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ORIGINAL ARTICLE

Cyclic DGR-peptidomimetic containing a bicyclic reverse turn inducer as a selective $\alpha_v\beta_5$ integrin ligand

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Abstract 3-Aza-6,8-dioxabicyclo[3.2.1]octane-based amino acids as reverse turn inducers have been introduced into cyclic peptidomimetics containing the RGD or DGR retro-sequence, in order to achieve a stereochemical scanning of the binding capability of the resulting molecules towards $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, resulting in retro-inverso DGR peptides as micromolar ligands. A comparative analysis between the conformational preferences of **4** and of its isomer **3**, having the opposite RGD sequence, was reported with respect to the binding activity, giving insight into the factors affecting the preferential binding of **4** to the $\alpha_v\beta_5$ integrin.

Keywords Amino acids · Peptides · Peptidomimetics · Bicyclic compounds · Conformational analysis

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Introduction

Integrins are a class of cellular receptors known to bind to extracellular matrix proteins, and therefore play a key role in cell adhesion events. The integrin receptors constitute a family of proteins with structural characteristics of non-covalent heterodimeric glycoprotein complexes formed by α and β subunits (Hynes, 2002). The vitronectin receptor is known to refer to three different integrins, named $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. While $\alpha_v\beta_3$ binds a large variety of ligands, $\alpha_v\beta_5$ binds exclusively vitronectin (Smiths et al. 1990), a plasma glycoprotein that is also distributed in the extracellular matrix of various tissues. Several biological processes are connected with a common binding of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to RGD sites, such as endothelial cell adhesion (Pedchenko et al. 2004), and whilst $\alpha_v\beta_3$ plays a major role in the angiogenic activity, other mechanisms involve exclusively the $\alpha_v\beta_5$ integrin. Asano et al. reported that the up-regulated $\alpha_v\beta_5$ integrin may contribute to the phenotypical alteration of scleroderma fibroblasts, leading to fibrotic disorders (Asano et al. 2004), and later, the same authors described a novel mechanism for the establishment of autocrine TGF-β signaling in dermal fibroblasts by the up-regulation of $\alpha_v\beta_5$, indicating the possibility of targeting such integrin for the treatment of fibrotic disorders (Asano et al. 2006). Also, $\alpha_v\beta_5$ integrin has been reported to play a role in neovascularization, particularly that induced by the growth factors, vascular endothelial growth factor (VEGF), transforming growth factor-α (TGF-α), and epidermal growth factor (EGF) (Brooks et al. 1997), indicating that neovascular disease in different tissues may induce two distinct integrin-mediated pathways: one induced by FGF2 and mediated by $\alpha_v\beta_3$, and another one induced mainly by VEGF and mediated by $\alpha_v\beta_5$. Finally, a recent paper indicated $\alpha_v\beta_5$ to influence the ability of circulating tumour

cells to adhere within the hepatic microvessels, suggesting a role of $\alpha_v\beta_5$ in the early steps of metastasis formation (Enns et al. 2005). Thus, the different role that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ display in different physiological and pathological pathways makes desirable to target them separately, so as to achieve a selective control of the biological events in which every integrin receptor is involved.

One important recognition site in a ligand for many integrins is the arginine-glycine-aspartic acid (RGD) tripeptide sequence, which is found in all of the ligands identified above for the vitronectin receptor integrins. The RGD recognition site can be mimicked by polypeptides that contain this RGD sequence, and the specificity of the inhibition can be altered by the sequence and by the structure of such peptides. Factors affecting the ligand binding specificity between integrin receptors include mainly the variable subunit composition of this class of proteins. In particular, integrins with the same α subunit, such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$, show different ligand binding specificities, and the selectivity between such integrins can be achieved by the conformational control of the RGD sequence in the ligand. Based on the lead cyclopeptide *c*[RGDf(Me)V] developed by Kessler et al. (Haubner et al. 1996; Sulyok et al. 2001), many cyclic Arg-Gly-Asp-containing peptides have been synthesized by substitution of the central D-Phe-Val residue with different cyclic and bicyclic turn mimetics, so as to increase the conformational restriction of the RGD sequence (Bach II et al. 1996; Dechantsreiter et al. 1999; Lohof et al. 2000; Belvisi et al. 2001; Casiraghi et al. 2005; Belvisi et al. 2006).

Over the years, our interest in peptidomimetic chemistry mainly focused on the generation of constrained bicyclic amino acids based on the 3-aza-6,8-dioxabicyclo[3.2.1] octane skeleton (Trabocchi et al. 2006). The core scaffold presents a quite rigid structure, as demonstrated by the presence of a unique energetic minimum in molecular mechanics calculations, and the possibility of amino acid side chains to be placed in different positions of the bicyclic scaffold is an evidence for these molecules as good templates for peptidomimetic design. Based on the cyclopeptide *c*[RGDf(Me)V], we envisioned some of these reverse turn inducers acting as templates for the construction of cyclic RGD-based peptidomimetics as ligands for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Specifically, we were interested in synthesizing cyclic peptides containing the two bicyclic 7-*endo*-BTG and 7-*endo*-BtG scaffolds (bicycles from tartaric acid and glycine),[1] as shown in Fig. 1, with the aim to study the effect of positional and stereochemical scanning on the binding affinity towards $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.

