Strain-promoted azide-alkyne cycloaddition-based PSMA-targeting ligands for multimodal intraoperative tumor detection of prostate cancer

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Supporting Information

Supplementary materials and methods

DIPCDI coupling of protected amino acids:

Fmoc-protected amino acid (3.0 eq.), 1- hydroxybenzotriazole hydrate (HOBt, 1M in DMF, 3.6 eq.), N, N'-Diisopropylcarbodiimide (DIPCDI, 1M in DMF, 3.3 eq.) were added to the resin and agitated until the Kaiser test was negative (~45 minutes) after which the resin was capped with a mixture of Ac_2O (10 eq) and pyridine (10 eq) in DMF for 5 minutes and subsequently washed with DMF (3x10 mL).

HATU coupling of protected amino acids:

Fmoc-protected amino acid (3.0 eq.), 1- hydroxybenztriazole hydrate (HOBt, 3.6 eq.), (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 2.9 eq.) and N, N'-diisopropylethylamine (DIPEA, 6 eq.) were dissolved in DMF. The solution was pre-activated for 2 minutes before it was added to the resin. The mixture was agitated until the Kaiser test was negative (~1.5 hrs.) after which the resin was washed with DMF (3x10 mL) and DCM (3x10 mL).

Fmoc removal:

The resin was treated with 20% piperidine in DMF 3x6 minutes. The product was washed with DMF (3x10 mL).

Alloc removal:

Phenylsilane (25eq) and tetrakis(triphenylphosphine)palladium(0) (0.3eq) in DCM were added to the resin. The mixture was agitated for 20-30 minutes under a stream of argon (upon which the color changed from yellow to dark brown). Next the resin was washed with DCM, DMF and sodium diethyldithiocarbamate (0.5% in DMF) until the brown color had completely disappeared (generally 3 times).

Synthesis of the PSMA binding motif and linker

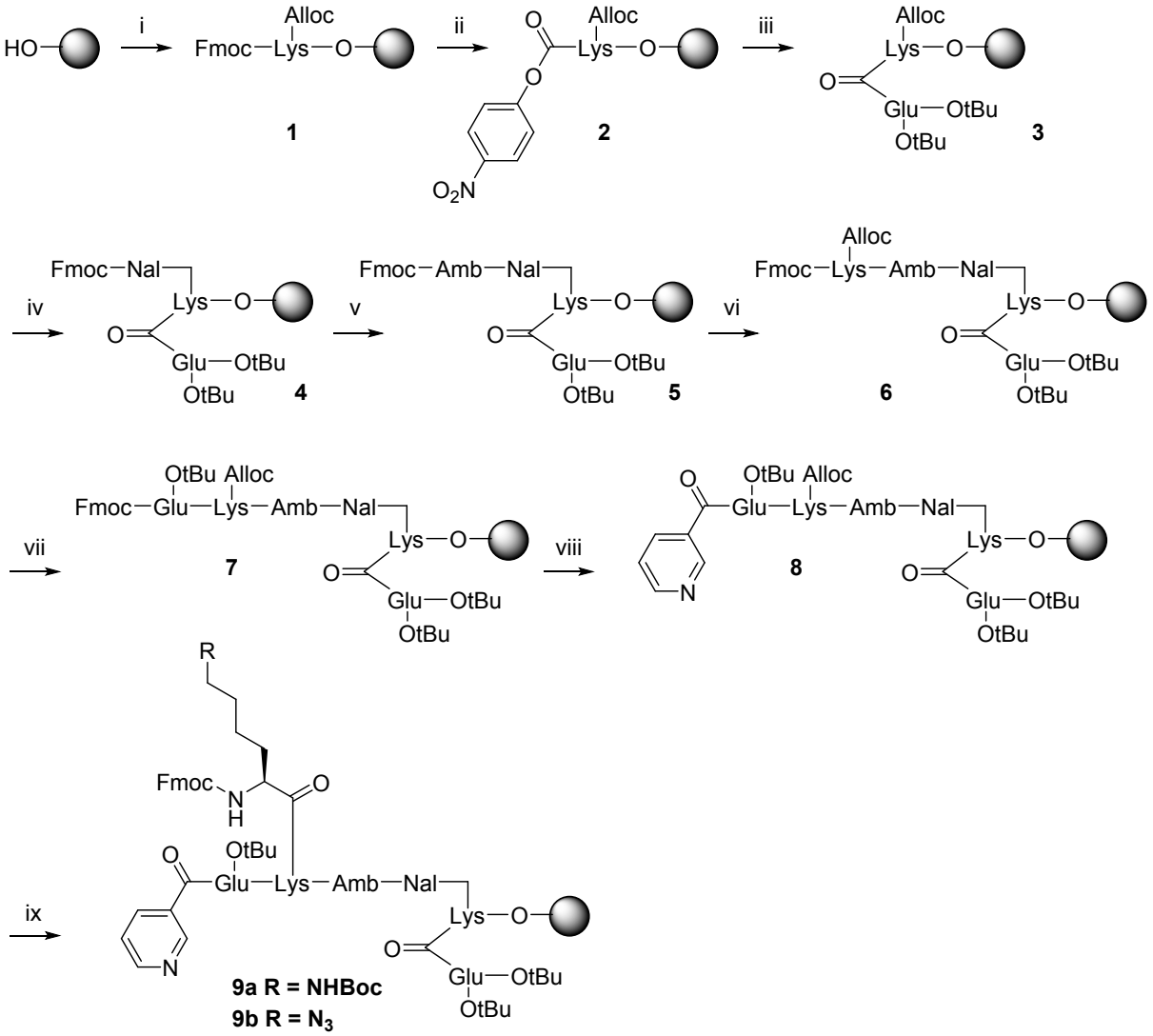


Fig. S1. **Synthesis of the common PSMA binding motif and linker.**

i) Wang resin (1 eq., 1.0 mmol/g, 1.00 g) was swollen in 10 mL DMF for 10 minutes. Fmoc-Lys(Alloc)-OH (3 eq., 3 mmol, 1.87 g), 4-dimethylaminopyridine (1 eq., 1 mmol, 122.2 mg), HOBt (3.6 eq., 3.6 mmol, 1M in DMF) and DIPCDI (3.3 eq., 3.3 mmol, 1M in DMF) were added to the resin and mixed on a bench roller for 20 hours. The reagents were removed from the resin by vacuum filtration. The resin was washed with DMF (3x10 mL) and DCM (3x10 mL). The Fmoc-loading was determined to be 0.5 mmol/g. Next, the resin was capped with a solution of pyridine (0.34 mL/g resin) and benzoyl chloride (0.34 mL/g resin) in DCM for 1 hour.

ii) The resin was washed with DCM (3x10 mL) and DMF (3x10 mL) and after Fmoc removal DIPEA (0.52 mL, 3 eq., 3 mmol,), 4-nitrophenyl chloroformate (2 eq., 2.0 mmol, 402 mg) in 2 mL DCM were added to the H-Lys(Mtt)-resin (1eq, 0.5 mmol/g, 2 g) and the resin was agitated for 1 hour. Consecutively a Kaiser test was performed to check for completion (39).

iii) Glutamic acid di-*tert*-butyl ester hydrochloride (3 eq., 3 mmol, 887.4 mg) and DIPEA (4 eq., 4 mmol, 0.70 mL) in DCM were added to the resin and the mixture was agitated for 1 hour. The resin was washed with DCM (3x10 mL) and DMF (3x10 mL).

iv) After Alloc removal, Fmoc-3-(2-naphthyl)-L-alanine (Fmoc-Nal) was coupled using HATU.

v) After Fmoc removal either Fmoc-(4-aminomethyl)benzoic acid (Fmoc-Amb) or trans-4-(aminomethyl)cyclohexane-1-carboxylic acid (Fmoc-Amc) was coupled using DIPCDI.

vi) After Fmoc removal Fmoc-Lys(Alloc)-OH was coupled using DIPCDI.

vii) After Fmoc removal Fmoc-Glu(OtBu)-OH was coupled using DIPCDI.

viii) After Fmoc removal nicotinic acid was coupled using DIPCDI

ix) After Alloc removal, Fmoc-Lys(Alloc) was coupled using DIPCDI.

x) DIPEA (2 eq.) and DOTA-OSu were added to the resin in NMP and mixed on a bench roller at room temperature respectively for 6-8 hrs. Upon a negative Kaiser test the resin was washed with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (3x).

