Supporting information

^{18}F-positron emitting/trimethine cyanine-fluorescent contrast for image-guided prostate cancer management

Harikrishna Kommidi[1]‡, Hua Guo[1]‡, Fuad Nurili[2], Yogindra Vedvyas[1], Moonsoo M. Jin[1], Timothy D. McClure[3], Behfar Ehdaie[4], Haluk Sayman[5], Oguz Akin[2], Omer Aras[2], Richard Ting[1*].*

1. Department of Radiology, Molecular Imaging Innovations Institute, Weill Cornell Medicine, New York, NY 10065, USA.

2. Department of Radiology, Memorial Sloan Kettering Cancer Center, New York, NY, 10065, USA.

3. Department of Urology, Weill Cornell Medicine, New York, NY 10065, USA.

4. Urology Service, Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, 10065, USA.

5. Department of Nuclear Medicine, Istanbul University, Cerrahpasa Medical Faculty, Fatih, Istanbul, 34303, Turkey.

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1 Cell culture and Cell line authentication and verification of PSMA expression

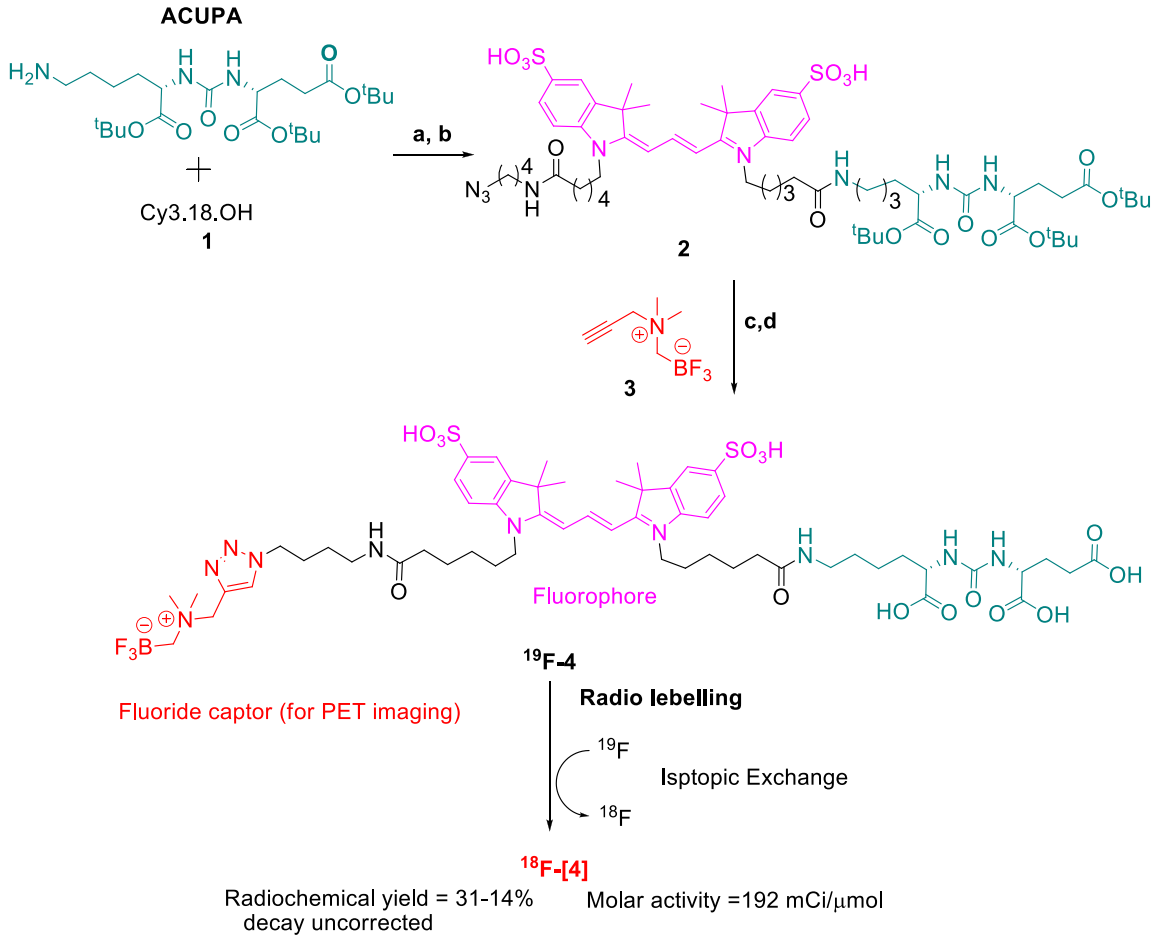
A PSMA-negative prostate cancer cell line (PSMA-, PC3, ATCC) and a PSMA-positive prostate cancer cell line (PSMA+, PC3-PIP, gift from Memorial Sloan Kettering Cancer Center) are transduced (lentivirus) to express Firefly Luciferase-F2A and green fluorescent protein genetic markers. Cells are cultured in RPMI 1640 medium (Corning, USA) supplemented with 10 % fetal bovine serum (FBS, Seradigm, USA) and 100 U/mL penicillin/streptomycin (Gibco, USA) at 37 °C in a humidified incubator. A 1.5x10^{5} quantity of PC3 and PC3-PIP cells, incubated with human-IgG monoclonal PSMA antibody (J591, gift from Dr. Neil Bander, 1:500 in PBS) on ice for 30 min, are mixed with an anti-human-IgG allophycocyanin-conjugated secondary antibody (1:500 in PBS, Cat#4093065, BioLegend, USA). Primary antibodies are labeled for 30 min at 4°C. Fluorescence assisted cell sorting is used to quantitate cells (FACS, Gallios, Beckman Coulter, USA). Collected data was analyzed using Beckman Coulter Kaluza software.

1.1 Procedure for quantitation of PSMA specific [^{19}F]-4 affinity and quantification of EC_{50} by fluorescence.

[^{19}F]-**4** EC_{50} (half-maximal response) cellular affinity is calculated by FACS mean fluorescence intensity and confirmed in gamma counter scintillated radioligand binding experiments. A 1.5x10^{5} quantity of PC3-PIP and PC3 (control) cells are allowed to adhere at 37°C overnight (16h) to 12-well plates. Trypsinized PC3 or PC3-PIP cells are incubated with 0.1-100 nM of [^{19}F]-**4** for 1 h at 37°C. A 2x10^{4} quantity of cells, twice washed with phosphate buffered saline (PBS), are analyzed by FACS. FACS data are verified, qualitatively, in epifluorescent imaging experiments performed on a EVOS microscope (Life Technologies, USA), performed at 0.1 and 10 µM [^{19}F]-**4**.

2.1 Synthetic details

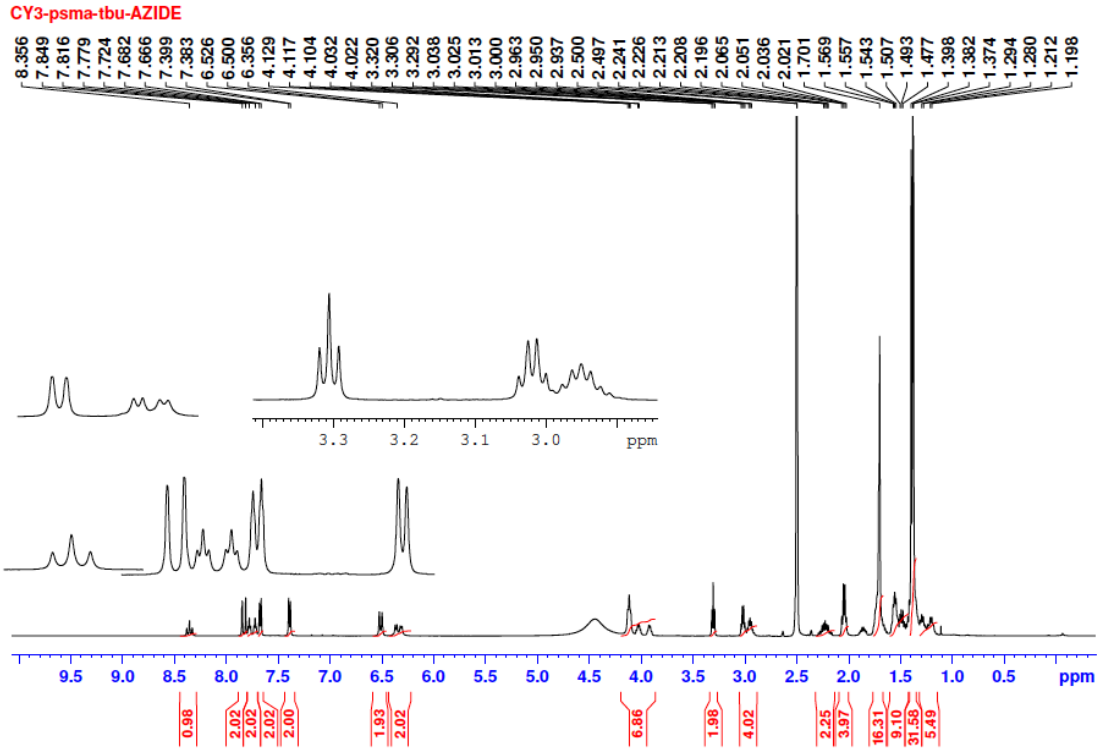
[^{18}F]-**4** was synthesized according to the following scheme:



