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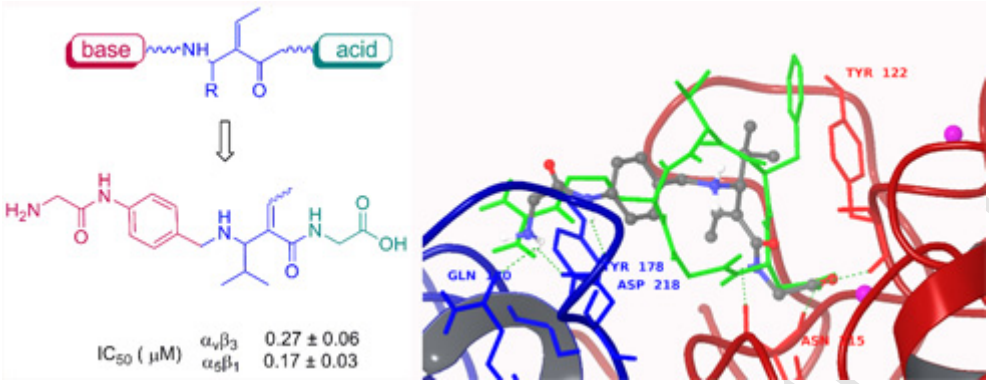
Graphical Abstract



Highlights

▶ Dehydro-β-amino acid derivatives were synthesized as RGD mimetics ▶ Cell adhesion assays suggested good affinity toward $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins ▶ Selected compounds showed ability to inhibit integrin-mediated signalling activation ▶ Docking experiments were performed to verify ligand-receptor interactions. ▶ The low molecular weight and the simple synthetic route may represent an advantage on other ligands

Modulation of $\alpha_v\beta_3$- and $\alpha_5\beta_1$-integrin mediated adhesion by dehydro-β-amino acids containing peptidomimetics

Alessandra Tolomelli,*[a] Monica Baiula,*[b] Laura Belvisi,[c] Angelo Viola,[a] Luca Gentilucci,[a] Stefano Troisi,[a] Samantha Deianira Dattoli,[b] Santi Spampinato,[b] Monica Civera[c], Eusebio Juaristi[d] and Margarita Escudero[d]

[a] Department of Chemistry "G.Ciamician", University of Bologna, Via Selmi 2, 40126, Bologna, Italy, Fax: (+39)0512099456, e-mail: alessandra.tolomelli@unibo.it

[b] Department of Pharmacology, University of Bologna, Via Irnerio 48, 40126 Bologna, Italy, e-mail: monica.baiula@unibo.it

[c] Department of Chemistry, University of Milan , Via Golgi 19, 20133, Milan, Italy

[d]Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Av. Instituto Politécnico Nacional 2508, Col. San Pedro Zacatenco, 07360 México

Abstract

A novel class of low molecular weight ligands of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, that possess a dehydro-β-amino acid as conformationally constrained core, linked to the pharmacophoric moieties mimicking the RGD recognition sequence, have been synthesized through a very simple protocol. Cell adhesion assays and integrin-mediated signaling activation experiments suggested a good affinity of these compounds toward both integrin receptors. Moreover, further elongation with two glycine units allowed to obtain an excellent dual inhibitor. Structural models for $\alpha_v\beta_3$ integrin-ligand binding confirmed that the dehydro-β-amino derivatives are able to act as an electrostatic clamp by establishing several stabilizing interactions with the receptor.

Keywords: • Dehydro-β-amino acids, Peptidomimetic, Cell adhesion, Signalling, Drug discovery

Introduction

In the last few decades, antiangiogenic therapy has been extensively studied since neovascularization is essential for tumour growth and development,[1] but the benefit of this approach is often quite limited, since inhibition of one angiogenetic pathway may turn into activation of a parallel mechanism.[2] It has been demonstrated that, during the angiogenesis process, spreading endothelial cells express different markers, which are completely absent in normal capillaries.[3] Among them, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins are over-expressed in novel vessels surrounding tumour cells, therefore drugs having these integrins as targets could lead to a synergistic effect in vessel generation without altering normal blood vessels.[4]

Integrin $\alpha_v\beta_3$ is not generally expressed on epithelial cells and is present only at low levels on a subset of B cells, some cells of macrophage lineage, smooth muscle cells, and activated endothelial

cells.[5] On the other hand, this integrin is also expressed on certain invasive tumours including metastatic melanoma and late-stage glioblastoma.[6] *In vivo,* integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ are simultaneously present on the endothelial cells of tumour blood vessels and of tissues exposed to angiogenic growth factors.[7] Integrin $\alpha_5\beta_1$ is largely selective for fibronectin, which is a component of the provisional matrix expressed by endothelial cells during wound healing or tumour angiogenesis.[7,8] Integrin $\alpha_v\beta_3$, in contrast, is a promiscuous integrin with the potential to mediate migration on a host of extracellular matrix proteins with arginine-glycine-aspartic acid moieties, such as vitronectin, fibrinogen, collagen, von Willebrand's factor, and others.[9] To maintain orderly outgrowth of blood vessels, this ability of $\alpha_v\beta_3$ to promote migration in most extracellular matrix contexts must be regulated at cellular level through precise mechanisms. Thus, ligation of integrin $\alpha_5\beta_1$ by endothelial cell fibronectin is one means by which $\alpha_v\beta_3$ functions are regulated during angiogenesis *in vivo*.[10] Involvement and cross-talking of the two receptors makes the discovery of ligands capable to tune or inhibit the activity of both of them (dual inhibitors) highly desirable.[11]

The identification of key recognition motifs within integrin ligands is the starting point for the development of antagonists. Both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins bind to ECM components through recognition of the Arg-Gly-Asp (RGD) tripeptide sequence. The X-ray analysis of the complex between $\alpha_v\beta_3$ integrin and c(RGDf-NMe-V) ligand (Cilengitide) shows that binding occurs mainly through electrostatic interactions.[12] Concerning $\alpha_5\beta_1$ integrin, the crystal structure of the receptor in complex with a Fab fragment of the anti-β1 inhibitory antibody SG/19, both in the presence and in the absence of an RGD containing peptide, has been published, revealing a high similarity with $\alpha_v\beta_3$ integrin complex.[13]

Recently, great interest has been paid also to integrin conformational changes and their role in activation and signaling. Although the exact mechanism is still not completely disclosed, it is generally accepted that ligand-receptor interaction induces switch from a low-affinity bent conformation, to an high-affinity extended one, passing through an intermediate inactive conformation having closed headpiece. It has been reported that small molecule ligands, that may only partially match with the binding site, show a different effect when compared with classical RGD ligands [14]. Carboxylate coordination with MIDAS (metal ion-dependent adhesion site) metal ion induces swing out to the active conformation, while no changes occurs for binding with molecules linking only to the α subunit.

In previous papers, we have identified a series of $\alpha_v\beta_3$/ $\alpha_5\beta_1$ integrin dual ligands, sharing as a common feature the presence of an unsaturated α- or β-amino acid fragment, inserted into a cyclic

backbone (Figure 1, compounds A and B).[15] The introduction of a β-amino acid into RGD-containing cyclic peptide has been already reported in the literature with the aim to stabilize distinct conformations, having a β-turn with centrally positioned glycine.[16] We recently reported isoxazoline-containing ligands having bioactivity in the nanomolar range and we hypothized that the restricted conformation introduced by the dehydro-β-amino acid and the cyclic structure could give a favorable alignment of both the basic and carboxylate moieties, meeting the crucial requirements for affinity and selectivity (Figure 1, compound B). Next, our aim was to minimize molecular complexity and synthetic steps, in order to obtain low molecular weight compounds.

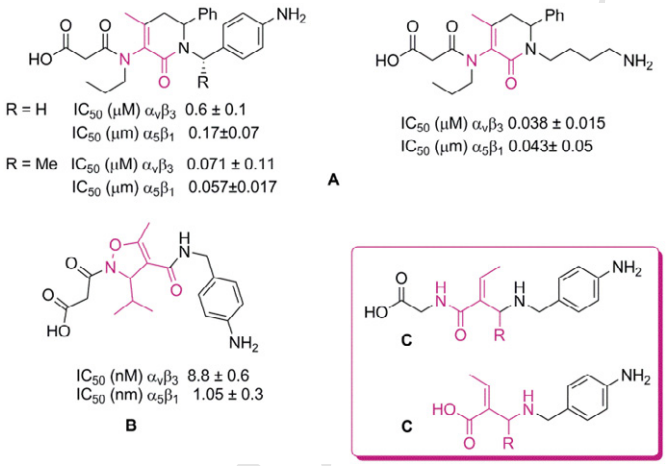


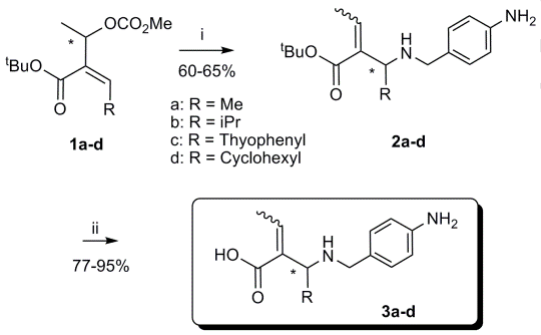
Figure 1. $\alpha_v\beta_3$/ $\alpha_5\beta_1$ integrin ligands containing an unsaturated α- or β-amino acid fragment (A,B) and novel dehydro-β-amino acid structures (C).

In the study reported herein, the synthesis and biological evaluation of a collection of dehydro-β-amino acid containing molecules has been developed (Figure 1, compound C). Some compounds from the library, that have a shorter length than that generally required for RGD mimicking, have been studied also with the aim to observe the effect of a partial ligand/receptor interaction on adhesion and intracellular signaling. Moreover, on the basis of our recent results in the design and synthesis of ligands containing cyclic dehydro-β-amino acids, we wanted to verify if the linear dehydro-β-amino acid could be a useful scaffold and lead to the preparation of bioactive derivatives, thus representing the minimal peptidomimetic structures for $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins mediated adhesion regulation.

