A localised tolerance in the substrate specificity of the fluorinase enables 'last step' [^{18}F]-fluorination of a RGD peptide under ambient aqueous conditions

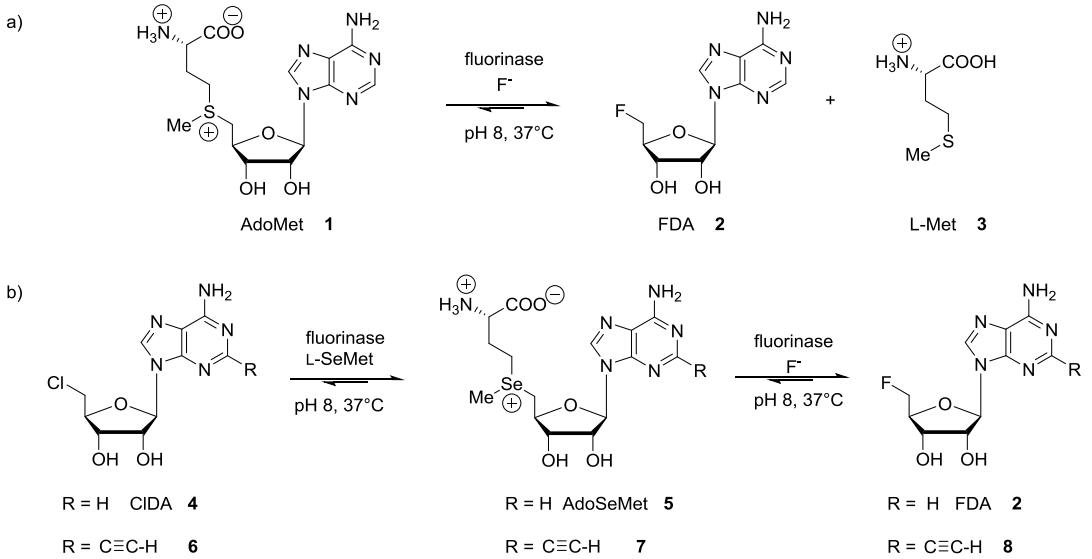
Stephen Thompson[a], Qingzhi Zhang[a], Mayca Onega[b], Stephen McMahon[a], Ian Fleming[c], Sharon Ashworth,[b] James H. Naismith[a], Jan Passchier[b], David O'Hagan[a]*.

[a]School of Chemistry, University of St Andrews, North Haugh, St Andrews, KY16 9ST, UK.
[b]Imanova, Burlington Danes Building, Imperial College London, Hammersmith Hospital, Du Cane Road, London,W12 0NN, UK
[c]Aberdeen Biomedical Imaging Centre, School of Medicine and Dentistry, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK.
[d]Imperial College London, Departent of Medicine, Burlington Danes Building, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN (United Kingdom)

‡Authors contributed equally.

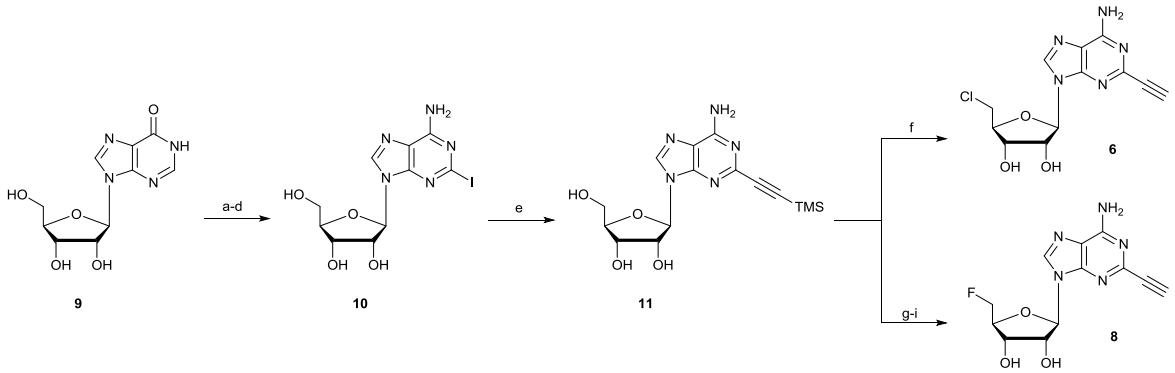
Abstract: A strategy for last step [^{18}F]-fluorination of bioconjugated peptides is reported that exploits an 'Achilles heel' in the substrate specificity of the fluorinase enzyme. An acetylene functionality at C-2 of the adenosine substrate projects from the active site into the solvent. The fluorinase catalyses a transhalogenation of 5'-chlorodeoxy-2-ethynyladenosine (ClDEA) to 5'-fluorodeoxy-2-ethynyladenosine [^{18}F]-FDEA. Extending a polyethelene glycol linker from the terminus of the acetylene allows bio-conjugation cargo to be presented to the enzyme for [^{18}F]-labeling. The method benefits from using an aqueous ($H_2{}^{18}O$) solution of [^{18}F]-fluoride, generated by the cyclotron and has the modular capacity to isotopically label peptides of choice for positron emission tomography (PET).

