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Cyclic *iso*DGR Peptidomimetics as Low-Nanomolar $\alpha_v\beta_3$ Integrin Ligands

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Integrins are a large family of heterodimeric transmembrane glycoprotein receptors, composed of two noncovalently associated α and β subunits.[1] Many integrins, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ recognize the tripeptide Arg-Gly-Asp (RGD) motif in their ligands. In particular, integrin $\alpha_v\beta_3$ was the first integrin to be characterized by X-ray structural determination and was shown to be one of the key regulators of angiogenesis.[2] Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have been found to be overexpressed on blood vessels in human tumors, but not on vessels in normal human tissues. For this reason, these integrins have become attractive targets for pharmacological studies mainly in the oncology area. Many peptide and peptidomimetic integrin ligands have been developed that contain the RGD tripeptide sequence with different flanking residues and three-dimensional presentation.[3] Recently, our group reported a new class of cyclic RGD peptidomimetics (Figure 1), containing bifunctional diketopiperazine (DKP) scaffolds, formally derived from 2,3-diaminopropionic acid and aspartic acid and differing in the configuration of the two DKP stereocenters and in the substitution at the DKP nitrogen atoms.[4] The *trans-cyclo*-[DKP-RGD] (Figure 1) are low nanomolar ligands of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$.

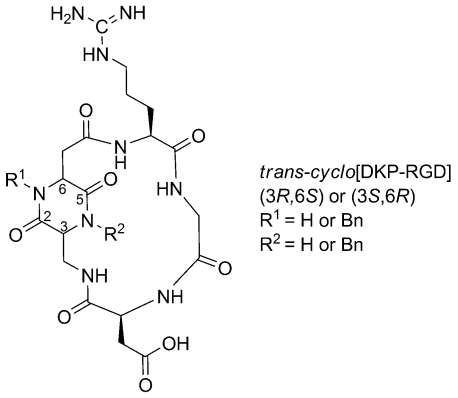

Figure 1. Cyclic RGD peptidomimetics containing the bifunctional DKP scaffold.

Recent biochemical studies have shown that a spontaneous post-translational modification, occurring at the Asn-Gly-Arg (NGR) motif of the extracellular matrix protein, fibronectin, leads to the *iso*Asp-Gly-Arg (*iso*DGR) sequence.[5] This rearrangement, which involves the attack of the protein backbone NH on the primary amide side chain of asparagine and ring opening of the succinimide, is a known side reaction normally leading to loss of biological activity.[6] In this case, however, the result is a gain of protein function and the creation of a new adhesion binding site for integrins.[7] Subsequent biochemical, spectroscopic and computational investigations have shown that the *iso*DGR sequence can fit into the RGD-binding pocket of $\alpha_v\beta_3$ integrin, establishing the same electrostatic clamp as well as additional polar interactions.[8] Based on these observations, a few conformationally constrained cyclopeptides containing the *iso*DGR sequence have been synthesized.[9] However, these ligands show a moderate affinity for $\alpha_v\beta_3$ integrin in competitive binding assays.[9b]

Prompted by these observations, we synthesized two cyclic *iso*DGR peptidomimetics (**1**, **2**; Figure 2) containing the bifunctional diketopiperazine scaffolds mentioned above, and investigated their conformation in solution, and their ability to compete with biotinylated vitronectin for binding to the purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors.

The synthesis was conveniently performed on solid phase (Fmoc strategy) using SASRIN™ resin, following the strategy depicted in Scheme 1. Fmoc-glycine was loaded onto the resin and Fmoc-aspartic acid α-*tert*-butyl ester was coupled. The *trans* DKP scaffolds (either 3*R*,6*S* or 3*S*,6*R*) were then coupled to the supported dipeptide. The DKP scaffolds are normally obtained as *N*-Boc protected amino acids,[4] and as such are unsuitable for solid phase synthetic applica-

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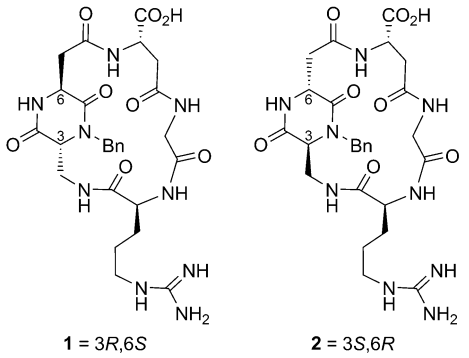


