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Synthesis of a Cross-Bridged Cyclam Derivative for Peptide Conjugation and ^{64}Cu Radiolabeling

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Abstract

The increased use of copper radioisotopes in radiopharmaceutical applications has created a need for bifunctional chelators (BFCs) that form stable radiocopper complexes and allow covalent attachment to biological molecules. Previous studies have established that 4,11-bis-(carbo-*tert*-butoxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane ($\text{H}_2\text{CB-TE2A}$), a member of the ethylene “cross-bridged” cyclam (CB-cyclam) class of bicyclic tetraaza macrocycles, forms highly kinetically stable complexes with Cu(II) and is less susceptible to *in vivo* transchelation than its non-bridged analog, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA). Herein, we report a convenient synthesis of a novel cross-bridged BFC that is structurally analogous to CB-TE2A in that it possesses two coordinating acetate arms, but in addition possesses a third orthogonally-protected arm for conjugation to peptides and other targeting agents. Application of this strategy to cross-bridged chelators may also enable the development of even further improved agents for ^{64}Cu -mediated diagnostic positron emission tomography (PET) imaging as well as for targeted radiotherapeutic applications.

INTRODUCTION

The syntheses of several novel “cross-bridged” tetraamine ligands such as 1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-cyclam) having non-adjacent nitrogens connected by an ethylene (CH_2CH_2) bridge (Figure 1) have been reported by Weisman and Wong (1–3). These ligands were designed to form complexes with small metal ions such as Cu(II), but are also remarkably efficient proton sponges due to their ability to adopt low-energy conformations having all four nitrogen lone pairs convergent upon a three-dimensional cleft (1). An extended series of related Cu(II) complexes of these ligands has been synthesized (2), including those featuring carboxymethyl arms that serve to fully envelop the six-coordinate cation and neutralize its dicationic charge (3). Additional Cu(II) complexes of ethylene cross-bridged ligands reported by Busch (4–7), Springborg (8–11), and Micheloni (12–16) have investigated

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Cu(II) complexes of related bicyclic ligands having other bridging units. Springborg has thoroughly reviewed the coordination chemistry of bicyclic tetraaza ligands (17).

Copper-64 has favorable properties as a radionuclide for use in both positron emission tomography (PET) imaging and targeted radiotherapy due to its half-life ($t_{1/2} = 12.7$ h), its decay characteristics (β^+ (19%); β^- (39%)) and its compatibility with large-scale production at high specific activities using a biomedical cyclotron (18). Increased use of ^{64}Cu and other copper radioisotopes in nuclear medicine applications has resulted in a need for copper chelators with high stability against metal loss (19–23). Development of optimal chelators for copper is of considerable importance when designing systems for the *in vivo* delivery of copper radioisotopes (21,23–25).

1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) has been extensively used as a BFC for copper radionuclides in clinical imaging and therapy studies involving both antibodies and peptides (Figure 1) (19,20). Seminal work performed by Moi, Denardo, Meares, and co-workers pursued the development of TETA-based bifunctional chelators for attaching the low-energy β -emitting isotope ^{67}Cu to monoclonal antibodies for targeted radioimmunotherapy of lymphoma (26–29). Despite widespread use, TETA is not an optimal BFC for biomedical applications, as Anderson and co-workers have demonstrated the dissociation of ^{64}Cu from TETA-D-Phe¹-octreotide (TETA-OC) in rat liver and subsequent binding to superoxide dismutase (SOD) (25,30). Copper complexes of the cross-bridged cyclam derivative CB-TE2A (Figure 1), were shown to possess superior *in vivo* stability over TETA (25), and a ^{64}Cu -labeled peptide-conjugate of CB-TE2A demonstrated superior imaging characteristics over the corresponding TETA-peptide conjugate (31). However, one of the two metal coordinating carboxylic acid arms was sacrificed in order to achieve CB-TE2A peptide conjugation (31), leaving the Cu(II) in a less than ideal coordination geometry as both carboxylic acid moieties would be necessary in order to fully saturate the coordination sphere of the six-coordinate cation and neutralize its +2 charge (3).

