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Last-Step Enzymatic [^{18}F]-Fluorination of Cysteine-Tethered RGD Peptides Using Modified Barbas Linkers

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Abstract: We report a last-step fluorinase-catalyzed [^{18}F]-fluorination of a cysteine-containing RGD peptide. The peptide was attached through sulfur to a modified and more hydrophilic variant of the recently disclosed Barbas linker which was itself linked to a chloroadenosine moiety via a PEGylated chain. The fluorinase was able to use this construct as a substrate for a transhalogenation reaction to generate [^{18}F]-radiolabeled RGD peptides, which retained high affinity to cancer-cell relevant $\alpha_v\beta_3$ integrins.

Introduction

During the past decade, bioactive peptides, in particular cyclic RGD peptides, have been increasingly identified as targets for radiolabeling as positron emission tomography (PET) tracers due to their high target-selectivity, non-immunogenicity, good tissue penetration and rapid metabolic clearance.[1,2]Fluoride [^{18}F] is the isotope of choice for PET as it has a moderate to long half-life (109.8 min) and is amenable to relatively extended synthesis and preparative manipulations. It also has a high percentage of β^+ decay (97%) and a low β^+ energy (635 Kev) and therefore a short positron range (2.3 mm in water). This leads to increased image resolution. Also the ease of production of [^{18}F] at nM levels and in high specific activity by proton (H^+) bombardment of an [^{18}O]H_2O target on a cyclotron, provides a ready source of the isotope in radiochemistry laboratories, particularly within hospital environments. [^{18}F]-Fluorination of peptides and other biomolecules is usually achieved by conjugation with small [^{18}F]-labeled prosthetic synthons through complimentary functionalization. The most frequently employed small [^{18}F] molecules are prepared by nucleophilic [^{18}F] fluoride chemistry to generate ^{18}F–C bonds. These include [^{18}F]fluorobenzaldehyde ([^{18}F]FB)[3,4] which is generally conjugated to aminoxy or hydrazine-decorated peptides through oximation or hydrazone formation, [^{18}F]-fluorobenzoate ([^{18}F]FBA)[5] or *N*-succinimidyl 4-[^{18}F]-fluorobenzoate ([^{18}F]SFB)[6] for reaction with the N^{α}-amino group of the backbone or N^{ε}-amino group of lysine residues by acylation or amidation, *N*-(2-[4-[^{18}F]fluorobenzamido]ethyl)maleimide ([^{18}F]FBEM)[7] which covalently modify at cysteine RSH residues, and [^{18}F]azide or [^{18}F]alkyne substrates[8,9] which combine with alkyne or azide modified peptides by "click" reactions. The aldehyde form of the sugars 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG)[10] and 5-deoxy-5-[^{18}F]fluoro-D-ribose ([^{18}F]FDR)[11,12] have also been used for aminooxy group conjugation to peptides and have some advantage in that they display increased hydrophilicity and a favorable radiopharmacokinetic profile. Also a new prosthetic group, 4-[^{18}F]fluorophenylboronic acid ([^{18}F]FPB)[13] was developed for Suzuki coupling with aryliodide derivatives of biomolecules.

While the best coupling reactions between the [^{18}F] prosthetic group and peptides are mild, biorthogonal and site-specific, the syntheses of the [^{18}F]-building blocks requires predrying of the [^{18}F]-fluoride, which is generated from [^{18}O]H_2O. Good nucleophilic reactions require anhydrous solvents to ensure fluoride nucleophilicity. This can often require harsh conditions incompatible with peptides, such as elevated temperatures. Therefore, a last-step [^{18}F]-fluorination of a substrate under ambient aqueous conditions is attractive for radiosynthesis and is a current research focus within the radiochemistry community. To this end [^{18}F]-fluoride has been reacted in last-step protocols with organosilicon decorated peptides to give [^{18}F-SiFA]-conjugate by ^{18}F–^{19}F isotopic exchange.[14,15] Similarly, arylfluoroboronate-modified peptides have been induced to capture carrier-added [^{18}F]-fluoride under aqueous acidic condition in a single step to generate [^{18}F]-ArBF_3-peptides.[16,17] Alternatively, [^{18}F]-fluoride can be locked into chelator (NODA or NOTA) functionalized peptides by coordination chemistry with Al^{3+} as a last step treatment, to generate Al–[^{18}F] tracers.[18,19]

