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RGD–cyclam conjugate: Synthesis and potential application for positron emission tomography

Mathieu Galibert [a], Zhao-Hui Jin [b], Takako Furukawa [b], Toshimitsu Fukumura [b], Tsuneo Saga [b], Yasuhisa Fujibayashi [b], Pascal Dumy [a], Didier Boturyn [a,*]

[a] Département de Chimie Moléculaire, UMR-5250, CNRS—Université Joseph Fourier, Grenoble, France
[b] Molecular Imaging Center, National Institute of Radiological Sciences, Chiba, Japan

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ABSTRACT

Cyclam and DOTA-containing positron emission tomography radiotracers were prepared by using a modular chemical strategy based on peptide synthesis and chemoselective ligations. These molecules encompass two functional domains, one a tumour 'homing' domain and the other a chelating ligand for copper allowing nuclear imaging of tumours.

In recent years, a series of RGD (Arg-Gly-Asp) peptide conjugates was developed for the non-invasive imaging of tumours through the molecular targeting of $\alpha_V\beta_3$ integrin.[1] Different techniques were used including positron emission tomography (PET),[2] single photon emission tomography (SPECT),[3] magnetic resonance imaging (MRI),[4] and optical imaging.[5] Among these methodologies, the most sensitive molecular imaging techniques are the radionuclide-based PET and SPECT imaging modalities.[6] In particular, PET has emerged as non-invasive imaging technique since the most successful commercial radiopharmaceutical 2-deoxy-2-[^{18}F]-fluoroglucose is routinely used for clinical evaluation (oncological imaging). At the same time, we and others designed and synthesized multivalent RGD (Arg-Gly-Asp) peptide ligands with enhanced binding affinity for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins.[7] We have shown that tetrameric RGD-containing scaffolds exhibit desirable biological properties for tumour imaging[8] and for targeted drug delivery.[9] These compounds contain a cluster of four copies of a *cyclo*[-RGDfK-] monomer grafted onto a cyclic decapeptide scaffold (Fig. 1). We recently measured the affinity constant (K_D) of RGD-containing compounds for purified integrins. K_D values rose from 3.87 nM for tetrameric RGD-containing compound to 41.70 nM for *cyclo*[-RGDfK-].[10] This result agreed well with electronic microscopy study of $\alpha_V\beta_3$/RGD-peptides. Tetrameric RGD peptide but not monomeric RGD peptide could form clusters of two integrins.[10] We also hypothesize that the observed multivalent effect could arise from a statistical rebinding of tetrameric-RGD peptide due to the high local concentration of RGD elements.

To further improve the in vivo imaging quality by enhancing the signal to noise ratio through the use of tetrameric RGD-containing scaffolds, we herein report the synthesis and applicability for PET imaging. Several reports have focused on the use of ^{18}F to label RGD-containing probes.[11] For instance, molecular imaging using

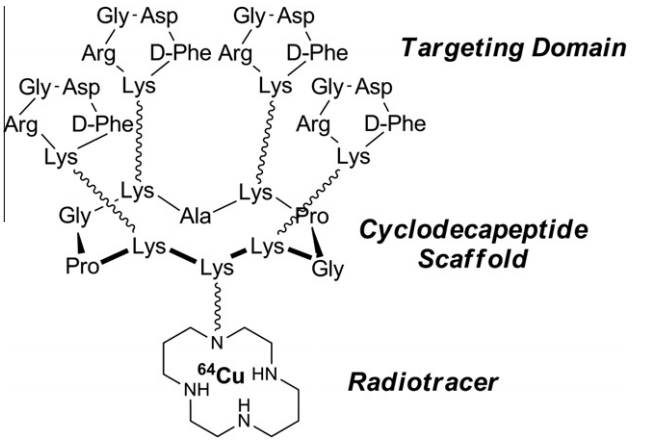

Figure 1. Structure of radiolabelled clustered RGD-containing compound.

