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Tumour Targeting with Radiolabelled Macrocycle–Antibody Conjugates

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1 Introduction

There is a good deal of interest in the development of monoclonal antibodies which may be linked to drugs, toxins, or radioisotopes as targeting agents in the diagnosis and therapy of cancer, cardiovascular, and other diseases. In tumour targeting with antibody-conjugated radioisotopes, diagnosis and therapy are closely related and may be achieved in a single treatment protocol. In addition, antibodies that are irreversibly labelled with metal ions may be used for *in vitro* immunoassays (*e.g.* with luminescent ions such as Tb^{3+}, Eu^{3+}), or for *in vivo* magnetic resonance imaging (*e.g.* using Gd^{3+} as a paramagnetic contrast agent).[1] Central to the success of such approaches is the development of bifunctional complexing agents which can be attached to a protein and can bind the given metal ion rapidly and selectively, yet form a complex which is kinetically inert with respect to dissociation either by acid or cation promoted pathways. The choice of complexing agent is determined by the nature of the metal ion to be bound;[2] no single ligand has been found (nor is likely to be found) that can form sufficiently stable complexes with, *e.g.* Cu^{2+}, In^{3+}, Ga^{3+}, and Y^{3+} to permit its use *in vivo*. Early work in this area, however, did tend to concentrate on a single ligand approach. A small number of similar C-functionalized EDTA[3] or DTPA[4] chelates were developed for use with many metal ions but the promiscuity of these acyclic chelating agents that bind several ions in solution was compromised by complex instability *in vivo*. The anionic complexes of EDTA or DTPA with copper, indium, and yttrium, for example, are either readily protonated or attract other competing metal ions (Ca^{2+} is 1.26 mmol dm^{-3}, Mg^{2+} is 0.8 mmol dm^{-3}, and Zn^{2+} is 10^{-5} mol dm^{-3} in human serum) to form mixed complexes of reduced kinetic stability. The ensuing dissociation of the metal ion *in vivo* is essentially irreversible as the metal ion will be rapidly sequestered by serum proteins (*e.g.* transferrin [10^{-5} mol dm^{-3}] or albumin [10^{-3} mol dm^{-3}]) and the complexing agent will be simultaneously occupied by one of the abundant serum cations. These problems of metal dissociation are compounded in the case of radioisotope based imaging or therapy, where a fast forward rate of complexation

[1] R. B. Lauffer, *Chem. Rev.*, 1987, **87**, 90.
[2] R. D. Hancock and A. E. Martell, *Chem. Rev.*, 1989, **89**, 1875.
[3] S. M. Yeh, D. G. Sherman, and C. F. Meares, *Anal. Biochem.*, 1979, **100**, 152; C. F. Meares, and T. G. Wensel, *Acc. Chem. Res.*, 1984, **17**, 202.
[4] M. W. Brechbiel, O. A. Gansow, R. W. Atcher, J. Schlom, J. Esteban, D. E. Simpson, and D. Colcher, *Inorg. Chem.*, 1986, **25**, 2772.

is essential in order to achieve a good radiolabelling yield. Furthermore, with antibody-conjugated complexes, non-specific binding of the metal ion to the protein must be minimized and the metal-binding conditions are defined by the need to avoid protein denaturation, *i.e.* work in the pH range 4—9 and a temperature of 4—40 °C.

The introduction of macrocyclic bifunctional complexing agents has gone some way towards solving these problems.[5,6] In general, macrocyclic complexes are less sensitive to acid catalysed dissociation pathways[7] and so are more kinetically inert at lower pH—as may be encountered in the stomach or liver, for example. An additional design feature is that the ligand (where possible) should form a neutral or cationic complex with the metal ion of interest, thereby minimizing any direct electrostatic interaction with protons or cations. Furthermore, the ligand should be as 'pre-organized' as possible,[8] *i.e.* the conformations of the free ligand and of the ligand in the complex should be very similar in order not to create an unfavourable contribution to both the entropy and the enthalpy of complexation.

2 Choice of Radioisotopes

There are two classes of isotopes which are distinguished according to whether the primary aim is diagnostic (radioimmunoscintigraphy) or therapeutic (radioimmunotherapy). For imaging, a minimal interaction of the radiation with tissue is desired and a maximum interaction with an external detector. Gamma-emitting and positron emitting radioisotopes are suitable provided that they emit photons of sufficient energy and intensity to permit the necessary resolution for tumour imaging. Single photon emission may be detected externally using a conventional Anger camera giving moderate resolution (1 cm) and improved information may be obtained using tomographic methods. In positron annihilation, two collinear photons are created (511 keV) and may be detected by a positron emission tomography scanner giving improved resolution (3 mm).[9] In radioimmunotherapy, as much energy as possible needs to be delivered to the target site in order to deliver a sterilizing dose of radiation (500—2000 rads) that will cleave cellular DNA. Suitable isotopes are either β- or α-emitters, with little or no gamma component. For both imaging and therapy, the half-life of the radionuclide needs to be sufficiently long to permit transportation to the tumour site This will be determined by the nature of the targeting antibody; before binding an antigen at a tumour site, the radiolabelled protein must be transported from the bloodstream through endothelial pores into the extracellular fluids of the target

[5] J R Morphy, D Parker, R Alexander, A Bains, M A W Eaton, A Harrison, A Millican, R Titmas, and D Weatherby, *J Chem Soc, Chem Commun*, 1988, 156, J P L Cox, J R Morphy, K J Jankowski, and D Parker, *Pure Appl Chem*, 1989, **61**, 1637

[6] 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

[7] D Parker, *Adv Inorg Chem Radiochem*, 1983, **26**, 1, L H Chen and C S Chung, *Inorg Chem*. 1988 **27**, 1880

[8] D J Cram, *Science*, 1983, **219**, 1177

[9] A Del Guerra, *Phys Scr*, 1987, **T19**, 481, M E Phelps and J C Mazziotta, *Science*, 1985, **228**, 799

Table 1 *Imaging radioisotopes for use in radioimmunoscintigraphy*

Radionuclide	$t_{\frac{1}{2}}$	E_{photon} keV, (%)	*Source*
^{99m}Tc	6.02 h	141 (89)	Generator
^{111}In	2.83 d	171 (88)	Cyclotron
		247 (94)	
^{67}Ga	3.25 d	184 (24)	Cyclotron
^{131}I [a]	8.05 d	364 (82)	Reactor
^{82}Rb	1.4 h	511	Generator
^{64}Cu [b]	12.8 h	511 (120)	Reactor
^{68}Ga	1.20 h	511 (178)	Generator

[a] ^{131}I has an accompanying β^- emission E_{max} = 0.188 MeV. [b] ^{64}Cu has an accompanying β^- emission (39%) E_{max} = 0.57 MeV.

tissue. For an intact antibody (IgG of molecular weight 150 000) diffusion is slow ($t_{\frac{1}{2}}$ 18—24 hours) although antibody fragments should localize more quickly. A physical half life of between 6 hours and 8 days is thereby defined. There are additional features common to imaging and therapeutic radioisotopes:

(1) the decay should produce a stable daughter isotope;
(2) the isotope should be cheap and readily available, preferably from a generator, in a carrier-free form, *i.e.* free from other stable isotopes of the given element and with good radiochemical and chemical purity;
(3) the radionuclide must have chemical properties that permit it to be attached to the protein (*NB* the exclusion of the α-emitter radon on these grounds).

