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To be cited as: *ChemBioChem* 10.1002/cbic.201600642

Link to VoR: http://dx.doi.org/10.1002/cbic.201600642

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Lineal and Cyclic Depsipeptidomimetics with a β-Lactam Core: A Class of new $\alpha_v\beta_3$ Integrin Receptor Inhibitors

Nerea Zabala-Uncilla,[a] José I. Miranda, [b] Antonio Laso, [c] Jose I. Ganboa,*[a] and Claudio Palomo*[a]

Abstract: The $\alpha_v\beta_3$ integrin receptor plays an important role in tumor metastasis and tumor-induced angiogenesis. The inhibition of this receptor using diverse ligands, antibodies or cyclic peptides, is a promising research field for the treatment of a variety of tumors. The replacement of Phe-(Me)Val dipeptide by a β-lactam ring in Cilengitide leads to new products that show higher inhibitory activity respect to parent cyclopeptide. In particular, substitution of a peptide bond β-lactam-NH–Asp by one β-lactam-O–Asp ester linkage, increases the activity of the new cyclodepsipeptide. In the same way it has been found that open-chain compounds, Asp–β-lactam–Arg, can interact with the receptor and inhibit its activity moderately. The integrin inhibitory activity of synthesized compounds has been established by using CGH array, a method that appears to be more reliable trial than the classical adhesion test.

Introduction

The discovery that tumor growth and metastasis are codependent on the formation of neovascularization revealed many potential protein targets for cancer treatment.[1] In other pathological states as diabetic retinopathy and chronic inflammatory disorders abnormal angiogenesis is observed too.[2] The angiogenic process depends on a family of highly conserved adhesion molecules, known as integrins, central compounds for regulation of these processes.[3,4] In particular, integrin $\alpha_v\beta_3$ is one of the most well-characterized integrin heterodimers and it is one of the several heterodimers that have been implicated in tumor-induced angiogenesis.[5] While sparingly expressed in mature blood vessels, is significantly upregulated during angiogenesis "in vivo".[6] The overexpression of $\alpha_v\beta_3$ correlates with the aggressiveness of the tumor.[7] The inhibition of this $\alpha_v\beta_3$-receptor is important, induces apoptosis of the proliferative vascular cells[8] and prevent tumor growth and metastasis.[9]

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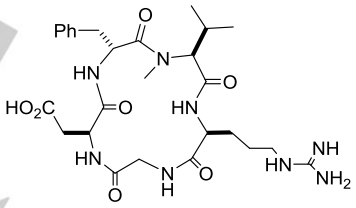
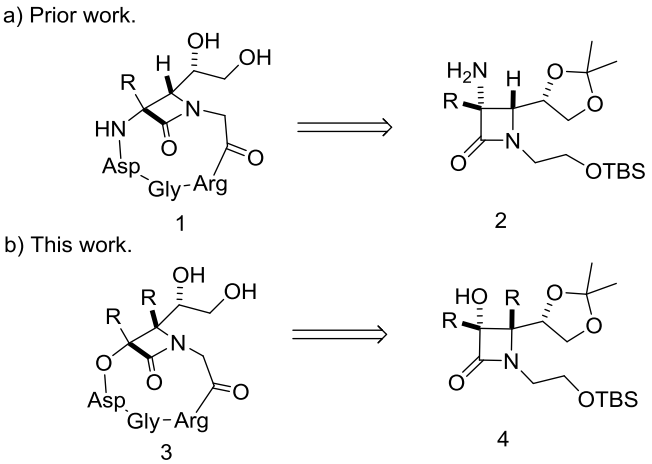
The most common integrins, including $\alpha_v\beta_3$, recognize the tripeptide Arg-Gly-Asp (RGD) sequence found in many extracellular matrix adhesive proteins.[10] The spatial layout of the RGD (rigidity, 3D disposal, possibility of interaction with integrin binding pockets…) determines the specificity and efficacy of interaction.[11] Whilst cyclic RGD peptides[12] and several RGD cyclic lactam mimetics[13] have been developed, the product EMD 121974 called Cilengitide[14] seems to be one of the most potent inducers (Scheme 1) of endothelial apoptosis,[15] inhibits angiogenesis and blocks metastasis[16].



Scheme 1. EMD 121974 (Cilengitide®) structure.

In an effort to found new bioactive compounds a prior work from this laboratory has documented that β-lactam based cyclic RGD peptides are promising candidates.[17] In particular cyclic product **1**, which is readily available from the α-amino β-lactam **2**, shows antagonist activity against $\alpha_v\beta_3$ somewhat higher than Cilengitide.[27] Herein we report that cyclodepsipeptides **3**, which can be prepared from the respective α-hydroxy β-lactam **4,** are also promising new candidates for a greater inhibitory capacity.



Scheme 2. RGD mimetics from β-lactams as constraining elements.

It has been reported that the replacement of an amide bond of the peptidomimetic by an ester linkage can modify the internal

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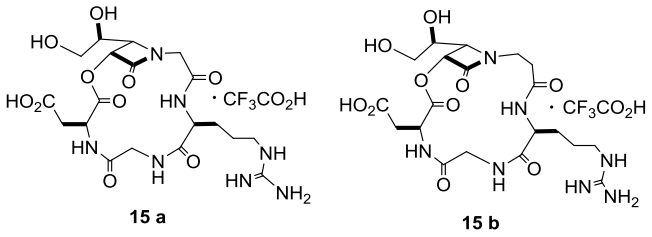
hydrogen bond interactions as well as the preferential torsion angles[18] and therefore the active conformations[19] of the native peptide. This approach, called "Ester-Scan" approach, has been applied to several cyclopeptidomimetics to modify the conformational ensemble and populations distribution, changing H-bonding pattern and torsional preferences.[20] Moreover, whilst increasing the structural flexibility of a given cyclopeptide a weaker interaction with the receptor can be produced[21], it has been proposed that a certain degree of flexibility is needed to adopt the bioactive conformation[22], in this latter case appropriate modulation of the architecture of depsipeptidomimetics is facilitated in order to achieve high receptor affinity.[19] Given these observations we decided to prepare the cyclodepsipeptides **15a** and **15b,** the first members of **3** type cyclodepsipeptides, to evaluate their activity profiles.



Scheme 3. Cyclodepsipeptides **15a,b** starting from a 3-HO-β-lactam**.**

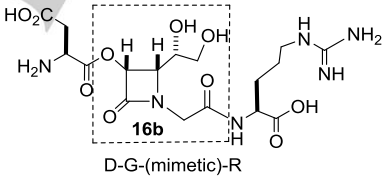
Results and Discussion.

Synthesis of cyclopeptides.

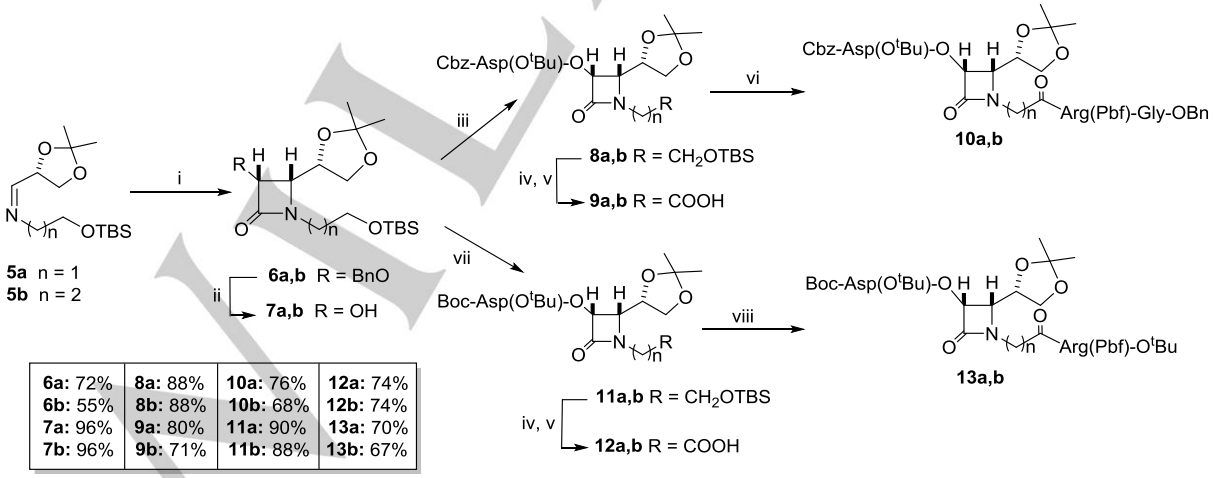
The starting β-lactams **6a** and **6b** were prepared as essentialy single diastereomers by using the standard cycloaddition between benzyloxyacetyl chloride and imines **5a** and **5b** in the presence of triethyamine followed by hydrogenolysis of the benzyloxy group[23]**.** With α-hydroxy-β-lactams **6a-b** in hand cyclodepsipeptides **15a**,**b** were synthesized as shown in Schemes 5 and 6. Each azetidin-2-one was coupled with Cbz-Asp(O^{t}Bu)-F[24] and the resulting products were subjected to desilylation and subsequent oxidation with BAIB/TEMPO to furnish the carboxylic acids **9a** and **9b**, respectively. Coupling of β-lactams **9a**,**b** with H_2N-Arg(Pbf)-Gly-OBn provided pseudopeptides **10a,b**. In a similar way **13a**,**b** were prepared from **7a**,**b** via coupling of carboxylic acids **12a**,**b** with H_2N-Arg-(Pbf)-O^{t}But.

Next, after deprotection of the Cbz and benzyloxy groups by hydrogenolysis, intramolecular HATU/HOBt[25] mediated peptide coupling between the glycine carboxy terminus and the aspartic amino group under high dilution conditions afforded the corresponding cyclized products **14a,b**, which were purified by column chromatography and isolated in yields between 45–65% range. The purity of cyclic and acyclic compounds was about 98% as determined using the UPLC/MS technique. After examining several conditions to perform complete deprotection in a single pot operation, a modification of the method described by Mehta et al[26]. was found to be the most appropriate. The resulting **15a,b** were isolated[27] by successive precipitations with diisopropyl ether, centrifugation and a final washing of the resulting white solid compound with more diisopropyl ether.

For comparative purpouses open-chain compounds, **16a**,**b**, were also prepared as shown in Scheme 6. These compounds (Scheme 6) as well as the cyclized products, **15a**,**b**, were then subjected to biological tests. The absence of recognition RGD sequence in open chain depsipeptides led us to expect poor or no activity. These compounds showed appreciable activity[28], as confirmed by qRT-PCR, and can be considered as DGR mimetic units, where the β-lactam is an extra residue.



Scheme 4. DGR compound with β-lactam as an extra residue.



6a: 72%	**8a:** 88%	**10a:** 76%	**12a:** 74%
6b: 55%	**8b:** 88%	**10b:** 68%	**12b:** 74%
7a: 96%	**9a:** 80%	**11a:** 90%	**13a:** 70%
7b: 96%	**9b:** 71%	**11b:** 88%	**13b:** 67%

Scheme 5. Preparation of macrocycle linear precursors. Reagents and conditions: i) $BnOCH_2COCl$, TEA, –78 °C → r.t., 16 h; ii) HCO_2NH_4, Pd/C (10%), MeOH reflux, 1 h; iii) Cbz-Asp(O^{t}Bu)-F, TEA, CH_2Cl_2, r.t., 30 min; iv) PyrHF, THF, 0 °C → r.t., 3 h; v) TEMPO, BAIB, MeCN/H_2O, r.t., 4 h; vi) H_2N-Arg(Pbf)-Gly-OBn (**17**), HOBt, EDC·HCl, TEA, CH_2Cl_2, 0 °C → r.t., 16 h; vii) Boc-Asp(O^{t}Bu)-F, Pyridine, THF, 0 °C → r.t.; viii) H_2N-Arg(Pbf)-O^{t}Bu, EDC·HCl, HOBt, TEA, CH_2Cl_2, 0 °C → r.t.

