Metal-directed Synthesis of Aminobenzyl Polyaza Macrocycles: Candidates for Attachment to Polymers and Biomolecules

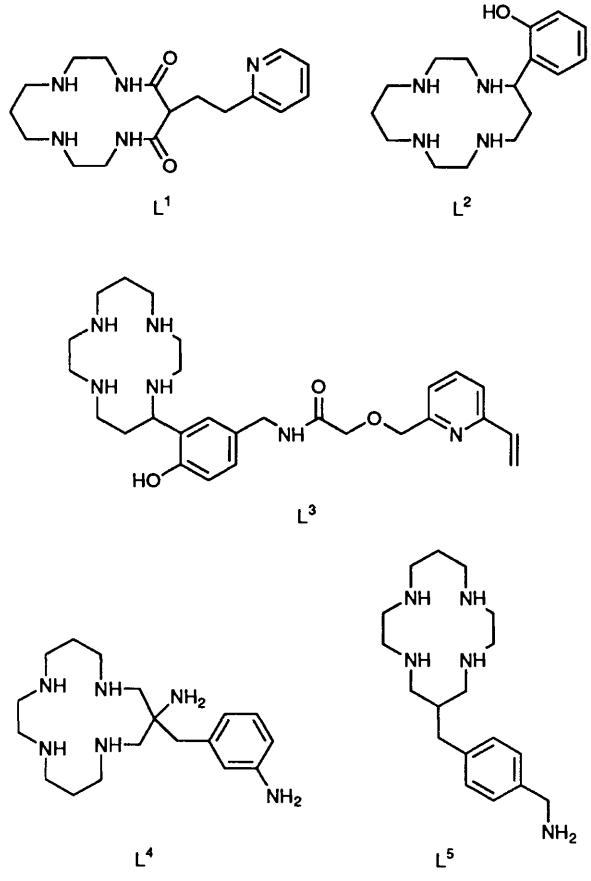
Anne Bayada, Geoffrey A. Lawrance,* Marcel Maeder and Margaret A. O'Leary
Department of Chemistry, The University of Newcastle, Callaghan 2308, Australia

Copper(II)-directed condensation between 4,7-diazadecane-1,10-diamine, formaldehyde and 1-nitro-3-(2-nitroethyl)benzene yielded the macrocyclic [10-nitro-10-(3-nitrobenzyl)-1,4,8,12-tetraazacyclopentadecane]copper(II) ion. Reduction (Zn,HCl) gave the pendant-arm macrocycle 10-(3-aminobenzyl)-1,4,8,12-tetraazacyclopentadec-10-ylamine as the hydrochloride salt. Condensation reactions with 1-nitro-4-(2-nitroethyl)benzene and 2-phenylnitroethane were also successful. The capacity of the aminobenzyl C-pendant introduced by this facile chemistry for covalent attachment has been examined by attachment of 10-(3-aminobenzyl)-1,4,8,12-tetraazacyclopentadec-10-ylamine to the acidic cation-exchange resin CM Bio-Gel A and to horse heart cytochrome c, employing a water-soluble carbodiimide coupling agent at pH 5 to promote amide formation. The attachment was probed by copper(II) complexation to the bound macrocycle and subsequent spectroscopic or voltammetric analysis.

Recent interest in the synthesis of both C- and N-functionalised polyaza macrocycles has been partly driven by their potential applications in such diverse areas as tumour targeting and selective cation binding.[1-3] The syntheses of N-functionalised tetraaza macrocycles have been studied in much greater depth than those of their C-functionalised analogues, largely due to the relative simplicity of the synthetic strategies.[4] However, studies of C-functionalised polyaza macrocycles have received growing attention even though their syntheses pose a greater challenge, since the steric influences of C-pendants are less intrusive than those of N-pendants on complexation behaviour.

Both metal-template reactions and conventional organic chemistry routes have been pursued in the synthesis of C-substituted pendant-arm macrocycles. Kimura *et al.*[5] have synthesised macrocycles such as L^1 by a condensation reaction of a linear tetramine with a pyridyl acrylate or a substituted malonic ester. Michael addition reactions have also been applied as part of the synthetic route to macrocycles such as L^2.[6] Parker and co-workers[2,7,8] have synthesised a range of both N- and C-functionalised macrocycles with a view towards covalent linking to biomolecules through a long and sterically less aggressive C-pendant arm. Tetraaza macrocycles such as L^3 have also been designed to bind metal ions including radioactive copper following linkage to monoclonal antibodies.[9]

In this paper, the synthesis of C-functionalised tetraaza macrocycles such as L^4 is reported. The macrocycle L^5, a close analogue of L^4, has been prepared by direct organic routes recently.[2] The present series of macrocycles are readily accessible *via* facile metal-directed formaldehyde condensation reactions with a nitroalkane precursor carrying an aromatic terminating group, followed by a simple reduction with zinc and hydrochloric acid. This represents the first application of this type of condensation reaction to produce molecules with aromatic pendants. Covalent binding to a polymer resin and the biomolecule cytochrome c and subsequent uptake of copper(II) ion are also examined.