A selection of imaging radioisotopes which fulfil most of these criteria is given in Table 1. Many commercial Anger cameras are based on the detection of ^{99m}Tc (141 keV), so that photon emissions close to this energy are desirable. This disfavours ^{131}I in particular and it also has an accompanying particulate emission which unnecessarily increases the dose to the patient. Furthermore, iodine isotopes when covalently bound to proteins tend to dehalogenate *in vivo*, build up in the thyroid (and hence their successful use in the treatment of thyroid carcinoma) or are excreted. Although ^{99m}Tc is the most widely used radioisotope in diagnostic nuclear medicine, it has a very short half-life which will limit its use to either antibody fragment or small-molecule carriers. In addition it is available as pertechnetate (TcO_4^-) and although stable complexes are attainable in lower oxidation states (*e.g.* Tc^V, Tc^I) a reduction step to a reactive precursor complex is needed for antibody labelling. The problem of efficiently and rapidly labelling a protein, that is modified with a suitable ligand to bind ^{99m}Tc, without non-specific coordination of reduced technetium species to the protein remains a challenge. This leaves ^{111}In and ^{67}Ga as strong candidates for antibody-based γ-imaging, while only ^{64}Cu has a sufficiently long half-life to be considered amongst the positron emitting isotopes.

Table 2 *Therapeutic radioisotopes*

Radionuclide	*Total*[a] *dose*	$t_{\frac{1}{2}}$ (hours)	β_{max} MeV, (%)	*Mean range in tissue* (mm)	*Gamma* keV, (%)
^{67}Cu	52 (30)	62	0.40 (45)	0.2	93 (17)
			0.48 (3)		184 (47)
			0.58 (20)		
^{90}Y	180 (180)	64	2.25 (100)	3.9	None
^{131}I	339 (115)	193	0.61 (90)	0.4	364 (79)
^{199}Au	58 (47)	75	0.25 (22)	0.1	158 (76)
			0.30 (72)		
^{111}Ag	212 (198)	179	1.04 (93)	1.1	342 (6)
			0.69 (6)		247 (1)
			0.79 (1)		
^{188}Re	47 (44)	17	1.96 (18)	3.3	155 (9)
			2.12 (80)		
^{161}Tb	119 (101)	166	0.45 (26)	0.3	75
			0 57 (64)		57 (21)
			0 58 (10)		

[a] Values in parentheses indicate β^- dose

For radioimmunotherapy, both α- and β-emitters need to be considered.[10] Although α-particles are particularly good cytotoxic agents since they dissipate a large amount of energy within one or two cell diameters, most α-emitters are heavy elements that decay to hazardous daughter products. Only ^{211}At and ^{212}Bi (^{212}Pb)[11] seem plausible candidates, but the ease of dehalogenation *in vivo* for astatine and the shortness of the physical half-life for ^{212}Bi (*ca.* 1 h) pose very significant practical problems. A good deal of interest has been kindled by the idea of generating an α-emitter *in situ* by the bombardment of ^{10}B by low energy thermal neutrons to give the α-emitter ^{12}B ($t_{\frac{1}{2}}$ 0.02s).[12] Unfortunately neutrons of this energy penetrate tissue very poorly, and the number of localized boron atoms required (>500) in order to achieve the desired resultant radiation dose may impose prohibitive chemical constraints. This leaves β^--emitting radioisotopes and a selection of the candidates is listed in Table 2. Although ^{131}I has been used for the treatment of cancer in humans,[13] its properties are far from ideal. It has a high intensity γ-component and it tends to dissociate *in vivo*. Terbium-161 has good nuclear properties but it is very difficult to obtain in a carrier-free form. Copper-67 with its lower β^- energy may be suited to the elimination of small metasteses or leukemias but it is produced in a linear accelerator (from ^{68}Zn by a p, 2p reaction) at a rather high cost (£40 per mCi)

[10] J L Humm, *J Nucl Med*, 1986, **27**, 1490

[11] K Kumar, M Magerstadt, and O A Gansow, *J Chem Soc, Chem Commun*, 1989, 145, R W Kozak, R W Atcher, O A Gansow, A M Friedmen, and T A Waldmann, *Proc Natl Acad Sci USA*, 1986, **83**, 474, O A Gansow, R W Atcher, D C Link, A M Friedman, R H Seevers, W Anderson, D A Scheinberg, and M Strand, *Am Chem Soc Symp Ser*, 1984, **346**, 215

[12] E A Mizusawa, M R Thompson, and M F Hawthorne, *Inorg Chem*, 1985, **24**, 1911

[13] S E Order, J L Klein, and D Ettinger, *Cancer Res*, 1980, **40**, 3001

and is apparently difficult to obtain 'carrier free'. Rhenium-188 and gold-199 are available from a generator and reactor resepctively as ReO_4^- and $AuCl_4^-$. The uncompromising nature of the aqueous solution coordination chemistry of the lower oxidation states of these elements has limited their application, although stable (pH 0—12) dioxo complexes of Re^V with 1,4,8,11-tetra-azaundecane have been defined.[14] Silver-111 is more amenable in this respect, although a ligand which binds it quickly to form a robust complex has yet to be found.[15] This analysis leaves ^{90}Y as the isotope of choice. It is a pure β-emitter of relatively high energy which enables it to penetrate larger tumours which may express low levels of surface antigen. It decays to stable zirconium, is relatively cheap and may be obtained from a ^{90}Sr generator. The most important feature of its behaviour *in vivo* is that the aquo-ion is a bone-localizing cation. The premature release of ^{90}Y from a radiolabelled antibody conjugate is a very serious limitation. The build-up of significant amounts of ^{90}Y in the bone may lead to myelosuppression (depletion of the immune cell population) due to irradiation of the proximate bone-marrow with a dramatically increased risk of infection that may prove fatal. Clearly the ligand chosen to bind ^{90}Y must form a complex that is resistant to yttrium dissociation *in vivo*.

3 Antibody Modification and Linkage

Murine (mouse) monoclonal antibodies may not be administered in repeat doses to human patients because of the recognition of the foreign protein by the human immune system. Accordingly the antibody needs to be modified in order to minimize the immunogenicity of mouse antibodies. There are two main approaches to this problem. One involves the development of 'chimeric' antibodies[16] in which the antigen recognizing mouse variable region is fused to DNA encoding human constant heavy and light chains (Figure 1). Interspecies antibody chimeras are obtained on expression with the same antigen specificity as the parent antibody. Preliminary studies in human patients have demonstrated that the chimeric molecule is substantially less immunogenic.[17] An alternative method uses CDR (complementarity determining region) grafting in which the complementarity determining regions of mouse antibodies are incorporated into an expression system encoding human variable and constant regions.[18] The resultant 'humanized' antibodies possess only a minor fraction of the original mouse amino-acid sequence, so that a minimal immune response to the antibody may be expected.

A second aspect of antibody modification relates to their speed and ability to penetrate target tissue and to their rate of clearance from the cardiovascular

[14] D. Parker and P. S. Roy, *Inorg. Chem.*, 1988, **27**, 4127.
[15] A. S. Craig, D. Parker, H. Adams, N. A. Bailey, and H. Schneider, *J. Chem. Soc., Chem. Commun.*, 1989, 1823; A. S. Craig, R. Kataky, R. C. Matthews, D. Parker, G. Ferguson, H. Schneider, H. Adams, and N. A. Bailey, *J. Chem. Soc., Perkin Trans. II*, 1990, 1523.
[16] S. L. Morrison, M. J. Johnson, L. A. Herzenberg, and V. T. Oi, *Proc. Nat. Acad. Sci. NY*, 1984, **81**, 6851.
[17] A. F. LoBuglio *et al.*, *Proc. Nat. Acad. Sci. NY*, 1989, **86**, 4220.
[18] P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, and G. Winter, *Nature*, 1986, **321**, 522.