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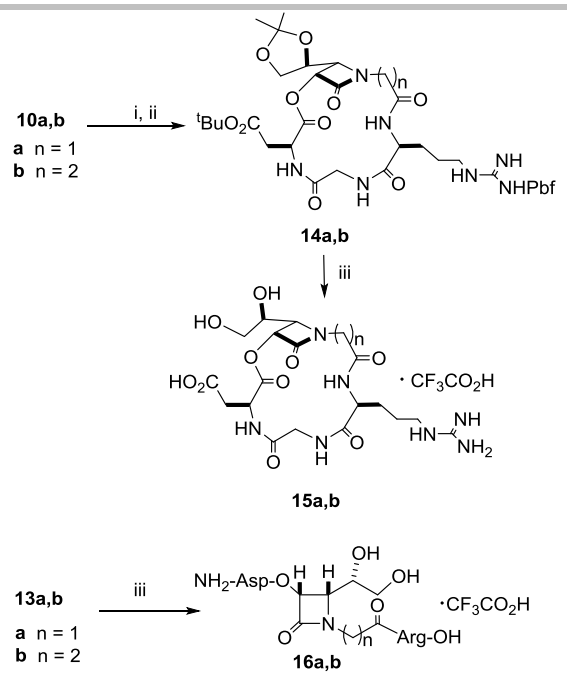


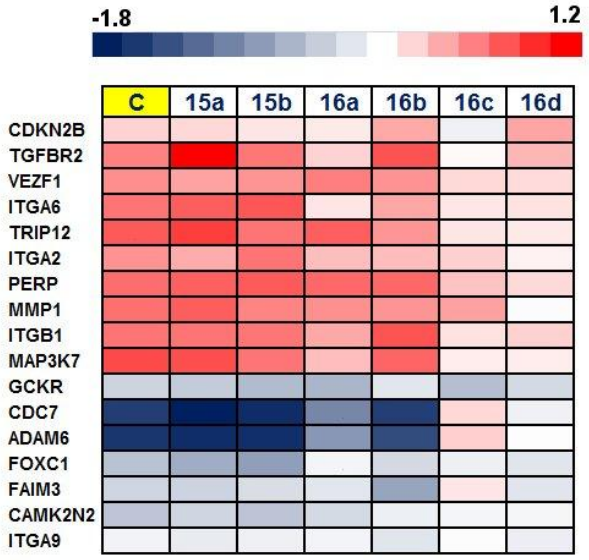
Scheme 6. Preparation of cyclodepsipeptidomimetics and depsipeptidomi metics. Reagents and conditions: i) H_2 (1 atm), Pd/C (10%), THF/EtOAc (1:1), r.t., 16 h; ii) HATU, HOAt, $KHCO_3$, DMF, –15 °C, 16 h; iii) Et_3SiH, TFA., H_2O, 4 °C, 16 h.

Bioactivity of the β-lactam based cyclopeptides

In order to determine the angiogenesis antagonist activity towards integrin $\alpha_v\beta_3$, many authors have used a binding test based on the capacity to inhibit the extracelular adhesion of a set of cellular line, typically HUVEC. [29] Recently we have observed that the inhibition assay of extracelular adhesion is not always directly related to the activation/deactivation of the genes responsible for controlling the inhibition of a specific integrin. Specifically, it was found a tetrapeptidomimetic β-lactam[30] that markedly inhibits the extracellular adhesion in a culture of HUVEC cells and, in its turn, acts as an angiogenic agonist of $\alpha_v\beta_3$ integrin. From these data it appears that quantification of the extracellular adhesion phenomenon is not enough to determine the inhibitory activity of a given compound. To evaluate the intracellular activity of genes involved in angiogenesis and inhibition of integrin $\alpha_v\beta_3$ the most accurate path would be the use of the comparative genomic hybridization (CGH)[31] technique. This technique enables the determination of the complete genome of HUVEC cells and the quantitative analysis of the activation/deactivation date in specific genes to compare these results with integrin inhibition related genes found in literature. Thus HUVEC cells were treated separately with a 10^{-2} mM concentration of compounds **15a,b**/**16a,b** and Cilengitide was used as a reference standard. After 48 h of incubation the cells were removed from culture and RNA was extracted. For data analysis of gene expression an Agilent G2505-B Array scanner was used.

The simultaneous analysis of all human gene expression by CGH array allowed us to identify the genes affected after the adhesion of compounds to be then tested on $\alpha_v\beta_3$ integrin receptors, overexpressed in HUVEC cells. Through analysis of the whole 20,500 human genes, the microarray assay provided us a two-color output image of normalized gene-expression data.[32] Genes that were at least 1.5 fold differentially expressed on 3 of 4 arrays were scored as significant. From these transcriptomics analysis we could identify up to 230/217 activated genes and 90/62 inhibited genes in analyzed compounds after the RGD mimetic cell-treatment. Among all the up or down-regulated genes, we selected for a more detailed analysis 17 genes, which can be seen in Table 1, known to be related to angiogenesis.[33] Ten of these genes were activated and seven were blocked after treatment with Cilengitide. The pattern of gene-expression was mainly conserved (17/15 genes) for the remaining compounds. On the other hand, the lack of correlation in the expression of 2/3 genes (CDC7, ADAM6 and FAIM3) demonstrated the highly specific "in-vivo" cell activity after ligand-receptor binding.

Table 1. Angiogenesis-related gene regulation of DNA samples extracted from HUVEC cells after treatment with Cilengitide® C, RGD β-lactam ligands **15a,b** and open chain compounds **16a-d**. Numbers refer to a binary logarithmic scale and colors refer to activation (red) or inhibition (blue). For numerical values, gene hierarchical clustering and gene function see ref. 29.



As positive control we chose the EMD 121974 (Cilengitide®), the magnitude of the activation or inhibition in each gene is determined by direct comparison with the pattern obtained from Cilengitide® (see supporting). Array gene expression qualitative data were reconfirmed using gene-specific quantitative mRNA assays, qRT-PCR, analyzing the pattern expression in a selected gene set (TGFBR2, TRIP12, MMP1, ITGA9, FOXC1), over each tested compound, using specific amplification primers and hybridization probes. Data consistency between quantitative qRT-PCR analysis and CGH array validates the process and results.[32] In addition, no significant changes were observed in the gene expression of the apoptosis associated tyrosine kinase (AATK)[34] and the apoptotic regulator BAX (BL2 associated X protein)[35] genes, suggesting that apoptosis was not induced after 72 h of treatment.

As can be seen in Table 2, K_i values obtained by theoretical calculations, Autodock 4.2 release 4.2.5,[36] show very similar values for cyclic compounds, **15a** and **15b**, indicating an

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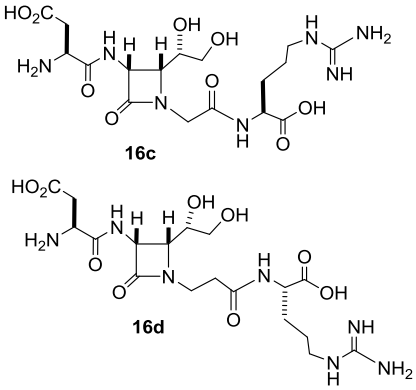
effective interaction of the substrates with the receptor in docking experiment. Cilengitide shows the best value for K_i, but this excellent data is not corroborated by IC_{50} and gene expression experimental data. Moreover open chain compounds show very low values of K_i that do not fit with the moderated experimental values of activity. We can conclude that experimental model of docking based on the use of extracelular domain of $\alpha_v\beta_3$ protein crystallized with Cilengitide[37] as receptor model is relatively effective with cyclic depsipeptides and has some predictive value on their activity but in open chain compounds docking predictions are far from experimental data and not allow anticipate real activity.

For comparative purposes we prepared the corresponding open chain cyclopeptidomimetics **16c** and **16d** from the respective 3-amino-β-lactams[32].



Scheme 7. Cyclopeptidomimetics **16c** and **16d** starting from a 3-NH-β-lactam.

As Table 2 shows IC_{50} values in compounds **16a-d** are very similar and adhesion test does not allow distinguish activity differences between them.

Table 2. Docking, adhesion test data and genic expression results in cyclic compounds **15a,b**; **16a–d** and Cilengitide.

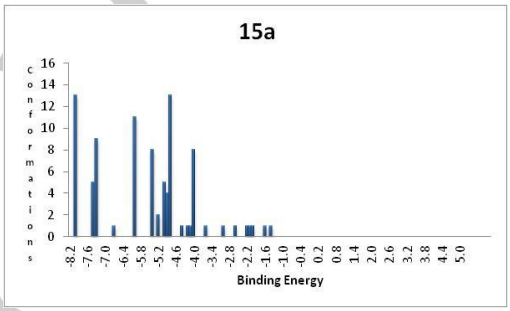
	Theoretical data			Experimental results[32]	
	Binding energy (Kcal)	Torsion energy (Kcal)	K_i (μM)	Adhesion test IC_{50} (μM)	Order of genic expression[a]
1[b]	–7.91	+2.98	1.58	3.1 ±0.3	3
C	–10.70	+ 2.68	0.014	4.0 ±0.1	4
15a	–8.04	+2.98	1.27	0.6 ±0.1	1
15b	–8.18	+2.98	1.01	2.5 ±0.3	2
16a	–5.94	+5.37	44.60	6.3 ±0.4	6
16b	–4.86	+5.67	272.59	5.0 ±0.3	5
16c	–5.48	+5.07	95.45	5.0 ±0.4	8
16d	–5.18	+5.37	159.16	6.3 ±0.2	7

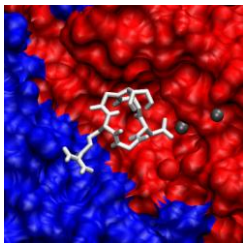
[a] The order is obtained comparing the relative values of each analyzed gene.

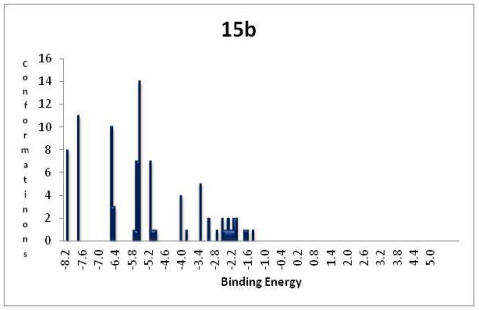
[b] Only for comparative purpouses.[30]

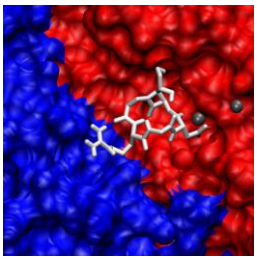
These differences are obvious using CGH Array technique that allows easily distinguish the most active compound. In both types of structures, peptidomimetics and depsipeptidomimetics **16b** and **16d**, which present higher chain length, are the most active.

To rationalize this result we analyzed the bioactive conformations of the compounds **15a**,**b**, **16a**,**b**, in their interaction with the extracellular domain of integrin receptor $\alpha_v\beta_3$ using VMD program.[38] Figure 1 shows four representative structures of the major conformational clusters in cyclic and open chain compounds, although it should be noted that the clusters of different compounds have very different populations. In both cyclic compounds **15a,b**, the interaction of the aspartic carboxylic group with the Ca^{2+} ion and guanidino group of arginine with a pocket of the β domain stabilizes the interaction, resulting in a distribution of low-energy clusters with abundant populations.