Experimental

Syntheses.—*1-Nitro-3-(2-nitroethyl)benzene.* A mixture of *m*-nitrobenzaldehyde (7.0 g, 47 mmol), potassium fluoride dihydrate (0.43 g, 4.6 mmol) and nitromethane (3.56 g, 58 mmol) was prepared in Pr^iOH (80 cm^3). Initially the *m*-nitrobenzaldehyde does not dissolve, but after warming for 20 min a clear orange solution was formed. The solution was warmed and stirred for 3 d. The reaction was monitored by TLC {R_f (in CH_2Cl_2) = 0.3 [*m*-$O_2NC_6H_4CH(OH)CH_2NO_2$] 0.8 (*m*-$O_2NC_6H_4CHO$)}. Most of the solvent was evaporated and the solution then extracted with diethyl ether. The ether layer was washed with water and then with sodium μ-oxo-disulfate(IV)

solution (12.4 g $Na_2S_2O_5$ in 80 cm^3 water) to remove any unreacted aldehyde. After washing with water again, the ether layer was dried over $MgSO_4$, filtered and evaporated to give a pale green oil, *m*-$O_2NC_6H_4CH(OH)CH_2NO_2$ (7.0 g, 71% yield). IR spectrum (thin film): 3520 (OH), 3090 (aromatic H), 2974, 2922, 2875 (CH), 1615 (aromatic C=C), 1553, 1531, 1353 cm^{-1} (NO_2).

Acetic anhydride (3.4 g, 33 mmol) and concentrated sulfuric acid (five drops) were added to the nitroalcohol (7.0 g, 33 mmol). The mixture became warm and was left to stand for 2 h at room temperature with occasional stirring. The oily solution of the nitroacetate *m*-$O_2NC_6H_4CH(O_2CMe)CH_2NO_2$ was taken up in Me_2SO (20 cm^3). A solution of $NaBH_4$ (1.59 g, 42 mmol) in Me_2SO (40 cm^3) was prepared and added dropwise to the stirred solution of the nitroacetate at 0 °C over 20 min. The reaction mixture foamed and turned brown. The pH was maintained at ≈4 with periodic additions of acetic acid. After stirring at room temperature for 1 h, ice (≈100 g) was added to give a pale yellow suspension. The suspension was taken up in ether and washed several times with water to remove Me_2SO. The ether extract was dried ($MgSO_4$) and evaporated to give an orange oil. The infrared spectrum still showed the presence of acetate, ν(C=O) 1754 cm^{-1}. The product was taken up in hot ethanol and then evaporated to give a yellow oil, *m*-$O_2NC_6H_4CH_2CH_2NO_2$ (3.86 g, 60% yield). IR spectrum (thin film): 3094 (aromatic H), 2925 (CH), 1545, 1532, 1350 cm^{-1} (NO_2). ^1H NMR ($CDCl_3$): δ 3.4 (t, $CH_2C_6H_4$), 4.6 (t, CH_2NO_2) and 7.4–8.4 (m, aromatic H).

[10-*Nitro*-10-(3-*nitrobenzyl*)-1,4,8,12-*tetraazacyclopentadecane*]*copper*(II) *nitrate hemihydrate*, $[CuL^7][NO_3]_2 \cdot 0.5H_2O$. 4,7-Diazadecane-1,10-diamine (3.43 g, 19.7 mmol) in AR methanol (60 cm^3) was added to a solution of copper nitrate trihydrate (4.75 g, 19.7 mmol) in AR methanol (100 cm^3). Formaldehyde (11 cm^3, 394 mmol), *m*-$O_2NC_6H_4CH_2CH_2NO_2$ (3.86 g, 19.7 mmol) in AR methanol (50 cm^3) and triethylamine (8.3 cm^3, 60 mmol) were then added. The solution was warmed and stirred for 3 h. A grey-green precipitate formed and the solution was left to stir at room temperature overnight. It was then filtered through Kieselguhr. A blue solid was deposited on the Kieselguhr which was collected and recrystallised from methanol–water to give the macrocyclic complex (2.3 g, 20% yield). Electronic spectrum (in water): λ_{max} 572 (ε 104) and 266 nm (ε 13 544 dm^3 mol^{-1} cm^{-1}). IR spectrum (KBr disc): 3450, 3190, 2945, 2881 (CH), 1547, 1384, 1350 cm^{-1} (NO_2) (Found: C, 36.6; H, 5.3; N, 18.7. Calc. for $C_{18}H_{30}CuN_8O_{10} \cdot 0.5H_2O$: C, 36.6; H, 5.3; N, 19.0%).

