C-Substituted Macrocycles as Candidates for Radioimmunotherapy

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The reaction between aryl aldehydes, the macrocyclic ligand 6-methyl-1,4,8,11-tetraazacyclotetradecane-6-amine (L^1), and $NaBH_3CN$ produces the corresponding benzyl-substituted ligands in good yield. Copper(II) complexes of the ligands derived from salicylaldehyde (L^2), *p*-hydroxybenzaldehyde (L^4), and *p*-carboxybenzaldehyde (L^5) were structurally characterized: $[CuL^2](ClO_4)_2 \cdot 3H_2O$ (monoclinic, $P2_1/c$, $a = 11.915(6)$ Å, $b = 13.861(2)$ Å, $c = 17.065(8)$ Å, $\beta = 102.14(2)°$, $Z = 4$); $[CuL^4](ClO_4)_2$ (monoclinic, $P2_1/n$, $a = 9.550(3)$ Å, $b = 17.977(2)$ Å, $c = 14.612(4)$ Å, β 96.76(1)°, $Z = 4$), and $[CuL^4](ClO_4)_2$ (monoclinic, $P2_1/n$, $a = 9.286(2)$ Å, $b = 11.294(1)$ Å, $c = 23.609(8)$ Å, β 93.68(1)°, $Z = 4$). Conjugation of several Cu^{II} complexes to a protein (bovine serum albumin) has been pursued with a view to the application of these macrocycles as bifunctional chelating agents in radioimmunotherapy.

Introduction

The application of immunological methods to cancer therapy has undergone intensive investigation in an attempt to achieve tumor-specific treatments.[1] Some of the technical difficulties in the use of antibodies (Abs), which impeded progress in this area, were overcome by the advent of monoclonal antibody (mAb) production by in vitro hybridization methods in 1975.[2] A number of antibodies have been found that localize to cancerous cells. Tumor-associated monoclonal antibodies are potential therapeutic or diagnostic agents of malignancies when used as selective carriers of cytotoxic or imaging reagents. Antibodies have been conjugated with drugs, toxins, or isotopes.[3] In the early 1950s, Pressman proposed that antibodies could be tagged with radioisotopes and the antibody could be the carrier to take the cytotoxic radiation source directly to the tumor.[4] The goal was greater selectivity with extended retention of the labeled antibody in the tumor for delivery of a higher dose to the tumor than to normal tissues.

The isotope ^{131}I has been the most utilized in clinical and preclinical radioimmunotherapy (RAIT) studies,[5] but with modest success.[6] This is thought to be due to the low dose and dose rates from the low-energy β-emitter. Iodination of tyrosine residues is the most common mode of radiolabeling for ^{131}I, but this can cause loss or impairment of the biological function of proteins, particularly when they are labeled at high specific activities.[7] Furthermore, directly radioiodinated mAbs, once localized to tumor tissue, are often only poorly retained by the tumor cells because of rapid endocytosis, intracellular degradation, and expulsion of low molecular weight ^{131}I metabolites.[8] This may lead to uptake in the thyroid, gut, and salivary glands, delivering undesirable nonspecific radiation doses. The disadvantages of ^{131}I as a therapeutic radioisotope have led to the investigation of other isotopes, including the γ-emitters ^{99m}Tc[9,10] and ^{111}In[11] for tumor imaging, and β-emitters such as ^{90}Y,[12,13] ^{186}Re, ^{188}Re,[14,15] ^{177}Lu,[16] and ^{67}Cu,[17,18] as well as α-emitters ^{212}Bi,[19,20] ^{212}Pb,[21,22] and ^{211}At.[23] The desirable properties of radionuclides for RAIT have been discussed in several reviews.[5,24,25]

