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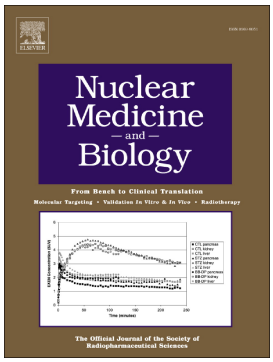

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A Trithiol Bifunctional Chelate for ^{72,77}As: a Matched Pair Theranostic Complex with High *in vivo* Stability

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Abstract

Introduction: Trithiol chelates are suitable for labeling radioarsenic (^{72}As: 2.49 MeV β^+, 26 h; ^{77}As: 0.683 MeV β^-, 38.8 h) to form potential theranostic radiopharmaceuticals for PET imaging and therapy. To investigate the *in vivo* stability of trithiol chelates complexed with no carrier added (nca) radioarsenic, a bifunctional trithiol chelate was developed, and conjugated to bombesin(7-14)NH_2 as a model peptide.

Methods: A trithiol-BBN(7-14)NH_2 bioconjugate and its arsenic complex were synthesized and characterized. The trithiol-BBN(7-14)NH_2 conjugate was radiolabeled with ^{77}As, its *in vitro* stability assessed, and biodistribution studies were performed in CF-1 normal mice of free [^{77}As]arsenate and ^{77}As-trithiol- BBN(7-14)NH_2.

Results: The trithiol-BBN(7-14)NH_2 conjugate, its precursors and its As-trithiol-BBN(7-14)NH_2 complex were fully characterized. Radiolabeling studies with nca ^{77}As resulted in over 90% radiochemical yield of ^{77}As-trithiol-BBN, which was stable for over 48 h. Biodistribution studies were performed with both free [^{77}As]arsenate and Sep-Pak® purified ^{77}As-trithiol-BBN(7-14)NH_2. Compared to the fast renal clearance of free [^{77}As]arsenate, ^{77}As-trithiol-BBN(7-14)NH_2 demonstrated increased retention with clearance mainly through the hepatobiliary system, consistent with the lipophilicity of the ^{77}As-trithiol-BBN(714)NH_2 complex.

Conclusion: The combined *in vitro* stability of ^{77}As-trithiol-BBN(7-14)NH_2 and the biodistribution results demonstrate its high *in vivo* stability, making the trithiol a promising platform for developing radioarsenic-based theranostic radiopharmaceuticals.

Running Title: A Trithiol Bifunctional Chelate for ^{72,77}As

Key Words: trithiol bioconjugate; arsenic trithiol; no carrier added ^{77}As; radiolabeling; biodistributions

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Introduction

Combined non-invasive radioimaging and radiotherapy (*i.e.,* theranostic) agents are believed to have many advantages because the diagnostic information provided by the imaging potentially guides the strategy for radiotherapy [1]. Ideally the radioimaging and radiotherapy counterparts are two radioisotopes of the same nuclide and thus behave identically *in vitro* and *in vivo*, making them a true "matched pair" [2]. Two arsenic radioisotopes (^{72,77}As) are a potential true matched pair for positron emission tomography (PET) and radiotherapy, respectively [3]. Arsenic-72 (3.33 MeV maximum β^+, 26 h half-life) and ^{77}As (0.683 MeV maximum β^-, 38.8 h half-life) have sufficiently long half-lives compared to most commonly used positron emitters (^{18}F, ^{11}C, ^{64}Cu, ^{68}Ga) making them useful in radiolabeling antibodies or peptides for radioimmunoimaging and therapy [4].

Arsenic-72 can be produced directly from proton irradiation of an enriched ^{72}Ge target via the p,n reaction [5]. Additionally, ^{72}Se decays by electron capture with an 8 day half-life to ^{72}As, which makes development of a ^{72}Se/^{72}As generator desirable [6-10]. Selenium-72 can be produced by a number of accelerator based routes on ^{70}Ge, ^{nat}Br or ^{75}As targets [7-11]. Several potential separation methods have been reported for such a generator [6-11]. Arsenic-77 is reactor produced by neutron irradiation of an enriched ^{76}Ge target to form ^{77}Ge, which beta decays to ^{77}As (**Equation 1**). The ^{77}As is then separated from the irradiated target as [^{77}As]arsenate using silica gel and the enriched ^{76}Ge is recovered and recycled [12].

$$^{76}\text{Ge} + {}^{1}\text{n} \rightarrow {}^{77}\text{Ge} \rightarrow {}^{77}\text{As} + \beta^- + \bar{\upsilon} \qquad \textbf{(1)}$$

Arsenic toxicity has been well discussed over the last few decades [13]. The high binding affinity between arsenic and thiols explains the *in vivo* arsenic accumulation in tissues with high cysteine content, and hence is the leading cause of arsenic toxicity [14]. Arsenic is typically observed in two oxidation states, namely +3 (arsenite) and +5 (arsenate), with trivalent arsenite believed to be the toxic form and reactive toward thiols [13,14].

The thiophilic nature of arsenic was recently used to develop potential no carrier added (nca) radioarsenic labeled radiopharmaceuticals. Monoclonal antibodies modified with *N*-succinimidyl S-acetylthioacetate (SATA) to increase the number of available thiol groups were directly radiolabeled with ^{72,74}As with high radiolabeling yields achieved (>99%) [15], excellent *in vitro* stability in serum [15,16], and rat biodistribution studies of radiolabeled bavituximab showed a

tumor:liver ratio of 22 at 72 h in tumor bearing rats with minimal release of radioarsenic from radiolabeled antibodies [16]. Thiol-containing *N*-(2-hydroxypropyl)-methacrylamide (HMPA) based polymers radiolabeled with nca $^{72/74}$As in high radiochemical yield showed good *in vitro* stability [17]. More recently, radioarsenic (^{72}As) labeling strategies using dithiol ligands and thiol-modified mesoporous silica nanoparticles were reported with reasonable yields [5]. Compared to the fast renal clearance of free ^{72}As, the blood circulation lifetime of ^{72}As labeled nanoparticles was enhanced providing a potential PET imaging radiopharmaceutical.

Aryldithiol [3] and trithiol [18] ligand frameworks and their nca radioarsenic complexes were recently reported. The trithiol ligand framework is being pursued to develop a bifunctional chelate because its radiochemistry is more straightforward. Since the nca radioarsenic is isolated as [$^{72/77}$As]arsenate with the arsenic in its highest oxidation state, the first step in radiolabeling is reduction of As(V) to As(III). The +3 oxidation state is required for radiolabeling. A monothiol, namely mercaptoacetate, was previously found to reduce [^{77}As]arsenate to ^{77}As (III) efficiently, and enabled trithiol radiolabeling to occur readily without interference [18]. The high *in vitro* stability observed for [^{77}As]arsenic-trithiol [18] led us to develop a bifunctional chelate based on the trithiol ligand framework. The initial trithiol bifunctional chelate selected was based on available starting materials and ease of synthesis (sulfurs are very reactive). Due to the high lipophilicity of the simple As-trithiol [18], the bifunctional trithiol was conjugated to a model peptide, namely bombesin(7-14)NH_2 (BBN(7-14)NH_2), via solid phase peptide synthesis. Its nca ^{77}As labeled analogue was evaluated *in vitro* in sterile saline and *in vivo* in mice for stability. Bombesin(7-14)NH_2 is not the ideal peptide for targeting gastrin-releasing peptide receptors (GRPr), which are highly expressed in human prostate cancer cells, but it is a well-characterized system at our institution [19-23]. This study reports the synthesis and characterization of a trithiol-BBN(7-14)NH_2 bioconjugate, the evaluation and biodistribution of nca ^{77}As radiolabeled trithiol-BBN(7-14)NH_2 in CF-1 normal mice, and comparison to free nca ^{77}As (arsenate) in CF-1 normal mice.

Experimental

Materials. Pentaerythritol, triethyl orthoacetate, *p*-toluene sulfonic acid monohydrate, propargyl bromide (80% in toluene), dioctyl phthalate, 3-bromopropionic acid, sodium azide, ethyl 3-bromopropionate, thioglycolic acid, *tris*(2-carboxyethyl)phosphine (TCEP), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxybenzotriazole (HOBt), *N*-ethyl-*N*-(propan-2-yl)propan-2-amine (DIEA), triisopropylsilane (TIS), protected amino acids,

silica gel TLC plates, silica gel 60Å, reversed phase C18 125Å, and aluminum backed C18-W TLC plates were purchased from Fisher Scientific or Sigma-Aldrich and used as received. Bacteriostatic saline was purchased from Hospira Inc. Sep-Pak® C18 Plus Light Cartridges were purchased from Waters. All solvents, and reagent grade acids and bases were purchased from Fisher Scientific or Sigma-Aldrich and used without further purification. Only 18 MΩ water was used. **CAUTION!** Arsenic is highly toxic and should be handled with care.

Arsenic-77 was prepared by irradiation of 2-5 mg of enriched $^{76}GeO_2$ (98.6%; Trace Sciences International) in a thermal neutron flux of 2.4 x 10^{14} n/cm^2-s at the University of Missouri Research Reactor (MURR). [^{77}As]arsenate was isolated in aqueous solution as previously reported [12]. **CAUTION!** ^{77}As and ^{77}Ge are radioactive and must be handled in laboratories outfitted and approved for work with radioactive materials. Arsenic-77: 38.8 h, 0.683 MeV maximum β^- , 239 keV γ (1.65%); ^{77}Ge: 11.3 h, 2.7 MeV maximum β^-, 211, 215.6, and 264.5 keV γ emissions.

