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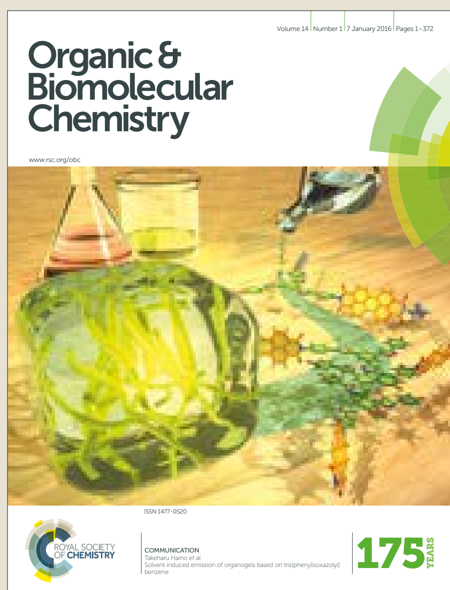

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Stereodivergent Synthesis of 5-Aminopipecolic Acids and Application in the Preparation of a Cyclic RGD Peptidomimetic as a nanomolar $\alpha_V\beta_3$ Integrin Ligand

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A stereodivergent strategy was devised to obtain enantiopure *cis* and *trans* 5-aminopipecolic acids (5-APAs) in suitably protected forms to be employed in peptide synthesis as conformationally constrained α- and δ-amino acids. The *cis* isomer was used as a δ-amino acid to construct a cyclic RGD-containing peptidomimetic, the ability of which to compete with biotinylated vitronectin for the binding to the isolated $\alpha_V\beta_3$ integrin was measured (IC_{50} = 4.2 ± 0.9 nM). A complete ^{1}H NMR and computational conformational analysis was performed to elucidate the reasons for the high affinity of this cyclic peptidomimetic in comparison with Cilengitide.

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Introduction

Integrins are heterodimeric transmembrane receptors for extracellular matrix (ECM) proteins which promote adhesion, migration, and proliferation of cells by recognizing binding motifs in ECM proteins.[1] The integrin family that recognizes endogenous arginine-glycine-aspartic acid (RGD) ligands are of great therapeutic interest, playing a role in thrombosis, fibrosis, and cancer. In cancer, integrins mediate adhesion events during various stages of the disease, *i.e.* angiogenesis, tumor growth and progression, invasion, and metastasis.[2–5] In particular, the $\alpha_V\beta_3$ integrin receptor, which recognizes the RGD sequence of vitronectin,[6–7] has a critical role in tumor-induced angiogenesis and metastasis formation.[8–13] Therefore, RGD-containing peptides and peptidomimetics[14–17] as well as RGD-mimetics[1a] are widely studied as antagonists to suppress the events mediated by this integrin and as shuttles for targeted delivery of drugs and diagnostics.[18]

To date, a few low-nanomolar-affinity $\alpha_V\beta_3$ receptor binders which present the RGD system installed on rigid hetero- and carbacyclic scaffolds have been reported. For example, bicyclic lactams,[19–21] γ-aminocyclopentanecarboxylic acids,[22] *cis*-β-aminocyclopropanecarboxylic acids,[23] 4-aminoprolines,[24,25] bifunctional diketopiperazines,[26] morpholine derivatives,[27,28] 7-aminoazocinone-2-carboxylate derivatives,[29] and both amino- and hydroxy-substituted 2,3-methanopipecolic acids (or CPA, cyclopropane pipecolic acid), the latter recently reported by us,[30-32] have all been used to generate potent $\alpha_V\beta_3$ integrin ligands.

Because of our interest in the synthesis[33-36] and biomedical applications[30-32] of pipecolic acid derivatives, in order to further expand the array of available scaffolds which can be grafted into the key $\alpha_V\beta_3$-recognizing peptide sequence, we decided to synthesize and evaluate 5-aminopipecolic acid, the homologue of 4-aminoproline,[24,25] as a δ-amino acid on which to install the RGD peptide sequence, thus generating new cyclic $\alpha_V\beta_3$ integrin ligands. Pipecolic acid derivatives bearing an amino group at position 3, 4 and 5 (Figure 1) are examples of endocyclic-N^{α}/exocyclic-N^{β}, -N^{γ}, and -N^{δ} constrained basic amino acids with a considerable potential in medicinal

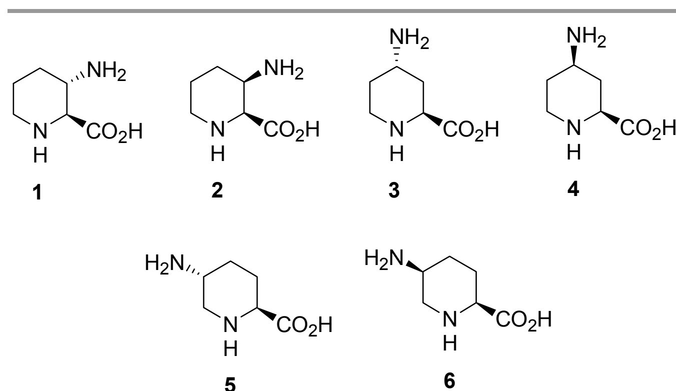

Figure 1. 3-,4-, and 5-Aminopipecolic Acids **1**-**6**.

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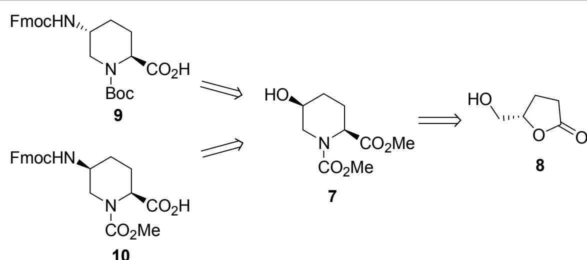
chemistry not only as α-amino acids with restricted ϕ, χ^1, χ^2, and/or χ^3 torsion angles, but also as rigid β-, γ- or δ-amino acids. While some examples of peptidomimetics incorporating 4-aminopicolic acids **3** and **4** as α-amino acids exist,[37,38] their regioisomers have rarely been introduced in amino acid sequences to generate conformationally restricted peptides or embedded in other bioactive compounds, and not many efforts have been devoted to their synthesis.[39] In particular, the synthesis of enantiopure *trans* 5-aminopipecolic acid (herein referred as 5-APA) **5**, used as a novel chiral PNA monomer, has been reported by Kumar.[40] Racemic *trans* and *cis* 5-APAs (**5** and **6**) were prepared by Dhimane as new conformationally constrained ornithine analogues, as well as intermediates for obtaining arginine analogues with simultaneous restriction of χ^i and ϕ torsion angles.[41] Racemic *cis* 5-APA **6** was also prepared by Harris[42] and Shono,[43] in both cases as an intermediate in the synthesis of (±)-Slaframine. Therefore, given the paucity of synthetic approaches to enantiopure *trans* and *cis* 5-APA **5** and **6**, and because of our interest in the incorporation of *cis* 5-APA in cyclic RGD ligands, we planned a stereodivergent strategy to obtain **5** and **6**, suitably protected for peptide synthesis, in enantiopure form.

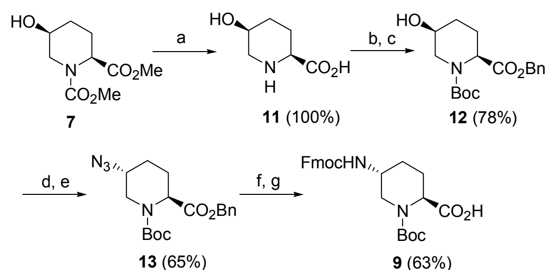
Results and Discussion

Chemistry

We have recently reported an efficient synthesis of both enantiomers of 4-hydroxypipecolic acid methyl ester derivative **7** (Scheme 1) in gram quantities starting from commercial γ-(hydroxymethyl)butyrolactone **8**.[35]



Scheme 1. Retrosynthetic scheme to *trans*- and *cis*-5-aminopipecolic acids **9** and **10**.



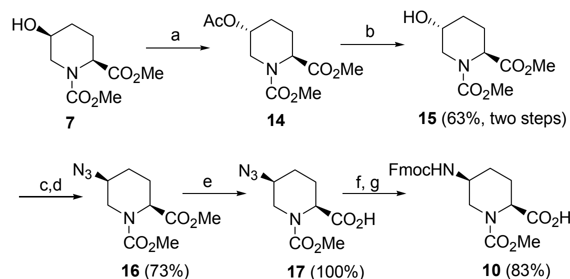
Scheme 2. *Reagents and conditions*: (a) 4 N HCl, reflux; (b) Boc_2O, Et_3N, MeOH, reflux, 24 h; (c) K_2CO_3, BnBr, DMF, 0 °C, then 25 °C, 24 h; (d) MsCl, Et_3N, CH_2Cl_2, -30 °C, then 25 °C, 2 h; (e) NaN_3, DMF, 100 °C, 3 h; (f) H_2, 10% Pd/C, MeOH, 25 °C, 24 h; (g) FmocOSu, Na_2CO_3 aq., THF, 0 °C then 25 °C, 16 h.

In analogy to both Kumar's[40] and Dhimane's[41] syntheses, this substrate is suitable to be converted into both *trans* and *cis* 5-APAs **9** and **10** (Scheme 1) by functional and protecting group manipulation, although the installation of the orthogonal exocyclic *N*-Fmoc and endocyclic *N*-Boc groups in *trans* 5-APA **9** required a different strategy.

