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# Introduction of the New Dipeptide Isostere *7-Endo*-BtA as Reverse Turn Inducer in a Bowman-Birk Proteinase Inhibitor: Synthesis and Conformational Analysis

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**Abstract**—Two dipeptide isosteres 7-*exo*-BTG (**1**) and 7-*endo*-BtA (**2**), belonging to the new class of γ/δ-bicyclic amino acid BTAa, were inserted into an 11-residue peptide deriving from the Bowman Birk Inhibitor (BBI) class of serine protease inhibitors, and the conformational properties of these modified peptides have been studied by NMR and molecular modelling. The dipeptide isostere 7-*endo*-BtA [(1*R*,4*S*,5*R*,7*R*)-4-*endo*-methyl-6,8-dioxa-3-azabicyclo[3.2.1]octane-7-*endo*-carboxylic acid] (**2**), derived from L-alanine and *meso* tartaric acid, gave rise to the modified BBI peptide **5** whose structure was very similar to that of the original peptide **3**, suggesting a possible reverse turn inducing property for this dipeptide isostere. 

## Introduction

Recently, we reported on a new class of bicyclic amino acids,[1] the 3-aza-6,8-dioxabicyclo[3.2.1]octane-7-carboxylic acids, named BTAa (Fig. 1), which are easily synthesized from α-amino aldehydes and tartaric acid derivatives.[1,2] These compounds can be functionalized in position 4 with a side chain from natural or unnatural amino acids and in position 7 with a carboxyl group. The distance between the secondary nitrogen atom and the carboxyl group is essentially the same as in a dipeptide, resulting in these derivatives being dipeptide isosteres. These compounds have semi-rigid structures, due to the bicyclic scaffold, and could therefore impose some conformational restrictions to a peptide chain. Moreover, the conformational properties of the modified peptide could be strongly dependent on the absolute configuration of the stereocenters, on the position *exo* or *endo* of the side chain at C4 and mainly on the disposition of the carboxyl group at C7. It is noteworthy that the latter group can be oriented in either *exo* or *endo* position simply by choosing as a starting material either chiral (*R,R* or *S,S*) or *meso* tartaric acid, respectively.

Since previous molecular modelling studies suggested that 7-*endo*-BTAa can act as reverse turn inducers,[1] with aim to explore and assess this possibility, we inserted two of our isosteres in an 11-residue peptide deriving from the Bowman Birk Inhibitor (BBI) class of serine protease inhibitors, and studied the conformational properties and the chymotrypsin inhibition of these modified peptides.

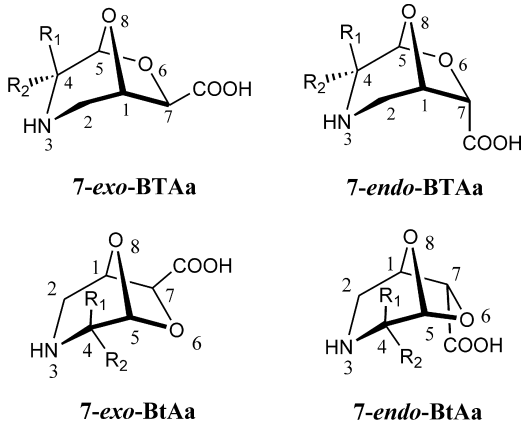


**Figure 1.** General structure of 7-*exo*- and 7-*endo*-BTAa (top), and 7-*exo*- and 7-*endo*-BtAa (bottom).

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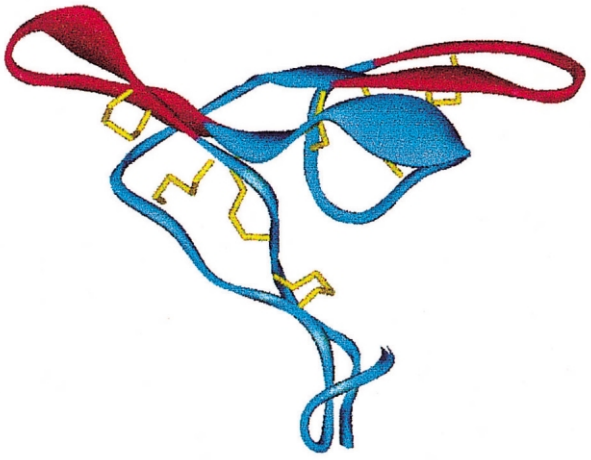


**Figure 2.** BBI structure.[9] In red are outlined the two solvent-exposed loops and in yellow the disulphide bonds.

The BBI is a rather small protein (Fig. 2)[3] in which there are two solvent-exposed loops of nine residues, including two cysteines, (in red in Figure 2) responsible for the inhibitory activity towards a wide range of proteases. The presence of these two loops allows the protein to interact simultaneously with two proteases. In the BBI, a typical sequence for the loop is Cys-Thr-X-Ser-Ile-Pro-Pro-Gln-Cys, where the $P_1$ residue X changes depending on the protease toward which the inhibitor must be active. Apart from the disulphide bond between the two cysteine residues, if we consider the sequence of various BBI proteins in the $P_4$–$P_7'$ region,[4] there are many conserved features that are important for structure or activity. In almost all cases there is a threonine residue at $P_2$ and a serine at $P_1'$; these are each involved in internal hydrogen bonding within the loop to create a stable organized structure. In all cases the presence of a *cis*-proline at $P_3'$ guarantees the formation of the VIb-type turn,[5] affording a two-strand antiparallel β-sheet structure.

