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Macrobicyclic Cage Amine Ligands for Copper Radiopharmaceuticals: A Single Bivalent Cage Amine Containing Two Lys^3-bombesin Targeting Peptides

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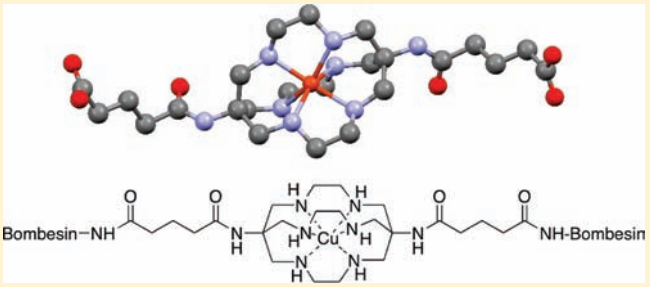
ABSTRACT: The synthesis of new cage amine macrobicyclic ligands with pendent carboxylate functional groups designed for application in copper radiopharmaceuticals is described. Reaction of $[Cu((NH_2)_2sar)]^{2+}$ (sar = 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane) with either succinic or glutaric anhydride results in selective acylation of the primary amine atoms of $[Cu((NH_2)_2sar)]^{2+}$ to give derivatives with either one or two aliphatic carboxylate functional groups separated from the cage amine framework by either a four- or five-atom linker. The Cu^{II} serves to protect the secondary amine nitrogen atoms from acylation, and can be removed to give the free ligands. The newly appended carboxylate functional groups can be used as sites of attachment for cancer-targeting peptides such as Lys^3-bombesin. The synthesis of the first dimeric sarcophagine-peptide conjugate, possessing two Lys^3-bombesin peptides tethered to a single cage amine, is presented. This species has been radiolabeled with copper-64 at ambient temperature and there is minimal dissociation of Cu^{II} from the conjugate even after two days of incubation in human serum.



INTRODUCTION

Positron emission tomography (PET) is an imaging technique that can provide noninvasive diagnostic information as a positron-emitting tracer 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 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. This is possible by coordinating $^{64}Cu^{II}$ to a bifunctional chelator that is conjugated to a targeting molecule.[3−5]

A wide range of tetraazamacrocycles based on the cyclam and cyclen frameworks, such as their tetracarboxymethylated derivatives TETA (1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (Figure 1), have been conjugated to targeting peptides and used to coordinate copper radioisotopes but certain derivatives suffer from dissociation of Cu from the chelate. This is a consequence of transfer of the copper from the macrocycle to biological ligands such as copper transport proteins either as Cu^{II} or following in vivo reduction to Cu^{I}.[6−12] Improved in vivo behavior is encountered for copper complexes of systems based on cross-bridged cyclam (CB-TE2A, 4,11-*bis*(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane) and triazacyclononane derivatives, such as NOTA (Figure 1).[13−21]

An alternative ligand framework is the hexaaminemacrobicyclic cage amine family of ligands, known by their trivial name of sarcophagines. The encapsulating nature of sarcophagines results in the formation of complexes with Cu^{II} that are remarkably thermodynamically stable and kinetically inert.[22] These ligands are capable of quantitatively binding Cu^{II} with fast complexation kinetics at remarkably low concentrations at room temperature over a pH range of 4−9.[23,24] These features make sarcophagine ligands particularly well suited for copper radiopharmaceutical applications. The development of cage amine ligands as bifunctional chelators relies on the ability to readily functionalize the ligand to incorporate targeting molecules without compromising the stability of the Cu^{II} chelate or the biological activity of the targeting molecule. Direct coupling reactions of the primary amines of the cage amine 'diaminosarcophagine', 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo-[6.6.6]icosane, $(NH_2)_2$sar (Figure 1), with peptides using standard coupling procedures are relatively inefficient, although a $\alpha_v\beta_3$ integrin-targeting molecule incorporating $(NH_2)_2$sar has been synthesized[25] as has the bioconjugate of $[Co((NH_2)_2sar)]^{3+}$ with

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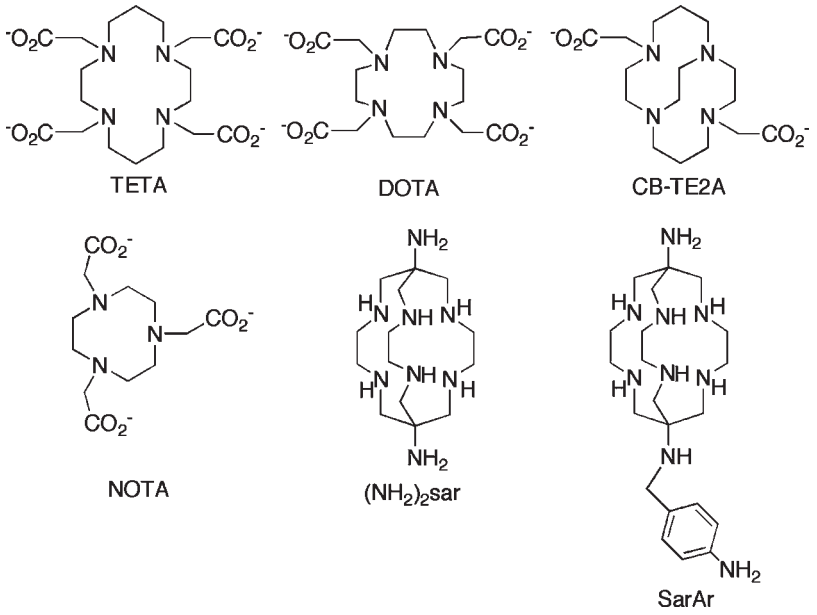

Figure 1. Chelators used to complex copper-64.

horse heart cytochrome C.[26] Pioneering work directed toward increasing the efficiency of coupling reactions between cage amine ligands and biomolecules has focused on the incorporation of an aromatic amine to produce SarAr (Figure 1).[24,27] The pendent aromatic amine can be used in conjugation reactions with the carboxylate residues of antibodies.[28] Alternatively, $(NH_2)_2$sar can be functionalized with carboxylate groups and subsequently attached to peptides or antibodies with similar coupling chemistry to that used to conjugate peptides to DOTA and TETA. This is of particular importance when the C-terminus is crucial to biological activity.

Functionalization of $(NH_2)_2$sar is complicated by the presence of eight amine groups: two primary and six secondary. Metal ion coordination is a useful method to effectively "protect" the six secondary amine groups and allows essentially selective reactions with the terminal primary amine groups of $(NH_2)_2$sar.[29−33] In this work copper ion coordination is used to "protect" the secondary amine atoms of $(NH_2)_2$sar from reaction with succinic anhydride and glutaric anhydride. This reaction results in selective acylation reactions on the primary amine atoms to give derivatives with either one or two aliphatic carboxylate functional groups separated from the cage amine framework by either a four or five atom linker. The metal ion can be removed from the ligands to give the metal-free ligands. The new derivatives with aliphatic linkers tethered to a carboxylate functional group are complementary to systems with aromatic carboxylate functional groups.[34,35] The conjugation of the bifunctional chelators with a single carboxylate functional group to representative cancer targeting peptides Tyr^3-octreotate and Lys^3-bombesin was reported in a preliminary communication.[36]

The following work describes the synthesis and isolation of ligands with two carboxylate functional groups that allow the synthesis of a system that bears two tumor-targeting peptides. There is growing interest in "multimeric" radiotracer constructs for PET imaging, as the incorporation of multiple targeting groups can impart higher affinity and selectivity.[13,17,37−39] A "polyvalent effect" that enhances receptor binding is achieved with multimeric constructs that possess two or more targeting peptides that are capable of simultaneously binding to more than one receptor on the surface on any given cell. Most studies demonstrating the benefits of "multimeric" peptides have focused on cyclic RGD-peptides that bind to $\alpha_v\beta_3$ integrins. Tetrameric and dimeric RGD-peptide motifs have a higher binding affinity than their monomeric counterparts.[40−42] This report outlines the synthesis of a dimeric Lys^3-bombesin sarcophagine ligand with two targeting peptides conjugated to a single cage amine ligand. The fourteen amino acid peptide Lys^3-bombesin targets the gastrin-releasing peptide receptor, which is overexpressed in human cancers including prostate,[43] breast[44] and small cell lung cancers.[45] Radiolabeled analogues of bombesin have displayed high tumor uptake in animal models and have potential for both diagnostic and therapeutic applications and the new derivatives described here have the potential to benefit from bivalent binding.