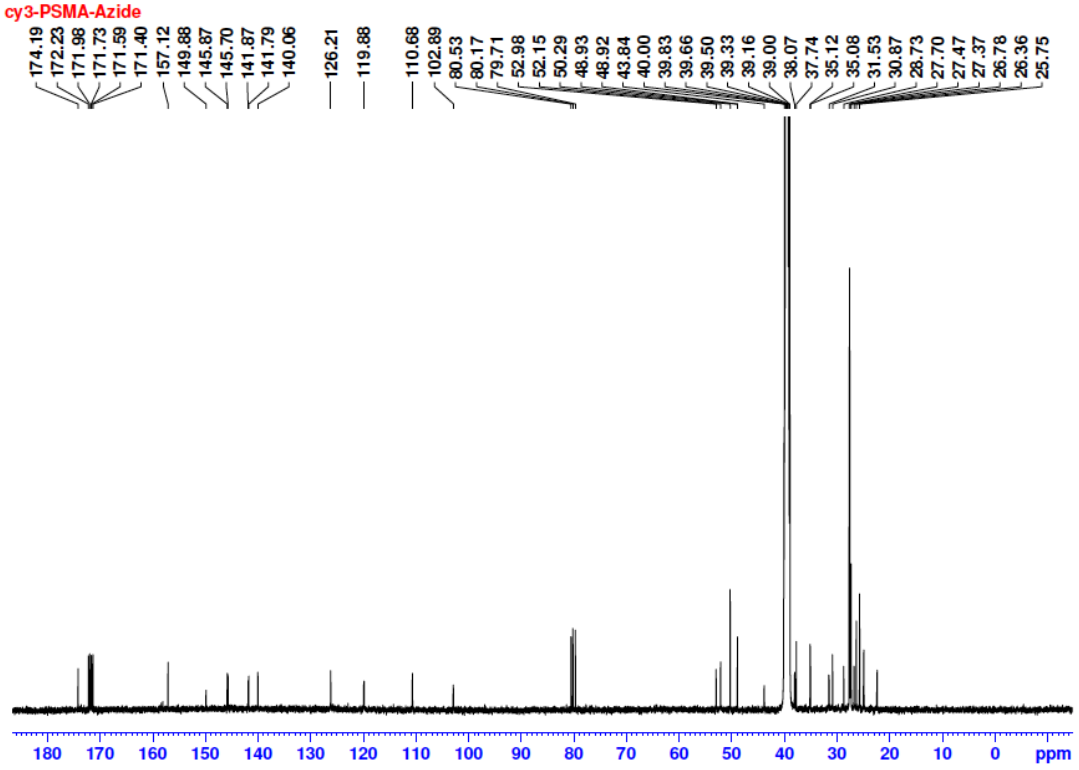
Reagent and Conditions: a) 1) 4.0 eq. EDC.HCl, 2.5 eq. HOBt, 2.5 eq. Pyridine, DMF, RT, N_2, 6h, 2) 1.2 eq 1-Azido butylamine, RT, 2 h; b) 1) 0.5 mL TFA, 1h, 2) 1.0 eq. 1M $CuSO_4.5H_2O$, 2.0 eq 1M Ascorbic acid, DMF, RT, 3h; **Radiolabeling:** c) 1M Pyridazine-HCl, pH = 2.5, ≤ 50 mCi aqueous [^{18}F]-fluoride ion (Specific concentration > 1.5 Ci/mL).

2.2 Characterization of (2)

2.2.1 1H NMR (DMSO-d6, 500 MHz, 21 °C)



2.2.2 ^{13}C NMR (DMSO-d6, 125 MHz, 21 °C)



2.2.3 HRMS (ESI)-

HRMS (ESI)$^-$: m/z calculated for [M]: $C_{63}H_{95}N_9O_{15}S_2$: 1281.6389; [M-H]$^-$: $[C_{63}H_{94}N_9O_{15}S_2]^-$: 1280.6311, found: 1280.6286 (Δ 2 ppm); [M-2H]$^{2-}$: $[C_{63}H_{93}N_9O_{15}S_2]^{2-}$: 639.8122, found: 639.8108 (Δ 2 ppm).

Qualitative Compound Report

Data File	RT_HK_170727_s01.d	**Sample Name**	HKCy3-PSMA-Az
Sample Type	Sample	**Position**	P1-F3
Instrument Name	HMS 6550 QToF	**User Name**	
Acq Method	by_FIA_MeOH.m	**Acquired Time**	7/27/2017 11:36:18 AM
IRM Calibration Status	Success	**DA Method**	by_Default.m
Comment			

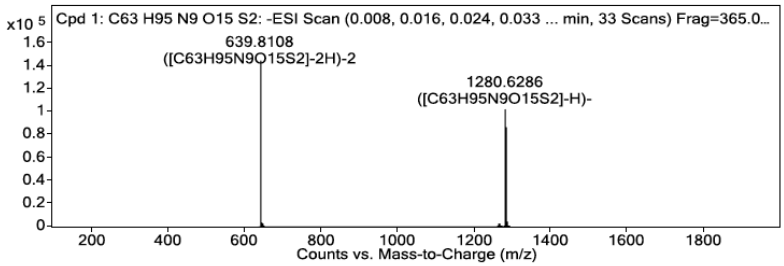
Sample Group	**Info.**
Acquisition SW	6200 series TOF/6500 series
Version	Q-TOF B.05.01 (B5125.3)

Compound Table

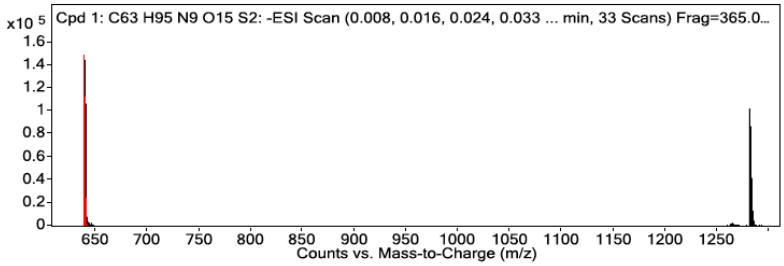
Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Diff (ppm)	MFG Formula	DB Formula
Cpd 1: C63 H95 N9 O15 S2	0.05	1281.6356	147611	C63 H95 N9 O15 S2	1281.6389	-2.59	C63 H95 N9 O15 S2	C63 H95 N9 O15 S2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 1: C63 H95 N9 O15 S2	639.8108	0.05	Find By Formula	1281.6356