The fluorinase enzyme, originally isolated from *Streptomyces cattleya* catalyses the conversion of *S*-adenosyl-L-methionine (AdoMet) **1** and fluoride ion to 5'-fluoro-5'-deoxyadenosine (FDA) **2** and L-methionine (L-Met) **3** as illustrated in Scheme 1a.[1] The enzyme has found application in positron emission tomography (PET) due to its ability to catalyse C-^{18}F bond formation if [^{18}F]-fluoride ion is presented as the nucleophile.[2] The enzymatic process has a technical advantage in the PET context because [^{18}F]-fluoride is generated in the cyclotron as a dilute solution in [^{18}O]-water, and the enzyme can use this form of aqueous fluoride directly. This obviates the usual requirement to secure dry [^{18}F]-fluoride ion by ion exchange chromatography and as a Kryptofix 222 formulation[3] and the enzymatic process therefore offers the possibility of directly radiolabelling biomolecules in the aqueous phase.



Scheme 1. a) The fluorinase catalyses the transformation of AdoMet **1** to FDA **2** and L-methionine **3**. b) A fluorinase catalyzed transhalogenation converts ClDA **4** and L-selenomethionine to FDA **2**, *via* the intermediacy of AdoSeMet **5**.

A constraint of the fluorinase technology is that the enzyme has a high substrate specificity. To date, [^{18}F]-radiolabelled compounds using the fluorinase have been prepared *via* [^{18}F]-FDA **2**, the product of the native enzymatic reaction.[4-6] However there is some substrate tolerance to halide. It has previously been shown that ClDA **4** is a better substrate than FDA **2** for the generation of AdoMet (Scheme 1b).[7] This is because chloride is a better leaving group than fluoride, but a poor nucleophile in the forward reaction. Also in these reactions L-selenomethionine (L-SeMet) is a better substrate than L-Met **3**, generating AdoSeMet **5** in place of AdoMet **1**. More generally the substrate specificity of the fluorinase is poor. All carbon skeletal modifications that have been explored so far lead to substantially reduced kinetics frustrating efficient biocatalysis. We now report a breakthrough in substrate specificity which exploits a structural modification to the substrate, incorporating an acetylene moiety at C-2 of the adenine ring. Close inspection of co-crystal structures of the fluorinase with FDA **2** (Figure 1)[8] indicated that the C-2 adenine hydrogen was positioned in the active site close to the enzyme surface and it was envisaged that a small linear functional group at C-2 should penetrate into the solvent. It appeared most strategic to place an acetylene functionality at C-2 due to its linearity and low steric impact. Thus 5'-chloro-5'-deoxy-2-ethynyladenosine (ClDEA) **6** became a candidate substrate for a transhalogenation reaction. The route developed for the synthesis of **6** is outlined in Scheme 2. Iodoadenosine **10** prepared from guanosine **9**,[9,10] was subject to Sonogashira cross-coupling to generate **11** and introduce the acetylenic functionality. TMS-protected ethynyladenosine (**11**) was then converted to the required, **6** in a one-pot procedure.[11] A sample of FDEA **8**, the anticipated product of enzymatic transhalogenation, was also prepared as a reference compound by protection of the 2',3'-diol of **11** as an acetonide, and then subsequent fluorination[12] of the primary alcohol and hydrolysis to furnish **8**.