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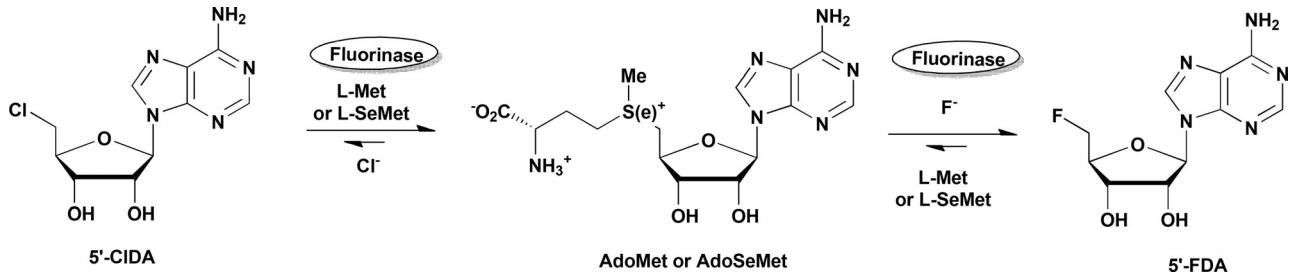


Scheme 1. Fluorinase-catalyzed fluorination and chlorination of AdoMet as well as the conversion of 5′-ClDA to 5′-FDA.

Our focus has been on enzymatic incorporation of [^{18}F] into candidate radiotracers under ambient conditions. The fluorinase enzyme, originally isolated from *Streptomyces cattleya*, catalyzes the conversion of *S*-adenosyl-L-methionine (AdoMet) and fluoride ion to 5′-fluoro-5′-deoxyadenosine (5′-FDA) and L-methionine (L-Met).[20] Fluorinase also functions as a chlorinase, particularly in the reverse direction.[21] The reversibility allows 5′-chloro-5′-deoxyadenosine (5′-ClDA) to be used as a substrate for fluorination in a one step reaction via AdoMet under ambient (pH 7.8 phosphate buffer, 37 °C) aqueous conditions (Scheme 1).

The reaction benefits from the fact that the equilibrium favors 5′-FDA in fluorination but AdoMet in chlorination. In addition, the use of L-selenomethione (L-SeMet) in place of L-Met increases the reaction rate and improves the efficiency of the transhalogenation of (5′-ClDA) to FDA. Fluorinase displays a high substrate specificity, however, we have recently demonstrated that the introduction of an acetylene group at C-2 of the adenine base of 5′-ClDA is tolerated by the enzyme.[22] In addition this acetylene can be extended at its terminus to carry a pegylated cargo. Decoration of 5′-ClDA in this way has allowed bioconjugation through "click" chemistry with azido functionalized peptides. In this context we have reported a "last-step" enzymatic [^{18}F]-fluorination of a 5′-ClDA-RGD peptide conjugate with the fluorinase.[22] This approach was good for lysine modified azido containing RGD peptides. It became an objective to develop a protocol compatible with cysteine ligation due to the general importance of cysteine in bioconjugation, often through an engineered cysteine thiol. A successful strategy would significantly broaden the utility of the enzymatic methodology for radiolabeling peptides and proteins. Conventional site-specific conjugates of cysteine with maleimide suffer from degradation *via* retro-Michael reaction, and competition from thio-containing peptides or proteins such as glutathione or serum albumin present in vivo.[23]

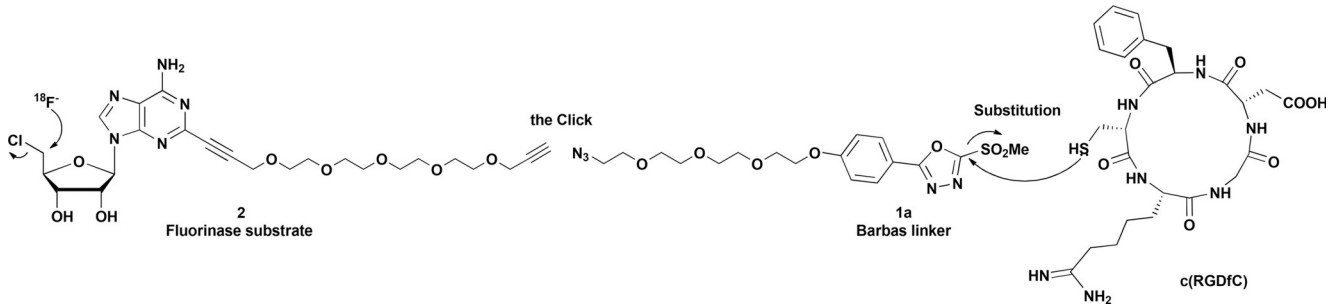
Barbas and co-workers recently introduced methylsulfonyl oxadiazole for bioconjugation through nucleophilic aromatic substitution by cysteine residues to generate a relatively stable oxadiazole-protein thiolate.[24,25] We saw an opportunity for combining this Barbas linker with our fluorinase technology in order to generate a thiol ligated peptides for last step enzymatic [^{18}F]-fluorination under ambient aqueous condition (Scheme 2).