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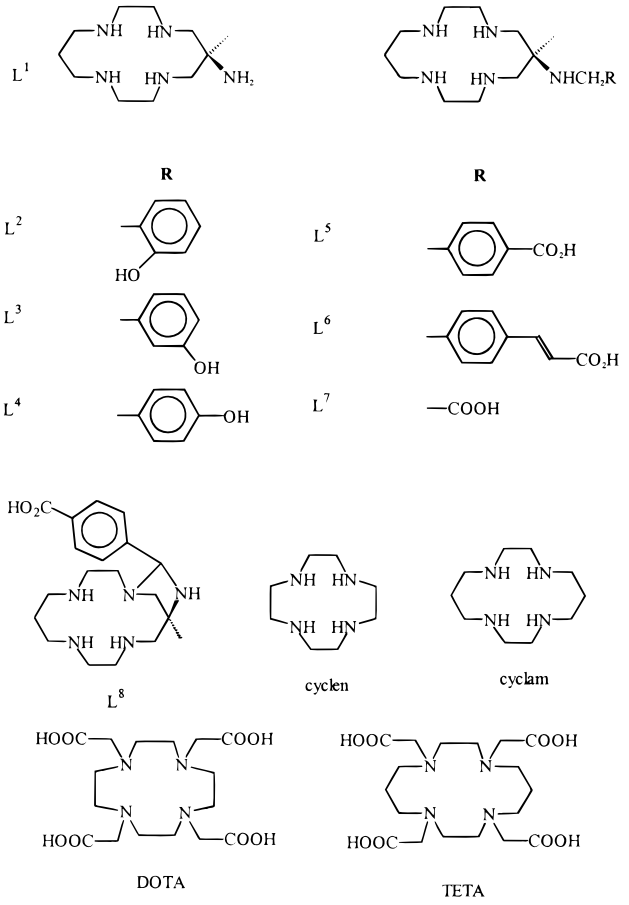
Two copper isotopes are of particular interest from the viewpoint of RAIT.[26,27] ^{64}Cu undergoes electron capture (41%), in addition to β^- (40%) and β^+ (19%) decay. It also has an Auger electron emission with therapeutic potential. The isotope has a half-life of 12.7 h, which is compatible with antibody pharmacokinetics, and its potential use in radioimmunotherapy has been demonstrated.[28,29] ^{67}Cu, with a half-life of 62 h, is the longest-lived radionuclide of copper and decays by β^- emission, accompanied by γ-ray emission at three different energies. These γ-rays are suitable for single photon imaging of the radionuclide distribution during therapy.[30] For Cu radionuclides, the use of a bifunctional chelating agent is necessary,[31] which can bind the metal ion rapidly, strongly, and selectively, while also covalently attached to a protein. There are a number of different methods available for the linkage of chelating ligands to proteins,[32] and radiolabeling may occur prior to or after conjugation of the ligand to the protein. Linear polyaminocarboxylate ligands (e.g., EDTA) display insufficient kinetic stability in vivo, and this has led to the use of macrocyclic ligands because of their increased thermodynamic stability and slower proton- and cation-assisted decomplexation kinetics.[26] Alkylation of the macrocyclic secondary amines is often synthetically simpler than the synthesis of C-substituted macrocycles,[33] and the carboxylate-substituted tertiary amines DOTA[34] and TETA[35] have been explored as candidates for radioimmunotherapy. Complexes of sterically crowded tertiary amine macrocycles are less stable than those of unsubstituted secondary amine analogues,[36] although coordination of carboxylate groups attached to the tertiary amines may offset this stability penalty.[37] The Cu^{II} complexes of DOTA and TETA (and their analogues) are anionic, which assists cation-associated decomplexation reactions. By contrast, unsubstituted or C-functionalized cyclam complexes of Cu^{II} are cationic and resist demetalation down to pH 1. Existing routes to functionalized macrocycles for use in RAIT are typically involved and expensive. There is a need for cheaper, more accessible macrocyclic agents. It was thus decided to pursue the C-functionalized cyclam derivatives shown in Chart 1 (L^2−L^7) and to assess their suitability for modification and attachment to proteins.

Chart 1



Experimental Section

Syntheses. L^1·5HCl[38] and (*E*)-4-(2′-carboxyallyl)benzaldehyde (*trans*-4-formylcinnamic acid)[39] were synthesized using literature procedures. The synthesis of β-[CuL^7](ClO_4)$_2$ has been reported in a separate publication.[40] All other chemicals were obtained commercially and were not purified further.

SAFETY NOTE. Although no problems were experienced with the new compounds described, perchlorate salts are potentially explosive. They should not be heated in the solid state nor scraped from glass frits with metal spatulas.

[CuL^2](ClO_4)$_2$. L^1·5HCl (2.0 g) was reacted with 5 equiv of Et_3N (3.3 mL) and 1 equiv of salicylaldehyde (0.56 g) in absolute EtOH (100 mL) under reflux for 1 h. The mixture was cooled to room temperature, and excess $NaBH_4$ (0.5 g) was added directly in small portions with stirring. The mixture was stirred for 1 h at room temperature; then water (100 mL) was added to hydrolyze any unreacted BH_4^-. Solid $Cu(NO_3)_2$·$3H_2O$ (1.1 g) was added; the solution was filtered and then sorbed onto a Sephadex C-25 cation exchange column (Na^+ form). A blue compound, present as a minor product, failed to adhere to the ion exchange column and was washed from the column with water.

Elution was then commenced with a 0.2 M $NaClO_4$ solution, adjusted to pH 8 with Na_2CO_3. A minor band eluted rapidly from the column (λ_{max} 530 nm) and was discarded. This was followed closely by the major band (λ_{max} 510 nm) containing the desired product. The major band was reduced in volume on the rotary evaporator until a pink-colored powder began to precipitate from the solution. This was collected by filtration and washed with two 5 mL portions of EtOH and one 5 mL portion of diethyl ether. The solid was air-dried (yield = 1.74 g, 30.0%). Red X-ray quality crystals were grown by the diffusion of diethyl ether into a saturated acetonitrile solution of the complex. (Found: C, 36.15; H, 5.6; N, 11.61. Calcd for $C_{18}H_{33}N_5Cl_2O_9Cu$: C, 36.10; H, 5.72; N, 11.69.) UV/vis (H_2O): λ_{max} (ϵ M^{-1}

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cm^{-1}) 505 nm (96.2), 261 (7540), shoulder 219 (7690). The remaining slow-moving purple bands on the column consisted of N-based isomers of $[CuL^1]^{2+}$.