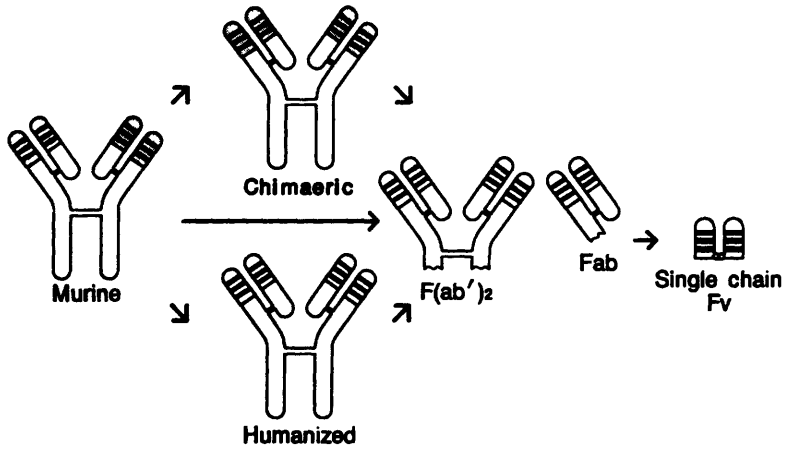

Figure 1 *Antibody modifications that may permit their use as targeting radiopharmaceuticals in humans*

system. Essentially this is size and shape dependent so that antigen-binding fragments (Fabs) should localize more quickly, penetrate more deeply and clear from the blood more rapidly than intact antibodies.[19] Of course this must not be offset by a loss in binding ability for the antigen. Ultimately antigen-binding Fv fragments (Fv's are specific variable region pairs[20]), or perhaps even a single complementarity determining region (*i.e.* small peptide) may mimic the binding ability of the parent antibody and serve as the targeting vehicle. Needless to say, this assumes that they possess sufficient stability to proteolysis and that they are non-toxic.

Finally, in order to irreversibly label the antibody or fragment with a metallic radioisotope, the bifunctional complexing agent must be covalently linked to the protein. Again there are two main approaches both of which involve attachment to lysine residues on the protein (Figure 2). Reaction of an aryl isothiocyanate directly yields a stable thiourea linkage, and this method has been used for benzyl-substituted EDTA[21] and DTPA[4] ligands. A limitation is the rather forcing conditions needed to convert the precursor aminophenyl group to the isothiocyanate, precluding, for example, the use of this method with substituted tetraazacycloalkane ligands.[5] Other workers have favoured the use of α-bromoacetamides, although it should be noted that their formation may be compromised by competitive *N*-alkylation instead of the desired acylation. A further variant uses active esters directly, *e.g.* dichlorophenyl or *N*-hydroxysuccinimide, and this method has been effected with polyaminocarboxylate ligand. The other generally-used approach is to link to a thiol residue either introduced onto the protein by

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[20] L. Riechmann and J. Foote, *J. Mol. Biol.*, 1988, **203**, 825.
[21] C. F. Meares, M. J. McCall, D. T. Reardan, D. A. Goodwin, C. I. Diamanti, and M. McTigue, *Anal. Biochem*, 1984, **142**, 68.

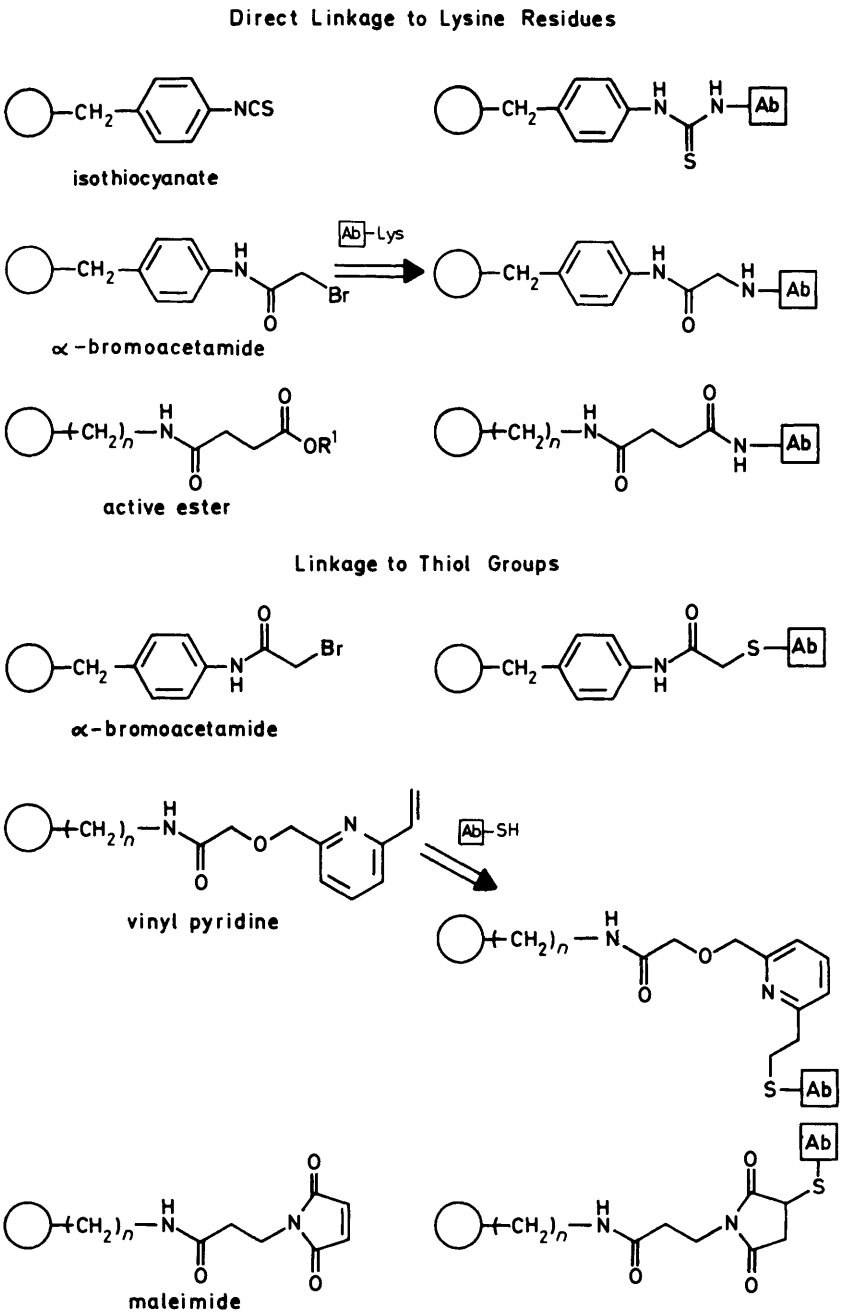
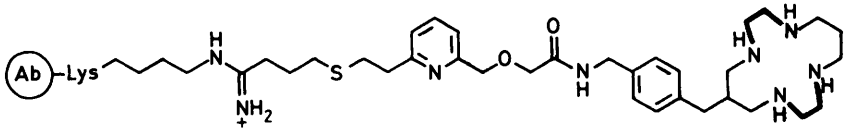


Figure 2 *Methods of linkage of complexing agents to antibodies*



(1)

reaction with Traut's reagent (2-iminothiolane), or engineered into an exposed part of the protein (as a Cys residue) using recombinant antibody methods. Maleimides show reasonable thiol selectivity, although they tend to undergo competitive hydrolysis above pH 7.5. Improved selectivity for thiol over primary amino groups may be obtained with vinyl-pyridine derivatives.[5,22] They react selectively in the pH range 5—9, although at a diminished rate with respect to malemide conjugation. These are not the only methods of linkage, of course, and a reverse proteolytic strategy has been used with an aminooxyacetyl derivative of deferrioxamine,[23] while linkage to the oxidized carbohydrate segment in a reductive amination has also been described.[24]

Once the bifunctional complexing agent has been bound to the antibody, it is important to quantify the level of derivatization and to demonstrate that the antibody has retained its immunoreactivity. The number of complexing agents linked may be assayed using ^{57}Co (or ^{58}Co) to bind the available sites[3,4,21] or with the aid of a ^{14}C-labelled ligand. An alternative fluorimetric method of analysis was used with (1):[22] exhaustive hydrolysis of the whole conjugate yielded the primary ammonium salt of the cyclam derivative. Reaction with *o*-phthalaldehyde in the presence of thioethanol generated the isoindole which possesses a convenient fluorophore and may be separated on cation-exchange HPLC by virtue of the dipositive charge of the diprotonated ring[5,25] (Scheme 1). As little as 5×10^{-11} mol dm^{-3} of ligand may be detected in this sensitive analysis.