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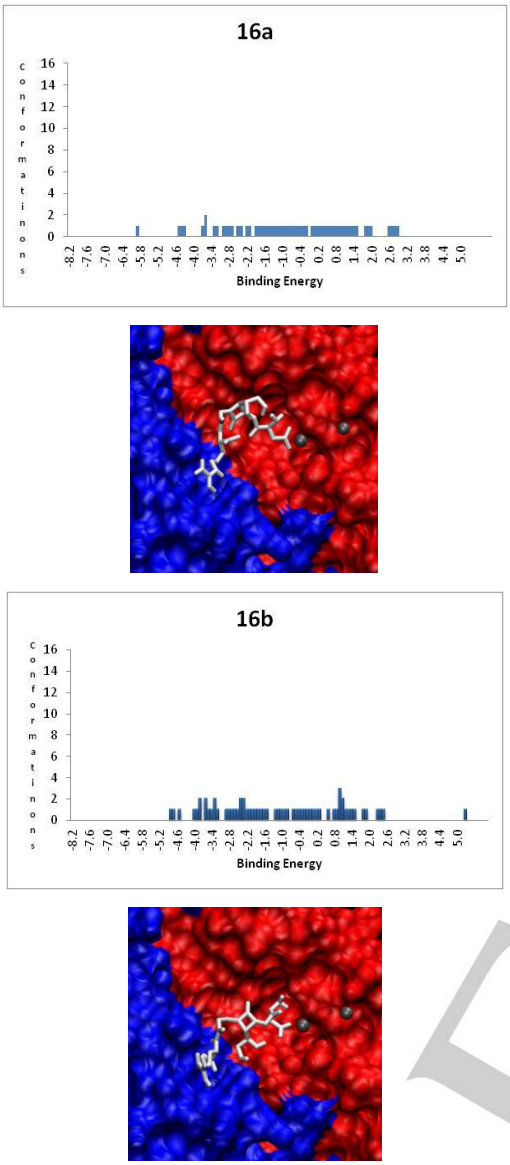

Figure 1. Docking simulation of the interaction of compounds **16a,b** with $\alpha_v\beta_3$ receptor. Only the major conformational clusters are represented (The calculations were performed with Xplor 2.35 program, and the dihedral angle restriction, determined by the *J* coupling of NH-Cα for different amino acids present on the macrocycle, was the only previous condition).[32] For easy viewing we only represent, in white, the conformation of majority cluster. For more details see supporting information.

In both open-chain compounds **16a,b**, the mentioned interactions are weak and cluster majority populations are not abundant. In compound **16a** MIDAS interaction is significant, with three structures interacting with the Ca^{2+} ion, but only the guanidino group of one of the clusters is able to access the pocket of the β domain. Regarding compound **16b** few structures show both interactions and consequently the interconnection between protein domains is weak; also the interaction with Ca^{2+} metal ion occurs with an atom located at a different position to **15a**, **15b** and **16a** MIDAS. These results are reflected in low interaction energies and greater K_i values, unlike what happens in cyclic compounds, resulting from the low interaction with the two domains (α and β) of the receptor. However experimental values show moderate activity therefore we relate the low Ki values with an inadequate docking receptor model for acyclic compounds, as previously mentioned.

Evaluating the results jointly we can conclude that application of an amide-ester substitution or "Ester scan" in designed cyclopeptides can be used to overpass the bioactivity of the synthesized compounds by fine conformational adjustment. Thus, compound **15a** seems to be the best one in terms of inhibitory activity and also shows to be relatively more potent than Cilengitide.

Conclusions

We have described an approach to $\alpha_v\beta_3$ inhibitors based on the generation of conformational restrictions introduced through the incorporation of a β-lactam framework into a RGD macrocycle. The effect AspCO–NH-β-lactam replacement by AspCO–O-β-lactam has also been studied and an increase in biological activity has been observed resulting in products that are appreciably more potent than Cilengitide, the reference compound in this field. Another interesting aspect emerged from this research, is the significant activity we have found in open-chain compounds, leading to a new family of open-chain RGD mimetics, which may be optimized very easily. However, the concept of an amide-ester substitution or "Ester scan" can be used in design of cyclopeptides to overpass the bioactivity of the synthesized compounds by fine conformational adjustment. This observation could be translated to another type of cyclic or acyclic structures, opening a new field of synthetic possibilities. In addition to these observations we also have stablished, for the first time, the CGH array pattern of Cilengitide, an aspect of this investigation that may be considered for further developments in the area.

Experimental Section

General Information. All reactions were carried out under an atmosphere of nitrogen in oven or flame-dried glassware with magnetic stirring. Solvents were distilled prior to use. Tetrahydrofuran (THF) was dried by Pure solv column and acetonitrile (MeCN) and methylene chloride (CH_2Cl_2) were dried by distillation from calcium hydride. Purification of reaction products was carried out by flash chromatography using silicagel 60 (230-400 mesh, from Merck 60F PF254). Analytical thin layer chromatography was performed on 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and phosphomolybdic acid-ammonium cerium (IV) nitrate sulfuric acid-water reagent, followed by heating. Analytical high performance liquid chromatography (HPLC) was performed on a Waters Acquity UPLC chromatograph equipped with a diode array UV detector (210-400 nm), using the analytical column BEH C18 1.7 un 2.1 x 50 mm with flow rates of 0.2 mL/min and 0.3 mL/min. Solvent: 99.9 (water, 0.1% HCO_2H) / 0.1 (MeCN, 0.1% HCO_2H). Infrared spectra were recorded on a Bruker Alpha 360 FT-IR spectrophotometer. Optical rotations were measured at 25 ± 0.2 °C in a Perkin-Elmer 243-B polarimeter using methylene chloride as solvent unless otherwise stated. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance500 spectrometer using a BBI probe at 500 MHz and 300 MHz respectively and are reported as δ values (ppm) relative to residual $CHCl_3$ δH (7.26 ppm) and $CDCl_3$ δC (77.16 ppm) as internal standards, respectively. Exact mass values were obtained by a Waters LCT Premier XE (TOF) after direct injection (HRMS) of sample. qRT-PCR analysis were recorded in a Roche Lightcycler 480, 96 well plate version equipment. Basic silicagel was prepared by mechanical stirring of the commercial

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acidic silica in saturated sodium bicarbonate solution (300 ml of solution per 100g of silicagel), followed by filtration and evaporation of the wastewater in a calefactor at 80 °C for 72h.

General procedure for the synthesis of β-lactams 6a,b. Step 1. To a stirred solution of (*R*)-2,2-dimethyl-1,3-dioxolane-4-carbaldehyde (0.79 g, 6.1 mmol) cooled to 0 °C in CH_2Cl_2 (30 mL) was added the corresponding silylated aminoalcohol (6.0 mmol) and molecular sieves (4 Å). The mixture was stirred over 60 minutes at same temperature, the solids were filtered off and the solvent was evaporated to give the intermediate imine **5a**,**b**. **Step 2.** The crude product was dissolved in CH_2Cl_2 (30 mL) and the solution was cooled to –78 °C. Then, Et_3N (1.7 mL, 12.0 mmol) was added at the same temperature, followed by a solution of the corresponding acid chloride (6.1 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred overnight, while slowly warming to room temperature. The resulting solution was washed successively with water (30 mL), 0.1 M HCl (2 x 30 mL) and saturated aqueous solution of $NaHCO_3$ (2 x 30 mL), it was dried ($MgSO_4$) and evaporated in "vacuo". Purification of the resulting crude oil was performed by flash column chromatography on basic silicagel (hexane/EtOAc 95/5) to afford the product **6a**,**b.**

(3*R*,4*S*)-3-Benzyloxy-1-[2-(*tert*-butyldimethylsilyloxy)ethyl]-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one (6a). The general procedure was followed by coupling of benzyloxyacetyl chloride (1 mL, 6.1 mmol) with the imine **1a**. Overall yield: 1.88 g (72%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 2985, 2953, 2928, 2856, 1761. ^{1}H-RMN (δ, ppm, $CDCl_3$): 7.43–7.25 (5H, m, arom); 4.91 (1H, d, CH_2-Ph, J = 11.7 Hz); 4.72–4.53 (2H, m, CH_2-Ph y H^{α}_R[β-lact]); 4.32 (1H, dt, CH_S[dioxol], J_1 = 8.9 Hz, J_2 = 6.5 Hz); 4.14 (1H, dd, CH_2[dioxol], J_1 = 8.7 Hz, J_2 = 6.5 Hz); 3.79 (2H, t, CH_2-O-Si, J = 6.0 Hz); 3.71 (1H, dd, H^{β}_S[β-lact], J_1 = 8.9 Hz, J_2 = 5.1 Hz); 3.63 (1H, dd, CH_2[dioxol], J_1 = 8.7 Hz, J_2 = 6.5 Hz); 3.60–3.49 (1H, m, N-CH_2); 3.35 (1H, dt, N-CH_2, J_1 = 13.7 Hz, J_2 = 6.0 Hz); 1.43 (3H, s, CH_3[dioxol]); 1.34 (3H, s, CH_3[dioxol]); 0.90 (9H, s, Si-^{t}Bu); 0.07 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 167.7, 137.0, 128.4, 127.9, 127.7, 109.4, 80.5, 77.2, 72.8, 66.8, 60.9, 59.9, 43.5, 26.8, 25.8, 25.1, 18.1, –5.4.

(3*R*,4*S*)-3-Benzyloxy-1-[3-(*tert*-butyldimethylsilyloxy)propyl]-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one (6b). The general procedure was followed by couplig of imine **1b** with benzyloxyacetyl chloride (1 mL, 6.1 mmol). Overall yield: 1.50 g (55%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 2985, 2929, 2884, 2857, 1755. ^{1}H-RMN (δ, ppm, $CDCl_3$): 7.41–7.20 (5H, m, arom.); 4.90 (1H, d, CH_2-Ph, J = 11.8 Hz); 4.63 (1H, d, CH_2-Ph, J = 11.8 Hz); 4.57 (1H, d, H^{α}_R[β-lact], J = 5.1 Hz); 4.30 (1H, dt, CH_S[dioxol], J_1 = 8.9 Hz, J_2 = 6.5 Hz); 4.14 (1H, dd, CH_2[dioxol], J_1 = 8.7 Hz, J_2 = 6.5 Hz); 3.74–3.58 (4H, m, 2H CH_2-O-Si, H^{β}_S[β-lact], 1H CH_2[dioxol]); 3.50 (1H, ddd, N-CH_2, J_1 = 13.9 Hz, J_2 = 8.3 Hz, J_3 = 6.7 Hz); 3.30 (1H, ddd, N-CH_2, J_1 = 13.9 Hz, J_2 = 8.1 Hz, J_3 = 6.0 Hz); 2.02–1.60 (2H, m, CH_2-CH_2-CH_2); 1.42 (3H, s, CH_3[dioxol]); 1.32 (3H, s, CH_3[dioxol]); 0.89 (9H, s, Si-^{t}Bu); 0.04 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 167.6, 137.1, 128.5, 128.0, 127.8, 109.5, 80.3, 77.2, 72.8, 66.9, 60.8, 60.5, 38.8, 30.6, 26.9, 25.9, 25.2, 18.3, –5.3.

General procedure for the synthesis of β-lactams 7b,e. Deprotection. The corresponding β-lactam **6a,b** (4.32 mmol) was dissolved in methanol (40 mL) under nitrogen atmosphere. Ammonium formate (1.63 g, 25.9 mmol) and 10% Pd/C (%15) were added and the solution was stirred under hydrogen atmospheric pressure during one hour. The catalyst was removed by filtration over celite and the resulting solution was washed with 0.1 M HCl (2 x 60 mL). The organic layer was dried with $MgSO_4$ and evaporated to give the product **7a**,**b**.

