Towards Tumour Targeting with Copper-radiolabelled Macrocycle–Antibody Conjugates: Synthesis, Antibody Linkage, and Complexation Behaviour

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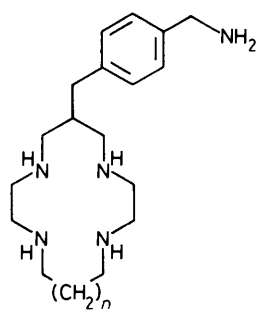
A set of four tetra-azamacrocyclic ligands bearing aminomethylphenyl substituents have been prepared and may be attached to a monoclonal antibody *via* an intermediate thiol-specific vinylpyridine linker molecule. The resultant conjugates may be efficiently radiolabelled with ^{64}Cu or ^{67}Cu at pH 4 to minimise non-specific protein binding, and the copper labelled antibody-conjugate is stable with respect to copper loss *in vivo*. The forward rate of copper binding has been optimised through a kinetic analysis using stopped-flow spectrophotometry. In succinate buffer, anionic copper species $Cu(succ)_2^{2-}$ ($\log \beta = 4.35$) and $HCu(succ)_2^-$ ($\log \beta = 9.64$) are the kinetically significant copper species in the pH range 3.6–5.6.

A challenging aspect of current clinical oncology involves the development of effective methods of radioimmunoimaging[1] and radioimmunotherapy.[2] The two techniques are complementary: *in vivo* imaging provides not only useful information about the size, morphology and location of tumour deposits but it is also vital for prognosis and for monitoring the effects of therapeutic radiation on the tumour following treatment. Large and accessible primary tumours are often best removed by surgery but smaller—more widely distributed—secondary metastases may be cleared following radioimmunotherapy. Most radioimaging applications use γ-ray emission from the nuclear decay of a given radioisotope (*e.g.* ^{99m}Tc, ^{131}I or ^{111}In). Notwithstanding the development of single photon-emitter computerised tomography, vastly superior high resolution images may be obtained with positron emission tomography (down to 3 mm).[3] The most common positron emitters ^{82}Rb and ^{68}Ga have half-lives (1.4 and 1.1 min) that are far too short to permit localisation of a radiolabelled antibody to the tumour. In this respect ^{64}Cu is more suitable ($t_{\frac{1}{2}} = 12.8$ h) and it was prepared for these studies 'carrier-free' from ^{64}ZnO by an (n,p) reaction followed by electrochemical extraction.

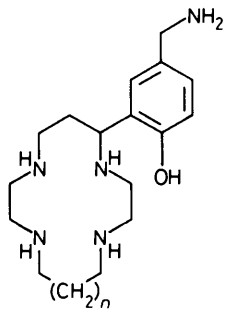
There is a particular interest in those monoclonal antibodies which bind specifically to tumour associated antigens for the treatment of the more common malignancies *e.g.* carcinoma of the breast, lung, or colon. The antibody B72.3 binds specifically to tumour associated glycoprotein (TAG-72) found with a high incidence in colon carcinoma tissue.[4] By irreversibly attaching a β^- emitting radioisotope to a suitably modified B72.3 antibody, effective radioimmunotherapy may be possible. In this respect, ^{67}Cu is an attractive isotope particularly for the treatment of smaller tumours. It has a sufficiently long half-life (62 h) to permit antibody localisation, decays to a stable product (^{64}Zn), has a mean range in tissue of 0.2 mm, and also emits γ-rays of an energy suitable for simultaneous imaging.[5] It is produced from ^{68}Zn by a (p,2p) reaction in a linear accelerator.

Clearly the successful application of copper-radiolabelled antibodies requires that the copper is irreversibly bound to a modified protein. The major kinetic pathway for metal loss from macrocyclic ligands involves an acid-promoted dissociation which is particularly slow for neutral tetra-aza ligands.[6] Previous workers have promulgated the efficacy of ligands such as DTPA (diethylenetriaminepenta-acetic acid) or TETA (1,4,8,11-tetra-azacyclotetradecanetetra-acetic acid) which form anionic complexes with copper at ambient pH. For TETA in particular it has been suggested that serum stability studies (pH 7.4)—which purport to demonstrate that the complex does not dissociate—vindicate its use *in vivo*.[7] These serum studies are of limited value as they fail to recognise that it is in regions of low pH (*e.g.* in the liver and stomach) that a metal–antibody conjugate is most susceptible to dissociation.

Copper is well known to form cationic and kinetically inert complexes with tetra-aza [13]- and [14]-N_4 coronands. Being positively charged the copper complexes are much less susceptible to acid (or cation) promoted dissociation.[8] Accordingly, two C-functionalised [13]-and [14]-membered tetra-aza macrocycles were prepared bearing an exocyclic amino group to facilitate linkage to the protein (**1**) and (**2**). The exocyclic

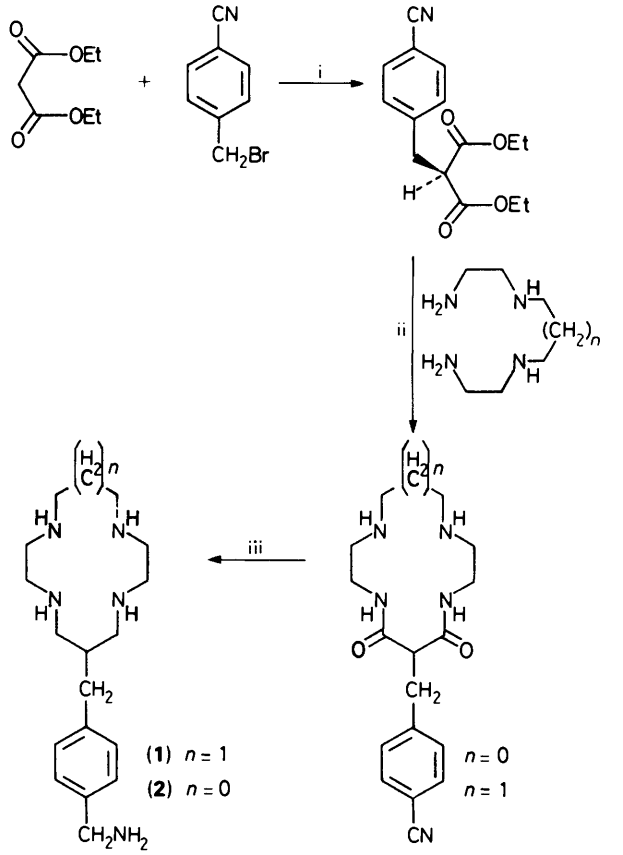


(**1**) $n = 1$
(**2**) $n = 0$



(**3**) $n = 1$
(**4**) $n = 0$

functional group was chosen to be a primary alkylamine group to permit the formation of a stable amide bond between the macrocyclic ligand and the modified protein. Previous studies have invoked a much less nucleophilic primary aminophenyl substituent as the linking group.[1,7] This has been converted either into an isocyanate through the action of thiophosgene or into an α-halo amide *via* reaction with an α-haloacyl chloride. Neither of these rather forcing methods is suitable in the presence of a nucleophilic tetra-amine macrocycle which is sensitive to electrophilic acylation. For purposes of comparison and following the work of Kimura with phenol *C*-substituted polyazamacrocycles,[9] the hydroxyphenyl ligands (**3**) and (**4**)



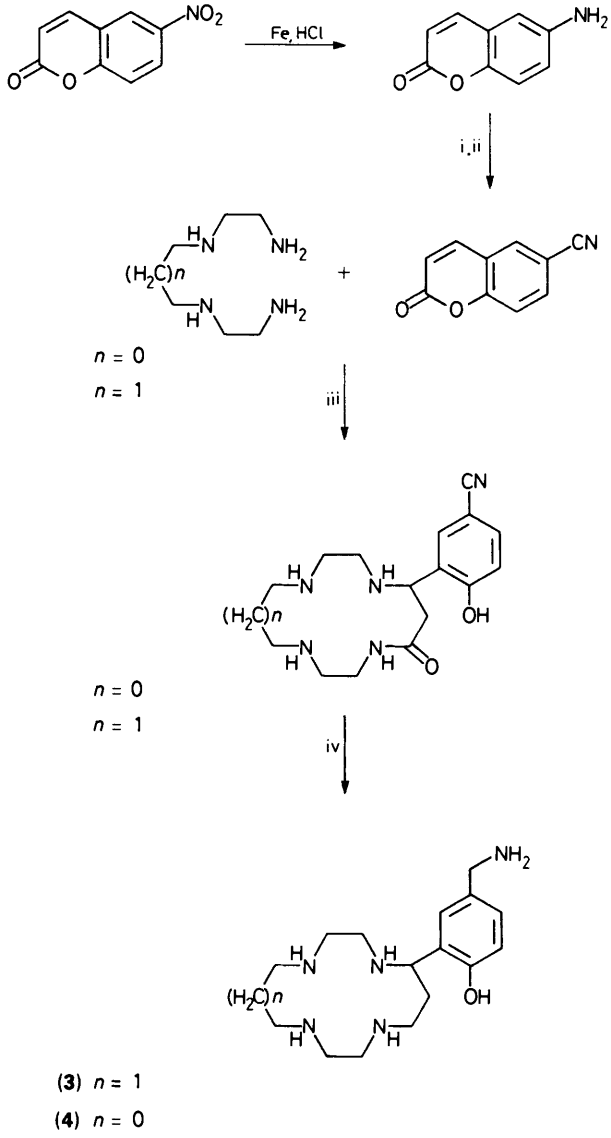
Scheme 1. Synthesis of *C*-functionalised [13]- and [14]-membered macrocycles. *Reagents and conditions:* i, NaOEt, EtOH; ii, EtOH, heat; iii, BH_3–THF, heat.

were prepared incorporating an exocyclic aminomethyl group. The phenol group has been shown to bind—albeit weakly—to copper sitting in the plane of the ring, and it was thought that this might also enhance the forward rates of association. Some of this work has been reported in two preliminary communications.[10,11]

Results and Discussion

Macrocycle Synthesis.—The synthesis of the non-phenolic tetra-amines (**1**) and (**2**) was undertaken following the method established by Tabushi[12] involving condensation of a linear tetra-amine with an appropriately substituted malonate ester. Reaction of diethyl *p*-cyanobenzylmalonate with 1,4,8,11-tetraazaundecane in boiling ethanol gave the macrocyclic diamide in moderate yield following careful chromatography on silica. The [13]-N_4 diamide was more conveniently isolated by crystallisation from hot ethanol. Reduction of the diamides with borane–tetrahydrofuran (TMF) proceeded smoothly to yield the desired penta-amines (Scheme 1). The synthesis of the phenolic macrocycles (**3**) and (**4**) involved condensation of 6-cyanocoumarin (prepared from 6-nitrocoumarin *via* a modification of Micklethwaite's procedure)[13] with the appropriate linear tetra-amine, followed by reduction of the resultant amide with borane–THF (Scheme 2).

Synthesis of Heterobifunctional Linker Molecules and Antibody Conjugation.—Attachment of the nucleophilic aminomethyl substituent on the macrocycle to thiol-modified lysine residues on the protein requires the use of a heterobifunctional cross-linker. This possesses one reactive group to permit amide bond formation to the macrocycle (*e.g.* an active ester) and



Scheme 2. Synthesis of [13]- and [14]-membered *p*-aminomethylphenol pendent macrocycles. *Reagents and conditions:* i, HNO_2/−5 °C; ii, Cu^ICN; iii, CH_3OH, heat; iv, BH_3–THF, heat.

another group permitting linkage to the modified protein (*e.g.* a thiol-selective electrophile). Heterobifunctional agents which employ maleimides *e.g.* (**5**) and (**6**) as the thiol-specific group are available commercially: they are highly reactive but do tend to be hydrolysed slowly above pH 8 to maleamic acid derivatives and generally show moderate selectivity for thiols over primary amines. Notwithstanding these limitations they have been linked to macrocycles (**1**) and (**2**), and the resultant maleimides may be stored as their trifluoroacetate salts over a period of months at −20 °C. A more thiol-selective heterobifunctional agent was sought and vinylpyridine linker molecules were considered. Previous work had established that the thiol group of cysteine is 300 times more reactive towards 4-vinylpyridine than the amino group,[14] and 4-vinylpyridine has been used as a specific marker for cysteine residues in protein identification using HPLC.[15] Both 2- and 4-vinylpyridine react selectively with thiols in the pH range 5–9: under these conditions both pyridines showed <3% reaction with primary amines (2 h). The behaviour of 2- and 4-vinylpyridine towards *N*-acetylcysteine and Ala-Pro-NH_2 is compared in Figure 1, in which the reaction was monitored by HPLC.

