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Article

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Preparation and Evaluation of Radiolabeled Antibody Recruiting Small Molecules that Target Prostate Specific Membrane Antigen (PSMA) for Combined Radiotherapy and Immunotherapy

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Abstract

The feasibility of developing a single agent that can deliver radioactive iodine and also direct cellular immune function by engaging endogenous antibodies, as an antibody recruiting small molecule (ARM), was determined. A library of new prostate-specific membrane antigen (PSMA)-binding ligands that contained antibody recruiting, 2,4-dinitrophenyl (DNP) groups and iodine, were synthesized and screened *in vitro* and *in vivo*. A lead compound (**9b**) showed high affinity for PSMA and the ability to bind anti-DNP antibodies. Biodistribution studies of the iodine-125 analogue showed 3% ID/g in LNCaP xenograft tumors at 1 h post-injection, with tumor-to-blood and tumor-to-muscle ratios of 10:1 and 44:1. The radiolabeled analogue was bound and internalized by LNCaP cells, with both functions blocked using a known PSMA inhibitor. A second candidate showed high tumor uptake (>10% ID/g), but had minimal binding to anti-DNP antibodies. The compounds reported represent the first examples of small molecules developed specifically for combination immunotherapy and radiotherapy for prostate cancer.

Introduction

Immunotherapies that activate, focus, or release immune mechanisms from aberrant regulation within tumors are changing cancer therapy paradigms, and introducing the prospect of immunization against recurrence. Agents such as anti-CTLA-4 and anti-PD-1 / PD-L1 antibodies are improving the prognosis of metastatic melanoma patients,[1] whose five year survival rate was previously estimated as only 16%.[2] For example, a pivotal trial of the anti-CTLA-4 antibody ipilumumab in metastatic melanoma patients demonstrated overall survival of 10 months versus 6.4 months in the control group.[3] As a result, there has been a subsequent surge in efforts to find new targets and molecules to stimulate anti-tumor immune response. One such method is the antibody recruiting small molecule (ARM) strategy. ARMs are designed to direct antibodies to bind specific cells and mark them for destruction by immune effector cells.[4]

One type of ARM used to promote immune response utilized 2,4-dinitrophenyl (DNP) groups, considering that anti-DNP antibodies exist in a large proportion of the North American population,[5] potentially as a result of environmental exposure to related hapten structures.[4] The ARM strategy has been employed to label the surface of prostate cancer cells with DNP groups, using prostate specific membrane antigen (PSMA) as the target (Figure 1).[6] PSMA is a cell surface protein that has significantly higher expression on prostate cancer cells compared to normal tissue.[7] The agents, referred to as ARM-P (ARM-targeting prostate cancer), were based on DNP linked to a potent PSMA inhibitor through a PEG-8 spacer (ARM-P8), which promoted antibody dependent killing of PSMA expressing LNCaP cells, in the presence of peripheral blood mononuclear cells and anti-DNP antibodies.[6] PSMA negative, DU145 cells were not susceptible to antibody dependent cell cytotoxicity (ADCC) under the same conditions. Subsequent, 14 day *in vivo* studies, using

human xenografts and an analogous DNP containing ARM, demonstrated target dependent ADCC, inhibiting the growth of PSMA positive tumors.[8]

The same construct that was used to prepare the PSMA targeting ARMs, has been used to develop radiotherapeutic agents for treating metastatic prostate cancer.[9-12] Babich, Haberkorn and coworkers showed that a ^{131}I-PSMA inhibitor (MIP-1095) significantly decreased prostate specific antigen levels and pain in men with metastatic, castration resistant prostate cancer.[12,13] We have noted that the amount of ARM needed to induce ADCC *in vivo* was comparable to the molar quantity of the radiopharmaceutical used during PSMA targeted radionuclide therapy. This suggested that a PSMA binding ARM containing a therapeutic medical isotope could offer a means of combining immunotherapy and radionuclide therapy within a single construct. There are an increasing number of reports that show both external beam radiotherapy and therapeutic radiopharmaceuticals induce an anti-tumor immune response. This "radiation vaccination" strategy offers not only the opportunity to kill the tumor, but to provide sustained immunity against tumor recurrence as well.[14-17] In addition, there are significant practical advantages to using a single construct with two therapeutic functions as opposed to two separate agents, in that it simplifies determining the optimal dosing schedule and the order of administration, and would decrease overall development costs.

To test the feasibility of developing a combination agent (a radiolabeled ARM-P), we prepared a series of PSMA-binding DNP derivatives, using a glutamine-urea-lysine targeting construct that contained iodine-125 within the backbone. The goal was to identify compounds that had high affinity for PSMA, would retain binding to anti-DNP antibodies, and would localize in PSMA expressing tumors.

Results and Discussion

Synthesis

The target library prepared was based on the ARM-P constructs reported previously[6] in which iodine is added at the 4 or 5 position of the triazole ring. Iodine was selected because iodine-131, a β-emitting isotope, is widely used clinically for the treatment of thyroid cancer and hyperthyroidism and because previous SAR and modeling data suggested that the addition of the halogen to the triazole would not have a detrimental impact on binding to PSMA.[18] For the present study, iodine-125 was used for cell uptake and biodistribution studies, after *in vitro* screening was performed using the non-radioactive iodinated analogues.

The initial class of compounds synthesized, contained the iodine atom at the 5-position of the triazole. Iodination and formation of the triazole was achieved through a concomitant reaction between a DNP alkyne, having a PEG-4 (**4a**) or a PEG-8 spacer (**4b**), and an azide derivative of the PSMA binding ligand (**5**), in the presence of copper iodide and N-iodosuccinimide (NIS, Scheme 1). The spacer group length was selected based on the PSMA binding data for the ARM-P4 and ARM-P8 reported previously.[6] The 5-iodo-triazoles **6a** and **6b** were obtained in 75% and 83% yields, respectively.

The identity of iodo-triazoles **6a** and **6b** were confirmed by a variety of characterization methods (see Supporting Information). The ^{1}H NMR indicated the NH proton linked to the DNP moiety appeared at 6.97 ppm, while the NH protons of urea linkage were at 5.07 ppm. In the ^{13}C NMR spectrum, the triazole carbon atom signals were at 148.5 and 156.8 ppm, and were shifted by 3 ppm compared to the corresponding *des*-iodo analogues. The presence of iodine at C5 was verified by the absence of triazole CH proton in ^{1}H NMR. Moreover, HRMS were consistent with the formation of both the I-PEG-4 and I-PEG-8 derivatives, as they showed the expected masses, matching the molecular formula of **6a** (*m/z* 1059.3464 $[M + Na]^+$) and **6b** (*m/z* 1213.4752 $[M + H]^+$).

The IR spectra where similarly characteristic with intense bands appearing at 3358, 2931 and 1730 cm^{-1} for **6a**, and 3358, 2867 and 1735 cm^{-1} for **6b**, corresponding to the NH and carbonyl stretches, respectively. Deprotection of **6a** and **6b** to the corresponding free acids **7a** and **7b** was achieved using a mixture of TFA and DCM, with microwave heating. Compounds **7a** and **7b** were isolated in 92% and 94% yield after HPLC purification, where the loss of the signals associated with the ^{t}Bu protecting groups was evident in the ^{1}H and ^{13}C NMR spectra.

A second family of 5-iodo-DNP derivatives were prepared as high affinity PSMA ligands using 2-azidoacetic acid as a spacer group, based on the work of Darwish *et al.*[19] (Scheme 2). The azide **8** was prepared in 90% yield by treatment of glutamate-urea-lysine[20] with methyl 2-azidoacetate in methanol at 60 ^{o}C for 48 h. A one-pot click reaction between **8** and the DNP-alkynes **4a** or **4b** in the presence of N-iodosuccinimide, followed by acidic deprotection of ^{t}Bu esters, produced two additional 5-iodo-triazoles **9a** and **9b** in 63% and 70% yield respectively. The ^{1}H and ^{13}C NMR spectra of **9a** and **9b** were similar to those of **7a** and **7b** respectively, with notable differences being the additional signals associated with the extra methylene protons that appeared at 5.10 ppm, and the carbonyl resonance at 176.2 ppm. HRMS data provided additional evidence to support the formation of the desired products **9a** and **9b**.

The 4-iodotriazole derivatives were prepared from the corresponding stannanes (Scheme 3). Here, the alkynes used to prepare the 5-iodo derivatives were converted to the alkynyl tin derivatives **10a** and **10b**, by treating **4a**/**4b** with Bu_3SnOMe in the presence of zinc bromide.[21] The PEG-4 and PEG-8 alkynes were isolated in 50% and 62% yield respectively. These were coupled to the azide **8** using a thermal click reaction to give **11a** and **11b** in 30% and 28 % yield respectively. It should also be noted that this reaction also led to the formation of approximately 10% of the *des*-iodotriazoles that can readily be separated

from the desired product by preparative TLC. ^{119}Sn NMR confirmed the presence of tin in the product with the ^{119}Sn chemical shifts of alkynes **10a** and **10b** observed at -64.7 ppm, and then shifted downfield to -58.7 ppm, upon formation of the triazoles **11a** and **11b**. The two sets of isomers could be distinguished by analytical HPLC, where the 5-iodo derivatives have shorter retention times compared to the 4-iodo analogues. The two desired compounds for *in vitro* screening, **12a** and **12b**, were prepared by iodination of the tin precursors, which proceeded in high yield, and deprotection of the t-butyl groups using TFA.

As controls, we prepared the 4-iodo derivatives without a DNP group and with DNP linked to the PSMA binding ligand via a short spacer group, such that binding to anti-DNP antibodies would be prevented. To accomplish this, an amino triazole was prepared using the pthalimide **13**, which was coupled to the azide **8**, and the product isolated in 50% yield as a single isomer (Scheme 4). For preparation of the iodine derivative **16**, compound **14** was treated with iodine, prior to deprotection with hydrazine. HRMS data for **14** was consistent with the expected mass (*m/z* 752.2842 $[M + H]^+$), and the ^{1}H NMR showed the three characteristic NH protons at 7.65, 5.94 and 5.61 ppm. Moreover, the triazole carbon atoms were evident in the ^{13}C NMR spectrum appearing at 157.6 and 141.0 ppm, and key carbonyl resonances at 172.1, 171.9, 171.8 and 171.6 ppm.

To prepare the DNP derivative, **16** was combined with 1-chloro-2,4-dinitrobenzene to produce **18** in 38% yield (Scheme 5). The appearance of signals associated with DNP (9.13, 8.32 and 7.05 ppm) and amide groups (8.93 and 7.77 ppm) in the ^{1}H NMR spectrum of **18** provided evidence of the successful coupling. Deprotection was accomplished by treatment of **18** with TFA over 10 h, where **19** was isolated in 87% yield.

***In Vitro* Screening**

The affinity (IC_{50}) of the reported compounds was assessed using an established competitive binding assay with ^{125}**I-23**; a known high affinity PSMA ligand (Figure 2).[19] All

compounds were assayed using PSMA expressing LNCaP cells under the same conditions. Experiments were performed in triplicate using 2-(phosphonomethyl)-pentanedioic acid (PMPA), a known PSMA inhibitor, as a positive control. The previously reported *des*-iodo ligands **1** and **2** were also tested to assess the impact of the addition of iodine to the triazoles, on the binding to PSMA.

The IC_{50} values of the 5-iodo derivatives ranged from 14 nM to 30 nM, with the most potent ligand being the acetamide linked, PEG-8 derivative **9b** (Table 1). The PEG-4 derivative **9a** was comparable with an IC_{50} of 15 nM. For compounds containing only the triazole linker (**7a**, **7b**), the products were still potent ligands, however the measured IC_{50} values nearly doubled compared to **9a**/**9b**. For the 4-iodo series, the PEG-4 derivative **12a** showed an IC_{50} of 19 nM, while the PEG-8 derivative **12b** was nearly 100 nM. Interestingly, the short chain derivative bearing no DNP was the least potent compound tested (123 nM), and its DNP derivative showed the lowest IC_{50} (3 nM), indicating the presence of an arene binding region.

The IC_{50} values measured for the parent ARM-Ps was 9 nM for the PEG-4 derivative versus 23 nM for the longer chain PEG-8 form. Although the affinities were higher in our assay than those reported using a different screening method,[6] the data are consistent with the literature in that they showed both constructs had good affinity for PSMA and that ARM-P4 was more potent than ARM-P8. In the case of the iodinated compounds, there was evidence of impact on affinity when the triazole alone was used as the linker between the PEG group and the PSMA binding portion of the molecule. When the acetamide linker was added, the affinities were similar or slightly better than that observed for ARM-P8 (**2**).[6]

Compound **9b** was selected as the lead compound and its ability to bind to both PSMA and anti-DNP antibodies was measured, using a previously reported *in vitro* fluorescence assay.[6] PSMA expressing LNCaP cells were incubated with **9b** or **2** (the *des*-

iodo analogue reported previously), with and without the PSMA inhibitor PMPA. This was followed by AlexaFluor488 conjugated, rabbit anti-DNP antibody, to detect the amount of DNP bound to the LNCaP cells. Antibody binding was evident for **9b**, which was reduced by approximately 70% when the assay was performed in the presence of PMPA (Figure 3A). Similar results were obtained with the positive control **2**.[6] A second test compared **9b** with **19**, a potent ligand that has no spacer group between the PSMA binding portion of the molecule and the DNP group. As expected, background levels fluorescence were obtained with **19**, indicating negligible anti-DNP binding, with no effect observed with PMPA blocking (Figure 3B).