If about one to two complexing agents are linked per antibody no diminution in immunoreactivity may be detected using an appropriate antigen-Elisa assay.[26]

4 Choice of the Bifunctional Complexing Agent

In selecting the parent ligand system to be functionalized the primary considerations are the nature of the metal ion to be bound and the need to form a kinetically inert complex *in vivo*. The following three examples illustrate these points for copper, indium and gallium (considered together), and yttrium. Until the mid-1980's the emphasis had been on the synthesis of C-functionalized derivatives of EDTA and DTPA, but their anionic metal complexes were not sufficiently stable at low pH or in the presence of serum cations to permit their

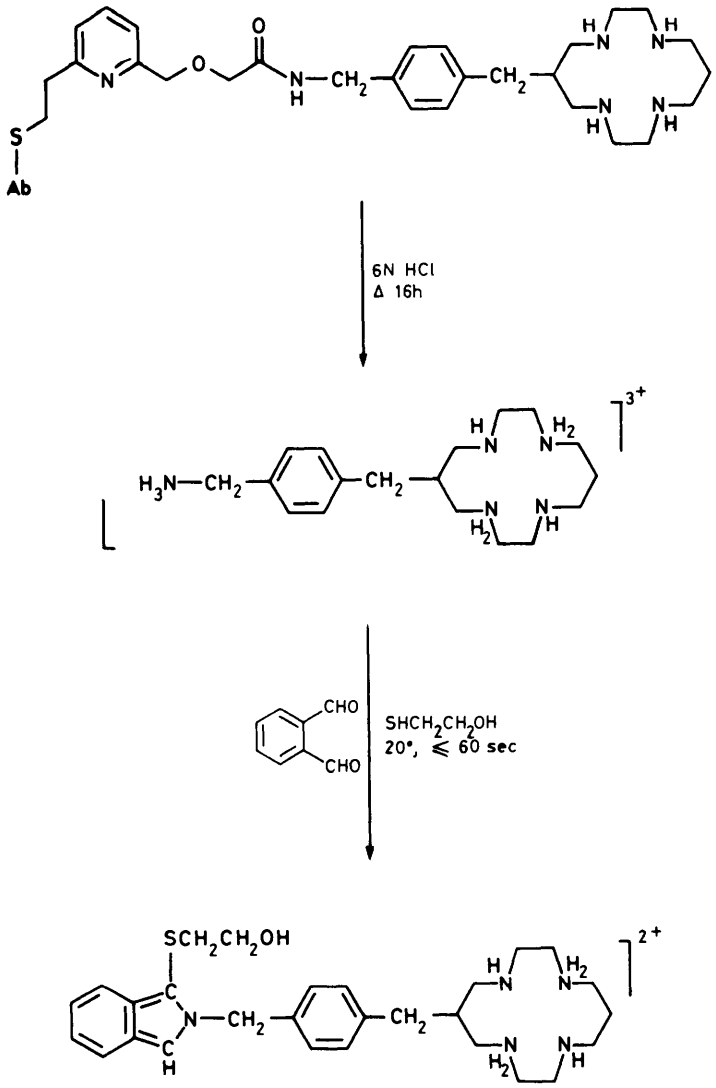
[22] J R Morphy, D Parker, R Kataky, M A W Eaton, A T Millican, R Alexander, A Harrison, and C Walker, *J Chem Soc*, *Perkin Trans 2*, 1990, 573

[23] J P Mach and R E Offord, unpublished results

[24] J D Rodwell, V L Alvarez, C Lee, A D Lopes, J W F Goers, H D King, H J Powsner, and T J McKeara, *Proc Nat Acad Sci USA*, 1986, **83**, 2632

[25] J R Morphy, D Parker, R Kataky, A Harrison, M A W Eaton, A T Millican, A Phipps, and C Walker, *J Chem Soc*, *Chem Commun*, 1989, 792

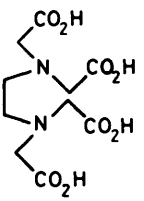
[26] D Colcher, M Zalutsky, W Kaplan, D Kufe, F Austin, and J Schlom, *Cancer Res*, 1983, **43**, 736



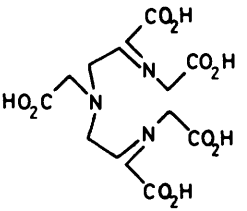
Scheme 1 ortho-*Phthaldehyde assay for the number of macrocycles bound per antibody*

successful use *in vivo*. Attention was switched to the functionalization of macrocyclic complexes which tend to undergo slower acid dissociation and cation exchange reactions.

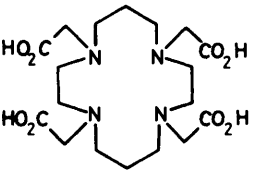
A. Copper.—There are several ligands which seem to form thermodynamically



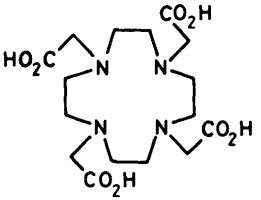
(2) (EDTA)



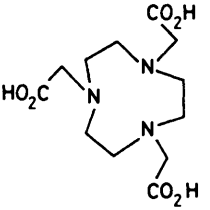
(3) (DTPA)



(4) (TETA)



(5) (DOTA)



(6) (NOTA)

stable complexes with copper (Table 3). Ligands (2) to (6) are mixed oxygen and nitrogen donors which form polyanionic complexes at ambient pH and are thereby inappropriate. Copper prefers nitrogen as a donor over oxygen and the most stable complexes (kinetically *and* thermodynamically) are those with cyclic N_4 donors which bind copper in an equatorial plane, with the elongated axial sites (arising from the Jahn–Teller distortion) playing no significant role in bonding. Crystal structures of these complexes bear out this simple analysis, (Figure 3).[31,32] The preferred parent ligand system is therefore that based on cyclam (14-N_4) (7) or the smaller 13-N_4 coronand, (8): copper complexes are cationic and resist decomplexation down to pH 1.[7] Despite this, other workers have pursued antibody conjugates of ^{64}Cu and ^{67}Cu based on complexes of TETA, (4), and DOTA, (5).[33] The approach adopted in Durham was to prepare C-functionalized [13]-N_4 and [14]-N_4 ligands (9), (10), bearing aminoalkyl substituents to permit antibody linkage.[5,22,25] Other workers have adopted a similar strategy using an *N*-alkylated cyclam, copper complexes of which are

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[28] A Bevilacqua, R I Gelb, W B Hebard, and L J Zompa, *Inorg Chem*, 1987, **26**, 2699, and references therein

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[30] A Risen, M Zehnder, and T A Kaden, *J Chem Soc, Chem Commun*, 1985, 1136, M K Moi, M Yanuck, S V Deshpande, H Hope, S J DeNardo, and C F Meares, *Inorg Chem*, 1987, **26**, 3458

[31] I M Helps, D Parker, J Chapman, and G Ferguson, *J Chem Soc, Chem Commun*, 1988, 1094

[32] P A Tasker and L Siklar, *J Cryst Mol Struct*, 1975, **5**, 329

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Table 3

Ligand	*log K*[c]	*Comment*
EDTA	18.9	Readily dissociates in serum, N_2O_2 bound
DTPA	21.4	Readily dissociates in serum, N_3O_3 bound
TETA	21.6	Anionic, primary binding is N_2O_2[d]
DOTA	22.2	Anionic, binding is N_2O_2[d] (+ 2 longer N_2)
NOTA[b]	21.6	Anionic complex, N_3O_3 bound[d]
14-N_4 (cyclam)[c]	27.2	cationic, decomplexation only in strong acid,
13-N_4[c]	29.1	N_4 bound[e]

