

Radiosynthesis and Biodistribution of Cyclic RGD Peptides Conjugated with Novel [¹⁸F]Fluorinated Aldehyde-Containing Prosthetic Groups

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Achieving high-yielding, robust, and reproducible chemistry is a prerequisite for the ¹⁸F-labeling of peptides for quantitative receptor imaging using positron emission tomography (PET). In this study, we extend the toolbox of oxime chemistry to include the novel prosthetic groups [¹⁸F]-(2-{2-[2-(2-fluoroethoxy)ethoxy]ethoxy}-acetaldehyde, [¹⁸F]**5**, and [¹⁸F]-4-(3-fluoropropoxy)benzaldehyde, [¹⁸F]**9**, in addition to the widely used 4-[¹⁸F]fluorobenzaldehyde, [¹⁸F]**12**. The three ¹⁸F-aldehydes were conjugated to the same aminooxy-bearing RGD peptide and the effect of the prosthetic group on biodistribution and tumor uptake studied in mice. The peptide conjugate [¹⁸F]**7** was found to possess superior *in vivo* pharmacokinetics with higher tumor to blood, tumor to liver, tumor to muscle, and tumor to lung ratios than either [¹⁸F]**10** or [¹⁸F]**13**. The radioactivity from the [¹⁸F]**7** conjugate excreted more extensively through the kidney route with 79%id passing through the urine and bladder at the 2 h time point compared to around 55%id for the more hydrophobic conjugates [¹⁸F]**10** and [¹⁸F]**13**. The chemical nature of a prosthetic group can be employed to tailor the overall biodistribution profile of the radiotracer. In this example, the hydrophilic nature of the ethylene glycol containing prosthetic group [¹⁸F]**5** clearly influences the overall excretion pattern for the RGD peptide conjugate.

INTRODUCTION

Integrins are heterodimeric glycoproteins involved in cell–cell and cell–matrix interactions (1). One of the most important members of this receptor class is the $\alpha_v\beta_3$ integrin, which is involved in the process of tumor-induced angiogenesis (2). We have previously reported on [^{99m}Tc]-NC100692, an RGD-containing peptide which has demonstrated clinical utility for imaging of breast cancer (3). The peptide pharmacophore binds strongly to the integrin receptors expressed on proliferating endothelial cells, and the structure allows for attachment of a variety of imaging reporter groups with no significant loss in binding affinity (4). These peptides therefore represent a new class of RGD-containing probes for the imaging of angiogenesis (5–8).

Although there are many examples of molecular imaging agents based on single photon emission computed tomography (SPECT), positron emission tomography (PET) represents a superior modality with respect to resolution, sensitivity, and the ability to acquire quantitative information from the imaging data (9). In addition, the widespread availability of [¹⁸F]fluoride, optimal half-life (110 min), and low positron energy (0.64 MeV) makes this isotope the first choice for PET imaging (10–13). Significant challenges, however, still need to be overcome before ¹⁸F-labeled peptides can be routinely manufactured for broad clinical application. Generic radiolabeling strategies using prosthetic groups offer the advantage that a significant part of the radiochemical process can be standardized and applied to multiple products. The prosthetic group must be unreactive toward the various functional groups found in peptides, forming instead a stable bond in a site-specific manner. The aforemen-

tioned criteria are met by utilizing oxime bond formation between an ¹⁸F-aldehyde and an aminooxy-modified peptide. This type of chemoselective ligation chemistry has been employed in the past by ourselves and others using 4-[¹⁸F]fluorobenzaldehyde with acceptable yields being reported for a range of peptides (14–18). We have previously observed, however, that the hydrophobic nature of this prosthetic group has an effect on *in vivo* pharmacokinetics, often increasing excretion of the tracer through the hepatobiliary system. The aim of this work was to further extend the toolbox of available ¹⁸F-aldehydes with the principle goal of improving the pharmacokinetic profile of our RGD tracer. To this end, we considered a design strategy utilizing a short poly(ethylene glycol) (PEG) spacer as an integral component of the prosthetic group. Short PEG spacers are known to increase hydrophilicity and have been shown to improve the biodistribution profile of tracers *in vivo* (19, 20). As predicted, the PEG-containing conjugate [¹⁸F]**7** demonstrated superior *in vivo* performance as compared to the more hydrophobic tracers [¹⁸F]**10** and [¹⁸F]**13** in the Lewis Lung Carcinoma mouse model (LLC). Micro-PET imaging studies were performed to examine the potential of this novel conjugate for the imaging of angiogenesis *in vivo*.

MATERIALS AND METHODS/EXPERIMENTAL PROCEDURES

Chemicals and solvents used were all reagent grade and used without further purification. The RGD core peptide **6** was synthesized as previously described in the literature from key intermediate NC100717 (4). ¹H NMR spectra were run at 25 °C on a Varian Unity Inova 500 MHz spectrometer equipped with a 5 mm ¹H-broadband PFG indirect detection probe. Mass analyses were recorded on a LCQ quadrupole ion-trap mass spectrometer (Thermo Finnigan). All chemicals used in radiolabeling were anhydrous solvents purchased from Sigma-Aldrich (Gillingham, UK). HPLC solvents were obtained from Fisher

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Scientific (Loughborough, UK). Fluorine-18 was produced by a Scanditronix MC 40 cyclotron using the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction. Enriched $[^{18}\text{O}]\text{H}_2\text{O}$ (10–30% ^{18}O) was irradiated by protons (19 MeV) with an integrated beam current of 5–10 μAh . The microwave oven was made in-house and consisted of a brass cavity [coaxial resonance system with aperture (1.3 cm) for microreaction vessels] and an external magnetron (Microtron 200, Electro-Medical Supplies Ltd., Wantage, England). The temperature was measured using an internal fiber-optic probe (FTP3-003H, system FT1310 by Takaoka Electric Ltd., Japan). The HPLC system was a Beckman System Gold instrument equipped with a gamma detector (Bioscan Flow-count). A Phenomenex Luna C18(2) column (50 $\times$ 4.6 mm, 3 μm ; flow rate 1 mL/min) was used for analytical HPLC. The preparative HPLC system utilized the semipreparative column Phenomenex Luna C18(2), 250 $\times$ 10 mm, 5 μm , at a flow rate of 4 mL/min unless otherwise stated. The following mobile phase/gradient systems were used. Gradient 1: solvent A, water (0.1% TFA); solvent B, acetonitrile (0.1% TFA); 1 min 10% B, 10–50% B in 20 min. Gradient 2: solvent A, water (0.5% TFA); solvent B, acetonitrile (0.5% TFA); 1 min 10% B, 10–50% B over 20 min.