For the synthesis of protected *trans* 5-APA **9** (Scheme 2) with *S* absolute configuration at C1, we exhaustively deprotected (1*S*, 5*S*)-**7** to obtain free amino acid **11**.[35] After protection of the N atom as *N*-Boc, benzylation of the carboxylic group provided ester **12** in 78% yield over two steps. Mesylation of the OH group, followed by nucleophilic displacement by sodium azide in DMF provided *trans* compound **13** in 65% yield (two steps). Eventually, hydrogenation over 10% Pd/C provided the free amino acid intermediate which was converted into the *N*-Fmoc protected derivative **9** in 63% yield over two steps and with orthogonal protection suitable for peptide synthesis.

For the synthesis of the *cis* 5-APA, since we wanted to introduce it in a cyclic peptide as a δ-amino acid, we decided to leave the original protection on the heterocycle N atom unaltered (Scheme 3). A first inversion of configuration was carried out on **7** by a Mitsunobu reaction which provided *trans* acetate **14**. This acetate could not be separated from a by-product but, after hydrolysis, alcohol **15**[35] was obtained in pure form by chromatography, in 63% yield over two steps. As before, the alcohol was converted into azide **16** (73% over two steps) and the methyl ester group of the latter hydrolyzed under basic conditions to give **17** (100%). This allowed us to reduce the azido to amino group without the risk of lactamisation during the hydrogenation step.[41b] The free amino acid intermediate was then protected as *N*-Fmoc derivative, furnishing *cis* 5-APA **10** in 83% yield ready for the incorporation into the cyclic peptide.

The *cis* relative stereochemistry was easily determined on azide **16** by ^{1}H NMR analysis. First, the carboxylic moiety at position 2 is axially oriented to remove the $A^{1,3}$-allylic strain present in α-substituted *N*-acylpiperidines,[44] as demonstrated by the small coupling constants (< 5.6 Hz) of 2-H with the protons at C3. The large coupling constant (12.4 Hz) of 5-H with the axially oriented 6-H is in accordance with the equatorial orientation of the azido group, which therefore is *cis* to the carboxylic moiety.



Scheme 3. *Reagents and conditions*: (a) Ph_3P, DIAD, AcOH, THF, 0 °C, 1 h; (b) MeONa, MeOH, 0 °C, 6 h; (c) MsCl, Et_3N, CH_2Cl_2, -30 °C, then 25 °C, 2 h; (d) NaN_3, DMF, 100 °C, 3 h; (e) 1 N NaOH, MeOH, 25 °C, 2 h; (f) H_2, 10% Pd/C, MeOH, 25 °C, 24 h; (g) FmocOSu, Na_2CO_3 aq., THF, 0 °C then 25 °C, 16 h.

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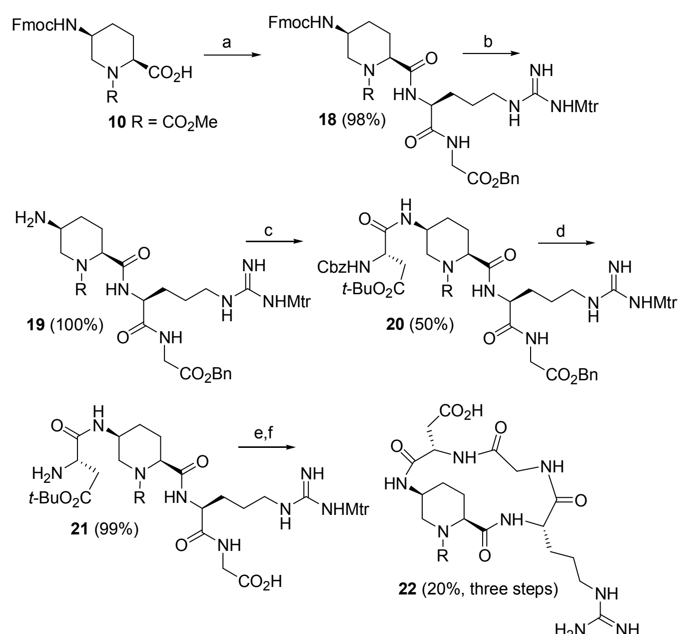

Scheme 4. *Reagents and conditions*: (a) DEPBT, DIPEA, H-Arg(Mtr)-Gly-OBn, THF, 35 °C, 4 d; (b) CH_2Cl_2/DEA 1 : 1, 3 h; (c) DEPBT, DIPEA, Z-Asp(OtBu)-OH, THF, 35 °C, 4 d; (d) H_2 (1 atm), 10% Pd/C, EtOH, 24 h; (e) DEPBT, DIPEA, THF, 35 °C, 4 d; (f) TFA/TIS/H_2O 95 : 2.5 : 2.5, 18 h.

In alcohol **15** (and in the corresponding mesylate) this coupling constant is instead low (< 5 Hz), because of the axial position of the substituent at position 5.

The synthesis of cyclopeptide **22** was carried out in solution (Scheme 4) as reported for 4-aminoproline derivatives[24] and 4-amino-substituted cyclopropane pipecolic acids.[32] Carboxylic acid **10** was coupled to dipeptide H-Arg(Mtr)-Gly-OBn by using DEPBT [3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one] as the coupling reagent in THF at 35 °C which provided, after four days, tripeptide **18** in 98% yield after chromatography. Deprotection of the 5-amino group in **18** by Et_2NH provided compound **19** quantitatively, which was then coupled to *N*-Z,*O*-*t*Bu-protected aspartic acid as above to give tetrapeptide **20** in 50% yield after chromatography. Catalytic hydrogenation over 10% Pd/C provided deprotected tetrapeptide **21**. This, as a crude reaction mixture, was subjected to cyclization (4 days) by using DEPBT as the coupling reagent in THF (3.5 mM), which furnished cyclopeptide **22** after exhaustive deprotection of the cyclization intermediate. Semi-preparative HPLC purification allowed us to obtain pure **22** (white solid) in 20% yield (over the last three steps) as a TFA salt.

Biological Studies

The RGD-containing peptidomimetic **22** was tested for its capacity to compete with vitronectin for the binding to $\alpha_V\beta_3$ integrin expressed in high level by M21 human melanoma cells as reported,[30,32] in comparison to the reference RGD ligand cyclo[RGDf(N-Me)V] (Cilengitide)[45] (Table 1).

Table 1. Binding affinity of compound **22** towards integrin $\alpha_V\beta_3$

Entry	Ligand	IC_{50} (nM)	
		M21 cells[a]	Isolated $\alpha_V\beta_3$[b]
1	**22**	228 ± 128	4.2 ± 0.9
2	c[RGDf(N-Me)V] (Cilengitide)	3.8 ± 1.7	0.71 ± 0.06[c]
3	c[RGDfV]	-	2.1 ± 0.9

[a] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of integrin-mediated M21 cell adhesion to vitronectin. Experiments were conducted in triplicate and all values are the arithmetic mean ± SD.
[b] IC_{50} values were calculated as the concentration of compound required for 50% inhibition of biotinylated vitronectin binding as estimated by GraphPad Prism software. All values are the arithmetic mean ± SD of duplicate determinations.
[c] Ref. 47

Tests were performed in the presence of 2 mM $MnCl_2$, in order to switch integrins of tumor cells into an activated form. The ability of compound **22** to compete with biotinylated vitronectin for the binding to $\alpha_V\beta_3$ was also tested towards the isolated receptor following a slightly modified procedure reported previously,[46] in comparison with both Cilengitide and cyclo[RGDfV], the parent peptide of Cilengitide. In the cell adhesion test, compound **22** was shown to inhibit the binding of M21 melanoma cells to vitronectin (IC_{50} = 228 ± 128 nM) but roughly sixty times less than Cilengitide (IC_{50} = 3.8 ± 1.7 nM). These data are in agreement with the results obtained in the test with the isolated receptor, with a less marked difference between the two ligands, that showed for **22** an IC_{50} value of 4.2 ± 0.9 nM and for Cilengitide an IC_{50} value of 0.71 ± 0.06 nM.[47] The comparatively lower inhibition activity of **22** measured on M21 cell line could be due to the presence of other integrins which are inhibited by Cilengitide.[17] We have already assessed by flow cytometry analysis that the M21 cell line, besides high level of $\alpha_V\beta_3$, expresses also low level (4%) of $\alpha_V\beta_5$ heterodimer.[32] In any case, as will be discussed later, these data suggest that the 5-APA structure induces a significant conformational asset towards the optimal presentation of the pharmacophoric groups of the ligand.

Conformational Analysis

^{1}H NMR analysis

The structure and connectivity of cyclopeptide **22** was unambiguously assigned by means of mono- and bi-dimensional ^{1}H NMR spectroscopy in aqueous solution (8 mM, in D_2O/H_2O 1:9) (all ^{1}H NMR data are reported in the ESI, Table S1) which revealed the presence of two sets of signals in a 1.4:1 ratio as a consequence of the existence of rotamers around the *N*-CO_2Me bond.

Most data suggest the existence of a preferred conformation for ligand **22**, even though the temperature coefficient values between −5.56 and −8.0 ppb K^{-1} (Figure S1, ESI) for the N-H protons of Asp, Arg and Gly indicate that none of these protons is tightly locked in an intramolecular H-bonded state. Instead a low coefficient value (-3.56 ppb K^{-1}) for 5-APA-NH seems to suggest the involvement of this proton in some H-bonding. Concerning the six-membered ring, a marked distortion of the initial chair after the cyclization, probably toward a twist-boat conformation, places the C-5 substituent

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in a pseudoaxial orientation, as shown by the decreasing coupling constant between 6-H_{ax} and 5-H from 12.4 to about 4 Hz. The analysis of the NOE contacts (see Figure 2) showed the presence of medium-strong sequential CHα(i)/NH(i + 1)

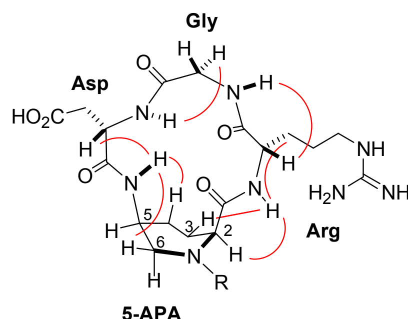

Figure 2. NOE correlations in compound **22**

cross-peaks along the Arg-Gly-Asp sequence, although AspNH has a NOE cross-peak only with one of the two CHα protons of Gly. Two strong contacts, very useful for the determination of the putative preferred conformation in solution, were found between ArgNH and the CHα of the pipecolic acid (i.e. 2-H) as well as with equatorial 3-H of the pipecolic ring, suggesting an "outward" orientation of the N–H bond. Moreover, 5-APA-NH seems oriented "inward" as it shows a strong cross-peak with equatorial 6-H (and a weaker one with 4-H), possibly forming an H-bond with the "inward" oriented pipecolic acid C=O group, as corroborated by the lowest temperature coefficient (−3.56 ppb K^{-1}) for this amide proton.