[a] Acronyms used TETA is 1,4,8,11-tetraazacyclotetradecane tetraacetic acid, DOTA is 1,4,7,10-tetracyclododecane tetraacetic acid, NOTA is 1,4,7-triazacyclononanetriacetic acid, Cyclam is 1,4,8,11-tetrazacyclotetradecane, 13-N_4 is 1,4,7,10-tetraazacyclotridecane [b] $I = 1.0$ [c] $I = 0.2$ [d] From references 27 and 28 [e] From references 29—31

somewhat less stable than the C-linked variants.[34a] Ligands (9) and (10) were prepared by condensation of the appropriate acyclic tetramine with either 6-cyanocoumarin or a mono-benzyl malonate followed by borane reduction. They were conjugated to the antibody B72.3[35] (which binds to the TAG-72 antigen found in 80% of human breast and colorectal cancers) *via* their vinyl pyridine (1), or malemide derivatives and copper radiolabelling studies were initiated.[22]

An important feature of radiolabelling antibodies is that non-specific binding of the isotope to the protein must be minimized. Copper tends to form very stable complexes with tetrapeptides but their formation is minimized by working at low pH (*ca.* 4.5 or below).[36] The forward rate of binding of Cu^{2+} with the ligands (7) and (8) has been optimized at this lower pH. Using a succinate or citrate buffer of minimal ionic strength at 37 °C, good radiolabelling yields were obtained. Any residual non-specifically bound ^{64}Cu or ^{67}Cu could be 'mopped-up' by addition of excess DTPA prior to gel filtration or dialysis. The kinetic analysis[22,25] revealed that *anionic* copper species (*e.g.* $[Cu(succinate)_2]^{2-}$ or $[HCu(succinate)_2]^-$) were reacting with the monoprotonated ligand. This conclusion was reached from the dependence of the rate of complexation with succinate concentration and with ionic strength: reaction was fastest at lower ionic strength implicating the interaction of species of opposite charge in the rate-limiting step.

The ultimate test of stability with respect to dissociation *in vivo*, involves analysis of the biodistribution of the copper radiolabel as a function of time. Free copper will tend to build-up in the liver and kidney. For the liver, in particular, a value of around 30% of the blood level is expected based on its blood content. Values above this indicate either metal loss or protein damage (over-derivatization or aggregation). Data for ^{67}Cu bound to B72.3 with the aid of (9b) (vinyl pyridine linked) reveal that there is no significant build-up of copper in the

[34] (*a*) J Franz, G M Freeman, E K Barefield, W A Vokert, G J Ehrhardt, and R A Holmes, *Int J Radiat Appl Instrum B*, 1987, **14**, 479 (*b*) J C Roberts, S L Newmyer, J A Mercer-Smith, S A Schreyer, and D K Lavallee, *Int J Radiat Appl Instrum B*, 1989, **40**, 775

[35] D Colcher, P Horan-Hand, M Nati, and J Schlom, *Proc Nat Acad Sci USA*, 1981, **78**, 3149

[36] A S Craig, I M Helps, K J Jankowski, D Parker, A Harrison, S K Rhind, M A W Eaton, N R A Beeley, A T Millican, and A Phipps, *J Chem Soc, Chem Commun*, 1989, 794

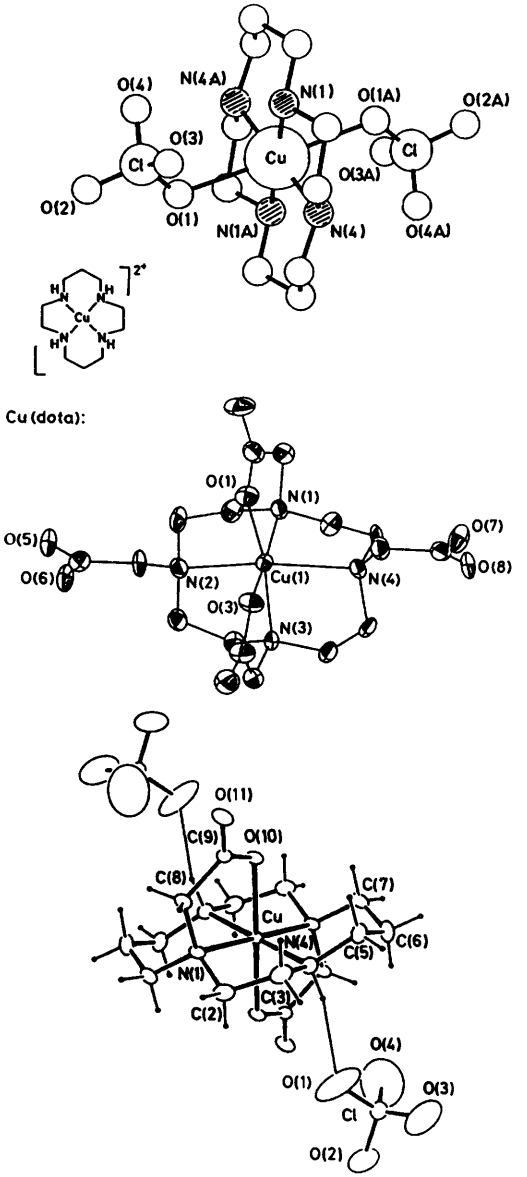
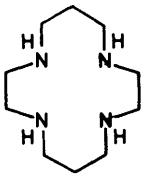
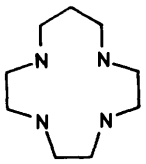


Figure 3 *Crystal structures of* [copper-cyclam] *(upper),* [copper-DOTA] *(middle) and* [copper-14-N_4 trans diacetate] *(lower)*

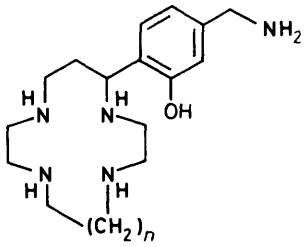
kidneys or liver over 72 h,[22] consistent with the good kinetic stability of the copper [14]-N_4 complex at low pH (Table 4).



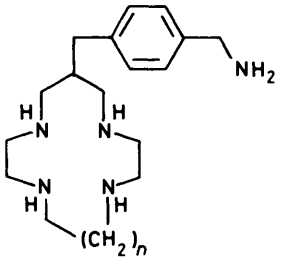
(7) (Cyclam)



(8)



(9) n = 1 or 2



(10) n = 1 or 2

Table 4 *Distribution of* ^{64}Cu *and* ^{67}Cu *labelled* B72.3*-macrocycle complex and* ^{64}Cu*-macrocycle labelled* B72.3 *in THY* 1.2 *mice* (% *I.D.* g^{-1} *tissue*)

	A(^{64}Cu)[a]	B(^{64}Cu)[b]	C(^{67}Cu)[c]		
Tissue	24 h	24 h	4 h	24 h	72 h
Blood	18.4	20.0	28.8	19.1	18.1
Kidneys	6.3	5.8	8.2	5.9	6.2
Liver	6.0	7.0	9.3	6.5	5.1
Lungs	8.1	7.8	10.1	7.7	8.6
Spleen	5.1	5.0	5.9	4.1	4.9

[a] Pre-conjugation labelled [14]-N_4-B72.3. [b] Post-conjugation labelled [14]-N_4-B72.3 (pH 4, 20 °C). [c] Post-conjugation labelled [14]-N_4-B72.3 (pH 4, 37 °C, DTPA wash).

B. Gallium and Indium.—Gallium(III) and indium(III) form readily hydrolysed aquo ions so the ligand used must be tribasic in order to satisfy the nuclear charge and be hexadentate in order to form coordinatively saturated complexes. The resultant neutral complexes should therefore resist acid or cation catalysed decomplexation over a wide pH range. Carboxylate donors are preferred over other less basic groups (*e.g.* phenols, pyridones) as they are ionized at ambient pH aiding fast complexation, and less sensitive to protonation at low pH inhibiting acid-catalysed dissociation. Of the four [9] to [12] ring triazacycloalkanetriacetates, the ligand NOTA, (6), bonds indium and gallium most rapidly under ambient conditions (pH 5, 37 °C, 0.1 mol dm^{-3} acetate).[36,37] Crystal

[37] C. J. Broan, A. S. Craig, J. P. L. Cox, R. Kataky, D. Parker, A. Harrison, A. M. Randall, and G. Ferguson, *J. Chem. Soc., Perkin Trans. 2*, 1991, in press.

