New Potent $\alpha_v\beta_3$ Integrin Ligands Based on Azabicycloalkane (γ,α)-Dipeptide Mimics

M. Pilkington-Miksa,[a] E.M.V. Araldi,[a] D. Arosio,[b] L. Belvisi,[c] M. Civera,[c] and L. Manzoni[b,*]

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Abstract We have designed a new synthetic strategy for the preparation of a new class of cyclic RGD integrin ligands in which the azabicycloalkane scaffold can be envisaged as a (γ,α) dipeptide mimic. The synthesis and in vitro biological evaluation of these RGD derivatives, as well as the computational study of their conformational properties and binding modes to $\alpha_v\beta_3$ integrin are described. Compound **3** has shown to be a promising candidate as $\alpha_v\beta_3$ integrin antagonist able to interfere with both cell adhesion and movement on vitronectin with no evidence of cytotoxic effects.

Introduction

The integrins, a large family of transmembrane heterodimeric receptors, have been identified as key regulators of different cellular processes related to tumour growth, such as cell adhesion and migration, cell proliferation and neo-angiogenesis, therefore their key functional role is well established in different physiological and pathological contexts.[1] The observation that $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_5\beta_1$ integrin subtypes are essential for tumour angiogenesis and can be successfully inhibited by small-molecule ligands has turned them into the focus of cancer research. The discovery of the structural basis of the recognition between integrins and their natural ligands together with crystallographic, electron microscopy, and computational analyses on selected integrin subfamilies provided a turning point for the rational design of a wide variety of integrin inhibitors.[2] The contribution of our group to the design of integrin ligands started from the discovery of RGD mimics in which the recognition motif is conformationally constrained by an 1-aza-2-oxobicyclo[X.3.0]alkane scaffold, resulting in potent and selective $\alpha_v\beta_3$ ligands.[3] Thereafter, we further progressed in structural modifications of the azabicycloalkane scaffold[4] providing novel related ligands with functionalized side chains, suitable for conjugation with imaging probes[5] and with therapeutic agents.[6]

In our work directed toward the synthesis of cyclic RGD containing functionalized azabicycloalkane we observed that deprotection reaction of the amino acid side chains of compound **1a** (Figure 1) led to the desired compound **1b** only in moderate yield (30%). The unexpected result was due to the formation of several by-products, the most relevant of which appeared to be compound **2**, obtained from a shift of the aspartic acid residue from the amine to the hydroxyl group of the side chain.[4b] Wondering to know if the structural change in compound **2** could have some effect on the activity toward the integrin receptors, the cyclic pentapeptide was examined *in vitro* for its ability to compete with biotinylated vitronectin for binding to the purified $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors, resulting in even stronger binder for $\alpha_v\beta_3$ (IC_{50} 2.3 ± 1.0 nM) in comparison to the parent compound **1b** ((IC_{50} 88 ± 7.3 nM). This good result forced us to design a new synthetic strategy for the preparation of this new class of cyclic RGD integrin inhibitors in which the azabicycloalkane scaffold can be envisaged as a (γ,α) dipeptide mimic.

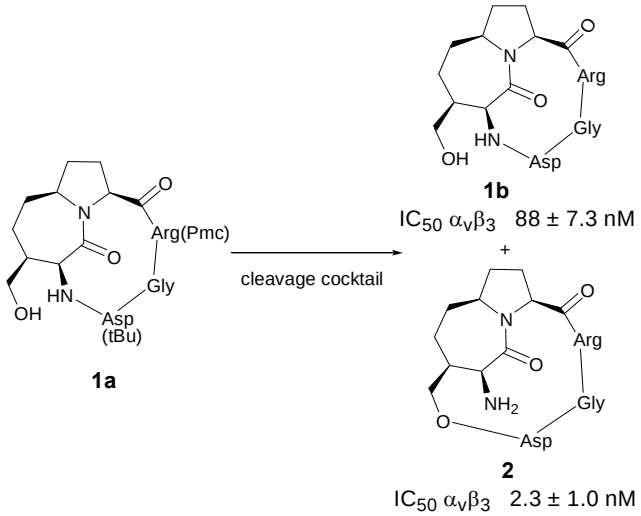


Figure 1. Nitrogen to oxygen shift.

Results and discussion

Chemistry

The synthesis of compounds **2-5** (Figure 2) required as common starting material compound **6** (Scheme 1), obtained through a known synthetic procedure.[4a] The synthetic plan for the preparation of the RGD pseudopeptides envisaged the synthesis of the linear peptides in solution employing the benzyloxycarbonyl (Cbz) protection strategy, followed by intramolecular cyclization and side chain deprotection. To minimize steric hindrance at the cyclization site and to avoid possible C-terminal epimerization, cyclization between the Gly and the Asp residues was considered. Arg and Asp were protected to the side chain with 4-methoxy-2,3,6-trimethyl-benzenesulfonyl (Mtr) and *tert*-butyl ester, respectively, which

can be removed by acidic conditions, and are therefore compatible with the Cbz strategy.

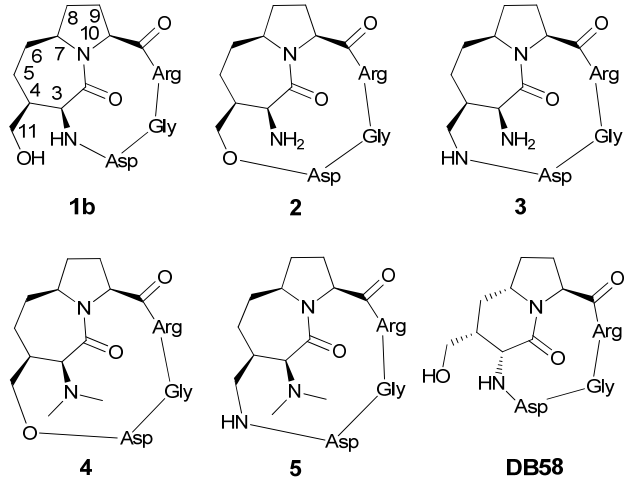
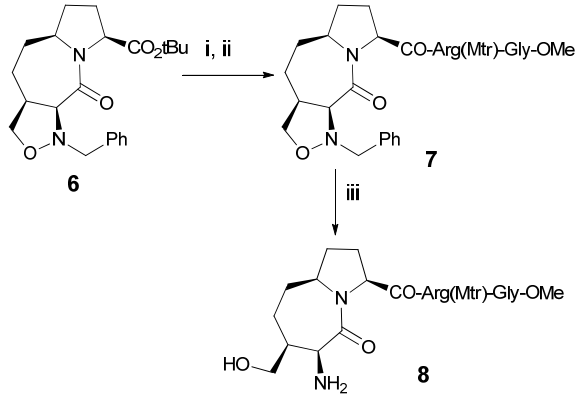


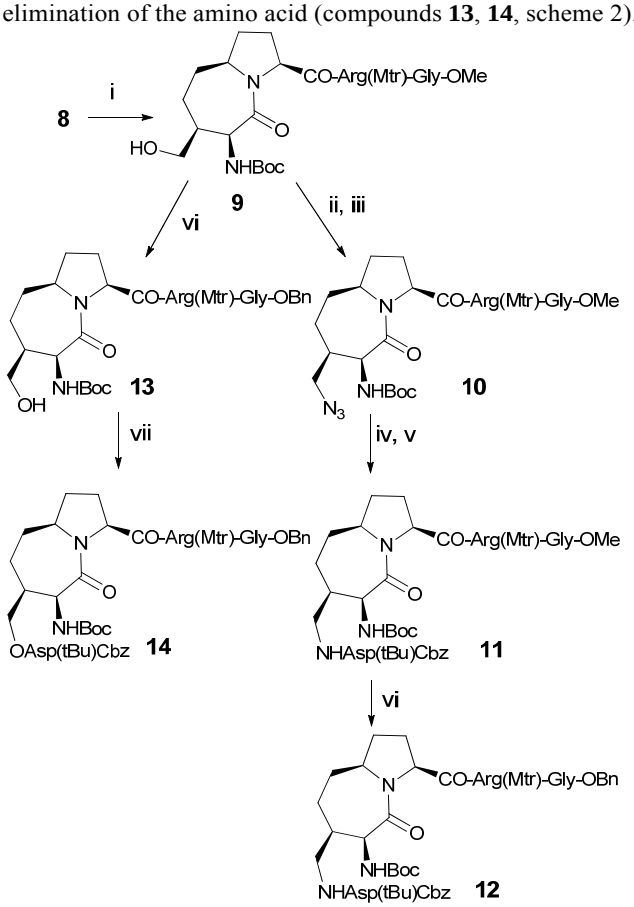
Figure 2. Cyclic RGD containing functionalized azabicycloalkane (γ,α)-dipeptide mimics and **DB58**.

The first step in the synthesis consisted in the cleavage of the *tert*-butyl ester of the bicyclic lactam **6** by treatment with trifluoroacetic acid (TFA) in dichloromethane in the presence of Et_3SiH, to give the corresponding carboxylic acid in quantitative yield (Scheme 1). This compound was used in the subsequent coupling step without further purification. The dipeptide H-Arg(Mtr)-Gly-OMe was coupled to the free carboxyl group of bicyclic lactam **6** using standard coupling reagents (HOBt, HBTU, DIPEA) to furnish the desired product **7** in 91% yield (Scheme 1). Hydrogenation of compound **7** on Pd/C cartridge by a continuous-flow hydrogenation reactor, afforded the conversion of the N-benzyloxazolidine function into the appropriate amino alcohol **8**, which is the common intermediate for the synthesis of all desired compounds.



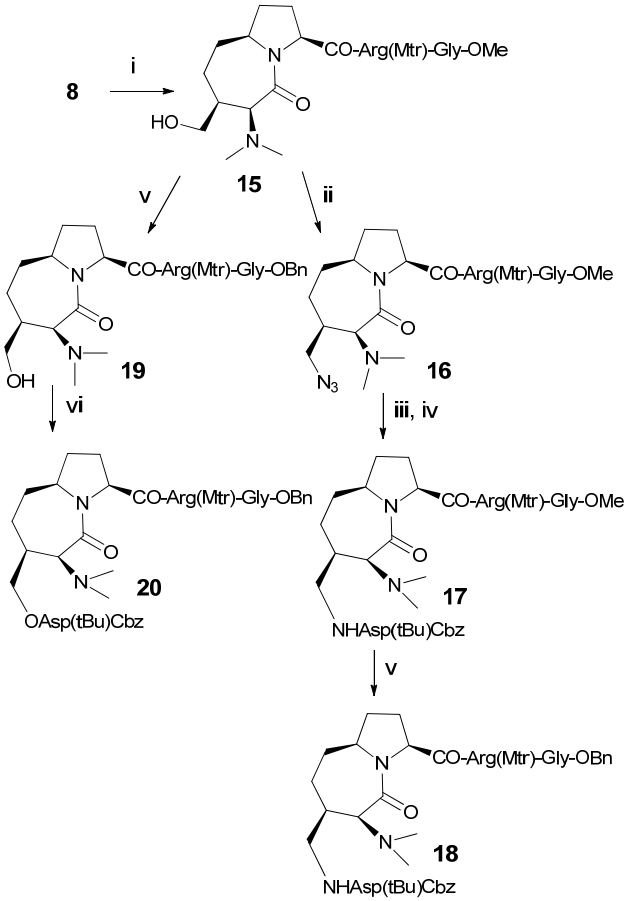
Scheme 1. i) TFA, DCM, Et_3SiH; ii) H-Arg(Mtr)GlyOMe, HOBt, HBTU, DIPEA, DCM, 91% over two steps; iii) H_2, Pd/C, EtOH:H_2O 9:1, 0.7 ml/min, 80 °C, 9.87 atm.