Because of these very specific and particular structural characteristics, the BBI peptide loop provides a good model to test the result of incorporating our dipeptide isosteres. Among the synthetic peptides[6] derived from the BBI loop and their $P_2$ variants, the sequence H-Ser-Cys-Thr-Phe-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-OH (**3**) (Fig. 3) had the highest inhibition potency against α-chymotrypsin, with a $K_i$ of 19 nM.[4a] This peptide has been structurally studied and its interaction with the protease rationalized.[4b] This sequence was thus chosen for the modification by our isosteres with aim to study the influence of the inserted BTAa, on the structural organization and functional characteristics of the peptide.

Specifically, we substituted the Ile-*cis*-Pro residues of peptide **3**, because of their presence in a key position of the β-turn (Fig. 3), using both 7-*exo*-BTG (**1**) and 7-*endo*-BtA (**2**), (Fig. 3), differing in the presence of the substituent at C4 and the stereochemistry of C7 carboxy group.

## Results and Discussion

### Chemistry

The synthesis of the two dipeptide isosteres **1** and **2** has been previously reported.[1]

Briefly, 7-*exo*-BTG (**1**) was prepared from a glycine derivative and a chiral *R,R*-tartaric acid derivative, providing the enantiopure final compound. However, as representing the 7-*endo* class we did not chose the corresponding 7-*endo*-BTG because, deriving it from glycine and *meso* tartaric acid, a racemic mixture would have been produced. For these reasons, we used L-alanine and *meso* tartaric acid as starting materials, obtaining a mixture of two diastereoisomeric intermediates, which were separated by chromatography. 7-*endo*-BtA (**2**) was obtained as a pure diastereomer.[1]

Both the dipeptide isosteres **1** and **2** were prepared as Fmoc-amino acids and inserted into the target peptides **4** and **5** by solid phase peptide synthesis. The two modified peptides were built on Wang type resin precoupled with the tyrosine C-terminal residue, using the HBTU-HOBt chemistry. The coupling between $P_4'$-proline and the isosteres was carried out manually, using DIPC-HOBt as the activating agent in DMF and prolonging the reaction time up to 3 days for the 7-*exo*-BTG (**1**), and using PyBOP in $CH_2Cl_2$ for 3 h for the 7-*endo*-BtA (**2**); the subsequent couplings were performed using again the HBTU-HOBt chemistry. After purification by HPLC the mass of the peptides was determined by $FAB^+$ spectrometry and the sequence verified by two-dimensional NMR spectroscopy (TOCSY and ROESY).[7]

## Conformational Analysis

### NMR

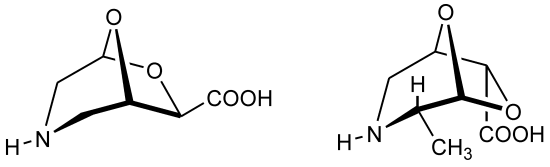
The NMR conformational analysis of peptides **3**–**5**, using the TOCSY and ROESY data, was carried out following the method described by Wishart et al.[8] In this paper, the authors reported a series of chemical shifts for amide and α-carbon protons of the amino acids residues present in many proteins. Chemical shift values were found to be dependent on secondary structure, allowing diagnostic identification of α-helix, β-sheet and random coil regions of a protein. Regions of α-helix are characterized by negative deviations from the random coil chemical shift whereas positive deviations indicate β-sheet. We have therefore used the chemical shifts of the amide and α-carbon protons as a fingerprint for structural organization of the peptide. Chemical shift deviation plot for the original sequence is shown in Figure 4a, which was used for comparison with the plots of the two modified BBI peptides **4** and **5** (Fig. 4b–d).

In the Wishart graph of the unmodified sequence H-Ser-Cys-Thr-Phe-Ser-Ile-Pro-Pro-Gln-Cys-Tyr-OH (**3**) (Fig. 4a) most of the $\Delta\delta$ values are positive, characteristic of a β-sheet like structure. The only exception is the

$P_1'$-serine that shows quite a negative $\Delta\delta$ for the amide proton. This negative value may be due to a hydrogen bond involving this residue, as is known to occur between the $P_1'$-serine and the $P_2$-threonine in the Bowman-Birk-type inhibitor structure reported by Lin et al.[9] For the peptide H-Ser-Cys-Thr-Phe-Ser-7-*exo*-BTG-Pro-Gln-Cys-Tyr-OH (**4**) (Fig. 3), modified by the introduction of 7-*exo*-BTG (**1**), two distinct sets of chemical shifts were found, indicating the presence of two different conformers in a 3:1 ratio, both of which were analyzed and characterized. The formation of two conformers has been already found in the NMR spectra of unmodified BBI peptides, and has been attributed to the *cis-trans* isomerism of the Pro-Pro amide bond.[10] Also in our case, the two distinct patterns of the TOCSY spectrum for the proline spin system of the two conformers seem due to *cis-trans* isomerism of the 7-*exo*-BTG-Pro amide bond. Since the ROESY spectrum of the major conformer shows a correlation between the two δ hydrogens of the proline residue and the H1 and H7 protons of the bicyclic amino acid, it is possible to assess that in this conformer the 7-*exo*-BTG-Pro amide bond assumes a *trans* geometry (Fig. 5, left). This is in agreement with the structure of the unmodified BBI peptide sequence **3**, wherein the Pro-Pro amide bond has a *trans* geometry too.