EXPERIMENTAL SECTION

Instrumentation. Mass spectra were recorded in the positive ion mode on an Agilent 6510 Q-TOF LC/MS Mass Spectrometer coupled to an Agilent 1100 LC system (Agilent, Palo Alto, CA). Data were acquired and reference mass corrected via a dual-spray electrospray ionization source, using the factory-defined calibration procedure. Each scan or data point on the Total Ion Chromatogram is an average of 9652 transients, producing 1.02 scans s^{-1}. Spectra were created by averaging the scans across each peak. Mass spectrometer conditions: fragmentor, 200−300 V; drying gas flow, 7 L/min; nebulizer, 30 psi; drying gas temp, 325 °C; V_{cap}, 4000 V; skimmer, 65 V; OCT R_fV, 750 V; scan range acquired, 150−3000 m/z.

HPLC-MS traces were recorded using an Agilent Poroshell 300SB-C18 column (5 μm, 2.1 × 75 mm) coupled to the Agilent 6510 Q-TOF LC/MS Mass Spectrometer described above. Aliquots of each sample (3−10 μL) were injected onto the column using the Agilent 1100 LC system, with a flow rate of 0.25 mL/min. Data acquisition parameters are the same as those described above for mass spectra.

Semipreparative HPLC purifications were performed using an Agilent 1200 Series HPLC system. Solvent gradients and column specifications are described below. An automated Agilent 1200 fraction collector collected 2.5 mL fractions and fraction collection was time based. Each

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Table 1. Crystallographic Data

	$[CuL^2](CF_3SO_3)Cl$	$[CuHL^3](NO_3)_3$	$[CuL^4\text{-}2H^+]\cdot 7H_2O$
formula	$CuC_{25}H_{44}N_8O_9SF_3Cl$	$CuC_{18}H_{39}N_{11}O_{12}$	$CuC_{22}H_{54}N_8O_{13}$
formula weight	788.73	665.14	702.27
crystal system	orthorhombic	monoclinic	monoclinic
space group	$C222_1$	$P2_1/c$	$P2_1/c$
a, *b*, *c* (Å)	12.4608 (13), 20.445 (2), 13.2263 (14)	8.48940 (10), 12.1436 (3), 26.3653 (5)	9.617 (5), 18.742 (5), 18.825 (5)
α, β, γ (deg)	90.00, 90.00, 90.00	90.00, 94.844 (2), 90.00	90.00, 102.433 (5), 90.00
cell volume (Å^3)	3369.6 (6)	2708.34 (9)	3313.48 (2)
Z	4	4	4
temperature (K)	130 (2)	130 (2)	130 (2)
λ (Å)	0.71073	1.54184	1.54180
reflections collected	8844	14675	11776
independent reflections	2973	5348	6245
R-factor (%)	5.44	4.69	5.90

fraction was analyzed using MS and analytical HPLC. Reverse phase HPLC method 1 employed a Phenomenex Synergi 4u Hydro-RP 80A 50 × 21.20 mm column with a flow rate of 5 mL min^{-1}. The gradient mobile phase started with 100% solvent A (0.1% trifluoroacetic acid in water) at 8 min to 100% solvent B (0.1% formic acid in acetonitrile) at 45 min. Reverse phase HPLC method 2 employed an Eclipse XDB-C18 5 μm 9.5 × 250 mm column with a flow rate of 3 mL min^{-1}. The gradient mobile phase started with 100% solvent A (0.1% trifluoroacetic acid in water) at 5 min to 100% solvent B (0.1% formic acid in acetonitrile) at 75 min. Reverse-phase HPLC method 3 employed an Eclipse XDB-C18 5 μm 9.5 × 250 mm column with a flow rate of 3 mL min^{-1}. The gradient mobile phase started with 100% solvent A (0.1% trifluoroacetic acid in water) at 5 min and increased at a rate of 1% B min^{-1} until all components had eluted.

Analytical reverse phase HPLC and radio-HPLC traces were acquired using an Agilent 1200 Series HPLC system and an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm) with a 1 mL/min flow rate and UV spectroscopic detection at 214 nm, 220 and 270 nm. Analytical size exclusion radio-HPLC traces were acquired using an Agilent 1200 Series HPLC system and a Phenomenex Biosep 2000 (300 × 7.8 mm) size exclusion column with phosphate buffer (0.1 M, pH 7) mobile phase. The radio-HPLC was coupled to a LabLogic Flow-Count detector with a sodium iodide probe (B-FC-3200). Aliquots (20 - 100 μL) of each radiolabeled sample were injected onto the column, using a flow rate of 1 mL/min.

Radio-TLC plates were analyzed using a Packard Instant Imager.

Reagents. SP Sephadex C25 and DOWEX 50wx2 200−400 mesh cation exchange resins were purchased from Aldrich. Fmoc-L-amino acids, HATU, HCTU and 2-chlorotrityl resin were purchased from GL Biochem Ltd. (Shanghai, China). Fmoc-D-amino acids were purchased from Bachem AG (Switzerland). Fmoc-Pal-PEG-PS resin was purchased from Applied Biosystems (Foster City, California). Nova PEG Rink Amide resin was purchased from NovaBiochem, Darmstadt, Germany.

$[Co((NO_2)_2sar)]Cl_3$, $[Co((NH_2)_2sar)]Cl_3$, $(NH_2)_2sar$, and $[Cu(NH_3)_2sar](CF_3SO_3)_4$ were prepared according to established procedures.[46,47]

^{64}Cu was produced at the PET Imaging Centre, St. Thomas' Hospital, London, U.K. by proton bombardment of 10−15 mg of ^{64}Ni, plated onto a gold disk, at 25 μA for 4−6 h in a CTI RDS 112 11 MeV cyclotron using purpose-built targetry. Purification of ^{64}Cu was undertaken using established procedures.[48] Radionuclidic purity was confirmed by gamma-ray spectroscopy (Ortec DSPEC Plus HPGe gamma-ray spectrometer) to be >99%. $^{64}CuCl_2$ was then obtained in HCl solution and was transformed into $^{64}Cu(OAc)_2$ by evaporation of the solution to a small volume and titrating it with small aliquots of a 1 M sodium acetate solution (99.995% trace metal basis) until the pH of the solution reached 6. Purity was confirmed by TLC (R_F = 0.66) with silica gel plates using 15 mM EDTA in 10% ammonium acetate/MeOH (50/50) as the mobile phase.

Synthesis and Characterization. Preliminary details describing the preparation of $[CuHL^1](NO_3)_3$, $[CuL^2](CF_3SO_3)Cl$, and L^1 have been reported in a communication.[36] Here, full descriptions of synthetic methods are included.

