Supplementary Material

Improved Metabolic Stability for ^{18}F PET Probes Rapidly Constructed via Tetrazine *trans*-Cyclooctene Ligation

Ramajeyam Selvaraj[1#], Benjamin Giglio[2#], Shuanglong Liu[3#], Hui Wang[2], Mengzhe Wang[2], Hong Yuan[2], Srinivasa R. Chintala[1], Li-Peng Yap[3], Peter S. Conti[3], Joseph M. Fox[1]*, Zibo Li[2]*

[1]Brown Laboratories, Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19803, USA;

[2] Department of Radiology and Biomedical Research Imaging Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

[3] Molecular Imaging Center, Department of Radiology, University of Southern California, Los Angeles, CA90033, USA;

[#]These authors contributed equally to the research work.

Corresponding Authors:

jmfox@udel.edu; ziboli@med.unc.edu

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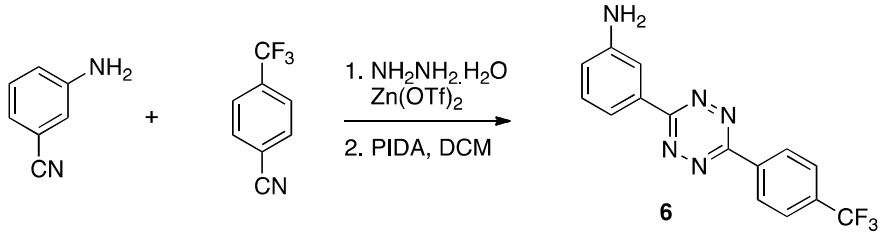
MATERIALS AND METHODS

All commercially available chemical reagents were used without further purification. The syringe filter and polyethersulfone membranes (pore size, 0.22 μm; diameter, 13 mm) were obtained from Nalge Nunc International (Rochester, NY). ^{125}I-Echistatin was purchased from PerkinElmer (Piscataway, NJ). c(RGDyK) was obtained from Peptides International (Louisville, KY). Semi-preparative reversed-phase HPLC using a Vydac protein and peptide column (218TP510; 5μm, 250 × 10 mm) was performed on a Dionex 680 chromatography system with a UVD 170U absorbance detector (Sunnyvale, CA) and model 105S single-channel radiation detector (Carroll & Ramsey Associates). HPLC conditions were as follows: with a flow rate of 4 mL/min, the mobile phase was maintained at 95% solvent A [0.1% trifluoroacetic acid (TFA) in water] and 5% B [0.1% TFA in acetonitrile (MeCN)] from 0–2 min, and then was changed with a gradient from 95% solvent A and 5% B at 2 min to 35% solvent A and 65% solvent B at 32 min. Analytical HPLC had the same gradient with flow rate of 1 mL/min using a Vydac protein and peptide column (218TP510; 5 μm, 250 × 4.6 mm). The UV absorbance was monitored at 218 nm and the identification of the peptides was confirmed by a LTQ FT mass spectrometer (Thermo Scientific). MicroPET scans were performed on a microPET R4 rodent model scanner

(Siemens Medical Solutions USA, Inc., Knoxville, TN). The scanner has a computer-controlled bed and 10.8-cm transaxial and 8-cm axial fields of view (FOVs). It has no septa and operates exclusively in the 3-dimensional (3D) list mode. Animals were placed near the center of the FOV of the scanner.

Chemistry

One-pot synthesis of 3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)aniline (6)

