H, $J = 17.2$, 5.4, β-H, Asp), 2.46–2.31 (m, 2H, H-3, Aze, 2′-H, Aze), 2.29–2.18 (m, 4H, H-3, Aze, 1′-H, 2′-H, Aze), 2.11 (m, 2H, γ-H, Gln), 1.96 (m, 1H, β-H, Gln), 1.79 (m, 1H, β-H, Gln), 1.73–1.49 (m, 4H, β-H and δ-H, Lys), 1.32 (m, 2H, γ-H, Lys), minor diastereoisomer: δ 8.26 (d, 1H, $J = 7.5$, α-NH-Gln), 7.97 (d, 1H, $J = 7.9$, α-NH-Lys), 7.84 (m, 2H, NH_2), 7.33 (s, 1H, $CONH_2$), 6.84 (s, 1H, $CONH_2$), 4.33 (m, 1H, α-H, Lys), 4.17 (m, 3H, α-H, Gln, H-4, Aze), 4.02 (m, 1H, α-H, Asp), 2.87 (dd, 1H, $J = 17.2$, 7.5, β-H, Asp), 2.76 (m, 3H, ε-H, Lys, β-H, Asp), 2.46–2.31 (m, 2H, H-3, Aze, 2′-H, Aze), 2.29–2.18 (m, 4H, H-3, Aze, 1′-H, 2′-H, Aze), 2.11 (m, 2H, γ-H, Gln), 1.96 (m, 1H, β-H, Gln), 1.79 (m, 1H, β-H, Gln), 1.73–1.49 (m, 4H, β-H and δ-H, Lys), 1.32 (m, 2H, γ-H, Lys). ES-MS: 545.3 $[M + 1]^+$.

H-Asp-(*RS*)-Azo-Lys-Gly-OH · 2 TFA [(*RS*)-5] Syrup, 53% yield. HPLC: $t_R = 9.68$ min. ^{1}H-NMR (400 MHz, DMSO-d_6): diastereoisomers ratio 1.2:1. Major diastereoisomer: δ 8.23 (t, 1H, $J = 5.1$, NH-Gly), 8.09 (d, 1H, $J = 8.2$, α-NH-Lys), 7.86 (m, 2H, NH_2), 4.29 (m, 1H, α-H, Lys), 4.05 (m, 3H, H-4, Azo, α-H, Asp), 3.73 (m, 2H, α-H, Gly), 2.82 (m, 1H, β-H, Asp), 2.72 (m, 2H, ε-H, Lys), 2.62 (m, 1H, β-H, Asp), 2.13 (m, 2H, H-3, Azo), 1.96 (m, 2H, 1′-H, Nle), 1.48 (m, 4H, β-H and δ-H, Lys), 1.22 (m, 6H, γ-H, Lys, 2′-H and 3′-H, Nle), 0.82 (t, 3H, $J = 6.5$, 4′-H, Nle), minor diastereoisomer: δ 8.21 (t, 1H, $J = 5.1$, NH-Gly), 8.12 (d, 1H, $J = 8.1$, α-NH-Lys), 7.86 (m, 2H, NH_2), 4.29 (m, 1H, α-H, Lys), 4.13 (m, 2H, H-4, Azo), 4.01 (m, 1H, α-H, Asp), 3.73 (m, 2H, α-H, Gly), 2.82 (m, 1H, β-H, Asp), 2.72 (m, 3H, ε-H, Lys, β-H, Asp), 2.40 (m, 2H, H-3, Azo), 1.96 (m, 2H, 1′-H, Nle), 1.48 (m, 4H, β-H and δ-H, Lys), 1.22 (m, 6H, γ-H, Lys, 2′-H and 3′-H, Nle), 0.82 (t, 3H, $J = 6.5$, 4′-H, Nle). ES-MS: 458.3 $[M + 1]^+$.

