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Versatile New Bis(thiosemicarbazone) Bifunctional Chelators: Synthesis, Conjugation to Bombesin(7−14)-NH_2, and Copper-64 Radiolabeling

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New bifunctional derivatives of diacetyl-*bis*(4-methylthiosemicarbazone) (H_2atsm) have been prepared by a selective transamination reaction of a new dissymmetric *bis*(thiosemicarbazone) precursor $H_2\mathbf{L^1}$. The new derivatives contain an aliphatic carboxylic acid ($H_2\mathbf{L^2}$ and $H_2\mathbf{L^3}$), *t*-butyl carbamate ($H_2\mathbf{L^4}$), or ammonium ion ($H_2\mathbf{L^5}$) functional group. The new ligands and copper(II) complexes have been characterized by NMR spectroscopy, mass spectrometry, and microanalysis. The complex $Cu^{II}(\mathbf{L^4})$ was structurally characterized by X-ray crystallography and shows the metal center to be in an N_2S_2 distorted square planar coordination geometry. Electrochemical measurements show that the copper(II) complexes undergo a reversible reduction attributable to a Cu(II)/Cu(I) process. The ligands and the copper(II) complexes featuring a carboxylic acid functional group have been conjugated to the tumor targeting peptide bombesin(7−14)-NH_2. The bifunctional peptide conjugates were radiolabeled with copper-64 in the interest of developing new positron emission tomography (PET) imaging agents. The conjugates were radiolabeled with copper-64 rapidly in high radiochemical purity (>95%) at room temperature under mild conditions and were stable in a cysteine and histidine challenge study.

Introduction

Positron emission tomography (PET) is an imaging technique that can provide valuable noninvasive diagnostic information. The technique relies on a positron-emitting tracer that is detected as it passes through the body. The decay profile of copper-64 includes positron-emission (β^+; E_{av} 278 keV, 17.9%) and β^- emission (37%) with a half-life of 12.7 h and consequently offers the potential for both PET imaging and radiotherapy.[1,2] The use of copper radioisotopes in radiopharmaceuticals is dependent on the ability to selectively deliver the radioisotope to target tissue. One approach is to incorporate the radioactive copper isotope into a coordination complex. The biodistribution of the copper complex is dictated by a variety of factors including lipophilicity, size of the complex, and redox properties. A wide range of *bis*(thiosemicarbazonato) ligands have been investigated as delivery vehicles for copper radioisotopes as they form stable ($K_a = 10^{18}$), neutral, membrane permeable copper complexes. The copper complex of the *bis*(thiosemicarbazone) derived from pyruvaldehyde, Cu^{II}(ptsm) has been investigated as a perfusion tracer[3] while more recent developments have focused on an ethyl derivative.[4] The stable, neutral complexes can diffuse into cells, which provide a reducing environment, whereupon the complexes are susceptible to intracellular reduction (Cu(II) to Cu(I)). The cellular metabolism of *bis*(thiosemicarbazonato)copper(II) complexes is remarkably sensitive to the substituents on the diimine backbone of the ligand. For example, the copper complex of the ligand with two methyl substituents on the diimine backbone, Cu^{II}(atsm), is harder to reduce than Cu^{II}(ptsm). Consequently, Cu^{II}(atsm) is only reduced in hypoxic cells and is being investigated as a hypoxia imaging agent.[5,6]

Another approach to achieve selectivity is to tether a biologically active targeting molecule to the coordination complex that targets the complex to the desired tissue or cells. Examples of targeting vectors include peptides such as bombesin (BBN) a 14 amino acid neuropeptide, which was first isolated from the skin of the frog *Bombina bombina*.[7,8] The mammalian analogue, gastrin-releasing peptide (GRP),

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has the same amino acid sequence of Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 in the C terminal region, which is referred to as BBN(7−14)-NH_2, and is responsible for their high affinity for GRP receptors.[9,10] GRP receptors are overexpressed in a number of human cancer cell lines including prostate, lung, and breast cancer tissue. Radiolabeled analogues of BBN and BBN(7−14)-NH_2 have displayed high tumor uptake in animal models and have shown potential for both diagnostic and therapeutic applications.[11−15] These systems usually make use of tetraazamacrocycles such as TETA (1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) and DOTA (1,4,7,10-tetraazadodecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) to act as the ligand for complexation of copper-64. Promising results have been obtained but some degree of loss of copper-64 from the ligand-BBN construct resulted in accumulation of copper-64 in nontarget tissue.[16] The stability of Cu(II) complexes in vivo depends not only on the thermodynamic and kinetic stability but also the Cu(II)/Cu(I) reduction potential. Recent studies have revealed a connection between the Cu(II)/Cu(I) reduction potential and complex stability.[17]

Since Cu^{II}(atsm) has sufficient stability for imaging applications, bifunctional chelates based on this framework could have potential as new targeted PET imaging agents. Importantly, due to the Cu(II)/Cu(I) reduction potential, reduction is only thought to occur intracellularly in hypoxic cells, hence reduction would not be expected when targeting cell surface receptors. Bifunctional chelates based on *bis*(thiosemicarbazones) have been prepared and radiolabeled with technetium-99 m, copper-64, and copper-67 where the carboxylate functional groups are situated on the backbone of the ligand.[18−20] However, the stability and reduction potentials of *bis*-(thiosemicarbazonato)copper(II) complexes is mainly dependent on the substituents on the diimine backbone than it is on the terminal N^4 substituents, particularly for alkyl substituents. Introduction of functional groups at the terminal N^4 positions and not the backbone would allow the conjugation of biological targeting peptides while retaining the stability and favorable redox properties of Cu^{II}(atsm). Bifunctional chelators based on Cu^{II}(atsm) bearing phenyl carboxylate groups have been prepared that retain the dimethyl backbone but the introduction of the aromatic phenyl group shifted the reduction potential to $E_{red} = -0.41$ V.[21] In this study we present a versatile new approach to synthesize new H_2atsm derivatives with aliphatic carboxylates and amines that form Cu(II) complexes in which the Cu(II)/Cu(I) reduction potential is closer to that encountered with Cu^{II}(atsm).

The new ligands have been prepared by a selective transamination reaction of a new dissymmetric *bis*(thiosemicarbazone) precursor called H_2atsm/m_2 (diacetyl-4,4-dimethyl-4′-methyl-*bis*(thiosemicarbazone), $H_2\mathbf{L}^1$). It is envisaged that this new method will allow the use of different bioconjugate linker systems, which will provide greater control over in vivo biodistribution. The new proligands and copper complexes with a carboxylic acid group have been conjugated to the tumor targeting bombesin derivative BBN(7−14)-NH_2 through the N-terminus of the peptide using standard solid phase peptide coupling procedures. The peptide-conjugates have been radiolabeled with copper-64 and the stability of the copper-64 radiolabeled conjugates has been measured.

Experimental Section

General Procedures. Fmoc-L-amino acids and 2-(7-aza-1 h-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from GL Biochem Ltd. (Shanghai, China). Fmoc-Pal-PEG-PS resin was purchased from Applied Biosystems (Foster City, CA). All other reagents and solvents were obtained from standard commercial sources and were used as received.

Nuclear magnetic resonance (NMR) spectra were acquired on a Varian FT-NMR 500 spectrometer and a Varian FT-NMR 400 spectrometer. ^{1}H NMR spectra were acquired at 500 or 400 MHz and $^{13}C\{^1H\}$ NMR spectra were acquired at 125.7 MHz. All NMR spectra were recorded at 25 °C. ^{1}H and $^{13}C\{^1H\}$ chemical shifts were referenced to residual solvent peaks and are quoted in ppm relative to TMS. ^{19}F chemical shifts are referenced to C_6F_6 in d_6-DMSO ($\delta = -164.9$ ppm). Mass spectra were recorded on an Agilent 6510 Q-TOF LC/MS mass spectrometer and a Waters-Micromass QuattroII triple quadrupole electrospray ionization mass spectrometer. Cyclic voltammograms were recorded on an AUTOLAB PGSTAT100 electrochemical workstation using GPES V4.9 software and employing a glassy carbon working electrode, a platinum counter electrode, and a Ag/Ag^+ reference electrode (silver wire in CH_3CN ($AgNO_3$ (0.01 M)). All measurements were carried out in DMF. All solutions were 5 mM of analyte in 0.1 M tetrabutylammoniumtetrafluoroborate solution. DMF was obtained from commercial sources and dried over 3 Å sieves before use. Each solution was purged with N_2 prior to analysis and measured at ambient temperatures under a N_2 atmosphere. Peak potentials, E_p, and half-wave potentials, $E_{1/2}$, were referenced to the ferrocene/ferrocinium couple, 0.54 V in DMF versus SCE. The ferrocene/ferrocinium half-wave potential under the conditions used was 0.07 V. Microanalyses for C, H, and N were carried out by Chemical & Microanalytical Services (CMAS) Pty. Ltd. Belmont, Vic.