Compound **8** was protected to the nitrogen as *tert*-butoxycarbonyl derivative to give intermediate **9** which can be used for the preparation of the final compounds **2** and **3** (Scheme 2). To synthesize the amide derivative **12,** the hydroxy group of compound **9** was transformed into azide via mesylate displacement. The azido compound **10** was subjected to standard hydrogenation on Pd/C affording the corresponding amine, which was condensed with Z-Asp(tBu)OH to give the desired amide **11** using the coupling methodology reported above. An acid-catalyzed transesterification mediated by $Ti(OiPr)_4$ in the presence of benzyl alcohol excess was performed to change the protecting group of the glycine in order to avoid base catalyzed methyl ester hydrolysis, that, in the case of similar compounds, resulted in low yieds.[4b] In this way, the resulting compound **12** has two simultaneously removable protecting groups. In the case of the ester series, the $Ti(OiPr)_4$ mediated transesterification was performed before the coupling with Z-Asp(tBu)OH in order to avoid the transesterification of the aspartic acid with consequent elimination of the amino acid (compounds **13**, **14**, scheme 2).



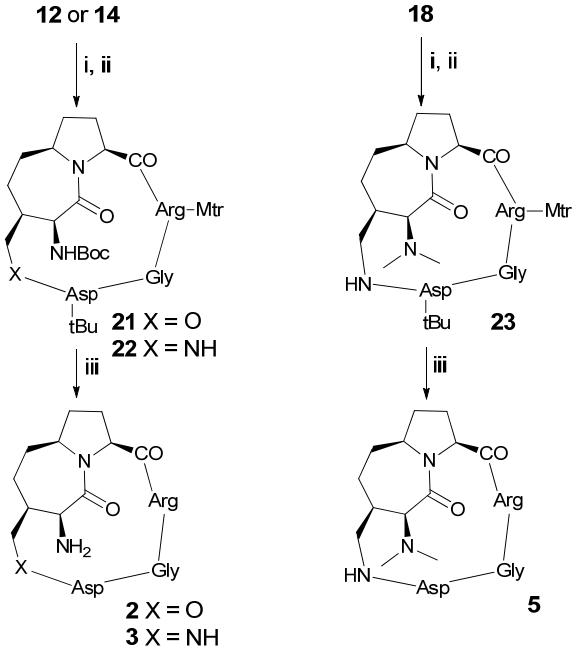
Scheme 2. i) Boc_2O, TEA, DCM, 85% over two steps; ii) methanesulfonyl chloride, TEA, DCM; iii), NaN_3, DMF, 80 °C, 89%; iv) H_2, Pd/C, MeOH; v) Z-Asp(tBu)OH, HOBt, HBTU, DIPEA, DMF, 87% over two steps; vi) benzyl alcohol, $Ti(iPrO)_4$, 80 °C, 83% for **12**, 91% for **13**; vii) Z-Asp(tBu)OH, DIC, DMAP, DCM, 91%.

For the synthesis of compounds **4** and **5**, compound **8** was submitted to a reductive amination by treatment with formaldehyde followed by hydrogenation to give the common intermediate **15** (Scheme 3). The transformation of the hydroxyl group into azide was performed by treatment with diphenylphosphoryl azide (DPPA). In this case, the well established mesylate displacement by NaN_3 didn't give the desired azide derivative but a dehydrated product. Compound **16** was subjected to standard hydrogenation on Pd/C to afford the corresponding amine, which was condensed with Z-Asp(tBu)OH to give, after transesterification, the desired amide **18**. The synthesis of the ester derivative **20** was performed by a similar synthetic approach.



Scheme 3. i) CH_2O, EtOH, H_2, Pd/C, 92%; ii) DPPA, THF, 0 °C, 99%; iii) H_2, Pd/C, MeOH; iv) Z-Asp(tBu)OH, HOBt, HBTU, DIPEA, DMF, 46% over two steps; v) benzyl alcohol, Ti(*i*PrO)$_4$, 75 °C, 60% for compound **18**, 69% for compound **19**; vi) Z-Asp(tBu)OH, HOBt, HBTU, DIPEA, DMF, 79%.

With the linear precursors **12**, **14**, **18**, and **20** in hands, the following steps were directed to obtain the cyclic compounds (Scheme 4). Hydrogenation on Pd/C was performed to remove the protecting groups of Gly and Asp amino acids. Surprisingly, hydrogenation of compound **20** led to the elimination of the aspartic acid giving, as the main product, compound **19**. The hydrogenation conducted in acidic conditions, to avoid the eventual participation of the neighbor dimethylamino group in the elimination reaction, gave the same result.
Cyclization of the three obtained deprotected intermediate using HATU and HOAt as condensing agents, afforded the protected cyclic pseudopentapeptides **21**-**23** in 67–77% yield over two steps. Finally, the side chain protecting groups were removed by TFA in the presence of cation scavengers giving compounds **2**, **3** and **5** in good yields (89%) after HPLC purification.



Scheme 4. i) H_2, Pd/C, MeOH; ii) HATU, HOAt, DMF, 77% for **21**, 73% for **22**, 67% for **23**, over two steps; iii) cleavage cocktail (TFA:phenol:1,2-ethandithiol:thioanisole:triisopropylsilane 80:5:5:5:5), 80% for **2**, 89% for **3**, 89% for **5**.

Biological evaluation

Solid phase receptor binding assay.
The synthesized cyclic pentapeptides **2, 3** and **5** were examined *in vitro* for their ability to compete with the binding of soluble biotinylated vitronectin to the immobilized $\alpha_v\beta_3$ and $\alpha_v\beta_5$ receptors (Table 1). The affinity of the new compounds for isolated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was compared with that of the commercially available compound *cyclo*-(RGDfV), and that of our reference compound **DB58**,[4b] whose affinity for the $\alpha_v\beta_3$ integrin was in the nanomolar range, as previously determined.

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

Compound	**IC_{50}(nM)±SD**	**IC_{50}(nM)±SD**
	$\alpha_v\beta_3$	**$\alpha_v\beta_5$**
c(RGDfV)	3.2 ± 1.3[b]	7.5 ± 4.8[b]
DB58	53.7 ± 17.3[b]	205 ± 33.5[b]
1b	88 ± 7.3[b]	929 ± 149[b]
2	2.3 ± 1.0	612 ± 335
3	2.2 ± 0.2	538 ± 219
5	11.6 ± 0.4	259 ± 98

[a]IC_{50} values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding as estimated by Prism GraphPad program. All values are the mean (± standard deviation) of triplicate determinations.
[b]ref. 4b.

Table 2. Effect of compounds **3** on HUVEC, T98G, and MDA-MB-231 cell adhesion to fibronectin, or vitronectin.[a]

	HUVEC $IC_{50}(\mu M)\pm SD$		T98G $IC_{50}(\mu M)\pm SD$		MDA-MB-231 $IC_{50}(\mu M)\pm SD$	
	VN	FN	VN	FN	VN	FN
c(RGDfV)			0.14 ± 0.03	0.12 ± 0.03	11.3± 0.1	3.4± 0.4
DB58	29.5 ± 1.1	26.2 ± 1.4[c]	3.2 ± 0.7	1.5 ± 0.3	21.8±5.4	9.4±2.6[c]
3	17.8 ± 0.4	18.3 ± 4.3	1.5 ± 0.6	1.0 ± 0.4[c]	31.0± 0.9	5.9± 4.1

[a] Each data point was performed in triplicate in two independent experiments.
VN= Cell adhesion on vitronectin
FN= Cell adhesion on fibronectin
HUVEC = Human Umbilical Vein Endothelial Cell
T98G = human glioblastoma tumor
MDA-MB-231 = human breast cancer cell line

All the new cyclic pseudopeptides tested showed a higher affinity toward integrin $\alpha_v\beta_3$ in comparison with the lead compound **1b**, with IC_{50} values between 2.2 and 11.6 nM similar to that of cRGDfV. Still low is the binding for integrin $\alpha_v\beta_5$ (IC_{50} values between 259 and 612 nM), evidencing a selectivity of the synthesized compounds towards the integrin $\alpha_v\beta_3$.

Cell adhesion assay.
Among the synthesized compounds, we selected compound **3** to assess the *in vitro* activity as integrin antagonist. The high affinity and its intrinsic stability due to the presence of an amido instead of an ester linkage induced us to select this molecule as a suitable candidate for further developments.
Cells were allowed to adhere to immobilized fibronectin or vitronectin in the presence of increasing concentrations of the tested compounds.
As shown in Table 2, compound **3** significantly inhibited cell adhesion of all cell types to either fibronectin or vitronectin in the low micromolar range with a slightly better result in the case of T98G cells.
Fibronectin, vitronectin, and the related integrin receptors play crucial and specific roles during angiogenic events. In particular, the fibronectin–$\alpha_5\beta_1$ complex is mainly involved in developmental angiogenesis, whereas vitronectin–$\alpha_v\beta_3$ and vitronectin–$\alpha_v\beta_5$ are related mainly to postnatal tumor angiogenesis. Therefore, to evaluate the suitability of compound **3** as integrin antagonist for tumor targeting, we performed wound assay experiments on HUVEC and T98G cells grown on immobilized vitronectin and fibronectin. Treatment with compound **3** at 10 μM significantly slows healing of the wounded area in the two cell types (Figures S1 and S2, SuppInfo). Taken together, these results indicate that compound **3** acts efficiently as an $\alpha_v\beta_3/\alpha_v\beta_5$ integrin antagonist by interfering with both cell adhesion and movement on vitronectin, with no evidence of cytotoxic activity.

Computational studies
To investigate the effects of the azabicycloalkane (γ,α)–dipeptide mimic on the ligand conformation and on the interaction with $\alpha_v\beta_3$ integrin, computational studies on ligand **3** were performed. As previously reported for the parent compound **1b**, Monte Carlo/energy minimization (MC/EM) conformational searches[7] of the cyclo-(Ala-Gly-Ala-lactam) pentapeptide analog followed by Monte Carlo/stochastic dynamics (MC/SD) simulations[8] of the cyclic RGD peptidomimetic ligand were run in water, as implicitly represented by the generalized Born/surface area (GB/SA) solvation model,[9] to detect the preferred backbone geometries. In line with previous work, the conformational constraints introduced by the cyclization and by the rigid scaffold forced the 17-membered cyclopeptide to adopt only few types of geometries, defined by specific β/γ turn arrangements. Notably, the low-energy conformers calculated for ligand **3** are very similar to the geometries previously detected for the 15-membered azabicycloalkane RGD cyclopeptides.[4b]
In particular, with respect to the parent compound **1b** (which was shown to preferentially adopt the extended RGD conformation denoted as type SIV geometry), owing to the larger cyclopeptide size, ligand **3** displayed more flexibility at the conformational equilibrium, by adopting three different backbone geometries (denoted as type SII, SIII and SIV geometries and shown in Figure 3). The SII and SIII structural types present a γ turn at Gly or an inverse γ turn at Asp, respectively, in combination with a distorted βII' turn at Gly-Asp, whereas the type SIV geometry is characterized by a βI turn at Pro-Arg (Pro residue of the lactam ring) and by an inverse γ turn at Asp (Figure 3).[10]

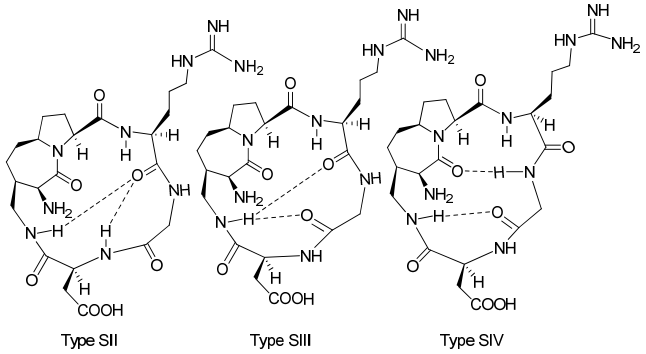


Figure 3. 2D representation of cyclopeptide geometries of compound **3** identified by conformational analysis.