Synthesis of Compound 1 (Scheme 1). To a stirring suspension of sodium hydride (248 mg, 5.15 mmol in mineral oil) in THF (5 mL) was added through a syringe a solution of 2-[2-(2-hydroxyethoxy)ethoxy]ethoxy ethanol (1.00 g, 5.15 mmol) in THF (5 mL) at ambient temperature. After gas evolution had ceased, the mixture was stirred for 45 min giving a clear slightly yellow solution. Bromoacetaldehyde dimethyl acetal (8.70 g, 51.5 mmol) was then added with stirring for 24 h followed by 3 mL of ethyl acetate for a further 2 h. The mixture was poured into ether (100 mL) and extracted with 10% aqueous K_2CO_3 (30 mL) and brine (30 mL). The organic phase was dried over Na_2SO_4 , filtered, and evaporated. Excess bromoacetaldehyde dimethyl acetal was removed from the mixture by distillation and the residual oil purified by flash chromatography using 100% ethyl acetate to afford the pure product (355 mg, 21%). ^1H NMR (CDCl_3): δ 4.52 (t, 1H, $^3J_{\text{HH}} = 5.2$ Hz, OCH_2CH), 4.20–4.24 (m, 2H, $\text{CH}_3\text{CO}_2\text{CH}_2\text{CH}_2\text{O}$), 3.69–3.72 (m, 2H, $\text{CH}_3\text{CO}_2\text{CH}_2\text{CH}_2\text{O}$), 3.63–3.69 (m, 12H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.54 (d, 2H, $^3J_{\text{HH}} = 5.2$ Hz, OCH_2CH), 3.39 (s, 6H, CH_3O), 2.08 (s, 3H, CH_3CO_2).

Synthesis of Compound 2. A solution of 1 N NaOH in methanol (1 mL) was added to a stirring solution of acetic acid 2-(2-[2-(2,2-dimethoxyethoxy)ethoxy]ethoxy)ethyl ester (110 mg, 0.34 mmol) in methanol (3 mL) at ambient temperature. The reaction was monitored by TLC ($\text{CHCl}_3/\text{MeOH}$, 9:1) and was found to be complete within 30 min. The solvent was evaporated and the residue purified by flash chromatography (silica, $\text{CHCl}_3/\text{MeOH}$, 9:1) to afford the product (73 mg, 76%) as a colorless oil. ^1H NMR (CDCl_3): δ 4.52 (t, 1H, $^3J_{\text{HH}} = 5.2$ Hz, OCH_2CH), 3.71–3.75 (m, 2H, $\text{HOCH}_2\text{CH}_2\text{O}$), 3.63–3.70 (m, 12H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.59–3.63 (m, 2H, $\text{HOCH}_2\text{CH}_2\text{O}$), 3.55 (d, 2H, $^3J_{\text{HH}} = 5.2$ Hz, OCH_2CH), 3.40 (s, 6H, CH_3O), 2.94 (broad s, 1H, $\text{HOCH}_2\text{CH}_2\text{O}$).

Synthesis of Compound 3. To a solution of alcohol 2 (60 mg, 0.21 mmol) in THF (1 mL), triethylamine (59 μL , 0.42 mmol), and methanesulfonyl chloride (24 μL , 0.30 mmol) were added with stirring. The reaction was monitored by TLC (silica, $\text{CHCl}_3/\text{MeOH}$, 9:1). After 2 h, the precipitated triethylamine hydrochloride salt was filtered off. The solvent was evaporated and the product obtained as an oil (75 mg, 99%) following flash chromatography (silica, chloroform/methanol (9:1)). ^1H NMR (CDCl_3): δ 4.52 (t, 1H, $^3J_{\text{HH}} = 5.2$ Hz, OCH_2CH), 4.37–4.40 (m, 2H, $\text{CH}_3\text{SO}_3\text{CH}_2\text{CH}_2\text{O}$), 3.75–3.78 (m, 2H, $\text{CH}_3\text{SO}_3\text{CH}_2\text{CH}_2\text{O}$), 3.62–3.69 (m, 12H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.54 (d, 2H, $^3J_{\text{HH}}$

$= 5.2$ Hz, OCH_2CH), 3.40 (s, 6H, CH_3O), 3.08 (s, 3H, $\text{CH}_3\text{SO}_3\text{CH}_2\text{CH}_2\text{O}$).

Synthesis of Protected Aldehyde [^{18}F]4. To a Wheaton vial (2 mL) charged with Kryptofix-222 (10 mg), potassium carbonate (1 mg dissolved in 50 μL water), and acetonitrile (0.8 mL), the fluorine-18 containing water (1 mL) was added. The solvent was removed by heating at 110 $^\circ\text{C}$ for 1 h under a stream of nitrogen. A 0.5 mL portion of anhydrous acetonitrile was added and immediately removed by evaporation. This step was repeated an additional two times, followed by addition of mesylate 3 (1.0 mg, 2.7 μmol) in anhydrous DMSO (0.2 mL). After heating by microwave (5 min, 160 $^\circ\text{C}$, 150 $\rightarrow$ 50 W) the reaction mixture was analyzed by HPLC (gradient 1, $t_{\text{R}} = 7.8$ min). After diluting with water (0.4 mL), the solution was applied to a SepPak C18-plus cartridge preconditioned with acetonitrile (5 mL) and water (20 mL). The cartridge was flushed with water (10 mL) and [^{18}F]4 eluted using acetonitrile (0.5 mL). The solvent was evaporated under a stream of nitrogen at 80 $^\circ\text{C}$.