10-(3-*Aminobenzyl*)-1,4,8,12-*tetraazacyclopentadec*-10-*ylamine hydrochloride*, $L^4 \cdot 6HCl$. A solution of the complex $[CuL^7][NO_3]_2 \cdot 0.5H_2O$ (0.54 g, 0.93 mmol) in water (100 cm^3) was added dropwise to zinc dust (5.0 g) with stirring over ≈30 min, while HCl (25 cm^3, 3.0 mol dm^{-3}) was added simultaneously. The clear solution was stirred for 30 min and then filtered through Kieselguhr and diluted to ≈400 cm^3. The pH was adjusted to ≈6 and the solution was loaded onto a Dowex 50WX2 (H^+ form) column (18 × 3 cm). Zinc salts were removed by washing with 2.0 mol dm^{-3} HCl (150 cm^3). The compound L^4 was then eluted with 5.0 mol dm^{-3} HCl. Evaporation and washing with ethanol, then ether, gave a cream powder (0.32 g, 62% yield). IR spectrum (KBr disc): 3374, 2946, 2740, 1575 and 1457 cm^{-1}. ^{13}C NMR ($CDCl_3$): δ 26.0 ($CH_2CH_2CH_2$), 41.8 ($CH_2C_6H_4$), 44.1, 46.9, 48.9, 55.7 (CH_2N), 126.1, 128.1, 134.4 (aromatic C) (Found: C, 35.5; H, 7.0; N, 13.1. Calc. for $C_{18}H_{40}Cl_6N_6 \cdot H_3O^+Cl^-$: C, 35.4; H, 7.1; N, 13.7%).

1-*Nitro*-4-(2-*nitroethyl*)*benzene*. A mixture of *p*-nitrobenzaldehyde (10.0 g, 67 mmol), potassium fluoride dihydrate (0.63 g, 6.7 mmol) and nitromethane (5.08 g, 83 mmol) was prepared in Pr^iOH (100 cm^3). The solution was warmed and stirred for 3 d, turning brown. It was taken up in ether and washed with water to remove Pr^iOH. The ether layer was washed several times with $Na_2S_2O_5$ solution (12.4 g in 80 cm^3 water) to remove any aldehyde. After washing with water and drying ($MgSO_4$), the ether was evaporated resulting in a yellowish oil, *p*-$O_2NC_6H_4CH(OH)CH_2NO_2$ (10.6 g, 75% yield). IR spectrum (thin film): 3528 (OH), 3116, 3080 (aromatic H), 2964, 2925, 2836 (CH), 1602 (aromatic C=C), 1559, 1521, 1349 cm^{-1} (NO_2). ^1H NMR ($CDCl_3$): δ 3.2 (d, OH, disappears on shaking with D_2O), 4.6 (d, CH_2), 5.6 (m, CH, resolves to triplet on shaking with D_2O) and 7.6–8.3 (ABq, aromatic H).

Acetic anhydride (5.12 g, 50 mmol) and concentrated sulfuric acid (five drops) were added to the nitroalcohol. The mixture became warm and was left to stand at room temperature for 2 h. The oily product was taken up in ether and washed well with water. The ether was evaporated affording a golden coloured oil *p*-$O_2NC_6H_4CH(O_2CMe)CH_2NO_2$ (10.8 g, 85% yield). IR spectrum (thin film): 3513, 3115, 3082 (aromatic H), 2964, 2925, 2863 (CH), 1756 (C=O), 1604 (aromatic C=C), 1556, 1524, 1348 cm^{-1} (NO_2).

A solution of $NaBH_4$ (2.14 g, 57 mmol) in Me_2SO (45 cm^3) was added dropwise over 20 min at 0 °C to a stirred solution of the nitroacetate (10.7 g, 42 mmol) dissolved in Me_2SO (20 cm^3). The pH was maintained at ≈4 with the addition of acetic acid, the reaction mixture was stirred at room temperature for 1 h and then ice (≈100 g) was added. The resulting brown residue was taken up in ether and washed well with water. Drying (over $MgSO_4$) and evaporation of the ether gave the product as a yellow oil (4.75 g, 58% yield). IR spectrum (thin film): 3408, 3107 (aromatic H), 2976, 2922, 2851 (CH), 1604 (aromatic C=C), 1555, 1517, 1344 cm^{-1} (NO_2). ^1H NMR ($CDCl_3$): δ 3.4 (t, $CH_2C_6H_4$), 4.7 (t, CH_2NO_2) and 7.3–8.3 (ABq, aromatic H).