Radiochemistry

A method for producing ^{125}**I-9b** was developed in order to assess the ability of the labeled construct to target PSMA *in vivo*, using PMSA-expressing xenograft tumors. Given its unusually high affinity for PSMA, the iodine-125 analogue of **19** was also prepared for comparison. The synthesis of ^{125}**I-9b** was performed using a methodology that is similar to the synthesis of the non-radioactive compound, in that the alkyne **4b** was treated with cupric chloride, followed by the azide **8** and Na^{125}I, in place of N-iodosuccinimide (Scheme 6). These conditions resulted in concomitant formation of the triazole and the addition of the iodine at the 5-position of the ring. The desired product was isolated by HPLC in 70% yield, and the retention time of the product matched that of the reference standard used to determine the IC_{50} value, with a measured radiochemical purity of 97% (Figure 4). The product was stable in saline up to 48 h, and for three weeks when stored as a solid at -10 ^{o}C. The log *P* value for ^{125}**I-9b** measured using the "shake-flask" method[22] was -2.54 ± 0.03, which was higher than the iodo-triazole **23**,[19] which had a log *P* of -3.23 ± 0.05. The log P value for ^{125}**I-9b** was however less than those for p-iodo-benzyl and iodo-phenylurea derivatives of

glutamate-urea-lysine (0.26 and 0.53 respectively), which have been evaluated in patients as PSMA targeting radiopharmaceuticals.[23]

For **19**, the corresponding stannane was prepared in order to introduce iodine-125 at the 4-position of the triazole. This was accomplished by treatment of **14** with hydrazine hydrate, followed by coupling with 1-chloro-2,4-dinitrobenzene to produce the stannane precursor **21** (Scheme 7). The radiolabeling of **21** to form ^{125}**I-19** was performed at in 5% AcOH/MeCN containing Na[^{125}I] and Iodogen® as an oxidant. After 15 min, the reaction was quenched with $Na_2S_2O_5$ and deprotected with TFA for 1 h and the product purified by HPLC to obtain ^{125}**I-19** in 72% yield and high (>98%) radiochemical purity (Figure 5). As expected the log *P* value of ^{125}**I-19** (-1.4 ± 0.05) was higher than ^{125}**I-9b** that contains a PEG spacer.

Biodistribution Studies in Mice with LNCaP Xenografts

Biodistribution studies on ^{125}**I-9b** and ^{125}**I-19** were performed using NCr nu/nu mice bearing LNCaP xenografts, at 4-7 weeks post inoculation. Mice were administered between 0.19 and 0.36 MBq of either ^{125}**I-9b** or ^{125}**I-19** formulated in 0.9% saline, via tail vein injection. Animals were sacrificed and tissues collected and counted at t = 1 and 6 h post injection. ^{125}**I-9b** showed tumor uptake of 3.01 ± 0.17 %ID/g at 1 h, and remained near that level (3.24 ± 0.06 %ID/g) at 6 h (Figure 6). Tumor-to-blood ratios were approximately 10:1 at 1 h but decreased to 3.4:1 at 6 h. Tumor-to-muscle ratios were 44:1 at 1 h and 24:1 at 6 h. High uptake at 1 h was observed in the gallbladder, which declined significantly by 6 h. There was also greatly increasing levels in the thyroid at the later time point, indicating deiodination of the product.

For ^{125}**I-19**, high tumor uptake (12.21 ± 1.85 %ID/g) was observed at 1 h, with only a small decrease (9.80 ± 1.55 %ID/g) by 6 h (Figure 7). The tumor-to-blood ratios were 35:1 at 1 h and increased to 59:1 at 6 h. There was a tumor-to-muscle ratio of 37:1 at 1 h and 73:1

at 6 h. Rapid blood clearance was observed by 1 h (0.36 ± 0.07 %ID/g), and similar to that observed for ^{125}**I-9b**, there was high gall bladder uptake at 1 h (92.46 ± 20.7 %ID/g) with a decrease to 7.89 ± 1.67 %ID/g at 6 h. High and sustained kidney uptake was also observed (68.35 ± 16.56 %ID/g at 1 h, and 66.46 ± 45.10 %ID/g, at 6 h), which is typical of radiolabeled, small molecule PSMA probes.[19,23] The small intestine showed high uptake (47.37 ± 7.86 %ID/g) at 1 h, but had decreased substantially to 2.18 ± 0.19 %ID/g at 6 h.

Cell Binding and Internalization

Despite the IC_{50} value, the extent of tumor uptake for ^{125}**I-9b** was modest when compared to ^{125}**I-19** and ^{123}**I-23**.[19] This could be due to non-specific binding *in vivo*, de-iodination, or possibly due to reduced internalization following binding to PSMA. To gain further insight, cell binding assays were performed for ^{125}**I-9b** and the known PSMA ligand ^{125}**I-23** (Figure 8). The total binding of ^{125}**I-23** to LNCaP cells was almost 9 fold higher than that of ^{125}**I-9b**. When the binding was performed in the presence of the cold analogues of each compound or PMPA as blocking agents, the measured binding of either radiolabeled compound to LNCaP cells was reduced to the same degree. This indicated that both agents bound PSMA specifically and had minimal non-specific binding to LNCaP cells. The extent of internalization of by LNCaP cells was also assessed for both compounds, over 2 h of incubation at 37 ^{o}C or 4 ^{o}C. Both radiolabeled compounds were continuously internalized over 2 h at 37 ^{o}C, with larger quantities of ^{125}**I-23** internalized compared to ^{125}**I-9b** (Figure 9). After 2 h approximately one half of the cell associated radioactivity was internalized for both compounds. Low amounts of internalization for both compounds occurred at 4 ^{o}C, indicating that uptake at 37 ^{o}C was an active process.

To assess the impact of internalization on the extent of DNP antigen available on the cell surface, flow cytometry experiments were performed using a fluorophore labeled, anti-DNP antibody. Compound **9b** was incubated with LNCaP cells for 0, 0.5, 1, 2 and 4 h at 37

^{o}C followed by the fluorescent anti-DNP antibody. Flow cytometry showed appreciable binding of the antibody with modest decrease between 2 and 4 h of incubation (Figure 10). This was likely due to internalization, as was observed for the radiolabeled analogue (Figure 9B). As a control, the studies were repeated in the presence of a known PSMA inhibitor (PMPA), which blocked all observed binding to background levels. This data indicates that despite internalization observed with the radiotracer there is sufficient antigen (DNP) available over time to allow for antibody recruitment, and that the binding is due to PSMA expression.

It is clear that the presence of the DNP-PEG spacer in **9b** reduces the extent of tumor binding and uptake, but is nonetheless necessary for binding to anti-PSMA antibodies. Spiegel and coworkers demonstrated that while their ARM compound with a PEG-4 linker (ARM-P4) had higher affinity for PSMA, the extent of anti-DNP binding compared to ARM-P8 was greatly diminished.[6] Notwithstanding, one therapeutic advantage of the ARM-P strategy is that it requires binding to PSMA and the anti-DNP antibody.[8] Consequently, the impact of lower than optimal target binding on the tumor may be reduced through the need to form a ternary complex (PSMA-DNP-antibody). Given the positive *in vitro* and *in vivo* data reported for DNP-derived PSMA ARMs,[8] Sufficient amounts of ligand must be getting to the tumors to induce an ADCC response. Unfortunately, the same benefit is not imparted to radiotherapy, where high off target binding can cause unwanted dose being delivered to normal tissue. Potential explanations for the modest cell and tumor uptake of ^{125}**I-9b** versus other radiolabeled PSMA derivatives are that the construct has inadequate affinity, the presence of the DNP-PEG group and/or addition of the iodine label decreases uptake, or that rapid deiodination occurs *in vivo*, prior to PSMA binding. To provide further insight, the tritiated analogue of **2** is being prepared and uptake and distribution in PSMA expressing cells and tumor models assessed. One of the immediate benefits of the reported approach is

that creation of radiolabeled ARMs provides a convenient means to determine the extent of tumor binding, internalization and overall biodistribution of novel constructs. This data can subsequently be used to fine tune pharmacokinetics and enhance the efficacy of the next generation of ARMs and analogues containing therapeutic radionuclides.

Conclusions

A series of radiolabeled antibody recruiting molecules were prepared and their binding to PSMA assessed, with several potent compounds identified. Two lead constructs were evaluated *in vivo* using LNCaP tumor models. One DNP derivative (**9b**) was capable of recruiting anti-DNP antibodies *in vitro*, and when iodinated, showed modest tumor uptake *in vivo*, with good tumor-to-blood ratios. In addition, a novel iodinated PSMA binding ligand (**19**) was also discovered, and showed high tumor uptake and good tumor-to-blood and tumor-to-muscle ratios. While combining radiotherapy and ARM in one construct will require additional work to find compounds with greater tumor uptake ratios, the availability of radioiodinated compounds **19** and **23**, along with other known PSMA-targeted radiotherapeutics, provides an opportunity to combine radiotherapy and immunotherapy using **2** and related analogues. In addition, access to radiolabeled ARMs provides the means to use *in vivo* imaging to support the development of new immune stimulating constructs in addition to seeking potent dual function therapeutics.

Experimental Section

General Procedures, Materials and Instrumentation. All chemicals and reagents for synthesis were purchased from Sigma-Aldrich. Solvents were purchased from Caledon and dried using a Pure-Solv drying apparatus (Innovative Technology). Compounds **1**-**5** were

prepared according to literature procedures.[6] Preparative TLC was carried out using 0.75 mm layers of silica gel 60 GF254 made from water slurries on glass plates of dimensions 20 × 20 cm^2, followed by drying in air at 100 ^oC. Iodine-125 was obtained from the McMaster Nuclear Reactor as Na[^{125}I] in 0.1 N NaOH. For labeling experiments, radioactivity was measured using a dose calibrator (Capintec, Ramsey, NJ, USA). *Caution: Iodine-125 is radioactive and should only be handled in an appropriately licensed and equipped laboratory.*

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance AV-600 spectrometer (^{1}H = 600.13 MHz, ^{13}C = 150.90 MHz, ^{119}Sn = 225 MHz). ^{1}H NMR, ^{119}Sn NMR and ^{13}C NMR chemical shifts are expressed in parts ppm (δ units), and coupling constants are expressed in Hz. $SnMe_4$ was used as an external reference in ^{119}Sn NMR. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (KBr disc). Low-resolution mass spectra were obtained on an Agilent 630 ion trap ESI instrument, using a 1200 series LC system and an eluent consisting of H_2O:MeOH (1:1). HRMS were obtained using a Waters Micromass Global Ultima Q-TOF in ESI mode. HPLC (analytical and semi-preparative) was performed on a Varian ProStar HPLC system fitted with an IN/US γ-RAM Model 3 detector or a Waters 1525 Binary HPLC system connected to a Bioscan γ-detector and a 2998 photodiode array detector monitoring at 254 nm. For analysis of compounds, a Phenomenex Gemini column (5 μm, 4.6 × 250 mm, C18) was used, eluting at a flow rate of 1.0 mL/min. For semi-preparative HPLC, a Phenomenex Luna column (5 μm, 10.0 × 250 mm, C18) was used, eluting at a flow rate of 4.0 mL/min. HPLC protocols were as follows: Analytical and semi-preparative HPLC: Solvent A = 0.1% trifluoroacetic acid (TFA) in water; Solvent B = 0.1% TFA in acetonitrile: gradient elution, 5% B (0-8 min), 39% B (8-20 min), 5% B (21-22 min).

Cell Culture. LNCaP cells were purchased from ATCC (CRL 1740). Cells were propagated using RPMI 1640 with 2mM glutamine, supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 10mM HEPES, 1% Penicillin

Streptomycin and 0.25% D-glucose, and grown at 37˚C and 5% CO_2. Cells were used between passage numbers 7 to 28.

Animal Studies. Animal studies were approved by the Animal Research Ethics Board at McMaster University in accordance with Canadian Council on Animal Care (CCAC) guidelines. Male, 4-5 week old NCr nu//nu mice were purchased from Taconic (Germantown, NY) and were maintained under SPF conditions in an established animal facility, with 12 h light/dark cycles, and given food and water ad libitum. Mice were injected with 2×10^6 LNCaP cells, in Matrigel:DPBS (1:1 v/v) subcutaneously in the right flank, to provide tumor xenografts.

PSMA Binding Studies

Binding studies were done as described by Hillier *et al.*[23] with some modifications. Briefly, equal numbers (3×10^5) of LNCaP cells in RPMI 1640 with 2 mM glutamine and 0.5% BSA, where incubated for 1 h at rt with either 10 nM ^{125}**I-9b** or 3 nM ^{125}**I-23** alone, or in the presence of 10 μM of the cold analogues (**9b** or **23**) or 10 μM PMPA. Cells and media were transferred to microcentrifuge tubes and were washed 3 times with centrifugation for 30 s, at 21,130 x g. Final pellets were counted for 10 min, using a Perkin Elmer Wizard 1470 Automatic Gamma Counter. The resulting CPM values were used to calculate fmol bound per sample.

Fluorescence Assay for Binding to PSMA Expressing Cells and anti-DNP Antibodies

Fluorescence based assessment of **2**, **9b**, and **19** binding to both PSMA and anti-DNP antibodies was performed as described,[6] with minor changes to the procedure. LNCaP cells were detached from the flask and re-suspended in TBS, having a density of 6.25×10^5 cells/mL. Aliquots of 0.2 mL of the cell solution were prepared in Eppendorf tubes. Three compound solutions were prepared: 220 nM solutions of **2**, **9b**, or **19**, containing either 887 μM PMPA (block) or no PMPA. AlexaFluor488 conjugated rabbit anti-DNP IgG (Invitrogen)

was added (final concentration, 580 nM) immediately before the mixture was added to LNCaP cells. A 57.3 μL volume of each solution was added to designated Eppendorf tubes containing the LNCaP cells. Aliquots were incubated at 37 °C for 1 h on a shaker. To each tube, 750 μL TBS was added, and the cells were washed an additional time with TBS. PBS was used to wash the cells once more. The aliquots were re-suspended in 0.5 mL lysing buffer and incubated at 37 °C. Three 150 μL aliquots of each 500 μL lysed cell sample was added to a 96-well plate, and fluorescence intensity was analyzed with the Tecan Infinite M1000 plate reader by exciting at 494 nm and reading the fluorescence intensity at 520 nm.