Results and Discussion

Chemistry

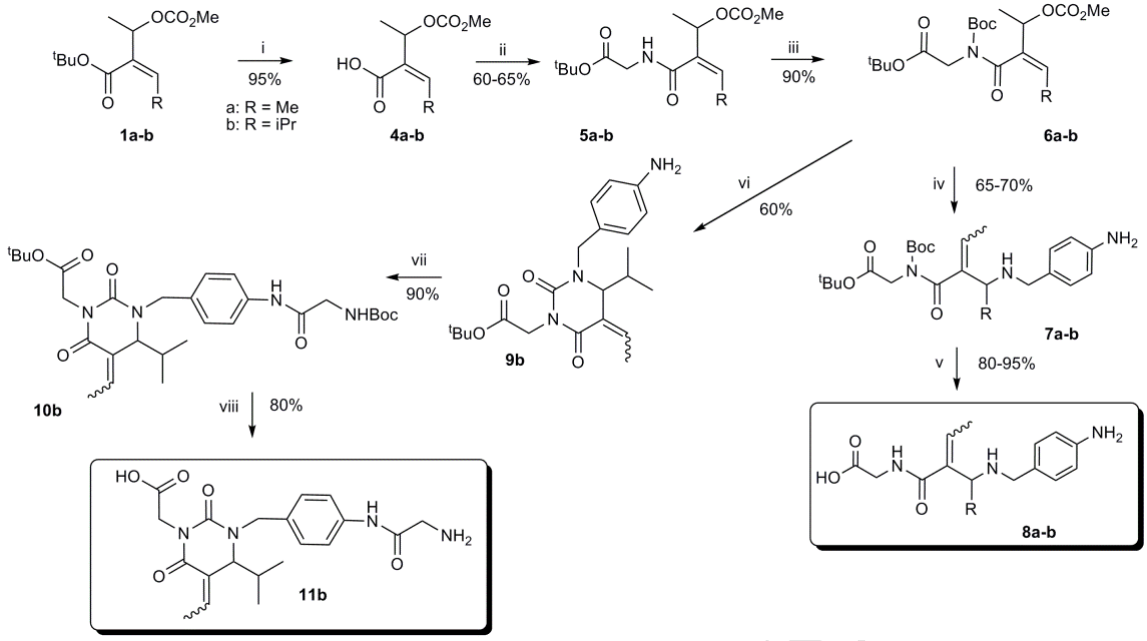
Dehydro-β-amino acid containing molecules, with different variations at the nature and length of the side chains were synthesized through S_N2' substitution of chiral carbonate-esters or amides and analyzed as integrin antagonists. At first, the small library of *N*-*p*-aminobenzyl-dehydro-β-amino acids **3a-d** was prepared through an easy two step procedure starting form carbonates **1a-d** (Scheme 1). The regio- and stereoselective synthesis of amino esters **2a-d** was based on our previously reported results and has been described in due papers, where each compound has been accurately analyzed.[17]



Scheme 1. i. *p*-aminobenzylamine, refluxing CH_3CN; ii. TFA, DCM, r.t.

Removal of the ester protection to obtain free amino acids **3a-d** was performed by treatment with an excess of trifluoroacetic acid in DCM at room temperature (77-95% yield). Although the compounds **3a-d** have a shorter length than that generally required for RGD mimetic ligands, their low molecular weight and the respect of the commonly accepted rules for bioavailability,[18] suggested that they could represent valuable lead compounds. On the other hand, the shorter length may lead to a partial ligand/receptor interaction and to an uncommon effect on adhesion and intracellular signaling.

The glycine containing derivatives **8a-b** have been obtained through the S_N2' substitution on the appropriate racemic Boc-amides **6a-b** (Scheme 2).



Scheme 2: i. TFA, DCM, r.t.; ii. Gly-OtBu, HBTU, TEA, DCM, r.t.; iii. Boc_2O, DMAP, TEA, THF, r.t.; iv. *p*-aminobenzylamine, refluxing CH_3CN, 16h; v. H_3PO_4, DCM, r.t.; vi. *p*-aminobenzylamine, refluxing CH_3CN, 4 days; vii. *N*-Boc- Gly, HBTU, DIPEA, DMF, r.t.; viii. H_3PO_4, DCM, r.t.

To this purpose, selective removal of the t-butyl ester moiety in **1a-b** (95% yield), followed by coupling with t-butyl glycinate afforded carbonates **5a-b** in 60÷65% yield. The Boc protection on the amide nitrogen was then introduced to obtain **6a-b** (90% yield), with the aim to enhance the reactivity of the double bond in the substitution reaction thanks to the electron withdrawing effect. The substitution reaction on the *N*-Boc-derivatives gave different results depending on the side chain. Treatment of **6b** with *p*-aminobenzylamine in refluxing acetonitrile afforded indeed the corresponding amino derivative **7b** in 65% yield when the reaction was stopped after 16h. After prolonged reaction times (4 days), spontaneous intramolecular cyclization to compound **9b** occurred (60% yield). On the contrary, starting from **6a**, compound **7a** was isolated in 70% yield, and no traces of the cyclic derivative could be detected even after longer refluxing times. Simultaneous removal of the ester and carbamate protections by treatment of **7a-b** in a phosphoric acid/dichloromethane biphasic mixture, allowed to obtain **8a-b** in 80÷95% yield.

The different behaviors of **7a** and **7b** in the intramolecular cyclization can be ascribed to the different conformational arrangement induced by the steric hinderance of methyl and isopropyl chains. The rotation of the C2-C3 bond in **7** that may occur to minimize steric hinderance, displays for **7b** the amine nitrogen in the proper position to act as nucleophile on the carbamate carboxylic group (Figure 2).

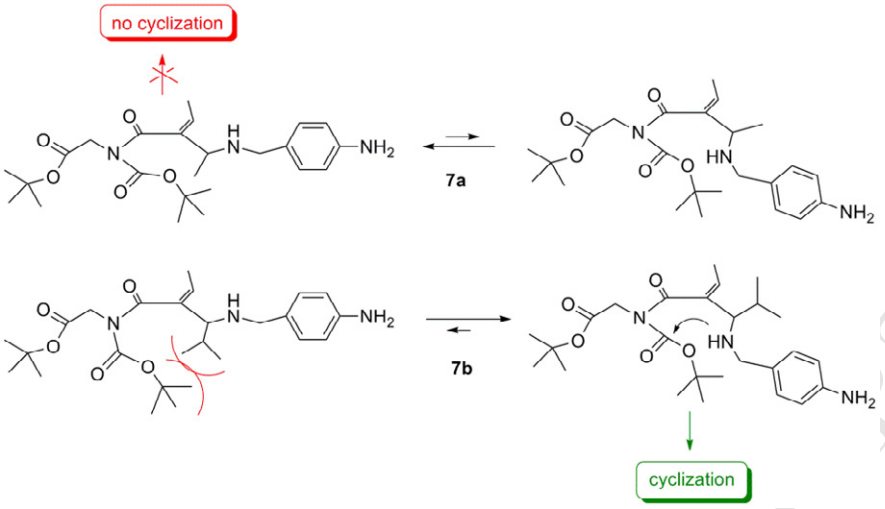
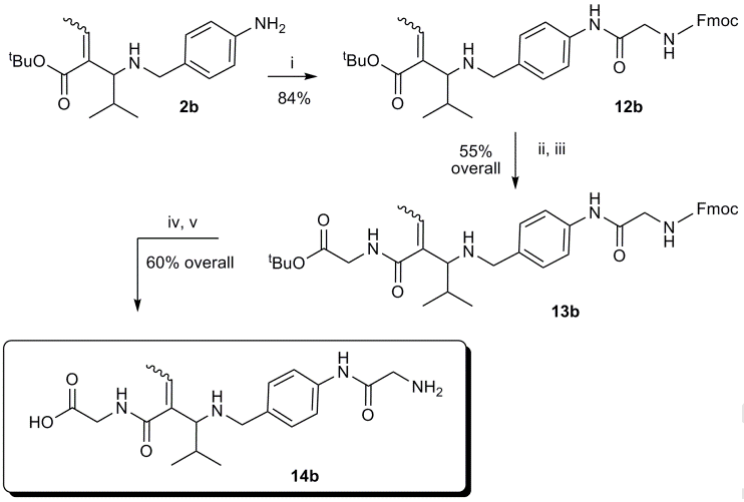


Figure 2: Proposed conformational arrangements of major isomers Z-**7a** and Z-**7b**, explaining the alternative behaviors in intramolecular cyclization.

Since this reaction affords dihydropyrimidindiones, that have been successfully introduced as rigid scaffolds in bioactive molecules of considerable interest for the pharmaceutical industry,[19] this process deserves further investigation. The effect of the carbamate leaving group, amine nucleophile, C3 stereochemistry and amino acid chain will be evaluated by computational studies and reported in due course.

To explore whether the cyclic structure could be suitable for the interaction with the receptor, dihydropyrimidindione **9b** was further decorated with a second glycine residue. Compound **10b** was isolated in 90% yield, following the usual coupling protocol and then deprotected to afford the free amino acid **11b** (80% yield). The spontaneous cyclization occurring during the substitution reaction on *N*-Boc amide, suggested that inversion of the steps in the synthetic protocol could allow the easier isolation of the linear compound. Thus, compound **12b** was isolated in 84% yield by treatment of **2b** with Fmoc-Gly-Cl in the presence of triethylamine. After removal of the tert-butyl ester, coupling with Gly-Otbu under the usual peptide synthesis conditions, allowed to obtain **13b** in 55% overall yield. Selective removal of Fmoc and t-Butyl protection afforded **14b** in 60% yield.



Scheme 3: i. Fmoc-Gly-Cl, TEA, DCM, r.t; ii. TFA, DCM, r.t.; iii. Gly-OtBu, HBTU, TEA, CH_3CN, r.t.; iv. H_3PO_4, DCM, r.t.; v. Piperidine, CH_3OH, r.t.

Pharmacology

Inhibition of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin-mediated cell adhesion

For adhesion assays, cells were seeded onto plates coated with different substrata and allowed to adhere to determine the number of adherent cells in the presence of increasing concentrations of test compounds. The mechanisms by which extracellular matrix components generate intracellular signals through integrins involves increased phosphorylation of intracellular messengers: phosphorylation of ERK plays a central role in fibronectin-mediated signalling through integrins.[20] Under these conditions, no significant cell adhesion was observed for bovine serum albumin (BSA)-coated plates (negative control) or nonspecific substrate-coated plates (i.e., collagen I for SK-MEL-24 expressing $\alpha_v\beta_3$ integrin and poly-l-lysine for K562 expressing $\alpha_5\beta_1$ integrin; data not shown). The ability of the new compounds to inhibit the adhesion of SK-MEL-24 and K562 cells to fibronectin was compared with that of the standard compounds, Gly-Arg-Gly-Asp-Thr-Pro and H-Gly-Arg-Gly-Asp-Asn-Pro-OH.[21] The results are summarized in Table 1.

The shorter molecules reported in entries 1-4 showed unexpected activity and selectivity depending on the substituent in position 3. Treatment with methyl derivative **3a** did not affect $\alpha_v\beta_3$ integrin-mediated cell adhesion, but excellent affinity towards $\alpha_5\beta_1$ integrin was observed. On the other hand, the introduction of the isopropylic group in **3b**, gave better results since comparable affinity could be observed towards both integrins (entry 2).

Table 1. $\alpha_v\beta_3$ integrin and $\alpha_5\beta_1$ integrin-mediated cell adhesion to fibronectin in the presence of dehydro-β-amino acid containing RGD mimetics.