H-Asp-(*RS*)-Azo-Arg-Gly-OH · 2 TFA [(*RS*)-6] White solid, 51% yield. HPLC: $t_R = 9.95$ min. ^{1}H-NMR (500 MHz, DMSO-d_6): diastereoisomers ratio 1.3:1. Major diastereoisomer: δ 9.29 (bs, 1H, NH-εArg), 8.45 (d, 1H, $J = 8.2$, NH-Arg), 7.92 (t, 1H, $J = 5.8$, NH-Gly), 7.16 (bs, 2H, NH_2-Arg), 4.26 (m, 1H, α-H, Arg), 4.12 (m, 2H, H-4, Azo), 3.95 (m, 1H, α-H, Asp), 3.72 (m, 2H, α-H, Gly), 3.09 (m, 1H, δ-H, Arg), 2.99 (m, 1H, δ-H, Arg), 2.66 (m, 1H, β-H, Asp), 2.51 (m, 1H, β-H, Asp), 2.34 (m, 1H, H-3, Azo), 2.20 (m, 1H, H-3, Azo), 1.91 (m, 2H, 1′-H, Nle), 1.60 (m, 2H, β-H, Arg), 1.52 (m, 2H, γ-H, Arg), 1.38-1.14 (m, 4H, 2′-H and 3′-H, Nle), 0.84 (t, 3H, $J = 7.0$, 4′-H, Nle), minor diastereoisomer: δ 8.52 (d, 1H, $J = 7.5$, NH-Arg), 8.44 (m, 1H, NH-Gly), 8.14 (bs, 1H, NH-εArg), 7.27 (bs, 2H, NH_2), 7.07 (bs, 2H, NH_2-Arg), 4.56 (m, 1H, H-4, Azo), 4.12 (m, 2H, α-H, Arg, H-4, Azo), 3.81 (m, 2H, α-H, Asp, α-H, Gly), 3.57 (m, 1H, α-H, Gly), 3.09 (m, 1H, δ-H, Arg), 2.99 (m, 1H, δ-H, Arg), 2.70 (m, 1H, β-H, Asp), 2.60

(m, 1H, β-H, Asp), 2.34 (m, 1H, H-3, Azo), 2.12 (m, 1H, H-3, Azo), 1.91 (m, 2H, 1′-H, Nle), 1.81 (m, 2H, β-H, Arg), 1.38 (m, 2H, γ-H, Arg), 1.38-1.14 (m, 4H, 2′-H and 3′-H, Nle), 0.84 (t, 3H, $J = 7.0$, 4′-H, Nle). ES-MS: 486.3 $[M + 1]^+$.

H-Asp-Gly-(*RS*)-Azk-Gln-OH · 2 TFA [(*RS*)-7] Amorphous solid, 66% yield. HPLC: $t_R = 4.96$ min (minor diastereoisomer) and 5.92 min (major diastereoisomer). ^{1}H-NMR (500 MHz, DMSO-d_6): diastereoisomers ratio 2:1. Major diastereoisomer: δ 8.70 (t, 1H, $J = 5.1$, NH-Gly), 8.32 (d, 1H, $J = 7.6$, α-NH-Gln), 7.88 (bs, 2H, NH_2), 7.34 (s, 1H, $CONH_2$), 6.82 (s, 1H, $CONH_2$), 4.16 (m, 1H, α-H, Gln), 4.12 (m, 1H, α-H, Asp), 4.00 (m, 2H, H-4, Azk), 3.84 (m, 2H, α-H, Gly), 2.82 (m, 1H, β-H, Asp), 2.76 (m, 2H, 4′-H, Azk), 2.72 (m, 1H, β-H, Asp), 2.39 (m, 1H, H-3, Azk), 2.15 (m, 1H, H-3, Azk), 2.08 (m, 2H, γ-H, Gln), 1.94 (m, 3H, 1′-H, Azk, β-H, Gln), 1.78 (m, 1H, β-H, Gln), 1.52 (m, 2H, 3′-H, Azk), 1.40 (m, 1H, 2′-H, Azk), 1.25 (m, 1H, 2′-H, Azk), minor diastereoisomer: δ 8.75 (m, 1H, NH-Gly), 8.36 (d, 1H, $J = 7.6$, α-NH-Gln), 7.88 (bs, 2H, NH_2), 7.36 (s, 1H, $CONH_2$), 6.83 (s, 1H, $CONH_2$), 4.16 (m, 1H, α-H, Gln), 4.12 (m, 1H, α-H, Asp), 4.00 (m, 2H, H-4, Azk), 3.84 (m, 1H, α-H, Gly), 2.82 (m, 1H, β-H, Asp), 2.76 (m, 2H, 4′-H, Azk), 2.72 (m, 1H, β-H, Asp), 2.44 (m, 1H, H-3, Azk), 2.15 (m, 1H, H-3, Azk), 2.08 (m, 2H, γ-H, Gln), 1.94 (m, 3H, 1′-H, Azk, β-H, Gln), 1.78 (m, 1H, β-H, Gln), 1.52 (m, 2H, 3′-H, Azk), 1.40 (m, 1H, 2′-H, Azk), 1.25 (m, 1H, 2′-H, Azk). ES-MS: 473.2 $[M + 1]^+$.