L^2. Ca. $[CuL^2](ClO_4)_2$ (0.1 g) was dissolved in 1 mL of D_2O. An excess of $Na_2S{\cdot}9H_2O$ was added and the solution heated gently. The solution was filtered twice and used directly for NMR spectroscopy. ^1H NMR (D_2O): 1.00 (s, 6H), 1.54 (mult, 2H), 1.93−1.99 (mult, 2H), 2.30−2.60 (mult, 14H), 3.46 (s, 2H), 6.35−6.50, 6.90−7.05 (mult, 4H). ^{13}C NMR: 21.1, 27.3, 41.5, 45.4, 46.3, 46.8, 54.8, 54.9, 113.8, 118.9, 128.5, 128.7, 129.8, 165.0.

General Procedure for Reductive Alkylation Using $NaBH_3CN$. $L^1{\cdot}5HCl$ (4.80 g, 11.6 mmol) was dissolved in 100 mL of H_2O. The pH was adjusted to 6.50 with Na_2CO_3. The aldehyde was added (11.6 mmol, 1 equiv), followed by 100 mL of EtOH to enable dissolution (if necessary). The pH was readjusted to 6.50. $NaBH_3CN$ (4.08 g, 65 mmol) was added, the flask was sealed, and the solution was stirred at room temperature for 5 days. EtOH was removed on the rotary evaporator. An excess of $CuCl_2{\cdot}2H_2O$ was added to the aqueous solution, which was stirred for 10 min. A cyanoborohydride salt of the copper complex precipitated, which was filtered off and suspended in 100 mL of H_2O. The pH was adjusted to 1 with concentrated HCl, and the solution was refluxed for 2 h in a well-ventilated fumehood to decompose the cyanoborohydride anion (**CAUTION**: HCN liberated). This solution was added to the filtrate obtained previously, and the combined solutions were diluted with water to a volume of 2 L. This diluted solution was applied to a Sephadex cation exchange column (Na^+ form). The elution conditions are listed below for each product.

$[CuL^3](ClO_4)_2{\cdot}0.75H_2O$. The procedure outlined above was carried out using 3-hydroxybenzaldehyde. After complexation with copper, the solution was applied to a Sephadex cation exchange column. The column was washed with water, and a very small amount of blue solution failed to adsorb to the column. Elution was commenced with 0.2 M $NaClO_4$ solution, pH 8. A second minor product, pink in color, eluted from the column. The first two bands were not present in sufficient amounts to identify. A third band slowly eluted from the column, this being the major product, which was collected and reduced in volume on the rotary evaporator to ca. 50 mL. The product precipitated as a pink solid (yield 2.71 g, 38%). (Found: C, 35.28; H, 5.49; N, 11.06. Calcd for $C_{18}H_{33}N_5O_9Cl_2Cu{\cdot}0.75H_2O$: C, 35.36; H, 5.69; N, 11.45.) The remaining bands moved only slowly on the column and consisted of copper complexes of the starting ligand. UV/vis (H_2O): λ_{max} (ϵ M^{-1} cm^{-1}) 504 nm (81.4), 258 (7770), shoulder 219 (8610).

$[CuL^4](ClO_4)_2{\cdot}2.5H_2O$. The procedure outlined above was carried out using 4-hydroxybenzaldehyde. After complexation with copper, the solution was applied to a Sephadex cation exchange column. Elution commenced with 0.2 M NaCl solution (pH 8). Two minor bands eluted from the column. These were discarded. A third, purple band (λ_{max} 527 nm) eluted from the column. It was followed closely by a fourth, pink band. Both bands were evaporated to dryness on the rotary evaporator. EtOH (200 mL) was added to each, and precipitated NaCl was removed by filtration. The pink product was redissolved in ca. 500 mL of H_2O, reapplied to a Sephadex column, and eluted with aqueous $NaClO_4$ solution. The solution was concentrated to ca. 50 mL, and orange-pink clusters formed. (Found: C, 33.36; H, 5.57; N, 10.88. Calcd for $C_{18}H_{33}N_5O_9Cl_2Cu{\cdot}2.5H_2O$: C, 33.62; H, 5.96; N, 10.89.) UV/vis (H_2O): λ_{max} (ϵ M^{-1} cm^{-1}) 504 nm (89.5), 254 (7860), shoulder 221 (13000). X-ray quality crystals were grown by diffusion of ethyl acetate into an acetonitrile solution of the complex.

$[Cu(HL^5)](ClO_4)_3{\cdot}5.5H_2O$. The reaction described above was carried out with 4-carboxybenzaldehyde. The copper complexes were eluted from a Sephadex cation exchange resin using 0.2 M NaCl solution, adjusted to pH 8 with Na_2CO_3, as the eluting solution. Three minor bands eluted rapidly from the column, but were not present in sufficient quantities to isolate. The fourth band was collected and the volume reduced on the rotary evaporator to ca. 40 mL. A purple powder, $[CuL^5]Cl_2{\cdot}4.5H_2O$, precipitated after several days (yield 0.13 g, 2.05%). (Found: C, 39.67; H, 6.59; N, 11.94. Calcd for $C_{19}H_{33}N_5O_2Cl_2Cu{\cdot}4.5H_2O$: C, 39.41; H, 7.31; N, 12.10.) UV/vis: λ_{max} nm (ϵ_M^{-1} cm^{-1}) 503 (88.1), shoulder 272 (1020), 235 (2760), 195 (7440). A fifth band was eluted from the column and concentrated to ca. 30 mL. The pH of the solution was adjusted to 3 by the dropwise addition of concentrated hydrochloric acid solution. A pink powder precipitated. This was collected by filtration, washed with EtOH and diethyl ether, and then air-dried (yield 1.15 g, 35.9%). (Found: C, 38.54; H, 6.19; N, 11.59. Calcd for $C_{19}H_{34}N_5O_2Cl_3Cu{\cdot}3.5H_2O$: C, 38.20; H, 6.92; N, 11.72.) X-ray quality crystals were grown by the diffusion of 2-propanol into an aqueous solution of the complex. The perchlorate salt was also formed by ion exchange. (Found: C, 26.66; H, 4.78; N, 7.34. Calcd for $C_{19}H_{34}N_5O_{14}Cl_3Cu{\cdot}7H_2O$: C, 26.77; H, 5.68; N, 8.21.) UV/vis: λ_{max} nm (ϵ_M^{-1} cm^{-1}) 513 (72.2). shoulder 267 (6570), 231 (14900), 196 (27200).