xi) The peptide was cleaved from the resin with trifluoroacetic acid/H_2O (95:5, v/v) for two hours after which the resin was filtered off and the peptide was precipitated in diethyl ether. After drying in air the crude peptide was lyophilized from water.

xii) After Fmoc removal, Fmoc-Gly-OH was coupled. This was repeated twice to couple two more glycines.

xiii) After Fmoc removal N-succinimidyl S-acetylthioacetate (SATA, 3eq.) in DMF was added to the resin. Upon a negative Kaiser test (~45 minutes) the resin was washed with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (3x)

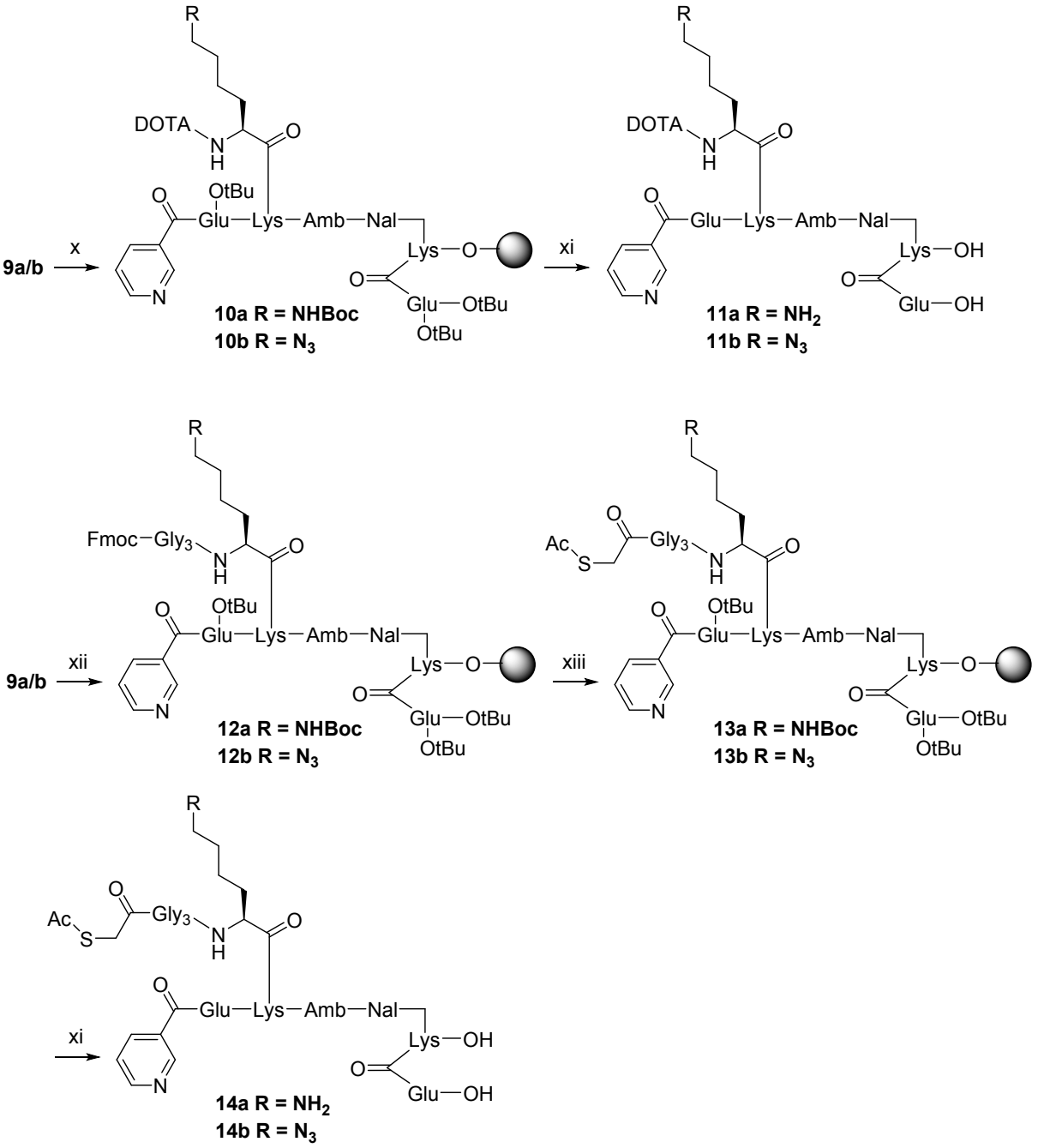


Fig. S2. **Synthesis of peptide ligands without dye.**

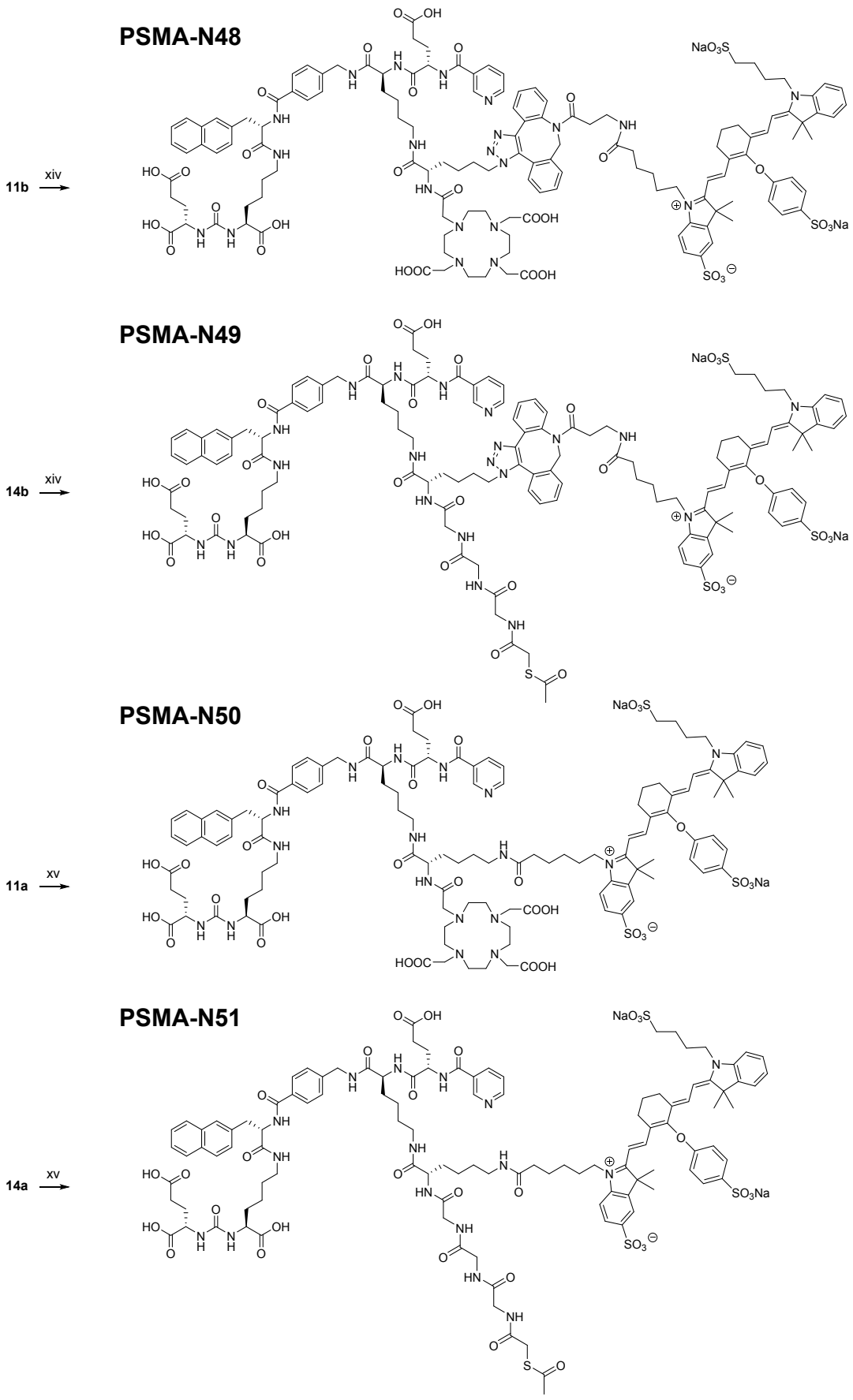


Fig. S3. **Conjugation of IRDye800CW to the ligands.**

Conjugation with IRDye800CW:

Peptide was dissolved in phosphate buffer (0.25 M, pH 8) after which the dye OSu ester (0.5-0.6 eq. in dry DMF) was added and shaken at rt for 4-6 hrs. The product was purified directly by preparative HPLC.