H-Asp-Glu-(*R*)-Azk-Gln-OH · 2 TFA [(*R*)-8] Syrup, 29% yield. HPLC: $t_R = 6.60$ min. $[\alpha]_D = -3.0$ ($c = 0.54$, H_2O). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.79 (d, 2H, $J = 6.6$, NH-Glu), 8.17 (d, 1H, $J = 7.1$, α-NH-Gln), 7.75 (bs, 2H, NH_2), 7.41 (s, 1H, $CONH_2$), 6.90 (s, 1H, $CONH_2$), 4.26 (m, 1H, α-H, Glu), 4.14 (m, 1H, α-H, Gln), 4.09 (m, 1H, α-H, Asp), 4.05 (m, 2H, H-4, Azk), 2.77 (m, 3H, β-H, Asp, 4′-H, Azk), 2.63 (dd, 1H, $J = 17.8$, 9.3, β-H, Asp), 2.30 (m, 3H, H-3, Azk, γ-H, Glu), 2.22 (m, 1H, H-3, Azk), 2.08 (m, 2H, γ-H, Gln), 1.95 (m, 3H, β-H, Gln, 1′-H, Azk), 1.83 (m, 2H, β-H, Glu and Gln), 1.72 (m, 1H, β-H, Glu), 1.55 (m, 2H, 3′-H, Azk), 1.45 (m, 1H, 2′-H, Azk), 1.22 (m, 1H, 2′-H, Azk). ES-MS: 545.3 $[M + 1]^+$.

H-Asp-Glu-(*S*)-Azk-Gln-OH · 2 TFA [(*S*)-8] Syrup, 19% yield. HPLC: $t_R = 10.82$ min. $[\alpha]_D = +15.7$ ($c = 0.54$, H_2O).^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.79 (d, 2H, $J = 6.6$, NH-Glu), 8.32 (d, 1H, $J = 7.8$, α-NH-Gln), 7.75 (bs, 2H, NH_2), 7.24 (s, 1H, $CONH_2$), 6.80 (s, 1H, $CONH_2$), 4.26 (m, 1H, α-H, Glu), 4.19 (m, 1H, α-H, Gln), 4.09 (m, 1H, α-H, Asp), 4.05 (m, 2H, H-4, Azk), 2.77 (m, 4H, β-H, Asp, 4′-H, Azk), 2.50 (m, 1H, H-3, Azk), 2.30 (m, 2H, γ-H, Glu), 2.08 (m, 3H, H-3, Azk, γ-H, Gln), 1.95 (m, 3H, β-H, Gln, 1′-H, Azk), 1.83 (m, 1H, β-H, Glu), 1.72 (m, 2H, β-H, Glu, β-H, Gln), 1.55 (m, 2H, 3′-H, Azk), 1.35 (m, 1H, 2′-H, Azk), 1.22 (m, 1H, 2′-H, Lys). ES-MS: 545.3 $[M + 1]^+$.

H-Asp-Ile-(*RS*)-Azk-Gly-OH · 2 TFA [(*RS*)-9] Syrup, 74% yield. HPLC: $t_R = 9.07$ min (major diastereoisomer) and 10.16 min (minor diastereoisomer). ^{1}H-NMR (500 MHz, DMSO-d_6): diastereoisomers ratio 2:1. Major diastereoisomer: δ 8.77 (d, 1H, $J = 7.8$, NH-Ile), 8.29 (t, 1H, $J = 5.9$, NH-Gly), 7.84 (bs, 2H, NH_2), 4.09 (m, 3H, α-H, Asp, H-4, Azk), 3.92 (m, 1H, α-H, Ile), 3.84 (m, 1H, α-H, Gly), 3.76 (m, 1H, α-H, Gly), 2.73 (m, 2H, 4′-H, Azk), 2.68 (m, 2H, β-H, Asp), 2.36 (m, 1H, H-3, Azk), 2.19 (m, 1H, H-3, Azk), 1.93 (m, 2H, 1′-H, Azk), 1.72 (m, 1H, β-H, Ile), 1.52 (m, 3H, 3′-H, Azk, γ-H, Ile), 1.42 (m, 1H, 2′-H, Azk), 1.25 (m, 1H, 2′-H, Azk), 1.09 (m, 1H, γ-H, Ile), 0.88 (d, 3H, $J = 6.8$, β-CH_3, Ile), 0.83 (t, 3H, $J = 7.3$, δ-H, Ile), minor diastereoisomer: δ 8.74 (d, 1H, $J = 8.3$, NH-Ile), 8.32 (t, 1H, $J = 5.8$, NH-Gly), 7.84 (bs, 2H, NH_2), 4.09 (m, 1H, α-H, Asp), 4.02 (m, 2H, H-4, Azk), 3.92 (m, 1H, α-H, Ile), 3.84 (m, 1H, α-H, Gly), 3.76 (m, 1H, α-H, Gly), 2.73 (m, 2H, 4′-H, Azk), 2.68 (m, 2H, β-H, Asp), 2.45 (m, 1H, H-3, Azk), 2.11 (m, 1H, H-3, Azk), 2.00 (m, 2H, 1′-H, Azk), 1.72 (m, 1H, β-H, Ile), 1.52 (m, 3H, 3′-H, Azk, γ-H, Ile), 1.42 (m, 1H, 2′-H, Azk), 1.25 (m, 1H, 2′-H, Azk), 1.09 (m, 1H, γ-H, Ile), 0.82 (d, 3H, $J = 8.3$, β-CH_3, Ile), 0.81 (t, 3H, $J = 7.3$, δ-H, Ile). ES-MS: 458.3 $[M + 1]^+$.