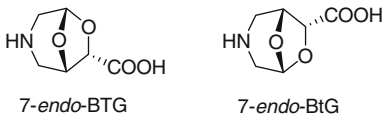

Fig. 1 Selected bicyclic amino acids as reverse turn inducers

Thus, four cyclic peptidomimetics (Fig. 2) were designed and synthesized, which differed by the presence of 7-*endo*-BTG or 7-*endo*-BtG scaffolds and by the RGD or DGR sequence. Also, the conformational analysis for selected compounds was carried out to give insight on the relationship between the binding affinity and the conformational preferences of 7-*endo*-BTG- and 7-*endo*-BtG-containing cyclic peptidomimetics.

Results and discussion

Synthesis

Fmoc-protected bicyclic scaffolds were prepared from glycine and erythrose derivatives, as reported (Trabocchi et al. 2003). The synthesis of the RGD- and DGR-cyclic peptidomimetics was carried out according to the procedure as reported by Scolastico et al. (Fig. 3) (Belvisi et al. 2001). Specifically, the linear sequence was prepared on solid phase using the Fmoc protocol, and starting from glycine bound to the acid-labile trityl resin. DIC/HOBt were used as carboxylic acid activators. In all cases, the coupling reaction of the scaffold to the peptidic fragment resulted in complete conversion, as shown by colourimetric monitoring with bromophenol blue (BB) (Krchnák et al. 1988), whereas coupling to the amine function of the bicyclic amino acid proved to be slow, and optimized reaction completion required three days reacting.

Side-chain protected linear peptidomimetic was cleaved from the resin with 1% TFA in dichloromethane, and successively allowed to cyclize in diluted DMF using HATU as COOH activator. After purification of the protected compounds by standard chromatography, the cyclic peptidomimetics were achieved by deprotection of Arg and Asp side chains with 90% TFA cleaving mixture containing catalytic amounts of thioanisole, 1,2-ethandithiol, and anisole. Subsequent HPLC purification and elution of the collected fractions over ion-exchange resin produced the title compounds as hydrochloride salts.

Biological assay

The ability of compounds **1–4** to compete with [^{125}I]-echistatin for binding to the isolated and purified $\alpha_v\beta_3$ and

[1] 7-*endo*-BTG: **B**icyclic compound from **T**artaric acid and **G**lycine; the prefix *endo* indicates the orientation of the carboxylic group at position 7 of the scaffolds, which originates from the use of *meso*-tartaric acid. 7-*endo*-BtG refers to the enantiomeric structure also originating from *meso*-tartaric acid.

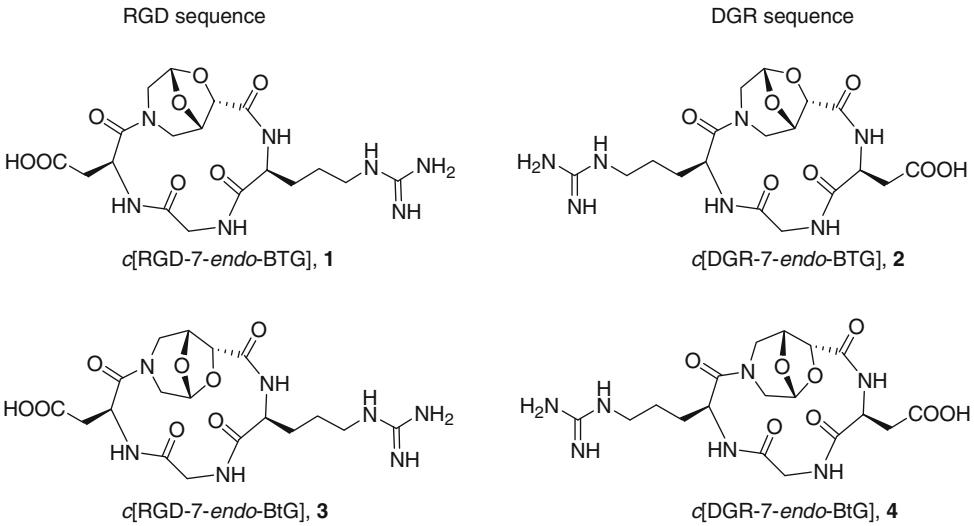

Fig. 2 Panel of DGR- and RGD-cyclic peptidomimetics having the bicyclic amino acids of Fig. 1