Physical Measurements. ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ on a Bruker ARX-500 MHz spectrometer using TMS as an internal standard. Electrospray Ionization Mass Spectra (ESI-MS) were obtained on a Thermo Finnigan TSQ7000 triple-quadrupole instrument with an API2 source. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). An ORTEC HPGe detector outfitted with Genie multichannel analysis software was used to assay ^{77}Ge and ^{77}As liquid samples. Reversed phase HPLC (RP-HPLC) was performed using a Shimadzu Prominence HPLC system equipped with a pump, controller, and Prominence UV-Vis detector (model SPD20-AV) set to 220 and 280 nm, and coupled to a Beckman 170 NaI(Tl) radioisotope detector. The gradient system used for RP-HPLC run on a Phenomenex Jupiter C 18 (5 μm, 150 mm x 4.6 mm) column was as follows: a linear gradient from 20/80 acetonitrile (ACN)/H_2O w/ 0.1 % trifluoroacetic acid (TFA) to 50/50 ACN/H_2O w/ 0.1 % TFA in 10 min, then from 50/50 ACN/H_2O to 90/10 ACN/H_2O in 3 min, followed by returning to 20/80 ACN/H_2O over 5 min, all at a flow rate of 1 mL/min. Peptide analysis and purification were performed on a Beckmann Coulter System Gold HPLC equipped with a 168 diode array detector, a 507e autoinjector and the 32 KARAT software package (Beckmann Coulter, Fullerton, CA) using a C-18 XBridge BEH, 250 x 4.6 mm, 5 μm, 130 Å from Waters, Milford, MA. All LC-MS analyses and MS assisted preparative purifications were performed with an LCQ Fleet from Thermo Fisher, Waltham, MA.

Syntheses

*(1-Methyl-2,6,7-trioxabicyclo[2.2.2]octan-4-yl)methanol [$C_7H_{12}O_4$], **2**.* Synthesis of compound **2** was accomplished using a modified literature procedure [24]. Pentaerythritol (60 g, 440 mmol), was added to a stirring solution of dioctyl phthalate (100 mL) containing *p*-toluene sulfonic acid monohydrate (~15 mg, 78 µmol). The reaction outfitted with a distillation apparatus, heated to 120 °C, and triethyl orthoacetate (71.685 g, 441.87 mmol) was added. After stirring for 22 h, 64 mL of ethanol had distilled, 83% of the theoretical amount. Triethylamine (TEA) (2 mL, 14 mmol) was added, and the reaction mixture was allowed to stir for 10 minutes. The reaction clarified upon heating to 160 °C, at which time a vacuum was applied to remove the TEA and any remaining ethanol. The final product, a white solid, was isolated by vacuum distillation at 185 °C and recrystallized from benzene. Yield: 83%, 58.64 g. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 1.453 (s, 3H, C**H$_3$**), 1.58 (t, 1H, OH), 3.454 (d, 2H, CC**H$_2$**OH), and 4.015 (s, 6 H, OC**H$_2$**C). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 23.53 (**C**H$_3$), 35.71 (CH$_2$**C**CH$_2$), 61.50 (C**C**H$_2$OH), 69.41 (O**C**H$_2$C), and 108.67 (O**C**CH$_3$). ESI-MS (*m/z*): 161.06 (161.07 calc'd for $C_7H_{12}O_4$ [M+H]$^+$). Elemental analysis calc'd (found) for $C_7H_{12}O_4$: C, 52.49 (51.58); H, 7.55 (7.67).

*1-Methyl-4-((prop-2-yn-1-yloxy)methyl)-2,6,7-trioxabicyclo[2.2.2]octane [$C_{10}H_{14}O_4$], **3**.* Synthesis of compound **3** was accomplished using a modified literature procedure [24]. Compound **2** (5.43 g, 33.9 mmol) was added to a stirring solution of anhydrous DMSO (30 mL) and powdered KOH (7.60 g, 136 mmol). After stirring for 10 minutes, the reaction mixture was cooled in an ice bath (0 °C). Propargyl bromide (4.04 g, 3.02 mL, 33.9 mmol) was added drop-wise (**Caution**! Reaction becomes very hot!), and the reaction mixture rapidly became dark brown. The reaction was stirred at room temperature for 95 minutes and poured into ice-cold water (200 mL). The off-white solid was collected by vacuum filtration, washed with water, and dried *in vacuo* to obtain the analytically pure product. X-ray quality crystals were obtained by slow evaporation from chloroform. Yield: 5.44 g, 81%. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 1.456 (s, 3H, C**H$_3$**), 2.442 (t, 1H, CC**H**), 3.286 (s, 2H, CC**H$_2$**O), 4.006 (s, 6 H, (C**H$_2$**)$_3$C), and 4.094 (d, 2H, OC**H$_2$**CCH). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 23.56 (**C**H$_3$), 34.85 (CH$_2$**C**CH$_2$), 58.88 (O**C**H$_2$CCH), 68.12 (C**C**H$_2$O), 69.55 (O**C**H$_2$C), 75.34 (**C**H), 78.97 (CH$_2$**C**CH), 108.72 (O**C**CH$_3$). ESI-MS (*m/z*): 199.10 (199.09 calc'd for $C_{10}H_{14}O_4$ [M+H]$^+$). Elemental analysis calc'd (found) for $C_7H_{12}O_4$: C, 60.59 (59.06); H, 7.12 (7.23).

*2-(Hydroxymethyl)-2-((prop-2-yn-1-yloxy)methyl)propane-1,3-diol [$C_8H_{14}O_4$], **4**.* Synthesis of compound **4** was prepared using a modified literature procedure [24]. Deprotection was accomplished by the addition of crude **3** (2.225 g, 11.22 mmol) to 6 M HCl (12 mL) in methanol (40 mL) at room temperature. After stirring overnight (16 h), potassium carbonate (5.6 g, 50

mmol) was added slowly. No starting material was observed after 24 hours, based on silica TLC using ethyl acetate as the eluent (**4**, $R_f \approx 0.25$; **3**, $R_f \approx 0.8$). The solvent was removed by vacuum distillation to give the crude product, a thick dark yellow oil (~3 g). This crude mixture was reconstituted in ethyl acetate, and filtered to remove any solids. The filtrate was taken to dryness, dissolved in ethyl acetate and loaded on a silica gel column. Ethyl acetate was used to elute the purified product. Removal of the solvent under vacuum gave the pure product, a thick light yellow oil. Yield: 84%, 1.6 g. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 2.148 (bs, 3H, OH), 2.465 (t, 1H, C**H**), 3.578 (s, 2H, CC**H$_2$**O), 3.722 (s, 6 H, OC**H$_2$**C), and 4.154 (d, 2H, OC**H$_2$**CCH). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 45.14 (**C**), 58.98 (C**C**H$_2$O), 64.70 (HO**C**H$_2$C), 71.60 (O**C**H$_2$CCH), 79.40 (CH$_2$**C**CH), and 75.15 (**C**H). ESI-MS (*m/z*): 174.99 (175.09 calc'd for $C_8H_{14}O_4$ $[M+H]^+$)

*2-((Prop-2-yn-1-yloxy)methyl)-2-((tosyloxy)methyl)propane-1,3-diyl bis(4-methylbenzenesulfonate) [$C_{29}H_{32}O_{10}S_3$], **5***. Tosylation of compound **4** was accomplished by the slow addition of *p*-toluene sulfonyl chloride (78.76 g, 413.1 mmol) to a stirring solution of pyridine (90.3 g, 92 mL, 1.135 mol), and **4** (14.38 g, 82.56 mmol) at -5 °C. The reaction was allowed to slowly come to room temperature, and stirred for approximately 2 days. The reaction progress was followed by silica gel TLC with dichloromethane as the mobile phase and visualized using $KMnO_4$ (**5**, $R_f \approx 0.55$). The reaction mixture was poured into 2 M HCl (400 mL) at 5 °C to give a white solid isolated by filtration. Solids were washed with 2 M HCl (2 x 100 mL), and cold water (2 x 100 mL). The solid was dissolved in ethyl acetate (200 mL) and washed with saturated sodium bicarbonate (2 x 50 mL), 2 M HCl (2 x 100 mL), and brine (1 x 50 mL). The organic layer was dried over magnesium sulfate, filtered, and taken to dryness yielding a clear light yellow oil of the crude product. The crude product was recrystallized from a mixture of hexane/dichloromethane (DCM) (70/30), and then washed with hot hexanes (50 °C) until the hexane wash revealed no UV active material. Residual hexane was removed *in vacuo* to give the product as a white solid. Yield: 92%, 48.21 g. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 2.408 (t, 1H, C**H**), 2.468 (s, 9H, C**H$_3$**), 3.358 (s, 2H, CC**H$_2$**O), 3.890 (m, 8H, OC**H$_2$**CCH and OC**H$_2$**C), 7.357 (d, 6H, Ar**H**), and 7.716 (d, 6H, Ar**H**). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 21.87 (**C**H$_3$), 43.76 (CH$_2$**C**CH$_2$), 66.11 (O**C**H$_2$CCH), 66.89 (O**C**H$_2$C), 75.51 (**C**H), 78.66 (CH$_2$**C**CH), 128.16 (Ar**C**), 130.22 (Ar**C**), 132.01 (Ar**C**), and 145.50 (Ar**C**). ESI-MS (*m/z*): 659.10 (659.11 calc'd for $C_{29}H_{32}O_{10}S_3$ $[M+Na]^+$). Elemental analysis calc'd (found) for $C_{29}H_{32}O_{10}S_3$: C, 54.70 (54.48); H, 5.07 (5.12); S, 15.10 (15.01).

