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Cyclic RGD-Peptidomimetics Containing Bifunctional Diketopiperazine Scaffolds as New Potent Integrin Ligands

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The tripeptide sequence arginine-glycine-aspartate (RGD) has been identified as the common motif used by several endogenous ligands to recognise and bind a group of integrins,[1] including $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$, which play key roles in angiogenesis, tumor progression and metastasis.[2] The context of the ligand RGD sequence (flanking residues, three-dimensional presentation) and individual features of the integrin binding pockets determine the recognition specificity and discrimination ability, that is, whether a productive interaction occurs. A major breakthrough for understanding this interaction came in 2002 from the X-ray structure determination of the complex of integrin $\alpha_V\beta_3$ with cyclo-[Arg-Gly-Asp-D-Phe-N(Me)-Val] (Cilengitide).[3] This potent $\alpha_V\beta_3$ ligand was developed by Kessler and co-workers,[4] and is currently in phase III clinical trials for patients with glioblastoma multiforme as an angiogenesisis inhibitor. The high activity and selectivity of this derivative has been attributed to an extended conformation of the RGD motif showing a distance of about 9 Å between the C_β atoms of Asp and Arg.[3,5] These observations prompted many other research groups to investigate the use of conformationally constrained cyclic RGD peptides and peptidomimetics as active and selective integrin ligands, encompassing a wide variety of rigid scaffolds and featuring 14-, 15- and even 16-membered rings. Among the successful approaches, we like to mention the γ-amino acid RGD-peptidomimetics, containing a γ-aminocyclopentane carboxylic acid or a 4-aminoproline residue (14-membered ring),[6] the azabicyclic lactam RGD-peptidomimetics (15-membered ring, with the scaffold mimicking a constrained dipeptide, see for example ST1646 in Table 1),[7] and the β-amino acid RGD-peptidomimetics, embodying β^3-homoamino acids[8] or a *cis*-β-aminocyclopropane carboxylic acid (16-membered ring).[9]

In a recent paper, we have reported the synthesis of a new bifunctional diketopiperazine scaffold (**DKP-1**, Figure 1), formally derived from L-aspartic acid and (*S*)-2,3-diaminopropionic acid, and bearing a carboxylic acid and an amino functionalities.[10] When inserted into an oligopeptide sequence, the DKP scaffold acts as a reverse turn inducer. In addition, **DKP-1**, while being derived from α-amino acids, can be seen as a conformationally constrained dipeptide formed by two β-amino acids and in particular a (*S*)-β^2- and a (*S*)-β^3-amino acids (following Seebach's nomenclature).[10]

In the frame of a program aimed at the rational design of peptidomimetics with a defined secondary structure, we envisioned the synthesis, conformational analysis and investigation of the biological activity of cyclic RGD-peptidomimetics **3** and **4** (Figure 2), containing the bifunctional diketopiperazine scaffolds **DKP-1** (*cis*) and **DKP-2** (*trans*).

The linear pentapeptide mimics were prepared by solution-phase synthesis using a Boc protection strategy and *t*Bu/Mtr in the side chains. The dipeptide Arg(Mtr)-GlyOMe was coupled to either **DKP-1** or **DKP-2** and the

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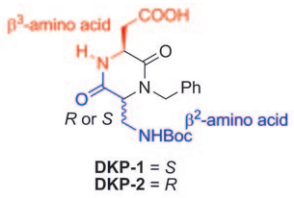


Figure 1. Structure of the bifunctional diketopiperazine scaffolds **DKP-1** and **DKP-2**, highlighting the conformationally constrained β^2–β^3 dipeptide sequence.

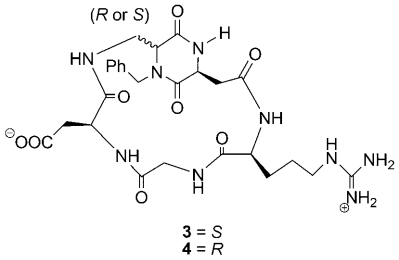


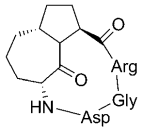
Figure 2. Structure of the cyclic RGD-peptidomimetics **3** and **4** containing the bifunctional diketopiperazine scaffolds **DKP-1** (*cis*) and **DKP-2** (*trans*), respectively.

resulting tetrapeptide mimics, after Boc cleavage, were attached to Cbz-Asp(O*t*Bu)-OH. Transesterification of the glycine methyl ester to the corresponding benzyl ester[11] and hydrogenolysis afforded the linear pentapeptide precursors which were subjected to macrolactamization (HATU,[12] HOAT, collidine, DMF). The *cis*-macrolactam (containing **DKP-1**) was obtained in good yield (61 %) under moderate dilution conditions (20 mM), while the *trans*-macrolactam (containing **DKP-2**) required high dilution (2 mM) and was obtained in a passable 41–45 % yield. The *cis*-substituted linear precursor is apparently better pre-organized for the intramolecular cyclization than the corresponding *trans*-isomer. Global deprotection gave cyclic RGD-peptidomimetics **3** and **4** (Figure 2; for details of the synthetic sequence, see the Supporting Information).