As previously reported, a key parameter for the RGD fitting into the active site of the $\alpha_v\beta_3$ integrin is the distance of about 9 Å between the Cβ atoms of Asp and Arg, imparted by an extended conformation of the RGD sequence.[2f] Only the type III and IV geometries feature such an extended arrangement of the recognition motif (with Cβ (Arg)-Cβ(Asp) distance values of about 9 Å), that should be able to properly place the carboxylate and guanidinium groups in the receptor active site to effectively exert their function of electrostatic clamp.
To investigate this issue and to interpret, on a molecular basis, the affinity of compound **3** for the $\alpha_v\beta_3$ receptor, docking studies were performed by starting from the three preferred cyclopeptide backbone geometries obtained from molecular mechanics conformational analysis. The Glide

program V4.5[11] was employed for docking calculations (see Experimental Section for computational details) and the crystal structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with the cyclic pentapeptide Cilengitide (PDB code 1L5G)[2f] was taken as a reference model for the interpretation of the results.

Docking runs starting from the type SIII geometry produced top-ranked poses conserving all the key interactions observed in the X-ray complex. In the best pose shown in Figure 4, the positively charged Arg guanidinium group of ligand **3** 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) of the β unit, and the second carboxylate oxygen forms hydrogen bonds with the backbone amides of Asn215 and Tyr122 in the β unit. Further stabilizing polar interactions involve residues Gln180 in the α unit and Arg216 in the β unit.

On the contrary, docking calculations starting from the type SII and SIV geometries provided binding poses poorly reproducing the crystallographic interactions. The results of the type SII geometry can be explained by considering the non-extended RGD arrangement that probably prevents the guanidine and carboxyl groups from achieving the optimal separation for binding to the $\alpha_v\beta_3$ integrin. As previously suggested for the parent compound **1b**, although the type SIV geometry ensures the suitable extended RGD arrangement, it is likely that it cannot properly fit into the active site due to some unfavorable contacts of the 7,5-fused bicyclic scaffold.

In summary, with respect to the parent compound **1b**, ligand **3** revealed higher conformational flexibility, adopting also the extended RGD type III geometry. Especially this conformation was shown by docking studies to fit unhindered into the receptor, thus offering a satisfactory rationalization of the improved affinity of **3** toward the $\alpha_v\beta_3$ integrin.

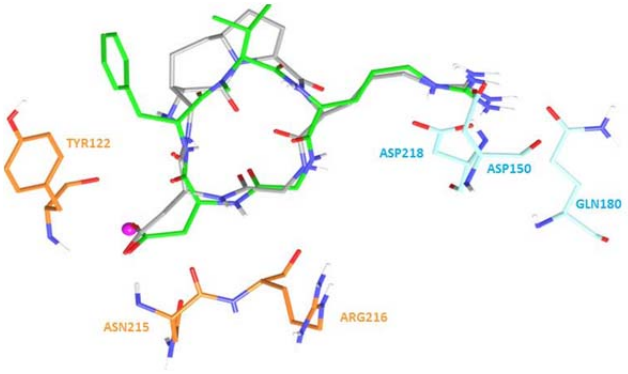


Figure 4. Docking best pose of compound **3** (type SIII conformation, grey carbon atoms) in the crystal structure of the extracellular domain of $\alpha_v\beta_3$ integrin overlaid on the bound conformation of Cilengitide (green carbon atoms). Selected integrin residues involved in the interactions with the ligand are shown (α unit cyan carbon atoms, β unit orange carbon atoms). The metal ion at MIDAS is shown as a magenta CPK sphere. Nonpolar hydrogen atoms are removed for clarity.

Conclusions

In this work, three novel cyclic RGD ligands have been synthesized, by exploiting a functionalized 7,5-fused azabicycloakane scaffold as a (γ,α)–dipeptide mimic. The RGD peptidomimetics have been evaluated in competitive binding assays for their ability to inhibit biotinylated vitronectin binding to the purified integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which are involved in tumor angiogenesis. All the texted compounds have been shown to be selective toward the integrin $\alpha_v\beta_3$ in this kind of assay, displaying low nanomolar IC_{50} values for the inhibition of vitronectin binding to this receptor. Among the three ligands, compound **3** was selected for further in vitro biological evaluation and for computational investigation. According to cell adhesion and wound assay experiments, compound **3** can be considered a promising candidate as integrin antagonist able to interfere with both cell adhesion and movement on vitronectin with no evidence of cytotoxic effects. In the conformational study by computational methods, the 17-membered cyclopeptide **3** revealed higher conformational flexibility compared to the corresponding 15-membered azabicycloalkane RGD cyclopeptide **1b**. In particular, a well-defined conformation of **3** featuring intramolecular hydrogen-bonded turn motifs and an extended arrangement of the RGD sequence was shown by docking studies to produce binding poses in $\alpha_v\beta_3$ very similar to the binding mode of the cyclic pentapeptide Cilengitide to the $\alpha_v\beta_3$ integrin in the X-ray complex.

Experimental Section

Chemistry

General. All chemicals and solvents were of reagent grade and were used without further purification. Solvents were dried by standard procedures and reactions requiring anhydrous conditions were performed under nitrogen or argon atmospheres. ^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker AVANCE-400 or a Bruker AVANCE-600 MHz spectrometer. Chemical shifts δ are expressed in ppm relative to internal Me_4Si as standard. For the assignment the numbering of the bicyclic scaffolds follow that reported in figure 2. Mass spectra were obtained with an ESI apparatus Bruker Esquire 3000 plus. Thin-layer chromatography (TLC) was carried out with precoated Merck F_{254} silica-gel plates. Flash chromatography was carried out with Macherey-Nagel silica gel 60 (230-400 mesh) or using SP1 Biotage flash purification system (with silica or C18 cartridges). Semi-preparative HPLC was carried out on a Waters Atlantis Prep T3 OBD 5μm 19x150 mm; eluents: A H_2O + 0.1% TFA, B MeCN + 0.1% TFA; gradient from 0% B to 30% B over 15 minutes, flow 15 mL/min. ^{1}H-, ^{13}C-NMR and MS analysis confirmed the purity and identity of all synthesized compounds.

Synthesis of methyl 2-(2-((3aS,5aS,8S,10aS)-1-benzyl-10-oxodecahydro-1H-isoxazolo[4,3-e]pyrrolo[1,2-a]azepine-8-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (7). Template **6** (1.1 g, 2.845 mmol) was dissolved in trifluoroacetic acid/DCM 3:1 (20 mL) and Et_3SiH (1 mL) was added. The resulting solution was left to stir at room temperature for 3 hours. After 3 hours the reaction was concentrated under reduced pressure. To the remaining residue was added toluene, which was then removed under reduced pressure once more. This was repeated once and the remaining residue was then dried under vacuum. A viscous yellow residue was finally obtained that was not purified further.

To the crude (1.1 g, 2.845 mmol) in dry DCM (12 mL) were added HOBt (0.442 g, 3.273 mmol) and HBTU (1.24 g, 3.273 mmol) followed by diisopropylethylamine (1.49 mL, 8.538 mmol). To this solution under argon at room

temperature was added the dipeptide H-Arg(Mtr)GlyOMe (1.8 g, 3.042 mmol). The resulting solution was left to stir for ca. 18 hours. After this time the organic solution was washed with 5% citric acid solution, then with saturated $NaHCO_3$ and finally with brine. The organic phase, dried on anhydrous Na_2SO_4, was evaporated under reduced pressure. The remaining residue was purified by flash chromatography with Isolera flash purification system by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10). Y: 91%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.52-1.59 (m, 4H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-5), 1.81-1.96 (m, 3H, 2 *H*-6, *H*-8), 1.97-2.10 (m, 2H, *H*-β-Arg, 2 *H*-9), 2.11-2.29 (m, 4H, *H*-5, C*H*$_3$ Mtr), 2.25 (m, 1H, *H*-8), 2.57 (m, 1H, *H*-4), 2.63 (s, 3H C*H*$_3$ Mtr), 2.64 (s, 3H C*H*$_3$ Mtr), 3.20 (m, 1H, *H*-δ-Arg), 3.27 (m, 1H, *H*-δ-Arg), 3.51-3.63 (m, 2H, *H*-11, *H*-3), 3.68 (s, 3H OC*H*$_3$),), 3.77 (d, 1H, J = 13.6 Hz, *H*CHPh), 3.86 (s, 3H OC*H*$_3$), 3.90-4.06 (m, 3H, 2 *H*-α-Gly, *H*-11), 4.09 (m, 1H, *H*-7), 4.24 (d, 1H, J = 13.6 Hz, *H*CHPh), 4.63 (m, 1H, *H*-α-Arg), 4.68 (m, 1H, *H*-10), 6.38 (m, 1H, N*H* guanidine), 6.67 (s, 1H, *aromatic proton* Mtr), 6.71 (m, 2H N*H* guanidine), 7.20-7.31 (m, 3H, *aromatic protons* Ph), 7.31-7.40 (m, 2H, *aromatic protons* Ph), 7.51 (m, 1H, N*H* Arg), 7.78 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.4, 171.1, 171.0, 169.8, 157.8, 156.7, 156.3, 138.2, 137.4, 136.0, 135.5, 129.2, 127.9, 127.1, 123.8, 111.6, 72.8, 71.2, 61.8, 61.6, 59.1, 55.0, 51.8, 51.3, 45.4, 40.6, 39.8, 33.2, 33.0, 31.0, 27.1, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 770.5 [M+H]$^+$, calcd for $C_{37}H_{51}N_7O_9S$: 769.35 [M].

Synthesis of methyl 2-(2-((3S,6S,7S,9aS)-6-amino-7-(hydroxymethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (8). Compound **7** (1.989 g, 2.583 mmol) dissolved in ethanol/water 9:1 (250 mL) was passed through a palladium on carbon cartridge in the H-Cube apparatus at a rate of 0.7 mL/min. The temperature of the cartridge chamber was set to 80 °C and the pressure was set to 9.87 atm. The reaction was monitored by LC-MS. Once complete, the recovered solution was concentrated *in vacuo* to give a white solid that was used in the following step without any further purification.