In the Wishart graph of the major conformer of peptide **4** (Fig. 4b) there are both positive and negative $\Delta\delta$, indicating a more random structure. In contrast, in the minor conformer most of the bars lies in the upper part of the graph (Fig. 4c), indicating a β-sheet like structure, even though the $\Delta\delta$ for the amide proton of the $P_1'$-serine is not as negative as in the unmodified sequence. This suggests that in the modified peptide **4** the hydrogen bond is weaker than in the original sequence, possibly due to the presence of the 7-*exo*-BTG (**1**) which could enlarge the peptide ring and consequently increase the distances between the various residues, thus breaking the hydrogen bond network.

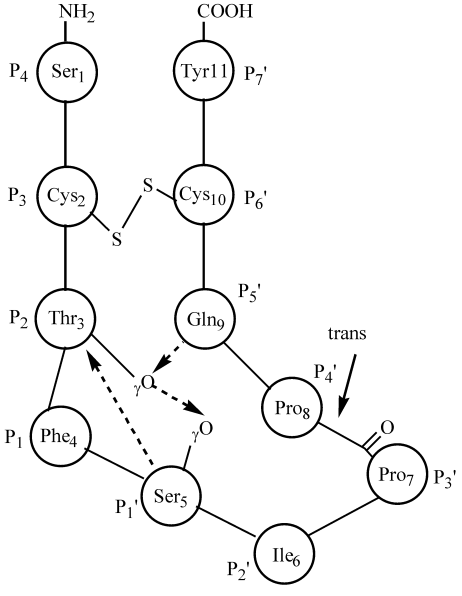
In the case of the sequence H-Ser-Cys-Thr-Phe-Ser-7-*endo*-BtA-Pro-Gln-Cys-Tyr-OH (**5**) only one conformer was detected by NMR spectroscopy. Even in this case it is possible to assign a *trans* geometry to the 7-*endo*-BtA-Pro amide bond by the analysis of the ROESY spectrum which shows a correlation between the H1 and H7 protons of the 7-*endo*-BtA amino acid and the δ protons of the proline residue (Fig. 5, right). Furthermore, from the analysis of the ROESY spectrum, strong sequential nOe correlations between the Cα-H(i) and NH(i + 1) protons, and Cβ-H(i) and the NH(i + 1) protons, respectively (Table 1) suggest the presence of two β-strands in



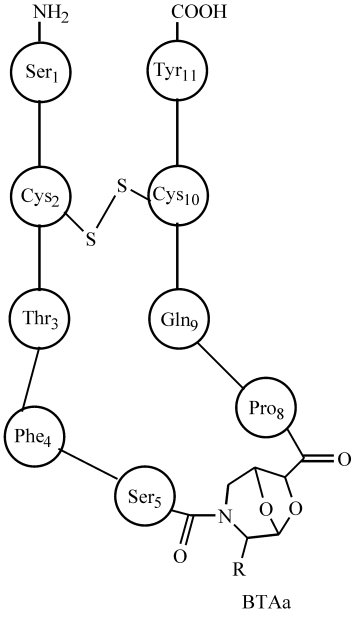

**1**




**2**




**3**




**4** BTAa = **1**
**5** BTAa = **2**

**Figure 3.** Representations of 7-*exo*-BTG (**1**) and 7-*endo*-BtA (**2**) structures (top), and of the unmodified BBI and BTAa-BBI sequences (bottom): the dashed arrows indicate the hydrogen bond donor to acceptor direction.

7-*endo*-BtA-BBI peptide (**5**). This is also supported by the vicinal $J_{NH,\alpha}$ values, which are all included in the 7.8–9.8 Hz range, as expected for extended peptide conformations.[11] Therefore, since in the Wishart graph of peptide **5**, all the $\Delta\delta$ values are positive (Fig. 4d), it appears that peptide **5** adopts a β-sheet like structure which reproduces the organisation found in the original loop. However, it lacks the negative $\Delta\delta$ value for the amide proton of the $P_1'$-serine, which could indicate detailed differences in the internal hydrogen bonding.