Results and Discussion

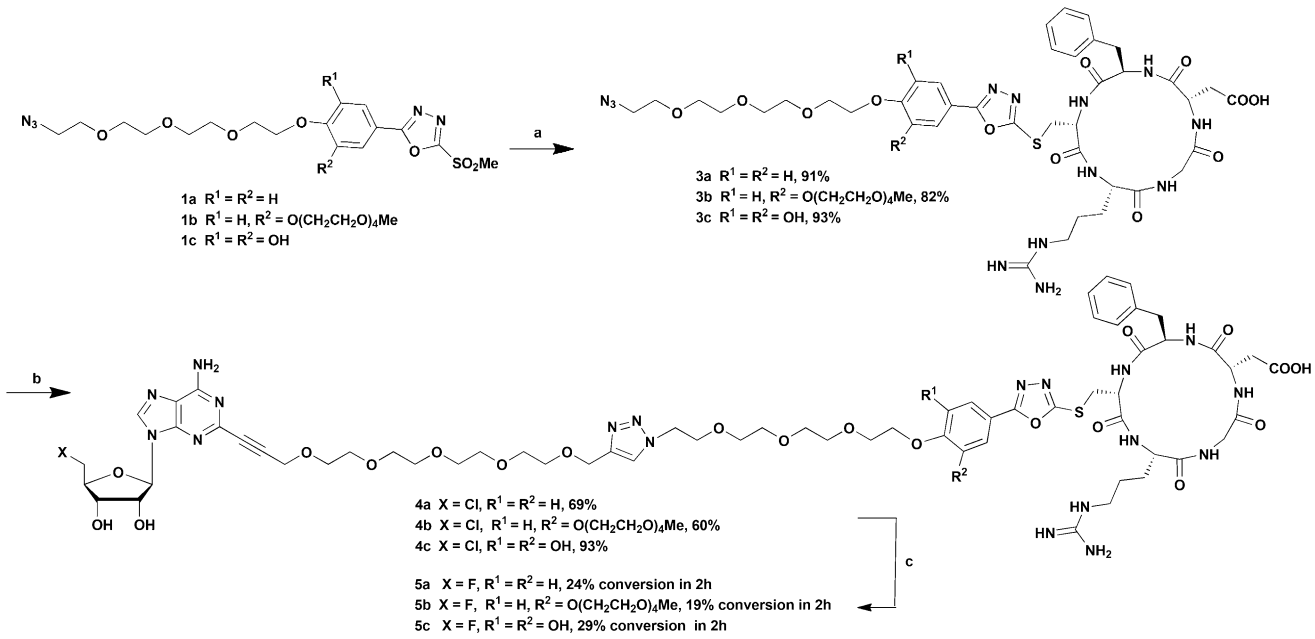
RGD conjugation to a chlorodeoxyadenosine through modified Barbas linkers

To obtain the conjugate, we attempted to click ClDA-TEG (**2**) and 2-methylsufonyl 5-(4-azidotetraethyleneglycoxyphenyl)oxadiazole (**1a**)[23] in the first step, but the free methylsulfonyl moiety was gradually hydrolyzed during work up and this was not progressed. However when the sequence was reversed (Scheme 3) by first reacting the methylsulfonate **1a** with the RGD, then the azido phenyloxadiazole thiolate (APOT-RGD) **3a** was obtained in excellent yield.

Conjugate **3a** is very insoluble in common solvents such as MeOH, CH_3CN and H_2O and was only partially soluble in DMSO. A dilute solution of conjugate **3a** in DMSO/H_2O with ClDA-TEG-acetylene (**2**)[22] under Cu-catalyzed "click" conditions generated ClDA-APOT-RGD (**4a**), which was purified by semi-



Scheme 2. Design of three components for last-step [^{18}F] labeling of cysteine-containing peptide: fluorinase substrate, Barbas linker and the cysteine-containing peptide.



Scheme 3. Successive conjugation of methylsulfonyl azido phenyloxadiazole, RGD and ClDA-TEG or FDA-TEG and fluorinase-catalyzed transhalogenation. Reagents and conditions: a) c(RGDfC), PBS buffer (pH 7.4), 2 h; b) compound **2**, CuTBTA in 55% DMSO, sodium ascorbate, H_2O, overnight; c) KF, L-SeMet, fluorinase, phosphate buffer (pH 7.8).

preparative HPLC. Again, **4a** is insoluble in common solvents with only partial solubility in DMSO.

To improve the solubility, a second PEGylated chain was introduced at the 3-position on the phenyl ring of the Barbas linker. This involved the selective deacylation and Williamson etherification at C-4 of the precursor benzoate in a one pot protocol to give **7b** (Supporting Information, Scheme S1).[26,27] Further etherification of 3-OH with tosylate **13** afforded **8b**. Product **8b** was condensed with hydrazine and then cyclized with carbon disulfide in the presence of KOH to generate oxadiazole **10b**. Finally thiol methylation and then oxidation with *m*-CPBA gave bisPEGlated azido phenyloxadiazole **1b** (Scheme S1).[24]

Conjugation of **1b** with c(RGDfC) as shown in Scheme 3 afforded **3b** (MAPOT-RGD), and this construct was "clicked" with **2** affording (ClDA-MAPOT-RGD) **4b**. The solubility of **4b** was not significantly improved relative to **4a** particularly in acetonitrile, methanol, ethanol and water.