* Corresponding author. Address: Département de Chimie Moléculaire, UMR-5250, CNRS—Université Joseph Fourier, 570 rue de la chimie, BP53, 38041 Grenoble cedex 9, France. Tel.: +33 4 56 52 08 32; fax: +33 4 56 52 08 05.
E-mail address: didier.boturyn@ujf-grenoble.fr (D. Boturyn).

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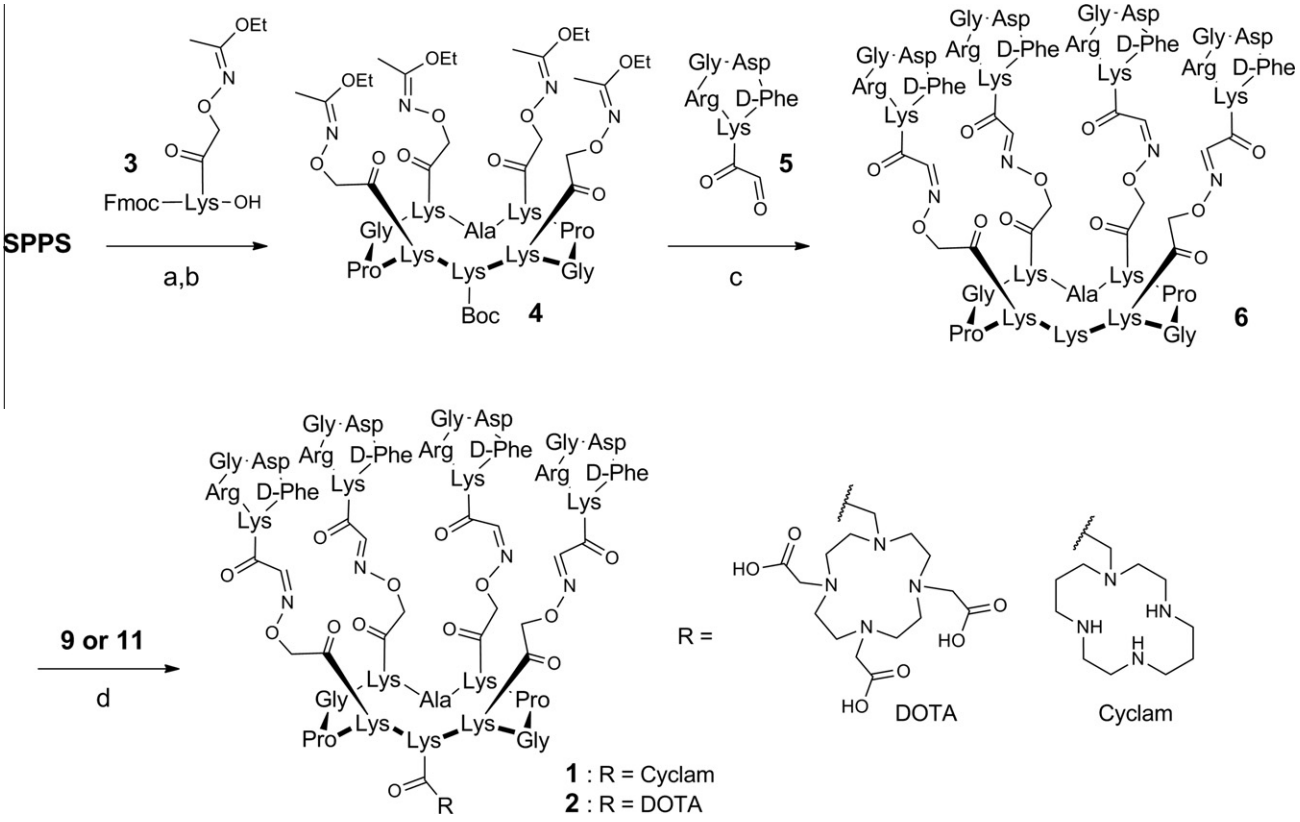


Scheme 1. Reagents and conditions: (a) Standard Fmoc SPPS chemistry; (b) PyBOP, DIPEA, DMF, 30 min; (c) **5** (6 equiv), TFA/H_20 (1:9), 1 h; (d) **9** or **11** (5 equiv), DIPEA (pH 9.0), DMF, 2 h, then TFA/TIS/H_2O (2 vol, 94:3:3), 2 h.