*3-(3-Thiocyanatomethyl)propoxy)prop-1-yne [$C_{11}H_{11}N_3OS_3$], **6**.* Synthesis of compound **6** was accomplished using a modified literature procedure [24]. To a 100 mL round bottom flask equipped with a condenser, **5** (10.02 g, 15.75 mmol), KSCN (19.86 g, 204.8 mmol) and anhydrous dimethylformamide (DMF) (70 mL) were heated to 110 °C for 18 hours, 120 °C for 2 hours, and then an additional 8 hours at 110 °C while vigorously stirring. The reaction was monitored by silica gel TLC, using DCM as the mobile phase, until no starting material remained (**6**, $R_f \approx 0.4$). The dark brown reaction mixture was poured over water containing crushed ice (800 mL) and placed in the freezer overnight (-13 °C) to precipitate the crude product, a dark brown solid. All of the solids were collected, combined, dissolved in ethyl acetate, and purified through a plug of silica gel. Pure product, a light yellow precipitate, was obtained by recrystallization from a mixture of ethyl ether and DCM using a dry ice/acetone bath. X-ray quality crystals were obtained by slow evaporation from ethyl ether. Yield: 2.79 g, 60%. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 2.53 (t, 1H, C**H**), 3.33 (s, 6 H, C$\mathbf{H_2}$SCN), 3.70 (s, 2H, CC$\mathbf{H_2}$O), and 4.24 (d, 2H, OC$\mathbf{H_2}$CCH). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 37.04 (S**C**H_2C), 46.17 (CH_2**C**CH_2), 58.69 (C**C**H_2O), 68.97 (O**C**H_2CCH), 76.42 (**C**H), 78.09 (CH_2**C**CH), and 111.53 (S**C**N). ESI-MS (*m/z*): 298.73 (299.01 calc'd for $C_{11}H_{11}N_3OS_3$ $[M+H]^+$). Elemental analysis calc'd (found) for $C_{11}H_{11}N_3OS_3$: C, 44.42 (44.72); H, 3.73 (3.67); N, 14.13 (13.86); S, 32.34 (32.51).

Ethyl 3-azidopropionate [$C_5H_9N_3O_2$], **9**. The product was prepared using a modified literature procedure [25]. To a stirring solution of DMSO (200 mL), and ethyl 3-bromopropionate (20.55 g, 113.5 mmol) at 0 °C, NaN_3 (9.71 g, 149.3 mmol) was added slowly. The reaction was allowed to slowly come to room temperature and stirred for 3 days. The reaction was poured into water (600 mL), and extracted with ethyl acetate (3 x 100 mL). The combined ethyl acetate fractions were washed with saturated sodium bicarbonate (2 x 50 mL), water (3 x 50 mL), brine (1 x 50 mL), dried over sodium sulfate, filtered, and taken to dryness to give the pure product, a light yellow liquid. Yield: 71%, 11.5 g. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 1.468 (t, 3H, C$\mathbf{H_3}$), 2.54 (t, 2H, C$\mathbf{H_2}$C), 3.54 (t, 2H, N_3C$\mathbf{H_2}$), and 4.16 (q, 2H, COOC$\mathbf{H_2}$). ^{13}C NMR ($CDCl_3$: 125.8 MHz) δ ppm: 14.28 (**C**H_3), 34.08 (**C**H_2COO), 46.87 (N_3**C**H_2), 61.02 (COO**C**H_2), and 170.91 (**C**).

*Ethyl 3-(4-((3-thiocyanato-2,2-bis(thiocyanatomethyl)propoxy)methyl)-1H-1,2,3-triazol-1-yl)propanoate [$C_{16}H_{20}N_6O_3S_3$], **7**.* Synthesis of compound **7** was accomplished using a modified literature procedure [26,27]. Compounds **6** (1.19 g, 4 mmol) and **9** (1.76 g, 12 mmol) were added to a stirring solution of copper (II) sulfate pentahydrate (21.9 mg, 0.08 mmol), sodium ascorbate (270 mg, 1.2 mmol), copper metal (1 g; cut from Cu sheet (Fisher Scientific)), tetrahydrofuran (THF) (4 mL), ACN (4 mL), H_2O (4 mL), and *tert*-butanol (*t*-BuOH) (4 mL) at 55

°C. The reaction was monitored by silica gel TLC using DCM as the mobile phase (**9**, $R_f \approx 1$; **6**, $R_f \approx 0.4$; **7**, $R_f \approx 0$). Once compound **6** was no longer observed, the solvents were removed by vacuum distillation. The residue was dissolved in DCM and loaded on a plug of silica gel, and then washed with DCM to remove impurities. Ethyl acetate was then added to elute the crude product. Further purification was accomplished by silica gel column chromatography (3 x 18 cm, 40 g) using 40/60 hexanes/ethyl acetate to 20/80 hexanes/ethyl acetate to 100% ethyl acetate to obtain the product in the fractions containing ≥80% ethyl acetate; removal of solvent under vacuum gave the product as a clear light yellow oil. Yield: 1.73 g, 98%. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 1.24 (t, 3H, C**H$_3$**), 2.96 (t, 2H, C**H$_2$**COO), 3.28 (s, 6H, NCSC**H$_2$**), 3.65 (s, 2H, CC**H$_2$**), 4.15 (q, 2H, COOC**H$_2$**), 4.64 (t, 2H, NC**H$_2$**), 4.67 (s, 2H, OC**H$_2$**C=C), and 7.71 (s, 1H, C**H**). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 14.22 (**C**H_3), 34.64 (**C**H_2COO), 36.92(NCS**C**H_2), 45.74 (N**C**H_2), 46.08 (**C**), 61.36 (COO**C**H_2), 64.18 (C**C**H_2O), 69.33 (O**C**H_2), 111.62 (S**C**N), 124.64 (C=**C**), 143.11 (**C**=C), and 170.53 (**C**=O). ESI-MS (*m/z*): 441.06 (440.08 calc'd for $C_{16}H_{20}N_6O_3S_3$ $[M+H]^+$).

3-(4-((3-Thiocyanato-2,2-bis(thiocyanatomethyl)propoxy)methyl)-1H-1,2,3-triazol-1-yl)propanoic acid [$C_{14}H_{16}N_6O_3S_3$], **8**. Concentrated sulfuric acid (0.5 mL) was added to a stirring solution of compound **7** (0.40 g, 0.908 mmol) in ACN (5 mL) and water (25 mL) at 70 °C. The reaction was equipped with a reflux condenser, and monitored by silica gel TLC (20/80 hexane/ethyl acetate : **9**, $R_f \approx 1$; **7**, $R_f \approx 0.5$; **8**, $R_f \approx 0.25$) until no starting material (compound **7**) was observed. The ACN was removed via vacuum distillation, and the remaining material was extracted in DCM (3 x 50 mL). The organic layers were combined, and taken to dryness by vacuum distillation. The residual oil was dissolved in DCM (10 mL) and applied to a plug of silica gel. DCM (200 mL) was added to remove any unwanted materials, and then ethyl acetate (200 mL) to elute the crude product. The ethyl acetate was removed and the product was further purified by reversed phase C18 chromatography (3 x 15 cm) using 40/60 ACN/H_2O as the mobile phase (**8**, $R_f \approx 0.6$ by reversed phase TLC with 40/60 ACN/H_2O). The solvent was removed *in vacuo* to afford a light yellow oil. X-ray quality crystals (pale yellow) were obtained by slow evaporation from a 40/60 mixture of ACN and H_2O. Yield: 50%, 0.187 g. ^{1}H NMR ($CDCl_3$; 500 MHz) δ ppm: 3.03 (t, 2H, C**H$_2$**COO), 3.29 (s, 6H, NCSC**H$_2$**), 3.64 (s, 2H, CC**H$_2$**), 4.65 (t, 2H, NC**H$_2$**), 4.67 (s, 2H, OC**H$_2$**C=C), and 7.76 (s, 1H, C**H**). ^{13}C NMR ($CDCl_3$; 125.8 MHz) δ ppm: 34.34 (**C**H_2COO), 36.95 (NCS**C**H_2), 45.79 (N**C**H_2), 46.05 (**C**), 63.94 (C**C**H_2O), 69.38 (O**C**H_2), 111.77 (S**C**N), 124.95 (C=**C**), 143.07 (**C**=C), and 173.92 (**C**=O). ESI-MS (*m/z*): 412.02 (412.04 calc'd for $C_{14}H_{16}N_6O_3S_3$ $[M+H]^+$). Elemental analysis calc'd (found) for $C_{14}H_{16}N_6O_3S_3$: C, 37.07 (37.15); H, 3.89 (3.94).

Trithiocyanate-BBN(7-14)NH_2 synthesis. Bombesin(7-14)NH_2 was synthesized by solid phase peptide synthesis (model AAPPTEC 396 Omega, Louisville, KY) using Fmoc chemistry on Sieber Resin. The protecting groups used for the amino acid side chains were: Trityl (Gln, His); tert-butyloxycarbonyl (Boc) (Trp). The Fmoc protecting groups were removed at each subsequent cycle by treatment with 20% piperidine for 10 min. The peptide chain was assembled by sequential acylation (20 min for coupling) with "*in situ*" activated Fmoc-amino acids. Re-coupling was automatically performed at every cycle. The "*in situ*" activation of Fmoc-amino acids (3 eq. compared to the resin amount) was carried out using uronium salts (HBTU, 2.7 eq., HOBt 3 eq.) and DIEA (6 eq.). Compound **8** (700 mg, 2.4 mmol) was conjugated to the N terminus of the Bombesin(7-14)NH_2 peptide on the resin using the same standard procedure used for the other amino acids [28]. The peptidyl-resin was cleaved and deprotected in a single reaction (2 hours) with the following mixture: TFA, phenol, water and TIS (85:5:5:5). Thiol scavengers were avoided as they react with thiocyanate groups. Precipitation and multiple washings with diethyl ether gave the final crude product. The product was HPLC purified as the trithiocyanate-BBN(7-14)NH_2 precursor, characterized by LC-MS (1333 (1333 calc'd for $C_{57}H_{79}O_{11}N_{19}S_4$ [M+H^+])), lyophilized and stored for further use.

Trithiol-BBN(7-14)NH_2. The trithiocyanate-BBN precursor (1 mg, 0.75 µmol, 0.75 mM) was dissolved in 20% ACN in water (1 mL), to which *tris*(2-carboxyethyl)phosphine (TCEP, 2 mg, 7.5 µM) was added. The solution was placed in a 55 °C water bath for 2 h to yield the trithiol-BBN(7-14)NH_2 (residual TCEP remained), which was characterized by LC-MS (1258 (1258 calc'd for $C_{54}H_{82}O_{11}N_{16}S_4$ [M+H^+]), 90% yield) and used without further purification as the trithiol-BBN stock solution.