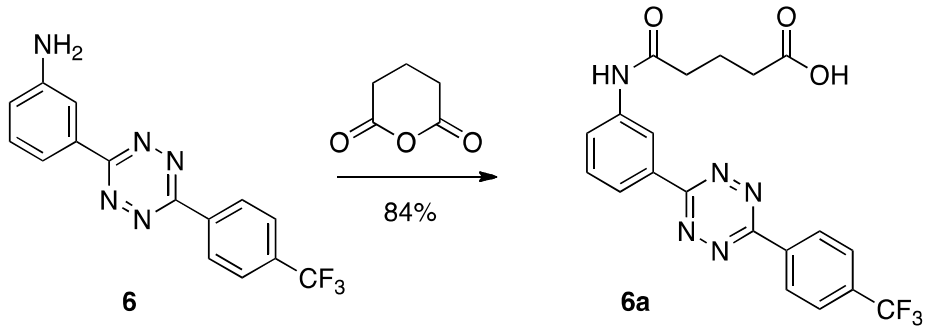


This procedure was adapted from the literature.[1] The reaction was carried out behind a blast shield. A 50 mL single neck round-bottomed flask was charged with 3-aminobenzonitrile (1.2 g, 10 mmol), 4-(trifluoromethyl)benzonitrile (0.85 g, 5.0 mmol), zinc (II) triflate (0.27 g, 0.75 mmol) and hydrazine hydrate (2.6 g, 2.5 mL, 52 mmol) and the flask was fitted with reflux condenser and heated to 65 °C for 24 h under an atmosphere of nitrogen. The reaction mixture was cooled to r.t. and 20 mL of ice cold water and 20 mL dichloromethane were added, and then filtered on a Büchner funnel and rinsed with dichloromethane (100 mL) followed by diethyl ether (3 x 20 mL). A yellow solid, which was mainly the dihydrotetrazine precursor to **6** and symmetrical dihydrotetrazines, was filtered and reserved. The filtrate was diluted with dichloromethane (150 mL) and washed with brine solution (3 x 100 mL), dried over anhydrous magnesium sulfate and concentrated. The residue was dissolved in dichloromethane (200 mL) and phenyliodonium diacetate (2.4 g, 7.5 mmol) was added. After stirring for 15 mins the

reserved solid from the earlier Büchner funnel filtration was added into the reaction mixture and the heterogeneous mixture was stirred for 8 h. The reaction mixture was absorbed onto silica gel and purified by column chromatography using a gradient of 100% hexanes to 100% dichloromethane to give the title compound (0.37 g, 1.2 mmol, 24%) as a red solid. An identical experiment provided 0.46 g (1.5 mmol, 29%). The spectral data for the title compound matches with the literature report.[2]

^{1}H NMR (DMSO-d_6, 400 MHz, δ): 8.72 (d, *J* = 8.2 Hz, 2H) 8.08 (d, *J* = 8.2 Hz, 2H), 7.82 (t, *J* = 2 Hz, 1H), 7.75-7.69 (m, 1H), 7.33 (t, *J* = 7.8 Hz, 1H), 6.92-6.86 (m, 1H), 5.59 (s br, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 163.8 (u), 162.5 (u), 149.8 (u), 136.0 (u), 132.1 (u), 132.0 (u) [q, ^{2}J(C-F) = 36.9 Hz], 130.1 (dn), 128.3 (dn), 126.4 (dn) [q, ^{3}J(CF) = 3.6 Hz], 124.1 (u)[q, ^{1}J(CF) = 266 Hz], 118.3 (dn), 115.3 (dn), 112.4 (dn).

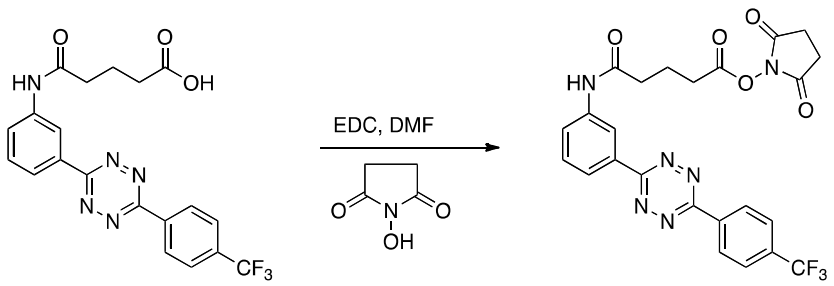
5-oxo-5-(3-(6-4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenylamino) pentanoic acid (6a)



This procedure was adapted from the literature.[2] A 50 mL round bottomed flask was charged with 3-(3-aminophenyl)-6-[4-(trifluoromethyl)phenyl]-*s*-tetrazine **6** (370 mg, 0.315 mmol), glutaric anhydride (180 mg, 1.58 mmol) and THF (20 mL). The reaction mixture was refluxed under nitrogen atmosphere with stirring for 4 h. The reaction mixture was cooled to rt, and filtered, residue was then rinsed with CH_2Cl_2 (3 x 10 mL) and dried to give the title compound (423 mg, 84%) as a pink solid. The spectral data of the title compound matches the literature report.[2]