The cyclic RGD peptidomimetics were examined in vitro for their abilities to compete with biotinylated vitronectin for binding to the purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Table 1). Screening assays were performed by measuring the effect of the RGD macrolactams **3** and **4** on the interaction between immobilized integrin receptors and biotinylated soluble ligands. The ability of the new compounds to inhibit the binding of vitronectin to the isolated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors was compared with that of the standard compound c(RGDfV)[13] and compound ST1646[7] (Table 1).

Interestingly, the cyclic RGD-peptidomimetic **4**, containing the *trans*-diketopiperazine scaffold **DKP-2**, exhibited a nanomolar affinity for the $\alpha_v\beta_3$ integrin, comparable to *c*-(RGDfV) and ST1646. On the contrary, RGD-peptidomimetic **3**, bearing the *cis*-diketopiperazine **DKP-1**, showed only a modest micromolar affinity towards the $\alpha_v\beta_3$ integrin. Moreover, unlike reference compounds c(RGDfV) and

Table 1. Inhibition of biotinylated vitronectin binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors.

Compound	$\alpha_v\beta_3$ IC_{50} [nM][a]	$\alpha_v\beta_5$ IC_{50} [nM][a]
3	3898 ± 418	$> 10^4$
4	3.2 ± 2.7	114 ± 99
c(RGDfV)	3.2 ± 1.3	7.5 ± 4.8
 ST1646	1.0 ± 0.5	1.4 ± 0.8

[a] IC_{50} values were calculated as the concentration of compound required for 50 % inhibition of biotinylated vitronectin binding as estimated by GraphPad Prism software; all values are the arithmetic mean ± SD of triplicate determinations.

ST1646, the RGD-peptidomimetic **4** was approximately 40-fold more selective for the $\alpha_v\beta_3$ integrin with respect to the $\alpha_v\beta_5$, in this kind of assay.

Two other cyclic RGD ligands containing a bifunctional diketopiperazine scaffold were reported in the literature,[14] namely cyclo-[aspartate-(2*S*,4*S*)-4-aminoproline] by Robinson and co-workers,[14a] and cyclo-(Asp-Lys) by Albericio and co-workers.[14b] In both cases the diketopiperazine substituents in the 3,6-positions were *cis*. The activities of these compounds in competitive binding assays towards the $\alpha_v\beta_3$ integrin receptor were only modest (in the micromolar range), and this was attributed to the loose conformational constraint exerted by the scaffold.

To investigate the origin of the strikingly different behavior of the RGD-peptidomimetics **3** and **4**, a conformational study was performed by ^{1}H NMR spectroscopy of dilute H_2O/D_2O 9:1 solutions and by computational methods.

Both compounds were fully characterized by ^{1}H- and ^{13}C NMR spectroscopy (the NMR data are reported in the Supporting Information). One-dimensional ^{1}H NMR experiments were conducted to detect intramolecular hydrogen bonds, by measuring the chemical shift of the N-H protons and their temperature coefficients ($\Delta\delta/\Delta T$). NOESY spectra were recorded to investigate both sequential and long-range NOE signals that provide evidences of preferred conformations. Three-dimensional structures satisfying long-range NOE contacts were generated by restrained mixed-mode Metropolis Monte Carlo/Stochastic Dynamics (MC/SD) simulations,[15] using the implicit water GB/SA solvation model.[16]

The RGD-peptidomimetic **3** exists in two different preferred conformations as reported in Figure 3 (A and B).

The NOESY spectrum of **3** shows two mutually exclusive long-range NOE contacts. The cross peak between DKP-NH10 and Asp-NH (strong) is indicative of a β-turn conformation stabilized by a hydrogen bond between DKP-NH10 and Arg-C=O. The chemical shift value (7.46 ppm) and the $\Delta\delta/\Delta T$ value (-2 ppb K^{-1}) of the amide proton DKP-NH10 indicate that this proton is strongly locked in an intramolecularly H-bonded state (Figure 3 A). The cross peak between Gly-NH and Asp-NH (medium) is indicative of a β-turn

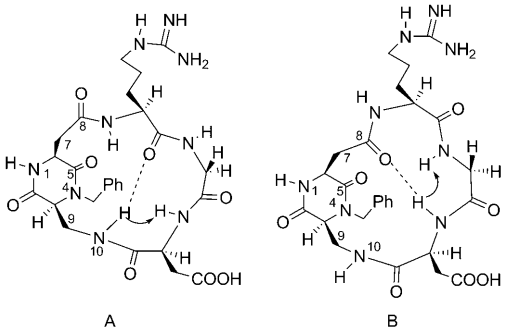


Figure 3. Preferred intramolecular hydrogen-bonded patterns proposed for compound **3** on the basis of spectroscopic data. A) β turn at Gly-Asp. B) β turn at Arg-Gly. The arrows indicate significant NOE contacts.