## Molecular modelling

In order to have additional structural information, molecular modelling calculations using AMBER*[12] on peptides **3**, **4** and **5** were performed. Conformational

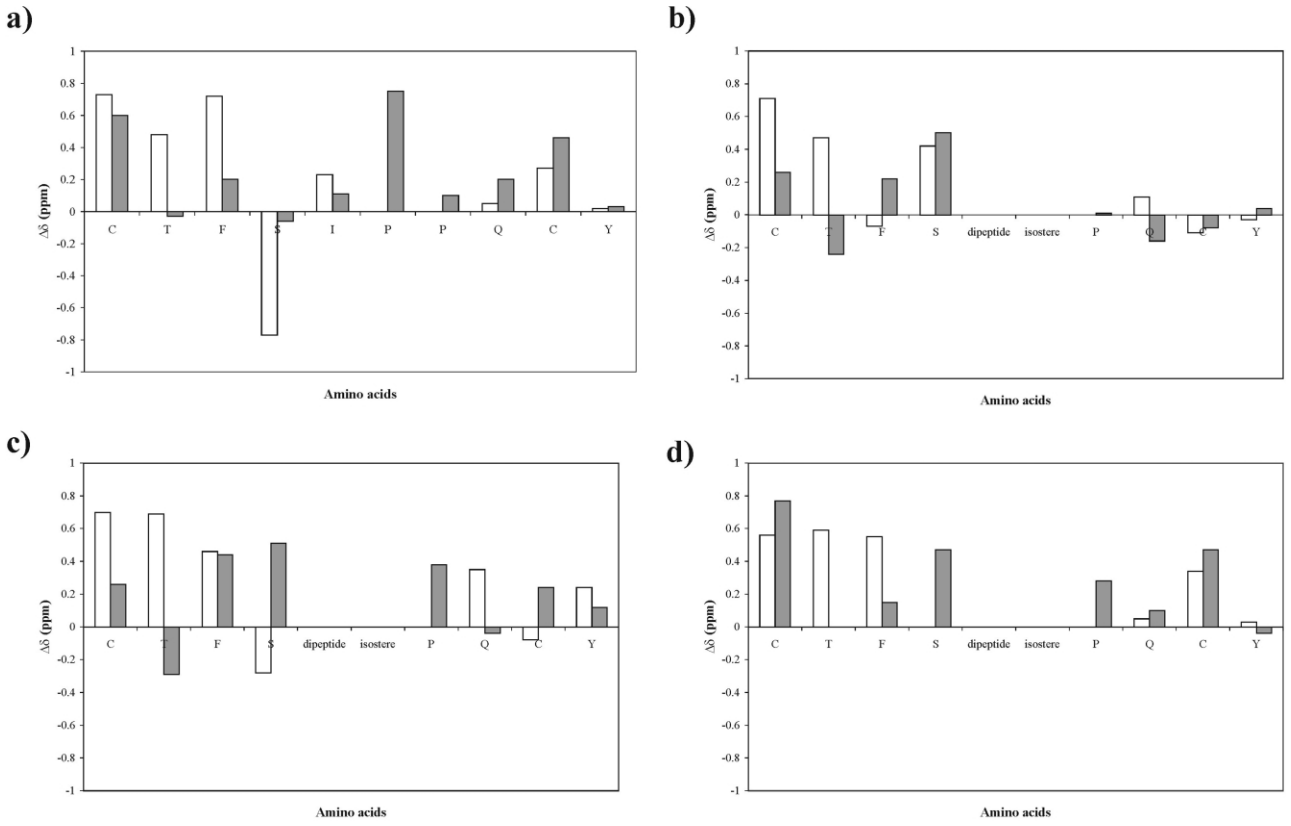



**Figure 4.** Chemical shift deviation from random coil values for the peptides studied. White bars refer to the $\Delta\delta$ for amide protons, while the grey ones to the α-carbon hydrogens: (a) unmodified BBI **3**; (b) 7-*exo*-BTG-BBI (**4**), major conformer; (c) 7-*exo*-BTG-BBI (**4**), minor conformer; (d) 7-*endo*-BtA-BBI (**5**), unique conformer.

**Table 1.** Summary of the nOes observed and vicinal coupling constants for 7-*endo*-BtA-BBI (**5**)[a]

| Amino acid | nOe[b] | | | $^3J_{NH,\alpha}$ (Hz) |
|---|---|---|---|---|
| | αN(i, i + 1) | βN(i, i + 1) | αδ(i, i + 1) | (i,i) |
| $Ser_1$ | ■ | ■ | | – |
| $Cys_2$ | ■ | ■ | | 8.6 |
| $Thr_3$ | ■ | ■ | | 9.6 |
| $Phe_4$ | ■ | | | 8.3 |
| $Ser_5$ | | | | 9.8 |
| 7-*endo*-BtA | | | ■ | – |
| $Pro_8$ | ■ | | | – |
| $Gln_9$ | ■ | | | 8.1 |
| $Cys_{10}$ | ■ | ■ | | 9.4 |
| $Tyr_{11}$ | | | | 7.8 |

[a]$^1$H NMR and ROESY spectra were recorded at 299.5 K in 90% $H_2O$–10% $D_2O$.
[b]Length of the bars is proportional to the integrated volume of the ROESY cross-peaks.