There was the option of introducing a second PEGylated chain at C-5 of the phenyl ring, however the molecular weight of the construct was getting large, and therefore it seemed more appropriate to explore the free diphenol **4c** as a potentially more soluble construct. The settled route to the analogous precursor **1c** is shown in Supporting Information (Scheme S2). Thiol conjugation of **1c** with peptide c(RGDfC) in THF/phosphate buffer afforded **3c**, which was then "clicked" with **2** under standard Cu-catalyzed conditions to give **4c** (Scheme 3). It is immediately apparent that **3c** and **4c** have increased solubility in MeOH and CH_3CN relative to **3a**/**b** and **4a**/**b**. They have moderate water solubility (up to 0.1 mM for **4c**). The increased solubility of **3c**/**4c** makes them easier to handle and they do not require [D_6]DMSO for characterization.

The increased polarity and hydrophilicity of **4c** is also reflected in reverse phase HPLC profiles. While **4a** and **4b** have almost the same retention time, **4c** elutes over 1 min earlier than **4a** and **4b** under the conditions (Figure 1).

Simultaneously, a variant of the Barbas linker was prepared without an aryl ring to explore solubility. Thus methylsulfonyl oxadiazole (**1d**) became a synthesis target (Supporting Information, Scheme S3). A key reaction involved nucleophilic substitution of the methylsulfonate **1d** with the thiol of (RGDfC) and this led to azido oxadiazole thiolate (AOT-RGD) **3d**. "Click"

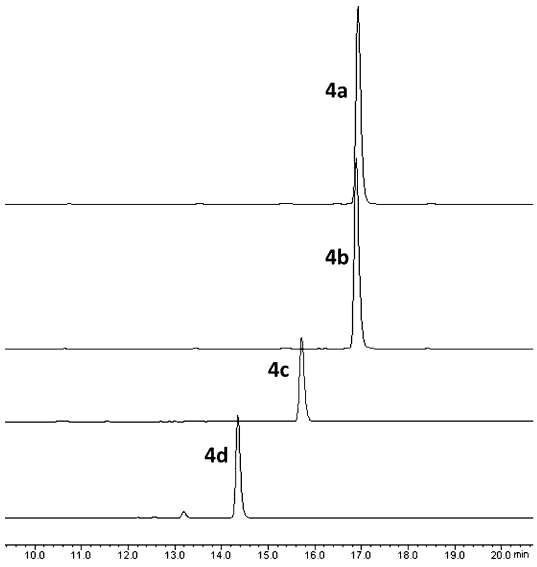
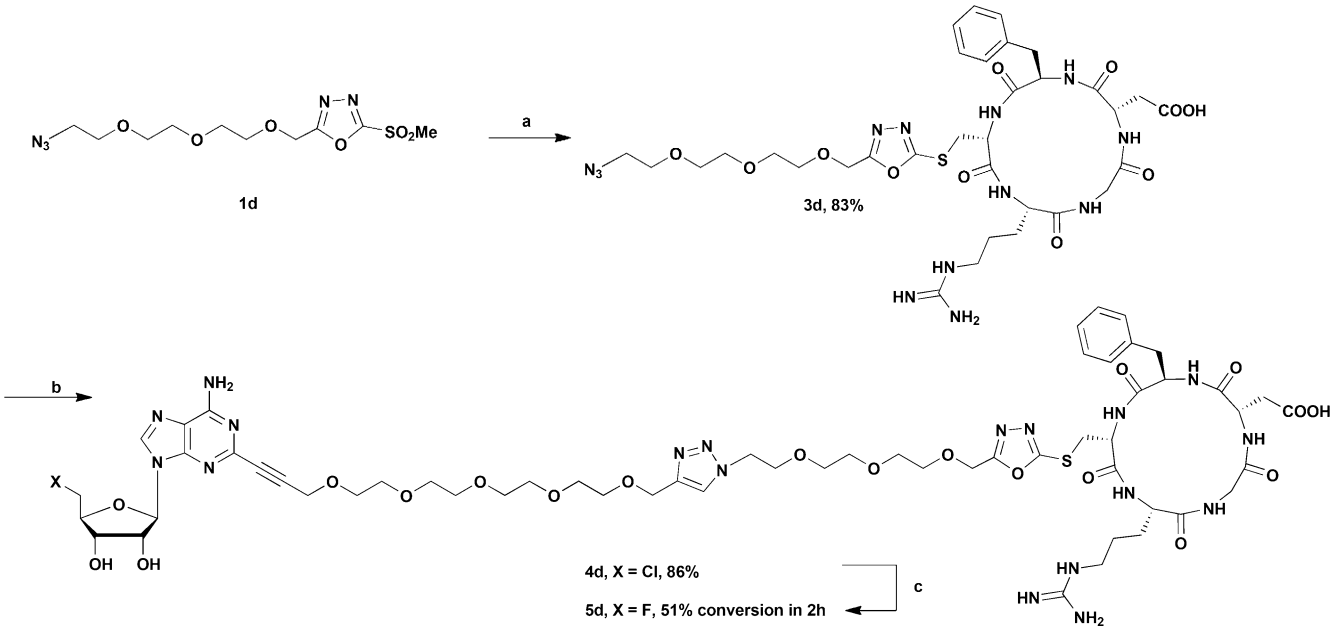