High-Performance Liquid Chromatography. Several chromatographic systems were used for the analytical experiments and the purification steps. **System A**: RP-HPLC (Agilent 1200 Series HPLC system using an Agilent Zorbax Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm) with a 1 mL/min flow rate, gradient elution of Buffer A = 0.1% TFA in H_2O and Buffer B = 0.1% TFA in acetonitrile (0 to 100% B in A at 20 min) and detection at 220, 254, and 275 nm. **System B**: System A with the following gradient (0 to 80% B in A at 20 min). **System C**: RP-HPLC

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(Shimadzu LC-20AT HPLC system with a Waters Cosmosil C18 column (4.6 × 150 mm, 5 μm) with a 1 mL/min flow rate, gradient elution of Buffer A = 0.1% TFA in H_2O and Buffer B = 0.1% TFA in acetonitrile (0 to 100% B in A at 20 min) and a sodium iodide scintillation detector and a UV−vis detector in series (detection at 254 and 275 nm). **System D**: System C with the following gradient (0 to 20% B in A at 1 min, 20 to 100% B in A at 12 min, 100% B at 16 min). **System E**: System C with the following gradient (0 to 100% B in A at 5 min, 100% B at 8 min, 100 to 0% B in A at 9 min). **System F**: Semipreparative RP-HPLC (Agilent 1200 series HPLC system on an Agilent Eclipse XDB C18 column, 9.4 × 250 mm, 5 μm) with a 5 mL/min flow rate, gradient elution of Buffer A = 0.1% TFA in H_2O and Buffer B = 0.1% TFA in acetonitrile (0 to 80% B in A at 1 h) and detection at 230 nm.

Small Molecule Synthesis. Diacetyl-*mono*-4-methyl-3-thiosemicarbazone, 1. This compound was prepared by a modification of a previously reported procedure.[21,22] A solution of 2,3-butanedione (2.39 g, 27.7 mmol) in distilled water (50 mL) was acidified with a few drops of conc. HCl (36%) and cooled to 5 °C. 4-Methyl-3-thiosemicarbazide (2.65 g, 25.2 mmol) was added in small portions to the stirred cold solution over 1.5 h to produce a white precipitate, which was further stirred for 40 min. The precipitate was extracted into chloroform (50 + 50 + 40 mL) and the extracts were combined, dried over $MgSO_4$, filtered, and concentrated. *N*-pentane was added to the solution until slight turbidity and then it was cooled to −20 °C to give white needles. The product was collected by filtration, washed with *n*-pentane and dried to give **1** (3.26 g, 18.82 mmol, 75%). ^{1}H NMR (d_6-DMSO, 500 MHz): δ = 1.96, 3H, s, CH_3; 2.42, 3H, s, CH_3; 3.05, 3H, d, $^3J_{HH}$ = 4.5 Hz, NH−CH_3; 8.61, 1H, m, NH; 10.61, 1H, s, NH. ESIMS: (+ve ion) m/z 100% [M + H$^+$] 174.07 (experimental), 174.07 (Calcd).

Diacetyl-4,4-dimethyl-4′-methyl*bis*(thiosemicarbazone), H_2L^1, H_2atsm/m_2. To a solution of **1** (0.92 g, 5.3 mmol) in DMF (3 mL) was added 4,4-dimethyl-3-thiosemicarbazide (0.76 g, 6.4 mmol, 1.2 equiv) and acetic acid (5 drops, glacial). The resulting solution was stirred at room temperature for 48 h. A yellow solid precipitated from solution upon addition of water (50 mL). The suspension was cooled in an ice bath before the bright yellow solid was collected by filtration, washed with water (1×), ethanol (2×), and diethyl ether (3×) and dried to give H_2L^1 (1.30 g, 4.7 mmol, 89%). (Found: C, 39.45; H, 6.63; N, 30.81; calcd for $C_9H_{18}N_6S_2$: C, 39.39; H, 6.61; N, 30.63). ^{1}H NMR (d_6-DMSO, 500 MHz): δ = 2.15, 3H, s, CH_3; 2.19, 3H, s, CH_3; 3.03, 3H, d, $^3J_{HH}$ = 4.5 Hz, NH−CH_3; 3.27, s, 6H, $(CH_3)_2$; 8.36, bq, 1H, $^3J_{HH}$ = 4.5 Hz, NH−CH_3; 9.49, bs, 1H, NH; 10.16, bs, 1H, NH. ^{13}C{^{1}H} NMR (125.7 MHz): δ = 11.1, CH_3; 11.4, CH_3; 31.2, NH−CH_3; 42.3, $(CH_3)_2$; 148.0, C=N; 149.4, C=N; 178.5, C=S; 181.7, C=S. ESIMS: (+ve ion) m/z 100% [M + H$^+$] 275.3 (experimental), 275.1 (Calcd), (-ve ion) m/z [M − H$^+$] 273.3 (experimental), 273.1 (calcd). Crystals suitable for single crystal X-ray crystallography were grown from a concentrated solution in DMSO.

Diacetyl-4-butyric acid-4′-methyl*bis*(thiosemicarbazone), H_2L^2. To a stirring suspension of H_2L^1 (0.28 g, 1.0 mmol) in acetonitrile (30 mL) was added 4-aminobutyric acid (0.21 g, 2.0 mmol, 2 equiv). The resulting yellow suspension was heated at reflux for 34 h under an atmosphere of N_2. The resulting light cream suspension was cooled to room temperature and the white solid was collected by filtration, washed with HCl (3%, 3 × 3.5 mL), acetonitrile (1×), and diethyl ether (3×), and dried to give H_2L^2 (0.28 g, 0.8 mmol, 81%). (Found: C, 39.91; H, 6.15; N, 25.39; calcd for $C_{11}H_{20}N_6O_2S_2$: C, 39.74; H, 6.06; N, 25.28). ^{1}H NMR (d_6-DMSO, 500 MHz): δ = 1.81, 2H, m, CH_2−CH_2−CH_2; 2.21, 6H, s, CH_3; 2.25, 2H, t, $^3J_{HH}$ = 7.5 Hz, CH_2−CO_2H; 3.02, 3H, d, $^3J_{HH}$ = 4.5 Hz, NH−CH_3; 3.58, 2H, m, NH−CH_2−CH_2; 8.38, 1H, q, $^3J_{HH}$ = 4.5 Hz, NH−CH_3; 8.44, 1H, t, $^3J_{HH}$ = 6 Hz, NH−CH_2; 10.18, 1H, s, NH; 10.23, 1H, s, NH. ^{13}C{^{1}H} NMR (125.7 MHz): δ = 11.6, CH_3; 11.7, CH_3; 24.2, CH_2−CH_2−CH_2; 31.2, NH−CH_3, CH_2−CO_2H; 43.2, NH−CH_2; 147.9, C=N; 148.1, C=N; 174.2, C=O; 177.8, C=S; 178.5, C=S. ESIMS: (+ve ion) m/z 100% [M + H$^+$] 333.4 (experimental), 333.1 (calcd), (-ve ion) m/z [M − H$^+$] 331.3 (experimental), 331.1 (calcd).

Diacetyl-4-butyric acid-4′-methyl*bis*(thiosemicarbazonato)-copper(II), $Cu^{II}(L^2)$. To a solution of H_2L^2 (0.11 g, 0.3 mmol) in DMF (2 mL) stirring at room temperature was added $Cu(OAc)_2 \cdot H_2O$ (0.07 g, 0.3 mmol). The red/brown solution was stirred at room temperature for 17 h. A brown solid was precipitated upon addition of water (40 mL) to the solution. The solid was collected by filtration, washed with water, ethanol, and diethyl ether, and dried to give $Cu^{II}(L^2)$ (0.09 g, 0.2 mmol, 70%). (Found: C, 33.40; H, 4.70; N, 21.27; calcd for $CuC_{11}H_{18}N_6O_2S_2$: C, 33.53; H, 4.61; N, 21.33). ESIMS: (+ve ion) [M + H$^+$] m/z 100% 394.03 (experimental), 394.03 (calcd). RP HPLC (System A): R_T = 9.879 min.