MS Spectrum



MS Zoomed Spectrum

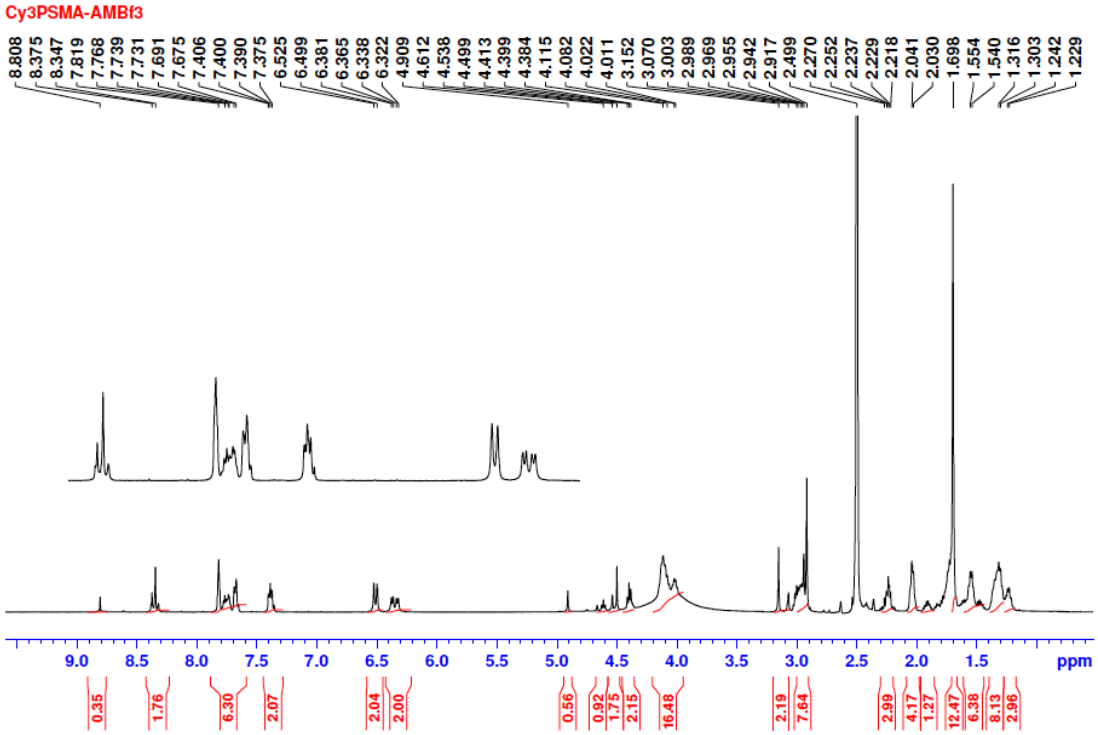


MS Spectrum Peak List

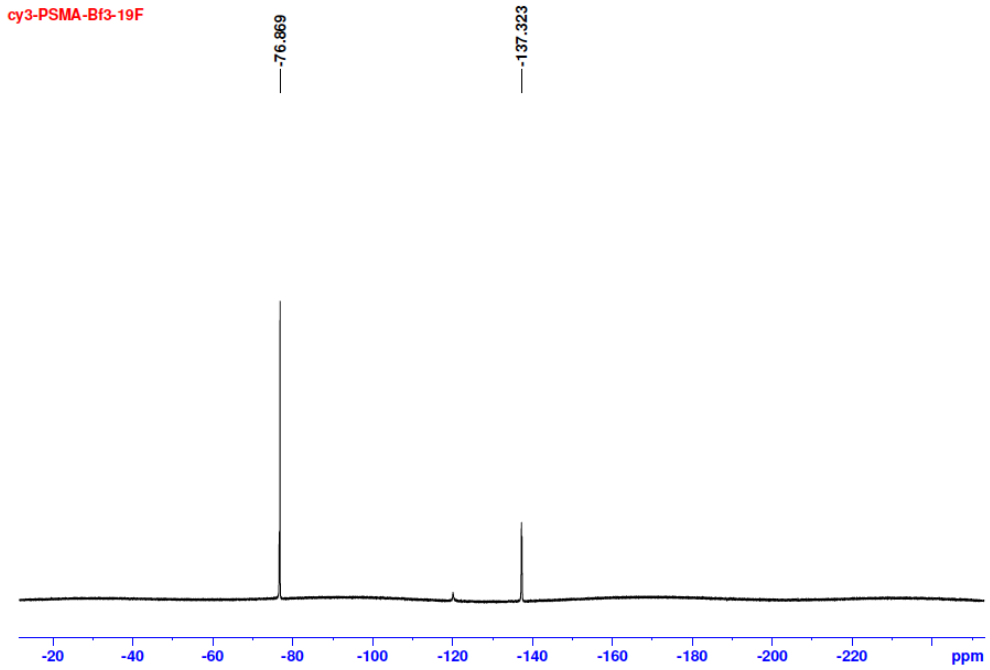
m/z	*Calc m/z*	Diff(ppm)	z	Abund	Formula	Ion
639.8108	639.8122	2.17	2	147611.03	C63H95N9O15S2	(M-2H)-2
640.3124	640.3137	2.01	2	108499.28	C63H95N9O15S2	(M-2H)-2
640.8126	640.814	2.21	2	60140.55	C63H95N9O15S2	(M-2H)-2
641.3131	641.3144	2.09	2	24277.42	C63H95N9O15S2	(M-2H)-2
641.8132	641.8148	2.51	2	8183.99	C63H95N9O15S2	(M-2H)-2
1263.5935	1263.6289	28	1	2180.27	C63H95N9O15S2	M-[-H2O]
1280.6286	1280.6316	2.39	1	102366.53	C63H95N9O15S2	(M-H)-
1281.6313	1281.6347	2.63	1	87969.03	C63H95N9O15S2	(M-H)-
1282.632	1282.6352	2.55	1	41222.05	C63H95N9O15S2	(M-H)-
1283.6323	1283.6361	2.96	1	13635.35	C63H95N9O15S2	(M-H)-

2.3 Characterization of (4)

2.3.1 ^{1}H NMR (DMSO-d6, 500 MHz, 21 °C)



1.3.2 ^{19}F NMR (vs. $CFCl_3$, 1x PBS pH 7.4, TFA reference, 470 MHz, 21 °C)



2.3.3 HRMS (ESI)-

HRMS $(ESI)^-$: m/z calculated for [M]: $C_{57}H_{82}BF_3N_{10}O_{15}S_2$: [M+2[-F]]: calculated 630.2770, found: 630.2765 (Δ 0.81 ppm).

Qualitative Compound Report

Data File	RT_HK_171002_s01.d	**Sample Name**	CY3-PSMABF3
Sample Type	Sample	**Position**	P1-B1
Instrument Name	HMS 6550 QToF	**User Name**	
Acq Method	by_FIA_ACN.m	**Acquired Time**	10/2/2017 12:48:41 PM
IRM Calibration Status	Success	**DA Method**	by_Default.m
Comment			

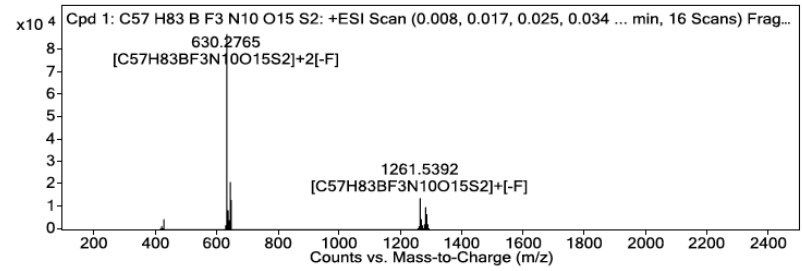
Sample Group	**Info.**
Acquisition SW	6200 series TOF/6500 series
Version	Q-TOF B.05.01 (B5125.3)

Compound Table

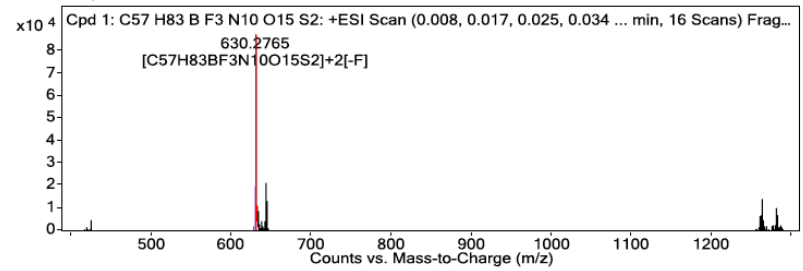
Compound Label	RT	Mass	Abund	Formula	Tgt Mass	Diff (ppm)	MFG Formula	DB Formula
Cpd 1: C57 H83 B F3 N10 O15 S2	0.034	1278.5512	86963	C57 H83 B F3 N10 O15 S2	1278.5562	-3.91	C57 H83 B F3 N10 O15 S2	C57 H83 B F3 N10 O15 S2

Compound Label	*m/z*	RT	Algorithm	Mass
Cpd 1: C57 H83 B F3 N10 O15 S2	630.2765	0.034	Find By Formula	1278.5512

MS Spectrum



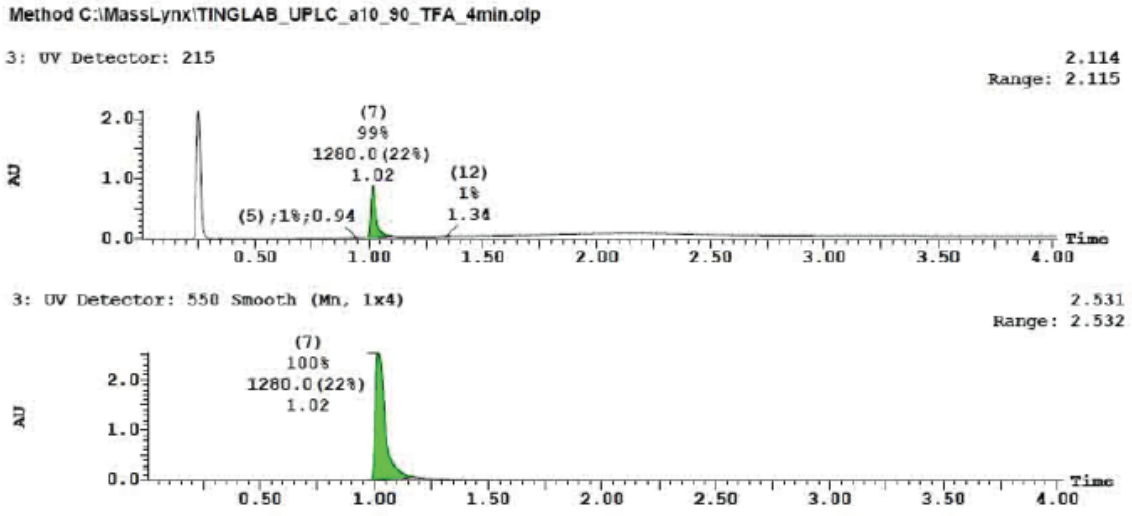
MS Zoomed Spectrum



MS Spectrum Peak List

m/z	*Calc m/z*	Diff(ppm)	z	Abund	Formula	Ion
420.8582	420.8599	4.11	3	57.51	C57H83BF3N10O15S2	(M+3H)+3[-F]
630.2765	630.277	0.81	2	86962.61	C57H83BF3N10O15S2	M+2[-F]
630.7777	630.7783	0.84	2	58680.43	C57H83BF3N10O15S2	M+2[-F]
631.2776	631.2783	1.25	2	27970.46	C57H83BF3N10O15S2	M+2[-F]
631.777	631.7787	2.68	2	10962.65	C57H83BF3N10O15S2	M+2[-F]
639.7905	639.7762	-22.29	2	99.74	C57H83BF3N10O15S2	M+2
1259.542	1259.5573	12.11	1	6464.77	C57H83BF3N10O15S2	M+[-F]
1261.5392	1261.5571	14.14	1	14388.94	C57H83BF3N10O15S2	M+[-F]
1262.54	1262.5572	13.62	1	9122.9	C57H83BF3N10O15S2	M+[-F]
1279.5484	1279.5635	11.81	1	10043.38	C57H83BF3N10O15S2	(M+H)+

