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β-Amino acids containing peptides and click-cyclized peptide as β-turn mimics: a comparative study with 'conventional' lactam- and disulfide-bridged hexapeptides

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The increasing interest in click chemistry and its use to stabilize turn structures led us to compare the propensity for β-turn stabilization of different analogs designed as mimics of the β-turn structure found in tendamistat. The β-turn conformation of linear β-amino acid-containing peptides and triazole-cyclized analogs were compared to 'conventional' lactam- and disulfide-bridged hexapeptide analogs. Their 3D structures and their propensity to fold in β-turns in solution, and for those not structured in solution in the presence of α-amylase, were analyzed by NMR spectroscopy and by restrained molecular dynamics with energy minimization. The linear tetrapeptide Ac-Ser-Trp-Arg-Tyr-NH_2 and both the amide bond-cyclized, c[Pro-Ser-Trp-Arg-Tyr-D-Ala] and the disulfide-bridged, Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 hexapeptides adopt dominantly in solution a β-turn conformation closely related to the one observed in tendamistat. On the contrary, the β-amino acid-containing peptides such as Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2, and the triazole cyclic peptide, c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2, both specifically designed to mimic this β-turn, do not adopt stable structures in solution and do not show any characteristics of β-turn conformation. However, these unstructured peptides specifically interact in the active site of α-amylase, as shown by TrNOESY and saturation transfer difference NMR experiments performed in the presence of the enzyme, and are displaced by acarbose, a specific α-amylase inhibitor. Thus, in contrast to amide-cyclized or disulfide-bridged hexapeptides, β-amino acid-containing peptides and click-cyclized peptides may not be regarded as β-turn stabilizers, but can be considered as potential β-turn inducers. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: β-turn; β-amino acids containing peptide; click-cyclized peptide; cyclic peptides; tendamistat; α-amylase; NMR

Introduction

The objective of structure-based design is to discover mimic(s) of a natural ligand, which will be a smaller, preferentially non-peptidic molecule, presenting the structural characteristics of the lead molecule and plausibly retaining its binding capacities. These two goals may sometimes be elusive, as the structural features of the lead compound, which can be determined by either NMR (solution or solid state) and/or X-ray (solid state), may not be related to its bound structure, the so-called bioactive conformation. Thus, the structure of the ligand (or substrate) remains the only tangible starting point in these structure-based strategies, notwithstanding the fact that mutual adaptation of both partners might occur upon formation of the ligand–receptor or substrate–enzyme complexes. In that context, β-turn structures within the ligand or substrate have been implicated as recognition domains [1,2], as in numerous cases this short motif (by definition four amino acids being required) stabilizes the pharmacophore, or the so-called hot spot contacts, in protein–protein interactions and in ligand–receptor or substrate–enzyme complexes.

Somatostatin [3,4], the 14-residue hypothalamic peptide, which interacts with nanomolar affinities with five isoforms of somatostatin receptors [5–7], and tendamistat, the 74-residue bacterial protein that inhibits α-amylase with a sub-nanomolar affinity [8–10], constitute attractive targets for designing β-turn models.

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Abbreviations used: *βtA, β-(1H-[1,2,3]triazol-4-yl)alanine; MES, 2-(N-morpholino)ethanesulfonic acid; N_3Lys, (S)-α-azidolysine; Pra, (S)-propargylglycine; (R)-β^2-hTrp, (R)-β^2-homotryptophan; (R)-β^3-hSer, (R)-β^3-homoserine; (S)-β^3-hArg, (S)-β^3-homoarginine; (S)-β^3-hTyr, (S)-β^3-homotyrosine.*

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Table 1. Sequences of synthesized peptides

Peptide	Sequence	Feature
(1)	Ac-Ser-Trp-Arg-Tyr-NH_2	Linear (α-peptide)
(2)	Ac-(*S*)-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*R*)-Tyr-NH_2	Linear (α/β-peptide)
(3)	Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2	Linear (α/β-peptide)
(4)	Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2	Linear (β-peptide)
(Control)	H-Arg-Tyr-D-Ala-Pro-Ser-Trp-OH	Linear precursor of **(5)**
(5)	c[Pro-Ser-Trp-Arg-Tyr-D-Ala]	Cyclic (amide bond)
(6)	Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2	Cyclic (disulfide bridge)
(lin-7)	N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2	Linear precursor of **(7)**
(7)	c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2	Cyclic (triazole link)

Indeed, both somatostatin and tendamistat interact with their protein targets, via a β-turn structure: β-II′ turn [11–13] and a slightly distorted β-I type turn [8–10], respectively. Numerous strategies and consequently structures have been proposed to stabilize β-turn conformations (for reviews see [14–17]). One of these strategies consists in incorporating the amino acids or side chain functions in a cyclic structure, as successfully shown with hexapeptide analogs of somatostatin [18–20]. Lactam-bridged and disulfide-bridged hexapeptide analogs of somatostatin led to molecules as potent as the parent hormone, and both types of cyclic analogs are now used for therapeutic applications, as these cyclizations have considerably improved the metabolic stability of the analogs compared to somatostatin ($t_{1/2}$ over a few hours compared with minutes) [21]. On the basis of this strategy, lactam-bridged and disulfide-bridged analogs of tendamistat have been described about 15 years ago, as well as analogs incorporating non-peptidic β-turn stabilizers/inducers [22–25]. However, the inhibitory potencies of the designed analogs remained low compared to tendamistat (15–500 μM vs 0.2 nM) [22–25]. The main conclusion of the structural analysis of these constrained analogs was that the β-turn in the vicinity of the Trp-Tyr-Arg tripeptide sequence was maintained in most of these hexapeptides and these templates were capable of mimicking the tendamistat structure 'qualitatively, if not quantitatively' [22]. Indeed, these three residues constitute only part of the domains of tendamistat that interact with α-amylase [8–10]. More recently, other strategies have been reported to stabilize a β-turn conformation, for example, using β-amino acid-containing peptides [26], triazole cyclic peptides as an application of click chemistry [27–29], and also chimeric proline amino acids [30,31].

Herein, we report the syntheses and structural analyses of different peptides analogs of the β-turn structure of tendamistat. The aim of this study was to compare two of the new strategies for restraining the conformational flexibility within a given conformational space with the classical stabilizations of β-turn conformation by conventional cyclization. The nanomolar activity of tendamistat was not within the scope of this study, considering the overall surface of interaction in the tendamistat–α-amylase complex. Starting from the initial β-turn structure of tendamistat Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) the following analogs have been synthesized and analyzed (Table 1): Ac-(*S*)-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*R*)-Tyr-NH_2 (**2**), Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**) and Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**4**), c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**), Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) and c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**). The β-turn conformation of linear β-amino acid-containing peptides (**2–4**) and the triazole-cyclized analog (**7**) were compared to 'conventional' lactam-bridged hexapeptide (**5**), and disulfide-bridged hexapeptide (**6**) analogs. Their 3D structures and their propensity to adopt β-turn conformations were analyzed by NMR spectroscopy in solution. The conformational properties of these peptides were first examined in methanol as a solvent to promote peptide structure. They were next studied in aqueous solution to analyze whether the conformational preferences were still retained in water. Finally the conformations of the most flexible peptides were investigated in the presence of α-amylase. The 3D structures of all these peptides were calculated by restrained molecular dynamics and energy minimization using interproton distance restraints derived from NOEs and dihedral angle restraints derived from homonuclear vicinal coupling constants measured in methanol or aqueous solvent.