(3*R*,4*S*)-3-Hydroxy-1-[2-(*tert*-butyldimethylsilyloxy)ethyl]-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one (7a). The general procedure was followed starting with the β-lactam **2a** (1.88 g, 4.32 mmol). Yield: 1.43 g (96%). $[\alpha]_D^{25}$ = –4.07 (c = 1, CH_2Cl_2). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3333, 2984, 2952, 2929, 2884, 2856, 1735. ^{1}H-RMN (δ, ppm, $CDCl_3$): 4.82 (1H, dd, H^{α}_R[β-lact], J_1 = 8.5 Hz, J_2 = 5.1 Hz); 4.40–4.30 (1H, m, CH_S[dioxol]); 4.19 (1H, dd, CH_2[dioxol], J_1 = 8.8 Hz, J_2 = 7.1 Hz); 4.00 (1H, dd, H^{β}_S[β-lact], J_1 = 8.8 Hz, J_2 = 5.1 Hz); 3.90–3.83 (1H, m, CH_2[dioxol]); 3.79–3.71 (2H, m, CH_2-O-Si); 3.66 (1H, dt, N-CH_2, J_1 = 14.0 Hz, J_2 = 4.8 Hz); 3.39 (1H, d, HO, J = 8.5 Hz); 3.24 (1H, dt, N-CH_2, J_1 = 14.0 Hz, J_2 = 4.8 Hz); 1.46 (3H, s, CH_3[dioxol]); 1.35 (3H, s, CH_3[dioxol]); 0.88 (9H, s, ^{t}Bu); 0.05 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.8, 109.7, 76.6, 75.3, 66.7, 62.1, 60.2, 43.6, 26.8, 25.9, 25.1, 18.2, –5.3, –5.4.

(3*R*,4*S*)-3-Hydroxy-1-[3-(*tert*-butyldimethylsilyloxy)propyl]-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one (7b). The general procedure was followed starting with the β-lactam **2b** (1.50 g, 3.30 mmol). Yield: 1.14 g (96%). $[\alpha]_D^{25}$ = +13.83 (c = 1, CH_2Cl_2). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3308, 2985, 2929, 2894, 2858, 1719. ^{1}H-RMN (δ, ppm, $CDCl_3$): 4.79 (1H, dd, H^{α}_R[β-lact], J_1 = 7.5 Hz, J_2 = 4.9 Hz); 4.34 (1H, dt, CH_S[dioxol], J_1 = 6.5 Hz, J_2 = 5.5 Hz); 4.21 (1H, dd, CH_2[dioxol], J_1 = 8.8 Hz, J_2 = 5.5 Hz); 3.97 (1H, d, OH, J = 7.5 Hz); 3.81 (1H, dd, CH_2[dioxol], J_1 = 8.8 Hz, J_2 = 5.5 Hz); 3.71 (1H, dd, H^{β}_S[β-lact], J_1 = 6.5 Hz, J_2 = 4.9 Hz); 3.63 (2H, t, CH_2-O-Si, J = 6.1 Hz); 3.55 (1H, ddd, N-CH_2, J_1 = 14.4 Hz, J_2 = 8.1 Hz, J_3 = 6.8 Hz); 3.25 (1H, ddd, N-CH_2, J_1 = 14.4 Hz, J_2 = 8.0 Hz, J_3 = 6.1 Hz); 1.96–1.66 (2H, m, CH_2-CH_2-CH_2); 1.45 (3H, s, CH_3[dioxol]); 1.35 (3H, s, CH_3[dioxol]); 0.89 (9H, s, Si-^{t}Bu); 0.04 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.6, 109.6, 76.9, 75.0, 66.9, 61.8, 60.8, 39.0, 30.6, 26.9, 26.0, 25.2, 18.3, –5.3.

General procedure for the acoplation of aspartic. Compounds 8a,b and 11a,b. Step 1. The corresponding *L*-aspartic acid-derived (2.4 mmol) was dissolved in CH_2Cl_2 (7 mL) at –15 °C and pyridine (0.2 mL, 2.4 mmol) and 2,4,6-trifluoro-1,3,5-triazine (0.3 mL, 3.6 mmol) were added slowly. The solution was stirred during one hour at same temperature and a white precipitate was formed. The mixture was quenched with water/ice solution (10 mL) and extracted with CH_2Cl_2 (3 x 10 mL). The organic layer was washed with water (3 x 20 mL), dried with $MgSO_4$, and the solvent was evaporated under reduced pressure to give the activated *L*-aspartic derived fluoride and after checking by NMR was used without further purification. **Step 2.** The HO-β-lactam **7a**,**b** (2.07 mmol) was dissolved in CH_2Cl_2 (20 mL) and Et_3N (0.58 mL, 4.14 mmol) was added. A solution of *L*-aspartic fluoride (0.66 g, 2.28 mmol) in CH_2Cl_2 (20 mL) was added slowly. The mixture was stirred for 30 minutes at room temperature. After this time the solution was washed with 0.1 M HCl (2 x 30 mL), saturated solution of $NaHCO_3$ (2 x 30 mL) and brine (2 x 30 mL). The organic layer was dried with $MgSO_4$ and evaporated to give **8a**,**b**, which were used in the next step without further purification.

Cbz-Asp(O^{t}Bu)-[(3R,4S)-3-oxy-1-(2-(tert-butyldimethylsilyl)ethyl)-4-((S)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (8a). Following the general procedure the title compound was obtained by coupling the HO-β-lactam **7a** (0.71 g, 2.07 mmol) with Cbz-*L*-aspartic acid-*tert*-butyl ester (0.78 g, 2.4 mmol). Overall yield: 1.18 g (88%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3442, 2984, 2931, 2856, 1752, 1726. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.40–7.29 (5H, m, arom.); 5.77–5.68 (2H, m, NH[Asp], H^{α}_R[β-lact]); 5.13 (2H, s, CH_2-Ph); 4.65 (1H, dt, H^{α}_S[Asp], J_1 = 8.9 Hz, J_2 = 4.4 Hz); 4.14–4.00 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.86 (1H, dd, H^{β}_S[β-lact], J_1 = 8.3 Hz, J_2 = 4.9 Hz); 3.78 (2H, dt, CH_2-O-Si, J_1 = 5.6 Hz, J_2 = 1.7 Hz); 3.68–3.58 (2H, m, N-CH_2, CH_2[dioxol]); 3.34 (1H, dt, N-CH_2, J_1 = 13.8 Hz, J_2 = 5.6 Hz); 2.97 (1H, dd, H^{β}[Asp], J_1 = 17.2 Hz, J_2 = 4.4 Hz); 2.79 (1H, dd, H^{β}[Asp], J_1 = 17.2 Hz, J_2 = 4.4 Hz); 1.43 (12H, s, ^{t}Bu, CH_3[dioxol]); 1.30 (3H, s, CH_3[dioxol]); 0.90 (9H, s, Si-^{t}Bu); 0.07 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 169.7, 163.8, 155.8, 136.1, 128.4, 128.0, 127.8, 109.5, 81.9, 75.7, 75.1, 66.9, 66.4, 60.6, 59.7, 50.4, 43.9, 37.1, 27.8, 26.6, 25.7, 24.8, 18.0, –5.5, –5.6.

Cbz-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-1-(3-(tert-butyldimethylsilyloxy) propyl)-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (8b). Following the general procedure the title compound was obtained by coupling the HO-β-lactam **7b** (0.85 g, 2.38 mmol) with Cbz-*L*-aspartic acid-*tert*-butyl ester (0.88 g, 2.73 mmol). Overall yield: 1.39 g (88%). IR

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(ν, cm^{-1}, CH_2Cl_2 solution): 3324, 2931, 2886, 2857, 1768, 1724. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.45–7.25 (5H, m, arom.); 5.77–5.64 (2H, m, NH[Asp], H^{α}_R[β-lact]); 5.13 (2H, m, CH_2-Ph); 4.65 (1H, dt, H^{α}_S[Asp], J_1 = 9.1 Hz, J_2 = 4.7 Hz); 4.20–3.93 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.78 (1H, dd, H^{β}_S[β-lact], J_1 = 8.6 Hz, J_2 = 4.8 Hz); 3.69–3.48 (4H, m, 2H CH_2-O-Si, 1H N-CH_2, 1H CH_2[dioxol]); 3.32 (1H, ddd, N-CH_2, J_1 = 13.9 Hz, J_2 = 8.2 Hz, J_3 = 5.9 Hz); 2.95 (1H, dd, H^{β}[Asp], J_1 = 17.3 Hz, J_2 = 4.7 Hz); 2.78 (1H, dd, H^{β}[Asp], J_1 = 17.3 Hz, J_2 = 4.7 Hz); 1.97–1.68 (2H, m, $CH_2CH_2CH_2$); 1.43 (12H, s, ^{t}Bu, CH_3[dioxol]); 1.30 (3H, s, CH_3[dioxol]); 0.89 (9H, s, Si-^{t}Bu); 0.04 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.0, 163.8, 156.0, 136.2, 128.7, 128.4, 128.2, 109.9, 82.4, 76.1, 75.3, 67.3, 66.8, 60.9, 60.4, 50.6, 38.8, 37.4, 30.6, 28.2, 27.0, 26.0, 25.1, 18.4, –5.3.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-1-(2-(*tert*-butyldimethylsilyloxy)ethyl)-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (11a). Following the general procedure the title compound was obtained by coupling the HO-β-lactam **7a** (0.36 g, 1.04 mmol) with Boc-*L*-aspartic acid-*tert*-butyl ester (0.34 g, 1.19 mmol). Overall yield: 0.58 g (90%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3443, 3346, 2979, 2930, 2857, 1774, 1719. ^{1}H-NMR (δ, ppm, $CDCl_3$): 5.72 (1H, d, H^{α}_R[β-lact], *J* = 4.8 Hz); 5.43 (1H, d, NH[Asp], *J* = 9.0 Hz); 4.59 (1H, dt, H^{α}_S[Asp], J_1 = 9.0 Hz, J_2 = 4.6 Hz); 4.19–4.03 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.85 (1H, dd, H^{β}_S[β-lact], J_1 = 8.4 Hz, J_2 = 4.8 Hz); 3.77 (2H, dt, CH_2-O-Si, J_1 = 5.6 Hz, J_2 = 1.4 Hz); 3.68–3.53 (2H, m, N-CH_2, CH_2[dioxol]); 3.34 (1H, dt, N-CH_2, J_1 = 13.9 Hz, J_2 = 5.6 Hz); 2.92 (1H, dd, H^{β}[Asp], J_1 = 17.0 Hz, J_2 = 4.6 Hz); 2.76 (1H, dd, H^{β}[Asp], J_1 = 17.0 Hz, J_2 = 4.6 Hz); 1.44 (21H, s, 2 x ^{t}Bu, CH_3[dioxol]); 1.31 (3H, s, CH_3[dioxol]); 0.88 (9H, s, Si-^{t}Bu); 0.05 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.2, 169.9, 164.0, 155.2, 109.7, 82.0, 80.1, 75.9, 75.1, 66.6, 60.7, 59.8, 50.0, 44.0, 37.4, 28.2, 28.0, 26.8, 25.8, 25.0, 18.1, –5.4, –5.5.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-1-(3-(*tert*-butyldimethylsilyloxyl) propyl)-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (11b). Following the general procedure the title compound was obtained by coupling the HO-β-lactam **7b** (0.28 g, 0.79 mmol) with Boc-*L*-aspartic acid-*tert*-butyl ester (0.26 g, 0.90 mmol). Overall yield: 0.44 g (88%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3433, 3347, 2978, 2953, 2929, 2894, 2856, 1770, 1718. ^{1}H-NMR (δ, ppm, $CDCl_3$): 5.70 (1H, d, H^{α}_R[β-lact], *J* = 4.8 Hz); 5.43 (1H, d, NH[Asp], *J* = 9.1 Hz); 4.59 (1H, dt, H^{α}_S[Asp], J_1 = 9.1 Hz, J_2 = 4.7 Hz); 4.18–4.03 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.78 (1H, dd, H^{β}_S[β-lact], J_1 = 8.6 Hz, J_2 = 4.8 Hz); 3.69–3.49 (4H, m, 2H CH_2-O-Si, 1H N-CH_2, 1H CH_2[dioxol]); 3.32 (1H, ddd, N-CH_2, J_1 = 14.0 Hz, J_2 = 8.2 Hz, J_3 = 5.9 Hz); 2.92 (1H, dd, H^{β}[Asp], J_1 = 17.0 Hz, J_2 = 4.7 Hz); 2.75 (1H, dd, H^{β}[Asp], J_1 = 17.0 Hz, J_2 = 4.7 Hz); 1.94–1.74 (2H, m, CH_2-CH_2-CH_2); 1.44 (21H, s, 2 x ^{t}Bu, CH_3[dioxol]); 1.31 (3H, s, CH_3[dioxol]); 0.88 (9H, s, Si-^{t}Bu); 0.04 (6H, s, CH_3-Si-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.3, 170.0, 163.8, 155.3, 109.8, 82.2, 80.3, 76.1, 75.0, 66.7, 60.8, 60.3, 50.1, 39.6, 37.5, 30.5, 28.3, 28.1, 26.9, 26.1, 25.0, 18.3, –5.3.