[10-*Nitro*-10-(4-*nitrobenzyl*)-1,4,8,12-*tetraazacyclopentadecane*]*copper*(II) *nitrate*, $[CuL^8][NO_3]_2$. 4,7-Diazadecane-1,10-diamine (0.22 g, 1.3 mmol) in AR methanol (5 cm^3) was added to a solution of copper nitrate trihydrate (0.3 g, 1.3 mmol) in AR methanol (20 cm^3). Formaldehyde (1.3 cm^3, 18 mmol), *p*-$O_2NC_6H_4CH_2CH_2NO_2$ (0.25 g, 1.3 mmol) in methanol (10 cm^3) and triethylamine (0.3 g, 3.0 mmol) were then added. The mixture was warmed and stirred for 2.5 h. It had turned brown after 30 min. After standing at room temperature overnight, crystals appeared in the reaction mixture. The brown solution was decanted and the crystals were washed with methanol then ether to give the product as a blue solid (0.3 g, 41% yield). Electronic spectrum (in water): λ_{max} 572 (ε 94) and 270 nm (ε 15 048 dm^3 mol^{-1} cm^{-1}). IR spectrum (KBr disc): 3440, 3195 (aromatic H), 2935, 2876 (CH), 1605 (aromatic C=C), 1548, 1383, 1348 cm^{-1} (NO_2). A small amount was converted into the perchlorate salt for analysis (Found: C, 32.7; H, 4.6; N, 12.5. Calc. for $C_{18}H_{30}Cl_2CuN_6O_{12}$: C, 32.9; H, 4.6; N, 12.8%).

2-*Phenylnitroethane*. A mixture of benzaldehyde (5.0 g, 47 mmol), nitromethane (2.9 g, 47.5 mmol) and potassium fluoride dihydrate (0.15 g, 1.6 mmol) in Pr^iOH (20 cm^3) was stirred at room temperature for 48 h. Most of the solvent was removed then the reaction mixture was extracted into ether and washed several times with $Na_2S_2O_5$ solution to remove benzaldehyde. The ether extract was dried ($MgSO_4$), filtered and evaporated to give $PhCH(OH)CH_2NO_2$ as a brown oil (5.8 g, 74% yield). IR spectrum (thin film): 3460 (OH), 3033 (aromatic H), 2920 (CH), 1554 (NO_2), 1495 (aromatic C=C) and 1377 cm^{-1} (NO_2).

Acetic anhydride (3.5 g, 34 mmol) and concentrated sulfuric acid (two drops) were added to the nitroalcohol (5.8 g, 35 mmol). The mixture was cooled in ice and stirred for 1 h to give a green solution. A solution of $NaBH_4$ (1.59 g, 42 mmol) in Me_2SO (40 cm^3) was then added to the ice-cooled solution over 20 min with stirring. The solution foamed and a yellow suspension was formed. The solution was left to stand at room temperature for 1 h. Ice (≈100 g) was added and the pH of the aqueous layer was adjusted to ≈4 with acetic acid. The mixture was extracted into ether and washed with water, dried ($MgSO_4$), filtered and then evaporated to give the product as an orange oil (4.7 g, 90% yield). IR spectrum (thin film): 3063, 3030 (aromatic H), 2918 (CH), 1600 (aromatic C=C) and 1553 cm^{-1} (NO_2). ^1H NMR

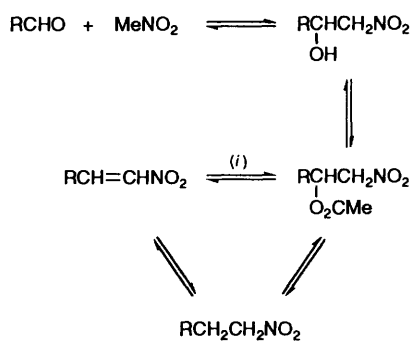
($CDCl_3$): δ 3.3 (t, CH_2Ph), 4.5 (t, CH_2NO_2) and 6.8–7.8 (m, aromatic H).