^{1}H NMR (DMSO-d_6, 400 MHz, δ): 10.4 (s, 1H), 8.92-8.88 (m, 1H), 8.72 (d, *J* = 8.2 Hz, 2H), 8.24-8.19 (m, 1H) 8.07 (d, *J* = 8.2 Hz, 2H), 7.94-7.88 (m, 1H), 7.61 (t, *J* = 8.2 Hz, 1H), 2.41 (t, *J* = 7.1 Hz, 2H), 2.26 (t, *J* = 7.4 Hz, 2H), 1.83 (quin., *J* = 7.0 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz, δ): 174.5 (u), 171.5 (u), 163.5 (u), 162.6 (u), 140.5 (u), 135.9 (u), 132.1 (u), 132.1 (u) [q, ^{2}J(C-F) = 34.5 Hz], 130.1 (dn), 128.4 (dn), 126.4 (dn) [q, ^{3}J(CF) = 4.0 Hz], 124.3 (u)[q, ^{1}J(CF) = 275 Hz], 123.1 (dn), 122.3 (dn), 118.0 (dn) ,35.8 (u) 33.8 (u), 20.8 (u).

2,5-dioxopyrrolidin-1-yl 5-oxo-5-((3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenyl)amino)pentanoate (6b)



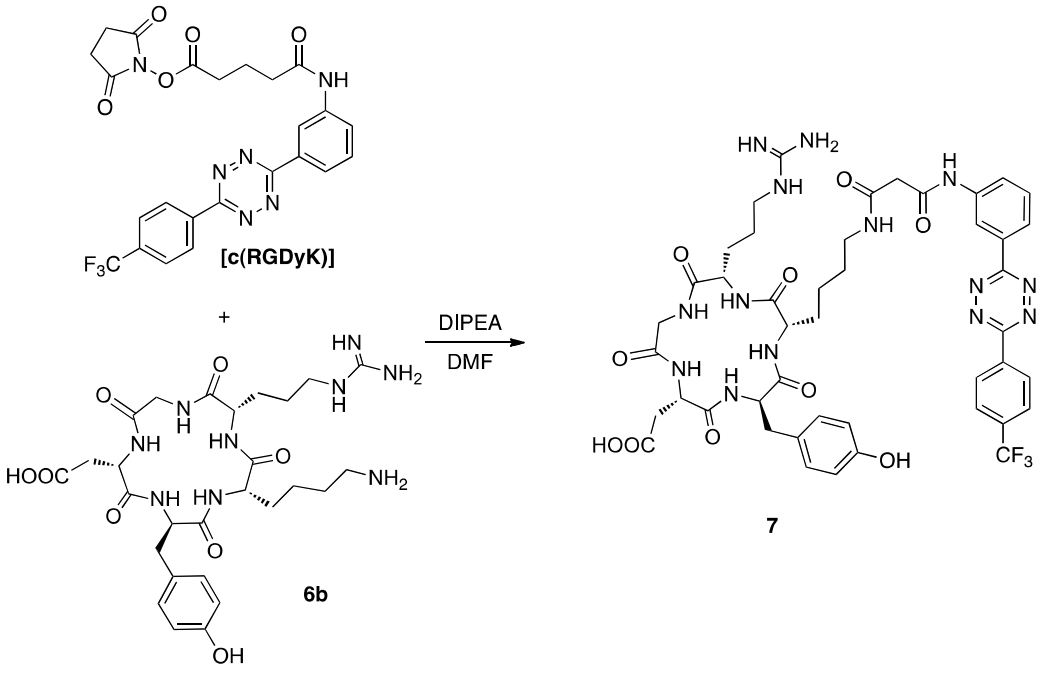
6a **6b**

A dry 3 mL vial was sequentially charged with **6a** (86 mg, 0.20 mmol), *N*-hydroxysuccinimide (46 mg, 0.40 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (77 mg, 0.40 mmol). The vial was capped by a screw cap with a teflon septum. The vial was swept with nitrogen, and anhydrous DMF (1.5 mL) was added via syringe. The reaction mixture was allowed to stir for 34 h at room temperature. The mixture was then diluted by CH_2Cl_2 (10 mL), centrifuged, and the supernatant was decanted. The purple solid was subjected to three further cycles of suspension in CH_2Cl_2 (10 mL), centrifugation, and decantation to provide the title compound as a purple solid (79 mg, 75%).