Diacetyl-4-hexanoic acid-4′-methyl*bis*(thiosemicarbazone), H_2L^3. Following the same procedure employed for the synthesis of H_2L^2, H_2L^1 (0.31 g, 1.1 mmol) and 6-aminohexanoic acid (0.30 g, 2.3 mmol) were used to prepare H_2L^3 (0.33 g, 81%). (Found: C, 43.33; H, 6.79; N, 23.29; calcd for $C_{13}H_{24}N_6O_2S_2$: C, 43.31; H, 6.71; N, 23.31). ^{1}H NMR (d_6 DMSO, 500 MHz): δ = 1.29, 2H, m, NH−CH_2−CH_2−CH_2; 1.55, 4H, m, NH−CH_2−CH_2, CH_2−CH_2−COOH; 2.20, 6H, s, CH_3; 2.21, 2H, t, $^3J_{HH}$ = 7.5 Hz, CH_2−COOH; 3.02, 3H, d, $^3J_{HH}$ = 5 Hz, NH−CH_3; 3.55, 2H, m, NH−CH_2; 8.37, 2H, m, NH−CH_2, NH−CH_3; 10.14, 1H, s, NH; 10.21, 1H, s, NH; 11.97, 1H, bs, OH. ^{13}C{^{1}H} NMR (125.7 MHz): δ = 11.7, CH_3; 24.2, NH−CH_2−CH_2; 25.9, NH−CH_2−CH_2−CH_2; 28.4, CH_2−CH_2−COOH; 31.2, NH−CH_3; 33.6, CH_2−COOH; 43.6, NH−CH_2; 147.9, C=N; 148.0, C=N; 174.4, C=O; 177.6, C=S; 178.5, C=S. ESIMS: (+ve ion) m/z 100% [M + H$^+$] 361.4 (experimental), 361.1 (calcd), (-ve ion) m/z [M − H$^+$] 359.4 (experimental), 359.1 (calcd).

Diacetyl-4-hexanoic acid-4′-methyl*bis*(thiosemicarbazonato)-copper(II), $Cu^{II}(L^3)$. Following the same procedure employed for the synthesis of $Cu^{II}(L^2)$, H_2L^3 (0.15 g, 0.4 mmol) and $Cu(OAc)_2 \cdot H_2O$ (0.08 g, 0.4 mmol) were used to prepare Cu^{II}-(L^3) (0.16 g, 88%). (Found: C, 36.94; H, 5.33; N, 20.00; calcd for $CuC_{13}H_{22}N_6O_2S_2$: C, 37.00; H, 5.25; N, 19.91). ESIMS: (+ve ion) [M + H$^+$] m/z 100% 422.06 (experimental), 422.06 (calcd). RP HPLC (System A): R_T = 11.023 min.

***tert*-Butyl 2-aminoethylcarbamate, 2.** The title compound was prepared according to the literature procedure method and was obtained as colorless oil (2.24 g, 97%).[23] ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.29, 2H, br s, NH_2; 1.43, 9H, s, $(CH_3)_3$; 2.78, 2H, t, $^3J_{HH}$ = 6 Hz, CH_2; 3.16, 2H, m, CH_2; 4.91, 1H, br s, NH.

Diacetyl *tert*-butyl 4-ethylcarbamate-4′-methyl*bis*(thiosemicarbazone), H_2L^4. To a stirring suspension of H_2L^1 (0.51 g, 1.9 mmol) in acetonitrile (8 mL) was added **2** (0.48 g, 3.0 mmol, 1.6 equiv) The resulting yellow suspension was heated at reflux for 3 h under an atmosphere of N_2 and was followed by TLC analysis (7.5% MeOH/CH_2Cl_2 v/v). Once the reaction was complete the resulting white suspension was cooled to room temperature and the white solid was collected by filtration, washed with acetonitrile (1×) and diethyl ether (3×), and dried to give H_2L^4 (0.59 g, 1.5 mmol, 81%). R_f (7.5% MeOH/CH_2Cl_2 v/v) 0.5. (Found: C, 43.08; H, 6.88; N, 25.02; calcd for $C_{14}H_{27}N_7O_2S_2$: C, 43.17; H, 6.99; N, 25.17). ^{1}H NMR (d_6-DMSO, 500 MHz): δ = 1.37, 9H, s, $(CH_3)_3$; 2.21, 3H, s, CH_3; 2.23, 3H, s, CH_3; 3.02, 3H, d, $^3J_{HH}$ = 4.5 Hz, CH_3−NH; 3.18, 2H, q, $^3J_{HH}$ = 5.5 Hz, CH_2NHC=O; 3.60, 2H, q, $^3J_{HH}$ = 5.5 Hz, NHCH_2; 6.99, 1H, t, $^3J_{HH}$ = 5 Hz, NHC=O; 8.37, 1H, q,

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$^3J_{HH}$ = 4.5 Hz, N*H*−CH_3; 8.44, 1H, t, $^3J_{HH}$ = 5 Hz, N*H*−CH_2; 10.24, 2H, br s, NH. $^{13}C\{^1H\}$ NMR (125.7 MHz): δ = 11.7, CH_3; 11.8, CH_3; 28.2, $(CH_3)_3$; 31.2, NH−CH_3; 39.1, *C*H_2NHC=O; δ 44.5, NHCH_2; 77.9, *C*$(CH_3)_3$; 147.8, C=N; 148.3, C=N; 156.2, C=O; 178.1, C=S; 178.5, C=S. ESIMS: (+ve ion) *m*/*z* 100% [M + H^+] 390.17 (experimental), 390.17 (calcd).

Diacetyl *tert*-butyl 4-ethylcarbamate-4′-methyl*bis*(thiosemicarbazonato)-copper(II), $Cu^{II}(L^4)$. To a suspension of H_2L^4 (0.08 g, 0.2 mmol) in ethanol (5 mL) was added Cu(OAc)·H_2O (0.04 g, 0.2 mmol). The red/brown solution was stirred at reflux for 4 h. The solvent was removed in vacuo and the brown residue was dissolved in dichloromethane (3 mL) and precipitated with hexane (30 mL). The solid was collected by filtration, washed with hexane, and dried to give $Cu^{II}(L^4)$ (0.08 g, 0.18 mmol, 0.88%). (Found: C, 37.30; H, 5.60; N, 21.65; calcd for $CuC_{14}H_{25}N_7O_2S_2$: C, 37.28; H, 5.59; N, 21.74). ESIMS: (+ve ion) [M + H^+] *m*/*z* 100% 451.09 (experimental), 451.08 (calcd). RP HPLC (System E): R_T = 8.002 min. Crystals suitable for single crystal X-ray crystallography were grown from a concentrated solution in acetone.

Diacetyl 4-ethyleneammonium-4′-methyl*bis*(thiosemicarbazone) trifluoroacetate, $[H_3L^5][CF_3CO_2]$. To a solution of trifluoroacetic acid (5 mL, 0 °C) cooled in an ice bath was added H_2L^4 (0.12 g, 0.3 mmol) in portions over 0.5 h under an atmosphere of nitrogen. The clear solution mixture was warmed to room temperature and stirred for 2 h. The solvent was removed to give yellow oil. Diethyl ether was added and a white solid precipitated, which was collected by filtration, washed with diethyl ether, and dried to give $[H_3L^5][CF_3CO_2]\cdot H_2O$ (0.096 g, 76%). (Found: C, 31.65; H, 4.86; N, 22.80; calcd for $C_{11}H_{20}F_3N_7O_2S_2.H_2O$: C, 31.35; H, 5.26; N, 23.26). ^1H NMR (d_6-DMSO, 500 MHz): δ = 2.22, 6H, s, CH_3; 3.02, 3H, d, $^3J_{HH}$ = 5.5 Hz, C*H*$_3$−NH; 3.05, 2H, t, $^3J_{HH}$ = 7.5 Hz, CH_2; 3.83, 2H, q, $^3J_{HH}$ = 7.5 Hz, CH_2; 7.83, 3H, br s, NH_3; 8.40, 1H, m, N*H*−CH_3; 8.48, 1H, t, $^3J_{HH}$ = 7.5 Hz, N*H*−CH_2; 10.25, 1H, br s, NH; 10.51, 1H, br s, NH. $^{13}C\{^1H\}$ NMR (125.7 MHz): δ = 11.8, CH_3; 11.9, CH_3; 31.2, NH−CH_3; 38.1, CH_2; δ 41.3, CH_2; 147.7, C=N; 148.9, C=N; 178.5, C=S. ESIMS: (+ve ion) *m*/*z* 100% [M^+] 290.12 (experimental), 290.12 (calcd). RP HPLC (System A): R_T = 7.497 min.