Figure 1. Comparative HPLC traces to illustrate the relative polarities of **4a** (least polar), **4b**, **4c** and **4d** (most polar) by retention time [min]. For HPLC conditions see the Experimental Section.



Scheme 4. Preparation of water soluble fluorinase substrate ClDA-AOT-RGD. Reagents and conditions: a) c(RGDfC), PBS buffer (pH 7.4), 2 h; b) compound **2**, CuTBTA in 55% DMSO, sodium ascorbate, H_2O, 12 h; c) KF, L-SeMet, fluorinase, phosphate buffer (pH 7.8).

reaction of **3d** with **2** gave conjugate ClDA-AOT-RGD (**4d**; Scheme 4).

The solubility of **4d** in water is significantly increased (2.3 mM) relative to **4c** (0.1 mM). Compounds **4a** and **4b**, are essentially insoluble. Reverse phase HPLC retention times were used as a proxy measure of solubility/polarity and **4d** and then **4c** eluted most rapidly, as illustrated Figure 1.

The corresponding fluorinated conjugates **5a–d** were prepared from reactions of **3a–d** and FDA-TEG **15**[22] (Supporting Information, Schemes S4 and S5).

"Cold" enzymatic fluorination of 4a–d to 5a–d

Non-radiolabeled fluorinase reactions (last step in Scheme 3 and 4) were generally performed in phosphate buffer (pH 7.8, 20 to 50 mM) at 37 °C with the appropriate 5′-chloro-5′-deoxyadenosine substrate (0.1 mM), L-SeMet (0.1 mM), fluorinase (1 mg mL^{-1}) and potassium fluoride (75 mM). For **4a**, DMSO 5% v/v was added for solubility, although DMSO is detrimental to enzyme activity and promotes gelling on heat denaturation of the enzyme on workup. The enzymatic conversion of **4a** proved sluggish (Supporting Information, Figure S1) and did not improve for **4b** with the added PEG chain (Figure S2).

Constructs **4c** (up to 0.1 mM) and **4d** (up to 2.3 mM) had improved water solubility, and reactions were carried out in phosphate buffer (pH 7.8, 50 mM) without added DMSO. Workup of the neat aqueous reaction mixture was more straightforward than that for **4a** and **4b** which contained DMSO. The fluorinase enzyme was cleanly precipitated on heating at 95 °C for 5 min and could be readily centrifuged (13000 rpm, which corresponds to 16060 g, 5 min).

The biotransformations of **4c** to **5c** and **4d** to **5d** (Figure 2) are more efficient than that of **4a** and **4b** (Supporting Information, Figures S1 and S2). Substrate **4d** was processed most efficiently and at about twice the rate of substrates **4a–c**. An additional HPLC peak (at 16.1 min in Figure 2A and 14.9 min in Figure 2B) was attributed to a decomposition product associated with the AdoSeMet intermediate of the transhalogenation reactions based on MS analysis (exampled by Supporting Information Figure S3).

Enzymatic hot [^{18}F]-fluorination of 4a–c to 5a–c

For hot labeling, the [^{18}F]-fluoride is generated in GBq and used in MBq aliquots for reactions. The actual [^{18}F]-fluoride concentration is very low in the picomolar range but the enzyme is in excess in the micromolar range and therefore the reactions are no longer catalytic.

This stoichiometry is dramatically reversed relative to the cold reactions which use a large molar excess of fluoride over enzyme. The very low [^{18}F] concentration requires a high substrate concentration, and the low solubility of **4a** and **4b** in 5% DMSO limited their reaction concentrations to 0.15 and 0.48 mM, respectively. The conversion of $^{18}F^-$ into **5a** and **5b** was only modest (Supporting Information, Figures S4 and S5, based on the integration of the peaks of product and unreacted $^{18}F^-$ ion by HPLC[28]). Substrate **4d**, has good solubility but proved to be hydrolytically unstable (Figure 1, bottom HPLC trace shows a degraded product at 13.2 min) relative to **4a–c**, and **5d** visibly decomposed on work up, therefore radiochemical trials were not explored further with this substrate.