Analytical HPLC:

Compounds were analyzed on a Shimadzu LC-20A Prominence system with a dual UV-Vis detector (Shimadzu, 's Hertogenbosch, The Netherlands) equipped with a C18 Gemini-NX column, 150 × 3 mm, particle size 3 µm (Phenomenex, Utrecht, The Netherlands) Solvent A was 0.1% trifluoroacetic acid (TFA) in H_2O and solvent B was 0.1% TFA in acetonitrile (MeCN). A gradient of 5-100% acetonitrile (30 min.) was applied.

Preparative HPLC:

All compounds were purified on a Shimadzu dual-pump LC-20A Prominence system (Shimadzu, 's Hertogenbosch, The Netherlands) equipped with a C18 Gemini-NX column, 150 × 10 mm, particle size 10 µm (Phenomenex, Utrecht, The Netherlands), applying a gradient of 20-80% methanol in triethylammonium acetate buffer (10 mM, pH 7) for all IRDye containing compounds or a gradient of 5-100% acetonitrile in water (0.1% TFA) for all others.

Supplementary results

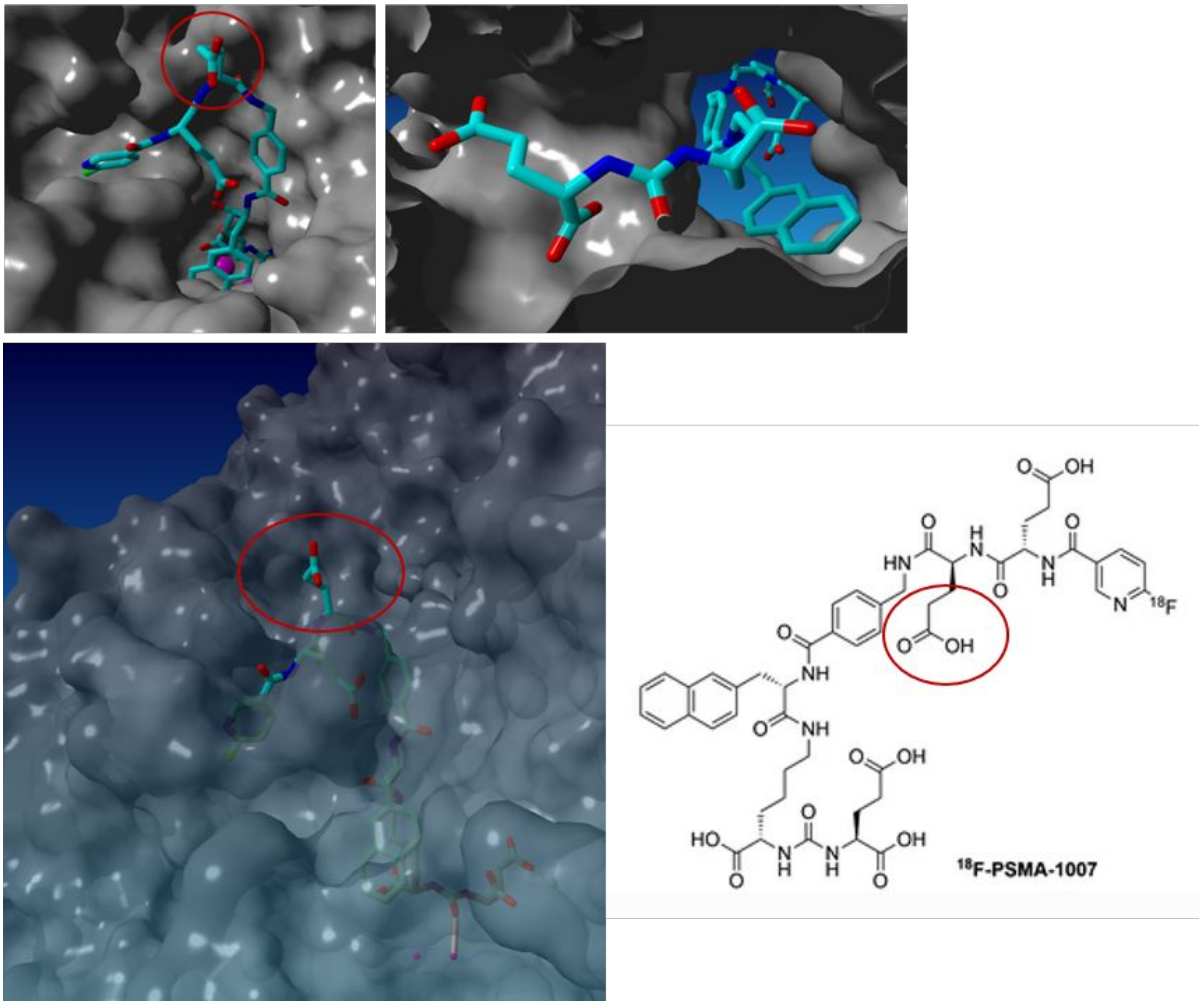


Fig. S4. Crystal structures of PSMA-1007 in the active site of PSMA. Top left: pockets in the entrance funnel of PSMA are occupied by naphtylalanine, aminomethyl benzoic acid, two glutamates and fluorinated nicotinic acid of PSMA-1007. Top right: PSMA binding motif Glu-urea-Lys in the active site of PSMA. Bottom left: View of the entrance of the PSMA binding pocket with the C-terminal glutamic acid side chain sticking out (red circle). Bottom right: Structure of PSMA-1007 with C-terminal glutamic acid circled in red. Surface of PSMA is indicated in grey. Oxygen atoms (red), nitrogen atoms (dark blue) and fluorine atom (green) of PSMA-1007 (bonds, light blue) are indicated, as well as active site Zn (purple). PDB file 5O5T (11, 24, 25).