Diacetyl 4-ethyleneamino-4′-methyl*bis*(thiosemicarbazonato-copper(II) ditrifluoroacetate, $[Cu^{II}(H_2L^5)][CF_3CO_2]_2$. Following the same procedure employed for the synthesis of $[H_3L^5][CF_3CO_2]$, $Cu^{II}(L^4)$ (0.04 g, 0.09 mmol) was used to prepare $[Cu^{II}(H_2L^5)][CF_3CO_2]_2$ (0.04 g, 77%). (Found: C, 26.80; H, 3.38; N, 16.65; calcd for $C_{13}H_{19}CuF_6N_7O_4S_2$: C, 26.97; H, 3.31; N, 16.93). ESIMS: (+ve ion) [M + H^+] *m*/*z* 100% 351.04 (experimental), 351.04 (calcd), [M + 2H^+] *m*/*z* 100% 176.02 (experimental), 176.02 (calcd). RP HPLC (System A): R_T = 7.227 min.

Solid-Phase Bis(thiosemicarbazone)−Peptide Conjugation. General Procedure: Side-chain protected BBN(7−14)-NH_2 (H-Gln(Trt)-Trp(Boc)-Ala-Val-Gly-His(Trt)-Leu-Met-NH-resin) was assembled on PAL-PEG resin (loading 0.2 mmol/g) using standard Fmoc solid-phase peptide synthesis procedures.[24] A solution of *bis*(thiosemicarbazone) or *bis*(thiosemicarbazonato)−copper(II) complex (2−3 equiv) was preactivated with HATU (2−3 equiv) and DIPEA (4−6 equiv) in DMF (1 mL) and added to the resin in a sintered glass funnel. The mixture was allowed to react for 1 h, with occasional stirring. The reaction solution was drained and the resin was washed with DMF (3 × 5 mL) and DCM (3 × 5 mL). Resin and protecting group cleavage was performed in a 50 mL tube using a 10.5 mL solution of TIPS (2.5%), distilled water (2.5%), and TFA (95%), and shaken for 3 h. The solution was then filtered and sparged with a steady stream of N_2 to reduce the volume to 20%. Cold ether (40 mL) was then added to precipitate the peptide, which was then centrifuged for 4 min at 4000 rpm. The ether layer was decanted and the peptide was allowed to air-dry, after which it was solubilized in 50% acetonitrile/H_2O and lyophilized. The crude peptide material was purified as described above (system F).

H_2L^2-BBN(7−14)-NH_2. To BBN(7−14)-NH_2 on PAL-PEG resin (0.3 g) was added a solution of H_2L^2 (35 mg, 0.13 mmol), HATU (50.7 mg, 0.13 mmol), and DIPEA (66 μL, 0.39 mmol) in DMF (1 mL). ESIMS: (+ve ion) [M + H^+] *m*/*z* 1254.58 (experimental), 1254.58 (calcd), [M + 2H^+] *m*/*z* 627.79 (experimental), 627.79 (calcd). RP HPLC (System A): R_T = 12.518 min.

H_2L^3-BBN(7−14)-NH_2. To BBN(7−14)-NH_2 on PAL-PEG resin (0.24 g) was added a solution of H_2L^3 (36 mg, 0.1 mmol), HATU (39 mg, 0.1 mmol), and DIPEA (34 μL, 0.2 mmol) in DMF (1 mL). ESIMS: (+ve ion) [M + H^+] *m*/*z* 1282.61 (experimental), 1282.61 (calcd), [M + 2H^+] *m*/*z* 641.81 (experimental), 641.81 (calcd). RP HPLC (System B): R_T = 19.097 min.

$Cu^{II}(L^2$-BBN(7−14)-NH_2). To BBN(7−14)-NH_2 on PAL-PEG resin (0.24 g) was added a solution of $Cu^{II}(L^2)$ (28 mg, 0.07 mmol), HATU (28 mg, 0.07 mmol), and DIPEA (25 μL, 0.14 mmol) in DMF (1 mL). ESIMS: (+ve ion) [M + H^+] *m*/*z* 1315.50 (experimental), 1315.50 (calcd), [M + 2H^+] *m*/*z* 658.25 (experimental), 658.25 (calcd). RP HPLC (System D): R_T = 9.359 min.

$Cu^{II}(L^3$-BBN(7−14)-NH_2). To BBN(7−14)-NH_2 on PAL-PEG resin (0.24 g, 0.2 mmol/g) was added a solution of $Cu^{II}(L^3)$ (31.5 mg, 0.08 mmol), HATU (30 mg, 0.08 mmol), and DIPEA (35 μL, 0.2 mmol) in DMF (1 mL). ESIMS: (+ve ion) [M + H^+] *m*/*z* 1343.53 (experimental), 1343.53 (calcd), [M + 2H^+] *m*/*z* 672.27 (experimental), 672.27 (calcd). RP HPLC (System C): R_T = 13.858 min.

^{64}Cu Radiolabeling. $^{64}CuCl_2$ (1.88 GBq/mL, pH 1) was purchased from ANSTO radiopharmaceuticals and industrials (ARI), Lucas Heights, NSW, Australia. The radionuclidic purity at calibration {(^{64}Cu)/(^{67}Cu)} was 100% and the radiochemical purity as Cu(II) was 100%. The chemical purities of copper, zinc, and iron were 1.1, 0.9, and 10 μg/mL, respectively.

General Procedure. An aliquot of $^{64}CuCl_2$ (20 μL, ∼35 MBq, pH 1.0) was added to a solution containing the ligand (10 μL, 1 mg/mL DMSO), sodium acetate (90 μL, 0.1 M), and Milli-Q water (390 μL). The reaction was left for 30 min at room temperature before 100 μL of the reaction solution was injected onto a reverse-phase C18 analytical HPLC column. A DMSO solution of the "cold" copper complex (1 mg/mL) was injected (8 μL) under the same conditions (λ = 275 nm) to verify the identity of the radiolabeled complex.

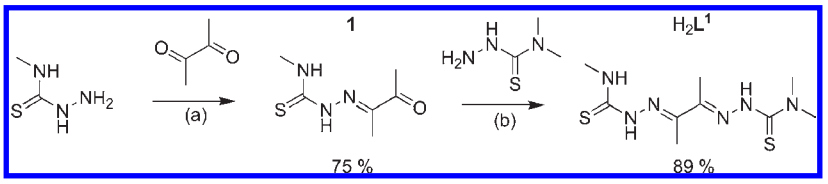
$^{64}Cu^{II}(L^3$-BBN(7−14)-NH_2): RP-HPLC (System C) R_T: 13.625 min and $Cu^{II}(L^3$-BBN(7−14)-NH_2), R_T: 13.858 min. $^{64}Cu^{II}(L^2$-BBN(7−14)-NH_2): RP-HPLC (System D) R_T: 9.377 min and $Cu^{II}(L^2$-BBN(7−14)-NH_2), R_T: 9.359 min. $^{64}Cu^{II}(L^3)$: RP-HPLC (System C) R_T: 13.278 min and $Cu^{II}(L^3)$, R_T: 13.197 min. $^{64}Cu^{II}(L^2)$: RP-HPLC (System C) R_T: 11.446 min and $Cu^{II}(L^2)$, R_T: 11.882 min. $^{64}Cu^{II}(L^5)$: RP-HPLC (System E) R_T: 7.251 min and $Cu^{II}(L^5)$, R_T: 6.601 min. $^{64}Cu^{II}(L^4)$: RP-HPLC (System E) R_T: 8.340 min and $Cu^{II}(L^4)$, R_T: 8.002 min.

Cysteine/Histidine Challenge. An aliquot (5 μL) of an aqueous solution of cysteine and histidine (5 mM) was added to a solution of $^{64}Cu^{II}(L^3$-BBN(7−14)-NH_2) (400 μL) and incubated at 37 °C. Aliquots (100 μL) of the mixture were injected onto the HPLC column at intermittent time periods (System C). At 2 h, R_T: 13.604 min; 4 h, R_T: 13.498 min; 6 h, R_T: 13.538 min.

X-ray Crystallography. Crystals of H_2L^1 and $Cu^{II}(L^4)\cdot(CH_3)_2CO$ respectively were mounted in low temperature oil then flash cooled to 130 K using an Oxford low temperature device. Intensity data were collected at 130 K with an Oxford XCalibur X-ray diffractometer with Sapphire CCD detector using Cu Kα radiation (graphite crystal monochromator λ = 1.54184 Å). Data were reduced and corrected for absorption.[25]

(24) Atherton, E.; Fox, H.; Harkiss, D.; Logan, C. J.; Sheppard, R. C.; Williams, B. J. *J. Chem. Soc., Chem. Commun.* **1978**, 537.

Scheme 1. Synthesis of diacetyl-*mono*-4-methyl-3-thiosemicarbazone (**1**) and H_2L^1 (a) distilled water, conc. HCl (36%), 5 °C; (b) DMF and acetic acid (glacial), 48 h.