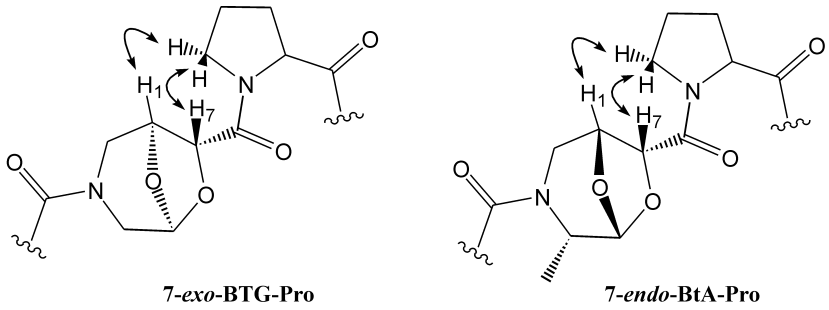



**Figure 5.** The arrows indicate the nOe correlations between proline δ-hydrogens and H1, H7 of 7-*exo*-BTG (**1**) (left) and 7-*endo*-BtA (**2**) (right) amino acids.

analysis on the three BBI structures furnished three minima that are shown in Figure 6.[13] The Monte Carlo conformational search carried out on the 7-*exo*-BTG-BBI peptide (**4**) (Fig. 6b) resulted in a series of conformers all having a *trans* 7-*exo*-BTG-Pro amide bond, differing only by the position of the side chains, while they presented well overlapping main chains. The superimposition of the global minimum conformer of the 7-*exo*-BTG-BBI peptide (**4**) with that of the unmodified BBI peptide **3** reported in Figure 6a shows that the two structures are very different (RMS = 2.39 Å). They are both loops, but the 7-*exo*-BTG-modified one, although being cyclic because of the disulphide bond, has been heavily distorted by the introduction of the isostere. In the diagrams of Figure 7 is represented the distribution of all conformers found in the Monte Carlo conformational search versus β and d values[14] reported for the bicyclic scaffold.[15] Dipeptide isostere 7-*exo*-BTG (**1**) should mimic the amino acid sequence (i + 1)-(i + 2),[16] however the calculated β (−67.9°) and d (8.69 Å) values are not in the range which is typical for a tight turn (Fig. 7a and b). Instead, if 7-*exo*-BTG (**1**) is considered mimic of the i-(i + 1) sequence,[17] the β and d values (β = 16.22°, d = 6.46 Å) are more consistent with the reported values[14] (Fig. 7c and d), but in this case the role of turn inducer has been shifted to the adjacent proline. Therefore, it seems confirmed that the presence of the 7-*exo* carboxylic group opens the peptide chain by increasing the distance among all the residues and breaks the hydrogen bonding network. This is in agreement with the NMR analysis of the major conformer in which a linearly organized structure is evident for the 7-*exo*-BTG modified peptide **4**. The second conformer observed in the NMR spectra, having probably a *cis* 7-*exo*-BTG-Pro amide bond was not detected by our conformational analysis. Why the minor conformer gives rise to a better organized structure, as suggested by NMR analysis, is yet unclear.

In the case of the 7-*endo*-BtA modified peptide **5**, the shape of the loop is very similar to that of the starting BBI peptide (Fig. 6c) in accordance with the NMR analysis above discussed and it is retained the anti-parallel β-sheet structure together with the three hydrogen bonds ($H_{Ser1}-O_{Tyr11}$, $H_{Tyr11}-O_{Ser1}$ and $H_{Thr3}-O_{Gln9}$). The presence in the ROESY spectrum of the long-range nOe between Ser5 βH and the methyl group at C4 of 7-*endo*-BtA indicates a Z configuration of the peptide bond between Ser5 and 7-*endo*-BtA, as found in the global minimum conformer of peptide **5** shown in Figure 6c. Moreover an excellent match (RMS = 0.703 Å) results from superimposition of the two structures (Fig. 6d), although in the modified BBI the proline points outward and the loop area is not as bent as in the Ile-Pro-Pro sequence of the original BBI. The β and d values, with the dipeptide isostere mimic of (i + 1)-(i + 2) residues[18] (Fig. 7e and f), agree with those of a tight turn (β = 7.73°, d = 6.03 Å), and are as good as the unmodified BBI ones (β = 24.5°, d = 4.61 Å). These results agree with those found in the NMR experiments, showing that the 7-*endo*-BtA derivative is able to induce the modified peptide to assume a stable structure that resembles those of the starting peptide.

A further observation is the relative position of the bicyclic isostere with respect to the BBI loop; in peptide