10-Benzyl-10-nitro-1,4,8,12-tetraazacyclopentadecane)-copper(II) nitrate, $[CuL^9][NO_3]_2$. 4,7-Diazadecane-1,10-diamine (2.3 g, 13.2 mmol) in AR methanol (5 cm^3) was added to a solution of copper nitrate trihydrate (3.2 g, 13.7 mmol) in AR methanol (80 cm^3). Formaldehyde (10.0 cm^3, 140 mmol), $PhCH_2CH_2NO_2$ (2.0 g, 13.2 mmol) in methanol (10 cm^3) and triethylamine (1.0 cm^3, 7.2 mmol) were then added. The mixture was warmed and stirred for 2 h and then left at room temperature for 2 d. The deep blue solution was concentrated to a viscous oil, which was washed well with ether. Some solid formed on the addition of ethanol and standing overnight. The solid was washed well with ethanol to give the product as a blue solid (1.34 g, 19% yield). Electronic spectrum (in water): λ_{max} 571 (ε 128) and 266 nm (ε 10 024 dm^3 mol^{-1} cm^{-1}). IR spectrum (KBr disc): 3200 (aromatic H), 2928, 2872 (CH) and 1562 cm^{-1} (NO_2). A small amount was converted into the perchlorate salt for analysis (Found: C, 35.3; H, 5.1; N, 11.6. Calc. for $C_{18}H_{31}Cl_2CuN_5O_{10}$: C, 35.3; H, 5.1; N, 11.4%).

10-Benzyl-1,4,8,12-tetraazacyclopentadec-10-ylamine hydrochloride, L^{10}·5HCl. A solution of the copper complex $[CuL^9][NO_3]_2$ (0.54 g, 1.0 mmol) in water (50 cm^3) was added dropwise to zinc dust (5.1 g) with stirring over 20 min. Hydrochloric acid (25 cm^3, 3.0 mol dm^{-3}) was added simultaneously. After completion of the addition the mixture was stirred for 30 min, the final pH being 6.7. It was filtered through Kieselguhr and the filtrate chromatographed on Dowex (8 × 2.5 cm). Zinc salts were removed by washing with 2.0 mol dm^{-3} HCl (100 cm^3) and the compound L^{10} was eluted with 5.0 mol dm^{-3} HCl. Evaporation and washing with ethanol then ether gave the macrocycle as a cream powder (0.145 g, 29% yield). IR spectrum (KBr disc): 3420 (NH), 2950, 2760 (CH), 1594, 1458 cm^{-1} (aromatic C=C). ^{13}C NMR ($CDCl_3$): δ 23.5 ($CH_2CH_2CH_2$), 40.0 (CH_2Ph), 42.4, 44.0, 45.9, 52.9 (CH_2N) and 57.1 (quaternary C).

Attachment of L^4 to CM Bio-Gel A.—The CM Bio-Gel A suspension (Bio-Rad, 10 cm^3) was diluted with water (10 cm^3). The macrocycle L^4·6HCl (0.061 g, 0.11 mmol) was added and the pH adjusted to 5.5 with a few drops of 2.5 mol dm^{-3} NaOH. The hydrochloride of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (0.085 g, 0.44 mmol) was then added and the pH adjusted to 5.0 with 2.0 mol dm^{-3} HCl. The reaction mixture was stirred at room temperature for 3 h. The gel was then poured into a small column (2 × 2.5 cm) and allowed to settle. It was then washed with water (200 cm^3) to remove excess of coupling reagent.

To test for attachment, a known concentration of copper(II) ions (0.1 mol dm^{-3}, 20 cm^3) was passed through the column. The gel turned deep blue and the eluent was collected and made up to a known volume (50 cm^3). The concentration of Cu^{2+} before and after elution was determined spectrophotometrically and the amount of Cu^{2+} retained was calculated. The capacity of the active sites on the resin was 11 μequivalents cm^{-3}, comparable with the commercial value for the acid sites on the parent resin. The bound copper could not be removed with salt solution (1.0 mol dm^{-3} NaCl) and the blue colour was still retained when a small volume (10 cm^3) of saturated NaCl solution was passed through the column. This is consistent with complexation to covalently bound macrocycle. A blank was also performed using the same procedure described above except that no macrocycle was added. The column turned very light blue and the colour was removed easily with a small volume (20 cm^3) of dilute salt solution (1.0 mol dm^{-3} NaCl). The capacity of the acid sites was calculated as 17 μequivalents cm^{-3}, similar to the previous result. A diffuse-reflectance spectrum was obtained from the blue coloured complexed gel. Maxima were found at 585 and 365 nm, very similar to the solution maxima of the copper complex of the precursor macrocycle L^4 at 580 and 381 nm.