Synthesis of Aldehyde [^{18}F]5. To the above product was added a mixture of 12 M HCl (34 μL , 0.41 mmol) and acetonitrile (34 μL) and the vial left at room temperature for 15 min. Complete deprotection of starting material was observed as evidenced by HPLC analysis.

Synthesis of Aminooxy–Peptide Precursor 6. To peptide precursor NC100717 (detailed synthesis described in ref (4)) (150 mg, 0.12 mmol) in DMF (5 mL) was added a solution of Boc-aminooxyacetic acid (34 mg, 0.18 mmol), 7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) (94 mg, 0.18 mmol), and *N*-methylmorpholine (NMM) (40 μL , 0.36 mmol) in DMF (3 mL) and the mixture left stirring for 12 h. Excess solvent was evaporated in vacuo and the crude product purified by preparative HPLC chromatography (Phenomenex Luna C18 column, 00G-4253-V0; solvents A = water/0.1% TFA and B = $\text{CH}_3\text{CN}/0.1\%$ TFA; gradient 10–50% B over 60 min; flow 50 mL/min; detection at 254 nm), affording 97 mg (57%) of pure compound (analytical HPLC: Phenomenex Luna C18 column, 00G-4252-E0; solvents: A = water + 0.1% TFA and B = CH_3CN + 0.1% TFA, gradient 10–50% B over 20 min; flow 1.0 mL/min; t_{R} 19.4 min, detected at 214 and 254 nm). Further characterization was carried out by mass spectrometry, giving an m/z value for the desired product at $[M - \text{H}^+]$ 1431.2.

Synthesis of Peptide Conjugate [^{18}F]7. To a small centrifuge vial (PP with screw cap, 1.5 mL) was added Boc-aminooxy RGD peptide precursor 6 (1 mg, 0.7 μmol) and 0.2 mL of a 95% TFA solution containing 5% water. After standing for 5 min at room temperature, the solvent was removed under a flow of nitrogen. To the vial containing [^{18}F]5 were added 28% ammonium hydroxide (48 μL , 0.71 mmol) and 0.5 M sodium acetate buffer pH 4.0 (50 μL). This mixture was transferred into the vial containing the peptide and the solution incubated at 75 $^\circ\text{C}$ for 7 min. The reaction mixture was cooled in an ice bath, quenched with 0.2 mL HPLC mobile phase (20% acetonitrile/ water), characterized by analytical HPLC (gradient 2, $t_{\text{R}} = 11.8$ and 12.0 min), and isolated by preparative HPLC (gradient 2, $t_{\text{R}} = 13.0$ min). The peptide conjugate [^{18}F]7 was formulated, following evaporation in vacuo of excess solvent at 40–50 $^\circ\text{C}$, by addition of PBS buffer containing 10% ethanol.

Synthesis of Compound 8 (Scheme 2). To a solution of 4-(3-hydroxypropoxy)benzaldehyde (1.4 g, 8.0 mmol) in dichloromethane (10 mL) was added triethylamine (1.2 mL, 8.5 mmol) and mesyl chloride (0.62 mL, 8.0 mmol). After stirring for 1.5 h at room temperature, the reaction mixture was washed with water and dried over Na_2SO_4 to give 1.8 g of crude material (yellow oil). An aliquot of 290 mg was purified by reverse-phase chromatography (column Phenomenex Luna C18(2) 5 μm

Scheme 1. Preparation of [¹⁸F](2-[2-[2-(2-Fluoroethoxy)ethoxy]ethoxy]ethoxy)acetaldehyde [¹⁸F]5 and Conjugation Reaction Forming Labeled RGD Peptide [¹⁸F]7