$[CuL^1](NO_3)_3$ and $[CuL^2](CF_3SO_3)Cl$. A solution of $[Cu(NH_3)_2sar](CF_3SO_3)_4$ (1.5 g, 1.53 mmol) in anhydrous *N*,*N*-dimethylacetamide (DMA) (12 mL) was heated to 70 °C under an atmosphere of nitrogen. Glutaric anhydride (0.19 g, 1.64 mmol) and diisopropylethylamine (600 μL) were added and heating continued at 70 °C for two hours. The solution was cooled and water (20 mL) added. The mixture was applied to a column of SP Sephadex C-25 cation exchange (Na^+ form, 30 × 5 cm) that was sodium citrate (0.05 M) to separate three components. (Chromatographic yield: Fraction 1−30%, fraction 2−30%, fraction 3−30%.) Each fraction was applied separately to a DOWEX 50W x 2 cation exhange column (H^+ form, 10 × 5 cm). The column was washed with water (500 mL) and 1 M HCl solution (500 mL) and then eluted with 4 M HCl (350 mL) and the eluent evaporated to dryness under reduced pressure at 40 °C. Fraction 1: $[CuL^2]Cl_2$ (1.00 g) MS: $[CuC_{24}H_{45}N_8O_6]^+$ m/z = 604.27 (experimental), 604.28 (calculated). Fraction 2: $[CuHL^1]Cl_3$ (0.82 g) MS: $[CuC_{19}H_{39}N_8O_3]^+$ m/z = 490.24 (experimental), 490.24 (calculated). The dark blue residue from fraction 2 was dissolved in deionized water (30 mL). Concentrated nitric acid (2 mL) was added and the solution was concentrated evaporation under reduced pressure until crystallization commenced. The mixture was then cooled at 5 °C for 30 min and sky blue crystals were collected by filtration. $[CuHL^1](NO_3)_3$: 0.13 g, 13% isolated yield.

Crystals suitable for X-ray diffraction were grown from evaporation of an aqueous solution of $[CuHL^1]Cl_3$ (20 mg) in ~1 M HNO_3 (2 mL) at ambient temperature.

Crystals of $[CuL^2](CF_3SO_3)Cl$ were suitable for X-ray diffraction studies and were formed as follows: $[CuL^2]Cl_2$ (0.45 g) was dissolved in water (2 mL) and a solution of silver triflate (0.33 g in water (2 mL)) was added. This solution was filtered twice (Milli-Q syringe filter (0.45 μm)) to remove precipitated AgCl, and the filtrate was evaporated to dryness under reduced pressure to give a dark blue-purple residue. The residue was dissolved in water (8 mL) and over the course of 10 min, blue crystals precipitated from solution. These were collected and dried by filtration. $[CuL^2](CF_3SO_3)Cl$: 0.13 g. Crystals suitable for X-ray diffraction were grown by evaporation of a solution of $[CuL^2](CF_3SO_3)Cl$ (30 mg) in water (6 mL) at ambient temperature: analysis confirmed that only one of the two chloride anions had been exchanged for triflate by the addition of silver triflate.

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Crystal data: $[CuL^1](NO_3)_3$ $C_{25}H_{41}N_{11}O_{12}Cu$, M = 679.17, T = 130.0(2) K, λ = 0.71069, monoclinic, space group $P2_1/c$ a = 8.345(5), b = 12.231(5), c =26.941(5) Å, β = 93.658(5)°, V = 2744(2) Å^3, Z = 4, D_c = 1.644 mg M^{-3} μ(Mo−Kα) 0.879 mm^{-1}, F(000) = 1428, crystal size 0.35 × 0.3 × 0.01 mm; 17656 reflections measured, 6107 independent reflections (R_{int} = 0.17). The final R was 0.067 [$I > 2\sigma(I)$], and wR(F^2) was 0.1524.

$[CuL^2](CF_3SO_3)Cl$ $C_{19}H_{46}ClCuF_3N_8O_9S$, M = 790.75, T = 130.0(2) K, λ = 0.71069, orthorhombic, space group $C222_1$, a = 12.4608(13), b = 20.445(2), c =13.2263(14) Å, V = 3369.6(6) Å^3, Z = 4, D_c = 1.559 mg M^{-3} μ(Mo−Kα) 0.879 mm^{-1}, F(000) = 1652, crystal size 0.40 × 0.30 × 0.20 mm; 8848 reflections measured, 2973 independent reflections (R_{int} = 0.0334). The final R was 0.0542 [$I > 2\sigma(I)$] and wR(F^2) was 0.1471.

UV−vis: $[CuL^1](NO_3)_3$ in water, pH 4, λ_{max} = 658 nm, ε = 140 M^{-1} cm^{-1}; $[CuL^2](CF_3SO_3)Cl$ in water, pH = 4, λ_{max} = 655 nm, ε = 146 M^{-1} cm^{-1}

$[CuHL^3](NO_3)_3$ and $[CuL^4]\cdot xH_2O$: A solution of $[Cu(NH_3)_2$-sar$](CF_3SO_3)_4$ (0.74 g, 0.76 mmol) in anhydrous *N*,*N*-dimethylacetamide (DMA) (10 mL) was heated under an atmosphere of nitrogen to 70 °C. Succinic anhydride (0.09 g, 0.91 mmol) and diisopropylethylamine (600 μL) were added and the solution was heated at 70 °C for two hours. The solution was cooled and water (20 mL) was added. The solution was applied to a column of SP Sephadex C-25 cation exchange (Na^+ form, 30 × 5 cm). The column was eluted with 0.05 M sodium citrate solution to separate three components. (Chromatographic yield: Fraction 1−30%, fraction 2−30%, fraction 3−30%.) Fraction 1: This fraction was evaporated under reduced pressure until crystallization commenced and then cooled for ∼1 min in an ice bath. The light blue crystals were then separated from the supernatant, washed with ethanol and dried. $[CuL^4].4H_2O$ (71 mg) MS: $[CuC_{22}H_{42}N_8O_6]^{2+}$ m/z = 288.63 (experimental), 288.63 (calculated). Fraction 2: The second fraction was applied to a DOWEX 50Wx2 cation exhange column (H^+ form, 10 × 5 cm). The column was washed with water (500 mL) and 1 M HCl solution (500 mL) and then eluted with 4 M HCl (350 mL) and the eluent was evaporated to dryness under reduced pressure at 40 °C. $[CuHL^3]Cl_3\cdot x$HCl (0.13 g) MS: $[CuC_{18}H_{38}N_8O_3]^{2+}$ m/z = 238.62 (experimental), 238.62 (calculated). The dark blue residue from fraction 2 was dissolved in deionized water (∼ 5 mL) and several drops of nitric acid were added and the solution left to crystallize overnight. Light blue crystals were collected by filtration. Isolated yield: $[CuHL^3](NO_3)_3$: 72 mg

Crystals of $[CuL^4]\cdot 7H_2O$ and $[CuHL^3](NO_3)_3$ suitable for X-ray diffraction were grown from evaporation of solutions of $[CuL^4]$ and $[CuHL^3](NO_3)_3$ at ambient temperature, respectively.

Crystal data: $[CuHL^3](NO_3)_3$ $CuC_{18}H_{39}N_{11}O_{12}$, M = 665.14, T = 130.0(2) K, λ = 1.54184, monoclinic, space group $P2_1/c$, a = 8.48940(10), b = 12.1436(3), c = 26.3653(5) Å, β = 94.884(2)°, V = 2708.34(9) Å^3, Z = 4, D_c = 1.631 mg M^{-3} μ(Cu−Kα) 1.870 mm^{-1}, F(000) = 1396, crystal size 0.3030 × 0.0970 × 0.0200 mm. 14675 reflections measured, 5348 independent reflections. The final R was 0.0469 [$I > 2\sigma(I)$] and wR(F^2) was 0.1078.

$[CuL^4].7H_2O$ $CuC_{22}H_{54}N_8O_{13}$, M = 702.27, T = 130.0(2) K, λ = 0.71069, monoclinic, space group $P2_1/c$, a = 9.617(5), b = 18.742(5), c =18.825(5) Å, V = 3313.48(2) Å^3, Z = 4, D_c = 1.408 mg mm^{-3} μ(Mo−Kα) 1.548 mm^{-1}, F(000) = 1500, crystal size 0.2939 × 0.1778 × 0.1085 mm. 11776 reflections measured, 6245 independent reflections. The final R was 0.0590 [$I > 2\sigma(I)$] and wR(F^2) was 0.2132.