As-trithiol-BBN(7-14)NH_2. As-trithiol-BBN(7-14)NH_2 was synthesized from natural As_2O_3 and characterized by LC-ESI-MS. The trithiol-BBN(7-14)NH_2 stock solution (500 µL, 0.5 mg, 0.4 µmol) was added to a vial. An arsenic trioxide stock solution was prepared by dissolving As_2O_3 (10.6 mg, 0.054 mmol) in water (13.4 mL, As_2O_3 concentration 0.004 M). NaOH solution (50 µL, 10 M) was added to assist the dissolution of As_2O_3. The arsenic trioxide stock solution (100 µL, 0.4 µmol) was added to the trithiol-BBN(7-14)NH_2 stock solution (0.4 µmol) and the reaction vial was placed in a 55 °C water bath for 40 min. The As-trithiol-BBN(7-14)NH_2 reaction solution was characterized by LC-ESI-MS (1330 (1330 calc'd for $C_{54}H_{79}O_{11}N_{16}S_4As$ for [M+H^+]), over 95% yield). HPLC: t_r = 12.3 min for trithiol-BBN(7-4)NH_2 and t_r = 13.3 min for As-trithiol-BBN(7-14)NH_2.

X-ray Crystal Structures. Intensity data for compound **3** was obtained at -173 °C on a Bruker Prospector equipped with a Cu IμS tube and an Apex II CCD detector. Data for **6** and **8** were collected at -173 ºC on a Bruker Smart system with an Apex II CCD detector and Mo Kα radiation from a sealed tube and a graphite monochromator. Intensities were corrected for Lorentz and polarization effects. Equivalent reflections were merged, and absorption corrections were made using the multi-scan method. The structures were solved by direct methods with full-matrix least-squares refinement, using the SHELX package [29]. All non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were placed at calculated positions and included in the refinement using a riding model, with fixed isotropic *U,* except for the OH and water H atoms of **8**, which were located and refined. Data were corrected for decay and absorption using the program SADABS [30]. Final difference maps contained no features of chemical significance.

Radiotracer synthesis of no carrier added ^{77}As-trithiol-BBN. No carrier added (nca) ^{77}As ([^{77}As]$H_2AsO_4^-$, 370 MBq/mL (10 mCi/mL)) in aqueous solution was obtained from the University of Missouri Research Reactor (MURR) as a stock solution. An aliquot of the ^{77}As stock solution (200 µL, 74 MBq (2 mCi)) was added to a 2 mL sterile centrifuge tube along with a 30% ethanol/water solution (581 µL). Ammonium mercaptoacetate (18.2 µL, 5.5 M, 100 µmol) was then added, and the reaction was placed in a 55 °C water bath for 45 min. The trithiol-BBN(7-14)NH_2 stock solution (100 µL, 0.75 mM, 0.1 mg) was added to the reaction mixture and heating continued in the 55 °C water bath for 45 min. The reaction was cooled to room temperature and diluted with 10 mL of water. A Sep-Pak® C18 Plus Light cartridge was preconditioned with ethanol and rinsed with water, and then the diluted reaction mixture was loaded on the cartridge, and washed with 10 mL of water. No carrier added ^{77}As-trithiol-BBN(7-14)NH_2 was eluted from the cartridge with 1 mL of ethanol to which ascorbic acid (30 µg) was added. The solution was brought to dryness under a gentle N_2 stream at 25 °C, and then reconstituted with 5 mL of sterile saline solution. The final yield was determined to be 44 MBq (1.2 mCi; 60%) in 5 mL of bacteriostatic saline solution. The specific activity of the Sep-Pak purified product was determined to be 20.91 MBq/nmol based on HPLC analysis.

Stability in Saline (sterile saline). An aliquot of the Sep-Pak® purified radiolabeled ^{77}As-trithiol-BBN in sterile saline solution at a concentration of 11.1 MBq/mL (300 µCi/mL) was set aside at room temperature. Ascorbic acid (6 µg/mL) was added to prevent radiolysis from the decay of ^{77}As. The ^{77}As-trithiol-BBN solution was monitored over time by HPLC. It remained intact at least to 48 h with no sign of free ^{77}As (arsenate) observed by HPLC (**Figure S3**).

Biodistribution studies of no carrier added ^{77}As-arsenate and ^{77}As-trithiol-BBN. Male CF-1 mice at 5-6 weeks of age (Charles River Laboratories, Wilmington, MA) were used for pharmacokinetic studies. Mice were fed ad libitum rodent chow (Lab Diet 5008; Lab Diet, Inc., St. Louis, MO) and ad libitum acidified water in a humidity-controlled environment while housed four mice per cage in a ventilated rack system on a 12-hour light/12-hour dark light cycle in an AAALAC accredited facility. All studies were approved by the HS Truman Memorial Veterans' Hospital Subcommittee for Animal Studies (SAS).

Biodistribution studies of both uncomplexed ^{77}As ([^{77}As]$H_2AsO_4^-$) and [^{77}As]As-trithiol-BBN(7-14)NH_2 were performed in CF-1 mice. An ^{77}As ([^{77}As]$H_2AsO_4^-$) solution was prepared by diluting 60 µL of nca ^{77}As ([^{77}As]$H_2AsO_4^-$, 370 MBq (10 mCi/mL)) stock solution with 2 mL of sterile saline solution. Animals were administered 100 µL via the lateral tail vein with 0.74 MBq (~20 µCi) administered of the uncomplexed ^{77}As ([^{77}As]$H_2AsO_4^-$) and 0.37 MBq (10 µCi) administered of the complexed [^{77}As]As-trithiol-BBN(7-14)NH_2. Mice were sacrificed at 15 min, 1 h, 4 h and 24 h post-injection for uncomplexed ^{77}As and 1 h and 4 h p.i. for ^{77}As-trithiol-BBN(7-14)NH_2. Tissues and organs were collected, weighed, and residual radioactivity was quantified using a NaI(Tl) well detector. Data were analyzed to determine the percent injected dose (%ID) and percent injected dose per gram (% ID/g) of each tissue/organ. Blood volume was calculated based on a blood volume of 6.5% of the total body weight.

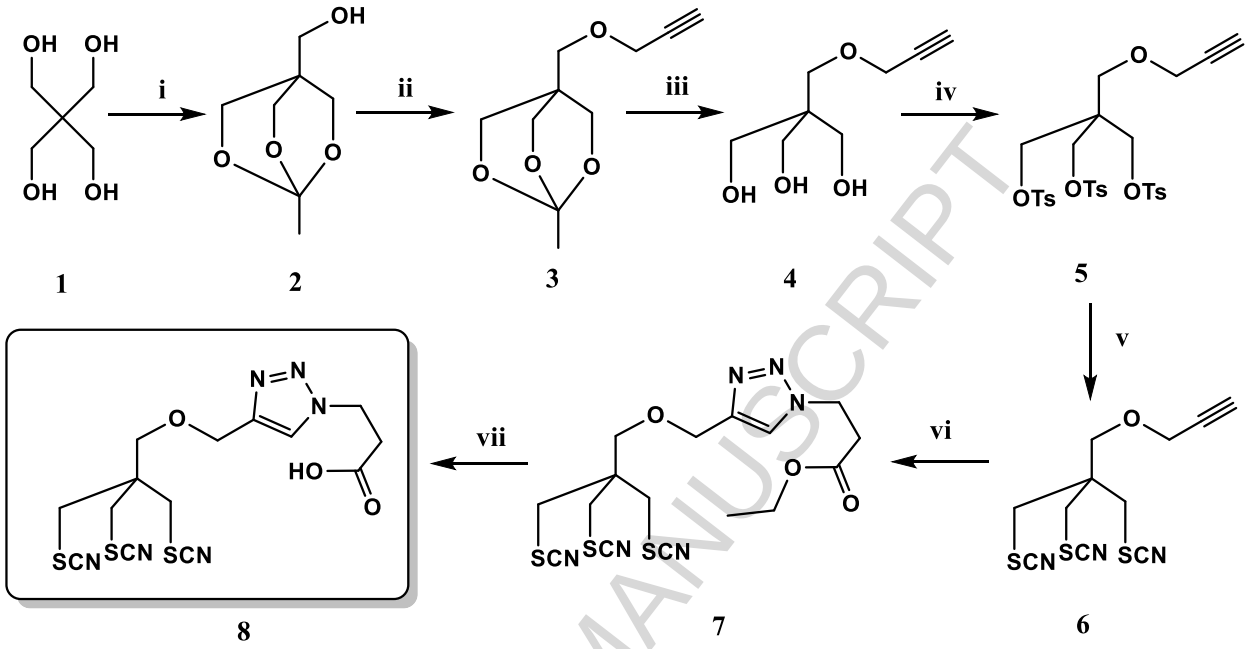
Results and Discussion

No carrier added ^{72}As and ^{77}As are of interest as a potential matched pair for theranostic radiopharmaceuticals [3,18]. Previously a trithiol ligand, its arsenic complex, and its nca ^{77}As complex were reported as the basis of potential theranostic radiopharmaceuticals [18]. Now a functionalized trithiol ligand conjugated to a model peptide (BBN(7-14)NH_2) and its arsenic (^{75}As and ^{77}As) complex were synthesized with the goal of determining the *in vivo* stability of the nca ^{77}As-trithiol framework. A linkable trithiol ligand was synthesized and then conjugated to the N terminus of the BBN(7-14)NH_2 peptide via solid phase peptide synthesis to afford the trithiocyanate-BBN(7-14)NH_2 precursor. The trithiol-BBN(7-14)NH_2 conjugate was readily generated by reducing the trithiocyanate-BBN(7-14)NH_2 precursor with TCEP, and then radiolabeled with ^{77}As for *in vivo* stability evaluation in normal mice.

Trithiol Precursor Syntheses. Synthesis of the first trithiol precursors, namely 3-(3-thiocyanatomethyl)propoxy)prop-1-yne, **6**, and 3-(4-((3-thiocyanato-2,2-

bis(thiocyanatomethyl)propoxy)methyl)-1H-1,2,3-triazol-1-yl)propanoic acid, **8**, were prepared in yields of 31% and 50%, respectively, using modified literature procedures (**Scheme 1**) [18].