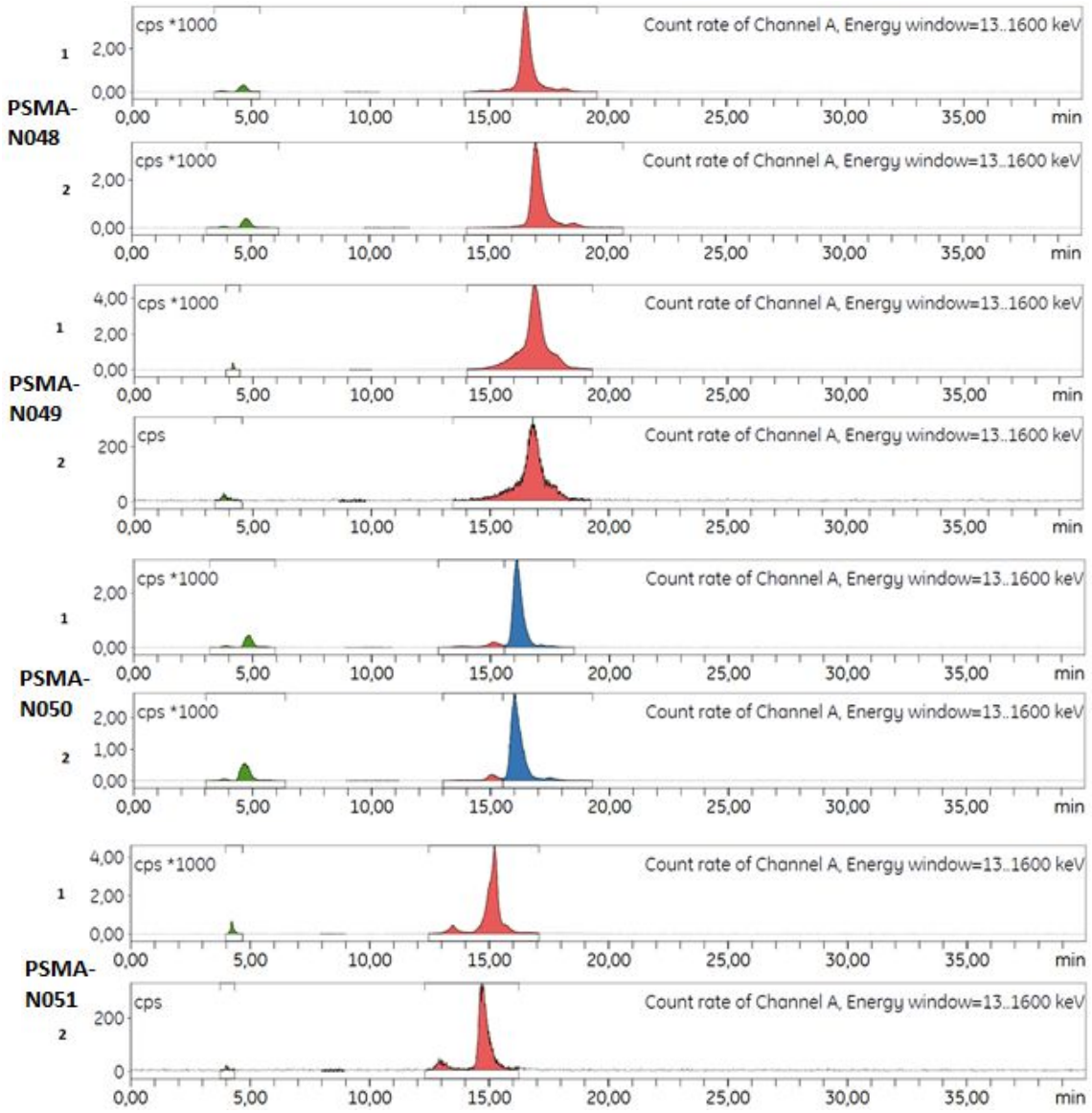


Fig. S5. Serum stability determination in human serum. Serum stability of four dual-labeled ligands was tested with use of RP-HPLC. Stability determination before (1) and after 2 hours of incubation in human serum (2).

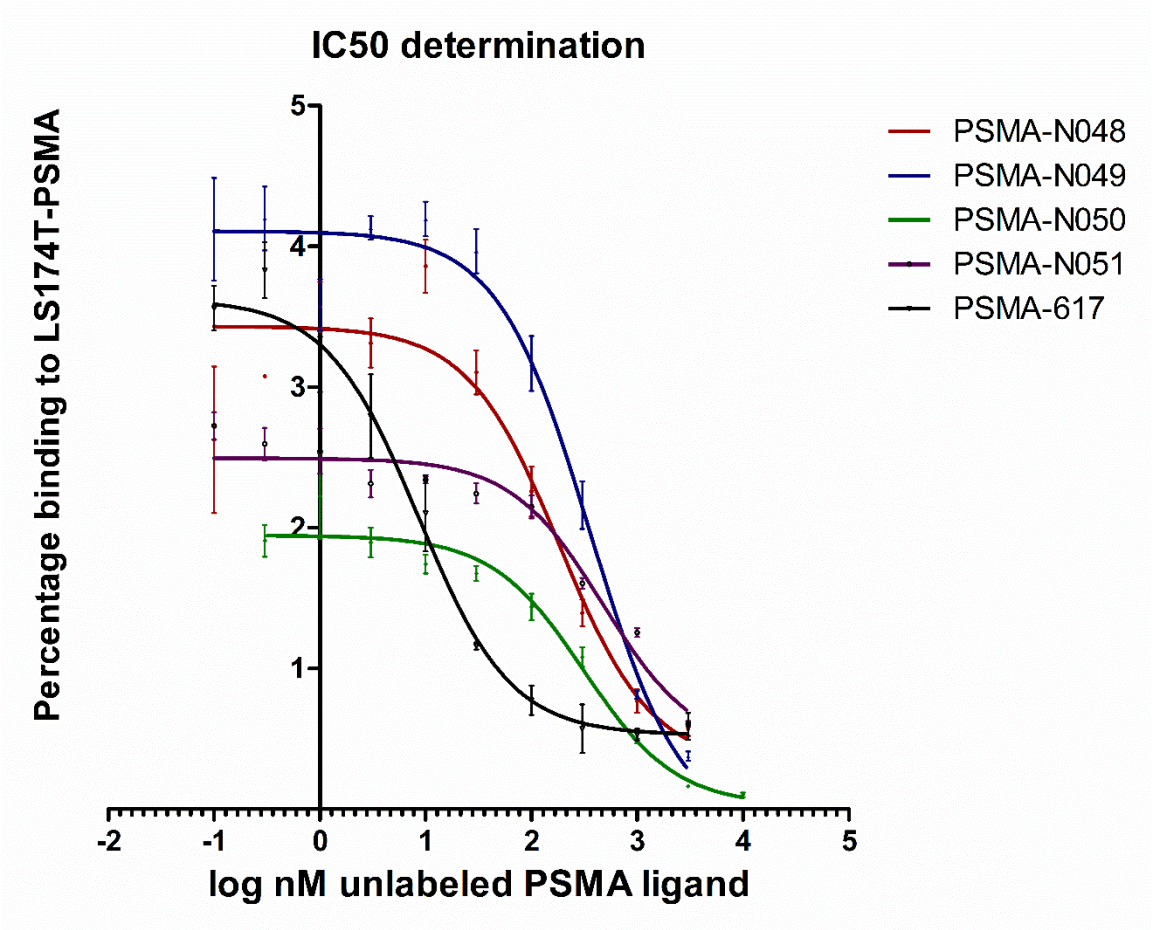


Fig. S6. IC50 determination of PSMA-N048, PSMA-N049, PSMA-N050, PSMA-N051 and PMSA-617 in PSMA-positive LS174T cells. IC_{50} graphs of PSMA-N048 (red), PSMA-N049 (blue), PSMA-N050 (green) and PSMA-N051 (purple). IC_{50} is defined as the concentration required to inhibit the binding of clinically available ^{111}In-PSMA-617 by 50%. Data is expressed as percentage binding of PSMA-617 (y-axis) and the log scale of unlabeled ligand (cold, x-axis). PSMA-617 was used as a positive control (black).