Figure 2. Cyclic *iso*DGR peptidomimetics containing the bifunctional diketopiperazine scaffolds.

tions. In order to avoid an exchange of the nitrogen protecting group, involving a deprotection–reprotection sequence, an intermediate of DKP synthesis, namely the azido-acids **3** and **4** were used in this case. The azide reduction on resin was performed by using trimethylphosphine in dioxane/water and Cbz-Arg(Mtr) was finally coupled. The linear precursors were cleaved from the resin under controlled acidic conditions (to avoid deprotection of the *tert*-butyl ester and the Mtr group) and, after hydrogenolysis of the benzyloxycarbonyl group, were subjected to macrolactamization. Final side chain deprotection and purification by RP-HPLC afforded the cyclic *iso*DGR peptidomimetics **1** and **2** ready for biological studies and conformational analysis.

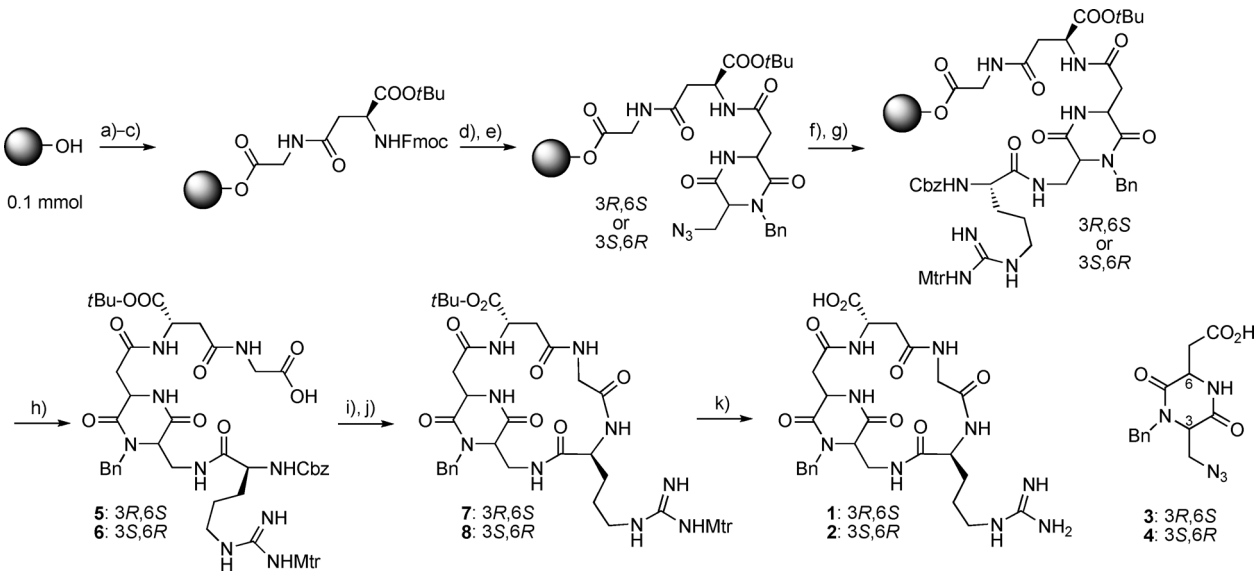
Cyclic *iso*DGR peptidomimetics **1** and **2** were examined in vitro for their abilities to compete with biotinylated vitronectin for binding to the purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Table 1). Screening assays were performed by incubating the immobilized integrins $\alpha_v\beta_3$, and $\alpha_v\beta_5$ with increasing concentrations (10^{-12}–10^{-5} M) of the two *iso*DGR ligands in the presence of biotinylated vitronectin (1 mg mL^{-1}), and measuring the concentration of bound vitronectin in the presence of the competitive ligands.

The IC_{50} values of compounds **1** and **2** reported in Table 1 (entries 1, 2) compare favorably with other *iso*DGR ligands, such as the cyclopentapeptide *cyclo*[G*iso*DGRphg] (entry 3) recently published by Kessler and co-workers,[9b,10] as well as with known RGD ligands, such as the cyclic peptidomimetic *cyclo*[(3*S*,6*R*)DKP-RGD],[4] cyclopentapeptides *cyclo*-(RGDfV)[11] and Cilengitide[11d] (entries 4–6). In particular, the new *iso*DGR ligand **2** is ten-times more potent in binding integrin $\alpha_v\beta_3$ than *cyclo*[G*iso*DGRphg], only 2–3-times less potent than the RGD ligands *cyclo*[(3*S*,6*R*)DKP-RGD] and *cyclo*[RGDfV], and fifteen-times less potent than the powerful RGD ligand Cilengitide.