Synthesis of methyl 2-(2-((3S,6S,7S,9aS)-6-((tert-butoxycarbonyl)amino)-7-(hydroxymethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (9). To a solution of **8** (0.800 g, 1.173 mmol) in dry DCM (40 mL) and TEA (490 µL, 3.520 mmol) at 0 °C and under nitrogen atmosphere, Boc_2O (540 µL, 2.346 mmol) was added dropwise. The reaction mixture was stirred for 6 h. After this time, the organic phase was washed with saturated NH_4Cl, dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified by flash chromatography with Isolera flash purification system by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10) yielding 0.782 g of derided product. Y: 85%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.29 (s, 9H, C(C*H*$_3$)$_3$), 1.40-1.59 (m, 5H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-6, *H*-4), 1.60-1.70 (m, 2H, *H*-6, *H*-5), 1.71-1.80 (m, 2H, *H*-β-Arg, *H*-8), 1.82-1.97 (m, 3H, 2 *H*-9, *H*-5), 2.00 (s, 3H C*H*$_3$ Mtr), 2.11 (m, 1H, *H*-8), 2.49 (s, 3H C*H*$_3$ Mtr), 2.54 (s, 3H C*H*$_3$ Mtr), 3.09 (m, 2H, 2 *H*-δ-Arg), 3.35 (m, 1H, *H*-11), 3.41 (m, 1H, *H*-11), 3.54 (s, 3H OC*H*$_3$), 3.71 (s, 3H OC*H*$_3$) 3.75-3.92 (m, 3H, 2 *H*-α-Gly, *H*-7), 4.14 (t, 1H, J = 9.0 Hz, *H*-3), 4.42 (m, 1H, *H*-α-Arg), 4.48 (m, 1H, *H*-10), 6.23 (m, 1H, N*H*Boc), 6.48 (m, 3H N*H* guanidine), 6.55 (s, 1H, *aromatic proton* Mtr), 7.53 (m, 1H, N*H* Arg), 7.65 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.7, 171.5, 171.4, 170.0, 158.4, 156.7, 156.3, 138.3, 136.2, 135.0, 123.9, 111.6, 78.9, 63.9, 61.7, 58.5, 55.5, 55.0, 52.2, 51.4, 41.4, 40.6, 40.5, 33.1, 32.9, 30.6, 29.6, 27.7, 27.2, 25.1, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 782.5 [M+H]$^+$, calcd for $C_{35}H_{55}N_7O_{11}S$: 781.37 [M].

Synthesis of methyl 2-(2-((3S,6S,7R,9aS)-7-(azidomethyl)-6-((tert-butoxycarbonyl)amino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (10). To a solution of compound **9** (0.300 g, 0.384 mmol) in dry CH_2Cl_2 (4 mL), under N_2 and at r.t., methanesulfonyl chloride (89 µL, 1.15 mmol) and TEA (267 µL, 1.92 mmol) were added. The solution was stirred for ca 1 h, then a saturated solution of NH_4Cl was added and the intermediate was extracted with CH_2Cl_2. The organic phase, dried with Na_2SO_4, was evaporated under reduced pressure. The crude was dissolved in dry DMF (4 mL) and, under N_2 and at r.t., NaN_3 (0.250 g, 3.84 mmol) was added. The reaction was stirred at 80 °C for ca. 18 h. After reaction completion, the resulting suspension was filtered through a pad of celite and washed with CH_2Cl_2. and the solvent was evaporated under reduced pressure. The crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage using a C18 cartridge and eluting with H_2O/CH_3CN (gradient from 90:10 to 0:100). Y: 89%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.43 (s, 9H, C(C*H*$_3$)$_3$), 1.57-1.65 (m, 2H, 2 *H*-γ-Arg), 1.66-1.75 (m, 3H *H*-β-Arg, *H*-6, *H*-5), 1.76-1.87 (m, 2H, *H*-8, *H*-4), 1.88-1.98 (m, 2H, *H*-β-Arg, *H*-6), 2.03-2.10 (m, 3H, 2 *H*-9, *H*-5), 2.13 (s, 3H C*H*$_3$ Mtr), 2.28 (m, 1H, *H*-8), 2.64 (s, 3H C*H*$_3$ Mtr), 2.70 (s, 3H C*H*$_3$ Mtr), 3.24 (m, 2H, 2 *H*-δ-Arg), 3.47 (m, 1H, *H*-11), 3.60 (m, 1H, *H*-11), 3.69 (s, 3H OC*H*$_3$), 3.86 (s, 3H OC*H*$_3$) 3.90-4.10 (m, 3H, 2 *H*-α-Gly, *H*-7), 4.33 (t, 1H, J = 9.0 Hz, *H*-3), 4.57 (m, 1H, *H*-α-Arg), 4.62 (m, 1H, *H*-10), 6.25 (m, 1H, N*H*Boc), 6.60 (m, 3H N*H* guanidine), 6.70 (s, 1H, *aromatic proton* Mtr), 7.65 (m, 1H, N*H* Arg), 7.77 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.6, 171.1, 170.0, 158.0, 156.7, 155.7, 138.3, 136.2, 135.1, 123.9, 111.6, 78.6, 61.7, 58.4, 55.3, 55.0, 54.1, 52.1, 51.3, 40.6, 40.5, 38.6, 32.9, 32.6, 31.1, 27.7, 27.2, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 807.5 [M+H]$^+$, calcd for $C_{35}H_{54}N_{10}O_{10}S$: 806.37 [M].

Synthesis of *tert*-butyl 3-(((benzyloxy)carbonyl)amino)-4-((((3S,6S,7R,9aS)-6-((tert-butoxycarbonyl)amino)-3-((5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-((2-methoxy-2-oxoethyl)amino)-1-oxopentan-2-yl)carbamoyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl)amino)-4-oxobutanoate, (11). To a solution of compound **10** (0.260 g, 0.322 mmol) in MeOH (12 mL) Pd-C 10% (25 mg, 10% w/w) was added. The suspension was stirred under hydrogen atmosphere for ca. 6 h. After this time the catalyst was filtered through a pad of celite and washed with MeOH. The collected organic phase was evaporated under reduced pressure to yield the desired compound as white foam that was used without any further purification. This solid (0.322 mmol, 1 mol eq.) was then dissolved in dry dimethylformamide (6 mL) and added to a solution of Z-Asp(tBu)OH (0.146 g, 0.45 mmol), HOBt (0.061 mg, 0.45 mmol), HBTU (0.171 g, 0.45 mmol) and diisopropylethylamine (168 µL, 0.966 mmol) in dimethylformamide (6 mL). The resulting solution was left to stir, under N_2 for 18 hours After this time the reaction was concentrated under reduced pressure and the crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage using a C18 cartridge and eluting with H_2O/CH_3CN (gradient from 90:10 to

0:100). Y: 87%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.42 (s, 9H, C(CH$_3$)$_3$), 1.45 (s, 9H, C(CH$_3$)$_3$), 1.50 (m, 1H, *H*-6), 1.53-1.74 (m, 5H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-4, *H*-5), 1.75-1.87 (m, 2H, *H*-8, *H*-6), 1.88-2.03 (m, 3H, *H*-β-Arg, *H*-5, *H*-9), 2.10-2.19 (m, 4H, *H*-9, C*H*$_3$ Mtr), 2.24 (m, 1H, *H*-8), 2.60-2.73 (m, 7H, 2 C*H*$_3$ Mtr, *H*-β-Asp), 2.83 (m, 1H *H*-β-Asp), 3.10-3.30 (m, 3H, 2 *H*-δ-Arg, *H*-11), 3.60 (m, 1H, *H*-11), 3.69 (s, 3H OC*H*$_3$), 3.82-3.89 (m, 4H OC*H*$_3$, *H*-7) 3.97 (m, 2H, 2 *H*-α-Gly,), 4.23 (m, 1H, *H*-3), 4.46-4.58 (m, 2H, *H*-α-Arg, *H*-α-Asp), 4.62 (m, 1H, *H*-10), 5.13 (m, 2H, C*H*$_2$Cbz), 6.25 (m, 1H, N*H*Boc), 6.62 (m, 3H N*H* guanidine), 6.70 (s, 1H, *aromatic proton* Mtr), 6.73 (m, 1H, N*H* Asp), 7.28-7.43 (m, 5H, *aromatic protons* Cbz), 7.56-7.67 (m, 2H, N*H* Arg, N*H*CH$_2$), 7.73 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.6, 171.2, 170.9, 169.8, 158.0, 156.8, 156.2, 138.3, 136.1, 135.1, 128.3, 127.7, 111.6, 80.3, 79.0, 66.2, 61.5, 58.5, 55.6, 55.0, 52.1, 51.3, 41.3, 40.6, 40.5, 39.8, 37.3, 33.2, 33.0, 30.8, 29.6, 27.7, 27.3, 26.7, 25.2, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 1086.7 [M+H]$^+$, calcd for $C_{51}H_{75}N_9O_{15}S$: 1085.51 [M].

Synthesis of *tert*-butyl 4-((((3S,6S,7R,9aS)-3-((1-((2-(benzyloxy)-2-oxoethyl)amino)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-oxopentan-2-yl)carbamoyl)-6-((tert-butoxycarbonyl)amino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl)amino)-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate, (12). To a solution of compound **11** (0.303 g, 0.279 mmol) in benzyl alcohol (433 µL, 4.18 mmol), Ti(*i*PrO)$_4$ (33 µL, 0.112 mmol) was added. The reaction mixture was stirred at 80°C under vacuum (0.013 atm) for ca. 24 h. After reaction completion, the reaction mixture was diluted with DCM and purified by flash chromatography with Isolera flash purification system by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10). Y: 83%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.42 (s, 9H, C(CH$_3$)$_3$), 1.45 (s, 9H, C(CH$_3$)$_3$), 1.48 (m, 1H, *H*-6), 1.50-1.68 (m, 4H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-5), 1.70-1.83 (m, 3H, *H*-4, *H*-8, *H*-6), 1.84-2.03 (m, 3H, *H*-β-Arg, *H*-5, *H*-9), 2.08-2.15 (m, 4H, *H*-9, C*H*$_3$ Mtr), 2.22 (m, 1H, *H*-8), 2.60-2.73 (m, 7H, 2 C*H*$_3$ Mtr, *H*-β-Asp), 2.82 (m, 1H *H*-β-Asp), 3.10-3.26 (m, 3H, 2 *H*-δ-Arg, *H*-11), 3.60 (m, 1H, *H*-11), 3.82-3.94 (m, 4H OC*H*$_3$, *H*-7), 4.05 (m, 2H, 2 *H*-α-Gly), 4.24 (m, 1H, *H*-3), 4.44-4.57 (m, 2H, *H*-α-Arg, *H*-α-Asp), 4.62 (m, 1H, *H*-10), 5.05-5.22 (m, 4H, C*H*$_2$Cbz, C*H*$_2$Ph), 6.25 (m, 1H, N*H*Boc), 6.62 (m, 3H N*H* guanidine), 6.69 (s, 1H, *aromatic proton* Mtr), 6.74 (m, 1H, N*H* Asp), 7.27-7.45 (m, 10H, *aromatic protons* Cbz, Ph), 7.55-7.65 (m, 2H, N*H* Arg, N*H*CH$_2$), 7.79 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.7, 171.2, 170.9, 169.8, 169.5, 158.0, 156.6, 156.2, 139.1, 138.3, 137.2, 136.1, 135.1, 128.4, 128.3, 128.1, 127.7, 123.9, 111.6, 80.3, 79.0, 66.7, 66.2, 61.5, 58.5, 55.6, 55.0, 52.1, 41.3, 40.8, 40.7, 40.1, 39.8, 37.3, 33.2, 33.0, 30.8, 27.7, 27.3, 26.7, 25.2, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 1162.8 [M+H]$^+$, calcd for $C_{57}H_{79}N_9O_{15}S$: 1161.54 [M].