Internalization Studies of Radiolabeled Compounds

Internalization studies were done as described by Hillier *et al.*[23] with some modifications. Briefly, aliquots (3 x 10^5) of LNCaP cells were incubated in HEPES buffered saline with 10 nM ^{125}I-**9b** or 5 nM ^{125}I-**23** and 90 nM **9b** or 95 nM **23** respectively, at 4 °C or 37 °C for 0 to 2 h. At designated times, cells in media were transferred to microcentrifuge tubes and washed twice with PBS using centrifugation for 30 s, at 21,130 x g, to remove unbound radioactivity. The cells were then washed (50 mM Glycine, 150 mM NaCl, pH 3.0) at 4 °C for 5 min, to release surface-bound radioactivity. Cell aliquots were then centrifuged at 20,000 × g for 5 min. Both the supernatant (released surface bound radioactivity) and the cell pellet (internalized radioactivity) were counted for 10 min with a Perkin Elmer Wizard 1470 Automatic Gamma Counter. The resulting cpm values were used to calculate surface bound and internalized fmol of each radiolabeled compound.

Flow Cytometry

Flow cytometry studies were conducted as described in Murelli *et al.*[6] with minor modifications. Briefly, LNCaP cells were detached, counted, washed, resuspended in TBS (25 mM Tris-HCl, 150 mM NaCl, 1.5% BSA, 5mM Glucose, 1.5 mM $MgCl_2$, pH 7.2) at a density of 5×10^5 cells/mL and 0.25 mL aliquots were added to Eppendorf tubes. Compound

9b in water (2.5 μL of 5 μM stock) was added to the aliquots followed by either vehicle (2.3 μL of saline) or the blocking PMPA solution (2.3 μL of 22.1 mM PMPA solution in saline). Samples were incubated with shaking at 37 °C prior to the addition of 2 mg/mL (2.5 μL) AlexaFluor488 conjugated rabbit anti-dinitrophenyl IgG (Invitrogen), at t=0, 0.5, 1, 2, and 4 h. Samples were protected from light and incubated at 37 °C with shaking for a further 1 h. Cells were diluted and washed with 0.75 mL of TBS and centrifuged for 30 s at max speed. The wash was repeated once more with 1 mL of TBS followed by a final wash with 1 mL of PBS. Cells were resuspended in 0.5 mL PBS and kept on ice until analyzed on a FACS Aria III (Becton-Dickinson). AlexaFluor488 (Fl-1 channel) was determined, after dead cells were excluded based on propidium iodide (500 μg/mL, 2 μL) staining (Fl-2). Data was analyzed using FlowJo software (Tree Star Inc.) and all experiments performed in triplicate.

Synthetic Procedures

For all compounds evaluated purity was >95% as determined by HPLC, HRMS and ^{1}H and ^{13}C NMR.

General Procedure for Synthesis of 6a and 6b. To a solution of copper(I) iodide (63.0 mg, 0.33 mmol) and triethylamine (19.7 mg, 0.33 mmol) in dry DMF (1.0 mL) were added alkyne (**4a** or **4b**, 0.33 mmol), azide **5** (190 mg, 0.33 mmol) and *N*-iodosuccinimide (82 mg, 0.37 mmol). The resulting mixture was stirred at rt under an atmosphere of argon for 4-8 h. After the specified reaction time, water (10 mL) was added and the product mixture extracted with DCM (3 × 10 mL). The organic layers were combined, extracted with 20 mL of brine and dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure and the desired product was purified by preparative TLC (5% MeOH/DCM).

6a: Yellow waxy solid (75% yield, 256 mg, R_f= 0.45). ^{1}H NMR ($CDCl_3$): δ 9.13 (d, *J* = 3 Hz, 1H, CH), 8.80 (bs, 1H, CH), 8.26 (d, *J* = 12 Hz, 1H, CH), 6.97 (d, *J* = 6 Hz, 1H, CH), 5.07 (bs, 2H, NH), 4.59 (s, 2H, CH_2), 4.34 (m, 4H, CH_2), 3.84 (d, *J* = 6 Hz, 2H, CH_2), 3.68 (m,

12H, CH_2), 3.60 (m, 2H, CH_2), 2.30 (m, 2H, CH_2), 2.07 (m, 1H, CH), 1.86 (m, 4H, CH_2), 1.65 (m, 1H, CH), 1.46, 1.44, 1.42 (s, 27H, CH_3), 1.30 (m, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 172.4, 172.2, 172.0, 156.8, 148.5, 148.1, 136.0, 130.4, 130.0, 124.4, 114.2, 81.9, 80.6, 80.2, 70.8, 70.7, 70.6, 70.4, 69.7, 68.6, 64.2, 53.2, 53.0, 50.4, 43.3, 32.6, 31.6, 29.5, 28.4, 28.1, 28.0, 22.1 ppm. IR (KBr): 3358, 2976, 1730, 1621 cm^{-1}. HRMS (ESI^+) m/z for $C_{41}H_{65}O_{15}N_8INa$: calcd 1059.3512, obsd 1059.3464 $[M + Na]^+$.

6b: Yellow waxy solid **(**83% yield, 332 mg, R_f = 0.38). ^{1}H NMR ($CDCl_3$): δ 9.13 (d, *J* = 3 Hz, 1H, CH), 8.80 (s, 1H, CH), 8.26 (dd, *J* = 3, 6 Hz 1H, CH), 6.97 (d, *J* = 6 Hz, 1H, CH), 5.07 (m, 2H, NH), 4.59 (s, 2H, CH_2), 4.31 (m, 4H, CH_2), 3.83 (t, *J* = 6 Hz, 2H, CH_2), 3.65 (m, 32H, CH_2), 2.29 (m, 2H, CH_2), 2.09 (m, 1H, CH), 1.83 (m, 4H, CH_2), 1.62 (m, 1H, CH), 1.46, 1.45, 1.42 (s, 27H, CH_3), 1.34 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$): δ 172.5, 172.2, 172.0, 156.8, 148.6, 136.2, 130.5, 130.2, 124.4, 114.2, 82.1, 82.0, 80.6, 70.8, 70.7, 70.6, 70.4, 69.6, 68.6, 64.3, 53.2, 53.1, 50.4, 43.3, 32.6, 31.6, 29.4, 28.4, 28.1, 28.0, 22.0 ppm. IR (KBr): 3359, 2868, 1736 cm^{-1}. HRMS (ESI^+) m/z for $C_{49}H_{82}O_{19}N_8I$: calcd 1213.4741, obsd 1213.4752 $[M + H]^+$.

Synthesis of 7a. Boc-protected **6a** (50.0 mg, 0.048 mmol) was dissolved in a mixture of TFA (2 mL) and CH_2Cl_2 (1 mL) in a 5 ml microwave vial that was capped and subjected to microwave heating for 5 min at 70 ^{o}C. The resulting reaction mixture was concentrated under reduced pressure. The desired product was purified by semi-preparative HPLC and lyophilized to give **7a**. Yellow waxy solid (92% yield, 38 mg, t_r = 14.1 min). ^{1}H NMR (CD_3OD): δ 8.91 (d, *J* = 3 Hz, 1H, CH), 8.17 (m, 1H, CH), 7.11 (d, *J* = 6 Hz, 1H, CH), 4.45 (s, 2H, CH_2), 4.33 (t, *J* = 6 Hz, 2H, CH_2), 4.19 (m, 2H, CH_2), 3.70 (t, *J* = 6 Hz, 2H, CH_2), 3.56 (m, 16H, CH_2), 3.21 (s, 4H, CH_2), 2.30 (m, 2H, CH_2), 2.04 (m, 1H, CH), 1.85–1.75 (m, 4H, CH_2), 1.60 (m, 1H, CH), 1.34 (m, 2H, CH_2) ppm. ^{13}C NMR (CD_3OD): δ 176.3, 160.1, 149.8, 137.0, 131.7, 131.0, 124.7, 116.2, 71.7, 71.6, 71.5, 70.9, 70.0, 65.0, 53.8, 51.7, 44.2,

32.9, 31.1, 30.5, 29.0, 23.5 ppm. IR (KBr): 3356, 2924, 2868, 1734 cm^{-1}. HRMS (ESI^+) m/z for $C_{29}H_{42}O_{15}N_8I$: calcd 869.1815, obsd 869.1805 $[M + H]^+$.

Synthesis of 7b. Compound **7b** was prepared from **6b** (12.0 mg, 0.01 mmol) following the procedure used to synthesize **7a**, to give **7b**. Yellow waxy solid (93% yield, 9.0 mg, t_r = 12.3 min). ^1H NMR (CD_3OD): δ 8.93 (d, *J* = 3 Hz, 1H, CH), 8.19 (dd, *J* = 6, 8 Hz, 1H, CH), 7.13 (d, *J* = 12 Hz, 1H, CH), 4.48 (s, 2H, CH_2), 4.34 (t, *J* = 6 Hz, 2H, CH_2), 4.22 (m, 1H, CH), 4.18 (m, 1H, CH), 3.71 (t, *J* = 6 Hz, 2H, CH_2), 3.55 (m, 34H, CH_2), 2.30 (m, 2H, CH_2), 2.04 (m, 1H, CH), 1.80 (m, 4H, CH_2), 1.59 (m, 1H, CH), 1.35 (m, 2H, CH_2) ppm. ^{13}C NMR (CD_3OD): δ 176.4, 176.2, 175.8, 160.1, 149.9, 149.5, 137.1, 131.6, 131.2, 124.8, 116.2, 83.4, 71.6, 71.5, 71.4, 71.3, 70.8, 70.0, 65.1, 53.8, 53.4, 51.6, 44.0, 32.9, 31.1, 30.4, 29.0, 23.5 ppm. IR (KBr): 3360, 2919, 2868, 1717, 1684 cm^{-1}. HRMS (ESI^+) m/z for $C_{37}H_{58}O_{19}N_8I$: calcd 1045.2863, obsd 1045.2814 $[M + H]^+$.

General Procedure for Synthesis of 9a and 9b. To a mixture of alkyne **4a** or **4b** (0.066 mmol), and azide **8** (31.0 mg, 0.054 mmol) in dry DMF (0.5 mL) was added copper(I) iodide (13.3 mg, 0.070 mmol), triethylamine (7.24 mg, 0.070 mmol) and *N*-iodosuccinimide (15.0 mg, 0.073 mmol). The resulting mixture was allowed to stir at rt under an atmosphere of argon for 3 h. The reaction was deemed complete by TLC (and mass spectrometry), filtered through a plug of celite, and concentrated to give a yellow oil, which was redissolved in CH_2Cl_2 (2 mL) and TFA (4 mL) in a microwave vial. The vial was capped and subjected to microwave irradiation for 5 min at 70 ^oC. The resulting reaction mixture was concentrated under reduced pressure, and chromatographed using semi-preparative HPLC and lyophilized.

9a: Yellow waxy solid (63% yield, 37 mg, t_r = 11.97 min). ^1H NMR (CD_3OD): δ 8.93 (d, *J* = 6 Hz, 1H, CH), 8.17 (dd, *J* = 6, 12 Hz, 1H, CH), 7.11 (d, *J* = 12 Hz, 1H, CH), 5.06 (m, 2H, CH_2) 4.48 (s, 2H, CH_2), 4.22 (m, 1H, CH), 4.17 (m, 1H, CH), 3.71 (t, *J* = 6 Hz, 2H, CH_2), 3.55 (m, 16H, CH_2), 3.16 (t, *J* = 6 Hz, 2H, CH_2), 2.31 (m, 2H, CH_2), 2.05 (m, 1H, CH), 1.78

(m, 2H, CH_2), 1.52 (m, 1H, CH), 1.42 (m, 2H, CH_2), 1.34 (m, 2H, CH_2) ppm. ^{13}C NMR (CD_3OD): δ 176.4, 176.3, 175.9, 167.1, 160.1, 150.0, 149.8, 137.1, 131.1, 124.8, 116.3, 85.2, 71.7, 71.6, 71.5, 70.9, 70.0, 65.1, 54.0, 53.7, 53.6, 44.1, 40.5, 33.2, 31.1, 30.0, 28.9, 24.0, 22.0 ppm. IR (KBr): 3356, 2925, 1676, 1622 cm^{-1}. HRMS (ESI^+) m/z for $C_{31}H_{45}O_{16}N_9I$: calcd 926.2029, obsd 926.2056 $[M + H]^+$.