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

Entry	Ligand	IC_{50} [nM][a]		IC_{50} ratio
		$\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_v\beta_5/\alpha_v\beta_3$
1	*cyclo*[(3*R*,6*S*)DKP-*iso*DGR] (**1**)	46.7 ± 18.2	220 ± 84	4.7
2	*cyclo*[(3*S*,6*R*)DKP-*iso*DGR] (**2**)	9.2 ± 1.1	312 ± 21	33.9
3	*cyclo*[G*iso*DGRphg]	89 ± 19[b]	n.d.	n.d.
4	*cyclo*[(3*S*,6*R*)DKP-RGD]	4.5 ± 1.1	149 ± 25	33.1
5	*cyclo*[RGDfV]	3.2 ± 1.3	7.5 ± 4.8	2.3
6	*cyclo*[RGDf(*N*-Me)V] (Cilengitide)	0.6 ± 0.1[c]	11.7 ± 1.5[c]	19.5

[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. [b] Determined by a solid phase binding assay by using supported vitronectin, soluble $\alpha_v\beta_3$ integrin, specific primary and secondary antibodies (see ref. [9b] and the note in ref. [10]). [c] Calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding.[11d]



Scheme 1. a) Fmoc-Gly-OH, DIC, cat. DMAP, DMF, 2 h; b) 20% piperidine in DMF; c) Fmoc-Asp(OH)-O*t*Bu, DIC, HOAt, DMF; d) 2% piperidine and 2% DBU in DMF; e) **3** or **4**, DIC, HOAt, DMF, 18 h; f) Me_3P, dioxane/water 4:1; g) Cbz-Arg(Mtr)-OH, DIC, HOAt, DMF; h) 1% TFA in DCM; i) H_2, 10% Pd/C, THF/water 1:1; j) HATU, HOAt, DIPEA, 1.4 mM in DMF; k) TFA/thioanisole/EDT/anisole 90:5:3:2.

The structure and connectivity of compounds **1** and **2** were unambiguously assigned from the mono- and bidimensional ^{1}H and ^{13}C NMR spectra. The preferred conformations of **1** and **2** were investigated by ^{1}H NMR spectroscopy of dilute H_2O/D_2O 9:1 solutions and by computational methods, with the aim of rationalizing the affinity of these compounds for the $\alpha_v\beta_3$ receptor at a molecular level. One-dimensional ^{1}H NMR spectroscopy experiments were conducted to detect intramolecular hydrogen bonds, by measuring the chemical shift of the amide N–H protons, their temperature coefficients ($\Delta\delta/\Delta T$) and their chemical exchange rate in the presence of D_2O. The results are reported in the Supporting Information. NOESY spectra were also 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,[12] using the implicit water GB/SA solvation model.[13]

The data obtained from the NMR experiments (see the Supporting Information) show that compound **1** (Figure 3) is characterized by a high conformational flexibility. In fact,

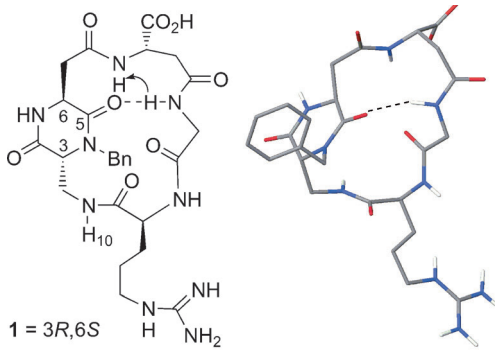


Figure 3. Left: preferred intramolecular hydrogen-bonded pattern proposed for the cyclic *iso*DGR peptidomimetic **1**, on the basis of spectroscopic data. The arrow indicates the NOE contact. Right: 3D structure of **1** as obtained by restrained MC/SD simulations based on experimental distance information, after energy minimization [pseudo-β-turn at DKP-*iso*Asp, Cβ(Arg)–COO^-(Asp) distance = 8.8 Å].

the $\Delta\delta/\Delta T$ values range from -4.7 ppb K^{-1} (Gly-NH) to -8.5 ppb K^{-1} (DKP-NH10) and the N–H chemical shifts indicate that the amide protons of **1** are all solvent exposed. However, the strong NOE contact between *iso*Asp-NH and Gly-NH (Figure 3, structure on the left) indicates a preferred folding through the formation of a pseudo-β-turn stabilized by an intramolecular hydrogen bond between Gly-NH and DKP-C(5)=O.