As a further indication of the existence of a preferred conformation in aqueous solution, the two diastereotopic CHα of Gly resonate as highly separated dds (4.04 and 3.50 ppm) and, moreover, only one of them (i.e. that at 4.04 ppm) shows a cross-peak with the AspNH.

Computational analysis

The conformational preference of compound **22** was also evaluated by replica exchange molecular dynamic (REMD) simulations,[48] using a previously optimized protocol[49] that proved to be successful in determining the preferred conformation of similar cyclopeptides.[30] The simulation consisted in 12 replica of 400 ns, with temperatures spanning from 300 to 860 K, without using any restraint derived from the observed NOE's.

Table 2. Population and Selected Dihedrals (deg.) of the Main Conformational Cluster of Compound **22** and Cilengitide.[a] The Corresponding Parameters Measured in the Cilengitide-$\alpha_V\beta_3$ Crystal Structure Are Also Reported for Comparison.

	22	Cilengitide	
		REMD	crystal
Pop%	93.3	64.4[b]	/
ϕ1	-57.9±9.3	-100.8±23.0	-114.5
ψ1	119.5±12.8	106.5±25.6	130.5
ϕ2	88.1±15.2	91.3±27.3	84.0
ψ2	-81.3±11.5	-103.1±25.0	-136.2
ϕ3	-145.5±10.8	-104.5±29.1	-87.1
ψ3	77.6±14.6	94.0±16.4	61.4
dih1	33.0±15.7	/	/
dih2	174.7±9.6	/	/

[a] Calculated from the cluster analysis of the final 50 ns of the 400 ns REMD trajectory obtained at 300 K.
[b] Additional conformations found for Cilengitide, together with corresponding populations, are shown in Figure S2 (ESI).

As a comparison, the same protocol was applied to Cilengitide, taken as a reference.[17] The most representative conformations of compound **22** and Cilengitide are shown in Figure 3, while selected geometrical parameters are reported in Table 2.

It can be observed that the obtained conformation for **22** perfectly matches the putative preferred conformation inferred by the NMR analysis (Figure 3A). As suggested by the latter, the six-membered ring is in a twist-boat conformation and the C5-NH bond is pseudoaxially oriented with the 5-APA-NH pointing slightly inward (C5-C6-N-H dihedral (dih1) = 33.0±15.7 deg.) and forming the aforementioned H-bond with the pipecolic acid C=O group (d1 = 2.3±0.3 Å). Its distance with 6-H_{eq} (d2) is 2.6±0.2 Å, and with 4-H_{eq} (d3) is 3.0±0.2 Å, in accordance with the NOE correlations and intensities. The C_{Arg}-N_{Arg} bond is instead oriented outward (N1-C2-C_{Arg}-N_{Arg} dihedral (dih2) = 174.7±9.6 deg.) with a distance between Arg NH and 2-H (d4) of 2.5±0.2 Å and between Arg NH and 3-H_{eq} of (d5) 2.2±0.2 Å, consistently with the NOEs found, with the pipecolic acid C=O group forming a dihedral angle of -5.3 deg with the C2-N1 bond.

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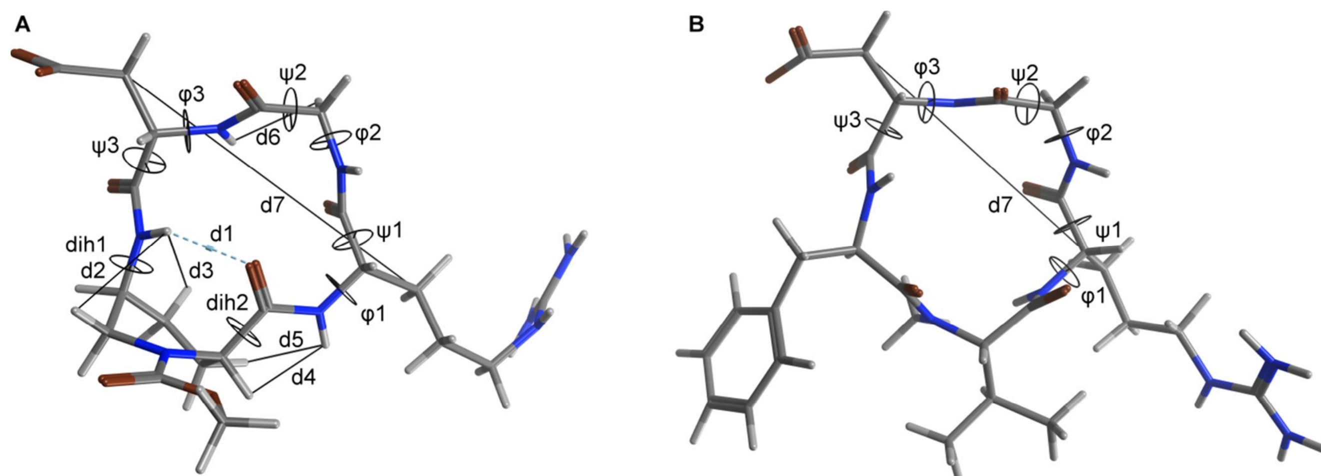

Figure 3. Representative conformations of the most populated cluster for compound **22** (panel A) and Cilengitide (panel B). Selected distances and dihedrals are shown accordingly to Table 2.

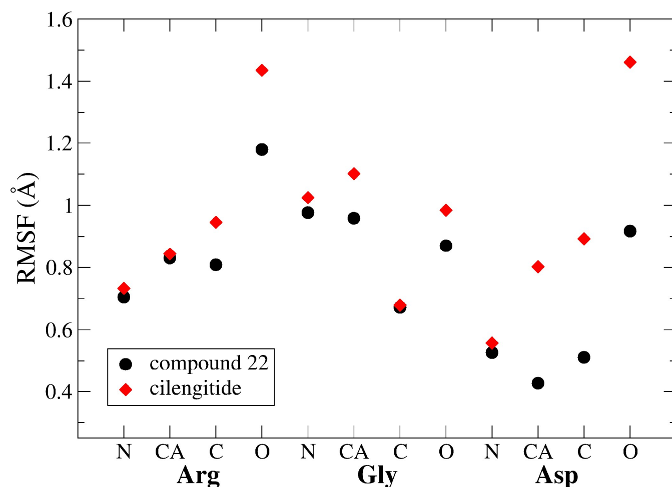

Figure 4. Comparison of the root mean squared fluctuations (RMSF) calculated for the backbone atoms of the RGD portion of compound **22** (black circle) and cilengitide (red diamond). The RMSF was obtained by analyzing a 100 ns MD trajectory obtained from the simulation of compound **22** and cilengitide in explicit water.

All the other distances are in accordance with the NOEs correlations (see Table S1, ESI), too. In particular, as found by NMR analysis, the AspNH is close to one (the pro-*S*, accordingly to calculations) of the Gly CHα protons (d6 = 2.4±0.2 Å). Because of its importance for the biological activity, the measured distance between the Cβ atoms of Asp and Arg (d7) in the global minimum conformer of **22** resulted in a value of 8.4±0.2 Å, which is comparable to the corresponding distance (8.9 Å) measured in the X-ray structure of the $\alpha_V\beta_3$-Cilengitide complex,[50] and very close to the value obtained by REMD simulations followed by cluster analysis of Cilengitide (8.5±0.5 Å) (Figure 3B). Similarly, the analysis of the ϕ and ψ dihedrals belonging to the RGD portion showed a good match between compound **22** and Cilengitide in both the free form, obtained by REMD, and bound form found in the crystal structure (Table 2). Interestingly, the lower standard deviations of the compared dihedrals and the higher population of the main conformational cluster (93.3% vs 64.4% for compound **22** and Cilengitide, respectively) suggest that the 5-APA scaffold is particularly effective in maintaining the RGD portion rigid and close to the bioactive conformation. To confirm this observation, we performed 100 ns of classical MD simulation in explicit water, starting from the representative conformation of the main cluster obtained from the REMD simulations of compound **22** and of Cilengitide (in this last case, starting from the conformation found in the crystal structure provided comparable results). The obtained trajectories were processed to perform a root-mean-squared-fluctuation (RMSF) analysis, which provides a picture of the atomic fluctuations during the simulation time and, consequently, of the molecule flexibility. A representation of the average fluctuations of the backbone atoms of the bioactive RGD portions of compound **22** and Cilengitide is provided in Figure 4. As expected, it can be observed that compound **22** is significantly more rigid, particularly at the Arg C=O and Asp Cα and C=O.