Molecular modeling

Theoretical calculations methodology is described in Baeza et al. 2008 and 2009.

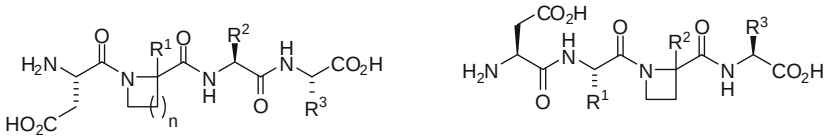
Results and discussions

To evaluate the ability of 2-carboxyazetidine derivatives to induce reverse turns we have prepared the azetidine-containing NGF fragments **3**–**9**, and the proline derivatives **1** and **2**, for comparative purposes (Fig. 1).

The conformational space available to the designed tetrapeptides was first studied by molecular modeling, using different initial conformations of each peptide. The different conformers obtained within +3 kcal mol^{-1} window from the global minimum were grouped into families according to the torsion angles of the peptide skeleton (ϕ and ψ).

To get insight into the reverse turns adopted by these tetrapeptides, we studied the existence of the intramolecular N–H···O = C hydrogen bonds characteristic of these turns. Peptides that incorporate the conformational restricted amino acid at the $i + 1$ position do not present, in general, the characteristic hydrogen bond of β-turns,

Fig. 1 Peptide derivatives that incorporate a conformationally restricted amino acid residue at the $i + 1$ or $i + 2$ position



1-6 **7-9**

Compound	n	R^1	R^2	R^3
1	2	H	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
2		CH_3	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
3	1	H	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
4		$(CH_2)_2CO_2H$	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
5		$(CH_2)_3CH_3$	$(CH_2)_4NH_2$	H
6		$(CH_2)_3CH_3$	$(CH_2)_3NHC(NH)NH_2$	H
7		H	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
8		$(CH_2)_2CO_2H$	$(CH_2)_4NH_2$	$(CH_2)_2CONH_2$
9		$CH(CH_3)CH_2CH_3$	$(CH_2)_4NH_2$	H

Table 1 Percentage of reverse turns in which NH^{i+2} or NH^{i+3} are involved as deduced by molecular dynamics studies, on peptides **1–9**

Compounds[a]		β-Turn (%)	γ-Turn (%)	
		NH^{i+3}	NH^{i+2}	NH^{i+3}
H-Asp-Pro-Lys-Gln-OH	**1**	2	27	65
H-Asp-α-MePro-Lys-Gln-OH	**2**	19	5	49
H-Asp-Azg-Lys-Gln-OH	**3**	4	19	31
H-Asp-(*S*)-Aze-Lys-Gln-OH	**(*S*)-4**	9	82	26
H-Asp-(*R*)-Aze-Lys-Gln-OH	**(*R*)-4**	29	100	0
H-Asp-(*S*)-Azo-Lys-Gly-OH	**(*S*)-5**	7	49	34
H-Asp-(*S*)-Azo-Arg-Gly-OH	**(*S*)-6**	0	82	23
H-Asp-(*R*)-Azo-Arg-Gly-OH	**(*R*)-6**	13	100	20
H-Asp-Gly-(*S*)-Azk-Gln-OH	**(*S*)-7**	3	–	94
H-Asp-Glu-(*S*)-Azk-Gln-OH	**(*S*)-8**	0	–	91
H-Asp-Ile-(*S*)-Azk-Gly-OH	**(*S*)-9**	0	–	76

[a] Azg (R^1 = H), Aze[$R^1 = (CH_2)_2CO_2H$], Azo ($R^1 = (CH_2)_3CH_3$), Azk[$R^1 = (CH_2)_4NH_2$]. R^1 refers to Fig. 1