Scheme 2. *Reagents and conditions*: a) Ac_2O, pyridine, DMF, 84%. b) $POCl_3$, Et_4NCl, dimethylaniline, CH_3CN, 77%. c) CuI, I_2, CH_2I_2, isoamyl nitrite, THF, 69%. d) NH_3, MeOH, 69%. e) $Pd(PPh_3)_2Cl_2$, Et_3N, CuI, DMF, trimethylsilylacetylene, 60%. f) $SOCl_2$, pyridine, CH_3CN, then NH_3, MeOH, H_2O 47%. g) 2,2-dimethoxypropane, Amberlyst-15 H^+. 53%. h) TBAF, TsF, THF, 71%. i) TFA, H_2O, 53%.

FDEA **8** was also subjected to co-crystallisation trials with the fluorinase and an X-ray structure was solved to 2.4 Å by molecular replacement using the historical fluorinase refinement.[8] The overlay of the co-crystal structures of **2** and **8** shows **8** (grey, Figure 1) bound in the active site in a similar manner to FDA **2** (yellow, with the acetylene at C-2 projecting into the solvent.

Incubation of ClDEA **6** with the fluorinase and L-SeMet, resulted in a conversion to FDEA **8** as illustrated in Scheme 1b (For HPLC profiles see Figure S1a). The reaction progressed at about 60% of the rate of that of ClDA **4** to FDA **2** (See SI Figure S1b). This transhalogenation demonstrated that the enzyme does indeed have a specificity weakness at C-2 of adenine. It became immediately attractive to explore extending a polyethylene glycol (PEG) chain from the terminus of the acetylene, as a tether onto a cargo such as a peptide, as a potential strategy for radiolabelling the peptide cargo with fluorine-18. RGD peptides are validated ligands with high affinity to $\alpha_V\beta_3$ integrin epitopes on the surface of a variety of cancer cells.[13] They have been the focus of many labelling studies for PET, in the development of tumor imaging agents for the clinic, so this seemed a good model to explore. To this

end ClDEA-TEG-RGD **15** (TEG = tetraethyleneglycol), emerged as a synthetic target (Scheme 3). The PEG chain was incorporated into the design to project the cyclic peptide into the bulk solvent. A key step involved the Sonogoshira cross-coupling between alkyne **12** and 2-iodoadenosine **10** to generate **13**. Reaction of **13** with thionyl chloride gave ClDEA-TEG **14**, with a HCl/acetylene addition product (See SI compound **33**). The co-product was readily removed from **14** by preparative HPLC. In order to attach the RGD peptide, **14** was subject to a 'click' reaction with c(RGDfK(N_3)), to generate **15**, a product which was purified by HPLC and fully characterised (see SI). With the required substrate in hand, **15** was incubated with the fluorinase under the previously established conditions (L-SeMet, F^-, fluorinase, pH 7.8) and proved to be a good substrate for the transhalogenation to FDEA-TEG-RGD **16**, as monitored by HPLC (Figure 2). No reaction was observed without enzyme.