9b: Yellow viscous oil (70% yield, 50 mg, t_r = 10.95 min). ^{1}H NMR (CD_3OD): δ 8.93 (d, *J* = 3 Hz, 1H, CH), 8.20 (dd, *J* = 6, 8 Hz, 1H, CH), 7.13 (d, *J* = 6 Hz, 1H, CH), 5.08 (m, 2H, CH_2), 4.50 (s, 2H, CH_2), 4.22 (m, 1H, CH), 4.17 (m, 1H, CH), 3.71 (t, *J* = 6 Hz, 2H, CH_2), 3.55 (m, 32H, CH_2), 3.16 (t, *J* = 6 Hz, 2H, CH_2), 2.31 (m, 2H, CH_2), 2.05 (m, 1H, CH), 1.84-1.73 (m, 2H, CH_2), 1.57 (m, 1H, CH), 1.49 (m, 2H, CH_2), 1.38 (m, 2H, CH_2) ppm. ^{13}C NMR (CD_3OD): δ 176.4, 176.4, 175.9, 167.1, 161.9, 161.7, 160.2, 149.9, 149.8, 137.1, 131.0, 124.7, 116.2, 85.3, 71.6, 71.5, 71.4, 71.3, 70.8, 70.0, 65.1, 54.0, 53.7, 53.6, 44.2, 40.4, 33.3, 31.2, 29.9, 28.9, 24.1 ppm. IR (KBr) 3359, 2919, 2870, 1726, 1683 cm^{-1}. HRMS (ESI^+) m/z for $C_{39}H_{61}O_{20}N_9I$: calcd 1102.3077, obsd 1102.3107 $[M + H]^+$.

Synthesis of 10a. To a solution of $ZnBr_2$ in THF (0.05 M, 2.0 mg/0.3 mL) and DNP-PEG4-alkyne[6] (50 mg, 0.12 mmol) was added dropwise Bu_3SnOMe (43 uL, 0.15 mmol). The mixture was stirred for 4 h at 60 ^oC, and then quenched by H_2O (5 mL). The mixture was extracted with diethyl ether (3 × 10 mL). The collected organic layers were dried ($MgSO_4$). Evaporation of volatiles gave a waxy residue which was diluted with EtOAc (20 mL) and washed by NH_4F (aq) (10%, 20 mL). The obtained white precipitate was filtered off, and the filtrate was dried ($MgSO_4$). Evaporation of volatiles gave an oily yellow product. Purification by prep-TLC using hexane/EtOAc (1:1) gave compound **10a**. Yellow oil (41% yield, 36 mg, R_f= 0.35). It is noteworthy that alkyne **10a** is unstable and must be kept in the refrigerator to prevent fast decomposition. Typically a freshly prepared sample of **10a** was used in all subsequent reactions. ^{1}H NMR (CD_3CN): δ 8.77 (d, *J* = 6 Hz, 1H, CH), 8.51 (bs, 1H, CH),

8.06 (dd, J = 6, 8 Hz, 1H, CH), 6.94 (d, J = 12 Hz, 1H, CH), 3.91 (m, 2H, CH_2), 3.57 (t, J = 6 Hz, 2H, CH_2), 3.38 (m, 16H, CH_2), 1.75 (m, 2H, CH_2), 1.36 (m, 4H, CH_2), 1.13 (m, 6H, CH_2), 0.79 (m, 4H, CH_2), 0.69 (t, J = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR (CD_3CN): δ 148.6, 123.5, 114.8, 105.9, 89.4, 70.0, 69.9, 69.7, 69.6, 68.6, 68.2, 68.1, 58.4, 42.8, 28.4, 26.4, 12.6, 10.4 ppm. ^{119}Sn NMR: (225 MHz, CD_3CN) -65.8. IR (KBr): 3326, 2798, 1731 cm^{-1}. HRMS (ESI$^+$) m/z for $C_{29}H_{49}O_8N3SnH$: calcd 688.2626, obsd 688.2623 $[M + H]^+$.

Synthesis of 10b. Compound **10b** was prepared as described for **10a** using $ZnBr_2$ (0.12 M, 5.0 mg/0.5 mL THF), DNP-PEG8-alkyne[6] (50 mg, 0.09 mmol) and Bu_3SnOMe (50 μL, 0.17 mmol). The reaction mixture was stirred for 3 days at 60 ^{o}C, and then quenched by H_2O (5 mL). Purification by preparative-TLC using 4% MeOH/DCM gave compound **10b**. Yellow oil (62% yield, 46 mg, R_f = 0.65). ^{1}H NMR (CD_3CN): δ 9.00 (d, J = 3 Hz, 1H, CH), 8.75 (bs, 1H, CH), 8.28 (dd, J = 3, 8 Hz, 1H, CH), 7.17 (d, J = 12 Hz, 1H, CH), 4.17 (m, 2H, CH_2), 3.80 (t, J = 6 Hz, 2H, CH_2), 3.62 (m, 32H, CH_2), 1.58 (m, 4H, CH_2), 1.37 (m, 6H, CH_2), 1.04 (m, 6H, CH_2), 0.92 (t, J = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR (CD_3CN): δ 148.6, 129.8, 123.4, 115.1, 105.8, 89.2, 74.3, 70.0, 69.8, 69.5, 68.7, 68.2, 68.1, 58.3, 57.4, 42.7, 28.4, 26.3, 12.7, 10.4 ppm. ^{119}Sn NMR (CD_3CN): δ -65.7 ppm. IR (KBr): 3112, 2960, 1734 cm^{-1}. HRMS (ESI$^+$) m/z for $C_{37}H_{65}O_{12}N_3SnNH_4$: calcd 881.3943, obsd 881.3953 $[M + NH_4]^+$.

General Procedure for Synthesis of 11a and 11b. Alkyne **10a** or **10b** (0.04 mmol) was dissolved in toluene (2 mL) followed by the addition of the azide **8** (24 mg, 0.04 mmol). The reaction mixture was stirred at 100 ^{o}C for 16-24 h. After cooling to rt, the reaction mixture was concentrated under reduced pressure and purified by prep-TLC.

11a. Ethyl acetate/hexane (1:1) was used as prep-TLC eluent and the product isolated. Yellow waxy solid (30% yield, 15 mg, R_f = 0.47). ^{1}H NMR (CD_3CN): δ 8.94 (d, J = 3 Hz, 1H, CH), 8.70 (bs, 1H, NH), 8.23 (dd, J = 3, 6 Hz, 1H, CH), 7.12 (d, J = 6 Hz, 1H, CH), 6.86 (t, J = 6 Hz, 1H, NH), 5.48 (d, J = 12 Hz, 1H, NH), 5.36 (d, J = 12 Hz, 1H, NH), 5.07 (s, 2H, CH_2),

4.52 (s, 2H, CH_2), 4.13 (m, 1H, CH), 4.03 (m, 1H, CH), 3.75 (t, J = 3 Hz, 2H, CH_2), 3.58 (m, 16H, CH_2), 3.16 (m, 2H, CH_2), 2.23 (m, 2H, CH_2), 1.93 (m, 1H, CH), 1.75 (m, 1H, CH), 1.65 (m, 1H, CH), 1.53 (m, 1H, CH), 1.46 (m, 6H, CH_2), 1.40 (m, 27H, CH_3), 1.30 (m, 9H, CH_2), 1.11 (m, 6H, CH_2), 0.86 (t, *J* = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR (CD_3CN): δ 172.1, 171.8, 171.6, 165.7, 157.0, 148.4, 144.1, 140.1, 140.6, 129.7, 123.4, 114.8, 80.8, 80.4, 79.6, 70.0, 69.9, 69.8, 69.5, 69.3, 68.1, 61.9, 53.3, 52.6, 50.4, 42.7, 38.4, 31.4, 30.9, 28.4, 28.3, 27.5, 27.0, 26.9, 26.6, 22.1, 12.7, 9.3 ppm. ^{119}Sn NMR (CD_3CN): δ -58.6 ppm. IR (KBr): 3334, 2978, 1730 cm^{-1}. HRMS (ESI^+) m/z for $C_{55}H_{95}N_9O_{16}SnH$: calcd 1258.6011, obsd 1258.6044 $[M + H]^+$.

11b. 5 % MeOH/DCM was used as prep-TLC eluent and the product isolated. Yellow viscous oil (28% yield, 16 mg, R_f = 0.32). ^{1}H NMR (CD_3CN): δ 9.00 (s, 1H, CH), 8.75 (bs, 1H, CH), 8.28 (dd, *J* = 6,6 Hz, 1H, CH), 7.17 (d, *J* = 12 Hz, 1H, CH), 6.95 (t, *J* = 6 Hz, 1H, CH), 5.53 (d, *J* = 12 Hz, 1H, CH), 5.42 (d, *J* = 12 Hz, 1H, CH), 5.13 (s, 2H, CH_2), 4.59 (s, 2H, CH_2), 4.18 (m, 1H, CH), 4.08 (m, 1H, CH), 3.80 (t, *J* = 6 Hz, 2H, CH_2), 3.66 (m, 4H, CH_2), 3.60 (m, 28H, CH_2), 3.21 (m, 2H, CH_2), 2.27 (m, 2H, CH_2), 1.80 (m, 1H, CH), 1.71 (m, 1H, CH), 1.57 (m, 8H, CH_2), 1.45 (m, 29H, CH_3, CH_2), 1.36 (m, 10H, CH_2), 1.17 (m, 2H, CH_2), 1.15 (m, 6H, CH_2), 0.90 (m, 9H, CH_3) ppm. ^{13}C NMR (CD_3CN): δ 172.2, 171.9, 171.6, 165.7, 157.1, 148.5, 144.1, 140.7, 129.7, 123.4, 114.8, 80.8, 80.4, 79.6, 69.9, 69.8, 69.5, 69.3, 68.1, 61.8, 53.3, 52.6, 50.4, 42.7, 38.4, 31.4, 30.9, 28.5, 28.3, 27.5, 27.0, 26.9, 26.6, 22.1, 12.7, 9.3 ppm. ^{119}Sn NMR (CD_3CN): δ -58.7 ppm. IR (KBr): 3326, 2930, 1731 cm^{-1}. HRMS (ESI^+) m/z for $C_{63}H_{112}O_{20}N_9Sn$: calcd 1434.7063, obsd 1434.7029 $[M + H]^+$

General Procedure for Synthesis of 12a and 12b. To a solution of **11a** or **11b** (0.006 mmol) in DCM (3 mL), I_2 (3 mg, 0.012 mmol) was added at rt and the mixture was stirred for 30 min. The reaction was passed through a silica plug (2 g, 10 wt% KF mixed in silica) and concentrated under vacuum to dryness. The resulting brown oily crude was redissolved in a

mixture of TFA and CH_2Cl_2 (2 mL, 1:1) in a 5 ml microwave vial. The vial was capped and subjected to microwave irradiation for 4 min at 70 ^oC. The resulting reaction mixture was concentrated under reduced pressure and the desired product was purified by semi-preparative HPLC and lyophilized.

12a: Yellow waxy solid, (97% yield, 5 mg, t_r = 12.45 min). ^1H NMR (CD_3OD): δ 9.02 (d, *J* = 3 Hz, 1H, CH), 8.28 (dd, *J* = 3, 6 Hz, 1H, CH), 7.18 (d, *J* = 12 Hz, 1H, CH), 5.29 (s, 2H, CH_2) 4.61 (s, 2H, CH_2), 4.31 (m, 1H, CH), 4.26 (m, 1H, CH), 3.82 (t, *J* = 6 Hz, 2H, CH_2), 3.65 (m, 16H, CH_2), 3.24 (m, 2H, CH_2), 2.42 (m, 2H, CH_2), 2.15 (m, 1H, CH), 1.87 (m, 2H, CH_2), 1.66 (m, 1H, CH), 1.57 (m, 1H, CH), 1.45 (m, 1H, CH), 1.32 (m, 4H, CH_2) ppm. ^{13}C NMR (CD_3OD): δ 176.4, 176.3, 175.9, 167.6, 162.3, 160.2, 149.8, 138.7, 137.3, 131.2, 124.7, 116.2, 91.1, 71.6, 71.5, 71.2, 71.1, 69.9, 62.7, 54.0, 53.6, 52.7, 44.1, 40.5, 33.2, 31.1, 29.9, 28.9, 24.0 ppm. IR (KBr): 3362, 2926, 1686 cm^{-1}. HRMS (ESI^+) m/z for $C_{31}H_{45}O_{16}N_9I$: calcd 926.2029, obsd 926.2039 $[M + H]^+$.

12b: Yellow waxy solid (93% yield, 6 mg, t_r = 12.92 min). ^1H NMR (CD_3CN): δ 8.99 (t, *J* = 6 Hz, 1H, CH), 8.72 (t, *J* = 6Hz, 1H, NH), 8.27 (dd, J = 3, 6 Hz, 1H, CH), 7.15 (m, 2H, CH, NH), 6.12 (m, 2H, NH), 5.11 (s, 2H, CH_2) 4.59 (s, 2H, CH_2), 4.27 (bs, 1H, CH), 4.19 (bs, 1H, CH), 3.80 (t, *J* = 6 Hz, 2H, CH_2), 3.64 (m, 34H, CH_2), 3.24 (d, *J* = 6 Hz, 2H, CH_2), 2.42 (m, 2H, CH_2), 2.11 (m, 1H, CH), 1.91 (m, 1H, CH), 1.80 (m, 1H, CH), 1.67 (m, 1H, CH), 1.58 (m, 2H, CH_2), 1.44 (m, 2H, CH_2) ppm. ^{13}C NMR (CD_3CN): δ 173.8, 173.7, 173.4, 164.6, 158.4, 148.4, 148.0, 135.5, 129.7, 123.4, 114.8, 82.9, 69.7, 69.5, 69.4, 69.1, 68.8, 68.6, 68.0, 63.5, 53.0, 52.4, 42.5, 38.5, 30.8, 29.7, 28.0, 26.7, 22.2 ppm. IR (KBr): 3327, 3058, 1693 cm^{-1}. HRMS (ESI^+) m/z for $C_{39}H_{60}O_{20}N_9INH_4$: calcd 1119.3344, obsd 1119.3333 $[M + NH_4]^+$.