L^2: A solution of $[Cu(L^2)]Cl_2\cdot x$HCl (1.0 g, ∼1.48 mmol based on x = 0) in water (20 mL) was deoxygenated by purging with N_2 gas for 20 min. Sodium sulfide (1.3 g) was added and the solution was stirred overnight at room temperature (under an atomosphere of nitrogen gas). After ∼16 h, the solution was green with a black-brown precipitate. Another portion of sodium sulfide (1.4 g) was added and the solution stirred overnight at room temperature. After this time, the solution appeared light yellow. This mixture was filtered (Whatman Filter Paper 1), and the filtrate was diluted with 1 M HCl (250 mL) resulting in the formation of a cloudy, white precipitate. The mixture was filtered (Milli-Q syringe filters (0.45 μm)) and applied to a DOWEX50Wx2 cation exhange column (H^+ form, 10 × 5 cm). The column was washed with 1 M HCl solution (750 mL) (to remove Na_2S) and then eluted with 4 M HCl solution (400 mL). The eluent was evaporated to dryness under reduced pressure to give a clear, colorless residue. ($L^2\cdot x$HCl, 0.255 g; ∼ 30% MS, $[C_{24}H_{47}N_8O_6]^+$ 543.37 (experimental), 543.36 (calculated); ^{1}H NMR δ 1.86, m, 4H, βCH_2 (with respect to COOH); 2.34, t, ^3J = 7.47, 4H, glutarate CH_2; 2.41, t, ^3J = 7.19, 4H glutarate CH_2; 3.21, broad s, 12H, cage CH_2; 3.44, broad s, 12H, cage CH_2; ^{13}C NMR δ 20.7, 33.4, 35.5 (glutarate CH_2); 47.4, 51.4, 56.2 (cage); 177.8, 178.4 (CO).

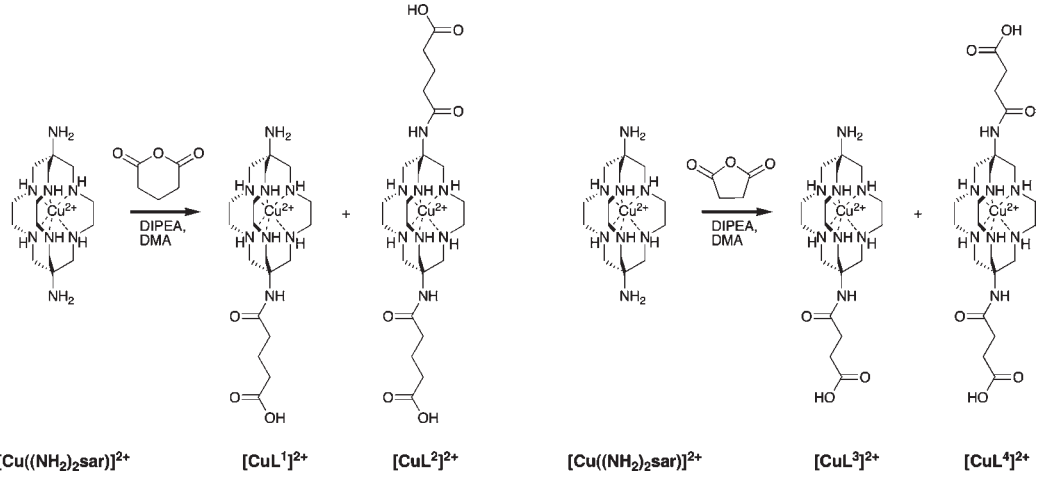
(t-Boc)$_{4-5}$-**L^2**: **L^2**$\cdot x(HCF_3SO_3)$ (1.78 g) was dissolved in acetonitrile. Di-*tert*-butyldicarbonate (2.05 g) and diisopropylethylamine (1.6 mL) were added. A white precipitate was observed upon addition of base. The reaction mixture was stirred vigorously for 1 h. The remaining white precipitate was separated from the solution, and the solution evaporated under reduced pressure. Mass spectral analysis indicated that the resulting residue from the supernatant contained the desired species, while the white precipitate contained unreacted **L^2**. The white precipitate was redissolved in deionized water (10 mL) and acetonitrile (10 mL); di-*tert*-butyldicarbonate (1.2 g) and diisopropylethylamine (0.8 mL) were added; and the reaction was stirred vigorously for 2 h. After this time, the solvent was removed under reduced pressure. Mass spectral analysis indicated that the desired species was present. Both sets of residues containing (t-Boc)$_{4-5}$-**L^2** were combined and washed with deionized water and diethyl ether (to remove low molecular weight species and excess di-*tert*-butyldicarbonate respectively). (t-Boc)$_{4-5}$-**L^2**: 670 mg, ∼ 30% yield. MS: $[C_{44}H_{79}N_8O_{14}]^+$ 943.57 (experimental), 943.57 (calculated) $[C_{49}H_{86}N_8O_{16}]^+$ 1043.62 (experimental), 1043.62 (calculated).

(t-Boc)$_4$(Succ)$_2$-**L^2** and (t-Boc)$_5$(Succ)$_2$-**L^2**: (t-Boc)$_{4-5}$-**L^2** (0.12 g) was suspended in acetonitrile (30 mL) and diisopropylcarbodiimide (200 μL) and N-hydroxysuccinimide (60 mg) were added. The reaction mixture was stirred at ambient temperature for five hours. After this time, the solution was clear and no undissolved material was present. The solvent was evaporated under a stream of dinitrogen and the dry material was dissolved in acetonitrile/water (50%/50%) and applied to a C18 semipreparative HPLC column and purified using HPLC method 1. Fractions containing pure material were combined and lyophilized. (t-Boc)$_4$(Succ)$_2$-**L^2** (13 mg) eluted at 28 − 31 min. MS: $[C_{52}H_{85}N_{10}O_{18}]^+$ 1137.61 (experimental), 1137.60 (calculated). (t-Boc)$_5$(Succ)$_2$-**L^2** (57 mg) eluted at 34−40 min. MS: $[C_{57}H_{93}N_{10}O_{20}]^+$ 1237.66 (experimental), 1237.66 (calculated).

L^2-BBN$_2$: Lys^3-bombesin (3 mg) and (t-Boc)$_5$(Succ)$_2$-**L^2** (4 mg) were dissolved in dimethylformamide (400 μL) and diisopropylethylamine (4 μL) was added. This solution was stirred at ambient temperature for 3 h. Acetonitrile/water (50%/50%) (4 mL) was added and the solution was applied to a C18 semipreparative HPLC column and separated using HPLC method 3. The fraction containing the desired species eluted at 56 min. This was frozen and lyophilized. MS: $[C_{191}H_{302}N_{52}O_{50}S_2]^{3+}$ 1397.75 (experimental), 1397.74 (calculated); Analytical HPLC: 13.97 min (0 to 100% B in 25 min).

The lyophilized product was dissolved in trifluoroacetic acid (500 μL) containing bromotrimethylsilane (5 μL) and 3,6-dioxa-1,8-octanedithiol (5 μL) (to reduce any methionine residiues that became oxidsed during peptide synthesis)[49] and the mixture was allowed to react for 1 h. Acetonitrile (1 mL) and water (1 mL) were then added to the solution, and the solution was frozen and lyophilized. The dried material was redissolved and purified using a C18 semipreparative HPLC column using HPLC method 3. **L^2-BBN$_2$**: MS $[C_{166}H_{264}N_{52}O_{40}S_2]^{2+}$ 1845.98 (experimental), 1845.98 (calculated); Analytical HPLC 15.80 min (0 to 60% B in 25 min).