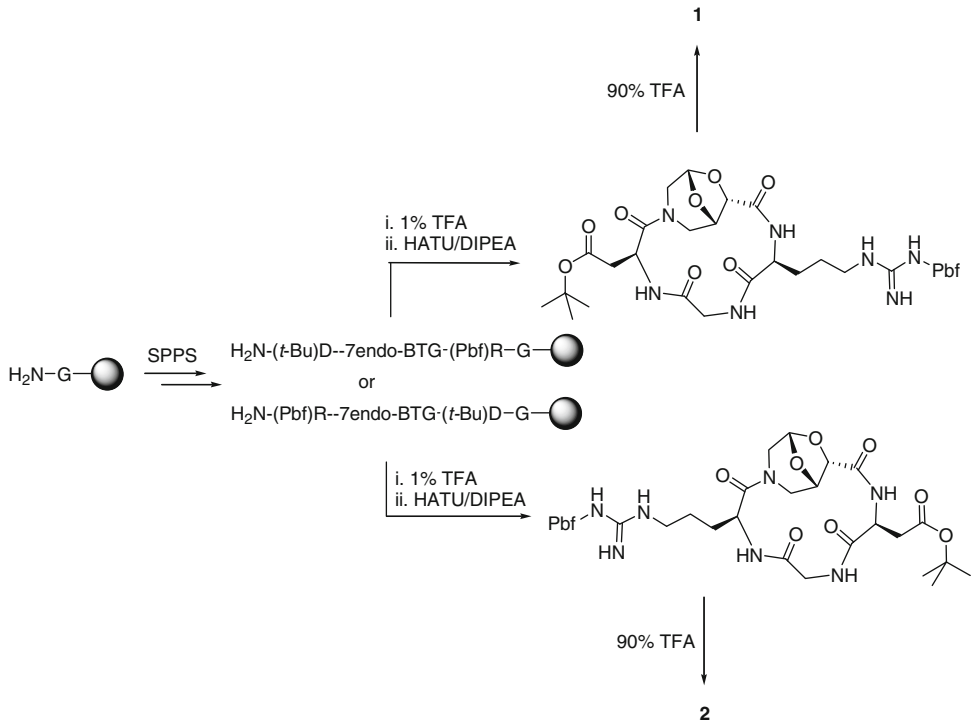

Fig. 3 Representative solid-phase synthesis of **1** and **2**

$\alpha_v\beta_5$ integrins originated from human placenta (Belkin et al. 1990; Pytela et al. 1987) was evaluated in solid-phase receptor assays (Kumar et al. 1998, 2001) and compared with that of ST1646, a known integrin ligand (Belvisi et al. 2005).[2] Competition studies were carried out using a fixed concentration of the radioligand (0.05 and 0.1 nM for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors, respectively), and a range of concentrations between 200 μM and 1 nM of the tested molecules. The $IC_{50} \pm$ SEM values (nM) were calculated as the concentration of the peptidomimetic required for

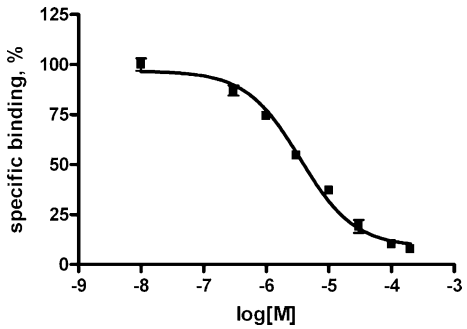

Fig. 4 Binding affinity curve for **4** relatively to $\alpha_v\beta_5$ ($IC_{50} = 3.49 \pm 1.13$ μM)

[2] IC_{50} values of ST1646 from our assays were 0.9 ± 0.1 nM for $\alpha_v\beta_5$ and 5.64 ± 0.4 nM for $\alpha_v\beta_3$.

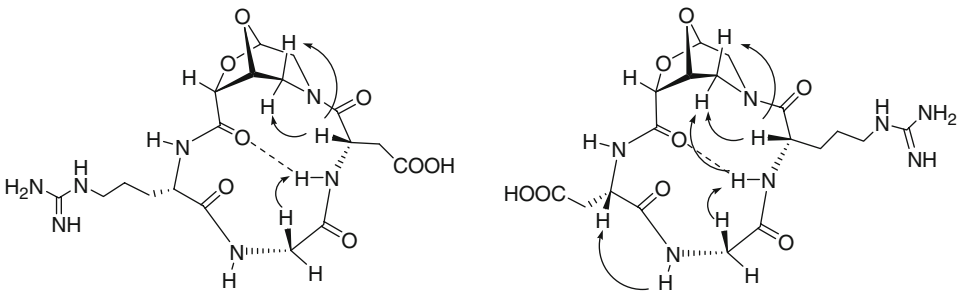

Fig. 5 Hydrogen bonding and key NOE connectivities found for compounds **3** and **4**

achieving 50% inhibition of radioligand binding, as estimated by the GraphPad Prism software.