Synthesis of 13. Method A: (3-Bromoprop-1-yn-1-yl)tributylstannane was prepared according to a literature method[21] and treated with potassium phthalimide as described previously[24] to give **13** in 33% yield. Method B: To a 0.5 M solution of $ZnBr_2$ in THF (0.5

mL) containing *N*-proparglphthalimide (185 mg, 1 mmol) was added dropwise Bu_3SnOMe (345 μL, 1.2 mmol). The mixture was stirred for 3 h at 60 ^oC, and then quenched by H_2O (10 mL). The mixture was extracted with diethyl ether (3 × 10 mL). The collected organic layers were dried ($MgSO_4$), and evaporated. The resulting residue was diluted with EtOAc (30 mL) and extracted with 10% m/v NH_4F (aq) (20 mL). The solution was filtered and the filtrate dried ($MgSO_4$) and evaporated producing an oily white product. Purification by preparative TLC using DCM gave compound **13**. Colorless oil (49% yield, 235 mg, R_f = 0.71). The alkyne **13** is unstable and must be kept in the refrigerator to prevent decomposition. It is advantageous to use a freshly prepared sample for further chemical transformations. ^1H NMR ($CDCl_3$): δ 7.89 (m, 2H, CH), 7.74 (m, 2H, CH) 4.48 (s, 2H, CH_2), 1.53 (m, 6H, CH_2), 1.31 (m, 6H, CH_2), 0.97 (m, 6H, CH_2), 0.87 (m, 9H, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 167.1, 134.0, 132.2, 123.4, 103.1, 86.7, 28.8, 28.2, 26.9, 13.6, 11.0 ppm. ^{119}Sn NMR ($CDCl_3$): δ -64.5 ppm. IR (KBr): 3245, 2917, 2113, 1734 cm^{-1}. HRMS (ESI^+) m/z for $C_{23}H_{33}O_2NSnH$: calcd 476.1616, obsd 474.1614 $[M + H]^+$.

Synthesis of 14. Azide **8** (300 mg, 0.53 mmol) was added to a solution of 1**3** (362 mg, 0.76 mmol) in toluene (5 mL) and the reaction mixture stirred at 80 ^oC for 16 h. After cooling to rt the reaction mixture was concentrated and the desired product was purified twice by preparative TLC, using ethyl acetate/hexane (2:1) as eluents and the product isolated. Colorless oil (50% yield, 275 mg, R_f = 0.50). ^1H NMR ($CDCl_3$): δ 7.83 (m, 2H, CH), 7.72 (m, 2H, CH), 6.98 (t, *J* = 6 Hz, 1H, NH), 5.86 (d, *J* = 12 Hz, 1H, NH), 5.56 (d, *J* = 6 Hz, 1H, NH), 5.35 (s, 2H, CH_2), 5.00 (m, 2H, CH_2), 4.36 (m, H, CH), 4.31 (m, H, CH), 3.22 (m, 1H, CH), 3.01 (m, 1H, CH), 2.34 (m, 2H, CH_2), 2.07 (m, H, CH), 1.84 (m, H, CH), 1.74 (m, 1H, CH), 1.48 (m, 29H, CH_3, CH_2), 1.31 (m, 6H, CH_2), 1.24 (m, 4H, CH_2), 0.86 (t, *J* = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR (CD_3CN): δ 172.1, 171.9, 171.8, 171.6, 167.5, 166.9, 165.4, 157.0, 145.9, 139.2, 134.1, 131.6, 122.8, 80.9, 80.6, 79.7, 53.2, 52.5, 51.3, 49.8, 38.3, 38.2, 31.5, 31.4, 30.9,

30.6, 28.6, 28.5, 28.4, 28.0, 27.5, 26.9, 22.1, 21.9, 12.7, 9.5 ppm. ^{119}Sn NMR (CD_3CN): δ -58.8 ppm. IR (KBr): 3315, 2979, 1733 cm^{-1}; HRMS (ESI^+) m/z for $C_{49}H_{79}O_{10}N_7SnH$: calcd 1046.5001, obsd 1046.5026 $[M + H]^+$.

Synthesis of 15. To a solution of **14** (240 mg, 0.23 mmol) in DCM (5 mL), I_2 (58 mg, 0.23 mmol) was added and the mixture was stirred for 15 min at rt. The reaction mixture was passed through a silica plug (2 g, 10 wt % KF mixed in silica), concentrated under vacuum to dryness and the desired product was purified by silica gel column chromatography using hexane/ethyl acetate as eluents (1:2) to provide compound **15**. Colorless oil (89% yield, 180 mg, R_f = 0.38). ^{1}H NMR ($CDCl_3$): δ 7.85 (m, 2H, 2CH), 7.74 (m, 2H, 2CH), 5.79 (m, 1H, NH), 5.47 (m, 3H, NH, CH_2), 5.02 (m, 2H, CH_2), 4.37 (m, 1H, CH), 4.30 (m, H, CH), 3.29 (m, H, CH), 3.08 (m, 1H, CH), 2.34 (m, 2H, CH_2), 2.08 (m, 1H, CH), 1.89 (m, 2H, CH_2), 1.75 (m, 1H, CH), 1.56 (m, 2H, CH_2), 1.47 (s, 9H, CH_3), 1.43 (s, 9H, CH_3), 1.41 (s, 9H, CH_3), 1.32 (m, 2H, CH_2) ppm. ^{13}C NMR ($CDCl_3$): δ 174.0, 172.5, 172.3, 167.8, 165.4, 157.6, 135.9, 134.5, 131.9, 123.8, 91.6, 82.4, 81.8, 80.8, 53.6, 53.4, 53.2, 52.6, 52.0, 39.4, 32.6, 32.1, 31.9, 30.8, 28.8, 28.2, 28.0, 22.7, 22.2 ppm. IR (KBr): 3345, 2976, 2928, 1721 cm^{-1}. HRMS (ESI^+) m/z for $C_{37}H_{52}IO_{10}N_7H$: calcd 882.2894, obsd 882.2899 $[M + H]^+$.

Synthesis of 16. Hydrazine hydrate (103 mg, 2.08 mmol) was added to a stirred solution of phthalimide **15** (180 mg, 0.204 mmol) in THF (5 mL), and the solution was heated to reflux for 5 h. After cooling to rt, the solvent was removed under reduced pressure. The resulting residue was dissolved in NaOH (5 mL, 20%), and the desired product extracted with DCM (3 × 10 mL). The combined organic layers were then dried over $MgSO_4$. The filtrate was evaporated under reduced pressure to leave the amine **16**. Colorless viscous oil (60% yield, 92 mg, R_f = 0.23). ^{1}H NMR ($CDCl_3$): δ 7.64 (t, *J* = 6 Hz,1H, NH), 5.94 (d, *J* = 6 Hz, 1H, NH), 5.61 (d, *J* = 6 Hz, 1H, NH), 5.25 (q, *J* = 18 Hz, 2H, CH_2), 4.35 (m, 1H, CH), 4.27 (m, 1H, CH), 3.94 (s, 2H, CH_2), 3.35 (m, 1H, CH), 3.19 (m, 1H, CH), 2.35 (m, 2H, CH_2), 2.05 (m,

5H, CH), 1.88 (m, 2H, CH_2), 1.75 (m, 2H, CH_2), 1.58 (m, 3H, CH), 1.47 (s, 9H, CH_3), 1.45 (s, 9H, CH_3), 1.43 (s, 9H, CH_3), 1.35 (m, 2H) ppm. ^{13}C NMR ($CDCl_3$): δ 173.5, 172.5, 172.3, 165.6, 157.6, 141.0, 89.2, 82.4, 81.6, 80.6, 53.4, 53.3, 51.6, 50.6, 39.3, 35.2, 32.2, 31.7, 28.3, 28.0, 27.9, 22.4 ppm. IR (KBr) 3289, 2926, 1735, 1652 cm^{-1}. HRMS (ESI^+) m/z for $C_{29}H_{50}IO_8N_7H$: calcd 752.2844, obsd 752.2842 $[M + H]^+$.

Synthesis of 17. Compound **16** (30 mg, 3.99 mmol) was dissolved in 1:1 (v/v) TFA:DCM (3 mL) and stirred at rt for 24 h. Evaporation of volatiles gave the crude product as a TFA salt, which was dissolved in water, lyophilized and analyzed by NMR. The free amine was obtained by dissolving the TFA salt in MeOH (1mL) and treating it with TEA (100 μL) at rt for 1 h. The solvent was removed under reduced pressure and the product was isolated by semi-preparative HPLC and lyophilized to provide **17**. Waxy white solid (74% yield, 17 mg, t_r = 6.25 min). ^{1}H NMR (DMSO-d_6): δ 12.48 (bs, 3H, COOH), 8.81 (s, 1H, NH), 8.28 (bs, 3H, NH_3), 6.34 (m, 2H, NH), 5.34 (s, 2H, CH_2), 4.20 (s, 2H, CH_2), 4.13 (m, 2H, CH), 3.12 (m, 2H, CH_2), 2.25 (m, 2H, CH_2), 1.91 (m, 1H), 1.70 (m, 2H), 1.51 (m, 4H), 1.27 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 174.5, 174.2, 173.7, 165.7, 157.4, 134.8, 95.4, 52.2, 51.7, 51.2, 31.7, 30.8, 29.8, 28.3, 28.0, 27.4, 22.6 ppm; IR (KBr): 3295, 2942, 1724, 1197 cm^{-1}. HRMS (ESI^-) m/z for $C_{17}H_{25}IO_8N_7$: calcd 582.0809, obsd 582.0805 $[M - H]^-$.

Synthesis of 18. 1-Chloro-2,4-dinitrobenzene (10 mg, 4.95 mmol) was added to a solution of **16** (30 mg, 3.99 mmol) in ethanol (2 mL). The reaction mixture was heated to reflux for 8 h and concentrated under reduced pressure. The desired product was isolated by preparative TLC using 9:1 DCM/MeOH. A yellow oil (38% yield, 14 mg, R_f = 0.62). ^{1}H NMR ($CDCl_3$): δ 9.13 (s, 1H, CH), 8.93 (s, 1H, NH), 8.32 (bs, 1H, CH), 7.77 (bs, 1H, NH), 7.05 (bs, 1H, CH), 5.84 (bs, 1H, NH), 5.47 (s, 1H, NH), 5.33 (m, 1H, CH), 5.15 (m, 1H, CH), 4.89 (m, 2H, CH_2), 4.35 (bs, 1H, CH), 4.22 (bs, 1H, CH), 3.49 (s, 2H, CH_2), 3.30 (bs, 1H, CH), 3.17 (bs, 1H, CH), 2.34 (s, 2H, CH_2), 2.07 (bs, 1H, CH), 2.01 (bs, 1H, CH), 1.87 (m, 1H, CH), 1.77 (m,

2H, CH_2), 1.46 (m, 29H, CH_2, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 173.9, 172.3, 172.2, 164.9, 157.7, 147.4, 137.1, 134.9, 134.8, 131.5, 130.5, 124.0, 114.5, 90.9, 82.9, 81.9, 80.8, 53.5, 53.4, 51.8, 50.8, 39.4, 37.4, 32.4, 31.6, 29.6, 28.0, 27.9, 27.8, 27.6, 22.7 ppm. IR (KBr): 3315, 3101, 2979, 1733 cm^{-1}. HRMS (ESI^+) m/z for $C_{35}H_{52}IO_{12}N_9H$: calcd 918.2858, obsd 918.2833 $[M + H]^+$.

Synthesis of 19. 10 mg (1.09 mmol) of **18** was dissolved in 1:1 (v/v) TFA:DCM (1 mL) and stirred at rt for 10 h. Evaporation of the solvent gave a dark yellow waxy solid that was dissolved in water, and lyophilized to provide **19**. Yellow solid (87% yield, 7 mg, tr = 7.89). ^1H NMR (DMSO-d_6): δ 12.39 (bs, 3H, COOH), 9.12 (t, J = 4 Hz, 1H, CH), 8.87 (d, J = 4 Hz, 1H, CH), 8.37 (t, J = 12 Hz, 1H, NH), 8.25 (dd, J = 4, 6 Hz, 1H, CH), 7.18 (d, J = 6 Hz, 1H, NH), 6.30 (dd, J = 6, 8 Hz, 2H, 2NH), 5.27 (s, 2H, CH_2), 4.84 (d, J = 6, 2H, CH_2), 4.08 (m, 1H, CH), 4.03 (m, 1H, CH), 3.01 (bs, 2H, CH_2), 2.28 (bs, 2H, CH_2), 1.91 (m, 1H, CH),1.65 (m, 2H, CH), 1.50 (m, 1H, CH), 1.38 (m, 2H, CH), 1.26 (bs, 3H, CH) ppm. ^{13}C NMR (DMSO-d_6): δ 176.5, 176.2, 175.8, 160.2, 150.0, 149.5, 137.2, 131.7, 131.1, 124.7, 116.2, 83.4, 71.6, 71.4, 71.3, 70.8, 70.0, 65.1, 53.8, 53.6, 51.6, 44.1, 32.9, 31.1, 30.5, 29.0, 23.5 ppm; IR: (neat) 3430, 3272, 2253, 1653, 1027 cm^{-1}; HRMS (ESI^-) m/z for $C_{23}H_{27}IO_{12}N_9$: calcd 748.0824, obsd 748.0788 $[M - H]^-$.