$CO^i \cdots NH^{i+3}$, being the percentage above 10% only for those that incorporate a residue of α-MePro **2**, and two of the tetrapeptides that contain a residue of 2(*R*) azetidine **(*R*)-4** and **(*R*)-6** (Table 1). On the other hand, a γ-turn centered on the restricted amino acid should imply a hydrogen bond between the CO (Asp) and the amide proton of the $(i + 2)$ residue. The population of this γ-turn is low for peptides with a residue of Pro **1** (27%) and Azg **3** (19%) whereas it increased for those that incorporated α,α-disustituted azetidines **4–6** (49–82%). Thus, the presence of an alkyl group on the chiral carbon of the azetidine ring favors the adoption of γ-turn-like structures, in agreement with the previous data (Baeza et al. 2008, 2009), while a greater conformational flexibility is seen at C-terminus (Fig. 2). Regarding the γ-turn in with the NH^{i+3} is involved, its percentage is rather low, being only above 50% for pro-derived dipeptides **1** and **2**. The existence of hydrogen bonds between the backbone NHs and the CO groups of the amino acid side chains were also observed, as the one between the NH^{i+2} and the carbonyl group of Asp side chain that is over 30% in compounds **1** (35%), **2** (40%) and **3** (31%). The differences between peptides that incorporate at the $i + 1$ position a residue of α-MePro, showing a certain tendency towards β-turn structures, and those with a residue of 2-alkyl-2-carboxyazetidines, which prefer γ-turn conformations, can be seen in Fig. 2.

The data on derivatives **7–9**, which incorporate the azetidine residue at the $i + 2$ position, show a high population of conformers (76–94%) with a hydrogen bond between NH^{i+3} and CO^{i+1}, characteristic of γ-turn conformations centered on the Azk residue, and a higher flexibility toward the N-terminus (Fig. 3). Regarding hydrogen bonds with amino acid side chains, the theoretical studies showed that the NH^{i+1} is involved in hydrogen bonds with the Gln or Asp side chain in derivatives **7** (40%) and **8** (17%).

According to previous reports on 2-alkyl-2-carboxyazetidine containing dipeptide models (Baeza et al. 2008, 2009), the theoretical studies here reported show that the preferred conformation of tetrapeptides **3–9** are γ-turns. Moreover, as previously observed, the tendency to adopt reverse turns depends on the presence of the 2-alkyl group (compounds **4–9** versus **3**). Regarding peptides incorporating Pro derivatives, only the α-MePro containing peptide, **2**, showed a certain tendency to adopt β-turn conformations.

Next, tetrapeptides **1–9** were synthesized to study whether the theoretical studies have a correlation with experimental conformational analysis. The starting non-commercial azetidines were prepared following a synthetic route previously developed in our laboratory (Gerona-Navarro et al. 2004), whereas Fmoc-α-MePro-OH was obtained by acylation of H-α-MePro-OH with Fmoc-Cl.

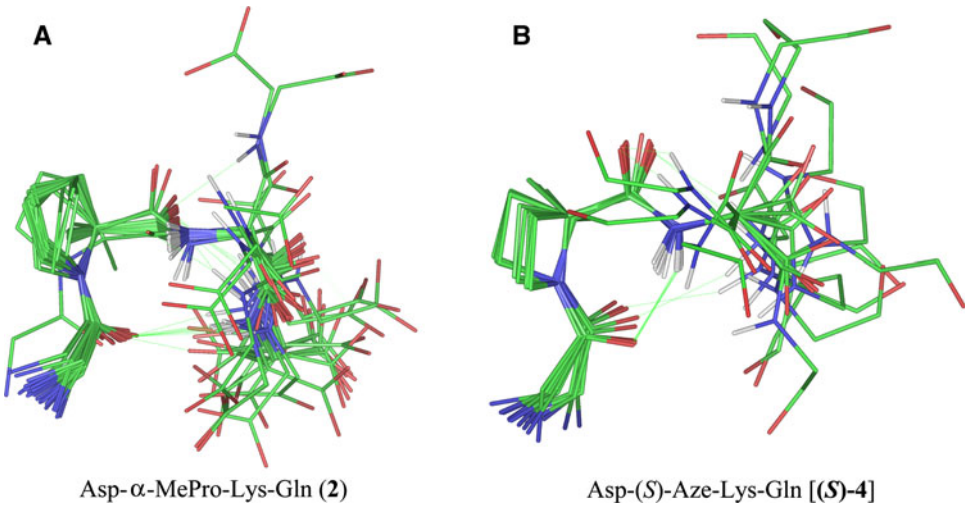


Fig. 2 Superposition of minimal energy conformers (within a 3 kcal mol^{-1} window from the global minimum) of representative peptides that incorporate restricted amino acids at the $i + 1$ position. Side chains and protons are not shown, except for the NH protons of amide groups