Finally, we ought to evaluate compound **22** in the binding site of the $\alpha_V\beta_3$ receptor. The models were generated starting from

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the crystal structure of Cilengitide in complex with the $\alpha_V\beta_3$ receptor as described in the Experimental section. The comparison of the geometries of compound **22** and Cilengitide in the binding site of $\alpha_V\beta_3$ is shown in Figure 5. It can be observed that the match between the principal pharmacophoric moiety of the two compounds (i.e. the sidechains of Arg and Asp) is rather good. In both compounds, the Asp carboxylate strongly bind the Mn^{2+} ion (C=O···Mn^{2+} distance = 2.2 Å) and forms an H-bond with the backbone NH of Asn215 (O···H-N distance = 2.3 and 2.1 Å; O···H-N angle = 174.9 and 174.3 deg. for **22** and Cilengitide, respectively). Two strong H-bonds are formed between the guanidine group and Asp218 (R-NH···O distance = 1.9 Å for both compounds; R-NH···O angle = 174.0 and 176.8 deg. for **22** and Cilengitide, respectively; C=NH···O distance = 1.8 for both compounds; C=NH···O angle = 167.7 and 172.8 deg. for **22** and Cilengitide, respectively). It can also be noted that compound **22** misses the dispersive interactions between Tyr122 and the benzyl group of D-Phe in Cilengitide, which was considered important for potency.[17,50] However, considering the comparable IC_{50} obtained for Cilengitide and compound **22** when tested on the isolated $\alpha_V\beta_3$ integrin receptor, we can gather that the rigidity induced by the 5-APA scaffold might be responsible for a reduced entropy loss upon binding. Thus, we can speculate that future functionalization of the pipecolic ring might lead to new $\alpha_V\beta_3$ ligands with increased potency.

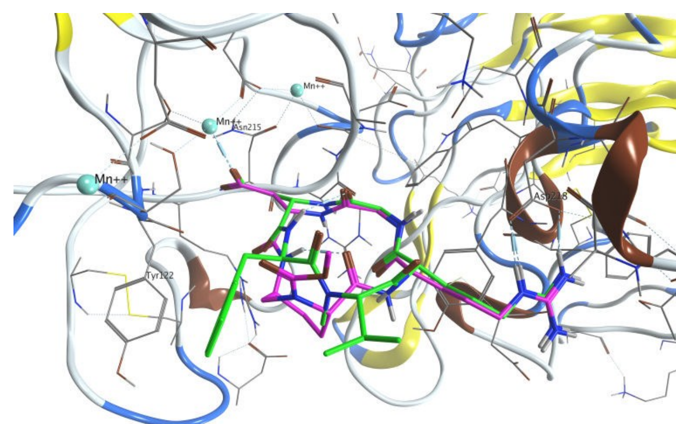

Figure 5. Superposition of compound **22** to the crystal structure of Cilengitide bound to $\alpha_V\beta_3$ integrin receptor.

Conclusions

In conclusion, a stereodivergent strategy to obtain enantiopure *cis* and *trans* 5-aminopipecolic acids (5-APA) in a suitably protected form for peptide synthesis has been devised starting from known 5-hydroxypipecolic acid as the common precursor. The two isomers were efficiently obtained in 38% and 32% overall yield, respectively, in seven steps. To demonstrate their usefulness in peptidomimetics, the *cis* isomer was embedded as a δ-amino acid in a cyclic RGD-containing sequence (compound **22**). The capacity of compound **22** to compete with biotinylated vitronectin for the binding to the isolated $\alpha_V\beta_3$ integrin (IC_{50} = 4.2 ± 0.9 nM) and to that expressed in M21 cells (IC_{50} = 228 ± 128 nM) was measured in comparison with Cilengitide. A conformational analysis by both NMR and REMD calculations confirmed that the ϕ and ψ dihedrals of the RGD portion of **22** are in good match with those of Cilengitide. Moreover, the 5-APA scaffold was shown to be particularly effective in maintaining the RGD portion rigid and close to the bioactive conformation. The comparison between compound **22** docked to the binding site of the $\alpha_V\beta_3$ receptor and the crystallographic pose of Cilengitide resulted in a rather good match between the principal pharmacophoric moieties of the two compounds (i.e. the sidechains of Arg and Asp). Despite the lack of the dispersive interaction presents between integrin's Tyr122 and the benzyl group of D-Phe in Cilengitide, the comparable IC_{50} obtained for Cilengitide and compound **22**, when tested on the isolated $\alpha_V\beta_3$ integrin receptor, suggests that the rigidity induced by the 5-APA scaffold might be responsible for a reduced entropy loss upon binding. Thus, we can envisage that proper substitutions on the pipecolic ring might lead to new $\alpha_V\beta_3$ ligands with increased potency.

Experimental

General Information

Melting points were recorded on a Büchi B-540 apparatus and are uncorrected. Chromatographic separations were performed under pressure on silica gel 60 (Merck,70–230 mesh) by flash-column techniques; R_f values refer to TLC carried out on 0.25-mm silica gel plates (Merck F_{254}), with the same eluent as indicated for the column chromatography. ^{1}H NMR (200, 400, 500 MHz) and ^{13}C NMR (100.4 MHz) spectra were recorded either on a Bruker Avance II 500 MHz Ultrashield or on a Varian Mercury 400 MHz or on a Varian Gemini 200 MHz spectrometers in the specified deuterated solvent at 25 °C. Solvent reference lines were set at 7.26 and 77.00 ($CDCl_3$), 3.31 and 49.00 (CD_3OD) in ^{1}H and ^{13}C NMR spectra, respectively. Mass spectra were carried out by direct inlet of a 20 ppm solution either in CH_3OH or CH_3OH + 0.1% HCO_2H on a LCQ Fleet™ Ion Trap LC/MS system (Thermo Fisher Scientific) with an electrospray ionization (ESI) interface in the positive mode. Microanalyses were carried out with a CHN Thermo FlashEA 1112 Series elemental analyzer. Purification of compound **22** was done by semi-preparative HPLC using a Dionex Ulltimate 3000 system equipped with an Alltech Alltima C18, 10 µm, 250 mm × 10 mm, reverse-phase column, using 5% acetonitrile in water buffered with 0.1% TFA as eluent and at a flow rate of 5 mL/min. HPLC analyses were carried out on a Dionex Acclaim 120, C18, 5 µm, 4.6 × 250 mm, reverse-phase analytical column at a flow rate of 1 mL/min and using a water–acetonitrile gradient eluent buffered with 0.1% TFA. Signals were monitored at 223 nm with a UV-detector. Optical rotations were determined with a JASCO DIP-370 instrument. Anhydrous solvents were either commercial or prepared accordingly to standard techniques. (2*S*,5*S*)-5-Hydroxypipecolic acid hydrochloride (**11**),[35] dimethyl (2*S*,5*S*)-5-hydroxypiperidine-1,2-dicarboxylate (**7**),[35] (2*S*,5*R*)-dimethyl 5-

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hydroxypiperidine-1,2-dicarboxylate (**15**),[35] and *rac*-dimethyl 5-azidopiperidine-1,2-dicarboxylate (**16**),[41a] are known.

Synthesis of (2*S*,5*R*)-5-(9-Fluorenylmethoxycarbonylamino)-1-(*tert*-butoxycarbonyl)piperidine-2-carboxylic acid [(−)-9]

(2S,5S)-2-Benzyl 1- *tert*-Butyl 5-Hydroxypiperidine-1,2-dicarboxylate [(-)-12]. To a solution of compound **11** (81 mg, 0.45 mmol) in anhydrous methanol (10 mL) triethylamine (188 µL, 1.34 mmol) and Boc_2O (197 mg, 0.89 mmol) were added, under nitrogen atmosphere, and the resulting reaction mixture was heated under reflux. After 24 h, the solvent was evaporated under reduced pressure and the residue dissolved in H_2O (5 mL). $NaH_2PO_4{\cdot}2H_2O$ (8 mg) was added and the resulting solution was cooled to 0 °C, acidified down to pH 3 by addition of 0.5 N HCl aqueous solution and then stirred for 30 min at 0 °C. The product was then extracted with EtOAc (4 x 7 mL) and the combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude N-Boc protected intermediate (109 mg) was obtained and used in the next step without further purification: ^1H NMR (200 MHz, CD_3OD) (1.1:1 mixture of rotamers) δ (ppm): 4.72 (br. d, *J* = 5.2 Hz, 1 H, major), 4.65 (br. d, *J* = 5.7 Hz, 1 H, minor), 4.13–4.02 (m, 1 H, both rotamers), 3.58–3.42 (m, 1 H, both rotamers), 2.78–2.57 (m, 1 H, both rotamers), 2.36–2.23 (m, 1 H, both rotamers), 1.96–1.89 (m, 1 H, both rotamers), 1.81–1.61 (m, 1 H, both rotamers), 1.48 (s, 9 H, minor), 1.43 (s, 9 H, major), 1.34–1.12 (m, 1 H, both rotamers).