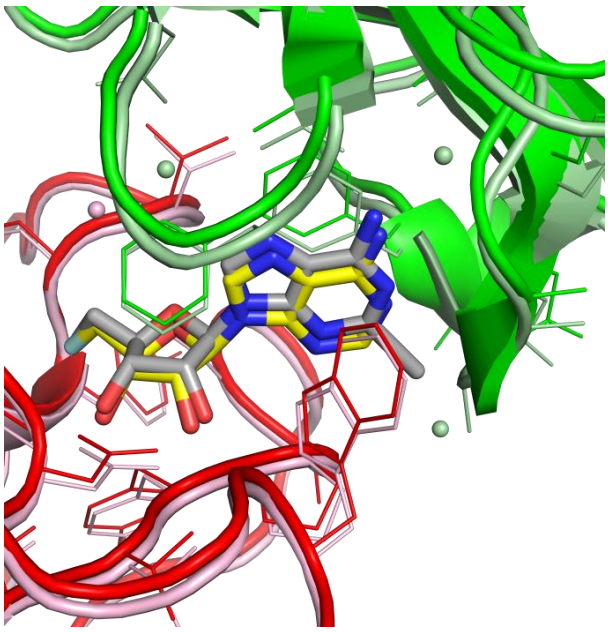
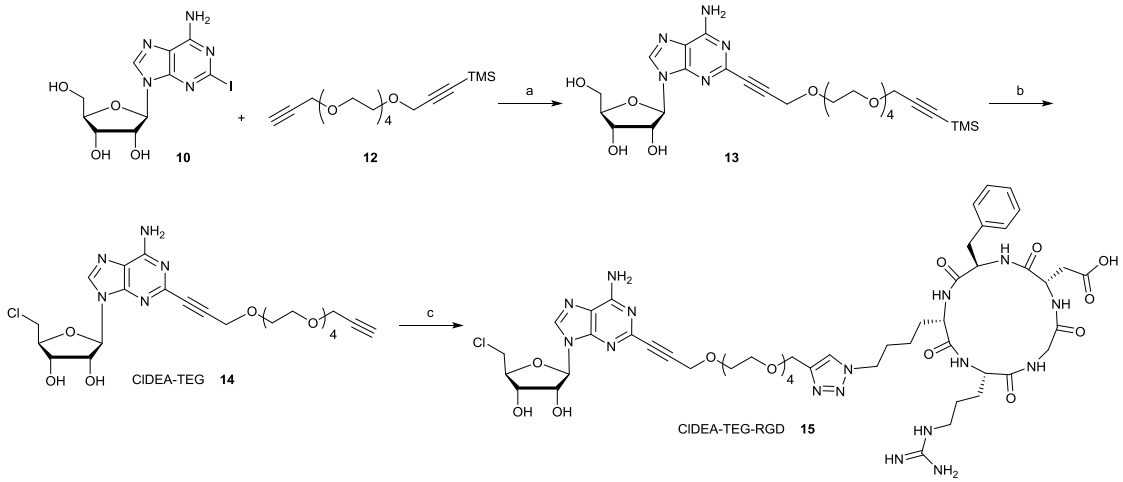


Figure 1. Overlay of co-crystal structures of FDA **2** (yellow, pink and pale green) and FDEA **8** (gray, red and bright green) showing projection of the acetylene of **8** between two subunits (red and green) of the fluorinase.



Scheme 3. *Reagents and conditions:* a) $Pd(PPh_3)_2Cl_2$, Et_3N, CuI, DMF, 67%. b) $SOCl_2$, pyridine, CH_3CN, then NH_3 (aq), 20%. c) c(RGDfK(N_3)), $CuSO_4$.TBTA (TBTA = tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine), sodium ascorbate, 67%.