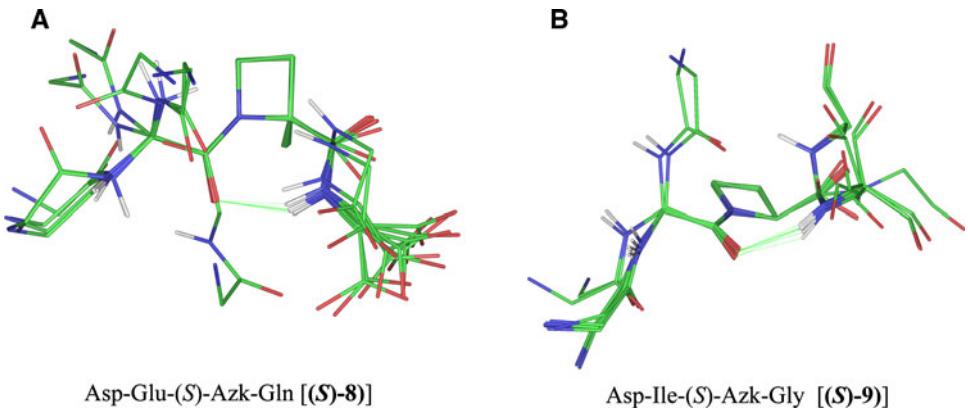
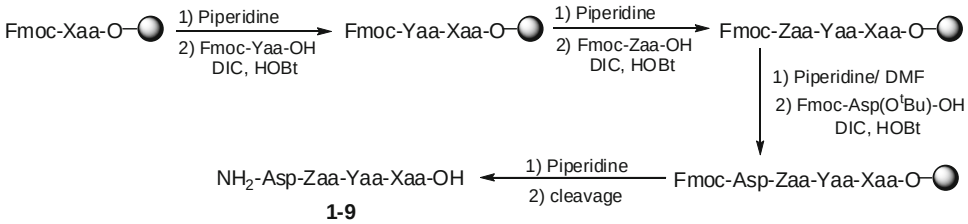


Fig. 3 Superposition of minimal energy conformers (within a 3 kcal mol^{-1} window from the global minimum) of representative peptides that incorporate an Azk residue at $i + 2$ position. Side chains are not shown and only the protons of the amide groups are indicated

Peptides **1–9** were prepared following standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide chemistry, using N-Fmoc-amino acids with the side chain adequately protected [*tert*-butoxy (OtBu) for Asp and Glu, trityl (Trt) for Gln and *tert*-butoxycarbonyl (Boc) for Lys] (Scheme 1). It is worth to point out that for the total formation of tertiary peptide bonds (coupling to Pro and Azx residues) it was necessary to repeat twice the coupling reaction.

Although the starting azetidines were racemic mixtures, tetrapeptides **4–9** have, in general, a different ratio between the two diastereoisomers, as deduced by a careful integration of the non overlapping signals in the ^{1}H-NMR spectra. These data are indicative of the existence of some kinetic resolution due to the different rate in the coupling of both enantiomers, in favor of peptides with an (*R*)-azetidine residue, as it will be discussed later. This different reaction ratio is more significant for peptides containing

Scheme 1 Solid-phase synthesis of compounds **1–9**



Compd.	1	2	3	4	5	6	7	8	9
Xaa	Gln	Gln	Gln	Gln	Gly	Gly	Gln	Gln	Gly
Yaa	Lys	Lys	Lys	Lys	Lys	Arg	Azk	Azk	Azk
Zaa	Pro	α-MePro	Azg	Aze	Azo	Azo	Gly	Glu	Ile

Azk (2:1) compared with those with Aze (1.1:1) or Azo (1.3:1). The results indicated that the kinetic resolution depends on the 2-substituent of the azetidine ring and not on the amino acid that is coupled to the secondary amine (compounds **7**–**9**).

To establish the absolute configuration on the asymmetric carbon (2-C) of the azetidine ring, NOESY and ROESY experiments were carried out on diastereoisomeric mixtures **4**–**9**. However, due to the complexity of the spectra, no correlations signals were obtained that would allow the unequivocal assignment. Then, the couple of diastereoisomers of compound **8** was separated and NOESY experiments were recorded on the isolated isomers. This study showed a weak nOe between the α-Glu proton and the 2′-H of Azk for the minor diastereoisomer, whereas this correlation signal is not observed in the major one. Molecular modeling studies indicated that for the (*S*)-Azk derivative [**(*S*)-8**], in which the α-Glu proton is on the same side than the side chain of Azk, the distance between the above-mentioned protons is lower than in **(*R*)-8**, and it is within nOe distance. These data suggested an S stereochemistry on the chiral carbon of the azetidine ring for the minor diastereoisomer. A close examination of the ^{1}H-NMR spectra of compounds **7**–**9**, showed that the chemical shifts are very similar between each couple of diastereoisomers, with small variations for azetidine H-3 proton and 3-C carbon. A subsequent careful comparison between the chemical shifts for **(*R*)-** and **(*S*)-8** and those for **7** and **9** allowed the assignments of the azetidine chiral atom as *S* for the minor diastereoisomers and *R* for the major ones. On the other hand, the absolute configuration of compounds **(*RS*)-4**–**6** could not be assigned through a careful inspection of their NMR spectra. However, the evaluation of the NMR data coupled with computational analysis allowed us to tentatively assign the chiral center of the azetidine ring as *R* for the major diastereoisomer and *S* for the minor one, as it will be discussed latter.