Scheme 1. Acylation of $[Cu(NH_3)_2sar]^{2+}$ with (Left) Glutaric Anhydride and (Right) Succinic Anhydride



HPLC-ESI-MS of L_1-Peptides. HPLC-ESI-MS traces were acquired for $\mathbf{L^2}$-$\mathbf{BBN_2}$ (<0.5 μg uL^{-1}) with and without $CuCl_2$ (0.02 μg uL^{-1}), to determine whether $\mathbf{L^2}$-$\mathbf{BBN_2}$ binds "free" Cu^{II} ion. A reverse phase C18 analytical HPLC column was employed with a linear gradient (0 → 60% B in A over 25 min) to determine retention times and molecular masses of peptide species. (Here, A = Milli-Q water with 0.1% formic acid; B = acetonitrile with 0.1% formic acid.)

^{64}Cu Radiolabeling of L^2-BBN_2. HPLC and serum stability studies: A solution of ^{64}Cu (19 MBq, 40 μL, 0.4 M ammonium acetate) was added to $\mathbf{L^2}$-$\mathbf{BBN_2}$ (~ 8 μg in 40 μL of 0.1 M ammonium acetate) and the solution was allowed to react at ambient temperature for twenty minutes. After this time, an aliquot was analyzed using analytical radio-HPLC (0 → 100% B in A over 12 min). The remainder of the solution was made up to 100 μL with ammonium acetate solution (0.1 M) and added to 400 μL of human serum. This sample was filtered and incubated at 37 °C for 2 days. After 1 day and 2 days, aliquots from this sample were subjected to both size exclusion HPLC and reverse phase HPLC analysis.

pH studies: A solution of ^{64}Cu (1.2 MBq, 1 μL, 1 M hydrochloric acid) was added to solutions (30 μL) containing ~4 μg of $\mathbf{L^2}$-$\mathbf{BBN_2}$ at pH 2 (1 M phosphate buffer), pH 4 (1 M acetate buffer), pH 5 (1 M acetate buffer), pH 6 (1 M ammonium acetate), pH 7.4 (1 M phosphate buffer), and pH 9 (1 M glycine buffer). Each reaction solution was subjected to TLC analysis (0.1 M sodium citrate mobile phase) at 5, 10, 30, and 60 min. R_F: $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ < 0.1; $^{64}\mathbf{Cu^{II}}$ R_f > 0.2.

Concentration studies: A solution of ^{64}Cu (1 MBq, 5 μL, 1.3 M ammonium acetate) was added to solutions (5 μL, 0.1 M ammonium acetate) containing ~10, 1, 0.1, 0.01, and 0.001 μg of $\mathbf{L^2}$-$\mathbf{BBN_2}$. Each reaction solution was subjected to TLC analysis (0.1 M sodium citrate mobile phase) at 5, 10, 30, and 60 min. R_f: $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ < 0.1; $^{64}\mathbf{Cu^{II}}$ R_f > 0.2.

RESULTS AND DISCUSSION

Synthesis and Characterization of Acylated Diaminosarcophagine Compounds. The synthesis of the acylated diaminosarcophagine species, $[Cu((1\text{-}NH_3)(8\text{-}NHCO(CH_2)_3CO_2H)sar)]^{3+}$ ($[CuH\mathbf{L^1}]^{3+}$) and $[Cu(1,8\text{-}NHCO(CH_2)_3CO_2H)_2sar]^{2+}$ ($[Cu\mathbf{L^2}]^{2+}$) were reported recently in a preliminary communication. Full characterization of the ligand $\mathbf{L^2}$ and its conjugation to two targeting peptides are reported here for the first time, as well as the synthesis and characterization of the homologous succinate-derived species. The complexes $[CuH\mathbf{L^1}]^{3+}$ and $[Cu\mathbf{L^2}]^{2+}$ were synthesized from reaction of $[Cu((NH_2)_2sar)]^{2+}$ and glutaric anhydride in *N*,*N*-dimethylacetamide (DMA) with diisopropylethylamine (DIPEA) at 70 − 80 °C (Scheme 1). The use of succinic anhydride instead of glutaric anhydride led to the formation of homologues $[Cu((1\text{-}NH_3)(8\text{-}NHCO(CH_2)_2CO_2H)Sar)]^{3+}$ ($[CuH\mathbf{L^3}]^{3+}$) and $[Cu(1,8\text{-}NHCO(CH_2)_2CO_2H)_2Sar]^{2+}$ ($[Cu\mathbf{L^4}]^{2+}$) (Scheme 1). Although a stoichiometric equivalent of cyclic anhydride was used both the monoacylated and the diacylated species were formed. The use of an excess of cyclic anhydride (>2 equivalents) results in the formation of predominantly diacylated compound.

The two products from each of these reactions, along with unreacted starting material, were separated using ion exchange chromatography to give the complexes as their chloride salts that were hygroscopic and were converted to either nitrate or mixed triflate-chloride salts to give crystalline materials. Coordination of Cu^{II} to the secondary amine atoms of diaminosarcophagine effectively protects them from reaction with glutaric anhydride and at 70−80 °C, and the acylation reaction occurs exclusively at the primary amine groups. The electronic spectra in water at pH 4 (for example, $[Cu\mathbf{L^2}]^{2+}$: λ_{max} = 655 nm, ε = 146 M^{-1} cm^{-1}) are similar to the parent compound, $[Cu((NH_2)_2sar)]^{2+}$ and confirm the Cu−N_6 coordination environment about the metal ion.

All four complexes were characterized by X-ray crystallography (the crystal structure of $[CuH\mathbf{L^1}]^{3+}$ was reported in a preliminary communication, the other three are reported here for the first time).[36] For the diacylated product isolated from the reaction of $[Cu((NH_2)sar)]^{2+}$ with glutaric anhydride crystals of $[Cu\mathbf{L^2}]Cl(CF_3SO_3)$ were grown from an aqueous solution of $[Cu(\mathbf{L^2})]Cl_2$ following addition of $AgCF_3SO_3$ and filtration to remove precipated AgCl: this precipitation only succeeded in exchanging one of the chloride anions for triflate. The unit cell of a selected crystal of $[Cu\mathbf{L^2}](CF_3SO_3)Cl$ contains a single enantiomer of $[Cu\mathbf{L^2}]^{2+}$ (Figure 2). The Cu^{II} ion, located on a 2-fold rotation axis, is coordinated to the six secondary cage amines in a six-coordinate environment, with a geometry halfway between trigonal prismatic and octahedral. All Cu−N bond lengths for $[Cu\mathbf{L^2}]^{2+}$ are similar but there is a small degree of tetragonal compression, where a pair of trans Cu−N3, Cu−N3 (x, 2 − y, 2 − z) bonds are slightly shorter than Cu−N1 and Cu−N2 bonds (Table 2). Both formerly primary amine groups have been acylated and the appended carboxylate functional

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groups extend away from the macrobicyclic cage that is present in the lel_3 conformation, where the C−C bonds of the five-membered chelate rings are parallel to the pseudo-C_3 axis.

The monoacylated product from the reaction of $[Cu((NH_2)_2\text{-sar})]^{2+}$ with succinic anhydride, isolated as $[Cu(HL^3](NO_3)_3$, has a six coordinate Cu^{II} in a distorted octahedral environment but trigonal distortion, similar to previously reported Cu^{II} complexes of sarcophagines, is apparent[47] (Figure 2). Tetragonal elongation as a result of Jahn−Teller distortion is evident with Cu−N bond lengths for one pair of trans axial secondary amines (Cu−N3, Cu−N4) longer than Cu−N bond lengths for equatorial secondary amines (Cu−N1, Cu−N2, Cu−N5, Cu−N6) (Table 2). Once again the cage is in the common lel_3 conformation. The diacylated product was isolated as a neutral complex, $[CuL^4]$ with both of the carboxylic acid groups deprotonated (Figure 2). A representation of the structure of $[CuL^4]$ is depicted in Figure 2. Both Δ and Λ isomers are present in the unit cell. Significant Jahn−Teller tetragonal distortion is observed, with a pair of trans Cu−N bonds (Cu−N2, Cu−N6,) significantly longer than Cu−N bond lengths for other equatorial secondary amines (Cu−N1, Cu−N3, Cu−N4, Cu−N6) (Table 2).

