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Template Assembled Cyclopeptides as Multimeric System for Integrin Targeting and Endocytosis

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Abstract: The $\alpha_V\beta_3$ integrin receptor plays an important role in human metastasis and tumor-induced angiogenesis. c[-RGDfV-] peptide represents a selective $\alpha_V\beta_3$ integrin ligand that has been extensively used for research, therapy, and diagnosis of neoangiogenesis. We report here the modular synthesis and biological characterization of template assembled cyclopeptides as a multimeric system for targeting and endocytosis of cells expressing $\alpha_V\beta_3$ integrin. c[-RGDfK-] was cleanly assembled in a multivalent mode by chemoselective oxime bond formation to a cyclodecapeptides template labeled by different reporter groups. Binding propensity to the $\alpha_V\beta_3$ receptor and the associated good uptake property displayed by the multivalent molecules demonstrated the interest in the RAFT molecule to design new multimeric system with hitherto unreported properties. These compounds offer an interesting perspective for the reevaluation of integrins as angiogenesis regulators (Hynes, R. O. *Nature Med.* **2003**, *9*, 918−921) as well as for the design of more sophisticated systems such as molecular conjugate vectors.

Introduction

Integrins are heterodimeric transmembrane cell surface receptors that play a central role in cell−cell and cell-extracellular matrix (ECM) adhesion processes.[1] The $\alpha_V\beta_3-\alpha_V\beta_5$ integrin subclass has received special attention as it is expressed in various cell types such as endothelial cells, platelets, osteoclasts, melanoma, and smooth muscle cells. They have a pivotal involvement in pathologies as diverse as osteoporosis, arthritis, retinopathy, tumor-induced angiogenesis, and metastasis formation.[2] Cells expressing integrins $\alpha_V\beta_3-\alpha_V\beta_5$ interact to the ECM bearing a wide variety of ligands such as fibronectin and vitronectin mainly through the recognition of the ubiquitous triad sequence RGD (Arg-Gly-Asp) that has served as basis for the development of high and selective integrin-peptide ligands. Rational screening of RGD-containing cyclopeptides has led Kessler's group to the discovery of the highly active first-generation peptide c[-RGDfV-] **1** (Figure 1)[3] whose *N*-alkylated analogue c[-RGDf(*N*Me)V-][4] is currently investigated in clinical phase II as an angiogenesis inhibitor (Cilengitide, code EMD 12974 Merck KGaA).[5] Interestingly, other conformationally constrained RGD-containing peptides have been selected in vivo as selective $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin ligands using phage display peptide libraries.[6] These highly selective RGD-containing peptides represent key compounds for targeting and tracing $\alpha_V\beta_3$-expressing tumor cells as well as endothelial cells during neoangiogenesis in the fields of research, therapy, and diagnosis as underlined by the abundant corresponding literature.[7−10]

Very recently, multivalent $\alpha_V\beta_3$ integrin ligands with an improved affinity have been developed by scaffolding multiple copies of an RGD-containing peptide to a polymer,[11] an artificial membrane,[12] as well as a protein[13] or a peptide.[14] Because the natural functional mode of integrins interaction involves multivalent interactions, this approach could provide not only more effective binding molecules but also systems that could improve the cell targeting and promote cellular uptake through the

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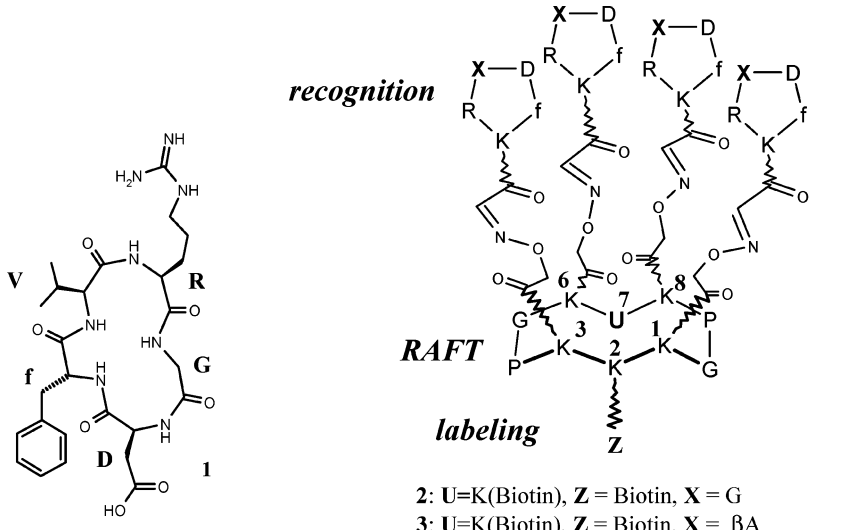

2: **U**=K(Biotin), **Z** = Biotin, **X** = G
3: **U**=K(Biotin), **Z** = Biotin, **X** = βA
4: **U**=K(Fluorescein), **Z** = Fluorescein, **X** = G
5: **U**=K(Fluorescein), **Z** = Fluorescein, **X** = βA
6: **U**=A, **Z** = Fluorescein, **X** = G
7: **U**=A, **Z** = Fluorescein, **X** = βA
8: **U**=A, **Z** = Y, **X** = G

Figure 1. RGD-assembled peptides derivatives **1**−**8** (f = D-Phe).

integrin-dependent endocytosis pathway thus offering new therapeutic avenues.[15−17]

There is however still a need for developing more appropriate chemical methods for the preparation of well-defined multivalent ligand architectures that may also incorporate effector molecules such as label groups as tools for biological evaluation or subsequently cytotoxic molecules for therapeutic applications. In this context, we describe herein the modular synthesis of compounds **2**−**8** based on a cyclic decapeptide scaffold (RAFT) that incorporates and presents in a spatially controlled manner two independent functional domains: a clustered ligand domain for integrin recognition and cell targeting and a labeling domain for detection and characterization of this binding. Preliminary study on the biological activity of the $(biotin)_2$RAFT(c[-RGDfK-])$_4$, **2**, $(fluorescein)_2$RAFT(c[-RGDfK-])$_4$, **4**, and (fluorescein)-RAFT(c[-RGDfK-])$_4$, **6**, have shown that these multivalent systems fully conserved the recognition and selectivity properties of the cognate monovalent cyclopentapeptide c[-RGDfK-] to cells expressing $\alpha_V\beta_3$ integrin receptors. Most notably, these systems were found to penetrate cells expressing $\alpha_V\beta_3$ integrins by endocytosis in a receptor-dependent manner more effectively than the cognate monovalent ligand. These results underline the effectiveness of this general strategy to provide biological active systems for selectively targeting cells expressing the $\alpha_V\beta_3$ integrin receptors. These molecules offer a wide range of possibility to characterize the binding interactions to cells expressing the $\alpha_V\beta_3$ integrin as well as the endocytosis process. For instance, biotinylated compounds **2** and **3** were designed to form stable complexes with avidin or streptavidin protein which are commercially available for applications such as surface functionalization or labeling. Fluorescein-containing molecules **4**−**6** represent useful compounds ready for use in experiments such as fluorescent or confocal miscroscopy. Compounds **7** and **8** were designed for radiolabeling with ^{125}I and are currently used in radiobinding assays as well as in vivo imaging in mice.[18] These molecules represent the first instance of a cyclopeptide presenting an array of cyclopeptides which emphasizes the efficiency of our approach to construct such new architectures for recognition. In addition, the usefulness of the chemical strategy presented here is currently extended to more complex systems presenting elements for cell recognition as well as cytotoxic elements for therapeutic evaluation.[19] It is expected that these bifunctional systems might be useful in the near future as reagents to study and modulate physiologically important integrin-extracellular matrix protein interactions and as drugs for disease-related applications as well as new nonviral targeting systems for drug delivery. In addition, it is worth noting that our approach is not limited to integrin targeting or detection applications. By adapting the corresponding domain on the template, it may be conceptually easily exploited to target different cells or tissues due to the increasing number of selective ligands selected in vivo, and the range of applications may be greatly extended by using the vast repertoire of available organic compounds.