General procedure to get de acid 9a,b. Deprotection and oxidation. Step 1. The desilylation of TBS group was conducted dissolving the corresponding Asp-β-lactam **8a–h** (3.47 mmol) in THF (17 mL) at 0 °C and adding hydrogen fluoride pyridine (0.27 mL, 13.88 mmol). The solution was stirred at room temperature about 3 hours and was neutralized with a saturated aqueous solution of $NaHCO_3$. The product was extracted with diethyl ether (3 x 15 mL) and the organic layer was washed with brine (3 x 10 mL) and dried with $MgSO_4$. The solvent was eliminated in "vacuo" to afford the terminal alcohol. **Step 2.** BAIB (2.24 g, 7.26 mmol), TEMPO (0.13 g, 0.82 mmol) and the corresponding alcohol (3.33 mmol) were added to a mixture of MeCN/water (8/8 mL). The reaction mixture was stirred for 4 hours. After this time the compound was extracted several times from the water solution using CH_2Cl_2 (3 x 15 mL). The organic layer was dried with $MgSO_4$ and evaporated under reduced pressure.

Cbz-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-1-carboxymethylen-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (9a). The general procedure was followed starting with the Cbz-Asp(O^{t}Bu)-O-β-lactam **8a** (1.18 g, 1.82 mmol). Overall yield: 0.79 g (80%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3339, 2982, 2934, 1719. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.41–7.29 (5H, m, arom.); 5.83–5.68 (2H, m, NH[Asp], H^{α}_R[β-lact]); 5.13 (2H, s, CH_2-Ph); 4.66 (1H, dt, H^{α}_S[Asp], J_1 = 8.8 Hz, J_2 = 4.3 Hz); 4.35 (1H, d, N-CH_2, *J* = 18.2 Hz); 4.20–3.94 (4H, m, CH_2[dioxol], CH_S[dioxol], H^{β}_S[β-lact], N-CH_2); 3.65 (1H, dd, CH_2[dioxol], J_1 = 9.1 Hz, J_2 = 4.4 Hz); 2.99 (1H, dd, H^{β}[Asp], J_1 = 17.5 Hz, J_2 = 4.6 Hz); 2.79 (1H, dd, H^{β}[Asp], J_1 = 17.5 Hz, J_2 = 4.6 Hz); 1.43 (9H, s, ^{t}Bu); 1.39 (3H, s, CH_3[dioxol]); 1.28 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 171.5, 170.1, 169.9, 164.3, 156.1, 136.1, 128.7, 128.3, 128.2, 109.9, 82.5, 75.8, 75.6, 67.4, 66.6, 60.6, 50.5, 43.0, 28.1, 26.8, 25.1.

Cbz-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-1-carboxyethylen-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (9b). The general procedure was followed starting with the Cbz-Asp(O^{t}Bu)-O-β-lactam **8b** (1.39 g, 2.09 mmol). Overall yield: 0.84 g (71%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3327, 3310, 3064, 3034, 2982, 2937, 1719. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.42–7.28 (5H, m, arom.); 5.77 (1H, d, NH[Asp], *J* = 9.3 Hz); 5.71 (1H, d, H^{α}_R[β-lact], *J* = 4.8 Hz); 5.13 (2H, s, CH_2-Ph); 4.66 (1H, dt, H^{α}_S[Asp], J_1 = 9.3 Hz, J_2 = 4.7 Hz); 4.17–3.99 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.84 (1H, dd, H^{β}_S[β-lact], J_1 = 8.8 Hz, J_2 = 4.8 Hz); 3.74 (1H, dt, N-CH_2, J_1 = 14.0 Hz, J_2 = 6.9 Hz); 4.67–3.47 (2H, m, N-CH_2, CH_2[dioxol]); 2.95 (1H, dd, H^{β}[Asp], J_1 = 17.1 Hz, J_2 = 4.7 Hz); 2.86–2.61 (3H, m, H^{β}[Asp], CH_2-COOH); 1.44 (12H, s, ^{t}Bu, CH_3[dioxol]); 1.31 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 175.3, 170.0, 169.9, 164.2, 156.1, 136.2, 128.6, 128.3, 128.1, 110.0, 82.4, 75.8, 75.1, 67.3, 66.6, 64.5, 60.6, 50.6, 37.9, 37.4, 31.9, 28.1, 26.8, 25.0.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxo-1-carboxymethylen-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (12a). The general procedure was followed starting with the Boc-Asp(O^{t}Bu)-O-β-lactam **11a** (0.58 g, 0.94 mmol). Overall yield: 0.36 g (74%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3338, 2979, 2933, 1760, 1717, 1502. ^{1}H-NMR (δ, ppm, $CDCl_3$): 5.79 (1H, d, H^{α}_R[β-lact], *J* = 4.7 Hz); 5.47 (1H, d, NH[Asp], *J* = 10.0 Hz); 4.57 (1H, dt, H^{α}_S[Asp], J_1 = 10.0 Hz, J_2 = 4.9 Hz); 4.33 (1H, d, N-CH_2, *J* = 18.1 Hz); 4.24–4.15 (1H, m, CH_2[dioxol]); 4.12–3.97 (3H, m, CH_S[dioxol], H^{β}_S[β-lact], N-CH_2); 3.64 (1H, dd, CH_2[dioxol], J_1 = 9.0 Hz, J_2 = 4.5 Hz); 2.92 (1H, dd, H^{β}[Asp], J_1 = 17.2 Hz, J_2 = 4.9 Hz); 2.75 (1H, dd, H^{β}[Asp], J_1 = 17.2 Hz, J_2 = 4.9 Hz); 1.44 (21H, s, 2 x ^{t}Bu, CH_3[dioxol]); 1.38 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 172.1, 171.5, 170.3, 164.3, 155.5 109.9, 82.4, 80.6, 75.7, 66.7, 60.6, 50.1, 42.9, 37.6, 28.4, 28.2, 20.2, 26.9, 25.1.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxo-1-carboxyethylen-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)azetidin-2-one] (12b). The general procedure was followed starting with the Boc-Asp(O^{t}Bu)-O-β-lactam **11b** (0.44 g, 0.69 mmol). Overall yield: 0.27 g (74%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3334, 2979, 2933, 1762, 1718. ^{1}H-NMR (δ, ppm, $CDCl_3$): 9.79 (1H, s, COOH); 5.70 (1H, d, H^{α}_R[β-lact], *J* = 5.0 Hz); 5.50–5.36 (1H, m, NH[Asp]); 4.67–4.52 (1H, m, H^{α}_S[Asp]); 4.26–4.00 (2H, m, CH_2[dioxol], CH_S[dioxol]); 3.79 (1H, dd, H^{β}_S[β-lact], J_1 = 8.8 Hz, J_2 = 5.0 Hz); 3.75–3.49 (3H, m, 2H N-CH_2, 1H CH_2[dioxol]); 3.03–2.66 (4H, m, 2H H^{β}[Asp], CH_2-COOH); 1.46 (21H, s, 2 x ^{t}Bu, CH_3[dioxol]); 1.30 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 199.7, 174.7, 170.3, 164.1, 155.5, 110.0, 82.3, 80.5, 75.9, 75.1, 66.7, 60.7, 50.1, 41.7, 37.9, 37.6, 28.4, 28.2, 26.9, 25.1.

General procedure for the synthesis of the linear precursors 10a,b and 13a,b. To a stirred solution of Asp-β-lactam-$(CH_2)_x$-COOH (X = 1,2) compounds **9a,b** (1.26 mmol) cooled at 0 °C in dry CH_2Cl_2 (60 mL) **17** (1.39 mmol), EDC·HCl (0.38 g, 2.01 mmol), HOBt (0.23 g, 1.76 mmol) and Et_3N (0.37 mL, 2.52 mmol) were added. The mixture was stirred for 20 hours at room temperature. After this time, the reaction was washed with HCl 0.1 M (2 x 40 mL) and saturated solution of $NaHCO_3$ (2 x 40 mL). The organic layer was dried with $MgSO_4$ and evaporated and the crude product was purified by flash chromatography MeOH/CH_2Cl_2 1/25.

Cbz-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-methylen carbonyl-azetidin-2-one]-Arg(Pbf)-Gly-OBn (10a).

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Following the general procedure the title compound was obtained by coupling the Cbz-Asp(O^{t}Bu)-O-β-lactam-CH_2-COOH **9a** (0.39 g, 0.72 mmol) with H_2N-Arg(Pbf)-Gly-OBn 12 (0.45 g, 0.79 mmol). Yield: 0.60 g (76%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3443, 3328, 2975, 2932, 1728, 1700, 1547. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.63 (1H, t, NH[Gly], *J* = 5.8 Hz); 7.40–7.26 (11H, m, arom., NH[Arg]); 6.35–6.17 (2H, m, NH-(C=N*H*)-N*H*); 6.05 (1H, b.s., N*H*-(C=NH)-NH); 5.86–5.74 (2H, m, H$^{\alpha}_R$[β-lact], NH[Asp]); 5.20–5.01 (4H, m, 2 x CH_2-Ph); 4.69–4.53 (2H, m, H$^{\alpha}_S$[Asp], H$^{\alpha}_S$[Arg]); 4.33–4.13 (2H, m, CH_S[dioxol], N-CH_2); 4.13–3.91 (5H, m, H$^{\beta}_S$[β-lact], 1H CH_2[dioxol], 1H N-CH_2, 2H H$^{\alpha}$[Gly]); 3.61 (1H, dd, CH_2[dioxol], J_1 = 9.1 Hz, J_2 = 4.6 Hz); 3.32–3.14 (2H, m, H$^{\delta}$[Arg]); 2.99–2.86 (3H, m, CH_2[Pbf], H$^{\beta}$[Asp]); 2.78 (1H, dd, H$^{\beta}$[Asp], J_1 = 17.3 Hz, J_2 = 4.8 Hz); 2.56 (3H, s, CH_3[Pbf]); 2.49 (3H, s, CH_3[Pbf]); 2.08 (3H, s, CH_3[Pbf]); 2.02–1.81 (1H, m, H$^{\beta}$[Arg]); 1.78–1.64 (1H, m, H$^{\beta}$[Arg]); 1.64–1.51 (2H, m, H$^{\gamma}$[Arg]); 1.45 (6H, s, CH_3-C-CH_3[Pbf]); 1.41 (9H, s, ^{t}Bu); 1.40 (3H, s, CH_3[dioxol]); 1.36 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 172.3, 170.1, 170.0, 169.9, 167.2, 165.2, 159.0, 156.6, 156.1, 138.5, 136.2, 135.4, 132.8, 132.4, 128.7, 128.6, 128.4, 128.3, 128.2, 124.8, 117.7, 110.1, 86.6, 82.4, 75.8, 75.6, 67.4, 67.2, 66.7, 61.0, 53.0, 50.6, 45.3, 43.4, 41.4, 40.6, 37.5, 30.0, 29.4, 28.7, 28.2, 26.9, 25.2, 19.4, 18.1, 12.6.