A recent investigation by Sprague and co-workers evaluated both the structure and *in vivo* behavior of 4-acetamido-11-carboxymethyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TEAMA), a hybrid monoamide analog of CB-TE2A, as a model for a CB-TE2A-peptide conjugate (32). X-ray crystallography studies have revealed a distorted octahedral coordination geometry for both Cu(II)-CB-TE2A³ and Cu(II)-CB-TEAMA (32), with coordination from the carbonyl oxygen of the amide pendant arm in the latter case (32). Interpretation of the corresponding *in vivo* data for ^{64}Cu -CB-TEAMA, however, must be approached with great caution due to differences in complex lipophilicity ($\log P$) as well as the potential scenario (proposed by the authors) of hepatic enzymatic hydrolysis of ^{64}Cu -CB-TEAMA to ^{64}Cu -CB-TE2A, secretion of the radio-metabolite(s) into blood, and subsequent clearance via the kidneys (32). In any event, an orthogonal bifunctional chelator having two pendant negatively charged carboxylic acid groups and a third arm for the sole purpose of conjugation would (1) saturate Cu(II)'s coordination sphere while neutralizing its dicationic charge and (2) simplify conjugation chemistry by providing a selective site for peptide conjugation. The current study addresses these issues through the synthesis and development of a novel cross-bridged bifunctional chelator *via* a mono-pendant-armed cross-bridged tetraamine intermediate. This approach allows greater control of conjugation chemistry and may also serve to enhance metal complex *in vivo* stability in diagnostic and therapeutic applications involving copper radionuclides.

EXPERIMENTAL SECTION

General Methods

^1H NMR and ^{13}C NMR spectra were obtained using a Varian Gemini 300 MHz instrument, and the chemical shifts are reported in ppm on the δ scale relative to internal TMS or TSP. Proton chemical shifts are annotated as follows: ppm (multiplicity, integration). Resonance assignments are established with the aid of NOESY, COSY, or HETCOR. Electrospray ionization time-of-flight-mass spectra were obtained on a Waters LCT Premier Time-of-Flight Mass Spectrometer using electrospray ionization (ESI/TOF/MS) operated in positive ion mode. The electrospray capillary voltage was 3 kV, and the sample cone voltage was 60 V. The desolvation temperature was 225 °C, and the desolvation nitrogen gas flow rate was 300 L/h. Accurate masses were obtained using the lock spray mode with Leu-Enkephalin as the external reference compound. Fast-atom bombardment (FAB) mass spectra were run on a VG 7070E-HF double-focusing mass spectrometer and were obtained in positive ion mode except where noted. A sample matrix of either glycerol (GLY) or 3-nitrobenzyl alcohol (NBA) was employed and ionization was effected by a beam of xenon atoms generated in a saddle-field ion gun at 8.0 ± 0.5 kV. Nominal mass MS were obtained at a resolution of 1200, while accurate mass analysis (high resolution FAB/MS) was carried out at a resolution of approximately 5000. For the latter, a limited-range V/E scan was employed under control of a MASPEC-II data system for Windows (MasCom GmbH, Bremen, Germany). For these analyses, 1.0 μL 1.0 N KCl in 90% methanol/ H_2O was added to the sample before mixing with the NBA matrix so that the matrix-derived ions, utilized as the internal mass references for accurate mass determinations, also included K^+ adducts. In these analyses, molecular identity could usually be confirmed by formation of a $[\text{M}+\text{K}]^+$ species. Both ^1H and ^{13}C NMR data, in conjunction with the observed isotopic distribution of $[\text{M}+\text{H}]^+$, were used to set constraints for the calculation of all possible elemental compositions within 20 ppm of the measured accurate mass. In all cases, a unique molecular formula could be determined by consideration of the molecular ion species and appropriate fragment ions. Positive ion MALDI mass spectra were obtained on a Shimadzu-Biotech Axima-CRF time-of-flight mass spectrometer using α -cyano-4-hydroxycinnamic acid as the sample matrix. Peptide standards of appropriate molecular weight to bracket the mass of the sample were used to provide internal mass reference peaks for accurate mass measurement. In all cases, the number of significant figures reported is determined by the experimental precision expressed in ppm and is related to the resolution and performance characteristics of the instrument used as well as the internal mass references employed in accurate mass measurement.