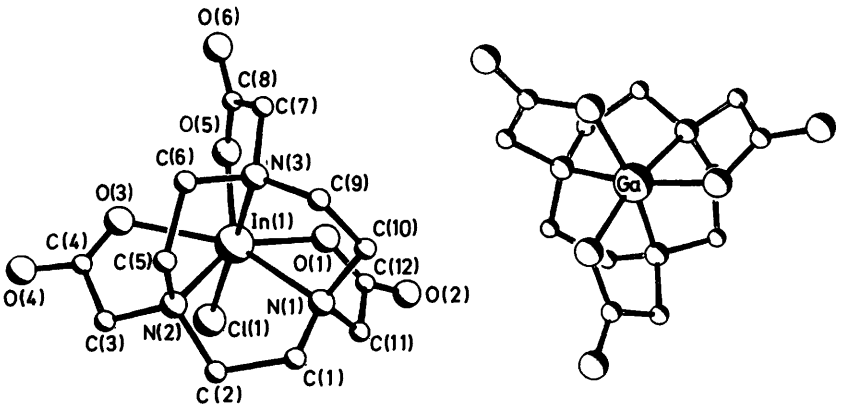


Figure 4 *Crystal structures of* [Ga-NOTA] *and* [InH-NOTA] *(as the chloro adduct)*

structures of the neutral gallium and of the monoprotonated indium complex of NOTA have been described (Figure 4).[38,39] The stability of the C_3-symmetric gallium complex at high acid concentration is particularly striking. The complex may be observed unchanged by ^{71}Ga NMR in 6M nitric acid over a period of 6 months. Moreover, the complex has been detected intact by ^{71}Ga NMR, in the liver region of a mouse following injection of a 1.4 mg sample of complex.[37] The dissociation of indium from NOTA has been monitored by ^{13}C NMR using ^{13}C labelled ligand (at the carbonyl carbon). Indium dissociation was observed in the pH range 0 to -0.7 with a second order rate constant (296 K) of 1.8×10^{-4} dm^3 mol^{-1} s^{-1}.[37,38] A kinetic scheme for the dissociation pathway was proposed involving two successive protonations to give a kinetically labile dicationic complex (Scheme 2). Direct evidence for this pathway comes from the structural characterization of the monoprotonated intermediate[39] and from ^{13}C and ^{1}H NMR analyses of the broadening and shifts in resonances of the complex as a function of pH.

Given the high stability, with respect to acid dissociation, of the gallium and indium complexes of NOTA, aminoalkyl-substituted ligands were required to permit protein linkage. Both C and N-functionalized variants were prepared.[40,38,41] The homochiral C-functionalized ligand (Scheme 3) was prepared from (2*S*)-lysine in an expeditious synthesis that relied upon copper protection of the triamine prior to tosylation and cyclization. The route is versatile, permitting, for example, the synthesis of the aminobutyl-substituted

[38] A. S. Craig, D. Parker, H. Adams, and N. A. Bailey, *J. Chem. Soc., Chem. Commun.*, 1989, 1793.
[39] A. S. Craig, I. M. Helps, D. Parker, H. Adams, N. A. Bailey, M. G. Williams, and G. Ferguson, *Polyhedron*, 1989, **8**, 2481.
[40] A. S. Craig and J. P. L. Cox, Ph.D. Theses, University of Durham, 1989.
[41] J. P. L. Cox, A. S. Craig, I. M. Helps, K. J. Jankowski, D. Parker, M. A. W. Eaton, N. R. A. Beeley, B. A. Boyce, A. J. Millican, and K. Millar, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2567.

$$H^+ + [In \cdot NOTA] \underset{A}{\overset{K_{a_1}}{\rightleftharpoons}} [H^+ In \cdot NOTA] \underset{B}{\overset{K_{a_2}}{\rightleftharpoons}} [H_2 In \cdot NOTA] \underset{C}{\xrightarrow{fast}} In^{3+}$$

Total substrate concentration $[S]_T = [A] + [B] + [C]$

$$\text{rate} = k[C] = \frac{k[B][H^+]}{K_{a_2}}$$

$$\text{as } [C] \longrightarrow 0, \text{ then } [B] = \frac{[S]_{tot}[H^+]}{([H^+] + K_{a_1})}$$

$$\longrightarrow \text{rate} = \frac{k[S]_{tot}[H^+]^2}{K_{a_2}([H^+] + K_{a_1})} \quad (1)$$

At high acid: $[H^+] \gg K_{a_1}$, equation 1 simplifies:

$$\text{rate} = \frac{k[S]_{tot}[H^+]}{K_{a_2}} \quad (2)$$

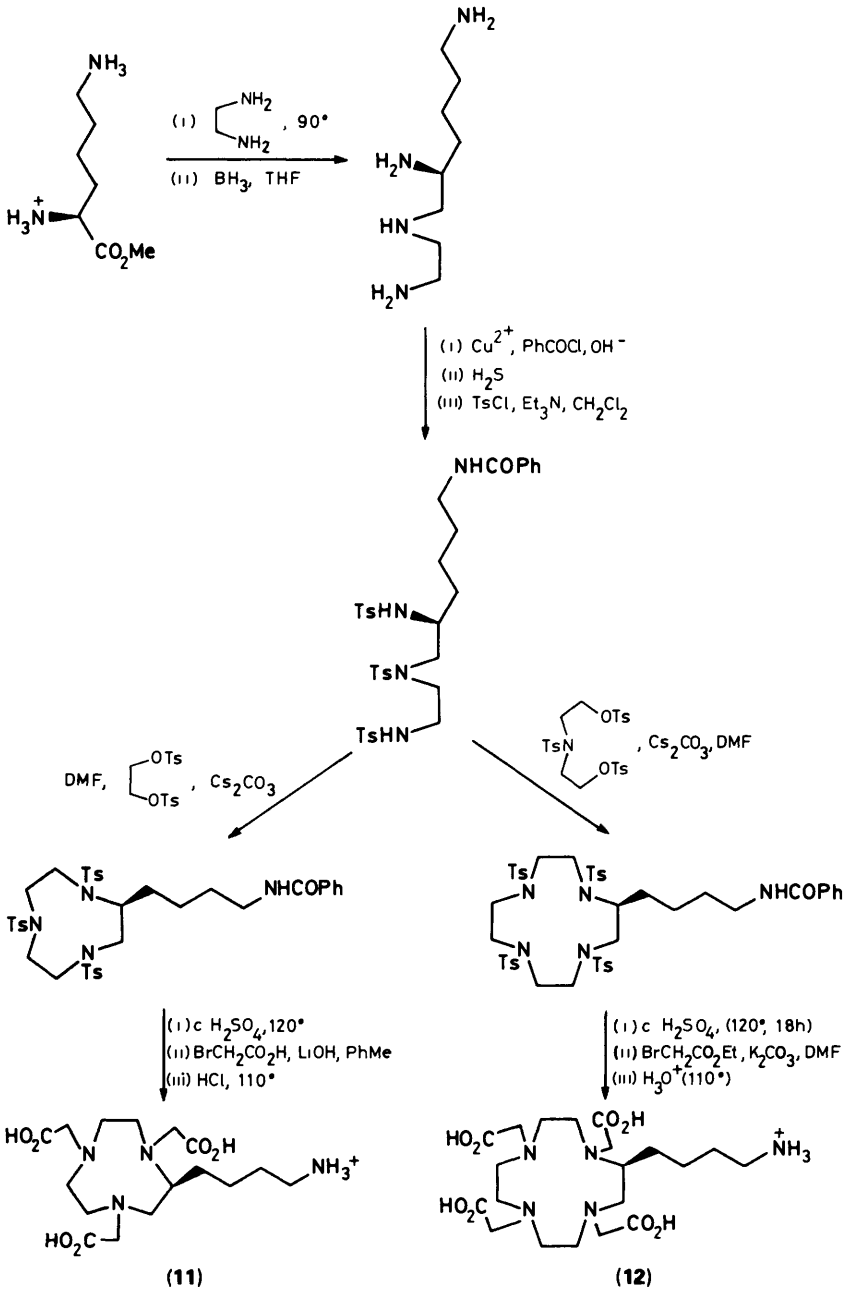
$$\longrightarrow k_{obs} = \frac{k[H^+]}{K_{a_2}}$$