To a solution of the *N*-Boc protected intermediate (109 mg, 0.45 mmol) and K_2CO_3 (68 mg, 0.49 mmol) in anhydrous DMF (10 mL), cooled at 0 °C and under nitrogen atmosphere, benzyl bromide (53 µL, 0.45 mmol) was added. The ice bath was removed and the resulting mixture stirred at room temperature for 24 h. Water (50 mL) was added, the product extracted with EtOAc (25 mL) and the organic extract dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/EtOAc, 2:1; R_f 0.23), to afford pure **12** (117 mg, 78%) as a white solid: m.p. 69.8-73.7 °C. $[\alpha]_D^{25}$ −31.8 (c 1.05, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.1:1 mixture of rotamers) δ (ppm): 7.38–7.30 (m, 5 H), 5.24–5.11 (m, 2 H), 4.89 (m, 1 H, major), 4.70 (m, 1 H, minor), 4.21–4.18 (m, 1 H, minor), 4.11–4.08 (m, 1 H, major), 3.63 (m, 1 H, both rotamers), 2.78–2.65 (m, 1 H, both rotamers), 2.35–2.26 (m, 1 H, both rotamers), 1.98–1.94 (m, 1 H, both rotamers), 1.78–1.73 (m, 1 H, both rotamers), 1.45 (s, 9 H, major), 1.37 (s, 9 H, minor). ^{13}C NMR (100.4 MHz, $CDCl_3$) (mixture of rotamers) δ (ppm): 171.3, 155.4 and 155.0, 135.5, 128.5, 128.4 and 128.2, 128.1 and 128.0, 80.5, 66.9, 66.7 and 66.5, 53.9 and 52.8, 48.4 and 47.5, 30.4 and 29.9, 28.2 and 28.1, 25.0 and 24.8. MS (ESI) *m/z* (%): 693 (100) [2M + Na]$^+$, 358 (61) [M + Na]$^+$, 336 (4) [M + 1]$^+$. $C_{18}H_{25}NO_5$ (335.39): calcd. C 64.46, H 7.51, N 4.18; found C 64.15, H 7.33, N 4.10.

(2S,5R)-2-Benzyl 1- tert-Butyl 5-Hydroxypiperidine-1,2-dicarboxylate [(-)-13]. Methanesulfonyl chloride (36 µL, 0.45 mmol) was added dropwise to a solution of **12** (117 mg, 0.35 mmol) and triethylamine (63 µL, 0.45 mmol) in anhydrous CH_2Cl_2 (3.7 mL), cooled at −30 °C and under nitrogen atmosphere. After 5 min the cooling bath was removed and the reaction mixture was left under stirring for 2 h at room temperature. Water (2 mL) was added followed by a dropwise addition of a 0.5 N solution of HCl (1.2 mL) and, after 10 min, the product was extracted with CH_2Cl_2 (3 x 3 mL). The combined organic layers were washed with water (3 x 5 mL) and dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude was purified by flash chromatography (*n*-hexane/Acetone, 1:3; R_f 0.29) to afford the intermediate O-mesylate (144 mg, quantitative) as a colourless oil: ^1H NMR (400 MHz, $CDCl_3$) (1.2:1 mixture of rotamers) δ (ppm): 7.42–7.34 (m, 5 H both rotamers), 5.25–5.12 (m, 3 H, both rotamers), 4.92 (d, *J* = 5.4 Hz, 1 H, major), 4.70 (d, *J* = 4.8 Hz, 1 H, minor), 4.61–4.52 (m, 1 H, both rotamers), 4.36 (dd, *J* = 12.8, 5.0 Hz, 1 H, minor), 4.25 (dd, *J* = 12.6, 5.0 Hz, 1 H, major), 3.03 (s, 3 H, both rotamers), 2.39–2.31 (m, 1 H, both rotamers), 2.39–2.31 (m, 1 H, both rotamers), 1.80–1.61 (m, 1 H, both rotamers), 1.45 (br s, 10 H, major + 1 H, minor), 1.36 (s, 9 H, minor).

Sodium azide (170 mg, 2.62 mmol) was added under nitrogen atmosphere to a solution of the O-mesylate (144 mg, 0.35 mmol) in anhydrous DMF (3.2 mL). The mixture was heated to 100 °C (external) for 3 h, diluted with water (32 mL) and the product extracted with Et_2O (3 x 32 mL). The combined organic extracts were dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The crude residue was then purified by flash chromatography (*n*-hexane/EtOAc, 8:1; R_f 0.23) and pure **13** (82 mg, 65%) was obtained as a white solid: m.p. 76.5-77.0 °C. $[\alpha]_D^{23}$ −13.2 (c 0.95, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.6:1 mixture of rotamers) δ (ppm): 7.38–7.30 (m, 5 H, both rotamers), 5.25–5.09 (m, 2 H, both rotamers), 5.03 (br s, 1 H, major), 4.78 (br s, 1 H, minor), 4.18–4.09 (m, 1 H, both rotamers), 3.79 (br s, 1 H, minor), 3.73 (br s, 1 H, major), 3.24 (d, *J* = 14.2 Hz, 1 H, major), 3.12 (d, *J* = 14.7 Hz, 1 H, minor), 2.08–2.03 (m, 3 H, both rotamers), 1.79–1.73 (m, 1 H, both rotamers), 1.47 (s, 9 H, major), 1.38 (s, 9 H, minor). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major) δ (ppm): 171.2, 155.3, 135.5, 128.5, 128.2, 128.1, 127.9, 80.7, 66.8, 54.8, 54.2, 52.9, 44.1, 43.5, 28.1, 24.8, 24.5, 20.9. MS (ESI) *m/z* (%): 743 (53) [2M + Na]$^+$, 383 (100) [M + Na]$^+$. $C_{18}H_{24}N_4O_4$ (360.18): calcd. C 59.99, H 6.71, N 15.55; found C 59.91, H 6.75, N 15.35.

(2S,5R)-5-(9-Fluorenylmethoxycarbonylamino)-1-(tert-butoxycarbonyl)piperidine-2-carboxylic acid [(−)-9]. To a solution of **13** (77 mg, 0.21 mmol) in anhydrous MeOH (4 mL), 10% Pd/C (7 mg, 6.6 µmol) was added under nitrogen atmosphere. The resulting suspension was first flushed with hydrogen under vigorous stirring and then left under hydrogen atmosphere (balloon) at room temperature. After 24 h, the mixture was filtered over a celite pad, and the residual solution evaporated under reduced pressure. The amino acid (52 mg, quantitative) was obtained as a white solid and immediately used in the next step without further purifications: ^1H NMR (400 MHz, CD_3OD) δ (ppm): 4.61–4.50 (m, 1 H), 3.57–3.42 (m, 2 H), 2.23–2.12 (m, 2 H), 1.94–1.74 (m, 4 H), 1.45 (s, 9 H).

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A 10% aqueous solution of Na_2CO_3 (640 μL) was added to a suspension of amino acid (52 mg, 0.21 mmol) in THF (510 μL) and the resulting mixture cooled to 0 °C. After the addition of a solution of Fmoc-OSu (72 mg, 0.21 mmol) in THF (1.5 mL), the ice bath was removed and the mixture was vigorously stirred at room temperature for 16 h. The solvent was evaporated under reduced pressure and the residue was taken up in EtOAc (3 mL). The organic solution was washed once with satd. NH_4Cl (3 mL) and, after separation of the phases, the aqueous one was extracted with EtOAc (5 x 3 mL). The aqueous layer was then acidified down to pH 2 by addition of aqueous 0.5 N HCl, and extracted again with EtOAc (5 x 3 mL). The combined organic extracts were dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude residue was purified by flash chromatography ($MeOH/CH_2Cl_2$ 1:20, R_f 0.14) and pure compound **9** (44 mg, 63%) was obtained as a clear syrup: $[\alpha]_D^{23}$ –21.7 (c 1.00, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.4:1 mixture of rotamers) δ (ppm): 7.77 (d, *J* = 7.5 Hz, 2 H, both rotamers), 7.59 (d, *J* = 7.1 Hz, 2 H, both rotamers), 7.41 (t, *J* = 7.4 Hz, 2 H, both rotamers), 7.32 (t, *J* = 7.4 Hz, 2 H, both rotamers), 5.17 (br s, 1 H, minor), 5.11 (br s, 1 H, major), 4.96 (br s, 1 H, major), 4.76 (br s, 1 H, minor), 4.51–4.47 (br s, 2 H, minor), 4.42–4.38 (br s, 2 H, major), 4.23–4.20 (m, 1 H, both rotamers), 4.03–3.88 (m, 2 H, both rotamers), 3.24–3.15 (m, 1 H, both rotamers), 2.18–2.03 (m, 1 H, both rotamers), 1.92–1.81 (m, 2 H, both rotamers), 1.46 (br s, 10 H, major + 1 H, minor), 1.24 (s, 9 H, minor). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major) δ (ppm): 176.4, 176.2, 156.3, 156.0, 155.6, 143.9, 143.8, 143.7, 143.5, 141.2, 127.6, 127.0, 124.9, 124.8, 119.9, 119.4, 107.9, 81.1, 67.2, 66.5, 58.4, 54.4, 53.1, 49.8, 47.2, 46.0, 45.0, 44.6, 31.8, 30.2, 29.6, 29.3, 29.3, 29.2, 29.0, 28.2, 27.2, 25.0, 24.6, 23.0, 22.6, 21.0, 20.8, 20.4, 18.2, 14.0. MS (ESI) *m/z* (%): 954 (9) $[2M + Na]^+$, 505 (23) $[M + K]^+$, 489 (100) $[M + Na]^+$. MS (ESI) *m/z* (%) (negative mode): 465 (100) $[M - 1]^-$. $C_{26}H_{30}N_2O_6$ (466.53): calcd. C 66.94, H 6.48, N 6.00; found C 67.22, H 6.70, N 5.93.

Synthesis of (2*S*,5*S*)-5-(9-Fluorenylmethoxycarbonylamino)-1-(methoxycarbonyl)piperidine-2-carboxylic acid [(–)-10]

(2S,5R)-Dimethyl 5-Hydroxypiperidine-1,2-dicarboxylate [15]. A solution of **7** (372 mg, 1.71 mmol) and Ph_3P (673 mg, 2.56 mmol) in dry THF (13 mL) was added to a solution of diisopropyl azodicarboxylate (DIAD) (505 μL, 2.56 mmol) and acetic acid (147 μL, 2.56 mmol) in dry THF (13 mL), cooled to 0 °C and under nitrogen atmosphere. After 1 h, the solvent was removed under reduced pressure and the residue taken up in a *n*-hexane-Et_2O 1:1 mixture (20 mL). After filtration on a Celite pad and evaporation of the solvent, the crude residue was purified by flash chromatography (*n*-hexane/EtOAc, 2:1; R_f 0.30) to afford acetate **14** (in a 1.2:1 ratio with a residual impurity of reduced DIAD) as a colourless oil: ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.08–4.98 (m, 1 H), 4.93–4.84 (m, 1 H), 4.23–4.07 (m, 1 H), 3.75 (s, 3 H), 3.72 (s, 3 H), 3.31–3.18 (m, 1 H), 2.15–2.01 (m, 2 H), 2.05 (s, 3 H), 1.96–1.79 (m, 1 H), 1.61–1.52 (m, 1 H).