2.3.4 UPLC absorbance and mass spectra of (4)



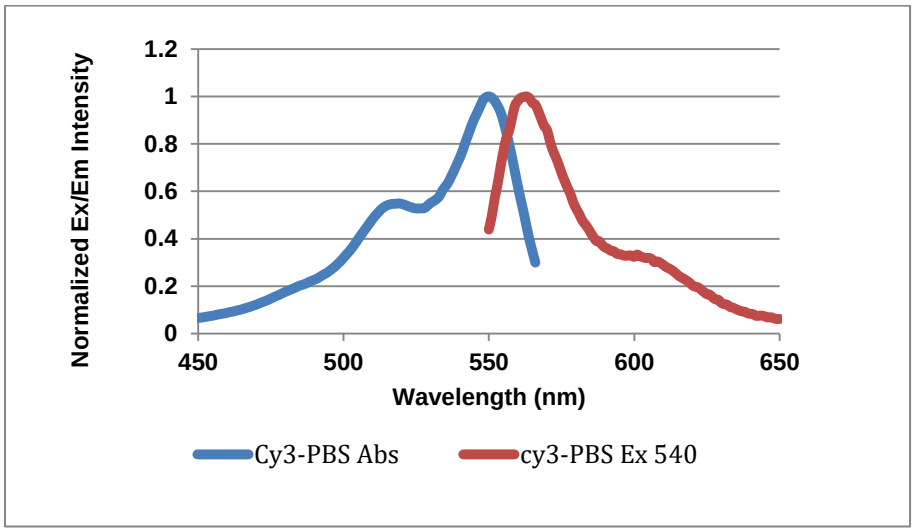
3. Radiochemical synthesis

Radiosynthesis (TOS = 0 min) was initiated with the addition of 15 to 20 µL of concentrated, but not fully evaporated [^{18}F]-fluoride-ion-containing water to [^{19}F]-**4**. Typically, ~35 mCi of [^{18}F]- water is added to a 1500 µL poly propylene tube containing 100 µg (78 nmol) of [^{19}F]-**4** in 10 µl of DMSO. The reaction was initiated with 10 µL of aqueous pyridazine HCl (1.25 M, pH 2.5). The resulting mixture is heated at 80-90°C for 25 min. The reaction is cooled to room temperature (end of synthesis, TOS = 25 min) by adding 1.0 mL of distilled water, which results in a suspension. The aqueous solution is loaded into a 10 mL syringe with an additional 7 mL of deionized water. The entire syringe containing 8.0 mL of crude solution and an additional 1.0 mL gap of air is flushed through a C-18 cartridge (waters Oasis HLB (30 mg) light cartridge, waters, #186005125) to separate [^{18}F]-**4** from contaminating [^{18}F]- fluoride ion. This is best achieved by placing the 10 mL syringe on a syringe pump that is placed perpendicular, and set to drive the syringe at a flow rate of 40 mL/hr. Following loading of [^{18}F]-**4** on the oasis cartridge, another 20 mL of deionized water is driven through the cartridge (using the syringe pump) to remove unreacted [^{18}F]- fluoride ion completely. Pure [^{18}F]-**4** is eluted from the cartridge and through a 0.2 micron sterile syringe filter using 3.0 mmol of 500 µL HCl/EtOH. The resulting solution (450 µL) was diluted 10 fold with 1x PBS (4.5 mL to give a neutral pH 7.0 solution, ready for in vivo use). We highly recommend the use of a syringe pump to automate [^{18}F]-**4** purification to avoid [^{18}F]-fluoride ion contamination that is sometimes observed upon manual preparation. We generally dilute PBS solutions of [^{18}F]-**4** 10-fold, to concentrations of ~1.5 mCi/mL for in vivo mouse use.

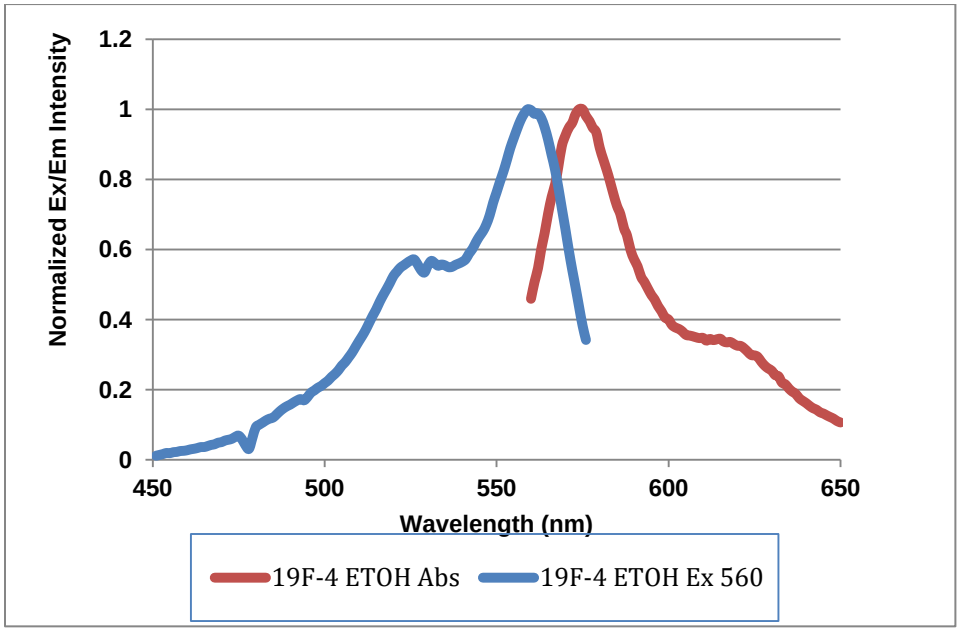
4. Fluorescence properties of (4)

[^{19}F]-**4** is fluorescent. The normalized excitation and emission spectra of [^{19}F]-**4** and a Cy3.18.OH (reference) measured in PBS (1 mM, pH 7.4), and EtOH are shown below and in table 1.

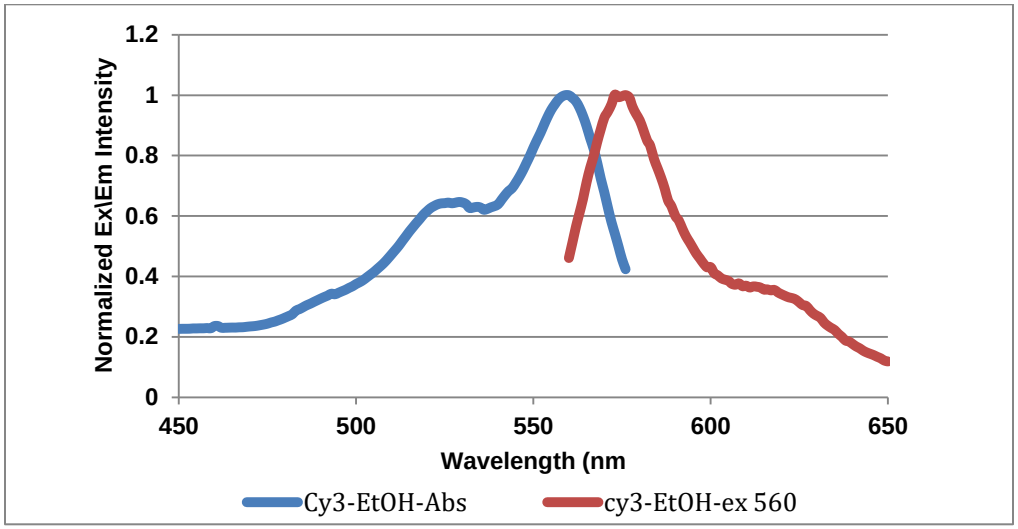
4.1 Fluorescence spectrum of CY3.18.OH in 1 mM phosphate buffered saline, pH 7.4



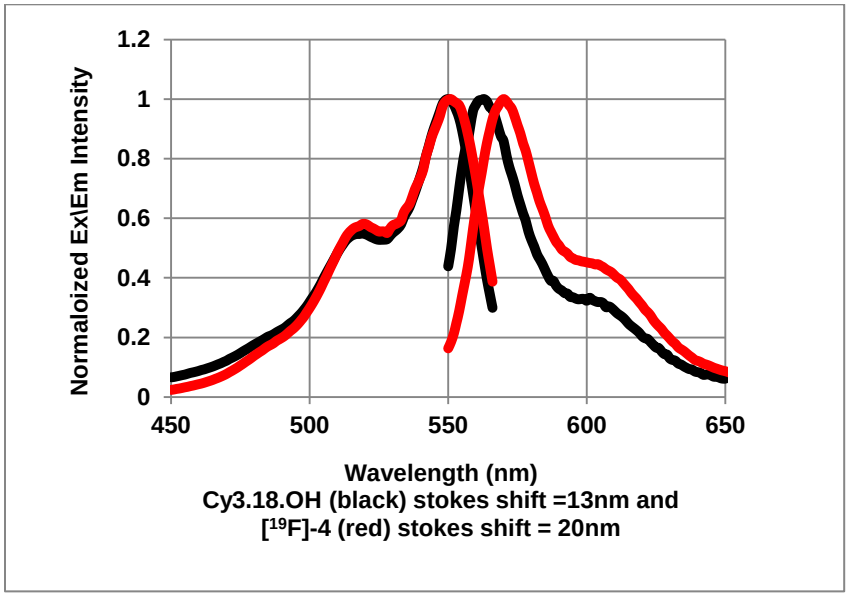
4.2 Fluorescence spectrum of (4) in EtOH,