The structures were solved by direct methods and difference Fourier synthesis using the SHELX suite of programs[26] as implemented within the WINGX software.[27] Thermal ellipsoid plots were generated using the program ORTEP-3 integrated within the WINGX suite of programs.

Results and Discussion

Synthesis. The dissymmetric *bis*(thiosemicarbazone) central to the selective transamination reactions (H_2L^1) was prepared by condensation of diacetyl-*mono*-4-methyl-3-thiosemicarbazone with 4,4-dimethyl-3-thiosemicarbazide and an acetic acid catalyst in DMF at room temperature (Scheme 1). The previously unreported compound was characterized by NMR spectroscopy, mass spectrometry, and microanalysis. The dissymmetry of H_2L^1 is evident in both the ^1H and $^{13}C\{^1H\}$ NMR spectra with the protons (δ = 2.15 and 2.19 ppm) and carbons (δ = 11.1 and 11.4 ppm) of the diimine backbone methyl groups having different chemical shifts respectively. The carbon atoms of each imine are also distinguishable (δ = 148.0 and 149.4) as are the thiocarbonyl carbons (δ = 178.5 and 181.7 ppm). Crystals of H_2L^1 suitable for X-ray crystal structure determination were grown from a concentrated solution of the ligand in dimethyl sulphoxide.

An ORTEP-3 representation of the X-ray crystal structure of H_2L^1 is shown in Figure 1. The structure shows that in the solid state the molecule adopts an (*E*, *E*)-configuration about the imine double bonds and an *s-trans* (antiperiplanar) conformation about the C(5)−C(4) bond much like the ligand H_2atsm.[28] There are small differences in the bond lengths between the thiosemicarbazone arms. The arm bearing a dimethyl substituent has a slightly shorter C(3)−S(1) (1.6802(19) Å) bond length and a longer C(3)−N(1) (1.341(2) Å) bond length than the arm with a single methyl substituent (1.693(2) and 1.323(3) Å, respectively). The bond lengths indicate that there is extensive delocalization throughout the molecule but the tautomeric form shown in Figure 1 dominates.

A transamination reaction has been reported previously with N^4,N^4-disubstituted α-(N)-heterocyclic thiosemicarbazones[29,30] but this is the first time this reaction has been applied to a molecule that is a *bis*(thiosemicarbazone) or contains two different thiosemicarbazone

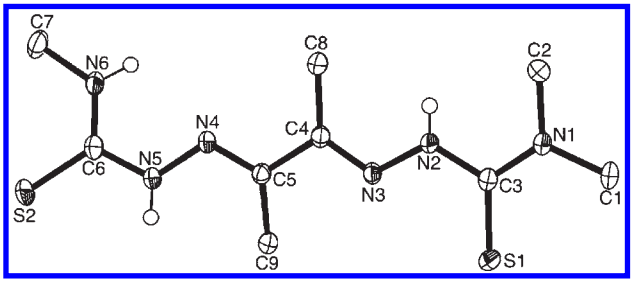


Figure 1. ORTEP-3 representation of H_2L^1. Hydrogen atoms are omitted for clarity (except hydrogen atoms bound to nitrogen). Ellipsoids are at the 50% level.

substituents. The reaction of H_2L^1 with nucleophilic amines resulted in the selective displacement of dimethylamine from the dimethyl substituted moiety of the *bis*-(thiosemicarbazone). This reaction occurs under mild conditions exclusively on the N^4,N^4-dimethyl thiosemicarbazone moiety as it contains a more electrophilic thiocarbonyl carbon atom than the N^4-monosubstituted moiety, which undergoes tautomerization.[29] The leaving group, which in the present cases is the secondary amine dimethylamine, is a weak base and is readily removed as a gas from the reaction mixture. The basicity and steric bulk of the amine nucleophile is also crucial for successful transamination.

The focus was to prepare new *bis*(thiosemicarbazone) derivatives with pendant functional groups that were appropriate for attaching a wide range of peptide based targeting groups. Consequently we focused on the preparation of substituents bearing a reactive carboxylic acid functional group for conjugating peptides through their N terminus or amine functional groups for conjugating peptides through their C terminus.

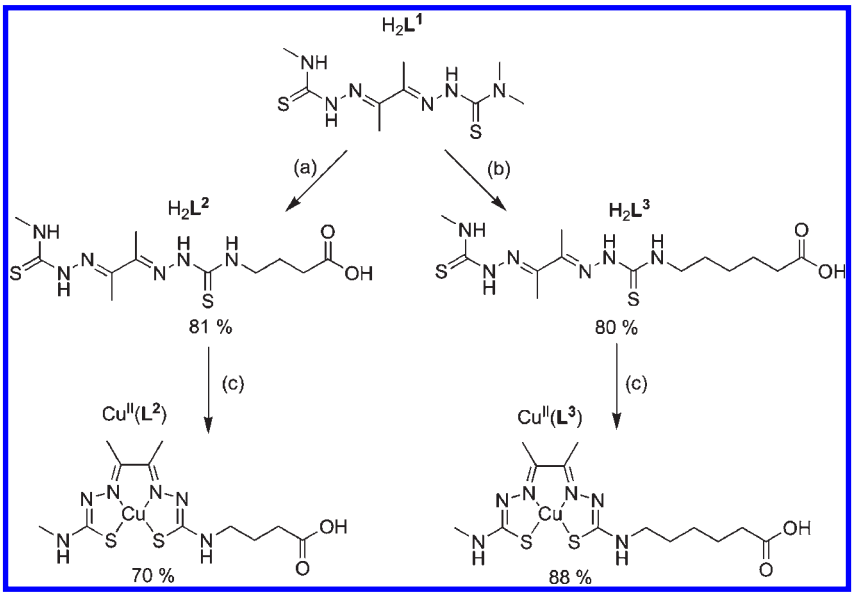
The reaction of H_2L^1 with either 4-aminobutyric acid or 6-aminohexanoic acid allows the isolation of new ligands with alkyl carboxylic acid substituents (H_2L^2 and H_2L^3 respectively, Scheme 2) in high yield.

The ^1H and $^{13}C\{^1H\}$ NMR spectra were assigned using hsqc and cosy experiments and indicated successful transamination due to the loss of the signals attributed to the dimethylamino functional group of H_2L^1. The ^1H NMR spectrum of H_2L^2 contains two multiplets (δ = 1.81 and 3.58 ppm) and a triplet (δ = 2.25 ppm) due to the three CH_2 groups. The spectrum also contains a quartet (δ = 8.38 ppm) and a triplet (δ = 8.44 ppm) due to the thioamide NH groups while the $^{13}C\{^1H\}$ spectrum indicated the presence of the carbonyl carbon (δ = 174.2 ppm). The ESIMS spectra (positive ion) gave a peak at m/z = 333.4 and 361.4 corresponding to the protonated species $[H_2L^2 + H^+]$ and $[H_2L^3 + H^+]$, respectively.

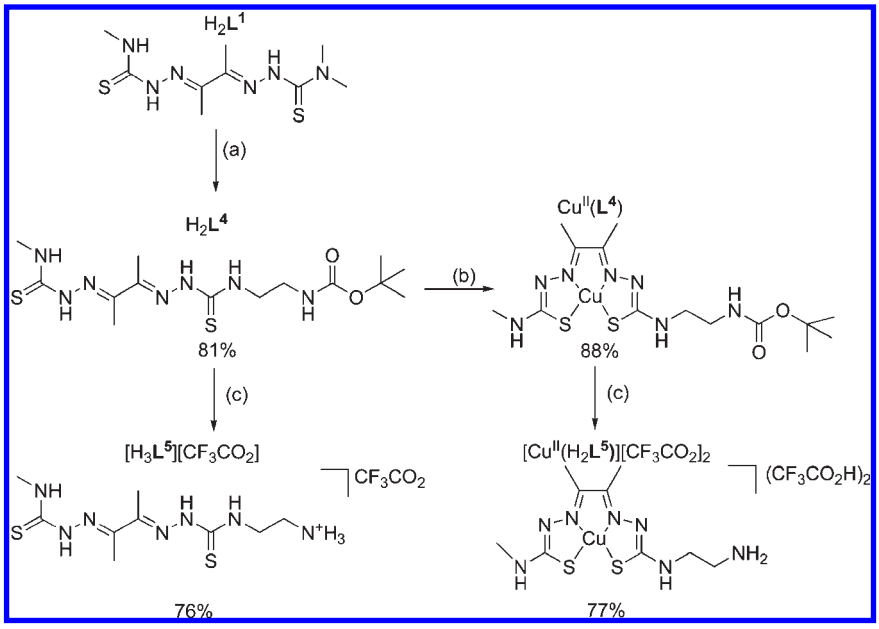
The copper complexes $Cu^{II}(L^2)$ and $Cu^{II}(L^3)$ were prepared by addition of copper acetate monohydrate to the ligand in DMF at room temperature (Scheme 2). The

(25) CrysAlis CCD, Oxford Diffraction Ltd., Version 1.171.32.5 (release 08-05-2007 CrysAlis171 .NET)(compiled May 8, 2007, 13:10:02).
(26) *SHELX97* [*Includes SHELXS97, SHELXL97*] − *Programs for Crystal Structure Analysis* (*Release 97−2*). Sheldrick, G. M. Institut fur Anorganische Chemie der Universitat, Tammanstrasse 4, D-3400 Gottingen, Germany, 1998.
(27) Farrugia, L. J. *J. Appl. Crystallogr.* **1999**, *32*, 837.
(28) Blower, P. J.; Castle, T. C.; Cowley, A. R.; Dilworth, J. R.; Donnelly, P. S.; Labisbal, E.; Sowrey, F. E.; Teat, S. J.; Went, M. J. *Dalton Trans.* **2003**, 4416.
(29) Klayman, D. L.; Lin, A. J. *Org. Prep. Proced. Int.* **1984**, *16*, 79.
(30) Scovill, J. P. *Phosphorus, Sulfur Silicon Relat. Elem.* **1991**, *60*, 15.