A solution of **14** in dry MeOH (10 mL) was cooled to 0 °C, under nitrogen atmosphere, and MeONa (93 mg, 1.73 mmol) was added. After 6 h, glacial acetic acid (769 μL) was added and the solvent evaporated. The residue was diluted with water (45 mL), the product extracted with EtOAc (6 x 60 mL) and the combined organic extracts dried over Na_2SO_4. After filtration and evaporation of the solvent, the crude residue was purified by flash chromatography (*n*-hexane/EtOAc, 2:3; R_f 0.18) to afford **15** (236 mg, 63% over two steps) as a colourless oil.

Spectroscopic data are identical to those already reported in the literature.[35]

(2S,5S)-Dimethyl 5-Azidopiperidine-1,2-dicarboxylate [(–)-16]. Prepared as reported for (–)-**13**, starting from **15** (236 mg, 1.09 mmol) and obtaining the intermediate O-mesylate (302 mg, 94%) after purification by flash chromatography (*n*-hexane/EtOAc, 1:1; R_f 0.21) as a colourless oil: ^1H NMR (400 MHz, $CDCl_3$) (1.2:1 mixture of rotamers) δ (ppm): 5.03 (br s, 1 H, major), 4.94–4.86 (m, 2 H, major + 1 H, minor), 4.42 (d, *J* = 15.0 Hz, 1 H, minor), 4.30 (d, *J* = 15.0 Hz, 1 H, major), 3.74 (s, 6 H major + 3 H, minor), 3.72 (s, 3 H, minor), 3.35 (d, *J* = 15.0 Hz, 1 H, major), 3.24 (d, *J* = 15.0 Hz, 1 H, minor), 3.05 (s, 3 H, minor), 3.03 (s, 3 H, major), 2.20–2.04 (m, 3 H, both rotamers), 1.67–1.57 (m, 1 H, both rotamers).

The intermediate O-mesylate was treated with NaN_3 as reported and pure compound (–)-**16** was obtained as a pale yellow oil (192 mg, 78%), after purification by flash chromatography (*n*-hexane/EtOAc, 1:4; R_f 0.18): $[\alpha]_D^{23}$ –10.2 (*c* 1.35, $CHCl_3$).

Spectroscopic data are identical to those already reported in the literature for the corresponding racemic compound.[41a]

(2S,5S)-5-Azido-1-(methoxycarbonyl)piperidine-2-carboxylic acid [(–)-17]. Aqueous 1 N NaOH (1.19 mL) was added to a solution of **16** (192 mg, 0.79 mmol) in methanol (2.3 mL) and the resulting mixture was vigorously stirred for 2 h at room temperature. The methanol was removed under reduced pressure and the residue diluted in water (15 mL). The resulting solution was acidified to pH 2 by addition of aqueous 1 N HCl and the product extracted with CH_2Cl_2 (3 x 30 mL). The aqueous layer was then further acidified to pH 1 and the product was extracted again with CH_2Cl_2 (3 x 30 mL). The combined organic extracts were dried over Na_2SO_4 and, after filtration and evaporation of the solvent, compound **17** (181 mg, quantitative) was obtained as a thick colourless oil: $[\alpha]_D^{23}$ –4.4 (*c* 0.78, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$) (1.5:1 mixture of rotamers) δ (ppm): 10.70 (bs, 1 H), 4.96 (d, *J* = 5.3 Hz, 1 H, major), 4.81 (d, *J* = 5.3 Hz, 1 H, minor), 4.32 (dd, *J* = 12.5, 4.2 Hz, 1 H, minor), 4.16 (dd, *J* = 12.5, 4.5 Hz, 1 H, major), 3.75 (s, 3 H, major), 3.72 (s, 3 H, minor), 3.42–3.31 (m, 1 H, both rotamers), 2.86 (t, *J* = 12.5 Hz, 1 H, major), 2.78 (t, *J* = 12.5 Hz, 1 H, minor), 2.43–2.32 (m, 1 H, both rotamers), 2.10–2.01 (m, 1 H, both rotamers), 1.84–1.73 (m, 1 H, both rotamers), 1.44–1.31 (m, 1 H, both rotamers). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major) δ (ppm): 176.0, 156.7, 56.2, 53.4, 45.4, 29.6, 26.8, 24.9. MS (ESI) *m/z* (%): 723 (51) $[3M + K]^+$, 495 (100) $[2M + K]^+$. MS (ESI) *m/z* (%) (negative mode): 227 (100) $[M - 1]^-$. $C_8H_{12}N_4O_4$ (228.21): calcd. C 42.10, H 5.30, N 24.55; found C 41.88, H 5.65, N 24.10.

(2S,5S)-5-(9-Fluorenylmethoxycarbonylamino)-1-(methoxycarbonyl)piperidine-2-carboxylic acid [(–)-10].

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Prepared as reported for (–)-**9**, starting from **17** (176 mg, 0.77 mmol) and obtaining the intermediate amino acid (156 mg) as a white solid: ^{1}H NMR (400 MHz, CD_3OD) (1.1:1 mixture of rotamers) δ (ppm): 4.60 (br s, 1 H, minor), 4.51 (br s, 1 H, major), 4.18 (d, J = 9.4 Hz, 1 H, both rotamers), 3.70 (br s, 3 H, minor), 3.68 (br s, 3 H, major), 3.38–3.25 (m, 1 H, both rotamers), 3.21–3.12 (m, 1 H, both rotamers), 2.43–2.31 (m, 1 H, both rotamers), 2.03–1.95 (m, 1 H, both rotamers), 1.78–1.66 (m, 1 H, both rotamers), 1.57–1.46 (m, 1 H, both rotamers). MS/MS (ESI of [M + 1]$^+$) m/z (%): 203 (8) [M + 1]$^+$, 185 (84), 157 (100).

The intermediate amino acid was treated with Fmoc-*O*Su as reported and pure compound **10** (272 mg, 83%) was obtained as a white solid, after purification by flash chromatography ($MeOH/CH_2Cl_2$ 1:10, R_f 0.28): $[\alpha]_D^{19}$ –16.5 (*c* 0.70, CH_3OH). ^{1}H NMR (400 MHz, CD_3OD) (6.5:1 mixture of rotamers) δ (ppm): 7.77 (d, J = 7.5 Hz, 2 H, both rotamers), 7.62 (d, J = 7.5 Hz, 2 H, major), 7.57 (d, J = 7.5 Hz, 2 H, minor), 7.37 (t, J = 7.5 Hz, 2 H, both rotamers), 7.29 (t, J = 7.5 Hz, 2 H, both rotamers), 4.64–4.53 (m, 1 H, both rotamers), 4.50–4.41 (m, 2 H, minor), 4.34 (br s, 2 H, major), 4.23–4.07 (m, 2 H, both rotamers), 3.66 (s, 3 H, both rotamers), 3.40 (br s, 1 H, major), 3.14 (br s, 1 H, minor), 2.92–2.80 (m, 1 H, both rotamers), 2.42–2.31 (m, 1 H, both rotamers), 1.83–1.76 (m, 1 H, both rotamers), 1.70–1.58 (m, 1 H, both rotamers), 1.38–1.29 (m, 1 H, both rotamers). ^{13}C NMR (100.4 MHz, CD_3OD) (major) δ (ppm): 178.2, 158.7, 158.1, 145.3, 142.6, 128.7, 128.1, 126.1, 120.9, 67.6, 53.4, 48.5, 47.1, 30.7, 28.7, 27.4, 27.2. MS (ESI) m/z (%): 871 (20) [2M + Na]$^+$, 447 (100) [M + Na]$^+$. $C_{23}H_{24}N_2O_6$ (424.45): calcd. C 65.08, H 5.70, N 6.60; found C 65.00, H 5.75, N 6.43.