Entry	Compound	IC_{50} (μM-10^{-6})[a]	
		$\alpha_v\beta_3$	$\alpha_5\beta_1$
1	**3a**	>100	0.019 ± 0.003
2	**3b**	1.49 ± 0.05	9.8 ± 0.6
3	**3c**	3.3 ± 0.3	4.7 ± 0.2
4	**3d**	0.83 ± 0.08	2.1 ± 0.8
5	**8a**	0.12 ± 0.03	9.8 ± 0.3
6	**8b**	2.0 ± 0.1	0.22 ± 0.05
7	**11b**	>100	>100
8	**14b**	0.27 ± 0.06	0.17 ± 0.03
9	Gly-Arg-Gly-Asp-Thr-Pro [b]	0.00062 ± 0.00003	0.93 ± 0.08
10	H-Gly-Arg-Gly-Asp-Asn-Pro-OH [b]	0.23 ± 0.03	>100

[a] Values are means of three experiments. [b] Reference compounds. See reference **15b** and **20**.

Increased activity towards $\alpha_5\beta_1$ integrin was observed for thiophenyl derivative **3c** (entry 3), while for **3d** a preferential affinity towards $\alpha_v\beta_3$ receptor could be observed (entry 4). Anyway, the difference in the affinity among the members of this class of compounds is not sufficient to deduce strong structure-activity relationship effects. Moreover, these molecules are quite smaller than the usual RGD-mimetics as cilengitide, and for this reason difference in the steric hinderance of the side chain may not be crucial for receptor affinity. Entries 5 and 6 exemplify elongated ligands anti-adhesion activity. Compound **8b** showed an increased $\alpha_5\beta_1$ integrin-mediated inhibitory activity respect to the corresponding shorter analogue **3b**. On the contrary, compound **8a** displayed good activity and a strong preference towards $\alpha_v\beta_3$ integrin, behaving as a selective not dual inhibitor.

Introduction of a second further glycine unit allowed elongation of the molecule. Moreover in this ligand the interaction with the α chain of the receptor should no longer occur through the aniline group but with the glycine amino moiety. These modification allowed to obtain the most potent dual antagonist **14b** (entry 8). Dramatic decrease in affinity towards both integrins occurred for compound **11b** (entry 7), thus suggesting that the conformational constraint introduced by the cyclic scaffold doesn't allow pharmacophor's proper orientation.

Effect of dehydro-β-amino acid containing RGD mimetics on fibronectin-induced ERK phosphorylation in DAOY or K562 cells

Cells are dependent upon integrin-mediated adhesion to the extracellular matrix for proliferation and survival. Integrins regulate these processes partially through control of extracellular signal-

regulated kinases 1 and 2 (ERK1/2). The ERK1/2 cascade impacts on cell proliferation and survival, and this pathway is regulated by cell adhesion signaling.[22] SK-MEL-24 melanoma cells harbour mutated B-Raf and this mutated form determines constitutively activation of ERK1/2 pathway and therefore adhesion-dependent regulation of ERK1/2 activity is by-passed.[23] As this pathway is impaired SK-MEL-24 cells cannot be used to study any influence of the assayed compounds on fibronectin-induced ERK phosphorylation.

Therefore, to investigate the effect of the most effective compounds **8a** and **14b** on intracellular integrin-mediated signaling activation we employed DAOY medulloblastoma cells, which express $\alpha_v\beta_3$ integrin (Figure 3).[24] Compounds **8a** and **14b** showed a similar potency as cell adhesion inhibitors in DAOY cells (data not shown).

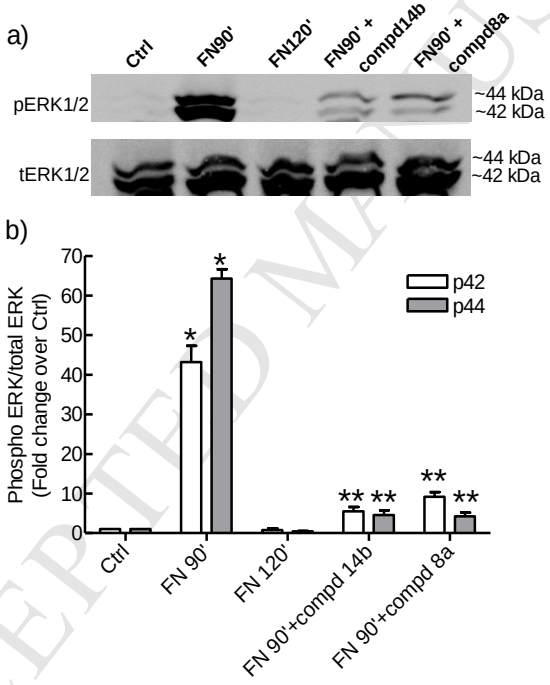


Figure 3. Compounds **8a** and **14b** prevent fibronectin-induced phosphorylation of ERK1/2. DAOY cells were serum-starved in DMEM for 16 h; cells were then pre-incubated with compounds **8a** and **14b** (10μM) for 30 min and then plated on fibronectin (FN, 10μg/ml) or kept in suspension without any compound (Ctrl). After 90 min, cells were lysed, and lysates were analysed by Western blotting using an antibody directed against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (tERK1/2). a) Western blotting shows that cells plated on FN for 90 min had a much stronger signal for phosphorylated ERK than control cells. Pre-incubation with compounds **8a** and **14b** caused a significant decrease in the amount of phosphorylated ERK. b) Densitometric analysis of the bands (mean ± SEM; n=6): p42 (□), p44 (■); the amount of pERK is normalized to the total amount of ERK. *p<0.001 vs Ctrl, **p<0.001 vs FN90' (Newman-Keuls test after ANOVA).

DAOY cells were serum-starved in DMEM for 16 h; thereafter, they were detached and pre-incubated with **8a** or **14b** integrin antagonists for 30 min and plated for 90 min on fibronectin. As shown in Figure 3, in cells exposed to fibronectin, a significant increase in phosphorylated ERK1/2 is observed after 90 min, while after 120 min ERK1/2 activation is completely abolished (ERK1/2 phosphorylation was not detected in DAOY cells exposed for 30 or 60 minutes to fibronectin; data not shown). Pre-incubation with both compounds **8a** or **14b** caused a significant decrease in the amount of fibronectin-induced ERK phosphorylation in DAOY cells.

To investigate the effect of the most effective compounds **3a** and **14b** on intracellular $\alpha_5\beta_1$ integrin-mediated signalling activation we employed K562 cells. As previously described, K562 cells were serum-starved in RPMI-1640 containing 1% fetal bovine serum (FBS) for 16 h; thereafter, they were pre-incubated with compound **3a** or **14b** (10μM) for 60 min in suspension and plated for 60 min on fibronectin. In agreement with other studies,[25] the ERK signaling is activated in K562 cells exposed to phorbol-12-myristate-13-acetate positive control (PMA, 65 nm; Figure 4).

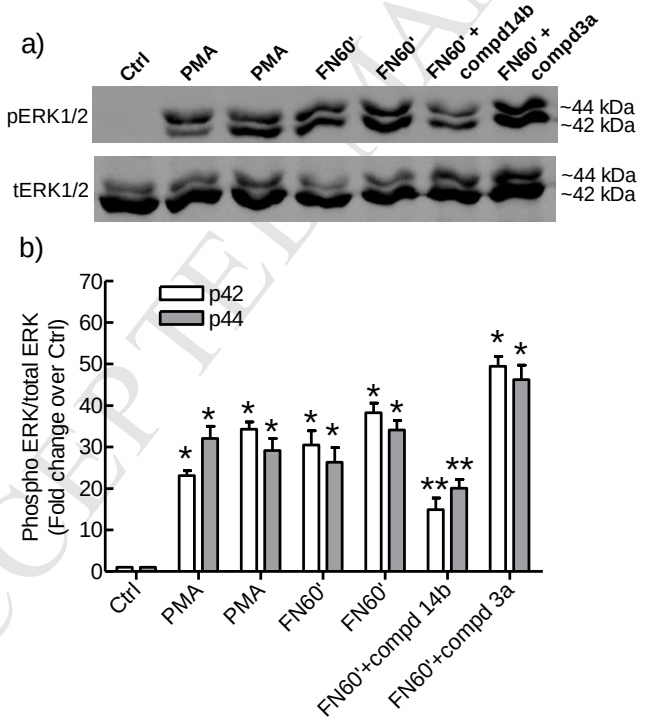


Figure 4. Compound **14b** and **3a** prevent fibronectin-induced phosphorylation of ERK1/2. K562 cells were serum-starved in RPMI+1% FBS for 16 h; cells were then pre-incubated with compound **14b** or **3a** (10μM) for 60 min and then plated on fibronectin (FN, 10μg/ml) or kept in suspension (Ctrl). PMA (65nM) treated cells were used as positive control. After 60 min, cells were lysed, and lysates were analysed by Western blotting using an antibody directed against phosphorylated ERK1/2 (pERK1/2) or total ERK1/2 (tERK1/2). a) Western blotting shows that cells plated on FN for 60 min had a much stronger signal for phosphorylated ERK than control cells. Pre-incubation with compound **14b** or **3a** caused a significant decrease in the amount of phosphorylated ERK in K562 cells. b) Densitometric analysis

of the bands (mean ± SEM; n=6): p42 (□), p44 (■); the amount of pERK is normalized to the total amount of ERK. *p<0.001 vs Ctrl, **p<0.001 vs FN60' (Newman-Keuls test after ANOVA).

Cells exposed to PMA or fibronectin showed a significant increase in phosphorylated ERK after 60 min whereas compound **14b** was able to revert fibronectin-induced ERK1/2 phosphorylation increment (Figure 4). On the contrary, compound **3a** was not able to block ERK pathway activation.

The behaviour of compound **3a**, that is ineffective towards $\alpha_v\beta_3$ integrin-mediated cell adhesion and shows high affinity for $\alpha_5\beta_1$ integrin but is not able to block signalling, suggest that probably, it could act through a different mechanism of action that deserves more investigation in future studies.

Integrins and their endogenous ligands are large and complex molecules and so there is the potential for competitive antagonists to interact with more than one site on the integrin to block ligand binding and to prevent the downstream sequela of integrin activation.[26] It is conceivable that small molecules, like **3a** described in this study, can prevent fibronectin-mediated cell adhesion in cells expressing $\alpha_5\beta_1$ integrin, whereas they are not capable to block fibronectin-induced cell signaling. Integrin-mediated adhesion often occurs under tensile forces such as fluid flow or myosin-mediated contractions that cells exert to sample the rigidity of their surroundings. Thus, to enable mechanosensing, integrins could not be constitutively active.[27] These events are mediated by fibronectin and seem to be antagonized by the compound **14b**. On the contrary, compound **3a** may not prevent fibronectin-initiated cell signaling which requires more complex integrin conformational modifications.