Scheme 1. Synthetic scheme for the linkable trithiol precursor, Compound **8**.



i: pentaerythritol, DOP, trace p-toluene sulfonic acid, triethyl orthoacetate (1 equiv.)
ii: DMSO, powdered KOH, propargyl bromide (1 equiv.)
iii: MeOH, 2 M HCl, then K_2CO_3
iv: pyridine (15.2 equiv.), p-toluene sulfonyl chloride (5.5 equiv.)
v: dry DMF, KSCN (13 equiv.)
vi: ACN, DI water, THF, t-butanol, copper (II) sulfate pentahydrate, sodium ascorbate, and ethyl 3-azidopropinoate (3 equiv)
vii: DI water, ACN, and H_2SO_4 (cat.)

(1-Methyl-2,6,7-trioxabicyclo[2.2.2]octan-4-yl)methanol, **2**, was synthesized by the reaction of pentaerythritol, **1**, with triethyl orthoacetate and a catalytic quantity (0.025% wt.) of *p*-toluene sulfonic acid monohydrate in dioctyl phthalate at 120 °C. A key detail omitted in many literature preparations is the addition of TEA or another trialkyl amine base prior to distillation of the product. This compound is acid sensitive; rapid decomposition occurs if the amine base is absent due to the catalytic quantity (0.025% wt.) of *p*-toluene sulfonic acid present. Reaction of propargyl bromide with the bicyclic orthoester in dry DMSO containing powdered KOH yielded the bicyclic orthoester alkyne, 1-methyl-4-((prop-2-yn-1-yloxy)methyl)-2,6,7-trioxabicyclo[2.2.2]octane, **3**. Attempts to purify this by silica gel proved futile because of the inherent acidity of silica. Recrystallization from benzene or ethyl ether is possible, however typically the crude product was immediately added to a stirring solution of methanol containing HCl followed by the addition of base, 24 hours later, to generate 2-(hydroxymethyl)-2-((prop-2-

yn-1-yloxy)methyl)propane-1,3-diol, **4**. Reaction of **4** with excess *p*-toluene sulfonyl chloride in pyridine yielded the 2-((prop-2-yn-1-yloxy)methyl)-2-((tosyloxy)methyl)propane-1,3-diyl bis(4-methylbenzenesulfonate), **5**. The first protected trithiol precursor, 3-(3-thiocyanatomethyl)propoxy)prop-1-yne, **6**, was synthesized by reacting the tris-tosylate (**5**) with excess potassium thiocyanate in dry DMF.

Further reaction of ethyl 3-azidopropionate, **9**, with the alkyne, **6**, through a Huisgen 1,3-dipolar cycloaddition or 'click' reaction provided ethyl 3-(4-((3-thiocyanato-2,2-bis(thiocyanatomethyl)propoxy)methyl)-1H-1,2,3-triazol-1-yl)propanoate, **7**, in good yield. Subsequent acid catalyzed hydrolysis of the ester was carried out to give 3-(4-((3-thiocyanato-2,2-bis(thiocyanatomethyl)propoxy)methyl)-1H-1,2,3-triazol-1-yl)propanoic acid, **8**. Saponification of the ester was avoided due to the instability of the thiocyanate in alkali base.

The key intermediates, **2**, **5**, **6,** and **8**, were characterized by elemental analysis, while compounds, **2**-**8** were characterized by ^{1}H and ^{13}C NMR spectroscopy and ESI-MS. Compound **9** was characterized by ^{1}H and ^{13}C NMR spectroscopy. The molecular ions for compounds **2-8** were observed in the ESI-MS spectra at the calculated *m/z* values. The ^{1}H and ^{13}C NMR spectra of the compounds reported, **2**-**9**, were characteristic of the functional groups present and comparable to available literature [31].

Single Crystal X-ray Structures. Compounds **3**, **6**, and **8** were characterized by single crystal X-ray diffraction analysis. Crystal refinement data, bond angles, and distances are summarized in **Tables S1** and **S2** (Supplemental Material). **Figures 1** show the structures of **8** (structures of **3** and **6** are shown in **Figures S1** and **S2**)

Figures 1

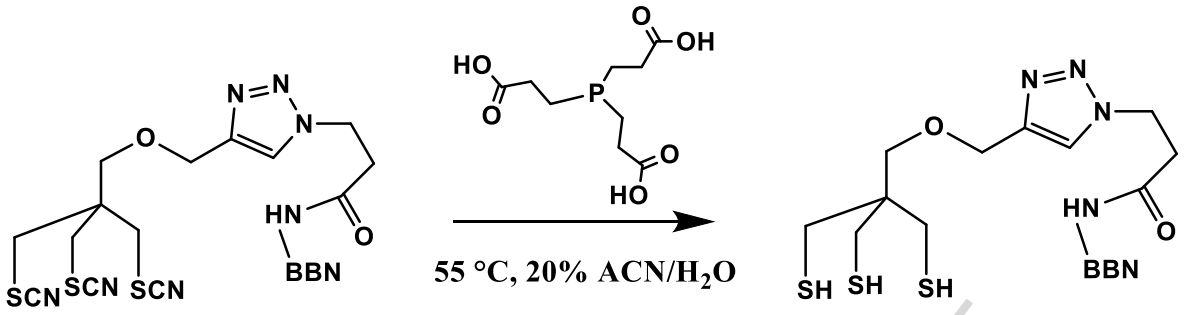
Bond distances and angles for compounds **3**, **6** and **8** were consistent with literature values [18]. The CN distances observed for compounds **6** and **8** ranged from 1.142(3) to 1.149(3) are comparable to another reported trithiocyanate (1.145(2) to 1.147(2)) [11]. The S-CN distances range from 1.693(2) to 1.7004(16) and are similar to the reported trithiocyanate (S-CN bond range of 1.6932(2) Å to 1.6977(2) Å) [18]. The CH_2-SCN distances for compounds **6** and **8** have a bond distance range of 1.827(2) Å to 1.8374(14) Å and are in agreement with the distances reported for the trithiocyanate (1.8289(1) to 1.8332(1) Å. The SCN angles reported here ranged from 176.4(2)° to 179.12(14)°, which fall within the range of the previously reported trithiocyanates (175.87(2)° to 179.19(2)°) [18].

Trithiol Precursor Bombesin analogue synthesis and Trithiol-Bombesin deprotection. Bombesin (BBN(7-14)NH_2) is an amphibian peptide analogue of the mammalian regulatory gastrin-releasing peptide (GRP) [32], which targets gastrin-releasing peptide receptors (GRPr) with high binding affinity, specificity and *in vivo* stability (**Figure 2**) [20]. GRPr are found highly expressed in human prostate cancer cells [19,33]. Although BBN(7-14)NH_2 is no longer considered the ideal GRP targeting peptide, it was used as the model peptide for our studies as we have a lot of pharmacokinetic data for this peptide with other chelators and metals [21-23,34]. A trithiol-BBN(7-14)NH_2 complex was synthesized for developing an ^{77}As radiolabeled BBN(7-14)NH_2 analogue (**Scheme 2**) for evaluation. The trithiol-BBN(7-14)NH_2 complex was stored as its protected precursor, trithiocyanate-BBN(714)NH_2 because thiol groups tend to oxidize over time. The synthesis of the trithiol-BBN(7-14)NH_2 precursor followed standard peptide synthesis procedures by coupling the protected trithiol ligand (compound **8**) to the Bombesin(7-14)NH_2 peptide on resin (**Figure 2**) [22]. The "click" chemistry to synthesize **8** was performed prior to peptide synthesis to maximize yield of the Bombesin(7-14)NH_2 bioconjugate on resin. An *in situ* activation with hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was carried out and the trithiol ligand was coupled twice with a three times stoichiometric excess to the resin to ensure maximum coupling. The final product was cleaved from the resin using a TFA, phenol, water and triisopropylsilane (TIS) (85:5:5:5) mixture, purified by HPLC and lyophilized for storage. The product was characterized by LC-MS ([$M+H^+$] 1333, 1333 calculated).

Figure 2

A trithiol-BBN(7-14)NH_2 solution was prepared by reducing the trithiocyanate-BBN(7-14)NH_2 precursor with *tris*(2-carboxyethyl)phosphine (TCEP), and was evaluated for quality control prior to radiolabeling (**Scheme 2**). The yield of the trithiol-BBN(7-14)NH_2 was determined to be greater than 90% and was thus used in radiolabeling without further purification, with the impurity identified as the trithiocyanate-BBN(7-14)NH_2 precursor.

Scheme 2. Synthesis of trithiol-BBN(7-14)NH_2 from the trithiocyanate precursor.



Trithiol-BBN precursor **Trithiol-BBN**

Trithiol-BBN(7-14)NH_2 radiolabeling. The nca ^{77}As radiolabeling of trithiol-BBN(7-14)NH_2 was carried out in 3 h in over 90% radiochemical yield (**Scheme 3**) [18]. The first step in the radiolabeling involves reduction of [^{77}As]arsenate from the +5 to the +3 oxidation state to allow synthesis of the desired complex. Separation of ^{77}As from its irradiated GeO_2 target yields [^{77}As]arsenate. Mercaptoacetate (SR^{-1}; 111 mM) is used to reduce and stabilize the nca ^{77}As in the +3 oxidation state as [^{77}As]As$(SR)_3$. The trithiol-BBN(7-14)NH_2 (0.075 mM) is then added to displace the mercaptoacetate groups (monothiols) taking advantage of the chelate effect. The radiolabeling reaction was analyzed by RP-HPLC and purified using a Sep-Pak® C18 cartridge. Compared to the concentration of nca ^{77}As, the ammonium mercaptoacetate (monothiol) and trithiol-BBN(7-14)NH_2 were in great excess. After radiolabeling, the solution was passed through a 0.2 µm syringe filter, and approximately 30% of the radioactivity remained on the filter due to the hydrophobic nature of the ^{77}As-trithiol-BBN(7-14)NH_2. The HPLC data is shown below (**Figure 3**). In **Figure 3**, purified ^{77}As-trithiol-BBN(7-14)NH_2 was injected and showed over 90% radiochemical yield. Peaks observed at 3.28 min, 4.18 min, 4.4 min and 6.3 min by UV analysis were associated with the excess ammonium mercaptoacetate and TCEP. The peak at 12.4 min was unreacted trithiol-BBN(7-14)NH_2, which was in good agreement with the standard trithiol-BBN(7-14)NH_2 solution (1 mg/mL). A Sep-Pak® C18 cartridge was used to purify the ^{77}As-trithiol-BBN(7-14)NH_2. Both radiolabeled ^{77}As-trithiol-BBN(7-14)NH_2 and trithiol-BBN(7-14)NH_2 were retained on the cartridge because of their high lipophilicities, while the mercaptoacetate and TCEP eluted in the mobile phase during loading and the water wash. Purified ^{77}As-trithiol-BBN(7-14)NH_2 was eluted from the cartridge with ethanol, and the peaks associated with the monothiol and TCEP were much less intense (less than 0.1% remaining). Surprisingly the concentration of unlabeled trithiol-BBN(7-14)NH_2 was also reduced because a significant amount of both labeled and unlabeled trithiol-BBN(7-14)NH_2 was retained on the cartridge with the ethanol elution (~40% of the activity also was retained). To further determine the total

