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A convenient access to $\alpha_V\beta_3/\alpha_V\beta_5$ integrin ligand conjugates: regioselective solid-phase functionalisation of an RGD based peptide

Didier Boturyn and Pascal Dumy*

LEDSS, Ingénierie Moléculaire et Chimie des Composés Bioorganiques, UMR CNRS 5616, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

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Abstract—The cyclopeptide (-RGDfK-) is a potent and selective $\alpha_V\beta_3/\alpha_V\beta_5$ integrin ligand. A methodology for the conjugation of cyclo(-RGDfK-) through the regioselective derivatisation of lysine side chains, either in solution or directly on the solid support, is described. This provides a rapid and flexible chemical entry to conjugated integrin ligands bearing reporter groups for biological investigations or reactive chemical functions for the preparation of new vector systems.

The integrin family of adhesion molecules participate in important cell–cell and cell–extracellular matrix interactions in a diverse range of biological processes.[1] The $\alpha_V\beta_3/\alpha_V\beta_5$ integrins are expressed in various cell types, such as endothelial cells, melanoma, platelets, osteoclasts and smooth muscle cells. Furthermore, they play an important role in angiogenesis as well as in tumour cell migration by interacting with vitronectin on the extracellular matrix mainly through the recognition of the tripeptide sequence RGD.[2] The search for highly selective ligands to target the endothelial cell integrin $\alpha_V\beta_3$ is currently the focus of many research groups as they may represent new therapeutics or valuable diagnosis in a number of areas such as metastasis, angiogenesis, arthritis and retinopathy.

To date, the cyclic (-RGDfX-) peptides[3] and related *N*-methylated analogues[4] developed by Kessler's group are among the most active and selective compounds for the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin subtype receptors. For instance, the combined use of c(-RGDf(*N*Me)V-) and an antibody–cytokine fusion protein led to an impressive in vivo tumour eradication in mice.[5] They are also under investigation in clinical studies; they are used especially in phase I/II in patients with progressive or recurrent anaplastic glioma, and in phase I in patients with HIV related Kaposi's sarcoma.[6] Moreover, the specificity of these peptides for the $\alpha_V\beta_3$ integrin is increasingly exploited to target endothelial cells for drugs or DNA delivery,[7] as well as for the diagnosis of tumour formation.[8]

For these reasons, there is a current need for selective RGD ligands, which may be readily synthesised and easily derivatised. The cyclopeptide (-Df**K**RG-), containing a lysine following a D-phenylalanine residue, displays an excellent affinity and selectivity toward $\alpha_V\beta_3/\alpha_V\beta_5$ receptors.[9] Most importantly, this peptide represents a very interesting ligand for biological investigations of integrin because it can be easily functionalised through the lysine side chain without significant modification of its binding capacity. Indeed, the lysine side-chain functionalisation has been used for surface-mediated osteoblast adhesion or for biological detection of the binding to the integrin.[10] However, there is no general synthesis described so far for this purpose. This communication describes a methodology suitable for the synthesis of cyclic (-DfKRG-) peptides as well as for the cognate peptide conjugates through the lysine side chain in solution and on solid phase.

The linear-protected peptides were assembled using standard Fmoc solid-phase chemistry with a highly acid-labile *o*-chlorotrityl chloride® as well as Sasrin® resins. The very mild acid conditions for the release of the linear-protected peptide from this support (1% TFA/CH_2Cl_2, 2 min or AcOH/TFE/CH_2Cl_2, 1/1/3, 2 h) are compatible with the side-chain protection stability.