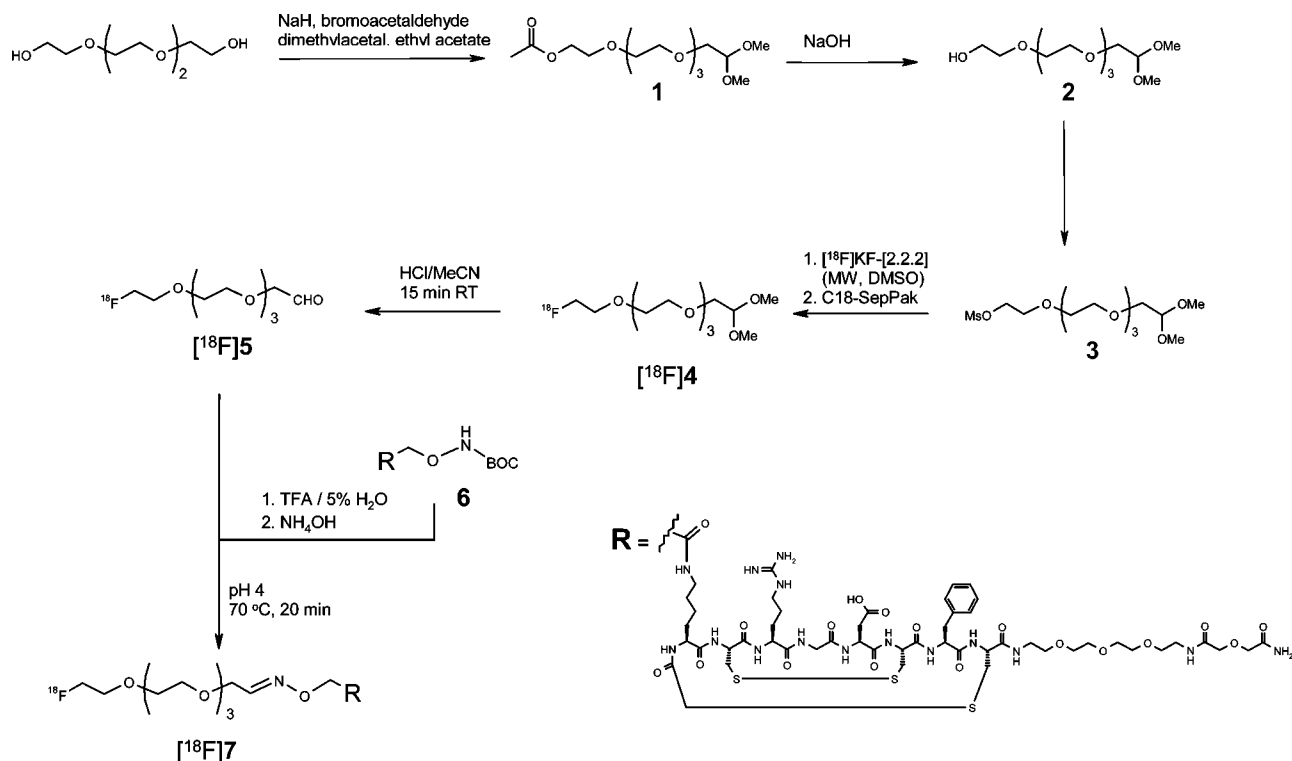


Table 1. Analytical and Isolated Radiochemical Yields of [¹⁸F]4

entry	solvent	temperature	reaction time	radiochemical yield, [^{18}F]
1	MeCN	80 °C	15 min	11% ^b
2	MeCN	80 °C	30 min	16% ^b
3	DMSO	80 °C	15 min	12% ^b
4	DMSO	80 °C	30 min	23% ^b
5 ^a	MeCN	80 °C	30 min	18% ^b
6	DMSO	160 °C	5 min	60% ^b (33%) ^c
7	DMSO	160 °C ^d	5 min	97% ^b (62%) ^c

^a [¹⁸F]TBAF vs [¹⁸F]KF-[2.2.2]. ^b Measured by HPLC. ^c Isolated and decay-corrected. ^d Microwave heating.

21.2 $\times$ 250 mm, solvents: A = water/0.1% TFA and B = acetonitrile/0.1% TFA; gradient 20–40% B over 60 min; flow 10.0 mL/min. The structure was confirmed by NMR analysis. ^1H NMR [CDCl_3]: δ 2.279 (q, 2H, $\text{MsOCH}_2\text{CH}_2\text{CH}_2$, $J = 6.0$ Hz), 3.012 (s, 3H, CH_3); 4.190 (t, 2H, $\text{MsOCH}_2\text{CH}_2\text{CH}_2$, $J = 5.9$ Hz), 4.462 (t, 2H, $\text{MsOCH}_2\text{CH}_2\text{CH}_2$, $J = 6.1$ Hz), 7.010 (pd, 2H, aromatic, $J = 8.8$ Hz), 7.845 (pd, 2H, aromatic, $J = 8.8$ Hz); 9.895 (s, 1H, CHO).

Synthesis of Aldehyde [¹⁸F]9. A Wheaton vial (2 mL) was charged with tetrabutylammonium hydroxide solution (1.0 M in MeOH, 13.5 μL) and water (0.1 mL). After saturation with gaseous CO₂, acetonitrile (0.4 mL) and ¹⁸F-containing water (178–485 MBq) were added. The water was removed azeotropically as described above, followed by addition of mesylate **8** (0.5 mg, 1.9 μmol) in anhydrous DMSO (0.2 mL). The mixture was heated in the microwave oven for 2 min at 110 °C. The product was identified by HPLC analysis (gradient 1, t_R = 16.1 min).

Synthesis of Peptide Conjugate [¹⁸F]10. Peptide **6** (4.0 mg, 2.8 μmol) was Boc-deprotected as described above and dissolved in 0.5 M ammonium acetate buffer pH 4.0 (0.1 mL). The reaction mixture containing [¹⁸F]**7** was added and the vial heated for 20 min at 70 °C. The coupling reaction was quenched by adding HPLC mobile phase (0.2 mL, 20% B). The labeled peptide [¹⁸F]**10** was identified by HPLC (gradient 1, t_R = 14.4

min), isolated by preparative HPLC (gradient 1, $t_R = 15.4$ min), and formulated by adding water (4 mL) followed by extraction using a SepPak C18-plus cartridge. The labeled peptide was eluted with ethanol (0.5 mL). After partial evaporation under a flow of nitrogen, the mixture was reconstituted with PBS.

Synthesis of Aldehyde [^{18}F]12 (Scheme 3). To a Wheaton vial (2.0 mL) was added Kryptofix–222 (5 mg), potassium carbonate (1 mg in 50 μL water), acetonitrile (0.8 mL), and water containing [^{18}F]fluoride. The water was removed azeotropically as described above. After addition of triflate **11** (0.5 mg, 1.6 μmol) in anhydrous DMSO, the vessel was heated for 15 min at 80 $^{\circ}\text{C}$.

Synthesis of Peptide Conjugate [¹⁸F]13. Peptide **6** (2.3 mg, 1.6 μmol) was Boc-protected as described above, dissolved in 0.5 M ammonium acetate buffer pH 4.0 (0.2 mL), mixed with the solution containing [¹⁸F]**10**, and then heated to 70 °C for 10 min. [¹⁸F]**13** was identified by analytical HPLC (gradient

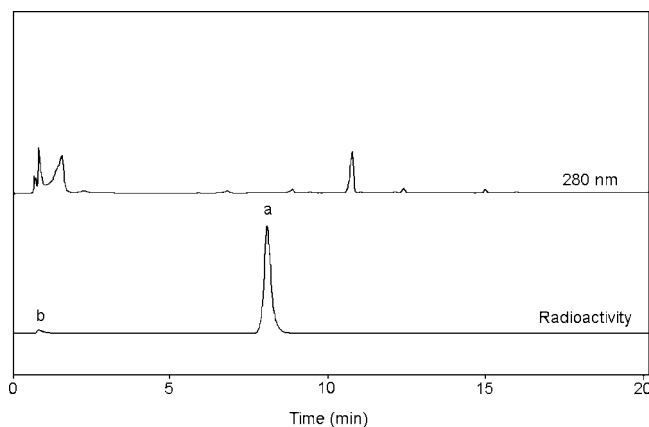


Figure 1. HPLC analysis of a reaction mixture containing diacetal peak (a) [^{18}F]**4** and (b) polar radioactive byproduct (microwave heating at 160 °C for 5 min in DMSO).