concentration of trithiol-BBN(7-14)NH_2 in the purified product solution, the UV profile at 280 nm of purified ^{77}As-trithiol-BBN(7-14)NH_2 solution was compared with a standard trithiol-BBN(7-14)NH_2 solution (1 mg/mL) and the total trithiol-BBN(7-14)NH_2 concentration was determined to be 5.5 µg/mL (below detection limit). Ascorbic acid was added to the Sep-Pak® purified ^{77}As-trithiol-BBN(7-14)NH_2 ethanol solution to prevent radiolysis; significant radiolysis resulting in the formation of ^{77}As-arsenate was previously observed when the volume was reduced to near dryness [18]. Following addition of ascorbic acid, the ^{77}As-trithiol-BBN(7-14)NH_2 solution was brought to near dryness under a gentle N_2 stream and reconstituted with bacteriostatic saline. Quality control was performed by RP-HPLC, with no sign of the free ^{77}As formation observed, and the retention time of the product peak was in good agreement with ^{77}As-trithiol-BBN(7-14)NH_2 standard (**Figure 3**).

Scheme 3. Radiotracer synthesis of no carrier added ^{77}As-trithiol-BBN(7-14)NH_2.

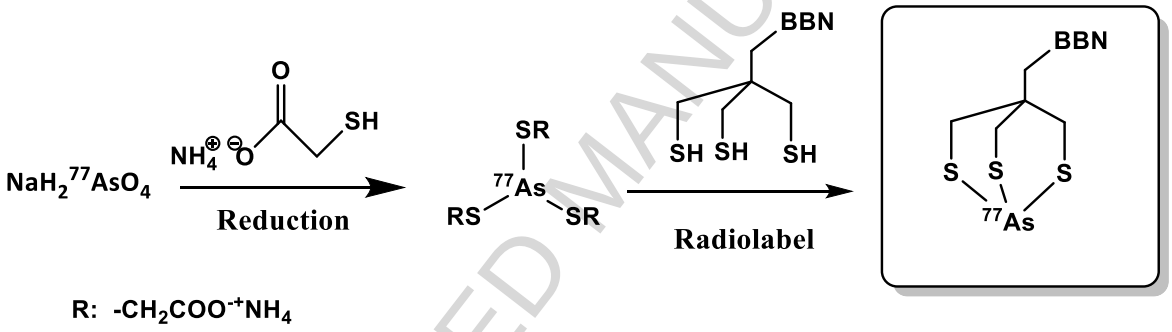


Figure 3

Stability of ^{77}As-trithiol-BBN(7-14)NH_2. The radiolabeling was carried out in an excess of monothiol (ammonium mercaptoacetate) to ensure that the ^{77}As was present in oxidation state +3 prior to addition of the trithiol-BBN(7-14)NH_2. Previously, we observed that the trithiol ligand was not sufficient for reducing arsenate to As(III) [18]. The ^{77}As-trithiol-BBN(7-14)NH_2 remained in the radiolabeling solution (monothiol concentration 100 mM) for up to 48 h. *In vitro* stability of the ^{77}As-trithiol-BBN in the radiolabeling solution (monothiol concentration = 100 mM) was assessed up to 48 hours by HPLC. The formation of free ^{77}As (arsenate) was not observed. Similar results were previously reported for the non-functionalized trithiol arsenic [18]. This result indicates that it is unlikely for thiols found *in vivo* (i.e., glutathione, cysteine, albumin) to displace the As from the conjugate.

Biodistribution studies of no carrier added free ^{77}As and ^{77}As-trithiol-BBN(7-14)NH$_2$. A summary of the biodistribution of ^{77}As-arsenate and ^{77}As-trithiol-BBN(7-14)NH$_2$ is shown in **Figure 4** and the detailed data are shown in **Tables 1** and **2**. A fast renal clearance of free As was expected [5]. Arsenate acts as a phosphate (HPO_4^-) mimic and can be reduced to As(III) *in vivo* [35,36]. Generally arsenite has better *in vivo* retention due to multiple protein binding sites compared to arsenate, however a fast renal excretion is typical for both species [36]. ^{77}As (arsenate) was excreted through the renal system with 86.90 ± 2.53% of the injected dose (ID) excreted by 4 h p.i. At 4 h, 1.00% ± 0.12% ID remained in the kidneys, which indicates the majority of ^{77}As (arsenate) was not binding to free thiols from proteins present in blood plasma. The rapid renal clearance of ^{77}As-arsenate could potentially avoid radiotoxicity because if ^{77}As was to dissociate from the chelate, it should be excreted in a short period of time ($\leq$4 h). The biodistribution of ^{77}As-trithiol-BBN(7-14)NH$_2$ is vastly different from that of ^{77}As (arsenate) as ^{77}As-trithiol-BBN(7-14)NH$_2$ was primarily cleared through the hepatobiliary system. By 4 h p.i., 39.83 ± 9.26% ID was located in the large intestine, 2.70 ± 1.71% ID in the small intestine, 6.84 ± 2.61% ID in the liver, 1.07 ± 0.68% ID in the kidneys, and 9.46 ± 2.25% ID in the residual carcass. Renal system excretion accounted for only 38.04 ± 8.76% of the injected dose, which is lower than typically observed [21-23,34].

Figure 4

Tables 1 and 2

The different clearance pathway of ^{77}As-trithiol-BBN(7-14)NH$_2$ suggests high *in vivo* stability of ^{77}As-trithiol-BBN(7-14)NH$_2$ complex. At 4 hr, 3.52% ± 1.14% ID/g was retained in the liver, compared with 0.42 ± 0.07% ID/g for ^{77}As-arsenate. However the effective clearance via liver and intestines was expected due to the high lipophilicity of ^{77}As-trithiol-BBN(7-14)NH$_2$. Additional indications of stability come from the fact that even in the presence of a 1300-fold excess of monothiol (mercaptoacetate) compared to trithiol-BBN97-14)NH$_2$ present in the formulation, no free [^{77}As]arsenate or [^{77}As]As(mercaptoacetate)$_3$ were observed over time by HPLC here or for the previously reported nca [^{77}As]As-trithiol [18]. The Sep-Pak® purified ^{77}As-trithiol-BBN(7-14)NH$_2$ was stable for at least 24 h as determined by HPLC analysis.

Although this was a proof of principle study to determine the stability of the trithiol-arsenic complex under *in vivo* conditions, some pancreatic uptake was anticipated but none (<2% ID/g) was observed at 1 h p.i. [23,36,37]. This was not due to excess unlabeled trithiol-BBN(7-14)NH$_2$ as only 0.1 mg were originally used and the HPLC trace (**Figure 3**) shows that following Sep-

Pak® purification, the amount remaining was below the detection limit (bottom vs upper UV trace). It has been reported that the spacer length between the bombesin(7-14)NH_2 and the radiolabeled chelate should be a chain length of 5-8 atoms with less than 5 or more than 8 being detrimental to receptor targeting and thus pancreatic uptake [34,37]. A spacer length of either 2 or 9 is present in the ^{77}As-trithiol-BBN(7-14)NH_2 complex depending on whether the spacer count begins at the bridgehead carbon or after the sterically rigid triazole ring. The crystal structure of **8** suggests that the spacer is quite rigid between **C5** and **C12** due to the bridgehead and planarity of the **C9** through **C12** triazole-containing unit and this may impact receptor binding.

Conclusion

A trithiol ligand and its ^{77}As radiochemistry were previously reported [18]. To investigate the *in vivo* utility of the trithiol ligand framework for nca radioarsenic, an ^{77}As trithiol complex, namely nca ^{77}As-trithiol-BBN(7-14)NH_2, was synthesized in >90% radiochemical yield. Following Sep-Pak® purification, normal mouse biodistribution studies of uncomplexed ^{77}As-arsenate and ^{77}As-trithiol-BBN(7-14)NH_2 demonstrated that the trithiol is a promising ligand for developing ^{72,77}As matched pair theranostic radiopharmaceuticals. The trithiol ligand forms an *in vivo* stable radioarsenic complex and development of a more hydrophilic linker that does not involve click chemistry (i.e., a triazole unit) to reduce the lipophilicity and rigidity of the ^{77}As complex is underway.

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References

[1] Del Vecchio S, Zannetti A, Fonti R, Pace L, Salvatore M. Nuclear imaging in cancer theranostics. Quart J Nucl Med Mol Imaging 2007**;** 51(2): 152-163.

[2] Bockisch A. Matched pairs for radionuclide-based imaging and therapy. Eur J Nucl Med Molec Imag 2011; 38(1): 1-3.

[3] DeGraffenreid AJ, Feng Y, Wycoff DE, Morrow R, Phipps MD, Cutler CS, Ketring AR, Barnes CL, Jurisson SS. Dithiol Aryl Arsenic Compounds as Potential Diagnostic and Therapeutic Radiopharmaceuticals. Inorg Chem 2016; 55(16): 8091-8098.

[4] Nayak TK, Brechbiel MW. Radioimmunoimaging with longer-lived positron-emitting radionuclides: potentials and challenges. Bioconjug Chem 2009; 20(5): 825-841.