Synthesis of benzyl 2-(2-((3S,6S,7S,9aS)-6-((tert-butoxycarbonyl)amino)-7-(hydroxymethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (13). To a solution of compound **9** (0.100 g, 0.128 mmol) in benzyl alcohol (198 µL, 1.918 mmol), Ti(*i*PrO)$_4$ (15 µL, 0.05 mmol) was added. The reaction mixture was stirred at 80°C under vacuum (0.013 atm) for ca. 24 h. After reaction completion, the reaction mixture was diluted with DCM and purified by flash chromatography with Isolera flash purification system by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10). Y: 91%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.44 (s, 9H, C(CH$_3$)$_3$), 1.53-1.73 (m, 5H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-6, *H*-4), 1.75-1.85 (m, 2H, *H*-5, *H*-8), 1.86-1.94 (m, 2H, *H*-β-Arg, *H*-6), 1.95-2.10 (m, 3H, 2 *H*-9, *H*-5), 2.12 (s, 3H C*H*$_3$ Mtr), 2.26 (m, 1H, *H*-8), 2.64 (s, 3H C*H*$_3$ Mtr), 2.69 (s, 3H C*H*$_3$ Mtr), 2.84 (m, 1H, O*H*), 3.22 (m, 2H, 2 *H*-δ-Arg), 3.56 (m, 1H, *H*-11), 3.65 (m, 1H, *H*-11), 3.86 (s, 3H OC*H*$_3$), 3.96-4.13 (m, 3H, 2 *H*-α-Gly, *H*-7), 4.28 (t, 1H, J = 8.8 Hz, *H*-3), 4.54 (m, 1H, *H*-α-Arg), 4.57 (m, 1H, *H*-10), 5.17 (s, 2H, C*H*$_2$Bn), 6.35 (d, 1H, J = 7.2 Hz, N*H*Boc), 6.62 (m, 3H N*H* guanidine), 6.69 (s, 1H, *aromatic proton* Mtr), 7.31-7.44 (m, 5H, *aromatic protons* Bn), 7.62 (d, 1H, J = 7.2 Hz, N*H* Arg), 7.80 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.5, 171.1, 169.5, 158.1, 156.5, 156.3, 138.2, 136.1, 135.1, 128.4, 128.1, 123.9, 119.4, 111.6, 78.9, 66.7, 66.2, 63.8, 61.6, 58.5, 55.4, 55.0, 52.0, 41.4, 40.8, 39.9, 33.1, 33.0, 30.6, 27.7, 27.0, 25.0, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 858.6 [M+H]$^+$, calcd for $C_{41}H_{59}N_7O_{11}S$: 857,40 [M].

Synthesis of 1-(((3S,6S,7S,9aS)-3-((1-((2-(benzyloxy)-2-oxoethyl)amino)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-oxopentan-2-yl)carbamoyl)-6-((tert-butoxycarbonyl)amino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl) 4-tert-butyl 2-(((benzyloxy)carbonyl)amino)succinate, (14). To a solution of Z-Asp(tBu)OH (0.151 g, 0.466 mmol) in dry DCM (6 mL), under N$_2$ and at 0 °C, DIC (54 µL, 0.349 mmol) was added. After 15 min. this solution was added to compound **13** (0.100 g, 0.116 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred for 2 h. After reaction completion the organic phase was washed with NaHCO$_3$, dried over Na$_2$SO$_4$ and evaporated under reduced pressure. The crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10). Y: 91%. ^{1}H NMR (400 MHz, *Acetone-d_6*): δ 1.43 (s, 18H, 2 C(CH$_3$)$_3$), 1.53-1.67 (m, 4H, 2 *H*-γ-Arg, *H*-β-Arg, *H*-6), 1.68-1.81 (m, 2H, *H*-5, *H*-8), 1.82-1.97 (m, 3H, *H*-β-Arg, *H*-6, *H*-4), 1.98-2.10 (m, 3H, 2 *H*-9 *H*-5), 2.12 (s, 3H, C*H*$_3$ Mtr), 2.22 (m, 1H, *H*-8), 2.64 (s, 3H, C*H*$_3$ Mtr), 2.70 (s, 3H, C*H*$_3$ Mtr), 2.83 (m, 2H, 2 *H*-β-Asp), 3.22 (m, 2H, 2 *H*-δ-Arg), 3.85 (s, 3H OC*H*$_3$), 3.97 (m, 1H, *H*-7), 4.50 (m, 2H, 2 *H*-α-Gly), 4.16 (m, 1H, *H*-11), 4.27 (m, 1H, *H*-11), 4.40 (t, 1H, J = 9.6 Hz, *H*-3), 4.56 (m, 1H, *H*-10), 4.61 (m, 1H, *H*-α-Arg), 4.67 (m, 1H, *H*-α-Asp), 5.18-5.22 (m, 4H, C*H*$_2$Cbz, C*H*$_2$Bn), 6.25 (d, 1H, J = 8.8 Hz, N*H*Boc), 6.60 (m, 3H N*H* guanidine), 6.67 (m, 1H, N*H* Asp), 6.69 (s, 1H, *aromatic proton* Mtr), 7.29-7.44 (m, 10H, *aromatic protons* Cbz, Bn), 7.63 (d, 1H, J = 7.6 Hz, N*H* Arg), 7.80 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d_6*): δ 171.7, 171.0, 170.6, 169.5, 169.4, 158.0, 156.7, 155.7, 138.3, 136.2, 135.1, 128.4, 128.1, 127.8, 123.9, 111.6, 80.6, 78.7, 66.8, 66.2, 66.1, 61.7, 58.4, 55.6, 55.0, 54.7, 52.1, 51.1, 40.8, 38.4, 37.5, 32.9, 32.8, 30.6, 27.8, 27.3, 27.1, 25.1, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 1163.7 [M+H]$^+$, 1185.6 [M+Na]$^+$, calcd for $C_{57}H_{78}N_8O_{16}S$: 1162.53 [M].

Synthesis of methyl 2-(2-((3S,6S,7S,9aS)-6-(dimethylamino)-7-(hydroxymethyl)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (15). Compound **8** (0.824 g, 1.208 mmol) and a 1.232 M aqueous solution of formaldehyde (2.10 mL, 2.587 mmol) were dissolved in ethanol (125 mL) and passed through a palladium on carbon cartridge in the H-Cube apparatus at a rate of 2.0 mL/min. The temperature of the cartridge chamber was set to 50 °C and the pressure was set to 9.87 atm. After 24 h the temperature of the cartridge chamber

was set to 80 °C and the solution was passed through the palladium on carbon cartridge for other 24h. The reaction was monitored by LC-MS. Once complete, the recovered solution was concentrated under reduced pressure and the crude was purified by flash chromatography with Isolera Flash Purification System by Biotage eluting with DCM/MeOH both containing 2% Et_3N (gradient from 100:0 to 90:10). Y: 92%. ^{1}H NMR (400 MHz, *Acetone-d$_6$*): δ 1.52-1.70 (m, 3H, 2 *H*-γ-Arg, *H*-6), 1.71-1.88 (m, 4H, *H*-β-Arg, *H*-5, *H*-6, *H*-8), 1.90-2.08 (m, 4H, *H*-4, *H*-5, *H*-β-Arg, *H*-9), 2.09-2.13 (m, 3H, *H*-9, C*H*$_3$ Mtr), 2.18 (m, 1H, *H*-8), 2.55 (s, 6H, (C*H*$_3$)$_2$N), 2.61 (s, 3H C*H*$_3$ Mtr), 2.68 (s, 3H C*H*$_3$ Mtr), 3.22-3.36 (m, 3H, 2 *H*-δ-Arg, *H*-3), 3.45 (m, 1H, *H*-11), 3.71 (s, 3H OC*H*$_3$), 3.76 (m, 1H, *H*-7), 3.83 (m, 3H OC*H*$_3$, *H*-11), 3.93 (dd, 1H, J = 5.2 Hz 2, J = 18.0 Hz, *H*-α-Gly), 4.06 (dd, 1H, J = 5.8 Hz 2, J = 17.8 Hz, *H*-α-Gly), 4.49 (m, 1H, *H*-α-Arg), 4.55 (m, 1H, *H*-10), 6.44 (bs, 2H N*H* guanidine), 6.52 (s, 1H, *aromatic proton* Mtr), 6.61 (bs, 1H N*H* guanidine), 7.61 (m, 1H, N*H* Gly), 7.73 (m, 1H, N*H* Arg). ^{13}C NMR (100.6 MHz, *Acetone-d$_6$*): δ 172.8, 172.4, 170.1, 158.4, 156.6, 156.3, 138.4, 136.3, 133.8, 124.8, 111.7, 68.3, 67.2, 61.5, 58.1, 55.5, 52.1, 41.1, 37.1, 33.3, 33.0, 30.6, 29.0, 27.7, 25.8, 24.1, 18.3, 12.0. MS (ESI$^+$) m/z = 710.5 [M+H]$^+$, calcd for $C_{32}H_{51}N_7O_9S$: 709.35 [M].

Synthesis of methyl 2-(2-((3S,6S,7R,9aS)-7-(azidomethyl)-6-(dimethylamino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (16). To a solution of compound **15** (0.300 g, 0.423 mmol) in dry THF (4 mL), under N_2 and at 0 °C, DPPA (114 μL, 0.528 mmol) followed by DBU (79 μL, 0.528 mmol) were added. The solution was stirred at 0 °C for ca 2 h, then was left to warm at room temperature and stirred for other 24 h. After reaction completion the solvent was evaporated and the crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage using a C18 cartridge and eluting with H_2O/CH_3CN both containing 0.2% AcOH (gradient from 90:10 to 0:100). The collected compound was finally desalted by running through a short pad of silica gel eluting with $CHCl_3$ + 2% Et_3N Y: 99%. ^{1}H NMR (400 MHz, *Acetone-d$_6$*): δ 1.46-1.70 (m, 4H, H-6, 2 *H*-γ-Arg, *H*-β-Arg), 1.70-1.96 (m, 5H, *H*-β-Arg, *H*-6, *H*-9 *H*-5, *H*-8), 1.99-2.08 (m, 3H, *H*-9 *H*-5, *H*-4), 2.08-2.23 (m, 4H, C*H*$_3$ Mtr, *H*-8), 2.59 (s, 6H, (C*H*$_3$)$_2$N), 2.63 (s, 3H C*H*$_3$ Mtr), 2.67 (s, 3H C*H*$_3$ Mtr), 3.15-3.25 (m, 2H, 2 *H*-δ-Arg), 3.37-3.53 (m, 2H, *H*-3, *H*-11), 3.56-3.67 (m, 4H, *H*-11, OC*H*$_3$), 3.71-3.95 (m, 6H, *H*-7, 2 *H*-α-Gly, OC*H*$_3$), 4.38-4.51 (m, 1H, *H*-α-Arg), 4.52-4.59 (m, 1H, *H*-10), 6.67 (s, 1H, *aromatic proton* Mtr), 6.69-6.76 (m, 2H N*H* guanidine), 6.77-6.89 (m, 1H, N*H* guanidine), 7.80 (d, 1H, J = 8.0 Hz, N*H* Arg), 7.95-8.11 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d$_6$*): δ 173.0, 172.5, 171.0, 158.9, 157.9, 154.6, 154.5, 139.2, 137.0, 136.3, 124.9, 112.6, 68.0, 62.2, 58.8, 58.4, 56.0, 53.1,52.2, 42.0, 41.5, 33.9, 27.7, 26.2, 24.4, 18.8, 12.2. MS (ESI$^+$) m/z = 735.6 [M+H]$^+$, calcd for $C_{32}H_{50}N_{10}O_8S$: 734.35 [M].

Synthesis of *tert*-butyl 4-((((3S,6S,7R,9aS)-3-((1-((2-(methoxy)-2-oxoethyl)amino)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-oxopentan-2-yl)carbamoyl)-6-(dimethylamino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl)amino)-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate, (17). To a solution of compound **16** (0.330 g, 0.449 mmol) in MeOH (12 mL) Pd-C 10% (30 mg, 10% w/w) was added. The suspension was stirred under hydrogen atmosphere for ca. 6 h. After this time the catalyst was filtered through a pad of celite and washed with MeOH. The collected organic phase was evaporated under reduced pressure to yield the desired compound as white foam that was used without any further purification.