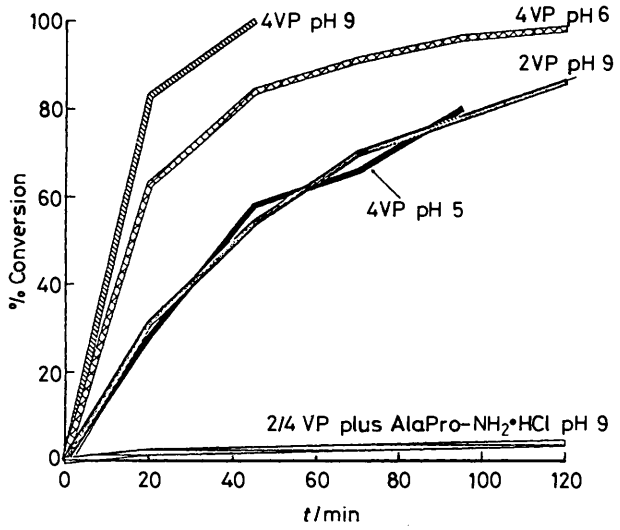
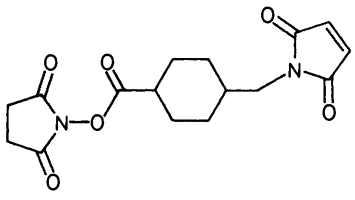


Figure 1. Reaction of 2- or 4-vinylpyridine (5 mmol dm^{-3}) with *N*-acetylcysteine (298 K, fivefold excess of thiol).



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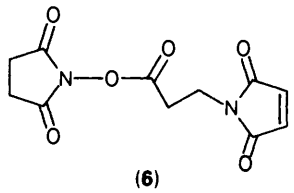

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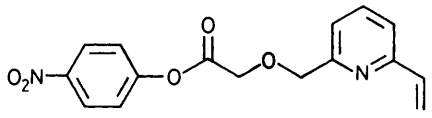
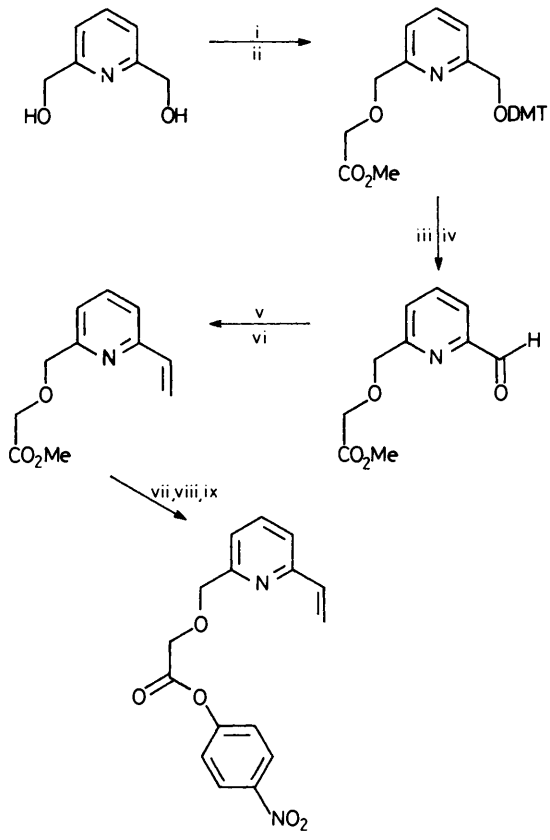


(7)

A suitable heterobifunctional vinylpyridine linker is (7) which incorporates a reactive *p*-nitrophenyl ester to aid linkage to a primary amine. It was synthesised in six steps from 2,6-bis(hydroxymethyl)pyridine. Reaction of the pyridine diol with dimethoxytrityl chloride afforded the mono-protected alcohol (Scheme 3) which was alkylated with ethyl bromoacetate, deprotected with zinc bromide and treated with manganese(IV) oxide to yield the aldehyde. Conversion of the aldehyde to a vinyl group proceeded in higher yield using a Peterson alkenation rather than a Wittig reaction. Ester exchange to give the desired *p*-nitrophenyl ester required that the lithium salt of the acid be converted into its pyridinium salt prior to the coupling reaction with *p*-nitrophenol. The resultant target ester (7) was stored at −20 °C under dry nitrogen.

In order to acylate selectively the exocyclic primary aminomethyl, the acylation of (1) [to (4)] by (7) was effected with careful pH control. Preliminary attempts to protect the tetra-aza ring with nickel(II) or copper(II) were successful, but deprotection of the resulting amide required unsuitable forcing conditions [*i.e.* boiling with 1 mol dm^{-3} NaCN cyanide to remove $Ni(CN)_2$ or treatment with 6 mol dm^{-3} HCl to remove



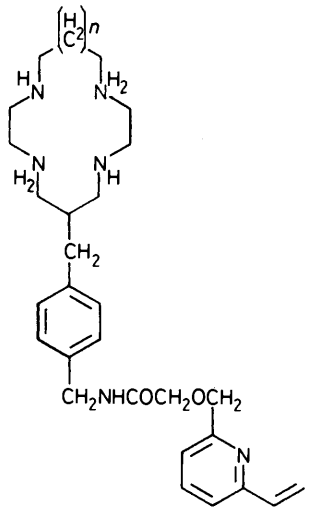
(7)

Scheme 3. *Reagents and conditions:* i, DMTCl/py; ii, $BrCH_2CO_2Me$/BuLi/THF; iii, $ZnBr_2/CH_2Cl_2$; iv, MnO_2/CH_2Cl_2; v, Me_3SiCH_2-MgCl/THF/−78 °C; vi, $SOCl_2$/THF; vii, LiOH, MeOH; viii, H^+; ix, DCC/CH_2Cl_2/p-$NO_2C_6H_4OH$.

copper as $CuCl_4^{2-}$]. At pH 7, the tetra-aza macrocyclic ring is diprotonated (pK_as are 11.5, 10.2, 1.7, and 1.0)[8] and effectively protected from electrophilic attack. Reaction of macrocycles (1)–(4) with (7) in aqueous dioxane (1:1) in a 0.5 mol dm^{-3} solution of piperazinylethanesulphonic acid buffer at 40 °C gave good yields of the desired amides (8)–(11) in <4 h. These amides are stable to long-term storage at 5 °C.

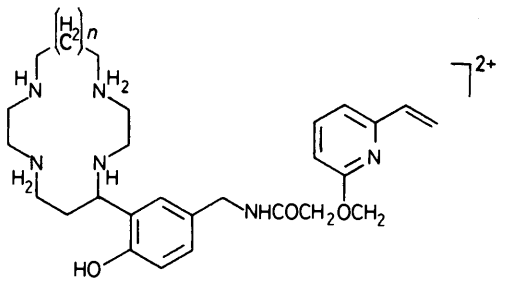
In order to link the macrocycles (8)–(11) to the protein, the antibody B72.3 was treated with 2-iminothiolane (Traut's reagent) [pH 7.4, 4 °C] and purified by filtration through Sephadex G-50 (0.1 mol dm^{-3} phosphate, pH 8). The number of free thiols introduced per antibody varied from 2–5 as determined by titration against Ellman's reagent, 6,6′-dinitro-3,3′-dithiobenzoic acid.[15] A solution of 'Trauted' antibody at pH 8, in the presence of 2 mmol dm^{-3} EDTA to sequester trace metals, was divided in half and was incubated at 4 °C for 18 h in the absence or presence of the vinylpyridine derivative (8)–(11). After centrifugation through Sephadex both fractions were assayed for free thiols using Ellman's reagent and the difference was taken to give an indication of the number of thiols capped by macrocycles. Before any radiolabelling studies were performed with these conjugates, those thiol groups which had been oxidised were reduced back to thiols (using dithiothreitol) and these free thiols were capped by reaction with excess 4-vinylpyridine to prevent their participation in metal binding.

Although the 'Ellman assay' method to determine the number of macrocycles per antibody gave reasonably consistent values, an independent and more direct technique was sought in order to confirm the values obtained. Other workers have used radioassay methods based on either ^{14}C labelled complexing



(8) $n = 1$

(9) $n = 0$

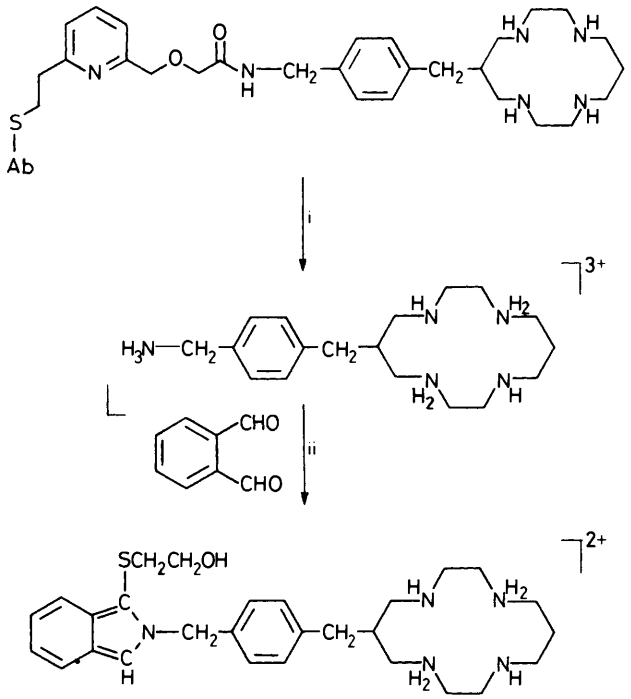


(10) $n = 1$

(11) $n = 0$

agents[1] or ^{57}Co-complexed antibody–chelate conjugates.[16] A fluorimetric method was sought based on the reaction of primary amines and *o*-phthalaldehyde in the presence of 2-thioethanol (OPA assay).[17] The resultant isoindole may be detected at low concentrations (*ca.* 10^{-11} mol dm^{-3}). Exhaustive hydrolysis of the antibody–macrocycle conjugate with 6 mol dm^{-3} HCl (18 h, 110 °C) yielded a mixture of amino acids and protonated macrocycles (**1**)–(**4**). Reaction of the liberated primary amines with *o*-phthalaldehyde and 2-mercaptoethanol gave a series of isoindole adducts which were detected spectrofluorimetrically. Separation of the macrocycle-isoindole compound [λ_{exc} 334 nm, λ_{fluor} 453 nm] (Scheme 4) from derivatised amino acids was achieved using cation exchange HPLC, taking advantage of its dipositive charge at pH 6.5. Typically derivatised antibodies were prepared with between 0.5 and 2 macrocycles per antibody, as determined by the OPA assay. This level of conjugation was consistent with that determined previously using the modified Ellman's assay. The immunoreactivity of the derivatised B72.3 antibody was assayed by affinity chromatography—measurement of the percentage of the total antibody which retains an affinity for an immobilised TAG-72 antigen. It was observed that up to three macrocycles per antibody could be tolerated before compromising the immunoreactivity, as noted by other workers.[1,2,16]

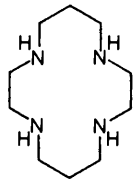
This system—outlined above—for attaching macrocycles to the lysine residues of a 'Trauted' antibody, leads to a random distribution of macrocycles on the protein. It is preferable to attach complexing agents outside the immunologically sensitive variable region of the antibody. Using recombinant antibody



Scheme 4. *o*-Phthalaldehyde assay for the number of macrocycles bound per antibody. *Reagents and conditions:* i, 6 mol dm^{-3} HCl, heat 16 h; ii, $SHCH_2CH_2OH$, 20 °C, ≤60 s.

methods, cysteine residues may be engineered into the antibody close to the hinge region thereby permitting site-specific attachment of the required macrocycles.