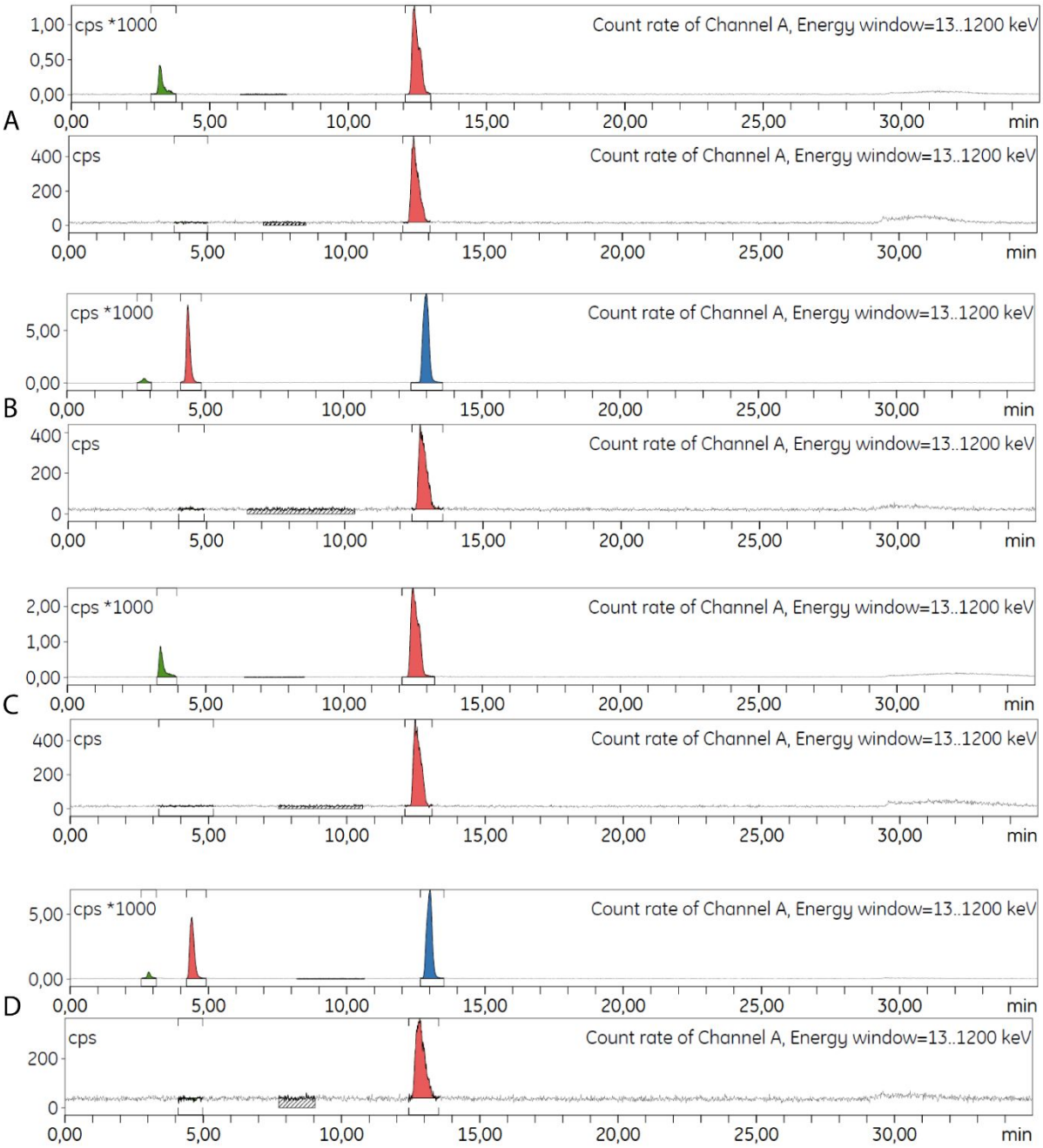


Fig. S7. Radio-HPLC before and after C18 purification. Radio-HPLC of **(A)** N48, **(B)** N49, **(C)** N50 and **(D)** N51 before and after Seppak C18 purification. Ligands were labeled with ^{111}In or ^{99m}Tc and purified before intravenous injection in mice.

Table S1. Pharmacokinetics of ^{99m}Tc-labeled PSMA-N049 and ^{111}In-labeled PSMA-N048 in mice bearing LS174T-PSMA and LS174T xenografts

	PSMA-N049 2h	***PSMA-N049 4h***	***PSMA-N049 24h***	***PSMA-N048 2h***	***PSMA-048 4h***	***PSMA-N048 24h***
Biodistribution						
Blood	2.8 ± 0.5	1.6 ± 0.2	0.3 ± 0.1	1.8 ± 0.2	0.8 ± 0.1	0.1 ± 0.0
Muscle	0.6 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.8 ± 0.1	0.3 ± 0.1	0.3 ± 0.0
Tumor- LS174T	1.7 ±0.4	1.3 ± 0.2	0.6 ± 0.2	1.9 ± 0.4	1.4 ± 0.3	1.0 ± 0.4
Tumor+ LS174T-PSMA	9.0 ± 1.0	10.5 ± 1.7	8.9 ± 0.7	23.0 ± 4.2	22.8 ± 1.6	17.8 ± 1.6
Heart	1.2 ± 0.1	0.9 ± 0.1	0.2 ± 0.0	1.2 ± 0.3	0.8 ± 0.1	0.4 ± 0.1
Lung	2.8 ±0.5	1.8 ± 0.2	0.5 ± 0.0	5.5 ± 1.6	2.4 ± 0.8	0.9 ± 0.4
Spleen	3.9 ±0.4	3.3 ± 0.7	1.6 ± 0.4	3.8 ± 0.6	2.9 ± 0.5	2.0 ± 0.5
Pancreas	0.8 ±0.4	0.6 ± 0.1	0.2 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.3 ± 0.0
Liver	5.1 ± 0.5	4.4 ± 0.6	2.6 ± 1.3	2.8 ± 0.3	2.5 ± 0.2	2.2 ± 0.2
Stomach	1.5 ± 0.2	1.1 ± 0.1	0.4 ± 0.0	1.3 ± 0.2	0.8 ± 0.1	0.5 ± 0.0
Kidney	43.4 ± 2.3	60.5 ± 13.5	43.1 ± 7.2	103.4 ± 7.1	103.1 ± 5.6	70.0 ± 5.9
Adrenals	3.3 ± 0.6	2.9 ± 1.1	0.9 ± 0.3	3.7 ± 1.4	3.1 ± 0.3	1.9 ± 0.4
Duodenum	1.5 ± 0.3	1.0 ± 0.2	0.4 ± 0.0	1.1 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
Prostate	1.1 ± 0.3	0.8 ± 0.8	0.4 ± 0.1	1.5 ± 0.4	0.9 ± 0.5	0.8 ± 0.2
Salivary glands	1.8 ± 0.1	1.4 ± 0.4	0.5 ± 0.1	1.9 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
Bone marrow	1.8 ± 0.1	1.5 ± 0.3	1.1 ± 0.2	1.2 ± 0.4	1.0 ± 0.2	1.6 ± 0.4
Bone	1.0 ± 0.2	0.7 ± 0.1	0.4 ± 0.0	1.3 ± 0.3	0.8 ± 0.1	0.6 ± 0.0
Tumor/Blood	3.1 ± 0.4	6.1 ± 1.1	33.0 ± 8.7	12.8 ± 1.9	12.8 ± 1.9	262.4 ± 28.7
Tumor/Kidney	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Tumor/ Tumor-	5.5 ± 1.2	7.4 ± 2.5	16.3 ± 4.7	14.3 ± 2.5	14.3 ± 2.5	20.7 ± 4.5
Tumor/Spleen	2.4± 0.4	3.3 ± 0.8	6.2 ± 2.2	6.2 ± 1.8	6.2 ± 1.8	9.8 ± 3.2
Tumor/Liver	1.7 ± 0.1	2.3 ± 0.5	3.8 ± 1.3	8.2 ± 1.0	8.2 ± 1.0	7.8 ± 1.3
Tumor/Salivary gland	4.6 ± 1.1	7.4 ± 1.9	17.8 ± 2.3	12.2 ± 2.2	12.2 ± 2.2	15.6 ± 2.0
Tumor/Prostate	9.2 ± 2.4	13.7 ± 4.2	24.7 ± 6.1	16.8 ± 4.2	16.8 ± 4.2	22.8 ± 5.6

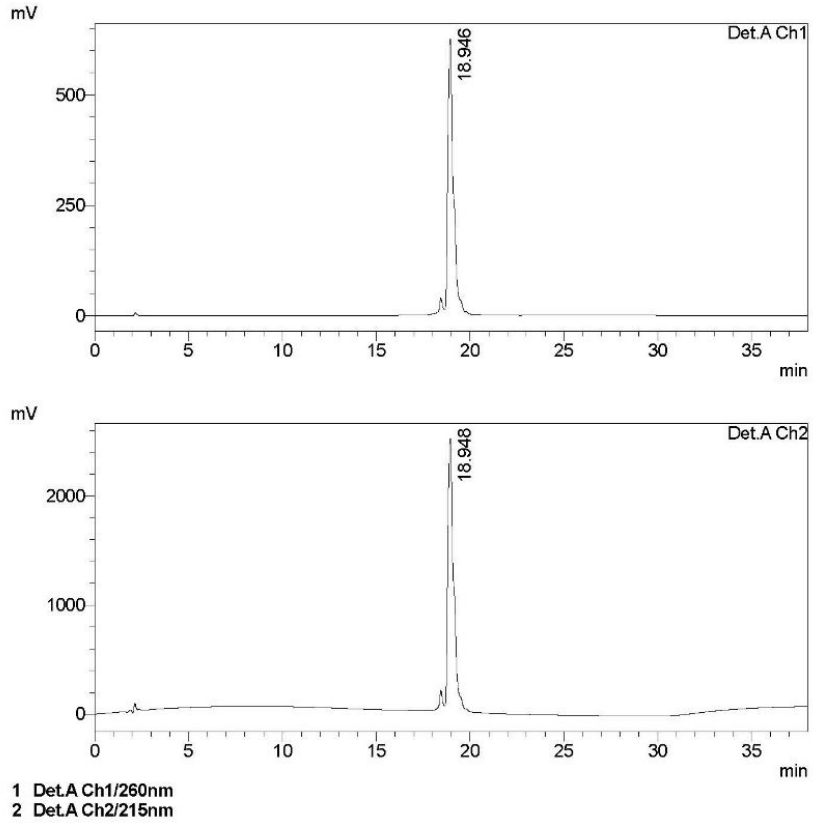
Table S2. Biodistribution of ^{111}In- or ^{99m}Tc labeled multimodal ligands in mice bearing LS174T-PSMA and LS174T xenografts 2 h p.i.