Results

Design. We reasoned that a controlled presentation of RGD moieties in a clustered format may result in more efficient

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clustering of the ligand-bound integrin compared to the same ligand presented individually. The recognition module consists of the c[-RGDfK-] ligand since it is known that an exchange of valine residue in the cognate cyclopentapeptide for lysine has no significant influence on biological activity[3]. c[-RGDfK-] can be readily functionalized through the chemistry of its lysine ϵ-amino group as emphasized by various applications such as tumor targeting[9] or imaging[20] and cell adhesion stimulation.[21] In addition, we reported that a lysine side chain functionalized by aminooxy or glyoxylyl groups[22] represents a powerful way to conjugation to a diversity of molecules by chemoselective oxime bond formation.[23] This ligation technique is highly efficient, compatible with a wide variety of chemical functions, and allows the oxime bond formation between unprotected fragments without any coupling reagent and with minimal chemical manipulation.[24] Moreover, the oxime linkage presents the further advantage of being stable in vitro and in vivo.[25] We selected the regioselectively addressable functionalized template (RAFT) as a suitable scaffold to direct independently and separately the cyclopentapeptide ligands as well as reporter groups (Figure 1). RAFT molecules represent topological cyclic decapeptides containing orthogonally protected attachment sites pointing to opposite faces of the template backbone that are chemically accessible at gram scale.[26] These addressable systems can readily exhibit various levels of regioselectivity thanks to an appropriate choice of the residues' side chain protecting groups encompassing the Pro-Gly sequence. They display homogeneous conformation control[27] that have been exploited successfully for protein mimic[28] or surface functionalization.[29] In addition, we recently reported successfully the chemoselective oxime assembly of aminooxy-carbohydrates to the RAFT molecule for the presentation of carbohydrate recognition motifs clusters.[30] The modular functionalization with c[-RGDfK-] as an $\alpha_V\beta_3$ integrin ligand or c[-RβADfK-] as a nonsense peptide, as well as fluorescein, biotin, or tyrosine molecules (Figure 1), thus provides series of molecules required for integrin targeting studies.

The ligand and technique for its chemical manipulation in hand, a strategy in three steps was devised, whereby the RAFT molecules formed the pivotal intermediate: (1) the solid phase peptide synthesis of linear protected peptide fragments, their cyclization in solution to provide the corresponding templates and cyclopentapeptides; (2) the sequential deprotection−functionalization of a lysine side chain with label molecules and subsequently four aminooxy groups on the template's face as well as the functionalization of the lysine side chain on the cyclopentapeptides by a glyoxylyl group; (3) the final oxime ligation of deprotected aminooxy-containing RAFT molecules and N^{ϵ}-glyoxylyl-lysyl cyclopentapeptides to provide the desired compounds.

RAFT Molecules. The RAFT with two functional domains formed the central intermediate required for a multivalent presentation with an integrin ligand as well as the chemical derivatization with labeling molecules. The convenient choice of lysine side chain protections and their relative positioning within the peptide primary sequence provide cyclodecapeptides **9** and **19** exhibiting two attachment faces (Scheme 1). The Boc and Alloc groups were selected because they are well-documented orthogonal protections and the corresponding protected lysine residues are commercially available. The linear precursor-containing side chain protected peptides, namely H-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-OH and H-Lys(Boc)-Ala-Lys(Boc)-Pro-Gly-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-OH, were obtained using a standard Fmoc/*t*Bu solid-phase chemistry on an acid-labile Sasrin resin by adapting the previously described procedures.[26,28b] It is worth noting that the mild acid conditions used for the release of the linear protected peptide from this support (e.g., 1% TFA/CH_2Cl_2, 2 min) was required to be compatible with the Boc side chain protection stability. Glycine as the C-terminal end was essential to secure the subsequent cyclization step with the N-terminal lysine α-amino group from epimerization. The corresponding head-to-tail cyclization was performed in DMF under high dilution with PyBOP reagent as reported[26] to provide the corresponding RAFT molecules **9** and **19** in quantitative yields. Removal of the Alloc group using the well-established $Pd^0/PhSiH_3$ procedure[31] afforded the desired derivatives **10** and **20** containing one and two free amino groups, respectively, in excellent yields. Acylation of the fluorescein or biotin groups to the latter amine functions was accomplished directly with FITC (isomer I) or PyBOP coupling conditions, respectively. The corresponding labeled derivatives $(biotin)_2RAFT(Boc)_4$ **11**, $(fluorescein)_2RAFT(Boc)_4$ **12**, and $(fluorescein)RAFT(Boc)_4$ **21** were isolated after ether precipitation in sufficient purity to carry out the subsequent step. Compounds **11**, **12**, and **21** were smoothly deprotected with 50% trifluoroacetic acid at room temperature in dichloromethane for 1 h and acylated with the succinimide ester of *N*-Boc-*O*-(carboxymethyl)hydroxylamine[32] to yield functionalized templates **15**, **16,** and **23**, respectively. Removal of the Boc groups was achieved by treatment with TFA containing triisopropylsilane (TIS) and water in CH_2Cl_2 (50/5/5/40) for 1 h. RP-HPLC purification provided the key aminooxy intermediates $(biotin)_2RAFT(COCH_2ONH_2)_4$ **17**, $(fluorescein)_2RAFT(COCH_2ONH_2)_4$ **18**, and $(fluorescein)RAFT(COCH_2ONH_2)_4$ **24** in excellent overall yields, 42%, 70%, and 36%, respectively, for four steps (Scheme 1). To introduce a