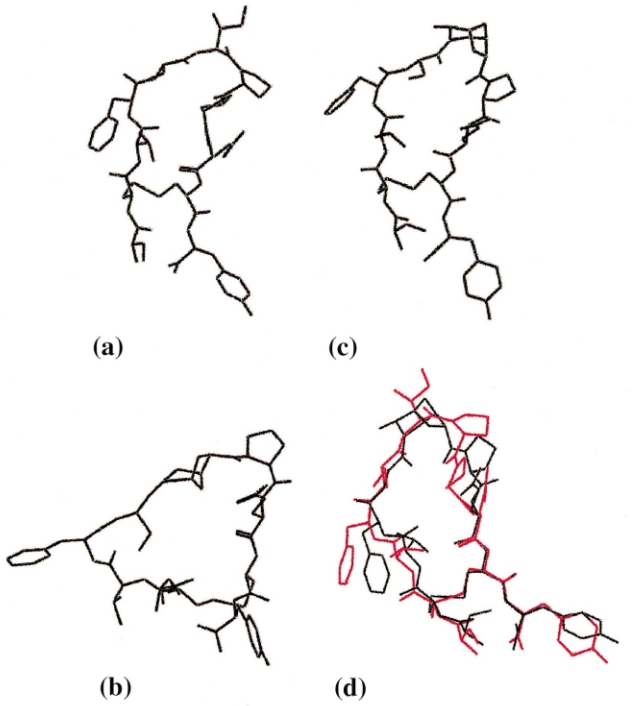



**Figure 6.** Global minimum conformer of (a) unmodified BBI **3**; (b) 7-*exo*-BTG-BBI (**4**); (c) 7-*endo*-BtA-BBI (**5**). Superimposition of 7-*endo*-BtA-BBI (**5**) and unmodified BBI **3**; (d) red: 7-*endo*-BtA-BBI (**5**); black: unmodified BBI **3**.

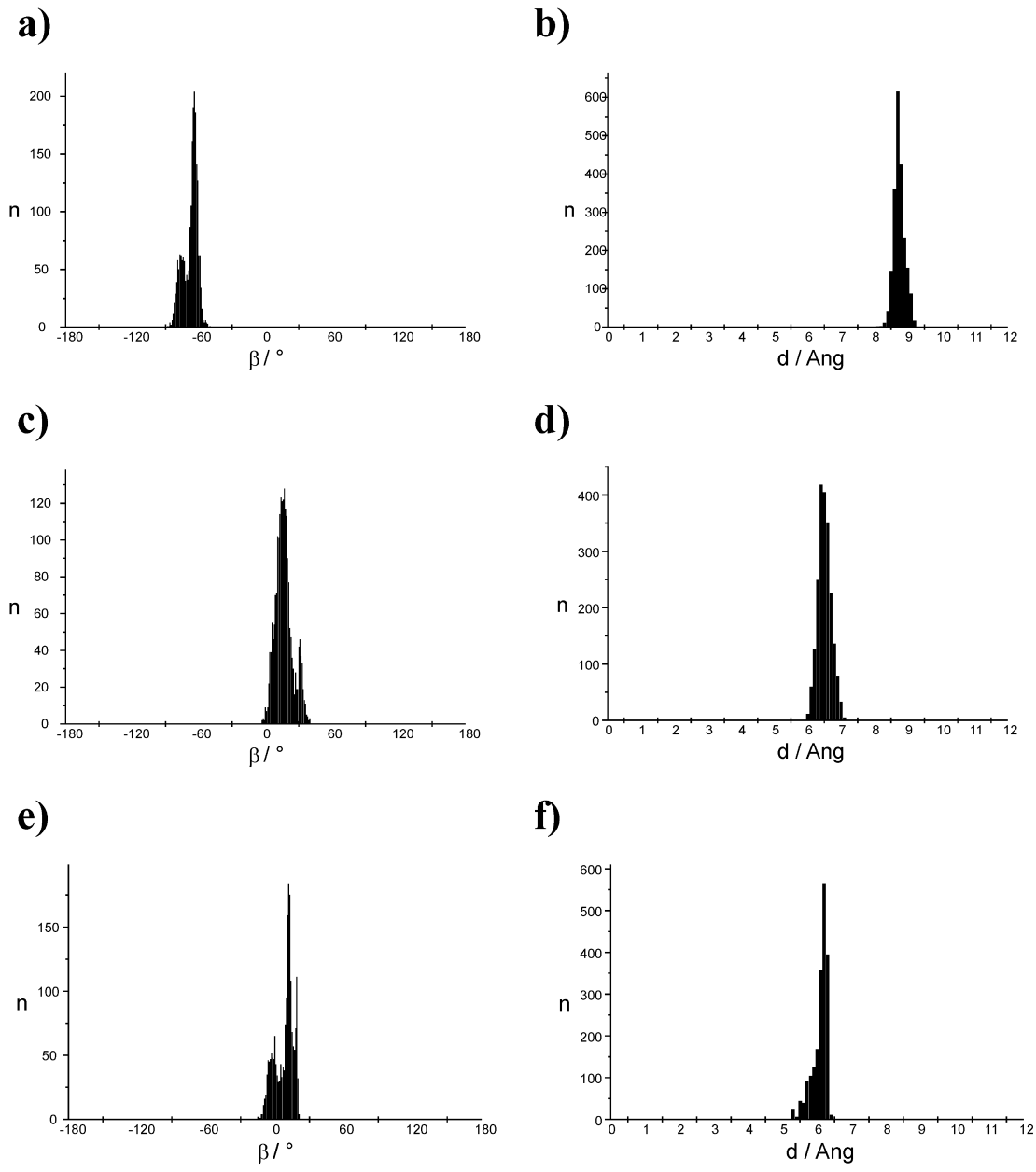



**Figure 7.** Plots of β and d parameters distribution among the conformers of the peptides studied: (a) β for 7-*exo*-BTG (**1**) as (i + 1)–(i + 2) mimetic; (b) d for 7-*exo*-BTG (**1**) as (i + 1)–(i + 2) mimetic; (c) β for 7-*exo*-BTG (**1**) as i–(i + 1) mimetic; (d) d for 7-*exo*-BTG (**1**) as i–(i + 1) mimetic; (e) β for 7-*endo*-BtA (**2**) as (i + 1)–(i + 2) mimetic; (f) d for 7-*endo*-BtA (**2**) as (i + 1)–(i + 2) mimetic.