	PSMA-N049	***PSMA-N051***	***PSMA-N048***	***PSMA-N050***	***PSMA-617***
Biodistribution					
Blood	3.5 ± 1.6	2.1 ± 0.5	1.9 ± 0.2	1.1 ± 0.1	0.1 ± 0.02
Muscle	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	0.4 ± 0.1	0.1 ± 0.1
Tumor- LS174T	1.9 ± 0.6	1.6 ± 1.4	2.1 ± 0.5	1.1 ± 0.3	0.2 ± 0.2
Tumor+ LS174T-PSMA	12.0 ± 1.4	17.7 ± 3.7	21.2 ± 1.2	25.3 ± 2.0	18.5 ± 1.9
Heart	1.6 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.6 ± 0.1	0.03 ± 0.01
Lung	4.0 ± 0.4	1.9 ± 0.8	2.8 ± 0.4	1.6 ± 0.3	0.1 ± 0.1
Spleen	5.4 ± 0.6	5.8 ± 1.2	5.7 ± 0.8	5.5 ± 1.7	0.5 ± 0.2
Pancreas	1.0 ± 0.1	1.0 ± 0.6	0.8 ± 0.1	0.7 ± 0.2	0.1 ± 0.1
Liver	4.3 ± 0.3	1.7 ± 0.3	3.3 ± 0.3	1.2 ± 0.1	0.1 ± 0.02
Stomach	1.8 ± 0.2	1.0 ± 0.3	1.5 ± 0.2	0.8 ± 0.1	0.1 ± 0.1
Kidney	73.9 ± 5.6	81.6 ± 13.5	121.6 ± 8.2	129.9 ± 15.3	7.1 ± 2.6
Adrenals	5.8 ± 1.6	6.9 ± 2.7	8.7 ± 0.5	8.8 ± 2.4	1.4 ± 0.6
Duodenum	1.9 ± 0.2	1.1 ± 0.2	1.3 ± 0.3	0.8 ± 0.2	0.1 ± 0.1
Prostate	1.5 ± 0.3	1.3 ± 0.5	1.5 ± 0.6	1.4 ± 0.7	1.5 ± 3.1
Salivary glands	1.9 ± 0.1	1.4 ± 0.3	2.0 ± 0.2	1.0 ± 0.1	0.1 ± 0.02
Bone marrow	3.0 ± 1.7	2.2 ± 0.9	2.9 ± 1.0	1.4 ± 0.4	0.9 ± 1.3
Bone	1.3 ± 0.2	0.6 ± 0.1	1.6 ± 0.2	0.6 ± 0.1	0.3 ± 0.5
Tumor/Organ ratios					
Tumor/Blood	6.0 ± 7.0	8.4 ± 0.9	11.3 ± 0.8	22.8 ± 2.6	424.4 ± 173.9
Tumor/Kidney	0.16 ± 0.01	0.22 ± 0.04	0.18 ± 0.01	0.20 ± 0.02	3.0 ± 1.5
Tumor/Negative tumor	6.8 ± 2.1	15.2 ± 6.2	10.4 ± 2.4	24.3 ± 6.6	141.5 ± 52.8
Tumor/Spleen	2.2 ± 0.3	3.0 ± 0.1	3.8 ± 0.6	4.9 ± 1.3	43.5 ± 25.0
Tumor/Liver	2.8 ± 0.3	10.3 ± 1.4	6.4 ± 0.3	21.1 ± 1.6	266.6 ± 73.5
Tumor/Salivary gland	6.2 ± 0.6	12.9 ± 1.7	10.8 ± 1.1	24.6 ± 1.6	232.9 ± 68.5
Tumor/Prostate	8.3 ± 1.4	13.9 ± 2.5	16.7 ± 7.3	22.3 ± 10.2	140.0 ± 95.5

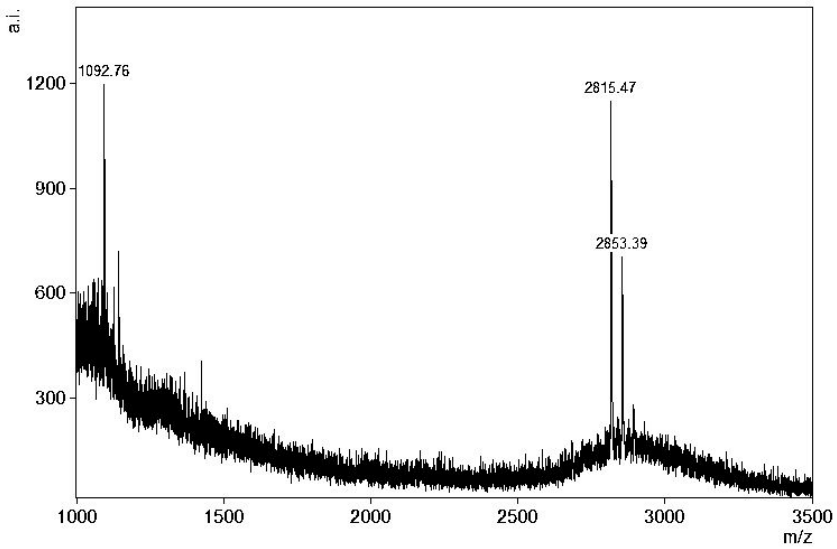
Fig. S8. HPLC and MALDI-ToF spectra of PSMA-N048, PSMA-N049, PSMA-N050 and PSMA-N051.

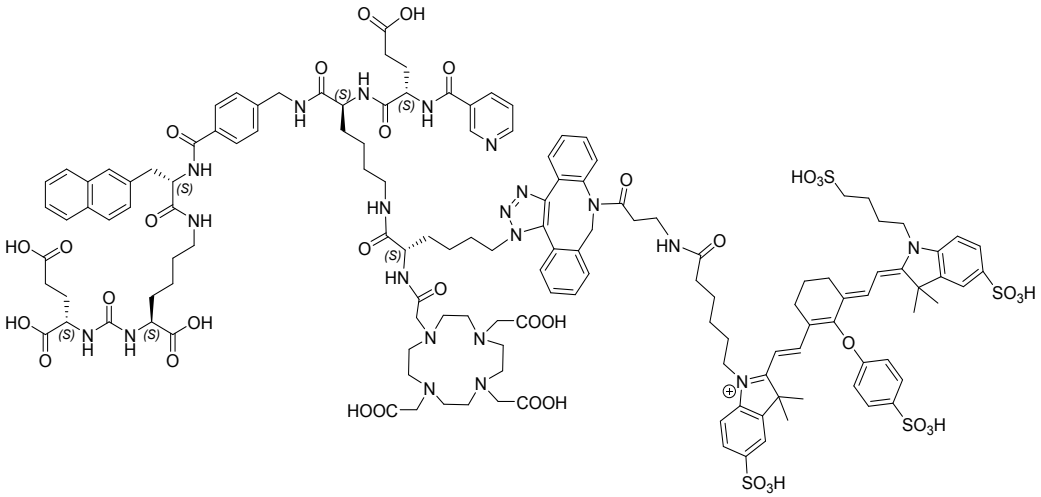
PSMA-N048

HPLC chromatogram



MALDI-ToF spectrum with matrix SA (Sinapinic acid)