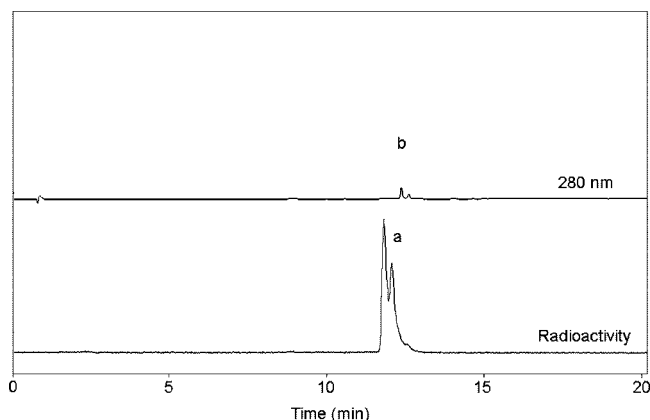
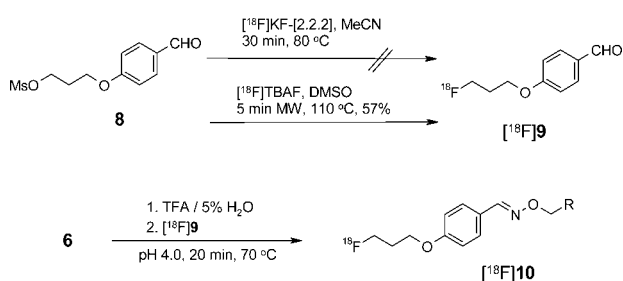


Figure 2. HPLC analysis of isolated labeled peptide (a) $[^{18}\text{F}]7$ and (b) trace amounts of starting aminooxy peptide precursor.

Scheme 2. Preparation of $[^{18}\text{F}]$ -4-(3-fluoropropoxy)benzaldehyde, $[^{18}\text{F}]9$, and Conjugation Reaction Forming Labeled RGD Peptide $[^{18}\text{F}]10$



1, $t_R = 13.7$ min). After quenching with 0.1 mL HPLC mobile phase (20% acetonitrile/water), the product $[^{18}\text{F}]13$ was isolated by preparative HPLC (gradient 1, $t_R = 14.5$ min) and formulated by SPE as described above.

The identities of all three ^{18}F -labeled peptide conjugates were confirmed by HPLC analysis in coelution experiments using authentic nonradioactive standards.

In Vitro Binding Assay. Membranes from the human endothelial adenocarcinoma cell line EA-Hy926 were prepared and the K_d calculated for the purified membrane fraction. A competitive binding assay was then established to measure inhibition constants for each test substance without the need for labeling of the substance itself (4). ^{125}I -Echistatin was used as the labeled ligand and nonradioactive echistatin as a reference standard. A total of sixteen dilutions of test compound (either echistatin or test substance) were prepared and mixed with a combination of the radioactive tracer and membrane prior to

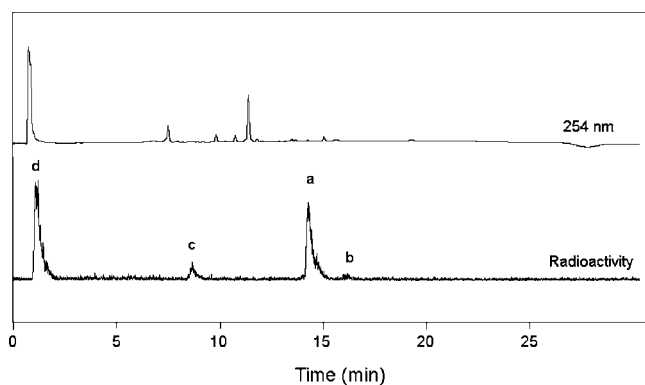


Figure 3. HPLC analysis of the one-pot peptide conjugation mixture: 5 min, 80 °C, pH 4.0, showing (a) the product $[^{18}\text{F}]10$ (b) a trace of synthon $[^{18}\text{F}]9$ (c) an unknown byproduct (d) $[^{18}\text{F}]$ fluoride.

Scheme 3. Preparation of $[^{18}\text{F}]$ -*p*-Fluorobenzaldehyde, $[^{18}\text{F}]12$, and Conjugation Reaction Forming Labeled RGD Peptide $[^{18}\text{F}]13$