Synthesis of 20. Hydrazine hydrate (70 mg, 1.46 mmol) was added to a stirred solution of phthalimide **14** (148 mg, 0.143 mmol) in THF (3 mL), and the solution was heated to reflux for 5 h. It was then cooled to rt, and the solvent removed under reduced pressure. The resulting residue was dissolved in NaOH (3 mL, 20%), and the desired product was extracted with DCM (3 × 5 mL). The combined organic solution was then dried over $MgSO_4$. The filtrate was evaporated to produce **20**. Colorless oil (54% yield, 70 mg, R_f = 0.27). ^1H NMR ($CDCl_3$): δ 7.16 (t, J = 6 Hz, 1H, NH), 5.82 (d, J = 12 Hz, 1H, NH), 5.48 (d, J = 6 Hz, 1H, NH), 5.16 (q, J = 18 Hz, 2H, CH_2), 4.33 (m, 1H, CH), 4.23 (m, 1H, CH), 3.92 (s, 2H, CH_2),

3.29 (m, 1H, CH), 3.10 (m, 1H, CH), 2.31 (m, 2H, CH_2), 2.04 (m, 1H, CH), 1.83 (m, 2H), 1.71 (m, 2H, CH_2), 1.51 (m, 6H, CH_2), 1.44 (m, 11H, CH_2), 1.40 (m, 12H, CH_3, CH_2), 1.39 (m, 11H, CH_3, CH_2), 1.29 (m, 6H, CH_2), 1.12 (m, 5H, CH_2), 0.85 (t, J = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 173.2, 172.4, 172.3, 167.4, 166.4, 157.7, 144.8, 144.3, 82.2, 81.5, 80.6, 53.3, 53.2, 52.4, 51.0, 39.0, 35.5, 31.8, 29.0, 28.4, 28.1, 28.0, 27.2, 22.1, 27.9, 27.5, 26.9, 22.1, 21.9, 13.7, 10.1 ppm. ^{119}Sn NMR ($CDCl_3$): δ -59.7 ppm. IR (KBr): 3314, 2956, 1771 cm^{-1}. HRMS (ESI^+) m/z for $C_{41}H_{77}O_8N_7SnH$: calcd 916.4943, obsd 916.4980 $[M + H]^+$.

Synthesis of 21. To a solution of **20** (20 mg, 2.18 mmol) in ethanol (2 mL) were added 1-chloro-2,4-dinitrobenzene (8 mg, 3.96 mmol) and TEA (0.1 mL, 1.36 mmol). The reaction mixture was heated to reflux for 12 h and concentrated under reduced pressure. Purification of the desired product by preparative TLC using 95:5 DCM/MeOH gave compound **21**. Yellow waxy solid (52% yield, 12 mg, R_f = 0.35). ^{1}H NMR ($CDCl_3$): δ 9.14 (d, J = 3 Hz, 1H, CH), 8.58 (t, J = 6 Hz, 1H, CH), 8.32 (m, 1H, CH), 7.40 (bs, 1H, NH), 7.06 (d, J = 6 Hz, 1H, NH), 5.79 (d, J = 6 Hz, 1H, CH), 5.36 (d, J = 12 Hz, 1H, CH), 5.24 (d, J = 12 Hz, 1H, CH), 5.00 (d, J = 12 Hz, 1H, CH), 4.84 (m, 1H, CH), 4.72 (m, 1H, CH), 4.32 (m, 1H, CH), 4.26 (m, 1H, CH), 3.31 (m, 1H, CH), 3.11 (m, 1H, CH), 2.34 (m, 2H, CH_2), 2.07 (m, 1H, CH), 1.87 (m, 1H, CH), 1.75 (bs, 4H, CH_2), 1.42 (m, 33H, CH_2, CH_3), 1.27 (m, 8H, CH_2), 1.11 (m, 6H, CH_2), 0.83 (t, J = 6 Hz, 9H, CH_3) ppm. ^{13}C NMR ($CDCl_3$): δ 173.7, 172.3, 172.2, 165.6, 157.6, 147.3, 146.7, 138.8, 137.0, 131.3, 130.6, 124.1, 114.0, 82.5, 81.8, 80.7, 53.5, 53.0, 51.2, 39.2, 38.0, 32.2, 31.8, 29.0, 28.1, 28.0, 27.8, 27.7, 27.2, 22.1, 13.6, 10.0 ppm. ^{119}Sn NMR ($CDCl_3$): δ -58.6 ppm. IR (KBr): 3325, 2931, 1732 cm^{-1}. HRMS (ESI^+) m/z for $C_{47}H_{79}N_9O_{12}SnH$: calcd 1082.4960, obsd 1082.4934 $[M + H]^+$.

Radiochemistry

For all radiolabeled compounds, purity was determined using radioHPLC in comparison to the authentic non-radioactive standard detected by UV-HPLC. Purity was >95% for all compounds tested.

Preparation of ^{125}I-9b.[25] To anhydrous copper(II) chloride (13.4 mg, 0.1 mmol) was added anhydrous acetonitrile (4 mL), followed by anhydrous TEA (15.1 mg, 0.15 mmol), and the mixture sonicated until a clear burgundy solution was obtained. To the alkyne **4b** (1.0 μmol) was added to the solution of the $CuCl_2$/TEA complex in acetonitrile (40 μL). After 5 min, the solution was added to $Na^{125}I$ (4 MBq, 6.0 μL) in an eppendorf tube (1.5 mL), to which the azide **8** (1.0 μmol) in anhydrous acetonitrile (20 μL) was added. The tube was capped and the reaction was heated at 60 °C for 90 min. The solvent was evaporated using a stream of air and 500 μL of ACN and 2 mL of TFA were added, followed by stirring for 1 h at rt. The reaction mixture was concentrated, diluted with acetonitrile and water (1:1 v/v, 1 mL) and the compound purified by repeated preparative HPLC until there was no *des*-iodo side product evident. The labeled compound was identified by co-injection with the non-radioactive reference standard. ^{125}**I-9b:** radiochemical yield 70% ± 12 (n = 3), and > 97% radiochemical purity, as measured by analytical HPLC (see Figure 4).

Preparation of ^{125}I-19. A 1 mg/mL solution of Iodogen® in $CHCl_3$ was prepared shortly before use and 25 μL of the solution added to an eppendorf tube. The solvent was removed using a rotary evaporator leaving a film of Iodogen®. To this tube, 100 μL of a 1 mg/mL solution of **21** in 5% AcOH in acetonitrile was added, followed by 92.5 MBq solution of $Na^{125}I$ in sodium hydroxide (10 μL, pH 8-11). The reaction was allowed to stand for 15 min, whereupon 0.1 M $Na_2S_2O_5$ was added (20 μL). The solvent was evaporated and 500 μL of ACN and 2 mL of TFA were added, followed by stirring for 1 h at rt. The reaction mixture was concentrated, diluted with acetonitrile/water (1:1 v/v, 1 mL) and purified by HPLC. The

labeled compound was identified by co-injection with the non-radioactive reference standard. ^{125}**I-19:** radiochemical yield 72% ± 5 (n = 3), and > 98% radiochemical purity, as measured by analytical HPLC (see Figure 5).

Log *P* Measurements:

Log *P* measurements were made using the "shake-flask" method.[21] Compounds ^{125}**I-9b** and ^{125}**I-19** were concentrated separately to dryness and reconstituted in PBS (1 mL). The PBS solution (100 µL) was added to nine vials containing 1 mL phosphate buffer (pH 7.4) and 1 mL 1-octanol. The amount of each tracer added was 0.37 MBq. The vials were shaken on a vortex mixer for 20 min. Aliquots (0.1 mL) of each layer were transferred to pre-weighed vials, counted for activity in a Perkin-Elmer Wizard 1470 automatic gamma counter, and the count·mL^{-1} were calculated from the weight of solution transferred. The Log *P* was determined using log {[counts·mL^{-1} (1-octanol)]/[counts·mL^{-1} (buffer)]}. The reported value corresponds to the mean of n=9 measurements ± SD: log *P* ^{125}**I-9b** = –2.54 ± 0.03, log *P* ^{125}**I-19** = –1.4 ± 0.05.

Associated Content:

Supporting Information

^{1}H, ^{13}C, and ^{119}Sn NMR, MS, analytical HPLC, *in vitro* assay methods and results, and biodistribution data are provided. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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Abbreviations Used:

ARM, antibody recruiting small molecule; ARM-P (ARM-targeting Prostate cancer); ADCC, antibody-dependent cell cytotoxicity; DNP, 2,4-dinitrophenyl; NIS, N-iodosuccinimide; PSMA, prostate specific membrane antigen; PMPA, 2-(phosphonomethyl)-pentanedioic acid.

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Tables:

Table 1. IC_{50} values for inhibition of ^{125}**I-23** binding to PSMA expressing LNCaP cells, comparing PMPA (a known PSMA inhibitor) with ARM-P4 (**1**), ARM-P8 (**2**) and compounds **7a**, **7b**, **9a**, **9b, 12a**, **12b**, **17** and **19**.

Compound	IC_{50} (nM)	95% Confidence Interval
PMPA	52	47 - 57
1 (ARM-P4)	9	7 - 10
2 (ARM-P8)	23	19 - 30
7a	30	24 - 38
7b	24	20 - 30
9a	15	12 - 19
9b	14	12 - 17
12a	19	14 - 25
12b	98	81 - 118
17	123	96 - 157
19	3	2 - 4

Figure Legends:

Figure 1. PSMA binding, antibody recruiting small molecules (ARMs) reported by Spiegel and coworkers.[6]

Figure 2. Structure of ^{125}**I-23** reported by Darwish et al.[19]

Figure 3. A. Fluorescence intensity of LNCaP cells incubated with 50 nM of either **2** or **9b**, and AlexaFluor488 conjugated, rabbit anti-DNP IgG (gray bars). Binding of **2** and **9b** was blocked (black bars) by incubation with 0.2 mM PMPA. B. Fluorescence intensity of LNCaP cells co-incubated with 50 nM of either **9b**, or **19** and AlexaFluor488 conjugated, rabbit anti-DNP IgG (gray bars). Binding of **9b** and **19** was blocked by co-incubation with 0.2 mM PMPA (black bars). Data are expressed as mean **±** SEM, for n = 3 measures.

Figure 4. (A) HPLC-γ-trace of ^{125}**I-9b**; (B) HPLC-UV chromatogram of the co-injected reference standard **9b**.

Figure 5. (A) HPLC-γ-trace of ^{125}**I-19**; (B) HPLC-UV chromatogram of the co-injected reference standard **19**.

Figure 6. Selected biodistribution data for ^{125}**I-9b** administered to NCr nu/nu mice with LNCaP xenografts. Mice (n=3 per group) were injected with ~ 0.36 MBq of ^{125}**I-9b** and sacrificed at 1 h (black bar) and 6 h (gray bar). Data are expressed as percent injected dose per gram (%ID/g of tissue ± SEM). Complete data for all fluids and tissues are available in Supporting Information.

Figure 7. Selected biodistribution data for ^{125}**I-19** administered to NCr nu/nu mice with LNCaP xenografts. Groups of mice (n=3, 1 h; n=2, 6 h) were injected with ~ 0.19 MBq of ^{125}**I-19** and sacrificed at 1 h (black bar) or 6 hr (gray bar). Data are expressed as percent injected dose per gram (%ID/g of tissue ± SEM). Complete data for all fluids and tissues are available in Supporting Information.

Figure 8. Binding of ^{125}**I-9b** and ^{125}**I-23** to PSMA expressing LNCaP cells. Samples of LNCaP cells were incubated for 1 h with ^{125}**I-9b** (black bars) or ^{125}**I-23** (gray bars) in the absence or presence of an excess of each cold analogue (**9b** or **23**) or PMPA. Data are reported as the total bound radioactivity (fmol) per cell sample (Data are mean ± SD, n = 3 for ^{125}**I-23**, n = 6 for ^{125}**I-9b**).

Figure 9. Internalization of ^{125}**I-23** (A) and ^{125}**I-9b** (B) by PSMA expressing LNCaP cells. Aliquots of LNCaP cells were incubated with 10 nM ^{125}**I-9b** or 5 nM ^{125}**I-23**, at 4℃ or 37℃, for the indicated times. The amounts (fmol) of compounds bound (open symbols) or internalized (solid symbols) are shown as mean +/- SEM (n = 4 for ^{125}**I-23** and n = 2 for ^{125}**I-9b**).

Figure 10. Flow cytometric analysis of fluorescent, anti-DNP antibody binding to PSMA positive cells after incubation with **9b**. LNCaP cells treated with 50 nM of **9b** were incubated at 37 ^{o}C for the indicated time (x axis), followed by the addition of AlexaFluor488 conjugated, rabbit anti-DNP. Data indicate mean +/- SEM fluorescence intensity (n = 3). Fluorescence indicates DNP antibody binding in the absence (gray bars) or presence of 200 μM PMPA (black bars) used to block **9b** binding.

