Cyclic RGD Peptidomimetics Containing 4- and 5-Amino-Cyclopropane Pipecolic Acid (CPA) Templates as Dual $\alpha_V\beta_3$ and $\alpha_5\beta_1$ Integrin Ligands

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Abstract

4-Amino and 5-amino-cyclopropane pipecolic acids (CPAs) with *cis* relative stereochemistry between the carboxylic and amino groups were used as templates to prepare cyclic peptidomimetics containing the RGD sequence as possible integrin binders. The peptidomimetic ***c*(RGD8)** built on the 5-amino-CPA displayed an inhibition activity (IC_{50} = 2.4 nM) toward the $\alpha_v\beta_3$ integrin receptor comparable to that of the most potent antagonists reported so far and it was ten times more active than the corresponding antagonist ***c*(RGD7)** derived from the isomeric 4-amino-CPA. Both compounds were also nanomolar ligands of the $\alpha_5\beta_1$ integrin. These results suggest that the CPA-derived templates are suitable for the preparation of dual $\alpha_v\beta_3$ and $\alpha_5\beta_1$ ligands to suppress integrin-mediated events as well as for targeted drug delivery in cancer therapy.

Introduction

Integrins are cell adhesion transmembrane receptors for extracellular matrix (ECM) proteins, growth factors, immunoglobulins, cytokines and matrix-degrading proteases that mediate adhesive events during various cancer stages (angiogenesis, tumor growth and progression, invasion, and metastasis).[1-3] The $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrin receptors in particular recognize ECM proteins (e.g. vitronectin and fibronectin) which contain an arginine-glycine-aspartic acid (RGD) peptide sequence.[4-7] Because of their critical role in tumor-induced angiogenesis and metastasis formation,[8-12] $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins have received increasing attention as therapeutic targets. It has been suggested that targeting $\alpha_5\beta_1$ in combination with $\alpha_V\beta_3$ is likely to prove more efficient in anticancer therapy. Amongst the various options,[13-16] RGD-containing linear and

cyclic peptides and peptidomimetics have been widely studied as potential antagonists to suppress the events mediated by these integrins.[14-17] Advantages of cyclic systems are the high stability to chemical degradation and, possibly, a higher potency and specificity than linear systems because of their marked conformational restraints. To date, a few low-nanomolar-affinity cyclic binders for the $\alpha_V\beta_3$ receptor have been reported which present the RGD system installed on rigid hetero- and carbacyclic scaffolds. For example, bicyclic lactams,[18-20] γ-aminocyclopentanecarboxylic acids,[21] *cis*-β-aminocyclopropanecarboxylic acids,[22] 4-aminoprolines,[23,24] bifunctional diketopiperazine,[25] and morpholine derivatives,[26,27] have all been used to generate potent $\alpha_V\beta_3$ integrin antagonists (compounds **1-6**, Figure 1). We have recently reported that the 4-amino-substituted CPA (CPA, cyclopropane pipecolic acid) **7** (Figure 2), a new conformationally constrained δ-amino acid prepared from (*S*)-(+)-γ-hydroxymethyl-γ-butyrolactone, could be successfully introduced in a cyclic peptidomimetic [***c*(RGD7)**, Figure 2] bearing the RGD sequence.[28,29] This derivative displayed nanomolar activity as ligand of the $\alpha_V\beta_3$ integrin in M21 human melanoma cells, suggesting that the rigid structure of CPA induces a significant conformational asset towards optimal presentation of the pharmacophoric groups of the ligand. Based on these results, since we wanted to establish amino-substituted CPAs as new rigid platforms for RGD-containing peptidic sequences, we decided to further extend our study to other isomeric CPAs and in particular to the corresponding 5-amino-CPA isomer **8** (Figure 2). We were in fact interested in evaluating whether the different relative position (and spatial orientation) of the amino and carboxylic groups on which the RGD sequence is fixed would affect the potency of the peptidomimetic against the $\alpha_V\beta_3$ integrin. At the same time, we were also interested in comparing the activity of both peptidomimetics towards the $\alpha_5\beta_1$ receptor.[30] In this way we could determine whether the two templates (**7** and **8**) would provide an optimal platform for the synthesis of integrin ligands as potential therapeutics or for targeted delivery of drugs or diagnostics.[13, 31] In this paper we thus report on the synthesis of 5-amino-CPA **8** and its cyclic RGD-containing peptidomimetic derivative ***c*(RGD8)** and the evaluation of both ***c*(RGD7)** and ***c*(RGD8)** as ligands of the $\alpha_V\beta_3$ and $\alpha_5\beta_1$ receptors followed by a comparative conformational analysis.

Results and Discussion

Synthesis

The particular absolute configuration of **7** (Figure 2) was chosen on the basis of preliminary molecular modeling studies which showed that the RGD sequence in ***c*(RGD7)** best overlapped with that of potent 4-aminoproline derivative **4** (Figure 1). With 5-amino-CPA derivative **8**, which is a γ-amino acid, it was the opposite stereochemistry at C-1 and at the NH_2-bearing C atom that provided the best superposition with peptidomimetic **4** and for this reason we opted for the inclusion in the cyclic peptidomimetic of this 5-