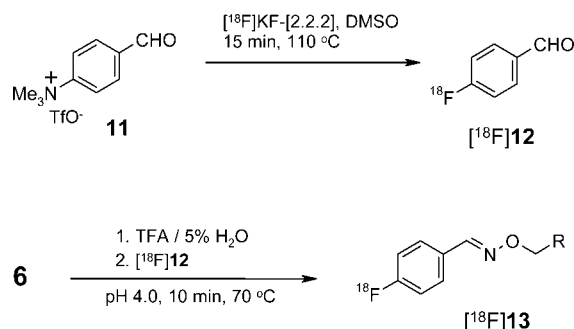


Table 2. Biodistribution of $[^{18}\text{F}]7$, $[^{18}\text{F}]10$, and $[^{18}\text{F}]13$ in the Mouse Lewis Lung Tumor Model^a

biodistribution (%id/g)	$[^{18}\text{F}]7$	$[^{18}\text{F}]10$	$[^{18}\text{F}]13$
blood 5 min	9.73 (3.1)	7.30 (1.3)	8.70 (3.7)
blood 120 min	0.28 (0.1)	0.71 (0.2)	0.5 (0.2)
muscle 120 min	0.24 (0.1)	0.44 (0.1)	0.32 (0.2)
liver 120 min	1.13 (0.1)	3.28 (0.9)	2.98 (0.4)
lung 120 min	1.06 (0.15)	2.03 (0.4)	1.61 (0.4)
tumor 120 min	1.64 (0.1)	1.12 (0.3)	1.20 (0.3)
tumor: blood	5.9	1.6	2.2
tumor: lung	1.6	0.6	0.7
tumor: liver	1.5	0.3	0.4
tumor: muscle	6.9	2.6	3.7
% retained over 120 min	53	27	38
clearance (%id)			
urine 120 min	79 (3.6)	53 (2.7)	56 (1.1)
GI	5 (0.9)	23 (1.4)	21 (3.7)

^a Summarized data at 5 and 120 min p.i. Average data ($n > 3$) of 2 independent experiments, presented as mean (SD).

incubation for 1 h at 37 °C. Following washing, the bound material was harvested on a filter using a Skatron microharvester. The filter spots were finally excised and counted in a Packard γ -counter. K_i values were calculated from the binding curves using *Prism* software.

Biodistribution Studies (LLC model). Lewis Lung carcinoma cells (LLC) (0.1 mL, 1×10^7 cells/mL in medium) were injected subcutaneously into the right inner thigh of C57/BL6 mice (*ca.* 20 g; Charles River UK Ltd.). At 15 days following inoculation of the tumor cells, animals were divided into 3 groups (3 mice per group). Each mouse received 1.0 MBq of $[^{18}\text{F}]7$, $[^{18}\text{F}]10$, or $[^{18}\text{F}]13$ as an intravenous bolus *via* the tail vein. Mice were sacrificed by cervical dislocation at 5, 60, and 120 min postinjection. Blood, tumor, and major organs were collected, weighed, and counted in a γ -counter (Wallac Wizard, Perkin-Elmer LAS (UK) Ltd.). The percentage of injected dose per gram (%id/g) or percentage of injected dose (%id) was determined for each sample.

The specificity of tumor uptake was determined by coinjecting an excess dose of nonradiolabeled RGD peptide with the $[^{18}\text{F}]$ RGD peptide. Six LLC tumor-bearing mice were coinjected with 0.4 mg RGD peptide per kilogram of bodyweight, and the uptake of $[^{18}\text{F}]$ RGD at 120 min postinjection in the blood, muscle, and tumor in these animals compared with that in control animals.

All animal experiments were approved by and carried out in compliance with the UK Home Office guidelines.

Imaging Studies. PET imaging was performed on a MicroPET-P4 system (Siemens Inc., Knoxville, USA). Images were generated from three-dimensional sinogram data, rebinned to two-dimensional format by the FORE algorithm followed by

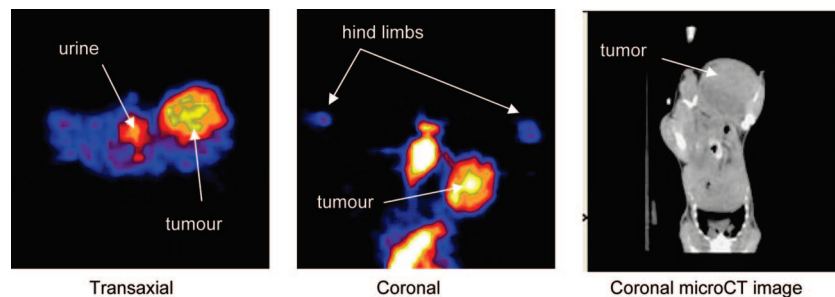


Figure 4. Transaxial and coronal MicroPET images of a mouse bearing a LLC tumor 2 h after tail vein injection of 8 MBq [^{18}F]7. The animal was anaesthetized in the prone position using isoflurane.

two-dimensional filtered back-projection. For imaging studies, the animal model described above was used, followed by injection of 8.0 MBq of [^{18}F]7 as an intravenous bolus *via* the tail vein. Imaging was performed dynamically up to 120 min postinjection, with the animal anaesthetized in the prone position by inhaled isoflurane. Ten minutes prior to the beginning of the CT image, each mouse received an intravenous bolus of 0.1 mL Omnipaque (GE Healthcare, UK) in order to give improved tumor contrast. MicroCT imaging was performed using the microCAT II system (Siemens Inc., Knoxville, USA) with manufacturer recommended settings. MicroPET data were analyzed by using Amide software (<http://amide.sourceforge.net>).

RESULTS AND DISCUSSION

Radiosynthesis and Conjugation. Scheme 1 outlines the steps involved in the radiosynthesis of [^{18}F]5 and subsequent conjugation to the aminoxy-RGD precursor peptide 6. Initial experiments to prepare the diacetal [^{18}F]4 using Kryptofix resulted in low labeling yields and so attempts were made to evaluate the effect of solvent and temperature on incorporation of [^{18}F]fluoride. Changing the solvent from acetonitrile to dimethylsulfoxide did not significantly increase the yield at a reaction temperature of 80 °C (Table 1, entries 1 and 3). In addition, only modest improvement was observed with 30 min heating compared to 15 min (Table 1, entries 2 and 4) due to the rapid degradation of mesylate 3. Although attempts at using the milder base [^{18}F]TBAF (21, 22) gave no further improvement (Table 1, entry 5), increasing the temperature further to 160 °C for 5 min did favor the fluoridation reaction giving 60% radiochemical yield as evidenced by HPLC analysis (Table 1, entry 6). Optimal conditions were, however, established using microwave heating with near-quantitative incorporation of [^{18}F]fluoride observed using an open vessel at 160 °C for 5 min (Table 1, entry 7). Figure 1 shows a typical HPLC analysis of the reaction mixture. Internal heating using microwaves gave better control of the [^{18}F]fluoridation step and appeared to prevent side reactions. This result supports the growing evidence that microwave activation is often beneficial in PET radiochemistry (23).