mp: 238 - 241 °C; ^{1}H NMR (400 MHz, DMSO-d_6, δ): 10.35 (s, 1H), 8.92 (t, 2.0 Hz, 1H), 8.74 (d, *J* = 8.0 Hz, 2H), 8.26-8.24 (m, 1H), 8.09 (d, *J* = 8.0 Hz, 2H), 7.92-7.95 (m, 1H), 7.64 (t, *J* =

8.0 Hz, 1H), 2.88 (s, 4H), 2.81 (t, J = 7.2 Hz, 2H), 2.54 (t, J = 7.2 Hz, 2H), 1.98 (quin, J = 7.2 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6, δ): 170.8 (C), 170.3 (C), 168.9 (C), 163.5 (C), 162.7 (C), 140.3 (C), 135.9 (C), 132.2 (C), 132.1 (C, q, ^{2}J(CF) = 32.4 Hz), 130.1 (CH), 128.4 (CH), 126. 4 (CH, q, ^{3}J(CF) = 3.8 Hz), 124.0 (C, q, ^{1}J(CF) = 271 Hz), 123.1 (CH), 122.4 (CH), 118.0 (CH), 34.7 (CH_2), 29.6 (CH_2), 25.5 (CH_2), 20.0 (CH_2); HRMS (LIFDI-TOF) (*m/z*) $[M]^+$: calcd for $[C_{24}H_{19}F_3N_6O_5]^+$ 528.1364; found: 528.1370.

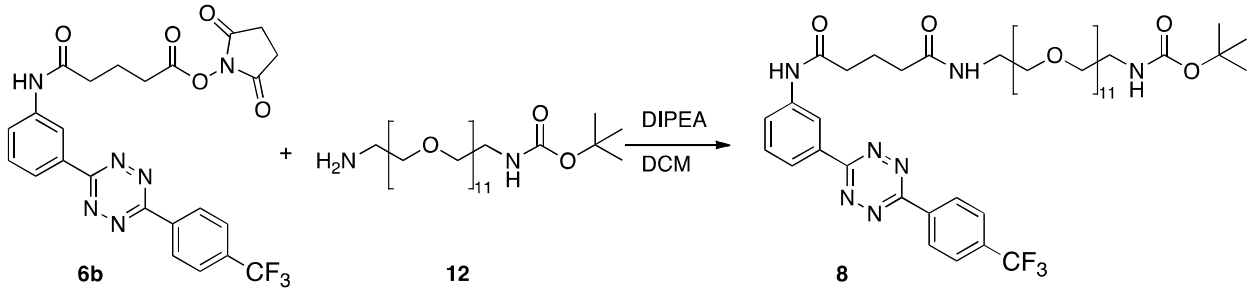
Tetrazine-RGD conjugate (7)



A dry 3 mL vial was charged with the above NHS-ester (10.6 mg, 0.020 mmol) **(6b)** and c(RGDyK) (6.2 mg, 0.010 mmol). The vial was capped by a screw cap with a teflon septum, and the vial was swept with nitrogen. Diisopropylethylamine (5.2 mg, 0.040 mmol, 7.0 □L) was added via syringe, followed by the addition of DMF (0.50 mL). The reaction mixture was allowed to stir for 18 h at room temperature. The mixture was then centrifuged and the

supernatant was decanted. The purple solid was dissolved in 0.8 mL of DMSO, centrifuged and the supernatant liquid was purified by reverse phase HPLC (Phenommenex C18 column, 4.6 mm x 250 mm, 6micron) using 50% acetonitrile/water that contained 0.1% formic acid as an eluant, eluting at the rate of 1.5 mL/min, to provide **3** as a purple solid (2.2 mg, 21%). The purity was judged to be 98% by HPLC analysis (Phenomenex C-18 column, 4.6 mm x 250 mm, 6 micron). HPLC analysis was performed with 50% acetonitrile/water that contained 0.1% formic acid as an eluant, eluting at the rate of 1.0 mL/min. HRMS-ESI (*m/z*) $[M+H]^+$: calcd for $C_{47}H_{56}F_3N_{14}O_{10}^+$, 1033.4256; found: 1033.4250.