The distance restraint corresponding to the NOE contact between *iso*Asp-NH and Gly-NH was applied in the MC/SD simulations of compound **1**. Approximately 50% of the conformations sampled during the simulations adopted an extended arrangement of the *iso*DGR sequence characterized by a pseudo-β-turn at DKP-*iso*Asp and the formation of the corresponding hydrogen bond between Gly-NH and C(5)=O. A Cβ(Arg)–COO^-(Asp) average distance of 8.6 Å was obtained during the MC/SD calculations. A representative energy minimized conformation featuring the 11-membered ring hydrogen bond between Gly-NH and C(5)=O is shown in Figure 3 (structure on the right).

NMR spectroscopy experiments performed on the *iso*DGR peptidomimetic **2** suggest a different conformational preference. In fact, compound **2** exists in two preferred conformations (Figure 4a and b). The NOESY cross-peak between DKP-NH10 and Arg-NH (strong) is indicative of a β-turn conformation stabilized by a hydrogen bond between DKP-NH10 and *iso*Asp-C=O (Figure 4a), whereas the NOESY cross-peak between Gly-NH and *iso*Asp-NH (weak) is indicative of a pseudo-β-turn motif (Figure 4b) similar to that observed for compound **1**.

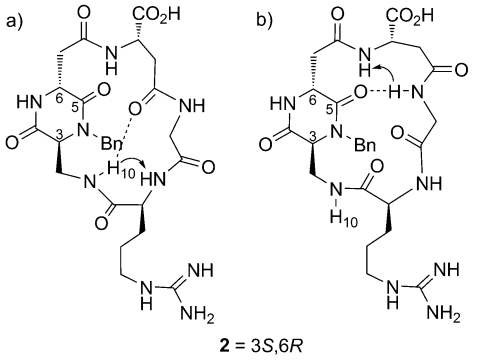


Figure 4. Preferred intramolecular hydrogen-bonded patterns proposed for the cyclic *iso*DGR peptidomimetic **2**, on the basis of spectroscopic data. The arrows indicate the NOE contacts. a) β-Turn at Gly-Arg; b) pseudo-β-turn at DKP-*iso*Asp.

Only the distance restraint corresponding to the strong NOE contact between DKP-NH10 and Arg-NH (Figure 4a) was applied in the MC/SD simulations of compound **2**. It is worth noting that approximately 80% of the conformations sampled during the simulations adopted an extended arrangement of the *iso*DGR sequence, characterized by a pseudo-β-turn at DKP-*iso*Asp and the formation of the corresponding hydrogen bond between Gly-NH and C(5)=O (Figure 4b). In addition, the formation of a distorted β-turn at Gly-Arg (Figure 4a), characterized by hydrogen bond distances of about 3.5 Å between DKP-NH10 and *iso*Asp-C=O, was observed for 10% of the simulations. A Cβ(Arg)–COO^-(Asp) average distance of 10.7 Å was obtained during the MC/SD calculations of compound **2**. Two representative energy-minimized conformations featuring the two hydrogen bond patterns compatible with the strong NOE contact between DKP-NH10 and Arg-NH are shown in Figure 5.

In order to rationalize, on a molecular basis, the affinity of compounds **1** and **2** 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 (PDB

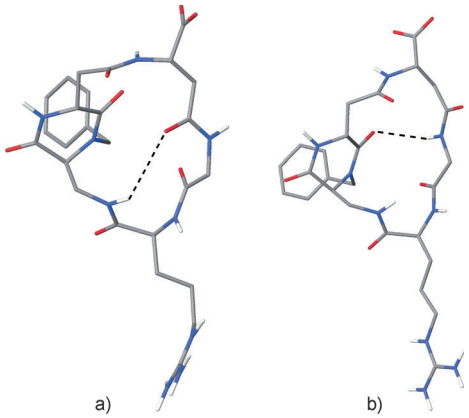


Figure 5. The 3D structures of compound **2** as obtained by restrained MC/SD simulations based on experimental distance information, after energy minimization. a) Distorted β-turn at Gly-Arg, populated for 10% of the simulation, Cβ(Arg)–COO^-(Asp) distance = 10.7 Å; b) pseudo-β-turn at DKP-*iso*Asp, populated for 80% of the simulation, Cβ(Arg)–COO^-(Asp) distance = 10.8 Å.