All the peptidomimetics **1**–**4** did not show any relevant binding affinity towards the $\alpha_v\beta_3$ integrin, whereas only the cyclic peptidomimetics **4** exhibited micromolar activity towards $\alpha_v\beta_5$ (3.49 μM IC_{50} value) (Fig. 4), suggesting the possibility to access DGR-based integrin ligands, and to act as a selective $\alpha_v\beta_5$ inhibitor. Further insight on the role of the structure of **4** on the binding affinity was achieved through the synthesis and binding affinity evaluation of a stereoisomer of **4** containing D-Arg and D-Asp amino acids, so as to investigate the topology of the DGR sequence by means of stereochemical inversion. Inactivity of such compound towards both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins confirmed the binding requirements to be strictly dependent on the conformational preferences of the ligand and on the stereochemistry of the key Arg and Asp amino acids, both contributing to the correct presentation of the acidic and basic moieties towards the receptor's site.

Conformational analysis

Structural features of peptidomimetics **3** and **4** were analysed by NMR in 4:1 H_2O/D_2O solutions, and by molecular modeling techniques, so as to assess the structural aspects leading to remarkable differences in binding affinity towards $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, and to gain information on the structural requirements leading to the selectivity displayed by **4** over $\alpha_v\beta_5$. We observed that the amide bonds Asp-(7-*endo*-BtG) in **3** and Arg-(7-*endo*-BtG) in **4** can adopt both the *E* and *Z* conformations in solution. Exchange between the conformers is slow in the NMR time scale, leading to the appearance of doubled resonances in the NMR spectra. In both compounds, the population of the major rotamer was greater than 95% and was attributable to the *Z* conformer (Fig. 5).

This preferred conformation was proved, for compound **4**, by NOESY cross peaks between Arg H-α and both H-2_{ax} and H-2_{eq} protons of 7-*endo*-BtG, and for compound **3** by NOESY interactions between Asp H-α and the H-2 protons of 7-*endo*-BtG (Fig. 5). Thus, 7-*endo*-BtG was found to mimic exactly the *trans*-proline commonly found at $i + 1$ position of a type II β-turn. In compound **4**, the chemical shift value of Arg NH ($\delta = 7.66$ ppm) suggested a participation in a moderate amount of intramolecular hydrogen-bonding. The behaviour of Arg NH and the (weak) NOE contact between Arg NH and 7-*endo*-BtG H-2_{eq} indicated that this amide proton is inside to the pseudopeptide ring and can experience a hydrogen-bond with the carbonyl group of the scaffold (Fig. 5). Moreover, NOESY spectra showed the following strong cross peaks (Fig. 5): Gly H-α/Arg NH, and Asp H-α/Gly NH. This pathway and the chemical shift values of Gly NH ($\delta = 8.41$ ppm) and Asp NH (8.65 ppm) indicated that these protons are solvent exposed and not involved in hydrogen-bonding.

Energy-minimized structure of compound **4**, *Z* rotamer, is shown in Fig. 6. The minimum energy *Z* conformer was 9 kcal/mol lower in energy than the corresponding *E* rotamer.[3] The existence of a type II β-turn having the scaffold at position $i + 1$ agreed with NMR data, and the sequential NOESY peaks as indicated above supported the calculated conformation. The distance between C-β-Asp and C-β-Arg of 7.45 Å suggested that the minimum energy structure of the *Z* rotamer assumes a flat conformation capable to stretch the two side chains far from the cyclic backbone, probably favouring the interaction with the MIDAS Ca^{2+} and Asp150/218 regions of the $\alpha_v\beta_5$ binding pocket. Nevertheless, comparison with the structure of Cilengitide taken from X-ray data of the complex with $\alpha_v\beta_3$ integrin, and having a distance between C-β-Asp and C-β-Arg of

[3] Energy minimization calculations in gas phase were performed with the CS Chem3D Ultra package software of CambridgeSoft, using MM2 as a forcefield with a minimum RMS gradient of 0.100 kcal/mol. The initial structure was build according to NMR data as shown in Fig. 5.