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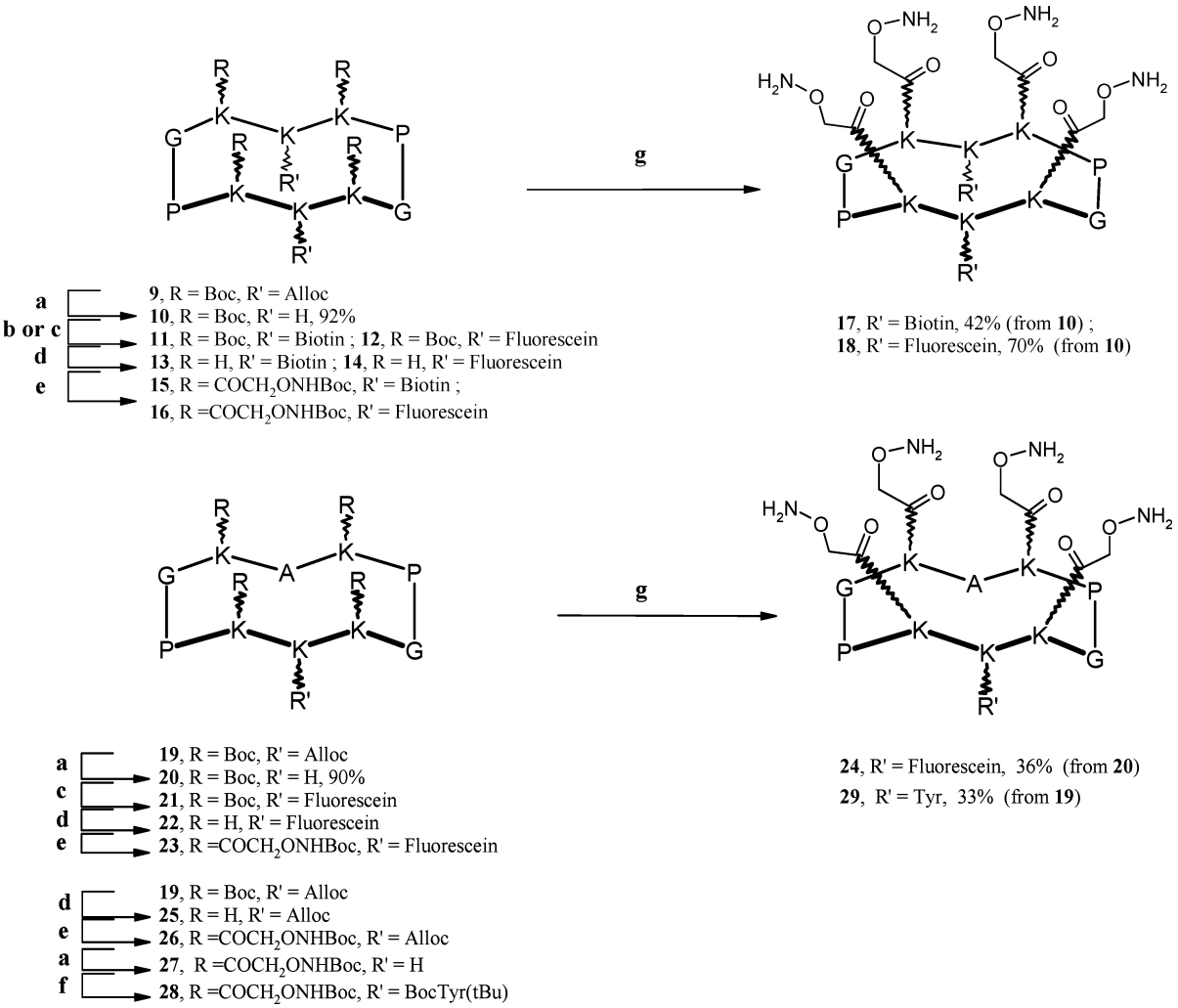
Scheme 1. Synthesis of Aminooxy-Containing RAFT **17**, **18**, **24**, and **29**[a]

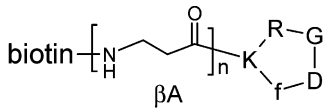


[a] (a) $(PPh_3)_4Pd$, $PhSiH_3$, CH_2Cl_2; (b) PyBOP, biotin, DIEA, DMF; (c) FITC (isomer I); (d) TFA/CH_2Cl_2 1:1; (e) $BocNHOCH_2COOSu$, DIEA, DMF; (f) PyBOP, BocY(*t*Bu)OH, DIEA, DMF; (g) TFA/TIS/H_2O/CH_2Cl_2 50:5:5:40.

tyrosine residue, a reverse functionalization strategy was required to satisfy the use of BocTyr(O*t*Bu)OH: first, removal of the Boc groups of **19** followed by a subsequent acylation with *N*-Boc-*O*-(carboxymethyl)hydroxylamine succinimide ester afforded compound **26**. Then, removal of the Alloc group and PyBOP acylation with BocTyr(O*t*Bu)OH provided Boc/*t*Bu protected template **28**. A total deprotection was accomplished using a concentrated TFA solution containing *p*-cresol, TIS, and water as scavengers providing compound **29** in 33% overall yield (Scheme 1).

Cyclopeptide Ligand and Control Peptide. The exchange of glycine residue for β-alanine in cyclic pentapeptide c[-RGDfK-] is known to totally abolish recognition of the peptide by $\alpha_V\beta_3$ integrin.[3] Therefore, c[-RβADfK-] was used as a negative control in our biological assays. The cyclic pentapeptide c[-RGDfK(S)-] **30** as well as c[-RβADfK(S)-] **31** derivatives acylated on the lysine side chain with a serine residue were prepared by two-dimensional solid-phase synthesis as described earlier.[22] Oxidative cleavage with sodium periodate of the amino-alcohol moiety of serine containing cyclopentapeptides afforded the desired glyoxylyl compounds c[-RGDfK-(COCHO)-] **32** and c[-RβADfK(COCHO)-] **33** in quantitative yields. Removal of formaldehyde byproduct and unreacted periodate was readily accomplished by RP-HPLC purification to give the pure compounds ready for oxime ligation.

Scheme 2. Structure of the Monovalent Biotinylated-RGD Ligands Containing β-Alanine Spacers **34**−**39**



34 :n=0; **35** :n=1; **36** :n=2; **37** :n=3; **38** :n=4; **39** :n=5

Access to monovalent RGD-control peptides bearing a biotin on the lysine side chain, c[-RGDfK([βA]$_n$-biotin)-] **34**−**39** (n = 0−5, Scheme 2) was achieved by a combination of SPPS and solution strategies.[22] Incorporation of β-alanine (n = 0−5) units as a spacer was used to optimize the separation between the ligand and biotin group required for the interaction with the integrin receptor as well as with streptavidin (vide supra). Fluorescent cyclopentapeptide c[-RGDfK(fluorescein)-] **41** was obtained by oxime ligation between the N^{ϵ}-glyoxylyl-lysyl peptide **32** and fluorescein containing oxyamine group[33] **40** in acetate solution at pH 4.6 in 77% yield. Acylation at the lysine side chain of protected cyclopentapeptide by succinic anhydride

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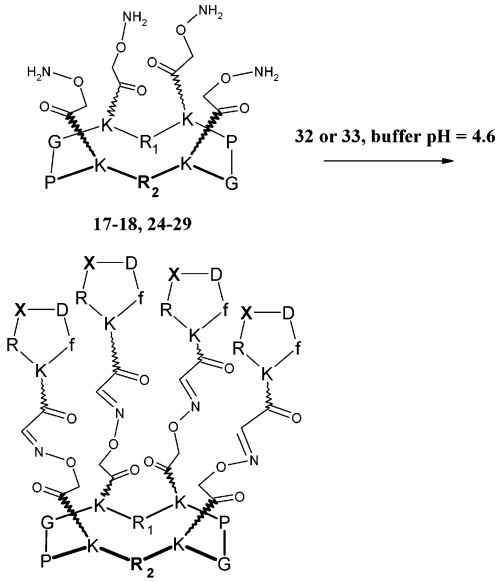
Scheme 3. Synthesis of Compounds **2**−**8** by Oxime Ligation of Glyoxylyl Cyclopentapeptides to Aminooxy-RAFT Molecules **32** and **33**



2 : $R_1 = R_2 =$ K(Biotin), X = G, 94%
3 : $R_1 = R_2 =$ K(Biotin), X = βA, 73%
4 : $R_1 = R_2 =$ K(Fluorescein), X = G, 51%
5 : $R_1 = R_2 =$ K(Fluorescein), X = βA, 75%
6 : $R_1 =$ A, $R_2 =$ K(Fluorescein), X = G, 75%
7 : $R_1 =$ A, $R_2 =$ K(Fluorescein), X = βA, 95%
8 : $R_1 =$ A, $R_2 =$ K(Y), X = G, 73%

followed by acid-mediated deprotection provided c[-RGDfK-($COCH_2CH_2CO_2H$)-] **42**, as the key compound for attempting direct RGD-peptide anchorage to the template molecule by conventional amide bond formation (vide supra).