***tert*-Butyl (37,41-dioxo-41-((3-(6-(4-(trifluoromethyl)phenyl)-1,2,4,5-tetrazin-3-yl)phenyl)amino)-3,6,9,12,15,18,21,24,27,30,33-undecaoxa-36-azahentetracontyl)carbamate (8)**



A dry 3 mL vial was charged with **6b** (7.0 mg, 0.013 mmol) and **12** (16.6 mg, 0.026 mmol). The vial was capped by a screw cap with a teflon septum, and the vial was swept with nitrogen. Diisopropylethylamine (8.3 mg, 0.065 mmol, 11 μL) was added via syringe, followed by the addition of CH_2Cl_2 (5 mL). The reaction mixture was allowed to stir for 16 h at room temperature. The mixture was then purified by column chromatography (0-5 % MeOH in CH_2Cl_2) to provide **8** as a purple waxy solid (9.4 mg, 68%).

^{1}H NMR (400 MHz, $CDCl_3$, δ): 9.18 (s, 1H), 8.80 (s, 1H), 8.79 (d, *J* = 8.0 Hz, 2H), 8.36 (d, *J* = 7.8 Hz, 1H), 8.08 (d, *J* = 8.3 Hz, 1H), 7.89 (d, *J* = 8.0 Hz, 2H), 7.57 (t, *J* = 8.0 Hz, 1H), 6.71 (br s, 1H), 5.11 (br s, 1H), 3.72 – 3.55 (m, 42H), 3.56 – 3.44 (m, 4H), 3.38-3.26 (m, 2H), 2.54 (t, *J* = 6.8 Hz, 2H), 2.37 (t, *J* = 6.8 Hz, 2H), 2.10 (p, *J* = 6.8 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 173.1 (C), 171.8 (C), 164.2 (C), 163.1 (C), 155.9, 139.9 (C), 135.1 (C), 134.1(C, q, ^{2}J(CF) = 32.6 Hz), 132.0 (C), 130.1 (CH), 128.3 (CH), 126.3 (CH, q, ^{3}J(CF) = 3.7 Hz), 124.2 (CH), 123.8 (C, q, ^{1}J(CF) = 271 Hz), 123.3 (CH), 118.9 (CH), 70.5 (CH_2), 70.2 (CH_2), 69.7 (C), 40.3 (CH_2), 39.3 (CH_2), 36.1 (CH_2), 35.0 (CH_2), 28.4 (CH_3), 21.9 (CH_2). HRMS (LIFDI-TOF) (*m/z*): $[M+Na]^+$: calcd for $[C_{49}H_{74}F_3N_7O_{15}Na]^+$ 1080.5088; found: 1080.5077.

The synthesis of **10**

^{19}F-**2** (378 μg, 2.2 μmol in acetonitrile) was added to the DMSO solution of compound **7** (2 mg, 1.9 μmol). The reaction mixture was vigorously vortexed for 1 min at room temperature, which was diluted with 1 ml water. The product was purified by semi-preparative HPLC, which was then lyophilized to afford **10** as off-white powder (2.2 mg, yield >99%).

The synthesis of ^{18}F-**10**

^{18}F-**2** was synthesized as reported. Approximately 2 mCi ^{18}F-**2** in 50–100 μL ethanol was added to 10 μg compound **7**. The reaction mixture was vigorously vortexed for 1 min at room temperature. The reaction mixture was diluted with 1 ml water before semi-preparative HPLC purification. The solvent in the collected HPLC fractions was removed under reduced pressure. ^{18}F-**10** was reconstituted with 1 × PBS and used for in vitro and in vivo study after passing through 0.22 μm filter.