* Corresponding author. Fax: 33(0)4.76.51.43.82; e-mail: pascal.dumy@ujf-grenoble.fr

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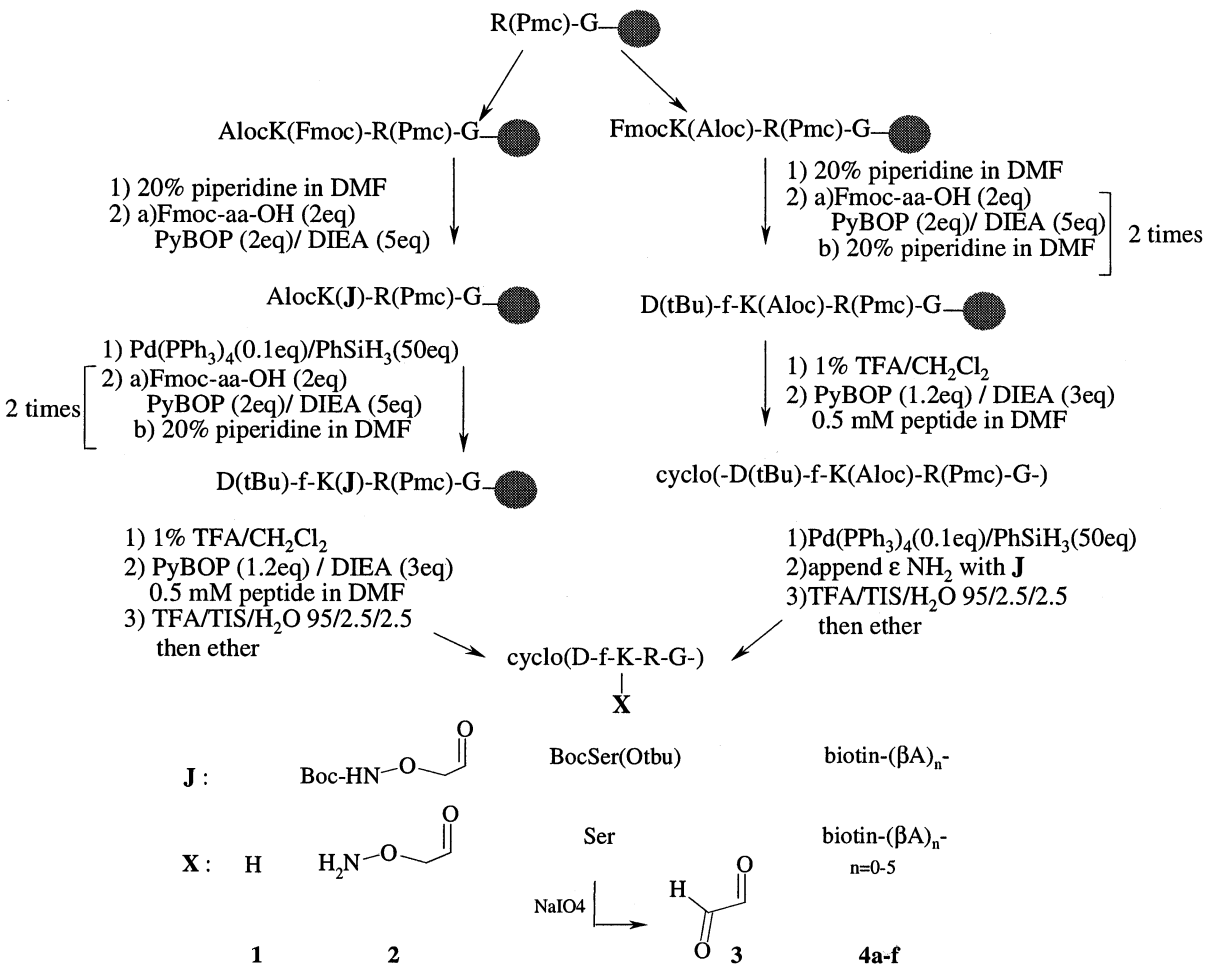
This is mandatory for the subsequent head-to-tail cyclisation performed in solution. Further, the use of glycine as a C-terminal end ensures that cyclisation between the lysine α-amino (head) to glycine α-carboxyl (tail) groups is racemisation free. Our synthetic plans have to fulfil the following synthetic aspects: (1) the removal of the protecting group of the lysine side chain has to be compatible with the stability of aspartic and arginine side-chain protection. (2) Modifications of the lysine side chain have to be feasible directly on the solid support before the release of the linear-protected peptide and its solution cyclisation. For this purpose, the Pd°-labile Aloc group on the lysine was preferred. In the first instance (Scheme 1, right side), Fmoc-K-(Aloc)-OH was introduced during the SPPS assembly in an orthogonal combination with *t*Bu and Pmc groups of aspartic and arginine, respectively.