[5] Ellison PA, Barnhart TE, Chen F, Hong H, Zhang Y, Theuer CP, Cai W, Nickles RJ, DeJesus OT. High yield production and radiochemical isolation of isotopically pure arsenic-72 and novel radioarsenic labeling strategies for the development of theranostic radiopharmaceuticals. Bioconjug Chem 2015; 27(1): 179-188.

[6] Wycoff DE, Gott MD, DeGraffenreid AJ, Morrow RP, Sisay N, Embree MF, Ballard B, Fassbender ME, Cutler CS, Ketring AR, Jurisson SS. Chromatographic separation of selenium and arsenic: A potential ^{72}Se/^{72}As generator. J. Chromatography A 2014**;** 1340: 109-114.

[7] Ballard B, Nortier FM, Birnbaum ER, John KD, Phillips DR, Fassbender ME. Radioarsenic from a Portable ^{72}Se/^{72}As Generator: A Current Perspective. Current Radiopharmaceuticals 2012**;** 5(3): 264-270.

[8] Jennewein M, Qaim SM, Kulkarni PV, Mason RP, Hermanne A, Rösch F. A no-carrier-added ^{72}Se/^{72}As radionuclide generator based on solid phase extraction. In Radiochim. Acta 2005; 93: 579-583.

[9] Chajduk E, Doner K, Polkowska-Motrenko H, Bilewicz A. Novel radiochemical separation of arsenic from selenium for ^{72}Se/^{72}As generator. Appl. Radiat. Isot. 2012; 70: 819-822.

[10] Jennewein M, Schmidt A, Novgorodov AF, Qaim SM, Roesch F. A no-carrier-added ^{72}Se/^{72}As radionuclide generator based on distillation. Radiochim Acta 2004; 92: 245-249.

[11] Mudrova B, Kopecky P, Svoboda K. The Separation of Carrier-Free ^{72}Se from a Germanium Oxide Target. Int. J. Appl. Radiat. Isot. 1973; 24: 610-612.

[12] Gott MD, DeGraffenreid AJ, Feng Y.; Phipps MD, Wycoff DE, Embree MF, Cutler CS, Ketring AR, Jurisson SS. Chromatographic separation of germanium and arsenic for the production of high purity ^{77}As. J Chromatogr A 2016; 1441: 68-74.

[13] Kitchin KT, Wallace K. The role of protein binding of trivalent arsenicals in arsenic carcinogenesis and toxicity. J Inorg Biochem 2008; 102(3): 532-539.

[14] Pott WA, Benjamin SA, Yang RS. Pharmacokinetics, metabolism, and carcinogenicity of arsenic. Reviews of Environmental Contamination and Toxicology 2001; 169: 165-214.

[15] Jennewein M, Hermanne A, Mason RP, Thorpe PE, Rosch, F. A new method for the labelling of proteins with radioactive arsenic isotopes. Nucl Instr Meth Phys Res A 2006; 569: 512-517.

[16] Jennewein M, Lewis MA, Zhao D, Tsyganov E, Slavine N, He J, Watkins L, Kodibagkar VD, O'Kelly S, Kulkarni P. Vascular imaging of solid tumors in rats with a radioactive arsenic-labeled antibody that binds exposed phosphatidylserine. Clin Cancer Res 2008; 14(5): 1377-1385.

[17] Herth MM, Barz M, Jahn M, Zentel R, Rösch F. $^{72/74}$As-labeling of HPMA based polymers for long-term in vivo PET imaging. Bioorg Med Chem Lett 2010; 20(18): 5454-5458.

[18] DeGraffenreid AJ, Feng Y, Barnes CL, Ketring AR, Cutler CS, Jurisson SS. Trithiols and their arsenic compounds for potential use in diagnostic and therapeutic radiopharmaceuticals. Nucl Med Biol 2016; 43(5): 288-295.

[19] Reubi JC. Neuropeptide receptors in health and disease: the molecular basis for in vivo imaging. J Nucl Med 1995; 36(10): 1825-1835.

[20] Ohki-Hamazaki H, Iwabuchi M, Maekawa F. Development and function of bombesin-like peptides and their receptors. Internat J Dev Biol 2003; 49(2-3): 293-300.

[21] Abd-Elgaliel WR, Gallazzi F, Garrison JC, Rold TL, Sieckman GL, Figueroa SD, Hoffman TJ, Lever S Z. Design, Synthesis, and Biological Evaluation of an Antagonist− Bombesin Analogue as Targeting Vector. Bioconjug Chem 2008; 19(10): 2040-2048.

[22] Hoffman TJ, Smith CJ. True radiotracers: Cu-64 targeting vectors based upon bombesin peptide. Nucl Med Biol 2009; 36(6): 579-585.

[23] Lane SR, Nanda P, Rold TL, Sieckman GL, Figueroa SD, Hoffman TJ, Jurisson SS, Smith CJ. Optimization, biological evaluation and microPET imaging of copper-64-labeled bombesin agonists, [64 Cu-NO2A-(X)-BBN (7–14) NH 2], in a prostate tumor xenografted mouse model. Nucl Med Biol 2010; 37(7): 751-761.

[24] Camerano JA, Casado MA, Ciriano MA, Lahoz FJ, Oro LA. A trithiol protio-ligand and its fixation to the periphery of a carbosilane dendrimer as scaffolds for polynuclear rhodium and iridium complexes and metallodendrimers. Organometallics 2005; 24(21): 5147-5156.

[25] Alvarez SG, Alvarez MT. A practical procedure for the synthesis of alkyl azides at ambient temperature in dimethyl sulfoxide in high purity and yield. Synthesis 1997; 1997(04): 413-414.

[26] Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise huisgen cycloaddition process: copper (I)-catalyzed regioselective “ligation” of azides and terminal alkynes. Angew Chem 2002; 114(14): 2708-2711.

[27] Meldal M, Tornøe CW. Cu-catalyzed azide− alkyne cycloaddition. Chem Rev 2008; 108(8): 2952-3015.

[28] Chan WC, White PD. Fmoc solid phase peptide synthesis. Oxford University Press: 2000.

[29] Sheldrick GM. A short history of SHELX. Acta Cryst Sect A 2008; 64(1): 112-122.

[30] Sheldrick G. SADABS. Germany: University of Göttingen; 1996.

[31] Field LD, Sternhell S, Kalman JR. Organic structures from spectra. John Wiley & Sons: 2012.

[32] Anastasi A, Erspamer V, Bucci M. Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians Bombina and Alytes. Experientia 1971; 27(2): 166-167.

[33] Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate relation to neoplastic transformation. Cancer Res 1999; 59(5): 1152-1159.

[34] Hoffman TJ, Gali H, Smith CJ, Sieckman GL, Hayes DL, Owen NK, Volkert WA. Novel Series of ^{111}In-Labeled Bombesin Analogs as Potential Radiopharmaceuticals for Specific Targeting of Gastrin-Releasing Peptide Receptors on Human Prostate Cancer Cells. J Nucl Med 2003; 44: 823-831.

[35] Hughes MF. Arsenic toxicity and potential mechanisms of action. Toxicol Lett 2002; 133(1): 1-16.

[36] Shen S, Li X-F, Cullen WR, Weinfeld M, Le XC. Arsenic binding to proteins. Chem Rev 2013; 113(10): 7769-7792.

[37] Li Y, Liu Z, Harwig CW, Pourghiasian M, Lau J, Lin K-S, Schaffer, P, Benard F, Perrin DM. ^{18}F-click labeling of a bombesin antagonist with an alkyne-^{18}F-ArBF_3^-: in vivo PET imaging of tumors expressing the GRP-receptor. Am J Nucl Mol Imaging 2013; 3(1): 57-70.

Tables

Table 1. Biodistribution of free ^{77}As ($[^{77}As]H_2AsO_4^-$) in CF-1 normal mice at 15 min, 1 h, 4 h and 24 h post injection, n=4. Data are presented as %ID/g ± SD.

Organ/Tissue	15 min	1 h	4 h	24 h
Heart	1.21 ± 1.73	1.33 ± 2.04	0.65 ± 1.14	0.31 ± 0.45
Lung	3.43 ± 2.81	2.87 ± 0.98	0.60 ± 1.10	0.67 ± 0.69
Liver	4.39 ± 0.73	3.31 ± 0.42	0.42 ± 0.07	0.15 ± 0.21
Kidneys	40.80 ± 5.52	13.77 ± 1.42	1.74 ± 0.42	0.66 ± 0.88
Spleen	0.00	1.07 ± 1.13	0.00	0.57 ± 1.14
Stomach	0.82 ± 1.03	0.62 ± 0.31	0.16 ± 0.32	0.11 ± 0.21
S. Intestine	1.67 ± 0.26	1.59 ± 0.17	0.40 ± 0.16	0.13 ± 0.18
L. Intestine	1.28 ± 0.28	1.31 ± 0.51	1.02 ± 0.37	0.03 ± 0.05
Muscle	2.04 ± 2.44	1.02 ± 0.70	0.30 ± 0.46	0.00
Bone	0.95 ± 1.89	0.63 ± 0.83	0.50 ± 0.99	0.00
Brain	0.00	0.35 ± 0.46	0.50 ± 0.38	0.16 ± 0.21
Pancreas	2.29 ± 1.95	1.30 ± 0.82	0.25 ± 0.25	0.00
Blood	1.95 ± 0.74	0.52 ± 0.19	0.28 ± 0.42	0.38 ± 0.32
Carcass	2.26 ± 0.19	1.25 ± 0.11	0.41 ± 0.15	0.04 ± 0.04
Excretion*	22.79 ± 2.20	54.82 ± 1.69	86.90 ± 2.53	97.13 ± 1.37

* Excretion includes bladder, urine, cage paper, and feces (24 h) and is presented as %ID ± SD.

Table 2. Biodistribution of ^{77}As-trithiol-BBN in CF-1 normal mice at 1 h and 4 h post injection, n=5. Data are presented as %ID/g ± SD.