Materials and Methods

Peptide Synthesis and Purification

All standard chemicals and protected amino acids were purchased from Sigma (Saint Quentin Fallavier, France), Merck Chemicals Ltd. (Nottingham, UK) or Iris Biotech (Marktredwitz, Germany). Solvents and reagents were used as commercially available without further purification. (*S*)-α-Azido-Lys(Boc)-OH [32], Boc-(*S*)-β^3-hArg(Z)$_2$-OH [33] and Boc-(*R*)-β^2-hTrp-OH [34] were prepared in the laboratory. All peptides were synthesized by SPPS for compounds (**1–4**) and (**6**) α-*p*-methylbenzylhydrylamine (MBHA resin, Merck Chemicals Ltd.), for compound (**5**) Boc-Trp(For)-PAM resin (Applied Biosystems, Foster City, CA, USA) and for compound (**7**) Rink amide MBHA resin (Merck Chemicals Ltd.). Peptides were purified by reverse phase HPLC on a SymmetryPrep™ C8 7 μm (7.8 mm × 300 mm) column (Waters, Saint Quentin en Yvelines, France) by eluting with a gradient [system A: 0.1% (v/v) TFA in acetonitrile or system B: 0.1% (v/v) TFA in methanol] in aqueous 0.1% (v/v) TFA. Homogeneous fractions were pooled and lyophilized after determination of more than 95% purity by analytical HPLC, that was performed on a Symmetry® C8 5 μm (4.6 mm × 250 mm) column (Waters) in isocratic mode with UV detection at 220 and 280 nm. Mass spectral analysis was performed by MALDI-TOF (Voyager-DE™ PRO Workstation, Applied Biosystems).

Ac-Ser-Trp-Arg-Tyr-NH_2 ***(1)***

The synthesis was carried out on an ABI Model 431A peptide synthesizer (Applied Biosystems) starting from MBHA resin (substitution: 0.51 mmol/g) in a 0.1 mmol scale. All N^{α}-Boc-amino acids (10 equiv.) were coupled by *N*, *N*′-dicyclohexylcarbodiimide

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(DCC)/1-hydroxybenzotriazole (HOBt) (10 equiv., 1 : 1). After acetylation (Ac_2O/DIEA, 20 equiv., 1 : 1), the resin was dried *in vacuo* before deformylation of Trp (piperidine/DMF, 1 : 10 at 0 °C). After drying *in vacuo*, the peptide was cleaved from the resin by HF for 2 h at 0 °C in the presence of 1.5 ml anisole and 0.25 ml dimethylsulfide/g of peptidyl-resin. The resin was subsequently washed three times with cold Et_2O and then extracted three times with 20% AcOH. After lyophilization and HPLC purification with system A, compound (**1**) was obtained in 53% yield. MALDI-TOF MS analysis: Ac-Ser-Trp-Arg-Tyr-NH_2 (MH^+) calculated: 652.3; (MH^+) found: 652.00.

*Ac-D-Cys-(R)-β^2-hTrp-(S)-β^3-hArg-D-Tyr-NH_2 (**2**)*

The synthesis was performed manually on MBHA resin (substitution: 0.51 mmol/g) in a 0.1 mmol scale. Boc-D-Tyr(2-Br-Z)-OH (5 equiv.) was coupled by DCC/HOBt (5 equiv., 1 : 1) and Boc-(*S*)-β^3-hArg$(Z)_2$-OH (3 equiv.), Boc-(*R*)-β^2-hTrp-OH (3 equiv.) and Boc-D-Cys(4-MeOBzl)-OH (5 equiv.) by *O*-benzotriazole-*N*, *N*, *N*′, *N*′-tetramethyl-uronium-hexafluorophosphate (HBTU)/DIEA (1 : 2). After acetylation as described for (**1**), the resin was dried *in vacuo* before HF cleavage for 1 h at 0 °C in the presence of 1.5 ml anisole, 0.25 ml dimethylsulfide and 300 mg *p*-thiocresol/g of peptidyl-resin. After HPLC purification by system A, compound (**2**) was obtained in 31% yield. MALDI-TOF MS: Ac-D-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-D-Tyr-NH_2 (MH^+) calculated: 696.32; (MH^+) found: 696.25.

*Ac-(R)-β^3-hSer-Trp-(S)-β^3-hArg-(S)-β^3-hTyr-NH_2 (**3**)*

The synthesis was carried out manually on MBHA resin (substitution: 0.51 mmol/g) in a 0.05 mmol scale. All N^{α}-Boc-amino acids (3 equiv., except Boc-Trp(For)-OH 5 equiv.) were coupled by HBTU/DIEA (1 : 2). After acetylation and deformylation as described for (**1**), the resin was dried *in vacuo* before HF cleavage for 3 h (benzyl protecting group of (*S*)-β^3-hTyr was not totally cleaved) at 0 °C in the presence of 1.5 ml anisole and 0.25 ml dimethylsulfide/g of peptide resin. After HPLC purification using system A, compound (**3**) was obtained in 15% yield. MALDI-TOF MS: Ac-(*R*)-β^3-hSer-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (MH^+) calculated: 694.36; (MH^+) found: 694.41.

*Ac-(R)-β^3-hSer-(R)-β^2-hTrp-(S)-β^3-hArg-(S)-β^3-hTyr-NH_2 (**4**)*

The synthesis was carried out manually on MBHA resin (substitution: 0.51 mmol/g) in a 0.05 mmol scale. All N^{α}-Boc-amino acids (3 equiv.) were coupled by HBTU/DIEA (1 : 2). After acetylation as described for (**1**), the resin was dried *in vacuo* before HF cleavage for 2 h at 0 °C in the presence of 1.5 ml anisole and 0.25 ml dimethylsulfide/g of peptide resin. After HPLC purification by system A, compound (**4**) was obtained in 8% yield. MALDI-TOF MS: Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (MH^+) calculated: 708.37; (MH^+) found: 708.37.

*c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**)*

The synthesis of the linear peptide H-Arg-Tyr-D-Ala-Pro-Ser-Trp-OH (control) was carried out manually on a preloaded Boc-Trp(For)-PAM resin (substitution: 0.7 mmol/g) in a 0.1 mmol scale. All N^{α}-Boc-amino acids (10 equiv.) were coupled by HBTU/DIEA (1 : 2). After removal of the terminal Boc protecting group, Trp was deformylated (piperidine/DMF 2 : 10 at 0 °C). After drying *in vacuo*, the peptide was cleaved from the resin by treatment with liquid anhydrous HF for 2 h at 0 °C in the presence of 1.5 ml anisole and 0.25 ml methylsulfide/g of peptidyl-resin. After HPLC purification by system B, the desired compound **(control)** was obtained in 46% yield. MALDI-TOF MS: H-Arg-Tyr-D-Ala-Pro-Ser-Trp-OH (MH^+) calculated: 779.38; (MH^+) found: 779.36.

To a solution of the linear peptide (10 mg, 9.9 µmol) in anhydrous DMF (9.5 ml), were added, under argon atmosphere and at 0 °C, HATU (1.2 equiv., solution at 10 mg/ml in anhydrous DMF) and DIEA (3 equiv.). The reaction was stirred at 0 °C for 2 h and at room temperature (rt) for 4 days before concentration by rotary evaporation in the presence of toluene. After lyophilization and HPLC purification by system B (25% yield), the cyclic peptide (**5**) was characterized by MALDI-TOF MS: c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (MH^+) calculated: 761.36; (MH^+) found: 761.18.

*Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**)*

The synthesis of the linear peptide Ac-Cys-Ser-Trp-Arg-Tyr-Cys-NH_2 was carried out manually on MBHA resin (substitution: 0.59 mmol/g) in a 0.1 mmol scale. All N^{α}-Boc-amino acids (10 equiv.) were coupled by HBTU/DIEA (1 : 2). After acetylation as described for **1**, the resin was dried *in vacuo* before deformylation of Trp (piperidine/DMF 2 : 10 at 0 °C). After drying *in vacuo*, the peptide was cleaved from the resin by treatment with liquid anhydrous HF for 2 h at 0 °C in the presence of 1.5 ml anisole, 0.25 ml methylsulfide and 300 mg *p*-toluenethiol/g of peptidyl-resin. After lyophilization of the extract, the intramolecular disulfide formation was carried out in the mixed solvent system coming from the HPLC purification with system B of 40 mg of crude linear peptide. Oxidation could be performed in presence of TFA thanks to 'activation' by 4,4′ dithiodipyridine. Pure collected fractions were pooled and 100 µl of a 4,4′-dithiodipyridine solution (1.2 mg/ml in distilled H_2O) were added *per* milliliter of solution before monitoring the reaction progress by analytical HPLC. Crude oxidation reaction mixture was concentrated by rotary evaporation at rt to remove methanol. After HPLC purification by system B (13% overall yield), the cyclic peptide (**6**) was characterized by MALDI-TOF: Ac-*c*[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (MH^+) calculated: 856.33; (MH^+) found: 856.24.