Synthesis of Cyclopeptide 22

(2S,5S)-5-(9-Fluorenylmethoxycarbonylamino)-1-(methoxycarbonyl)piperidine-2-Arg(Mtr)-Gly-OBn [(–)-18]. DEPBT (334 mg, 1.12 mmol) and DIPEA (194 µL, 1.12 mmol) were added under nitrogen atmosphere to a solution of **10** (237 mg, 0.56 mmol) in anhydrous THF (4 mL) cooled to 0°C, and the resulting mixture was allowed to warm to room temperature. After 15 min the reaction was cooled again to 0°C, and H-Arg(Mtr)-Gly-OBn (387 mg, 0.73 mmol) was added. The ice bath was removed and the mixture was stirred at 35°C (external) for 4 days. The mixture was then diluted with EtOAc (90 mL), washed with satd NH_4Cl (2 x 23 mL), satd $NaHCO_3$ (2 x 23 mL), and finally with H_2O (2 x 23 mL). The organic extract was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude residue was purified by flash chromatography ($MeOH/CH_2Cl_2$ 1:25, R_f 0.23) and pure **18** (514 mg, 98%) was so obtained as a colourless gummy solid: $[\alpha]_D^{20}$ –18.2 (*c* 0.91, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (4:1 mixture of rotamers) (major) δ (ppm): 7.72 (d, J = 8.0 Hz, 2 H), 7.55 (d, J = 8.0 Hz, 3 H), 7.36 (t, J = 5.4 Hz, 2 H), 7.31–7.22 (m, 7 H), 6.49 (s, 1 H), 6.28 (br s, 4 H), 5.56 (br s, 1 H), 5.09 (s, 2 H), 4.80 (br s, 1 H), 4.58 (br s, 1 H), 4.37–4.29 (m, 2 H), 4.19–3.98 (m, 4 H), 3.79 (s, 3 H), 3.67 (s, 3 H), 3.67–3.60 (m, 1 H), 3.36–3.14 (m, 2 H), 2.95 (m, 1 H), 2.66 (s, 3 H), 2.59 (s, 3 H), 2.33–2.25 (m, 1 H), 2.10 (s, 3 H), 2.34–2.24 (m, 1 H), 1.88–1.51 (m, 5 H), 1.47–1.38 (m, 1 H). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major) δ (ppm): 175.0, 172.1, 171.5, 169.8, 158.4, 157.3, 156.5, 155.7, 143.8, 141.1, 138.3, 136.3, 134.9, 133.1, 128.5, 128.3, 128.1, 127.5, 126.9, 124.9, 124.8, 119.8, 111.6, 67.0, 66.6, 55.3, 53.2, 49.3, 47.1, 46.8, 46.2, 41.1, 30.6, 29.5, 25.5, 24.0, 18.3, 17.5, 11.9. MS (ESI) m/z (%): 1901 (13) [2M + Na]$^+$, 962 (100) [M + Na]$^+$, 940 (11) [M + 1]$^+$. $C_{48}H_{57}N_7O_{11}S$ (940.07): calcd. C 61.33, H 6.11, N 10.43; found C 61.51, H 5.95, N 10.35.

(2S,5S)-5-[Z-Asp(OtBu)]-1-(methoxycarbonyl)-5-APA-2-Arg(Mtr)-Gly-OBn [(–)-20]. Compound **18** (514 mg, 0.55 mmol) was dissolved in a 1:1 CH_2Cl_2/diethylamine mixture (7 mL) under nitrogen atmosphere. The resulting solution was stirred at room temperature for 4 h, meanwhile additional 1:1 CH_2Cl_2/diethylamine mixture (4 mL) was added. The solution was concentrated under reduced pressure, the residue was taken up in CH_2Cl_2 (5 mL) and then concentrated again. The crude **19** (584 mg) was obtained as a yellow solid: ^{1}H NMR (400 MHz, $CDCl_3$) (5:1 mixture of rotamers) (major) δ (ppm): 7.52 (br s, 1 H), 7.41–7.24 (m, 5 H), 6.51 (s, 1 H), 6.36 (br s, 1 H), 6.33–6.20 (m, 3 H), 5.12 (s, 2 H), 4.81–4.50 (m, 2 H), 4.17–3.89 (m, 3 H), 3.81 (s, 3 H), 3.67 (s, 3 H), 3.32–3.12 (m, 3 H), 2.73–2.65 (m, 1 H), 2.67 (s, 3 H), 2.60 (s, 3 H), 2.33–2.21 (m, 1 H), 2.11 (s, 3 H), 2.03–1.77 (m, 2 H), 1.77–1.46 (m, 4 H), 1.28–1.22 (m, 1 H). MS (ESI) m/z (%): 740 (9) [M + Na]$^+$, 718 (100) [M + 1]$^+$.

DEPBT (433 mg, 1.45 mmol) and DIPEA (253 µL, 1.45 mmol) were added under nitrogen atmosphere to a solution of Z-Asp(OtBu)-OH monohydrate (247 mg, 0.72 mmol) in anhydrous THF (8.5 mL), cooled at 0 °C, and the resulting mixture was allowed to warm to room temperature. After 15 min this solution was slowly added to a solution of compound **19** (0.55 mmol) in anhydrous THF (1 mL) precooled at 0 °C. The ice bath was removed and the resulting mixture was stirred at 35 °C. After 24 h a second portion of Z-Asp(OtBu)-OH monohydrate (95 mg, 0.28 mmol), DEPBT (167 mg, 0.56 mmol) and DIPEA (97 µL, 0.56 mmol) was added and the mixture stirred at 35°C for additional 3 days. The mixture was then diluted with EtOAc (90 mL) and washed with satd. NH_4Cl (2 x 25 mL), satd. $NaHCO_3$ (2 x 25 mL) and finally with H_2O (2 x 25 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude residue was purified by flash chromatography ($MeOH/CH_2Cl_2$ 1:20, R_f 0.24) and pure **20** (285 mg, 50%) was obtained as a white solid: $[\alpha]_D^{20}$ –11.4 (*c* 1.00, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) (8:1 mixture of rotamers) (major) δ (ppm): 7.48 (br s, 1 H), 7.38–7.27 (m, 10 H), 6.84 (br s, 1 H), 6.51 (s, 1 H), 6.27 (br s, 3 H), 5.97 (m, 1 H), 5.13 (s, 2 H), 5.12–5.01 (m, 2 H), 4.84–4.69 (m, 1 H), 4.63–4.52 (m, 1 H), 4.51–4.41 (m, 1 H), 4.14–3.91 (m, 3 H), 3.84 (s, 3 H), 3.66 (s, 3 H), 3.31–3.12 (m, 3 H), 3.04–2.92 (m, 1 H), 2.86–2.71 (m, 1 H), 2.66 (s, 3 H), 2.65–2.58 (m, 1 H), 2.59 (s, 3 H), 2.31–2.21 (m, 1 H), 2.11 (s, 3 H), 1.95–1.85 (m, 1 H), 1.84–1.49 (m, 5 H), 1.46–1.40 (m, 1 H), 1.39 (s, 9 H). ^{13}C NMR (100.4 MHz, $CDCl_3$) (major) δ (ppm): 172.1, 171.6, 170.6, 170.2, 169.8, 158.5, 157.2, 156.6, 156.0, 138.5, 136.5, 136.1, 135.2, 135.1, 133.3, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 124.9, 111.7, 81.6, 67.2, 67.1, 55.4, 53.7, 53.3, 53.0, 52.9, 51.4, 45.6, 41.2, 40.4, 37.7, 29.7, 29.6, 28.0, 26.4, 25.4, 24.2, 18.4,

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12.0. MS (ESI) *m/z* (%): 1045 (100) $[M + Na]^+$, 1023 (10) $[M + 1]^+$. $C_{49}H_{66}N_8O_{14}S{\cdot}2,5H_2O$ (1068.20): calcd. C 55.10, H 6.70, N 10.49; found C 55.45, H 6.76, N 10.10.

Cyclo{Arg-Gly-Asp-[1-(methoxycarbonyl)-5-APA]}·TFA (22). To a solution of **20** (282 mg, 0.28 mmol) in ethanol (16 mL), 10% Pd/C (138 mg, 0.13 mmol) was added under nitrogen atmosphere. The resulting suspension was first flushed with hydrogen under vigorous stirring and then left under hydrogen atmosphere (balloon) at room temperature. After 18 h, the mixture was filtered over a celite pad, and the residual solution evaporated under reduced pressure. Compound **21** (220 mg, 99%) was obtained as a white solid and used in the next step without further purifications: ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 6.67 (s, 1 H), 4.77 (m, 1 H), 4.41 (dd, *J* = 8.7, 4.9 Hz, 1 H), 4.20–4.10 (m, 1 H), 4.11 (dd, *J* = 7.9, 4.7 Hz, 1 H), 3.84 (s, 3 H), 3.80–3.70 (m, 3 H), 3.71 (s, 3 H), 3.23–3.15 (m, 2 H), 2.99–2.79 (m, 3 H), 2.68 (s, 3 H), 2.62 (s, 3 H), 2.36–2.32 (m, 1 H), 2.13 (s, 3 H), 1.91–1.76 (m, 3 H), 1.75–1.52 (m, 3 H), 1.51–1.43 (m, 1 H), 1.47 (s, 9 H). MS (ESI) *m/z* (%): 837 (7) $[M + K]^+$, 821 (400) $[M + Na]^+$, 799 (100) $[M + 1]^+$. MS (ESI) *m/z* (%) (negative mode): 797 (100) $[M - 1]^-$.

The crude **21** (218 mg, 0.27 mmol) was suspended in THF (80 mL) under nitrogen atmosphere. The suspension was cooled to 0 °C and DEPBT (245 mg, 0.82 mmol) and DIPEA (143 µL, 0.82 mmol) were added. The resulting mixture was stirred at 35 °C for 4 days and then diluted with EtOAc (40 mL); the organic solution was washed with satd. NH_4Cl (2 x 15 mL), satd. $NaHCO_3$ (2 x 15 mL), H_2O (2 x 15 mL) and finally dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was eluted through a short pad of silica gel (CH_2Cl_2/MeOH, 25:2) and used directly in the next step: MS (ESI) *m/z* (%): 1583 (23) $[2M + Na]^+$, 819 (21) $[M + K]^+$, 803 (100) $[M + Na]^+$, 781 (7) $[M + 1]^+$.