4.3 Fluorescence spectrum of CY3.18.OH in EtOH.



4.4 Excitation and emission spectrum of (4) in 1 mM phosphate buffered saline, pH 7.4

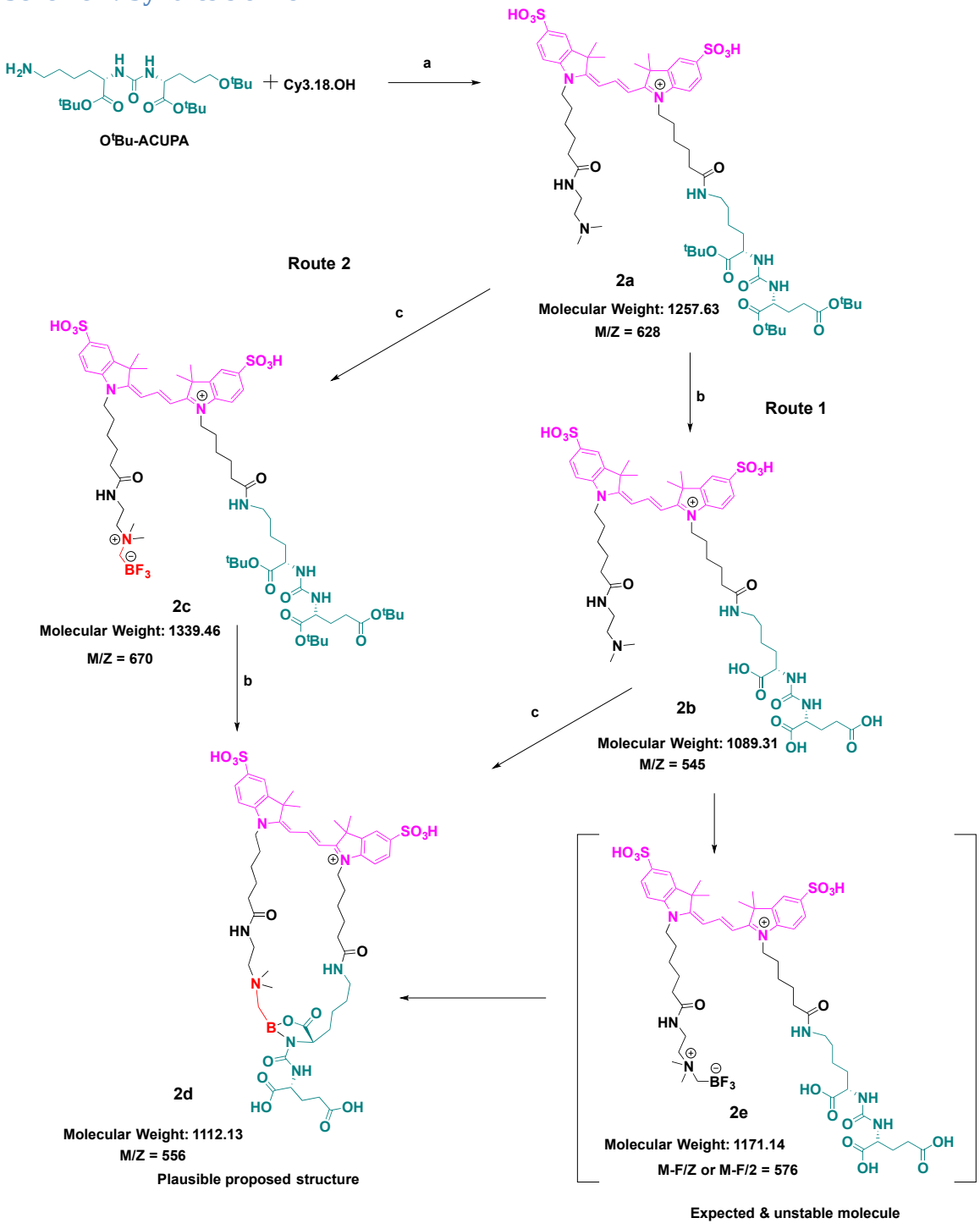


Absorption and emission spectra of 2.0 µM solutions of Cy3.18.OH (black) and [^{19}F]-**4** (red) in PBS, as measured on a Cary 60 absorption spectrometer and Cary eclipse spectrophotometer.

5. Failed attempts towards the synthesis of a triazole-free derivative of (4) through N,N-dimethylethylenediamine alkylation.

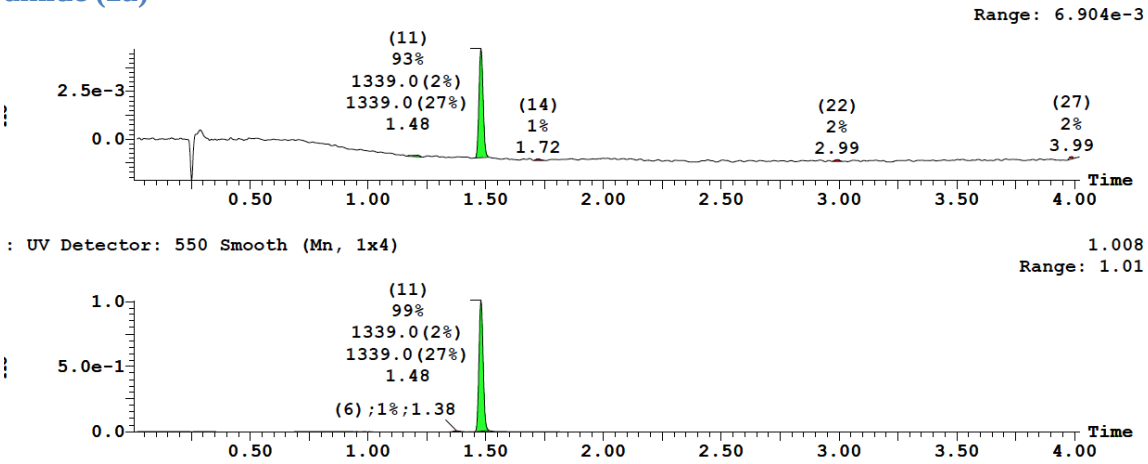
Cy3.18.OH (1.0 mole) was reacted with 1 mole of tert-butyl protected (O^tBu)-glutamate–urea-lysine in presence of EDC.HCl and HOBt for 5 hours at room temperature to generate a Cy3-(O^tBu)-glutamate–urea-lysine intermediate. *In situ* addition of 1 mole of *N*,*N*-dimethylethylenediamine and additional EDC.HCl gives (O^tBu)glutamate–urea–lysine-Cy3-amide **2a**, which was purified by preparative HPLC. We attempted to synthesize target **2e** from precursor **2a** two ways (routes 1 & 2, Scheme 2). Tert-butyl ester deprotection of **2a** with neat trifluoroacetic acid gave **2b**. Attempted alkylation of **2b** in the same pot with bromomethylboronic acid pinacol ester, followed by HF/KHF_2 workup did not give **2e**. Alternatively, N- alkylation of **2a** with bromomethylboronic acid pinacol ester and fluoride workup resulted in the desired trifluoroborate **2c**. Unfortunately, tert-butyl ester deprotection of **2c** with neat trifluoroacetic acid did not give **2e**. Both strategies failed to give desired **2e**. Instead, LC-MS analysis show the formation of a single product with the same retention time (1.38 min) and mass (556 m/z) (Fig. 4.4). This product, **2d**, does not undergo isotopic exchange radiolabeling when subject to reported ammoniomethyl trifluoroborate aqueous radiolabeling conditions. Characterization of the failed scheme to give **2e**, and the unexpected molecule is shown below.

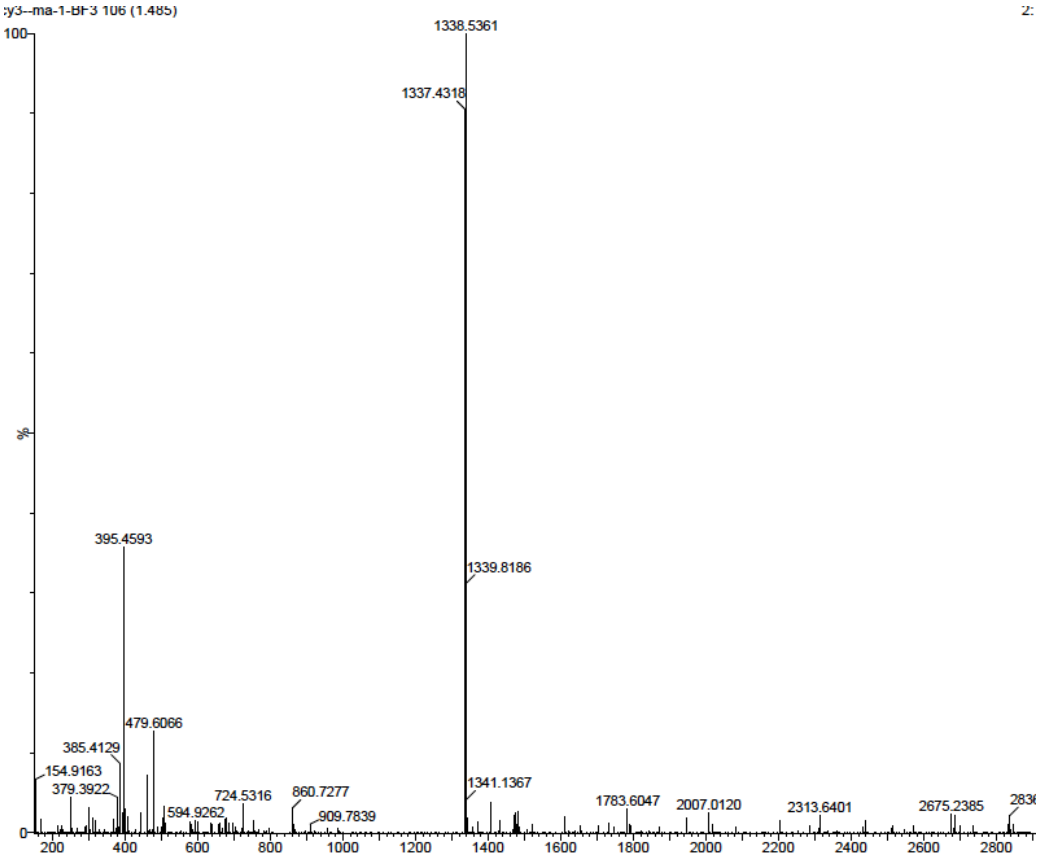
Scheme 2: Synthesis of 2e



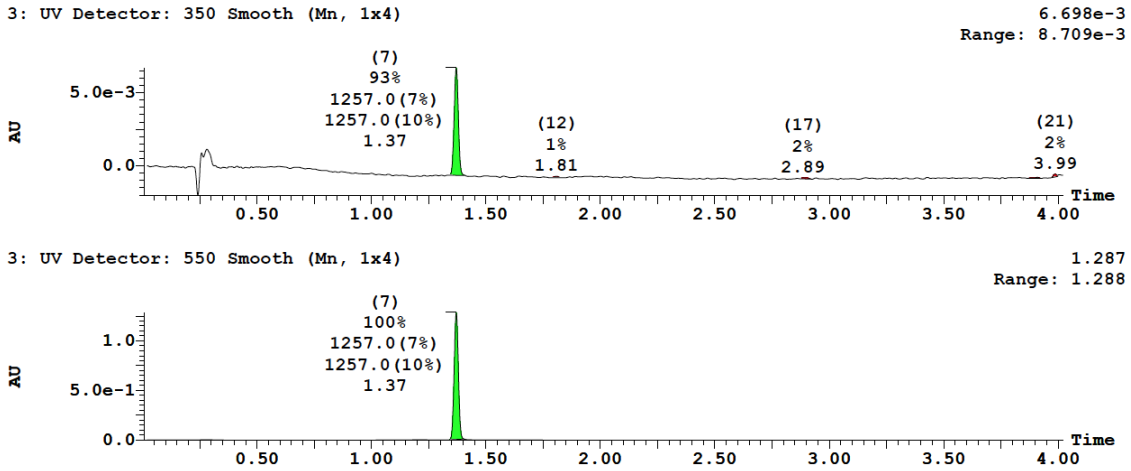
Reagent and Conditions: a) 1) 4.0 eq. EDC.HCl, 2.5 eq. HOBt, 2.5 eq. Pyridine, DMF, RT, N_2, 5h, 2) 1.2 eq *N*,*N*-dimethylethylenediamine, RT, 2 h; b) 0.5 mL TFA, c) 1) 1h, 1.1 equiv of bromomethylboronic acid pinacol ester, DIPEA, DMF/THF (2:1), rt, (2) 3 M KHF2, 1 M HCl, 0 °C to rt, 1 h.