*c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**)*

The synthesis of the linear peptide N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2 (**lin-7**) was carried out manually on Rink amide MBHA resin (substitution: 0.7 mmol/g) in a 0.1 mmol scale. All N^{α}-Fmoc-amino acids (5 equiv.) were coupled by HBTU/DIEA (1 : 2), except for Fmoc-L-Pra-OH and N_3Lys(Boc)-OH where a three-fold excess was used. After drying *in vacuo*, the peptide was cleaved from the resin by treatment with a TFA/TIS/H_2O (95 : 2.5 : 2.5 v/v) mixture during 2 h at rt. The filtrate from the cleavage reaction was evaporated, precipitated in cold Et_2O, collected by centrifugation and lyophilized. After HPLC purification with system B (**lin-7**) was obtained in 32% yield. HRMS analysis: N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2 (MH^+) calculated: 859.4322; (MH^+) found: 859.4321.

To a solution of linear peptide **lin-7** (17.3 mg, 15.9 µmol) in H_2O/tBuOH (1 : 1 v/v, 15 ml), $Cu(OAc)_2$ (12 equiv.) and ascorbic acid (23 equiv.) were added. The reaction was stirred at rt for 80 min before centrifugation and lyophilization of the supernatant. After HPLC purification with system B, the cyclic peptide (**7**) was obtained in 40% yield. MALDI-TOF MS: c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (MH^+) calculated: 859.40; (MH^+) found: 859.53.

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Enzyme Assays

The buffer used for all assays was 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) containing 250 mM NaCl and 5 mM $CaCl_2$ at pH 6.25 ± 0.03. Stock solutions of 2-chloro-4-nitrophenyl-α-maltotrioside ($CNPG_3$, Genzyme, Liestal, Switzerland) (10 mM) were prepared in the same buffer. Stock solutions of porcine pancreatic α-amylase type I-A (Sigma,) were freshly prepared in the same buffer before quantification with a Bradford test. Peptide concentrations in the stock solutions were determined by UV at 277 nm, with $\varepsilon = 7020\ M^{-1}\ cm^{-1}$. The assays were conducted at 30 °C using a UVIKON 930 UV/vis spectrophotometer (Muenchen, Germany). Buffer and stock solutions were kept at 0 °C but aliquots of each stock solution were incubated for 5 min at 30 °C before initiation of the enzymatic reaction. α-Amylase activity was determined by monitoring the rate of formation of 2-chloro-4-nitrophenol at 405 nm over a 10 min period. For inhibition studies with peptides, all tubes were precoated with BSA 0.1% in Tris 500 mM pH 6.8 and washed twice with the buffer before incubation of α-amylase with the peptides. Five to 138 µl of the peptide stock solutions (final concentration 0.84 mM) were incubated 1 h at 30 °C with 20 µl of α-amylase stock solution (270 nM) and buffer. The reaction was initiated by addition of 25 µl of $CNPG_3$ stock solution (total volume 0.5 ml).

Molecular Mechanics Calculations

Peptides were built-up using InsightII package (Accelrys Inc., San Diego, CA, USA). All peptide bonds were fixed in a *trans* conformation. Molecular mechanics calculations were performed with the Discover program and cff91 force field as for NMR structure calculation. The electrostatic potential was calculated *in vacuo* with a distance-dependant dielectric screening of 4r. The non-bonded interactions were evaluated with an 8 Å cut-off. Twenty to 100 structures were generated by molecular dynamics at 1000 K, saving snapshots every 2 ps. Each structure was then minimized using steepest descent, conjugate gradient and Raphson algorithms until the gradient was less than 0.001 kcal/mol/Å.

NMR Spectroscopy

NMR experiments were recorded on Bruker Avance spectrometers (Wissembourg, France) equipped with a conventional TXI probe or a cryogenic TCI probe at a ^1H frequency of 500 MHz. Lyophilized peptides were dissolved in 550 µl CD_3OH or 90% H_2O/10% D_2O in the presence of sodium succinate buffer (20 mM, pH 5.3) at 1–5 mM concentrations. Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 0.11 mM) was used as an internal reference for chemical shift calibration in water. ^1H and ^{13}C resonance assignments were obtained from the analysis of 2D TOCSY (DIPSI2 isotropic scheme of 22–67 ms duration), 2D ROESY (300 ms mixing time), 2D NOESY (500 ms mixing time) and ^1H–^{13}C HSQC spectra. NMR experiments were processed with XWIN-NMR or TOPSPIN softwares (Bruker). Spectra were analyzed with the aid of XEASY program [35]. $J_{HN-H\alpha}$ and $^3J_{H\alpha-H\beta}$ coupling constants were measured on 1D spectra or extracted with INFIT program from 2D experiments [36]. The chemical shift deviations of H_α protons and C_α carbons were calculated as the differences between observed chemical shifts and random coil values reported in water [37]. The temperature gradients of the amide proton chemical shifts were derived from 1D spectra recorded between 278 and 313 K with an increment of 5 K.

NMR Restraints

Interproton distance restraints were derived from 2D ROESY or NOESY experiments. ROESY or NOESY cross-peaks were integrated using XEASY, and peak volumes were converted into upper distance bounds using the two-spin approximation (with additional 10% tolerance). The lower bounds were set to the sum of the Van der Waals radii of two protons (1.8 Å). Pseudoatoms were introduced for distances involving equivalent protons and upper limits were corrected appropriately. The constraints on the backbone ϕ torsion angle were determined from the analysis of intraresidual $d\alpha$N ROE and $^3J_{HN-H\alpha}$ coupling constants.

NMR structure Calculations

Structures were calculated with InsightII/Discover programs (Accelrys Inc.), running on SGI O2 R10000 workstations, using the cff91 forcefield, as previously described [31]. Initial structures were submitted to 15 ps simulated annealing at 1000 K during which the experimental restraints were gradually increased. The nonbond interaction, defined by a simple quartic repulsive potential, was slowly increased during the next 10 ps of dynamics. Temperature was then gradually decreased from 1000 to 0 K over 40 ps. The structures were subsequently minimized by steepest descent and conjugated gradient methods, using a Lennard–Jones potential for the Van der Waals interaction and a distance-dependent dielectric function for the electrostatic term. Depending on the peptide, a set of 20–100 structures were calculated and the final structures were selected on the basis of lowest potential energy.

TrNOESY and Saturation Transfer Difference Experiments

NMR experiments were recorded for peptides (**1**), (**3**), (**6**), (**7**) in the presence of α-amylase. The experiments were carried out at 283K in 90% H_2O/10% D_2O in the presence of 10 mM sodium succinate (pH ~5.8–6.3), 1 mM $CaCl_2$, 0.02% (w/v) NaN_3 and 0.1 mM DSS. The concentration of peptide was around 1 mM and the concentration of added α-amylase was typically 20 µM (this concentration may be overestimated since precipitation of α-amylase was observed in the samples). Acarbose (generous gift from Dr H. Driguez, Grenoble) was added at a final concentration of 1 mM. 2D NOESY experiments were acquired with mixing times ranging from 100 to 400 ms. 1D saturation transfer difference (STD) experiments were recorded with 2 s irradiation (Gaussian pulses) at −1 and +30 ppm alternatively.