Substrate **4c** has good solubility in water/DMSO and an efficient transhalogenation of **4c** (reaction conc. $\leq$0.66 mM) to [^{18}F]**5c** (final conc. of DMSO 2%, Supporting Information, Figure S6) was accomplished (87% by HPLC).[28] At 2% DMSO, the enzyme precipitated cleanly after heat denature without gel

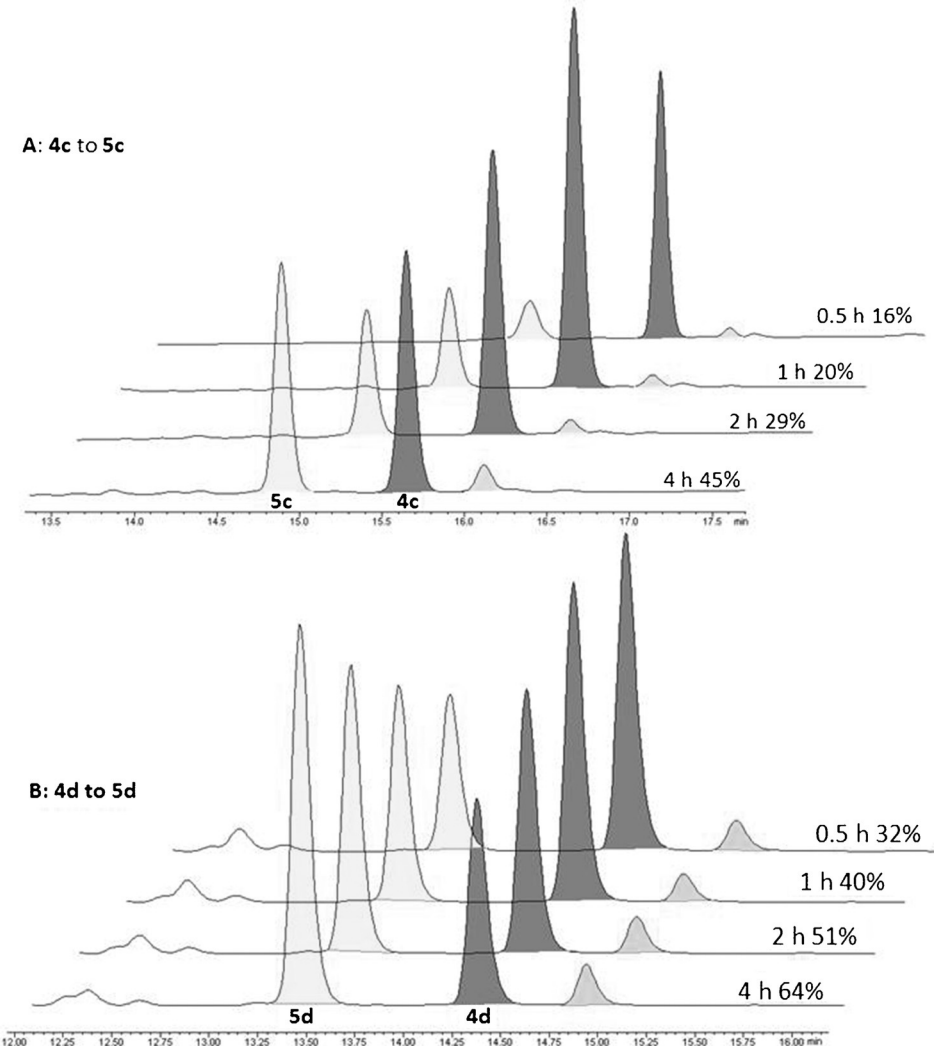


Figure 2. HPLC traces monitoring fluorinase-catalyzed conversions [%] of: A) **4c** (t_R = 15.7 min) to **5c** (t_R = 14.9 min) via AdoSeMet intermediate (t_R = 16.1 min). B) **4d** (t_R = 14.4 min) to **5d** (t_R = 13.4 min) via AdoSeMet intermediate (t_R = 14.9 min). For full conditions see the Experimental Section and Supporting Information, Figures S1 and S2.

formation. The semi-prep HPLC purified [^{18}F]**5c** was diluted with water and then secured on a C_{18} reverse phase cartridge. The product was finally eluted with EtOH (Figure 3 and Supporting Information, Figure S7) for bioaffinity experiment. The total procedure from [^{18}F]-fluoride (422 MBq) to EtOH elution of [^{18}F]**5c** (22.5 MBq) took 1.5 h, and with a radiochemical yield of 5% (decay uncorrected).