Scheme 2 *Dissociation pathway for indium loss from* [NOTA-In] *at low pH*

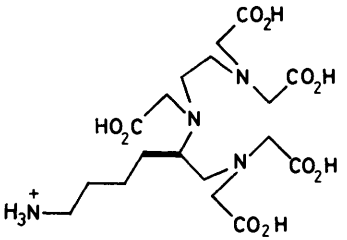
DOTA, (12)[42] and the acyclic DTPA derivative, (13).[41] Other workers have reported related syntheses of C-linked DTPA or EDTA starting from *p*-nitrophenylalanine with the intention of using an aryl isothiocyanate linkage.[3,4] The shorter syntheses of the racemic mono-substituted N-linked ligand (14) and the pair of diastereoisomeric di-substituted ligands (15) involve alkylation of the parent 1,4,7-triazacyclononane ring with the requisite α-bromo ester (Scheme 4).[41] These ligands have been conjugated to chimeric B72-3 antibody, the biodistribution of the indium-111 radiolabelled conjugates examined in animals, and are currently being evaluated in limited trials in human patients in order to assist in the diagnosis of human colon carcinoma.

C. Yttrium.—In seeking a suitable ligand to bind yttrium-90 for radioimmunotherapy, similar criteria to those required for In binding were imposed. The ligand was required to bind yttrium quickly under ambient conditions and at low ligand concentrations (in the range 10—30 μmol dm^{-3}), yet form a complex which was kinetically inert (in the pH range 2—8) with respect to acid or cation promoted dissociation. Given the well-defined tendency of yttrium to form

[42] J. P. L. Cox, K. J. Jankowski, R. Kataky, D. Parker, M. A. W. Eaton, N. R. A. Beeley, A. T. Millican, A. Harrison, and C. Walker, *J. Chem. Soc., Chem. Commun.*, 1989, 797.


NH3
CO2Me
(i) , 90°
(ii) BH3, THF
NH2
H2N
HN
H2N
(i) Cu2+, PhCOCl, OH−
(ii) H2S
(iii) TsCl, Et3N, CH2Cl2
NHCOPh
TsHN
TsN
TsHN
DMF, , Cs2CO3
, Cs2CO3, DMF
Ts
N
TsN
NHCOPh
(i) c H2SO4, 120°
(ii) BrCH2CO2H, LiOH, PhMe
(iii) HCl, 110°
(i) c H2SO4, (120°, 18h)
(ii) BrCH2CO2Et, K2CO3, DMF
(iii) H3O+ (110°)
HO2C
CO2H
NH3+
(11)
(12)

Scheme 3



(13)

octadentate complexes, ligands (4), (5), and [(16—(18)] were screened. Of these (4), (5), (13), and (16) have been reported to form relatively stable complexes with trivalent metals, particularly the lanthanides[43,44] although the sluggishness with which (5) in particular was reported[43] to bind Gd^{3+} did not augur well at first inspection. A further reason why (18), (17), and (16) were considered is that they should form neutral complexes with yttrium and hence could be less sensitive to acid/cation promoted dissociation. It has been demonstrated very clearly[45–47] that DTPA-antibody conjugates are not sufficiently stable for ^{90}Y-based radioimmunotherapy in man.[46,47] Although treatment revealed significant tumour regression, the dissociation of ^{90}Y and its localization in bone led to major bone-marrow toxicity which severely limited the therapeutic efficacy of systemically administrated ^{90}Y-antibody.

Of all the ligands examined in terms of the optimization of association and the minimization of dissociation at low pH, we[40,42] and others[48] concluded that ligands based on DOTA were superior in both respects. The stability constant for 1:1 complex formation gave a value of log $K = 24.8$[37,41] which compares to log $K = 22.1$ for [Y·DTPA]. Stability studies in serum (pH 7.4, 37 °C) using [^{88}Y·DOTA] showed that less than 0.5% of the ^{88}Y had dissociated from the ligand over 18 days.[48] The dissociation of yttrium has also been examined at low pH (<2.5) by two independent methods using ^{13}C NMR with ^{13}C-carbonyl labelled and with ^{90}Y-labelled complex (using HPLC-radiometric methods).[37] Dissociation occurred by an acid-dependent pathway (thereby highlighting the limitations of serum stability studies at pH 7.4) *via* successive protonation (Figure 5). Optimal conditions for the binding of ^{90}Y to the DOTA–antibody conjugate have been carefully developed. By working in an ammonium acetate buffer (pH 5.8) at 37 °C, a radiolabelling yield of in excess of 70% may be obtained within 30

[43] J. F. Desreux, M. E. Loncin, and E. Merciny, *Inorg. Chem.*, 1986, **25**, 2646; 1981, **20**, 987; J. F. Desreux, *Inorg. Chem.*, 1980, **19**, 1319.

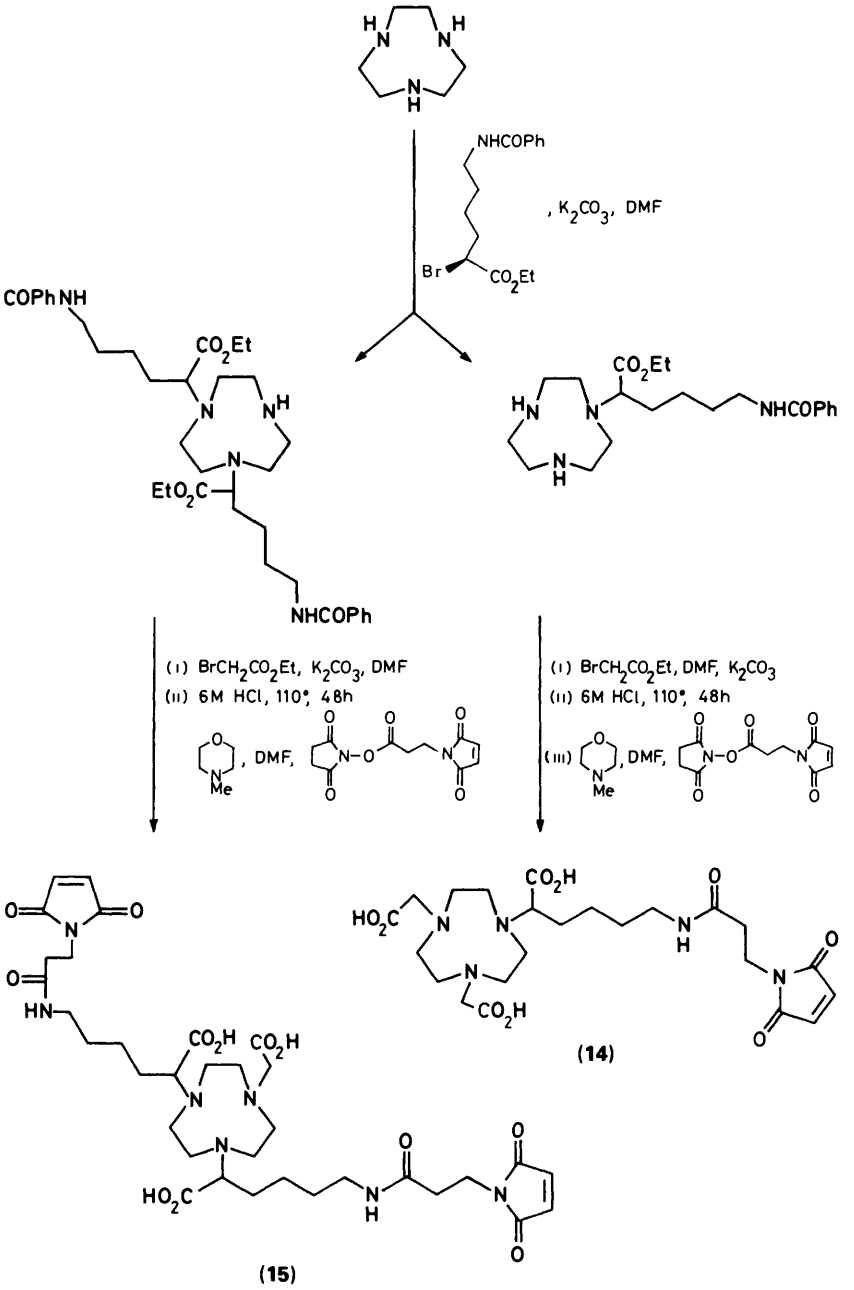
[44] M. T. S. Amorim, R. Delgado, J. J. R. Frausto da Silva, M. C. T. Vaz, and M. F. Vilhena, *Talanta*, 1988, **35**, 741.