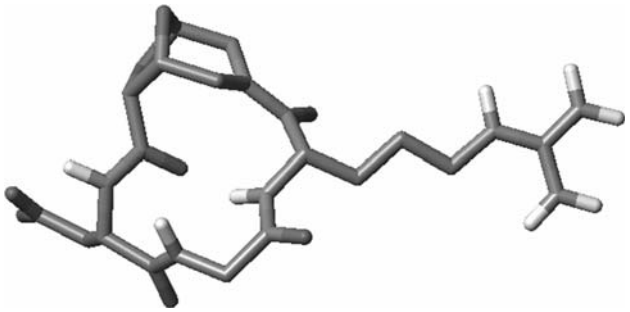

Fig. 6 Energy-minimized structure of **4**

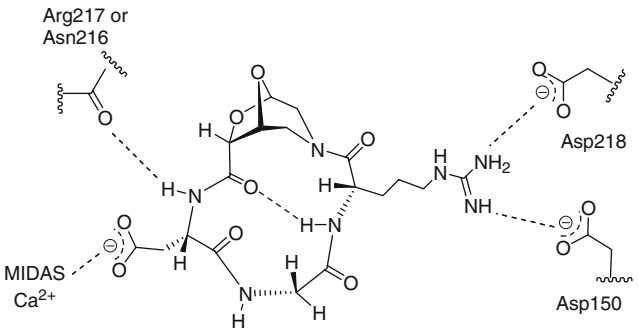

Fig. 7 Proposed binding mode for DGR-peptidomimetic **4** to $\alpha_v\beta_5$

8.87 Å (Xiong et al. 2002), proved that compound **4** cannot reach the optimal distance to interact correctly with the $\alpha_v\beta_3$ integrin binding pocket, thus justifying the lack of any binding affinity towards such integrin.

We observed the same conformational behaviour for compound **3**, the only difference being the inversion between the Arg and Asp amino acids in the sequence. Thus, in compound **3**, Asp–NH is involved in an intramolecular hydrogen-bond whereas Arg–NH is solvent exposed (Fig. 5), thus resulting in compounds **3** and **4** to show the same conformational preferences with respect to the cyclic backbone. Therefore, we cannot ascribe the different behaviour of **3** and **4** towards $\alpha_v\beta_5$ integrin to their conformational features. Probably, their different activity depends on the correct presentation of the 7-*endo*-BtG moiety towards the receptor's site. In fact, ligand **4** approaches the integrin surface with the hydrophobic side, and exposes the acetal oxygen atoms out of the receptor' surface. Moreover, the amide proton of the aspartic acid in **4** is solvent exposed and prone to interact with the amino acids of the receptor' surface.

A proposed binding mode of DGR-peptidomimetic **4** to the $\alpha_v\beta_5$ integrin was assumed, as shown in Fig. 7, according to a modeling study about the $\alpha_v\beta_5$ receptor and the structure of dual ligands, as reported (Marinelli et al. 2004).

In particular, the reported structural basis for the $\alpha_v\beta_5$ ligand recognition agrees with the assumption that a hydrogen-bond donor in the proximity of the Asp carboxylate group of peptidomimetic **4** can participate in a stabilizing interaction with the backbone CO group of Arg217 or the side chain CO group of Asn216, thus accounting for the different binding preferences of **3** and **4** towards $\alpha_v\beta_5$. Moreover, the minor affinity with respect to the $\alpha_v\beta_3$ integrin may account for a different topology of the β subunit in which such stabilizing interaction occurs, indicating a less prone binding asset between compound **4** and the binding site environment of the $\alpha_v\beta_3$ integrin. Finally, the absence of binding affinity for the parent DGR-peptidomimetic **2**, possessing the 7-*endo*-BTG structure, indicated a mismatched presentation of the bicyclic scaffold towards the binding site of the receptor, whereas compound **1** showed both unfavourable structural features, having the 7-*endo*-BTG amino acid embedded in the RGD sequence.

Conclusions

In summary, it has been reported on the synthesis, biological evaluation, and conformational analysis by NMR of a class of cyclic peptidomimetics containing the RGD or DGR sequence and two bicyclic amino acids acting as reverse turn inducers, indicating compound **4**, having the DGR retro-sequence, as a low micromolar ligand for the $\alpha_v\beta_5$ integrin. Detailed conformational analysis of **4** compared to **3** was assessed to achieve information on the factors leading to the preferential binding to the $\alpha_v\beta_5$ integrin. The conformation of the cyclic ligands influenced the distance between the two functional moieties, and the key role of peptidomimetic templates was assessed in provoking the overall ligand to assume the correct conformation, which is a stringent condition for optimal binding. The DGR-peptidomimetic, having the 7-*endo*-BtG as a turn inducer, experienced the required topology for the interaction with the binding site of the $\alpha_v\beta_5$ integrin, due to the correct presentation of the pharmacophoric side chains of Arg and Asp amino acids, and probably taking into advantage the possibility of an additional stabilizing interaction between the solvent exposed amide proton of the aspartic acid and the carbonyl group of Arg217 or of Asn216, as suggested in the literature. Given the poor structural data regarding the free $\alpha_v\beta_5$ receptor or in complex with a ligand, with the exception to the modeling study reported by Kessler and collaborators (Marinelli et al. 2004), the stereochemical and conformational study of the herein reported cyclic peptidomimetics may serve as a tool for the design of new selective inhibitors of the $\alpha_v\beta_5$ integrin.