Importantly, the Cu^{II} used to 'protect' the secondary amine atoms from acylation can be removed from the cage ligand by reacting $[CuL^2]^{2+}$ with an excess of sodium sulfide (Scheme 2). Initial reduction of Cu^{II} to Cu^{I} results in metal extrusion from the cage. A combination of precipitation of insoluble copper sulfides and disproportionation of extruded Cu^{I} results in precipitation of all copper-containing species. The 'free' ligand, L^2, can be isolated by filtration to remove insoluble material followed by purification by ion-exchange chromatography. The ^{13}C NMR spectrum of L^2 displays the expected eight signals with carbon atoms of the cage ligand framework giving three signals at δ 47.4, 51.4, and 56.2 ppm while the amide and carboxylate carbon atoms give signals at δ 177.8 and 178.4 ppm.

Attachment of Acylated Diaminosarcophagine Derivatives to Cancer Targeting Peptides. The bifunctional chelator L^2 possesses two carboxylate groups and can be derivatized to give a sarcophagine-peptide species containing two biologically active peptide groups. So-called "bivalent conjugates" could show improved biological activity relative to their corresponding "monovalent" analogues. The presence of six uncoordinated secondary amine groups in L^2 leads to the possibility that any coupling reaction undertaken on the pendant carboxylic acid functional group of L^2 could lead to partial coupling of L^2 with

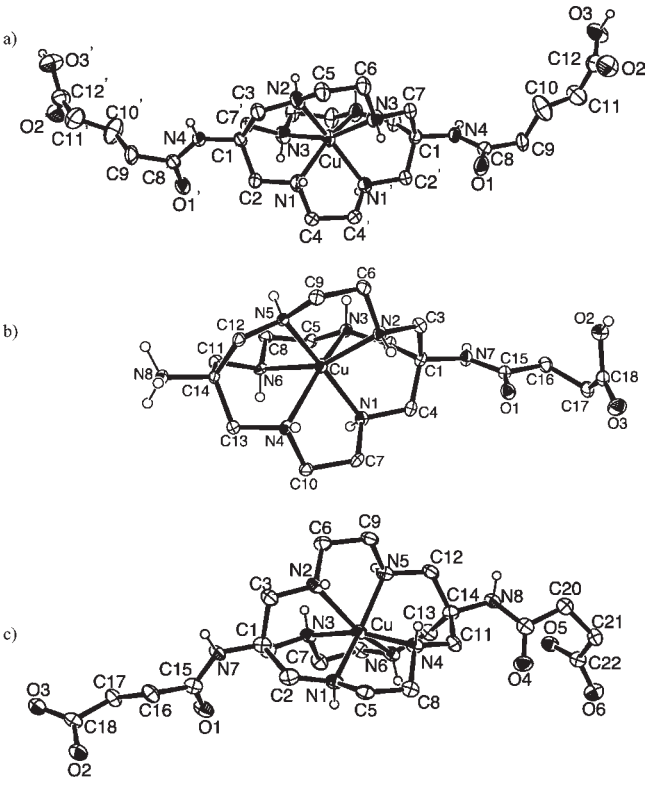

Figure 2. ORTEP representations of: (a) the cation present in $[CuL^2](CF_3SO_3)Cl$; (b) the cation present in $[CuHL^3](NO_3)_3$; (c) the copper complex present in $[CuL^4\text{-}2H^+]\cdot 7H_2O$: the two carboxylic acid functional groups are both deprotonated in this molecule giving the neutral zwitterion. Ellipsoids are at the 40% probability level. Hydrogen atoms attached to carbon, solvent, and anions (for a and b) are omitted for clarity.

Scheme 2. Synthesis of $(t\text{-Boc})_x(Succ)_2\text{-}L^2$ (x = 5)

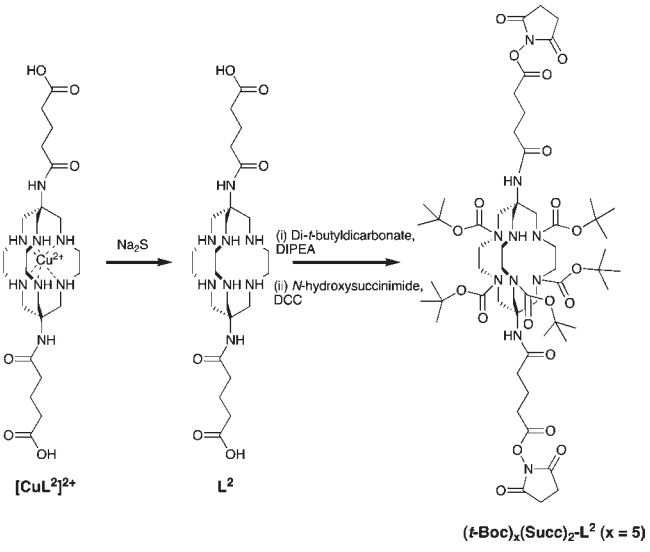

Table 2. Cu−N Bond Lengths

	$[CuHL^1](NO_3)_3$ bond lengths (Å)	$[CuL^2](CF_3SO_3)Cl$ bond lengths (Å)	$[CuHL^3](NO_3)_3$ bond lengths (Å)	$[CuL^4].7H_2O$ bond lengths (Å)
Cu−N1	2.407(5)	2.158(4)	2.054(3)	2.048(3)
Cu−N2	2.033(6)	2.197(4)	2.055(3)	2.336(4)
Cu−N3	2.028(6)	2.111(4)	2.430(3)	2.082(3)
Cu−N4	2.088(5)		2.283(3)	2.033(4)
Cu−N5	2.290(5)		2.029(3)	2.122(3)
Cu−N6	2.018(5)		2.188(3)	2.336(3)