Copper Binding to Tetra-aza Macrocycles.—It is of great importance to optimise the forward rate of copper association to a macrocycle–antibody conjugate, to bind selectively the copper in the macrocycle and minimise the degree of non-specific (protein-bound) labelling. This is dictated by the short half-lives of the copper isotopes—particularly ^{64}Cu (12.8 h), by the need to minimise the dose to the protein when in a concentrated form and by the tendency of protein-bound copper to dissociate *in vivo* and accumulate in the liver and kidney. Accordingly a series of semi-quantitative studies was undertaken with a view to maximising the rate of copper binding by the parent macrocycles (**12**)–(**15**). This work was



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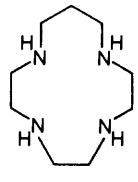

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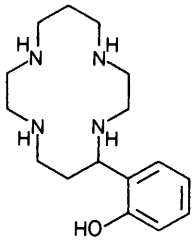

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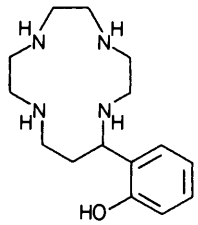

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Table 1. Percentages of ^{64}Cu activity bound (293 K, 30 min).

Buffer	pH	Stripping agent	Mac-Ab X	Control Ab Y	Ratio X:Y
0.3 mol dm^{-3} phosphate	7	—	38.6	4.7	8.2
0.2 mol dm^{-3} succinate	4	—	57.6	0.65	88.6
0.2 mol dm^{-3} acetate	4	—	33.2	0.64	52.0
0.2 mol dm^{-3} acetate	4	cyclam	19.6	0.15	130.0
0.2 mol dm^{-3} succinate	4	cyclam	35.3	0.20	176.0
0.3 mol dm^{-3} phosphate	5.5	—	60.4	8.8	6.9
0.3 mol dm^{-3} phosphate	5.5	EDTA	55.1	4.3	12.8

guided by the observations of Pettit[18] who noted that oligopeptides do not bind copper(II) ions significantly in aqueous solution <pH 4.5. Initially, the effects of low pH (4) and of a post-labelling wash of the protein with a 'stripping agent' (*i.e.* cyclam) upon the incorporation of ^{64}Cu by a control antibody and a macrocycle–antibody conjugate were investigated. A macrocycle–antibody (B72.3) conjugate [using (**3**) with 0.24 macrocycles per antibody] was incubated at pH 7 or pH 4 for 30 min and the radiolabelled conjugate was purified using a PD10 column in order to determine the percentage of the ^{64}Cu activity associated with the protein fraction. This was repeated for the control antibody (B72.3). The effect of a 'stripping agent' was studied by incubating the labelled protein with a 150-fold excess (over Ab) of cyclam (**12**) for 30 min. Results are summarised in Table 1, and these show the benefits of working at a lower pH to minimise non-specific binding of copper to the protein and the advantage of using a 'stripping agent' in minimising this further.

Having established the preference for working at low pH, the rate of copper binding by (**12**)–(**15**) was compared as a function of pH, ionic strength, temperature and buffer. Prior work had concluded that in binding copper and nickel ions at low pH, the monoprotonated form of the macrocyclic tetra-amines was much more reactive (by *ca.* 10^6) than the more abundant diprotonated form.[19] In addition, incorporation of copper at low pH was significantly faster in the presence of acetate, and this was interpreted in terms of the higher reactivity of $[Cu(OAc)]^+$ compared to aqueous Cu^{2+}. It was therefore evident that electrostatic repulsion between the protonated ligand and the copper species should be minimised in formation of the initial outer-sphere complex. Moreover it was recently noted that copper was bound by cyclam, (**12**), most quickly in the presence of succinate and this was interpreted in terms of the enhanced reactivity of the neutral [Cu(succ)] species.[20]

The effect of temperature on the rate of incorporation of Cu^{2+} by (**13**) was studied by stopped-flow spectrophotometry, with the ligand in tenfold excess (*i.e.* pseudo-first-order conditions for copper). In an 0.2 mol dm^{-3} succinate buffer at pH 4, the observed pseudo-first-order rate constant increased from 2.30 s^{-1} at 298 K to 12.55 s^{-1} at 318 K, with an Arrhenius activation energy of 63(±3) kJ mol^{-1}. Antibodies are denatured at temperatures >40 °C, so a temperature of 37 °C is appropriate for protein labelling.

The effects of varying ring size and buffer-type (acetate *vs.* succinate *vs.* citrate) were studied under the same experimental conditions (see the Experimental for details). Pseudo-first-order constants are given in Table 2. As expected the rate was faster for (**13**) than for (**12**), and succinate and citrate buffers gave the faster rates for (**13**) and (**12**), respectively. At pH 4.0 and in 0.2 mol dm^{-1} succinate the ligand (**13**) bound copper most quickly (Table 3). The behaviour of the [13]-membered phenolic ligand, (**15**), was unique. It appeared to react by a more complex mechanism since no single exponential curve could be resolved from the absorbance profile. Instead, the profile was fitted to three separate exponentials, comprising a fast reaction complete

Table 2.[a]

Ligand	k_{obs}/s^{-1} acetate	succinate	citrate
(**13**)	0.530	2.550	1.670
(**12**)	0.077	0.422	0.627

[a] Rate of copper association: pH 4.0, [buffer] = 0.20, 298 K, [Cu] = 5×10^{-4} mol dm^{-3}, [L] = 5×10^{-3} mol dm^{-3}.

Table 3.

Ligand	(**12**)	(**13**)	(**14**)	(**15**)
k_{obs}/s^{-1}	0.422	2.55	0.387	complex

[a] Rates of copper association: pH 4.0, [succinate] = 0.20 mol dm^{-3}, 298 K.

Table 4. Formation constants for the copper succinic acid (L) system.[a]

	SCOGS	SUPERQUAD
$\log \beta_L$	5.20	5.20
$\log \beta_{HL}$	9.19	9.19
$\log \beta_{CuL}$	2.59	2.58
$\log \beta_{CuHL}$	7.03	7.03
$\log \beta_{CuL_2}$	4.30	4.35
$\log \beta_{CuHL_2}$	9.59	9.64

[a] [Cu] = 2×10^{-3} mol dm^{-3}, [H_2succ] = 4×10^{-3} mol dm^{-3}, [$HClO_4$] = 4×10^{-3} mol dm^{-3}, [$NaClO_4$] = 0.1 mol dm^{-3}, 25 °C.

within 50 ms (k_{obs} = 110 s^{-1}), a slower reaction (k_{obs} = 0.532 s^{-1}) and a much slower step (k_{obs} = 0.047 s^{-1}). As this was not observed for the non-phenolic analogue, a step-wise mechanism involving transient phenolic participation is likely. The phenolic ligands evidently did not offer any advantage over (**13**) and (**12**) in terms of rate enhancement of copper binding. Finally, at pH 4 and in an 0.2 mol dm^{-3} succinate the effect of ionic strength on the rate of copper binding by (**13**) was examined. On varying the ionic strength (with added sodium perchlorate) from 1.0–0.2, and finally to *ca.* 0.02 (by diminishing the succinate concentration), the rate increased with k_{obs} of 1.00, 1.77, and 2.42 s^{-1}, respectively. Such an effect rules out the postulation of two cationic species interacting in the kinetically significant rate-determining step and implicates the encounter of oppositely charged species *i.e.* anionic copper species. This behaviour was examined more closely for (**13**) in a succinate buffer of varying composition.

Kinetic Analysis of [13]-N_4 *Copper Complexation in Succinate Buffer.*—In order to clarify the role of copper succinate species in binding to tetra-aza macrocycles, the copper(II)-succinic acid was investigated carefully by pH potentiometric analysis. Although prior work by Kaden had not considered the role of anionic copper species,[20] a polarographic analysis of the copper–succinate system had been reported[21] with stepwise formation constants of 4.00 and 2.57 for Cu(succ) and $Cu(succ)_2^{2-}$. This contrasted with other values determined potentiometrically for Cu(succ) *e.g.* 2.61 for Cu(succ) and 1.86 for $HCu(succ)^+$.[22]

A standard pH-metric analysis of the copper–succinic acid system was effected and the data were analysed using the non-linear least-squares programs, SCOGS and SUPERQUAD.[23] The pK_a values for succinic acid of pK_1 = 3.99, pK_2 = 5.20 (I = 0.1, 298 K) were taken as constant. Agreement between the two methods of analysis was good (Table 4),

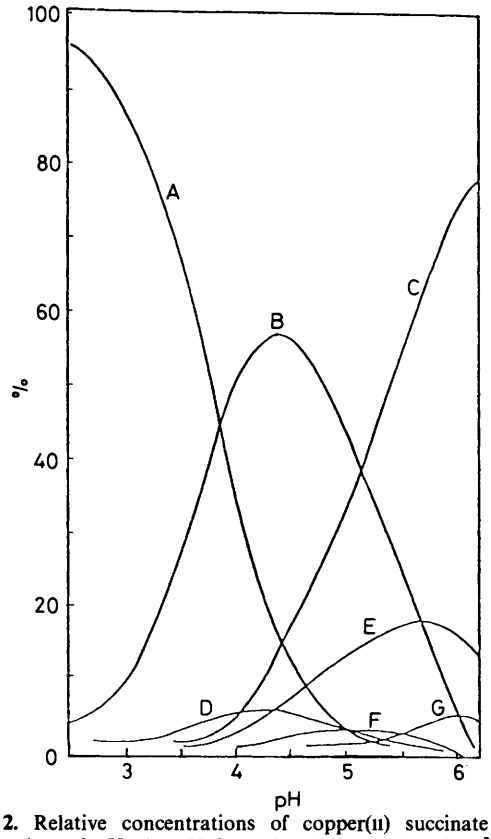
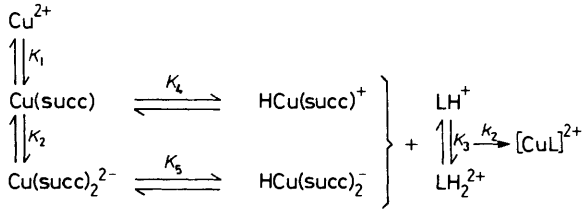


Figure 2. Relative concentrations of copper(II) succinate species as a function of pH. A = H_2Succ; B = $HSucc^-$; C = $Succ^{2-}$; D = $CuHSucc^+$; E = CuSucc; F = $CuHSucc_2^-$; G = $CuSucc_2^{2-}$; [Cu] = 2 mmol dm^{-3}; [H_2Succ] = 4 mmol dm^{-3}; [$HClO_4$] = 2 mmol dm^{-3}; [$NaClO_4$] = 0.1 mol dm^{-3}.

and the species distribution as a function of pH (Figure 2) shows clearly the presence of $Cu(succ)_2^{2-}$ and $HCu(succ)_2^-$ in small quantities even with a twofold excess of succinate to copper. Their relative concentration in a 0.2 mol dm^{-3} succinate buffer would obviously be much greater.

Mechanistically, the reaction between Cu^{II} ions and [13]-N_4 in a succinate buffer is more complex than in acetate. In acetate, it has been established[19] that $[CuOAc]^+$ is the kinetically significant species at low pH (3.2–3.7) and that the mono-protonated ligand was 10^6 more reactive than the diprotonated species. In succinate a full analysis based on the dependence of rate on pH is not possible since both ligand and copper–succinate equilibria are pH dependent (Scheme 5). However it



Scheme 5.

is most instructive to consider the relative importance of the different copper species as a function of pH and succinate concentration. The slope of the ionic strength dependence of the rate at a given pH indicates the product of the charge of the species involved in the rate-determining step: the relative contributions of Cu^{2+} and its mono- and di-succinate complexes may be evaluated from the variation of rate with succinate concentration.