This solid (0.449 mmol) was then dissolved in dry dimethylformamide (5 mL) and added to a solution of Z-Asp(tBu)OH (0.181 g, 0.561 mmol), HOBt (0.076 g, 0.561 mmol), HBTU (0.213 g, 0.561 mmol) and diisopropylethylamine (235 μL, 1.347 mmol) in dimethylformamide (5 mL). The resulting solution was left to stir, under N_2 for 18 hours After this time the reaction was concentrated under reduced pressure and the crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage using a C18 cartrige and eluting with H_2O/CH_3CN both containing 0.2% AcOH (gradient from 90:10 to 0:100). Y: 46%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.41 (s, 9H, C(CH$_3$)$_3$), 1.51-1.67 (m, 3H, *H*-6, 2 *H*-γ-Arg), 1.68-1.86 (m, 4 *H*-β-Arg, *H*-6, *H*-8, *H*-5), 1.87-2.10 (m, 5H, *H*-β-Arg, *H*-5, *H*-8, 2 *H*-9), 2.13 (m, 3H, C*H*$_3$ Mtr), 2.34 (m, 1H, *H*-4), 2.60 (s, 3H, C*H*$_3$ Mtr), 2.66 (s, 3H, C*H*$_3$ Mtr), 2.68-2.79 (m, 2H, 2 *H*-β-Asp), 2.82 (bs, 3H, NC*H*$_3$), 3.00 (bs, 3H, NC*H*$_3$), 3.17 (m, 2H, 2 *H*-δ-Arg), 3.41 (m, 2H, 2 *H*-11), 3.65 (s, 3H OC*H*$_3$), 3.73-3.85 (m, 5H, *H*-7, *H*-α-Gly, OC*H*$_3$), 3.88-4.03 (m, 2H, *H*-α-Gly, *H*-3), 4.41-4.53 (m, 3H, *H*-α-Arg, *H*-α-Asp, *H*-10), 4.98 (m, 1H, HC*H*Cbz), 5.12 (m, 1H, HC*H*Cbz), 6.31 (m, 1H, N*H* Asp), 6.52 (s, 1H, *aromatic proton* Mtr), 6.56 (bs, 3H N*H* guanidinio), 7.03 (m, 1H, *aromatic proton* Cbz), 7.15-7.25 (m, 2H, *aromatic protons* Cbz), 7.27-7.34 (m, 2H, *aromatic protons* Cbz), 7.73 (m, 1H, N*H* Gly), 7.88 (m, 1H, N*H* Arg). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 172.5, 171.5, 170.5, 170.1, 158.4, 156.7, 156.7, 138.4, 136.3, 129.3, 128.3, 128.1 127.9, 124.8, 123.5, 120.2, 111.7, 81.7, 67.1, 61.9, 55.5, 52.9, 52.1, 51.8, 41.0, 37.2, 32.0, 29.4, 28.0, 27.3, 18.4, 12.0. MS (ESI$^+$) m/z = 1014.7 [M+H]$^+$, 507.9 [M+2H]$^+$, calcd for $C_{48}H_{71}N_9O_{13}S$: 1013.49 [M].

Synthesis of *tert*-butyl 4-((((3S,6S,7R,9aS)-3-((1-((2-(benzyloxy)-2-oxoethyl)amino)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-oxopentan-2-yl)carbamoyl)-6-(dimethylamino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl)amino)-3-(((benzyloxy)carbonyl)amino)-4-oxobutanoate, (18).

To a solution of **17** (0.209 g, 0.206 mmol) in benzyl alcohol (320 μL, 3.09 mmol), Ti(*i*PrO)$_4$ (24 μL, 0.804 mmol) was added. The reaction mixture was stirred at 75°C under vacuum (9.87 atm) for ca. 30 h. After reaction completion, the reaction mixture was diluted with DCM and purified by flash chromatography with Isolera flash purification system by Biotage eluting with $CHCl_3$/MeOH both containing 1% Et_3N (gradient from 100:0 to 90:10). Y: 60%. ^{1}H NMR (400 MHz, *Acetone-d$_6$*): δ 1.44 (s, 9H, C(CH$_3$)$_3$), 1.42-1.47 (m, 1H, *H*-6), 1.50-1.70 (m, 3H, 2 *H*-γ-Arg, *H*-5), 1.70-2.00 (m, 5H, 2 *H*-β-Arg, *H*-6, *H*-8, *H*-5, *H*-9), 2.00-2.14 (m, 5H, *H*-9, C*H*$_3$ Mtr, *H*-4), 2.14-2.22 (m, 1H, *H*-8), 2.56 (bs, 6H, (C*H*$_3$)$_2$N), 2.63 (s, 3H, C*H*$_3$ Mtr), 2.64-2.68 (m, 4H, C*H*$_3$ Mtr, *H*-β-Asp), 2.75-2.90 (m, 1H, *H*-β-Asp), 3.10-3.28 (m, 3H, 2 *H*-δ-Arg, *H*CH-NH), 3.30-3.45 (m, 1H, *H*-3), 3.50-3.61 (m, 1H, *H*CH-NH), 3.80-3.90 (m, 4H, *H*-7, OC*H*$_3$), 3.91-4.08 (m, 2H, 2 *H*-α-Gly), 4.39-4.54 (m, 2H, *H*-α-Arg, *H*-α-Asp), 4.54-4.62 (m, 1H, *H*-10), 5.00-5.18 (m, 4H, 2 C*H*$_2$Ph), 6.33-6.58 (bs, 1H N*H* guanidine), 6.59-6.71 (m, 2H, N*H* guanidine, *aromatic proton* Mtr), 6.78 (d, 1H, J = 8 Hz, N*H* Asp), 7.27-7.42 (m, 10H, *aromatic protons*), 7.67-7.78 (m, 2H, N*H* Arg, N*H*CH$_2$), 7.79-7.88 (m, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, *Acetone-d$_6$*): δ 171.8, 169.8, 169.5, 158.0, 156.7, 156.1, 138.2, 136.2, 136.1, 128.4, 128.1, 128.0, 123.9, 111.6, 66.8, 66.2, 61.0, 57.3, 55.0, 52.2, 42.7, 41.1, 40.8, 37.5, 35.9, 33.2, 27.3, 26.4, 25.3, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 1090.7 [M+H]$^+$, calcd for $C_{54}H_{75}N_9O_{13}S$: 1089.52 [M].

Synthesis of benzyl 2-(2-((3S,6S,7S,9aS)-6-(dimethylamino)-7-(hydroxymethyl)-5-oxooctahydro-

1H-pyrrolo[1,2-a]azepine-3-carboxamido)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)pentanamido)acetate, (19). To a solution of **15** (0.100 g, 0.141 mmol) in benzyl alcohol (219 µL, 2.113 mmol), Ti(*i*PrO)$_4$ (16.7 µL, 0.056 mmol) was added. The reaction mixture was stirred at 75°C under vacuum (9.87 atm) for ca. 30 h. After reaction completion, the reaction mixture was diluted with DCM and purified by flash chromatography with Isolera flash purification system by Biotage eluting with $CHCl_3$/MeOH both containing 1% Et_3N (gradient from 100:0 to 90:10). Y: 69%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.50-1.90 (7H, 2 *H*-γ-Arg, 2 *H*-6, *H*-8, *H*-5, *H*-β-Arg), 1.90-2.21 (m, 9H, *H*-β-Arg, *H*-5, 2 *H*-9, *H*-4, *H*-8, C*H*$_3$ Mtr), 2.56-2.65 (s, 9H, C*H*$_3$ Mtr, NC*H*$_3$), 2.69 (s, 3H, C*H*$_3$ Mtr), 3.21-3.32 (m, 2H, 2 *H*-δ-Arg), 3.40-3.52 (m, 2H, *H*-3, *H*CHOH), 3.75-3.88 (m, 5H, HC*H*OH, *H*-7, OC*H*$_3$), 3.97 (dd, 1H, J = 4.4 Hz, J = 18 Hz, *H*-α-Arg), 4.14 (dd, 1H, J = 4.4 Hz, J = 18 Hz, *H*-α-Arg), 4.45-4.60 (m, 2H, *H*-α-Arg, *H*-10), 5.15 (s, 2H, C*H*$_2$Ph), 6.36 (bs, 1H, N*H* guanidine), 6.50-6.58 (m, 2H, N*H* guanidine, *aromatic proton* Mtr), 7.30-7.40 (m, 5H, *aromatic protons*), 7.50 (bs, 1H, N*H* Arg), 7.81 (bs, 1H, N*H* Gly). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 172.5, 172.3, 169.5, 158.5, 156.5, 138.4, 136.3, 135.3, 133.6, 128.6, 128.4, 128.3, 124.5, 111.7, 68.3, 67.1, 67.0, 66.8, 61.7, 58.0, 55.5, 53.4, 41.3, 40.4, 36.7, 33.1, 31.5, 30.3, 30.2, 28.9, 27.9, 25.9, 24.1, 18.3, 12.0. MS (ESI$^+$) m/z = 786.5 [M+H]$^+$, calcd for $C_{38}H_{55}N_7O_9S$: 785.38 [M].

Synthesis of 1-(((3S,6S,7S,9aS)-3-((1-((2-(benzyloxy)-2-oxoethyl)amino)-5-(3-((4-methoxy-2,3,6-trimethylphenyl)sulfonyl)guanidino)-1-oxopentan-2-yl)carbamoyl)-6-(dimethylamino)-5-oxooctahydro-1H-pyrrolo[1,2-a]azepin-7-yl)methyl) 4-tert-butyl 2-(((benzyloxy)carbonyl)amino)succinate, (20). To a solution of Z-Asp(tBu)OH (0.293 g, 0.905 mmol) in dry DCM (5 mL), under N_2 and at 0 °C, DIC (105 µL, 0.679 mmol) was added. After 15 min. this solution was added to compound **13** (0.100 g, 0.116 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred for 2 h. After reaction completion the organic phase was washed with $NaHCO_3$, dried over Na_2SO_4 and evaporated under reduced pressure. The crude was purified by reverse phase chromatography with Isolera Flash Purification System by Biotage eluting with DCM/MeOH (gradient from 100:0 to 90:10). Y: 79%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.43 (s, 9H, C(C*H*$_3$)$_3$), 1.53-1.62 (m, 2H, *H*-5, *H*-γ-Arg), 1.62-1.71 (m, 2H, *H*-γ-Arg, *H*-6), 1.71-1.85 (m, *H*-β-Arg, *H*-6, *H*-8), 1.80-2.20 (m, 9H, *H*-β-Arg, *H*-6, *H*-5, 2 *H*-9, *H*-4, C*H*$_3$ Mtr), 2.5 (s, 6H, (C*H*$_3$)$_2$N), 2.62 (s, 3H, C*H*$_3$ Mtr), 2.69 (s, 3H, C*H*$_3$ Mtr), 2.72-2.81 (m, 1H, *H*-β-Asp), 2.88-2.96 (m, 1H, *H*-β-Asp), 3.22-3.35 (m, 3H, 2 *H*-δ-Arg, *H*-3), 3.70-3.76 (m, 1H, *H*-7), 3.84 (s, 3H, OC*H*$_3$), 3.96-4.08 (m, 1H, *H*-α-Gly), 4.10-4.18 (m, 2H, *H*-α-Gly, *H*CHO), 4.18-4.31 (m, 1H, HC*H*O), 4.47 (m, 1H, *H*-α-Arg), 4.53 (m, 1H, *H*-10), 4.61 (m, 1H, *H*-α-Asp), 5.08-5.20 (m, 4H, 2 C*H*$_2$Ph), 5.81 (m, 1H, N*H* Asp), 6.30-6.50 (m, 2H, N*H* guanidine), 6.54 (s, 1H, *aromatic proton* Mtr), 7.30-7.42 (m, 11H, *aromatic protons*, N*H* Gly), 7.75 (bs, 1H, N*H* Arg). ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 172.4, 172.2, 171.0, 170.2, 169.5, 158.4, 156.5, 156.0, 138.5, 136.4, 136.2, 135.3, 133.7, 128.6, 128.4, 128.3, 128.1, 128.0, 124.8, 111.7, 81.9, 68.0, 67.1, 67.0, 66.5, 61.7, 57.9, 55.4, 51.8, 50.6, 41.3, 40.5, 37.7, 35.1, 33.0, 28.9, 28.1, 27.7, 24.2, 18.4, 12.0. MS (ESI$^+$) m/z = 1091.8 [M+H]$^+$, calcd for $C_{54}H_{74}N_8O_{14}S$: 1090.50 [M].