[45] D. J. Hnatowich, M. Chinol, D. A. Siebecker, M. Gionet, T. Griffin, P. W. Doherty, R. Hunter, and K. A. Kase, *J. Nucl. Med.*, 1988, **29**, 1428.

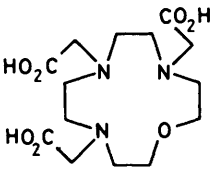
[46] M. Roselli, J. Schlom, O. A. Gansow, S. M. Raubitschek, M. W. Brechbiel, and D. Colcher, *J. Nucl. Med.*, 1989, **30**, 672.

[47] R. M. Sharkey, F. A. Kaltovich, L. B. Shih, I. Fand, G. Govelitz, and D. M. Goldenberg, *Cancer Res.*, 1988, **48**, 3270.

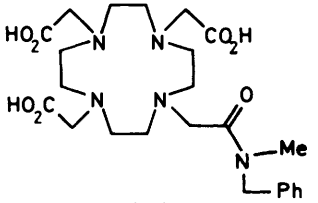
[48] M. K. Moi, C. F. Meares, and S. J. DeNardo, *J. Am. Chem. Soc.*, 1988, **110**, 6266.


NHCOPh
, K_2CO_3, DMF
Br
CO_2Et
COPhNH
CO_2Et
EtO_2C
NHCOPh
(i) $BrCH_2CO_2Et$, K_2CO_3, DMF
(ii) 6M HCl, 110°, 48h
, DMF,
Me
(i) $BrCH_2CO_2Et$, DMF, K_2CO_3
(ii) 6M HCl, 110°, 48h
(iii)
, DMF,
Me
CO_2H
HO_2C
(14)
(15)

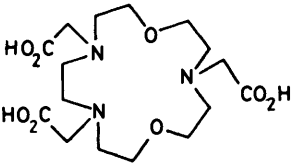
Scheme 4



(16)



(17)



(18)

minutes. The choice of buffer cation is important: the use of sodium acetate, for example, inhibits ^{90}Y labelling since sodium itself forms a complex with DOTA ($\log K = 4.4$). As in any radiochemical experiment precautions must be taken to ensure that the concentrations of contaminant metal cations are minimised.[3,21,37] The use of pure water, reagents, and clean equipment is essential in order to suppress competitive ligand complexation by trace metal ions. The ligand DOTA for example is well known to form relatively stable complexes with Cu^{2+} (Table 3, p. 281), Ni^{2+} and Zn^{2+} ($\log K = 22.2$, 20.0, and 21.0 respectively): nickel spatulas are strictly forbidden!

Functionalized DOTA ligands have been prepared bearing either C-substituents or an N-substituent.[41,42,48] The aminobutyl compound (19) was prepared from 2*S*-lysine (Scheme 3),[41,42] while the *p*-nitrophenyl substituted ligand (20) has been prepared from the tetrapeptide (2*S*)-NO_2Phe–Gly–Gly–Gly.[48] Linkage through one of the ring nitrogens provided a shorter synthetic route to (21),[41] while retaining the octadentate nature of the ligand. The related tetraphosphinic acid (22) has also been prepared in which the basic PO_2H group ($pK_a \sim 1.6$) binds strongly to yttrium creating a new stereogenic centre at phosphorus. Remarkably one diastereoisomer only was observed in the yttrium complex with the parent ligand (^1H, ^{31}P NMR),[49] suggesting that the complex may be resolved with a suitable chiral base.

Ligands (21), (19), and (22) have been conjugated to chimeric B72.3 antibody either *via* maleimide or active ester intermediates and the biodistribution of the ^{90}Y-radiolabelled conjugate examined in tumour and non-tumour bearing animals. The most significant feature of the preliminary studies is that there is a much reduced deposition of ^{90}Y in the bone compared to analogous experiments

[49] C. J. Broan, K. J. Jankowski, and D. Parker, unpublished work.

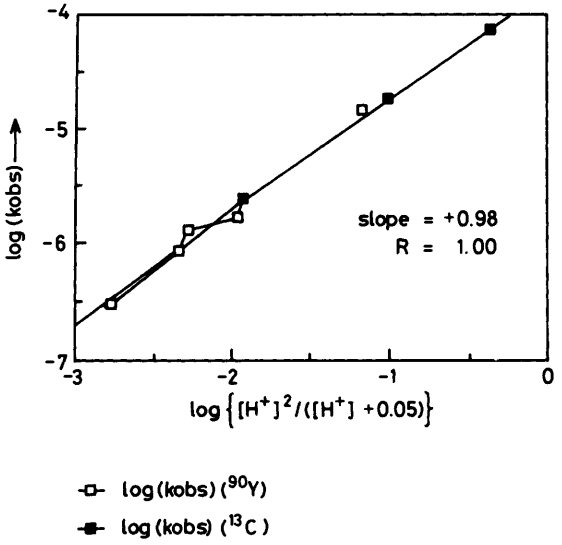
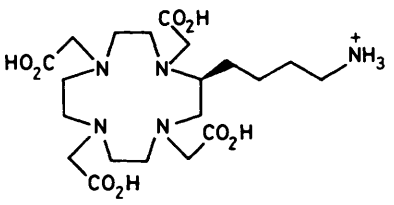


Figure 5 *Variation of the observed rate of dissociation of yttrium* (310 K) *from* [Y-DOTA] *with log* $\{[H^+]^2/[H^+] + K_{a_1}\}$ *for* $K_{a_1} = 0.05$. *Data were determined using* ^{90}Y*-labelled complex or with* ^{13}C-NMR *using* ^{13}C*-labelled ligand*



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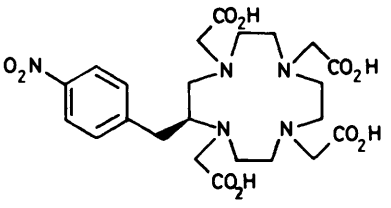

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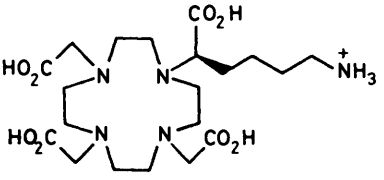
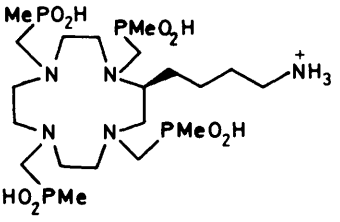


(21)



(22)

with C- or N-conjugated DTPA.[50] This augurs well for the planned clinical trials which will define whether ^{90}Y-based radioimmunotherapy is indeed feasible.

Acknowledgements. It is a pleasure to thank co-workers in Durham and collaborators at Slough (Celltech Ltd) and Harwell (MRC—Radiobiology Unit) without whom none of the work described would have been possible.

[50] A. Harrison, C. Walker, J. Sansom, J. P. L. Cox, A. S. Craig, K. J. Jankowski, A. T. Millican, and A. Farnsworth, *Int. J. Nucl. Med. Biol.*, submitted.