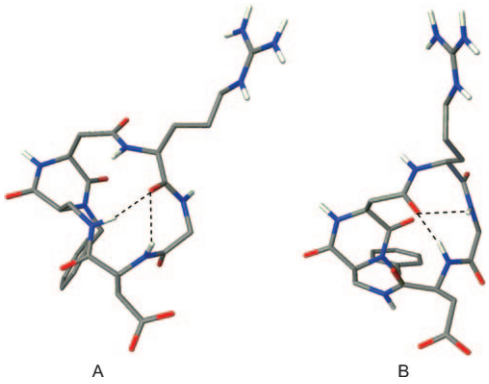


Figure 4. Structures of **3** as obtained by restrained MC/SD simulations based on experimental distance information. A) γ turn at Gly and βII′-turn at Gly-Asp [C_β(Arg)–C_β(Asp) = 7.9 Å]. B) γ turn at Arg and βII′ turn at Arg-Gly [C_β(Arg)–C_β(Asp) = 5.9 Å].

conformation stabilized by a hydrogen bond between Asp-NH and C(8)=O (Figure 3B).

Two 10 ns MC/SD simulations were performed for compound **3** applying the DKP-NH10/Asp-NH or the Asp-NH/Gly-NH distance restraint derived from NOESY spectra. Approximately 70% of the conformations sampled during the first simulation adopted a non-extended arrangement of the RGD sequence characterized by a β turn at Gly-Asp and the presence of the corresponding hydrogen bond between DKP-NH10 and Arg-C=O. In addition, the formation of a γ turn at Gly stabilized by the hydrogen bond between Asp-NH and Arg-C=O was observed for 50% of the simulation. A C_β(Arg)–C_β(Asp) average distance of 7.4 Å was obtained during this MC/SD calculation. A representative energy minimized conformation selected by cluster analysis and featuring both H-bonds is shown in Figure 4A. More than 90% of the conformations sampled during the simulation of **3** featuring the Asp-NH/Gly-NH distance restraint, adopted a non-extended arrangement of the RGD sequence characterized by a β turn at Arg-Gly and the corresponding hydrogen bond between Asp-NH and C(8)=O. In addition, the formation of a γ turn at Arg stabilized by the hydrogen bond between Gly-NH and C(8) = O was observed for 50% of the simulation. The C_β(Arg)–C_β(Asp) average distance in this MC/SD calculation was 6.5 Å. A representative energy minimized conformation selected by cluster analysis and featuring both H-bonds is shown in Figure 4B.

On the contrary, the NOESY spectrum of compound **4** shows only one relevant long-range interaction between Gly-NH and Arg-NH: this NOE is indicative of a β-turn motif stabilized by a hydrogen bond between Gly-NH and C(5)=O (confirmed by the chemical shift and the $\Delta\delta/\Delta T$ value of Gly-NH) (Figure 5). The distance restraint corresponding to the NOE contact between Gly-NH and Arg-NH was applied in the 10 ns MC/SD simulation of compound **4**. More than 90% of the conformations sampled during the simulation adopted an extended arrangement of the RGD sequence characterized by a pseudo β turn at DKP-Arg and the formation of the corresponding hydrogen bond between the Gly-NH and C(5)=O. In addition, the formation of a γ turn at Asp stabilized by the hydrogen bond between DKP-NH10 and Gly-C=O was observed for 23% of the simulation. A C_β(Arg)–C_β(Asp) average distance of 9.3 Å was obtained during this MC/SD calculation. A representative energy minimized conformation selected by cluster analysis and featuring the H-bond between the Gly-NH and C(5)=O is shown in Figure 5.

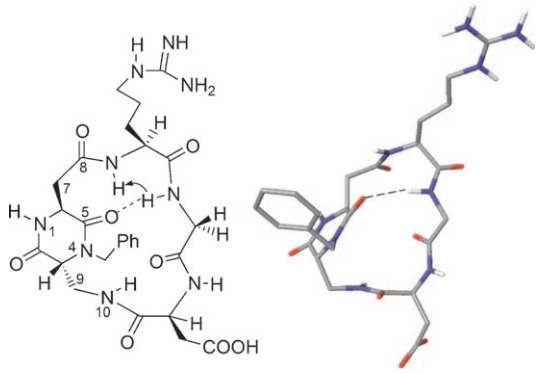


Figure 5. Left: Preferred intramolecular hydrogen-bonded pattern proposed for compound **4** on the basis of spectroscopic data. The arrow indicates a significant NOE contact. Right: Structure of **4** as obtained by restrained MC/SD simulations based on experimental distance information (distorted inverse γ turn at Asp and pseudo β turn at DKP-Arg, C_β-(Arg)–C_β(Asp) = 9.4 Å).