**4** the O8 atom points inside the BBI loop while in peptide **5** it points outward, thus having the overall isostere orientation reversed in the two modified peptides **4** and **5**.

## Biological Evaluation

As regarding to the ability of the modified BBIs to act as protease inhibitor, we evaluated the biological activity against α-chymotrypsin as described by McBride et al.[4] In the same experiment, by comparison, the normal sequence has been tested ($K_i = 19$ nM). Both the 7-*exo*-BTG-BBI (**4**) and 7-*endo*-BtA-BBI (**5**) do not inhibit the enzyme even with a 126,000:1 concentration ratio ([E] = 5 nM). Furthermore, the tests have been repeated with different pre-incubation times (5, 15 and 30 min) and the results do not change. Although in particular peptide **5** matches quite well the conformation of the original BBI sequence, the lack of inhibitory activity is not very surprising to us, since it is known that even slight modifications of the BBI original sequence[19] determine important losses of inhibitory activity.

## Conclusion

By synthesizing the two modified sequences, we verified the compatibility of our isosteres with the peptide synthesis on solid support. Furthermore we demonstrated that in the modification of the BBI sequence the 7-*endo* isosteres can act as possible reverse turn inducer. Although the BBI-BTAa derived compounds failed to

**Table 2.** Chemical shifts of NH and α-H of each residue in the modified BBI sequences

| Sequence | SCTFS-**X-Y**-PQCY δ (ppm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| X–Y[a] | 7-*exo*-BTG (major) | | 7-*exo*-BTG (minor) | | 7-*endo*-BtA | | Ile-Pro | |
| | NH | α-H | NH | α-H | NH | α-H | NH | α-H |
| Ser | — | — | — | — | — | — | — | 4.02 |
| Cys | 8.89 | 4.78 | 8.87 | 4.78 | 8.74 | 5.29 | 8.91 | 5.12 |
| Thr | 8.64 | 4.13 | 8.86 | 4.08 | 8.66 | 4.37 | 8.65 | 4.34 |
| Phe | 7.86 | 4.63 | 8.39 | 4.86 | 8.48 | 4.57 | 8.65 | 4.62 |
| Ser | 8.72 | 4.88 | 8.02 | 4.89 | 8.30 | 4.85 | 7.53 | 4.32 |
| X[b] | — | 3.27<br>2.73 | — | –[c] | — | 3.73 | 8.22 | 4.20 |
| Y[b] | — | 4.48 | — | –[c] | — | 4.72 | — | 5.08 |
| Pro | — | 4.34 | — | 4.71 | — | 4.61 | — | 4.43 |
| Gln | 8.30 | 4.12 | 8.54 | 4.24 | 8.24 | 4.38 | 8.24 | 4.48 |
| Cys | 8.07 | 4.44 | 8.10 | 4.76 | 8.52 | 4.99 | 8.45 | 4.98 |
| Tyr | 7.80 | 4.47 | 8.34 | 4.55 | 8.13 | 4.39 | 8.12 | 4.46 |

[a]X–Y stands for 7-*exo*-BTG amino acid (**1**) in 7-*exo*-BTG-BBI (**4**) (major) and in 7-*exo*-BTG-BBI (**4**) (minor), for 7-*endo*-BtA (**2**) in 7-*endo*-BtA-BBI (**5**), and for Ile-Pro in the unmodified BBI **3**.
[b]α-H is H4 for X and H7 for Y.
[c]Not detectable values.

have an inhibition toward chymotripsin, the informations gained from this detailed structure analysis will help to design new dipeptide isosteres, having reverse turn properties and optimized activity toward biological targets. A more detailed study on structural aspects of the 7-*endo*-BTAa acting as β-turn or hairpin inducers is in progress.

## Experimental

### Chemistry

Solid phase peptide synthesis was performed by a multiple synthesizer Shimadzu PSSM-8. Purification of the peptides was done by preparative HPLC using a Gilson 712 system equipped with a Waters Nova-Pak, $C_{18}$, Radial, 6 micron, 100×25 mm, reverse-phase column, using a water–acetonitrile gradient eluant buffered with 0.1% TFA. Signals were monitored at 223 nm with an ABI759A UV-detector. All peptides were characterized by $FAB^+$ and analytical HPLC equipped with Vydac $C_{18}$, 5 micron, 150×4.6 mm, reverse-phase column. All *N*-Fmoc-amino acids and Wang-*N*-Fmoc-amino acids resins were purchased from Calbiochem-Novabiochem Ltd (UK) and from Bachem Ltd (UK) with the following side chain protecting groups: Cys (Trt), Gln (Trt), Ser (tBu), Thr (tBu), Tyr (tBu).