Semi-preparative reversed-phase HPLC was performed using two Gilson Model 303 pumps, a Gilson 803C manometric module, a Gilson 811B dynamic mixer, a Knauer ultraviolet detector, and an INUS Ram radioactive detector all connected through a Gilson 506C system interface module and operated by UniPoint version 1.65 software. Analytical HPLC of synthetic products **4**, **5**, **6**, and all peptide conjugates was achieved with a Hamilton Prp-1 polymer reversed-phase semi-preparative column (10 μm , 7.0 mm $\times$ 30.5 cm) using a flow rate of 3 mL/min; purification of all peptide conjugates was performed using identical conditions. Size-exclusion HPLC (SE-HPLC) was performed using the same system in a single pump mode using a Tosohaas G3000SW, 10 μm , 7.8 mm $\times$ 30 cm column with phosphate buffered saline solution as the eluent (0.5 mL/min).

Materials

Solvents were used as purchased. *Tert*-butyl bromoacetate, *N*-hydroxysuccinimide, and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), peptide sequence grade trifluoroacetic acid were purchased from the Aldrich Chemical Company. Sodium borohydride powder and 10% Pd/C were purchased from Lancaster Synthesis, Incorporated.

CB-cyclam (**3**) and 2-bromo-pentanedioic acid 5-benzyl ester 1-*tert*-butyl ester (**33**) were prepared as reported in the literature. The integrin $\alpha_v\beta_3$ -binding peptide c(RGDfK(S)) was purchased from Peptides International. All experiments with moisture- and/or air-sensitive compounds were carried out under a dry Ar atmosphere. For column chromatography, Merck 60 Silica Gel (70–230 mesh) was used. Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates from EM Reagents. All water used was purified using a Hydro Ultrapure Water Purification system.

2-(1,4,8,11-Tetraaza-bicyclo[6.6.2]hexadec-4-yl)-pentanedioic acid 5-benzyl ester 1-*tert*-butyl ester (3**)**

To a solution of CB-cyclam (**1**) (1 g, 4.42 mmol) in anhydrous CH₃CN was added K₂CO₃ (0.914 g, 6.61 mmol). The solution was shielded from light, and a solution of 2.36 g (6.63 mmol) of 2-bromo-pentanedioic acid 5-benzyl ester 1-*tert*-butyl ester in CH₃CN was added drop-wise *via* an addition funnel over 1 h with constant stirring. The resulting mixture was allowed to react for 24 hr in darkness under argon with constant stirring. Excess salt was removed by filtration and solvent removed by rotary evaporation. The resulting residue was dissolved in minimal CH₂Cl₂ and subjected to flash chromatography using a slowly increasing gradient from 100% CH₂Cl₂ to 15% MeOH in CH₂Cl₂. The relevant fractions were identified by ¹H NMR spectroscopy, combined, and solvent removed (0.43 g of protonated (HBr) product). The resulting residue was dissolved in CHCl₃, chilled, and quickly extracted once with 20% NaOH (aq) at 0 °C. The organic phase was dried with Na₂SO₄ and solvent removed to yield **3** as a pale yellow oil (0.36 g, 16.2% yield). ¹H NMR (300 MHz, CDCl₃, TMS) δ 1.45 (s, 9H), 1.38–1.62 (m, 7H), 1.80–1.96 (m, 1H) 1.97–2.12 (m, 1H), 2.13–2.24 (m, 2H), 2.28–3.40 (m, 16H), 3.12–3.34 (m, 2H), 4.78–4.98 (br s, 1H), 5.12 (s, 2H), 7.33–7.37 (m, 5H); ¹³C NMR (75 Hz, CDCl₃, TMS) δ 25.08, 25.91, 27.46, 28.26, 31.36, 48.63, 48.68, 48.86, 49.17, 51.74, 53.89, 54.05, 57.65, 58.88, 60.17, 61.63, 66.12, 80.79, 128.12, 128.45, 135.88, 172.22, 172.85. High resolution ESI/TOF/MS: [M+H]⁺ for C₂₈H₄₇N₄O₄ 503.3597, found, 503.3604.