General procedure A. To compounds **12**, **14** or **18** (1 eq) in methanol (0.02 M) 10 % palladium on carbon (10% w/w) was added. The resulting suspension was hydrogenated at atmospheric pressure, at room temperature for 18 h. After this time the suspension was filtered through a bed of celite and the resulting filtrate was concentrated under reduced pressure to give a white solid. DMF (0.001 M) was passed through a cartridge of polymer bound *para*-toluenesulphonic acid under argon into a round bottomed flask. A solution of the aforementioned white solid (1 eq) was prepared using the 1/15 volume of the treated DMF. Similarly HATU (1.1 eq) and HOAt (1.1 eq) were dissolved in the same volume of DMF. Each of the solutions was then drawn into a Hamilton Gastight syringe. The two syringes were then fitted to the syringe pump. To the remaining DMF in the round bottomed flask, under argon, was added diisopropylethylamine (3 eq). The two solutions of linear deprotected peptide in one syringe, and coupling reagents in the other syringe were then added to this solution drop-wise over 18 h by means of the syringe pump. Once addition of the contents of the two syringes was complete, the reaction was left to stir for a further 6 hours. The reaction was then concentrated under reduced pressure to give a brown residue. The remaining brown residue was purified by reverse phase column chromatography with Isolera Flash Purification System by Biotage using a C18 cartridge and eluting with H_2O/CH_3CN (gradient from 90:10 to 0:100).

Synthesis of compound 21. Compound **21** has been prepared starting from compound **12** (0.100 g, 0.086 mmol) following the general procedure A. Y: 77%. ^{1}H NMR (400 MHz, *Acetone-d$_6$*): δ 1.42 (s, 9H, C(C*H*$_3$)$_3$), 1.44 (s, 9H, C(C*H*$_3$)$_3$), 1.53 (m, 1H, *H*-γ-Arg), 1.60 (m, 1H, *H*-γ-Arg), 1.66-1.79 (m, 2H, *H*-β-Arg, *H*-5), 1.85 (m, 1H, *H*-5), 1.85-1.95 (m, 3H, 2 *H*-6, *H*-β-Arg), 1.86-2.04 (m, 2H, *H*-8, *H*-9), 2.13 (s, 3H, C*H*$_3$ Mtr), 2.15-2.25 (m, 2H, *H*-4, *H*-9), 2.33 (m, 1H, *H*-8), 2.64 (s, 3H, C*H*$_3$ Mtr), 2.70 (s, 3H, C*H*$_3$ Mtr), 2.73 (m, 1H, *H*-β-Asp), 2.83 (m, 1H, *H*-β-Asp), 3.23 (m, 2H, 2 *H*-δ-Arg), 3.65 (d, 1H, J = 10.4 Hz, *H*-α-Gly), 3.72 (d, 1H, J = 7.2 Hz, *H*-11), 3.86 (s, 3H OC*H*$_3$), 3.99 (m, 1H, *H*-7), 4.12 (d, 1H, J = 10.8 Hz, *H*-α-Gly), 4.24-4.32 (m, 3H, *H*-11, *H*-10, 1H, *H*-α-Arg), 4.45 (m, 1H, *H*-3), 4.89 (m, 1H, *H*-α-Asp), 6.50 (m, 1H, N*H*Boc), 6.70 (s, 1H, *aromatic proton* Mtr), 6.97 (m, 1H, N*H* Arg), 7.15 (m, 1H, N*H*). ^{13}C NMR (100.6 MHz, *Acetone-d$_6$*): δ 172.2, 171.9, 169.9, 169.6, 169.5, 158.0, 156.4, 155.8, 138.3, 136.1, 135.2, 123.9, 111.6, 80.7, 78.3, 68.0, 66.7, 64.0, 59.0, 56.9, 55.0, 49.2, 43.2, 40.2, 36.0, 33.8, 32.5, 29.8, 27.7, 27.5, 27.3, 23.4, 17.8, 11.2. MS (ESI$^+$) m/z = 921.7 [M+H]$^+$, calcd for $C_{42}H_{64}N_8O_{13}S$: 920.43.

Synthesis of compound 22. Compound **22** has been prepared starting from compound **14** (0.100 g, 0.086 mmol) following the general procedure A. Y: 73%. ^{1}H NMR (400 MHz, *Acetone-d$_6$*): δ 1.43 (s, 9H, C(C*H*$_3$)$_3$), 1.46 (s, 9H, C(C*H*$_3$)$_3$), 1.55 (m, 2H, 2 *H*-γ-Arg), 1.71 (m, 1H, *H*-β-Arg), 1.75-1.88 (m, 4H, *H*-5, *H*-8, *H*-6, *H*-β-Arg), 1,95 (m, 1H, *H*-5), 1.97-2.06 (m, 3H, *H*-9, *H*-6, *H*-4), 2.13 (m, 3H, C*H*$_3$ Mtr), 2.24 (m, 1H, *H*-9), 2.33 (m, 1H, *H*-8), 2.60 (dd, 1H, J = 4.6 Hz, J = 11.0 Hz, *H*-β-Asp), 2.65 (s, 3H, C*H*$_3$ Mtr), 2.70 (s, 3H, C*H*$_3$ Mtr), 2.87 (m, 1H *H*-β-Asp), 3.01 (m, 1H, *H*-11), 3.24 (m, 1H, *H*-δ-Arg), 3.29 (m, 1H, *H*-δ-Arg), 3.38 (m, 1H, *H*-11), 3.55 (d, 1H, J = 10.0 Hz, *H*-α-Gly), 3.87 (m, 3H OC*H*$_3$), 4.08 (d, 1H, J = 10.0 Hz, *H*-α-Gly), 4.14 (m, 1H, *H*-7), 4.26 (m, 1H, *H*-10), 4.43 (m, 1H, *H*-α-Arg), 4.45 (d, 1H, J = 6.0 Hz, *H*-3), 4.69 (m, 1H, *H*-α-Asp), 6.70 (s, 1H, *aromatic proton* Mtr), 6.80 (m, 1H, N*H* Arg), 6.99 (m, 1H, N*H* Asp). ^{13}C NMR (100.6 MHz, *Acetone-d$_6$*): δ 172.6, 172.4, 171.6, 169.8, 169.4, 168.9, 158.0, 156.5, 155.7, 138.3, 136.2, 135.0, 123.9, 111.6, 80.2, 78.5, 66.7, 63.8, 59.1, 57.1, 55.0, 51.8, 43.9, 41.8, 39.9, 35.7, 35.0, 34.3, 32.4, 29.9, 27.7, 27.3, 25.6, 23.4, 22.3, 17.8, 11.2. MS (ESI$^+$) m/z = 920.8 [M+H]$^+$, calcd for $C_{42}H_{65}N_9O_{12}S$: 919.45 [M].

Synthesis of compound 23. Compound **23** has been preparated starting from compound **18** (0.120 g, 0.110 mmol) following the general procedure A. Y: 67%. ^{1}H NMR (400 MHz, CD_3CN + D_2O): δ 1.32-1.48 (8, 11H, $C(CH_3)_3$, 2 *H*-γ-Arg), 1.58-1.76 (m, 3H, *H*-β-Arg, *H*-5, *H*-6), 1.79 (m, 1H, *H*-8), 1.81-1.97 (m, 3H *H*-β-Arg, *H*-9, *H*-6), 2.02 (m, 1H, *H*-4), 2.13 (s, 3H, C*H*$_3$ Mtr) 2.16-2.27 (m, 3H, *H*-5, 1H, *H*-9, *H*-8), 2.43 (s, 6H, $N(CH_3)_2$), 2.58 (s, 3H, C*H*$_3$ Mtr), 2.62-2.72 (m, 5H, C*H*$_3$ Mtr, *H*-β-Asp, *H*-11), 2.77 (dd, 1H, J = 4.4 Hz, J = 16.4 Hz, *H*-β-Asp), 3.11 (m, 2H, 2 *H*-δ-Arg) 3.37 (d, 1H, J = 8.8 Hz, *H*-3), 3.54 (d, 1H, J = 15.2 Hz, *H*-α-Gly), 3.77 (d, 1H, J = 14.8 Hz, *H*-α-Gly), 3.83 (s, 3H OCH_3), 3.97 (m, 1H, *H*-7), 4.02 (m, 1H, *H*-11), 4.28 (m, 1H, *H*-10), 4.36 (m, 1H, *H*-α-Arg), 4.48 (m, 1H, *H*-α-Asp), 6.68 (s, 1H, *aromatic proton* Mtr). ^{13}C NMR (100.6 MHz, CD_3CN + D_2O): δ 175.4, 172.9, 172.4, 170.3, 170.2, 170.1, 158.3, 156.5, 138.3, 136.4, 134.0, 124.5, 112.0, 81.2, 69.4, 62.8, 58.2, 55.3, 51.5, 50.7, 44.1, 41.8, 40.1, 37.7, 36.3, 34.8, 32.5, 30.2, 27.6, 27.2, 24.1, 23.1, 20.3, 17.7, 11.2. MS (ESI^+) m/z = 848.5 $[M+H]^+$, calcd for $C_{39}H_{61}N_9O_{10}S$: 847.43.

General procedure B. To compounds **21**, **22** or **23** (1 eq) a solution of phenol (5 % v/v), 1,2-ethanedithiol (5 % v/v), thioanisole (5 % v/v), and triisopropylsilane (5 % v/v) in trifluoroacetic acid (0.088 M) was added. The resulting solution was left to stir at room temperature for 6 hours. After this time the reaction was concentrated *in vacuo* and to the resulting residue was added diisopropyl ether. The resulting suspension was centrifuged and the supernatant liquid was carefully decanted. The remaining solid was dried under vacuum to remove any residual diisopropyl ether and then dissolved in water and freeze-dried. The freeze-dried powder was dissolved in water containing 1 % trifluoroacetic acid and then purified by reverse-phase HPLC, eluting with acetonitrile/water both containing 0.1 % trifluoroacetic acid.