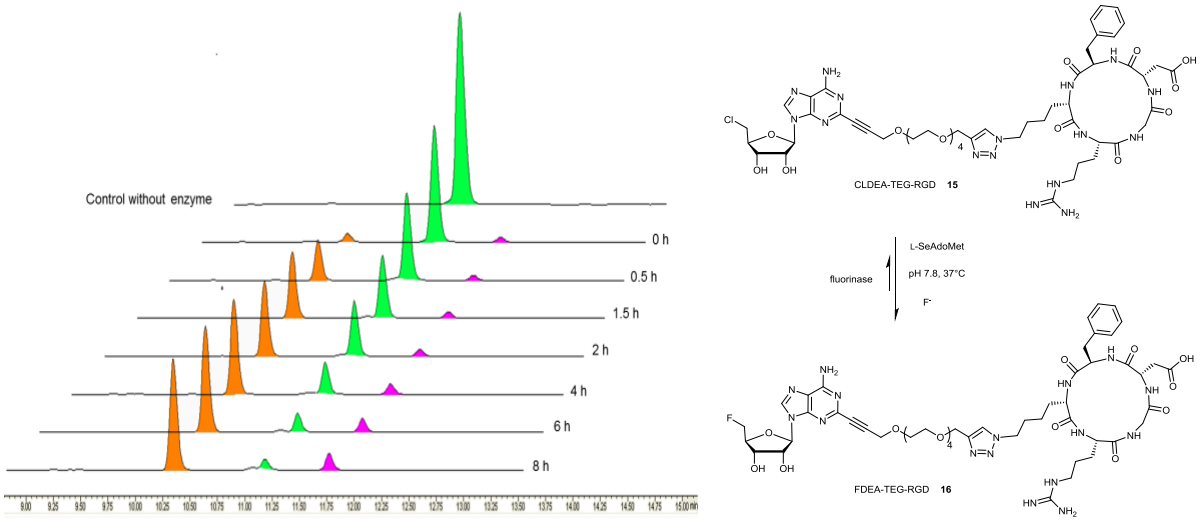
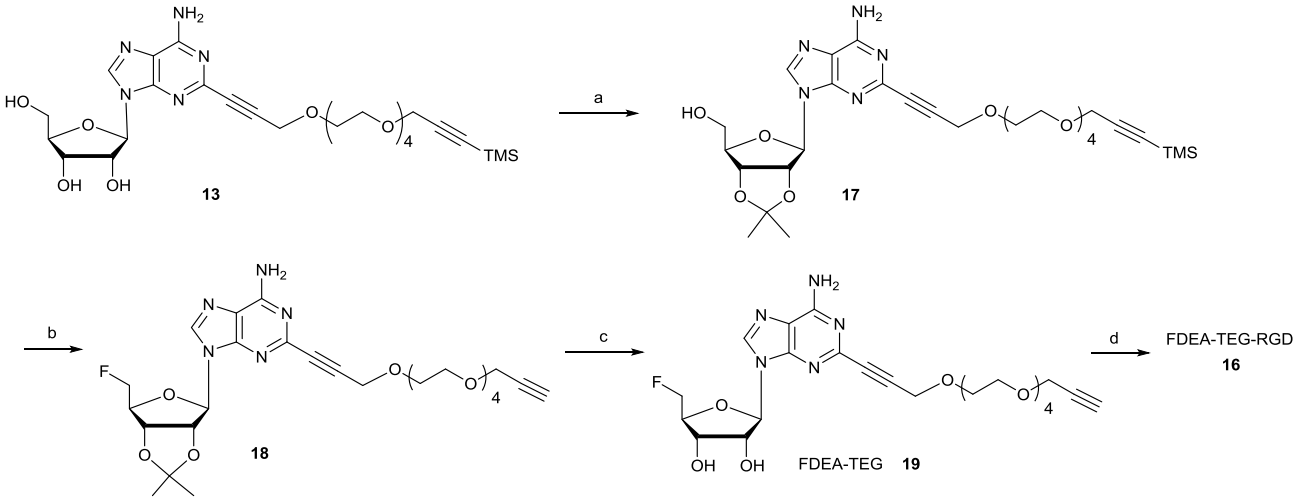


Figure 2. HPLC monitoring **15** to **16** by the fluorinase. *Conditions:* KF, L-SeMet, fluorinase, phosphate buffer, pH 7.8, 37°C. Traces show the formation of the FDEA-TEG-RGD at t_R = 10.2 min (orange), while ClDEA -TEG-RGD (t_R = 11.0 min) (green) is consumed. Some accumulation of SeAdoMet is observed, evidenced by the presence of a SeAdoMet breakdown product (purple).

The identity of **16** was confirmed by both mass spectrometry (See Figure S4), and against an authentic reference sample which was prepared by synthesis (Scheme 4). The success of this reaction demonstrates that fluorinase mediated transhalogenation can be accomplished with a peptide 'cargo' attached to the terminus of the C-2 acetylenic functional group. This opens up prospects for attaching a variety of peptides to a ClDEA motif, for direct 'last step' fluorine-18 labelling using fluorinase catalysis for PET.



Scheme 4. *Reagents and conditions:* a) 2,2-Dimethoxypropane, $HClO_4$, MeOH, 90%. b) TBAF, TsF, 55%. c) CF_3CO_2H, MeOH/H_2O, 69%. d) c(RGDfK(N_3)), $CuSO_4$.TBTA (TBTA = tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine), sodium ascorbate, 70%.