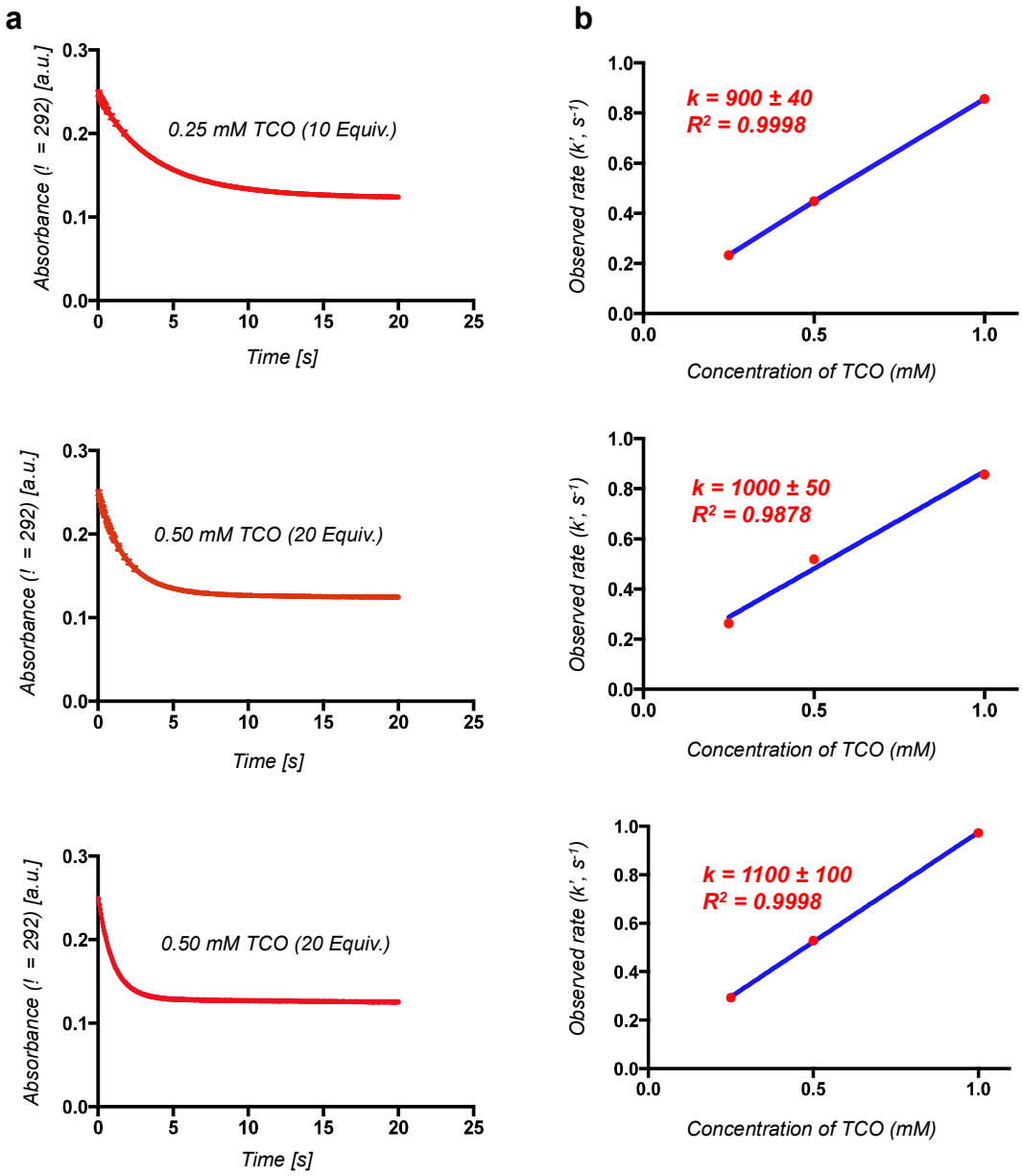


Figure S1 (a) The exponential plot of the reaction of **8** (25 μM) and **9** (1 mM, 0.50 mM and 0.25 mM) in 40:60 MeOH:water monitored at 292 nm. Data was recorded for 20 s at 298 K, with triplicate runs on three independent samples at three different concentrations (27 runs total). (d) The observed rates k' vs concentration of **9** of triplicate sets of measurements using stopped-flow kinetics for the reaction between **8** and **9.** Under these pseudo-first order conditions the mean second order rate constant (k_2) was determined by nonlinear regression to be 1000 +/- 100 $M^{-1}s^{-1}$.

Metabolic Stability

The metabolic stability of ^{18}F-RGD conjugate **10** was evaluated in an athymic nude mouse bearing a U87MG tumor. The procedure was as reported previously.[3] Briefly, 2 h after the intravenous injection of 7.4 MBq of ^{18}F-**10**, the mouse was sacrificed, and the organs of interest were harvested. The blood was collected immediately and centrifuged for 5 min at 14,000 rpm. Then, 50 μL TFA in 100 μL of PBS was added to the upper serum solution, followed by mixing and centrifugation for 5 min. The supernatant was then taken for HPLC analysis. The liver, kidneys, and tumor were homogenized using a homogenizer, suspended in 1 mL of PBS buffer, and then centrifuged for 5 min at 14,000 rpm. For each sample, to the supernatant was added 50 μL TFA in 100 μL PBS, followed by centrifugation for 5 min. The supernatant was then taken for HPLC analysis. The eluent was collected with a fraction collector (1.0 min/fraction), and the radioactivity of each fraction was measured with a gamma counter.