The intermediate [^{18}F]4 was isolated by solid-phase extraction and following facile removal of the acetal groups aldehyde [^{18}F]5 was ready for conjugation with the aminoxy RGD peptide. Starting with 2 mg of peptide precursor, the desired compound [^{18}F]7 was obtained following preparative HPLC with a decay-corrected radiochemical conjugation yield of $24 \pm 6\%$ ($n = 10$) and a total radiochemical yield (decay-corrected referring to starting activity of [^{18}F]fluoride) of $14 \pm 6\%$ ($n = 10$). Doubling the amount of precursor, however, to 4 mg increased the conjugation yield further to 51% with a total isolated yield of 38%. The analytical HPLC profile of the [^{18}F]7 revealed two peaks with identical retention times to the nonradioactive standard (Figure 2) formed by the isomerism of

the oxime bond. The final product was formulated in PBS on a rotary evaporator with a recovery of $93 \pm 7\%$ ($n = 5$) in a total synthesis time of 3 h.

The radiosynthesis of aldehyde [^{18}F]9 was first attempted using the standard Kryptofix protocol in acetonitrile for 30 min at 80 °C as shown in Scheme 2. Surprisingly, only two byproducts with longer retention times were generated during the reaction as evidenced by HPLC analysis. In this case, the less basic [^{18}F]TBAF system produced the desired aldehyde [^{18}F]9 in acceptable yields. Radiochemical yields could again be enhanced by microwave heating, the aldehyde [^{18}F]9 obtained in 57% yield after 5 min at 110 °C in DMSO, while with conventional heating, only 36% yield was obtained. In both experiments, the thermal lability of the mesylate 8 was found to be a limiting factor with complete decomposition occurring after 5 min. The Kryptofix/oxalate method, an alternative mild fluoridation approach, was tested under microwave conditions in DMSO (24). Although the mesylate precursor 8 appeared to have better stability, [^{18}F]9 was produced with a radiochemical yield of only 14% after 5 min heating at 110 °C. Optimal conditions were achieved with a combination of [^{18}F]TBAF and microwave activation. [^{18}F]9 was isolated from the reaction mixture by solid-phase extraction with a radiochemical yield of 38% (decay corrected) and a radiochemical purity of 70%. Figure 3 shows the radio-HPLC of the conjugation step demonstrating that the aldehyde [^{18}F]9 was near-quantitatively consumed to give product [^{18}F]10. Using the one-pot conjugation method, [^{18}F]10 was isolated following HPLC with an overall radiochemical yield of $14 \pm 7\%$ (decay-corrected, $n = 4$) and a radiochemical purity of 94%. The tracer was formulated by C18 SepPak extraction with a total synthesis time of 170 min.

[^{18}F]12 was obtained from 4-*N,N,N*-trimethylammonium benzaldehyde trifluoromethanesulfonate 11 following a literature method (25). On the basis of the labeling results for the fluoroaldehydes [^{18}F]5 and [^{18}F]9, [^{18}F]12 was used in the conjugation step without isolation as shown in Scheme 3. The corresponding product [^{18}F]13 was obtained with a decay-corrected radiochemical yield of $23 \pm 5\%$ ($n = 3$) and a radiochemical purity of 96%. The radio-HPLC analysis of the reaction mixture after 10 min incubation indicated an almost quantitative coupling efficiency with only a trace of [^{18}F]12 remaining (5%).

In Vitro Affinity. The competition binding assay (4) was established to measure the binding affinity for the integrin $\alpha_v\beta_3$ of the three conjugates. The nonradioactive compounds [^{19}F]7, [^{19}F]10, and [^{19}F]13 were found to compete with [^{125}I]-echistatin with K_i values calculated to be 2.3, 1.1, and 13.3 nM, respectively. In comparison, the α_v selective inhibitor cyclo(-Arg-Gly Asp-D-Phe-Lys-), a widely used RGD pharmacophore (26), had a K_i value in this assay of 27.8 nM. We would therefore suggest that differences observed in the biodistribution profiles

of the three conjugates could not be explained on the grounds of differences in the binding affinity for the integrin receptors *in vivo*.

Biodistribution and Imaging Studies. The conjugate [^{18}F]7 demonstrated favorable biodistribution profile with good tumor uptake and retention at 120 min postinjection (1.64%id/g and 53%, respectively) as shown in Table 2. Additionally, due to the reduced background tissue uptake (e.g., in lung and liver) the ratios against tumor were improved with a tumor to blood ratio of ~ 6 and tumor to muscle ratio of ~ 7 at 120 min postinjection. The majority of the radioactivity was excreted via the urine, with 79%id in the urine and bladder 120 min postinjection. The localization of [^{18}F]7 in LLC tumors as determined by MicroPET imaging is shown in Figure 4 alongside a microCT slice for anatomical reference. Representative transaxial and coronal images are shown from one mouse. There is clear visualization of the LLC tumor in the animal, along with clearance of [^{18}F]7 through the bladder and gastrointestinal tract, which was consistent with the biodistribution data.

Although the blood kinetic profiles for [^{18}F]13 and [^{18}F]7 are similar, as summarized in Table 2, the increased lipophilicity of [^{18}F]13 results in higher liver uptake compared to that of [^{18}F]7 (data shown 120 min postinjection). Consequently, the amount of activity excreted via the liver into the gastrointestinal tract was around 21%id for [^{18}F]13 compared to only 5%id for [^{18}F]7. Similarly, [^{18}F]10 had significant gastrointestinal tract excretion (23%id) and lower urinary excretion (53%id).