Template Presenting Tetravalent Cyclopeptides. Compounds **17**, **18**, **24**, and **29** exhibiting four aminooxy functions provide the key intermediates to assemble by chemoselective oxime formation an array of cyclopentapeptides on the template. (biotin)$_2$RAFT(c[-RGDfK-])$_4$ **2**, (biotin)$_2$RAFT(c[-RβADfK-])$_4$ **3**, (fluorescein)$_2$RAFT(c[-RGDfK-])$_4$ **4**, (fluorescein)$_2$RAFT-(c[-RβADfK-])$_4$ **5**, (fluorescein)RAFT(c[-RGDfK-])$_4$ **6**, (fluorescein)RAFT(c[-RβADfK-])$_4$ **7**, and (tyrosine)RAFT(c[-RGDfK-])$_4$ **8** were easily obtained by oxime ligation of the appropriate cyclopentapeptide-containing aldehyde, namely c[-RGDfK-(COCHO)-] **32** and c[-RβADfK(COCHO)-] **33**, to free aminooxy-containing RAFTs **17**, **18**, **24**, and **29** under classical conditions at pH 4.6 (Scheme 3). This provided labeled molecules with a clustered presentation of the integrin RGD ligand **2**, **4**, **6**, and **8** as well as comparable molecules as negative controls **3**, **5**, and **7**. As inferred by HPLC, the ligation step proceeded cleanly and the reactions were generally complete after 6 h by using a slight 1.5-fold excess of aldehyde compounds relative to the aminooxy-containing template. The yields of the reactions were satisfactory ranging from 51 to 95% after purification steps. In addition, a reaction was carried out using an equimolar ratio of compounds **17** and **32** to probe the putative intermediates formed along the oxime coupling reaction. The HPLC profile (Figure 2) obtained after a 1 h reaction time with these conditions showed the presence of intermediate products.[34] They disappeared on further addition of aldehyde

(34) Intermediates were identified and studied by LC-MS technique to be the mono, bis, and tri ligated compounds.

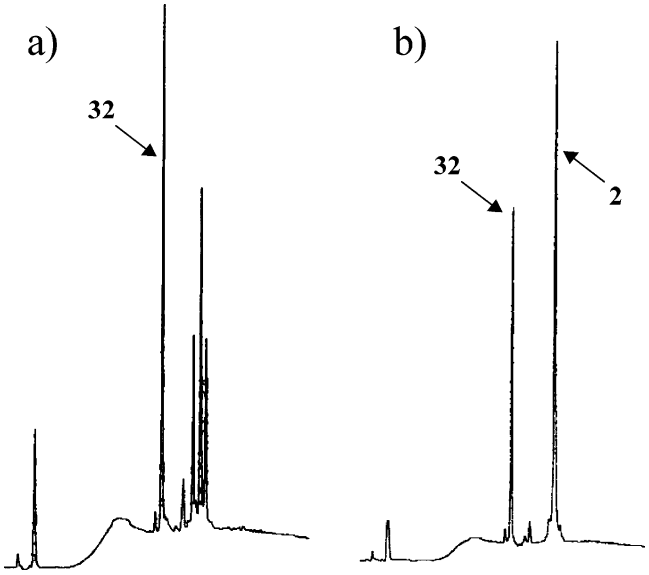


Figure 2. HPLC profiles of (a) a reaction between **17** and 1 equiv of **32** after 1 h and (b) a reaction between **17** and 6 equiv of **32** after 6 h.

compound to provide eventually the HPLC signal corresponding to the desired product. It is important to note that attempts to assemble the cyclopentapeptide by conventional amide formation using protected compounds **42** and template molecule **13** were totally unsuccessful[35] thus emphasizing the efficacy of the oxime ligation.

Biological Data. First, oxime-containing compounds **2**−**8** were incubated in phosphate buffer at pH 7.0 to assess on their stability. Neither hydrolysis nor degradation phenomenon was observed by HPLC even after several days (data not shown) which indicates that these compounds remain fully stable under the biological experimental conditions used in this study.

A. Cell Adhesion Assays. Binding studies of RGD derivatives were first realized on different cell lines (data not shown).[36] Among all cell lines investigated, CHO3a was selected as the model for our in vitro experiments because it is a stable transfectant of the chinese hamster ovary (CHO) cell line expressing a good level of $\alpha_V\beta_3$ integrin. To test the functional integrity of the c[-RGDfK-]-containing peptides, we studied first the adhesion of CHO3a cells on a surface coated by biotinylated peptides **2** and **34**−**39** and second the inhibition of CHO3a attachment to vitronectin-coated wells by the same peptides. Previously, we investigated the importance of the linker length between the control RGD ligand and the biotin moiety. Biotinylated-peptides **34**−**39** were loaded onto streptavidin-coated 96-well plates. CHO3a cells were then incubated for 45 min at 37 °C, and their ability to adhere on these surfaces was measured after staining (Figure 3). As expected the negative control peptide **3** containing the RβAD sequence did not allow cell adhesion, while the cells adhered normally on a fibronectin-coated surface (FN). Derivatives **34** and **35** containing shorter linkers between RGD and biotin motifs were not able to bridge the streptavidin surface and the cells. Increasing gradually the length of the linker from two up to five, β-alanine (respectively,

(35) This is attributed to tedious solubility problems and presumed steric hindrance imposed by the presence of protecting groups required during the coupling reaction.

(36) HEL, PMA-induced HEL, CHOT, K562, HeLa, and CHO3a were used for preliminary experiments. K562 devoid of $\alpha_V\beta_3$ integrin was used along this work as control.

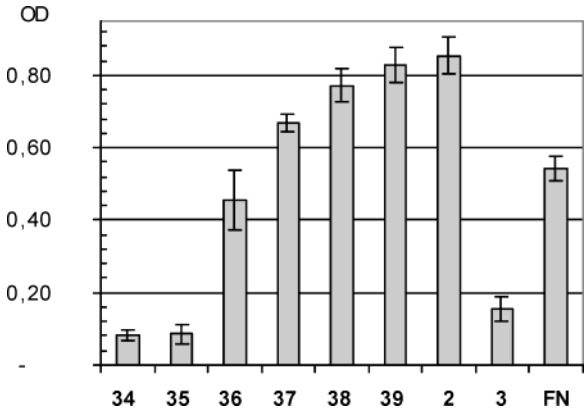


Figure 3. Adhesion assay. Streptavidin-coated 96-well dishes were loaded with 1 μM **2**, **3**, and **34**−**39** peptides. Fibronectin (FN) was directly coated on MaxiSorp 96-well plates at a concentration of 0.5 μg/well. CHO3a cells (100 000) were then incubated for 45 min at 37 °C in PBS containing 1 mM $MgCl_2$ and rinsed 3 times, and the remaining adherent cells were fixed, stained with methylene blue, and resuspended in 100 μL HCl 0.1 N. The OD at 630 nm was measured. The mean ± SEM ($n = 8$ for each peptides) of one representative experiment repeated 3 times is presented.

peptides **36**, **37**, **38**, and **39**) was associated with better cell adhesion processes probably due to an increased accessibility of the RGD motifs. Remarkably, a surface coated with the multivalent compound **2** exhibited an adhesion pattern comparable to those displayed by the optimized monovalent RGD ligand.

Next, we compared the multivalent compound's efficiency to compete with vitronectin toward CHO3a adhesion. A suspension of cells was mixed with different concentrations of peptides and allowed to adhere on vitronectin for 45 min at 37 °C before being rinsed 3 times in PBS, fixed, and stained. The inhibitory ability of each peptide was quantified by the measuring of its IC_{50} (concentration of peptide necessary to inhibit 50% of the cell attachment). As expected the negative control peptide **3** had no effect on vitronectin-mediated adhesion. In contrast, the monovalent compound **38** as well as the multivalent compound **2** inhibited strongly the attachment of CHO3a on vitronectin-coated wells. An IC_{50} of 0.5 ± 0.05 μM was calculated for compound **2**, while a lower IC_{50} value around 2.5 μM was found for **38**.