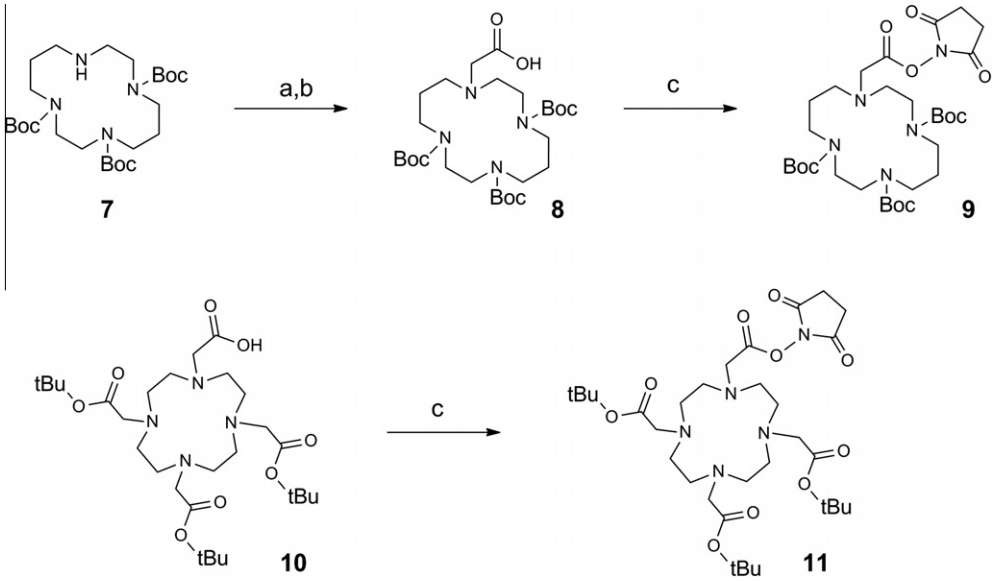
[^{18}F]-galacto-RGD compound can efficiently show the level of $\alpha_V\beta_3$ expression in man and therefore this radiotracer can be potentially used for planning and controlling $\alpha_V\beta_3$-targeted therapies.[11a] Copper-based radiopharmaceuticals are also extensively used for PET. Cu-radionuclides offer a varying range of half-lives and decay forms that make copper an ideal radioisotope for PET imaging and radiotherapy. We decided to use ^{64}Cu radiotracer ($t_{1/2}$ = 12.7 h), and RGD-containing compounds that exhibit DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) or Cyclam (1,4,8,11-tetraazacyclotetradecane) chelate agents were designed (Fig. 1).

Scheme 1 gives an overview of the route to construct RGD conjugates **1**–**2**. A modular synthesis was adopted: stable oxime bonds were utilized to append aldehyde-bearing RGD motifs to the cyclodecapeptide scaffold prior to grafting copper chelators. All peptides were prepared by means of solid-phase peptide syntheses (SPPS) according to methods already developed by our group.[12] Aminooxy groups were directly incorporated during the SPPS by using the building block **3**. We have previously shown that the 1-ethoxyethylidene protecting group is entirely compatible with the SPPS and prevents the N-overacylation side reaction obtained with Boc protecting group.[12] The following head-to-tail cyclisation was performed in DMF under high dilution with PyBOP reagent as reported[7d] providing the fully protected cyclodecapeptide **4**. By using a similar synthesis scheme,[13] RGD compound **5** bearing a glyoxylyl aldehyde group at the lysine side chain was prepared. To append RGD targeting elements to the cyclodecapeptide scaffold, we performed full deprotection of **4** under acidic conditions and its chemoselective ligation with the peptide **5**. RP-HPLC purification afforded the key intermediate **6** in satisfying 70% yield. Compound **6** was characterized by ESI-MS and the observed molecular weight (3877.0) was found in excellent agreement with the calculated values (3876.9).