Perhaps the most striking observation of the effect of the prosthetic group on biodistribution was seen on kidney excretion rates. In all, 79%id of [^{18}F]7 activity passed into the urine and bladder and 5.4%id into the gastrointestinal (g.i.) tract at 2 h postinjection. This was compared to $>20\%$ g.i. excretion for both [^{18}F]10 and [^{18}F]13.

In order to look at specificity, an *in vivo* blocking study with 10 μg excess unlabeled RGD peptide per animal resulted in a significantly reduced [^{18}F]7 uptake in the tumor at 120 min postinjection. Tumor uptake was decreased from $1.48 \pm 0.3\%$ id/g to $0.76 \pm 0.2\%$ id/g ($p < 0.01$). Although this represents a 49% reduction of tumor uptake, further studies are needed with higher doses of nonradioactive peptide in order to achieve full blocking.

In summary, this work demonstrates that a rational approach to prosthetic group design can be successfully employed to optimize key *in vivo* characteristics of peptide-based tracers. Although localization of all three conjugates to the tumor was observed, [^{18}F]7 possessed superior tumor to blood, lung, liver, and muscle ratios compared to the more hydrophobic conjugates [^{18}F]10 and [^{18}F]13.

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Supporting Information Available: Supporting data for the synthesis of nonradioactive standards. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supplemental Information

Synthesis of non-radioactive peptide conjugates **7**, **10** and **13** as HPLC standards.

Boc-deprotection of peptide 6. A solution of Boc protected aminooxy derivatised peptide **6** (28 mg, 0.020 mmol) in 95% TFA (10 mL) was stirred for 15 min. After concentration the residue was triturated with ether and dried affording 20 mg of aminooxy peptide (77 % yield). Analytical HPLC (Phenomenex Luna C18(2), 4,6 x 250 mm, 5 μ m; solvent A: water/0.1% TFA, solvent B: acetonitrile/0.1% TFA gradient 10-50% B over 20 min; flow 1.0 mL/min; UV 214 nm): t_R = 11.05 min. ESI-MS: found m/z = 1331.3 (MH)⁺, calculated m/z = 1331.5.

Synthesis of non-radioactive precursor **4**

To mesylate **3** (75 mg, 0.21 mmol) was added TBAF (1.1 M in THF, 0.57 mL, 0.63 mmol) and 8 mL of acetonitrile and the mixture heated to 90 °C for 30 minutes. TLC (ethyl acetate) monitoring showed that the reaction had gone to completion. After cooling to ambient temperature and evaporation of solvent the residue was purified on a silica column using ethyl acetate affording of the desired product as a colorless oil (56 mg, 96% yield).

Synthesis of non-radioactive peptide conjugate **7**.

The protecting groups were liberated from 0.68 mg of **4** (0.0028 mmol) in 1N HCl/ acetonitrile (1:1, 2 mL) for 30 min yielding aldehyde **5**, before addition of the solution to peptide **6** (4 mg, 0.0028 mmol). The pH was then adjusted to 4 with dilute ammonia solution and the mixture heated to ~ 65 degrees for 1 h. The product was purified by HPLC (Phenomenex Luna C18(2), 21.2 x 250 mm, 5 μ m; solvent A: water/0.1% formic acid, solvent B: acetonitrile/0.1% formic acid, gradient 15-40% B over 60 min; flow 10.0 mL/min; UV 214 nm) to give pure material (2.3 mg, 53% yield). Analytical HPLC (Vydac C18, 4,6 x 250 mm, 5 μ m; solvent A: water/ 0.1% TFA, solvent B: acetonitrile/ 0.1% TFA gradient 10-50% B over 20 min; flow 1.0 mL/ min; UV 214 nm): t_R = 12.9 and 13.2 min. ESI-MS: found m/z = 1550.1 (MH)⁺, calculated m/z = 1550.6.

Synthesis of non-radioactive peptide conjugate 10. To a solution of deprotected aminooxy peptide (27 mg, 0,020 mmol) in water (3 mL) was added a solution of 4-(3-fluoropropoxy)benzaldehyde (20 mg, 0.11 mmol) mmol) in acetonitrile (3 mL). The reaction mixture was stirred at 70 °C for 30 min,

cooled and purified by HPLC (Phenomenex Luna C18(2), 21.2 x 250 mm, 5 μ m; solvent A: water/0.1% formic acid, solvent B: acetonitrile/0.1% formic acid, gradient 15-40% B over 60 min; flow 10.0 mL/min; UV 214 nm) to give 7.3 mg (24%) of pure material. Analytical HPLC (Phenomenex Luna C18(2), 2.0 x 50 mm, 3 μ m; solvent A: water/ 0.1% formic acid, solvent B: acetonitrile/0.1% formic acid, gradient 5-50% B over 10 min; flow 1.0 mL/min; UV 214 nm): t_R = 7.5 min. ESI-MS: found m/z = 1495.5 (MH)⁺, calculated m/z = 1495.6.

Synthesis of peptide conjugate 13. To a solution of deprotected aminooxy peptide (5.9 mg, 4.4 μ mol) in water (1 mL) was added a solution of 4-fluorobenzaldehyde (1.1 mg, 8.9 μ mol) in acetonitrile (1 mL). The reaction mixture was stirred at 70 °C for 45 min, cooled and purified by HPLC (Phenomenex Luna C18(2), 21.2 x 250 mm, 5 μ m; gradient 10-40% B over 30 min; flow 5.0 mL/min; UV 214 nm) to give 2.0 mg (31%) of pure material. Analytical HPLC (Phenomenex Luna C18(2), 4,6 x 250 mm, 5 μ m; solvent A: water/0.1% TFA, solvent B: acetonitrile/0.1% TFA, gradient 10-50% B over 20 min; flow 1.0 mL/min; UV 214 nm): t_R = 16.3 min. ESI-MS: found m/z = 1437.0 (MH)⁺, calculated m/z = 1436.5.