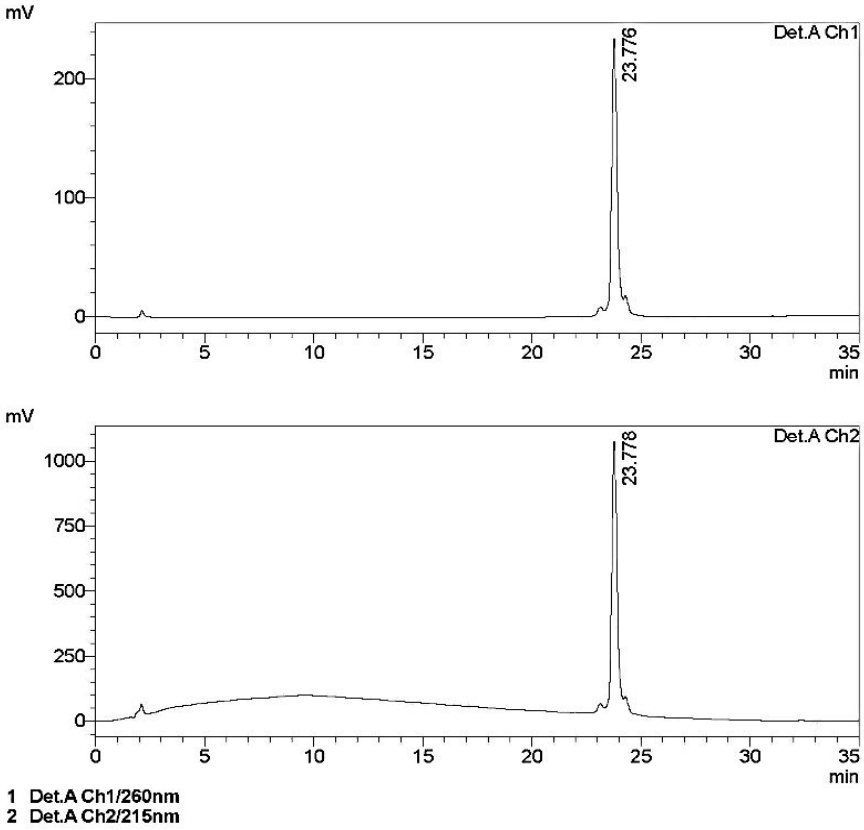




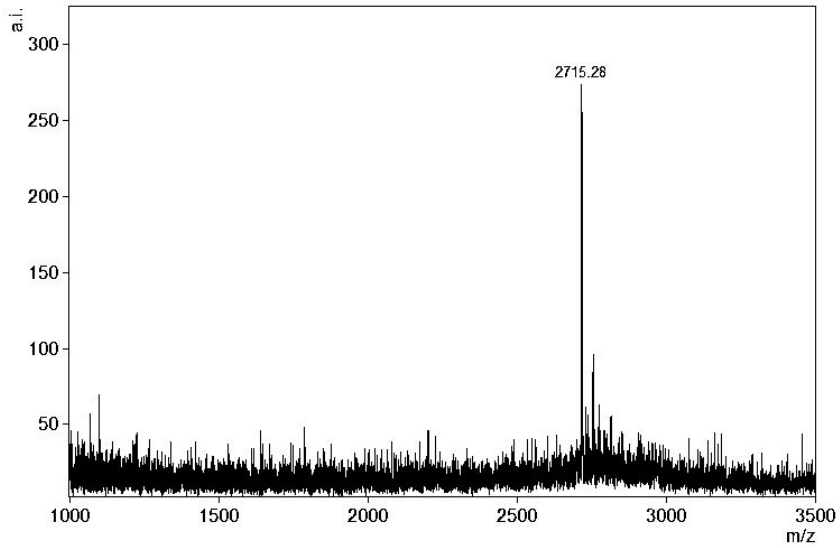
Chemical Formula: $C_{136}H_{166}N_{21}O_{37}S_4^+$
m/z: 2814.07 (100.0%), 2815.07 (73.0%), 2813.06 (68.0%), 2816.07 (24.5%)

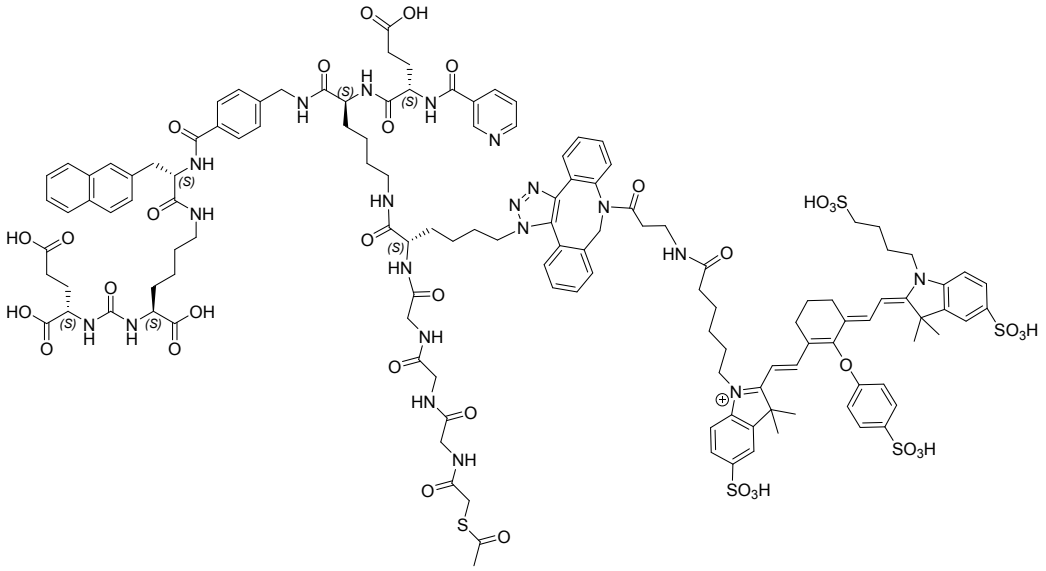
PSMA-N049

HPLC chromatogram



MALDI-ToF spectrum with matrix SA (Sinapinic acid)



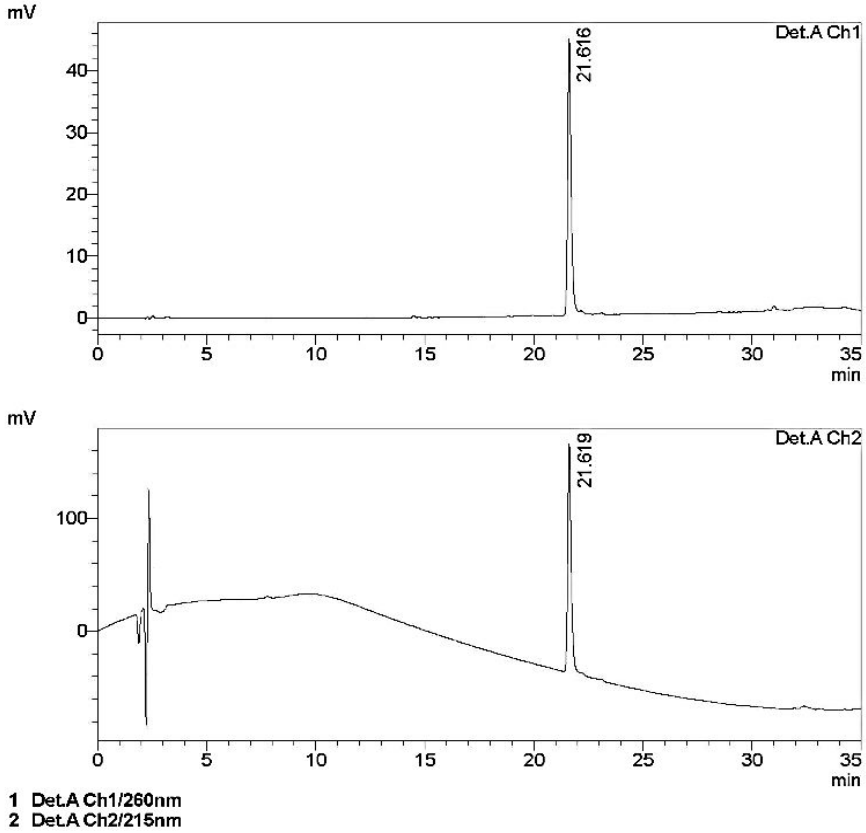


Chemical Formula: $C_{130}H_{153}N_{20}O_{35}S_5^+$

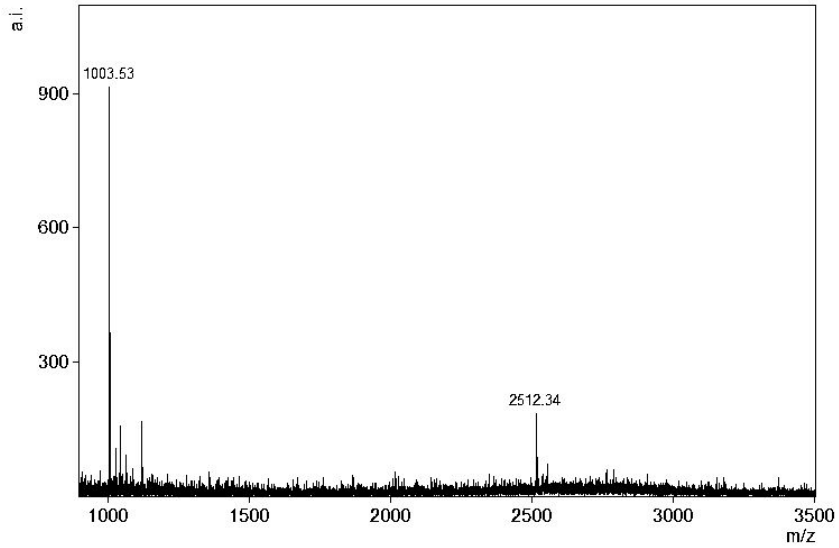
m/z: 2714.94 (100.0%), 2713.94 (71.1%), 2715.95 (39.4%), 2715.95 (30.4%), 2716.95 (23.7%), 2716.94 (22.6%)