Scheme 1 R = *m*-$O_2NC_6H_4$, *p*-$O_2NC_6H_4$ or Ph. (*i*) $NaBH_4$

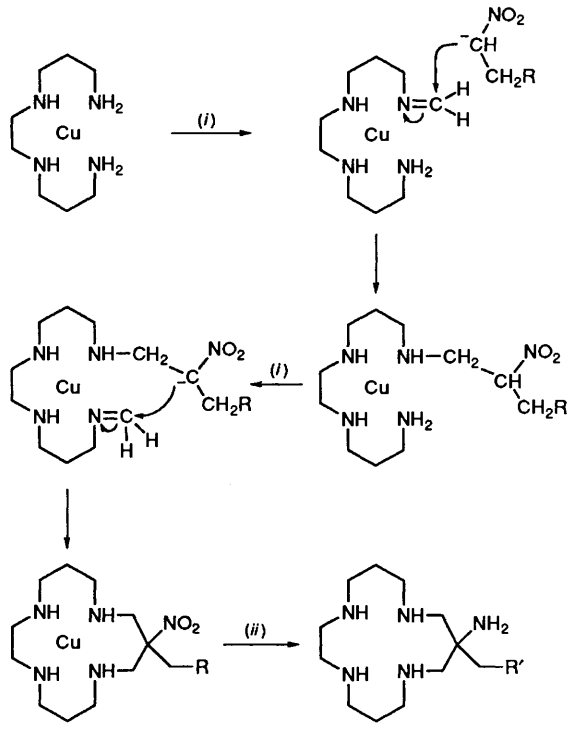
Attachment of L^4 to Horse Heart Cytochrome c.—Horse heart cytochrome c (sigma, 0.0495 g, 4.0 μmol) was dissolved in water (25 cm^3). The macrocycle L^4·6HCl (0.009 g, 16.0 μmol) and the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.005 g, 24.0 μmol) were then added. The pH of the solution was 5 and the solution was left to stir at room temperature overnight. It was then made up to a final volume of 50.0 cm^3. Aqueous Cu^{2+} (0.3 cm^3, 0.1 mol dm^{-3}) was added to a portion of this solution (15 cm^3). It was then passed through a small column of CM Bio-Gel A (2 × 2.5 cm) and made up to 25 cm^3 in 0.1 mol dm^{-3} KCl and 5 mmol dm^{-3} hepes [*N'*-(2-hydroxyethyl)piperazine-*N*-ethane-2-sulfonic acid] buffer (pH 7.0). A cyclic voltammogram was recorded from 0.3 to −1.0 V *versus* Ag–AgCl. Cyclic voltammograms of the cytochrome c solution alone and the copper(II) macrocycle solution alone were also recorded. The presence of the covalently bound copper macrocycle on the cytochrome c also caused a significant reduction in frothing compared with the unadulterated cytochrome c solution.

Physical Methods.—Proton and ^{13}C NMR spectra were recorded on a JEOL FX90Q spectrophotometer, infrared spectra with a Bio-Rad FTS-7 Fourier-transform spectrometer. Microanalyses were performed by the Research School of Chemistry Microanalytical Service at the Australian National University. Absorption spectra were recorded on Hitachi 150-20 and 220A spectrophotometers, diffuse-reflectance spectra using a 60 mm diameter integrating sphere accessory with the latter. Cyclic voltammograms were recorded using a BAS CV-27 controller employing a standard three-electrode configuration with a freshly polished glassy carbon working electrode, silver–silver chloride reference electrode and platinum-wire auxiliary electrode. Solutions were purged with nitrogen. Voltammetry involving cytochrome c employed aminosugar promotors as described previously.[10]