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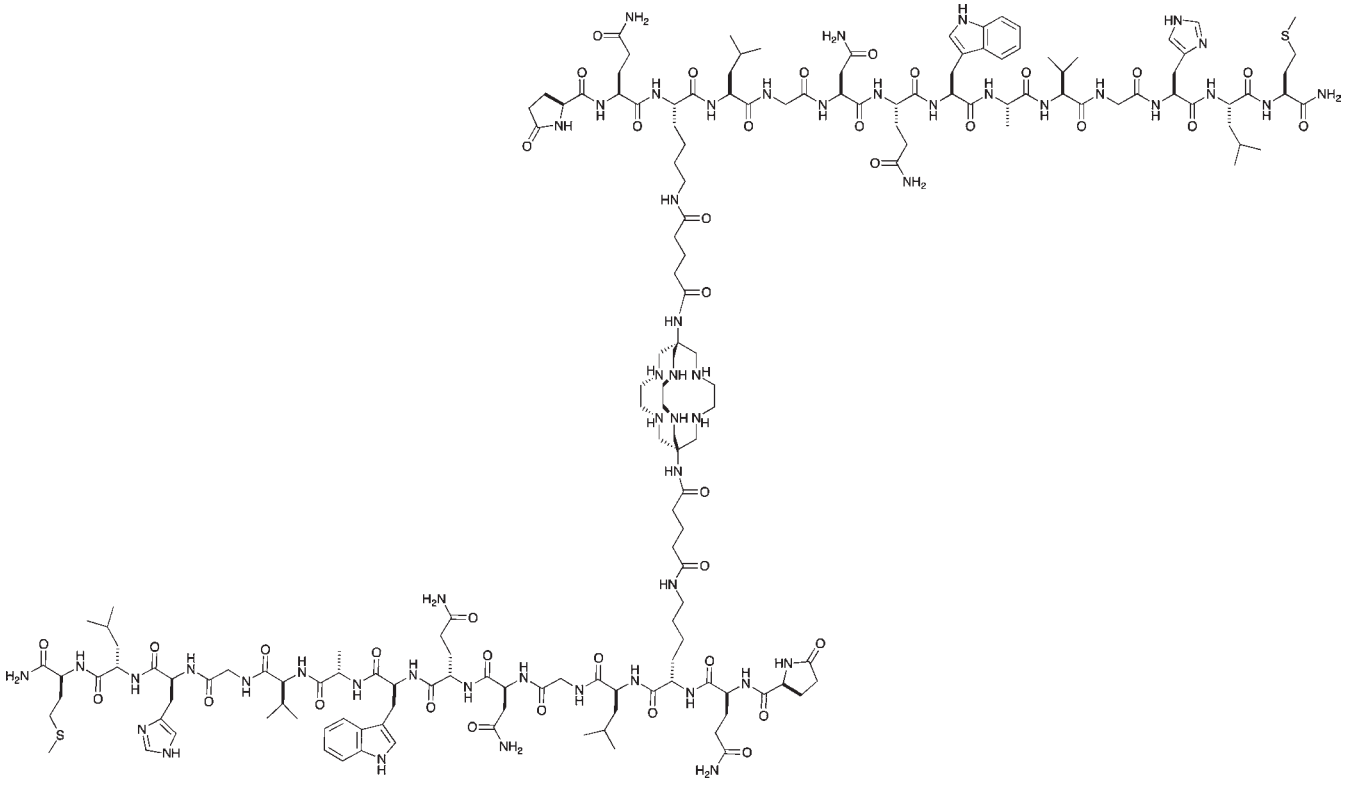

Figure 3. Structure of $\mathbf{L^2}$-$\mathbf{BBN_2}$.

itself. To mitigate this possibility, $\mathbf{L^2}$ was reacted with ditert-butyldicarbonate, to give the *t*-Boc protected cage species, $(t\text{-Boc})_x\mathbf{L^2}$ (x = 4−5) (Scheme 2). This reaction produced a mixture of *t*-Boc-protected species, where the number of *t*-Boc groups per cage ranged most commonly from four to five. The N-hydroxysuccinimide ester of $(t\text{-Boc})_x\mathbf{L^2}$ was prepared by the reaction of N-hydroxysuccinimide with $(t\text{-Boc})_x\mathbf{L^2}$ in the presence of the coupling agent diisopropylcarbodiimide to give $(t\text{-Boc})_x(\text{Succ})_2\text{-}\mathbf{L^2}$ (x = 4, 5, Succ = succinimide) (Scheme 2). The two major products, $(t\text{-Boc})_4(\text{Succ})_2\mathbf{L^2}$ and $(t\text{-Boc})_5(\text{Succ})_2\mathbf{L^2}$, were separated and purified by semipreparative HPLC. The 14 amino acid peptide, Lys^3-bombesin was prepared using standard solid phase peptide synthesis and reacted with the activated ester, $(t\text{-Boc})_5(\text{Succ})_2\text{-}\mathbf{L^2}$, to give a homodimeric sarcophagine conjugate possessing two bombesin groups. Deprotection and purification by semipreparative HPLC allowed isolation of the peptide conjugate $\mathbf{L^2}$-$\mathbf{BBN_2}$ (Figure 3). The copper complex was prepared by addition of Cu^{II} to give $[\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ characterized by LC-MS to give the expected signal at m/z = 1876.95 corresponding to $[\text{Cu}(\text{C}_{166}\text{H}_{262}\text{N}_{52}\text{O}_{40}\text{S}_2]^{2+}$ (Figure 4, Table 3). This is the first synthesis of a dimeric sarcophagine-peptide conjugate and its copper complex.

Radiolabeling $\mathbf{L^2}$-$\mathbf{BBN_2}$ with ^{64}Cu. The $^{64}\text{Cu}^{\text{II}}$ complex, $[^{64}\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ can be prepared with high radiochemical purity by addition of $^{64}\text{Cu}^{\text{II}}\text{Cl}_2$ (19 MBq, 40 μL 0.4 M ammonium acetate, pH 6) to $\mathbf{L^2}$-$\mathbf{BBN_2}$ (0.2 mg/mL peptide, 40 μL 0.1 M ammonium acetate, pH 6) at ambient tempertature. A radio-HPLC trace of this solution 20 min after addition of the activity indicates a radiochemical yield >99% (Figure 5). The HPLC retention time of this signal (7.04 min) corresponds with the retention time of $\mathbf{L^2}$-$\mathbf{BBN_2}$ (detected by λ_{214} at 6.43 min). TLC

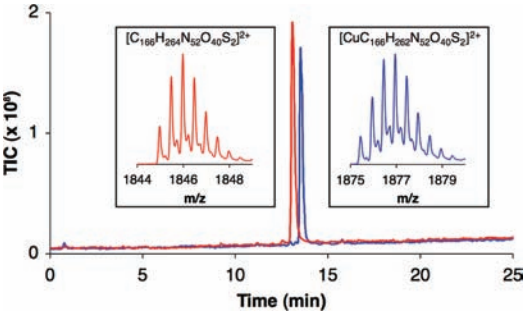

Figure 4. LC-MS chromatogram of $\mathbf{L^2}$-$\mathbf{BBN_2}$ (red) and $[\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ (blue). Inset: ESMS of peaks in LC chromamtogram corresponding to m/z = 1845.98 for $[\mathbf{L^2}\text{-BBN}_2]^{2+}$ (red) and m/z = 1876.95 for $[\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ (blue).

analyses of solutions of $[^{64}\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ at similar peptide concentrations confirm radiochemical yields >97%. The stability of $[^{64}\text{Cu}(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ in human serum at 37 °C was assessed by analysis by size exclusion HPLC and C18 HPLC and revealed that <2% $^{64}\text{Cu}^{\text{II}}$ dissociates from the sarcophagine-peptide conjugate, and no ^{64}Cu is associated with serum proteins after two days (Figure 5). Although the $^{64}\text{Cu}^{\text{II}}$ remains bound to the cage amine ligand the peptide fragment undergoes some decomposition under these conditions presumably by endogenous serum proteases.[50]

The complexation of $^{64}\text{Cu}^{\text{II}}$ by $\mathbf{L^2}$-$\mathbf{BBN_2}$ is pH dependent. Unsurprisingly, at pH 2, $\mathbf{L^2}$-$\mathbf{BBN_2}$ does not bind $^{64}\text{Cu}^{\text{II}}$ appreciably even after one hour (1.2 MBq $^{64}\text{Cu}^{\text{II}}$, 30 μL 0.13 mg/mL $\mathbf{L^2}$-$\mathbf{BBN_2}$ solution) (Figure 6). As the pH is raised to pH 4, < 75% of ^{64}Cu is bound to $\mathbf{L^2}$-$\mathbf{BBN_2}$ after one hour. In contrast, at pH

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Table 3. LCMS Data for L^2-BBN_2 and Its Cu^{II} Complex

compound	retention time (min)	experimental (m/z)	calculated (m/z)
L^2-BBN_2	13.086	$[C_{166}H_{264}N_{52}O_{40}S_2]^{2+}$: 1845.98	1845.98
$[Cu(L^2\text{-}BBN_2)]^{2+}$	13.533	$[CuC_{166}H_{262}N_{52}O_{40}S_2]^{2+}$: 1876.95	1876.95