As few and efficient radiolabelling transformations are desired in PET radiochemistry, due to the relatively short half-life of ^{18}F ($t_{1/2}$ = 109.7 min). This generally requires that [^{18}F]-fluoride, generated by the cyclotron from [^{18}O]-water, is dried by ion exchange chromatography, and rendered good for nucleophilic chemistry. Water soluble peptides are most commonly labelled with fluorine-18 *after* the anhydrous synthesis of reactive small molecules followed by a conjugation to the peptide. These protocols require at least two transformations after securing the [^{18}F]-fluoride ion by ion exchange chromatography. However 'last step' fluorination protocols for peptides are less well developed due to the incompatibility of peptides with non-aqueous solvents and a requirement for heating to improve the rate of reactions. Current strategies for the direct labelling of peptides have used fluoride-sequestering strategies. Radiolabelled trifluoroborates[14] have been prepared using aqueous [^{18}F]-fluoride and KFH_2, but at low pH (pH 2), while aluminium hydroxide complexes[16] require heating at elevated temperatures

(105 °C) for fluorination. A silicon-based fluoride acceptor approach[15] and direct nucleophilic fluorination reactions[17,18] at carbon require anhydrous conditions and heating to accomplish fluoride incorporation. It is in this context that fluorinase catalysis presents an attractive strategy for last step fluorination of peptides as no drying of fluoride ion is required and reactions occur at ambient temperature and neutral pH in buffer.

Accordingly a transhalogenation reaction is now explored with **15** using [^{18}F]-fluoride for conversion to [^{18}F]-**16**. The reaction progressed at a modest rate under 'cold' fluoride ion conditions as illustrated in Figure 2, however fluorinase catalysed radiochemical reactions progress to higher levels of conversion at very low levels of fluoride ion. The cyclotron generates a solution of [^{18}F]-fluoride at picomolar (10^{-12}M) concentration. The fluorinase is added to this solution at a micromolar (10^{-6}M, 15-20 mg.ml^{-1}) concentration, with a 10^6 molar excess of enzyme over fluoride ion in the radiochemical biotransformation. Under these conditions the enzyme is not catalytic. The success of the radiochemical experiments lies in converting the picomolar aqueous [^{18}F]-fluoride into organic [^{18}F]-fluorine and generally very good radiochemical conversions are achieved due to the heavily biased stoichiometry. The radiochemical conversion was excellent. Figure 3 shows the simultaneous radiochemical and UV detection HPLC traces of the supernatant of the reaction mixture (30 min, rt) after denaturation of the enzyme. The supernatant displays an almost neat signal of [^{18}F]-**16** in the radio trace, while the UV trace reveals the excess of **15**. The identity of [^{18}F]-**16** was confirmed by HPLC spiked with cold **16** (See SI Figure S5).

Peptide **16** was evaluated[19] for its ability to bind to $\alpha_V\beta_3$ integrins. It is clear from Table 1 that **16** retains high affinity to $\alpha_V\beta_3$ integrin compared with structurally less elaborate cyclic RGD peptides and has significantly higher affinity that the RGD motif itself.