In parallel, succinimidyl esters of Cyclam and DOTA were prepared (Scheme 2). Commercially available Cyclam **7** was coupled with ethyl bromoacetate and then treated under alkaline conditions to give the acid **8**. Cyclam **9** was then obtained by using *N*-hydroxysuccinimide and *N,N′*-dicyclohexylcarbodiimide. Starting with acid **10**, we prepared the DOTA compound **11** using the same synthetic strategy. Finally, peptide intermediate **6** was coupled with succinimidyl esters of Cyclam or DOTA under mild condition (pH 8.0) to provide, respectively, the novel RGD-containing compounds **1** and **2** in 70% yields after RP-HPLC. The two conjugates **1** and **2** were characterised without ambiguity by ESI-MS as the deconvoluted masses were in total agreement with the calculated values.

As shown in Figure 2 (right chromatogram), the labelling using the DOTA-containing compound **2** was not complete after 1 h of incubation with $^{64}CuCl_2$ at 37 °C: a significant amount of free copper remained in the reaction mixture. In contrast, the labelling via cyclam (Fig. 2, left chromatogram) gave no free copper under the same labelling condition. Labelling efficiencies obtained for radiotracers **1** and **2** were approximately 100% and 25%, respectively. Similar results were also revealed from autoradiography of TLC plate (see Supplementary data). Therefore, the use of cyclam as chelating agent for ^{64}Cu is far more advantageous than the use of DOTA.

For this reason, we next examined if ^{64}Cu radiotracer **1** is applicable for PET. The evaluation was performed on HEK(β_3) or HEK(β_1) ($\alpha_V\beta_3$-negative control) tumour-bearing mice. The PET image at 5 h post-injection showed a strong tumour uptake in mouse who received a single dose of 3.7 MBq of ^{64}Cu radiotracer **1** (Fig. 3). As



Scheme 2. Reagents and conditions: (a) $BrCH_2CO_2Et$, Na_2CO_3, CH_3CN, 85 °C, 16 h; (b) NaOH, MeOH, 5 h; (c) NHS (1.4 equiv), DCC (1 equiv), AcOH/dioxane (1:1), 5 h.

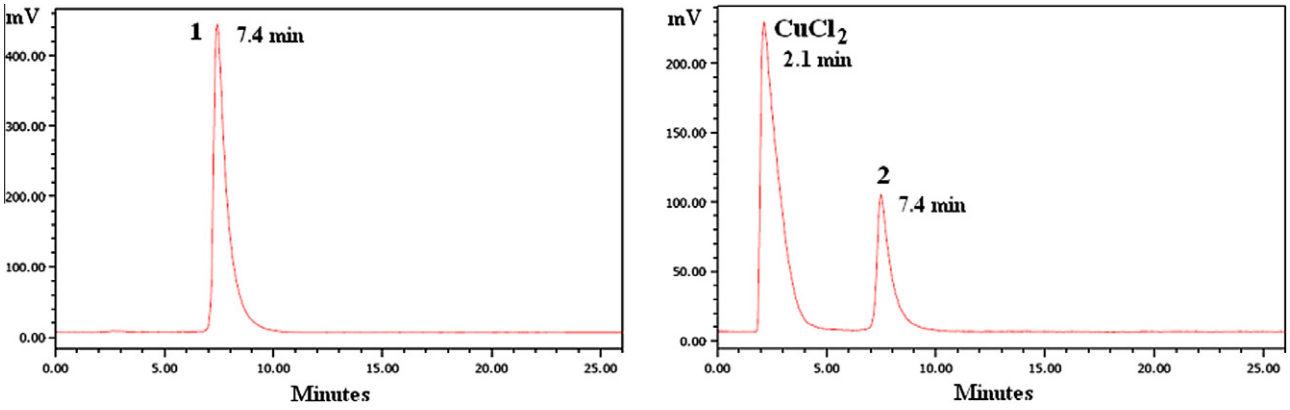


Figure 2. HPLC traces at 1 h for ^{64}Cu labelling of **1** (left chromatogram) and **2** (right chromatogram).