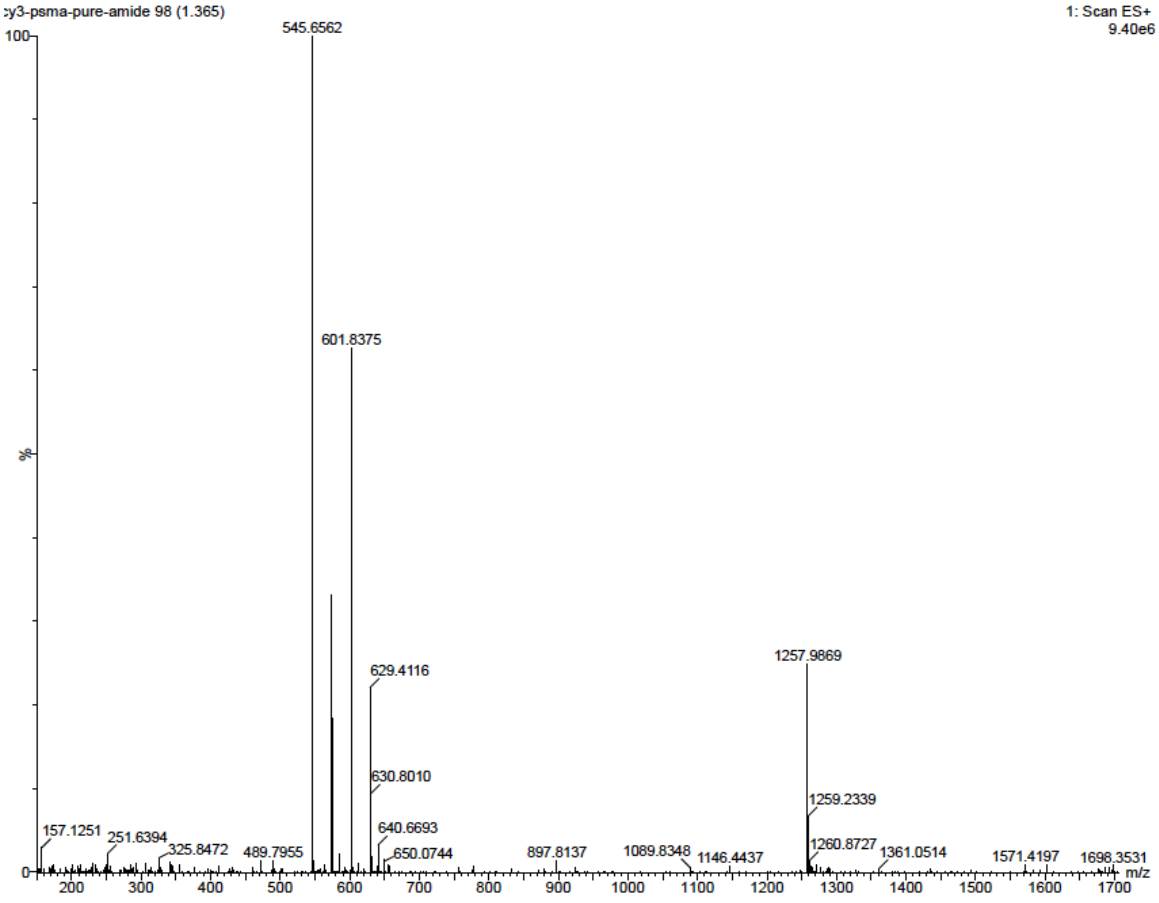
5.1 UPLC fluorescence and mass spectra of (O^{t}Bu)glutamate–urea– lysine-Cy3-amide (2a)



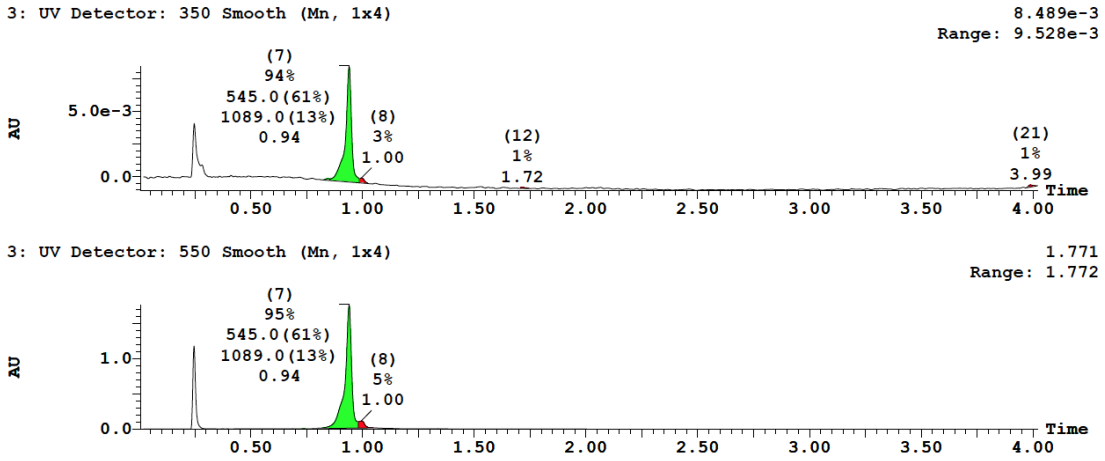


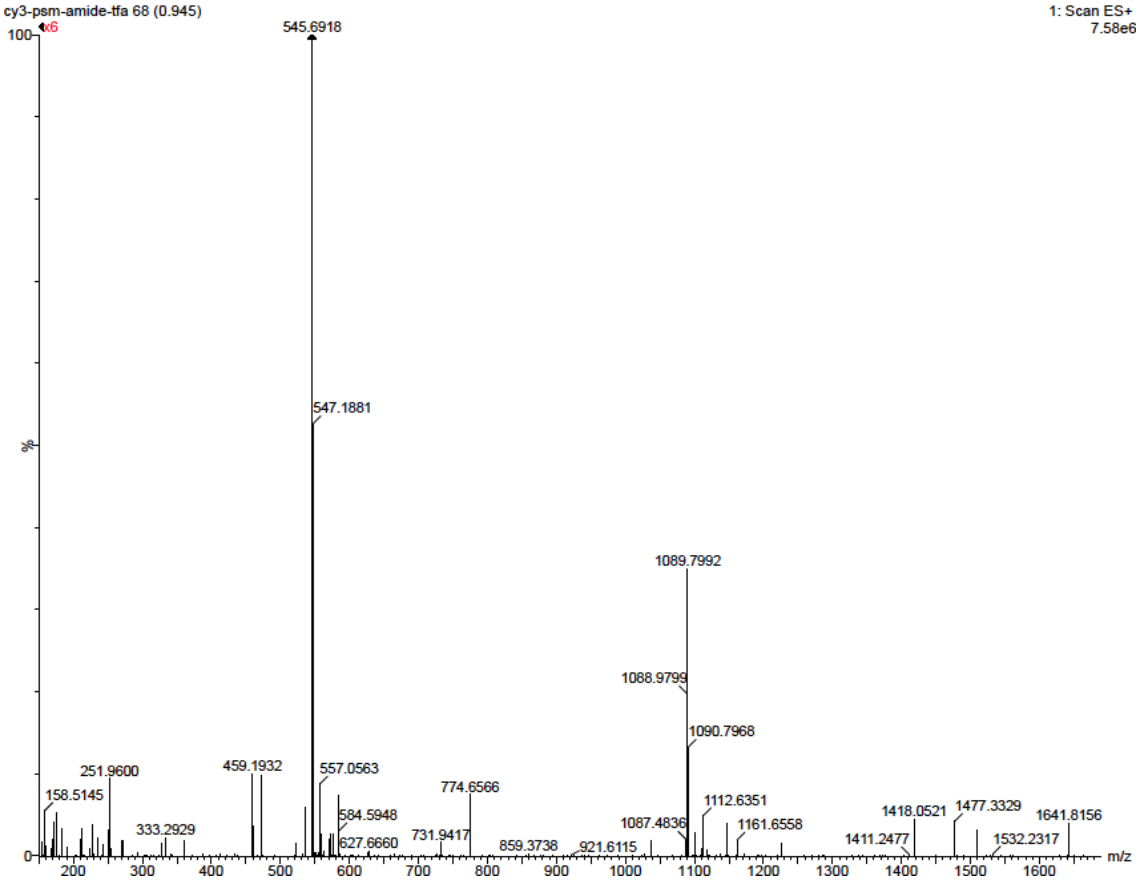
5.2 UPLC fluorescence and mass spectra of glutamate–urea– lysine-Cy3-amide (2b)