Cbz-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-ethylencarbonyl-azetidin-2-one]-Arg(Pbf)-Gly-OBn (10b). Following the general procedure the title compound was obtained by coupling the Cbz-Asp(O^{t}Bu)-O-β-lactam-$(CH_2)_2$-COOH **9b** (0.42 g, 0.74 mmol) with H_2N-Arg(Pbf)-Gly-OBn **12** (0.47 g, 0.81 mmol). Yield: 0.56 g (68%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3434, 3319, 2974, 2934, 1757, 1729, 1541. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.63 (1H, t, NH[Gly], *J* = 5.7 Hz); 7.43–7.23 (10H, m, arom.); 7.21–7.10 (1H, m, NH[Arg]); 6.39–6.25 (2H, m, NH-(C=N*H*)-N*H*); 6.02 (1H, b.s., N*H*-(C=NH)-NH); 5.88 (1H, d, NH[Asp], *J* = 9.2 Hz); 5.68 (1H, d, H$^{\alpha}_R$[β-lact], *J* = 5.1 Hz); 5.19–5.01 (4H, m, 2 x CH_2-Ph); 4.65 (1H, dt, H$^{\alpha}_S$[Asp], J_1 = 9.2 Hz, J_2 = 5.0 Hz); 4.59–4.51 (1H, m, H$^{\alpha}_S$[Arg]); 4.17–4.09 (1H, m, CH_S[dioxol]); 4.09–3.96 (3H, m, 1H CH_2[dioxol], 2H H$^{\alpha}$[Gly]); 3.92 (1H, dd, H$^{\beta}_S$[β-lact], J_1 = 9.0 Hz, J_2 = 5.1 Hz); 3.76 (1H, dt, N-CH_2, J_1 = 13.6 Hz, J_2 = 6.1 Hz); 3.65–3.58 (1H, m, CH_2[dioxol]); 3.50 (1H, dt, N-CH_2, J_1 = 13.6 Hz, J_2 = 6.1 Hz); 3.30–3.18 (2H, m, H$^{\delta}$[Arg]); 3.00–2.83 (3H, m, CH_2[Pbf], H$^{\beta}$[Asp]); 2.77 (1H, dd, H$^{\beta}$[Asp], J_1 = 17.2 Hz, J_2 = 5.0 Hz); 2.67–2.53 (5H, m, 2H N-CH_2-C*H*$_2$, CH_3[Pbf]); 2.50 (3H, s, CH_3[Pbf]); 2.09 (3H, s, CH_3[Pbf]); 2.00–1.86 (1H, m, H$^{\beta}$[Arg]); 1.75–1.64 (1H, m, H$^{\beta}$[Arg]); 1.64–1.50 (2H, m, H$^{\gamma}$[Arg]); 1.46 (6H, s, CH_3-C-CH_3[Pbf]); 1.43 (3H, s, CH_3[dioxol]); 1.41 (9H, s, ^{t}Bu); 1.31 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 172.6, 170.9, 170.1, 170.0, 169.9, 164.6, 158.8, 156.6, 156.0, 138.4, 136.1, 135.4, 133.0, 132.3, 128.7, 128.5, 128.4, 128.3, 128.2, 124.8, 117.6, 110.0, 86.5, 82.4, 75.8, 75.2, 67.3, 67.1, 66.6, 60.1, 52.7, 50.5, 43.3, 41.4, 40.5, 39.2, 37.4, 33.7, 29.7, 28.7, 28.1, 26.9, 25.1, 19.4, 18.1, 12.6.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-methylencar bonyl-azetidin-2-one]-Arg(Pbf)-O^{t}Bu (13a). Following the general procedure the title compound was obtained by coupling the commercial NH_2-Arg(Pbf)-O^{t}Bu (0.19 g, 0.38 mmol) with Boc-Asp(O^{t}Bu)-O-β-lactam-CH_2-COOH **12a** (0.18 g, 0.35 mmol). Yield: 0.26 g (70%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3430, 3335, 2978, 2945, 1773, 1719, 1617, 1543. ^{1}H-NMR (δ, ppm, $CDCl_3$): 6.87 (1H, d, NH[Arg], *J* = 7.8 Hz); 6.17–6.00 (2H, m, NH-(C=N*H*)-N*H*); 5.87 (1H, b.s., N*H*-(C=NH)-NH); 5.78 (1H, d, H$^{\alpha}_R$[β-lact], *J* = 4.9 Hz); 5.51 (1H, d, NH[Asp], *J* = 8.8 Hz); 4.59 (1H, dt, H$^{\alpha}_S$[Asp], J_1 = 8.8 Hz, J_2 = 4.6 Hz); 4.45 (1H, dt, H$^{\alpha}_S$[Arg], J_1 = 7.8 Hz, J_2 = 4.7 Hz); 4.24 (1H, ddd, CH_S[dioxol], J_1 = 9.2 Hz, J_2 = 6.3 Hz, J_3 = 4.7 Hz); 4.19–4.01 (3H, m, 2H N-CH_2, CH_2[dioxol]); 3.98 (1H, dd, H$^{\beta}_S$[β-lact], J_1 = 9.2 Hz, J_2 = 4.9 Hz); 3.64 (1H, dd, CH_2[dioxol], J_1 = 9.0 Hz, J_2 = 4.7 Hz); 3.28–3.17 (2H, m, H$^{\delta}$[Arg]); 2.99–2.84 (3H, m, CH_2[Pbf], H$^{\beta}$[Asp]); 2.76 (1H, dd, H$^{\beta}$[Asp], J_1 = 17.0 Hz, J_2 = 4.6 Hz); 2.59 (3H, m, CH_3[Pbf]); 2.52 (3H, s, CH_3[Pbf]); 2.09 (3H, s, CH_3[Pbf]); 1.96–1.83 (1H, m, H$^{\beta}$[Arg]); 1.77–1.50 (3H, m, 1H H$^{\beta}$[Arg], 2H H$^{\gamma}$[Arg]); 1.49–1.42 (33H, m, CH_3-C-CH_3[Pbf], 3 x ^{t}Bu); 1.39 (3H, s, CH_3[dioxol]); 1.30 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 170.9, 170.1, 170.0, 166.9, 164.9, 158.6, 156.3, 155.3, 138.2, 133.0, 132.2, 124.5, 117.4, 109.8, 86.3, 82.2, 82.0, 80.2, 75.5, 75.5, 66.5, 60.8, 52.6, 50.0, 44.7, 43.2, 40.6, 37.4, 29.5, 28.6, 28.2, 28.0, 27.9, 26.7, 25.2, 25.0, 19.2, 17.9, 12.4.

Boc-Asp(O^{t}Bu)-[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-ethylencarbonyl-azetidin-2-one]-Arg(Pbf)-O^{t}Bu (13b). Following the general procedure the title compound was obtained by coupling the commercial NH_2-Arg(Pbf)-O^{t}Bu (0.13 g, 0.28 mmol) with Boc-Asp(O^{t}Bu)-O-β-lactam-$(CH_2)_2$-COOH **12b** (0.13 g, 0.25 mmol). Yield: 0.17 g (67%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3443, 3333, 2976, 2932, 1761, 1722, 1662, 1618, 1545. ^{1}H-NMR (δ, ppm, $CDCl_3$): 6.47–6.32 (1H, m, NH[Arg]); 6.20–6.13 (2H, m, NH-(C=N*H*)-N*H*); 5.81 (1H, b.s., N*H*-(C=NH)-NH); 5.67 (1H, d, H$^{\alpha}_R$[β-lact], *J* = 4.9 Hz); 5.50 (1H, d, NH[Asp], *J* = 8.8 Hz); 4.57 (1H, dt, H$^{\alpha}_S$[Asp], J_1 = 8.8 Hz, J_2 = 4.5 Hz); 4.50–4.41 (1H, m, H$^{\alpha}_S$[Arg]); 4.20 (1H, dt, CH_S[dioxol], J_1 = 9.0 Hz, J_2 = 4.2 Hz); 4.11 (1H, d, CH_2[dioxol], J_1 = 8.8 Hz, J_2 = 4.2 Hz); 3.99 (1H, dd, H$^{\beta}_S$[β-lact], J_1 = 9.0 Hz, J_2 = 4.9 Hz); 3.78–3.67 (1H, m, N-CH_2); 3.63 (1H, dd, CH_2[dioxol], J_1 = 8.8 Hz, J_2 = 4.2 Hz); 3.60–3.47 (1H, m, N-CH_2); 3.37–3.12 (2H, m, H$^{\delta}$[Arg]); 2.95 (3H, m, CH_2[Pbf]); 2.87 (1H, dd, H$^{\beta}$[Asp], J_1 = 17.2 Hz, J_2 = 4.5 Hz); 2.73 (1H, dd, H$^{\beta}$[Asp], J_1 = 17.2 Hz, J_2 = 4.5 Hz); 2.60 (3H, m, CH_3[Pbf]); 2.57–2.49 (5H, m, CH_3[Pbf], N-CH_2-C*H*$_2$); 2.09 (3H, s, CH_3[Pbf]); 1.94–1.76 (1H, m, H$^{\beta}$[Arg]); 1.71–1.51 (3H, m, 1H H$^{\beta}$[Arg], 2H H$^{\gamma}$[Arg]); 1.51–1.37 (36H, m, CH_3-C-CH_3[Pbf], 3 x ^{t}Bu, CH_3[dioxol]); 1.34 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 171.4, 170.6, 170.2, 170.1, 164.9, 158.6, 156.3, 155.4, 138.4, 133.6, 132.4, 124.6, 117.4, 110.1, 86.4, 82.7, 82.4, 80.6, 76.0, 75.1, 66.7, 60.0, 52.1, 50.1, 43.4, 40.5, 39.4, 37.6, 33.8, 29.7, 28.7, 28.4, 28.2, 28.1, 26.9, 25.2, 24.6, 19.4, 18.1, 12.6.

General procedure for the cyclation. Step 1. A suspension of the linear precursor **10a,b** (0.49 mmol) and Pd/C (0.08 g, 15%) in MeOH (or THF/EtOAc 1/1 v/v) (20 mL) was stirred for 16 hours in a H_2 atmosphere, generated by bubbling gas through the mixture. After this time the mixture was filtered through celite. The solvent was evaporated in "vacuo". **Step 2.** The intermediate N,C-deprotected pseudopeptide (yield > 95 %) was immediately dissolved under N_2 atmosphere in dry DMF (180 mL) and the solution was cooled to –15 °C. Anhydrous $KHCO_3$ (0.46 g, 4.6 mmol), HOAt (0.10 g, 0.74 mmol) and HATU (0.23 g, 0.60 mmol) were added at –15 °C, and the mixture was stirred for 20 hours at the same temperature. Evaporation of the solvent under vacuum provided a crude product which was dissolved in EtOAc (60 mL), and the solution was washed consecutively with 1 M HCl (40 mL) and 5% aqueous $NaHCO_3$ (40 mL), dried and evaporated. The crude product was purified by preparative TLC (EtOAc/acetone 4/6).