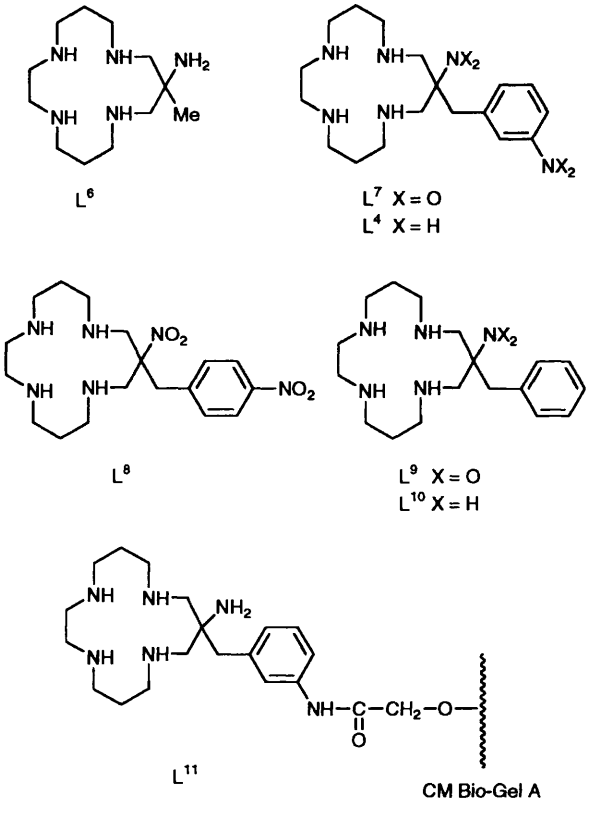
Results and Discussion

The synthetic strategy employs copper(II)-directed condensation between a polyamine, formaldehyde and a nitroalkane developed earlier for simple nitroalkanes such as nitroethane.[11] Earlier[11] we found that the nature of the R group in RCH_2NO_2 has some, but not necessarily a major, influence on the success of the condensation reaction, which suggested that reaction of nitroalkanes with nitroaromatic substituents would be feasible. With the facility of the chemistry established in this study, the method offers a useful route to C-pendant aminobenzyl macrocyclic polyamines with potential for attachment to polymers or biomolecules.

The syntheses of the precursor nitroalkanes was achieved by adaptation of a literature method[12] involving an initial base-catalysed condensation of an aromatic aldehyde and nitromethane, followed by acylation and reductive elimination (Scheme 1). The initial condensation step is catalysed by the base HF_2^- which is formed from F^- in a protic solvent such as



Scheme 2 R = *m*-$O_2NC_6H_4$, *p*-$O_2NC_6H_4$ or Ph; R′ = *m*-$H_2NC_6H_4$ or Ph(*i*) HCHO; (*ii*) Zn, HCl



Pr^iOH.[13] The resulting nitroalcohol is then converted into the acetate, and a sodium tetrahydroborate reduction in dimethyl sulfoxide results in the nitroalkane. The reduction is thought to proceed *via* a nitroalkene.[14] It is sensitive to pH, and dimerisation can occur *via* a Michael addition if basic conditions exist. This can occur *via* reaction of the carbanion of the reduced product with the intermediate nitroalkene giving unsymmetrical 1,3-dinitroalkanes. A high-yielding one-pot procedure has also been reported;[14] however, this method uses no solvent and is not suitable for solid aldehydes.

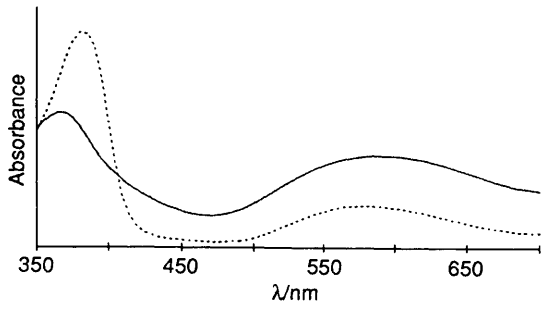


Fig. 1 Diffuse-reflectance spectrum (– – –) of the polymer-bound complex ion $[CuL^{11}]^{2+}$ and electronic spectrum in water of the $[CuL^4]^{2+}$ precursor (——)

The chemistry leading to the metal-free aminobenzyl C-pendant macrocycles is summarised in Scheme 2. The resultant macrocycles present both a pendant primary amine and a pendant aminobenzyl or benzyl group attached to the same carbon of the macrocycle. Analogues such as L^6 act as quinquedentate ligands to metal ions, involving the pendant primary amine in co-ordination,[11] but this primary amine was considered too sterically hindered to permit ready covalent binding to other organic entities. Consequently, we have introduced the aminobenzyl group in L^4 in place of the methyl group in L^6, illustrating techniques which can result in a functionality more appropriate for covalent linking. The syntheses are performed simply, under mild conditions, and with reasonable yields. The spectroscopic properties of both the copper(II) complexes and metal-free macrocycles prepared are consistent with their formulation. In particular, the complexes are very similar to those of L^6 and analogues, which have been exhaustively characterised.[11]

Carbon-substituted macrocycles such as L^4 have the potential for covalent attachment to proteins and antibodies *via* the additional functionality of the reactive aromatic amine. The fifteen-membered macrocycle is also a viable carrier for radiolabelled copper ion, the copper(II) complexes being of high thermodynamic stability and highly resistant to acid or base hydrolysis. Although we have not pursued it here, attachment of acetate arms *via* the secondary amine to extend and amend the metal-ion preference is well established chemistry.[15] The introduction of the appropriate aromatic group may also provide a 'molecular antenna' for intramolecular energy transfer to lanthanide metals such as europium(III), serving as a fluorescent label.[16] At present we have examined the binding of macrocycle L^4 to polymeric or biomolecular species employing a mild carbodiimide coupling agent.