Then, it was analyzed the possible existence of *cis*/*trans* isomery around the amide bond between Pro or azetidine derivatives and the preceding amino acid. For these assignments, a series of criteria widely used for Xaa-Pro peptides was considered (Kessler 1982; Ishimoto et al. 2000; Schubert et al. 2002). Only derivatives **1** and **3** showed two set of homologous signals in the NMR spectra that can be attributed to *cis*/*trans* isomers around CO-Pro or CO-Azg amide bond, as previously observed in peptides than contain these residues (Baeza et al. 2008, 2009; Ishimoto et al. 2000; Kessler 1982; Schubert et al. 2002). The population of *cis* isomer is higher for compound **3** (40%), which incorporates a residue of Azg, in comparison with derivative **1** (17%) with a Pro residue. Although for diastereoisomeric mixtures **4**–**9** the rate of *cis*/*trans* isomers could not be quantitative measured, a detailed inspection of their NMR spectra suggested that the percentage of *cis* isomer is below 15%, whereas only the *trans* isomer is observed for the MePro derivative **2**.

To study whether peptides **1**–**9** were able to adopt reverse turns structures stabilized by hydrogen bonds, temperature-dependent NMR measurements were performed. In Table 2, there are collected values of the temperature coefficients for derivatives **1**–**9**. These values indicated that when the restricted amino acid is at the $i + 1$ position (derivatives **1**–**6**) the temperature coefficients of the amide proton $i + 2$ are, in general, compatible with the existence of an intramolecular hydrogen bond ($\Delta\delta/\Delta T \leq 3$ in absolute value), whereas when the azetidine residue is located at the $i + 2$ position (compounds **7**–**9**) it is the NH^{i+3} the one that is solvent shielded, likely due to the formation of an intramolecular hydrogen bond.

It is interesting to note that tetrapeptide **2** (α-MePro at $i + 1$ position) and the major diastereoisomers of **4** and **6**, with a residue of Aze or Azo at the $i + 1$ position, respectively, have low temperature coefficients for the NH^{i+3}, indicating their involvement in intramolecular hydrogen bonds. It is worth to mention the different behavior for the two diastereoisomers of compound **6** (Asp-Azo-Arg-Gly). Thus, while for the major isomer the temperature coefficient of both of its amide protons are in the range of hydrogen-bonded amide protons, these protons are

Table 2 Temperature coefficients of the amide NH protons of compounds **1**–**9**

Compounds[a]		$\Delta\delta/\Delta T$[b]		
		NH^{i+1}	NH^{i+2}	NH^{i+3}
H-Asp-Pro-Lys-Gln-OH	**1**	–	−5.7	−4.2
H-Asp-α-MePro-Lys-Gln-OH	**2**	–	−2.1	−3.0
H-Asp-Azg-Lys-Gln-OH	**3**	–	−3.7	−3.7
H-Asp-Aze-Lys-Gln-OH	**(*R*)-4**[a]	–	−0.6	−1.8
	(*S*)-4[a]	–	−2.8	−4.9
H-Asp-Azo-Lys-Gly-OH	**(*R*)-5**[a]	–	−1.8	−4.4
	(*S*)-5[a]	–	−0.8	n.d.
H-Asp-Azo-Arg-Gly-OH	**(*R*)-6**[a]	–	−1.8	−2.7
	(*S*)-6[a]	–	−3.9	−7.7
H-Asp-Gly-Azk-Gln-OH	**(*R*)-7**	−3.6	–	−2.3
	(*S*)-7	−3.8	–	−1.5
H-Asp-Glu-Azk-Gln-OH	**(*R*)-8**	−4.6	–	−1.9
	(*S*)-8	n.d.	–	−2.0
H-Asp-Ile-Azk-Gly-OH	**(*R*)-9**	−4.1	–	−1.1
	(*S*)-9	−4.4	–	−1.6

[a] The stereochemistry has been tentatively assigned on the base of molecular modeling studies [(*R*), major diastereoisomer, (*S*) minor diastereoisomer]

[b] $\Delta\delta$ measured in DMSO-d_6, 30°–60° (5° intervals)

n.d. not determined

solvent exposed for the minor diastereoisomer ($\Delta\delta/\Delta T \geq 4$ in absolute value). A similar behavior, although with no so pronounced differences, is observed for the diastereoisomers **4** (H-Asp-Aze-Lys-Gln-OH). The different behavior regarding the capacity to form hydrogen bonds of the couple of diastereoisomers of compounds **4** and **6**, which parallelized the results obtained in the theoretical studies, support the tentative assignment of the major diastereoisomer as *R*, and the minor as *S*.