Cyclo-{[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-methylencarbonyl-azetidin-2-one]-Arg(Pbf)-Gly-Asp(O^{t}Bu)} (14a). Following the general procedure the title compound was obtained starting with the Cbz-Asp(O^{t}Bu)-(O-β-lactam-1-methylencarbonyl)-Gly-Arg(Pbf)-Gly-OBn **10a** (0.42 g, 0.38 mmol). Overall yield: 0.20 g (60%). IR (ν, cm^{-1} CH_2Cl_2 solution): 3440, 3337, 3063, 2971, 2924, 2853, 1763, 1662, 1545. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.99–7.92 (1H, m, NH[Gly]); 7.88 (1H, d, NH[Asp], *J* = 9.1 Hz); 7.08 (1H, d, NH[Arg], *J* = 8.5 Hz); 6.37–6.24 (2H, m, NH-(C=N*H*)-N*H*); 6.13 (1H, b.s., N*H*-(C=NH)-NH); 5.85 (1H, d, H$^{\alpha}_R$[β-lact], *J* = 4.2 Hz); 4.90 (1H, dt, H$^{\alpha}_S$[Asp], J_1 = 9.1 Hz, J_2 = 6.1 Hz); 4.54 (1H, dt, H$^{\alpha}_S$[Arg], J_1 = 8.5 Hz, J_2 = 5.5 Hz); 4.40 (1H, d, N-CH_2, *J* = 15.5 Hz); 4.20–4.04 (3H, m, CH_2[dioxol], H$^{\alpha}$[Gly], CH_S[dioxol]); 3.88 (1H, dd, H$^{\beta}_S$[β-lact], J_1 = 8.5 Hz, J_2 = 4.2 Hz); 3.67–3.42 (3H, m, CH_2[dioxol], N-CH_2, H$^{\alpha}$[Gly]); 3.43–3.31 (1H, m, H$^{\delta}$[Arg]); 3.29–3.16 (1H, m, H$^{\delta}$[Arg]); 2.95 (2H, s, CH_2[Pbf]); 2.79 (1H, d, H$^{\beta}$[Asp], *J* = 6.1 Hz); 2.57 (3H, s, CH_3[Pbf]); 2.49 (3H, s, CH_3[Pbf]); 2.08 (3H, s, CH_3[Pbf]); 2.01–1.88 (1H, m, H$^{\beta}$[Arg]); 1.86–1.69 (1H, m, H$^{\beta}$[Arg]); 1.67–1.53 (2H, m, H$^{\gamma}$[Arg]); 1.46 (6H, s, CH_3-C-CH_3[Pbf]); 1.44 (9H, s, ^{t}Bu); 1.43 (3H, s, CH_3[dioxol]); 1.31 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 172.8, 169.4, 169.2, 169.1, 169.0, 164.9, 159.0, 156.6, 138.5, 132.7, 132.4, 124.8, 117.7, 110.5, 86.6, 82.4, 76.2, 74.3, 66.6, 62.0, 53.7, 48.8, 47.2, 44.7, 43.4, 40.5, 36.7, 29.5, 28.7, 28.2, 26.9, 25.6, 24.9, 19.4, 18.1, 12.6.

Cyclo-{[(3*R*,4*S*)-3-oxy-4-((*S*)-2,2-dimethyl-1,3-dioxolan-4-yl)-1-ethylencarbonyl-azetidin-2-one]-Arg(Pbf)-Gly-Asp(O^{t}Bu)} (14b). Following the general procedure the title compound was obtained starting with the Cbz-Asp(O^{t}Bu)-(O-β-lactam-1-methylencarbonyl)-Arg(Pbf)-Gly-OBn **10b** (0.39 g, 0.35 mmol). Overall yield: 0.14 g (45%). IR (ν, cm^{-1},

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CH_2Cl_2 solution): 3564, 3435, 3325, 3075, 2954, 2922, 2852, 1759, 1652, 1546. ^{1}H-NMR (δ, ppm, $CDCl_3$): 7.97 (1H, d, NH[Asp], *J* = 8.9 Hz); 7.82 (1H, t, NH[Gly], *J* = 3.2 Hz); 7.38 (1H, d, NH[Arg], *J* = 7.6 Hz); 6.44–6.15 (3H, m, NH-(C=NH)-NH); 5.76 (1H, d, H^{α}_R[β-lact], *J* = 4.7 Hz); 4.80 (1H, dt, H^{α}_S[Asp], J_1 = 8.9 Hz, J_2 = 5.8 Hz); 4.55 (1H, ddd, H^{α}_S[Arg], J_1 = 7.6 Hz, J_2 = 5.6 Hz, J_3 = 4.9 Hz); 4.17–4.09 (3H, m, CH_2[dioxol], H^{α}[Gly], CH_S[dioxol]); 3.72 (1H, dd, H^{β}_S[β-lact], J_1 = 8.4 Hz, J_2 = 4.7 Hz); 3.69–3.53 (3H, m, 2H N-CH_2, 1H H^{α}[Gly]); 3.47–3.39 (1H, m, CH_2[dioxol]); 3.33–3.09 (3H, m, 2H H^{δ}[Arg], 1H N-CH_2-CH_2); 2.94 (2H, s, CH_2[Pbf]); 2.81 (2H, d, H^{β}[Asp], *J* = 5.8 Hz); 2.56 (3H, s, CH_3[Pbf]); 2.53–2.41 (4H, m, CH_3[Pbf], N-CH_2-CH_2); 2.08 (3H, s, CH_3[Pbf]); 1.95–1.79 (1H, m, H^{β}[Arg]); 1.77–1.47 (3H, m, 1H H^{β}[Arg], 2H H^{γ}[Arg]); 1.45 (6H, s, CH_3-C-CH_3[Pbf]); 1.43 (9H, s, ^{t}Bu); 1.40 (3H, s, CH_3[dioxol]); 1.33 (3H, s, CH_3[dioxol]). ^{13}C-NMR (δ, ppm, $CDCl_3$): 173.0, 171.3, 169.7, 169.6, 169.30, 164.8, 158.9, 156.6, 138.4, 132.9, 132.4, 124.8, 117.6, 110.1, 86.5, 82.2, 76.0, 74.7, 66.3, 61.5, 52.8, 48.9, 44.6, 43.4, 41.0, 40.6, 36.8, 34.2, 29.5, 28.7, 28.2, 26.9, 25.7, 25.2, 19.4, 18.1, 12.6.

General procedure for the deprotection of the macrocycles 15a,b and 16a,b. The final deprotection was carried out dissolving the macrocycle **14a,b** or **13a,b** (0.035 mmol) in (CF_3CO_2H/H_2O/Et_3SiH (1.8 mL/ 0.2 mL/ 0.3 mL) at 4 °C and stirring the mixture at same temperature during 16 hours. After this time the product was precipitated adding diisopropylether (10 mL) at 0 °C and centrifuging at 2.2 RCF. The solid was washed again with diisopropylether (4 x 2 mL) and dried under vacuum to provide the corresponding RGD cyclic mimetic as a trifluoracetate salt.

Cyclo-{[(3*R*,4*S*)-3-oxy-4-((*S*)-1,2-dihidroxy-ethyl)-1-methylencarbonyl-azetidin-2-one]-Arg-Gly-Asp} trifluoroacetic salt (15a). Following the general procedure the title compound was obtained starting with the macrocycle **14a** (30 mg, 0.035 mmol). Yield: 15 mg (80%). IR (ν, cm^{-1}, solid): 3277, 2921, 1735, 1648, 1542. ^{1}H-NMR (δ, ppm, D_2O): 5.94 (1H, d, H^{α}_R[β-lact], *J* = 4.4 Hz); 4.92–4.79 (1H, m, H^{α}_S[Asp]); 4.53 (1H, d, N-CH_2, *J* = 15.3 Hz); 4.42 (1H, dd, H^{α}_S[Arg], J_1 = 8.3 Hz, J_2 = 6.5 Hz); 4.16 (1H, dd, H^{β}_S[β-lact], J_1 = 7.9 Hz, J_2 = 4.4 Hz); 4.07 (1H, d, H^{α}[Gly], *J* = 14.7 Hz); 3.86 (1H, ddd, CH_SOH, J_1 = 8.2 Hz, J_2 = 6.2 Hz, J_3 = 4.0 Hz); 3.79 (1H, d, H^{α}[Gly], *J* = 14.7 Hz); 3.70–3.61 (2H, m, CH_2OH, N-CH_2); 3.57 (1H, dd, CH_2OH, J_1 = 12.1 Hz, J_2 = 6.1 Hz); 3.26 (2H, t, H^{δ}[Arg], *J* = 6.8 Hz); 3.12 (1H, dd, H^{β}[Asp], J_1 = 17.3 Hz, J_2 = 6.0 Hz); 2.95 (1H, dd, H^{β}[Asp], J_1 = 17.3 Hz, J_2 = 7.3 Hz); 1.99–1.73 (2H, m, H^{β}[Arg]); 1.73–1.58 (2H, m, H^{γ}[Arg]). ^{1}H-RMN (δ, ppm, D_2O/H_2O (1/9)): 8.86 (1H, d, NH[Asp], *J* = 8.1 Hz); 8.06 (2H, m, NH[Arg], NH[Gly]); 7.23 (1H, t, N*H*-(C=NH)-NH_2, *J* = 6.1 Hz) 6.79–6.53 (3H, b.s, NH-(C=N*H*)-NH_2). ^{13}C-NMR (δ, ppm, D_2O): 174.4, 173.1, 170.6, 170.2, 169.8, 165.7, 156.8, 76.8, 69.7, 62.6, 60.2, 54.2, 49.4, 46.3, 43.0, 40.5, 34.3, 27.4, 24.4. HRMS (ESI): m/z calcd for $C_{19}H_{29}N_7O_{10}$ $(M + H)^+$ 516.2054, found 516.2051.

Cyclo-{[(3*R*,4*S*)-3-oxo-4-((*S*)-1,2-dihidroxy-ethyl)-1-ethylencarbonyl-azetidin-2-one]-Arg-Gly-Asp} trifluoroacetic salt (15b). Following the general procedure the title compound was obtained starting with the macrocycle **14b** (30 mg, 0.034 mmol). Yield: 15 mg (80%). IR (ν, cm^{-1}, solid): 3500–2700, 1743, 1654, 1542. ^{1}H-NMR (δ, ppm, D_2O): 5.91 (1H, d, H^{α}_R[β-lact], *J* = 4.7 Hz); 4.96–4.86 (1H, m, H^{α}_S[Asp]); 4.60 (1H, dd, H^{α}_S[Arg], J_1 = 8.2 Hz, J_2 = 6.1 Hz); 4.13 (1H, d, H^{α}[Gly], *J* = 14.9 Hz); 4.02 (1H, dd, H^{β}_S[β-lact], J_1 = 8.9 Hz, J_2 = 4.7 Hz); 3.81 (1H, d, H^{α}[Gly], *J* = 14.9 Hz); 3.76–3.60 (4H, m, 2H N-CH_2, CH_SOH, CH_2OH); 3.55 (1H, dd, CH_2[dioxol], J_1 = 12.2 Hz, J_2 = 6.0 Hz); 3.30 (2H, t, H$^{\delta}$[Arg], *J* = 6.7 Hz); 3.19 (2H, ddd, N-CH_2-CH_2, J_1 = 15.7 Hz, J_2 = 12.4 Hz, J_3 = 5.1 Hz); 3.09–2.98 (2H, m, H^{β}[Asp]); 2.66 (1H, dt, N-CH_2-CH_2, J_1 = 15.7 Hz, J_2 = 3.1 Hz); 2.02–1.80 (2H, m, H^{β}[Arg]); 1.77–1.60 (2H, m, H^{γ}[Arg]). ^{1}H-RMN (δ, ppm, D_2O/H_2O (1/9)): 8.70 (1H, d, NH[Asp], *J* = 8.1 Hz); 8.58 (1H, t, NH[Gly], *J* = 8.3 Hz); 8.37 (1H, d, NH[Arg], *J* = 8.6 Hz); 7.10 (3H, m, NH-(C=NH)-NH_2). ^{13}C-NMR (δ, ppm, D_2O): 175.3, 173.5, 173.4, 167.9, 166.5, 156.8, 74.8, 69.9, 63.3, 58.1, 52.4, 49.2, 40.5, 39.0, 33.7, 32.8, 27.7, 24.4. HRMS (ESI): m/z calcd for $C_{20}H_{31}N_7O_{10}$ $(M + H)^+$ 530.2210, found 530.2210