Results

Peptide Design and Syntheses

*Linear tetrapeptide (**1**)*

The linear tetrapeptide Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) was synthesized as the control peptide, i.e. a minimal mimic of the tendamistat β-turn [8–10,22–25] bearing the pharmacophore implicated in the interaction with α-amylase. The peptide was acetylated at the *N*-terminus and amidated at the *C*-terminus, as surrogates of amide bonds. Peptide (**1**) prepared by automatic SPPS was obtained with a 53% overall yield.

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*β-Amino acid-containing peptides (**2**), (**3**), (**4**)*

First, modelling studies were performed on β-amino acid-containing peptides to identify the most pertinent sequence that could mimic the tendamistat β-turn. Six β-amino acid-containing tetrapeptides were built-up according to the following criteria: (i) (*R*)-β^2-hTrp and (*S*)-β^3-hArg were chosen as central amino acids because their configurations allow keeping side chain orientations of the tendamistat pharmacophore, (ii) all the amide bonds were constrained in *trans* orientation, (iii) all sequences contained an *N*-acetyl and a *C*-terminal carboxamide and (iv) Ser residue of the tendamistat β-turn was replaced by a (*S*)-Cys (i.e. D-Cys) to (i) allow further functionalization (thiol function) and (ii) increase proteolytic stability (D-stereochemistry). The nature of the fourth amino acid varied between the six modelled tetrapeptides: (*S*)-Tyr, (*R*)-Tyr, (*R*)-β^2-hTyr, (*S*)-β^2-hTyr, α-homotyrosine or peptoid-tyrosine (i.e. *N*-alkylated amino acid analog). Each modelled sequence was subjected to restrained molecular dynamics calculation and the generated structures were superimposed to the β-turn of tendamistat (α-amylase-tendamistat complex, PDB entry 1BVN). The best fit was observed for Ac-(*S*)-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*R*)-Tyr-NH_2 (**2**) and the peptide was therefore synthesized (Fig. S1, Supporting information). Two other β-amino acid-containing sequences were also analyzed. These peptides were inspired from the active somatostatin analogs constituted of β^3–α^2 – β^3 – β^3 or β^3 – β^3 – β^3 sequences [26]. The peptides Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**) and Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**4**) were synthesized as these sequences allowed to maintain the correct side chain orientations as present in tendamistat. Peptides (**2**), (**3**) and (**4**) were prepared by manual SPPS, using HBTU to ensure complete coupling of β-amino acids derivatives, and were obtained in 31, 15 and 8% overall yields, respectively.

Cyclic peptides

Cyclic hexapeptides were also synthetized to stabilize a β-turn conformation and mimic the tendamistat pharmacophore. The four residues of the tendamistat β-turn, Ser-Trp-Arg-Tyr, were conserved while the two other amino acids varied depending on the cyclization strategy. The 3D structure observed with the 'conventional' cyclization methods involving either an amide bond or a disulfide bridge (Fig. 1) were compared to that obtained with the triazole 'click' chemistry-cyclization.

The linear peptide H-Arg-Tyr-D-Ala-Pro-Ser-Trp-OH was obtained by manual SPPS. After HPLC purification, the intramolecular cyclization was performed in dilute solution. HATU was found to be the best activating agent to minimize epimerization of Trp. The cyclic peptide c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**) was obtained in 11% overall yield (Fig. 1b).

The linear peptide Ac-Cys-Ser-Trp-Arg-Tyr-Cys-NH_2 was prepared by manual SPPS. After purification, this peptide was directly subjected to intramolecular oxidation in the HPLC solvents (i.e. in the presence of 0.1% TFA) using 4,4′-dithiodipyridine as an 'activating' agent [38]. The cyclic peptide Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) was obtained in 13% overall yield (Fig. 1c).

*'Click' chemistry-cyclized peptide (**7**)*

As for the β-amino acid-containing linear peptides, modelling studies were performed *prior* to the syntheses to select the sequence to be cyclized. Fmoc-Pra being commercially available, was chosen for introducing the alkyne component of the Huisgen reaction [39–41], the azido partner coming from either an *N*-terminal α-azido acid, readily accessible by known procedures [42–44] or from 3-azido-alanine, both enantiomers being commercially available (Fig. 2).

The structure induced by the triazole cyclization was studied by molecular dynamics (Discover with cff91 force field). Four model molecules (Fig. 3) were built-up with Insight II, their methyl groups corresponding to $C\alpha_i$ and $C\alpha_{i+3}$. The CO–HN distances were determined and the energies of the different structures were compared after generating 100 structures (simulated annealing *in vacuum*). For each case the lowest energy structures were analyzed to identify the privileged conformation. Only triazole-cyclized molecules generated from *N*-terminal-(*S*)-α–azido-'alanine' led to conformations compatible with a hairpin structure and plausibly with a β-turn structure for the pharmacophore residues, the CO–HN distance d_1 being longer than d_2 (0.4 vs 0.3 nm). The opposite orientation, i.e. $d_1 < d_2$, must disfavour the hydrogen bonding network required for a β-turn structure centred on Trp-Arg. Finally, in the most stable conformations, the distance between $C\alpha_i$ and $C\alpha_{i+3}$ was shorter than 0.7 nm, a distance compatible with a β-turn structure for the following residues (Fig. 4). Thus, a 'click' cyclization with a Pra residue on one side and a *N*-terminal-(*S*)-α–azido-'alanine' on the other side should favour a β-turn structure on the other side of the cyclized peptide. In this study, (*S*)-α–azido-lysine (Fig. 2) was used to allow further functionalization for other studies.

The triazole cyclic peptide c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) was prepared as summarized in Fig. 5. Fmoc-Pra was commercially available, while N_3Lys had to be prepared [32] for the synthesis of the linear peptide N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2 by manual SPPS. After HPLC purification, triazole 'click' chemistry-cyclization was performed in dilute solution to produce c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) in 11% overall yield.

α-Amylase Assay

The inhibitory potencies of these peptides for porcine pancreatic α-amylase were determined with $CNPG_3$ as substrate [45]. All peptides were studied at a single concentration of 840 μM with preincubation with the enzyme for 5 min or 1 h. The results were similar under both conditions. The most potent inhibitors were the lactam- and the disulfide-bridged cyclic hexapeptides, c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**) and Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**), with over 80% inhibition of α-amylase activity. The inhibition potencies, expressed as a percentage of the inhibition of α-amylase activity, are reported in Fig. 6.

The linear peptide Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**), the β-amino acid-containing peptides Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**) and Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**4**) and the triazole-cyclized peptide c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) are weak inhibitors of α-amylase with an inhibition around 30–40%. The linear precursor N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2 (**lin-7**) of the click-cyclized peptide shows the same inhibitory potency as its cyclic congener (**7**).

Structural analysis of peptides (*1*) to (*7*)

*Linear tetrapeptide (**1**)*

1D proton, 2D homonuclear TOCSY and ROESY, and 2D ^1H–^{13}C HSQC experiments were carried out in methanol (CD_3OH) and in water (H_2O/D_2O: 9/1, sodium succinate 20 mM, pH 5.3). The linear tetrapeptide Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) adopts a β-I turn

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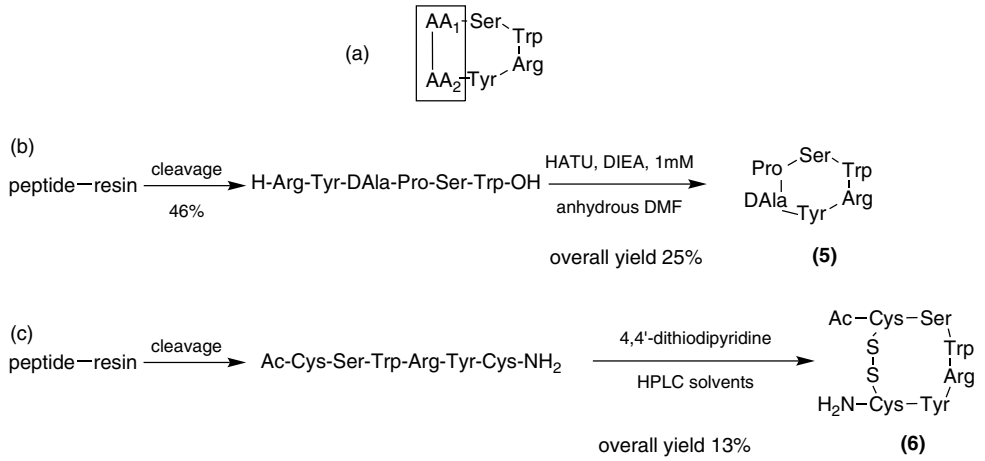

Figure 1. Cyclic hexapeptides. (a) Schematic representation of the four residues of the tendamistat β-turn. (b) Synthesis of the backbone-cyclized peptide c[Arg-Tyr-D-Ala-Pro-Ser-Trp] (**5**). (c) Synthesis of the disulfide-bridged cyclic peptide Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**).