2-(11-*tert*-Butoxycarbonylmethyl-1,4,8,11-tetraaza-bicyclo[6.6.2]hexadec-4-yl)-pentanedioic acid 5-benzyl ester 1-*tert*-butyl ester (4**)**

To a solution of **3** (0.180 g, 0.360 mmol) in dry CH₃CN was added K₂CO₃ (0.0494 g, 0.357 mmol) and *tert*-butyl bromoacetate (0.070 g, 0.359 mmol) in one portion. The mixture was constantly stirred in darkness for 24 h under argon. Salts were removed by filtration, and the solvent removed by rotary evaporation. The residue was placed under reduced pressure to give the HBr salt of **4** with no further purification necessary to proceed to the next step in the synthesis. For characterization, the residue was dissolved in CHCl₃ and quickly extracted once with 20% NaOH (aq) at 0 °C. The organic phase was dried with Na₂SO₄ and solvent removed to yield **4** as a pale yellow oil (0.21 g, 95.0% yield). ¹H NMR (300 MHz, CDCl₃, TMS) δ 1.45 (d, 18H), 1.22–1.52 (m, 7H), 1.78–1.92 (m, 1H) 1.93–2.10 (m, 1H), 2.30–2.25 (m, 22H), 5.12 (s, 2H), 7.27–7.37 (m, 5H); ¹³C NMR (75 Hz, CDCl₃, TMS) δ 26.14, 27.84, 27.88, 28.32, 28.44, 31.45, 51.13, 51.38, 52.83, 53.01, 55.99, 56.39, 56.57, 57.69, 59.13, 59.18, 61.72, 66.23, 80.43, 80.73, 128.24, 128.26, 128.59, 136.08, 171.80, 172.62, 173.20. High-resolution FAB/MS: [M+H]⁺ for C₃₄H₅₇N₄O₆ 617.4281, found, 617.4276.

2-(11-*tert*-Butoxycarbonylmethyl-1,4,8,11-tetraaza-bicyclo[6.6.2]hexadec-4-yl)-pentanedioic acid 1-*tert*-butyl ester (5**)**

To a solution of **4** (0.350 g, 0.568 mmol) in dry MeOH (15 mL) was added 88% HCO₂H (0.0594 g, 1.3 mmol) followed by 0.10 g of Pd/C (Degussa type E101 NE/W). The round bottom flask with magnetic stir bar was sealed using a rubber septum. The flask was evacuated and recharged three times using a balloon filled with H₂ gas. The mixture was maintained under the balloon of H₂ for 4 h with constant stirring. The mixture was filtered through Celite 535

pre-wetted with MeOH, and solvent removed to yield **5** as a pale yellow solid (0.300 g, >99% yield). ^1H NMR (300 MHz, CDCl_3 , TMS) δ 1.46 (d, 18H), 1.45–2.11 (m, 7H), 2.48–3.59 (m, 22H), 3.90–4.08 (br m, 1H), 4.10–4.80 (br s, 2H); ^{13}C NMR (75 Hz, CDCl_3 , TMS) δ 22.77, 24.96, 27.81, 27.87, 31.21, 47.82, 48.25, 49.46, 50.20, 50.67, 52.89, 55.39, 55.85, 58.10, 59.24, 61.75, 81.94, 81.76, 169.98, 171.10, 174.96. High-resolution FAB/MS: $[\text{M}+\text{H}]^+$ for $\text{C}_{27}\text{H}_{51}\text{N}_4\text{O}_6$ 527.381, found 527.3794.

2-(11-*tert*-Butoxycarbonylmethyl-1,4,8,11-tetraaza-bicyclo[6.6.2]hexadec-4-yl)-pentanedioic acid 5-*tert*-butyl ester 1-(2,5-dioxo-pyrrolidin-1-yl) ester (6**)**

To a solution of **5** (0.100 g, 0.190 mmol) in dry CH_3CN was added *N*-hydroxysuccinimide (0.026 g, 0.23 mmol) and EDCI (0.044 g, 0.23 mmol) in one portion. The resulting solution was maintained under argon for 10 h with constant stirring. Solvent was removed under reduced pressure, and the residue was dissolved in CHCl_3 . The solution was extracted twice with brine and once with water. Solvent was removed from the organic phase by rotary evaporation to yield **6** as a pale yellow solid (0.102 g, 86.1% yield). ^1H NMR (300 MHz, CDCl_3 , TMS) δ 1.41 (d, 18H), 1.50–2.15 (m, 5H), 2.40–3.65 (m, 28H), 3.65–3.80 (m, 1H), 3.80–4.00 (m, 1H); ^{13}C NMR (75 Hz, CDCl_3 , TMS) δ 22.80, 23.23, 25.59, 25.60, 25.73, 28.13, 28.21, 28.32, 48.14, 48.38, 50.21, 50.42, 51.23, 53.55, 53.70, 55.54, 55.95, 56.43, 57.62, 59.89, 61.47, 82.15, 82.36, 168.20, 169.22, 170.40, 170.70. High-resolution FAB/MS: $[\text{M}+\text{H}]^+$ for $\text{C}_{31}\text{H}_{54}\text{N}_5\text{O}_8$ 624.3973, found 624.3941; $[\text{MH}-\text{C}_4\text{H}_3\text{O}_2\text{N}]^+$ 527.383, found 527.381; $[\text{MH}-2\text{C}_4\text{H}_8]^+$ 512.271, found 512.272.