Scheme 2. Synthesis of H_2L^2, H_2L^3, and the neutral copper complexes $Cu^{II}(L^2)$ and $Cu^{II}(L^3)$: (a) 4-aminobutyric acid (2 equiv), acetonitrile, reflux, 34 h; (b) 6-aminohexanoic acid (2 equiv), acetonitrile, reflux, 34 h; (c) $Cu(OAc)_2 \cdot H_2O$ (1 equiv), DMF, rt.



Scheme 3. Synthesis of H_2L^4 and $Cu^{II}(L^4)$ and the deprotected compounds $[H_3L^5][CF_3CO_2]$ and $[Cu^{II}(H_2L^5)][CF_3CO_2]_2$: (a) *tert*-butyl 2-aminoethylcarbamate (1.6 equiv), acetonitrile, reflux, 3 h; (b) $Cu(OAc)_2 \cdot H_2O$ (1 equiv), ethanol, reflux, 4 h; (c) TFA, 0 °C to rt, diethyl ether.



ESIMS spectra (positive ion) for $Cu^{II}(L^2)$ and $Cu^{II}(L^3)$ possess an isotopic pattern corresponding to $[Cu^{II}(L^2 + H^+)]$ at m/z = 394.03 and $[Cu^{II}(L^3 + H^+)]$ at m/z = 422.06, respectively. Purity was confirmed by reverse-phase HPLC and microanalysis.

The transamination of H_2L^1 with *t*-Boc-protected amine *tert*-butyl 2-aminoethylcarbamate produced H_2L^4 (Scheme 3). The ^1H NMR spectrum showed the resonances corresponding to two CH_2 (δ = 3.18 and 3.60 ppm) as well as a singlet indicating the presence of the nine protons of the *tert*-butyl substituent at δ = 1.37 ppm. The enhanced solubility of H_2L^4 allowed for complexation of Cu(II) in ethanol. The resulting complex $Cu^{II}(L^4)$ was recrystallized from dichloromethane/hexane.

Crystals of $Cu^{II}(L^4)$ suitable for X-ray crystal structure determination were grown from a concentrated solution of the complex in acetone. An ORTEP-3 representation of the X-ray crystal structure of $Cu^{II}(L^4)$ is shown in Figure 2. The Cu atom is four coordinate and sits 0.052 Å out of the plane of the N_2S_2 square planar donor system. The Cu−N and Cu−S bond distances are similar to Cu^{II}(atsm) (Table 1).[28] The distortion from ideal square planar geometry is highlighted by the bond angle S(1)−Cu(1)−S(2) of 109.23(4)°.

The ligand is dianionic as shown by the increase in the C−S bond distances (1.763(4) and 1.759(3) Å) compared to the neutral proligand H_2L^1 (1.6802(19) and 1.693(2) Å). The ligand adopts an s-*cis* (synperiplanar) conformation about the C(3)−C(4) bond upon complexation. The flexibility of the side chain is demonstrated by the dihedral angle defined by the atoms N(6)−C(6)−C(7)−N(7) of 60.02°. This angle is influenced by the packing and

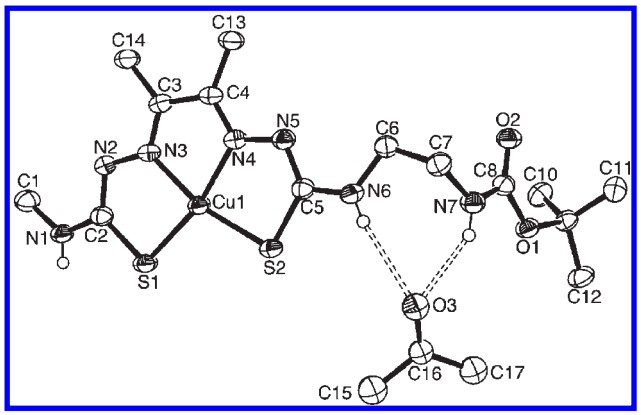


Figure 2. ORTEP-3 representation of $Cu^{II}(\mathbf{L^4})$. Hydrogen atoms are omitted for clarity (except hydrogen atoms bound to nitrogen). Ellipsoids are at the 50% level.

Table 1. Selected Bond Distances (Å) and Angles (°) in $Cu^{II}(\mathbf{L^4})\cdot(CH_3)_2CO$

$Cu^{II}(\mathbf{L^4})\cdot(CH_3)_2CO$			
Cu1−N3	1.965(3)	S1−Cu1−S2	109.23(4)
Cu1−N4	1.965(3)	N3−Cu1−N4	80.67(12)
Cu1−S1	2.2396(10)	N3−Cu1−S1	84.81(8)
Cu1−S2	2.2517(10)	N4−Cu1−S2	85.16(9)

hydrogen bonding to a solvent molecule of acetone and a second molecule of $Cu^{II}(\mathbf{L^4})$. One of the NH groups forms a hydrogen bond to the carbamate O atom of a second molecule of the Cu complex (N(1)···O(2)′ is 2.876 Å, N(1)−H(1)···O(2)′ 165.34°, symmetry operator $x + 1$, $y, -1$). The other NH groups form hydrogen bonds to the O atom of the molecule of solvent acetone. The stronger of the hydrogen bonds involves the carbamate NH group (N(7)···O(3) 2.866 Å, N(7)−H(7)···O(3) 150.28°) while the weaker hydrogen bond involves the thioamide NH group (N(6)···O(3) 3.166 Å, N(6)−H(6)···O(3) 164.03°).

The compound featuring an ammonium ion $[H_3\mathbf{L^5}][CF_3CO_2]$ was synthesized by deprotection of $H_2\mathbf{L^4}$ with trifluoroacetic acid (Scheme 3). The compound was analyzed by NMR, ESIMS, reverse-phase HPLC, and microanalysis. Loss of the signals for the *tert*-butyl functional group in the ^1H and $^{13}C\{^1H\}$ NMR spectra of $[H_3\mathbf{L^5}][CF_3CO_2]$ indicated successful deprotection. The ^1H NMR spectrum of $[H_3\mathbf{L^5}][CF_3CO_2]$ contained a broad singlet centered at $\delta = 7.83$ ppm attributed to the ammonium group. The trifluoroacetate counterion of $[H_3\mathbf{L^5}][CF_3CO_2]$ was present in the ^{19}F NMR spectrum at $\delta = -75.8$ ppm. Crystallographic data are provided in Table 2.