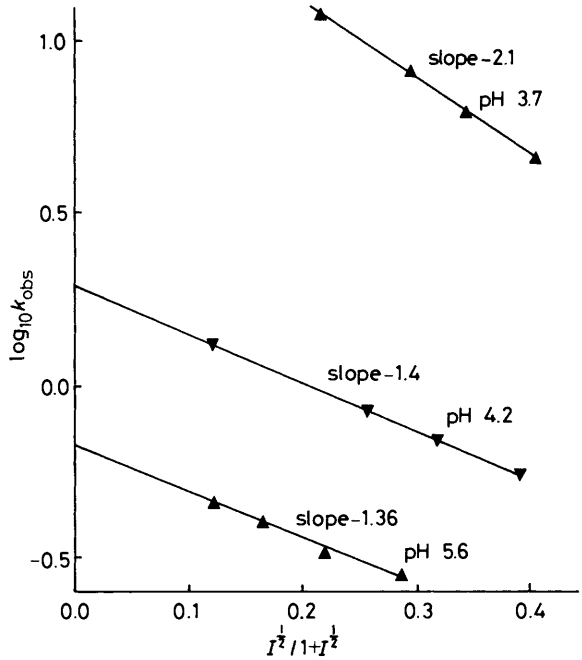


Figure 3. Ionic strength dependence. The rate-determining step involves species of opposite charge. LH with $[Cu(succ)_2]^{2-}$ and $[CuH(succ)_2]^-$ are involved.

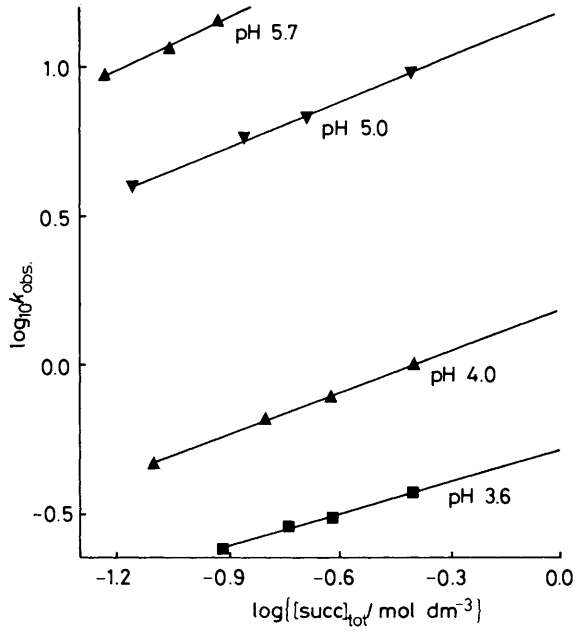


Figure 4. Rate dependence on [succinate].

For the ionic strength dependence, the rate was observed at three pH values (5.6, 4.2, and 3.7) and in all three cases the rate of complexation decreased with increased ionic strength (I = 0.02–0.48), the slope being steepest at pH 5.6. A plot of $\log k_{obs}$ *vs.* ($I^{\frac{1}{2}}/1 + I^{\frac{1}{2}}$) gave a linear dependence (Figure 3) with a negative slope ($2A'Z_AZ_B$ where A' = 0.509 $dm^{\frac{3}{2}}$ $mol^{-\frac{1}{2}}$, and Z_A and Z_B are the charges on the reactant species) rising from −1.36 (pH 4) to −2.1 (pH 5.6). Given the kinetic dominance of the monoprotonated (LH^+) ligand, both mono-anionic and dianionic copper species are implicated *i.e.* $HCu(succ)_2^-$ and $Cu(succ)_2^{2-}$. It may be noted that previous studies of metal complexation in carboxylate buffers have not addressed this issue,[20] and the results presented may therefore be open to other mechanistic interpretation.

The rate of reaction accelerates with increasing succinate concentration ($I = 0.3$) and a plot of $\log k_{obs}$ *vs.* $\log [succ]^{2-}$ shows that the dependence is steeper at higher pH (Figure 4). At high pH, such a dependence may reflect the conversion of Cu(succ) into the more reactive $Cu(succ)_2^{2-}$ species while at lower pH the more gentle slope may arise from the conversion of free Cu^{2+} into the more reactive neutral copper–monosuccinate complex. For a more thorough analysis, the equilibria in Scheme 5 all need to be considered [equation (1)].

$$\text{velocity} = k_2[Cu]_{tot}\{[13]\text{-}N_4\}_{tot} = k_{obs}[Cu]_{tot} \quad (\textit{ligand is in excess})$$

$$\therefore\ k_{obs} = \frac{v}{[Cu]_{tot}} = \{k_{Cu}[Cu^{2+}] + k_{CuL}[CuL] + k_{CuLH}[CuLH] + k_{CuL_2}[CuL_2] + k_{CuL_2H}[CuL_2H]\}/[Cu]_{tot} \quad (1)$$

Expressing the concentration of each copper species in terms of known quantities, *i.e.* K_1, K_2, K_4, and K_5 (Scheme 5) and the concentrations of $[succ^{2-}]$ and $[H^+]$ gives equation (2),

$$K_1 = \frac{[CuL]}{[Cu^{2+}][L]},\ K_2 = \frac{[CuL_2]}{[CuL][L]},\ K_4 = \frac{[CuHL]}{[CuL][H^+]},\ K_5 = \frac{[CuHL_2]}{[CuL_2][H^+]}$$

and

$$Cu_{tot} = [Cu^{2+}] + [CuL] + [CuHL] + [CuL_2] + [CuL_2H] \quad (2)$$

so that in terms of [Cusucc], equation (2) becomes equation (3).

$$[Cu_{tot}] = \frac{[CuL]}{K_1L} + [CuL] + K_4[CuL][H^+] + K_2[CuL][L] + K_2K_5[CuL][L][H^+]$$

$$\therefore\ [Cu]_{tot} = [CuL]\left\{\frac{1}{K_1[L]} + 1 + K_4[H^+] + K_2[L] + K_2K_5[L][H^+]\right\} \quad (3)$$

Writing [CuL] as a fraction of the total copper gives equation (4).

$$\frac{[CuL]}{[Cu]_{tot}} = \frac{K_1[L]}{\delta_1}$$

where

$$\delta_1 = 1 + K_1[L]\{1 + K_4[H^+] + K_2[L](1 + K_5[H^+])\} \quad (4)$$

Since each concentration is known in terms of [CuL], the other fractions may be written as follows [equation (5)].

$$\frac{[Cu^{2+}]}{[Cu]_{tot}} = \frac{1}{\delta_1}$$

$$\frac{[CuHL]}{[Cu]_{tot}} = \frac{K_1K_4[L][H^+]}{\delta_1}$$

$$\frac{[CuL_2]}{[Cu]_{tot}} = \frac{K_1K_2[L]^2}{\delta_1}$$

$$\frac{[CuHL_2]}{[Cu]_{tot}} = \frac{K_1K_2K_5[L]^2[H^+]}{\delta_1} \quad (5)$$

Table 5. Equilibrium constants (298 K) for formation of copper succinate species as a function of ionic strength (I/mol dm^{-3}).

I	K_1	K_2	K_4	K_5
0.1	380	60	2.75×10^4	1.94×10^5
0.3	150	60	2.75×10^4	1.17×10^5

Substituting for each fraction in equation (1) gives equation (6).

$$k_{obs} = \{k_{Cu} + k_{CuL}K_1[L] + k_{CuHL}K_1K_4[L][H^+] + k_{CuL_2}K_1K_2[L]^2 + k_{CuHL_2}K_1K_2K_5[L]^2[H^+]\}/\delta_1$$

$$\therefore\ \frac{k_{obs}\delta_1}{[L]} = \frac{k_{Cu}}{[L]} + k_{CuL}K_1 + k_{CuHL}K_1K_4[H^+] + \{k_{CuL_2}K_1K_2 + k_{CuHL_2}K_1K_2K_5[H^+]\}[L] \quad (6)$$

According to equation (6), a plot of $k_{obs}\delta_1/[L]$ *vs.* [L] provides information about the role of copper–monosuccinate species from the intercept and copper–disuccinate species from the slope.

For the pH 5.7 and pH 5.0 experiments, the concentration of $succ^{2-}$ (L) can be calculated directly from the amounts of NaOH and free ligand added to the buffer solution. Since $[succ^{2-}]$ is so low at pH 4.0 and pH 3.6, it must be calculated from $[HSucc^-]$ using equation (7).

$$pH = pK_a + \log\frac{[succ^{2-}]}{[Hsucc^-]} \quad (7)$$

The pK_a values of succinic acid at ionic strength 0.3 mol dm^{-3} are *ca.* 4.0 and 5.2. Since the equilibrium constants K_1, K_2, K_4, and K_5 are known at $I = 0.1$ (Table 5), their values at $I = 0.3$ may be calculated using the Debye–Hückel, equation (8).

$$\log K_I = \log K_0 + \frac{2AZ_AZ_BI^{\frac{1}{2}}}{1 + I^{\frac{1}{2}}}$$

i.e. $\log K_1 = 2.58$ at $I = 0.1$ ($Z_AZ_B = -4$) (8)

From equation (8):

$$\log K_{0.1} = \log K_0 - 0.96$$

$$\log K_{0.3} = \log K_0 - 1.42$$

$$\log K_1 = 2.58 - 0.46$$

and $K_1 \approx 150$ (at $I = 0.3$)

The complete set of equilibrium constants is given in Table 5.

K_2 and K_4 are assumed to be independent of ionic strength since the net charge is equal on both sides of the equilibrium. According to Debye–Hückel theory, neither products nor reactants will be stabilised more than the other by the presence of a more intense ionic atmosphere of equal but opposite charge at $I = 0.3$ mol dm^{-3}. Plots of $k_{obs}\delta_1[succ]$ *vs.* [succ] are shown in Figures 5 and 6. At lower pH (Figure 5) a positive intercept and slope are obtained, indicating that both mono- and di-succinate copper species are kinetically significant. At higher pH (Figure 6) the lack of a positive intercept suggests that monosuccinate species are no longer of major importance and that disuccinate species make an important contribution, in accord with the ionic strength dependence results.

In summary, the dependence of rate upon pH is a complex function of the degrees of protonation of both copper and ligand species and it is not possible to evaluate unique second order rate constants. It is possible to define the role played by

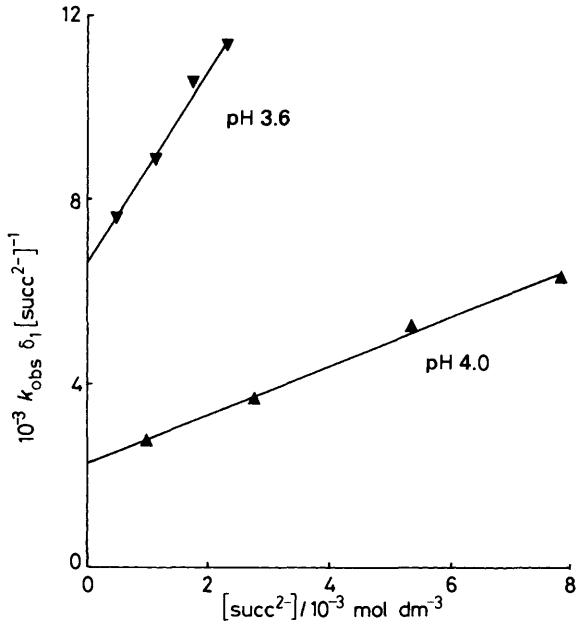


Figure 5. Dependence on succinate concentration. Cu(succ) and $Cu(succ)_2$ species are kinetically significant.