L^5. $[CuL^5]Cl_2{\cdot}4.5H_2O$ and $[Cu(HL^5)](ClO_4)_3{\cdot}5.5H_2O$ were demetalated as described above. Both compounds gave identical spectra. ^1H NMR (D_2O): 1.15 (3H, s), 1.67 (2H, mult), 2.42−2.75 (16H, mult), 3.69 (2H, s), 7.42 and 7.86 (4H, AB pattern). ^{13}C NMR: 20.8, 26.8, 44.5, 45.2, 45.9, 46.6, 54.3, 55.1, 128.1, 128.8, 134.4, 142.7, 175.0.

$[CuL^6](ClO_4)_2{\cdot}4H_2O$. Reaction of L^1 with (*E*)-4-(2′-carboxyallyl)-benzaldehyde was carried out as outlined above. After addition of $Cu(NO_3)_2{\cdot}3H_2O$ and filtration, the solution was diluted and applied to a Sephadex column. NaCl (0.2 M), pH 8, was used as the eluting agent. Three minor bands eluted rapidly from the column. These were discarded. A fourth, purple band, well separated from the first three bands, eluted next. No solid was obtained from this band. A fifth, pink band (λ_{max} 513 nm) eluted from the column. It was diluted and reapplied to a Sephadex column. The compound was eluted with 1 M $NaClO_4$ solution. The volume of the solution was reduced on the rotary evaporator. The desired product precipitated from aqueous solution after careful dropwise addition of $HClO_4$. It was collected by filtration and washed with two 5 mL portions of EtOH and one 5 mL portion of diethyl ether. The solid was air-dried (yield 1.48 g, 27.3%). (Found: C, 34.43; H, 5.13; N, 9.53. Calcd for $C_{21}H_{35}N_5O_{10}Cl_2Cu{\cdot}4H_2O$: C, 34.84; H, 5.99; N, 9.67.) UV/vis (H_2O): 510 nm (85.5 M^{-1} cm^{-1}), 271 (26500), 254 (17890), 217 (16670), 208 (17300).

L^6. $[CuL^6](ClO_4)_2{\cdot}4H_2O$ was demetalated with $Na_2S{\cdot}9H_2O$ as above. ^1H NMR (D_2O): 1.13 (s, 3H), 1.68 (mult, 2H), 2.50−2.63 (mult, 16H), 3.60 (s, 2H), 6.51 (1H, d), 7.33−7.61 (5H, AB + one part of doublet at 7.43). ^{13}C NMR: 23.7, 30.0, 47.4, 48.2, 49.0, 49.5, 57.4, 58.0, 126.7, 130.6, 131.7, 136.6, 143.1, 143.9, 178.1.

Protein Conjugation. All solutions for protein conjugation were made using Millipore water. Values reported are the average of four experiments. β-$[CuL^7](ClO_4)_2$, $[Cu(HL^5)](ClO_4)_3{\cdot}5.5H_2O$, or $[CuL^6](ClO_4)_2{\cdot}4H_2O$ (15, 16, and 17 mg respectively) was dissolved in 2 mL of 0.01M KH_2PO_4 buffer (pH 6.50) and 0.4 mL of NaOH solution (0.1 M). The solution was stirred until the copper complex had completely dissolved. *N*-Hydroxysuccinimide (NHS) (5 mg) and 1-(3′-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) (20 mg) were added, and the solution was stirred for 15 min. This solution was then added dropwise over 15 min to a stirred solution of bovine serum albumin (BSA) (100 mg) in 100 mL of phosphate buffer. The solution was stirred overnight at room temperature. The solution was purified by size exclusion chromatography over a Sephadex G-25 column (30 × 2 cm), with 10 mL fractions being taken. The first four fractions were discarded. The protein-containing fractions were identified by their absorbance at 280 nm. These fractions were pooled and accurately diluted to 50 mL with phosphate buffer solution. The absorbances at 280 nm were measured. The copper concentrations of the solutions were determined by atomic absorption spectroscopy, as absorbance in the visible region was too low to be measured accurately. The protein concentrations were calculated by assuming that the absorption at 280 nm was due to contributions from BSA (ϵ_{280} 63000 M^{-1} cm^{-1}) and the copper macrocycle complex (ϵ_{280} $[CuL^7]^{2+}$ 3225 M^{-1} cm^{-1}; $[CuL^5]^{2+}$ 4135 M^{-1} cm^{-1}; $[CuL^6]^{2+}$ 24780 M^{-1} cm^{-1}) and that the intensity of neither contribution was appreciably changed by the conjugation of the copper complex to the protein, i.e.,

$$A_{280}(\text{BSA}-\text{macrocyclic conjugate}) = A_{280}(\text{BSA}) + A_{280}(\text{macrocyclic complex})$$