2-(11-Carboxymethyl-1,4,8,11-tetraaza-bicyclo[6.6.2]hexadec-4-yl)-pentanedioic acid 5-benzyl ester (7**)**

Neat trifluoroacetic acid (15 mL) was added to **4** (0.100 g, 0.162 mmol) in a round bottom flask. The mixture was stirred for 10 h under argon. Excess acid was removed by rotary evaporation. The residue was dissolved in CH_2Cl_2 (20 mL) and solvent removed by rotary evaporation; this process was repeated exhaustively to remove residual acid. The resulting yellow oil was triturated with Et_2O and by submerging the flask into an ultra-sonicating bath. Upon solidification, the solid was allowed to settle, and the ethereal wash was carefully pipetted away from the product. Residual solvent was removed to give a hygroscopic pale yellow solid **7** (0.109 g, 91.6% yield including 1.5 associated TFA molecules and 1 H_2O). ^1H NMR (300 MHz, CD_3CN) δ 1.60–2.35 (m, 8H), 2.45–3.40 (m, 20H), 3.40–3.85 (m, 3H), 5.24 (s, 2H), 7.48–7.53 (m, 5H), 9.00–9.60 (br s, 2H); ^{13}C NMR (75 Hz, CD_3CN) δ 23.11, 24.53, 25.16, 32.33, 48.79, 49.61, 51.49, 52.75, 53.98, 56.74, 57.84, 58.22, 60.24, 65.03, 67.35, 116.13, 118.69, 120.00, 129.50, 129.92, 137.84, 161.25, 172.83, 173.96, 174.11. Elemental analysis calculated for $\text{C}_{26}\text{H}_{40}\text{N}_4\text{O}_6\text{w}(\text{TFA})_{1.5}(\text{H}_2\text{O})$: C, 50.20; H, 6.32; N, 8.08, found: C, 49.95; H, 6.09; N, 7.99.

CB-TE2A di-*tert*-butyl ester-(propionamide linker)-c(RGDfK(S))

To c(RGDfK(S)) (0.025 g, 0.036 mmol) in DMF (250 μL) was added **6** (0.0282 g, 0.045 mmol) in one portion. The reaction mixture was stirred at 25 $^\circ\text{C}$ for 16 h under argon. The mixture was subjected to purification by semi-preparative reversed-phase HPLC using a gradient from 100% 0.1% TFA in H_2O (pH 2.5) to 60% 0.1% TFA in CH_3CN over 30 min. Solvent was removed under reduced pressure, and the remaining aqueous solution was lyophilized to give fluffy white solid CB-TE2A di-*tert*-butyl ester-(propionamide linker)-c(RGDfK(S)) (0.017 g, 39.2% yield). ^1H NMR (300 MHz, D_2O , TSP) δ 0.95–1.05 (br s, 3H), 1.12–2.05 (m, 7H), 1.53 (d, 18H), 2.18–2.40 (br s, 4H), 2.40–2.65 (br s, 3H), 2.75–2.90 (m, 2H), 2.90–4.00 (m, 33H), 4.15–4.25 (m, 1H), 4.30–4.8 (m, 5H), 7.18–7.45 (m, 5H). High resolution MALDI/MS: $[\text{M}+\text{H}]^+$ for $\text{C}_{57}\text{H}_{95}\text{N}_{14}\text{O}_{14}$ 1199.72, found 1199.74.