Although the temperature coefficients provided information regarding the NH involved in a hydrogen bond, there is no data concerning the acceptor carbonyl oxygen. Thus, there are several possible hydrogen bonding arrangements for compounds **1–9**, that might lead to the adoption of β- or γ-turn conformations. Alternatively, there might be hydrogen bonds in which participate the side chains of Asp, Glu and/or Gln. To obtain clues of the 3D disposition of these tetrapeptides, NOESY and ROESY experiments have been carried out. Unfortunately, the data obtained did not show nOe signals that might be of help to discriminate between the different possibilities.

Finally, to establish the 3D disposition of these peptides, a carefully comparison between experimental NMR data and molecular modeling results was done. Although theoretical studies have shown the presence of hydrogen bonds between backbone NH and amino acids side chains, in general, NMR studies do not support their existence, with the possible exception of those peptides with the highest percentages in the molecular dynamics studies, as derivative **7** (40% with Gln side chain), which an NH^{i+1} temperature coefficient in the uncertainty range, and **2** (40% with Asp side chain), in which the NH^{i+2} is involved in a hydrogen bond.

Molecular modeling and NMR studies also showed the existence of families with reverse turns. In this sense, for peptides that incorporate the restricted amino acid at position $i + 1$ (**1–6**), the existence of β-turns would imply protection from the solvent of the NH^{i+3}, as in peptide **2** having α-MePro, and **(*R*)-4**, **(*R*)-6** with 2(*R*) azetidine residues. In contrast, β-turn-like conformations are not observed in peptides containing 2(*S*)-2-alkyl-2-carboxyazetidines at $i + 1$ (**4–6**), as well as **(*R*)-5**, despite having an Asp residue at position *i*, which has a high tendency to be at this position in β-turns. With respect to the NH^{i+2} proton, in general, it is solvent shielded in tetrapeptides **4–6**, which might be due to the presence of the hydrogen bond characteristic of γ-turns, or one in which the Asp, Glu or Gln side chain are involved. Theoretical studies indicated low percentage (<30%) of hydrogen bonds with side chains, whereas the percentage of γ-turn-like conformers is high. Although it is known that theoretical studies tend to overestimate γ-turns, it is worth pointing out that the methodology and force field used in this study are identical to the ones applied for related simplified peptides, which results have been validated by NMR and X-ray studies (Baeza et al. 2008, 2009). Thus, these data support the adoption of γ-turn structures within peptides **4–6**.

The results of the temperature studies of derivatives **7–9** incorporating Azk at the $i + 2$ position indicated protection from the solvent of the NH^{i+3} proton. However, there are at least three hydrogen bonding possibilities, namely the formation of a β- or a γ-turn, or a hydrogen bond with an amino acid side chain. Molecular dynamic studies on these tetrapeptides showed the existence of a hydrogen bond $CO^{i+1}\cdots NH^{i+3}$, characteristic of γ-turns (over 75% of the low energy families), whereas the percentage were much lower for the other possible hydrogen bonds. Once more, the rather high percentage of γ-turn populations indicated by theoretical studies, varying from 76 to 91%, together with the good predictions of our previous studies, are indicative of γ-turn conformations center on the azetidine ring for tetrapeptides **7–9**.

On the whole, the results of the experimental studies support the molecular modeling data, indicating that 2-carboxyazetidines derivatives have a tendency to induce γ-turns when incorporated into tetrapeptide sequences, being essential the presence of a 2-alkyl substituent on the azetidine ring.

Conclusions

Conformational studies on NGF tetrapeptide fragments incorporating 2-alkyl-2-carboxyazetidine residues, here reported, indicate a tendency toward γ-turn conformations, and support previous results on simplified dipeptide models. It is worth noticing the ability of these α,α-disubstituted azetidines to bias the conformational preferences towards γ-turn-like structures versus β-turns, even when an Asp residue is at the i position, a location highly preferred for β-turns, but not for γ-bends. The γ-turn inducing tendency of the 2-alkyl-2-carboxyazetidines in these tetrapeptides, with rather no influence either of its location in the peptide chain or the proteinogenic amino acids of the peptide sequences, strength the value of these azetidines as γ-turns inducers able to stabilize this natural motif in peptides.

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