(for purposes of comparison, ϵ_{268} *trans*-cinnamic acid (EtOH) 20420

Table 1. Crystal Data

	$[CuL^2](ClO_4)_2 \cdot 3H_2O$	$[CuL^4](ClO_4)_2$	$[Cu(HL^5)]Cl_3 \cdot H_2O$
formula	$C_{18}H_{39}Cl_2CuN_5O_{12}$	$C_{18}H_{33}Cl_2CuN_5O_9$	$C_{19}H_{36}Cl_3CuN_5O_3$
fw	651.98	597.93	552.42
space group	$P2_1/c$ (No. 14)	$P2_1/n$ (No. 14[a])	$P2_1/n$ (No. 14[a])
a, Å	11.915(6)	9.550(3)	9.286(2)
b, Å	13.861(2)	17.977(2)	11.294(1)
c, Å	17.065(8)	14.612(4)	23.609(8)
β, deg	102.14(2)	96.76(1)	93.68(1)
V, Å^3	2755(2)	2491(1)	2471(1)
Z	4	4	4
T, K	296	296	296
λ, Å	0.71073	0.71073	0.71073
μ, cm^{-1}	10.53	11.49	12.38
ρ_{calcd}, g cm^{-3}	1.572	1.594	1.485
$R(F_o)$[b]	0.0419	0.0507	0.0678
wR2(F_o^2)[c]	0.1305	0.1595	0.2003

[a] Variant of $P2_1/c$. [b] $R(F_o) = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [c] $wR2(F_o^2) = (\Sigma w(F_o^2 - F_c^2)/\Sigma wF_o^2)^{1/2}$.

M^{-1} cm^{-1}, *trans*-cinnamide (H_2O) ϵ_{273} 25120 M^{-1} cm^{-1}).[41] It was also assumed that the concentration of the copper complex was equal to the copper concentration determined by AAS, i.e., there is no loss of the metal from the macrocycle to the protein. The validity of this assumption was tested by carrying out a blank experiment, with BSA and $[CuL^5](ClO_4)_2$ mixed under conditions identical to those of the protein conjugation experiments, but omitting the coupling reagents NHS and EDC·HCl. No copper was detected in the blank experiments, indicating that nonspecific binding of the macrocyclic complex or of free copper to the protein may be neglected.

Physical Methods. Solution UV/vis spectra were measured on a Beckman DU 7500 spectrometer. Nuclear magnetic resonance (NMR) spectra were measured at 200 (^{1}H) and 50.3 MHz (^{13}C) on a Bruker AC200 spectrometer. Spectra in D_2O were referenced with tetradeuterated sodium trimethylsilylpropionate (TSP) or 1,4-dioxane (^{13}C), and all chemical shifts are cited versus trimethylsilane. EPR spectra of Cu^{II} complexes were measured on a Bruker ER200 D spectrometer as frozen 1 mM solutions (1:2 DMF/water, 77 K). Spin Hamiltonian parameters were obtained by spectral simulation.[42] A Varian SpectrAA-300 atomic absorption spectrometer was used for measurements of Cu concentrations in solutions. An air/acetylene flame was used and a detection wavelength of 324.8 nm.

X-ray Crystal Structure Analyses. Intensity data for all compounds were measured on an Enraf-Nonius CAD4 four-circle diffractometer using graphite-monochromated Mo Kα radiation and operating in the $\omega-2\theta$ scan mode. Lattice dimensions were determined by a least-squares fit of the setting parameters of 25 independent reflections. Data reduction and empirical absorption corrections were performed with the XTAL package.[43] Structures were solved by heavy-atom methods with SHELXS-86[44] and refined by full matrix least-squares analysis with SHELXL-97.[45] All non-H atoms were refined with anisotropic thermal parameters, except for minor contributors to disorder. In the structure of $[CuL^4](ClO_4)_2$ perchlorate O-atom disorder (attached to Cl2) was modeled with the aid of tetrahedral restraints applied to the two contributors. In the structure of $[Cu(HL^5)]Cl_3 \cdot H_2O$, the carboxybenzyl group was disordered over two sites, which were refined with complementary occupancies. Crystallographic data appear in Table 1, and selected bond lengths are given in the text. The atomic nomenclature is defined in Figures 1−3 drawn with the graphics program PLATON.[46]

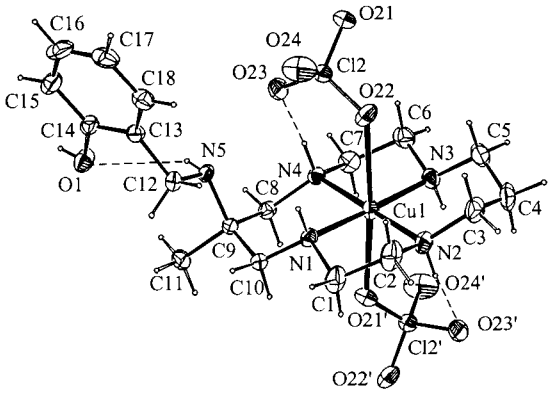

Figure 1. View of the molecule $[CuL^2](ClO_4)_2$ (30% probability ellipsoids shown).