CB-TE2A-(propionamide linker)-c(RGDfK(S))

Neat trifluoroacetic acid (15 mL) was added to CB-TE2A di-*tert*-butyl ester-(propionamide linker)-c(RGDfK(S)) (0.012 g, 0.010 mmol), and the reaction was stirred at 25 °C under for 16 h under argon. Excess acid was removed by rotary evaporation. The residue was dissolved in CH₂Cl₂ (20 mL) of and solvent removed by rotary evaporation; this process was repeated exhaustively to remove residual acid. A 20-mL aliquot of Et₂O was added to the residue to induce solidification. Solvent removal under reduced pressure gave 0.013 g (>99% assuming two associated TFA molecules) of fluffy white solid CB-TE2A-(propionamide linker)-c(RGDfK(S)). ¹H NMR (300 MHz, D₂O, TSP) δ 0.80–1.10 (br s, 2H), 1.30–2.00 (m, 12H), 2.22–2.45 (m, 3H), 2.45–2.60 (m, 2H), 2.65–2.85 (m, 3H), 2.90–4.00 (m, 33H), 4.00–4.30 (m, 2H), 4.35–4.45 (m, 2H), 4.65–4.75 (m, 1H), 7.20–7.45 (m, 5H). High resolution MALDI/MS: [M+H]⁺ for C₄₉H₇₉N₁₄O₁₄ 1087.59, found 1087.55.

Radiochemistry – Synthesis of ⁶⁴Cu-CB-TE2A-(propionamide linker)-c(RGDfK(S))

Caution: ⁶⁴Cu (t_{1/2} = 12.7 h) is a (β⁺ (19%); β[−] (39%)) emitting radionuclide. **Appropriate shielding and handling protocols should be in place when using this radionuclide**—⁶⁴CuCl₂ was purchased from MDS Nordion (Vancouver, BC, Canada). To CB-TE2A-(propionamide linker)-c(RGDfK(S)) (15 μg) in 0.1 M NH₄OAc (200 μL) (pH 8.0) was added ⁶⁴CuCl₂ (1 mCi in 0.1 M HCl). The reaction mixture was heated for 1 h at 95 °C. Unchelated ⁶⁴Cu was removed from ⁶⁴Cu-CB-TE2A-(propionamide linker)-c(RGDfK(S)) by ion exchange chromatography using Chelex-100 (100–200 mesh, Na⁺ form, Bio-Rad Labs, Hercules, CA). The radiochemical yield was 64%. To generate analytical radio-HPLC data, a portion of the purified radiolabeled peptide was subjected to reversed-phase HPLC using a gradient from 100% 0.1% TFA in H₂O (pH 2.5) to 60% 0.1% TFA in CH₃CN over 30 min. In addition, the resulting peak fraction was collected, frozen, lyophilized, and subjected to analytical radio-HPLC using the same column and flow rate but different solvent conditions: 0.15 M NH₄OAc (pH 7.0) with 0% to 80% MeOH over 30 min (Supporting Information).

Serum Stability

An aliquot of ⁶⁴Cu-CB-TE2A-(propionamide linker)-c(RGDfK(S)) (3 μCi) was added to 25 μL of human serum (NHS Gemini Cat# 100–110) or to 25 μL of saline in a 100 μL microcentrifuge tube for each time point. The samples were capped and placed in a 37 °C incubator. At the appropriate time points samples were analyzed by SE-HPLC. The % radioactivity associated with protein versus low-molecular weight species was determined by integration.

RESULTS AND DISCUSSION

Ligand Synthesis and Rationale

The overall synthetic approach is presented in Scheme 1, wherein the bifunctional chelator derivative of CB-TE2A is synthesized for the ultimate purpose of conjugation to peptides and subsequent targeted PET imaging of tumor receptors using ⁶⁴Cu. It should be noted, however, that targeted radiotherapy of small to medium tumor burden may also be realized through use of the low energy β-emitting sister isotope, ⁶⁷Cu (t_{1/2} = 61.92 h; β[−] (E_{β[avg]} = 141 keV; R_{mean} = 0.24 mm)). For the synthesis of a bifunctional chelator derivative of CB-TE2A compatible with solid-phase peptide synthesis, an orthogonally-protected, bromide-functionalized glutamic acid derivative (**2**) was synthesized as previously described by Mäcke and co-workers (33). Reaction of cross-bridged cyclam (**3**), **1**, with **2** resulted in the formation of the desired hybrid-arm construct (**3**) (Scheme 1). Our previous attempts at coupling **2** with the bisaminal resulting from condensation of cyclam with glyoxal were unsuccessful (data not shown). Reversing the sequence, alkylating **1** with **2** after reacting with *tert*-butyl

bromoacetate, was found to be less desirable (data not shown). Steric effects greatly reduced the reaction yield, and the initial alkylation results in a statistical mixture of products. Unlike the more statistical product distribution achieved by alkylation with *tert*-butyl bromoacetate, alkylation of **1** using **2** tends to greatly favor the 1:1 macrocycle-arm product, even in the presence of excess alkylating agent. Interestingly, the alkylation of **3** proceeds even in CHCl_3 without the use of a carbonate base (data not shown); however, use of K_2CO_3 in CH_3CN reduces the reaction time from 3 days to 1 day and increases the yield of **4**.