In the literature examples of small molecule ligands that may only partially interact with the binding site and for this reason show an uncommon effect when compared with classical RGD ligands, have been reported [14a]. Moreover, several studies have also described compounds that could be classified as allosteric antagonist or allosteric effector: these molecules could not directly compete with the binding of integrin specific ligands.[28]

Molecular Docking

In order to rationalize, on a molecular basis, the inhibition activity observed for the dehydro-β-amino acid containing RGD mimetics in the $\alpha_v\beta_3$-mediated cell adhesion assays, computational models for the binding of **8b** and **14b** to the $\alpha_v\beta_3$ integrin were built using a docking approach previously set up and validated.[29]

Starting from the X-ray structure of the extracellular segment of integrin $\alpha_v\beta_3$ in complex with the cyclic pentapeptide ligand Cilengitide (Protein Data Bank entry 1L5G),[12a] automated docking calculations were carried out using the Glide program,[30] according to the procedure described in

the experimental section. The docking protocol was successful in reproducing the crystallographic binding mode of Cilengitide at the interface of the α and β subunits, forming all the specific electrostatic and H-bond interactions. The docking procedure was then applied to small libraries of cyclic and linear RGD peptidomimetics to evaluate their ability to properly fit the receptor site. A nice agreement between docking results and experimental biological data was observed.[29,31] In all the calculations, the experimentally observed binding mode of Cilengitide with $\alpha_v\beta_3$ integrin was taken as a reference model for the analysis of the docking results in terms of ligand-protein interactions.

In this work, the Glide docking protocol was applied to compounds **8b** and **14b** to generate structural models for the interaction with the ligand binding site of the $\alpha_v\beta_3$ integrin receptor. Automated docking calculations of the (*3R,Z*) isomers of both compounds produced top-ranked poses conserving the key polar interactions of the X-ray complex.

In the best pose of compound (*3R,Z*)-**8b** (Figure 5), the ligand carboxylate group is coordinated to the metal cation in the metal-ion-dependent adhesion site (MIDAS) region of the β_3 subunit, and the ligand aromatic amine moiety interacts with the negatively charged side chain of Asp218 in the α_v subunit, although the fit with the Cilengitide Arg guanidinium group is lacking.

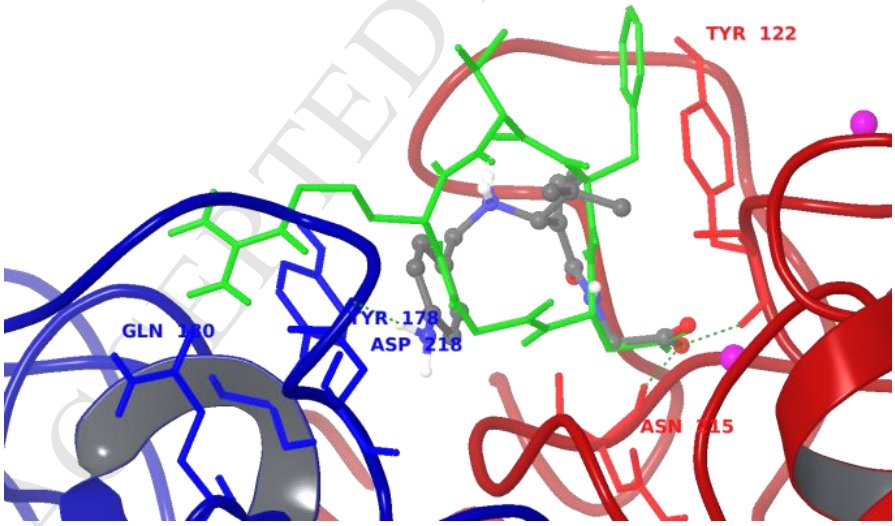


Figure 5. Docking best pose of compound (*3R,Z*)-**8b** (atom colour ball and stick representation) into the crystal structure of the extracellular domain of αvβ3 integrin (α unit blue and β unit red ribbon representation) overlaid on the bound conformation of Cilengitide (green tube representation). Only selected integrin residues involved in the interactions with the ligand are shown. The metal ions at MIDAS and ADMIDAS are shown as magenta CPK spheres. Non polar hydrogens hidden for better visual representation, intermolecular hydrogen bonds visible (green dashed lines).

Further stabilizing interactions occur in the docking poses of **8b**, involving the formation of hydrogen bonds between the ligand carboxylate group and the backbone amide hydrogens of Asn215 and Tyr122 in the β unit. A ring stacking effect between the ligand 4-amino aromatic group and the α_v -Tyr178 side chain is also observed in the binding modes calculated for compound **8b**.

In the best pose of compound (*3R,Z*)-**14b** (Figure 6), the acid and basic pharmacophoric groups act like an electrostatic clamp, interacting with charged regions of the receptor binding site. The elongation of the molecule by the introduction of an additional glycine unit on the aromatic amine of **8b** allows to achieve the pharmacophoric distance required for RGD mimicking and a good fit with the receptor-bound structure of Cilengitide. Moreover, in ligand **14b** the interaction with the negatively charged carboxylate of Asp218 in the α_v subunit not only occurs through the aromatic amide moiety but also through the positively charged glycine amino terminal group. In addition, this amino group forms a hydrogen bond with the side chain amide carbonyl of Gln180. Similarly to what is observed for compound **8b**, one carboxylate oxygen of ligand **14b** is coordinated to the metal cation in the MIDAS region of the β_3 unit, while the second carboxylate oxygen forms hydrogen bonds with the backbone amides of Asn215 and Tyr122 in the β unit. Further stabilizing interactions observed in the calculated binding modes of **14b** involve a H-bond between the ligand Gly amide and the backbone carbonyl of Asn215, and ring stacking between the ligand aromatic group and the α_v -Tyr178 side chain.

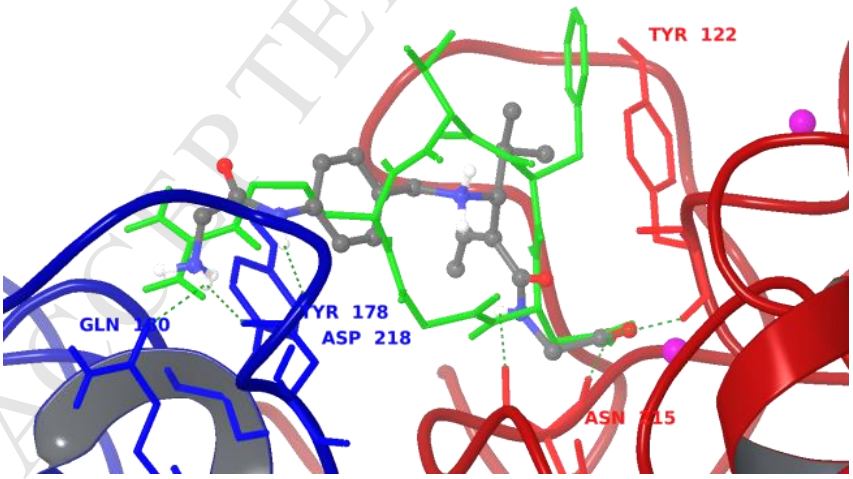


Figure 6. Docking best pose of compound (*3R,Z*)-**14b** (atom colour ball and stick representation) into the crystal structure of the extracellular domain of αvβ3 integrin (α unit blue and β unit red ribbon representation) overlaid on the bound conformation of Cilengitide (green tube representation). Only selected integrin residues involved in the interactions with the ligand are shown. The metal ions at MIDAS and ADMIDAS are shown as magenta CPK spheres. Non polar hydrogens hidden for better visual representation, intermolecular hydrogen bonds visible (green dashed lines).

Conclusion

We have reported herein a novel class of low molecular weight ligands, possessing a dehydro-β-amino acid as conformationally constrained core linked to the pharmacophoric moieties mimicking the RGD recognition sequence. The shortest ligands **3a-d** were obtained through a a very simple synthetic procedure. Although **3a** showed an unusual behaviour, the other members of this class were able to inhibit cell adhesion and integrin-mediated signalling activation with satisfactory values. Althoug the affinity is not excellent, the low molecular weight and the minimization of synthetic steps, may represent an advantage on other more complex ligands.

On the other hand, enhanced affinity and dual inhibition could be observed by adding two further glycine units to the central core, as confirmed by docking experiments showing that the dehydro-β-amino derivatives are able to act as electrostatic clamps by establishing several stabilizing interactions with the receptor. These observation suggest a potential exploitation of these molecules as lead compounds for antiangiogenetic tools development. The important contribution of integrins to the biology of both tumour cells and tumour-associated cell types has made them an appealing target for cancer therapy and for this reason the use of peptides containing the sequence RGD or structural analogues able to mimic it, represents a promising strategy for anticancer therapy.

Experimental Section

*General***:** All chemicals were purchased from commercial suppliers and used without further purification. Anhydrous solvents were purchased in sure seal bottles over molecular sieves and used without further drying. Flash chromatography was performed on silica gel (230-400 mesh). DOWEX® 50WX2-200(H) ion exchange resin was used for purification of free amino acids. NMR Spectra were recorded with Varian Gemini 200, Mercury Plus 400 or Unity Inova 600 MHz spectrometers. Chemical shifts were reported as δ values (ppm) relative to the solvent peak of $CDCl_3$ set at δ = 7.27 (^{1}H NMR) or δ = 77.0 (^{13}C NMR), CD_3OD set at δ = 3.31 (^{1}H NMR) or δ = 49.0 (^{13}C NMR), D_2O set at δ = 4.79 (^{1}H NMR). Coupling constants are given in Hz. Purity of compounds was established by means of HPLC-MS analyses was performed on a HP1100 liquid chromatograph equipped with Phenomenex Gemini 3μ-C18 column (3μ particle size, 110Å pore size, 100 X 3 mm), coupled with an electrospray ionization-mass spectrometer (LC-ESI-MS), using H_2O/CH_3CN as solvent at 25 °C (positive scan 100-500 m/z, fragmentor 70V). Compounds **1a-d** and **2a-d** were prepared according to a previously reported procedure. Complete characterization can be found in ref. 17.

*General procedure for the synthesis of dehydro-β-amino acids **3a-d**:* Trifluoroacetic acid (7 mmol, 7 equiv.) was added to a solution of **2a-d** (1 mmol) in CH_2Cl_2 (5mL) and the reaction mixture was stirred at room temperature until complete consumption of the starting material. The solvent and the excess of reagent were removed under reduced pressure. The crude residue was treated with Dowex 50WX2-200 ion exchange resin, eluting with NH_4OH 0.5 M. Compounds **3a-d** were isolated in 77-95% yield after removal of the aqueous solvent.