B. Internalization Assays. Internalization assays were performed directly using fluorescent peptides **4**−**7** and **41** or after the formation of an FITC-labeled streptavidin complex with the biotinylated peptides **2** and **3**. After a 15 min incubation at 37 °C in the presence of 10 μM peptides, the CHO3a cells were washed and fixed, and the fluorescence detected directly in the case of **2** or **3** or after permeabilization and amplification. As shown in Figure 4, RGD-containing compound **6** is more particularly internalized by the CHO3a cells than the control peptide **49**. At 4 °C, cellular uptake was inhibited, the peptides being located outside the cell membrane thus suggesting a typical endocytic pathway (Figure 4b). For molecules **2**, **4**, and **6**, similar internalization results were obtained demonstrating that the detection domain does not interfere with the recognition domain. The streptavidin−**2** complex was also internalized pointing out the ability of our system to bring selectively the exogenous protein into cells expressing the $\alpha_V\beta_3$ integrin. The same experiments were also performed on primary human umbilical vein endothelial cells (HUVEC) which naturally express the $\alpha_V\beta_3$ integrin and the fluorescence observed using a biphoton confocal microscope (see experimental part). The results also indicate a massive uptake of the multimeric RGD-containing peptides compared to the monovalent cyclopentapeptide. In addition, neither cell binding nor internalization of the multivalent peptides was observed with K562 cells devoid of $\alpha_V\beta_3$ integrin. Internalization experiments were also done on CHO3a cells using peptides **4** and **5** containing two fluorescein residues. The same tendency was broadly observed, namely a higher uptake of the multivalent RGD-containing peptide **4**. It is worthy to note that the use of compound **4** containing two fluorophores is invaluable because this latter ensures directly a sufficient level of fluorescence to allow the observations without method of amplification.

Discussion

Our approach aims at developing multivalent ligands of $\alpha_V\beta_3$ integrins for improving cell targeting. Indeed, the selectivity and efficiency of c[RGDfK] for targeting as well as its capacity to penetrate cells expressing $\alpha_V\beta_3$ was called into question recently thus emphasizing the need for multimeric RGD-containing molecules.[16] This is corroborated by the fact that multivalency is a general strategy used by nature to mediate and control recognition processes.[15] Moreover, the shape of a multivalent ligand controls its activity: its ability to cluster surface receptors can promote a specific biological response via signal transduction.[37] Diverse scaffolds have been used to generate multivalent ligands such as linear polymers,[38] dendrimers,[39] calixarenes,[40] multiple antigenic peptides (MAP),[14,41] or globular proteins.[42] The importance of ligand density in RGD peptide−integrin interactions was assessed by scaffolding several RGD recognition motifs from a polymer chain[11,17] or after conjugation from a humanized antibody protein.[13] Although these multidentate compounds were found to be more active in competitive inhibition experiments than the cognate monovalent ligand, the difficulties in predicting and controlling the homogeneity of the protein- or polymer-conjugate remain a problem that may complicate the data interpretation and limit the clinical

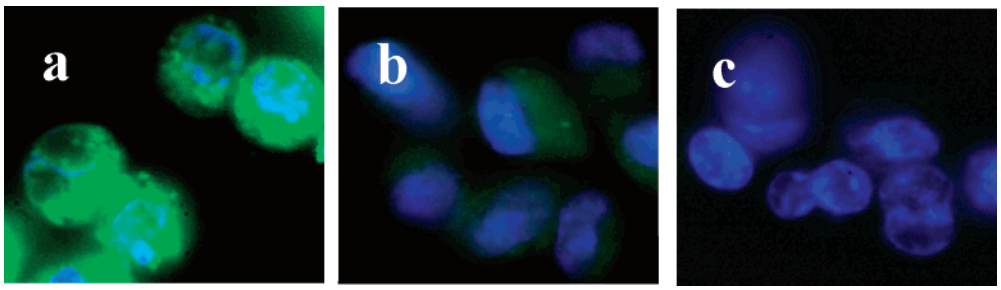


Figure 4. Internalization of RGD-containing peptides: (a) RGD-containing RAFT **6** at 37 °C, (b) RGD-containing RAFT **6** at 4 °C, (c) RGD-containing peptide **41** at 37 °C.

exploitation of these compounds. During the preparation of this work, Kessler et al. reported a modular synthesis of tetravalent RGD molecules that displayed improved binding to the isolated integrin receptor.[14] This approach, based on an MAP-lysine core as scaffold to direct radially the c[-RGDfK-] pendants, represents conceptual progress for controlling the ligand density. However, an MAP system suffers from the lack of conformational control which renders the spatial presentation difficult to manage. Indeed, besides the importance of the ligand density, its local spatial distribution and presentation were shown to be essential for integrin-mediated signal transduction.[17] Here, we chose a decapeptide named RAFT as a pivotal element to assemble $\alpha_V\beta_3$ integrin-peptide ligands in a multivalent mode and different reporter groups. A RAFT molecule having two addressable domains allows functional components to be assembled and directed in well-defined and controlled spatial orientations.[27] Separation of each functional domain in space is required in order for them to function in an independent manner. The advantage of such conformational control by related template molecules has been emphasized in the case of carbohydrate-recognition elements and a malaria-epitopes presentation.[30,43] In particular, an array of ligand molecules can be assembled and directed on one face for the cell-receptor targeting, the other being functionalized by reporter groups or drugs. Therefore, the use of such a RAFT molecule capitalizes in a row the requirement of multimeric ligand presentation to improve ligand−receptor interaction, the presence of label groups for detecting and tracing the corresponding biological activity, and the guarantee of the control of their spatial presentation. In addition, because the assembly is convergent and the domains separated, this allows the synthesis of series of compounds that are required for biological studies by only varying the functionalization at one template face. To address regioselectively the lysine side chains, an appropriate orthogonally protecting group strategy has been devised. The Boc and Alloc protecting groups used in this work are well-documented and exhibit an excellent level of orthogonality. Successive adequate deprotection and coupling reaction cycles by using a combined solid-phase and solution approach provided the key template compounds **17**, **18**, **24**, and **29** containing aminooxy groups (Scheme 1) ready for chemoselective oxime formation. The oxime ligation technique between unprotected molecules functionalized by aminooxy and aldehyde groups was selected because of its high efficiency and chemoselectivity level as well as its in vivo stability.[25] Moreover, it requires minimal chemical manipulations in aqueous conditions, since neither coupling reagent nor protecting group is used. This ligation has been used to circumvent inherent chemical incompatibility and proved very effective for the conjugation of various molecules such as peptides,[24,43] DNA,[23] carbohydrates,[44] and labels.[14] Recently, it has also been adapted to the RAFT molecule for presenting clusters of carbohydrate recognition motifs.[30] In addition, a preliminary attempt of amide coupling in solution between RGD derivative **42** and template **13** failed in our hands.[35] Herein, chemoselective oxime ligation has proved to be very efficient to prepare our conjugated molecules. The target compounds were obtained cleanly without side reactions providing the tetravalent RGD-containing RAFTs **2**, **4**, **6**, and **8** as well as the control peptides **3**, **5**, and **7** in good yields. Binding studies of the RGD derivatives to different cell lines demonstrated that our molecules displayed selectivity for cells expressing $\alpha_V\beta_3$ integrin.[36] An adhesion assay with CHO3a cells (Figure 3) shows the importance of an appropriate linker length required between the RGD-binding motif and the biotin for the monovalent RGD peptides: the increase of the spacer length, namely from three to five β-alanine residues, improves the binding efficiency to CHO3a cells. For molecule **2**, the lysine side chains used on the template as well as on the c[-RGDfK] peptide provide a suitable linker length to promote efficient surface and cell attachment. Together, these observations correlate well with those reported on the adhesion of osteoblasts to a surface functionalized with c[-RGDfK] where a minimum linker distance of 3.5 nm was reported.[45] However, these experiments do not make it possible to conclude from the improvement brought by the multivalent compound compared with the monovalent one. This is certainly due to a ligand density problem; beyond a critical value exhibited on the surface, similar cell attachments occur preventing the differentiation of the compounds. To circumvent this problem, we studied the inhibition of CHO3a adhesion to a vitronectin-coated surface by our multimeric compound **2**. The inhibitory effects of compounds **2** and **38** were similar at high concentration (10 μM). At lower concentrations ($C < 1$ μM), a significant difference was observed, showing that the binding affinity is increased when several copies of RGD peptide are presented to the integrin receptor (IC_{50} of 0.5 ± 0.05 μM for **2** and 2.5 μM for **38**). This observation is consistent with those obtained with other multivalent c[-RGDfK]-containing systems.[13,14] Together, these experiments demonstrate the functional activity of our construct. Although the IC_{50} value of **2** corrected for the number of RGD elements (i.e., 2 μM) is close to that of **38**, this is certainly due to an overvaluation of the effective concentration for inhibition. In fact, it is important to note that a part of the multivalent compound is internalized by the $\alpha_V\beta_3$ expressing cells during the incubation period.[46] Indeed, internalization of multivalent RGD-containing peptides **2**, **4**, and **6** were visualized at 37 °C in CHO3a and HUVEC cells but very few for the peptide **41** (Figure 4c). No internalization was detected at 4 °C suggesting an energy-dependent process. In addition, neither cell binding nor internalization of the multivalent peptides was observed with cells devoid of the $\alpha_V\beta_3$ integrin. These observations are consistent with an $\alpha_V\beta_3$ integrin receptor-mediated endocytosis pathway that might have been triggered by integrin clustering resulting from the binding with our multimeric RGD ligands.[19] In addition, these results also suggest that the proximity in space between the c[-RGDfK] moieties is a prerequisite for such biological effects. Interestingly, since the same observations were obtained with com-