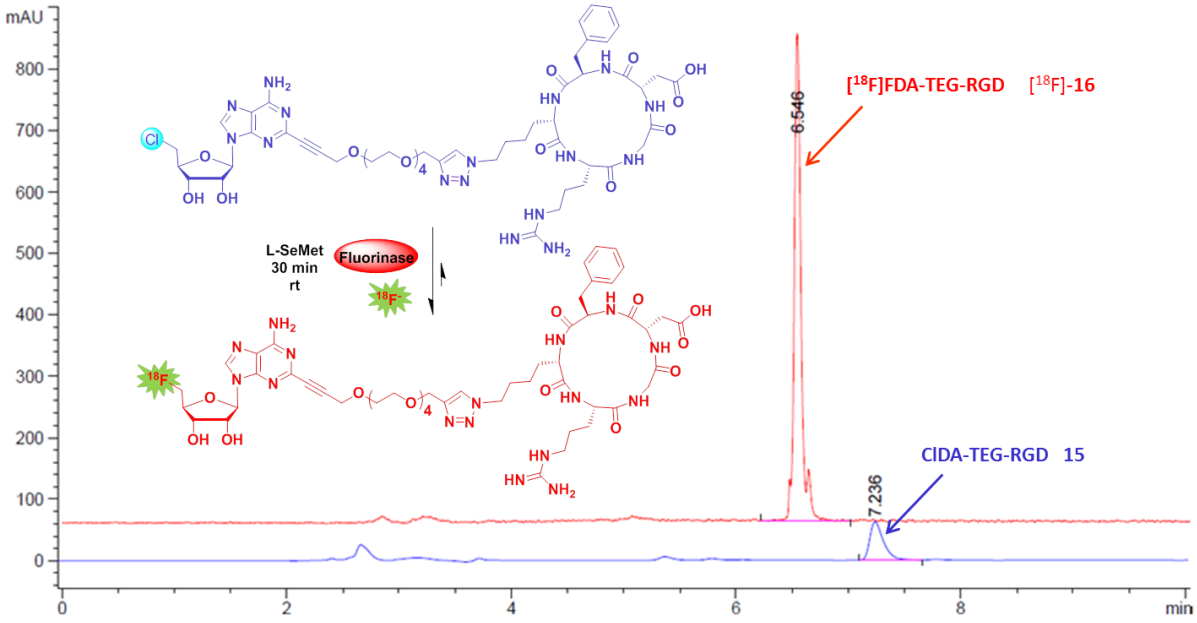


Figure 3. HPLC (radio and UV) traces of enzymatic transformation of **15** to [^{18}F]-**16** (30 min, rt). [^{18}F]-**16** at 6.5 min (radiochemical detector, red); UV trace (blue) shows excess **15** at 7.2 min.

Table 1: IC_{50} binding affinity of RGD-containing peptide motifs to immobilized $\alpha_V\beta_3$ integrin. Q is the normalised activity of the peptides referenced to linear GRGDSPK (Q=1).

Compound	*IC_{50} (μM)*	*Q*
RGD	8.56	4.019
c(RGDfK)[a]	0.11	0.052
c(RGDfC)[a]	0.08	0.038
FDEA-TEG-RGD	0.07	0.033

[a] IC_{50} values reported previously using an identical assay.[20]

Peptide **16** was then injected into a healthy rat to explore if it was robust to defluorination. There was some evidence of metabolism of the peptide, however a combination PET/CT whole body image averaging scans over a 1 h period (Figure 4) did not show radioactivity uptake in the bone indicating that **16** is stable to [^{18}F]-fluoride ion release, an important pre-requisite of a PET-tracer. The peptide distribution in different organs was assessed after dissection (see SI Figure S7) and had a profile (eg. highest radioactivity in kidney, intestine, spleen and lung) consistent with distribution of an intact RGD motif[21].

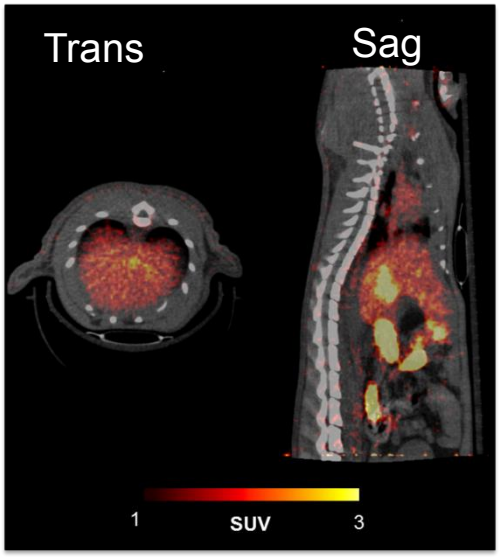


Figure 4 Figure 4: Summed transaxial (Trans, L) and saggital (Sag, R) PET images of a rat, 5-60min following administration of 5MBq of [18F]FDA-TEG RGD, co-registered with corresponding CT images (grey scale). No evidence of bone uptake could be observed, suggesting good *in vivo* stability of the C-F bond of the 5'-[^{18}F]-fluorodeoxyadenosine fragment. PET images are presented as standard uptake values {(tissue activity/mL)/(injected activity/animal weight[g])}.

This study demonstrates a breakthrough in fluorinase substrate specificity and its application to enzymatic 'last step' [^{18}F]-labelling of peptides from aqueous cyclotron generated [^{18}F]-fluoride. We are currently investigating a range of other peptides and linkers and exploring the metabolim of such constructs, in order to develop this approach as a strategy for tumor imaging studies.