3a: Yield 77%, (75:25, *Z/E* mixture), pale yellow solid, m.p. 96-98 °C (dec.) ^{1}H NMR (CD_3OD, 200 MHz) *Z* isomer δ 1.53 (d, *J*=6.8 Hz, 3H, CH_3CHN),1.88 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 3.91 (d, *J*=12.8 Hz, 1H, $NHCH_2Ph$), 4.01 (d, *J*=12.8Hz, 1H, $NHCH_2Ph$), 4.31 (q, *J*=6.8 Hz, 1H, CH_3CHN), 6.80 (d, *J*=8.4 Hz, 2H, Ph), 7.02 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$), 7.23 (d, *J*=8.4 Hz, 2H, Ph); *E* isomer δ 1.53 (d, *J*=6.8 Hz, 3H, CH_3CHN), 2.12 (d, *J*=7.0 Hz, 3H, CH_3CH=C), 3.81 (q, *J*=6.6 Hz, 1H, CH_3CHN), 3.91 (d, *J*=12.8 Hz, 1H, $NHCH_2Ph$), 4.01 (d, *J*=12.8 Hz, 1H, $NHCH_2Ph$), 6.05 (q, *J*=7.0 Hz, 1H, C=$CHCH_3$), 6.80 (d, *J*=8.4 Hz, 2H, Ph), 7.23 (d, *J*=8.4 Hz, 2H, Ph). ^{13}C NMR (CD_3OD, 50 MHz) δ *Z* isomer 14.3, 19.5, 50.9, 53.6, 117.1, 122.1, 130.6, 132.6, 139.6, 151.3, 174.3; IR (neat, cm^{-1}) 1081, 1392, 1590, 1651, 1688, 2848, 2921, 2950, 3368, 3399. LC-MS-ESI rt 1.18 min (*Z* isomer), 1.53 min (*E* isomer), 235 (M+1), 257 (M+23), 491 (2M+23). Anal. cald. for $C_{13}H_{18}N_2O_2$ (234.29): C 66.64, H 7.74, N 11.96; found C 66.40, H 7.76, N 11.99.

3b: Yield 95%, (70:30, *Z/E* mixture), white solid, m.p. 108-110 °C (dec.) ^{1}H NMR (CD_3OD, 200 MHz) Z isomer δ 0.80 (d, *J*=7.0 Hz, 3H, CH_3CHCH_3), 0.91 (d, *J*=7.0 Hz, 3H, CH_3CHCH_3), 1.66 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 2.01 -2.16 (m,1H, CH_3CHCH_3), 3.69-3.95 (m, 3H, CHCHN e $NHCH_2Ph$), 6.61 (d, *J*=8.4 Hz, 2H, Ph), 6.94 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$), 7.04 (d, *J*=8.4 Hz, 2H, Ph); *E* isomer δ 0.81 (d, *J*=7.0 Hz, 3H, CH_3CHCH_3), 0.89 (d, *J*=7.0 Hz, 3H, CH_3CHCH_3), 1.94 (d, *J*=7.0 Hz, 3H, CH_3CH=C), 2.01-2.16 (m,1H, CH_3CHCH_3), 3.69-3.95 (m, 3H, CHCHN e $NHCH_2Ph$), 5.77 (q, J=7.0 Hz, 1H, C=$CHCH_3$), 6.61 (d, *J*=8.4 Hz, 2H, Ph), 7.06 (d, J=8.4 Hz, 2H, Ph). ^{13}C NMR (CD_3OD, 50 MHz) δ *Z* isomer 14.3, 19.0, 32.6, 50.2, 62.0, 116.3, 121.0, 131.9, 132.0, 142.0, 150.3, 176.1; *E* isomer 14.9, 20.0, 31.8, 50.3, 62.0, 116.3, 121.0, 131.8, 132.0, 142.1, 151.0, 177.0. IR (neat, cm^{-1}) 846, 1372, 1610, 1684, 2852, 2957, 3366, 3421. LC-MS-ESI rt 1.13 min (*Z* isomer), 1.59 min (*E* isomer), 263 (M+1), 547 (2M+23). Anal. cald. for $C_{15}H_{22}N_2O_2$ (262.35): C 68.67, H 8.45, N 10.68; found C 68.88, H 8.45, N 10.71.

3c: Yield 88%; (70:30, *Z/E* mixture), yellow solid, m.p. 195-197°C (dec.) ^{1}H NMR (DMSO, 200 MHz) *Z* isomer δ 1.74 (d, *J*=7.4 Hz, 3H, CH_3CH=C), 3.60 (d, *J*=13.2 Hz, 1H, $NHCH_2Ph$), 3.72 (d, *J*=13.2 Hz, 1H, $NHCH_2Ph$), 5.02 (s, 1H,CHNH), 6.53 (d, *J*=8.6 Hz, 2H, Ph), 6.78 (q, *J*=7.4 Hz, 1H, C=$CHCH_3$), 7.01 (d, *J*=8.4Hz, 2H, Ph), 7.10 (d, 1H, thiophenyl), 7.47 (bs, 1H, thiophenyl), 7.50-

7.54 (m, 1H, thiophenyl); *E* isomer δ 1.96 (d, *J*=6.8 Hz, 3H, CH_3CH=C), 3.60 (d, *J*=13.2 Hz, 1H, $NHCH_2Ph$), 3.72 (d, *J*=13.2 Hz, 1H, $NHCH_2Ph$), 4.60 (s, 1H,CHNH), 5.89 (q, *J*=6.8 Hz, 1H, C=$CHCH_3$), 6.53 (d, *J*=8.6 Hz, 2H, Ph), 7.01 (d, *J*=8.4Hz, 2H, Ph), 7.10 (d, 1H, thiophenyl), 7.47 (bs, 1H, thiophenyl), 7.50-7.54 (m, 1H, thiophenyl); ^{13}C NMR (CD_3OD, 50 MHz) δ *Z* isomer 14.4, 51.2, 56.4, 117.1, 121.7, 126.2, 126.7, 128.7, 130.0, 132.8, 132.9, 134.3, 138.8, 140.5, 151.3, 174.6; *E* isomer 16.7, 50.5, 56.4, 117.1, 122.0, 126.3, 126.7, 128.7, 130.0, 132.6, 132.9, 134.3, 138.6, 140.5, 153.3, 170.7; IR (neat, cm^{-1}) 760, 823, 1027, 1160, 1368, 1442, 1678, 1741, 2824, 2924, 2978, 3364, 3437. LC-MS-ESI rt 1.22 min (*E* isomer), 1.63 min (*Z* isomer), 303 (M+1). Anal. cald. for $C_{16}H_{18}N_2O_2S$ (302.39): C 63.55, H 6.00, N 9.26, S 10.60; found C 63.32, H 5.99, N 9.24, S 10.57.

3d: Yield 78%; (75:25, *Z/E* mixture), pale yellow solid, m.p. 134-136 °C (dec.) ^{1}H NMR (CD_3OD, 200 MHz) *Z* isomer δ 0.80-1.36 (m, 6H, cyclohexyl), 1.64-1.88 (m, 4H, cyclohexyl), 1.77 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 1.99-2.01 (m, 1H, cyclohexyl), 3.89-4.11 (m, 3H, CHCHN, $NHCH_2Ph$), 6.83 (d, *J*=7.0 Hz, 2H, Ph), 7.22 (d, *J*=7.0 Hz, 2H, Ph), 7.34 (q, *J*=7.4 Hz,1H, C=$CHCH_3$); *E* isomer δ 0.80-1.36 (m, 6H, cyclohexyl), 1.64-1.88 (m, 4H, cyclohexyl), 1.99-2.01 (m, 1H, cyclohexyl), 2.13 (d, *J*=6.8 Hz, 3H, CH_3CH=C), 3.89-4.11 (m, 3H, CHCHN,$NHCH_2Ph$), 6.33 (q, *J*=6.8 Hz,1H, C=$CHCH_3$), 6.83 (d, *J*=7.0 Hz, 2H, Ph), 7.22 (d, *J*=7.0 Hz, 2H, Ph). ^{13}C NMR (CD_3OD, 50 MHz) *Z* isomer δ 15.1, 27.8 (2C), 27.9 (2C), 32.0, 43.1, 51.1, 62.3, 117.1, 121.9, 131.3, 132.7, 141.7, 151.2, 175.7; *E* isomer δ 15.6, 27.8 (2C), 28.0 (2C), 30.7, 42.1, 51.0, 62.3, 117.1, 122.1, 131.3, 132.7, 140.8, 151.2, 175.7; IR (neat, cm^{-1}) 1095, 1178, 1370, 1455, 1614, 1651, 2841, 2922, 2939, 3307, 3409. LC-MS-ESI rt 1.68 min (*Z* isomer), 1.80 min (*E* isomer), 303 (M+1), 325 (M+23), 341 (M+39), 627 (2M+23). Anal. cald. for $C_{18}H_{26}N_2O_2$ (302.41): C 71.49, H 8.67, N 9.26; found C 71.25, H 8.69, N 9.24.

*Procedure for the preparation of the acid **4a-b***. To a stirred solution of the carbonate **1a-b** (0.2 mmol) in CH_2Cl_2 (2 mL) at 0°C, trifluoroacetic acid (15 equiv, 3 mmol, 223 μL) was added. After 2h, the mixture was washed twice with water (5 mL), the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure.

4a: Yield 95%, pale yellow solid, (75:25, *Z/E* mixture), ^{1}H NMR ($CDCl_3$, 400 MHz) *Z* isomer δ 1.57 (d, *J*=6.8 Hz, 3H, CH_3CHO), 2.05 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 3.79 (s, 3H, OCH_3), 5.76 (q, *J*=6.8 Hz, 1H, CH_3CHO), 7.20 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$); *E* isomer δ 1.49 (d, *J*=6.8 Hz, 3H, CH_3CHO), 1.92 (d, *J*=7.2Hz, 3H, CH_3CH=C), 5.53 (q, *J*=6.8 Hz, 1H, CH_3CHO), 6.67 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$). IR (neat, cm^{-1}) 1105, 1172, 1288, 1463, 1671, 1750, 2855, 2915, 2961, 3317. ^{13}C NMR ($CDCl_3$, 200 MHz) δ *Z* isomer 14.3, 19.5, 50.9, 53.6, 117.1, 130.6, 139.6, 151.3, 174.3. LC-

MS-ESI rt 1.22 min , 189 (M+1). Anal. cald. for $C_8H_{12}O_5$ (188.18): C 51.06, H 6.43; found C 50.95, H 6.45.

4b: Yield 95%; (>99:1, *Z/E* mixture), pale yellow solid, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.97 (d, *J*=5.4 Hz, 6H, CH_3CHCH_3), 1.40 (d, *J*=6.6 Hz, 3H, CH_3CHO), 3.25-3.42 (m, 1H, CH_3CHCH_3), 3.70 (s, 3H, CH_3OCO), 5.46 (q, *J*=6.6 Hz, 1H, CH_3CHO), 6.11 (d, *J*=10.0 Hz, 1H, =CHCH), 10.41 (bs, 1H, CO_2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 20.1, 21.9, 31.8, 54.4, 73.3, 128.9, 142.5, 154.7, 171.4; IR (neat, cm^{-1}) 1010, 1168, 1287, 1691, 1742, 2845, 2921, 2961, 3323. LC-ESI-MS rt 6.65 min, m/z 216 (M), 239 (M+Na). Anal. cald. for $C_{10}H_{16}O_5$ (216.23): C 55.55, H 7.46; found C 55.57, H 7.45.