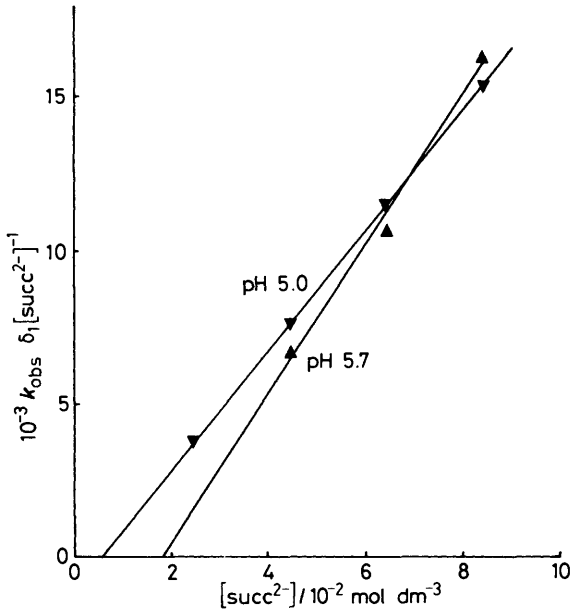


Figure 6. Dependence on succinate concentration. Disuccinate copper species are reactive at higher pH.

anionic copper species: at higher pH both $Cu(succ)_2^{2-}$ and $HCu(succ)_2^{2-}$ are implicated from the ionic strength dependence and from the succinate dependence, and the absence of a positive intercept negates a significant role for monosuccinate species. At lower pH, monosuccinate species are clearly involved but the ionic strength dependence suggests that $CuH(succ)_2^-$ is the dominant species.

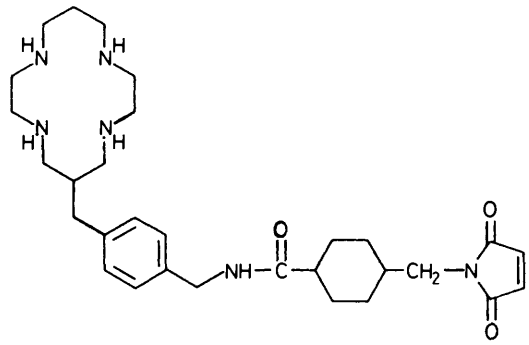
Biodistribution Studies.—The positron-emitting isotope ^{64}Cu ($t_{\frac{1}{2}}$ = 12.8 h) was prepared at Harwell by an (n, p) reaction by bombarding spectral grade zinc oxide with neutrons. The ^{64}Cu was extracted electrochemically in a 'carrier-free' form (*i.e.* in the absence of 'cold' copper), the electrode used being washed with 40 mmol dm^{-3} HCl to yield a solution of 6 mCi of $^{64}CuCl_2$.

Table 6. Biodistribution data for pre-labelled [^{64}Cu-(**1**)-B72.3] conjugate at 18 h. Number of mice per data set = 3.

Tissue	PD-10 purified % dose gm^{-1} tissue	HPLC purified % d gm^{-1}
Blood	17.5 ± 0.7	18.4 ± 0.2
Kidneys	5.7 ± 0.8	6.3 ± 1.2
Liver	8.9 ± 0.4	6.0 ± 0.5
Spleen	5.2 ± 0.3	5.1 ± 0.5
Lungs	7.8 ± 1.0	8.1 ± 0.6

Table 7. Biodistribution data for 'post-labelled' [^{64}Cu-(**3**)-B72.3] conjugate at 21 h. No. of mice per data set = 2.

Tissue	PD-10 purified % dose gm^{-1} tissue	HPLC purified % d gm^{-1}
Blood	18.5 ± 0.4	20.0 (±0.0)
Kidneys	5.7 ± 0.8	5.8 (±0.1)
Liver	8.3 ± 1.2	8.0 (±0.2)
Spleen	5.8 ± 0.1	5.0 (±0.4)
Lungs	7.5 ± 0.4	7.8 (±0.5)



The first biodistribution study was undertaken to assess the stability of the copper–macrocycle conjugate *in vivo*. A maleimide conjugate, (**16**), was prepared following reaction of (**1**) with sulphosuccinimido-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate at pH 6.8. The conjugate (**16**) was then labelled with $^{64}CuCl_2$ at pH 6.5 and subsequently linked to the 'Trauted' antibody. In this control experiment therefore, no non-specific labelling of the protein by copper can occur. After 2 h at 20 °C more than 60% of the total activity was associated with the protein. The protein fraction was purified either on a PD-10 column (Sephadex G-25) or using size-exclusion HPLC (Dupont GF-250) and each sample was injected into several mice and biodistribution data were obtained after 18 h (Table 6). The HPLC purification more efficiently removes protein aggregates and fragments (PD-10 columns are less efficient). The blood level at 55.8% of the injected dose (18 h) is close to the theoretical maximum value predicted by equilibration of IgG with the extracellular fluid. Moreover the liver level is less than one third of the cardiovascular level consistent with its blood content (30% perfused by blood). Since free $^{64}CuCl_2$ tends to accumulate in the liver and kidney, the macrocycle–antibody conjugate is clearly stable *in vivo* with respect to copper loss.

A sample of (**10**) coupled to a 'Trauted' B72.3 antibody was labelled in succinate buffer (pH 4) at 37 °C. The radiolabelled conjugate was purified either by gel filtration (PD-10) or HPLC (GF-250). Biodistribution data at 21 h (Table 7) revealed that the PD-10 purified material gave values apparently superior to that obtained using pre-labelling (Table 6): a higher blood to liver ratio is apparent. Again, HPLC-purified protein gave a superior biodistribution profile probably reflecting the removal

Table 8. Biodistribution data for [^{67}Cu-(3)-B72.3]. Two mice for 4 and 72 h; 7 mice for 24 h time point.

Tissue	4 h % d gm^{-1}	24 h % d gm^{-1}	72 h % d gm^{-1}
Blood	28.8 ± 1.9	19.1 ± 1.2	18.1 ± 0.9
Kidneys	8.2 ± 0.1	5.9 ± 0.4	6.2 ± 0.5
Liver	9.3 ± 0.4	6.5 ± 0.6	5.1 ± 0.3
Lungs	10.1 ± 0.1	7.7 ± 0.8	8.6 ± 0.3
Spleen	5.9 ± 0.2	4.1 ± 0.5	4.9 ± 0.5

of aggregated protein which tends to accumulate in the liver. These data certainly vindicate the choice of the low pH regime for direct labelling of the antibody–macrocycle conjugate. It should be noted that the level of activity in the liver (HPLC purified) is still slightly higher than it should be. Even HPLC may fail to separate aggregates cleanly.

The same antibody–macrocycle conjugate was also labelled with ^{67}Cu (supplied from Los Alamos, USA) (pH 4.0, 30 min, 37 °C, succinate buffer 0.02 mol dm^{-3}) and the labelled protein was washed with an aqueous solution of diethylenetriaminepenta-acetic acid (5 min). The conjugate was purified using size-exclusion HPLC and biodistribution profiles were obtained at 4, 24, and 72 h (Table 8). The most important feature of these data is that the blood to liver ratio does not decrease between 4 and 72 h. This indicates that the activity in the liver can be attributed entirely to its blood content. The apparent decrease in blood activity is due to normal antibody catabolism in the liver and radiolabel excretion. The blood: organ ratios for the other tissues also do not change appreciably with time.

These biodistribution data provide unequivocal evidence for the stability *in vivo* of copper-radiolabelled tetra-azamacrocycle–antibody conjugates. Such behaviour is essential for the usage of radiolabelled antibodies in diagnostic medicine (*i.e.* tumour imaging with ^{64}Cu positron emission tomography) or for the therapeutic treatment of certain cancer types using ^{67}Cu-labelled antibodies.

Experimental

HPLC analyses and purifications were performed using a Varian Vista 5560/Polychrom 9060 system with a cation exchange column (CM300 Synchropak). For each run, the flow rate was 1.4 cm^3 min^{-1} and the eluant gradient was varied as follows: (A = H_2O; B = 1 mol dm^{-3} NH_4OAc; C = CH_3CN), t = 0, A = 70%, B = 10%, C = 20%; t = 20 min, A = 80%, C = 20%. The retention time and eluant pH is specified for each different macrocyclic polyamine. Column chromatography was effected using silica gel (Merck 7734 and 9385). For the purification of macrocyclic amides and diamides, the standard conditions specified were [A = aq. NH_3 (0.89); B = CH_3OH; C = CH_2Cl_2]: t 0–1 h, (A = 1%, B = 12%, C = 87%); t 1–3 h (A = 2%, B = 23%, C = 75%); t 3–6 h (A = 4%, B = 36%, C = 60%); t > 6 h (A = 6%, B = 44%, C = 50%).

UV–VIS spectra were recorded with a Perkin-Elmer Lambda 3 or Uvikon 930 spectrophotometer (λ_{max}/nm, ε/dm^3 mol^{-1} cm^{-1}). Mass spectra were recorded using a VG7070E spectrometer operating in either CI, DCI, or FAB positive ion mode, unless otherwise indicated. IR spectra were recorded using a Perkin-Elmer 577 spectrometer, and ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC250 spectrometer operating at 250.1 and 62.9 MHz, respectively. Solvents were dried from an appropriate drying agent and dimethylformamide was used as received (Aldrich HPLC).

For the kinetics experiments, rates of reaction were measured using a Hitech SF-3L/30C stopped-flow spectrophotometer, on line with an Apple IIe microcomputer with 80 column card. Hi-tech ADS-1 software was used to analyse the raw data and compute the pseudo-first-order rate constant. pH measurements were made using a Jenway 3020 pH meter in conjunction with a Russell microelectrode. Equilibrium constants were determined by pH potentiometry using a Mettler DL20 titrator fitted with a 1 cm^3 burette (to add the base in 0.001 cm^3 increments or as desired) and a Mettler DG112 combination electrode was used in conjunction with a BBC microcomputer. The titration data was analysed by the two non-linear least-squares programs, SCOGS and SUPERQUAD.[23]

6-Cyanocoumarin.—To a suspension of 6-aminocoumarin hydrochloride[13] (22.7 g, 115 mmol) in hydrochloric acid (3 mol dm^{-3}, 100 cm^3) at 0 °C, was added a solution of sodium nitrite (8.36 g, 121 mmol) in water (18 cm^3) over 20 min. Following neutralisation with sodium carbonate, this solution was added in 5 cm^3 aliquots to a cold aqueous (50 cm^3) solution of copper(I) cyanide (12.3 g, 137 mmol) and potassium cyanide (16.9 g, 260 mmol). Chloroform was added (400 cm^3) to prevent excessive foaming and the reaction mixture was allowed to warm to room temperature over a period of 3 h. After further warming to 50 °C for 30 min, more chloroform (100 cm^3) was added, the mixture was filtered and the organic layer was separated, dried (K_2CO_3) and evaporated to yield an orange solid, homogeneous by TLC 15.9 g (81%), m.p. 214–215 °C (Found: C, 70.3; H, 2.8; N, 8.0. $C_{10}H_5NO_2$ requires: C, 70.2; H, 2.92; N, 8.19%); m/z (CI) 171 (M^+) and 143 (M^+ − CO); ν(KBr) 2 225 (CN) and 1 730 cm^{-1} (CO); δ_H($CDCl_3$) 7.85 (1 H, d), 7.79 (1 H, dd), 7.73 (1 H, d), 7.43 (1 H, d), and 6.55 (1 H, d); δ_C($CDCl_3$) 159.0 (s, CO), 156.4 (s, C–O), 141.7(5), 134.7, 132.3, 118.8, 118.4, 117.5, and 108.7.