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(46) Endocytosis of compound **2** occurred after 5 min at 37 °C.

pounds with different detection domains such as **2** or **4**, this strongly supports the idea that the template also serves to prevent the detection and the binding domain to interfere together and thus preserves the cell attachment as well as the endocytosis of the molecules. This feature is important for the future design of more sophisticated molecules. Therefore, the strong binding to specific integrin receptors and the associated good internalization property displayed by the multivalent molecules presented here offer interesting perspectives for in vivo studies as well as for the design of more sophisticated systems such as molecular conjugate vectors.[19]

Conclusion

All together, the preliminary biological results obtained here validate the concept based on RAFT molecules to incorporate and present in a spatially controlled manner two independent functional domains: a clustered-ligand domain for integrin recognition and cell targeting and a labeling domain for detection and characterization of this event. The strategy described is based on solid-phase peptide synthesis and chemoselective oxime ligation. It is very flexible, modular, and adapted to the synthesis of a series of compounds such as those presented here. The multimeric RGD-containing compounds **2**, **4**, **6**, and **8** are currently used for studying integrin clustering as well as in vivo experiments, and the corresponding results will be reported in due time.[47] Since the role of this integrin needs reevalution as regulators of angiogenesis,[48] it is expected that these bifunctional systems might be useful in the near future as reagents to study and modulate physiologically important integrin-extracellular matrix protein interactions and as drugs for disease-related applications as well as new nonviral targeting systems for drug delivery.[19] In addition, as demonstrated earlier,[30] it is worth noting that our approach is not limited to integrin ligand or for detection applications. By adapting the corresponding domain on the template, it may be conceptually exploited easily to target different cells or tissues due to the increasing number of selective ligands selected in vivo[49] and the range of applications such as drug delivery may be greatly extended by using the vast repertoire of available organic compounds.

Experimental Section

Materials. Protected amino acids, Sasrin, and ChloroTrityl resins were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Voivins-Le-Bretonneux, France), and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem, and other reagents were purchased from Aldrich (Saint Quentin Fallavier, France) and Acros (Noisy-Le-Grand, France). RP-HPLC analyses were performed on Waters equipment consisting of a Waters 600 controller, a Waters 2487 Dual absorbance detector, and a Waters in-line degasser. The analytical column (Nucleosil 120 Å 3 μm C_{18} particles, 30 × 4.6 mm^2) was operated at 1.3 mL/min, and the preparative column (Delta-Pak 300 Å 15 μm C_{18} particles, 200 × 25 mm^2), at 22 mL/min, with UV monitoring at 214 and 250 nm. Solvent B consisted of 0.09% TFA in 90% acetonitrile, and solvent A, of 0.09% TFA. Mass spectra were obtained by electron spray ionization (ES-MS) on a VG Platform II (Micromass).

General Procedure for Solid-Phase Peptide Synthesis. Assembly of all protected peptides was carried out using Fmoc/*t*Bu strategy manually in a glass reaction vessel fitted with a sintered glass frit or automatically on a synthesizer (348 Ω synthesizer, Advance ChemTech). Coupling reactions were performed using, relative to the resin loading, 1.5−2 equiv of *N*-α-Fmoc-protected amino acid activated in situ with 1.5−2 equiv of PyBOP and 3−4 equiv of DIEA in DMF (10 mL/g resin) for 30 min. Manual syntheses were controlled by Kaiser and/or TNBS tests. *N*-α-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4) (10 mL/g resin) for 10 min. The process was repeated 3 times, and the completeness of deprotection was verified by the UV absorption of the piperidine washings at 299 nm.

Synthetic linear peptides were recovered directly upon acid cleavage (1% TFA in CH_2Cl_2). The resins were treated for 3 min repeatedly until the resin beads became dark purple. The combined washings were concentrated under reduced pressure, and white solid peptides were obtained by precipitation from ether. They were analyzed by RP-HPLC and, if necessary, purified on a preparative column.

General Procedure for Cyclization Reactions. All linear peptides (0.5 mM) were dissolved in DMF, and the pH was adjusted to 8−9 by addition of DIEA. PyBOP (1.2 equiv) was added, and the solution was stirred at room temperature for 1 h as described.[22,26] Solvent was removed under reduced pressure, and the residue dissolved in the minimum of CH_2Cl_2. Ether was added to precipitate the peptide. Then it was triturated and washed 3 times with ether to yield crude material without further purification.

c[Lys(Boc)-Lys-Lys(Boc)-Pro-Gly-Lys(Boc)-Lys-Lys(Boc)-Pro-Gly] 10. The linear peptide H-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-OH was assembled on Sasrin resin (1 g) affording 1.068 g (0.6 mmol) of a white solid powder. The cyclization reaction was carried out using linear peptide (490 mg, 0.3 mmol) as described above. Alloc groups were removed using cyclic peptide **9** (450 mg, 0.27 mmol) dissolved in 30 mL of dry DCM under argon by adding phenylsilane (2.0 g, 18.5 mmol) for 3 min and then $Pd(PPh_3)_4$ (62.4 mg, 54 μmol) for 1 h at room temperature.[31] The solvent was removed under reduced pressure. The crude product was dissolved in the minimum of a solution containing a mixture of CH_2Cl_2 and CH_3-OH (1:1). Ether was added to precipitate the crude product. Then it was triturated and washed 3 times with ether. The materials were dissolved in a solution containing 50% CH_3CN in water and lyophilized to afford the product **10** as a white powder (366 mg, 0.25 mmol, 92%). Mass spectrum (ES-MS, positive mode) calcd 1477.9, found 1477.4.

c[Lys-Lys(Biotin)-Lys-Pro-Gly-Lys-Lys(Biotin)-Lys-Pro-Gly] 13. Biotin (22.9 mg, 93.6 μmol), PyBOP (48.7 mg, 93.6 μmol), and DIEA were added to a solution containing the compound **10** (58 mg, 39 μmol) in 10 mL of DMF to adjust the pH at 8.0. The reaction was stirred for 1 h at room temperature and then concentrated under diminished pressure. The crude product was triturated and washed with ether to yield compound **11** as a white powder (33 mg, 22.3 μmol, 57%).