Synthesis of compound 2. Compound **2** has been prepared starting from compound **21** (0.020 g, 0.022 mmol) following the general procedure B. Y: 89%. ^{1}H NMR (600 MHz, D_2O + H_2O): δ 1.36 (m, 2H, 2 *H*-γ-Arg), 1.50 (m, 1H, *H*-β-Arg), 1.55 (m, 1H, *H*-5), 1.59 (m, 1H, *H*-β-Arg), 1.63 (m, 1H, *H*-6), 1.72 (m, 1H, *H*-8), 1.78 (m, 1H, *H*-6), 1.80 (m, 1H, *H*-9), 1.87 (m, 1H, *H*-5), 2.04 (m, 1H, *H*-4), 2.10 (m, 1H, *H*-9), 2.12 (m, 1H, *H*-8), 2.62 (dd, 1H, J = 6.6 Hz, J = 17.4 Hz *H*-β-Asp), 2.78 (dd, 1H, J = 7.2, Hz, J = 17.4 Hz *H*-β-Asp), 3.00 (m, 2H, 2 *H*-δ-Arg), 3.54 (dd, 1H, J = 5.3 Hz, J = 16.8 Hz, *H*-α-Gly), 3.71 (dd, 1H, J = 4.8 Hz, J = 16.8 Hz, *H*-α-Gly), 3.84 (m, 1H, *H*-7), 3.98 (7, 1H, J = 11.4 Hz, *H*-11), 4.08 (m, 1H, *H*-10), 4.14-4.23 (m, 2H, *H*-3, *H*-α-Arg), 4.27 (m, 1H, *H*-11), 4.57 (m, 1H, *H*-α-Asp), 6.86 (d, 1H, J = 7.8 Hz, N*H* Arg), 6.99 (m, 1H NH guanidinio), 8.34 (m, 1H, N*H* Gly), 8.51 (d, 1H, J = 7.2 Hz, N*H* Asp). ^{13}C NMR (100.6 MHz, D_2O + H_2O): δ 174.5, 173.9, 173.2, 171.2, 171.0, 169.1, 157.0, 67.5, 64.6, 59.5, 57.5, 52.8, 49.7, 43.2, 40.7, 37.4, 34.2, 32.9, 32.2, 30.7, 29.3, 27.3, 24.3. MS (ESI^+) m/z = 553.9 $[M+H]^+$, 277.3 $[M+2H]^+$, calcd for $C_{23}H_{36}N_8O_8$: 552.27.

Synthesis of compound 3. Compound **3** has been prepared starting from compound **22** (0.020 g, 0.022 mmol) following the general procedure B. Y: 89%. ^{1}H NMR (600 MHz, D_2O + H_2O): δ 1.41 (m, 2H, 2 *H*-γ-Arg), 1.57 (m, 1H, *H*-β-Arg), 1.63-1.78 (m, 4H, *H*-5, *H*-8, *H*-6, *H*-β-Arg), 1.79-1.90 (m, 2H, *H*-5, *H*-9), 1.94 (m, 1H, *H*-4), 2.00 (m, 1H, *H*-6), 2.08-2.12 (m, 2H, *H*-9, *H*-8), 2.75 (m, 2H, 2 *H*-β-Asp), 2.95 (d, 1H, J = 16.2 Hz, *H*-11), 3.05 (m, 2H, 2 *H*-δ-Arg), 3.61 (dd, 1H, J = 6.0 Hz, J = 16.8 Hz, *H*-α-Gly), 3.66 (m, 1H, *H*-11), 3.83 (dd, 1H, J = 4.5 Hz, J = 16.5 Hz, *H*-α-Gly), 3.92 (m, 1H, *H*-7), 4.12 (d, 1H, J = 10.2 Hz, *H*-3), 4.14 (m, 1H, *H*-10), 4.23-4.32 (m, 2H, *H*-α-Arg, *H*-α-Asp), 6.92 (m, 1H, N*H* Arg), 7.05 (m, 1H NH guanidinio),7,81 (d, 1H, J = 8.4 Hz, CH_2NH), 8.50 (m, 1H, N*H* Gly), 8.83 (d, 1H, J = 4.8 Hz, N*H* Asp). ^{13}C NMR (150.95 MHz, D_2O + H_2O): δ 173.8, 173.7, 173.6, 172.1, 169.3, 163.0, 162.8, 156.9, 64.2, 59.3, 56.9, 52.5, 52.2, 43.2, 42.7, 40.5, 38.8, 34.4, 34.1, 33.3, 32.0, 29.3, 27.4, 24.3. MS (ESI^+) m/z = 552.9 $[M+H]^+$, 276.9 $[M+2H]^+$, calcd for $C_{23}H_{37}N_9O_7$: 551.28.

Synthesis of compound 5. Compound **5** has been prepared from compound **23** (0.020 g, 0.022 mmol) following the general procedure B. Y: 89%. ^{1}H NMR (600 MHz, D_2O + H_2O): δ 1.26 (m, 1H, *H*-γ-Arg), 1.31 (m, 1H, *H*-γ-Arg), 1.52-1.69 (m, 3H, *H*-β-Arg, *H*-6, *H*-8), 1.75-1.83 (m, 3H, *H*-β-Arg, *H*-5, *H*-9), 1.88 (m, 1H, *H*-6), 2.04 (m, 1H, *H*-4), 2.08-2.23 (m, 3H, *H*-9, *H*-8, *H*-5), 2.70 (d, 2H, J = 7.2 Hz, 2 *H*-β-Asp), 2.83 (s, 6H, $N(CH_3)_2$), 2.84 (m, 1H, *H*-11), 2.98 (m, 2H, 2 *H*-δ-Arg), 3.57 (dd, 1H, J = 6.6 Hz, J = 16.8 Hz, *H*-α-Gly), 3.65 (m, 1H, *H*-11), 3.79 (dd, 1H, J = 3.6 Hz, J = 15.6 Hz, *H*-α-Gly), 3.96 (m, 1H, *H*-7), 4.20 (m, 1H, *H*-10), 4.25(d, 1H, J = 10.2 Hz, *H*-3), 4.27-4.34 (m, 2H, *H*-α-Arg, *H*-α-Asp), 6.94 (d, 1H, J = 6.0 Hz, N*H* Asp), 7.0 (m, 1H N*H* guanidinio), 7.38 (d, 1H, J = 7.2 Hz, CH_2NH), 8.60 (d, 1H, J = 6.9 Hz, N*H* Arg), 8.79 (m, 1H, N*H* Gly). ^{13}C NMR (150.95 MHz, D_2O + H_2O): δ 174.3, 173.3, 173.0, 172.4, 172.0, 168.3, 156.9, 68.9, 63.6, 58.9, 52.0, 51.6, 43.9, 42.7, 40.4, 38.6, 36.3, 34.9, 33.6, 32.1, 29.3, 27.5, 23.1. MS (ESI^+) m/z = 580.7 $[M+H]^+$, 290.9 $[M+2H]^+$, calculated for $C_{25}H_{41}N_9O_7$: 579.31.

Biology

Solid-phase receptor binding assay

Purified αvβ3 and αvβ5 receptors (Chemicon International, Inc., Temecula,CA, USA) were diluted to 0.5 μg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. An aliquot of diluted receptors (100 μL/well) was added to 96-well microtiter plates (NUNC MW 96F Medisorp Straight) and incubated overnight at 4°C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for an additional 2 h at room temperature to block nonspecific binding, followed by 3 h incubation at room temperature with various concentrations (10^{-5}–10^{-12} M) of test compounds in the presence of biotinylated vitronectin (1 μg/mL). Biotinilation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After washing, the plates were incubated for 1 h at room temperature with biotinylated streptavidin–peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 min incubation with 100 μL/well Substrate Reagent Solution (R&D Systems, Minneapolis, MN) before stopping the reaction with the addition of 50 μL/well 2N H_2SO_4. Absorbance at 415 nm was read in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represents the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software. Each experiment was repeated in triplicate.

Adhesion assay

Round bottomed 96-well plates were coated with vitronectin (Duotech) or fibronectin (Sigma) at 10 μg/mL in PBS, overnight at 37 °C. Before starting the adhesion assay, wells were incubated with cell specific culture medium supplemented with 1% w/v BSA, for 30 minutes at 37 °C. During this incubation, $7.8x10^4$ cells/cm^2/well were harvested by trypsinisation and resuspended in culture medium alone (negative control) or containing increasing

concentrations of the tested compound (100 nM, 250 nM, 500 nM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM and 200 μM). Then, 50 μL of cell/compound suspension were seeded in each well and allowed to adhere for 2 hours at 37 °C. Nonadherent cells were removed with PBS. The remaining adherent cells were stained with a 0.5 w/v Crystal Violet solution, for 10 minutes at room temperature. Plates were finally rinsed with H_2O and dried overnight at room temperature. Stained cells were solubilized with 10% w/v SDS and quantified on a microtiter plate reader at 595 nM. Experiments were carried out in triplicate. Results are expressed as IC50 ± SD, calculated using GraphPad Prism software.

Computational studies

All calculations were run using the Schrödinger suite of programs through the Maestro graphical interface.[12] Conformational preferences of compound **3** were investigated by molecular mechanics calculations using the MacroModel v9.1[13] implementation of the Amber all-atom force field[14] (denoted AMBER*) and the implicit water GB/SA solvation model.[9] Monte Carlo/energy minimization (MC/EM) conformational search[7] of the Ala-Gly-Ala cyclopeptide analogue containing methyl groups instead of the Arg and Asp side chains was performed as the first step. For the search, 1000 starting structures for each variable torsion angle were generated and minimized until the gradient was <0.05 $kJÅ^{-1}mol^{-1}$ using the truncated Newton–Raphson method implemented in MacroModel.[15] Duplicate conformations and those with energy >6 $kcalmol^{-1}$ above the global minimum were discarded. Free simulations of the RGD cyclic peptide (Asp and Arg side chains were considered ionized) were then performed at 300 K using the metropolis Monte Carlo/stochastic dynamics (MC/SD) hybrid simulation algorithm,[8] starting from the cyclopeptide backbone geometries located by the previous MC/EM step. RGD side chain dihedral angles were defined as internal coordinate degrees of freedom in the Monte Carlo part of the algorithm. A time step of 1 fs was used for the stochastic dynamics part of the algorithm for 10 ns of simulation time.

The recently solved crystal structure of the extracellular domain of the integrin $\alpha_v\beta_3$ in complex with Cilengitide (PDB code 1L5G) was used for docking studies.[2f] Docking was performed only on the globular head of integrin because the head group of integrin has been identified in the crystal structure as the ligand binding region. The protein structure was set up for docking as follows: the protein was truncated to residue sequences 41–342 for chain α and 114–347 for chain β. Due to a lack of parameters, the Mn^{2+} ions in the experimental protein structure were modeled by replacing them with Ca^{2+} ions. The resulting structure was prepared by using the Protein Preparation Wizard of the graphical user interface Maestro and the OPLSAA force field.

The automated docking calculations were performed by using Glide (Grid-based Ligand Docking with Energetics).[11] The grid generation step started from the extracellular fragment of X-ray structure of $\alpha_v\beta_3$ complex with Cilengitide, prepared as previously described. The center of the grid enclosing box was defined by the center of the bound ligand, as described in the original PDB entry. The enclosing box dimensions, which are automatically deduced from the ligand size, fit the entire active site. For the docking step, the size of the bounding box for placing the ligand center was set to 12 Å. No further modifications were applied to the default settings. The GlideScore function[16] was used to select 20 poses for each ligand. The Glide program was initially tested for its ability to reproduce the crystallized binding geometry of Cilengitide. The program was successful in reproducing the experimentally determined binding mode of this compound, as it corresponds to the best-scored pose.

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Notes and references

[a] Centro Interdisciplinare Studi biomolecolari e applicazioni Industriali, Università degli Studi di Milano, Via Fantoli 16/15, I-20138, Milano.

[b] Istituto di Scienze e Tecnologie Molecolari, Consiglio Nazionale delle Ricerche, Via C. Golgi 19, I-20133, Milano, Fax: +39 0250314072 E-mail: Leonardo.manzoni@istm.cnr.it

[c] Dipartimento di Chimica, Università degli Studi di Milano, Via C. Golgi 19, I-20133, Milano.

Electronic Supplementary Information (ESI) available: [^{1}H and ^{13}C NMR spectra of new synthesized compounds; biological data]. See DOI: 10.1039/b000000x/

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