*Procedure for the preparation of the amide **5a-b**.* To a stirred solution of the acid **4a-b** (0.2 mmol) in dry CH_2Cl_2 (2 mL), under nitrogen atmosphere, EDCI (1.2 equiv, 0,24 mmol, 46 mg) and triethylamine (2.4 equiv, 0.48 mmol, 67 µL) were added. After 30 min HOBT (1.2 equiv, 0,24 mmol, 33 mg) and glycine t-butyl ester hydrochloride (1.2 equiv, 0,24 mmol, 41 mg) were added. The solution was stirred overnight and then the mixture was diluited with CH_2Cl_2 and washed twice with acidic water (5 mL) and twice with basic water (5 mL). The two phases were separated, the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **5a-b** was isolated by flash chromatography on silica gel (cyclohexane/ethyl acetate 80/20 as eluent).

5a: Yield 60%; (75:25, *Z/E* mixture), yellow oil, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.41 (s, 9H, tBu), 1.43 (d, *J*=6.6 Hz, 3H, CH_3CHO), 1.83 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 3.74 (s, 3H, OCH_3), 3.94-4.01 (m, 2H, $NHCH_2CO$), 5.25 (q, *J*=6.8 Hz, 1H, CHO), 5.96 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$), 6.61 (bt, 1H, NH). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 15.0, 20.0, 28.0 (3C), 42.0, 54.8, 76.1, 82.2, 131.8, 137.3, 155.1, 167.3, 168.8. IR (neat, cm^{-1}) 938, 1040, 1158, 1267, 1371, 1524, 1612, 1674, 1744, 1750, 28661, 2926, 2971, 3336. LC-MS-ESI rt 6.8 min, m/z 301 (M+1). Anal. cald. for $C_{14}H_{23}NO_6$ (301.34): C 55.80, H 7.69, N 4.65; found C 55.60, H 7.68, N 4.66.

5b: Yield 65%; (>99:1, *Z/E* mixture), yellow oil, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.01 (d, *J*=6.6 Hz, 6H, CH_3CHCH_3), 1.45 (d, *J*=7.0 Hz, 3H, CH_3CHO), 1.49 (s, 9H, $OC(CH_3)_3$), 2.70-2.88 (m, 1H, CH_3CHCH_3), 3.79 (s, 3H, CH_3OCO), 4.00 (d, *J*=4.8 Hz , 2H, $NHCH_2$), 5.27 (q, *J*=6.6 Hz, 1H, CH_3CHO), 5.67 (d, *J*=10.2 Hz, 1H, CHCHC), 6.57 (bt, 1H, NH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 19.9, 22.7 (2C), 26.8, 28.0 (3C), 41.9, 54.8, 76.0, 82.2, 131.8, 137.3, 155.1, 167.3, 168.8; IR (neat, cm^{-1}) 942, 1046, 1157, 1267, 1367, 1442, 1519, 1605, 1667, 1736, 1750, 2861, 2924, 2960, 3348. LC-ESI-MS rt 10.82 min, m/z 329 (M), 352 (M+Na); Anal. cald. for $C_{16}H_{27}NO_6$ (329.39): C 58.34, H 8.26, N 4.25; found C 58.20, H 8.28, N 4.26.

*Procedure for the preparation of the N-BOC-protected amide **6a-b***. To a stirred solution of the amide **5a-b** (0.2 mmol) in dry THF (1 mL), DMAP (0.2 equiv, 0.04 mmol, 5mg), triethylamine (1.2 equiv, 0.24 mmol, 34 μL) and $(BOC)_2O$ (1.3 equiv, 0.26 mmol, 60 μL) were added. The solution was stirred overnight and then the solvent was removed under reduced pressure. The residue was diluted with ethyl acetate (10 mL) and washed twice with water (5 mL). The two phases were separated, the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **6a-b** was isolated by flash chromatography on silica gel (cyclohexane/ethyl acetate 95/5 as eluent).

6a: Yield 88%; (70:30, *Z/E* mixture), yellow oil, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.42 (s, 18H, tBu), 1.46 (d, *J*=6.6 Hz, 3H, CH_3CHO), 1.72 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 3.69 (s, 3H, OCH_3), 4.19-4.41 (m, 2H, $NHCH_2CO$), 5.41 (q, *J*=6.6 Hz, 1H, CHO), 5.80 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$). ^{13}C NMR ($CDCl_3$, 50 MHz) δ14.6, 19.2, 27.7 (3C), 28.0 (3C), 45.9, 54.6, 74.8, 81.8, 83.8, 126.7, 137.6, 151.4, 155.1, 167.6, 169.8; IR (neat, cm^{-1}) 790, 849, 1034, 1160, 1448, 1592, 1657, 1679, 1746, 1762, 2866, 2928, 2979. LC-MS-ESI rt 11.0 min, m/z 424 (M+Na), 440 (M+K). Anal. cald. for $C_{19}H_{31}NO_8$ (401.45): C 56.84, H 7.78, N 3.49; found C 57.00, H 7.76, N 3.48.

6b: Yield 90%; (70:30, *Z/E* mixture), yellow oil, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.99 (d, *J*=6.6 Hz, 6H, CH_3CHCH_3), 1.42 (d, *J*=6.6 Hz, 3H, CH_3CHO), 1.48 (s, 18H, $OC(CH_3)_3$), 2.41-2.49 (m, 1H, CH_3CHCH_3), 3.75 (s, 3H, CH_3OCO), 4.28 (d, J=16.8 Hz , 1H, NCH_2), 4.45 (d, *J*=16.8 Hz , 1H, NCH_2), 5.42-5.53 (m, 2H, CH_3CHO, CHCHC); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 19.2, 22.6 (2C), 27.2, 27.8 (3C), 28.0 (3C), 45.9, 54.5, 74.7, 81.8, 83.8, 126.7, 137.6, 151.4, 155.1, 167.6, 169.8; IR (neat, cm^{-1}) 745, 848, 1030, 1159, 1442, 1588, 1652, 1677, 1743, 1756, 2859, 2928, 2978. LC-ESI-MS rt 12.26 min, m/z 429 (M), 452 (M+Na); Anal. cald. for $C_{21}H_{35}NO_8$ (429.50): C 58.72, H 8.21, N 3.26; found C 58.78, H 8.19, N 3.25.

*Procedure for the preparation of the amino derivative **7a-b***. To a stirred solution of **6a-b** (0.2 mmol) in dry CH_3CN (2 mL), under nitrogen atmosphere, 4-aminomethylaniline (1.5 equiv, 0.3 mmol, 34 μL) was added. The solution was refluxed for 16h and then the solvent was removed under reduced pressure. Compound **7a-b** was isolated by flash chromatography on silica gel (cyclohexane/ethyl acetate 95/5 as eluent).

7a: Yield 70%; (81:19, *Z/E* mixture), yellow sticky oil, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.39 (s, 18H, tBu), 1.48 (d, *J*=7.2 Hz, 3H, CH_3CHN),1.73 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 3.90 (t, *J*=6.2 Hz, 2H, $NHCH_2CO$), 4.44 (s, 2H, CH_2Ph), 5.03 (q, *J*=7.2 Hz, 1H, CHN), 6.15 (q, *J*=7.2 Hz, 1H, C=$CHCH_3$), 6.50 (bt, NH), 6.59 (d, *J*=8.4 Hz, 2H, Ph), 7.01 (d, *J*=8.4 Hz, 2H, Ph). ^{13}C NMR

($CDCl_3$, 50 MHz) δ 13.6, 18.0, 28.1(3C), 28.4 (3C), 42.2, 47.3, 50.1, 79.8, 82.1, 115.0, 127.9, 130.7, 131.3, 139.6, 144.9, 155.9, 169.1, 169.9. IR (neat, cm^{-1}) 741, 848, 1163, 1371, 1458, 1519, 1673, 1718, 1741, 2864, 2887, 2907, 2962, 3061, 3379, 3464. LC-MS-ESI rt 9.1 min, m/z 448 (M+1); Anal. cald. for $C_{24}H_{37}N_3O_5$ (447.57): C 64.41, H 8.33, N 9.39; found C 64.35, H 8.33, N 9.36.

7b: Yield 65%; (78:22, *Z/E* mixture), yellow sticky oil, ^1H NMR ($CDCl_3$, 200 MHz) δ 0.83 (d, *J*=6.6 Hz, 6H, CH_3CHCH_3), 1.42 (d, *J*=7.4 Hz, 3H, CH_3CHC), 1.47 (s, 18H, $OC(CH_3)_3$), 2.60-2.81 (m, 1H, CH_3CHCH_3), 3.59 (bs, 2H, NH_2), 3.83-3.98 (m, 3H, NCH_2, CHNHCO), 4.34-4.61 (m, 2H, CH_2Ph), 6.61 (d, *J*=8.0 Hz, 2H, Ph), 6.83 (q, *J*=7.4 Hz, 1H, $CCHCH_3$), 7.01 (d, *J*=8.0 Hz, 2H, Ph); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 13.5, 20.2 (2C), 28.1 (3C), 28.5 (3C), 31.8, 42.2, 47.3, 50.1, 79.8, 82.1, 115.0, 127.9, 130.7, 131.3, 139.6, 144.9, 156.0, 169.1, 169.9; IR (neat, cm^{-1}) 737, 847, 1157, 1367, 1463, 1519, 1674, 1715, 1738, 2863, 2902, 2962, 3047, 3373, 3467. LC-ESI-MS rt 9.15 min, m/z 476 (M+1), 498 (M+Na); Anal. cald. for $C_{26}H_{41}N_3O_5$ (475.62): C 65.66, H 8.69, N 8.83; found C 65.58, H 8.70, N 8.86.

*Procedure for the preparation of the amino acid **8a-b***. To a stirred solution of compound **7a-b** (0.2 mmol), in CH_2Cl_2 (0.5mL), H_3PO_4 (85% solution in water, 5 equiv, 1 mmol, 69 μL) was added. After 3h the organic solvent was removed under reduced pressure and the residue was treated with Dowex 50WX2-200 ion exchange resin, eluting with NH_4OH 0.5 M. Compound **8a-b** was isolated after removal of the aqueous solvent.

8a: Yield 80%; (77:23, *Z/E* mixture), pale yellow solid, m.p. 92-94 °C (dec.) ^1H NMR (CD_3OD, 200 MHz) δ 1.38 (d, *J*=7.0 Hz, 3H, CH_3CHN),1.79 (d, *J*=6.8 Hz, 3H, $CH_3CH=C$), 3.70-4.25 (m, 5H, $NHCH_2CO$ + CH_2Ph + CHN), 6.70-6.74 (m, 3H, Ph + $C=CHCH_3$), 7.01-7.25 (m, 3H, NH+ Ph). ^{13}C NMR (CD_3OD, 50 MHz) δ 14.1, 18.7 , 44.7, 49.8, 53.7, 115.1 (2C), 128.0, 130.4, 132.0 (2C), 137.8, 144.6, 164.2, 179.7; IR (neat, cm^{-1}) 1109, 1215, 1299, 1467, 1578, 1662, 1746, 2824, 2956, 3026, 3325, 3366. LC-MS-ESI rt 0.98 min, m/z 292 (M+1). Anal. cald. for $C_{15}H_{21}N_3O_3$ (291.35): C 61.84, H 7.27, N 14.42; found C 62.02, H 7.25, N 14.43.