Bioactivity of FDA-AOT-RGD conjugate 5a–c

Bioaffinity assays were conducted on cell lines expressing $\alpha_v\beta_3$ integrins. The inhibitory concentrations (IC_{50}'s) for **5a**–**c**, competing with a biotinylated peptide binding to immobilized $\alpha_v\beta_3$ integrins were explored. This is a proxy measure of binding affinity to $\alpha_v\beta_3$ integrins on the surface of cancer cells. The IC_{50} values were compared with that of the cyclic peptide, cRGDfC (85.0 nM). The values, **5a** (IC_{50} 85.9(±50.2) nM), **5b** (IC_{50} 80.1(±14.2) nM) and **5c** (IC_{50} 177(±23) nM) proved to be similar, indicating that modification of RGD pep-

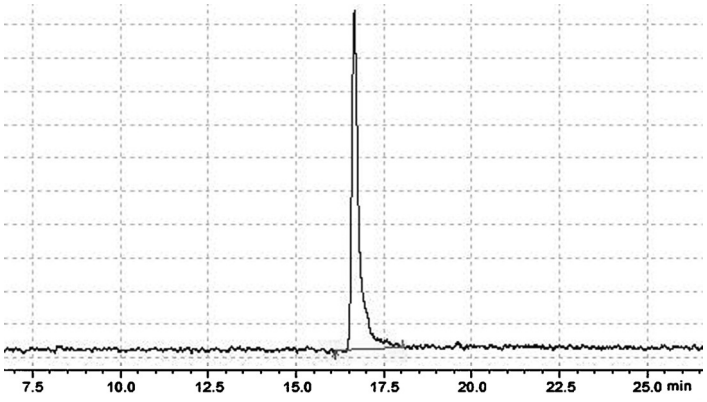


Figure 3. Analytical HPLC radio trace of purified [^{18}F]**5c**. For full conditions see the Experimental Section and Supporting Information, Figure S7.

tide with the fluoroadenosine/Barbas linker construct does not significantly affect its binding affinity.

Binding of [^{18}F]**5c** to $\alpha_v\beta_3$ integrins in cancer cells was evaluated in U87MG and PC3 cells, which are known to express high and medium levels of receptor.[29] Binding of [^{18}F]**5c** to U87MG cells was higher than to PC3 cells. Cold c(RGDfK) (10 μM) was included in the binding assay to permit identification of specific radiotracer binding to $\alpha_v\beta_3$ integrin (decreased by c(RGDfK). Inclusion of cold peptide decreased [^{18}F]**5c** binding to both U87MG and PC3 cells by approximately 75 and 50%, respectively (Figure 4). These data are consistent with the literature[29] and flow cytometry analysis which demonstrated that our U87MG cells contained 9-times more $\alpha_v\beta_3$ than PC3 (data not shown). This data provide strong evidence that [^{18}F]**5c** is binding selectively to $\alpha_v\beta_3$ integrin in cancer cells.

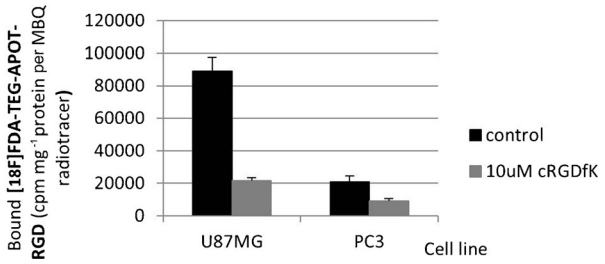


Figure 4. Bound [^{18}F]**5c** [cpm mg^{-1}] protein per MBq radiotracer.

Conclusions

We have modified a cysteine-containing RGD peptide with a fluorinase-recognized chloroadenosine for fluorinase mediated radiolabeling with the fluorine-18 isotope. The recently introduced Barbas bioconjugation strategy was variously modified to improve solubility. The diphenol **4c**, which derived from the sulfonyl oxadiazole **1c**, proved to be the most soluble construct and performed best for enzymatic mediated transhalogenation to generate radiolabeled [^{18}F]**5c**. Sulfonyl oxadiazole **1c** may find more general utility due to its relatively high aqueous solubility.

Experimental Section

Bioconjugation of Barbas linker with c(RGDfC)

Compound **1a–d** (1.5–4 equiv) in THF (400 μL) was added to a suspension of c(RGDfC) (1 equiv) in in PBS buffer (200 μL, 100 mM, pH 7.4). The mixture was stirred at RT for 0.5–2 h and then concentrated under reduced pressure to remove the organic solvent, leaving an aqueous suspension containing product and excess of starting material. The excess **1a–d** was removed effectively by extraction with diethyl ether; the aqueous suspension was dissolved/diluted with H_2O/DMSO and purified by semi-preparative HPLC to give **3a–d**.