Asp-[(3*R*,4*S*)-3-oxy-4-((*S*)-1,2-dihidroxy-ethyl)-1-methylencarbonyl-azetidin-2-one]-Arg trifluoroacetic salt (16a). Following the general procedure the title compound was obtained starting with the product **13a** (35 mg, 0.036 mmol). Yield: 14 mg (80%). IR (ν, cm^{-1}, solid): 3370–2939 (b.s.), 1771, 1659, 1416. ^{1}H-NMR (δ, ppm, D_2O): 6.13 (1H, d, H^{α}_R[β-lact], *J* = 5.0 Hz); 4.60 (1H, t, H^{α}_S[Asp], *J* = 4.6 Hz); 4.42 (1H, dd, H^{α}_S[Arg], J_1 = 8.6 Hz, J_2 = 5.0 Hz); 4.35–4.22 (2H, m, H^{β}_R[β-lact], N-CH_2); 4.18 (1H, d, N-CH_2, *J* = 17.3 Hz); 4.02–3.91 (1H, m, CH_SOH); 3.69 (1H, dd, CH_2OH, J_1 = 12.2 Hz, J_2 = 3.6 Hz); 3.58 (1H, dd, CH_2OH, J_1 = 12.2 Hz, J_2 = 5.4 Hz); 3.29–3.17 (3H, m, H^{δ}[Arg], H^{β}[Asp]); 3.09 (1H, dd, H^{β}[Asp], J_1 = 18.3 Hz, J_2 = 4.6 Hz); 2.04–1.89 (1H, m, H^{β}[Arg]); 1.89–1.74 (1H, m, H^{β}[Arg]); 1.74–1.61 (2H, m, H^{γ}[Arg]). ^{1}H-RMN (δ, ppm, D_2O/H_2O (1/9)): 8.73 (1H, d, NH[Arg], *J* = 7.2 Hz); 7.27–7.21 (1H, m, N*H*-(C=NH)-NH_2); 6.70 (3H, b.s., NH-(C=N*H*)-NH_2). ^{13}C-NMR (δ, ppm, D_2O): 175.2, 172.6, 169.6, 167.6, 167.0, 156.5, 74.9, 68.9, 63.4, 60.1, 52.4, 48.9, 44.8, 40.2, 33.4, 27.4, 24.1. HRMS (ESI): m/z calcd for $C_{17}H_{28}N_6O_{10}$ $(M + H)^+$ 477.1945, found 477.1942.

Asp-[(3*R*,4*S*)-3-oxo-4-((*S*)-1,2-dihidroxy-ethyl)-1-ethylencarbonyl-azetidin-2-one]-Arg trifluoroacetic salt (16b). Following the general procedure the title compound was obtained starting with the product **13b** (35 mg, 0.035 mmol). Yield: 14 mg (80%). IR (ν, cm^{-1}, solid): 3359–2500 (b.s.), 1742, 1654, 1559, 1388. ^{1}H-NMR (δ, ppm, D_2O): 5.96 (1H, d, H^{α}_R[β-lact], *J* = 4.5 Hz); 4.55 (1H, t, H^{α}_S[Asp], *J* = 4.9 Hz); 4.36 (1H, dd, H^{α}_S[Arg], J_1 = 8.7 Hz, J_2 = 5.0 Hz); 4.21–4.18 (1H, m, H^{β}_R[β-lact]); 3.98–3.92 (1H, m, CH_SOH); 3.91–3.82 (1H, m, N-CH_2); 3.66 (1H, dd, CH_2OH, J_1 = 12.1 Hz, J_2 = 3.8 Hz); 3.59 (1H, dd, CH_2OH, J_1 = 12.1 Hz, J_2 = 6.6 Hz); 3.53 (1H, td, N-CH_2, J_1 = 13.9 Hz, J_2 = 5.6 Hz); 3.24 (2H, t, H^{δ}[Arg], *J* = 6.8 Hz); 3.17 (1H, dd, H^{β}[Asp], J_1 = 18.1 Hz, J_2 = 4.9 Hz); 3.05 (1H, dd, H^{β}[Asp], J_1 = 18.1 Hz, J_2 = 4.9 Hz); 2.68 (2H, t, N-CH_2-CH_2, *J* = 6.6 Hz); 2.00–1.87 (1H, m, H^{β}[Arg]); 1.85–1.73 (1H, m, H^{β}[Arg]); 1.73–1.61 (2H, m, H^{γ}[Arg]). ^{1}H-RMN (δ, ppm, D_2O/H_2O (1/9)): 8.53 (1H, d NH[Arg], *J* = 7.6 Hz); 7.20 (1H, t, N*H*-(C=NH)-NH_2, *J* = 5.0 Hz); 6.67 (3H, b.s., NH-(C=N*H*)-NH_2). ^{13}C-NMR (δ, ppm, D_2O): 175.7, 173.7, 173.1, 168.0, 166.4, 156.4, 74.3, 69.0, 63.0, 57.7, 52.5, 49.2, 40.1, 38.7, 34.1, 32.5, 27.5, 24.1. HRMS (ESI): m/z calcd for $C_{18}H_{30}N_6O_{10}$ $(M + H)^+$ 491.2101, found 491.2103.

NH_2-Arg(Pbf)-Gly-Obn (17). Step 1. The commercial Boc-*L*-Arg(Pbf)-OH (4 g, 7.6 mmol) was dissolved in dry CH_2Cl_2 (180 mL) at 0 °C and Et_3N (2 mL, 15.2 mmol), EDC·HCl (2.33 g, 12.16 mmol), HOBt (1.44 g, 10.6 mmol) and NH_2-Gly-OBn·HCl (1.76 g, 8.4 mmol) was added. The mixture was stirred at room temperature about 16 hours. After this time, the reaction was washed with HCl 0.1 M (2 x 80 mL) and saturated solution of $NaHCO_3$ (2 x 80 mL). The organic layer was dried with $MgSO_4$, evaporated and the crude product was purified by flash chromatography (hexane/EtOAc 1/2). **Step 2.** The deprotection of Boc group was carried out dissolving the compound in HCOOH (40 mL) at room temperature during 5 hours getting the desired product. This product was used in the next step without purification process. Overall yield: 4.23 g (84%). IR (ν, cm^{-1}, CH_2Cl_2 solution): 3433, 3334, 2972, 1748, 1669, 1616, 1548. ^{1}H-NMR (δ, ppm, $CDCl_3$): 8.00 (1H, t, NH[Gly], *J* = 5.3 Hz); 7.42–7.21 (5H, m, arom.); 6.42–6.22 (3H, m, NH(C=NH)NH); 5.13 (2H, s, CH_2-Ph); 4.03 (2H, dd, H^{α}[Gly], J_1 = 5.3 Hz, J_2 = 2.7 Hz); 3.51–3.37 (1H, m, H^{α}_S[Arg]); 3.30–3.12 (2H, m, H^{δ}[Arg]); 3.00 (2H, s, CH_2[Pbf]); 2.60 (3H, s, CH_3[Pbf]); 2.52 (3H, s, CH_3[Pbf]); 2.10 (3H, s, CH_3[Pbf]); 1.95–1.74 (2H, m, H^{β}[Arg]); 1.74–1.53 (2H, m, H^{γ}[Arg]); 1.48 (6H, s, CH_3-C-CH_3). ^{13}C-NMR (δ, ppm, $CDCl_3$): 176.1, 170.0, 158.7, 156.5, 138.2, 135.3, 133.0, 132.2, 128.6, 128.4, 128.3, 128.2, 124.7, 117.5, 86.4, 67.0, 54.3, 43.2, 41.1, 40.6, 32.0, 28.6, 25.4, 19.2, 17.9, 12.4.

"In vitro" HUVEC Cell Adhesion Inhibition Test.

Cell lines and reagents Human umbilical endothelial cell (HUVEC) was purchased from Cambrex BioScience (USA). Cells were grown on the 0.5% gelatin coated plate in CS-C Complete Medium (Sigma) and were used for experiments after three passages. Human vitronectin was from

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Sigma. Cyclopentapeptides were dissolved in double-distilled water at a concentration of 10 mg/mL and stored in the dark at –80°C.

Endothelial cell adhesion assay HUVEC cells were grown to subconfluent state and then harvested by 0.025% trypsin/EDTA (Sigma) in one minute. Cells were preincubated with various concentrations of peptides on ice for 15 min in CS-C Medium. Same volume of vehicle was added to cells as a control. Peptide-treated cells (5 x 10^4 cells /100µL/well) were plated onto a 96-well microplate which was precoated with vitronectin (10 mg/mL in PBS), and incubated for 1h at 37°C, 5% CO_2/95% air to allow cell attachment. Cells were washed gently with PBS for three times to remove detached cells. Number of adherent cells was measured by Fluorescent Cell counting Kit (FCCK) at 535 nm (excitation at 485 nm) using a fluorescent plate reader (Bioscan). Experiments were done in triplicate wells and repeated four times.

Gene Regulation Assay

Cell culture and compound treatment. HUVEC cells were maintained in CS-C Complete Medium, grown at 50% of confluency and then treated with 10^{-5} M final concentration of compounds C (Cilengitide®), 15a,b and 16a-d during 48 h.To verify non-apoptotic induction in the RGD-treated cells, the apoptosis-associated tyrosine kinase (AATK) (a), and the apoptosis regulator BAX (BCL2-associated X protein) (b) gene-expression were measured. No significant changes were observed after 72 hours treatment with any of the compounds.[39]

RNA isolation and Gene-Expresion microarray assays Total RNA was extracted from untreated control or RGD-compounds treated cells using TRIzol (Invitrogen) according to standard protocol. RNA was further purified with RNeasy Mini Kit (Qiagen) and the concentration and quality was assessed by NanoDrop spectrophotometer and Agilent 2100 Bioanalyzer, respectively. Only RNA samples with a RIN number between 9-10 were used in the microarray assays.

Gene-Expresion microarray assay. In order to study the intracellular effect of all RGD-binding compounds, a cell-culture whole gene-expression assay was performed. HUVEC cells were treated separately with a 10^{-5} M concentration of the test compounds during 48 h. The simultaneous analysis of all human gene-expression by microarray let us identify which human gene(s) were induced, inhibited or unaffected after the test compounds were bound to the receptors of the cells. Whole human gene expression microarray analysis was performed using Agilent Technologies (Santa Clara, CA). This platform comprises a two-color design with two RNA samples labeled with either Cy3 or Cy5 and hybridized to the same 44K Agilent microarray. Each array contains about 41,000 unique noncontrol 60-mer probes. RNAs from untreated and peptide-treated HUVEC cells were two-color labeled and hybridized to randomly chosen arrays a total of four times. A dye swap was performed for each sample on a different slide to remove any bias from the labeling dyes. For each sample, 800 ng total RNA was reverse transcripted, linear amplified and labeled with either Cy3 or Cy5 using Agilent's Low RNA Input Linear amplification Kit PLUS, according to manufacturer's instructions. After labeling, samples were measured on a Nanodrop microarray module for labeling efficiency and quantification. Samples were then hybridized on Agilent 4 x 44K whole human genome GE arrays at 65ºC for 17 h. After washing in GE washing buffer, the slide was scanned with Agilent Microarray Scanner. Feature extraction software (version 9.5.3) was used to convert the image into expession data. Data were normalized by the Linear Lowess method. Genes that were 1.5-fold differentially expressed on 3 of 4 arrays were scored as significant. Furthermore, only genes with a p-value ≤ 0.05 based on the Student's t-test were selected. Mean fold change is mean of 4 arrays. Angiogenesis-related genes were classified according to Gene Ontology (Figure S6 and Table S8)[40].

Acknowledgements

We thank the Ministerio de Economía y Competividad (MEC, Spain) (Project: IPT-010000-2011-2014), UPV/EHU (UFI QOSYC 11/22) and Gobierno Vasco/Eusko Jaurlaritza (GAITEK IG-2011/0000489, GV grant IT-628-13) for financial support and SGIker UPV/EHU for NMR facilities.

Keywords cyclopeptidomimetics, cyclodepsipeptidomimetics, β-lactams, αvβ3 integrin, CGH array

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