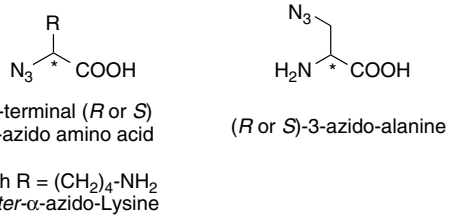

Figure 2. α-Azido acids for triazole-cyclization.

as a major conformation in methanol at 278 K, this structure being present both in methanol and aqueous solvent at 298 K (Tables S1–S8, supporting information). Indeed, the following characteristic ROE correlations are observed at 278 K in methanol: (i) a weak H_α Ser-HN Tyr ROE and a strong H_α Ser-H_δ Tyr ROE, which sign the folding of (**1**) in solution; (ii) the HN Arg-HN Tyr and H_α Trp-HN Tyr ROEs, which are characteristic of a β-turn [46]; and (iii) the intensity of HN Trp-HN Arg ROE, stronger than other ROEs between other amide protons being also characteristic of a β-turn. In addition, the chemical shift of the amide proton of Tyr is weakly dependent on temperature variation in methanol ($\Delta\delta_{HN}/\Delta T = -4$ ppb/°C) as well as in water ($\Delta\delta_{HN}/\Delta T = -4.4$ ppb/°C). The chemical shift deviation (CSD) parameters of Arg supports an α conformation (CSD $C^\alpha = +1.1$ ppm, CSD $H^\alpha = -0.35$ ppm) in methanol, however this propensity is less marked in water (CSD $C^\alpha = +0.5$, CSD $H^\alpha = -0.38$). Moreover, the chemical shifts of H_β and H_γ protons of Arg are more shielded than in a random coil state. The Arg side chain must consequently lie close to the Trp or Tyr side chains, an orientation only compatible with a folded structure. Furthermore, the coupling constant $^3J_{HN-H\alpha}$ of Tyr is greater than 8 Hz in methanol (and close to 8 Hz in water), indicating an extended conformation for this residue. On the contrary, the $^3J_{HN-H\alpha}$ coupling constants of Ser and Trp are <6 Hz in methanol (and close to 6 Hz in water), showing that these residues adopt mainly an α conformation. Finally, the χ_1 dihedral angle of the Tyr side chain is predominantly in a *gauche* (−) conformation ($\chi_1 \sim -60°$).

Twenty structures were generated by molecular dynamics calculations, taking into account 18 ROE correlations observed in methanol at 278 K and five dihedral angle restraints (Table S5). During the preliminary structure calculations, systematic violations were observed for distance restraints involving sequential HN/H_α correlations, leading to structures with high energies. These violations can be accounted for by the presence of minor conformers adopting extended structures that give a significant contribution to sequential H_α/HN ROE intensities. Furthermore, the amide proton distances inferred from ROE intensities are slightly longer than expected for a canonical β-turn structure (experimental HN_{i+2}/HN_{i+3} and HN_{i+1}/HN_{i+3} distances of 0.36 and 0.42 nm respectively, vs 0.24 and 0.36 nm in a canonical β-turn), indicating that the β-turn structure is not the unique populated conformation. In order to generate the folded conformers with low energies, the weight of sequential HN/H_α distance restraints was decreased in the final structure calculations. For the 20 obtained structures, the preferred conformation is a type I β–turn, the stabilizing hydrogen bond ($CO_i/HN_{i+3} \leq 0.23$ nm) being present in 10 structures. Thus, the major conformation of the peptide Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) is a β-turn structure closely related to the one observed in tendamistat (Fig. 7). In the lowest energy structure of (**1**) the (ϕ, ψ) angles of $i+1$ and $i+2$ residues correspond to a β-I turn, with values of (−90°, −46°) and (−92°, 48°), respectively.

*β-Amino acid-containing peptides (**2**), (**3**), (**4**)*

As for peptide (**1**) a β-turn structure was stabilized in methanol, NMR studies of the β-amino acid-containing tetrapeptides were also performed in this solvent. Unexpectedly, none of these peptides adopt a β-turn structure in methanol (Tables S9–S21). Indeed, no ROE correlations could be observed between i and $i+3$ residues and all amide proton chemical shifts show strong temperature coefficients ($\Delta\delta_{HN}/\Delta T < -5$ ppb/°C). Moreover, the NMR restraints were insufficient to identify a preferential backbone conformation of these peptides by molecular dynamics calculations. In conclusion, the three peptides Ac-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*R*)-Tyr-NH_2 (**2**), Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**) and Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**4**) are flexible in solution.

Cyclic peptides

*Amide bond-cyclized peptide (**5**)*

The NMR analysis of c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**) was performed in methanol at 298 K and in water (H_2O/D_2O: 9/1) at

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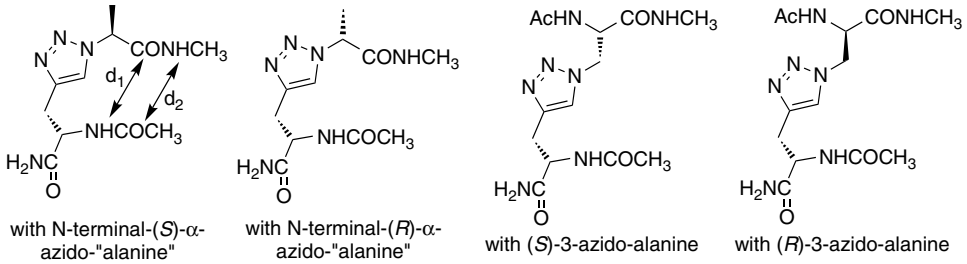

Figure 3. Pseudotetrapeptides used for modelling studies, the arrows representing the C═O–HN distances.

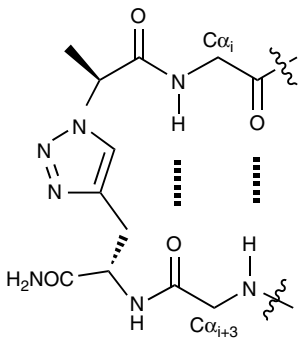

Figure 4. Privileged conformation for a click-cyclized molecule with Pra and N-terminal-(*S*)-α-azido 'alanine', stabilized by a hydrogen bond (d_2). This folding should favour the β-turn structure for the peptide.