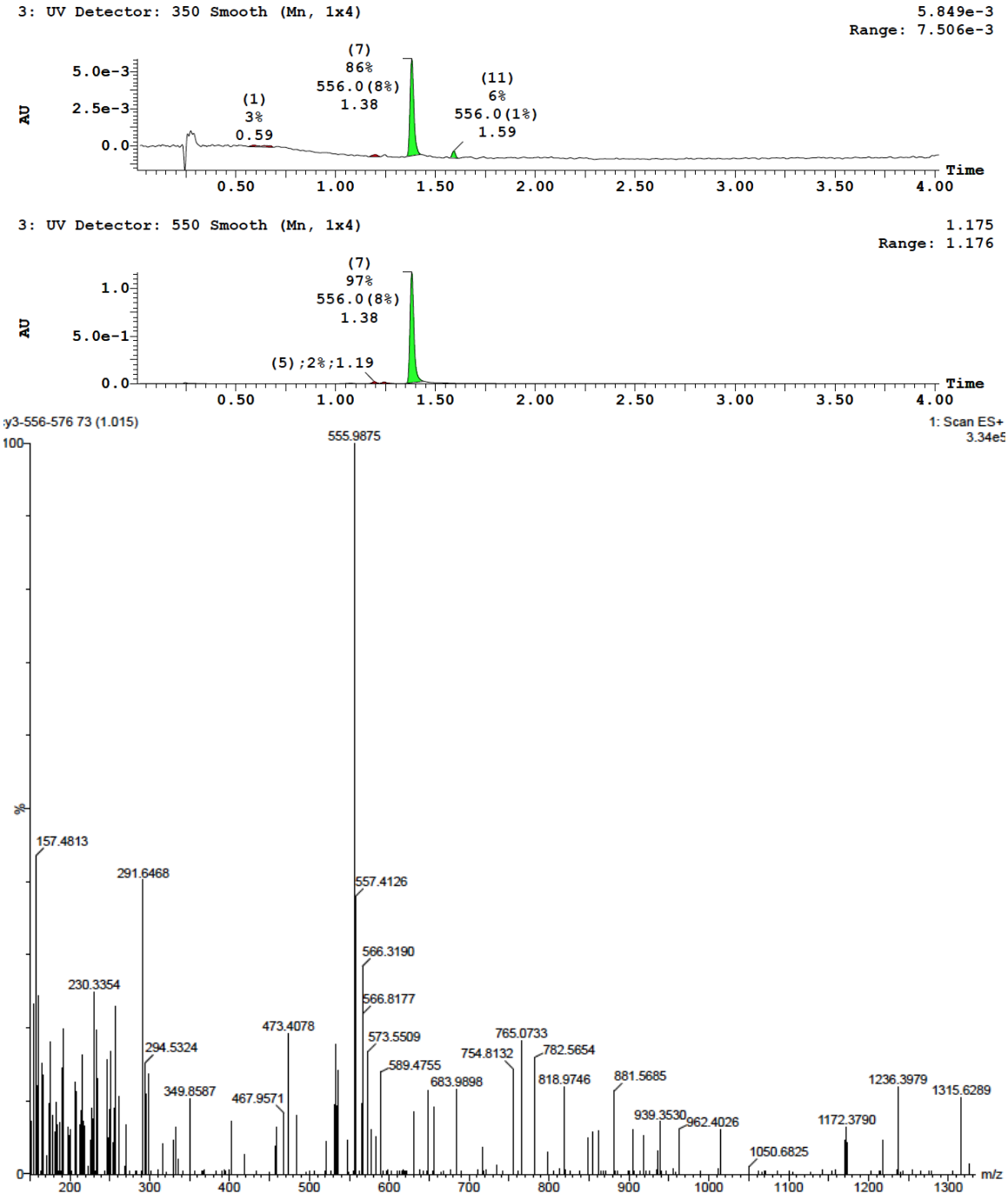


5.3 UPLC fluorescence and mass spectra of (2c)

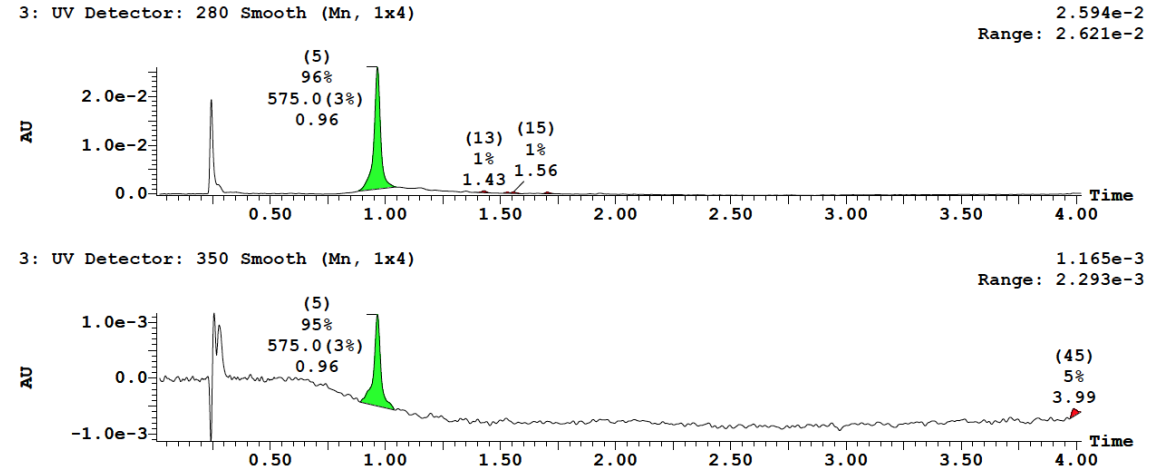


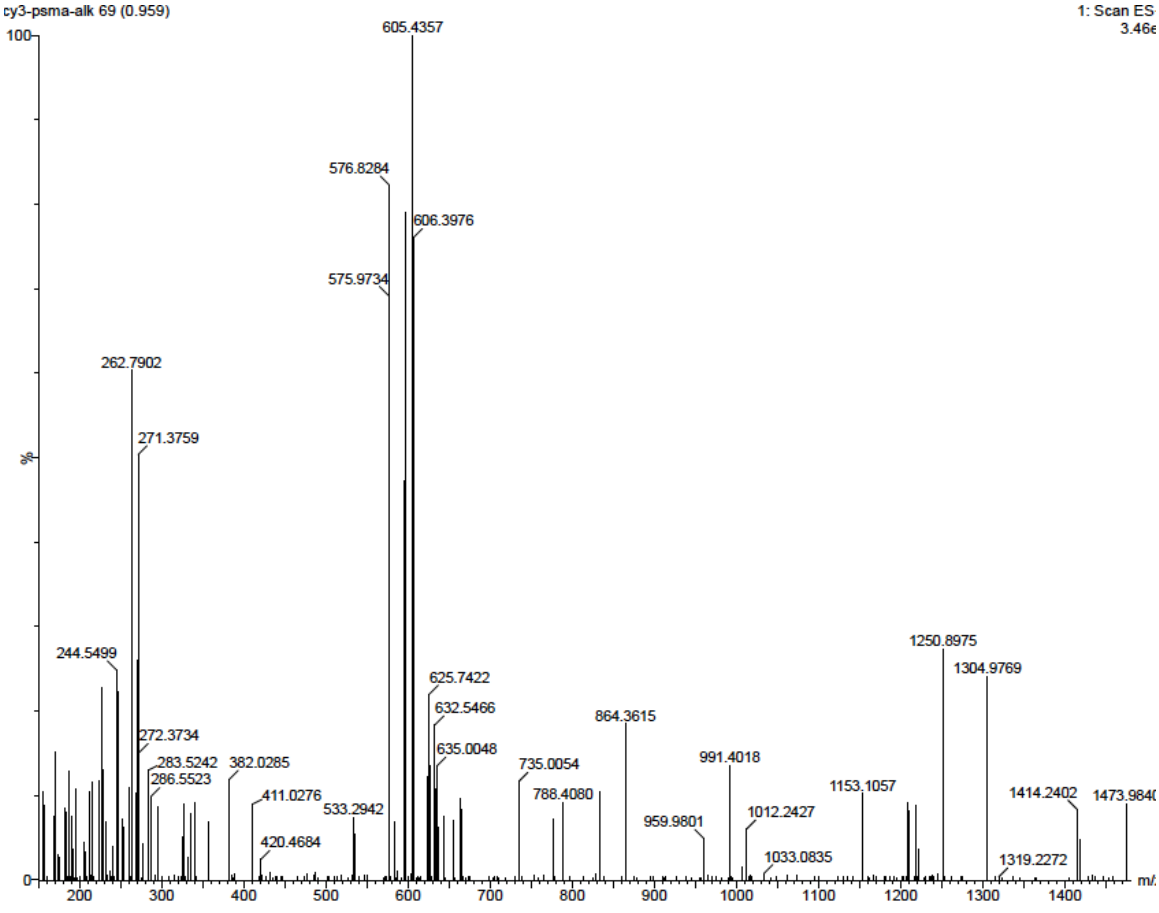


5.4 UPLC fluorescence and mass spectra of (2d)



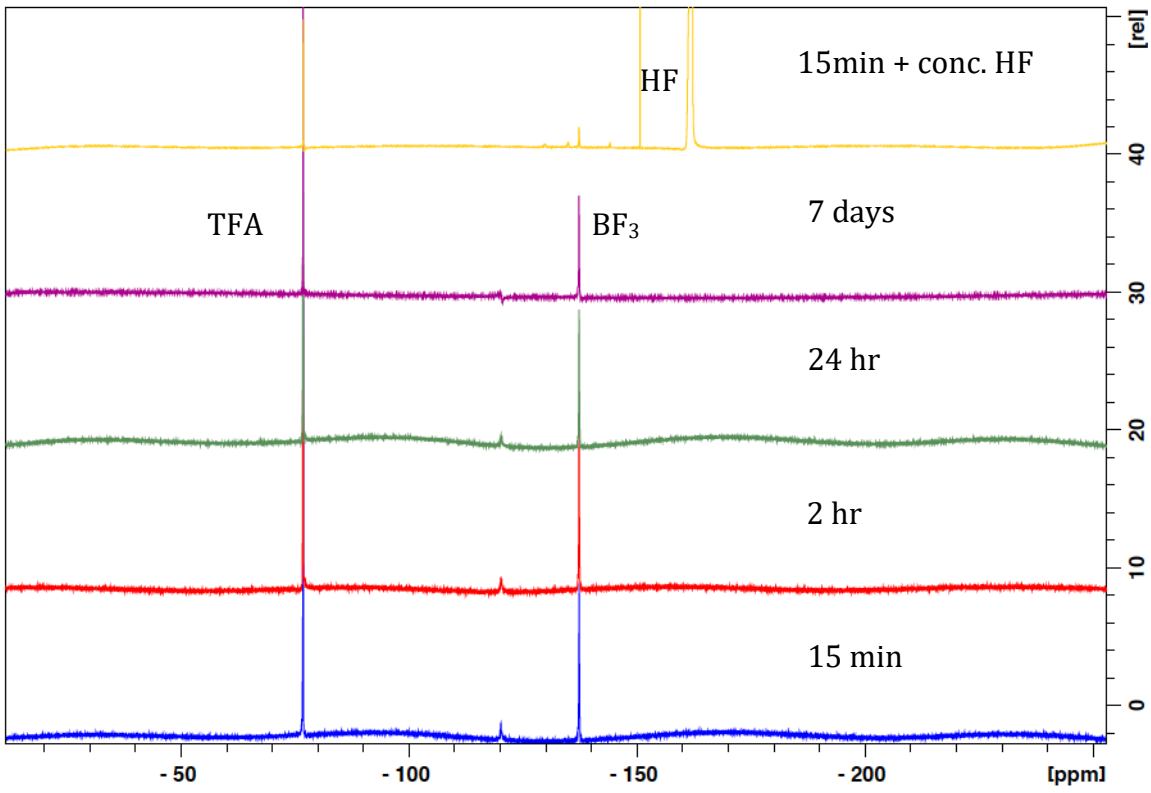
5.5 UPLC fluorescence and mass spectra of (2e)