Experimental

Chemistry

Fmoc-Gly-2-Cl-Trityl-resin (0.4–0.6 mmol/g) was purchased from Novabiochem. Chromatographic separations were performed on silica gel (Kieselgel 60, Merck) using flash-column techniques; R_f values refer to TLC carried out on 25-mm silica gel plates (Merck F_{254}) with the same eluant indicated for column chromatography. All the solid-phase reactions were carried out on a shaker, using solvent of HPLC quality. ^{1}H spectra were recorded with NMR instruments operating at 200 and 400 MHz for proton and using $CDCl_3$ solutions unless otherwise stated. EI mass spectra were carried out at 70-eV ionizing voltage. Bromophenol blue (BB) test was performed following this procedure: a few resin beads were put in a vial and suspended in 0.5 mL of DMF; two drops of a 1% solution of bromophenol blue in dimethylacetamide were added and then the sample was observed. The BB test is considered to be positive (presence of free amino groups) when the resin beads turn blue immediately and negative (absence of free amino groups) when the beads remain colourless. Peptidomimetics were characterized by ESI-MS and HPLC. HPLC analyses were carried out using a HPLC system equipped with an analytical C18 10 μm, 250 × 4.6-mm reverse-phase column, using H_2O/CH_3CN gradient eluant buffered with 0.1% TFA. Program: 1% $CH_3CN/5'$, 1–10% $CH_3CN/5'$ then 10–90% $CH_3CN/20'$ as gradient. Compounds **1**–**4** were purified by a HPLC system equipped with semi-preparative C18 10 μm, 250 × 10 mm reverse-phase column, using 0–10% $CH_3CN/5'$, then 10–90% $CH_3CN/25'$ as gradient.

c[7-*endo*-BtG-Asp-Gly-Arg] (4) *N*-Fmoc-Gly-2-Cl-trityl resin (500 mg, 0.3 mmol) was used as starting material. Fmoc deprotection was performed twice with 30% piperidine/DMF solution (10 mL) for 10 min, followed by resin washings with DMF (3 × 10 mL) and DCM (3 × 10 mL). The deprotection and the success of coupling was assessed by performing a BB test. After deprotection, the coupling of the first amino acid was performed with a solution of *N*-Fmoc-Asp(*t*Bu)-OH (370 mg, 0.9 mmol, 3 eq), HOBt (122 mg, 0.9 mmol, 3 eq) in DCM/DMF 2:1 (10 mL) and, at 0°C, DIC (140 μL, 0.9 mmol, 3 eq) that was added dropwise. The resulting mixture was stirred for 10 min at this temperature and for a further 10 min at room temperature, then added to the resin. This mixture was shaken at room temperature for 2.5 h. The solution was drained and the resin was washed with DMF (3 × 10 mL) and DCM (3 × 10 mL). After deprotection, the *N*-Fmoc-Asp(*t*Bu)-Gly-2-Cl-Trityl resin was suspended in a solution of HBTU (570 mg, 1.5 mmol, 5 eq), HOBt (203 mg, 1.5 mmol, 5 eq), *N*-Fmoc-7-*endo*-BtG-OH (229 mg, 0.6 mmol, 2 eq) and DIPEA (514 μL, 3 mmol, 10 eq) in DMF (10 mL) for 1.5 h. The solution was drained and the resin was washed with DMF and DCM. After deprotection, the *N*-Fmoc-7-*endo*-BtG-Asp(*t*Bu)-Gly-2-Cl-Trityl resin was suspended in a solution of HOBt (203 mg, 1.5 mmol, 5 eq), DIC (234 μL, 1.5 mmol, 5 eq), *N*-Fmoc-Arg(PMC)-OH (994 mg, 1.5 mmol, 5 eq) and DIPEA (514 μL, 3 mmol, 10 eq) in DMF (10 mL) for 3 days. The solution was drained and the resin was washed with DMF and DCM. The *N*-Fmoc-Arg(PMC)-7-*endo*-BtG-Asp(*t*Bu)-Gly-2-Cl-Trityl resin obtained was finally deprotected and washed with DMF and DCM.

In a solid-phase reaction vessel H_2N-Arg(PMC)-7-*endo*-BtG-Asp(*t*Bu)-Gly-2Cl-Trityl resin was treated with 5 mL of 1% TFA/DCM solution (10 × 2 min). The filtrates were immediately neutralized with a 10% pyridine/MeOH solution (1 mL) and then the resin was washed with DCM (3 × 10 mL), MeOH (3 × 10 mL), DCM (3 × 10 mL) and MeOH (3 × 10 mL). The fractions containing the product (TLC: DCM/MeOH 4:1, $R_f = 0.6$) were combined and concentrated under reduced pressure to yield a residue, which was dissolved in H_2O and purified from the pyridinium salts by size-exclusion chromatography (AMBERLITE XAD-2 resin, H_2O then MeOH). Evaporation of the combined MeOH fractions containing the product afforded the side-chain protected peptidomimetic (240 mg, 98%) as a white solid. HPLC: $t_R = 25.18$ (purity: 80%). ESI-MS: (*m*/*z*) 810.50 $[M + H]^+$, 833.50 $[M + Na]^+$.