The copper complex $[Cu^{II}(H_2\mathbf{L^5})][CF_3CO_2]_2$ was prepared by deprotection of $Cu^{II}(\mathbf{L^4})$ with trifluoroacetic acid (Scheme 3). The ESIMS spectrum (positive ion) possesses an isotopic pattern corresponding to $[Cu^{II}(\mathbf{L^5} + H^+)]$ at $m/z = 351.04$ and $[Cu^{II}(\mathbf{L^5} + 2H^+)]$ at $m/z = 176.02$, respectively. Microanalysis indicated the presence of two anions of trifluoroacetate, which strongly suggests that the isolated complex is a doubly protonated dication. The position of one of the protons is likely to be on the terminal amine similar to $[H_3\mathbf{L^5}][CF_3CO_2]$ with the other proton on one of the hydrazinic nitrogen atoms similar to a cationic thiosemicarbazone−pyridylhydrazine Cu(II) complex.[31]

Table 2. Crystallographic Data

crystal identification	$H_2\mathbf{L^1}$	$Cu^{II}(\mathbf{L^4})\cdot(CH_3)_2CO$
chemical formula	$C_9H_{18}N_6S_2$	$C_{17}H_{31}CuN_7O_3S_2$
M	274.41	509.15
crystal system	monoclinic	triclinic
space group	P 2_1/c	P$\bar{1}$
a/Å	15.4720(2)	9.0904(6)
b/Å	6.81020(10)	11.5022(9)
c/Å	13.66470(10)	12.4161(9)
α/°	90.00	78.916(6)
β/°	109.1160(10)	72.470(6)
γ/°	90.00	85.134(6)
V/Å^3	1360.42(3)	1214.34(15)
Z	4	4
independent reflections	2679	4699
R_{int}	0.0298	0.0402
R ($I > 2s(I)$)	0.0400	0.0488
wR (all data)	0.1124	0.1502

Electrochemistry. Recent studies have shown that in vivo stability of bifunctional chelators of copper is not only dependent on the thermodynamic stability and kinetic inertness of the complex but also the susceptibility to reduction of Cu(II) to Cu(I).[32] Intracellular reductants with thiols, such as cysteine-rich metallothioneins, may act as effective reductants of Cu(II) and scavengers of the labile Cu(I) ions. Reduction potentials have been hypothesized to be a good way of predicting in vivo stability where complexes that are harder to reduce are more stable.[17,33] The stability of the resulting Cu(I) ion upon reduction has been shown to be influential in the hypoxia selectivity exhibited by the *bis*(thiosemicarbazonato)−copper(II) complex Cu^{II}(atsm). It is proposed that this neutral complex can cross cell membranes and is retained selectively in cells experiencing hypoxia but is able to be "washed out" of cells under normal oxygen conditions.[5,34] The alkyl substituents on the diimine backbone seem to be of most importance regarding redox potentials whereas substitution at the N^4-terminus does not influence redox potentials significantly.[35] The complex Cu^{II}(atsm) undergoes a quasi-reversible reduction at $E_{1/2} = -0.63$ V (vs SCE, where $E_{1/2} = [E_{pc} + E_{pa}]/2$ and $Fc/Fc^+ = 0.54$ V) in anhydrous DMF at a glassy carbon working electrode. Cyclic voltammetry of the new Cu(II) complexes indicated that the structural change at the N^4-terminus has not altered the electrochemistry of the parent complex Cu^{II}(atsm) significantly (Table 3).

$Cu^{II}(\mathbf{L^3})$ for example, in DMF at a glassy carbon electrode, has a quasi-reversible reduction at $E_{1/2} = -0.63$ V with an anodic to cathodic peak separation of 101 mV which was attributed to a Cu(II)/Cu(I) reduction process (Figure 3). Under the same conditions the ferrocene/ferrocinium couple has a peak separation of 104 mV. There was also a quasi-reversible process at $E_{1/2} = 0.74$ V (vs SCE) with an anodic to cathodic peak separation of 101 mV, which is tentatively attributed to a Cu(II)/Cu(III) process.

The isolated dicationic complex $[Cu^{II}(H_2\mathbf{L^5})]^{2+}$ exhibits an electrochemically irreversible reduction, however

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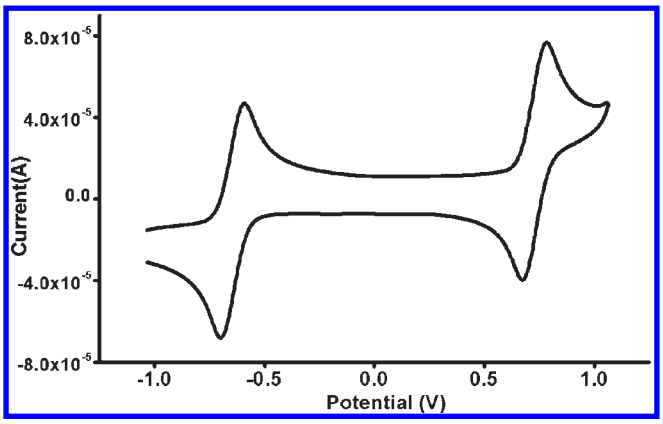

Figure 3. Cyclic voltammogram of $Cu^{II}(\mathbf{L^3})$. Scan rate 0.1 V s^{-1}. Potentials are quoted relative to a SCE.

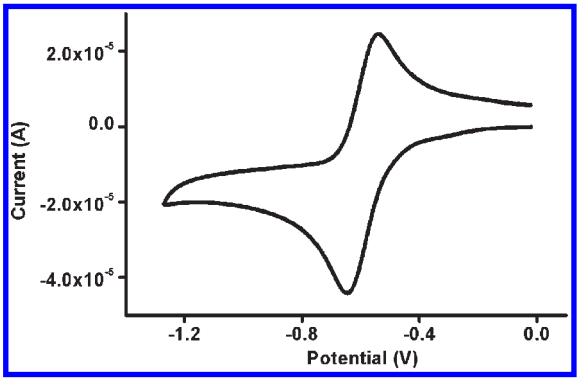

Figure 4. Cyclic voltammogram of $Cu^{II}(\mathbf{L^5})$ with triethylamine. Scan rate 0.1 V s^{-1}. Potentials are quoted relative to a SCE.

Table 3. Table of Half-Wave Potentials and Peak Separations of the Cyclic Voltammograms (Scan Rate 0.1 V s^{-1}; Potentials Are Quoted Relative to a SCE)

compound	Cu^{II}/Cu^{I} $E_{1/2}$ (V)	Cu^{II}/Cu^{I} $E_{pa} - E_{pc}$ (mV)	Cu^{III}/Cu^{II} $E_{1/2}$ (V)	Cu^{III}/Cu^{II} $E_{pa}-E_{pc}$ (mV)
Cu^{II}(atsm)	−0.63	102	0.75	94
$Cu^{II}(\mathbf{L^2})$	−0.63	99	0.75	104
$Cu^{II}(\mathbf{L^3})$	−0.63	101	0.74	101
$Cu^{II}(\mathbf{L^4})$	−0.61	113	0.76	113
$Cu^{II}(\mathbf{L^5})$	−0.58	101	0.78	91

the addition of triethylamine to the analyte solution to neutralize the complex to give $Cu^{II}(\mathbf{L^5})$ restores the expected electrochemistry (Figure 4). Protonation significantly alters the reduction potential of the Cu(II) complex, which has also been observed with a cationic thiosemicarbazone−pyridylhydrazine Cu(II) complex.[31] As in the present case deprotonation of that complex with a base restored the quasi-reversibility of the Cu(II)/Cu(I) reduction process.

Radiolabeling with Copper-64. Incorporation of a spacer such as 4-aminobutyric acid or 6-aminohexanoic acid between the coordination complex and the bioactive molecule is preferable to avoid compromising biological activity. The linker can also affect the biodistribution of the complex. Longer alkyl linkers can impart added lipophilicity resulting in higher liver uptake, although countering this with shorter linkers can reduce target-tumor binding due presumably to steric hindrance.[36]

(36) Parry, J. J.; Andrews, R.; Rogers, B. E. *Breast Cancer Res. Treat.* **2007**, *101*, 175.