This provided the flexibility required to regioselectively address the lysine side chain of **1** (Scheme 1) in solution subsequently to the cyclisation step. On the other hand, the converse combination, e.g. Aloc-K(Fmoc)-OH,[11] allowed the regioselective elongation from the side chain directly on the solid support by Fmoc chemistry (Scheme 1, left side). Most notably, the normal backbone elongation of the peptide is performed subsequently by the same Fmoc chemistry following the Nα-Aloc removal. The added advantage of this two directional approach is that all the steps are carried out on the resin, thus opening the way to automation of the whole process, including the cyclisation as well as the final side-chain deprotection.

The assembly of all RGD containing peptides was carried out as outlined in Scheme 1. The Aloc cleavage on the resin or in solution was readily performed by the well-established $Pd(PPh_3)_4/PhSiH_3$ procedure.[12] In general, the linear-protected peptides were obtained in good yield and high purity. The cyclisation step, performed at high dilution in DMF using PyBOP reagent, provided the corresponding RGD cyclic peptides in almost quantitative yields after a simple ether precipitation. It is interesting to note that the presence of chemical appendage on the lysine side chain does not seem to interfere with the cyclisation reaction.

Finally, deprotection using a solution of 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% water afforded, after 2 hours, the desired deprotected peptides **1**–**4**. It is worthy of note that usually only one final purification was required at the last step for the whole synthesis.



Scheme 1. Regioselective modification of the lysine side chain directly on the solid support (left) before cyclisation or in solution after cyclisation (right) to afford cyclopeptides **2**–**4** conjugated with **X**.

The simplicity and versatility of these two approaches are exemplified by appending to the RGD cyclopeptide a biotin containing β-alanine linker (compounds **4a**–**f**), an aminooxy or a glyoxylaldehyde group through the lysine side chain (Scheme 1). Compounds **4** bear a biotin tag for detecting the binding of the ligand to cell presenting integrins during biological assays. Our approach permitted to readily assess different linker lengths ($n=0$–5) between the biotin and the RGD ligand by parallel SPPS elongation of the lysine side chain with Fmoc-βAla-OH. Compounds **2** and **3** represent important functional groups required for chemoselective oxime formation with the corresponding complementary molecule. They open up the possibility for further chemical manipulation of RGD cyclic peptide without protecting groups. Such an approach is widely exploited with success in our laboratory for multivalent presentation of cyclic RGD peptides as well as DNA derivatisation.[13]

Aminooxy containing peptide **2** was introduced using the activated *N*-hydroxysuccinimide (*O*-carboxymethyl)-*N*-Boc-hydroxylamine derivative (Scheme 1, $J=\mathrm{BocNHOCH_2CO}$). Peptide **3**, bearing an aldehyde function, was generated after serine residue sodium periodate oxidation[14] of unprotected cyclo(-D-f-K(ε-S)-R-G-). For this purpose, Boc-Ser(*t*Bu)-OH was previously coupled with PyBOP/DIEA to the lysine ε-amino group (Scheme 1, $J=\mathrm{BocSer(O}t\mathrm{Bu)}$). Following the same procedure, biotin was also conjugated to the peptide subsequently to elongate the lysine side chain with β-alanine residues as spacer molecules. Products **1**–**4** were purified by preparative RP-HPLC after the final deprotection and the structure of peptides confirmed by ES-MS.[15] Compounds obtained either by solid-phase or solution derivatisation of the lysine were found to be identical. As anticipated, the solid-phase approach is much more advantageous than the solution derivatisation in terms of rapidity, product purity and quantity (110–190 mg, 60–80% overall yield relative to the initial resin loading versus 30% in solution).