The CM Bio-Gel A is a weakly acidic cation exchanger composed of carboxymethyl groups in a polymer matrix. In a similar manner to peptide linking, its acid group can be attached to the pendant aromatic amine group of the macrocycle L^4 through the use of the water-soluble coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide at modest pH (5.5). The carboxylic acid adds to the diimide carbon atom to produce an unstable intermediate. This intermediate is an activated carboxylic acid derivative which is reactive towards nucleophilic substitution. The coupling reagent is then released as its urea derivative, with an amide bond formed from the carboxylate and aromatic amine groups to form the functionalised resin L^{11}. The functionalised polymer binds copper(II) ion strongly, forming a blue-purple resin which exhibits a diffuse-reflectance spectrum with maxima very similar to those observed for the $[CuL^4]^{2+}$ complex in solution (Fig. 1). The capacity of the functionalised resin equates well with the known capacity of the parent resin, which binds copper ion reversibly compared with the functionalised resin.

Comparable attachment chemistry to that employed with the

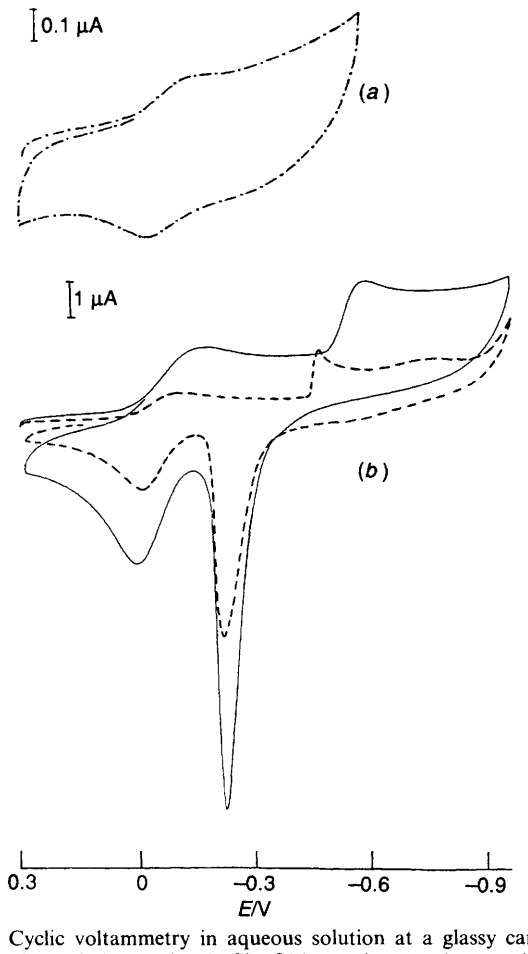


Fig. 2 Cyclic voltammetry in aqueous solution at a glassy carbon working electrode (*versus* Ag–AgCl) of (*a*) cytochrome c (– · – · –) and (*b*) $[CuL^4]^{2+}$ ion alone (– – –) and covalently linked to cytochrome c (——)

resin was pursued with horse heart cytochrome c, employing a 4:1 ratio of macrocycle to cytochrome c. Following treatment with copper ion and chromatography under acidic conditions to dissociate and separate excess of copper ion not bound irreversibly by presumably covalently attached macrocycle, the cyclic voltammetry of the amended cytochrome c was measured. The indistinct reversible wave detected with unmodified cytochrome c is masked in the modified cytochrome c sample by a strong irreversible wave associated with the macrocycle-bound copper ions. The cyclic voltammogram observed is very similar to that recorded for $[CuL^4]^{2+}$ ion alone (Fig. 2). In turn, the cyclic voltammogram of $[CuL^4]^{2+}$ is similar to that observed for the analogue $[CuL^6]^{2+}$,[17] with small shifts in peaks resulting from electronic influences of the different C-pendant groups. Analysis of Cu:Fe ratios in the modified cytochrome c determined by atomic absorption spectroscopy are indicative both of complete retention of the iron in the cytochrome unit and multiple attachment of macrocycle units (greater than three macrocycles per cytochrome) to the cytochrome c. Changes in the electronic spectrum are small, but this is consistent with the high molar absorptivity of the cytochrome unit compared with the copper macrocycle. Overall, there is good evidence of successful covalent attachment, although strong ion-pairing attachment cannot be entirely discounted. The relative simplicity of the synthetic chemistry described for L^4 suggests that this molecule and close analogues are candidates for covalent attachment to more complex biomolecules.

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