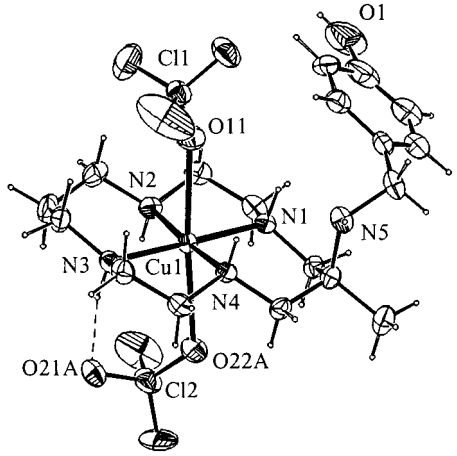

Figure 2. View of the molecule $[CuL^4](ClO_4)_2$ (30% probability ellipsoids shown). The C-atom numbering is the same as in Figure 1.

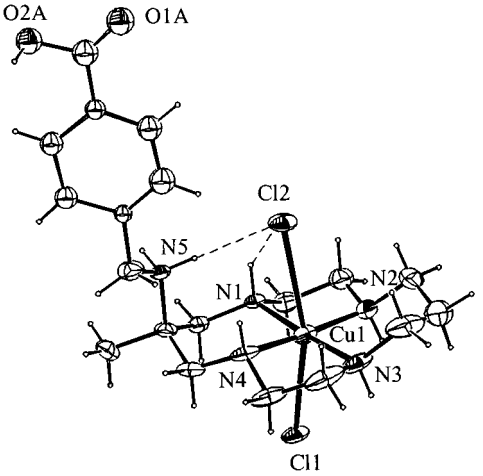

Figure 3. View of the cation $[Cu(HL^5)Cl_2]^+$ (30% probability ellipsoids shown). The C-atom numbering is the same as in Figure 1.

Results

The BH_3CN^- anion was employed successfully as a reductant in the reactions of the parent amine L^1 with a number of aromatic aldehydes. The yields of each reaction were not optimized. However, in all cases, more than 25% of the desired complex precipitated upon concentration of the chromatographic

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eluate, with additional crops being obtained from the filtrate. Given the inexpensive nature of the starting materials and the straightforward procedures, this is a highly economical route toward C-substituted macrocyclic bifunctional chelating agents. Notably, very little of the cyclic imidazolidine byproducts such as L^8 was observed in contrast to our earlier observations when using BH_4^- as a reductant in alcohol.[47]

The crystal structure of $[CuL^2](ClO_4)_2 \cdot 3H_2O$ is polymeric, with one of the perchlorate anions coordinating as a bridging bidentate to separate Cu atoms through O21 and O22. One fragment of this polymeric chain is shown in Figure 1. The metal center lies within an N_4O_2 tetragonally elongated octahedral coordination sphere, comprising four macrocyclic N donors and the *trans* perchlorate O atoms. The Cu−N bond distances (2.003(3)−2.020(3) Å) lie in the expected range,[48] as do the weak axial Cu−O bond lengths (2.417(3) and 2.680(3) Å) in this Jahn−Teller distorted d^9 complex. The pendant amine adopts an axial configuration in relation to the six-membered chelate ring, and the phenolic oxygen is protonated. There are several notable intramolecular H bonds in the structure. The orientation of the perchlorate anions is directed by H-bonding interactions with the amine H atoms, the most significant contact being N4−H4···O23 2.07 Å (N···O 2.934(4) Å) with a weaker interaction at 2.64 Å for N2−H2···O23′ (N···O) 3.423(4) Å. There is also a cyclic H bond involving the *o*-hydroxybenzylamine pendant: N5−H5···O1 2.39 Å (N···O 2.952(4) Å).

The crystal structure of the para isomer $[CuL^4](ClO_4)_2$ (Figure 2) again revealed a tetragonally elongated octahedral coordination geometry comprising the equatorial macrocyclic N donors and axially coordinated perchlorate anions. The Cu−N bond lengths (1.995(4)−2.015(4) Å) are again in the range expected and do not differ significantly. As expected, the axial Cu−O bonds are much weaker (2.486(5) and 2.598(5) Å). The pendant amine is not protonated in the crystal structure and adopts an axial position in relation to the six-membered chelate ring, and the phenolic oxygen remains protonated. The disposition of the phenyl ring with respect to the macrocycle is different from that found in the ortho isomer $[CuL^2](ClO_4)_2 \cdot H_2O$ (Figure 1). The two conformations are related by a 120° rotation of the N5−C12 bond. It emerges that rotation of the phenyl ring in $[CuL^2](ClO_4)_2 \cdot H_2O$ about this bond (to generate the same conformation found in $[CuL^4](ClO_4)_2$) would break the intramolecular H bond shown in Figure 1. This H-bonding interaction is impossible in the para isomer, which accounts for the conformational difference.