8b: Yield 95%; (80:20, *Z/E* mixture), pale yellow solid, m.p. 96-98 °C (dec.) ^1H NMR (CD_3OD, 200 MHz) δ 0.93 (d, *J*=6.6 Hz, 6H, CH_3CHCH_3), 1.82 (d, *J*=7.2 Hz, 3H, CH_3CHC), 2.18-2.28 (m, 1H, CH_3CHCH_3), 3.83-4.38 (m, 5H, CHNH, $NHCH_2$, CH_2Ph), 6.74 (d, *J*=8.4 Hz, 2H, Ph), 6.90 (q, *J*=7.2 Hz, 1H, $CCHCH_3$), 7.21 (d, *J*=8.4 Hz, 2H, Ph); ^{13}C NMR (CD_3OD, 50 MHz) δ 14.3, 19.9 (2C), 31.9, 46.9, 50.9, 62.4, 116.3 (2C), 121.1, 128.8, 132.0 (2C), 140.7, 148.5, 161.6, 178.2; IR (neat, cm^{-1}) 1107, 1206, 1289, 1455, 1574, 1662, 1750, 2821, 2842, 2926, 2981, 3015, 3317, 3368.

LC-ESI-MS rt 1.06 min, m/z 320 (M+1), 342 (M+Na). Anal. cald. for $C_{17}H_{25}N_3O_3$ (319.40): C 63.93, H 7.89, N 13.16; found C 64.12, H 7.90, N 13.20.

*Procedure for the preparation of the dihydropyrimidindione **9b***. To a stirred solution of **6b** (0.2 mmol) in dry CH_3CN (2 mL), under nitrogen atmosphere, 4-aminomethylaniline (1.5 equiv, 0.3 mmol, 34 μL) was added. The solution was refluxed for 4 days and then the solvent was removed under reduced pressure. Compound **9b** was isolated by flash chromatography on silica gel (DCM/ethyl acetate 90/10 as eluent).

9b: Yield 60%; (89:11, *Z/E* mixture), yellow sticky oil, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.79 (d, *J*=6.8 Hz, 3H, CH_3CHCH_3), 0.96 (d, *J*=6.8 Hz, 3H, CH_3CHCH_3),1.42 (s, 9H, tBu), 1.48 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 2.13 (m, 1H, CH_3CHCH_3), 3.77 (d, *J*= 7.6 Hz, 1H, CHN), 3.90 (d, J= 14.8 Hz, 1H, CH_2Ph), 4.38 (d, *J*= 16.8 Hz, 1H, NCH_2CO), 4.45 (d, *J*= 16.8 Hz, 1H, NCH_2CO), 5.28 (d, *J*= 14.8 Hz, 1H, CH_2Ph), 6.57 (d, *J*=6.8 Hz, 2H, Ph), 6.77 (q, *J*=7.2 Hz, 1H, $CCHCH_3$), 6.94 (d, *J*=6.8 Hz, 2H, Ph); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 18.5, 19.7, 28.0 (3C), 33.0, 42.9, 51.0, 57.0, 81.6, 115.2 (2C), 126.1, 129.0 (2C), 130.7, 137.2, 146.0, 153.1, 164.9, 167.6. IR (neat, cm^{-1}) 948, 1196, 1345, 1463, 1581, 1658, 1705, 2853, 2922, 2950, 3366. LC-ESI-MS rt 9.5 min, m/z 424 (M+Na), 825 (2M+Na); Anal. cald. for $C_{22}H_{31}N_3O_4$ (401.50): C 65.81, H 7.78, N 10.47; found C 65.84, H 7.81, N 10.51.

*Procedure for the preparation of the **10b***. To a stirred solution of the *N*-Boc-Gly (0.2 mmol, 35 mg) in dry DMF (2 mL), under nitrogen atmosphere, N,N-diisopropylethylamine (2 equiv, 0.4 mmol, 69 μL) and HBTU (1 equiv, 0.2 mmol, 76 mg) were added. After 30 min, compound **9b** (1.2 equiv, 0,24 mmol, 96 mg) was added. The solution was stirred for 3h and then the mixture was diluited with ethyl acetate and washed twice with acidic water (5 mL) and twice with basic water (5 mL). The two phases were separated, the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **10b** was isolated by flash chromatography on silica gel (DCM/ethyl acetate 80/20 as eluent).

10b: Yield 90%; (91:9, *Z/E* mixture), yellow oil, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.77 (d, *J*=7.2 Hz, 3H, CH_3CHCH_3), 0.94 (d, *J*=7.2 Hz, 3H, CH_3CHCH_3),1.39 (s, 9H, tBu), 1.41 (s, 9H, tBu), 1.49 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 2.10 (m, 1H, CH_3CHCH_3), 3.73 (d, *J*= 7.6 Hz, 1H, CHN), 3.84 (d, *J*= 6.0 Hz, 2H, CH_2NHBoc), 3.98 (d, J= 15.6 Hz, 1H, NCH_2Ph), 4.36 (d, *J*= 16.6 Hz, 1H, NCH_2CO), 4.42 (d, *J*= 16.6 Hz, 1H, NCH_2CO), 5.20 (bs, 1H, NHBoc), 5.32 (d, *J*= 15.6Hz, 1H, CH_2Ph), 6.79 (q, *J*=7.2 Hz, 1H, $CCHCH_3$), 7.09 (d, J=8.4 Hz, 2H, Ph), 7.40 (d, J=8.4 Hz, 2H, Ph), 8.10 (bs, NHGlyBoc); ^{13}C NMR ($CDCl_3$, 100 MHz) δC 14.2, 18.4, 19.7, 28.0 (3C), 28.2 (3C), 33.1, 42.9,

45.4, 51.0, 57.8, 81.7, 120.1 (2C), 127.8, 128.1 (2C), 132.5, 137.0, 137.6, 152.7, 156.5, 164.8, 167.5, 167.9. IR (neat, cm^{-1}) 961, 1200, 1377, 1463, 1552, 1632, 1651, 1660, 1700, 2884, 2924, 2956, 3500. LC-ESI-MS rt 9.97 min, m/z 581 (M+Na). Anal. cald. for $C_{29}H_{42}N_4O_7$ (558.67): C 62.35, H 7.58, N 10.03; found C 62.53, H 7.58, N 10.01.

*Procedure for the preparation of the amino acid **11b***. To a stirred solution of compound **10b** (0.2 mmol, 112 mg), in CH_2Cl_2 (0.5mL), H_3PO_4 (85% solution in water, 7.5 equiv, 1.5 mmol, 207 μL) was added. After 12h the organic solvent was removed under reduced pressure and the residue was treated with Dowex 50WX2-200 ion exchange resin, eluting with NH_4OH 0.5 M. Compound **11b** was isolated after removal of the aqueous solvent.

11b: Yield 80%; (88:12, *Z/E* mixture), yellow solid, m.p. 130-132 °C (dec.) ^{1}H NMR (D_2O, 400 MHz) δ 1.68 (d, *J*=6.8 Hz, 3H, CH_3CHCH_3), 1.85 (d, *J*=6.8 Hz, 3H, CH_3CHCH_3), 2.55 (d, *J*=7.6 Hz, 3H, CH_3CH=C), 3.03 (m, 1H, CH_3CHCH_3), 4.79 (s, 2H, CH_2NH_2), 5.01 (d, *J*= 6.8 Hz, 1H, CHN), 5.09 (d, *J*= 16.4 Hz, 1H, CH_2COOH), 5.11 (d, *J*= 16.4 Hz, 1H, CH_2COOH), 5.23 (d, *J*= 15.8Hz, 1H, CH_2Ph), 6.02 (d, *J*= 15.8Hz, 1H, CH_2Ph), 7.79 (q, *J*=7.6 Hz, 1H, $CCHCH_3$), 8.18 (d, *J*=8.4 Hz, 2H, Ph), 8.33 (d, *J*=8.4 Hz, 2H, Ph); ^{13}C NMR (D_2O, 100 MHz) δ 13.9, 17.1, 18.3, 32.7, 41.3, 44.6, 51.2, 58.4, 121.4 (2C), 126.6, 128.0 (2C), 133.7, 135.7, 140.6, 154.0, 166.3, 167.2, 175.3. IR (neat, cm^{-1}) 846, 1157, 1367, 1470, 1538, 1635, 1667, 1698, 2841, 2875, 2924, 2948, 3363, 3421. LC-ESI-MS rt 1.23 min, m/z 403 (M+1), 425 (M+Na). Anal. cald. for $C_{20}H_{26}N_4O_5$ (402.44): C 59.69, H 6.51, N 13.92; found C 59.75, H 6.48, N 13.96.

*Procedure for the preparation of **12b***. To a stirred solution of the **2b** (0.2 mmol) in dry DCM (5 mL), triethylamine (1.2 equiv, 0.24 mmol, 34 μL) and Fmoc-Gly-Cl (1.1 equiv, 0.22 mmol, 69 μL) were added. The solution was stirred for 12h and then washed twice with water (5 mL). The two phases were separated, the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **12b** was isolated by flash chromatography on silica gel (cyclohexane/ethyl acetate 90/10 as eluent).

12b: Yield 84%; (75:25, *Z/E* mixture), pale yellow solid, m.p. 95-97 °C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.74 (d, *J*=6.6 Hz, 3H, CH_3CHCH_3), 1.14 (d, *J*=6.6 Hz, 3H, CH_3CHCH_3),1.49 (s, 9H, tBu), 1.66 (d, *J*=7.2 Hz, 3H, CH_3CH=C), 1.93 (m, 1H, CH_3CHCH_3), 3.08 (d, *J*=9.2 Hz, 1H CHN), 3.52 (d, *J*=12.8 Hz, 1H, $CH_2NHFmoc$), 3.81 (d, *J*=12.8 Hz, 1H, $CH_2NHFmoc$) 4.00 (bs, 2H, NCH_2Ph), 4.25 (t, *J*= 9.6 Hz, 1H, Fmoc), 4.42 (d, *J*= 9.6 Hz, 2H, Fmoc), 7.14 (q, *J*=7.2 Hz, 1H, $CCHCH_3$), 7.30-7.43 (m, 8H, Fmoc), 7.70 (d, *J*=7.2 Hz, 2H, Ph), 7.74 (d, *J*=7.2 Hz, 2H, Ph); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 20.1, 21.2, 28.2 (3C), 32.0, 46.1, 50.4, 53.4, 65.0, 80.3, 114.9, 120.6, 122.2,

123.3, 124.5, 128.8, 129.2, 130.2, 130.6, 138.0, 144.0, 149.0, 156.2, 167.0, 168.8. IR (neat, cm^{-1}) 1159, 1276, 1367, 1455, 1651, 1682, 1698, 1738, 2823, 2861, 2923, 2982, 3018, 3450. LC-ESI-MS rt 5.46 min, m/z 620 (M+Na). Anal. cald. for $C_{35}H_{41}N_3O_5$ (597.32): C 72.34; H 7.25; N 7.03; found C 72.62, H 7.28, N 7.05.