The linear peptidomimetic (186 mg, 0.23 mmol) was dissolved in DMF (50 mL) under N_2, a solution of HOBt (94 mg, 0.66 mmol, 3 eq), HBTU (250 mg, 0.66 mmol, 3 eq) and 2,4,6-collidine (88 μL, 0.66 mmol, 3 eq) in DMF (5 mL) was added and the resulting mixture was stirred for 24 h at room temperature. The solvent was evaporated under reduced pressure and the residue was dissolved in H_2O (60 mL) and extracted with AcOEt (80 × 3 mL); the organic phase was washed twice with 5% $NaHCO_3$, dried with Na_2SO_4 and evaporated under reduced pressure. The crude residue was purified by flash column chromatography (DCM/MeOH from 95:5 to 9:1, $R_f = 0.3$) to afford the protected cyclic peptidomimetic (83 mg, 46%), as a white solid. ESI-MS: (*m*/*z*) 792.35 $[M + H]^+$, 814.33 $[M + Na]^+$, 830.30 $[M + K]^+$. HPLC: $t_R = 24.98$, purity: 81%.

Side chain protected cyclic peptidomimetic (69 mg, 0.086 mmol), was treated with TFA/thioanisole/1,2-ethandithiol/anisole 90:5:3:2 (31 mL) for 2 h. The reaction mixture was evaporated under reduced pressure and the residue was dissolved in H_2O (40 mL). The aqueous phase was washed twice with iPr_2O (40 mL × 3) and freeze-dried. The crude residue was purified by semi-preparative

HPLC to give the side-chain deprotected **4** (38 mg, 95%), as a white solid. Trifluoroacetate ion was substituted with chloride by ion-exchange chromatography (AMBERLITE IRA-93 resin, chloride form). ESI-MS: (*m/z*) 470.3 $[M + H]^+$, 492.2 $[M + Na]^+$. HPLC: $t_R = 15.6$, purity >99%. See Table 1 for ^{1}H NMR data.

***c*[7-*endo*-BtG-Arg-Gly-Asp] (3)** Compound **3** was prepared following the procedure as for **4**, exchanging the amino acids Asp and Arg. Cleavage from the solid support afforded the linear protected peptidomimetic in 70% yield, as a white solid. HPLC: $t_R = 25.18$, purity >99%. ESI-MS: (*m/z*) 810.34 $[M + H]^+$, 832.33 $[M + Na]^+$, 848.30 $[M + K]^+$. Cyclization using HATU methodology afforded the protected cyclic peptidomimetic in 40% yield, as a white solid. HPLC: $t_R = 25.0$ (purity: 81%). ESI-MS: (*m/z*) 792.35 $[M + H]^+$, 814.33 $[M + Na]^+$, 830.30 $[M + K]^+$. Final deprotection in analogy with **4** afforded **3** in 16% yield, as a white solid. HPLC: $t_R = 16.5$ (purity: >99%). ESI-MS: (m/z) 470.2 $[M + H]^{+,}$ 492.1 $[M + Na]^+$. See Table 1 for ^{1}H NMR data.

***c*[7-*endo*-BTG-Asp-Gly-Arg] (2)** Cyclic peptidomimetic **2** was prepared following the procedure as for **4**, using the scaffold Fmoc-BTG-OH instead of Fmoc-BtG-OH. Cleavage from the solid support afforded the linear protected peptidomimetic in 85% yield, as a white solid. HPLC: $t_R = 24.1$, purity 86%. ESI-MS: (*m/z*) 810.34 $[M + H]^+$, 832.33 $[M + Na]^+$, 848.30 $[M + K]^+$. Cyclization using HATU methodology afforded the protected cyclic peptidomimetic in 28% yield, as a white solid. HPLC: $t_R = 24.3$ (purity: >99%). ESI-MS: (*m/z*) 792.35 $[M + H]^+$, 814.33 $[M + Na]^+$, 830.30 $[M + K]^+$. Final deprotection in analogy with **4** afforded **2** in 51% yield, as a white solid. HPLC: $t_R = 14.6$ (purity: >99%). ESI-MS: (*m/z*) 470.2 $[M + H]^{+,}$ 492.1 $[M + Na]^+$.