In conclusion, the strategy described here provides a convenient synthetic access to various conjugates of the bioactive cyclo(-D-f-K-R-G-) peptides. Most importantly, the derivatisation is achieved directly on the solid support, the cyclisation and final deprotection remaining the sole steps performed in solution. These conditions are flexible and easily prone to automation for peptide synthesis. This is an important point one has to consider with respect to the biological impact these compounds may represent in the near future. The solid-phase method should also be suitable for large-scale synthesis given some similarity to those recently reported.[16] Biological exploitations of such conjugates are currently underway in our laboratory.[13b]

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11. To a solution containing 2 g (4.2 mmol) of H-K(Fmoc)OH and 2.2 g of Na_2CO_3 (20.7 mmol) in 50 mL of H_2O/dioxan (3/2) at 0–4°C, 500 μL (4.7 mmol) of allyl chloroformate in 10 mL of dioxan was added over a 1 h period. After 4 h, the reaction mixture was washed three times with 10 mL of ether. The aqueous solution was added dropwise to a cold 1 M HCl solution and the product was subsequently extracted with ethyl acetate and filtered over Na_2SO_4 to afford 1.80 g (4 mmol, 95%) of the desired product as a white powder. NMR (300 MHz for ^{1}H, DMSO-d_6 solvent, J in Hz): 12.50 (1H, s, CO_2H), 7.88 (2H, d, $J=7.3$, ArH-Fmoc), 7.68 (2H, d, $J=7.3$, ArH-Fmoc), 7.46 (1H, d, $J=8.0$, NH-Aloc), 7.41 (2H, t, $J=7.3$, ArH-Fmoc), 7.32 (2H, t, $J=7.3$, ArH-Fmoc), 7.27 (1H, t, $J=5.9$, NH-Fmoc), 5.89 (1H, m, C**H**=CH$_2$), 5.29 (1H, d, $J=15.6$, CH=C**H**$_2$), 5.17 (1H, d, $J=10.4$, CH=C**H**$_2$), 4.47 (2H, d, $J=5.3$, C**H**$_2$-CH=CH$_2$), 4.29 (2H, d, $J=6.9$, CH_2-Fmoc), 4.20 (1H, t, $J=6.3$, CH-Fmoc), 3.89 (1H, m, CHα), 2.96 (2H, m, $CH_2\delta$), 1.61 (2H, m, $CH_2\beta$), 1.35 (4H, m, $CH_2\gamma$, $CH_2\varepsilon$) ppm.
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15. HPLC characterisation (Nucleosil 100 Å 5 μm C_{18} particles, 250×4.6 mm; linear gradient 95:5 A:B to 0:100 A:B in 30 min; solvent B: 0.09% TFA in 90% acetonitrile and solvent A 0.09% TFA, 1 mL/min, detection $\lambda = 214$ and 250 nm), **2**, $t_R = 13.9$ min; **3**, $t_R = 15.2$ min; **4a**, $t_R = 17.7$ min; **4b**, $t_R = 16.9$ min; **4c**, $t_R = 16.7$ min; **4d**, $t_R = 16.4$ min; **4e**, $t_R = 16.1$ min; **4f**, $t_R = 16.0$ min. ES-MS (positive mode): **2**, $M+H]^+ = 677.0$ ($C_{29}H_{44}N_{10}O_9$, m/z calcd 676.7); **3**, $M+H]^+ = 678.4$ ($C_{29}H_{43}N_9O_{10}$, m/z calcd 677.7); **4a**, $M+H]^+ = 830.8$ ($C_{37}H_{55}N_{11}O_9S$, m/z calcd 829.9); **4b**, $M+H]^+ = 902.2$ ($C_{40}H_{60}N_{12}O_{10}S$, m/z calcd 901.1); **4c**, $M+H]^+ = 973.2$ ($C_{43}H_{65}N_{13}O_{11}S$, m/z calcd 972.1); **4d**, $M+H]^+ = 1044.3$ ($C_{46}H_{70}N_{14}O_{12}S$, m/z calcd 1043.2); **4e**, $M+H]^+ = 1114.7$ ($C_{49}H_{75}N_{15}O_{13}S$, m/z calcd 1113.5); **4f**, $M+H]^+ = 1186.0$ ($C_{52}H_{80}N_{16}O_{14}S$, m/z calcd 1185.4).
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