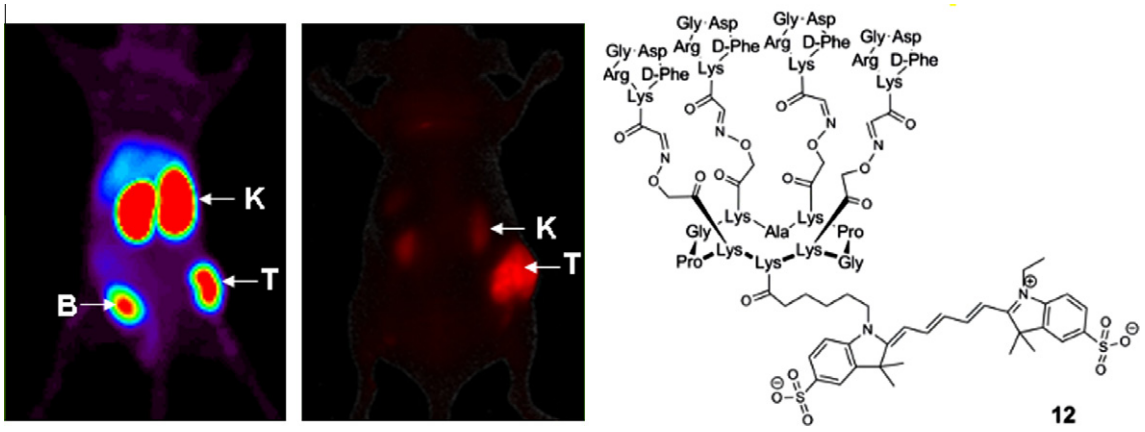


Figure 3. PET and optical image at 5 h of athymic nude mouse bearing subcutaneous $\alpha_V\beta_3$ (+) HEK(β_3) tumour. Mouse received tail vein injection (iv) of 3.7 MBq of ^{64}Cu radiotracer **1** (left) and 3 days later, iv injection of 10 nM of **12** (right). K = kidney, B = bladder, T = tumour.

expected, significant retention of radioactivity was found in kidneys and bladder. This result corroborates previous findings on Cy5-containing fluorescent probes **12** rapidly evacuated with urine.[14,15] Urine samples collected from mice at 4 h post-injection were analyzed and no free copper or Cu–cyclam complex was detected but a major compound that we assume to be a partially degraded peptide and a small amount of radiotracer **1**. Detailed in vivo metabolic stability studies will be evaluated in further study.

After 3 days, the same mouse was injected with RGD-containing fluorescent probe **12** (Fig. 3). The latter was extensively used for the in vivo optical imaging of tumour in mice[8] and successfully exploited for the intraoperative near-infrared image-guided surgery.[16] Optical imaging at 5 h post-injection of **12** shows the accumulation of clustered RGD compound within subcutaneous HEK(β_3) tumour and confirms that the selective targeting of tumour arises from RGD moieties. Further biodistribution studies in mice bearing HEK(β_3) or HEK(β_1) tumours demonstrate that ^{64}Cu radiotracer **1** had much lower radioactivity accumulation in $\alpha_V\beta_3$-negative HEK(β_1) tumours (see Supplementary data). This result reinforces the selectivity of the targeting domain of ^{64}Cu radiotracer **1** for $\alpha_V\beta_3$ integrin.

In conclusion, we used a flexible and modular chemical strategy that gives access to the synthesis of radiotracers in good yields. From basic in vivo studies, ^{64}Cu radiotracer **1** allowed the non-invasive nuclear imaging of $\alpha_V\beta_3$ integrin-expressing tumours. This study emphasizes the use of Copper-64-based radiopharmaceuticals as potential oncological PET imaging agent, and further studies are currently carried out to determine the potential of ^{64}Cu radiotracer **1** for clinical imaging.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.114.

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