Organ/Tissue	1 h	4 h
Heart	0.88 ± 0.36	0.28 ± 0.39
Lung	2.06 ± 0.25	2.65 ± 0.63
Liver	8.03 ± 1.39	3.53 ± 1.14
Kidneys	4.08 ± 0.72	1.67 ± 0.85
Spleen	2.09 ± 0.53	2.87 ± 1.09
Stomach	0.44 ± 0.15	0.80 ± 1.04
S. Intestine	13.77 ± 8.24	1.51 ± 0.93
L. Intestine	27.12 ± 13.76	30.45 ± 10.05
Muscle	0.60 ± 0.27	0.20 ± 0.18
Bone	0.50 ± 0.60	0.21 ± 0.46
Brain	0.54 ± 0.78	0.04 ± 0.08
Pancreas	1.51 ± 0.52	0.66 ± 0.67
Blood	0.58 ± 0.21	0.24 ± 0.25
Carcass	0.42 ± 0.03	0.44 ± 0.11
Excretion*	8.07 ± 0.93	38.04 ± 8.76

* Excretion includes bladder, urine and cage paper and is presented as %ID ± SD. Feces included in intestinal values.

Figures

Figure 1. X-Seed representation of (**8**) (CCDC# 1585889) with 50% probability ellipsoids.

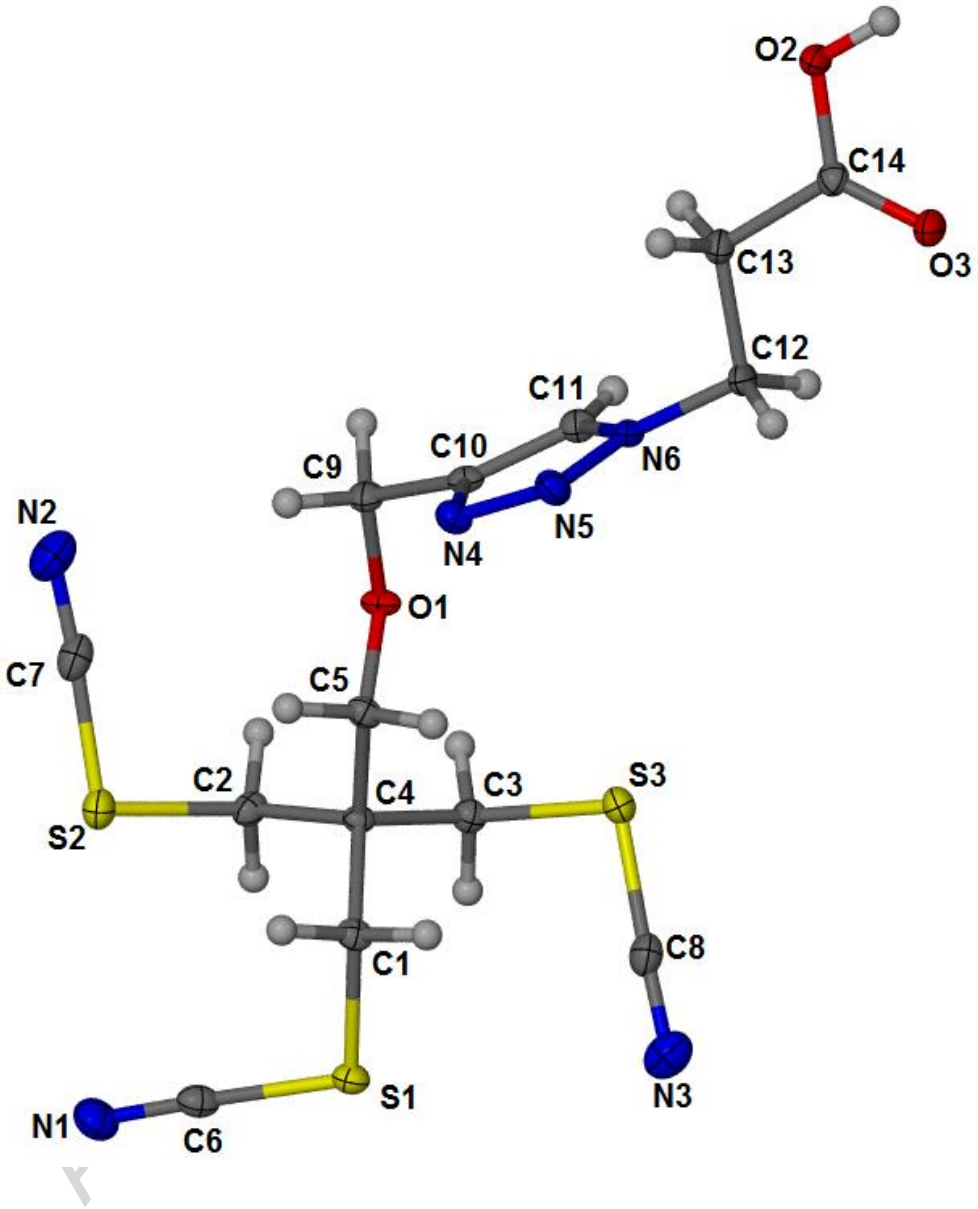
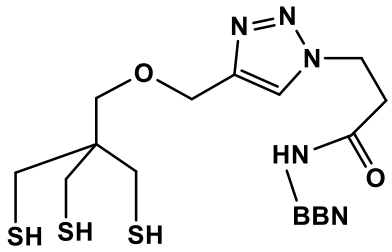


Figure 2. The structure of trithiol-BBN (7-14)NH_2 conjugate.



BBN: QWAVGHLM-CONH$_2$

Figure 3. RP-HPLC chromatograms of ^{77}As-trithiol-BBN using both gamma and UV detectors (wavelengths 220 nm and 280 nm) and comparison with trithiocyanate-BBN, trithiol-BBN and As-trithiol-BBN standards. Upper left diagram: γ peak of ^{77}As-trithiol-BBN (top trace) matched the UV peak of As-trithiol-BBN standard (middle trace) and was distinguished from the UV peak of trithiol-BBN standard (bottom trace). Lower diagram: UV signals of monothiols and TCEP impurities were reduced after purification (second and third traces); no UV signals related to the γ signals of ^{77}As-trithiol-BBN (first trace) were observed, indicating the low concentration of ^{77}As-trithiol-BBN in the product solution.

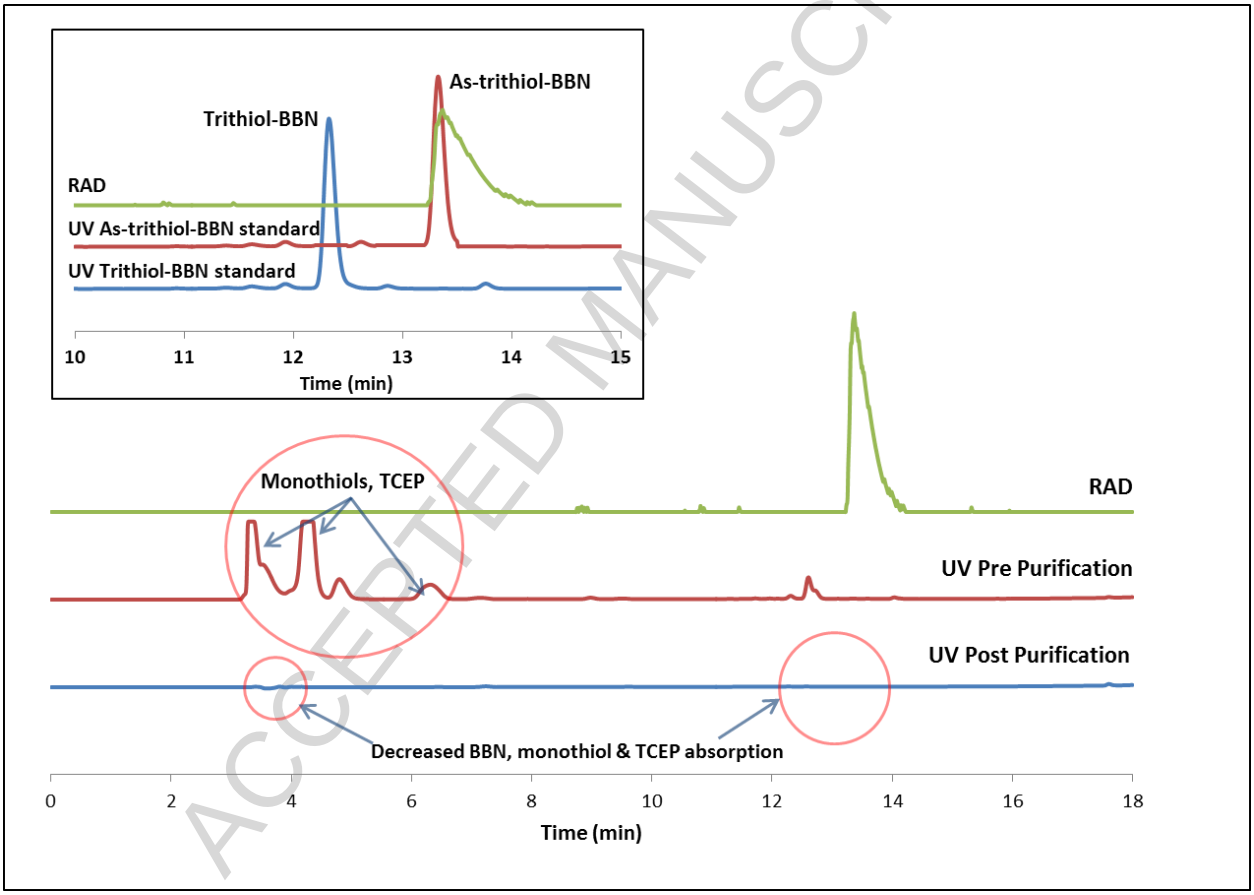


Figure 4. Biodistribution of free ^{77}As ($[^{77}As]H_2AsO_4^-$) and ^{77}As-Trithiol-Bombesin in CF-1 normal mice. (A) Free ^{77}As at 15 min, 1 h, 4 h and 24 h, n=4. (B) ^{77}As-Trithiol-Bombesin at 1 h and 4 h, n=5.