The crystal structure of $[Cu(HL^5)]Cl_3 \cdot H_2O$ is shown in Figure 3. The pendant amine is axial with respect to the six-membered chelate ring. The 4-carboxybenzyl group was disordered over two sites (one contributor is shown in Figure 3). A tetragonally distorted CuN_4Cl_2 coordination geometry was found, with typical equatorial Cu−N bond lengths (2.006(5)−2.021(5) Å). There are significant differences between the two Cu−Cl axial coordinate bonds (2.680(1) and 2.974(2) Å). The more weakly bound Cl2 also makes H-bonding interactions with the secondary amine H atoms attached to N1 and N5 (Figure 3): N1−H1···Cl2 2.58 Å (N···Cl 3.182(4) Å) and N5−H5A···Cl2 2.36 Å (N···Cl 3.259(5) Å). The carboxylic acid group is hydrogen bonded to a solvent water molecule. Both the pendant secondary amine and the carboxylic acid group are protonated.

The visible electronic spectra of all Cu^{II} complexes synthesized are virtually identical to each other and to that of the parent $[CuL^1]^{2+}$. This reinforces the crystallographic results which show that modification of the pendant amine has little effect, if any, on the coordination sphere of the metal ion. In all cases the complexes exhibit a single visible electronic maximum in the range 500−515 nm with an extinction coefficient 80−100 $M^{-1}\,cm^{-1}$. This is characteristic of a 14-membered macrocyclic secondary tetraamine. Previously, we noted[47] that N-alkylation of one macrocyclic amine results in a marked increase (by a factor of 2 or more) in the extinction coefficient through breaking the pseudo-centrosymmetry of the ligand field, but the wavelength at which this maximum occurs is little affected. The EPR spectra are also identical and typical of a C-substituted Cu^{II} cyclam complex ($g_{\|} = 2.182$, $g_{\perp} = 2.038$, $A_{\|} = 197.4$ G, $A_{\perp} = 23.8$ G).

Protein Modification. Attempts to esterify the hydroxybenzyl-appended macrocycles (L^2−L^4) with acetyl chloride or succinic anhydride were unsuccessful. The purpose of these reactions was to activate the phenolic O atom for conjugation with a protein. Either starting materials were recovered or complexes of L^1 were found, indicating instability of the hydroxybenzyl group under the reaction conditions. It appears that the presence of the hydroxyl group decreases the stability of the substituted benzylamine as no such instability was observed for the complexes of L^5 or L.[6] Instability of L^2 was also observed under basic conditions.

Successful protein conjugation experiments were carried out with the Cu^{II} complexes of the macrocycles bearing pendant carboxylic acid groups (L,[5] L^6, and L^7). Under the labeling conditions used there was no release of copper from the macrocyclic ligands to side chains on the protein, as shown by a blank experiment where $[CuL^5]^{2+}$ was incubated in the presence of BSA, but without coupling reagents. The labeling yield was poor (0.36 macrocycle per BSA) for $[CuL^7]^{2+}$; $[CuL^5]^{2+}$ was slightly better (0.9 macrocycle per BSA), but the best of this series was cinnamic acid derivative $[CuL^6]^{2+}$ (2.3 macrocycles per BSA).

Discussion

In this work, there was a large variation in the reactivity of substituted benzaldehydes with L^1. Salicylaldehyde reacts with L^1 in EtOH to form L^2 as the major product after reduction with $NaBH_4$. This is in contrast to the behavior observed for 4-carboxybenzaldehyde, which gives the imidazolidine derivative L^8 as the major product (even in the presence of $NaBH_4$) as a result of rapid cyclization of the imine intermediate.[47] Only small amounts of the imidazolidine analogue of L^2 were obtained from the reaction of salicylaldehyde, L^1, and $NaBH_4$. The general stability of salicyl-imines has been attributed[49] to the formation of an intramolecular H-bonding interaction similar to that seen in the crystal structure of the amine derivative $[CuL^2](ClO_4)_2 \cdot 3H_2O$ (Figure 1), and in this case it appears to prevent intramolecular attack by an adjacent macrocyclic secondary amine on the imine intermediate. Steric effects also appear to be important in determining the fate of the imine intermediate, i.e., intramolecular nucleophilic attack by an amine or intermolecular reduction by BH_4^-. We found no evidence of imidazolidine formation when the bulky 9-anthraldehyde was used.[50] Therefore, if intramolecular H-bonding or significant

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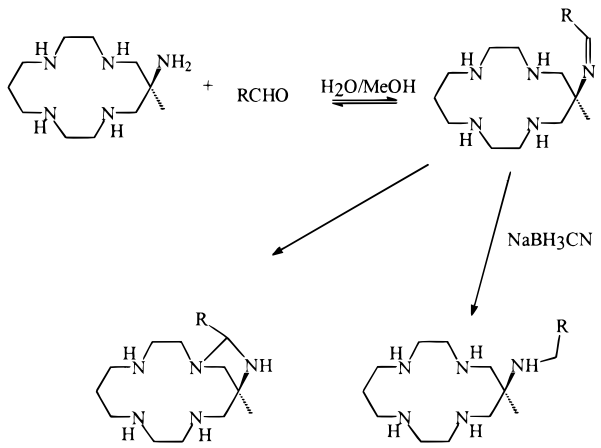
Scheme 1



steric effects are present, cyclization to the imidazolidine analogue is not a significant problem.