In order to rationalize, on a molecular basis, the affinity of compounds **3** and **4** for the $\alpha_v\beta_3$ receptor, docking studies were performed starting from the representative conformations obtained from the restrained MC/SD simulations. The crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ complexed with the cyclic pentapeptide Cilengitide (1L5G, pdb code) was taken as a reference model for the interpretation of the docking results in terms of ligand-protein interactions.[3] In the X-ray complex, Cilengitide binds to the interface of the α and β units forming specific electrostatic interactions. The acid and basic pharmacophoric groups and their orientation are essential for binding to $\alpha_v\beta_3$ because

they act like an electrostatic clamp, interacting with charged regions of the receptor binding site.[3]

For peptidomimetic **3**, docking calculations starting from both the representative geometries of Figure 4 produced top-ranked poses conserving optimal interactions only with the α subunit of the $\alpha_v\beta_3$ receptor. Probably, the short C_β-(Arg)–C_β(Asp) distances of these geometries prevent the guanidine and carboxylic groups from achieving the required separation for binding to the $\alpha_v\beta_3$ integrin.[17] On the other hand, docking calculations starting from the extended geometry of peptidomimetic **4** reported in Figure 5 produced top-ranked poses conserving all the important interactions of the X-ray complex (see Figure 6). The positively

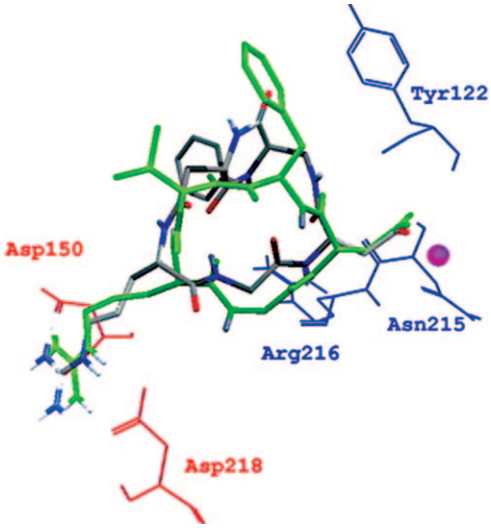


Figure 6. Best pose of compound **4** (atom colour tube representation) into the crystal structure of the extracellular domain of $\alpha_v\beta_3$ integrin (α unit red and β unit blue wire representation) overlaid on the bound conformation of Cilengitide (green tube representation). Only selected integrin residues involved in the interactions with the ligand are shown. The Mn^{2+} ion at MIDAS is shown as a magenta CPK sphere. Non-polar hydrogens were removed for clarity.

charged Arg guanidinium group of the ligand interacts with the negatively charged side chains of Asp218 and Asp150 in the α unit, one carboxylate oxygen of the ligand Asp side chain is coordinated to the metal cation in the metal-ion-dependent adhesion site (MIDAS) region of the β unit, while the second carboxylate oxygen forms hydrogen bonds with the backbone amides of Asn215 and Tyr122 in the β unit. Further stabilizing interaction involves the formation of a hydrogen bond between the ligand backbone NH of the Asp residue and the backbone carbonyl group of Arg216 in the β unit.

In light of all these considerations, the micromolar affinity of macrolactam **3** for $\alpha_v\beta_3$ (Table 1) can be explained in terms of its low pre-organization for binding. In fact, as determined by the computational and NMR studies, compound **3** in solution mainly features non-extended RGD conformations which, according to the docking results, are not able to properly fit into the $\alpha_v\beta_3$ receptor. On the contrary, the nanomolar affinity of macrolactam **4** for $\alpha_v\beta_3$ can be attributed to its high structural pre-organization. The *trans* **DKP-2** scaffold induces a preferred conformation with Arg in the i+1 position of a pseudo β turn which determines an extended RGD disposition similar to the RGD bound conformation of Cilengitide.

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Keywords: conformation analysis · diketopiperazines · integrin ligands · molecular modeling · peptides

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[17] As pointed out by a referee, a non-extended RGD conformation with a relatively short distance (6.7–7.4 Å) between C_β(Arg)–C_β-(Asp) has been proposed for c(RGDfV), see reference [13]. c-(RGDfV), which is conformationally much more flexible than compound **3**, probably also adopts more extended RGD arrangements which are able to properly fit into the $\alpha_V\beta_3$ receptor; this would explain its nanomolar affinity. A more thorough discussion will be reported in a forthcoming full paper.

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