11-(2-Hydroxy-5-cyanophenyl)-1,4,7,10-tetra-azacyclotridecan-13-one.—A solution of 1,4,7,10-tetra-azadecane (4.39 g, 30 mmol) and 6-cyanocoumarin (5.12 g, 29.9 mmol) in dry methanol (165 cm^3) was heated under reflux for 6 days. The methanol was evaporated and the crude residue was chromatographed on silica gel (standard conditions as above) to give the amide as a pale orange solid (1.57 g, 17%), m.p. 119–121 °C; R_f (aq.NH_3/CH_3OH/CH_2Cl_2, 5:45:50 on SiO_2) 0.27; m/z (DCI, NH_3) [Found: M, 318.19386. $C_{16}H_{23}N_5O_2$ requires 318.19298, (M^+ + 1)]; ν(KBr) 3 250 (NH), 3 050 (CH), 2 930 (CH), 2 840 (CH), 2 208 (CN), 1 640 (CO), 1 600 (Ar), 1 470, and 1 430 cm^{-1}; δ_H($CDCl_3$) 7.69 (1 H, br s, NHCO), 7.37 (1 H, d, J 8.6 Hz), 7.35 (1 H, s), 6.76 (1 H, d, J 8.3 Hz), 4.18 (1 H, d, ArCHN, J 11.5 Hz), and 3.73–2.44 (18 H, m, CH_2N + CH_2CO + NH); δ_C(D_2O) 175.3 (CO), 169.6 (C–O), 133.9 (s); 131.8, 130.5, 122.8, 120.2; 94.5 (CN), 55.2 (ArCHN); 47.7, 45.8, 45.1, 44.2, 43.0, 42.3, and 39.3 (CH_2N + CH_2CO).

11-(2-Hydroxy-5-aminomethylphenyl)-1,4,7,10-tetra-azacyclotridecane (**4**).—The amide prepared above (1.308 g, 4.13 mmol), was treated with borane–tetrahydrofuran (1 mol dm^{-3}, 40 cm^3) and heated to reflux for 60 h. After being cooled, quenched with methanol (10 cm^3) and evaporated, the residue was treated with hydrochloric acid (6 mol dm^{-3}, 30 cm^3) and the solution was boiled (3 h). After removal of solvent, the residue was re-dissolved in water (30 cm^3), washed with ether (2 × 50 cm^3), basified (15 g, KOH) and extracted into chloroform (2 × 200 cm^3) to yield the free amine (370 mg, 30%). HPLC: t_R 17.5 min (pH 6.5); m/z (DCI), 310 (M^+ + 2), 309 (M^+ + 1), 292, 204, and 147; δ_H($CDCl_3$) 7.01 (1 H, dd, J 8.3 Hz), 6.87 (1 H, s), 6.71 (1 H, d, J 8.3 Hz), 3.81 (1 H, dd, ArCHN, J 10.8 and 2.4 Hz), 3.72 (2 H, s, $ArCH_2N$), 3.09–2.42 (20 H, m, CH_2N + NH), and 2.03–1.70 (2 H, m, CH_2C); δ_C($CDCl_3$)

156.8 (C–O), 133.4 (d), 126.8, 116.5 (CH), 66.5 (ArCHN), 49.6, 48.5, 48.1, 47.5, 47.3, 46.7, 46.5, 46.0 (CH_2N), and 36.1 (CH_2C).

5-(2-*Hydroxy*-5-*cyanophenyl*)-1,4,8,11-*tetra-azacyclotetradecan*-7-*one*.—This was prepared in a similar manner to that described for (**4**) from 1,4,8,11-tetra-azaundecane (9.68 g, 60.5 mmol) to yield the amide 4.0 g (20%), m.p. 136–138 °C; R_f [SiO_2: aq.NH_3/CH_3OH/CH_2Cl_2 (5:45:50)] 0.32; *m/z* (DCI) [Found: 332.207 57. $C_{17}H_{25}N_5O_2$ requires 332.208 65 (M^+ + 1)]; ν(KBr) 3 040, 2 925, 2 830 (CH); 2 205 (CN), 1 635 (CO), 1 588, and 1 470; $\delta_H(D_2O)$ 7.42 (1 H, s), 7.40 (1 H, dd), 6.63 (1 H, d), 4.28 (1 H, dd, ArCHN), 3.55–2.38 (14 H, m, CH_2N + CH_2CO), and 1.85 (2 H, quint, CH_2C); $\delta_C(D_2O)$ 174.9 (CO), 170.3 (C–O), 133.5 (CH), 131.3 (CH), 130.8 (s), 122.8 (s), 120.2, 93.5 (CN), 53.5 (CHAr), 49.1, 48.9, 47.2, 46.6, 42.0 (CH_2N), 38.5 (CH_2N), and 24.9 (CH_2C).

5-(2-*Hydroxy*-5-*aminomethylphenyl*)-1,4,8,11-*tetra-azacyclotetradecane* (**3**).—The amide prepared above (2.87 g, 8.67 mmol), was reduced as described above for (**4**) to yield a colourless oil (1.93 g, 63%); HPLC: t_R 13.7 min (pH 7.4); *m/z* (DCI, Bu^iH) [Found: 332.268 83. $C_{17}H_{31}N_5O$ requires 322.260 69 (M^+ + 1)]; ν($CHCl_3$) 3 250 (OH, NH); 2 910, 2 830 (CH), and 1 660 cm^{-1} (Ar); $\delta_H(CDCl_3)$ 7.04 (1 H, dd), 6.91 (1 H, s), 6.73 (1 H, d), 3.81 (1 H, dd, ArCHN), 3.75 (2 H, s, $ArCH_2N$), 3.11–2.51 (14 H, m, CH_2N + NH), and 1.94–1.75 (19 H, m, CH_2C); $\delta_C(CDCl_3)$ 156.6 (C–O), 133.5 (d), 126.8, 126.6, 116.4; 66.8 (ArCHN); 51.2, 51.0, 50.1, 49.7, 49.4, 49.2, 47.4, 46.0 (CH_2N); 36.4 and 29.4 (CH_2C).

Diethyl 4-*Cyanobenzylmalonate*.—A solution of diethyl malonate (20 g, 125 mmol) in dry ethanol (50 cm^3) was added dropwise to sodium ethoxide (65 mmol) in dry ethanol 120 cm^3. After the reaction had been stirred for 0.5 h, a solution of *p*-cyanobenzyl bromide (12 g, 61 mmol) in dry dimethylformamide (60 cm^3) was added dropwise, and the mixture was heated to reflux for 24 h. Water (150 cm^3) was added to the cooled reaction mixture and the colourless precipitate of the dibenzyl malonate which formed was filtered off and the filtrate was extracted with diethyl ether (5 × 60 cm^3). After removal of ether, the residue was distilled under reduced pressure [150 °C (bath temp.), 0.1 mmHg] to yield a colourless oil 7.84 g (47%); *m/z* (CI) 277 (M^+ + 2), 276 (M^+ + 1), 275 (M^+), 230 and 184; ν($CHCl_3$) 3 010, 2 980 (CH), 2 235 (CN), and 1 730 cm^{-1} (CO); $\delta_H(CDCl_3)$ 7.59 (2 H, d), 7.33 (2 H, d), 4.16 (4 H, q, CH_2O), 3.36 (1 H, t, CH), 3.27 (2 H, d, CH_2), and 1.22 (6 H, t, CH_3); $\delta_C(CDCl_3)$ 167.3 (CO), 143.3, 132.1, 129.5, 116.5 (Ar), 110.5 (CN), 61.6 (CH_2O), 53.0 (CH), 34.4 (CH_2), and 13.8 (CH_3).

12-(4-*Cyanobenzyl*)-1,4,7,10-*tetra-azacyclotridecan*-11,13-*dione*.—A solution of 1,4,7,10-tetra-azadecane (4.18 g, 28.6 mmol) and diethyl 4-cyanobenzylmalonate (7.84 g, 28.5 mmol) in dry ethanol (60 cm^3) was heated to reflux for 10 days. A colourless precipitate was removed by filtration and recrystallised from hot ethanol, 1.41 g (15%), m.p. 245–247 °C (decomp.) (Found: C, 62.2; H, 7.03; N, 20.9. $C_{17}H_{23}N_5O_2$ requires C, 62.0; H, 6.99; N, 21.3%) R_f (SiO_2: aq.NH_3/CH_3OH/CH_2Cl_2, 6:44:50) 0.27; *m/z* (CI) 331 (M^+ + 2), 330 (M^+ + 1), 312, and 286; ν(KBr) 3 320 (NH), 2 930, 2 880, 2 810 (CH), 2 225 (CN), 1 665 (NH bend), 1 640 (CO), 1 550, and 1 525 cm^{-1}; $\delta_H(CD_3OD)$ 7.63 (2 H, d, *J* 8.0 Hz), 7.41 (2 H, d), 3.64 (2 H, m, CH_2N), 3.47 (1 H, t, CHCO), 3.26 (2 H, d, CH_2Ar), 3.02 (2 H, m, CH_2N), and 2.67 (8 H, m, CH_2N).

12-(4-*Aminomethylbenzyl*)-1,4,7,10-*tetra-azacyclotridecane* (**2**).—The diamide prepared above (1.3 g, 3.95 mmol) was treated with borane–tetrahydrofuran (80 cm^3, 80 mmol) and the mixture was heated to reflux for 3 days. After being quenched with methanol (5 cm^3) and removal of solvent, the residue was hydrolysed with hydrochloric acid (6 mol dm^{-3}; 40 cm^3) by boiling for 3 h. After removal of solvent, re-dissolving in water (30 cm^3), washing with ether (2 × 30 cm^3), basifying (8 g, KOH), extracting into chloroform (3 × 50 cm^3) and removal of solvents under reduced pressure a colourless oil was obtained (1.19 g, 99%) which was analysed as the hydrochloride salt (from aqueous ethanol) (Found: C, 36.7; H, 7.8; N, 12.1. $C_{17}H_{31}N_5$·5HCl·4H_2O requires: C, 36.5; H, 7.92; N, 12.5%); *m/z* (DCI) 307 (M^+ + 2), 306 (M^+ + 1), 305 (M^+), and 258; $\delta_H(CDCl_3)$ 7.22 (2 H, d, *J* 8.1 Hz), 7.74 (2 H, d), 3.84 (2 H, s, CH_2Ar), 2.84–2.49 (19 H, m, CH_2Ar + CH_2N + CH), and 2.04 (6 H, br s, NH); $\delta_C(CDCl_3)$ 140.7, 138.8 (s, Ar); 128.9, 126.8 (d); 54.6 (CH_2N), 48.8, 47.4, 47.2, 46.0 (CH_2N), 40.2, and 38.3 (CH_2C + CH).

6-(4-*Cyanobenzyl*)-1,4,8,11-*tetra-azacyclotetradecan*-5,7-*dione*.—A solution of 1,4,8,11-tetra-azaundecane (2.77 g, 17.3 mmol) and diethyl 4-cyanobenzylmalonate (4.75 g, 17.3 mmol) in dry ethanol (35 cm^3) was heated under reflux for 5 days. The solvent was evaporated and the residue purified by silica gel column chromatography under standard conditions, (1.1 g, 19%), m.p. 209–211 °C (Found: C, 60.1; H, 7.15; N, 18.9. $C_{18}H_{25}N_5O_2$·H_2O requires: C, 59.8; H, 7.53; N, 19.4%); *m/z* (DCI, NH_3), 345 (M^+ + 2), 344 (M^+ + 1), 326, 309, and 308; ν(KBr) 3 290 (NH), 2 910, 2 805 (CH), 2 225 (CN), 1 638 (CO), and 1 530 cm^{-1}; $\delta_H(CDCl_3)$ 7.56 (2 H, d), 7.33 (2 H, d), 6.55 (2 H, br s, NHCO), 3.50 (2 H, m, CH_2Ar), 3.25 (5 H, m, CHCO + CH_2NCO), 2.82–2.57 (8 H, m, CH_2N), 1.78 (2 H, br s, NH), and 1.65 (2 H, m, CH_2C); $\delta_C(CD_3OD)$ 171.2 (CO), 146.3 (s), 133.3, 131.3, 119.7 (Ar), 111.3 (CN), 56.5 (CH_2N), 50.9, 39.4, 35.1, and 28.3 (CH_2C).