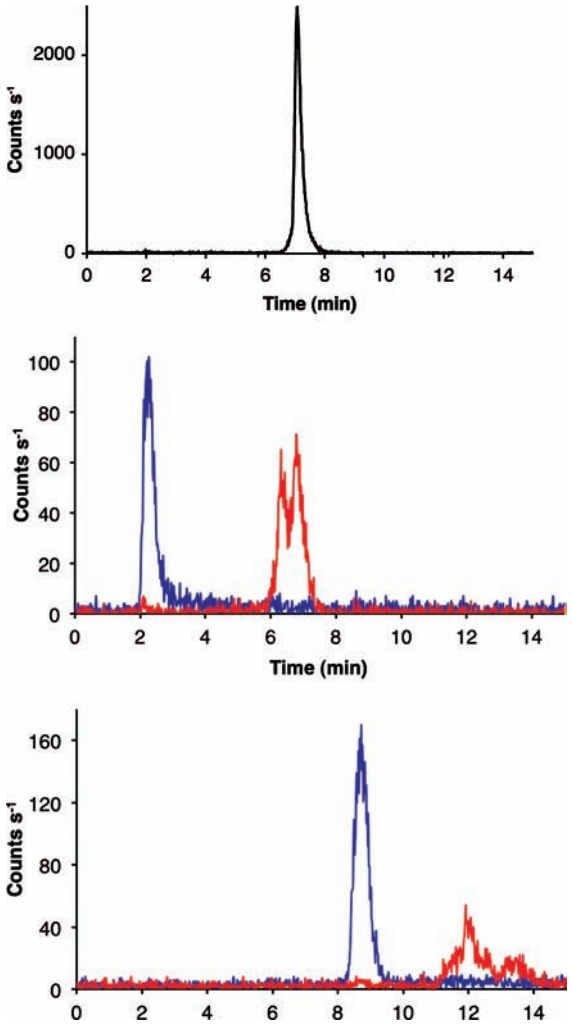

Figure 5. Top: C18 radio-HPLC of $[^{64}Cu(L^2\text{-}BBN_2)]^{2+}$. Middle: C18 radio-HPLC of $[^{64}Cu(L^2\text{-}BBN_2)]^{2+}$ after 2 days incubation in human serum (red trace) and supernatant separated from precipitated serum proteins following incubation of serum with $^{64}CuCl_2$ (blue trace). Bottom: Size exclusion radio-HPLC traces of $[^{64}Cu(L^2\text{-}BBN_2)]^{2+}$ in human serum after 2 days of incubation (red trace) and $^{64}CuCl_2$ in human serum after 2 days of incubation (blue trace).

5−7, at the same $^{64}Cu^{II}$ and ligand−peptide conjuagte concentrations, L^2-BBN_2 binds >93% of $^{64}Cu^{II}$, as determined by analysis by radio-TLC. At pH 9, < 60% of ^{64}Cu is complexed to L^2-BBN_2 after one hour, presumably due to the formation of insoluble hydroxide species. This confirms that the ideal radiolabeling conditions for sarcophagine ligands are at room temperature and pH 5−7: conditions that are compatible with most targeting molecules.[24,27]

The performance of radiolabeling reactions between $^{64}Cu^{II}$ and L^2-BBN_2 under more challenging conditions of reduced concentrations of chelate-peptide conjugate was investigated.

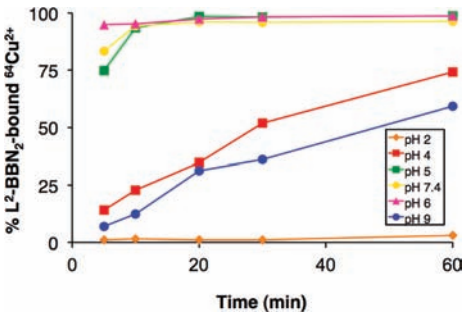

Figure 6. pH dependence of $^{64}Cu^{II}$ complexation by L^2-BBN_2.

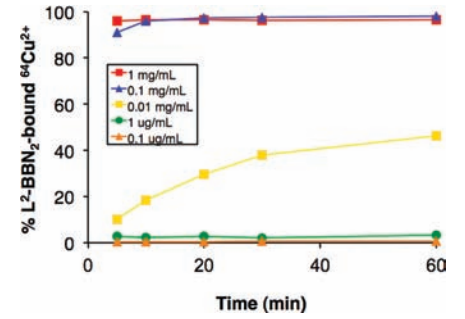

Figure 7. Concentration and time dependence of $^{64}Cu^{II}$ complexation by L^2-BBN_2.

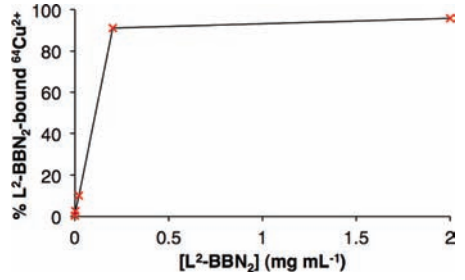

Figure 8. Concentration dependence of $^{64}Cu^{II}$ complexation by L^2-BBN_2 after a reaction time of 5 min.

The radiochemical yields for the synthesis of $[^{64}Cu(L^2\text{-}BBN_2)]^{2+}$ from solutions containing varying concentrations of peptide with $^{64}Cu^{II}$ (1 MBq ^{64}Cu, 10 μL solution, pH 6) were analyzed with radio-TLC over the time period of one hour (Figure 7). After 10 min incubation at room temperature, > 96% radiochemical yield was observed for solutions containing ≥0.1 mg/mL L^2-BBN_2, equivalent to >0.2 nmol in 10 μL. For reaction solutions containing <0.2 nmol in 10 μL, L^2-BBN_2 did not complex $^{64}Cu^{II}$ quantitatively, with <50% radiochemical yield for the solution containing 0.01 mg/mL L^2-BBN_2. Furthermore, at this concentration, the ^{64}Cu complexation is slower compared to that of reactions containing higher concentrations

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of $\mathbf{L^2}$-$\mathbf{BBN_2}$. For reaction solutions containing <0.02 nmol of $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ in 10 μL, very little $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ was detectable.

CONCLUDING REMARKS

Reaction of $[Cu((NH_2)_2sar)]^{2+}$ with either succinic or glutaric anhydride results in selective acylation of the primary amine atoms of $[Cu((NH_2)_2sar)]^{2+}$ to give derivatives with either one or two aliphatic carboxylate functional groups separated from the cage amine framework by either a four or five atom linker. The Cu^{II} serves to protect the secondary amine atoms from acylation and can be removed to give the free ligands. The newly appended carboxylate functional groups can be used as sites of attachment for cancer-targeting peptides using standard peptide coupling methodologies. A dimeric sarcophagine-peptide conjugate has been synthesized that possesses two Lys^3-bombesin peptides tethered to a single cage amine ligand, where the cage amine chelator has been incorporated into the third amino acid residue of the fourteen amino acid peptide. The radiolabeled complex $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ can be prepared in high radiochemical yields at ambient temperature at pH 5−7. The radiolabeled complex, $[^{64}Cu(\mathbf{L^2}\text{-}\mathbf{BBN_2})]^{2+}$ is stable with respect to dissociation of Cu^{II} from the cage amine ligand in human serum. The new dimeric constructs efficiently form extraordinarily stable complexes with the radioisotope, copper-64, and could overcome the limitations of other systems that suffer from loss of the metal ion from the ligand in vivo. In principle, these new cage amine derivatives with carboxylate functional groups could be incorporated into a wide range of peptide sequences. The new ligands with two carboxylate functional groups are ideally suited for tethering two targeting peptides to a single chelator to form dimeric constructs that are of interest in the design of new targeted diagnostic or therapeutic copper radiopharmaceuticals that have the potential to exploit bivalent interactions.

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