Removal of Boc moieties was carried out in a solution containing 50% TFA in DCM for 1 h at room temperature. The crude product was concentrated, triturated, and washed with ether to yield compound **13** as a white powder (32 mg, 21 μmol, 94%). Mass spectrum (ES-MS, positive mode) calcd 1530.0, found 1530.0.

c[Lys(−$COCH_2ONHBoc$)-Lys(Biotin)-Lys(-$COCH_2ONHBoc$)-Pro-Gly-Lys(-$COCH_2ONHBoc$)-Lys(Biotin)-Lys(-$COCH_2ONHBoc$)-Pro-Gly] 15. $BocNHOCH_2COSu$[32] (26.5 mg, 92.0 μmol) and DIEA were added to a solution of compound **13** (32 mg, 21 μmol) in 4 mL of DMF to adjust the pH at 8.0. The reaction was stirred for 10 min at room temperature and then concentrated under reduced pressure. The crude product was triturated and washed with ether to yield compound **15** as a white powder (40 mg, 18 μmol, 86%). Mass spectrum (ES-MS, positive mode) calcd 2222.7, found 2221.6.

c[Lys(-$COCH_2ONH_2$)-Lys(Biotin)-Lys(-$COCH_2ONH_2$)-Pro-Gly-Lys(−$COCH_2ONH_2$)-Lys(Biotin)-Lys(-$COCH_2ONH_2$)-Pro-Gly] 17. Removal of Boc moieties from compound **15** (40 mg, 18 μmol) was carried out in a solution containing 50% TFA/5% TIS/5% H_2O in DCM

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for 1 h at room temperature. The crude product was concentrated, triturated, and washed with ether. The product was then purified by RP-HPLC to afford compound **17** as a white powder (30 mg, 16.3 μmol, 91%). Mass spectrum (ES-MS, positive mode) calcd 1822.2, found 1821.8.

c[Lys(-COCH$_2$ONH$_2$)-Lys(Fluorescein)-Lys(-COCH$_2$ONH$_2$)-Pro-Gly-Lys(-COCH$_2$ONH$_2$)-Lys(Fluorescein)-Lys(-COCH$_2$ONH$_2$)-Pro-Gly] 18. To a solution containing compound **10** (10 mg, 6.8 μmol) in 1.5 mL of DMF was added FITC (8 mg, 18.5 μmol). The reaction was stirred for 1 h at room temperature and then concentrated under diminished pressure. The crude product was triturated and washed with ether to yield compound **12** as a white powder. Removal of Boc moieties was carried out in a solution containing 50% TFA in DCM for 1 h at room temperature. The crude product was concentrated, triturated, and washed with ether to yield compound **14** as a white powder. Then, BocNHOCH$_2$COSu[32] (10.0 mg, 35.0 μmol) and DIEA were added to a solution containing compound **14** in 2.5 mL of DMF to adjust the pH at 8.0. The reaction was stirred for 1 h at room temperature and then concentrated under reduced pressure. The crude product was triturated and washed with ether to yield compound **16** as a white powder. Removal of Boc moieties from compound **16** was carried out in a solution containing 50% TFA/5% TIS/5% H_2O in DCM for 1 h at room temperature. The crude product was concentrated, triturated, and washed with ether. The product was then purified by RP-HPLC to afford compound **18** as a yellow powder (10.2 mg, 4.8 μmol, 70% overall yield). Mass spectrum (ES-MS, positive mode) calcd 2148.4, found 2148.0.

c[Lys(Boc)-Lys-Lys(Boc)-Pro-Gly-Lys(Boc)-Ala-Lys(Boc)-Pro-Gly] 20. The linear peptide H-Lys(Boc)-Lys(Alloc)-Lys(Boc)-Pro-Gly-Lys(Boc)-Ala-Lys(Boc)-Pro-Gly-OH was assembled on Sasrin resin (300 mg) affording 255 mg (0.18 mmol) of a white solid powder. The cyclization reaction and Alloc group removal were accomplished using the procedure described above to yield the product **20** as a white powder (227 mg, 0.16 mmol, 90%). Mass spectrum (ES-MS, positive mode) calcd 1420.8, found 1420.4.

c[Lys(-COCH$_2$ONH$_2$)-Lys(Fluorescein)-Lys(-COCH$_2$ONH$_2$)-Pro-Gly-Lys(-COCH$_2$ONH$_2$)-Ala-Lys(-COCH$_2$ONH$_2$)-Pro-Gly] 24. Starting with compound **20** (138.5 mg, 97.5 μmol), compound **24** was isolated after a purification by RP-HPLC as a yellow powder using the procedure described to yield the product **18** (25.7 mg, 15.1 μmol, 36% overall yield). Mass spectrum (ES-MS, positive mode) calcd 1701.9, found 1702.0.

c[Lys(-COCH$_2$ONH$_2$)-Lys(-Tyrosin)-Lys(-COCH$_2$ONH$_2$)-Pro-Gly-Lys(-COCH$_2$ONH$_2$)-Ala-Lys(-COCH$_2$ONH$_2$)-Pro-Gly] 29. Starting with compound **19** (52.3 mg, 34.7 μmol), compound **29** was isolated after a purification by RP-HPLC as a white powder using the procedure described to yield the product **17** (17 mg, 11.5 μmol, 33% overall yield). Mass spectrum (ES-MS, positive mode) calcd 1475.7, found 1475.2.

Peptide Derivatives 32, 34−39. The compounds **32** and **34−39** were prepared as described in the literature by a combination of SPPS and solution strategy.[22]

Peptide Derivatives 33. Using the same procedure,[22] the linear peptide H-Asp(*t*Bu)-D-Phe-Lys[Boc-Ser(*t*Bu)]-Arg(Pmc)-βAla-OH was assembled on ChloroTrityl resin (150 mg) affording 72.8 mg (60.6 μmol) of a white solid powder. The cyclization reaction was carried out following the procedures described above. Full deprotection was carried out using a solution containing 10 mL of TFA/CH_2Cl_2 (95:5) at room temperature during 2 h. The product **31** was isolated after removal of solvents under reduced pressure and precipitation from ether. A serine oxidation of derivative **31** (10^{-2} M) by an aqueous solution containing 3 equiv of $NaIO_4$ afforded the aldehyde component **33**. The product was directly purified by RP-HPLC to yield compound **33** as a white powder (24 mg, 35.6 μmol, overall yield 60%). Mass spectrum (ES-MS, positive mode) $[M + H_3O]^+$ calcd 692.8, found 692.3.

c[Arg-Gly-Asp-D-Phe-Lys(Fluorescein)] 41. To a solution containing the derivative **32** (4 mg, 6.5 μmol) in 2 mL of sodium acetate buffer (0.1 mM, pH 4.0) was added the fluorescein derivative **40** (11 mg, 20 μmol) prepared as described.[33] The reaction was stirred for 24 h at 25 °C. Conjugate **41** was isolated after a purification by RP-HPLC as a yellow powder (5.4 mg, 5 μmol, 77%). Mass spectrum (ES-MS, positive mode) calcd 1178.3, found 1177.7.

c[Arg(Pmc)-Gly-Asp(*t*Bu)-D-Phe-Lys(-CO(CH$_2$)$_2$CO$_2$H)] 42. Succinimic anhydride (10 mg, 100 μmol) and DIEA (20 μL, 115 μmol) were added at room temperature to a solution containing the c[Arg-(Pmc)-Gly-Asp(*t*Bu)-D-Phe-Lys] (73 mg, 78.7 μmol)[22] in 5 mL of DMF. The reaction was stirred for 1 h, and the solvent was removed under reduced pressure. The residue was washed with ether, and compound **42** was isolated after purification by RP-HPLC as a white powder (80 mg, 77.9 μmol, 99%). Mass spectrum (ES-MS, positive mode) calcd 1026.2, found 1026.7.