293 K (Tables S22–S29). The results are similar in both solvents. All amide bonds are *trans*, according to strong $H_{\alpha i-1}/HN_i$ ROEs and strong $H_{\alpha i-1}/H_{\delta i}$ ROEs for the amide bond preceding proline. The CSDs of Hα and Cα resonances were informative for Ser and Trp residues. Ser adopts an extended conformation, confirmed by its high $^3J_{HN-H\alpha}$ coupling constant in methanol and water (8.6 and 9.1 Hz, respectively), while Trp is in an α conformation, confirmed by the small $^3J_{HN-H\alpha}$ coupling constants in methanol and water (3.6 and 3.0 Hz, respectively). For Arg, the $^3J_{HN-H\alpha}$ coupling constant in methanol (7.7 Hz) allows to restrain its φ angle around $-90^\circ \pm 20^\circ$. Moreover, the small $^3J_{HN-H\alpha}$ coupling constants of D-Ala in methanol and water (3.7 and 2.5 Hz, respectively) leads to a φ angle restriction around $+60^\circ \pm 30^\circ$, according to the Karplus equation adapted to D-amino acids. Finally, the χ_1 angle of the Ser side chain corresponds to a major *gauche* (+) conformation ($\chi_1 \sim +60^\circ$).

To obtain the structure of c[Pro-Ser-Trp-Arg-Tyr-D-Ala] in methanol and water, 30 structures were generated by molecular dynamics calculations by taking into account 5 and 4 dihedral angle restraints, 26 and 24 ROE connectivities, respectively (Tables S25, S29), and the 20 lowest energy conformers were selected to represent the structure families. c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**) adopts a β-hairpin conformation in both solvents, in agreement with the characteristic HN Ser-HN Tyr ROE observed in solution. The D-Ala-Pro sequence folds into a β-II′ turn conformation (hydrogen bond CO_i-HN_{i+3}), whereas a β-I turn is present around the Trp and Arg residues, this turn being similar to the tendamistat β-turn. Indeed, specific ROEs between the hydroxyl group of Ser and the Trp and Arg amide protons indicate that the Ser side chain is located above the turn. Thus, the oxygen atom of the hydroxyl group is probably involved in a bifurcated hydrogen bond with Tyr and also Arg amide groups. This hypothesis is corroborated by the temperature coefficients of Ser, Tyr and Arg amide protons indicating sequestration from the solvent. Thus, the amide bond cyclization is an efficient tool to generate a stable β-hairpin structure both in methanol and water (Fig. 8).

*Disulfide bridge-cyclized peptide (**6**)*

The conformational analysis of the cyclic hexapeptide Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-$CONH_2$ (**6**) was performed in methanol at 298 K and in water (H_2O/D_2O: 9/1) at 283 K (Tables S30–S36).

Conformational analysis in methanol at 298 K: All amide bonds are *trans* as shown by the $H_{\alpha i-1}/HN_i$ ROEs. The CSD values are informative for Ser and both Cys residues, which all adopt an extended conformation as confirmed by the high $^3J_{HN-H\alpha}$ coupling constants (~8 Hz). Moreover, the small $^3J_{HN-H\alpha}$ coupling constant of Trp (4.4 Hz) features an α conformation. Finally, the χ_1 angle of the Ser side chain adopts a major *gauche* (+) conformation ($\chi_1 \sim +60^\circ$). The Cys $^3J_{H\alpha-H\beta}$ coupling constants are averaged, consequently the disulfide bridge does not adopt a unique conformation in solution (Tables S30–S32).

Thirty structures were generated by molecular mechanics calculation, based on five dihedral angle restraints and 24 ROEs (Table S33). Owing to a conformational equilibrium some restraints could not be simultaneously satisfied, thus the weights of four of them were reduced in order to obtain the major conformation of the peptide in methanol. The 20 lowest energy structures present a β-hairpin conformation compatible with the observed HN Ser-HN Tyr ROE. The four Ser-Trp-Arg-Tyr residues adopt a type I β-turn conformation with the side chain of Ser pointing above the turn. However, this major conformation is in equilibrium with a non-β-hairpin minor conformation because the $^3J_{HN-H\alpha}$ coupling constant of Tyr (7.3 Hz) is too small to correspond to a unique extended conformation. Thus this residue is likely to adopt an alternative α conformation. In addition, the temperature coefficient of the Ser amide proton is too low (−7.7 ppb/°C) to be compatible with a unique β-hairpin conformation in methanol. The hydrogen bond between the CO group of Tyr and the Ser amide proton is therefore transient. In contrast, the amide protons of Arg and Tyr were strongly sequestered from the solvent ($\Delta\delta_{HN}/\Delta T = -0.4$ and -0.5 ppb/°C, respectively). The two corresponding hydrogen bonds are likely to be present in all conformations of the peptide, indicating the presence of a βI-turn similar to the tendamistat structure. Thus, the disulfide bridge-cyclization allows stabilizing a major β-hairpin conformation in methanol (Fig. 9).

Conformational analysis in water at 283 K: The β-turn characteristic ROE connectivities between amide protons show a strong decrease in intensity in water, indicative of a conformational equilibrium displaced toward unstructured forms (Tables S34–S36). The averaged $^3J_{HN-H\alpha}$ coupling constants also indicate that this peptide is very flexible in aqueous solution. The only common

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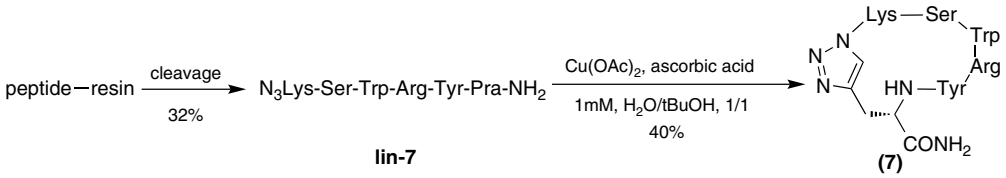

Figure 5. Synthesis of the triazole-cyclized peptide c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**).

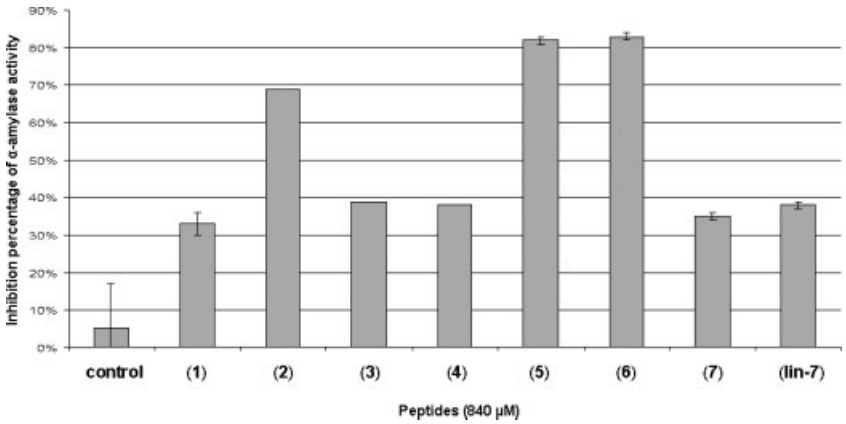

Figure 6. Inhibition potencies of peptides expressed as percentage of α-amylase activity after 1 h preincubation with the enzyme. $CNPG_3$ was used as substrate. Peptides: H-Arg-Tyr-D-Ala-Pro-Ser-Trp-OH as negative control, Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**), Ac-D-Cys-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*R*)-Tyr-NH_2 (**2**), Ac-(*R*)-β^3-hSer-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**), Ac-(*R*)-β^3-hSer-(*R*)-β^2-hTrp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**4**), c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**), Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**), c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) and its linear precursor N_3Lys-Ser-Trp-Arg-Tyr-Pra-NH_2 **lin-7**.

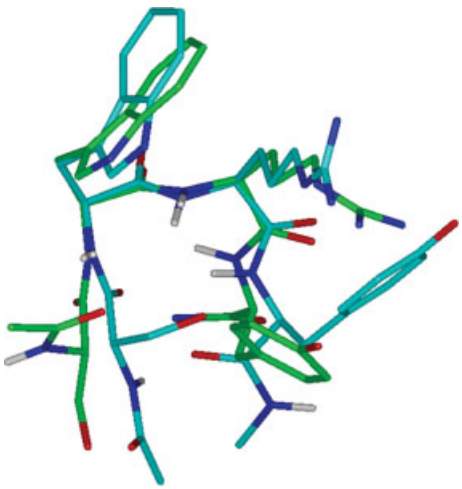

Figure 7. Superimposition of the tendamistat β-turn structure (Cα atoms in cyan, PDB structure: 1BVN) and of the lowest energy structure derived from NMR data for Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) (Cα atoms in green).