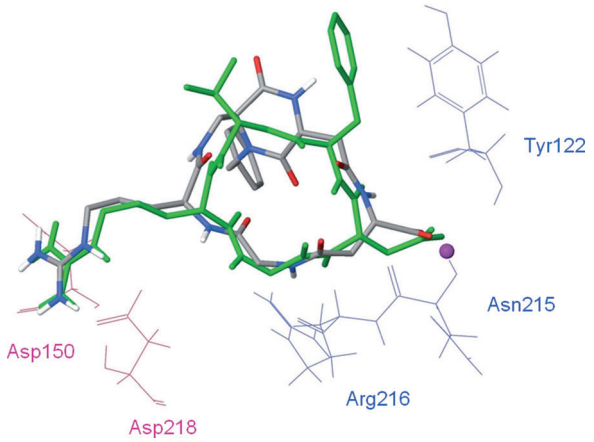


Figure 6. Best pose of compound **2** (atom color tube representation, pseudo-β-turn at DKP-*iso*Asp) into the crystal structure of the extracellular domain of $\alpha_v\beta_3$ integrin (α unit red and β unit blue wire representation) overlaid on the bound conformation of Cilengitide (green tube representation). Only selected integrin residues involved in the interactions with the ligand are shown. The metal ion in the MIDAS region is represented by a magenta sphere. Nonpolar hydrogen atoms were removed for clarity.

ID: 1L5G) was taken as a reference model for the interpretation of the docking results in terms of ligand–protein interactions.[14] 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 and interact with charged regions of the receptor binding site.

Docking calculations starting from the pseudo-β-turn DKP-*iso*Asp geometries of compound **1** (Figure 3, right) and from the distorted β-turn Gly-Arg conformations of compound **2** (Figure 5a), produced top-ranked poses conserving the key electrostatic interactions but lacking further stabilizing hydrogen bond interactions with the β subunit (e.g., hydrogen bonds of ligand carboxylate oxygen with the backbone amides of Asn215 and Tyr122, and hydrogen bonds between ligand backbone N–H and protein backbone carbonyl groups).

On the contrary, docking calculations starting from the pseudo-β-turn DKP-*iso*Asp geometries of compound **2** (Figure 5b) produced top-ranked poses conserving all the important interactions of the X-ray complex (Figure 6).

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

In conclusion, the cyclic *iso*DGR peptidomimetic **2** was identified as a low-nanomolar $\alpha_v\beta_3$ integrin ligand. The *iso*DGR sequence of compound **2** can fit into the RGD binding pocket of $\alpha_v\beta_3$ integrin, establishing the same electrostatic clamp as well as additional key interactions.

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Keywords: conformational analysis · diketopiperazines · molecular modeling · peptides · peptidomimetics