Peptide Conjugate 2. To a solution containing the derivative **17** (10 mg, 5.5 μmol) in 2 mL of sodium acetate buffer (0.1 mM, pH 4.0)/acetonitrile (1:1) was added the peptide **32** (22.5 mg, 33.5 μmol). The reaction was stirred for 3 h at 25 °C. Conjugate **2** was isolated after a purification by RP-HPLC as a white powder (22.6 mg, 5.1 μmol, 94%). Mass spectrum (ES-MS, positive mode) calcd 4390.0, found 4389.6.

Peptide Conjugate 3. To a solution containing the derivative **17** (3.5 mg, 1.5 μmol) in 3 mL of sodium acetate buffer (0.1 mM, pH 4.0)/acetonitrile (1:1) was added the peptide **33** (15 mg, 16.7 μmol). The reaction was stirred for 6 h at 25 °C. Conjugate **3** was isolated after purification by RP-HPLC as a white powder (5.5 mg, 1.1 μmol, 73%). Mass spectrum (ES-MS, positive mode) calcd 4445.1, found 4444.1.

Peptide Conjugate 4. To a solution containing the derivative **18** (21.5 mg, 10 μmol) in 10 mL of sodium acetate buffer (0.1 mM, pH 4.0) was added the peptide **32** (32 mg, 48 μmol). The reaction was stirred for 6 h at 25 °C. Conjugate **4** was isolated after purification by RP-HPLC as a yellow powder (24 mg, 5.1 μmol, 51%). Mass spectrum (ES-MS, positive mode) calcd 4715.1, found 4715.3.

Peptide Conjugate 5. To a solution containing the derivative **18** (5.8 mg, 2.7 μmol) in 7 mL of sodium acetate buffer (0.1 mM, pH 4.0) was added the peptide **33** (14.8 mg, 22 μmol). The reaction was stirred for 6 h at 25 °C. Conjugate **5** was isolated after purification by RP-HPLC as a yellow powder (8.5 mg, 1.8 μmol, 75%). Mass spectrum (ES-MS, positive mode) calcd 4771.2, found 4770.8.

Peptide Conjugate 6. To a solution containing the derivative **24** (10 mg, 5.9 μmol) in 2 mL of sodium acetate buffer (0.1 mM, pH 4.0)/acetonitrile (1:1) was added the peptide **32** (23 mg, 35 μmol). The reaction was stirred for 2 h at 25 °C. Conjugate **6** was isolated after purification by RP-HPLC as a yellow powder (18.8 mg, 4.4 μmol, 75%). Mass spectrum (ES-MS, positive mode) calcd 4268.7, found 4269.0.

Peptide Conjugate 7. To a solution containing the derivative **24** (7.8 mg, 4.6 μmol) in 1 mL of sodium acetate buffer (0.1 mM, pH 4.0) was added the peptide **33** (21.7 mg, 32.2 μmol). The reaction was stirred for 15 min at 25 °C. Conjugate **7** was isolated after purification by RP-HPLC as a yellow powder (16.8 mg, 3.9 μmol, 95%). Mass spectrum (ES-MS, positive mode) calcd 4324.8, found 4324.7.

Peptide Conjugate 8. To a solution containing the derivative **29** (4.4 mg, 3 μmol) in 2 mL of sodium acetate buffer (0.1 mM, pH 4.6)/acetonitrile (5:1) was added the peptide **32** (15.7 mg, 23.8 μmol). The reaction was stirred for 2 h at 25 °C. Conjugate **8** was isolated after purification by RP-HPLC as a white powder (10.3 mg, 2.2 μmol, 73%). Mass spectrum (ES-MS, positive mode) calcd 4042.4, found 4042.0.

Sample Preparation. Peptides were dissolved in phosphate buffer saline (PBS, 140 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl) at a final concentration of 1 mM.

Cell Culture. CHO3a cells (kind gift from Dr A.Duperray, Institut Albert Bonniot, Grenoble) are stable transfectants of the chinese hamster ovary (CHO) cell line. The clone was obtained by transfection of a human β_3 integrin encoding plasmid. Cells were cultured as adherent monolayers in a DMEM 1640 medium supplemented with 10% (v/v)

heat-inactivated fetal calf serum (FCS) and Geneticin (G418: 100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. All products were purchased from Gibco (Cergy Pontoise, France). HUVEC were cultured in medium 199 supplemented with 20% FCS, 100 μg/mL heparin, and 50 μg/mL ECGs (gift of Dr A. Duperray).

Adhesion Assay. Streptavidin-coated 96-well culture plates (Labsystem, Helsinki, Finland) were coated extemporaneously by adding 100 μL of a 1 μM solution of each biotinylated peptide diluted in PBS for 1 h at room temperature (rt). Nonspecific binding sites were saturated with 3% bovine serum albumin in PBS for 30 min at rt. Trypsinized CHO3a cells were resuspended at a concentration of 10^6 cells/mL in PBS containing 1 mM $MgCl_2$. 100 μL/well (10^5 cells) were added to the wells and incubated subsequently for 45 min at 37 °C. Wells were rinsed 3 times with PBS. Adherent cells were then fixed for 15 min with ethanol and stained with methylene blue in borate buffer. Wells were abundantly rinsed with water and dried overnight, and stained cells were resuspended in 100 μL of 0.1 N HCl. The number of attached cells was then evaluated by measuring the OD at 630 nm. Each value represents the mean ± SEM of eight independent wells.

Competitive Adhesion Assay. MaxiSorp immunoplates (Nunc) were incubated with 100 μL/well of a solution of vitronectin (Becton Dickinson, Meylan, France) at 5 μg/mL in PBS. After 1 h at rt, the solution was replaced by a 3% BSA solution in PBS for another 1 h. Trypsinized CHO3a cells (10^5/well) were then mixed with the indicated concentrations of peptides in PBS containing 1mM $MgCl_2$ and incubated for 45 min at 37 °C on the vitronectin-coated wells. The wells were then rinsed, and cells were fixed and stained as described for the adhesion assay. Each value represents the mean ± SEM of four independent wells.

Internalization Assay. Trypsined CHO3a cells were washed in PBS and incubated with 10 μM of FITC-labeled peptides in PBS 1mM $MgCl_2$ for 45 min at 37 °C or 4 °C. After being rinsed once in PBS and fixed for 10 min with 4% paraformaldehyde, cells were incubated for 5 min in a solution of Hoescht 33342 to stain the DNA. Cell suspensions were mounted in Moeviol (Sigma, Saint Quentin Fallavier, France), and slides examined using an Olympus AX 70 epifluorescence microscope (60× lens).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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