6-(4-*Aminomethylbenzyl*)-1,4,8,11-*tetra-azacyclotetradecane* (**1**).—The diamide prepared above (0.97 g, 2.82 mmol) was reduced with borane–THF (47 cm^3, 47 mmol) by boiling the solution under reflux for 24 h. After being quenched with methanol (5 cm^3) the product was isolated as described above, and the amine was recrystallised from toluene to yield a colourless solid (0.75 g, 83%), m.p. 149–150 °C (Found: C, 64.5; H, 10.1; N, 20.3. $C_{18}H_{33}H_5$·H_2O requires C, 64.1; H, 10.4; N, 20.7%); *m/z* (DCI, NH_3), 321 (M^+ + 2), and 320 (M^+ + 1); $\delta_H(CDCl_3)$ 7.22 (2 H, d), 7.14 (2 H, d), 3.83 (2 H, s, $ArCH_2N$), 2.85–2.40 (19 H, m, CH_2N_1 + CH_2Ar + $CHCH_2Ar$), 2.20 (6 H, br s, NH), and 1.71 (2 H, quint, CH_2C); $\delta_C(CDCl_3)$ 140.9, 139.0 (s), 129.1, 127.0 (d), 55.8 (CH_2NH_2), 50.7, 49.3, 46.2 (CH_2N), 40.8 (CH), 38.6 (CH_2Ar), and 29.3 (CH_2C).

Synthesis of Vinylpyridine Linker Molecule.—*2-Dimethoxytrityloxymethyl*-6-*hydroxymethylpyridine*. A solution of 2,6-bis(hydroxymethyl)pyridine (16.1 g, 116 mmol) in dry pyridine (200 cm^3) was added to a solution of dimethoxytrityl chloride (39.2 g, 116 mmol) in pyridine (120 cm^3) over 4 h. After a further hour of stirring, the solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (400 cm^3), washed with sodium hydrogen carbonate solution (2 × 200 cm^3), dried (K_2CO_3) and the solvent was removed under reduced pressure to yield a residue which was purified by flash chromatography (diethyl ether–hexane, 8:2) to give the alcohol as a pale yellow glass, 31.2 g (61%), m.p. 38–40 °C; R_f (Et_2O–hexane, 8:2) 0.20; $\delta_H(CDCl_3)$ 7.30 (4 H, d, *J* 8.4 Hz, trityl), 6.73 (4 H, d, trityl), 7.73–6.60 (8 H, m, Ar), 4.55 (2 H, s, CH_2OH), 4.27 (2 H, s, CH_2OCAr_3), and 3.63 (6 H, s, OCH_3).

2-*Hydroxymethyl*-6-*methoxycarbonylmethyloxymethylpyri-*

dine.—The alcohol (31 g, 70.2 mmol) was dissolved in dry THF (200 cm^3) and butyl-lithium (48 cm^3; 1.6 mol dm^{-3} solution in hexane, 77.2 mmol) was added by syringe at −78 °C under nitrogen. The brown suspension that formed was added by means of a steel cannula to a solution of methyl bromoacetate (26.85 g, 175 mmol) in dry THF (50 cm^3). The mixture was allowed to reach room temperature and was then stirred for 18 h. The solution was poured into a saturated solution of sodium hydrogen carbonate (250 cm^3) and extracted with dichloromethane (3 × 150 cm^3), dried ($MgSO_4$), and the remaining solution was concentrated to 250 cm^3. Solid zinc bromide (78.7 g, 350 mmol) was added and after stirring for 15 min, the mixture was poured into saturated disodium EDTA solution (300 cm^3). The aqueous layer was separated, basified with sodium hydrogen carbonate and extracted with chloroform (3 × 500 cm^3). Removal of solvent under reduced pressure yielded a glass (3 g, 20%); R_f (SiO_2: CH_3OH–CH_2Cl_2, 1:9) 0.46; *m/z* (DCI) (Found: 211.084 82. $C_{10}H_{13}NO_4$ requires 211.084 46); $\delta_H(CDCl_3)$ 7.72 (1 H, t, py), 7.41 (1 H, d, py), 7.18 (1 H, d, py), 4.76 (4 H, s, CH_2O), 4.22 (2 H, s, CH_2O), 3.75 (3 H, s, OCH_3), and 3.07 (1 H, br s, OH); $\delta_C(CDCl_3)$ 170.9 (CO), 158.3, 156.3, 137.5, 120.2, 119.4 (py), 73.9 (CH_2C), 67.9, 63.9 (CH_2OH), and 51.9 (OCH_3).

2-*Methanoyl*-6-*methoxycarbonylmethyloxymethylpyridine*.—The alcohol (2.6 g, 12.3 mmol) in dry dichloromethane (50 cm^3) was treated with manganese dioxide (25 g, 288 mmol). After being stirred at room temperature for 15 h and filtered through Celite, solvent was removed and the residue purified by flash chromatography (ether–hexane, 8:2) to yield a colourless gum, 1.3 g (51%) (Found: C, 57.4; H, 5.30; N, 6.70. $C_{10}H_{11}NO_4$ requires: C, 56.6; H, 5.00; N, 6.39%) *m/z* (DCI) [Found: 210.076 55. $C_{10}H_{11}NO_4$ requires 210.076 63 (M^+ + 1)]; $\delta_H(CDCl_3)$ 10.05 (1 H, s, CHO), 7.90 (2 H, mult, py), 7.78 (1 H, dd, py), 4.86 (2 H, s, CH_2O), 4.29 (2 H, s, CH_2O), and 3.80 (3 H, s, OCH_3); $\delta_C(CDCl_3)$ 193.2 (CO), 170.7 (CO_2Me), 158.4, 152.0, 137.7, 125.9, 120.6 (py), 73.7 (CH_2O), 68.0 (CH_2O), and 51.9 (OCH_3).

2-*Ethenyl*-6-*methoxycarbonylmethyloxymethylpyridine*.—To a solution of the aldehyde (1.3 g, 6.22 mmol) in dry THF (20 cm^3) was added a solution of trimethylsilylmethylmagnesium chloride in ether (1 mol dm^{-3}; 6.84 mmol) at −78 °C under nitrogen. After being stirred for 15 min at −78 °C, the mixture was allowed to warm to 0 °C and thionyl chloride (0.81 g, 6.81 mmol) was added dropwise. The solution was stirred at 0 °C for 20 min then warmed to room temperature, basified with saturated sodium hydrogen carbonate solution and extracted with dichloromethane (3 × 30 cm^3). After drying ($MgSO_4$) and evaporation of solvent the residue was purified by flash chromatography (SiO_2; ether–hexane, 1:1) to yield a colourless oil (0.91 g, 71%); R_f (SiO_2:Et_2O–hexane, 1:1) 0.19; *m/z* (DCI) 208.0974 [$C_{11}H_{14}NO_3$ requires 208.0974 (M^+ + 1)]; $\delta_H(CDCl_3)$ 7.68 (1 H, t, py), 7.41 (1 H, d, py), 7.29 (1 H, d, py), 6.82 (1 H, dd), 6.19 (1 H, d), 5.49 (1 H, d), 4.76 (2 H, s, $pyCH_2O$), 4.25 (2 H, s, CH_2O), and 3.78 (3 H, s, OCH_3); $\delta_C(CDCl_3)$ 170.6 (CO), 163.0, 156.4, 137.2, 136.6, 122.2, 120.3, 118.4, 73.9, 67.9, and 51.8.

2-*Ethenyl*-6-p-*nitrophenoxymethyloxymethylpyridine* (**7**).—The methyl ester (0.80 g, 3.87 mmol) was dissolved in methanol (40 cm^3) and aqueous lithium hydroxide (0.175 g, 13 cm^3) and stirred at room temperature for 15 min. After removal of solvent, the residue was passed down a pyridinium cation exchange column (20 g, Dowex 50 W) eluting with pyridine. After removal of pyridine, the residue was dissolved in dichloromethane (50 cm^3) and *p*-nitrophenol (840 mg, 6 mmol). Dicyclohexylcarbodi-imide (830 mg, 4 mmol) was then added as a solution in dichloromethane (25 cm^3). After being stirred for 1 h and filtered, the reaction was concentrated to yield a residue which was purified by silica gel flash chromatography (ether–CH_2Cl_2, 1:40) to yield a colourless solid, (0.81 g, 63%); R_f (SiO_2: ether–CH_2Cl_2, 1:40) 0.32; *m/z* (DCI) [Found: 315.098 10. $C_{16}H_{14}N_2O_5$ requires 315.098 09 (M^+ + 1)]; $\delta_H(CDCl_3)$ 8.28 (2 H, d, Ar), 7.76 (1 H, t, py), 7.41 (2 H, m, py), 7.32 (2 H, d, Ar), 6.84 (1 H, dd, vinyl, 6.18 (1 H, dd), 5.53 (1 H, dd), 4.84 (2 H, s, $pyCH_2O$), and 4.51 (2 H, s, CH_2O).

Formation of Macrocycle–Vinylpyridine Amides. 2-*Ethenyl*-6-[4-(1,4,7,10-*tetra-azacyclotridecan*-12-*ylmethyl*)*benzylcarbamoylmethoxymethyl*]*pyridine* (**9**).—The following procedure is generally applicable to the exocyclic acylation of the macrocyclic amines using the *p*-nitrophenyl ester (**7**). The macrocycle (**4**) (10.5 mg, 34.4 μmol) was dissolved in a buffer solution [pH 6.8, 1 cm^3, 0.5 mol dm^{-3} in piperazine-1,4-diylbis(ethanesulphonic acid)] and a solution of the ester (**7**) [21 mg, 69 μmol] in dioxane (1 cm^3) was added and the mixture stirred at 40 °C for 4–8 h. Washing with chloroform (10 × 3 cm^3), followed by basification (pH ≥ 12), extraction with chloroform (3 × 5 cm^3) and removal of solvent yielded a residue which was purified by cation exchange HPLC (t_R = 13 min, pH 6.5), 9 mg (55%); *m/z* (FAB, glycerol), 482 (M^+ + 1) 481 (M^+) and 243; $\lambda_{max}(H_2O)$ 281 nm; $\delta_H(D_2O)$ 7.82 (1 H, t, *J* 7.9 Hz), 7.54 (1 H, d), 7.36 (1 H, d), 7.22 (4 H, s, Ar), 6.79 (1 H, dd), 6.09 (1 H, d, *J* 17.7 Hz), 5.56 (1 H, d, *J* 11.0 Hz), 4.70 (2 H, s, $pyCH_2O$), 4.38 (2 H, s, CH_2O), 4.20 (2 H, s, $ArCH_2N$), 3.09–2.59 (18 H, m, CH_2N + CH_2Ar), 2.21 (1 H, m, $CHCH_2$), and 1.89 (6 H, s, $CH_3CO_2^-$ *i.e.* diacetate salt). The following were prepared in an analogous manner (as their diacetate salts).

2-*Ethenyl*-6-[4-(1,4,8,11-*tetra-azacyclotetradecan*-6-*ylmethyl*)*benzylcarbamoylmethoxymethyl*]*pyridine* (**8**). Yield 80%, HPLC: t_R 13 min (pH 6.5), *m/z* (FAB) 495 (M^+ + 1), 494 (M^+), 304, 185, and 115; $\lambda_{max}(H_3O)$ 280 nm; $\delta_H(D_2O)$ 7.76 (1 H, t, py), 7.48 (1 H, d, py), 7.30 (1 H, d, py), 7.17 (4 H, br s, Ar), 6.74 (1 H, dd), 6.03 (1 H, d), 5.50 (1 H, d), 4.64 (2 H, s, $pyCH_2O$), 4.33 (2 H, s, CH_2O), 4.14 (2 H, s, $ArCH_2N$), 2.98–2.32 (19 H, m, CH_2N + CH_2Ar + $CHCH_2$), and 1.84 (2 H, m, CH_2C).