The crude cyclic tetrapeptide was dissolved in a 95:2.5:2.5 trifluoroacetic acid/triisopropylsilane/H_2O mixture (8.4 mL) and the resulting solution was stirred at room temperature for 18 h. The mixture was concentrated under reduced pressure and the residue taken up in H_2O (10 mL) and washed with Et_2O (4 x 12 mL). The aqueous layer was then concentrated under reduced pressure to afford the deprotected cyclic tetrapeptide as a trifluoroacetate salt. This crude was purified by semi-preparative HPLC (R_t = 13.56 min) and pure **22** (33 mg, 20% over 2 steps) was obtained as a colourless glassy solid by lyophilization of the HPLC sample. Purity of the final compound was checked by analytical HPLC: $[\alpha]_D^{21}$ +15.2 (*c* 0.23, H_2O). ^{1}H NMR (500 MHz, H_2O/D_2O 9:1) (1.4:1 mixture of rotamers) δ (ppm): 8.73–8.66 (m, 1 H, both rotamers), 8.56 (d, *J* = 12 Hz, 1 H, major), 8.55 (d, *J* = 12 Hz, 1 H, minor), 8.23 (d, *J* = 8.5 Hz, 1 H, minor), 8.22 (d, *J* = 8.5 Hz, 1 H, major), 7.96 (d, *J* = 9.0 Hz, 1 H, minor), 7.94 (d, *J* = 9.0 Hz, major), 7.11 (t, *J* = 6.0 Hz, 1 H, both rotamers), 4.58–4.52 (m, 2 H major + 1 H minor), 4.50 (br s, 1 H, minor), 4.32–4.22 (m, 1 H, both rotamers), 4.06–4.00 (m, 1 H, both rotamers), 4.00–3.94 (m, 1 H, both rotamers), 3.71 (d, *J* = 13.5 Hz, 1 H, major), 3.68 (d, *J* = 13.5 Hz, 1 H, minor), 3.61 (s, 3 H, major), 3.56 (s, 3 H, minor), 3.50 (dd, *J* = 15.0, 4.5 Hz, 1 H, both rotamers), 3.35 (dd, *J* = 14.0, 4.0 Hz, 1 H, major), 3.30 (dd, *J* = 14.0, 4.0 Hz, 1 H, minor), 3.17–3.11 (m, 2 H, both rotamers), 2.84 (dd, *J* = 17.0, 7.0 Hz, 1 H, minor), 2.83 (dd, *J* = 17.0, 7.0 Hz, 1 H, major), 2.70 (dd, *J* = 17.0, 6.0 Hz, 1 H, both rotamers), 2.13–2.00 (m, 3 H, both rotamers), 1.77–1.57 (m, 3 H, both botamers), 1.56–1.46 (m, 1 H, both rotamers), 1.21–1.11 (m, 1 H, both rotamers). ^{13}C NMR (100.4 MHz, D_2O) (mixture of rotamers) δ (ppm): 175.9 and 175.5, 175.3 and 175.2, 175.0, 170.8 and 170.7, 169.9 and 169.8, 158.4 and 158.3, 156.7, 55.9 and 55.8, 54.0 and 53.9, 53.2 and 53.1, 49.9 and 49.8, 44.1, 43.3 and 43.2, 42.7, 40.43 and 40.40, 34.3 and 34.2, 26.4 and 26.3, 24.3 and 24.2, 23.6 and 23.4, 23.0. MS (ESI) *m/z* (%): 513 (100) $[M + 1]^+$.

Biological studies

Materials and methods

Tests with isolated $\alpha_v\beta_3$ receptor. Recombinant human integrin $\alpha_v\beta_3$ receptor (R&D Systems, Minneapolis, MN, USA) was diluted to 0.5 µg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. An aliquot of diluted receptor (100 µL/well) was added to 96-well microtiter plates (Nunc MaxiSorp) and incubated overnight at 4 °C. The plates were then incubated with blocking solution (coating buffer plus 1% bovine serum albumin) for an additional 2 h at room temperature to block nonspecific binding. After washing 2 times with blocking solution, plates were incubated shaking for 3 h at room temperature with various concentrations (10^{-12} –10^{-5} M) of test compound in the presence of 1 µg/mL biotinylated vitronectin (Molecular Innovations, Novi, MI, USA). Biotinylation was performed using an EZ-Link Sulfo-NHS-Biotinylation kit (Pierce, Rockford, IL, USA). After being washed 3 times, the plates were incubated shaking for 1 h at room temperature with streptavidin biotinylated peroxidase complex (Amersham Biosciences, Uppsala, Sweden). Then the plates were washed again and finally incubated for 30 min with Substrate Reagent Solution (100 µL/well; R&D Systems, Minneapolis, MN), before the reaction was stopped by addition of 2 N H_2SO_4 (50 µL/well). The absorbance at 415 nm was read in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point is the result of the average of triplicate wells; data analysis was carried out by nonlinear regression analysis with GraphPad Prism software (GraphPad Prism, San Diego, CA). Each experiment was repeated in duplicate.

Tests with M21 Cells.

Cell lines and culture conditions. The M21 human melanoma cell line 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. $5.0{\times}10^5$ melanoma cells were seeded in 100 mm Sarstedt dishes and propagated every 3 days by incubation with a trypsin–EDTA solution. Cultures were periodically monitored for mycoplasma contamination. For the use in the experiments, cells (passages 2-4) were grown to confluence in plates coated with 1% bovine gelatin (Sigma, St. Louis).

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Cell adhesion assay. Plates (96 wells) were coated with vitronectin (10 μg/mL) 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 tumor cells were counted and resuspended in serum free medium, and exposed to the compound (final concentration was 0.1, 1, and 10 pM, 0.1, 1, and 10 nM, 0.1, 1, and 10 μM) 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).

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Computational studies

Material and methods

Parameterization of nonstandard residues. Charge parameterization for (2*S*,5*S*)-5-APA, protected at N1 as a methyl carbamate, was performed using the R.E.D. procedure.[51] The structure was capped by a acetyl and a NHMe at the 5-amino and at the C-2 carboxy group, respectively, and subjected to a conformational search using the low mode method and the MMFF94x force field, implemented in MOE (other parameters were left as default).[52] The two lowest energy conformations were used for charge parameterization. Moreover, for each conformation, two different orientations were used to derive RESP charges. The Gaussian09 software was used for quantum mechanical calculations,[53] at the HF/6-31G* level, as requested for force field compatibility.

REMD simulations. Starting conformations of compound **22** were generated by a preliminary conformational search performed with MOE.[52] Independent REMD runs were started from two different conformations, selected to maximize geometrical difference. The ff96 force field coupled with the GB-OBC(II) solvent model[54] was adopted accordingly to a protocol previously applied to similar synthetic peptides.[30,49d] Geometries were minimized by 500 steps of steepest-descendant followed by 500 steps of conjugated-gradient and twelve replicas were run at 300.00, 330.69, 364.36, 401.31, 441.89, 486.42, 535.31, 588.95, 647.82, 712.41, 783.26, and 860.93 K. After a short equilibration (200 ps), production runs were conducted for 400 ns at constant temperature, using the Langevin thermostat with a collision frequency of with a collision frequency of 1.0 ps^{-1}.[55] A time step of 0.002 ps and an infinite cut-off for electrostatic were adopted. Exchanges were attempted every 2 ps and an acceptance rate above 50% was observed. To evaluate convergence, the 300 K trajectories were subjected to a cluster analysis every 50 ns. The simulation was considered converged when the population of the three principal clusters differed by less than 15% between consecutive 50 ns batches. After 300 ns of simulation, no significant differences were observed in the population of the three main clusters and in their representative geometries. Moreover, no significant differences were observed between results obtained by independent simulations starting from divergent geometries. REMD calculations and cluster analyses were performed with the *pmemd.MPI* and *cpptraj* executables of the Amber16/AmberTools17 packages, respectively.[56] A maximum of 10 clusters was requested, using the average-linkage algorithm, the pairwise mass-weighted RMSD on backbone heavy atoms as a metric and sampling one frame every 4 ps.

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MD simulations. Classical MD simulations were performed by starting from the reference geometry of the most populated cluster, as obtained from cluster analysis. In the case of Cilengitide, the simulations were also repeated by starting from the crystal structure reported in ref. 50. The system was prepared using *tleap*, setting an octahedral solvent box of TIP3P water extending up to 10 Å from the solute. Volume and density were equilibrated trough subsequent NVT (100 ps) and NPT (100 ps) simulations, where the solute was kept restrained. The system was then geometry minimized and gently heated up to the final temperature of 300 K. Further equilibration was performed by NVT (200 ps) and NPT (1000 ps) simulations, where solute restraints were gradually removed. A NVT production run of 100 ns was then performed using a cutoff of 8 Å was for electrostatics and PME for long-range interactions.[57] All bonds involving hydrogens atoms were constrained by using the SHAKE algorithm.[58] Production runs were performed on a GPU card using the *pmemd.cuda* engine[59] and trajectories were analyzed using *cpptraj* (AmberTools17).[56]

Modelling of ligand-receptor complexes. The ligand-receptor models for compound **22** and Cilengitide were obtained with MOE[52] starting from the 1L5G pdb file.[50] Only chains B (the receptor monomer) and C (Cilengitide) were maintained and the structure was processed with the Structure Preparation module and protonated accordingly to a pH = 7. The representative geometry of compound **22**, as obtained by REMD simulations followed by cluster analysis, was manually docked to $\alpha_V\beta_3$ by superposing the Cα of the RGD portion to the corresponding atoms of the co-crystallized Cilengitide, that was then removed. The resulting geometry was then minimized up to a gradient of 0.1 kcal/mol/$Å^2$, using the Amber10EHT force field and the Born solvation model for water, keeping the receptor backbone atoms restrained. For comparison, the same protocol was applied to the complex of Cilengitide.

Conflicts of interest

There are no conflicts of interest to declare.

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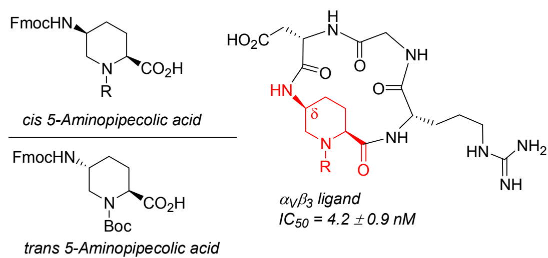

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