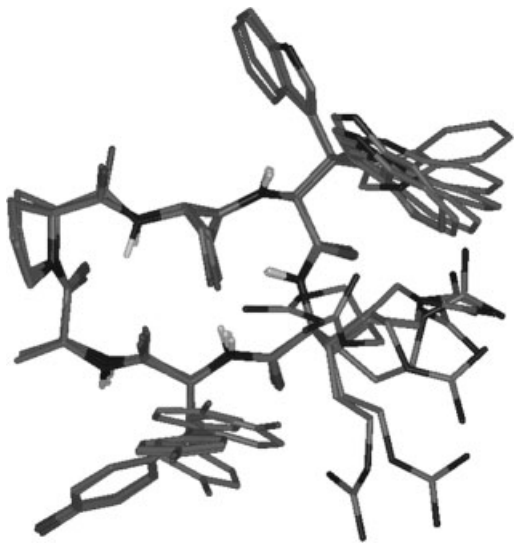

Figure 8. Superimposition of the lowest energy structures of c[Arg-Tyr-D-Ala-Pro-Ser-Trp] determined in methanol.

feature between the results in methanol and water is the *gauche* (+) conformation of the χ_1 angle of the Ser side chain ($\chi_1 \sim +60^\circ$). Thus, the β-hairpin structure observed in methanol for Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) is not conserved in water.

*'Click' chemistry-cyclized peptide (**7**)*

None of the β-turn characteristic connectivities could be observed for the peptide c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) in methanol or in water (Tables S38–S43). Moreover, the distance restraints cannot be simultaneously satisfied within a unique conformation. In addition, not only the unusual temperature coefficients of the amide protons, but also the chemical shift variations of the triazole proton and aliphatic protons indicate that there were important conformational changes with temperature. Thus, the peptide backbone presents a complex conformational equilibrium in both solvents.

Structural analysis in the presence of α-amylase

TrNOESY and STD NMR experiments [47] were performed in the presence of α-amylase in aqueous solution (10 mM sodium succinate, 1 mM $CaCl_2$, D_2O 10%, pH 6.2) in order to observe

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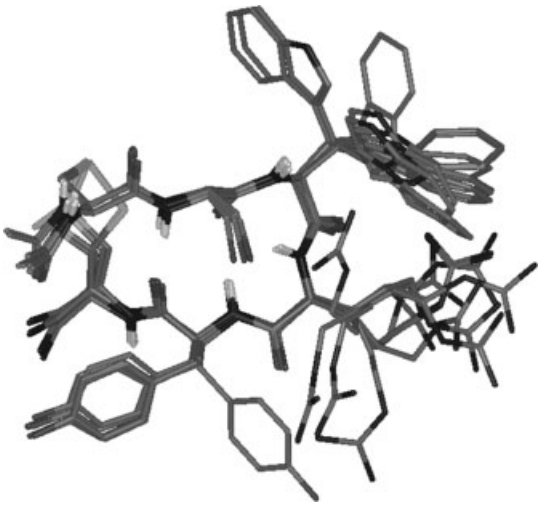

Figure 9. Superimposition of the lowest energy structures of Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) determined in methanol.

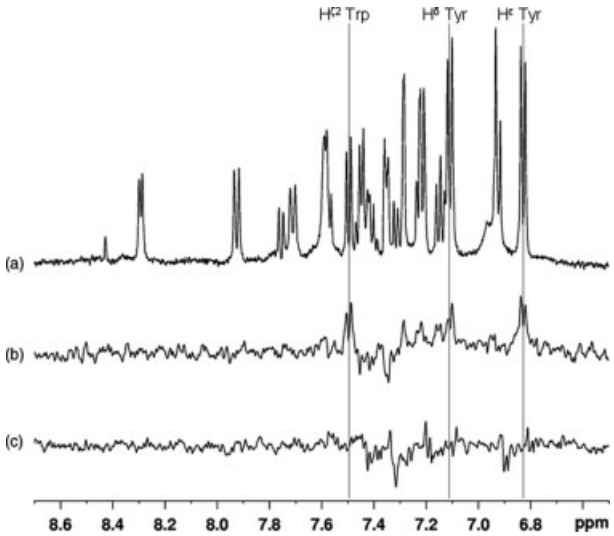

Figure 10. 1D ^{1}H NMR spectrum (a) and STD spectra (b, c) of Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**) in the presence of α-amylase. The STD spectrum shown in (c) was recorded after addition of acarbose, an inhibitor of α-amylase.

if unstructured peptides in solution may interact with porcine pancreatic α-amylase and if the interaction induces β-turn folding of the peptide.

*Ac-(R)-β^3-hSer-(S)-Trp-(S)-β^3-hArg-(S)-β^3-hTyr-NH2 (**3**)*

After addition of α-amylase to a solution of Ac-(*R*)-β^3-hSer-(*S*)-Trp-(*S*)-β^3-hArg-(*S*)-β^3-hTyr-NH_2 (**3**), a change of sign of several NOE correlations (from positive to negative) was observed, proving that peptide (**3**) interacts with the enzyme in aqueous solution. However, the small number of negative NOEs observed in the presence of α-amylase, and of HN–HN NOEs in particular, prevented calculation of the peptide structure bound to α-amylase. Additionally, STD experiments were performed to identify which peptide protons are involved in the interaction with α-amylase and to confirm the specificity of the interaction. In the presence of α-amylase STD signals were observed for several resonances of peptide (**3**), corresponding to H_δ and H_ε protons of (*S*)-β^3hTyr and to $H_{\zeta 2}$ proton of Trp (Fig. 10b). Upon addition of acarbose [48], a known inhibitor of α-amylase, the STD signals disappeared, indicating that peptide (**3**) and acarbose compete for the same binding site within α-amylase (Fig. 10). Altogether these results show that the side chains of both aromatic residues (Trp and Tyr) of peptide (**3**) bind to α-amylase in a specific manner. In addition, this indicates that the peptide (**3**) likely binds to the active site of the enzyme as acarbose does.

Cyclic peptides

Disulfide bridge-cyclized peptide (**6**): The presence of negative NOEs in the TrNOESY experiment after addition of α-amylase indicates that Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) interacts with α-amylase in aqueous solution (Table S37). Thirty NOE connectivities were used to generate 100 structures by molecular mechanics calculations. A β-turn centred on Trp and Arg is observed as evidenced by the observation of sequential HN Trp-HN Arg and HN Arg-HN Tyr NOEs (of higher intensity than HN Ser-HN Arg and HN Tyr-HN Cys NOEs). In addition, several NOEs were observed between the Trp and Arg side chains and between the Arg and Tyr side chains. These results are compatible with π-cation interactions and indicate that the side chains of Trp, Arg and Tyr may adopt similar orientations as in the tendamistat in interaction with α-amylase. Analysis of the peptide backbone conformation indicates that the calculated structures can be sorted in two families (Fig. 11): a regular β-hairpin structure (corresponding to the observed weak HN Ser-HN Tyr NOE) and a distorted hairpin structure with the presence of a β-bulge at Tyr position, which could account for the observation of the medium intensity HN Tyr–HN Cys NOE. The superimposition of the lowest energy structure of each family to the tendamistat complexed to α-amylase indicates that both structures can be accomodated within the active site of the enzyme, with no sterical hindrance. The STD spectrum of Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) recorded in the presence of enzyme, shows signals corresponding to the acetyl protons, Arg side chain protons (H_β, H_γ and H_δ), and the aromatic protons of Tyr (H_δ and H_ε) and Trp ($H_{\delta 1}$, $H_{\varepsilon 1}$, $H_{\varepsilon 3}$, $H_{\eta 2}$, $H_{\zeta 2}$ and $H_{\zeta 3}$) side chains. Thus, the Trp, Arg and Tyr side chains and also the *N*-terminal acetyl group must be involved in the interaction of this peptide with α-amylase.