*Procedure for the preparation of the acid **13b***. To a stirred solution of **12b** (0.2 mmol) in CH_2Cl_2 (2 mL) at 0°C, trifluoroacetic acid (15 equiv, 3 mmol, 223 μL) was added. After 2h, the mixture was washed twice with water (5 mL), the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. The obtained acid was diluted in dry CH_2Cl_2 (2 mL), under nitrogen atmosphere, and EDCI (1.2 equiv, 0,24 mmol, 46 mg) and triethylamine (2.4 equiv, 0.48 mmol, 67 μL) were added. After 30 min HOBT (1.2 equiv, 0,24 mmol, 33 mg) and glycine t-butyl ester hydrochloride (1.2 equiv, 0,24 mmol, 41 mg) were added. The solution was stirred overnight and then the mixture was diluted with CH_2Cl_2 and washed twice with acidic water (5 mL) and twice with basic water (5 mL). The two phases were separated, the organic layer was dried over Na_2SO_4, and solvent was removed under reduced pressure. Compound **13b** was isolated by flash chromatography on silica gel (cyclohexane/ethyl acetate 90/10 as eluent).

13b: Yield 55%; (95:5, *Z/E* mixture), yellow solid, m.p. 119-121 °C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.82 (d, *J*=7.2 Hz, 3H, CH_3CHCH_3), 1.07 (d, *J*=7.2 Hz, 3H, CH_3CHCH_3), 1.49 (s, 9H, tBu), 1.71 (d, *J*=7.6 Hz, 3H, CH_3CH=C), 2.10 (m, 1H, CH_3CHCH_3), 3.34-3.95 (m, 7H, CHN, $CH_2NHFmoc$, NCH_2Ph, NCH_2CO), 4.28 (t, *J*= 6.4 Hz, 1H, Fmoc), 4.42 (d, *J*= 6.4 Hz, 2H, Fmoc), 6.85 (q, *J*=7.6 Hz, 1H, $CCHCH_3$), 7.31-7.44 (m, 6H, Fmoc), 7.55 (d, *J*=8.0 Hz, 2H, Ph), 7.71 (d, *J*=7.6 Hz, 2H, Fmoc), 7.83 (d, *J*=8.0 Hz, 2H, Ph); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 20.1, 20.4, 28.3 (3C), 32.2, 38.8, 42.8, 48.3, 51.2, 62.4, 68.2, 82.9, 120.6, 121.0, 121.3, 122.0, 126.2, 128.7, 129.8, 130.2, 130.3, 138.9, 142.5, 145.2, 158.3, 170.5, 171.4 (2C); IR (neat, cm^{-1}) 1161, 1278, 1368, 1463, 1651, 1673, 1684, 1698, 1732, 2822, 2861, 2918, 2980, 3020, 3391. LC-ESI-MS rt 6.54 min, m/z 655 (M+1). Anal. cald. for $C_{38}H_{46}N_4O_6$ (654.80): C 69.70; H 7.08; N 8.56; found C 69.89, H 7.05, N 8.58.

*Procedure for the preparation of the amino acid **14b***. To a stirred solution of compound **13b** (0.2 mmol, 112 mg), in CH_2Cl_2 (0.5mL), H_3PO_4 (85% solution in water, 3 equiv, 0.6 mmol, 83 μL) was added. After 16h the organic solvent was removed under reduced pressure. The residue was diluted in methanol (2 mL), and piperidine (0.1 equiv., 0.02 mmol, 2 μL) was added. After 8h the organic solvent was removed under reduced pressure, the residue was diluted in 1N HCl (0,5 mL) and with treated with Dowex 50WX2-200 ion exchange resin, eluting with NH_4OH 0.5 M. Compound **14b** was isolated after removal of the aqueous solvent.

14b: Yield 60%; (90:10, *Z/E* mixture), yellow solid, m.p. 141-143°C (dec.) ^{1}H NMR (CD_3OD, 400 MHz) δ 0.78 (d, *J*=7.2 Hz, 3H, CH_3CHCH_3), 0.91 (d, J=7.2 Hz, 3H, CH_3CHCH_3), 1.64 (d, J=7.2 Hz, 3H, CH_3CH=C), 2.21 (m, 1H, CH_3CHCH_3), 3.33-3.39 (m, 2H, NCH_2CO), 3.94-4.02 (m, 2H, CH_2NH_2) 4.04 (s, 2H, NCH_2Ph,), 4.41 (d, *J*=7.8Hz, 1H, CHN), 6.78 (q, *J*=7.2 Hz, 1H, $CCHCH_3$), 7.33 (d, *J*=8.4 Hz, 2H, Ph), 7.40 (d, *J*=8.4 Hz, 2H, Ph); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.7, 19.7, 19.9, 31.9, 42.9, 43.4, 52.6, 67.1, 121.3 (2C), 128.5 (2C), 132.1, 132.4, 138.1, 154.0, 165.3, 168.6, 171.9. IR (neat, cm^{-1}) 1160, 1276, 1367, 1444, 1671, 1682, 1698, 1701, 2824, 2855, 2921, 3368, 3410. LC-ESI-MS rt 1.04 min, m/z 377(M+1), 399 (M+Na). Anal. cald. for $C_{19}H_{28}N_4O_4$ (376.45): C 60.62, H 7.50, N 14.88; found C 60.43, H 7.51, N 14.91.

Cell culture: SK-MEL-24 cells (American Type Culture Collection, ATCC, Rockville, MD, USA) were routinely grown in minimum essential medium (MEM, Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Life technologies, Carlsbad, CA, USA), nonessential amino acids, and sodium pyruvate. K562 cells (ATCC) were maintained as a stationary suspension culture in RPMI-1640 and glutamine with 10% FBS. Cells were kept at 37°C under a 5% CO2 humidified atmosphere; 40 h prior to experiments, K562 cells were treated with 25 ng/mL PMA (Phorbol 12-myristate 13-acetate, Sigma–Aldrich SRL, Milan, Italy) to induce differentiation and to increase expression of cell surface antigens.[32] DAOY human medulloblastoma cell line (a kind gift from Dr G. Cenacchi, University of Bologna) was propagated in Dulbecco's Modified Eagle's Medium (DMEM; Life technologies, Carlsbad, CA, USA) supplemented with 20% (v/v) FBS, 1% penicillin-streptomycin and 1mM glutamine (Cambrex, Walkersville, MD, USA). For all experiments, cells were used between passage 3 and 15.

Cell adhesion assays: 96-well plates (Corning Costar, Celbio, Milan, Italy) were coated by passive adsorption with fibronectin (10 μg/mL) or nonspecific substrate poly-l-lysine (0.002 %; Sigma–Aldrich SRL) overnight at 4°C. Cells were counted with a hemocytometer and pre-incubated with various concentrations of each compound for 30 min at room temperature to reach a ligand–receptor equilibrium. At the end of the incubation time, the cells were plated (50000 cells per well) and incubated at room temperature for 1 h. All the wells were then washed with PBS to remove nonadherent cells, and 50 μL hexosaminidase [4-nitrophenyl-N-acetyl-β-d-glucosaminide dissolved at a concentration of 7.5 mM in 0.09 M citrate buffer (pH 5) and mixed with an equal volume of 0.5% Triton X-100 in H2O] was added. This product is a chromogenic substrate for β-N-acetylglucosaminidase, whereby it is transformed into 4-nitrophenol; absorbance was measured at 405 nm. The reaction was blocked by the addition of 100 μL of a stopping solution [50 mM glycine and 5 mM EDTA (pH 10.4)], and the plates were read in a Victor2 Multilabel Counter

(PerkinElmer, Waltham, MA, USA). Experiments were carried out in quadruplicate and repeated at least three times. Data analysis and IC50 values were calculated using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

Western blot analysis: K562 or DAOY cells were incubated in RPMI-1640 with 1% FBS or DMEM respectively for 16 h. Plates were coated with fibronectin (10 μg/mL) and blocked with 1% BSA (Sigma–Aldrich SRL). Subsequently, 4x106 cells were pre-incubated with the various compounds for 30 min and then cells were allowed to adhere for 60-90 min on fibronectin. The cells were then lysed in mammalian protein extraction reagent (M-PER; Pierce, Rockford, IL, USA) supplemented with Phosphatase Inhibitor Cocktail (Sigma–Aldrich SRL) for 10 min at 4°C by gentle shaking. Cell debris was removed by centrifugation (14000 g for 15 min at 4°C), and protein concentrations were estimated by BCA assay (Pierce, Rockford, IL, USA). Protein extracts were denatured at 95°C for 3 min before loading and separation by 12% SDS-PAGE. The membranes were blocked in 1% BSA and incubated for 2 h with anti-phospho-ERK 1/2 (extracellular signal-regulated kinase 1/2) (1:2500) (Cell Signaling Technology, Danvers, MA, USA) or anti-total ERK1/2 antibodies (1:5000) (Promega, Madison, WI, USA), and, thereafter, with anti-rabbit HRP-conjugated secondary antibody. Digital images were acquired and analyzed according to a previously reported method.

Molecular Docking

All calculations were run using the Schrödinger suite of programs (http://www.schrodinger.com) through the Maestro graphical interface.

Protein Setup: The recently determined crystal structure of the extracellular domain of the integrin αvβ3 receptor in complex with cilengitide and in the presence of the proadhesive ion Mn2+ (PDB entry 1L5G)[12a] was used for docking studies. Docking was performed only on the globular head of the integrin because the headgroup of integrin has been identified in the X-ray structure as the ligand-binding region. The protein structure was set up for docking as follows; the protein was truncated to residues 41-342 for chain α and residues 114-347 for chain β. Due to a lack of parameters, the Mn2+ ions in the experimental protein structure were modeled via replacement with Ca2+ ions. The resulting structure was prepared using the Protein Preparation Wizard of the graphical user interface Maestro and the OPLSAA force field.

Docking: The automated docking calculations were performed using Glide (Grid-based Ligand Docking with Energetics)[30] within the framework of Impact version 4.5 in a standard precision mode (SP). The grid generation step started from the extracellular fragment of the X-ray structure of

the αvβ3 complex with cilengitide, prepared as described in Protein Setup. The center of the grid-enclosing box was defined by the center of the bound ligand. The enclosing box dimensions, which are automatically deduced from the ligand size, fit the entire active site. For the docking step, the size of the bounding box for placing the ligand center was set to 12 Å. No further modifications were applied to the default settings. The Glide-Score function was used to select 20 poses for each ligand. Glide was initially tested for its ability to reproduce the crystallized binding geometry of cilengitide. The program was successful in reproducing the experimentally found binding mode of this compound, as it corresponds to the best-scored pose.

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