Figures:

Figure 1

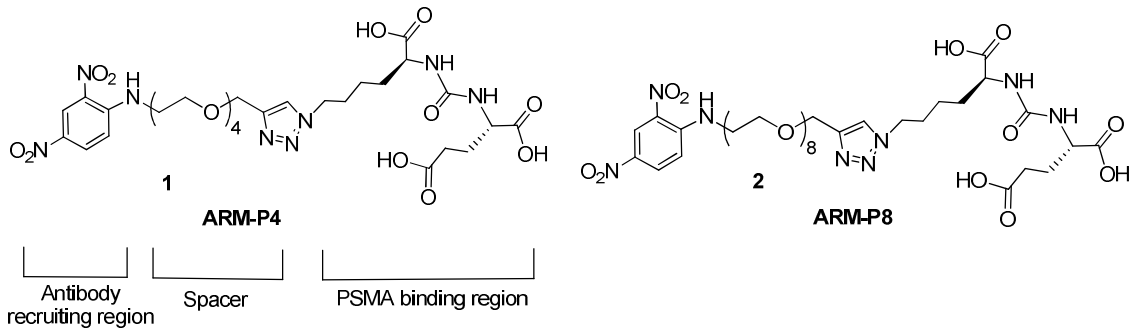


Figure 2

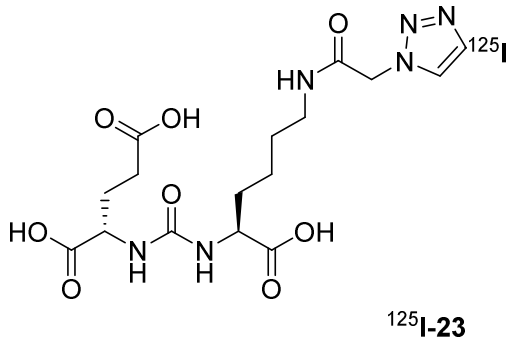
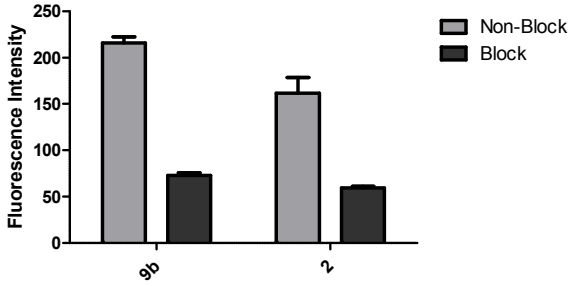


Figure 3

A



B

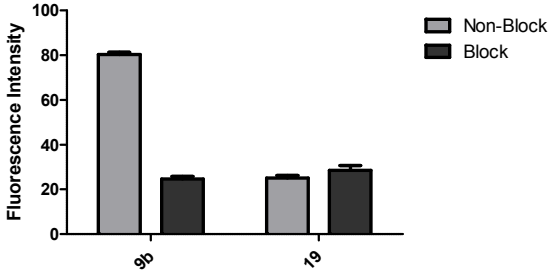
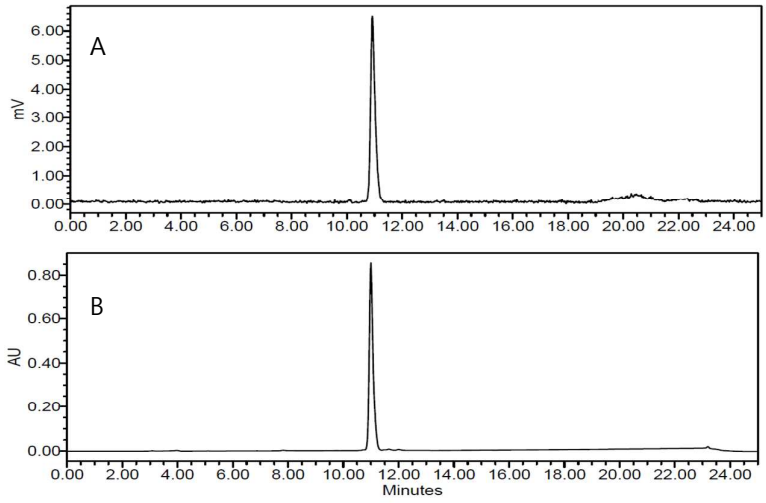


Figure 4



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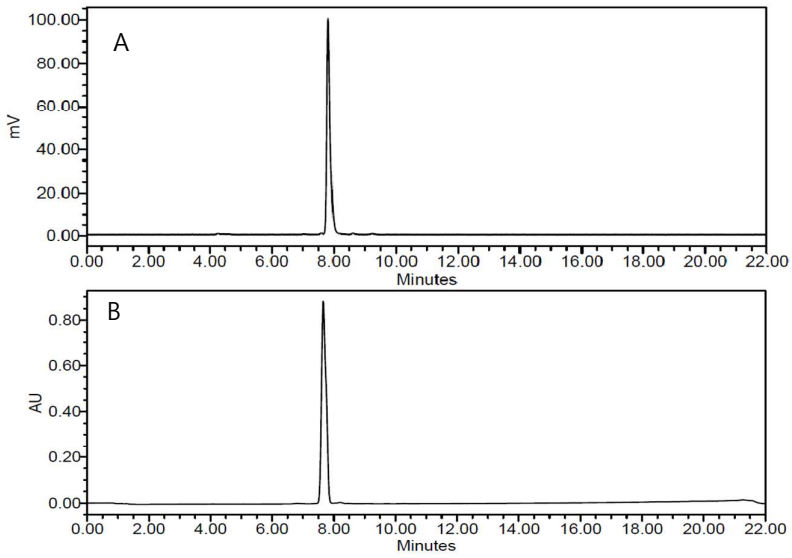

A
mV
100.00
80.00
60.00
40.00
20.00
0.00
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00
Minutes
B
AU
0.80
0.60
0.40
0.20
0.00
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00
Minutes

Figure 6

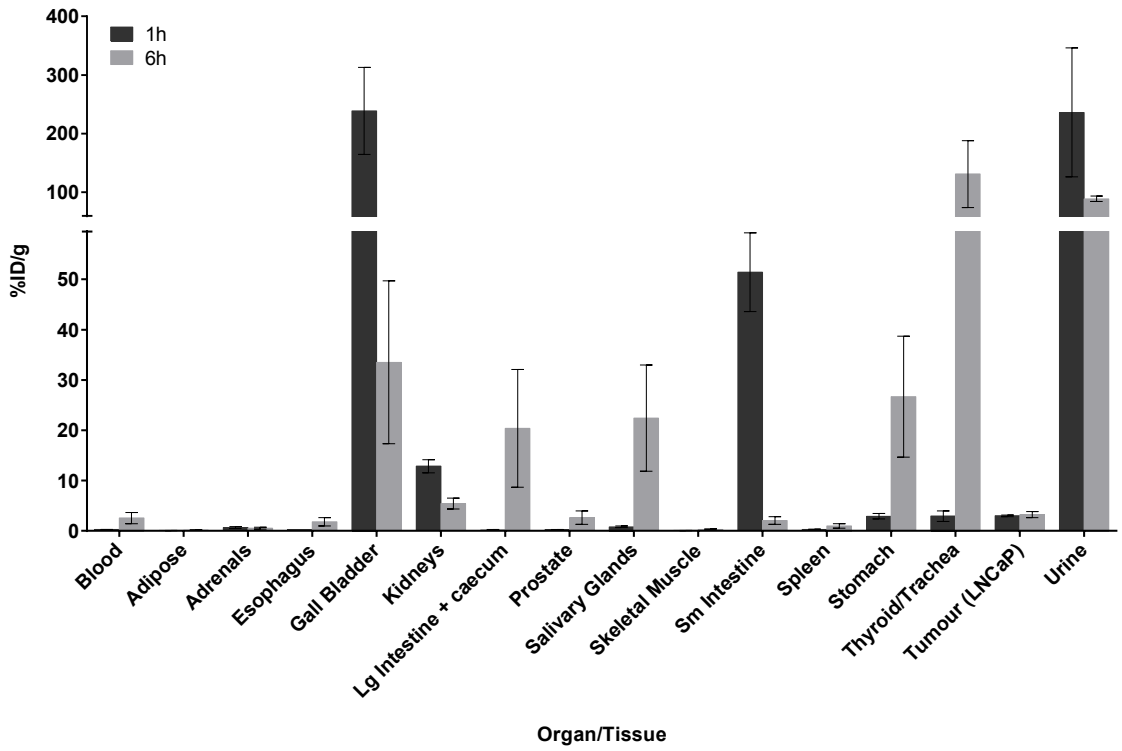


Figure 7

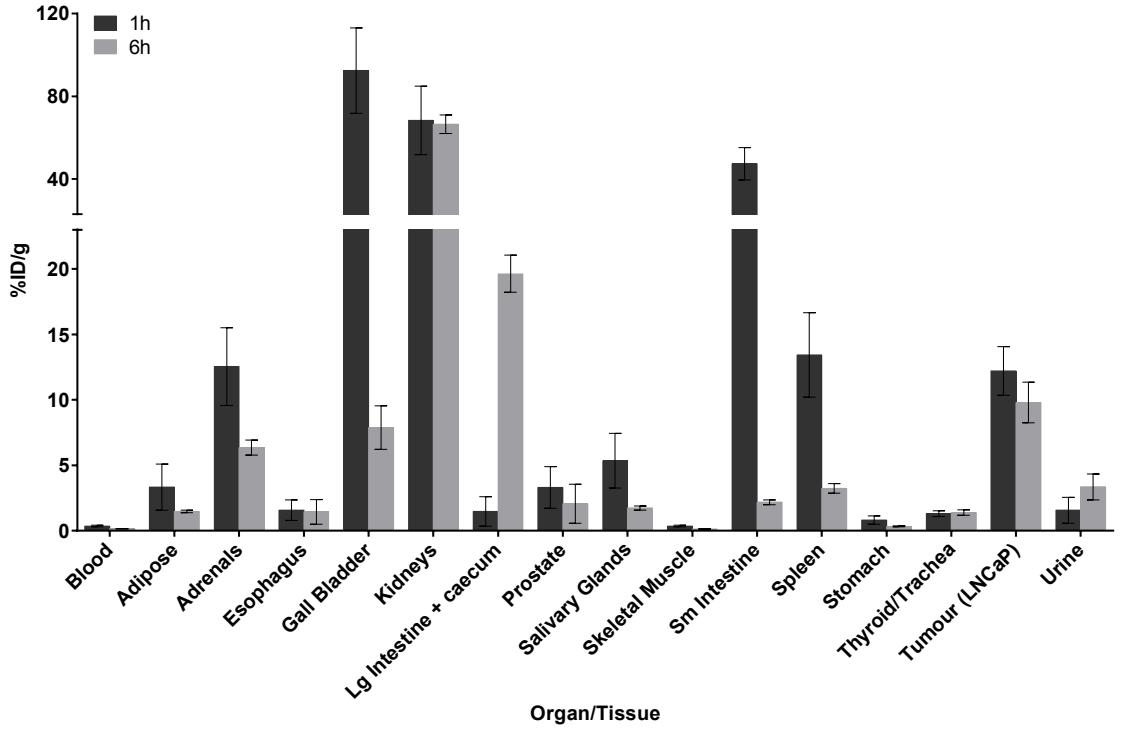


Figure 8

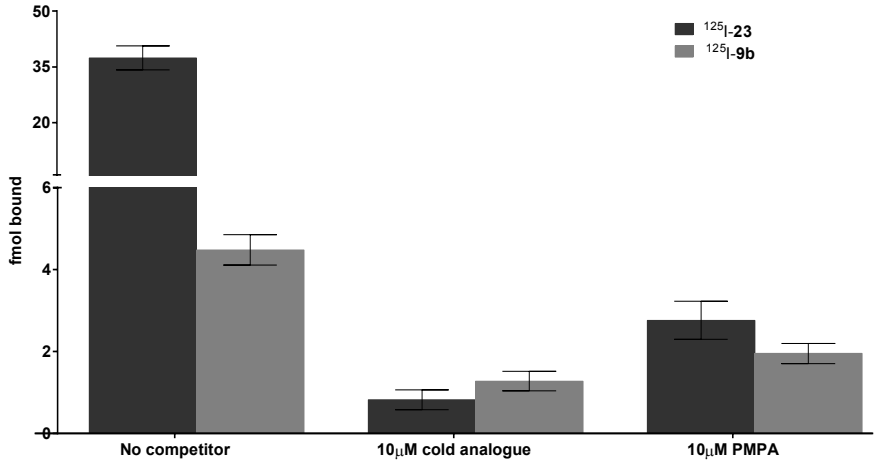
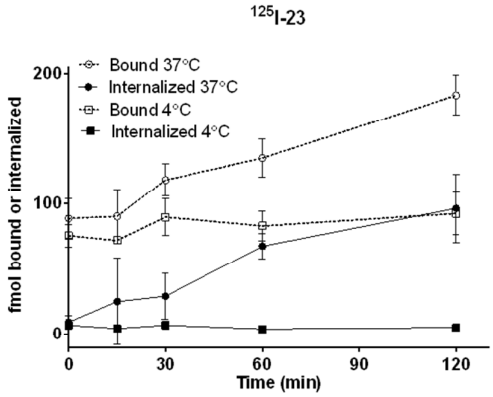