amino CPA stereoisomer with (1*S*,5*R*) absolute configuration. The synthesis of the ***c*(RGD8)** peptidomimetic is reported in Schemes 1 and 2. The synthesis of racemic alcohol **9** and its enzymatic kinetic resolution (EKR) was carried out as already reported.[32] Since the resolution provided just less than 50% of the requisite enantiopure stereoisomer (*S*)-**9** from the racemic alcohol, butyrate (*R*)-**10** was hydrolyzed and subjected to Mitsunobu reaction to invert the C4 configuration and thus increase the amount of the desired 4*S* enantiomer. However, as this reaction was carried out on an allylic alcohol, racemization took place to a certain extent and it furnished alcohol (*S*)-**9** with 36% e.e. Thus, for the synthesis of 5-amino-CPA **8** and its cyclic peptidomimetic derivative we used (*S*)-**9** as obtained from the EKR, that is with a 94% e.e. (97:3 e.r.) relying on the fact that HPLC purification of the final peptidomimetic product would provide a diastereopure compound. We first converted (*S*)-**9** into 5-amino CPA methyl ester **11** as reported[28] and tried to use directly this for assembling the target compound (Scheme 1). However, whilst the coupling of the 5-amino group with Z-Asp(O*t*Bu)OH was successful, the selective hydrolysis of the pipecolic CO_2Me group (necessary to link the Arg-Gly dipeptide) was troublesome. Despite the two protections (methyl and *t*-butyl esters) are generally considered orthogonal, in this molecule this proved untrue and we never managed to obtain the free carboxylic group at C1. Thus we changed approach and converted (*S*)-**9** into known azide **13**[28] (Scheme 2), then we carried out the hydrolysis of the methyl ester, the hydrogenation of the azide into the amino group and finally the *N*-Fmoc protection to obtain **16**. In this way we could obtain an amount of suitable protected amino acid **16** sufficient to accomplish the synthesis of the cyclic peptidomimetic. This was carried out (Scheme 2) as already reported for the corresponding 4-amino CPA derivative ***c*(RGD7)**.[28] Compared to that, only the final ring closure proved troublesome as it provided, besides compound **21**, a mixture of other compounds which we were unable to identify but which likely contained the minor diastereoisomer deriving from (*R*)-**9** and two dimers. At this stage of the study, we did not carry out any other experiment to optimize the final cyclization step by using other coupling methodologies and so exhaustive deprotection of the mixture containing compound **21** was carried out. As we hoped, we managed to obtain, by semipreparative HPLC, a pure fraction (12% yield over the last two steps) of major cyclopeptide ***c*(RGD8)** (molecular ion at m/z 525 [M^++1]), which was sufficient for the tests and the NMR analysis.

Biological tests

The RGD-containing peptidomimetic ***c*(RGD8)** was tested for its integrin binding affinity towards M21 human melanoma cells expressing high levels of $\alpha_v\beta_3$ heterodimer[33] as reported for its isomer ***c*(RGD7)**.[28] The test was carried out in agreement with similar cell-based screening methods, using the same $\alpha_v\beta_3$ expressing cell line M21 as reported in the literature, where the reference RGD ligand *c*[RGDf(Me)V] showed an IC_{50} value of 0.4 nM.[34] Tests were performed in the presence of 2 mM $MnCl_2$, in order to switch

integrins of tumor cells into an activated form. Both peptidomimetics were also screened for their capacity to compete with fibronectin for the binding to $\alpha_5\beta_1$ integrin expressed by human erythroleukemia cell line K562.[33] The results are reported in Table 1 and Figure 3. We were pleased to find that the new peptidomimetic ***c*(RGD8)** proved around ten times more potent than ***c*(RGD7)** in inhibiting the binding of M21 melanoma cells to vitronectin (IC_{50} = 2.4 ± 1.8 nM). Interestingly, when both compounds were tested as $\alpha_5\beta_1$ integrin binders, for 5-amino-CPA derivative ***c*(RGD8)** we measured an IC_{50} value of 26 ± 18 nM close to the IC_{50} value obtained for the ***c*(RGD7)** isomer (IC_{50} = 16 ± 23 nM).

Conformational analysis

Cyclopeptide ***c*(RGD8)** was subjected to conformational analysis to assess the structural determinants leading to binding activity towards the integrins. Diluted DMSO-d_6 solutions of ***c*(RGD8)** were used for the NMR analysis in order to prevent aggregation. TOCSY, ROESY and variable temperature ^{1}H NMR experiments were carried out for the NMR analysis (Table 2 and Figure 4). Also, molecular modelling calculations were carried out to get more insight into the conformational preferences of peptide ***c*(RGD8)**.

^{1}H NMR data of diluted DMSO-d_6 solution of ***c*(RGD8)** showed two sets of signals in a 5 : 1 ratio, as a consequence of the existence of rotamers around the *N*-CO_2Me bond (Table S1, Supporting Information). The major rotamer showed amide proton chemical shift values between 7.2 and 8.9 ppm, with Asp amide proton being the more deshielded, thus suggesting its hydrogen-bonded status (Table 2). Variable temperature experiments (Figure 4) indicated low $\Delta\delta/\Delta T$ coefficients for all amide protons, suggesting the existence of intramolecular hydrogen-bonds. In contrast to ***c*(RGD7)**, Asp and Arg amide protons in ***c*(RGD8)** showed an inverted profile in terms of chemical shift and temperature coefficients, as Asp NH possesses a more deshielded chemical shift value and higher $\Delta\delta/\Delta T$ coefficient, possibly resulting from equilibrating hydrogen bonding species. Gly NH shows the lowest $\Delta\delta/\Delta T$ coefficient, indicating the involvement in strong intramolecular hydrogen bonds. Such data suggest a different intramolecular hydrogen bonding network within the two cyclic peptides ***c*(RGD7)** and ***c*(RGD8)**, contributing to a diverse arrangement of the cyclic frameworks induced by the two isomeric CPAs (Figure 5).

Molecular modelling analysis resulted in a global minimum conformer for compound ***c*(RGD8)** which indicates the role of Gly, CPA and Asp amide protons in establishing equilibrating intramolecular hydrogen-bonds (Figure 5, bottom right). Specifically, Gly NH experienced a hydrogen-bond with CPA C=O, Asp NH with Arg C=O, and CPA NH with Gly C=O, although with variable strength, thus generating two γ-turns within the cyclopeptide. This is in contrast to the conformational asset of ***c*(RGD7)**, which displayed a similarly Arg-centered γ-turn although stabilized by a β-turn having a hydrogen bond experienced by Asp NH with CPA C=O (Figure 5, bottom left). ROESY analysis of ***c*(RGD8)** (Figure 5, top right) showed a ROESY

peak between CPA NH and CPA 3-H_{ax}, and sequential ROESY peaks between Asp NH and Gly H-α, between CPA NH and Asp H-α, and between Gly NH and Arg H-α, consistent with the preferred conformation of ***c*(RGD8)** found by the modelling and in which the CPA NH points "inwards" below the scaffold's six-membered ring. Thus, ROESY peaks together with the variable temperature experiments suggest a conformation of the cyclopeptide characterized by the presentation of Asp and Arg side chains as showed in Figure 5, right. Although temperature coefficient values indicate that the hydrogen-bonded states could be in equilibrium with non hydrogen-bonded states, the existence of a conformation displaying γ-turns suggests CPA nucleating a compact structure in all equilibrating conformations. The results of the conformational analysis and the IC_{50} values for ***c*(RGD8)** and ***c*(RGD7)** indicate a close connection between conformational preferences and ligand binding affinity. Specifically, higher ligand binding affinity of ***c*(RGD8)** resulted from equilibrium between correct conformations for the RGD recognition site of the integrin, whereas ***c*(RGD7)** showed more rigid structure though not as optimal as the isomeric ***c*(RGD8)**, producing lower affinity towards $\alpha_v\beta_3$. Such differences in binding affinity was not evinced for $\alpha_5\beta_1$ integrin, suggesting that this integrin best accommodates both RGD peptidomimetics in its recognition site. In all cases, the role of CPA in nucleating the correct conformation is due to the existence of the rigid *cis* orientation of amino and carboxylic groups, which allows maintaining the required torsional angles between Asp and Arg for an optimal binding to the integrin.

Based on NMR and conformational studies on integrin ligands, some prerequisites for a robust binding have been established amongst which the distance between Arg and Asp beta carbons.[14] For $\alpha_v\beta_3$ integrin antagonists, for instance, this distance has an optimal value of 8.9 Å[35] which is the distance between the beta C atoms in potent antagonist cilengitide c(RGDf[NMe]V) as measured in the X-ray crystal structure of the complex with the $\alpha_v\beta_3$[35,36] and which leads to an extended conformation of the RGD motif and a stretched arrangement of the charged side chains. Based on our modelling, the measured distance between the two beta C atoms in ***c*(RGD8)** is 7.9 Å, that is longer than that calculated for ***c*(RGD7)** (7.1 Å) and closer to the optimal value, which could account for the higher activity of **c(RGD8)**. On the other hand, the high potency of both ***c*(RGD7)** and ***c*(RGD8)** towards $\alpha_5\beta_1$ integrin is justified by the fact that the binding mode is the same as in $\alpha_v\beta_3$ and that the requisite distance between the beta C atoms for an optimal binding with $\alpha_5\beta_1$ is in the same range.[7,14,37-39]

It is worth to notice that $\alpha_v\beta_3$ *vs.* $\alpha_5\beta_1$ selectivity is observed in many RGD-containing cyclic peptides and peptidomimetics. For example *cyclo*-(-Arg-Gly-Asp-D-Phe-Val-),[40] and compounds **1**[19] and **3**[22] (Figure 1) are all $\alpha_v\beta_3$ selective ligands, and the same Cilengitide *cyclo*-[-Arg-Gly-Asp-D-Phe-(*N*-Me)Val-] shows sub-nanomolar (0.65 nM) activity for the $\alpha_v\beta_3$ receptor and nanomolar (13.2 nM) affinity for $\alpha_5\beta_1$. Extra N-methylation of Cilengitide further increases the $\alpha_v\beta_3$ / $\alpha_5\beta_1$ selectivity by 2-3 orders of magnitudes.[41] In all these cases the selectivity has been shown to depend on the overall conformation of the cyclopeptide. Our

compounds are instead not selective, as they display the same nanomolar potency toward both integrins.[42] For cyclopeptides like **3** (Figure 1) incorporating a β-aminocyclopropane amino acid, $\alpha_v\beta_3$ selectivity has been explained on the basis of a less stretched conformation of the RGD sequence, as suggested by a shorter distance between the Cα atoms of Arg and Asp (from 5.25 to 6.50 Å in **3**). In a non-selective isomer of **3**, this distance is 6.00-7.00 Å corresponding to a more stretched conformation for the RGD sequence.[22] For **c(RGD7)** and **c(RGD8)** the distances between the Cα atoms of Arg and Asp are 6.01 and 6.07 Å, which are at the borderline of the two ranges above mentioned, and therefore the lack of selectivity cannot be correlated with such a parameter.

Conclusion

In conclusion, both 4-amino and 5-amino-cyclopropane pipecolic acids (CPAs) with *cis* relative stereochemistry between the carboxylic and the amino groups are suitable templates to prepare cyclic peptidomimetics containing the RGD sequence and which are potent ligands of both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin receptors. The RGD-containing peptidomimetic ***c*(RGD8)** built on the 5-amino-CPA displayed an inhibition activity toward $\alpha_v\beta_3$ (IC_{50} = 2.4 nM) comparable to that of the most potent antagonists reported so far and it was also around ten times more active than the corresponding ligand ***c*(RGD7)** derived from the isomeric 4-amino-CPA. Since the activity of both compounds was in the nanomolar range towards both integrins, ***c*(RGD7)** and ***c*(RGD8)** can be considered as dual ligands, which could be an advantage in these compounds finding applications in anticancer therapy. These results are encouraging in further employing these CPA templates for the synthesis of new integrin binders and, by exploiting the scaffold nitrogen atom as an anchoring point, for targeted delivery of drugs and diagnostics.

Experimental section

Chemistry General.

Chromatographic separations were performed under pressure on silica gel 60 (Merck,70–230 mesh) using flash column techniques; R_f values refer to TLC carried out on 0.25 mm silica gel plates with the same eluent indicated for column chromatography. ^{1}H NMR (500, 400 and 200 MHz) and ^{13}C NMR (50.33 and 100.4 MHz) spectra were recorded on Bruker Avance II 500 MHz Ultrashield, and Varian Inova and Mercury spectrometers in $CDCl_3$ at 25 °C unless otherwise stated. Mass spectra were carried out by direct inlet on a LCQ Fleet™ Ion Trap LC/MS system (Thermo Fisher Scientific) with an ESI interface in the positive mode. Ligand ***c*(RGD8)** was purified by Beckman-Gold HPLC system equipped with a reverse-phase semi-preparative column (Alltima C18 10 µm, 250 mm × 10 mm, Alltech). Analytical HPLC analyses were

performed on Dionex Ulltimate 3000 system equipped with a reverse-phase column (Acclaim 120, C18, 5 μm, 4.6-250 mm). Anhydrous solvents were either commercial or prepared according to standard techniques.

5-[Z-Asp(O*t*Bu)]-2-(methoxycarbonyl)-5-NHCPA-OMe [12]

DEPBT (347 mg, 1.16 mmol) and DIPEA (202 μL, 1.16 mmol) were added to a solution of Z-Asp(O*t*Bu)-OH·H_2O (273 mg, 0.80 mmol) in anhydrous THF (3.5 mL) cooled to 0 °C, under N_2 atmosphere, and the resulting mixture was allowed to rise to room temperature. After 15 min this solution was slowly added to a solution of compound **11** (92 mg, 0.40 mmol) in anhydrous THF (3 mL) pre-cooled to 0 °C. The resulting reaction mixture was stirred at 35 °C for 6 days. Afterward, EtOAc (20 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (10 mL), a satd. solution of $NaHCO_3$ (10 mL) and H_2O (10 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (*n*-hexane/EtOAc 1:1, R_f 0.23) affording pure **12** (178 mg, 84 %) as a white solid.
M.p. 62.7-66.0 °C. $[\alpha]_D^{21}$ +16.6 (*c* 1.40, $CHCl_3$) (ee 94%). ^{1}H NMR (400 MHz, $CDCl_3$) (mixture of rotamers) δ (ppm): 7.38-7.30 (m, 5 H, Ph, both rotamers), 7.00-6.90 (m, 1 H, NH CPA, both rotamers), 6.02-5.90 (m, 1 H, NH Asp, both rotamers), 5.11 (s, 2 H, CH_2Ph, both rotamers), 4.55-4.44 (m, 1 H, H_α Asp, both rotamers), 4.18-4.05 (m, 1 H, 5-H, both rotamers), 3.88-3.81 (m, 1 H, 3-H_{eq}, major rotamer), 3.75-3.62 (m, 7 H, 3-H_{eq} minor rotamer + 2 x OCH_3 both rotamers), 3.06-2.98 (m, 1 H, 3-H_{ax}, minor rotamer), 2.94-2.84 (m, 2 H, 3-H_{ax} major rotamer + H_β Asp both rotamers), 2.59 (dd, *J* = 17.2, 7.4 Hz, 1 H, H_β' Asp, both rotamers), 1.97 (dd, *J* = 10.0, 5.7 Hz, 1 H, 7-H, minor rotamer), 1.90 (dd, *J* = 10.1, 5.4 Hz, 1 H, 7-H, major rotamer), 1.68-1.56 (m, 3 H, 4-H + 4-H' + 6-H, both rotamers), 1.42 [s, 9 H, $OC(CH_3)_3$], 0.92-0.84 (m, 1 H, 7-H', both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.2, 171.2, 169.9, 156.9, 156.1, 135.9 (C_{arom}), 128.6 (2 C, C_{arom}), 128.3 (2 C, C_{arom}), 128.1 (C_{arom}), 82.0 [$OC(CH_3)_3$], 67.3 (CH_2Ph), 53.0 (OCH_3), 52.5 (OCH_3), 50.9 (C_α Asp), 43.1, 38.3, 37.9, 37.7 (C_β Asp), 29.1, 28.0 [3 C, $OC(CH_3)_3$], 27.8, 21.3. MS (ESI) m/z (%): 1089 (22) $[2M + Na]^+$, 556 (100) $[M + Na]^+$. $C_{26}H_{35}N_3O_9$ (533.57): calcd. C, 58.53; H, 6.61; N, 7.88. Found: C, 58.64; H, 6.33; N, 7.62.

(1*S*,5*R*,6*S*)-5-Azido-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [14]

A 1 N solution of NaOH (1.23 mL) was added to a solution of **13** (208 mg, 0.82 mmol) in MeOH (2 mL) and the resulting mixture was vigorously stirred for 24 h at room temperature. Afterward, the methanol was evaporated and the remaining aqueous layer was washed with Et_2O (5 mL). Then, the aqueous layer was acidified to pH 3, adding a 1 N solution of HCl, and the product was extracted with $CHCl_3$ (5 x 5 mL). Finally, the aqueous layer was further acidified to pH 1 and the product was extracted again with $CHCl_3$ (5 x 5 mL). The combined organic layers were dried over Na_2SO_4 and after filtration and evaporation of the solvent, compound **14** (183 mg, 93%) was obtained as a white solid.

$[\alpha]_D^{21}$ +30.7 (*c* 0.98, $CHCl_3$) (e.e. 94%). ^{1}H NMR (400 MHz, $CDCl_3$) (1.5:1 mixture of rotamers) δ (ppm): 10.21 (bs, 1 H, COOH), 4.04-4.00 (m, 1 H, 5-H, both rotamers), 3.89 (dt, *J* = 11.3, 4.1 Hz, 1 H, 3-H_{eq}, major rotamer), 3.78-3.69 (m, 4 H, 3-H_{eq} minor rotamer + OCH_3 both rotamers), 3.07 (t, *J* = 11.9 Hz, 3-H_{ax}, minor rotamer), 2.97 (t, *J* = 11.3 Hz, 3-H_{ax}, major rotamer), 2.06-1.87 (m, 2 H, 6-H + 7-H_{exo}, both rotamers), 1.82-1.75 (m, 1 H, 4-H, both rotamers), 1.60-1.52 (m, 1 H, 4-H', both rotamers), 0.95-0.88 (m, 1 H, 7-H_{endo}, both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 177.2 (CO), 156.9 (NCO), 54.7 (C-5), 53.2 (OCH_3), 38.0, 36.7, 28.6, 27.6, 21.5. MS (ESI) m/z (%): 239 (100) $[M - 1]^-$. $C_9H_{12}N_4O_4$ (240.22): calcd. C, 45.00; H, 5.04; N, 23.32. Found: C, 45.21; H, 4.87; N, 23.01.

(1*S*,5*R*,6*S*)-5-Amino-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [15]

10% Pd/C (24 mg) was added to a solution of **14** (183 mg, 0.76 mmol) in anhydrous MeOH (15 mL), under N_2 atmosphere. The resulting suspension was stirred vigorously under H_2 atmosphere (balloon) at room temperature. During the reaction, the resulting product precipitated, due to the formation of the zwitterion. After 24 h, H_2O (100 mL) was added and the resulting mixture was filtered over a celite layer. After evaporation of the solvent, pure **15** (123 mg, 67%) was obtained as a white solid, directly used in the next step without further purifications.

^{1}H NMR (400 MHz, D_2O) (2:1 mixture of rotamers) δ (ppm): 3.79-3.72 (m, 1 H, 3-H_{eq}, both rotamers), 3.71 (s, 3 H, OCH_3, minor rotamer), 3.70 (s, 3 H, OCH_3, major rotamer), 3.62-3.58 (m, 1 H, 5-H, major rotamer), 3.57-3.54 (m, 1 H, 5-H, minor rotamer), 3.20-3.07 (m, 1 H, 3-Hax, both rotamers), 1.84-1.77 (m, 3 H, 4-H + 4-H' + 7-H, both rotamers), 1.69-1.61 (m, 1 H, 6-H, both rotamers), 0.94-0.87 (m, 1 H, 7-H', both rotamers). MS (ESI) m/z (%): 215 (67) $[M + 1]^+$.

(1*S*,5*R*,6*S*)-5-(9-Fluorenylmethoxycarbonylamino)-2-(methoxycarbonyl)-2-azabicyclo[4.1.0]heptane-1-carboxylic acid [16]

A 10% aqueous solution of Na_2CO_3 (1.6 mL) was added to a suspension of amino acid **15** (120 mg, 0.56 mmol) in THF (1.3 mL). The resulting mixture was cooled to 0 °C and added with a solution of Fmoc-OSu (189 mg, 0.56 mmol) in THF (3.8 mL). The reaction mixture was vigorously stirred at room temperature for 24 h. Afterward, the solvent was evaporated under *vacuum* and the residue was taken up in EtOAc (6 mL). Then, a satd. solution of NH_4Cl (6 mL) was added and the product was extracted with EtOAc (5 x 6 mL). The aqueous layer was acidified to pH 2, adding a 1 N solution of HCl, and the product was extracted again with EtOAc (5 x 6 mL). The combined organic layers were dried over Na_2SO_4. After filtration and evaporation of the solvent the crude was purified by flash chromatography (MeOH/CH_2Cl_2 1:20, then MeOH/CH_2Cl_2 1:10, R_f 0.38) to afford compound **16** (197 mg, 81%) as a white solid.

M.p. 171-173 °C (dec). $[\alpha]_D^{22}$ –16.1 (*c* 1.60, $CHCl_3$) (e.e. 94%). ^{1}H NMR (400 MHz, CD_3OD) (mixture of rotamers) δ (ppm): 7.76 (d, *J* = 7.5 Hz, 2 H, Fmoc), 7.63-7.61 (m, 2 H, Fmoc), 7.36 (t, *J* = 7.5 Hz, 2 H, Fmoc), 7.28 (t, *J* = 7.5 Hz, 2 H, Fmoc), 4.40-4.32 (m, 2 H, CH_2 Fmoc), 4.16 (bs, 1 H, CH Fmoc), 3.81 (bs, 1 H, 5-H), 3.75-3.60 (m, 4 H, 3-H_{eq} + OCH_3), 3.09-3.00 (m, 1 H, 3-H_{ax}), 1.79-1.76 (m, 1 H, 7-H), 1.66-1.56 (m, 3 H, 4-H + 4-H' + 6-H), 0.78-0.70 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD_3OD) (major rotamer) δ (ppm): 159.3 (C_q), 158.8 (C_q), 158.1 (C_q), 145.3 (2 C, $C_{q,arom}$), 142.6 (2 C, $C_{q,arom}$), 128.7 (2 C, C_{arom}), 128.1 (2 C, C_{arom}), 126.1 (2 C, C_{arom}), 121.0 (2 C, C_{arom}), 67.4 (CH_2 Fmoc), 53.3 (OCH_3), 48.5 (CH Fmoc), 46.1 (C-5), 40.5 (C-4), 39.0 (C-3), 29.2 (C-6), 29.0 (C-4), 21.0 (C-7). MS (ESI) m/z (%): 435 (100) $[M - 1]^-$. $C_{24}H_{24}N_2O_6$ (436.46): calcd. C, 66.04; H, 5.54; N, 6.42. Found: C, 66.23; H, 5.29; N, 6.09.

5-Fmoc-[2-(Methoxycarbonyl)-5-NHCPA]-Arg(Mtr)-Gly-OBn [17]

DEPBT (138 mg, 0.46 mmol) and DIPEA (80 μL, 0.46 mmol) were added to a solution of **16** (100 mg, 0.23 mmol) in anhydrous THF (2 mL) cooled to 0 °C, under N_2 atmosphere, and the resulting mixture was allowed to rise to room temperature. After 15 min the reaction was cooled again to 0 °C and a solution of H-Arg(Mtr)-Gly-OBn (184 mg, 0.35 mmol) in anhydrous THF (2.5 mL) was added. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (35 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (2 x 9 mL), a satd. solution of $NaHCO_3$ (2 x 9 mL) and H_2O (2 x 9 mL). The organic layer was dried on Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (EtOAc, R_f 0.59) affording pure **17** (172 mg, 79 %) as a white solid.

M.p. 110-130 °C (dec.). $[\alpha]_D^{21}$ –5.8 (*c* 0.80, $CHCl_3$). ^{1}H NMR (400 MHz, CD_3OD) (2:1 mixture of rotamers) δ (ppm): 8.41-8.36 (m, 1 H, NH Gly, minor rotamer), 8.15 (bs, 2 H, NH Gly + NH Arg, major rotamer), 7.90-7.86 (m, 1 H, NH Arg, minor rotamer), 7.76 (d, *J* = 7.4 Hz, 2 H, Fmoc, both rotamers), 7.60 (t, *J* = 7.1 Hz, 2 H, Fmoc, both rotamers), 7.37-7.25 (m, 9 H, Fmoc + Ph, both rotamers), 6.65 (s, 1 H, CH Mtr, minor rotamer), 6.60 (s, 1 H, CH Mtr, major rotamer), 5.17-5.12 (m, 2 H, C*H*$_2$Ph, both rotamers), 4.41-4.35 (m, 3 H, H_α Arg + CH_2 Fmoc, both rotamers), 4.17-4.05 (m, 2 H, H_α Gly + CH Fmoc, both rotamers), 4.02-3.84 (m, 2 H, H_α Gly + 5-H, both rotamers), 3.81 (s, 3 H, OCH_3 Mtr, minor rotamer), 3.79-3.74 (m, 4 H, 3-H_{eq} both rotamers + OCH_3 Mtr major rotamer), 3.66 (bs, 3 H, OCH_3, both rotamers), 3.23-2.90 (m, 3 H, 3-H_{ax} + H_δ Arg, both rotamers), 2.67 (s, 3 H, CH_3 Mtr, minor rotamer), 2.66 (s, 3 H, CH_3 Mtr, major rotamer), 2.61 (s, 3 H, CH_3 Mtr, minor rotamer), 2.59 (s, 3 H, CH_3 Mtr, major rotamer), 2.11 (s, 3 H, CH_3 Mtr, minor rotamer), 2.07 (s, 3 H, CH_3 Mtr, major rotamer), 1.99-1.42 (m, 8 H, 4-H + 4-H' + 6-H + 7-H + H_β Arg + H_γ Arg, both rotamers), 0.81-0.72 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD_3OD) (major rotamer) δ (ppm): 174.8, 173.8, 171.7, 159.8, 159.1, 158.1, 157.9, 145.3 (2 C, C_{arom}), 142.6 (2 C, C_{arom}), 139.5 (C_{arom}), 137.8 (C_{arom}), 137.1 (C_{arom}), 134.8 (C_{arom}), 129.6 (2 C, C_{arom}), 129.3 (C_{arom}), 128.7 (2 C, C_{arom}), 128.3 (C_{arom}), 128.1 (C_{arom}), 128.0 (2 C, C_{arom}), 126.1 (2 C, C_{arom}), 125.7 (C_{arom}), 120.9 (2 C, C_{arom}), 112.7 (C_{arom}), 68.0 (*C*H_2Ph), 67.5 (CH_2 Fmoc), 55.9 (OCH_3 Mtr), 54.6, 53.9 (OCH_3),

48.5 (CH Fmoc), 45.2, 44.8, 42.0 (C_α Gly), 41.7, 39.5, 29.8, 29.3, 28.9, 27.1, 24.4 (CH_3 Mtr), 20.8, 18.9 (CH_3 Mtr), 12.1 (CH_3 Mtr). MS (ESI) m/z (%): 1926 (9) $[2M + Na]^+$, 974 (100) $[M + Na]^+$.

5-Amino-[2-(methoxycarbonyl)-5-NHCPA]-Arg(Mtr)-Gly-OBn [18]

Compound **17** (170 mg, 0.18 mmol), was dissolved in a 1:1 CH_2Cl_2/DEA mixture (2.25 mL), under N_2 atmosphere. The resulting solution was stirred at room temperature for 4 h, meanwhile additional 1:1 DCM/DEA mixture (1.13 mL) was added. Afterward, the solution was concentrated under *vacuum*, the residue was taken up in CH_2Cl_2 (6 mL) and then concentrated again. The crude was purified by flash chromatography (EtOAc, then MeOH/EtOAc 1:1, R_f 0.18), affording pure **18** (129 mg, 99 %) as a white solid.
M.p. 160 °C (dec.). $[\alpha]_D^{19}$ –3.6 (*c* 0.92, CH_3OH). ^{1}H NMR (400 MHz, CD_3OD) (major rotamer) δ (ppm): 7.35-7.30 (m, 5 H, Ph), 6.65 (s, 1 H, CH Mtr), 5.16 (s, 2 H, C*H*$_2$Ph), 4.42-4.39 (m, 1 H, H_α Arg), 4.12-3.90 (m, 2 H, H_α Gly), 3.82 (s, 3 H, OCH_3 Mtr), 3.71-3.60 (m, 4 H, 3-H_{eq} + OCH_3), 3.35-3.10 (m, 4 H, 3-H_{ax} + 5-H + H_δ Arg), 2.67 (s, 3 H, CH_3 Mtr), 2.61 (s, 3 H, CH_3 Mtr), 2.12 (s, 3 H, CH_3 Mtr), 1.98-1.82 (m, 3 H, 7-H + H_γ Arg), 1.71-1.48 (m, 5-H, 4-H + 4-H' + 6-H + H_β Arg), 0.80-0.72 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, CD_3OD) (major rotamer) δ (ppm): 174.8, 174.0, 171.9, 159.8, 158.2, 142.7 (C_{arom}), 139.4 (C_{arom}), 137.8 (C_{arom}), 137.1 (C_{arom}),, 134.8 (C_{arom}), 129.5 (2 C, C_{arom}), 129.3 (C_{arom}), 128.3 (C_{arom}), 125.7 (2 C, C_{arom}), 112.7 (C_{arom}), 68.0 (*C*H_2Ph), 56.0 (OCH_3 Mtr), 54.5, 53.9 (OCH_3), 45.0, 42.0, 41.8, 39.0, 31.4, 30.7, 30.3, 29.3, 27.0, 24.3 (CH_3 Mtr), 20.9, 18.8 (CH_3 Mtr), 12.1 (CH_3 Mtr). MS (ESI) m/z (%): 1459 (18) $[2M + 1]^+$, 752 (17) $[M + Na]^+$, 731 (42), 730 (100) $[M + 1]^+$.

5-[Z-Asp(O*t*Bu)]-2-(methoxycarbonyl)-5-NHCPA-Arg(Mtr)-Gly-OBn [19]

DEPBT (108 mg, 0.36 mmol) and DIPEA (63 μL, 0.36 mmol) were added to a solution of Z-Asp(O*t*Bu)-OH·H_2O (85 mg, 0.25 mmol) in anhydrous THF (2 mL) cooled to 0 °C, under N_2 atmosphere, and the resulting mixture was allowed to rise to room temperature. After 15 min this solution was slowly added to a solution of compound **18** (120 mg, 0.16 mmol) in anhydrous THF (1 mL) pre-cooled to 0 °C. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (20 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (2 x 5 mL), a satd. solution of $NaHCO_3$ (2 x 5 mL) and H_2O (2 x 5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (EtOAc, R_f 0.40) affording pure **19** (84 mg, 49 %) as a white solid.
M.p. 110 °C (dec.). $[\alpha]_D^{21}$ +22.4 (*c* 0.84, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (major rotamer) δ (ppm): 7.78 (bs, 1 H, NH Gly), 7.68-7.40 (m, 1 H, NH CPA), 7.36-7.12 (m, 10 H, 2 x Ph), 7.18-7.05 (m, 1 H, NH Arg), 6.50 (s, 1 H, CH Mtr), 6.28-5.96 (m, 4 H, NH_ε Arg + NH Asp), 5.16-4.96 (m, 4 H, 2 x C*H*$_2$Ph), 4.61-4.40 (m, 2 H, H_α Asp + H_α Arg), 4.26 (bs, 1 H, 5-H), 4.16-3.98 (m, 2 H, H_α Gly), 3.90-3.74 (m, 4 H, 3-H_{eq} + OCH_3 Mtr), 3.64 (s, 3 H, OCH_3), 3.28-2.90 (m, 3 H, 3-H_{ax} + H_δ Arg), 2.75-2.65 (m, 5 H, H_β Asp + CH_3 Mtr), 2.60 (s, 3 H, CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 2.04-1.86 (m, 3 H, 7-H + H_β Arg), 1.78-1.50 (m, 5 H, 4-H + 4-H' + 6-H + H_γ Arg), 1.37 (s, 9 H,