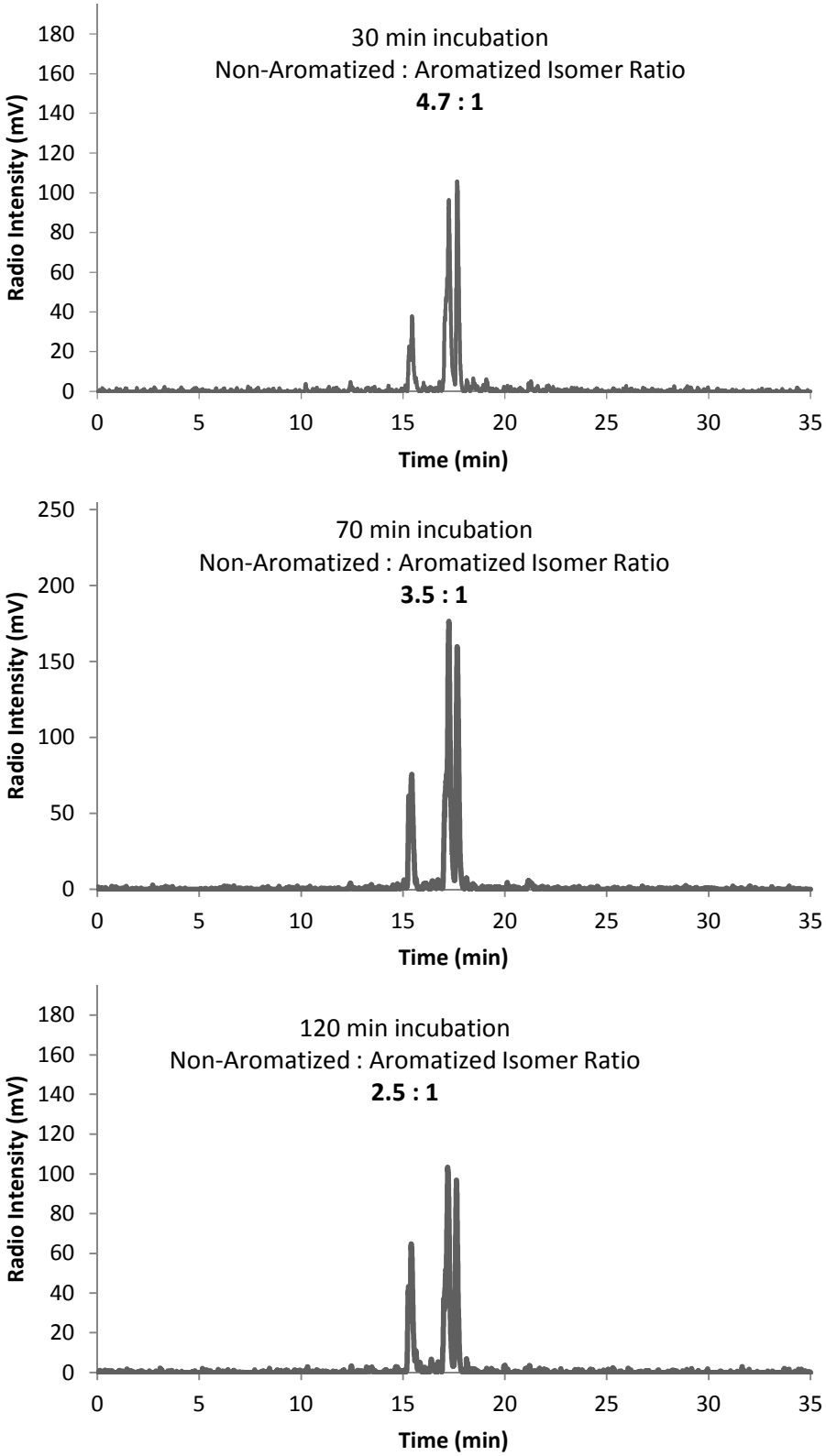


Figure S2 PBS stability study of ^{18}F-**10.**

References

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