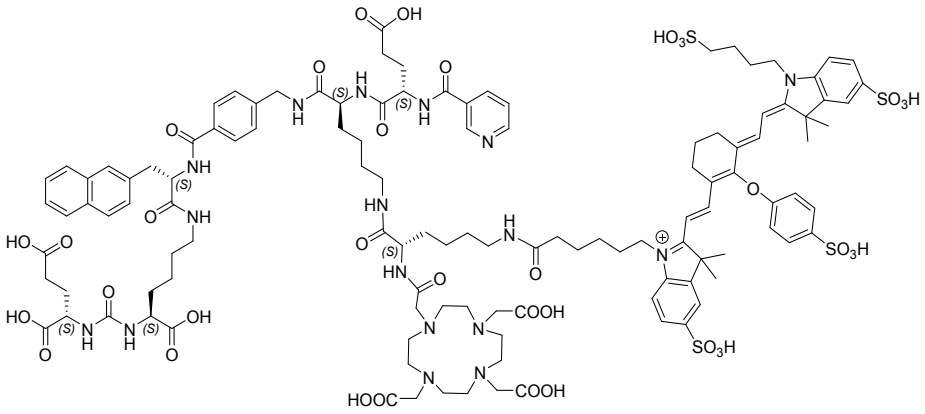
PSMA-N050

HPLC chromatogram



MALDI-ToF spectrum with matrix SA (Sinapinic acid)

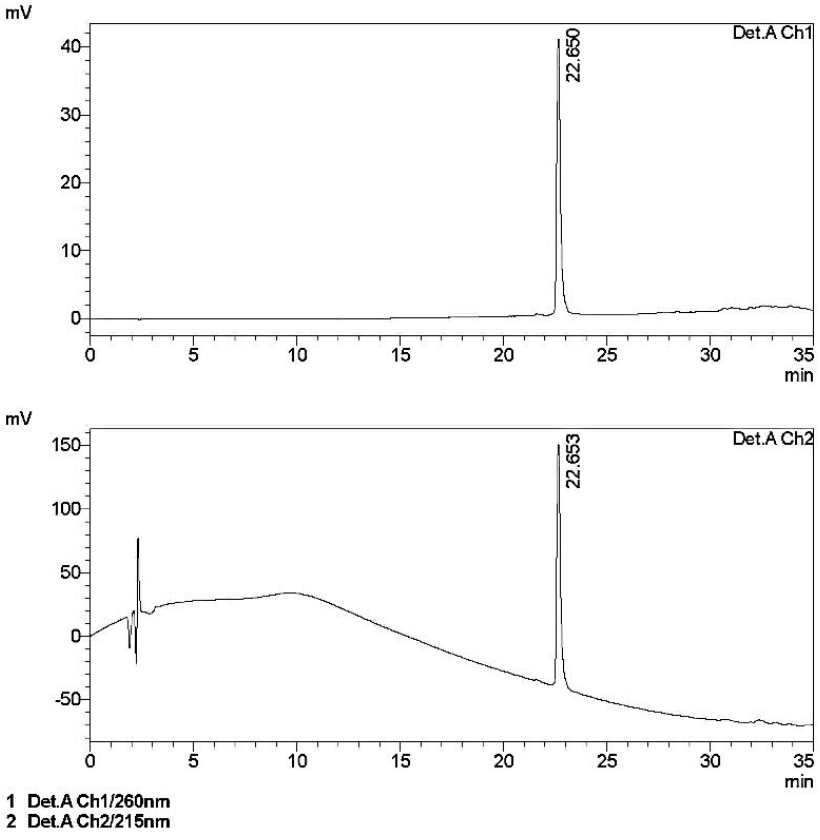




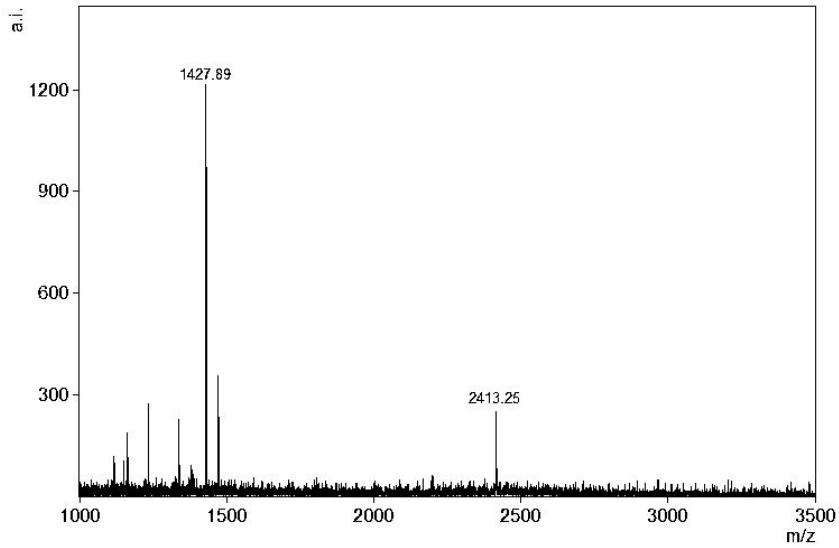
Chemical Formula: $C_{118}H_{152}N_{17}O_{36}S_4^+$
m/z: 2511.95 (100.0%), 2510.95 (78.4%), 2512.95 (63.3%), 2513.95 (18.1%)

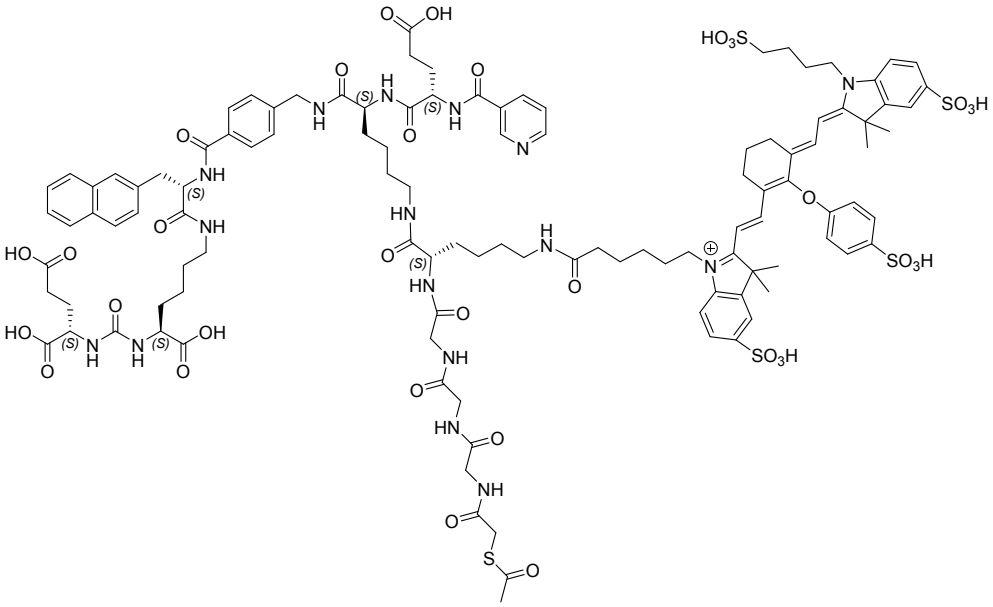
PSMA-N051

HPLC chromatogram



MALDI-ToF spectrum with matrix SA (Sinapinic acid)





Chemical Formula: $C_{112}H_{139}N_{16}O_{34}S_5^+$

m/z: 2412.83 (100.0%), 2411.82 (82.6%), 2413.83 (35.3%), 2413.83 (24.7%), 2414.82 (22.6%)