In the absence of H-bonding or steric effects, which stabilize the imine intermediate, a different synthetic procedure is required if one wishes to avoid formation of imidazolidine-containing products (Scheme 1). In these cases, reductive alkylation was carried out with water as the solvent, with some alcohol to enhance reactant solubility in some cases. This hinders formation of the five-membered imidazolidine rings, through solvation and protonation of the secondary amines in the macrocyclic ring. However, the more traditional reductant $NaBH_4$ is rapidly hydrolyzed under these conditions so the analogue $NaBH_3CN$ must be used instead. The presence of the electron-withdrawing cyano group decreases the rate of hydrolysis of BH_3CN^- by 10 orders of magnitude compared with BH_4^-. The formation of alcohols from the reduction of unreacted aldehyde is an unwanted reaction, but this can be minimized by keeping the reaction pH value above 4.5. Others have employed a similar procedure for the modification of amino-substituted ligands.[51] The ligands with substituted pendant amines form in reasonable yields using this method, and imidazolidine formation is minimal. The versatility of the method has been demonstrated by the variety of substituted macrocycles (L^2-L^7) that have been synthesized.

In this work, it was our hope that the primary amine of L^1 would be capable of being selectively modified with a bifunctional linker, to enable attachment to a protein. It was also decided to concentrate upon agents with a view to their eventual use with $^{64}Cu/^{67}Cu$. The serum stability of the unsubstituted relative $[Cu(cyclam)]^{2+}$ has already been demonstrated, with this complex being kinetically very stable under biological conditions.[52] The ^{64}Cu-labeled cyclam complex loses less than 0.5% of the copper after 48 h incubation with 1 mM EDTA or 24 h incubation with serum. The (6-*p*-thiocyanatobenzyl)TETA complex of ^{67}Cu, conjugated to a mAb, retained 94% of its original Cu label after 3 day incubation in serum.[35] The site of placement of the benzyl group is important. The 2-benzyl substituted TETA has a 10-fold less stable Cu complex, when compared to the 6-benzyl isomer.[53] A study comparing the serum stability of a number of $^{67}Cu^{II}$ complexes showed that 2-(*p*-nitrobenzyl)DOTA, 2-(*p*-nitrobenzyl)cyclen, and 2(*p*-nitrobenzyl)cyclam were the most stable.[54]

Successful protein conjugation experiments were carried out with the copper complexes of the macrocycles bearing pendant carboxylic acid functions (L,[5] L^6, and L^7), but the phenolic substituted isomers L^2, L^3, and L^4 could not be attached to BSA, as suitable activated precursors could not be prepared. The protein labeling yields were not maximized. It is thus likely that the reaction time can be reduced without reducing the labeling yield. A shorter reaction time would be desirable for labeling with radiometals. It was also observed that the labeling yield was strongly influenced by the nature of the functional group attached to the pendant amine of the macrocycle. In a simple manner, this can be related to the distance of the carboxylic acid group, the site of protein attachment, from the macrocyclic ring. The labeling yield was extremely poor for β-$[CuL^7]^{2+}$, the complex with the shortest distance between the macrocyclic ring and the carboxylic acid group (ca. 3.6 Å). The best result was obtained for the cinnamic acid derivative, $[CuL^6]^{2+}$ (ca. 9.8 Å separation), which showed high labeling yields.

A dependence of labeling yield on the length of the spacer group separating the site of copper binding from protein attachment (via a carboxylic acid group) was observed for a series of bis(thiosemicarbazone) (TSC) copper complexes.[55] It was found that protein coupling of the activated copper bis-(TSC) complexes increased slightly when the alkyl chain length was increased. The use of cinnamic acid derivatives for protein attachment does not appear to have been carried out previously and bears further investigation because of the excellent labeling yields obtained in this case.

The formation of the *N*-hydroxysuccinimide esters of the parent carboxylic acid copper complexes was carried out in aqueous solution. This active ester is susceptible to hydrolysis, and thus the labeling yield could be increased by synthesizing the active ester in a nonaqueous solvent and then reacting it with protein. BSA has 55 lysine residues, 3−4 of which are accessible to chemical reagents.[56] The cinnamic acid derivative $[CuL^6]^{2+}$ was attached to an average of 2.3 of these surface accessible residues. A typical IgG has 6−9 surface accessible lysine residues,[56] and thus the labeling yield (number of macrocyclic residues per protein molecule) would likely be greater in the case of labeling experiments involving that protein, although impairment of immunoreactivity has been observed at higher labeling ratios. The labeling yield could also be increased by adopting the approach advocated by Gansow[57] and others,[58,59] where the bifunctional chelating agent is first attached to a dendrimer, with a large number of surface amino groups. The modified dendrimer is then attached to the therapeutic antibody or antibody fragment.

An obvious further step is isolation of the free ligands and conjugation of the ligands to protein, followed by complexation with copper. This would enable evaluation of the pre- versus postconjugation labeling approaches. This should also reduce the time taken for purification following labeling.

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Conclusions

The synthetic methods presented in this work represent a valuable opportunity for the synthesis of a number of modified macrocyclic ligands. The variety of ligands synthesized has shown the versatility of this approach. Protein labeling experiments have confirmed that the length of the spacer unit between the protein conjugation site and the metal binding site is an important variable in the success of the labeling experiment. The macrocyclic ligand with a pendant cinnamic acid group (L^6) shows excellent promise as a protein labeling agent and should be investigated further for this purpose.

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

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