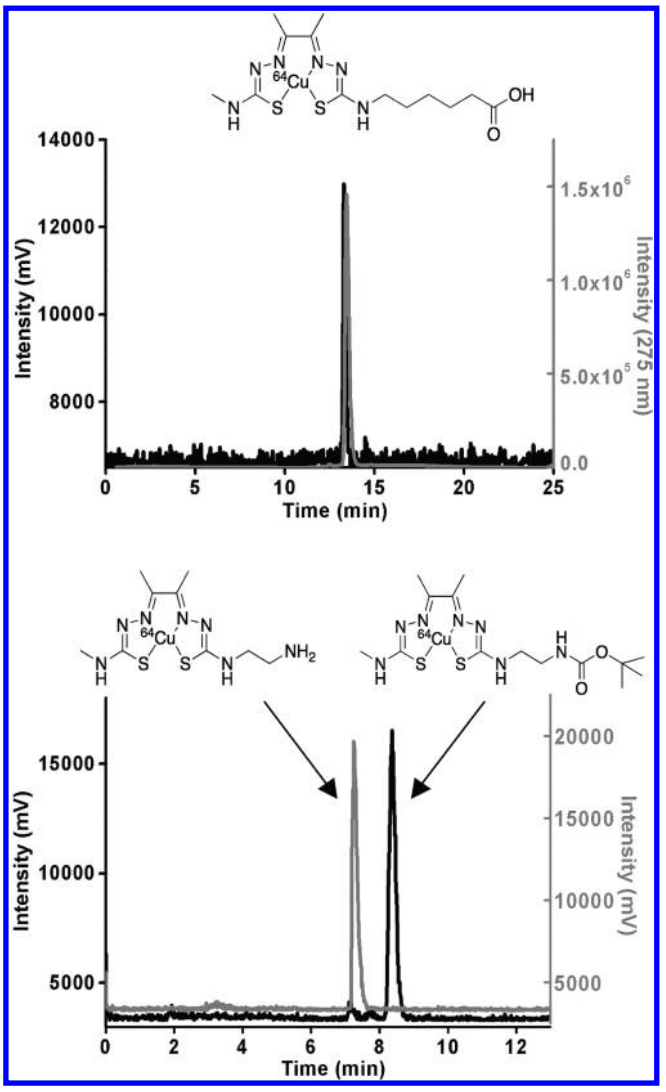

Figure 5. (Top) Radio-HPLC of $^{64}Cu^{II}(\mathbf{L^3})$ (System C, R_T: 13.278 min, black) compared with "cold" $Cu^{II}(\mathbf{L^3})$ with UV detection at 275 nm (R_T: 13.197 min, gray). The small difference in retention times reflects the detector configurations. (Bottom) Radio-HPLC of $^{64}Cu^{II}(\mathbf{L^5})$ (System E, R_T: 7.251 min, gray) and $^{64}Cu^{II}(\mathbf{L^4})$ (R_T: 8.340 min, black).

As derivatives of Cu^{II}(atsm), a known hypoxia imaging agent, the new bifunctional chelators are of interest in their own right as potential imaging agents. All the radiolabeled complexes could be prepared at room temperature under mild conditions with >95% radiochemical purity making them ideal candidates for in vivo imaging. The proligands $H_2\mathbf{L^2}$ and $H_2\mathbf{L^3}$ were radiolabeled with copper-64 with the RP-HPLC traces highlighting the difference in lipophilicity between the two complexes (System C, 11.446 and 13.278 min, respectively). Figure 5 shows the excellent agreement between the retention time of $^{64}Cu^{II}(\mathbf{L^3})$ and its nonradioactive analogue $Cu^{II}(\mathbf{L^3})$ (R_T: 13.197 min) under the same conditions.

$H_2\mathbf{L^4}$ and $[H_3\mathbf{L^5}][CF_3CO_2]$ were also radiolabeled and the retention times (System E, 8.340 and 7.251 min, respectively) demonstrate the increase in hydrophilicity imparted by the terminal amine group compared to the *t*-Boc group. This difference is likely to be reflected in the biodistribution of the compounds while retaining hypoxia selectivity and will be subject to further investigation.

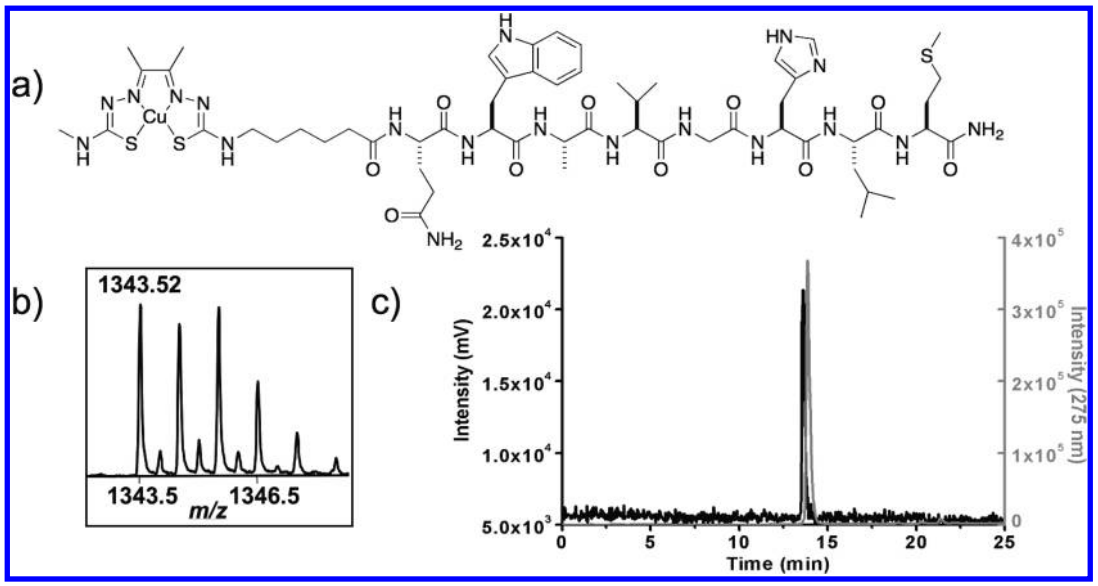

Figure 6. (a) Structure of $Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$; (b) ESIMS of $[Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2 + H^+)]$; (c) radio-HPLC of $^{64}Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ (R_T 13.625 min, black) compared with "cold" $Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ with UV detection at 275 nm (R_T 13.858 min, gray). The small difference in retention times reflects the detector configurations.

Peptide Conjugation. Conjugating the new derivatives to BBN(7−14)-NH_2 at the N terminus is crucial as the C terminus is necessary for high affinity of the peptide for the GRP receptor.[9] The carboxylic acid containing ligands $H_2\mathbf{L^2}$ and $H_2\mathbf{L^3}$ and their Cu(II) complexes were conjugated to side-chain protected BBN(7−14)-NH_2 on PAL-PEG resin using standard Fmoc solid-phase peptide chemistry to give the peptide conjugates $H_2\mathbf{L^2}$-BBN(7−14)-NH_2, $Cu^{II}(\mathbf{L^2}$-BBN(7−14)-$NH_2)$, $H_2\mathbf{L^3}$-BBN(7−14)-NH_2, and $Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ (Figure 6a). Conjugation was achieved at room temperature over 1 h. Cleavage from the resin and protecting groups was performed using a solution of TIPS/H_2O/TFA (2.5/2/5/95%) with shaking for 3 h. The Cu(II) complexes appear stable to the acidic conditions. The crude conjugates were purified using semipreparative HPLC (System F) and were characterized using ESIMS. For example, the ESIMS spectra of $Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ contained peaks corresponding to $[M + H^+]$ (1343.52, Figure 6b) and $[M + 2H^+]$ (672.27) that matched the calculated values.

Radiolabeling Conjugates with Copper-64. The *bis*-(thiosemicarbazone)-BBN(7−14)-NH_2 conjugates were readily radiolabeled with copper-64 at room temperature in aqueous sodium acetate buffer with a radiochemical purity of >95% as determined by radio-HPLC. The ability to radiolabel under these mild conditions and ambient temperatures is important for certain targeting peptides that are susceptible to elevated temperatures. This represents potential advantages over other systems that require heating to 55−75 °C to achieve complexation in high radiochemical yields.[37] Further studies are required to determine if quantitative labeling is still achieved at room temperature with lower concentrations of peptide. Product purity and identity were confirmed by comparing retention times with their nonradioactive analogues using sequential radio and UV reverse phase HPLC. The radio-HPLC trace of $^{64}Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ has a retention time of 13.625 min (System C) compared with its nonradioactive analogue $Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ which has a retention time of 13.858 min (Figure 6c). The small difference in retention times reflects the detector configuration.

Serum stability of $^{64}Cu^{II}(\mathbf{L^3}$-BBN(7−14)-$NH_2)$ was predicted from a cysteine/histidine challenge. Incubation with an excess of cysteine and histidine in aqueous sodium acetate buffer at 37 °C for 6 h indicated no significant losses of copper-64 from the conjugate. The cysteine/histidine challenge and the electrochemistry suggest that the complexes display the same high affinity for Cu(II) and resistance to reductively assisted transmetalation that has given Cu^{II}(atsm) sufficient stability for diagnostic applications.[38]

Conclusion

The transamination method for preparing new *bis*-(thiosemicarbazone) proligands is extremely versatile and has been used to prepare a wide range of derivatives that will be published in the future. The versatility of the reaction allows for the ready manipulation of the solubility, lipophilicity, and ultimately the biodistribution of *bis*(thiosemicarbazonato) metal complexes and as such offers considerable promise in the development of new pharmaceuticals. The present *bis*(thiosemicarbazone) compounds have ideal properties for targeting membrane-bound peptide receptors such as the GRP-R as they can be radiolabeled with copper-64 quickly in high radiochemical purity at room temperature under mild conditions, thus ensuring the integrity of the bioactive peptide is not compromised. It is expected that these new derivatives will retain the extracellular stability of the hypoxia selective diagnostic agent Cu^{II}(atsm) as the dimethyl backbone has been retained and is supported by electrochemical and cysteine/histidine challenge experiments.

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Supporting Information Available: X-ray crystallographic data in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.