'Click' chemistry-cyclized peptide (**7**): After addition of α-amylase, inversion of the sign of NOEs was observed, indicating that c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**) interacts with the enzyme in aqueous solution. However, no NOE connectivities characteristic of a β-turn structure could be identified. Nevertheless, STD experiments showed that this interaction is specific because signal intensities decreased after addition of acarbose. The protons of peptide (**7**) involved in the interaction were identified as the triazole proton, and the side chain protons of Lys (H_β, H_γ, H_δ and H_ε), Arg (H_β, H_γ and H_δ), Tyr (H_δ and H_ε) and Trp ($H_{\delta 1}$, $H_{\varepsilon 1}$, $H_{\varepsilon 3}$, $H_{\eta 2}$, $H_{\zeta 2}$ and $H_{\zeta 3}$). Thus, the Trp, Arg and Tyr side chains are mainly involved in the interaction of c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 with the enzyme, noteworthy the triazole group and Lys residue also seem to interact with α-amylase.

Discussion and Conclusion

The most successful conformational restraints into a β-turn structure have been achieved with hexapeptides cyclized on the opposite side of the β-turn by either amide bond or disulfide

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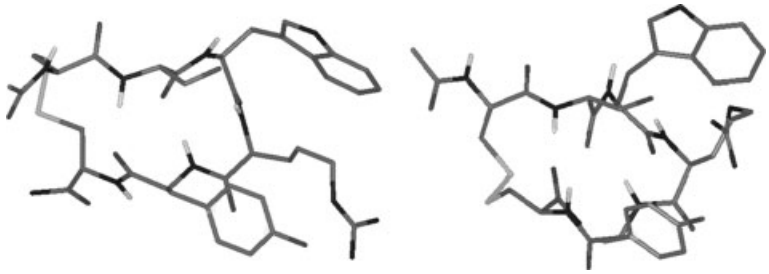

Figure 11. Lowest energies structures of Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) in interaction with α-amylase: regular β-hairpin conformation (left) and distorted β-hairpin conformation with a β-bulge at Tyr position (right). In both conformations a β-turn centred on Trp and Arg residues is observed.

bridge. The success story with cyclic analogs of somatostatin [11–13,18–20] has been tentatively extended to other lead compounds with in some cases a large decrease in potency, as shown in the tendamistat family. Nevertheless, the α-amylase/tendamistat complex remains a valuable tool to study conformational restriction in β-turn structure, as both 3D NMR structure of the ligand and X-ray structure of the complex have been reported. In that line of studies the work of Bartlett's [22,24] and Kessler's [23] teams should be mentioned, as well as the more recent work of Ono *et al*. [25]. However, in this last study the cyclic compounds that have been prepared are larger and with an odd number of residues, which is a cumbersome choice considering the works of Schwyzer and Hodges with the 'famous Schwyzer's rule' for hairpin formation of lactam-cyclic peptide containing only $2(2n+1)$ residues [49,50]. Starting from the initial β-turn structure of tendamistat: Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**), we have designed and synthesized: (i) internal β-turn mimics containing β-amino acids to constrain the turn conformation, peptides (**2**), (**3**) and (**4**), and (ii) external mimics cyclized by an amide bond (**5**), a disulfide bridge (**6**) or a triazole (**7**). The sequences of the β-amino acids containing peptides and the triazole cyclic peptide have been first deduced from modelling studies as the best fits with the β-turn structure of tendamistat.

NMR studies in water and in methanol, whose dielectric constant may better mimic the hydrophobic environment of the binding pocket, associated to restrained molecular dynamics and energy minimization using interproton distance restraints and dihedral angle restraints proved that the β-turn structure is found in some, but not all, of these analogs. Unexpectedly for a so short peptide, the linear tetrapeptide Ac-Ser-Trp-Arg-Tyr-NH_2 (**1**) adopts in both solvents a major β-turn conformation, closely related to the one observed in tendamistat. In contrast, the β-amino acid-containing peptides, specifically designed to mimic this conformation, are flexible in solution and do not adopt a β-turn structure or as a minor component of a complex equilibrium. Concerning the cyclic analogs, both the backbone-cyclized and the disulfide-bridged hexapeptides are structured in β-turn conformation. However, while the major conformation of c[Pro-Ser-Trp-Arg-Tyr-D-Ala] (**5**) is definitively in both solvents the β-hairpin, which is observed in the tendamistat, the disulfide-bridged Ac-c[Cys-Ser-Trp-Arg-Tyr-Cys]-NH_2 (**6**) presents two structures in methanol, a predominant βI-turn conformation and a non hairpin minor conformation; however, in water an equilibrium with unstructured forms is observed. Thus, the amide constraint (18-membered cycle) restrains more firmly the β-turn structure than the disulfide-bridged hexapeptide (20-membered cycle). The peptidic backbone of the triazole cyclic peptide, c[Lys-Ser-Trp-Arg-Tyr-βtA]-NH_2 (**7**), (18-membered cycle) presents a complex conformational equilibrium in both solvents, like the β-amino acid-containing peptides.

The β-amino acid-containing peptide (**3**), the disulfide-bridged hexapeptide (**6**) and the triazole cyclic peptide (**7**) present inhibitory potencies for α-amylase in the same range of concentrations as the linear tetrapeptide (**1**) and the lactam-cyclic hexapeptide (**5**), respectively (from 400 to 1000 μM), even though in solution the β-turn structure was not observed for the former (**3, 6** and **7**). With these three peptides (**3, 6** and **7**) we performed TrNOESY and STD NMR experiments in the presence of α-amylase and we found that in buffer solution these three peptides interact specifically within the active site of α-amylase, as this interaction may be displaced by acarbose, a specific α-amylase inhibitor [47]. In the case of the disulfide-bridged hexapeptide (**6**) trNOEs indicate that the bound structures are characterized by a β-turn centred on Trp and Arg residues. STD experiments allowed us to identify the protons involved in the interaction of the click peptide (**7**) within the active site of α-amylase; the Trp, Arg and Tyr side chain are definitively involved in this interaction as well as the triazole moiety and the Lys residue.

Applications in various fields of the CuI-catalyzed azide-alkyne reaction have been reviewed [39–44]. Only very recently a few studies on conformational constraints by triazole intramolecular cyclization have been reported, i.e. parathyroid hormone-related peptide (PTHrP) analogs cyclized, *via* $i-(i+4)$ positions to stabilize an α-helical structure [51–53] and melanocortin MTII analogs, *via* i - i+5 positions to stabilize a βII-turn conformation [54]. The triazole-constrained analogs of MTII are even more potent than the MTII, a long-acting superagonist of melanocortin receptors. The authors reach the conclusion that the Cu(I)-catalyzed azide-alkyne side chain-to-side chain reaction is 'a new and powerful approach for generating stable helix mimetic structures' [27,28]. From our study, we can conclude that the cyclization by triazole, as well as the introduction of β-amino acids, does not stabilize β-turn structure as lactam or disulfide-bridged cyclization, however β-turn can be potentially induced. With disulfide and triazole restraints the β-turn structure in solution is not detectable, however from the equilibrium of unordered structures the β-turn conformation may be induced during the binding of the ligand either *via* an induced fit mechanism or being part of a mutual adaptation process. Thus, we should be aware of an *effet de mode* for β-amino acid-containing peptides and click-cyclized peptides and keep in mind that amide-cyclized or disulfide-bridged hexapeptides remain highly competitive restraints to stabilize a β-turn structure, especially considering the costs and the length of the syntheses with β-amino acids or the precursors required for the click reaction.

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Supporting information

Supporting information may be found in the online version of this article.

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