Acknowledgements

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References

[1] D. O'Hagan, C. Schaffrath, S. L. Cobb, and J. T. G. Hamilton, *Nature* **2002**, *416*, 279.
[2] S. Dall'Angelo, N. Bandaranayaka, A. D. Windhorst, D. J. Vugts, D. van der Born, M. Onega, L. F. Schweiger, M. Zanda, D. O'Hagan, *Nuclear Med. Biol.*, **2013**, *40*, 464-470.
[3] R. Schirrmacher, C. Wängler, and E. Schirrmacher, *Mini-Rev. Org. Chem.* **2007**, *4*, 317–329.
[4] M. Winkler, J. Domarkas, L. F. Schweiger, and D. O'Hagan, *Angew. Chem. Int. Ed.* **2008**, *47*, 10141–10143.
[5] X.-G. Li, S. Dall'Angelo, L. F. Schweiger, M. Zanda, and D. O'Hagan, *Chem. Commun. (Camb.)* **2012**, *48*, 5247–5249.
[6] X.-G. Li, J. Domarkas, and D. O'Hagan, *Chem. Commun. (Camb.)* **2010**, *46*, 7819–7821.

[7] H. Deng, S. L. Cobb, A. R. McEwan, R. P. McGlinchey, J. H. Naismith, D. O'Hagan, D. A. Robinson, and J. B. Spencer, *Angew. Chem., Int. Ed.* **2006**, *45*, 759–762.
[8] C. Dong, F. Huang, H. Deng, C. Schaffrath, J. B. Spencer, D. O'Hagan, and J. H. Naismith, *Nature* **2004**, *427*, 561–565.
[9] V. Nair and S. Richardson, *Synthesis (Stuttg.)* **1982**, 670–672.
[10] A. Matsuda, M. Shinozaki, M. Tadashim, H. Machida, and T. Abiru, *Chem. Pharm. Bull.* **1985**, *33*, 1766–1769.
[11] M. J. Robins, F. Hansske, S. F. Wnuk, and T. Kanai, *Can. J. Chem.* **1991**, *69*, 1468–1474.
[12] M. Shimizu, Y. Nakahara, and H. Yoshioka, *Tetrahedron Lett.* **1985**, 4207–4210.
[13] H. Cai and P. S. Conti, *J. Label. Compd. Radiopharm.* **2013**, *56*, 264–279.
[14] Y. Li, Z. Liu, J. Lozada, M. Q. Wong, K.-S. Lin, D. Yapp, and D. M. Perrin, *Nucl. Med. Biol.* **2013**, *40*, 959–966.
[15] R. Schirrmacher, G. Bradtmöller, E. Schirrmacher, O. Thews, J. Tillmanns, T. Siessmeier, H. G. Buchholz, P. Bartenstein, B. Wängler, C. M. Niemeyer, and K. Jurkschat, *Angew. Chem. Int. Ed.* **2006**, *45*, 6047–6050.
[16] C. A. D'Souza, W. J. McBride, R. M. Sharkey, L. J. Todaro, and D. M. Goldenberg, *Bioconjug. Chem.* **2011**, *22*, 1793–1803.
[17] J. Becaud, L. Mu, M. Karramkam, P. a Schubiger, S. M. Ametamey, K. Graham, T. Stellfeld, L. Lehmann, S. Borkowski, D. Berndorff, L. Dinkelborg, A. Srinivasan, R. Smits, and B. Koksch, *Bioconjug. Chem.* **2009**, *20*, 2254–2261.
[18] O. Jacobson, L. Zhu, Y. Ma, I. D. Weiss, X. Sun, G. Niu, D. O. Kiesewetter, and X. Chen, *Bioconjug. Chem.* **2011**, *22*, 422–428.
[19] S. Dall'Angelo, Q. Z. Zhang, I. Fleming, M. Piras, L. F. Schweiger, D. O'Hagan and M. Zanda, *Org. Bio. Chem*. **2013**, 11, 4551 – 4558
[20] M. Piras, I. Fleming, W. Harrison, and M. Zanda, *Synlett* **2012**, *23*, 2899–2902.
[21] J. Oxboel, M. Brandt-Larsen, C. Schjoeth-Eskesen, R. Myschetsky, H. H. El-Ali, J. Madsen, A. Kjaer, *Nuclear Med. Biol*., **2014**, *41*, 259-267.

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