[1] a) R. O. Hynes, *Cell* **2002**, *110*, 673–687; b) Y. Takada, X. Ye, S. Simon, *Genome Biol.* **2007**, *8*, 215.1–215.9.
[2] C. J. Avraamides, B. Garmy-Susini, J. A. Varner, *Nat. Rev. Cancer* **2008**, *8*, 604–617.
[3] L. Auzzas, F. Zanardi, L. Battistini, P. Burreddu, P. Carta, G. Rassu, C. Curti, G. Casiraghi, *Curr. Med. Chem.* **2010**, *17*, 1255–1299.
[4] a) A. S. M. da Ressurreição, A. Vidu, M. Civera, L. Belvisi, D. Potenza, L. Manzoni, S. Ongeri, C. Gennari, U. Piarulli, *Chem. Eur. J.* **2009**, *15*, 12184–12188; b) M. Marchini, M. Mingozzi, R. Colombo, I. Guzzetti, L. Belvisi, F. Vasile, D. Potenza, U. Piarulli, D. Arosio, C. Gennari, *Chem. Eur. J.* **2012**, *18*, 6195–6207; c) R. Colombo, M. Mingozzi, L. Belvisi, D. Arosio, U. Piarulli, N. Carenini, P. Perego, N. Zaffaroni, M. De Cesare, V. Castiglioni, E. Scanziani, C. Gennari, *J. Med. Chem.* **2012**, *55*, 10460–10474.
[5] a) F. Curnis, R. Longhi, L. Crippa, A. Cattaneo, E. Dondossola, A. Bachi, A. Corti, *J. Biol. Chem.* **2006**, *281*, 36466–36476; b) A. Corti, F. Curnis, *J. Cell Sci.* **2011**, *124*, 515–522.
[6] S. Clarke, *Int. J. Peptide Protein Res.* **1987**, *30*, 808–821.
[7] F. Curnis, A. Sacchi, A. Gasparri, R. Longhi, A. Bachi, C. Doglioni, C. Bordignon, C. Traversari, G.-P. Rizzardi, A. Corti, *Cancer Res.* **2008**, *68*, 7073–7082.

[8] a) A. Spitaleri, S. Mari, F. Curnis, C. Traversari, R. Longhi, C. Bordignon, A. Corti, G.-P. Rizzardi, G. Musco, *J. Biol. Chem.* **2008**, *283*, 19757–19768; b) F. Curnis, A. Cattaneo, R. Longhi, A. Sacchi, A. M. Gasparri, F. Pastorino, P. Di Matteo, C. Traversari, A. Bachi, M. Ponzoni, G.-P. Rizzardi, A. Corti, *J. Biol. Chem.* **2010**, *285*, 9114–9123; c) M. Ghitti, A. Spitaleri, B. Valentinis, S. Mari, C. Asperti, C. Traversari, G.-P. Rizzardi, G. Musco, *Angew. Chem.* **2012**, *124*, 7822–7825; *Angew. Chem. Int. Ed.* **2012**, *51*, 7702–7705.

[9] a) A. Spitaleri, M. Ghitti, S. Mari, L. Alberici, C. Traversari, G.-P. Rizzardi, G. Musco, *Angew. Chem.* **2011**, *123*, 1872–1876; *Angew. Chem. Int. Ed.* **2011**, *50*, 1832–1836; b) A. O. Frank, E. Otto, C. Mas-Moruno, H. B. Schiller, L. Marinelli, S. Cosconati, A. Bochen, D. Vossmeyer, G. Zahn, R. Stragies, E. Novellino, H. Kessler, *Angew. Chem.* **2010**, *122*, 9465–9468; *Angew. Chem. Int. Ed.* **2010**, *49*, 9278–9281.

[10] The IC_{50} value of *c*[G*iso*DGRphg] can be compared with the IC_{50} values of **1** and **2** notwithstanding that the binding assays were realized by using two different protocols. In fact, 1) vitronectin is used as a competitive natural ligand in both assays, and 2) Cilengitide, that is, *c*[RGDf(*N*-Me)V], gives the same IC_{50} value for $\alpha_v\beta_3$ integrin with both assays (0.54 and 0.58 nM: see ref. [9b], and M. A. Dechantsreiter, E. Planker, B. Mathä, E. Lohof, G. Hölzemann, A. Jonczyk, S. L. Goodman, H. Kessler, *J. Med. Chem.* **1999**, *42*, 3033–3040, respectively).

[11] a) G. Müller, M. Gurrath, H. Kessler, *J. Comput.-Aided Mol. Des.* **1994**, *8*, 709–730; b) R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461–7472; c) R. Haubner, W. Schmitt, G. Hölzemann, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7881–7891; d) C. Mas-Moruno, J. G. Beck, L. Doedens, A. O. Frank, L. Marinelli, S. Cosconati, E. Novellino, H. Kessler, *Angew. Chem.* **2011**, *123*, 9668–9672; *Angew. Chem. Int. Ed.* **2011**, *50*, 9496–9500.

[12] F. Guarnieri, W. C. Still, *J. Comput. Chem.* **1994**, *15*, 1302–1310.

[13] W. C. Still, A. Tempczyk, R. C. Hawley, T. Hendrickson, *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.

[14] J.-P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* **2002**, *296*, 151–155.

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