Table 1 ^{1}H chemical shifts of compounds **3**–**4** recorded in 4:1 H_2O/D_2O solutions at 298 K

	3 δ (ppm)	**4** δ (ppm)
7-*endo*-BtG H-1	4.57	4.51
7-*endo*-BtG H-2	3.81–3.56	3.78–3.44
7-*endo*-BtG H-4	3.77–3.09	3.80–3.1
7-*endo*-BtG H-5	5.78	5.77
7-*endo*-BtG H-7	4.88	4.85
Gly NH	8.43	8.40
Arg NH	8.43	7.65
Asp NH	7.61	8.57
Gly H-α	3.77–3.09	4.31–3.32
Arg H-α	4.26	4.50
Arg H-β	1.51–1.58	1.51–1.42
Arg H-γ	1.77	1.68
Arg H-δ	3.16	3.12
Asp H-α	4.81	4.55
Asp H-β	2.70–2.50	2.78–2.50

***c*[7-*endo*-BTG-Arg-Gly-Asp] (1)** Compound **1** was prepared following the procedure as for **4**, using the scaffold Fmoc-BTG-OH instead of Fmoc-BtG-OH, and exchanging the amino acids Asp and Arg. Cleavage from the solid support afforded the linear protected peptidomimetic in 86% yield, as a white solid. HPLC: $t_R = 24.1$, purity 86%. ESI-MS: (*m/z*) 810.34 $[M + H]^+$, 832.33 $[M + Na]^+$. Cyclization using HATU methodology afforded the protected cyclic peptidomimetic in 29% yield, as a white solid. HPLC: $t_R = 24.3$ (purity >99%). ESI-MS: (*m/z*) 792.3 $[M + H]^{+,}$ 814.3 $[M + Na]^+$, 830.3 $[M + K]^+$. Final deprotection in analogy with **4** afforded **1** in 56% yield, as a white solid. HPLC: $t_R = 15.4$ (purity: >99%). ESI-MS: (*m/z*) 470.2 $[M + H]^{+,}$ 492.1 $[M + Na]^+$.

NMR methods

For the conformational analysis of **3** and **4**, NMR experiments were performed at a temperature of 300 K on Bruker Avance 400 and 600 MHz spectrometers. All proton and carbon chemical shifts were assigned unambiguously for **3** and **4**. The NMR experiments were carried out in a D_2O/H_2O (1:4) mixture, in order to observe amide protons; the water resonance was saturated with excitation sculpting sequence from the Bruker library. Two-dimensional experiments (TOCSY, COSY, NOESY and HSQC) were carried out on samples of **3** and **4** at the sample concentration of 3 mM where aggregation was not observed. ^{1}H chemical shifts of **3** and **4** are reported in Table 1. NOESY spectra were performed at 0.6 and 0.8 s. The conformation of the two peptidomimetics was first analysed with respect to hydrogen bonding of amide protons and NOE contacts.

Solid-phase receptor binding assay

[^{125}I]-Echistatin, labelled by the lactoperoxidase method (Kumar et al. 1998) to a specific activity of 2,000 Ci/mmol, was purchased from GE Healthcare. Integrin proteins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ purified from human placenta were purchased from Chemicon International Inc, Temecula, CA. The receptor binding assay was performed as described (Kumar et al. 1997, 2001). Purified receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were diluted, respectively, at 500 and 1,000 ng/mL in coating buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$]. An aliquot of the diluted receptors (100 µL/well) was added to a 96-well microtiter plate (Optiplate-96 HB, PerkinElmer Life Sciences, Boston, MA) and incubated overnight at 4°C. The plate was washed once with blocking/binding buffer [20 mM

Tris (pH 7.4), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1% BSA] and incubated an additional 2 h at room temperature. The plate was rinsed twice with the same buffer, then competition binding studies were performed with a fixed concentration of [^{125}I]-Echistatin (0.05 and 0.1 nM for $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively), and concentrations ranging from 0.01 nM and 100 μM of the tested compounds. All assays were performed in triplicate in a final volume of 0.2 mL, each containing the following species: 0.05 mL of [^{125}I]-Echistatin, 0.04 mL of the tested compound and 0.11 mL of blocking/binding buffer. Non-specific binding was defined as [^{125}I]-Echistatin bound in the presence of an excess (1 μM) of unlabelled echistatin. After incubation for 3 h at room temperature, the plate was washed three times with blocking/binding buffer, then counted in a Top-Count NXT microplate scintillation counter (PerkinElmer Life Sciences, Boston, MA) using 200 μL/well of MicroScint-40 liquid scintillation (PerkinElmer Life Sciences, Boston, MA). Relativity affinity values (IC_{50}) were determined by fitting binding inhibition values by non-linear regression using GraphPad curve fitting software (PRISM, San Diego, California). The reproducibility of the IC_{50} values was confirmed by performing the same tests on the reference compound ST1646 (Belvisi et al. 2001, 2005).

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