Debenzylation of **4** was achieved by catalytic hydrogenolysis in the presence of formic acid to yield mono-acid **5**. Our initial hydrogenation attempts in the absence of formic acid were completely unsuccessful, resulting in unexpected partial intramolecular transfer of the benzyl group to macrocyclic amines with concomitant formation of ethyl ester when the reaction was performed in EtOH (data not shown). This behavior was attributed to the remarkable basicity of the amines, and formic acid was chosen to acidify the reaction and to act as an additional hydrogen source for the hydrogenation. Subsequent reaction of **5** with NHS and EDCI gave the desired bifunctional chelating agent, succinimidyl ester **6**, ready for immediate conjugation to peptides. Purification of **6** was achieved by solvent extraction as described in the Experimental Methods section. The purity of this ester was acceptable by NMR and HPLC; therefore, no further purification was pursued. We observed a broad peak at ~10 ppm in the ^1H NMR spectrum of **6**; a similar peak was also observed for the HBr salts of **3** and **4** following column chromatography but prior to basic extraction into CHCl_3 (data not shown). We therefore attribute this peak to protonation of these remarkably basic amines. We are unsure of whether the proton is inside the 'clamshell' cavity or not, but this is a possibility. Following the basic extraction workup of **3** and **4**, the peak at ~10 ppm was not present (Supporting Information). Unfortunately, it is impossible to use extraction to deprotonate **5** and **6** due to a polar carboxylic acid and a base-sensitive succinimidyl ester, respectively.

Compound **7** was synthesized for two reasons: (1) to further demonstrate the orthogonality of our protection scheme and (2) in hopes that crystals of Cu(II)-7 might be obtained for structure determination. Successful deprotection of the *tert*-butyl esters using neat TFA was achieved with retention of the benzyl protection on the third pendant carboxylic acid arm. Unfortunately, all attempts at forming Cu(II)-7 for crystallization were thwarted by partial benzyl deprotection due to the requisite complexation conditions, namely, the addition of NaOH followed by reflux in ethanol.

We have previously used a similar approach to synthesize a bifunctional chelator derivative of the acyclic ligand, CHX-A''-DTPA , that is likewise compatible with off-line tagging of peptides following Fmoc solid-phase peptide synthesis (34). Synthesis of a carbon backbone-functionalized isothiocyanate-bearing bifunctional chelator derivative of CB-TE2A has been reported by Archibald and co-workers (35,36). This work involved a relatively high-yielding 6-step synthesis of a C-functionalized nitrobenzyl bisaminal intermediate (35), that was further reacted to a diacid, mono-isothiocyanate bifunctional chelating agent in 7 additional steps (*ca.* 20% overall yield from the bisaminal) followed by conjugation to biotin (36). While isothiocyanate derivatives of chelators are routinely used by our lab and others for antibody/protein conjugation, the orthogonally protected bifunctional chelator presented here may offer advantages in peptide synthesis studies due to the clean reactions afforded by succinimidyl ester coupling chemistry. In addition, fewer synthetic steps are involved in the current approach: only 4 steps from **1**, which is accessible from commercially available cyclam in only 4 steps (3). The only major disadvantage of the current approach is the low yield obtained for the coupling reaction of CB-cyclam to the bifunctional glutamic acid-derived arm (*ca.* 16%). This low yield reflects the necessity of column chromatography to separate the desired product **3** from a structurally related analog that is also formed during the alkylation (data not shown). This side product is a quaternary ammonium compound having the same mass where alkylation

on one of the bridgehead (tertiary) amines instead of on a secondary (NH) amine has occurred. Formation of significant amounts of this product is attributed to steric factors as the alkylating agent **2**, a secondary bromide, is somewhat bulky.