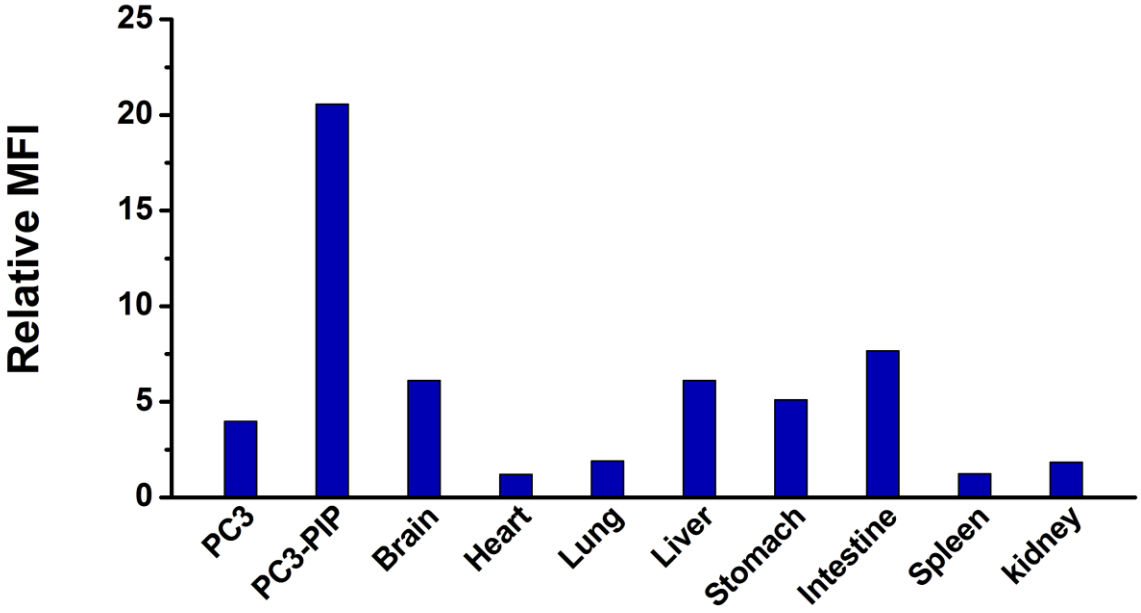


Supporting Figure S1. Proof of [^{19}F]-4 trifluoroborate stability in physiological pH:

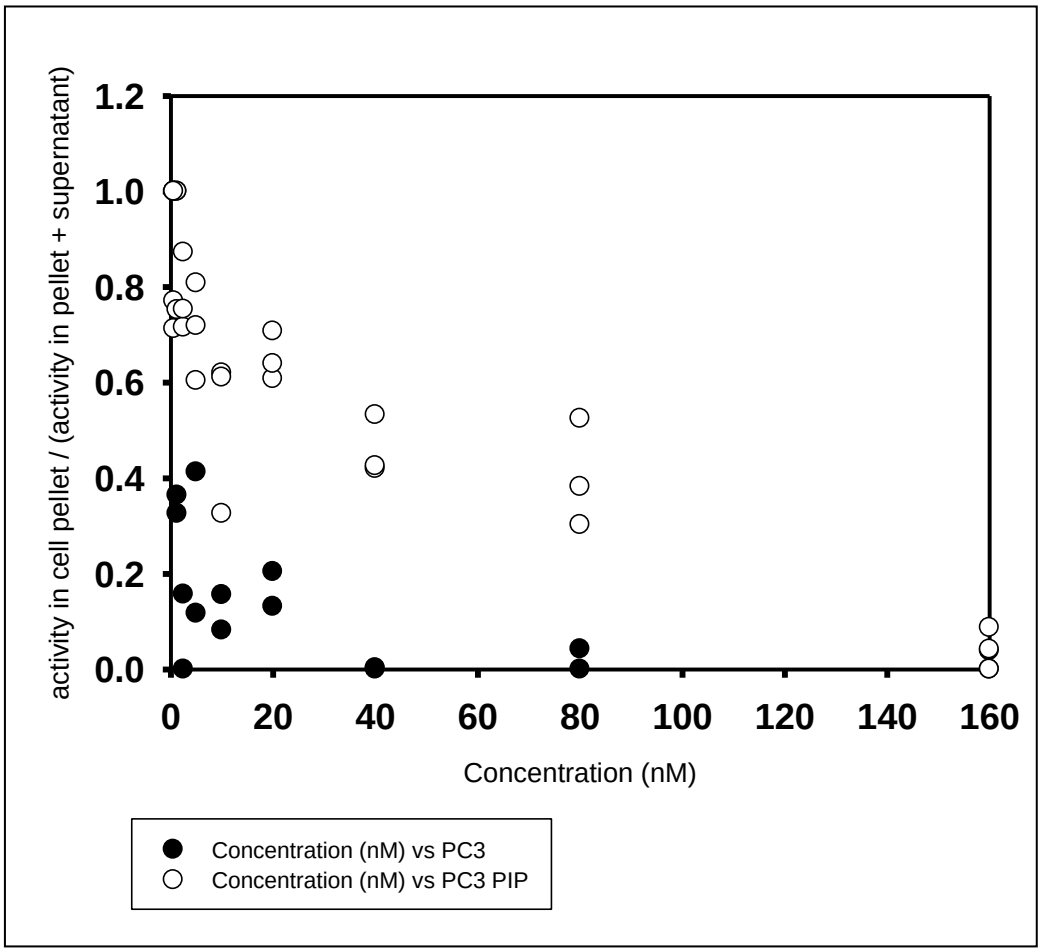
The stability of [^{19}F]-**4** was evaluated by ^{19}F-NMR in a 50% DMSO-d_6:FBS (fetal bovine serum) solution (1:1) at pH = 7.5. Compound [^{19}F]-**4** is stable even after 7 days of incubation at 21°C ($t_{1/2}$ defluoridation >7 days). This is clear from the lack of change in the ^{19}F-NMR spectra indicating no defluoridation.



Supporting Figure S2. Relative mean fluorescence intensity (MFI) quantitated from *ex* vivo tissue shown in Figure 5, mouse iii.



Supporting Figure S3. Scintillation analysis of [^{18}F]-4 binding to PC3 and PC3-PIP cells.



[^{18}F]-**4** affinity to PC3 and PC3-PIP cells are analyzed by gamma scintillation. Suspensions of 2.5x10^5 PC3 or PC3-PIP cells are incubated with 1.25-160 nM solutions of non-radiolabeled [^{19}F]-**4**, where each aliquot also contained 1 µCi of [^{18}F]-**4**. After 1 h incubation at room temperature, cells are washed, then pelleted by centrifuge. Isolated pellets and supernatants are collected and analyzed using a Wallac Wizard 3.0 gamma counter. Data was calculated by dividing the activity in the cell pellet by the sum of the activity in the cell pellet and supernatant. Each cell line was analyzed in duplicate. No washes were performed and observed noise is attributed to supernatant that could not be separated from cell pellets.

Supporting Table 1. Spectral properties of [^{19}F]-4 were identified along-side Cy3.18.OH[4] in pH=7.4 (1X PBS buffer) and in absolute ethanol.

	Buffer	λ_{max} (nm) (1-2) μM Solution	ε (M^{-1} cm^{-1}) (1-4 μM solution)	Excitation (nm)	Emission (nm)	Stokes shift (nm)	Quantum yield (ϕ)
Cy3.18.OH	1x PBS	550	159,559	540	563	13	0.04
^{19}F-4	1x PBS	550	159,441	540	570	20	0.06
Cy3.18.OH	EtOH	559	161,523	560	576	17	0.09
^{19}F-4	EtOH	559	159,471	560	575	16	0.18

Supporting Table 2. Yield and activity data from multiple radiolabelings of [^{19}F]-4

Date	Initial Volume of [^{19}F]-**4** in DMSO (8 mM)	Activity of [^{18}F]-fluoride ion added to [^{19}F]-**4**	Quantity of reacted [^{19}F]-**4**	1.0 M Pyridazine -HCl pH= 2.5	Isolated activity of [^{18}F]-**4**	Total time of synthesis (reaction and purification)	Decay uncorrected Radio chemical yield (post-chromatography)	Specific activity assuming 60% yield of [^{18}F]-**4** (Ci/μmol)
07/10/2017	10 μL	30 mCi	78 nmol	10 μL	4.3 mCi	55 min	14.3%	0.092
08/14/2017	10 μL	35 mCi	93 nmol	10 μL	5.1 mCi	60 min	14.5%	0.091
08/21/2017	10 μL	39 mCi	78 nmol	10 μL	7.2 mCi	60 min	18.4%	0.151
08/28/2017	10 μL	56 mCi	101 nmol	10 μL	15.4 mCi	60 min	27.5%	0.254
08/31/2017	10 μL	41 mCi	78 nmol	10 μL	12.8 mCi	70 min	31.2%	0.273
09/05/2017	10 μL	34 mCi	78 nmol	10 μL	6.1 mCi	70 min	17.9%	0.130
09/21/2017	10 μL	19 mCi	78 nmol	10 μL	3.8 mCi	70 min	20.0%	0.081
09/25/2017	10 μL	32 mCi	78 nmol	10 μL	5.9 mCi	70 min	18.4%	0.126