$OC(CH_3)_3$), 0.72-0.68 (m, 1 H, 7-H'). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major rotamer) δ (ppm): 172.4, 171.6, 170.2, 169.8, 158.3, 157.5, 156.3, 156.0, 138.4 (C_{arom}), 136.4 (C_{arom}), 136.1 (C_{arom}), 135.1 (C_{arom}), 135.0 (C_{arom}), 133.3 (C_{arom}), 128.5 (2 C, C_{arom}), 128.4 (2 C, C_{arom}), 128.3 (C_{arom}), 128.2 (2 C, C_{arom}), 128.1 (2 C, C_{arom}), 127.8 (C_{arom}), 124.7 (C_{arom}), 111.6 (CH_{arom}), 81.4 [$OC(CH_3)_3$], 67.0 (CH_2Ph), 66.8 (CH_2Ph), 55.3 (OCH_3 Mtr), 53.3 (OCH_3), 52.8 (C_α Arg), 51.3 (C_α Asp), 42.7 (C-5), 41.1 (C_α Gly), 40.4 (C_δ Arg), 38.3 (C-3), 37.9 (C_β Asp), 29.6 (C_β Arg), 29.1, 28.3 (C-6), 27.8 [3 C, $OC(CH_3)_3$], 27.4 (C-4), 25.1 (C_γ Arg), 24.0 (CH_3 Mtr), 20.0 (C-7), 18.3 (CH_3 Mtr), 11.8 (CH_3 Mtr). MS (ESI) m/z (%): 1057 (100) $[M + Na]^+$, 1035 (16) $[M + 1]^+$.

Cyclo[Arg(Mtr)-Gly-Asp(O*t*Bu)-2-(methoxycarbonyl)-5-NHCPA] [21]

10% Pd/C (30 mg) was added to a solution of **19** (84 mg, 0.08 mmol) in ethanol (4 mL), under N_2 atmosphere. The resulting suspension was stirred under H_2 atmosphere (balloon) at room temperature for 24 h. After filtration over a celite layer and evaporation of the solvent, compound **20** (59 mg, 91%) was obtained as a white solid. This crude was suspended in THF (20 mL), under N_2 atmosphere. The suspension was cooled to 0 °C and DEPBT (64 mg, 0.21 mmol) and DIPEA (37 μL, 0.21 mmol) were added. The resulting reaction mixture was stirred at 35 °C for 4 days. Afterward, EtOAc (10 mL) was added and the mixture was washed with a satd. solution of NH_4Cl (5 mL), a satd. solution of $NaHCO_3$ (5 mL) and H_2O (5 mL). The organic layer was dried on Na_2SO_4, filtered and evaporated under *vacuum*. The residue was purified by flash chromatography (EtOAc, then MeOH/EtOAc 1:4, R_f 0.60) affording **21** in mixture with other unidentified products (21 mg) and as a white solid.

^1H NMR (400 MHz, CD_3OD) (major product, major rotamer) δ (ppm): 6.66 (s, 1 H, CH Mtr), 4.69-4.61 (m, 1 H, H_α Asp), 4.53-4.43 (m, 1 H, 5-H), 4.30-4.23 (m, 1 H, H_α Arg), 4.07-3.96 (m, 1 H, H_α Gly), 3.93-3.80 (m, 4 H, 3-H_{eq} + OCH_3 Mtr), 3.78-3.63 (m, 3 H, OCH_3), 3.46 (d, *J* = 13.6 Hz, 1 H, H_α Gly), 3.23-3.07 (m, 2 H, H_δ Arg), 2.94-2.76 (m, 3 H, 3-H_{ax} + H_β Asp), 2.65 (s, 3 H, CH_3 Mtr), 2.62-2.45 (m, 4 H, H_β Asp + CH_3 Mtr), 2.11 (s, 3 H, CH_3 Mtr), 1.96-1.47 (m, 7 H, 4-H + 4-H' + 7-H + H_β Arg + H_γ Arg), 1.41 (s, 9 H, $OC(CH_3)_3$), 1.33-1.22 (m, 1 H, 6-H), 0.87-0.66 (m, 1 H, 7-H'). MS (ESI) m/z (%): 815 (36), 794 (37), 793 (100) $[M + 1]^+$.

Cyclo[Arg-Gly-Asp-2-(methoxycarbonyl)-5-NHCPA]·TFA [c(RGD8)]

Protected tetrapeptide **21** (21 mg, 0.026 mmol) was dissolved in a 95:2.5:2.5 TFA/TIS/H_2O mixture (1.25 mL) and the resulting solution was stirred at room temperature for 18 h. Afterward, the mixture was evaporated under vacuum and the residue was taken up in H_2O (2 mL) and washed with Et_2O (4 x 700 μL). Then, the aqueous layer was concentrated under *vacuum*, affording the deprotected cyclic tetrapeptide as a trifluoroacetate salt. This crude was purified by semi-preparative HPLC (C_{18} column, 10 μm, 250 mm × 10 mm) using acetonitrile (0.1% TFA) in H_2O (0.1% TFA), 0-50% linear gradient over 35 min at room temperature. A flow rate of 2 mL/min was used and detection was at 223 nm. HPLC R_t = 25.4 min. The HPLC

sample was concentrated under vacuum and lyophilized, affording pure ***c*(RGD8)** (5.6 mg, 12% from **20**) as a colourless glassy solid. Purity checked by HPLC analysis (C18 column, 5 µm , 4.6-250 mm), using acetonitrile (0.1% TFA) in water (0.1% TFA) as eluant, 5-35% linear gradient over 35 min at room temperature.
^{1}H NMR (500 MHz, DMSO) (5:1 mixture of rotamers) (major rotamer) δ (ppm): 8.88 (d, *J* = 7.6 Hz, NH Asp), 7.77 (d, *J* = 8.1 Hz, NH CPA), 7.53-7.47 (m, NH Arg), 7.24-7.20 (m, NH Gly), 7.19-6.64 (m, 4 H, NH_ε Arg), 4.52-4.46 (m, 1 H, H_α Asp), 4.16-4.11 (m, 1 H, 5-H), 4.04-3.95 (m, 1 H, H_α Gly), 3.86-3.79 (m, 1 H, 3-H_{eq}), 3.66-3.59 (m, 4 H, H_α Arg + OCH_3), 3.34-3.26 (m, 1 H, H_α Gly), 3.12-3.02 (m, 2 H, H_δ Arg), 2.99-2.87 (m, 1 H, 3-H_{ax}), 2.82 (dd, *J* = 16.5, 9.0 Hz, 1 H, H_β Asp), 2.45 (dd, *J* = 16.5, 6.1 Hz, 1 H, H_β Asp), 1.99-1.81 (m, 2 H, H_β Arg), 1.67-1.60 (m, 1 H, 7-H), 1.55-1.46 (m, 2 H, 4-H), 1.44-1.36 (m, 2 H, H_γ Arg), 1.36-1.29 (m, 1 H, 6-H), 0.83-0.71 (m, 1 H, 7-H'). MS (ESI) m/z (%): 526 (26), 525 (100) $[M + 1]^+$. $C_{23}H_{33}F_3N_8O_{10}$ (638.55): calcd. C, 43.26; H, 5.21; N, 17.55. Found: C, 43.48; H, 5.02; N, 17.23.