2-*Ethenyl*-6-[4-*hydroxy*-3-(1,4,7,10-*tetra-azacyclotridecan*-11-*ylbenzylcarbamoylmethoxymethyl*]*pyridine* (**11**). Yield 50%, HPLC: t_R 12.0 min (pH 6.5); *m/z* (FAB) 484 (M^+ + 1), 483 (M^+), 453, 378, and 325; $\lambda_{max}(H_2O)$ 280 nm; $\delta_H(D_2O)$ 7.79 (1 H, t, py), 7.52 (1 H, d, py), 7.32 (1 H, d, py), 7.07 (2 H, m, Ar), 6.86 (1 H, d, Ar), 6.72 (1 H, dd), 6.05 (1 H, d), 5.53 (1 H, d), 4.67 (2 H, s, $pyCH_2O$), 4.31 (2 H, s, CH_2O), 4.18 (2 H, s, CH_2N), 4.06 (1 H, dd, ArCHN), 3.32–2.54 (14 H, m, CH_2N), and 2.14–1.89 [8 H, m, CH_2C + $(CH_3CO_2^-)_2$].

2-*Ethenyl*-6-[4-*hydroxy*-3-(1,4,8,11-*tetra-azacyclotetradecan*-5-*yl*)*benzylcarbamoylmethoxymethyl*]*pyridine* (**10**). Yield 70%, HPLC: t_R 11.2 min (pH 6.5); $\delta_H(D_2O)$ 7.78 (1 H, t, py), 7.51 (1 H, d, py), 7.30 (1 H, d, py), 7.07 (2 H, m, Ar), 6.85 (1 H, d, Ar), 6.71 (1 H, dd, vinyl), 6.04 (1 H, d), 5.52 (1 H, d), 4.66 (2 H, s, $pyCH_2O$), 4.29 (2 H, s, CH_2O), 4.12 (3 H, m, ArCHN), 3.31–2.60 (14 H, m, CH_2N), and 2.12–1.80 [10 H, m, CH_2C + $(OAc^-)_2$].

Kinetics Experiments.—The formation of the copper complexes of cyclam (**12**), [13]-N_4, (**13**) and the phenolic ligands (**14**) and (**15**) was followed at λ_{max} 516, 561, 506, and 557 nm, respectively. For each separate reaction a minimum of five injections were made and k_{obs} was taken to be the mean of these values. A typical data set is given below: $[succ]_{tot}$ = 2 × 10^{-1} mol dm^{-3}, [13]-N_4 = 5 × 10^{-3} mol dm^{-3}, $[Cu^{2+}]$ = 5 × 10^{-4} mol dm^{-3}. k_{obs} = 2.633, 2.571, 2.403, 2.569, 2.583, 2.542 s^{-1} (mean = 2.550 s^{-1}, σ = 0.071).

Typically, a minimum of 50 cm^3 of each of the copper and ligand solutions was used, and the concentrations of copper and

ligand after mixing were 5×10^{-4} and 5×10^{-3} mol dm^{-3}. The hygroscopic copper perchlorate used (Johnson-Matthey, 99.9% purity) was analysed by atomic absorption and found to be $Cu(ClO_4)_2 \cdot 9.09H_2O$. Sodium perchlorate monohydrate (Fluka purum) used to adjust the ionic strength was analysed by atomic absorption and halide analysis and was $NaClO_4 \cdot 1.88H_2O$. Distilled water was used throughout.

For the equilibrium measurements, a solution containing 2 mmol dm^{-3} $Cu(ClO_4)_2 \cdot 9.09H_2O$, 4 mmol dm^{-3} succinic acid (Fluka puriss) and sodium perchlorate 0.1 mol dm^{-3} was adjusted to pH 2.5 with perchloric acid (Aldrich) and the volume was made up to 500 cm^3 with distilled water. The titrant was a standardised 0.05 mol dm^{-3} NaOH solution (BDH), the temperature was maintained at 298 K (Techne Tempette) and base was added in small increments to a stirred solution until the pH reached 6.

Biodistribution Studies.—Normal (non-tumour bearing) male CBA/H mice with a mean age of 100 days and a mean body-weight of 33(± 1) g were injected *via* the tail vein with ^{64}Cu or ^{67}Cu labelled murine B72.3 antibody. Typically 75 μg of B72.3 was administered labelled with 3.5 μCi of ^{64}Cu or 1 μCi of ^{67}Cu. Mice were examined at a given time interval (4, 24, 72 h) following injection with sodium pentabarbitone. Samples of cardiac blood were taken and the liver, spleen, lungs, and kidney removed and freed from adherent fat. Each tissue was placed in a 5 cm^3 vial and weighed before radioanalysis. Measurement of the 510 keV gamma (^{64}Cu) or 90/92 keV gamma emitted during the decay of ^{67}Cu was carried out using an LKB Compugamma (LKB Pharmacia) equipped with a 3 inch NaI (T1) crystal.

Macrocycle Conjugation to the Antibody.—Free thiol groups were introduced onto the B72.3 antibody following the procedures of Meares.[7,16] The number of thiol groups introduced was measured by titration with Ellman's reagent, as described in the literature.[15] The crude 'Trauted' antibody was desalted into phosphate buffer (0.15 mol dm^{-3}, pH 8.0) containing a 2.0 mmol dm^{-3} ethylenediaminetetra-acetic acid solution and was purified on a PD10 column equilibrated with running buffer. The protein fraction was incubated with a fortyfold excess of (**10**) (with respect to the free thiol concentration, which was typically four per antibody) at 4 °C for 15 h under nitrogen. The number of thiol groups per antibody was measured thereafter and compared with a control incubation performed in the absence of (**10**): the difference yielded the number of macrocycles bound per antibody. Any remaining free thiol groups were capped by reaction with a fortyfold excess of 4-vinylpyridine (or *N*-ethylmaleimide) and the conjugated protein was desalted into a phosphate buffer at pH 7.4.

Radiolabelling of Macrocycles and Macrocycle–Antibody Conjugates [^{64}Cu (**16**)] *and* [^{64}Cu (**16**)-B72.3].—To 150 mm^3 of a 0.63 mmol dm^{-3} solution of (**16**) in 0.4 cm^3 of a sodium phosphate buffer (0.3 mol dm^{-3}, pH 7.0) was added 0.4 cm^3 of a $^{64}CuCl_2$ solution (containing 143 μCi ^{64}Cu). The mixture was vortexed, incubated at room temperature for 15 min and the radiolabelled ligand (87% yield) was separated from $^{64}Cu^{2+}$ by reversed-phase HPLC. After reduction to small volume (50 mm^3) this solution was added to 100 mm^3 of phosphate-buffered saline (pH 8.0) and 200 mm^3 of a 60 μmol dm^{-3} solution of 'Trauted' B72.3 antibody [pH 8.0, phosphate (0.3 mol dm^{-3}) buffer] was added and the mixture held at room temperature for 2 h. Under these conditions the concentration of [^{64}Cu (**16**)] was 0.27 mmol dm^{-3} and the antibody B72.3 was 0.034 mmol dm^{-3} with a free thiol concentration of 0.13 mmol dm^{-3} (following reaction with Traut's reagent as described previously).[7,16] The mixture was vortexed, centrifuged and purified either by size-exclusion HPLC (100 mm^3 aliquots) or by PD-10 size exclusion chromatography. The HPLC separation (DuPont GF 250 column) was carried out with phosphate elution (0.2 mol dm^{-3}, pH 6.8) and the IgG protein peak was separated from smaller fragments and aggregates to yield purified protein (1.2 cm^3) with 16 μCi of ^{64}Cu (60%). This material was used directly in biodistribution studies. For purposes of comparison the antibody conjugate was also purified with a PD 10 Sephadex G25 column eluting with phosphate-buffered saline to yield 18 μCi of protein in a 1 cm^3 volume used for biodistribution experiments.

Direct Labelling of Macrocycle–Antibody Conjugates.—A solution of $^{64}CuCl_2$ (250 mm^3, 400 μCi) was brought to pH 4.0 following addition of succinate buffer (20 mm^3, 0.2 mol dm^{-3}). An aliquot (37 mm^3, 55 μCi ^{64}Cu) of this solution was added to 27 mm^3 of a solution containing [B72.3-(**10**)] [63 μmol dm^{-3} in antibody, 15.8 μmol dm^{-3} in (**10**)] and the mixture was vortexed and incubated at 37 °C for 30 min. Samples were purified by HPLC or using a PD10 column as described above, giving protein labelled with 15 μCi ^{64}Cu with a 33% labelling efficiency.

Labelling with ^{67}Cu was performed with $^{67}CuCl_2$ (in 2 mol dm^{-3} HCl) obtained from Los Alamos, USA. This material was not carrier free, containing (initially) *ca.* 600 atoms of 'cold' copper for each atom of ^{67}Cu. Labelling and purification was effected as described above except that DTPA (diethylenetriaminepenta-acetic acid) (100 mm^3, 0.2 mmol dm^{-3}), was added 5 min prior to chromatographic purification to remove any traces of non-specifically bound copper. Further details of these radiolabelling experiments will be reported elsewhere.

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References

1 M. W. Brechbiel, O. A. Gansow, R. W. Atcher, J. Schom, J. Esteban, D. E. Simpson, and D. Colcher, *Inorg. Chem.*, 1986, **25**, 2772.
2 D. J. Hnatowich, M. Chinol, D. A. Siebecker, M. Gionet, T. Griffin, P. W. Doherty, R. Hunter, and K. R. Kase, *J. Nucl. Med.*, 1988, **29**, 1428.
3 A. Del Guerra, *Phys. Scr.*, 1987, **T19**, 481; M. E. Phelps and J. C. Mazziotta, *Science*, 1985, **228**, 799.
5 J. L. Humm, *J. Nucl. Med.*, 1986, **27**, 1490.
6 L. H. Chen and C. S. Chung, *Inorg. Chem.*, 1988, **27**, 1880.
7 S. V. Deshpande, S. J. DeNardo, C. F. Meares, M. J. McCall, G. P. Adams, M. K. Moi, and G. L. DeNardo, *J. Nucl. Med.*, 1988, **29**, 217.
8 M. Kodama and E. Kimura, *J. Chem. Soc., Dalton Trans.*, 1976, 116, 1720.
9 E. Kimura, T. Koike, and M. Takahashi, *J. Chem. Soc., Chem. Commun.*, 1985, 385.
10 J. R. Morphy, D. Parker, R. Alexander, A. Bains, A. F. Carne, M. W. Eaton, A. Harrison, A. Millican, A. Phipps, R. Titmas, and D. Weatherby, *J. Chem. Soc., Chem. Commun.*, 1988, 156.
11 J. R. Morphy, D. Parker, R. Kataky, A. Harrison, M. A. W. Eaton, A. Millican, A. Phipps, and C. Walker, *J. Chem. Soc., Chem. Commun.*, 1989, 792.
12 I. Tabushi, Y. Tanigrechi, and H. Kato, *Tetrahedron Lett.*, 1977, 1049.
13 G. T. Morgan and F. M. G. Micklethwaite, *J. Chem. Soc.*, 1904, **85**, 1230.
14 J. F. Cavins and M. Friedman, *Anal. Biochem.*, 1970, **35**, 489.
15 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70.
16 C. F. Meares, M. J. McCall, D. T. Rearden, D. A. Goodwin, C. I. Diamenti, and M. McTigue, *Anal. Biochem.*, 1984, **142**, 68.
17 J. R. Benson and P. E. Hare, *Proc. Natl. Acad. Sci. USA*, 1975, **72(2)**, 619.

18 L. D. Pettit, I. Steel, G. Formicka-Kozlowska, T. Tatarowski, and M. Bataille, *J. Chem. Soc., Dalton Trans.*, 1985, 535.
19 M. Kodama and E. Kimura, *J. Chem. Soc., Dalton Trans.*, 1976, 1473.
20 E. Wu and T. A. Kaden, *Helv. Chim. Acta*, 1985, **68**, 6611.
21 J. K. Garg, L. K. Agarwal, P. S. Verma, and D. S. Jain, *J. Electrochem. Soc. India*, 1982, **31**, 141, 153.
22 G. Arena, R. Cali, E. Rizzarelli, and S. Sammartano, *Transition Met. Chem. (Weinheim, Ger.)*, 1978, **3**, 147.
23 P. Gans, A. Sabatini, and A. Vacca, *J. Chem. Soc., Dalton Trans.*, 1985, 1195.

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