"Click" reaction of 3a–d with 2 or 15 for 4a–d or 5a–d

ClDA-TEG (**2**) or FDA-TEG (**15**; 1.2–2.0 equiv) in a suitable amount of water was added to **3a–d** (1 equiv) in a suitable amount of DMSO. Sodium ascorbate (fresh aqueous degassed solution 100 mM or solid, 2–5 equiv) was added to the mixture. The mixture was degassed with argon before CuTBTA (10 mM in 55% DMSO, 0.2–0.5 equiv) was added. The mixture was heated at 60 °C for 10 min and stirred at RT, overnight. After dilution with suitable amount of DMSO/H_2O, the mixture was centrifuged at 13000 rpm (which corresponds to 16060 g) for 10 min to remove the Cu^I precipitate. The supernatant was isolated by semi-preparative HPLC to give the product **4a–d** or **5a–d** after concentration and freeze drying.

Fluorinase assay of 4a–c to 5a–c

In a total reaction volume of 200 μL, recombinant fluorinase (1 mg mL^{-1}, in phosphate buffer) was incubated with L-SeMet (0.1 mM), KF (75 mM) and **4a** (0.1 mM) or **4b** (0.1 mM) in 5% DMSO/phosphate buffer (pH 7.8, 50 mM) or **4c** or **4d** (0.1 mM) in phosphate buffer (pH 7.8, 50 mM) at 37 °C. Samples (20 μL) were periodically removed, denatured by heating at 95 °C for 5 min, diluted with phosphate buffer (80 μL, 20 mM) before being clarified by centrifugation (13000 rpm, which corresponds to 16060 g, 10 min). Samples of the supernatant (80 μL) were removed for analysis by HPLC. HPLC analysis (Figure 2, and Supporting Information, Figures S2 and S3) was performed on a Shimadzu Prominence HPLC system fitted with a SIL-20A HT autosampler, LC-20AT solvent delivery system, SPD-20 UV/Vis detector using a Phenomenex Luna 5 μm, C-18 100A (250×4.6 mm) column and a guard cartridge. Mobile phase: 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B); Linear gradient: 20–50% B in 20 min, 95% B in 25 min followed by equilibration of the column with initial condition; flow rate of 1 mL min^{-1}; detection: 254 nm; injection volume: 50 μL.

[^{18}F] Labelling of 4a–c to [^{18}F]5a–c

The typical $^{18}F^-$ labeling of **4** is exampled with **4c**: L-selenomethionine (40 μL), phosphate (50 mM, 30 μL) and fluorinase (5 mg in 50 mM phosphate buffer, 125 μL) were added successively to an Eppendorf tube containing substrate of **4c** (5 μL, 33 mM in DMSO). The contents were mixed well with a pipette. To this mixture was added 50 μL of [^{18}F]-fluoride in [^{18}O]water (380 MBq). The contents were well mixed and incubated at 37 °C for 30 min. After this time, an aliquot of the sample (20 μL) was taken for HPLC analysis. Once the conversion of **4c** to [^{18}F]**5c** was confirmed by the analysis, the remaining mixture was denatured by heating at 95 °C for 5 min and the precipitated protein was removed by centrifugation (13000 rpm, which corresponds to 16060 g, 5 min). The supernatant was injected into a Shimadzu Prominence HPLC system equipped with a quaternary pump, a degasser, a flow cell detector and a diode array detector using a Phenomenex Luna 5 μm, C-18 100 A (250×10 mm) column and a guard cartridge; Mobile phase: 0.05% TFA in water (solvent A) and 0.05% TFA in acetonitrile (solvent B); linear gradient: 0–4 min, 0% B, 4–10 min, 30%, 10–25 min 33%, 28 min 90% B, 35 min 0% B, 40 min stop. Flow rate: 4 mL min^{-1}. The radioactive fraction corresponding to the reference of [^{18}F]**5c** was collected, diluted with water (10 mL) and loaded onto a preactivated Waters Oasis® HLB Cartridge (conditioning with 2 mL EtOH, 5 mL water). The cartridge was washed with 20 mL of water. The desired product was collected by eluting with 1 mL of ethanol, to give about 22.5 MBq (5%, decay uncorrected) of pure product of [^{18}F]**5c** as evidenced by analytical HPLC using a Phenomenex Luna 5 μm, C-18 100A (250×4.6 mm, 5 μm) column and a guard cartridge; mobile phase: A (H_2O+0.05% TFA), B (MeCN+0.05% TFA); linear gradient: 0–4 min 0% B, 4–10 min 0–30% B,

10–25 min 30–33% B, 25–30 min 33–90% B, 30–35 min 0% B, 40 min stop. Flow rate: 1.0 mL min^{-1}.

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Keywords: ^{18}F labeling · Barbas linker · bioconjugation · chemical biology · RGD peptide

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