Biological assays

$\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin binding assays

Cell lines and culture conditions. The M21 human melanoma cell line (for $\alpha_v\beta_3$ binding assays) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Melanoma cells were grown in Dulbecco's modified Eagle medium, containing 4500 mg/L glucose (DMEM 4500, GIBCO) supplemented with 10% foetal calf serum (FCS) at 37 °C in a humidified incubator containing 10% CO_2. $5x10^5$ melanoma cells were seeded in 100 mm Sarstedt dishes and propagated every 3 days by incubation with a trypsin−EDTA solution. The human erythroleukemia cell line K562 (for $\alpha_5\beta_1$ binding assays) was maintained in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO) supplemented with 10% FCS in T25 culture flasks (Sarstedt) in humidified incubator at 37°C, 5% CO_2. When cultures reached a cell density between $1x10^5$ and $1x10^6$ cells/mL cells were re-suspended in warm fresh media at a volume to yield a density of $2x10^5$ cells/mL. Both M21 and K562 cultures were periodically monitored for mycoplasma contamination.

Citofluorimetric assay. M21 cells were detached by gentle treatment with Accutase (Lonza), a 0.5 mM EDTA solution. K562 cells were removed from culture flasks and re-suspended in fresh medium. Cells were then washed, and incubated for 1 h at 4°C in the presence of anti-$\alpha_v\beta_3$ monoclonal antibody (1 µg/50 µL, anti-integrin $\alpha_v\beta_3$, clone LM609, Millipore), anti-$\alpha_v\beta_5$ monoclonal antibody (1 µg/50 µL, anti-integrin $\alpha_v\beta_5$, Santa Cruz 13588) and anti $\alpha_5\beta_1$ monoclonal antibody (1 µg/50 µL, anti-integrin $\alpha_5\beta_1$, Abcam ab75472) . Cells were then washed and incubated for 1 h at 4 °C with a specific secondary antibody, 5 µg/mL goat antimouse IgG conjugated with FITC (Santa Cruz Biotecnology, Inc., Santa Cruz, CA). Integrin-Positive cells were analyzed at 488 nm on the flow cytometer FACScan system (BD-FACS Canto).

Cell adhesion assay. The plates (96 wells) containing M21 (for $\alpha_v\beta_3$ binding assays) or K562 (for $\alpha_5\beta_1$ binding assays) cells were coated with vitronectin (for $\alpha_v\beta_3$ binding assays, 10 μg/mL) or fibronectin (for $\alpha_5\beta_1$ binding assays, 10 μg/mL) (both from Sigma) by overnight incubation at 4 °C. Plates were washed with PBS and then incubated at 37 °C for 1 h with PBS−1% BSA. After being washed cells were counted and re-suspended in serum free medium, and exposed to compound (final concentration was in the 30 μM to 1 nM range for $\alpha_5\beta_1$ binding assays, and in the 30 μM to 10 nM range for $\alpha_5\beta_1$ binding assays) at 37 °C for 30 min to allow the ligand-receptor equilibrium to be reached. Assays were performed in the presence of 2 mmol/L $MnCl_2$. Cells were then plated (4−5×10^4 cells/well) and incubated at 37 °C for 1 h. All the wells were washed with PBS to remove the non-adherent cells, and 0.5% crystal violet solution in 20% methanol was added. After 2 h of incubation at 4 °C, plates were examined at 540 nm in a counter ELX800 (Bio TEK Instruments). Experiments were conducted in triplicate and were repeated at least three times. The values are expressed as % inhibition ± SEM of cell adhesion relative to untreated cells.

Data Analysis. The IC_{50} values were determined by fitting binding inhibition data by non-linear regression using GraphPad Prism 4.0 Software Package (GraphPad Prism, San Diego, CA).

NMR Methods

NMR experiments on diluted DMSO-d_6 solutions of ***c*(RGD8)** were performed at a temperature of 298 K on a Varian Mercury 400 MHz NMR spectrometer and on a Bruker Avance II 500 MHz Ultrashield. All proton chemical shifts were assigned unambiguously for ***c*(RGD8)**. Variable temperature 1D, and 2D experiments (TOCSY, gCOSY, ROESY) were carried out at the sample concentration of 3 mM for ***c*(RGD8)**. One-dimensional ^{1}H NMR spectra for determining temperature coefficients were obtained at 298–323 K with increments of 5 K. Sample temperatures were controlled with the variable-temperature unit of the instrument. Proton signals were assigned via TOCSY spectra, and ROESY spectra provided the data discussed in the conformational analysis. TOCSY spectra were recorded with 2048 points in t1, 256 points in t2, and 16 scans per t2 increment and using a mixing time of 80 ms. ROESY spectra were recorded with a similar number of t1 and t2 points unless otherwise noted, and 64 scans per t2 increment, and using a spinlock of 0.2 s.

Molecular Modelling

Molecular modeling calculations were carried out within the framework of Macromodel v6.5,[43] using Amber*[44] as a force field and the implicit water GB/SA solvation model of Still et al.[45] Monte Carlo energy minimization (MCEM)[46] conformational searches of the peptide analogue containing methyl groups instead of the Arg and Asp side chains were performed as the first step. The torsional space of each AGA

cyclopeptide was randomly varied with the usage-directed Monte Carlo conformational search. Ring-closure bonds were defined in the CPA ring and in the cyclopeptide ring. Amide bonds were included among the rotatable bonds. For each search, at least 1000 starting structures for each variable torsion angle were generated and minimized until the gradient was less than 0.05 kJ/Åmol using the truncated Newton-Raphson method implemented in Macromodel. Duplicate conformations and those with an energy greater than 6 kcal/mol above the global minimum were discarded.

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