Peptide Conjugation, Radiolabeling with ^{64}Cu , and Serum Stability Studies

To validate the utility of our agent, we chose to conjugate our novel bifunctional cross-bridged cyclam chelator **6** to a cyclic RGD peptide, c(RGDfK(S)) (Scheme 2). The peptide-chelator conjugate readily formed in DMF and, following chromatographic purification, was characterized by NMR spectroscopy (Figure 2). This conjugate was amenable to acid deprotection of the *tert*-butyl esters and subsequent radiolabeling with ^{64}Cu (Scheme 2) as demonstrated by the ^1H NMR spectra (Figure 2) and HPLC data (see Figure 3 and Supporting Information), respectively. In addition, *in vitro* evaluation of the radiolabeled peptide conjugate in human serum demonstrated a stably complexed radiocopper ion with no evidence of transchelation of ^{64}Cu into serum proteins for up to 48 h (Supporting Information).

The radiochemical yield obtained was 64%. We did not attempt to optimize the radiolabeling since our overall goal in demonstrating the conceptual functionality of our bifunctional chelator was achieved. The incorporation of ^{64}Cu into our peptide conjugate was lower than previously reported for a CB-TE2A-somatostatin analogue under the same conditions (31). Many factors may have contributed to our lower yield, including the nature of the peptide (structure, solubility, etc.), the structural difference in our novel chelate relative to the parent CB-TE2A and possible non-covalent intramolecular interactions between the chelate and peptide.

Identical radiolabeling conditions (1 hour, pH 8, 95 °C) were previously used to radiolabel the CB-TE2A conjugate of a somatostatin peptide analogue (31). Although many immunoglobulins can tolerate pH 8 and even slightly more alkaline conditions, this temperature requirement unfortunately precludes use of cross-bridged cyclam chelators for conjugation and subsequent radiolabeling of antibodies and other heat-sensitive biological macromolecules. Using our bifunctional chelator, however, it may be possible to first form the ^{64}Cu complex using the two closest free acids and then activate the third, more distant carboxylate group unused for Cu chelation for subsequent coupling to proteins; the possibility of this approach warrants further investigation.

In vitro evaluation of the radiolabeled peptide conjugate in human serum demonstrated a stably complexed radiocopper ion with no evidence of transchelation of ^{64}Cu into serum proteins for up to 48 h (Supporting Information). This is consistent with *in vitro* stability data for the structurally-related complex, ^{64}Cu -CB-TE2A, which showed no evidence of transchelation of ^{64}Cu into serum proteins for up to 24 h (37). A true test of *in vivo* kinetic stability, however, would require future *in vivo* metabolism studies to test for radiometal transchelation into blood and liver proteins. For example, previous studies within our laboratory have revealed that ^{64}Cu -TETA exhibits high *in vitro* serum stability (38); however, the situation for ^{64}Cu -TETA *in vivo* is much different in that transchelation of ^{64}Cu to superoxide dismutase and other proteins has been reported (25,30,39).

CONCLUSIONS

A cross-bridged BFC derivative of CB-TE2A has been synthesized in 4 steps with reasonable (*ca.* 13 %) overall yield from CB-cyclam. The novel agent is directly structurally analogous to the parental CB-TE2A in that it possesses the identical ligand donor set arranged in the same geometry for high kinetic stability with Cu(II) radionuclides, while also featuring a third orthogonally-protected arm for conjugation to peptides and other targeting agents. This work may also enable the development of improved agents for diagnostic positron emission tomography (PET) imaging as well as for targeted radiotherapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of cyclam, TETA, CB-cyclam, CB-TE2A, and 6.

Figure 2. ^1H -NMR spectra in D_2O of (a) c(RGDfK(S)) , (b) $\text{CB-TE2A-c(RGDfK(S))}$ di-*tert*-butyl ester and (c) $\text{CB-TE2A-c(RGDfK(S))}$ after TFA deprotection.

Figure 3.

Radio-HPLC chromatogram of ^{64}Cu -CB-TE2A-c(RGDfK(S)) including the radioactive trace (top) and the corresponding UV trace due to CB-TE2A-c(RGDfK(S)) (bottom) on a C18 column at 3 mL/min flow rate with the following gradient: 0.1% TFA in H₂O with 0% to 60% 0.1% TFA in CH₃CN over 30 min.

Scheme 1.
Synthetic route to the cross-bridged cyclam bifunctional chelator.

Scheme 2.

Conjugation of cyclic peptide, c(RGDfK(S)) , to the succinimidyl ester **6** and subsequent TFA deprotection of the resulting peptide-chelate conjugate.