**H-Ser-Cys-Thr-Phe-Ser-7-*exo*-BTG-Pro-Gln-Cys-Tyr-OH (4).** H-Pro-Gln-Cys-Tyr-Wang was prepared by the synthesizer starting from *N*-Fmoc-Tyr-Wang resin (35 mg, 0.028 mmol). Fmoc deprotection was performed twice using 30% piperidine solution in DMF and leaving the solution under nitrogen bubbling for 5 min, followed by successive resin washings with DMF. The peptide couplings were performed using a five-fold amino acid excess, a mixture of 0.5 M HBTU and 0.5 M HOBt as coupling reagents (0.28 mL, 0.14 mmol), and 1M DIEA (0.28 mL, 0.28 mmol) as base, allowing the mixture to react for 30 min for each coupling step.

The 7-*exo*-BTG coupling to the peptide bound to the resin was performed manually by addition to the preswollen resin in DMF (1 mL) of an activating mixture of 0.15 M HOBt and 0.16 M DIPC in DMF (0.69 mL, 0.11 mmol), Fmoc-7-*exo*-BTG-OH (42.5 mg, 0.11 mmol) and DIEA (23.5 μL, 0.14 mmol) and leaving the mixture reacting for three days. After resin washings with DMF, Fmoc-piperidine test was done to assess the completion of the coupling step, and the residual free amino groups were capped by reaction of the resin with acetic anhydride (26 μL, 0.28 mmol) in DMF (1 mL) and 0.1 M DMAP in DMF (28 μL, 2.8 μmol) for 1 h. After resin washings with DMF the final peptide couplings were performed automatically as described before. The peptide was cleaved off the resin by treating the resin with a solution of 95% TFA, 2.5% $H_2O$, 2.5% ethanedithiol (1 mL) for 3 h at rt. After filtering the solution, the peptide was precipitated out by addition of cold methyl-*t*-butyl ether (10 mL) and centrifuged to give a white solid which was washed with additional cold methyl-*t*-butyl ether (10 mL) and dried under vacuum. The crude peptide was dissolved in water (200 mL) and DMSO (50 mL), then glacial acetic acid (12.5 mL) was added and the pH adjusted to 6 with 34% $NH_3$ solution, and the solution was left stirring overnight. Then water was added (800 mL) and the solution was injected to preparative HPLC eluting with 5% acetonitrile and then using 5–80% acetonitrile/35 min as gradient (rt = 10.51 min), thus giving the desired peptide: analytical HPLC rt = 10.51 min; $FAB^+$–MS (1175, M + 1); NMR data are shown in Table 2.

**H-Ser-Cys-Thr-Phe-Ser-7-*endo*-BtA-Pro-Gln-Cys-Tyr-OH (5).** The synthesis of the 7-*endo*-BtA-BBI (**5**) was performed as reported for peptide **4**, but DIPC was replaced with PyBOP and $CH_2Cl_2$ was used as solvent, leaving the mixture reacting for 3 h at rt, and giving pure product after HPLC purification using 2–50% acetonitrile/39 min as gradient (rt = 14.30 min); $FAB^+$-MS (1189, M + 1); NMR data are shown in Table 2.

## Biology

α-Chymotrypsin, EC 3.4.21.1, Type VII from bovine pancreas, was purchased from Sigma-Aldrich C-3142 and its activity was determined by active site titration as reported.[20] *N*-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (Suc-AAPF-AMC) was purchased from Sigma. All inhibitory activities were measured on a CytoFluor Series 4000 PerSeptive Biosystem microplate reader.

**Chymotrypsin assay.** α-Chymotrypsin was diluted in 144 mM Tris HCl pH 7.8 buffer to give a working solution of 5.23 nM. The substrate Suc-AAPF-AMC was prepared as 20 μM stock solution in the same buffer. Peptides 7-*exo*-BTG-BBI (**4**) and 7-*endo*-BtA-BBI (**5**) to be tested were prepared as 660 and 356 μM stock solutions respectively.

Inhibition assay was carried out in a total volume of 200 μL of the above buffer wherein α-chymotripsin (50 μL, final concentration 1.30 nM) was incubated with various concentrations of the peptide (50 μL) to be tested for 5–30 min. The reaction was started by the addition of substrate Suc-AAPF-AMC (100 μL, final concentration 10 μM) and the residual activity was measured on the microplate reader at room temperature over 15 min. The initial rates obtained were plotted against the peptide concentration and no inhibition was found.

The range of concentrations used for the 7-*exo*-BTG-BBI (**4**) started from 0.2 up to 165 μM (Max I:E ratio 126,850:1) and for the 7-*endo*-BtA-BBI (**5**) from 0.2 up to 89 μM (Max I:E ratio 68460:1).

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## References and Notes

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17. In this case i + (i + 1) = 7-*exo*-BTG, i + 2 = P, i + 3 = Q.
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