Figure 9

A



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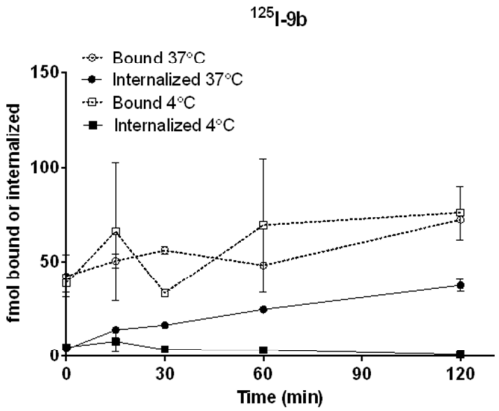
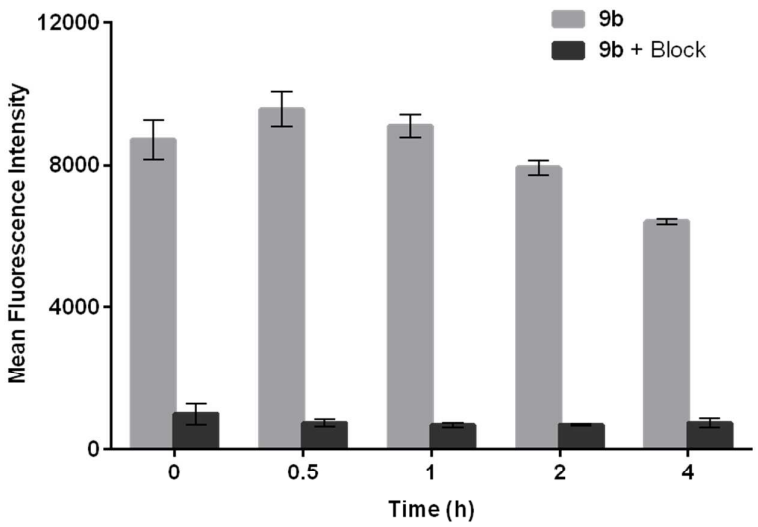
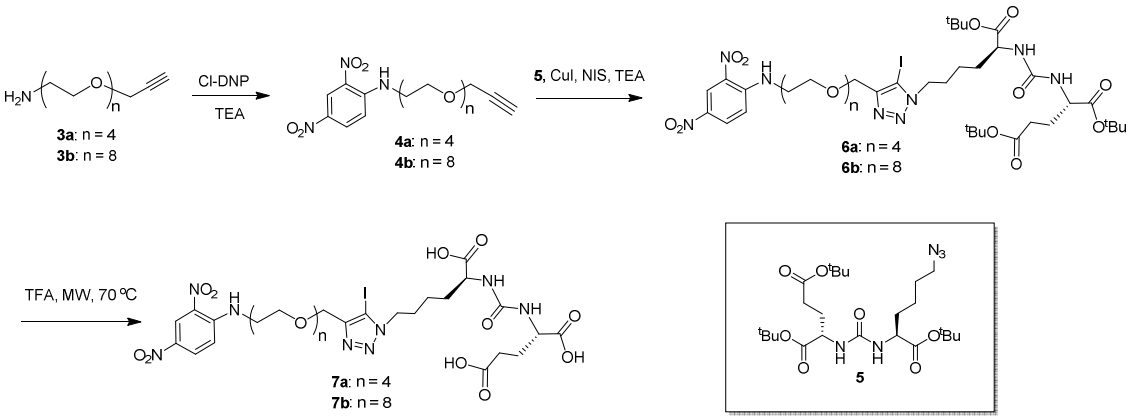


Figure 10

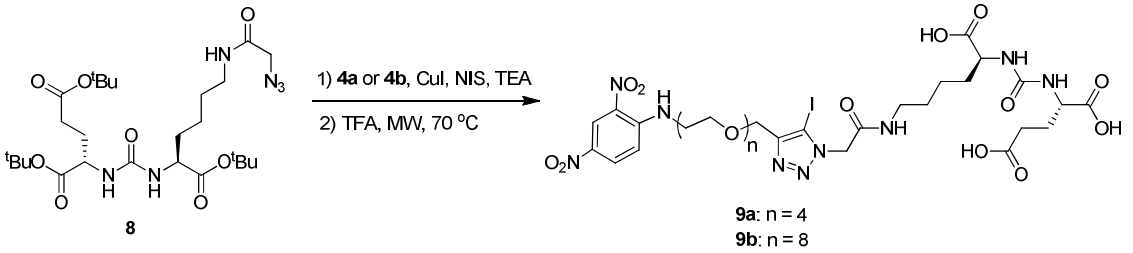

9b
9b + Block
12000
8000
4000
0
Mean Fluorescence Intensity
0
0.5
1
2
4
Time (h)

Schemes:

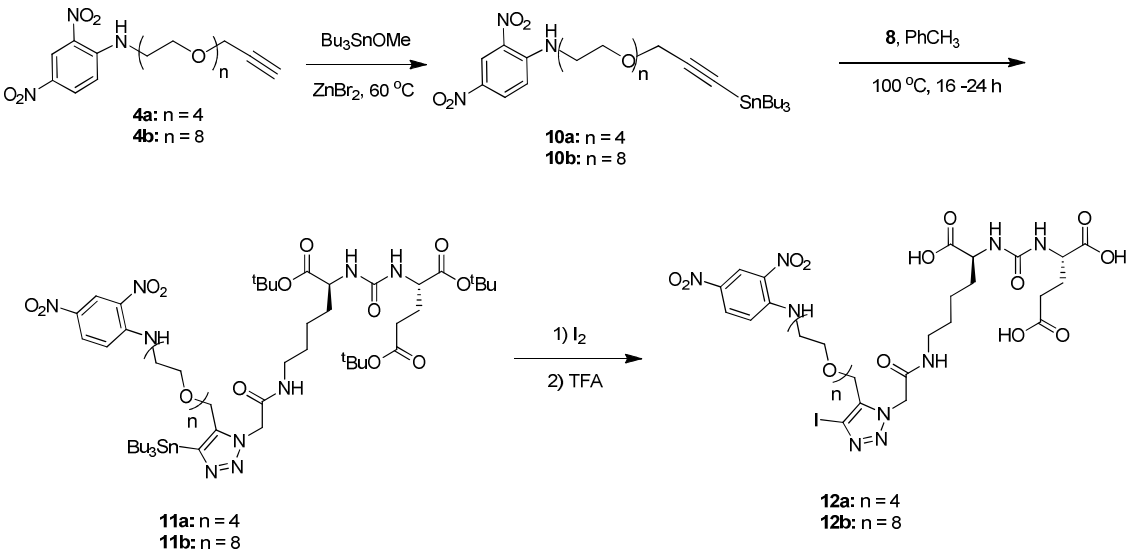
Scheme 1. Synthesis of 5-iodo-triazoles **7a** and **7b**.



Scheme 2. Synthesis of **9a** and **9b** from **8**.



Scheme 3. Synthesis of **12a** and **12b**.



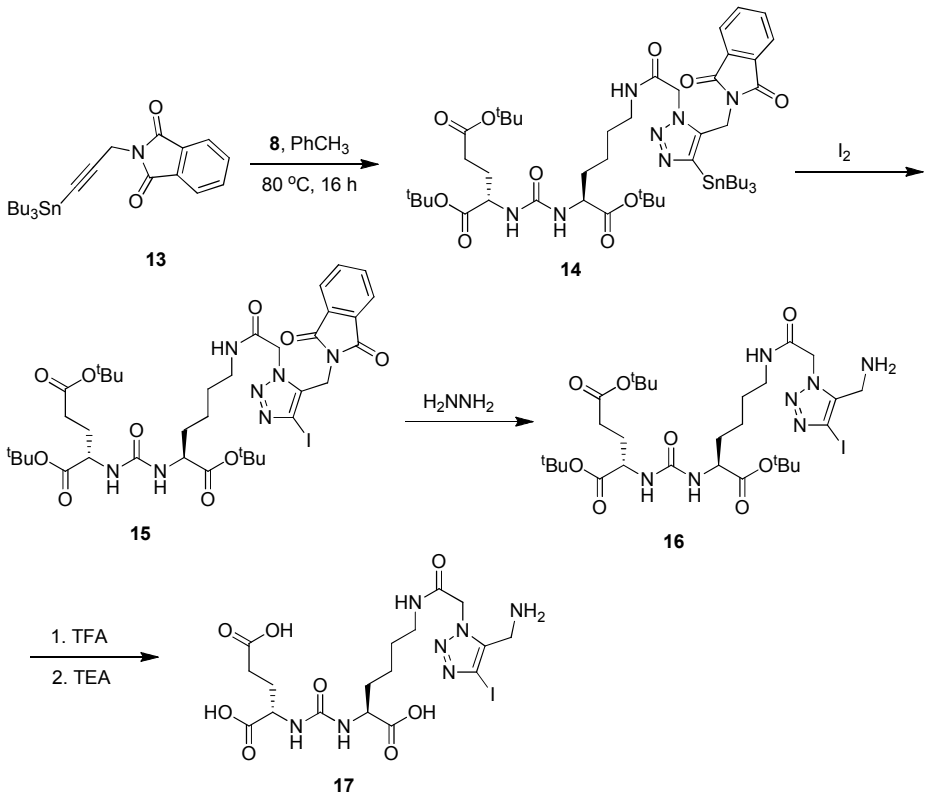
4a: n = 4
4b: n = 8

10a: n = 4
10b: n = 8

11a: n = 4
11b: n = 8

12a: n = 4
12b: n = 8

Scheme 4. Synthesis of **17**.



13

14

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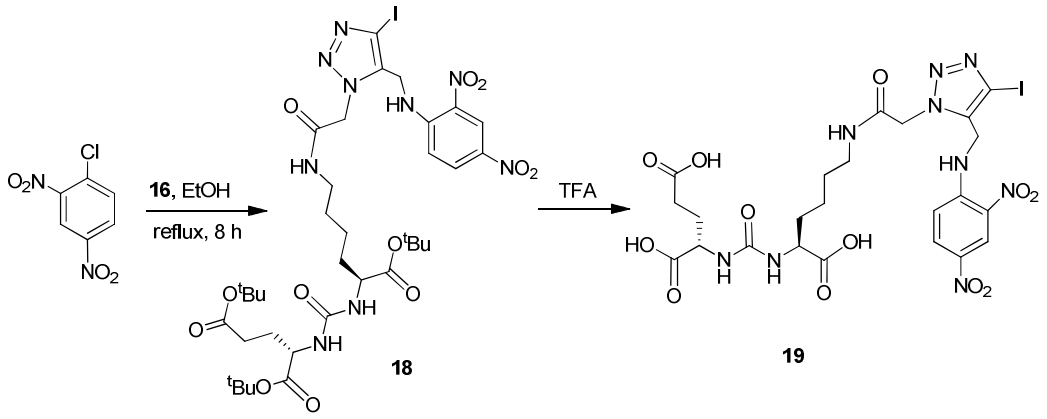
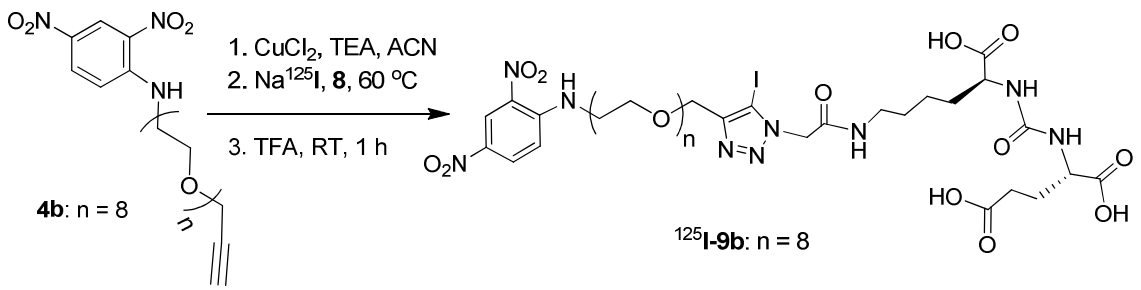
16

17

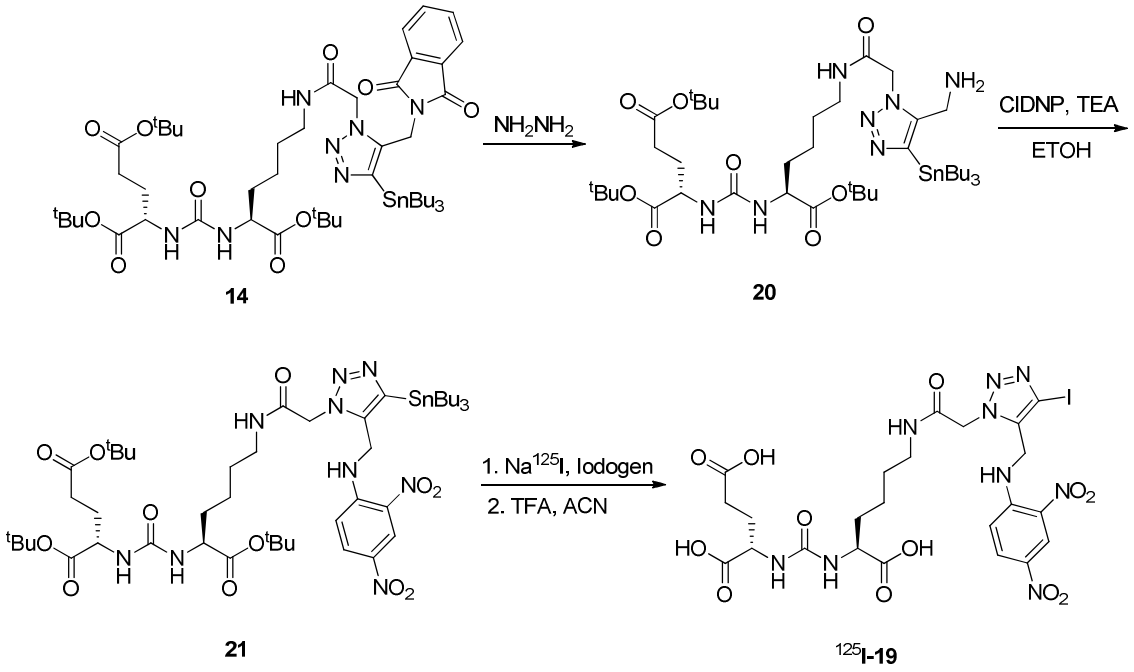
Scheme 5. Synthesis of **19** from **16.**



Scheme 6. Radiochemical synthesis of ^{125}**I-9b**.



Scheme 7. Synthesis of ^{125}**I-19** from **14** (ClDNP = 1-chloro-2,4-dinitrobenzene).



TOC Graphic: