Yttrium macrocycles and their use in the treatment of cancer

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YTTRIUM MACROCYCLES AND THEIR USE IN THE TREATMENT OF CANCER

by

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ABSTRACT

YTTRIUM MACROCYCLES AND THEIR USE IN THE TREATMENT OF CANCER.

Six macrocyclic ligands have been synthesized for binding yttrium(III). Of the six, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) forms the most stable yttrium(III) complex in aqueous solution, log K(ML) = 24.9 and half life ~ 2 weeks at pH 1.0. In addition to an acid-dependent dissociative mechanism, release of Y^{3+} from Y(DOTA) may, it is tentatively suggested, be promoted by metal ions. DOTA also demonstrates rapid uptake of Y^{3+} (98% labelling efficiency at 310 K with [DOTA] = 10^{-5} M and [Y^{3+}] ~ 10^{-9} M, pH 5.5 [0.1 M ammonium acetate]).

Accordingly, an aminobutyl C-functionalised derivative of DOTA has been made and coupled to a monoclonal antibody. Once labelled with $^{90}Y^{3+}$, a long range β^--emitter, the conjugate can be used to selectively deliver a sterilising dose of radiation to a tumour. Preliminary experiments have indicated that the radiolabelled MoAb conjugate remains relatively inert *in vivo*. Tumour regression studies are in progress.

Jonathan Paul Lawrence Cox (September 1989)

ABBREVIATIONS

EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
DTPA	Diethylenetriamine-N,N,N',N",N"-pentaacetic acid
NTA	Nitrilotriacetic acid
PCA	Polyaminocarboxylate complexing agent
DOTA	1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid
TRITA	1,4,7,10-Tetraaazacyclotridecane-1,4,7,10-tetraacetic acid
TETA	1,4,8,11-Tetraazacyclotetradecane-1,4,8,11-tetraacetic acid
DOTA-BMA	4-(N-Benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triacetic acid
ODOTRA	1-Oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid
DTCTA	1,7-Dioxa-4,10,13-triazacyclopentadecane-4,10,13-triacetic acid
NOTA	1,4,7-Triazacyclononane-1,4,7-triacetic acid
RIT	Radioimmunotherapy
DNA	Deoxyribonucleic acid
MoAb	Monoclonal antibody
IgG	Immunoglobulin G
3,2,3-tet	4,7-Diaza-1,10-dodecanediamine
cyclam	1,4,8,11-Tetraazacyclotetradecane
mal	Maleimide
Me	Methyl
Et	Ethyl
Ac	Acetyl
Ph	Phenyl
Bz	Benzyl
Ts (Tosyl)	4-Toluenesulphonyl
Ar	Aromatic
THF	Tetrahydrofuran
Ln	Lanthanide
MPt	Melting point
dec	Decomposes
IR	Infrared
UV	Ultraviolet
NMR	Nuclear magnetic resonance
HPLC	High performance liquid chromatography

MS	Mass spectroscopy
CI	Chemical ionisation
DCI	Desorption chemical ionisation
FAB	Fast atom bombardment
TLC	Thin layer chromatography

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CHAPTER ONE

INTRODUCTION

1.1 CANCER[1]

Cancer is a cellular disorder, of variable severity, arising from two or more distinct genetic events[2,3]. Factors triggering these events are partly inherited, partly environmental and not well understood.

The disorder manifests itself through an affected cell's inability to respond to normal growth control mechanisms. Division of that cell leads to a group of cells called a tumour. The tumour may remain where it is (in which case it is a benign tumour) or it may fragment and spread to other tissues (in which case it is a malignant tumour). The latter process, metastasis, is the more difficult to control and is consequently the major cause of cancer death.

1.2 THE INCIDENCE OF CANCER[3,4]

Cancers are normally classified according to the organ in which they originate. Of the hundred or so generated by this system, cancer of the colon, lung and breast are the commonest. Indeed, cancer of the colon is the second most common malignancy in Western Europe, killing 20,000 people a year in the United Kingdom alone. Figure 1.1 illustrates the situation in the United States, where cancer is responsible for nearly a fifth of the deaths each year.

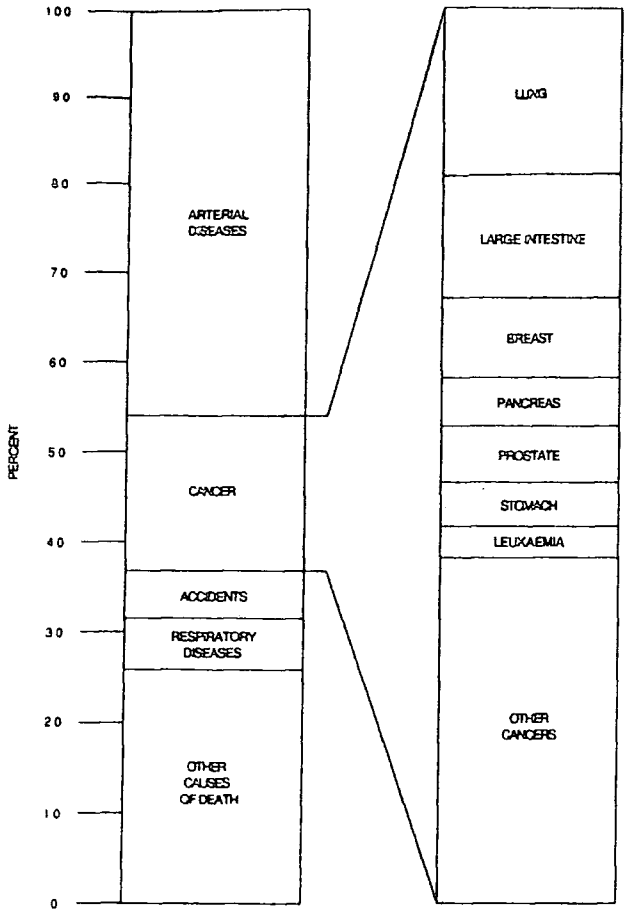


Figure 1.1: *The incidence of cancer in the United States [pre-AIDS]. (Source: Ref.4).*

1.3 THE TREATMENT OF CANCER[1]

There are two categories of treatment, surgery and therapy. Surgery involves the direct removal of the tumour and is effective against benign tumours. Therapy employs agents which attack the tumour *in situ*, *eg.* radiation in the form of X-rays and γ-rays (radiotherapy), and drugs such as 5-fluorouracil[5] and cisplatin[6] (chemotherapy). Radiotherapy, like surgery, is successful only against benign tumours. Chemotherapeutics, on the other hand, have had some success against metastatic disease: results with cisplatin have been particularly impressive, with complete remissions of testicular cancer in 85% of all patients treated, and a high efficacy against ovarian and bladder

cancers. There are still many cancers, however, which are in need of an improved therapy. In order of increasing priority, these are cancer of the breast, prostrate gland, colon, rectum, stomach, lung and pancreas.

There is another reason for improvement. Therapy works by damaging tumour cell DNA. As a large proportion of tumour cells in a single tumour are rapidly dividing, their DNA is particularly sensitive to this damage which results in cell, and eventually tumour, destruction. Unfortunately, it follows that the rapidly dividing cells of other tissues such as the gastro-intestinal tract and bone marrow are equally prone to this treatment, giving rise to unpleasant side-effects.

For an effective and painless treatment therefore, the anti-tumour agent must have some means of discriminating tumour cells from normal cells. Discrimination may be achieved using antibodies.

1.4 RADIOIMMUNOTHERAPY (RIT)

1.4.1 Antibodies[5]

Antibodies are glycoproteins, more specifically immunoglobulins (Ig), which the body generates in response to the presence of a foreign material, or antigen. A typical antibody such as IgG (Figure 1.2) consists of four polypeptide chains, two light (L) and two heavy (H), linked by disulphide bridges between cysteine residues on adjacent chains.

The polypeptide chains can be divided into variable and constant regions. There are specific sites in the variable regions, hyper-variable regions, which are responsible for antigen binding. The constant regions mediate effector functions, *eg.* initiating a sequence of immunological reactions terminating in the elimination of the

antigen. Figure 1.2 also shows another useful division of the antibody into two Fab fragments (F for fragment, ab for antigen binding) and an Fc fragment (c pertains to the fact that this fragment is readily crystallisable).

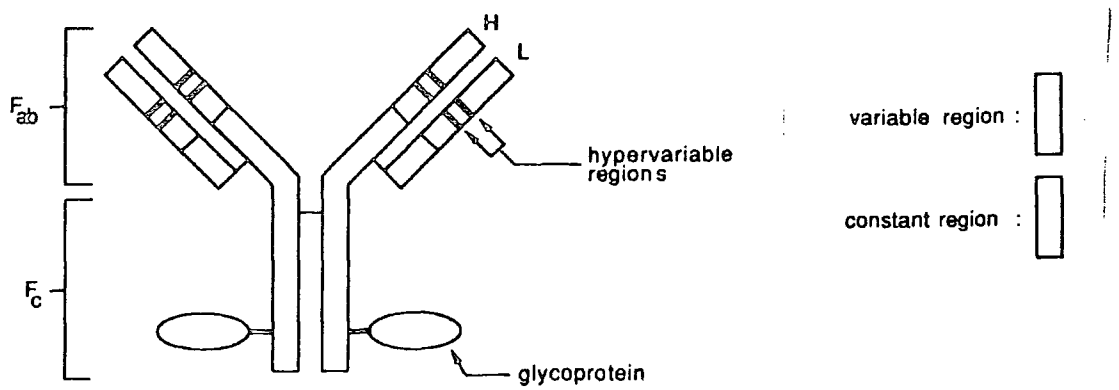


Figure 1.2: *The structure of a typical antibody (IgG).*

The antigen binding sites combine specifically with a particular molecular determinant, known as a hapten, on the surface of the antigen. Only one particular type of antibody can combine with a particular hapten (Figure 1.3).

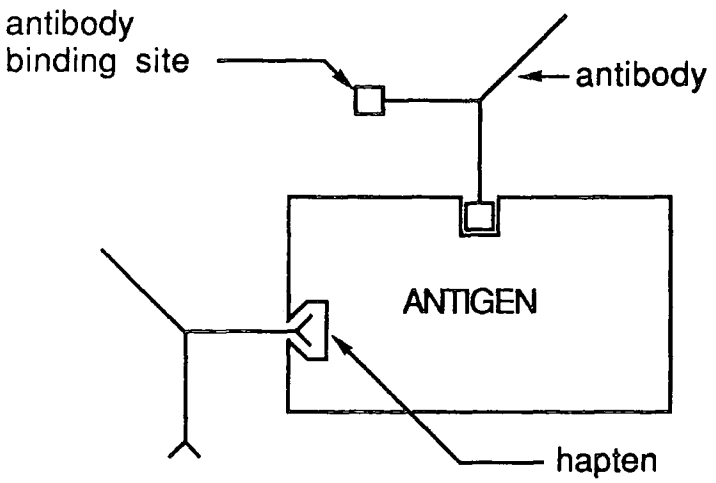


Figure 1.3: *Antibody-antigen binding specificity.*

1.4.2 Antibodies and RIT[1]

A tumour cell is coated with glycoproteins and glycolipids, some of which are foreign to the body and therefore constitute antigens. By developing antibodies specific for the various haptens (hereafter referred to as antigens themselves) on these antigens, a tumour cell can be distinguished from a normal cell. If the antibody is labelled with a suitable radionuclide, it can deliver a sterilising dose of radiation to the tumour cell to which it attaches itself (Figure 1.4). This is the basis of radioimmunotherapy (RIT).

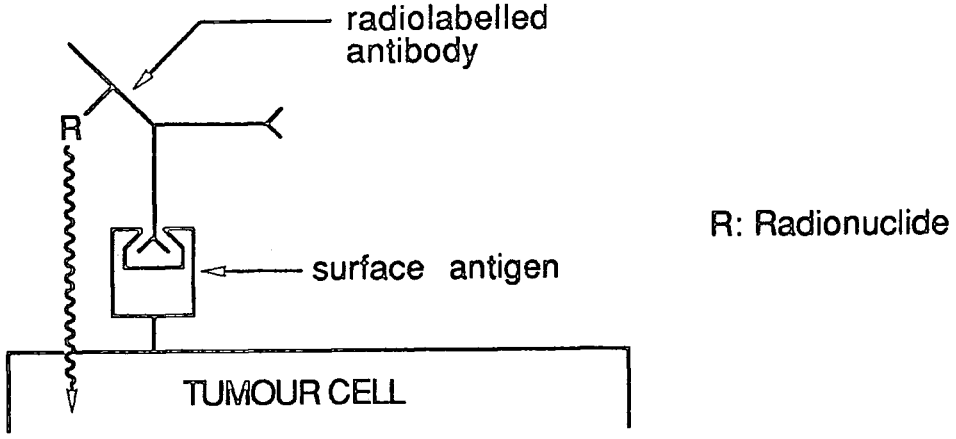


Figure 1.4: ***Radioimmunotherapy.***

1.4.3 Monoclonal Antibodies[7]

For thirty years after its initial conception[8], the implementation of RIT was scotched because it was not possible to generate a specific antibody for a specific antigen. The development of monoclonal antibodies by Köhler and Milstein[9] in 1975 rectified this state of affairs.

Antibodies are made by plasma cells. If one plasma cell is immunised against one particular antigen, it will secrete antibodies

specific only for that antigen. Growing one such cell in culture would result in a colony of identical cells (a clone) all producing the same antibody. However, plasma cells do not survive in culture.

Köhler and Milstein found that if they fused a plasma cell immunised against a specific antigen with a myeloma cell (ironically, a myeloma is a tumour of the immune system) - which has the property that it can be cultured indefinitely - the hybrid cell, or hybridoma, preserved the individual properties of its parent cells, namely that it was immortal and produced large amounts of identical, or *monoclonal*, antibody (Figure 1.5). Hence an ample supply of monoclonal antibody could be raised for any given tumour antigen.

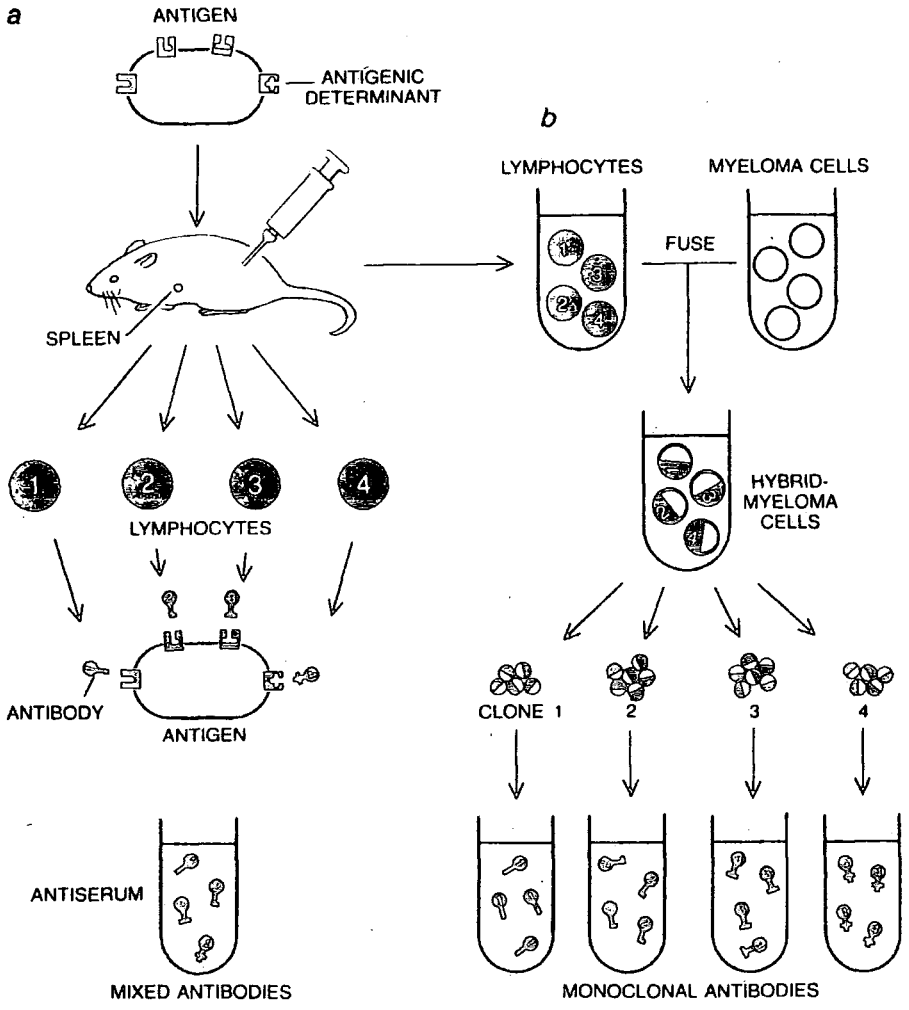


Figure 1.5: *Monoclonal antibody technology - A lymphocyte is the precursor of a plasma cell (Reproduced from Ref.7).*

1.4.4 Chimaeric Antibodies[10,11]

Despite the above development, making monoclonal antibodies for human tumour antigens has proved difficult.

With mice, which Köhler and Milstein used for their seminal work, the plasma cells for a specific antigen were obtained by first injecting the mice with the antigen, and then removing the cells from the spleen of the animal. Injecting humans with tumour antigens is obviously an unsatisfactory way of obtaining the required antibody. The alternative is to use murine (mouse) antibodies. But murine antibodies, since they are foreign to the body, evoke an immune response which can seriously interfere with therapy, or cause allergic side-effects.

These problems have been partly overcome by using chimaeric antibodies, antibodies with the hypervariable regions of a rat or mouse specific for the tumour antigen, and human constant and variable regions. Such antibodies, although still not ideal, should evoke a lesser immune response than the murine antibodies.

1.4.5 Choice of Radionuclide for RIT[12]

Radionuclides for RIT should have the following properties:

(a) A half-life ($T_{\frac{1}{2}}$) between 6-200 h., long enough for tumour localisation while minimising the risk to organs involved in antibody breakdown;

(b) Be carrier-free, to maximise the amount of "hot" antibody and therefore the dose received by the tumour;

(c) Low yields of penetrating radiation (X-rays, γ-rays);

(d) Stable daughter products;

(e) Be cheap and readily available.

According to the particle emitted and its range, radionuclides which satisfy the above criteria can be divided into five categories:

(1) Alpha emitters (range 50-90 μm), *eg.* ^{211}At;

(2) Short range beta emitters (mean range < 200 μm), *eg.* ^{199}Au;

(3) Medium range beta emitters (200 μm < mean range < 1mm), *eg.* ^{67}Cu;

(4) Long range beta emitters (mean range > 1 mm), *eg.* ^{90}Y;

(5) Radionuclides decaying by electron capture and/or internal conversion, *eg.* ^{131}Cs.

It is also important to match tumour morphology with the range of the radionuclide's particulate emission. For instance, circulating single tumour cells, as found in leukaemia, would be best eliminated by a source with short range (approximately one cell diameter) emissions - alpha and short range beta emitters fall into this category - damage to normal cells would therefore be minimal. Large, dense tumours, on the other hand, may have a non-uniform distribution of antigen, and cross-fire from long range (100 - 1000 cell diameters) beta emitters, such as ^{90}Y, would make a positive contribution to the absorbed dose (Figure 1.6).

1.4.6 Radionuclear Properties of Yttrium-90

Attributes which make ^{90}Y highly desirable as a therapeutic radionuclide[13] are: (a) $T_{\frac{1}{2}}$ = 64 hours; (b) pure β^--emitter of intermediate energy (2.3 MeV); (c) stable daughter product; (d) readily obtainable from its long-lived parent, ^{90}Sr ($T_{\frac{1}{2}}$ = 28 years) and (e) exhibits good complexation chemistry (see Section 1.4.8). ^{90}Y decays as follows:

$$^{90}_{39}Y \xrightarrow{\beta^-} {}^{90}_{40}Zr \text{ (stable)}$$

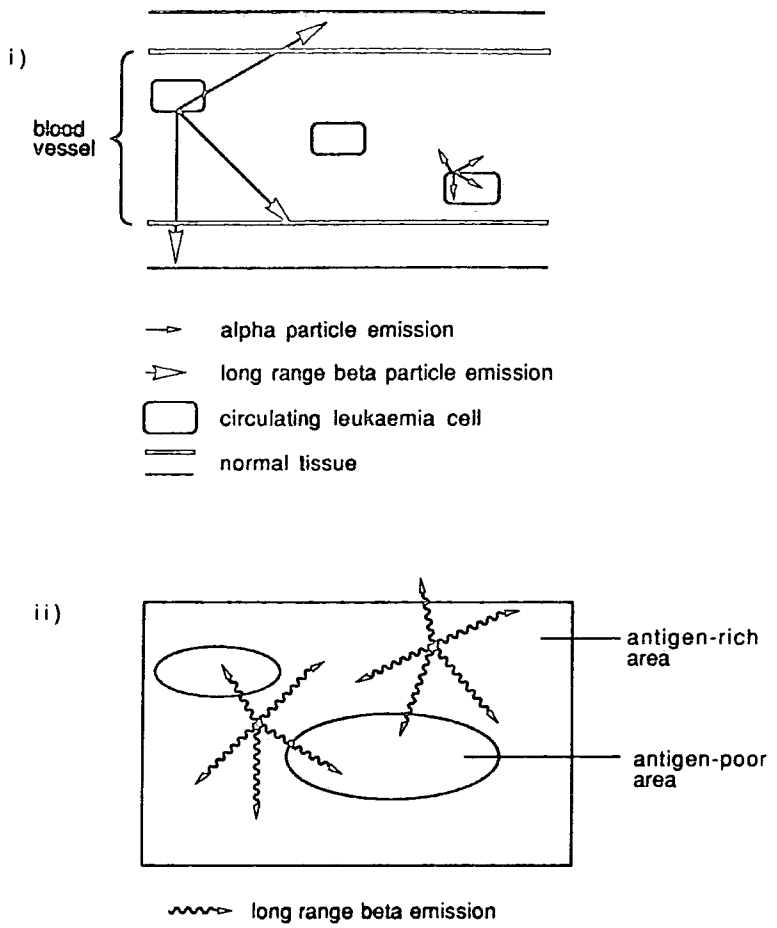


Figure 1.6: *Schematic diagram showing the importance of matching tumour morphology to choice of radionuclide: (i) circulating leukaemia cells; (ii) a densely packed tumour.*

As a long range (3.9 mm) β^--emitter, ^{90}Y is ideal for stomach, lung, ovarian[14], colorectal[15,16] and breast cancers. Its major disadvantages are, however, the damage caused to normal tissue as a result of these long range emissions[12] (see Figure 1.6) and the lack of γ-rays in its decay process with which to image the tumour[14]. However, ^{86}Y, a positron emitter ($T_{\frac{1}{2}}$ 14.6 hours) could be used. [A positron combining with an electron produces two γ-rays which can be detected with a gamma camera. This is the basis of *positron emission tomography* (PET), a diagnostic technique].

1.4.7 Bifunctional Complexing Agents

Yttrium-90 and the other radionuclides discussed so far (except for ^{211}At) are all metals and as such cannot be attached to an antibody *via* a stable covalent bond. Instead, radiolabelling is realised using a bifunctional complexing agent[17,18]. This consists of a chemically reactive functional group and a complexing moiety. The chemically reactive group facilitates conjugation to the antibody *via*, for example, the $-NH_2$ group of a lysine side chain, while the complexing moiety is responsible for binding the radioactive metal ion, *eg.* 1-(4-isothiocyanatobenzyl)-EDTA(1) (Figure 1.7).

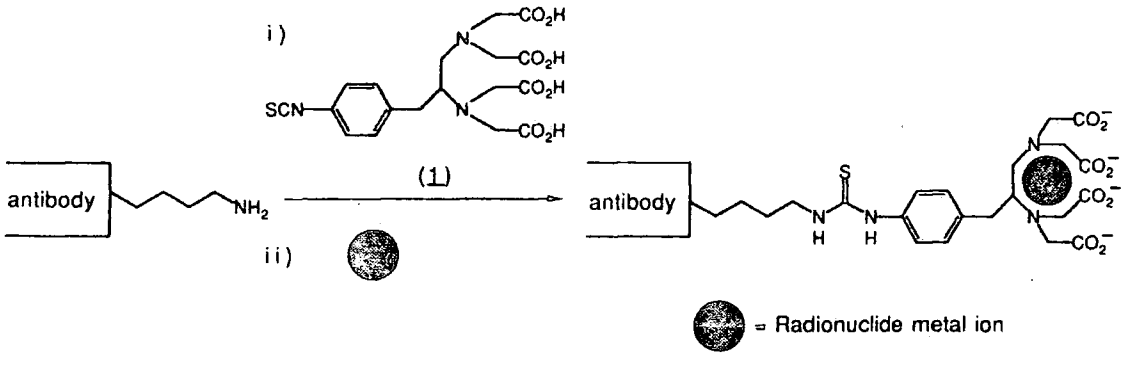


Figure 1.7: *Radiolabelling a monoclonal antibody using a bifunctional complexing agent such as 1-(4-isothiocyanatobenzyl)-EDTA.*

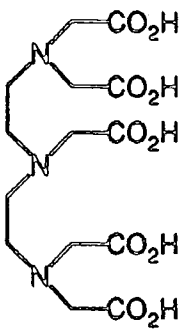
1.4.8 Chemical Properties of Yttrium(III)

As a result of the lanthanide contraction, yttrium(III) has similar ionic radii (8 and 9 coordinate) and hence displays a similar complexation chemistry to the trivalent lanthanides, particularly those in the middle of the series such as gadolinium(III), terbium(III) and holmium(III)[19]. It is a spherical ion, radius (9 coordinate) = 1.07Å [20] (*cf.* 9 coordinate ionic radius of holmium(III), also 1.07Å)[20], of low polarisability, which tends to form non-directional and electrostatic bonds with hard donors, *eg.* O and N. Its relatively large radius allows for large coordination numbers, typically 8-9, and its complexes show a preference for square antiprismatic and dodecahedral geometries (Figure 1.8).

Figure 1.8: *The favoured i) square antiprismatic and ii) dodecahedral stereochemistries of yttrium(III).*

1.4.9 Acyclic Bifunctional Complexing Agents for Yttrium(III)

DTPA (2), with eight donor atoms (5 oxygens, 3 nitrogens) and the ability to adopt a dodecahedral geometry, forms a very thermodynamically stable complex with yttrium(III) (log K_{ML} = 22.1)[21].



(2)

The origin of this stability lies also in the *chelate effect*[19], a phenomenon best explained by considering what happens when one molecule of DTPA complexes Y^{3+} (which will probably have a solvation number of 9) in aqueous solution:

$$DTPA^{5-} + Y(H_2O)_9^{3+} \rightleftharpoons Y(DTPA)^{2-} + 9H_2O \qquad \log K_{ML} = 22.1$$

and by recalling that:

$$\Delta G = -RT \log K_{ML} = \Delta H - T\Delta S$$

Thus one molecule of DTPA displaces nine water molecules resulting in a net increase in entropy such that ΔG becomes more negative, and $\log K_{ML}$ becomes more positive. Another way of looking at this would be to think of a second binding site (and third and fourth *etc*) being brought into closer proximity with the metal ion by the initial complexation between metal ion and ligand than if it were a separate entity in solution.

Because of this striking thermodynamic stability, and also the kinetic stability of the Y(DTPA) complex in serum, several workers have employed DTPA conjugated to an antibody *via* its dianhydride derivative (Figure 1.9) as a complexing agent for $^{90}Y^{3+}$ for use in RIT[14-15,22-24].

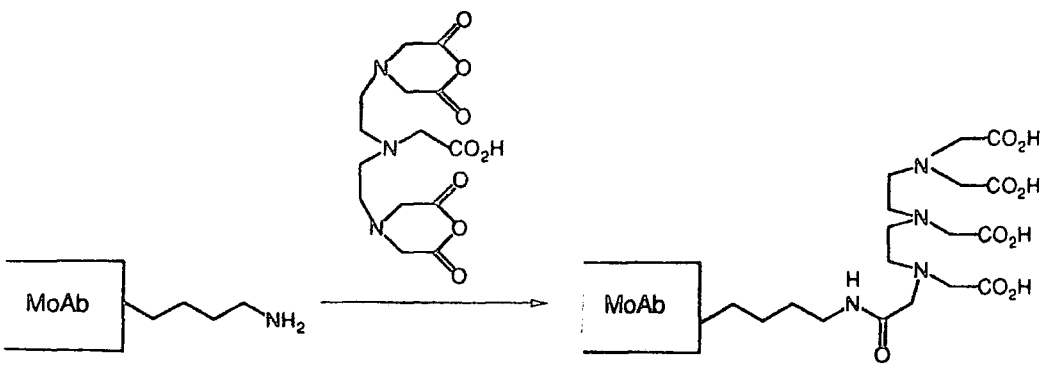


Figure 1.9: *The DTPA dianhydride derivative and its conjugation to a monoclonal antibody.*

However, radiolabelled monoclonal antibody (MoAb) conjugates of acyclic polyaminocarboxylate complexing agents are unstable *in vivo* for two reasons. Firstly, there is competition for the radionuclide from other complexing agents such as the blood proteins transferrin and haemoglobin[18]. Secondly, there is a small amount of acid-promoted (and possibly metal ion promoted - see Chapter 3) dissociation of the radionuclide from the chelate, the chelate-radionuclide complex being sufficiently flexible to allow, for example, the attack of a proton at one of the binding nitrogens[25]. Since the pH of serum is 7.4 and that of the body can be as low as 1.0, it is unsurprising that studies conducted in the former medium have failed to identify an acid-dependent dissociative mechanism. So, although the kinetic stability of the radiolabelled MoAb conjugate in serum is a prerequisite for its use in RIT, it is not an adequate assessment of its kinetic stability *in vivo*.

Sites left vacant by these leakage effects will almost certainly *not* be reoccupied by the free radionuclide, but will instead take up other metal ions such as Zn^{2+}, Ca^{2+} or Mg^{2+}, all present in significant concentrations in the blood (10^{-5}, 10^{-3} and 10^{-3} M respectively)[26]. A potentially lethal distribution of radionuclide results. Free Y^{3+}, for

instance, localises in the bone marrow (Section 4.8) where it can cause myelosuppression (depletion of the immune cell population).

Clearly for RIT to be successful, there is a need for a bifunctional complexing agent which binds $^{90}Y^{3+}$ quantitatively to form a thermodynamically *and* a kinetically stable complex. The other limiting factor is that complexation should be fast ($\leq$ 2 hours) between pH 4-6 at $37^{o}C$.

1.4.10 Macrocyclic Complexing Agents and the Macrocyclic Effect

Macrocycles, organic heterocycles of ring size $\geq$ 9 atoms, form complexes with metal ions of even greater thermodynamic stability than those formed by acyclic complexing agents such as EDTA and DTPA[27]. Examples of the various macrocyclic classes are shown in Figure 1.10.

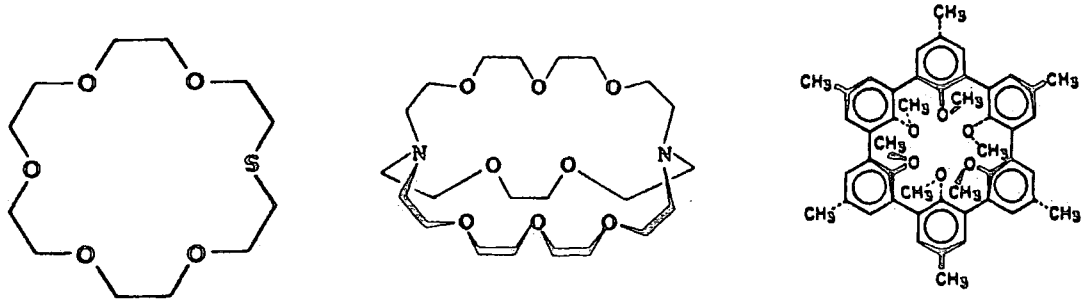
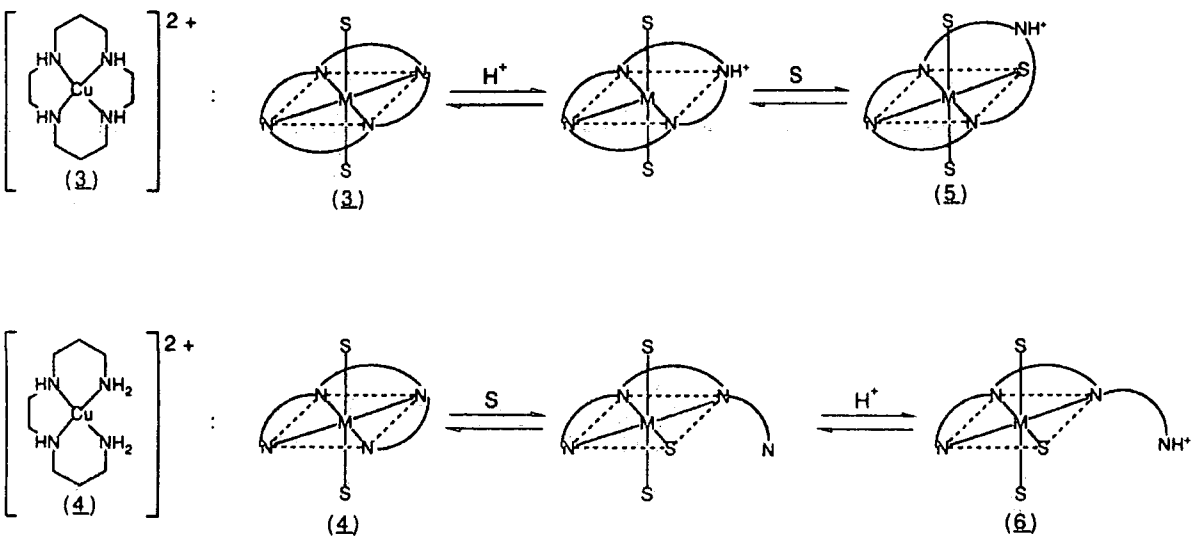


Figure 1.10: *Examples of some macrocyclic compounds: i) coronand; ii) cryptand; iii) spherand.*

Although enthalpy plays a part, it is likely that, for monomacrocyclics at least, the origin of this so-called *macrocyclic effect*[19] is mostly entropic: whereas an acyclic complexing agent must undergo considerable

conformational change to form a stable complex with the metal ion, the macrocyclic ring must undergo relatively few - it is said to be *pre-organised*.

Another ramification of the macrocyclic effect is the enhanced kinetic stability of the macrocyclic complex relative to its acyclic counterpart. Consider, for instance, the dissociation of $Cu(cyclam)^{2+}$ (3) and its acyclic analogue, $Cu(3,2,3\text{-tet})^{2+}$ (4) in strongly acidic media (Scheme 1.1)[28]. In both cases dissociation proceeds *via* the breakage of a Cu-N bond to give the intermediates (5) and (6). But the rate at which the Cu-N bond is broken in $Cu(cyclam)^{2+}$ is much slower than the rate at which the Cu-N bond is broken in $Cu(3,2,3\text{-tet})^{2+}$. The latter breakage is effected by the displacement of a nitrogen donor by a solvent molecule S. The donor subsequently swings out of the primary coordination sphere of the Cu^{2+} ion and becomes protonated to give the intermediate (6), which is further stabilised by solvation of the NH^+ group.



Scheme 1.1: *Breakage of the first C-N bond during the dissociation of $Cu(cyclam)^{2+}$ (3) and $Cu(3,2,3\text{-tet})^{2+}$ (4) in strongly acidic media.*

The $Cu(cyclam)^{2+}$ intermediate (5) is produced by the protonation of a nitrogen donor and the occupation of the vacant coordination site by a solvent molecule. However, by the twisting and folding of the ring, the leaving group is held in the primary coordination sphere, hindering the coordination of the solvent molecule and also the solvation of the NH^{+} group. The formation of this intermediate is thus considerably slower than that of the $Cu(3,2,3\text{-tet})^{2+}$ intermediate (6). The enhanced kinetic stability of $Cu(cyclam)^{2+}$ is therefore a property of the macrocyclic ring.

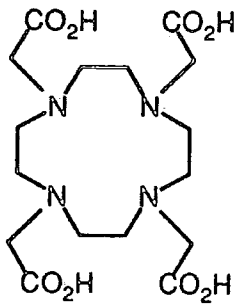
1.4.11 Macrocyclic Complexing Agents for Yttrium(III) and the Scope of this Work

Judicious choice of macrocycle can lead to a metal ion complex which is both thermodynamically and kinetically stable. With this in mind, the following macrocyclic ligands were proposed as yttrium(III) complexing agents (for their structures, see Figure 1.11): 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (7), 1,4,7,10-tetraazacyclotridecane-1,4,7,10-tetraacetic acid (TRITA) (8), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) (9), 4-(N-benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DOTA-BMA) (10), 1-oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid (ODOTRA) (11) and 1,7-dioxa-4,10,13-triazacyclopentadecane-4,10,13-triacetic acid (DTCTA) (12). As such, they possess the following virtues:

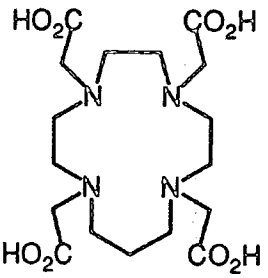
(a) A coordination number of eight(seven in the case of (11)).

(b) N and O hard donor sites.

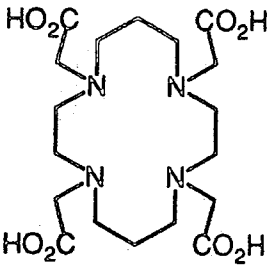
(c) At least three ligating carboxylate groups with which to reduce the charge density on Y^{3+}. In addition, DOTA-BMA (10)



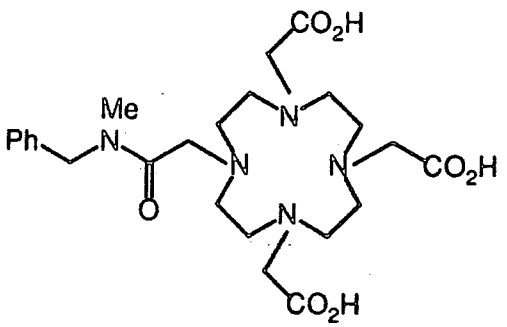
DOTA
(7)



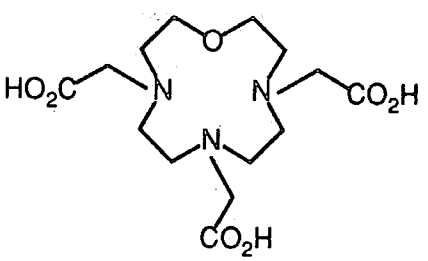
TRITA
(8)



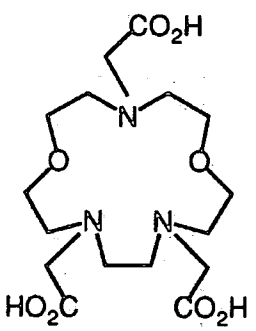
TETA
(9)



DOTA-BMA
(10)



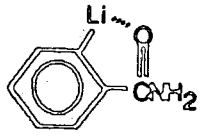
ODOTRA
(11)



DTCTA
(12)

Figure 1.11: *Macrocyclic candidates for complexing yttrium(III).*

has a neutral ligating amide arm. Amides are known to stabilise cations of high charge density such as Li^+ and Ca^{2+} (and Y^{3+} has a relatively high charge density), *eg.* in the ortho lithiation of benzamide:



Stabilisation is achieved principally through an electrostatic interaction, amides having appreciable ground state dipole moments (3.70 - 3.85 debye)[29,30].

The aims of this research were to synthesize each of the above macrocyclic ligands and to gauge, where possible, the thermodynamic stability of their yttrium(III) complexes, and their rate of uptake of Y^{3+} in aqueous solution, the latter with the aid of ^1H NMR spectroscopy and HPLC radiometry (Chapter 2). It was then undertaken to measure the rate of dissociation of Y(DTPA) as a function of pH and to attempt a comparison with the yttrium(III) macrocyclic complexes of promising candidates from ligands (7) - (12) (Chapter 3). The ligand which exhibited rapid Y^{3+} uptake and which in addition formed an yttrium(III) complex which was both thermodynamically and kinetically stable at low pH (0-3) would then be functionalised for linkage to a monoclonal antibody in order that the radiolabelled MoAb conjugate might be used in radioimmunotherapy (Chapter 4).

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CHAPTER TWO

SYNTHESIS OF THE MACROCYCLIC LIGANDS AND ASPECTS OF THE STABILITY, STEREOCHEMISTRY AND RATE OF FORMATION OF THEIR YTTRIUM(III) COMPLEXES

2.1 INTRODUCTION

The use of gadolinium complexes as contrast-enhancing agents for nuclear magnetic resonance imaging[1] has led to a boom in lanthanide macrocyclic chemistry[1-3]. Consequently, several of the ligands chosen for this work had considerable precedent.

DOTA, TRITA and TETA were first made in 1976 by Stetter and Frank[6]. Notably they found that DOTA forms the most thermodynamically stable calcium complex in aqueous solution known to date ($\log K_{ML} = 17.2$)[7]. Other workers have since shown that the $Ln(DOTA)^-$ complexes possess even larger stability constants [*eg.* $\log K = 24.6$ for $Gd(DOTA)^-$][8,9]. Also encouraging was the fast complexation kinetics (k_{obs} of the order of s^{-1}) observed for DOTA and TETA-alkaline earth systems[10].

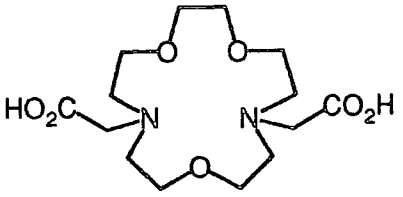
DOTA-BMA and ODOTRA can be thought of as being derived from DOTA by replacing an N-acetate group with an amide arm and an ether linkage respectively. The loss of a charged donor to a neutral one should give a neutral yttrium(III) complex - the yttrium(III) complexes of DOTA, TRITA and TETA on the other hand all bear a single negative charge. Being neutral should make the complex less sensitive to acid-promoted

---

[1]Nuclear magnetic resonance imaging (Ref.4) is a technique used to locate tumours as opposed to destroying them (therapy). It relies on the difference in the values of T_1 (spin-lattice relaxation time) and T_2 (spin-spin relaxation time) of the water protons in normal and malignant tissue. If a uniform magnetic field is applied across a section of tissue *eg.* in the leg, all the water protons in that section will resonate at one particular frequency. If, however, a gradient field is applied across the section, water protons at different points along the gradient will resonate at different frequencies and a correlation can be established between proton frequency and distance along the gradient. Thus the position of tumour-associated water protons, and therefore the tumours themselves, can be picked out. Paramagnetic contrast-enhancing agents (Ref.5) shorten the T_1 and T_2 times of water protons which in turn affects signal intensity. If the contrast-enhancing agents can be directed to the site of a tumour, by linking them to antibodies for instance, they will increase the difference between the signal intensities of normal tissue and those of malignant tissue and the position of the tumour will therefore become more distinct.

dissociation and therefore kinetically more stable.

Although there was no precedent for DTCTA, the stability constants of the lanthanide complexes of a macrocyclic ligand similar to it, (13), had been reported[11].



(13)

The macrocyclic ligand (13) exhibited a selectivity for lanthanides in the middle of the series (Figure 2.1) which was ascribed to the match between the size and charge density of the metal ion. Incorporating a third carboxylate group into a similar 15-membered ring, it was reasoned, could only improve this match.

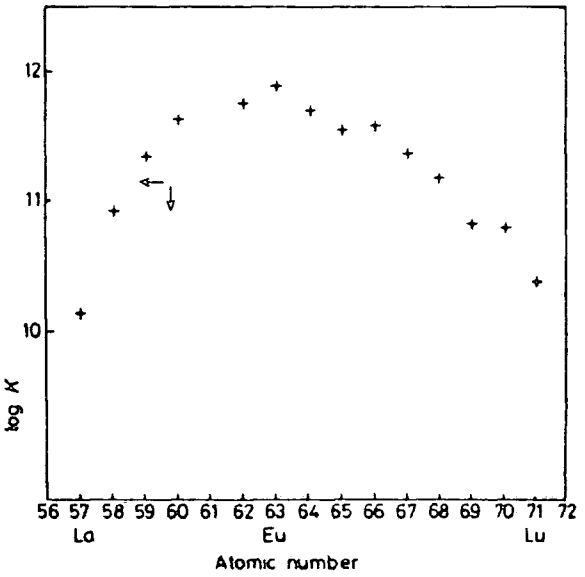


Figure 2.1: *Logarithmic stability constants for the lanthanide complexes of (13) (Ref.11).*

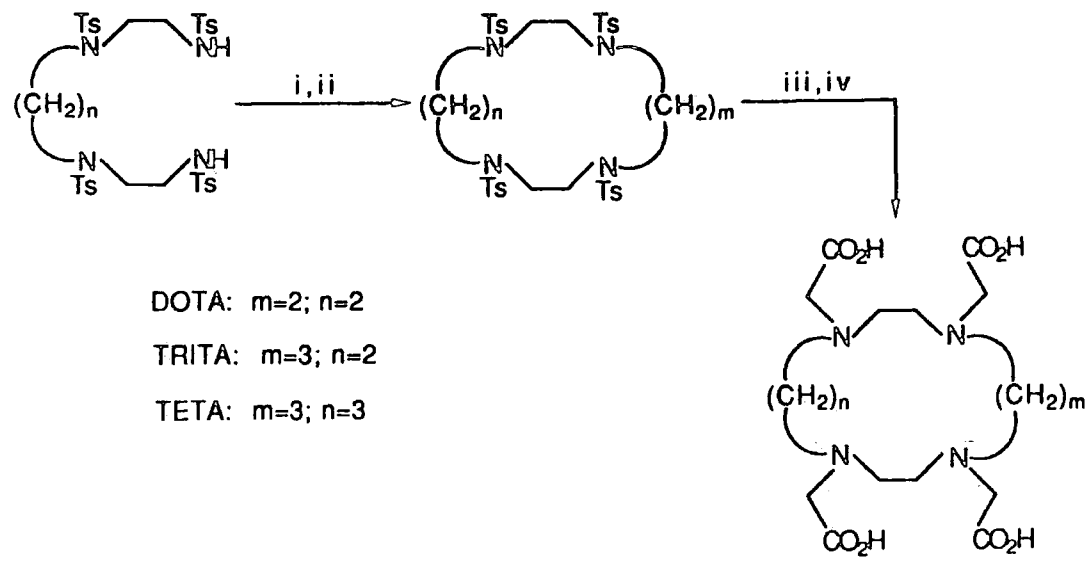
Like ODOTRA and DOTA-BMA, DTCTA should form a neutral complex with Y^{3+}.

After outlining the syntheses of the ligands, the emphasis of this chapter is placed on the thermodynamic stability and the rate of formation of their yttrium complexes. Kinetic stability is discussed in Chapter 3.

2.2 SYNTHESIS

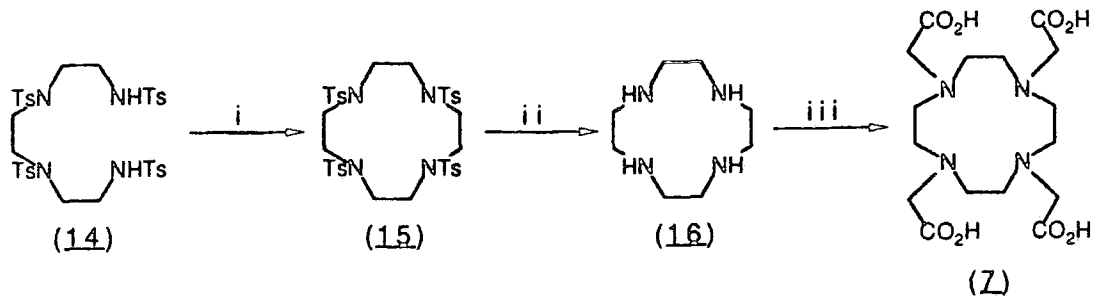
2.2.1 Synthesis of the Tetraaza Tetracarboxylic Acid Macrocycles

The general synthesis of the tetraaza tetracarboxylic acid macrocycles as used by Stetter and Frank[6] is given in Scheme 2.1.



Scheme 2.1: *Reagents: i, NaOEt; ii, $TsO(CH_2)_mOTs$, DMF; iii, H_2SO_4 or HBr, CH_3CO_2H, PhOH; iv, $ClCH_2CO_2H$, NaOH.*

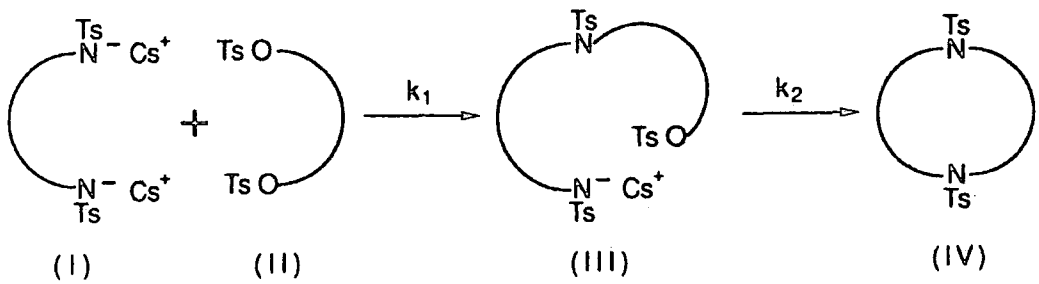
In the synthesis of DOTA, our own work employed two modifications to this route. The first was to use caesium carbonate/DMF in the cyclisation step and the second was to use a dissolving metal reduction to deprotect the 12N4 ring (Scheme 2.2).



(14) (15) (16) (7)

Scheme 2.2: *Reagents: i, $TsO(CH_2)_2OTs$, Cs_2CO_3, DMF; ii, Li, NH_3, EtOH, THF; iii, $ClCH_2CO_2H$, NaOH.*

The caesium carbonate reaction[12,13] has two advantages over the sodium ethoxide cyclisation method: it can be applied to the synthesis of large rings (up to 28-membered) with high yields and it is not messy. The reaction is carried out in a single vessel in DMF in which caesium carbonate forms a suspension. One equivalent of caesium carbonate is required per tosylamide to be deprotonated - HCO_3^- is not a strong enough base to deprotonate the second tosylamide either cleanly or at a reasonable rate. As a dipolar aprotic solvent with good cation and poor anion solvating abilities, DMF promotes S_N2 reactions. Two S_N2 displacements are involved in this particular cyclisation (Figure 2.2).



(I) (II) (III) (IV)

Figure 2.2: *The caesium carbonate reaction.*

The effectiveness of caesium carbonate in mediating these displacements is a result of the caesium ion being highly solvated in DMF. This leaves two reactive species in solution, the doubly deprotonated tosylamide (I) and the singly deprotonated tosylamide (III). As a dinucleophile, (I) will be approximately twice as reactive as (III), a mononucleophile. Thus (II) will prefer to react with (I), *ie.* $k_1 > k_2$, and oligomerisation, the major side reaction in cyclisations, will be minimised. Other alkali metal carbonate salts are less effective in promoting cyclisation, implying that their cations are less well solvated by DMF.

Reagents used for the dissolving metal reduction were lithium in ammonia with ethanol as the proton source and THF as the co-solvent. This procedure was adopted because it gave 1,4,7,10-tetraazacyclododecane, cyclen (16), as a white crystalline solid, corresponding to its tetrahydrochloride monohydrate salt, in good yield (72%) while avoiding the laborious work-up of the concentrated sulphuric acid method. Also the yields obtained using the H_2SO_4 method (<20%) were never as high as reported (>90%)[14] and inconsistent.

Alkylation of cyclen was achieved with chloroacetic acid. During the course of this reaction it was important to maintain the pH between 9 and 10 with periodic addition of sodium hydroxide in order to neutralise the HCl generated and ensure that the nitrogen lone pairs were unprotonated and free to act as nucleophiles.

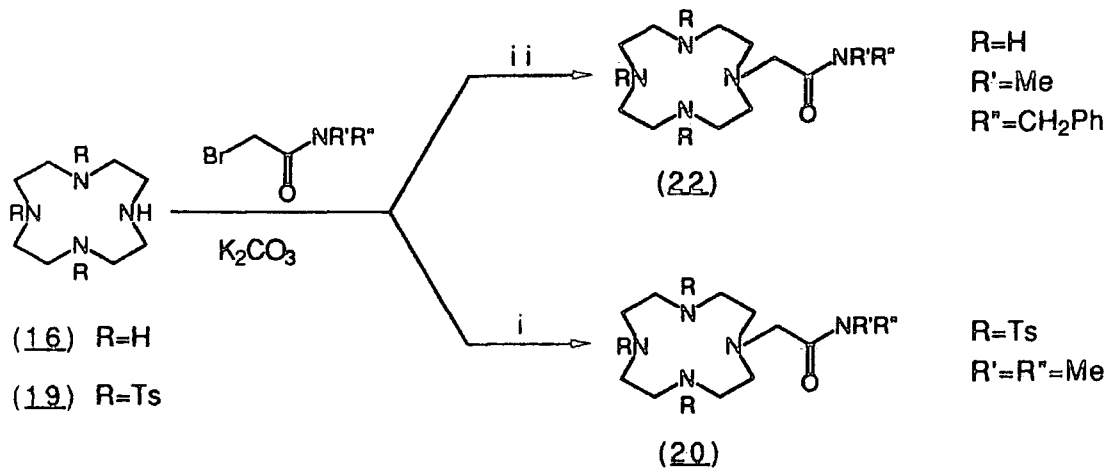
TRITA was synthesized according to Scheme 2.1, substituting the caesium carbonate method for the sodium ethoxide method in step i. Hydrobromic acid, glacial acetic acid and phenol were used to reductively deprotect the tosylated ring since the resultant amine was easily isolated as its 4HBr salt.

As the 14-membered tetraamine, cyclam, was commercially available, the synthesis of TETA was effected according to literature precedent[6].

2.2.2 Synthesis of DOTA-BMA

The strategy adopted to synthesize DOTA-BMA was to first introduce the pendent amide arm onto the 12N4 ring by a suitable monoalkylation reaction and *then* to attach the three carboxylic acid groups.

Of the several methodologies available[15] for monoalkylating polyazamacrocycles, two were attempted: alkylation of tri-protected cyclen [Scheme 2.3(i)] and selective alkylation of cyclen [Scheme 2.3(ii)].

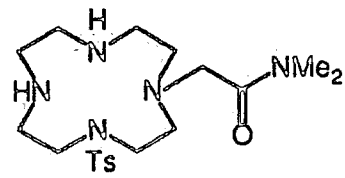


Scheme 2.3

Alkylation of Tri-protected Cyclen

Perversely, the tri-protected cyclen was obtained by a selective alkylation. This involved reacting cyclen with two equivalents of tosyl chloride in the presence of triethylamine and separating the ditosylated and tritosylated derivatives by column chromatography. After reacting the tritosylated cycle (19) with 1-bromo-N,N-dimethylethanamide, deprotection was attempted with a hydrobromic acid, glacial acetic acid and phenol mixture. After two weeks, the monotosylated derivative (21) was isolated (as confirmed by CI mass spectroscopy and ^{1}H NMR) as its

HBr salt.



(21)

Prolonged treatment under the same conditions failed to remove the tosylate group. Lithium, ammonia and ethanol may have been successful, but competitive tertiary amide reduction was deemed likely to occur.

Selective Alkylation

By choosing an alkylating agent with a UV active chromophore, the alkylation reaction could be monitored by analytical cation exchange HPLC. The alkylating agent in this case was 2-bromo-N-benzyl-N-methyl-ethanamide. Two peaks developed over 24 h. (Figure 2.3). Separation of these peaks by semi-preparative cation exchange HPLC and analysis by ^{1}H NMR and DCI mass spectroscopy indicated that the first ($\underline{R}_t$ 7.6 min) was the dialkylated cyclen derivative and that the second ($\underline{R}_t$ 8.1 min) was the monoalkylated derivative (20).

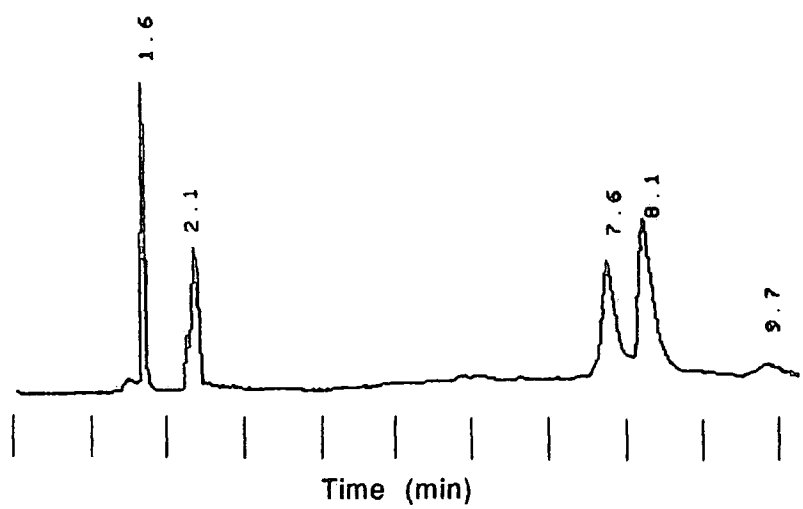
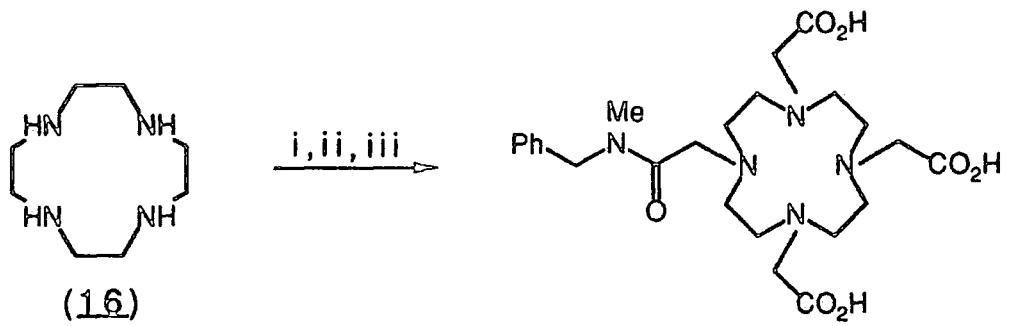


Figure 2.3: *HPLC trace showing appearance of mono- and dialkylated products (at 8.1 and 7.6 min respectively) in the treatment of cyclen with 2-bromo-N-benzyl-N-methylethanamide.*

The carboxylic acid groups were *not* introduced at this stage because the conditions of attachment ($ClCH_2CO_2H/NaOH$) would have resulted in alkaline hydrolysis of the amide group. Instead, ester functionalities were attached using ethyl bromoacetate in DMF in the presence of potassium carbonate. After purification, the ester groups were selectively hydrolysed under mildly basic conditions to give DOTA-BMA (Scheme 2.4).



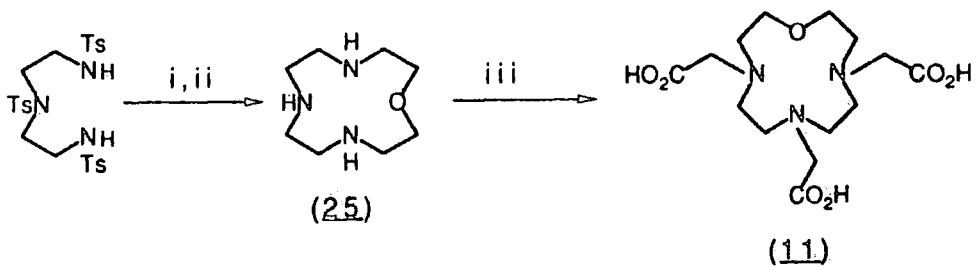
(16)

DOTA-BMA
(10)

Scheme 2.4: *Reagents: i, $BrCH_2CONMeBz$, K_2CO_3, DMF; ii, $BrCH_2CO_2Et$, K_2CO_3, DMF; iii, Me_4NOH, MeOH, H_2O.*

2.2.3 Synthesis of the Polyoxa Polyaza Tricarboxylic Acid Macrocycles

Scheme 2.5 outlines the synthesis of ODOTRA.



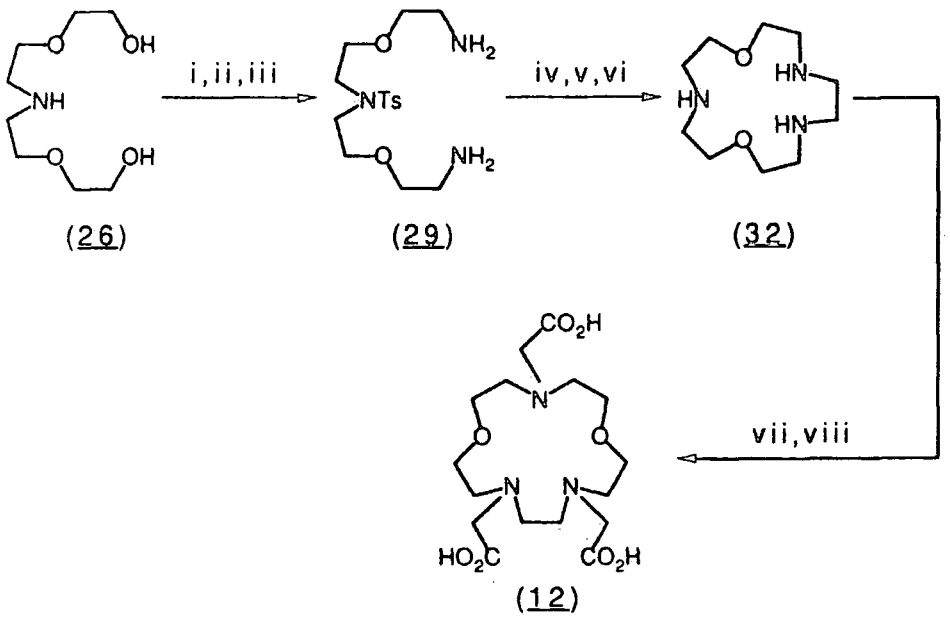
(25)

(11)

Scheme 2.5: *Reagents: i, $Ts(OCH_2CH_2)_2OTs$, Cs_2CO_3, DMF; ii, HBr, CH_3CO_2H, PhOH; iii, $ClCH_2CO_2H$, Me_4NOH.*

The only point of note here is the purification of the HBr salt of (25). The anion exchange resin used for this was converted to the OH^- form with a tetramethylammonium hydroxide solution as opposed to a sodium hydroxide or potassium hydroxide solution, thereby avoiding the possibility of complexation of Na^+ or K^+ by the liberated free amine. For the same reason, tetramethylammonium hydroxide was used as the base in the subsequent alkylation reaction.

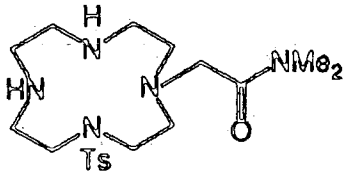
The synthesis of DTCTA (Scheme 2.6) began with the tosylation of the known diol (26)[16] followed by a Gabriel synthesis to convert the two -OTs groups into primary amines.



Scheme 2.6: *Reagents: i, TsCl, pyridine; ii, potassium phthalimide, DMF; iii, N_2H_4, HCl; iv, TsCl, K_2CO_3, H_2O; v, $TsOCH_2CH_2OTs$, Cs_2CO_3, DMF; vi, HBr, CH_3CO_2H, PhOH; vii, $BrCH_2CO_2Bz$, Cs_2CO_3, DMF; viii, HCl.*

The second tosylation, step iv, was found to give better yields (60% vs. <50%) using potassium carbonate rather than pyridine as the base. Steps v and vi were as previously outlined. Caesium carbonate was used as the base in the alkylation reaction, step vii, to avoid any metal ion

HBr salt.



(21)

Prolonged treatment under the same conditions failed to remove the tosylate group. Lithium, ammonia and ethanol may have been successful, but competitive tertiary amide reduction was deemed likely to occur.

Selective Alkylation

By choosing an alkylating agent with a UV active chromophore, the alkylation reaction could be monitored by analytical cation exchange HPLC. The alkylating agent in this case was 2-bromo-N-benzyl-N-methylethanamide. Two peaks developed over 24 h. (Figure 2.3). Separation of these peaks by semi-preparative cation exchange HPLC and analysis by ^{1}H NMR and DCI mass spectroscopy indicated that the first (R_t 7.6 min) was the dialkylated cyclen derivative and that the second (R_t 8.1 min) was the monoalkylated derivative (20).

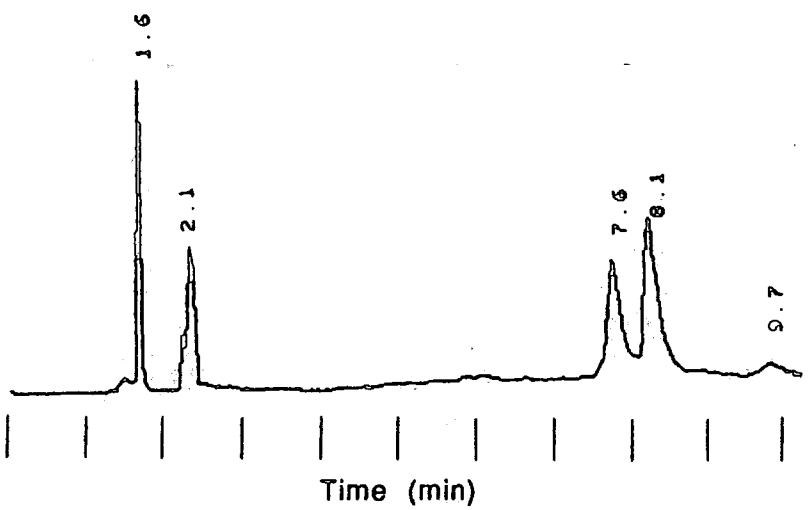
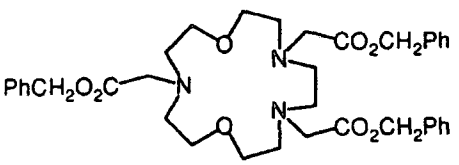


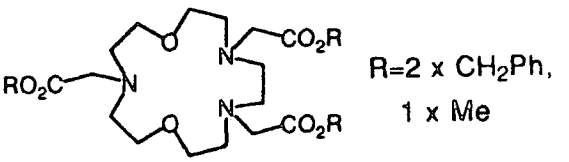
Figure 2.3: *HPLC trace showing appearance of mono- and dialkylated products (at 8.1 and 7.6 min respectively) in the treatment of cyclen with 2-bromo-N-benzyl-N-methylethanamide.*

complexation by the ring, the caesium ion being too large (6-coordinate ionic radius = 1.67Å) to be incorporated into its internal cavity (diameter approximately 1.70 - 2.20Å)[17]. The reaction was monitored by analytical cation exchange HPLC, the benzyl group of 2-bromobenzyl acetate providing a convenient UV chromophore.

During the course of the alkylation, methanol was adventitiously introduced into the reaction mixture. Semi-preparative cation exchange HPLC succeeded in isolating two products: (a) the tribenzyl ester (33) and (b) a dibenzyl methyl ester (34), as characterised by DCI mass spectroscopy and ^{1}H NMR. Presumably (34) had arisen from some methanolic transesterification of (33).



(33)



(34)

It was not possible to specify the precise substitution pattern of (34).

The tribenzyl ester (33) appeared to be unstable even when preserved under a nitrogen atmosphere, changing from a colourless to an orange oil. Acid hydrolysis, as adduced by ^{1}H NMR, did not give clean product. Curiously, acid hydrolysis of the mixed ester (34), by the same criterion, did.

2.3 STABILITY CONSTANTS OF THE TETRAAZA TETRACARBOXYLIC ACID MACROCYCLIC COMPLEXES OF YTTRIUM (III)

The stability constants of the 1:1 yttrium (III) complexes of DOTA, TRITA and TETA were measured[2] by slow (up to 48h. in the case of DOTA) potentiometric titrations.

There are two experimental details worthy of note. First, a potentiometric titration with the pre-formed $Y(DOTA)^-$ complex allowed calculation of the protonation constant for the latter, $pK_a = 3.08$. Second, tetramethylammonium nitrate as opposed to potassium nitrate or sodium chloride was used to keep the ionic strength constant during the titrations. DOTA in particular has been shown to form stable complexes with K^+ ($\log K_{ML} = 1.6$) and Na^+ ($\log K_{ML} = 4.4$)[7]. [The $Ln(DOTA)^-$ stability constants measured by several groups[8,9] must therefore be treated with some suspicion].

The titration data were analysed by the computer program SUPERQUAD[18]. This is able to minimise the errors encountered during a titration, *eg.* from electrode readings and ligand impurities, by a non-linear least squares treatment. The stability constants obtained by this method are given in Table 2.1 together with the stability constants of $Y(DTPA)^{2-}$ and $Y(EDTA)^-$ for comparison.

SPECIES	DOTA[a]	TRITA[a]	TETA[a]	DTPA[19]	EDTA[19]
YL	24.9	19.6	16.3	22.1[b]	18.1[b]

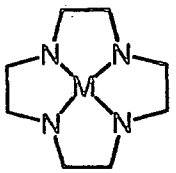
Table 2.1: *Stability constants (log K) of the yttrium(III) complexes of DOTA, TRITA, TETA, DTPA and EDTA; a) T=298±0.1K, I=0.1M [$(CH_3)_4NNO_3$]; b) T=298K, I=0.1M [KNO_3].*

Therefore of these five complexes, $Y(DOTA)^-$ is the most stable by almost

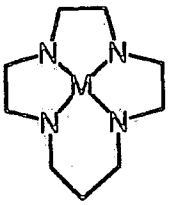
[2]by Ritu Kataky.

three orders of magnitude.

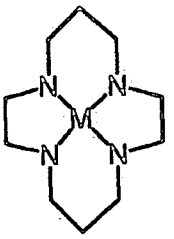
Within the tetraaza tetracarboxylic acid macrocyclic series, the trend in stability is $Y(DOTA)^-$ > $Y(TRITA)^-$ > $Y(TETA)^-$. Factors influencing this trend might be the steric crowding and torsional strain within the metal chelate rings of the complexes:



DOTA



TRITA



TETA

It is known that the propylenediamine groups of 6-membered chelates are more sterically crowded (*ie.* there are more unfavourable eclipsing interactions) than the ethylenediamine groups of 5-membered chelates[9]. Since steric crowding results in a loss of stability, $Y(TETA)^-$, with two 6-membered and two 5-membered chelates, will be less stable than $Y(TRITA)^-$ with three 5-membered and one 6-membered chelate, which in turn will be less stable than $Y(DOTA)^-$ with four 5-membered chelates. Furthermore, the torsional strain imposed on the 5-membered ethylenediamine chelate ring is less than that imposed on the 6-membered propylenediamine chelate ring for the chelation of a relatively large metal ion such as yttrium (III), *ie.* the "bite" of the ethylenediamine unit is better suited to the ionic radius of yttrium (here 8-9 co-ordinate) than is the "bite" of the propylenediamine unit (the situation is reversed with relatively small ions such as Li^+ and Be^{2+})[20].

Another factor influencing the trend in stabilities is molecular geometry, in which the conformation of the macrocyclic ring plays a vital part. Macrocyclic conformation is usually described in terms of

the number of bonds between corners in a ring[21]. To define a corner it is necessary to introduce the concepts of the gauche bond, the anti bond and the torsional angle. The torsional angle, ϕ, is the angle between the neighbouring bonds which propagate the hydrocarbon chain in the macrocyclic ring (Figure 2.4).

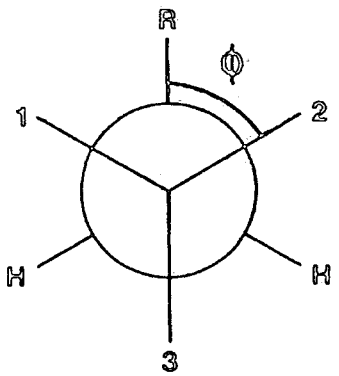
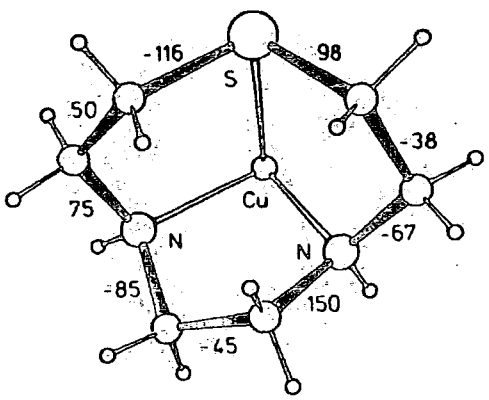


Figure 2.4: *Newman projection depicting the torsional angle ϕ for a macrocyclic ring.*

Gauche bonds are defined as those bonds with $|\phi| < 90^0$. Thus anti bonds are those with $|\phi| > 90^0$. Corners occur (a) at the junction of two gauche bonds, (b) at the junction of an isolated gauche bond and the adjacent bond with the smaller $|\phi|$ and (c) where an isolated gauche bond has a two-fold axis. Thus the 9-membered N_2S macrocyclic ring adopts a [234] conformation in its Cu^{2+} complex[21], there being two, three and four bonds between its three corners:



[234]

From a crystal structure of the $Eu(DOTA)H_2O^-$ complex[22] and from NMR studies, our own (see Section 2.4) and those of Desreux[23], it is likely that the geometry of the donors in the $Y(DOTA)^-$ complex is square antiprismatic, with the four oxygen atoms of the carboxylate groups in one plane of the square antiprism, and the four nitrogen atoms of the ring in the other (Figure 2.5).

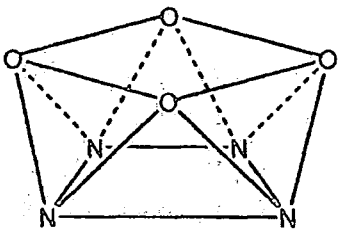
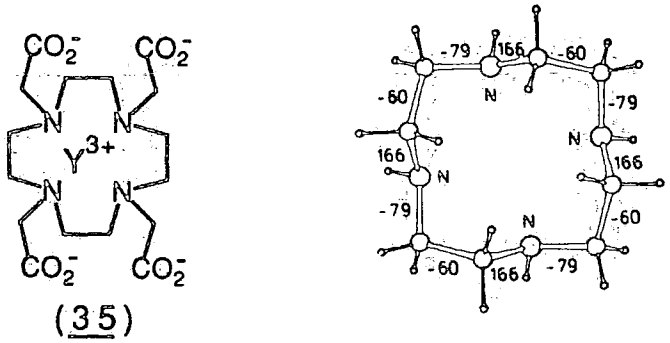


Figure 2.5: *Proposed square antiprismatic geometry of the $Y(DOTA)^-$ complex.*

The repulsion energy of the square antiprism is the smallest for any 8-membered polyhedron.

It is also likely that the 12N4 ring, both in solution and in the solid state, adopts a square quadrangular [3333] conformation. Of the two conformations a 12-membered ring may adopt (the other being a rectangular [2424] arrangement), this is the more stable. In this conformation the nitrogen atoms are directed towards the same side of the ring and the protons of the methylenic groups are fully staggered, *ie.* in their gauche forms (Figure 2.6).



(35)

Figure 2.6: *Proposed [3333] conformation of the DOTA macrocyclic ring in Y(DOTA).*

In the absence of Ln(TRITA)$^-$ crystal structures[3] and interpretable ^{1}H NMR data (see Section 2.4), it is not possible to say whether the arrangement of donors in the Y(TRITA)$^-$ complex is square antiprismatic or dodecahedral. However, from the little that is known about the 13N4 system it is probable that the macrocyclic ring exists in a [3334] conformation. If it does, the protons in the ethylene unit directly opposite the propylene unit will be eclipsed, leading to a less stable structure than that of the 12N4 system (Figure 2.7).

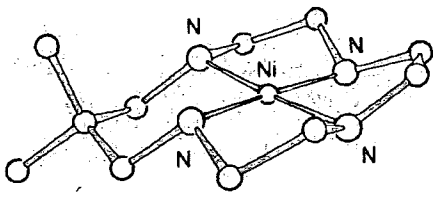


Figure 2.7: *[3334] conformation of the Ni(12,12-dimethyl-1,4,7,10-tetraazacyclotridecane)$^{2+}$ complex (Ref.21).*

The crystal structure[25] of Tb(TETA)$^-$ and ^{1}H and ^{13}C NMR studies of the lanthanide TETA complexes[26] suggest that the Y(TETA)$^-$ complex will

[3]Although a suitable crystal of Y(TRITA)$^-$ was obtained, the R factor of the complex was 29%, making distinction between ethylene and propylene units impossible (Ref.24).

be dodecahedral and the ring will assume a stable [3434] geometry. As implied above, the dodecahedral arrangement of donor atoms is less stable than the square antiprismatic. Figure 2.8 depicts this dodecahedral arrangement in the crystal structure of $Tb(TETA)^-$. Note that the four carboxylate oxygens are no longer co-planar, and nor are the four nitrogen atoms.

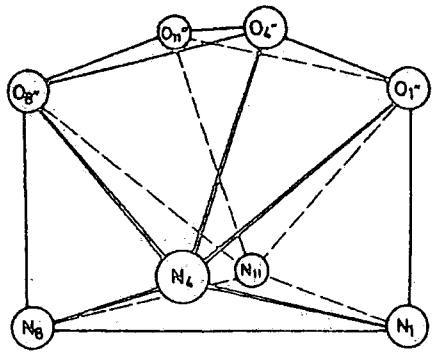


Figure 2.8: *Dodecahedral arrangement of donor atoms in the Tb(TETA)⁻ complex (Ref.26).*

Finally it is important to stress that the high thermodynamic stabilities of $Y(DOTA)^-$, $Y(TRITA)^-$ and $Y(TETA)^-$ are *not* a result of the macrocyclic effect. The cavities of these ligands are too small (radii < 0.75Å)[17,23] to accommodate Y^{3+}, which will therefore lie above the plane of the nitrogens. The importance of the macrocyclic ring lies more in its ability to act as a frame to constrain the nitrogens and carboxylate groups in a near spherical arrangement and to preorganise the nitrogen lone pairs for binding Y^{3+} such that they are all directed towards the centre of this arrangement. The preorganisation leads to a favourable entropy term in the free energy of complexation.

2.4 ^{1}H NMR COMPLEXATION EXPERIMENTS

The purpose of the ^{1}H NMR complexation experiments was two-fold: firstly to obtain a qualitative measure of the rate of uptake of Y^{3+} by the individual macrocyclic ligands in aqueous solution and secondly to infer the stereochemistries of the complexes from the ^{1}H NMR spectra.

Spectra (250 MHz) of the ligands and their yttrium(III) complexes (0.028 M) in D_2O buffered at pD 5.0 with deuterated sodium acetate (0.14 M) and acetic acid (0.06 M) at 293 K are shown in Figure 2.9.

DOTA, TRITA, ODOTRA and DTCTA[4] all formed 1:1 yttrium complexes (subsequently confirmed by FAB mass spectroscopy) in less than 10 minutes. Adding another equivalent of Y^{3+} and heating the solution to 50^{o}C for 1 hour produced no further change.

The $Y(TETA)^-$ spectrum did not appear to differ from the spectrum of TETA alone. This observation seems to contradict the stability constant measurement of log K $[Y(TETA)^-]$ = 16.3 (Section 2.3), *ie.* that the formation of the $Y(TETA)^-$ complex is thermodynamically favourable. But this value does not take into account either the protonation of TETA [at pH[5] 5.0 the significant species will be $(TETA)H^{3-}$][10] or the competition for Y^{3+} from other ligands, in this case a 10-fold excess of AcO^- with respect to both metal ion and ligand (*NB.* log K $[Y(AcO)^{2+}]$ = 1.68 and log K $[Y(AcO)_2^+]$ = 3.17)[19]. Both factors will lead to a reduction in the thermodynamic stability of $Y(TETA)^-$. Also, at pH 5.0, the $Y(TETA)^-$ complex could become protonated to form Y(TETA)H, the stability constant, log K_{MLH}, of which will be considerably lower than log K

---

[4]As the charge on a polyaminocarboxylate ligand and on its yttrium(III) complex depends on their degree of protonation, in turn depending on the pH, the convention in this and subsequent chapters has been to omit the charge, other than where it has been possible to be more precise.

[5]pH rather than pD is considered here, simply for clarity.

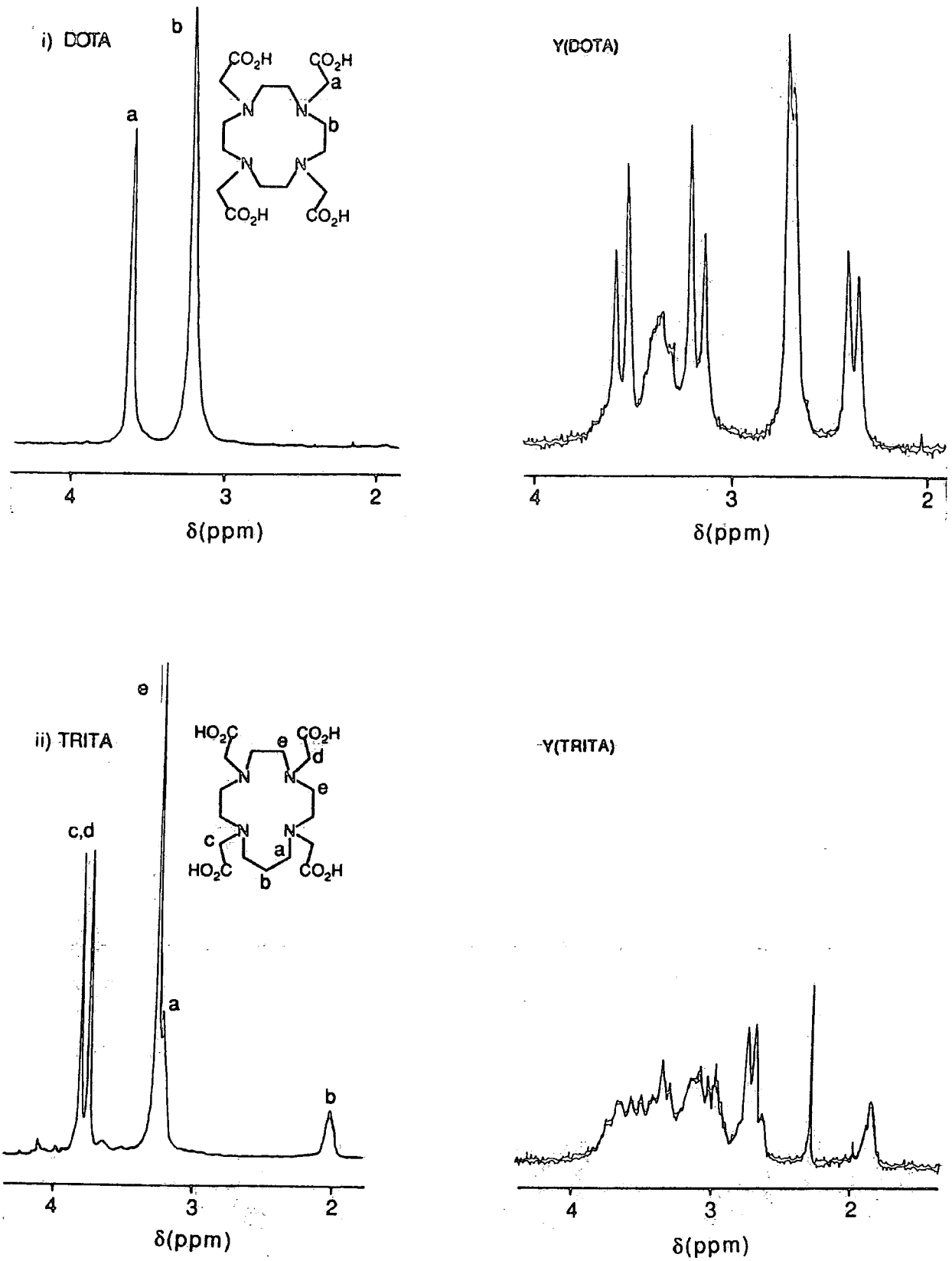


Figure 2.9: ^1H *NMR spectra (250 MHz) of the ligands (7)-(12) and their 1:1 complexes with* Y^{3+} *(D_2O, pD 5.0, 293K): i) DOTA and Y(DOTA); ii) TRITA and Y(TRITA).*

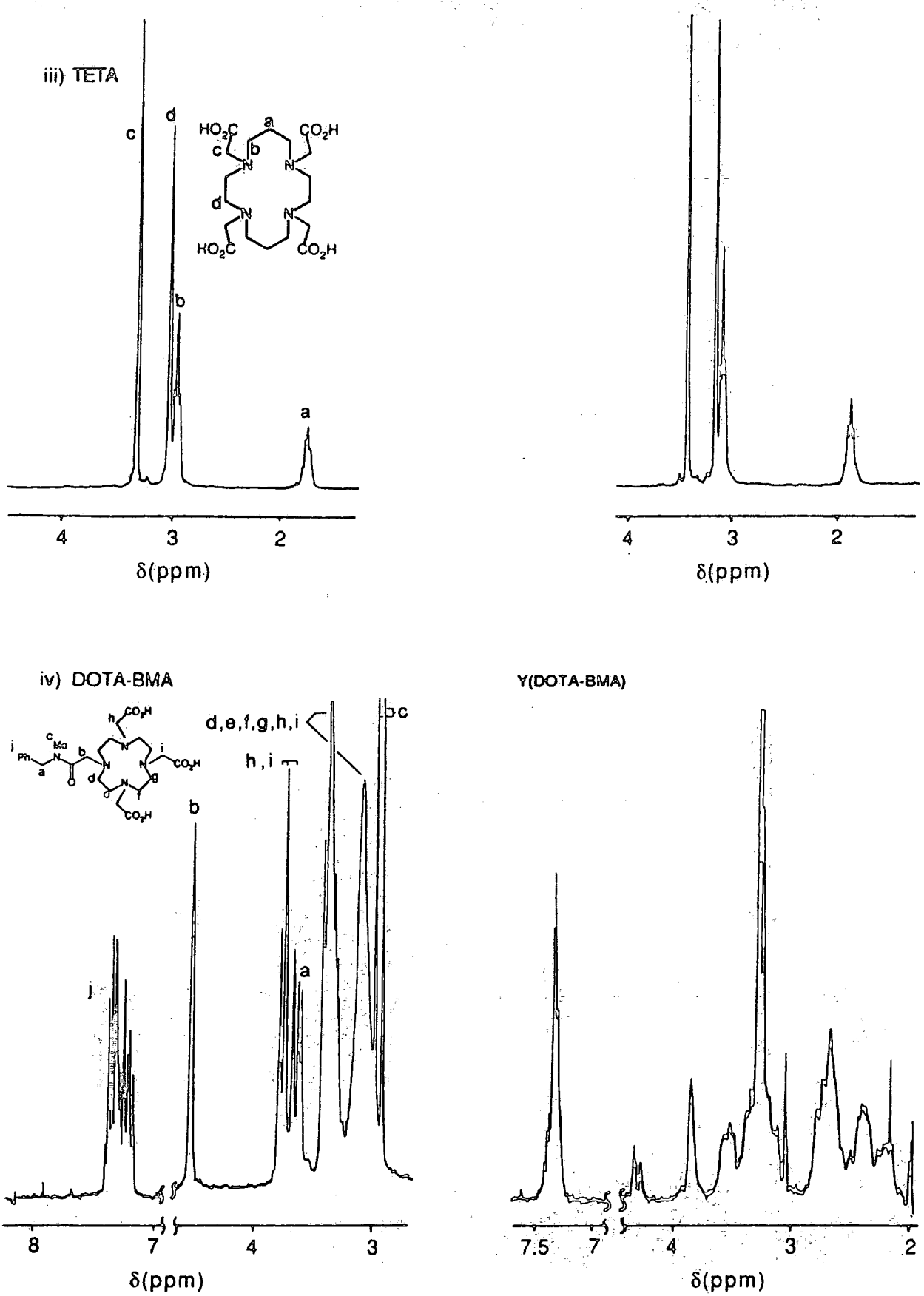


Figure 2.9: *iii) TETA and TETA in the presence of Y^{3+};*
(cont'd) *iv) DOTA-BMA and Y(DOTA-BMA).*

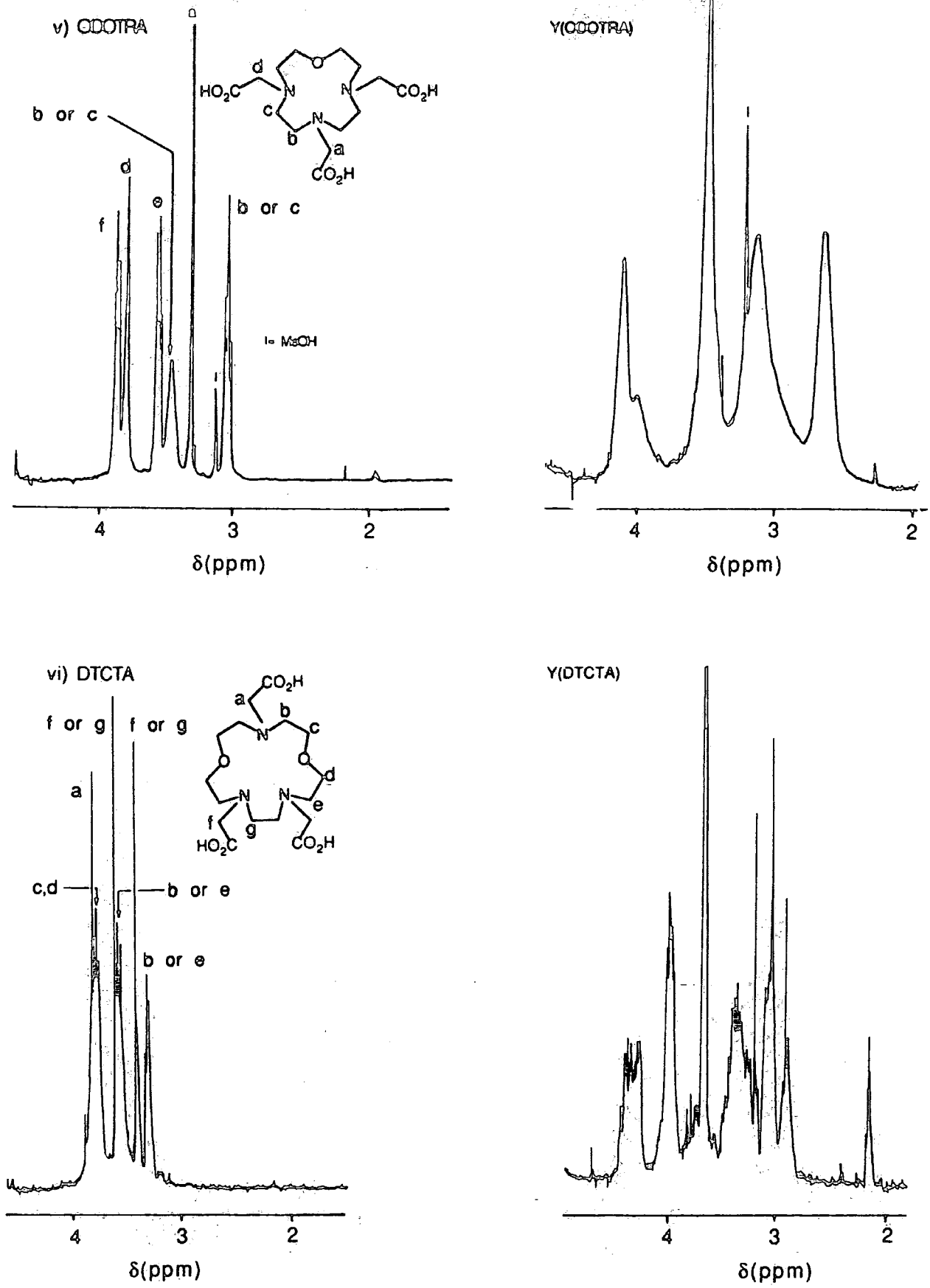


Figure 2.9: *v) ODOTRA and Y(ODOTRA); vi) DTCTA and Y(DTCTA)* **(cont'd)**

[Y(TETA)$^-$], probably less than 10.

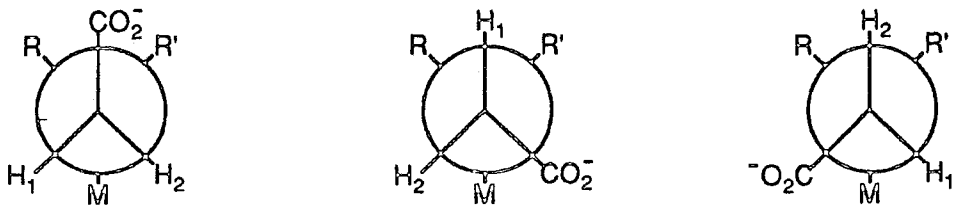
DOTA-BMA complexed Y^{3+} more slowly. After 1 week and heating to 50^oC for 1 hour, complexation was still not complete. Addition of another equivalent of Y^{3+} gave the 1:1 complex.

The complexity of the Y(TRITA), Y(DOTA-BMA) and Y(DTCTA) spectra precludes any detailed analysis. However, the sharpness of the latter two spectra indicates that at room temperature the DOTA-BMA and DTCTA rings are rigid. On the other hand, the broad Y(TRITA) spectrum suggests that the 13N4 ring is conformationally mobile at the same temperature. The validity of these comments will become apparent in the following discussion of the Y(DOTA) and Y(ODOTRA) spectra.

2.4.1 Y(DOTA)

The spectrum of Y(DOTA) [Figure 2.9(i) and Figure 2.11] shows a distinct AB splitting pattern to high frequency [(a) and (c), J_{AB} = 16 Hz], half of another AB system to low frequency [(e), J_{AB} = 14 Hz] and what are essentially two multiplets [(b) and (d)]. By analogy with Desreux's work on the diamagnetic lanthanide (La^{3+} and Lu^{3+}) complexes of DOTA[23], the AB system to high frequency can be assigned to the acetate CH_2 protons. They appear as an AB system according to the following rationale.

The acetate CH_2 protons of aminocarboxylate complexes can appear as a singlet or as an AB quartet. Which depends on the relative lifetimes of the metal-nitrogen and metal-oxygen bonds. If both are short a singlet is obtained. But if the lifetime of the metal-nitrogen bond is long relative to that of the metal-oxygen bond, an AB pattern is observed. Newman projections along the acetate CH_2-N bond with the carboxylate group rotating help to demonstrate this:



When R ≠ R', *eg.* in EDTA where R = an ethylene group and R' = H, then H_1 and H_2 are non-equivalent (diastereotopic) in all three orientations and an AB pattern arises. When R = R', as in NTA (R = $CH_2CO_2^-$), H_1 and H_2 are equivalent in all three orientations and a singlet results (Figure 2.10).

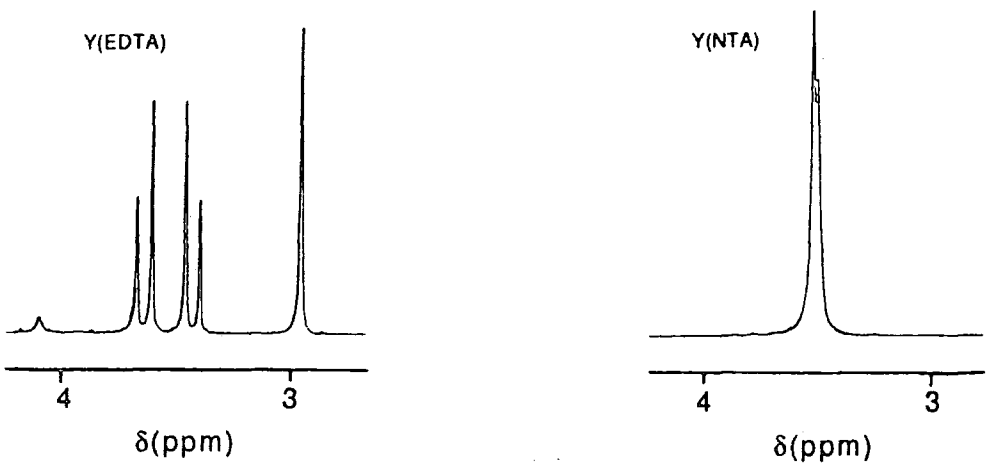


Figure 2.10: *^1H NMR spectra (250 MHz) of the 1:1 Y^{3+}:EDTA and NTA complexes.*

In DOTA, R = R' = an ethylene group, and therefore the acetate CH_2 protons should appear as a singlet. However, the square [3333] conformation of the ring means that although the four ethylene groups are in identical conformations, their disposition relative to each other on either side of a coordinating nitrogen atom will be different. Thus R ≠ R', the nitrogen atom is a stereogenic centre[28] and the acetate CH_2 protons will be diastereotopic, appearing as the AB quartet (a) and (c).

The protons at (b), (d) and (e) integrate in the ratio 4:8:4 respectively. Figure 2.11 shows the 12N4 ring in its [3333] conformation and the probable locations of these protons.

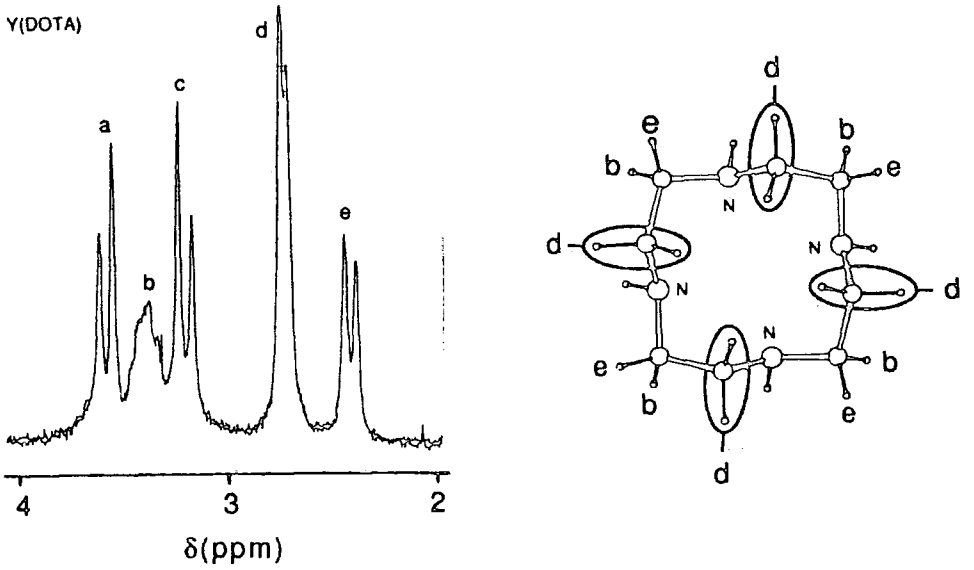
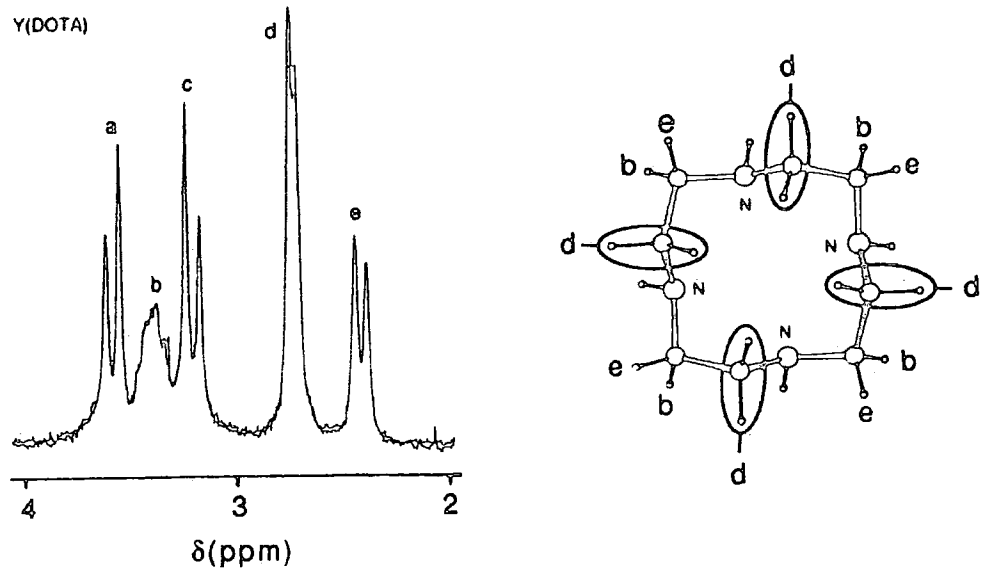


Figure 2.11: *Assignment of peaks in the ^1H NMR spectrum of Y(DOTA).*

The four protons (b) are assigned to those at the corners of the structure and directed into the plane of the paper because in these positions they will be near the carbonyls of the acetate groups and therefore subject to the carbonyl anisotropic deshielding effect.

The AB doublet at (e) is assigned to the four protons geminal to the (b) protons. These are directed out of the plane of the paper and as such are remote from the influence of the carbonyl groups. A spin-spin decoupling experiment indirectly confirmed this assignment. The decoupling power was applied to the multiplet at (d). Following irradiation, the protons at (b) collapsed to an AB doublet with the same coupling constant (for that particular spectrum), 13 Hz, as the AB doublet at (e):

The protons at (b), (d) and (e) integrate in the ratio 4:8:4 respectively. Figure 2.11 shows the 12N4 ring in its [3333] conformation and the probable locations of these protons.



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At room temperature, the energy barrier for interconversion of the ethylene groups in Y(DOTA) is obviously too high.

Thus invoking the preferential adoption of the [3333] 12N4 ring conformation permits an interpretation of the Y(DOTA) spectrum.

2.4.2 Y(ODOTRA)

Unlike Y(DOTA), the Y(ODOTRA) complex is conformationally mobile at room temperature [Figure 2.13(iv)]. Cooling down to 278K resulted in further broadening [Figure 2.13(i)-(iii)]. Because of the possibility of D_2O freezing, the behaviour of the complex below 278K was not investigated. Raising the temperature above 293K, to 333K, resulted in considerable sharpening and the appearance of several AA'BB' systems. The origin of these AA'BB' systems can be explained with reference to Figure 2.12. At high temperatures the ethylene groups rapidly interconvert between the two forms shown, with the result that the two positions which each proton can occupy become indistinguishable - an AA'BB' pattern arises.

Peaks (a)-(f) [Figure 2.13(vi)] integrate in the ratio of 2:2:6:6:2:4 respectively. Spin-spin decoupling experiments indicate that the peak at (c) corresponds to the three acetate CH_2 protons (*ie.* they are unaffected by any decoupling). Peaks (a) and (b) are coupled to (e) and two protons hidden under (d). The four protons corresponding to (f), probably NCH_2 protons, are coupled to the other four protons at (d). These relationships are summarised in Figure 2.14.

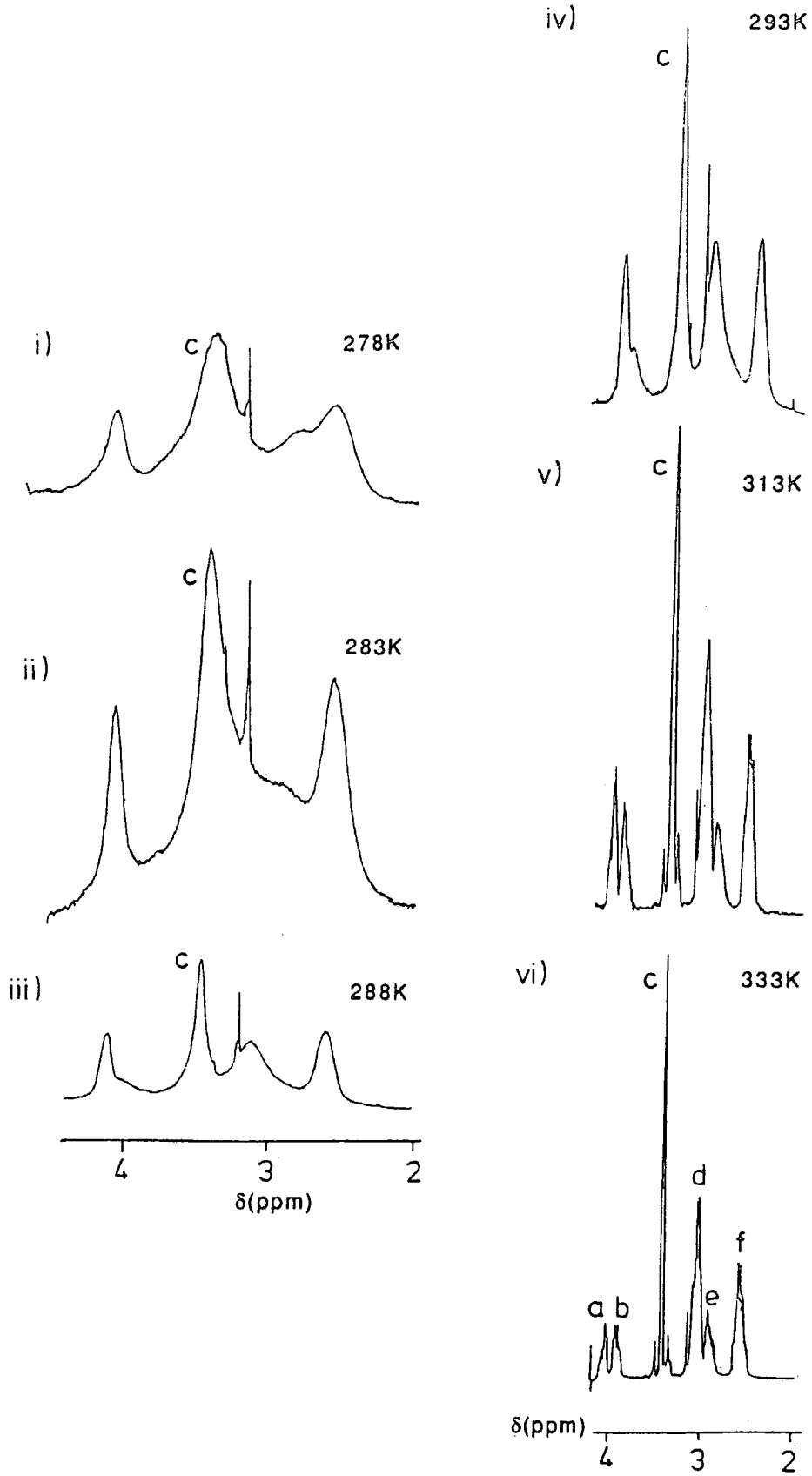

Figure 2.13: *^1H NMR spectra (250 MHz) of Y(ODOTRA) at various temperatures in D_2O (pD 5.0).*

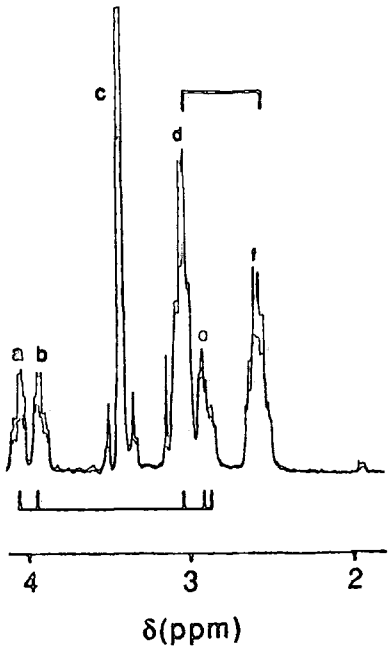

Figure 2.14: *^{1}H NMR spectrum (250 MHz) showing coupling within Y(ODOTRA) at 333K.*

At high temperatures, the ethylene groups of the lanthanum(III) and lutetium(III) complexes of DOTA also exhibit AA'BB' splitting patterns while the acetate CH_2 protons appear as a singlet. This suggests that if it were possible to lower the temperature to below 278K, the broad peaks of the Y(ODOTRA) spectrum would resolve into AB (acetate CH_2 protons) and ABCD (ethylenic protons) systems, as observed for the two lanthanide complexes. Arbitrarily taking 278 ± 5K as the coalescence temperature, T_c, of the Y(ODOTRA) ethylenic protons, and estimating $\Delta\nu$ to be 33 ± 2 Hz gives a free energy of activation of 58 ± 2 kJ mol^{-1}, according to the following equation:

$$\Delta G^{\ddagger} = RT_c(22.96 + \ln [T_c/\Delta\nu])$$

This figure is very close to the free energy of activation of the ethylenic protons of La(DOTA), $\Delta G^{\ddagger}$ = 61 ± 1 kJ mol^{-1}. Thus Y(ODOTRA) shows a similar low temperature rigidity to the lanthanide DOTA complexes.

2.5 RATE OF FORWARD BINDING AS ASSESSED BY HPLC RADIOMETRY[6]

The concentrations of macrocyclic ligand and Y^{3+} used in the ^{1}H NMR experiments above, 0.028 M, are much greater than those of monoclonal antibody conjugate (*ie.* the MoAb linked to a macrocycle) and $^{90}Y^{3+}$ which would be used in radioimmunotherapy: if administration of 20 mCi of ^{90}Y is required to destroy a tumour of, for example, 5g, and if the initial radiolabelling of the MoAb conjugate were effected in 1 ml of solution with a MoAb conjugate:$^{90}Y^{3+}$ ratio of 10:1, the concentrations of the latter would be 4×10^{-6} M and 4×10^{-7} M respectively (1 mCi of $^{90}Y \equiv 2 \times 10^{-11}$ mol). The concentrations of macrocyclic ligand and $^{90}Y^{3+}$ used in these semi-quantitative HPLC radiometric experiments, 1×10^{-3} - 5×10^{-6} M and (initially) $\approx 10^{-8}$ - 10^{-10} M respectively, were therefore more representative of clinical conditions. A 10:1 ratio of ligand : $^{90}Y^{3+}$ could not be realised under normal laboratory circumstances because of the safety hazard incurred in using activities of greater than 2 mCi.

In a typical experiment, the extent of complexation (labelling efficiency) was estimated by removing an aliquot of the reaction mixture, adding a solution of DTPA (the solution so formed being 5×10^{-3} M with respect to DTPA and 1×10^{-6} M with respect to macrocyclic ligand) to complex any free $^{90}Y^{3+}$, and analysing the relative amounts of ^{90}Y (macrocyclic ligand) complex and ^{90}Y(DTPA) by anion exchange HPLC (Hichrom AX 300 column coupled to a Beckman 170 Radioisotope Detector, eluting with 0.2 M ammonium acetate : 10% acetonitrile) - see, for example, Figure 2.15.

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[6]work done by Alice Harrison and Karl Jankowski, Medical Research Council Radiobiology Unit, Harwell.

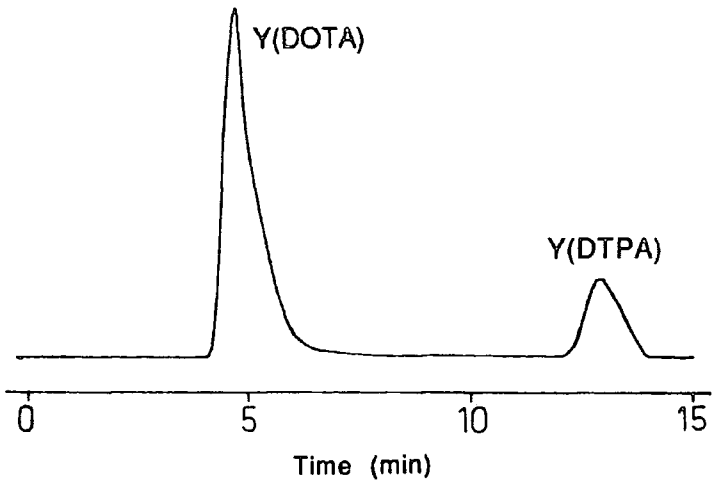


Figure 2.15: *HPLC analysis of a typical ^{90}Y-DOTA complexation reaction.*

Addition of DTPA, with the possibility of metal exchange between it and the ^{90}Y(macrocyclic ligand) complex, also provided a measure of the kinetic stability of the latter in the presence of a complexing agent (DTPA) also forming a thermodynamically stable complex with Y^{3+}.

Initially, with the ^{90}Y-DOTA system as the model, several factors were varied in order to optimise labelling efficiency : buffer anion and cation, pH, ionic strength, temperature and incubation period. Buffers were chosen with an operating range of between 4 and 6. This would be the range used when radiolabelling the MoAb conjugate, chosen so as to minimise non-specific binding (Section 4.6).

A direct correlation seemed to exist between labelling efficiency and the stability constants for the 1:1 Y(buffer anion) and buffer cation (DOTA) complexes. Thus for a given counterion and at pH 5.5, the labelling efficiency decreased according to the following sequence (stability constants, [log K_{ML}], are in parentheses)[19]:

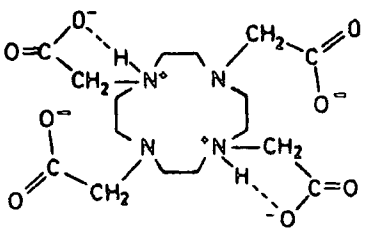
AcO^- (1.7) > $Succinate^{2-}$ (1.8)[7] > $Citrate^{3-}$ (7.9) and

[7]Figure for the protonated gadolinium(III) complex, which may be similar to that for the protonated yttrium(III) complex.

NH_4^+ (negligible) > K^+ (1.6) > Na^+ (4.4)

Ensuing experiments therefore favoured an ammonium acetate buffer.

Increasing the pH from 4.0 to 5.5 (pH 5.6 is the upper limit for the buffering action of NH_4OAc) produced an increased labelling efficiency. The pK_a values for DOTA are 11.1, 9.2, 4.2, 4.2, 1.9 and 1.7[27]. Thus at pH 4.0 the distribution of protonated species will favour $(DOTA)H_4$. At pH 5.5, $(DOTA)H_2^{2-}$ will predominate. However, between pH 4.0 and 5.5, the kinetically reactive species will probably be $(DOTA)H^{3-}$, despite there being, for instance, only 0.02% of this species relative to $(DOTA)H_2^{2-}$ at pH 5.5. The increase in labelling efficiency with increasing pH can then be ascribed to the increasing $[(DOTA)H^{3-}]$ concentration. That $(DOTA)H^{3-}$ rather than $(DOTA)H_2^{2-}$ is the kinetically reactive species in this pH regime has been rationalised in terms of electrostatic repulsion between macrocyclic ligand and metal ion[10]. In the diprotonated DOTA species, the two protons are situated on diagonally opposed nitrogen atoms[27]:



Only one of these nitrogen atoms will be protonated in $(DOTA)H^{3-}$. As the nitrogen atoms are directly involved in binding, electrostatic repulsion between metal ion and macrocyclic ligand will be less between metal ion and $(DOTA)H^{3-}$ than between metal ion and $(DOTA)H_2^{2-}$.

Increasing the ionic strength (0.02 to 0.5 M), temperature (291 to 310 K) and incubation period (5 to 45 min) also increased the labelling

efficiency. Optimum conditions for the labelling reaction were therefore considered to be NH_4OAc (0.1 M), pH 5.5, 310 K and an incubation time of 30 min. 0.1 M NH_4OAc as opposed to 0.5 M NH_4OAc was used for reasons which will become apparent at the end of this section.

Labelling efficiencies under these conditions for DOTA, TRITA, TETA, DOTA-BMA, ODOTRA and DTCTA at various concentrations with $[^{90}Y^{3+}]$ = 10^{-8} - 10^{-10} M are given in Table 2.2.

MOLARITY (x 10^{-6} M)	LIGAND					
	DOTA	TRITA	TETA	DOTA-BMA	ODOTRA	DTCTA
5	84[a]					
10	98[a]			48[a]		
100	98[a]			94[a]		
500	99[a]					72[a]
1000	99[a]	52[b]	0[c]	98[a]	82[a]	

Table 2.2: *Percentage labelling efficiency for various $^{90}Y^{3+}$-macrocyclic ligand systems incubated for 30 mins. at 310 K in 0.1 M NH_4OAc (pH 5.5), $[^{90}Y^{3+}]$ = 10^{-8}-10^{-10} M, [macrocyclic ligand] = (5-1000) x 10^{-6} M. Reactions quenched with DTPA (5 x 10^{-3} M final concentration); a) 10-15 min after DTPA addition; b) 40 min after DTPA addition; c) 1h after DTPA addition.*

From Table 2.2, it can be seen that for ligand concentrations $\geq$ 5 x 10^{-4} M the approximate order for the extent of uptake of $^{90}Y^{3+}$ decreases according to:

$$\text{DOTA} > \text{DOTA-BMA} \approx \text{DTCTA} > \text{ODOTRA} \approx \text{TRITA} >> \text{TETA}$$

with DOTA showing 98% incorporation even at 10^{-5} M. The failure of TETA to complex $^{90}Y^{3+}$ under these conditions, supports the ^1H NMR result of the previous section (and also the result obtained in Section 3.3).

The fall in labelling efficiency when [DOTA] becomes less than 10^{-5} M is probably due to metal ion impurities in $^{90}Y^{3+}$ (supplied by the Atomic Energy Authority, Harwell). Inductively coupled plasma source

mass spectroscopy[8] has shown that $^{90}Y^{3+}$ contains approximately 200 times as much Zn^{2+} and Ca^{2+} and 10 times as much Cu^{2+} - all three of these metal ions form thermodynamically stable complexes with DOTA, log K_{ML} = 21.0, 17.2 and 22.2 respectively[7]. Therefore, in a solution which is $\approx 10^{-9}$ M with respect to $^{90}Y^{3+}$, the concentration of Zn^{2+}, for instance, will be 2×10^{-7} M. If [DOTA] = 10^{-5} M, then the ratio of DOTA:Zn^{2+}:Y^{3+} will be 10^4:200:1 and competition between Y^{3+} and Zn^{2+} for DOTA will be negligible. But if [DOTA] = 10^{-6} M, the corresponding ratio will be 1000:200:1, and competition between Y^{3+} and Zn^{2+} for DOTA will become significant. Likewise, increasing the activity (and therefore the concentration) of $^{90}Y^{3+}$ will, if the metal ion contaminants are in excess of $^{90}Y^{3+}$, result in a reduced labelling efficiency. A striking example of this was seen in one particular experiment when, with [DOTA] = 10^{-5} M, increasing the initial concentration of $^{90}Y^{3+}$ from $\approx 10^{-9}$ to $\approx 10^{-7}$ M (equivalent to decreasing the ratio of DOTA:Zn^{2+}:Y^{3+} from 10^4:200:1 to 100:200:1) reduced the labelling efficiency from 98 to 51%.

Another possible source of metal ion contaminants may be the acetic acid (Aldrich) and ammonium acetate (Aldrich, Gold Label) used to make the ammonium acetate buffer. Analysis by atomic absorption spectroscopy has shown that the significant metal ion contaminant in both is calcium (0.008% in acetic acid and 0.0003% in ammonium acetate). To minimise interference with the ^{90}Y-macrocyclic ligand complexation reaction, the molarity (in this case essentially the ionic strength of the solution) of the ammonium acetate was maintained as low as possible without reducing the labelling efficiency, *ie.* 0.1 M.

In addition to displaying the highest labelling efficiency, ^{90}Y(DOTA) remained kinetically inert in the presence of DTPA over 72 hours. ^{90}Y(DOTA-BMA) remained reasonably kinetically stable in the

[8]Performed at the Mountjoy Research Centre, Durham.

presence of DTPA over 24 hours, showing 2% dissociation with [DOTA-BMA] = 10^{-5} M and 10% dissociation with [DOTA-BMA] = 10^{-4} M; at a ligand concentration of 5 x 10^{-4} M over 48h ^{90}Y(DTCTA) was kinetically unstable, undergoing complete dissociation.

2.6 CONCLUSIONS

^{1}H NMR experiments and HPLC radiometry demonstrate that of the six candidates put forward as yttrium binders, the rate of uptake of Y^{3+} is fastest for DOTA. The mechanism for uptake is probably a two-stage process[23]: the first involves the formation of an intermediate complex in which the carboxylate groups co-ordinate to the metal ion, which nevertheless remains outside the DOTA "cage" of nitrogen atoms and carboxylate groups; slow rearrangement then leads to complete encapsulation of the metal ion.

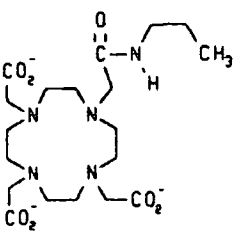
Of the tetraaza tetracarboxylic acid macrocyclic yttrium(III) complexes, $Y(DOTA)^-$ is the most thermodynamically stable in aqueous solution (log K_{ML} = 24.9) and is almost three orders of magnitude more stable than $Y(DTPA)^{2-}$. It is also likely that it is more stable than the yttrium complexes of DOTA-BMA, ODOTRA and DTCTA, according to the following arguments.

During the course of this work, Delgado *et al.*[29] prepared ODOTRA and measured the stability constants of its alkaline earth complexes. In each case the corresponding DOTA complexes were two to four orders of magnitude more stable (Table 2.3). Replacement of a hard donor (the nitrogen atom) by a harder donor (the oxygen atom) would therefore seem *not* to compensate for the loss of a charged donor (the carboxylate group) when comparing the relative thermodynamic stabilities of the ODOTRA and DOTA complexes.

ION	ODOTRA	DOTA
Mg^{2+}	10.3	11.9
Ca^{2+}	13.0	17.2
Sr^{2+}	11.4	15.2
Ba^{2+}	9.9	12.9

Table 2.3: *Stability constants, log K, of 1:1 ODOTRA and DOTA alkaline earth metal complexes; T=298K, I=0.1M $[(CH_3)_4NNO_3]$ (Ref.29).*

Sherry *et al.*[2] have made a compound similar to DOTA-BMA, a secondary amide derivative of DOTA, DOTA-PA (36).



(36)

They found that the log K_{ML} of its gadolinium complex [which may be similar to Y(DOTA-BMA)] was 20.1, approximately five orders of magnitude less stable than $Y(DOTA)^-$. Again, the loss of a charged donor to a neutral one appears to be the cause of this decreased stability, for it is unlikely that the postulated square antiprismatic arrangement of donor atoms in $Y(DOTA)^-$ will be altered in Gd(DOTA-PA).

DTCTA has not been synthesized before, so comparisons of stability constants are not possible. However, on charge considerations alone its yttrium(III) complex would be expected to be less stable than $Y(DOTA)^-$.

Chapter 3 compares the favourable binding and thermodynamic properties of the Y-DOTA system with its kinetic stability and also discusses the kinetic stability of the DTPA, TRITA and TETA yttrium(III)

complexes in aqueous solution.

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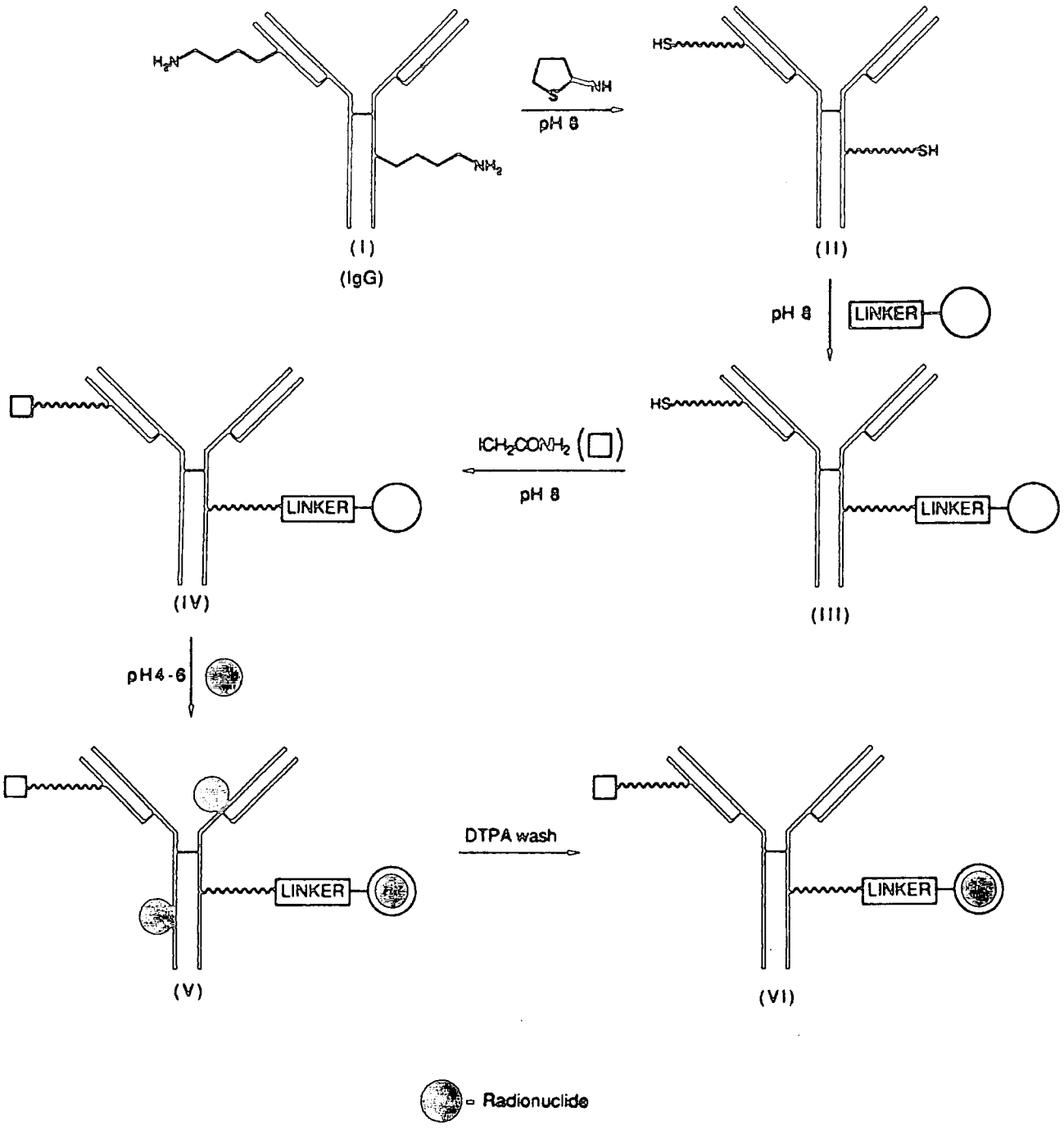

Figure 4.7: *A typical monoclonal antibody radiolabelling procedure.*

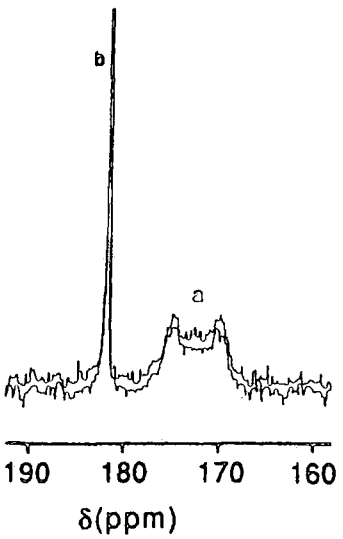

Figure 3.5: *Partial ^{13}C NMR spectrum (62.9 MHz) showing the carbonyl peaks corresponding to the Y(DOTA) complex (a) and DOTA alone [(b) and (c)] at pH 0, 293K.*

3.5 SUMMARY AND CONCLUSIONS

Between pH 4.0 and 5.0, in the presence of a 5-fold excess of Ni^{2+}, the kinetic stability of the yttrium(III)-polyaminocarboxylate complexes discussed in this chapter decreases according to:

$$Y(DOTA) >> Y(DTPA) \approx Y(TRITA) >> Y(TETA)$$

The Y(DOTA) complex is inert over a period of months, Y(DTPA) and Y(TRITA) have half lives of the order of 4 hours and Y(TETA) does not apparently form at all.

In the case of Y(DTPA), the dissociation is promoted by both hydrogen ions and nickel(II) ions. The existence of such a metal ion-dependent pathway suggests that the kinetic instability of the ^{90}Y-DTPA-MoAb conjugate *in vivo* may arise not only from hydrogen ion attack, as was proposed in Section 3.1, but also from metal ion attack, *eg.* Ca^{2+}, Mg^{2+} and Zn^{2+}. These ions are all present in considerable excess of the radiolabelled conjugate in blood ($[Mg^{2+}] = [Ca^{2+}] = 10^{-3}$ M, $[Zn^{2+}] = 10^{-5}$ M,[7] [^{90}Y-DTPA-MoAb] ~ 10^{-11} M) and all form thermodynamically

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CHAPTER THREE

THE KINETICS OF DISSOCIATION OF THE YTTRIUM(III) COMPLEXES OF DTPA, TRITA, TETA AND DOTA IN AQUEOUS SOLUTION

3.1 INTRODUCTION

Despite improvements in the conjugation of DTPA to a monoclonal antibody (Section 4.2.2), the subsequently radiolabelled MoAb remains relatively kinetically unstable *in vivo* (Section 4.10) probably due to acid-catalysed release of the radionuclide. It therefore seemed germane to quantify the rate of dissociation of the unattached yttrium(III)-DTPA complex as a function of pH at a fixed ionic strength and to compare it with the rate of dissociation of Y(DOTA) under similar conditions. In addition, it was hoped to assess the kinetic stabilities of Y(TRITA) and Y(TETA). This chapter describes the methodology and results of these investigations.

3.2 DISSOCIATION KINETICS OF THE YTTRIUM(III)-DTPA COMPLEX

The yttrium(III)-DTPA complex, like other yttrium(III)-polyamino-carboxylate complexes, does not have a chromophore in the UV/visible region (190 - 800 nm) with which to monitor its rate of dissociation spectrophotometrically. To circumvent this problem, another metal ion (M^{n+}) which *did* form a DTPA complex with a convenient chromophore[1,2] was used to scavenge any free ligand arising from the dissociation of Y(DTPA):

$$\underset{\substack{\textit{no UV-active}\\ \textit{chromophore}}}{Y(DTPA)} \longrightarrow Y^{3+} + DTPA$$

$$DTPA + M^{n+} \longrightarrow \underset{\substack{\textit{UV-active}\\ \textit{chromophore}}}{M(DTPA)}$$

The success of this method relies on the free ligand binding the scavenger metal ion M^{n+} in preference to free Y^{3+} to form a stable complex. To this end, an excess of scavenger metal ion was employed. Also, the rate of dissociation should be independent of the concentration of M^{n+}, *ie.* the latter should not assist the dissociation of Y(DTPA). The rate equation for dissociation would then be:

$$\frac{d[Y(DTPA)]}{dt} = k_d[Y(DTPA)^{2-}] + \sum_{1}^{n} k_n[Y(DTPA)H_n] + \sum_{0}^{m} k_{H_m}[Y(DTPA)H_m][H^+]^p$$

The rate constant for the dissociation of the unprotonated complex in the absence of an acid-dependent mechanism, k_d, will be small, of the order of 10^{-12} - 10^{-13} s^{-1} (this figure originates from a consideration of:

$$Y^{3+} + DTPA^{5-} \underset{k_d}{\overset{k_f}{\rightleftharpoons}} Y(DTPA)^{2-}$$

$$K_{[Y(DTPA)^{2-}]} = \frac{[Y(DTPA)^{2-}]}{[Y^{3+}][DTPA^{5-}]}$$

where the rate of association is probably diffusion-controlled, $k_f = 10^9$ - 10^{10} $\underline{M}^{-1}$ s^{-1}, and $K_{[Y(DTPA)^{2-}]} = k_f/k_d \sim 10^{22}$ $\underline{M}^{-1}$) - the first term for d[Y(DTPA)]/dt will therefore be negligible. The protonated and unprotonated forms of Y(DTPA) are likely to be in equilibrium with each other in acidic solution and therefore n is likely to be 1 in the second term. In the third term, m will probably be 0 and 1, the unprotonated and protonated complexes being subject to acid-promoted dissociation (with a first or second order dependence on $[H^+]$, p = 1 or 2).

We used nickel(II) to scavenge the free DTPA, the rate of dissociation of Y(DTPA) being monitored by the increase in absorbance at

385 - 390 nm due to the build up of Ni(DTPA) (Figure 3.1).

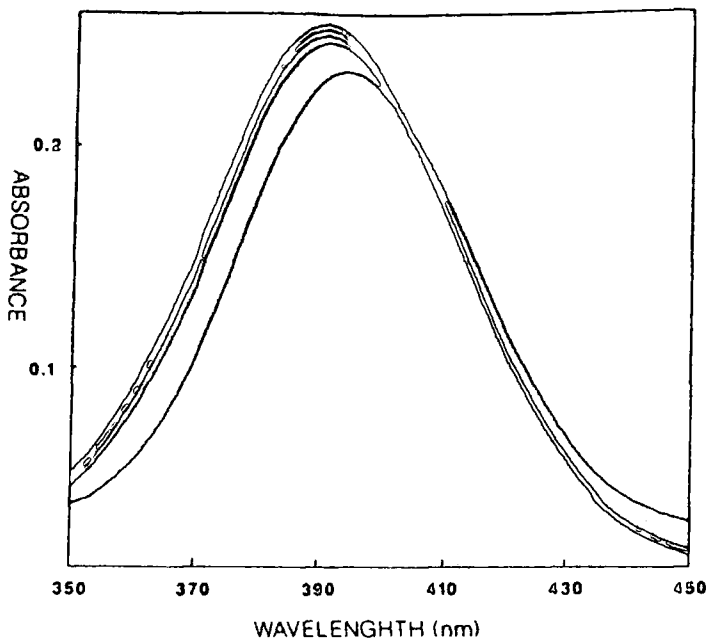


Figure 3.1: *Scans at 3 hourly intervals showing the build up of Ni(DTPA) at 390 nm due to the dissociation of Y(DTPA), pH 4.6, 298K.*

Inspection of the stability constants of Y(DTPA) and Ni(DTPA), log K_{ML} = 22.1 and 20.2 respectively[3], might suggest that the formation of any Ni(DTPA) is thermodynamically improbable, the free DTPA resulting from dissociation preferring to reassociate with Y^{3+}. However, as explained in Section 2.4, these stability constants refer to the formation of the species $Y(DTPA)^{2-}$ and $Ni(DTPA)^{3-}$ in non-competing media - the existence of other species such as $Y(DTPA)H^{-}$, $Ni(DTPA)H^{2-}$ and $Ni(DTPA)H_2^{-}$ is not considered (neutral complexes are unlikely to form), protonation of the ligands is neglected and the effect of other ligands competing for Ni^{2+} and Y^{3+}, *eg.* acetate, is ignored. But a *conditional stability constant*[4], log K'_{ML}, can be calculated for both Y(DTPA) and Ni(DTPA) which takes the latter two factors into account:

$$\log K'_{ML} = \log K_{ML} - \log \alpha_M - \log \alpha_H$$

where α_M represents the extent of competition for the metal ion from a ligand other than L, A, say, while α_H represents the extent of protonation of L at a given pH.

α_M can be written in terms of the overall stability constants (β_n) for the formation of MA, MA_2 ... MA_n and [A]:

$$\alpha_M = 1 + \beta_1[A] + \beta_2[A]^2 + \beta_3[A]^3 + \ldots + \beta_n[A]^n$$

and α_H can be written in terms of the protonation constants, K_1, K_2 ... K_n, for the ligand L:

$$\alpha_H = 1 + \frac{[H^+]}{K_1} + \frac{[H^+]^2}{K_1K_2} + \ldots + \frac{[H^+]^n}{K_1K_2..K_n}$$

For a system buffered with sodium acetate/acetic acid at pH 4.6, A = AcO^-, $[AcO^-] = 9.8 \times 10^{-2}$ M and $[H^+] = 2.5 \times 10^{-5}$ M; for $Y(AcO)^{2+}$ and $Y(AcO)_2^+$, $\beta_1 = 47.9$ and $\beta_2 = 1479$; for $Ni(AcO)^+$, $\beta_1 = 5.5$; for DTPA the protonation constants[3] are $K_1 = 3.2 \times 10^{-11}$ M, $K_2 = 2.5 \times 10^{-9}$ M, $K_3 = 5.5 \times 10^{-5}$ M, $K_4 = 3.9 \times 10^{-3}$ M, and $K_5 = 8 \times 10^{-3}$ M. Using the above expressions, log K'[Y(DTPA)] = 10.7 and log K'[Ni(DTPA)] = 10.0, *ie.* Y(DTPA) and Ni(DTPA) have comparable thermodynamic stabilities under these conditions.

Dissociation was monitored at pH 3.2, 3.7, 4.6 and 5.4. Solutions were buffered using sodium acetate and acetic acid, ionic strength (I) being maintained at 0.42 M with sodium perchlorate. At each pH $[Y^{3+}]$ = [DTPA] = 4×10^{-3} M, and the rate of dissociation was measured for $[Ni^{2+}] = 1 \times 10^{-2}$, 2×10^{-2} and 4×10^{-2} M, two and a half, five and ten times the concentration of Y(DTPA) respectively.

k_{obs} values for the dissociation of Y(DTPA) were calculated by taking absorbance measurements at equal time intervals over one half

life and are given in Table 3.1. These values show good agreement with first order kinetics.

pH	$[Ni^{2+}]$ (x 10^{-2} M)	k(obs) (x 10^{-5} s^{-1})
3.2	1	12.2
	2	17.9
	4	40.8
3.7	1	8.79
	2	9.82
	4	19.5
4.6	1	4.70
	2	6.22
	4	7.06
5.4	1	2.81
	2	3.11
	4	4.84

Table 3.1: *k(obs) for the dissociation of Y(DTPA) in the presence of Ni^{2+}, λ = 385 nm, I = 0.42 M ($NaClO_4$), [DTPA] = $[Y^{3+}]$ = 4 x 10^{-3} M, $[AcO^-]$ = 3 x 10^{-3} M (pH 3.2), 2 x 10^{-2} M (pH 3.7), 0.098 M (pH 4.6) and 0.172 M (pH 5.4), 298 K.*

From these results it can be seen that the rate of dissociation of Y(DTPA) is clearly *not* independent of $[Ni^{2+}]$ over the range of Ni^{2+} concentrations investigated. The nickel(II) ion must therefore assist the dissociation of Y^{3+} from Y(DTPA). The invalidity of using Ni^{2+} as a non-intrusive scavenger for measuring the rate of dissociation of Y(DTPA) notwithstanding, an analysis of the results was attempted.

A plot of k_{obs} against $[Ni^{2+}]$ (Figure 3.2) would seem to suggest that k_{obs} is a composite of several terms:

(a) The points for the two lower pH values, 3.2 and 3.7, seem better fitted by curves rather than straight lines, these curves having a strong dependence on both $[H^+]$ and $[Ni^{2+}]$. That k_{obs} doubles for a two-fold increase in $[Ni^{2+}]$ suggests that the rate of dissociation is first rather than second order with respect to $[Ni^{2+}]$. It would be reasonable to

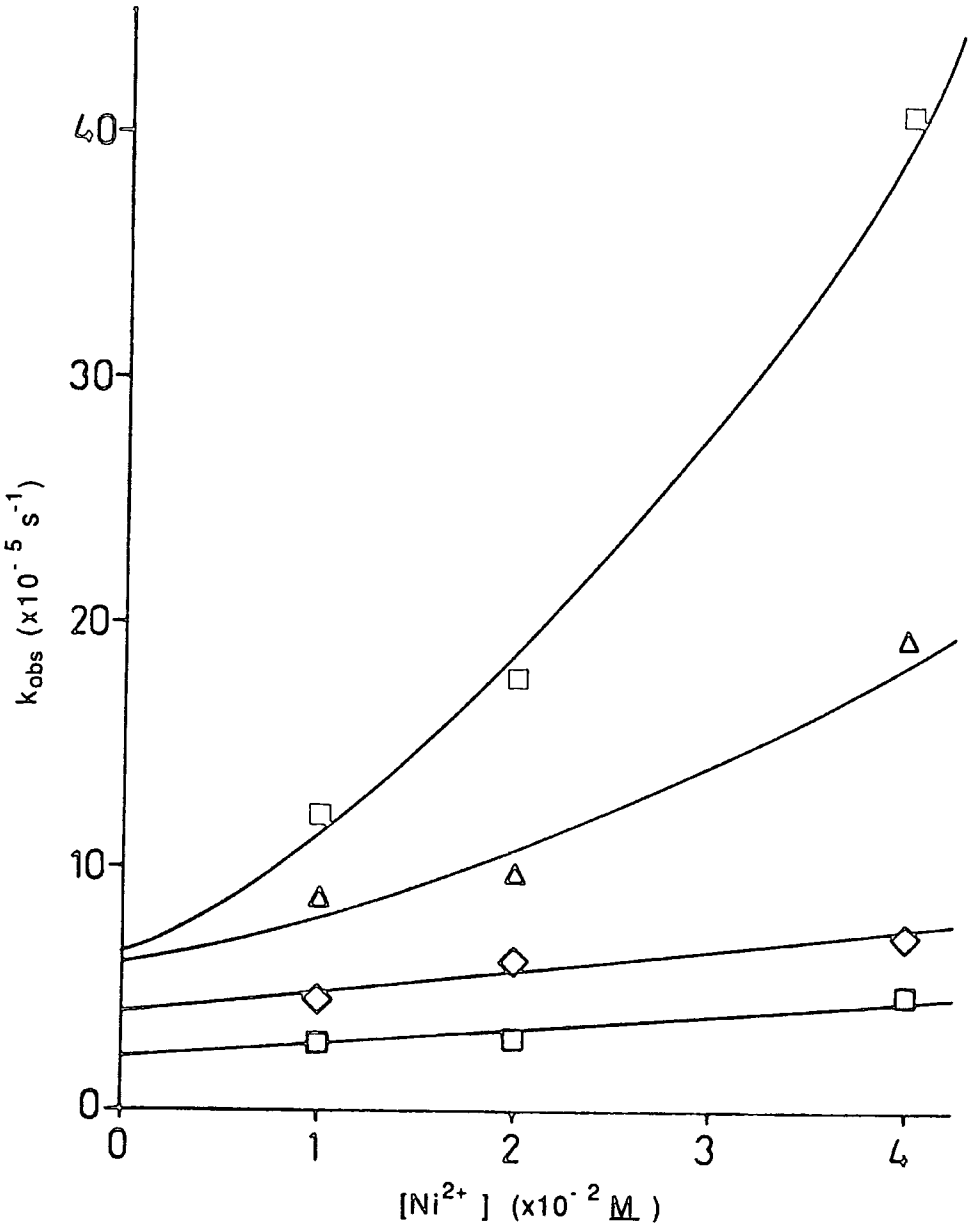


Figure 3.2: *Plot of k(obs) versus [Ni^{2+}] for the dissociation of Y(DTPA) in the presence of Ni^{2+} at pH 3.2 (□), 3.7 (Δ), 4.6 (◇) and 5.4 (□), 298 K, I = 0.42 M.*

propose, therefore, $k'_{Ni}{}^{2+}[Ni^{2+}][H^+]$ as a contributing term (with possible contributions from higher terms such as $k''_{Ni}{}^{2+}[Ni^{2+}][H^+]^2$).

(b) At the two higher pH values, 4.6 and 5.4, the curves tend towards straight lines with shallow gradients, which implies that as $[H^+]$ becomes smaller, the rate of dissociation becomes proportional to $[Ni^{2+}]$ alone. Another term might then be $k_{Ni}{}^{2+}[Ni^{2+}]$.

(c) By tentatively extrapolating the curves and lines to $[Ni^{2+}] = 0$, k_{obs} values of $(2.2 \pm 0.5) \times 10^{-5}\ s^{-1}$ (pH 5.4); $(4.0 \pm 1.0) \times 10^{-5}\ s^{-1}$ (pH 4.6); $(6.1 \pm 1.0) \times 10^{-5}\ s^{-1}$ (pH 3.7) and $(6.6 \pm 2.0) \times 10^{-5}\ s^{-1}$ (pH 3.2) are obtained. The increase in these values with increasing $[H^+]$ indicates that there is a nickel(II) independent term involved in the overall rate constant which is dependent on $[H^+]$ (and probably also on $[H^+]^2$). The large errors of the k_{obs} values at $[Ni^{2+}] = 0$ make the precise dependence on $[H^+]$ difficult to determine.

The terms in (a), (b) and (c) can be collected to give the complete (and qualitative) expression for k_{obs}:

$$k_{obs} = k_d + k'[H^+] + k_{Ni}{}^{2+}[Ni^{2+}] + k'_{Ni}{}^{2+}[Ni^{2+}][H^+] + k''_{Ni}{}^{2+}[Ni^{2+}][H^+]^2$$

Two rate-determining pathways would therefore seem to be operative in the dissociation of Y(DTPA) in the presence of a 2.5 - 10-fold excess of Ni^{2+}. The first, represented by the first two terms in the rate expression for k_{obs}, involves the direct dissociation of the protonated and unprotonated forms of Y(DTPA), which will probably be in rapid equilibrium with each other:

$$\left[\begin{array}{c} Y(DTPA)^{2-} \\ \updownarrow \\ Y(DTPA)H^{-} \end{array}\right] \longrightarrow \left[\begin{array}{c} (DTPA)H_2^{3-} \\ \updownarrow \\ (DTPA)H_3^{2-} \end{array}\right] + Y^{3+}$$

Subsequently, the free DTPA will rapidly complex Ni^{2+}.

The second pathway, represented by the last three terms of k_{obs}, involves the direct displacement of Y^{3+} from Y(DTPA) by Ni^{2+}. Again this is likely to proceed *via* the unprotonated and protonated species:

$$\left[\begin{array}{c} Y(DTPA)^{2-} \\ \updownarrow \\ Y(DTPA)H^{-} \end{array}\right] + Ni^{2+} \longrightarrow \left[\begin{array}{c} Ni(DTPA)^{3-} \\ \updownarrow \\ Ni(DTPA)H^{2-} \end{array}\right] + Y^{3+}$$

3.3 KINETIC STABILITY OF THE TRITA, TETA AND DOTA COMPLEXES OF YTTRIUM(III)

Using Ni^{2+} to scavenge free ligand was also the method of choice for monitoring the dissociation of Y(TRITA), Y(TETA) and, initially, Y(DOTA). The relevant absorption band for Ni(TRITA) and Ni(TETA) occurs at 580 nm, that of Ni(DOTA) at 540 nm, conveniently removed from the absorption bands of aqueous nickel (395 and 725 nm).

No attempt was made to examine the dependence or independence of the rates of dissociation on $[Ni^{2+}]$. We were interested only in determining the kinetic stability of the yttrium(III) complexes in the presence of excess Ni^{2+} at a given pH.

Under identical conditions ([complex] = 4×10^{-3} M, $[Ni^{2+}] = 2 \times 10^{-2}$ M, pH 4.6), the kinetic half life ($t_{\frac{1}{2}}$) of Y(TRITA) was comparable to the $t_{\frac{1}{2}}$ for Y(DTPA), both being of the order of 4 hours. On addition of Ni^{2+} to Y^{3+} and TETA to give a solution 2×10^{-2} M in Ni^{2+} and 4×10^{-3} M in Y^{3+} and TETA at pH 4.0 ($[AcO^{-}] = 3.6 \times 10^{-2}$ M), the appearance

of the Ni(TETA) absorption band was rapid, reaching a maximum in < 5 min. This suggests that either Y(TETA) had not formed and that the rate of appearance of the band merely reflected the rate of uptake of Ni^{2+} by the completely uncomplexed TETA, or that the rate of dissociation of Y(TETA) was fast, in which case the complex would have a $t_{\frac{1}{2}}$ < 5 min under these conditions. The ^{1}H NMR result of Section 2.4 and radiometry result of Section 2.5 support the former explanation, the existence of the protonated complex and competition from a nine-fold excess of AcO^{-} for yttrium(III) contributing to make Y(TETA) unstable with respect to dissociation at this pH.

In the presence of a five-fold excess of nickel(II) (2×10^{-2} M), the Y(DOTA) complex (in this particular experiment the preformed $Y(DOTA)Na.5H_2O$ complex as a solid was added to the Ni(II) solution at pH 3.0 to give a solution 4×10^{-3} M in complex) remains stable over at least six months at pH 3.0 (maintained using glycine (5×10^{-2} M) - hydrochloric acid (1.1×10^{-2} M) as a buffer). It was not possible to investigate the stability of Y(DOTA) at a lower pH because Ni(DOTA) does not form below pH 3.0.

3.4 DISSOCIATION OF THE YTTRIUM(III)-DOTA COMPLEX AT LOW pH

Since the physiological pH can be as low as 1.0, for instance in the stomach, it was important to examine the kinetic stability of Y(DOTA) below pH 3.0. This was done in two separate ways, by HPLC radiometry and by ^{13}C NMR.

3.4.1 Rate of Dissociation of Y(DOTA) as Monitored by HPLC Radiometry[1]

In this technique the extent of decomplexation of ^{90}Y(DOTA) ([DOTA] = 2.5 x 10^{-4} M, [$^{90}Y^{3+}$] ~ 10^{-9} M) at a given pH was estimated radiometrically by separating any unbound $^{90}Y^{3+}$ from complexed $^{90}Y^{3+}$ by analytical anion exchange HPLC, the relative amounts of each species being given by their corresponding peak areas (*cf.* Figure 2.15).

The ^{90}Y(DOTA) complex was formed at pH 5.5 (0.1 M ammonium acetate), incubating at 310K for 30 mins. An aliquot of this solution was removed and rebuffered to the required pH within the region 1.7 - 2.8 with glycine-hydrochloric acid. Aliquots of the resultant solutions were taken at given time intervals, treated with DTPA (to give a 5 x 10^{-3} M solution of the latter) - previous control experiments had established that ^{90}Y(DOTA) was stable at pH 5.5 with respect to ^{90}Y exchange with DTPA (5 x 10^{-3} M), see Section 2.5 - to bind any free $^{90}Y^{3+}$, quenched with 0.1 M ammonium acetate (pH 5.5) and analysed by anion exchange HPLC. The percentage ^{90}Y(DOTA) remaining at a given time was corrected for the decay of ^{90}Y.

No dissociation was observed over 10 days above pH 2.6. Dissociation between pH 1.7 and 2.3 was first order with respect to complex; k_{obs} values are given in Table 3.2.

pH	k(obs) (x 10^{-8} s^{-1})
2.3	5.43
2.0	30.8
1.8	84.4
1.7	130

Table 3.2: *k(obs) for the dissociation of ^{90}Y(DOTA) between pH 1.7 and 2.3, 298K.*

[1]Work carried out by Mandy Randall, MRC Radiobiology Unit, Harwell.

From a graph of k_{obs} against $[H^+]^2$ (Figure 3.3) it appears that:

$$k_{obs} = k\,[H^+]^2$$

A possible mechanism for the dissociation of Y(DOTA) might then be as follows:

$$[Y(DOTA)^-]^* \xrightarrow{H^+} [Y(DOTA)H]^* \xrightarrow{H^+} [Y(DOTA)H_2^+]^* \longrightarrow$$

$$\{[Y(DOTA)H_2^+]^*\}^{\ddagger} \xrightarrow{fast} Y^{3+} + H_n(DOTA)^{m+}$$

(m = 0, 1 or 2, n = 4, 5 or 6 for pH $\leq$ 2.3)

It is proposed that the first proton attacks the anionic complex, $[Y(DOTA)^-]^*$ [the pK_a of $Y(DOTA)^-$ is 3.08 (Section 2.3), therefore at pH 2.0 for every molecule of $Y(DOTA)^-$ there will be 12 molecules of Y(DOTA)H], starred to acknowledge the possible involvement of other species in the dissociative mechanism, *eg.* metal ions. As the $^{90}Y^{3+}$ used in these experiments contains approximately 200 times as much Zn^{2+} and Ca^{2+} as Y^{3+} (Section 2.5), such involvement is not unreasonable. It would perhaps be better then to rewrite k_{obs} as:

$$k_{obs} = k[H^+]^x + k'[M^{n+}]^x + k''[M^{n+}]^x[H^+]^x$$

$$x = 1 \text{ or } 2$$

Attack of the second proton in the proposed mechanism produces $[Y(DOTA)H_2^+]^*$, implicating this as the kinetically unstable species.

It is of passing interest with respect to this mechanism that the stability constants of the protonated and diprotonated Y(DOTA) complexes have been estimated as 16.9 and 7.7 respectively. (The estimations were made using the equation:[5]

$$\log K_{MLH_n} = \log K_{MLH_{n-1}} - pK_n(DOTA) + pK_n[(DOTA)H_n]$$

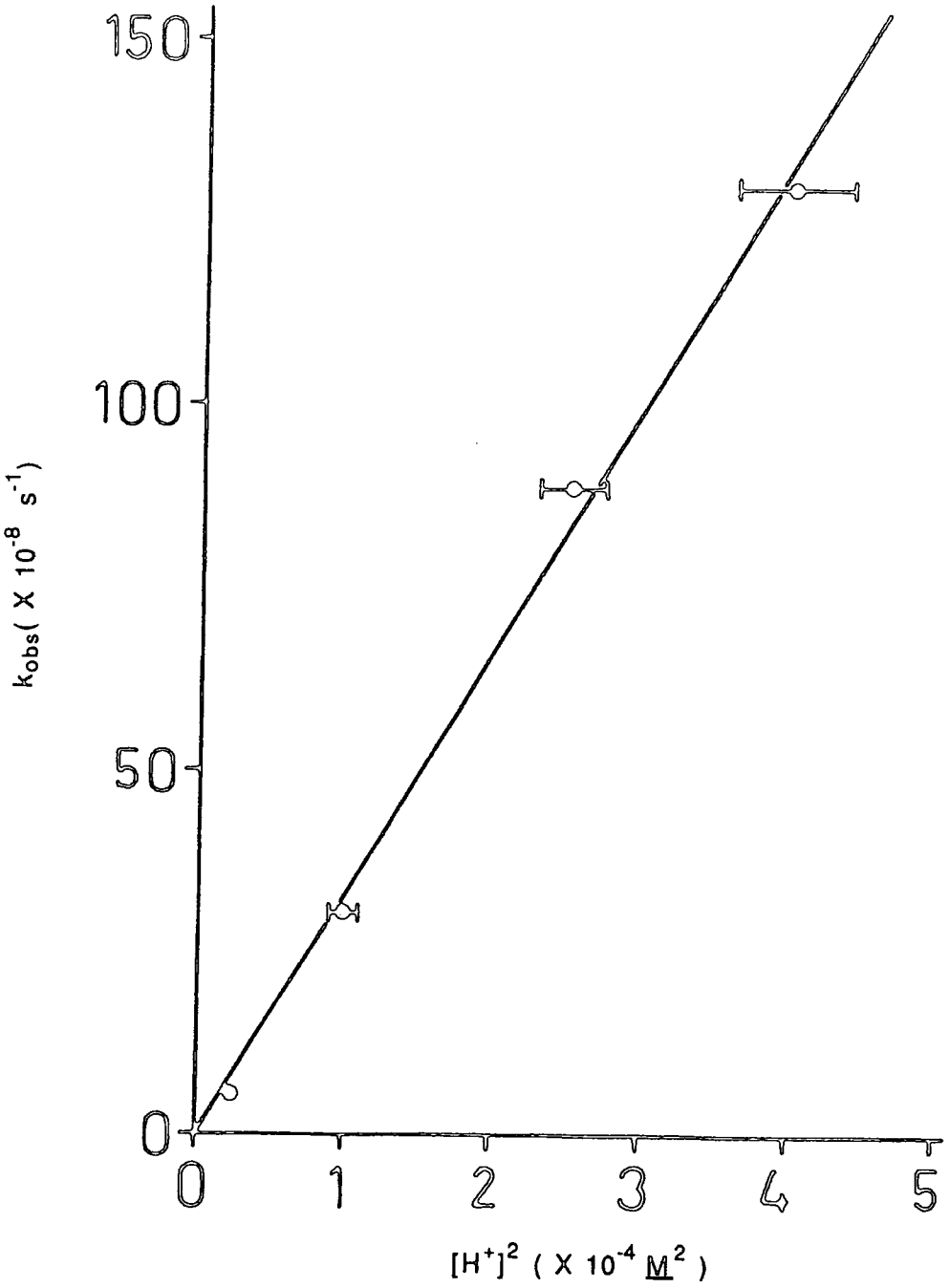


Figure 3.3: *k(obs) versus $[H^+]^2$ for the dissociation of ^{90}Y(DOTA), 298 K.*

Here log K_{ML} = 24.9 and pK_1[(DOTA)H] = 3.08 (Section 2.3), pK_1(DOTA) = 11.1 and pK_2 (DOTA) = 9.2[6] and pK_2[$(DOTA)H_2$] was assumed to be zero).

3.4.2 Rate of Dissociation of Y(DOTA) as Monitored by ^{13}C NMR

Accurate determination of the rate of dissociation of the yttrium(III)-DOTA complex by integration of the ^1H NMR spectrum of the reaction mixture was impeded by the large line widths of both complex and free ligand (Section 2.4) and also the overlap of their peaks. The line widths for the carbonyl signals of complex and ligand in the ^{13}C NMR spectrum, however, are considerably smaller; furthermore, these peaks are separated by a $\Delta\delta$ of approximately 9 ppm (Figure 3.4). Therefore, providing the relative amounts of these two species could be measured, a non-invasive means of monitoring the rate of dissociation of the Y(DOTA) complex was available.

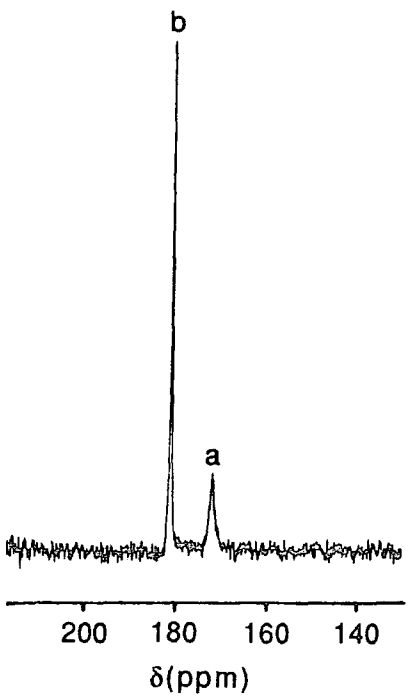
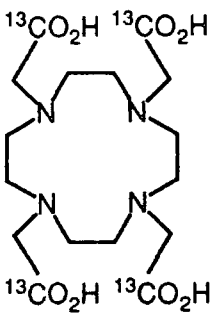


Figure 3.4: *Partial ^{13}C NMR spectrum (62.9 MHz) showing the carbonyl peaks corresponding to a) DOTA and b) Y(DOTA), pH 1.0, 293 K.*

The relative amounts of complex and ligand were estimated by integration. This was considered valid because their structurally identical and environmentally similar carbonyl atoms ought to have similar spin-lattice relaxation times, T_1, and thus the integrals ought to be a faithful representation of the relative amounts of complex and free ligand in solution. In acquiring the spectrum a two second interval between pulses ensured that the carbonyl carbon atoms of complex and ligand were fully relaxed. (Decreasing the interval to 1 second did alter the integrals whereas increasing the interval to 4 seconds did not. The optimum interval was therefore considered to be 2 seconds).

The ^{13}C NMR method posed two surmountable problems. The first was the potential error introduced by the time taken to accumulate a spectrum (say 30 minutes for a concentrated sample). If the dissociation occurred in a matter of a few hours, this error would become significant. To reduce the accumulation time and therefore the error, a ^{13}C carbonyl-labelled derivative (37) was used in the decomplexation experiments.



(37)

This was made by substituting $BrCH_2{}^{13}CO_2H$ for $ClCH_2CO_2H$ in the final step of the synthesis of unlabelled DOTA (Scheme 2.2). Using the ^{13}C-derivative of DOTA, it took 4-8 minutes to accumulate a spectrum (62.9 MHz, 293K, acquisition time 0.27s, relaxation delay 2s, 200-400 scans,

8K data storage). Time points were taken at the middle of this period.

The second problem was as follows. Routine irradiation of protons with a separate radiofrequency during a ^{13}C broad band decoupling experiment can lead to warming of the sample, caused by the power of the decoupling irradiation. In a kinetic experiment, the temperature of the sample should remain constant. To avoid the heating effect, therefore, *inverse gated decoupling* was employed. In this pulse sequence, the decoupling power is applied for a short time interval (~ 1 ms) rather than continuously. Warming is then minimised and the temperature of the sample will remain that of the probe (293K in these experiments) and of the thermostatted room.

The Y(DOTA) complex (1.2×10^{-2} M) did not dissociate over 2 days at pH 1.0. At pH 0, the complex had a half life between 10 and 20 hours. At pH -0.3 and -0.8 the rate of dissociation was first order with respect to complex, the rate constants for dissociation (k_{obs}) being $(4.9 \pm 1.0) \times 10^{-5}$ and $(7.2 \pm 1.0) \times 10^{-5}$ s^{-1} respectively. The large error associated with these values arose because the peak due to the free ligand in the ^{13}C spectrum became resolved into two separate peaks below pH 1.0 (Figure 3.5) - possibly due to a conformational change in the ring - reducing the signal to noise ratio and producing an error in the integral of these two free ligand peaks.

Unfortunately, two values for k_{obs} are insufficient to deduce unequivocally the relationship between k_{obs} and $[H^+]$, although they correspond quite closely to a first order dependence on $[H^+]$.

A 10-fold excess of Ca^{2+} (as $CaCl_2$) was added to the Y(DOTA) complex at pH 1.0 to determine whether or not a metal-assisted decomplexation mechanism existed for Y(DOTA). Calcium(II) is the predominant free divalent metal ion in blood, along with Mg^{2+} (both 10^{-3} M)[7]. Under these conditions the complex has a half life of approximately 2 weeks.

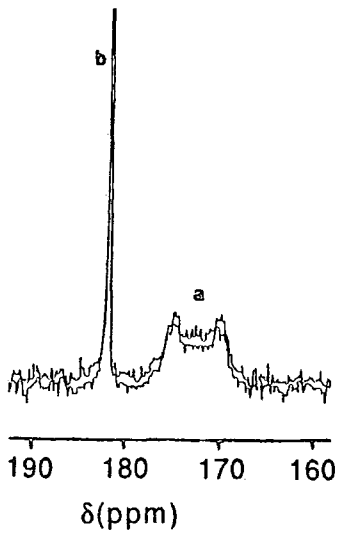


Figure 3.5: *Partial ^{13}C NMR spectrum (62.9 MHz) showing the carbonyl peaks corresponding to the Y(DOTA) complex (a) and DOTA alone [(b) and (c)] at pH 0, 293K.*

3.5 SUMMARY AND CONCLUSIONS

Between pH 4.0 and 5.0, in the presence of a 5-fold excess of Ni^{2+}, the kinetic stability of the yttrium(III)-polyaminocarboxylate complexes discussed in this chapter decreases according to:

$$Y(DOTA) >> Y(DTPA) \approx Y(TRITA) >> Y(TETA)$$

The Y(DOTA) complex is inert over a period of months, Y(DTPA) and Y(TRITA) have half lives of the order of 4 hours and Y(TETA) does not apparently form at all.

In the case of Y(DTPA), the dissociation is promoted by both hydrogen ions and nickel(II) ions. The existence of such a metal ion-dependent pathway suggests that the kinetic instability of the ^{90}Y-DTPA-MoAb conjugate *in vivo* may arise not only from hydrogen ion attack, as was proposed in Section 3.1, but also from metal ion attack, *eg.* Ca^{2+}, Mg^{2+} and Zn^{2+}. These ions are all present in considerable excess of the radiolabelled conjugate in blood ($[Mg^{2+}] = [Ca^{2+}] = 10^{-3}$ M, $[Zn^{2+}] = 10^{-5}$ M,[7] [^{90}Y-DTPA-MoAb] ~ 10^{-11} M) and all form thermodynamically

stable complexes with DTPA in aqueous solution (log K $[Mg(DTPA)^{3-}]$ = 9.3, log K $[Ca(DTPA)^{3-}]$ = 10.7, log K $[Zn(DTPA)^{3-}]$ = 18.8)[3].

At pH 3.0, again in the presence of a 5-fold excess of Ni^{2+}, Y(DOTA) is stable over at least six months. At pH 1.0, in the presence of a 10-fold excess of Ca^{2+}, ^{13}C NMR experiments show that the complex has a $t_{\frac{1}{2}}$ of approximately 2 weeks. But radiometric experiments show that at pH 1.7, in the presence of a 200-fold excess of Ca^{2+} *and* Zn^{2+}, Y(DOTA) has a $t_{\frac{1}{2}}$ of 6 days, *ie.* the rate of dissociation has increased, contrary to what might be expected for an increase in $[H^+]$. It would therefore appear that a metal-assisted decomplexation pathway does exist in the dissociation of Y(DOTA). With Ca^{2+} and Zn^{2+} in, respectively, a 10^8 and 10^6-fold excess of the injected ^{90}Y-DOTA-MoAb conjugate in the blood and the relatively large stability constants of the unprotonated, protonated and diprotonated Ca^{2+} and Zn^{2+} DOTA complexes (Table 3.3), displacement of the radionuclide from the radiolabelled conjugate by one of these metal ions *in vivo*, where the pH may be as low as 1.0, might become important.

METAL ION	SPECIES	STABILITY CONSTANT
Ca^{2+}	MH_2L	3.1
	MHL	8.7
	ML	17.2
Zn^{2+}	MH_2L	7.0
	MHL	13.1
	ML	21.0
Y^{3+}	MH_2L	7.7
	MHL	16.9
	ML	24.9

Table 3.3: *Stability constants [log K] for the calcium(II) and zinc(II) complexes of DOTA, 298K, I = 0.1 M $[(CH_3)_4NNO_3]$ (Ref.8) with the measured (Section 2.3) and estimated (Section 3.4.1) stability constants of the yttrium(III) complexes of DOTA for comparison.*

However, the rate of release of radionuclide at pH 1.0 would be sufficiently slow ($t_{\frac{1}{2}}$ ~ 100h) as to allow the radiolabelled conjugate to localise in the tumour (~ 6h) and deliver a sterilising dose of radiation (~ 120 hr).

3.6 REFERENCES

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CHAPTER FOUR

SYNTHESIS OF A FUNCTIONALISED DOTA DERIVATIVE, ITS CONJUGATION TO AN ANTIBODY AND THE PERFORMANCE OF THE RADIOLABELLED ANTIBODY *IN VIVO*

4.1 INTRODUCTION

Having identified DOTA as the macrocyclic ligand which forms the most kinetically and thermodynamically stable yttrium(III) complex of the six candidates originally proposed, we endeavoured to make its functionalised derivative for attachment to a monoclonal antibody.

This chapter is divided into three parts. Part 1 concerns the three attempts to synthesize the functionalised DOTA derivative. Part 2 outlines the attachment of the derivative to the monoclonal antibody and the radiolabelling of the resultant conjugate. Part 3 discusses the results obtained thus far with the radiolabelled conjugate.

PART 1: FUNCTIONALISATION OF DOTA

4.2 SYNTHETIC STRATEGIES FOR FUNCTIONALISING ACYCLIC AND MACROCYCLIC COMPLEXING AGENTS

There are two approaches to functionalising polyaza acyclic and macrocyclic complexing agents: one is through a nitrogen atom, the other is through the hydrocarbon skeleton, N-functionalisation and C-functionalisation respectively.

4.2.1 N-Functionalisation

A polyaminocarboxylate complexing agent (PCA) such as DTPA can be conjugated to a monoclonal antibody (MoAb) through the activated carbonyl of a carboxylate group. Activation can be achieved by converting the PCA to an acid anhydride derivative. Thus DTPA has been linked to a MoAb *via* an asymmetrical isobutylcarboxylic anhydride

derivative (38) (Figure 4.1)[1] and *via* a symmetrical dianhydride (see Figure 1.9).

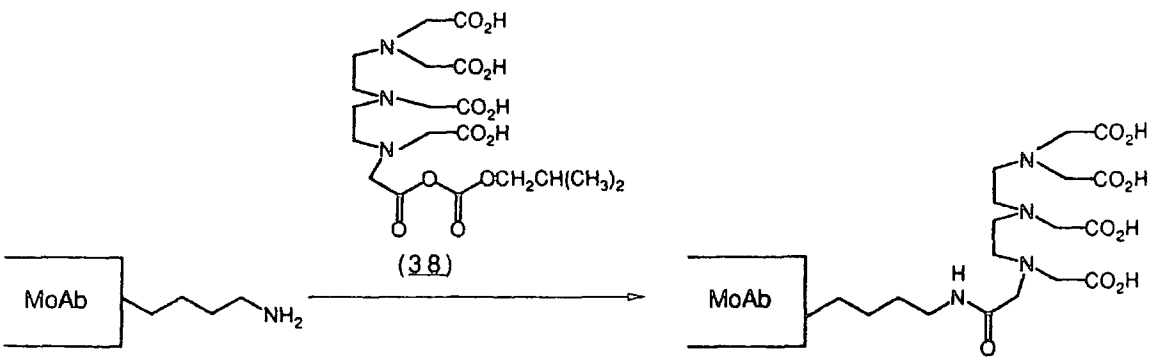


Figure 4.1: *DTPA conjugation to a monoclonal antibody* via *its isobutylcarboxylic anhydride derivative.*

The disadvantage of the anhydride linkage method is that a carboxylate binding site is lost on forming the amide bond. This leads to a drastic drop in the stability of the Y(DTPA) complex, even in serum, as Figure 4.2 demonstrates for the model complex ^{88}Y(DTPA-monobutylamide) (^{88}Y is a positron emitter, $T_{\frac{1}{2}}$ = 107 days)[2].

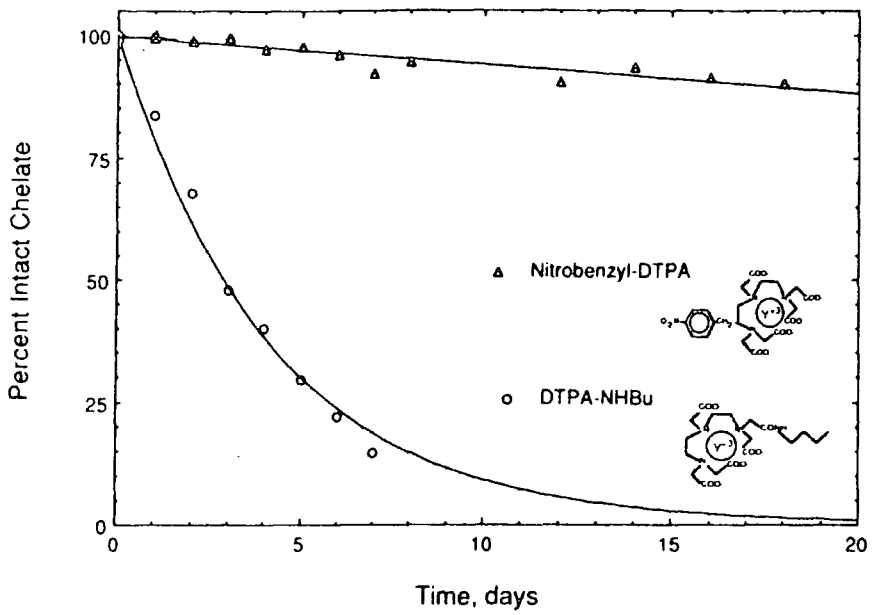
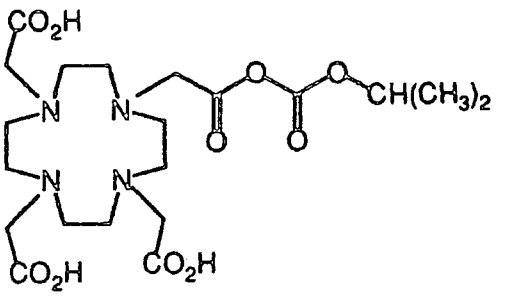


Figure 4.2: *Loss of $^{88}Y^{3+}$ from N-functionalised and C-functionalised derivatives of DTPA in serum, pH 7.4, 37°C (Ref.3).*

[Conjugation with the dianhydride DTPA derivative (Figure 1.9) can also lead to inter- and intramolecular cross-linking of the MoAb and a loss of immunoreactivity][4]. So although it is possible to make the asymmetrical anhydride of DOTA (39)[5], it would be unwise to use such a derivative for conjugation to a MoAb.

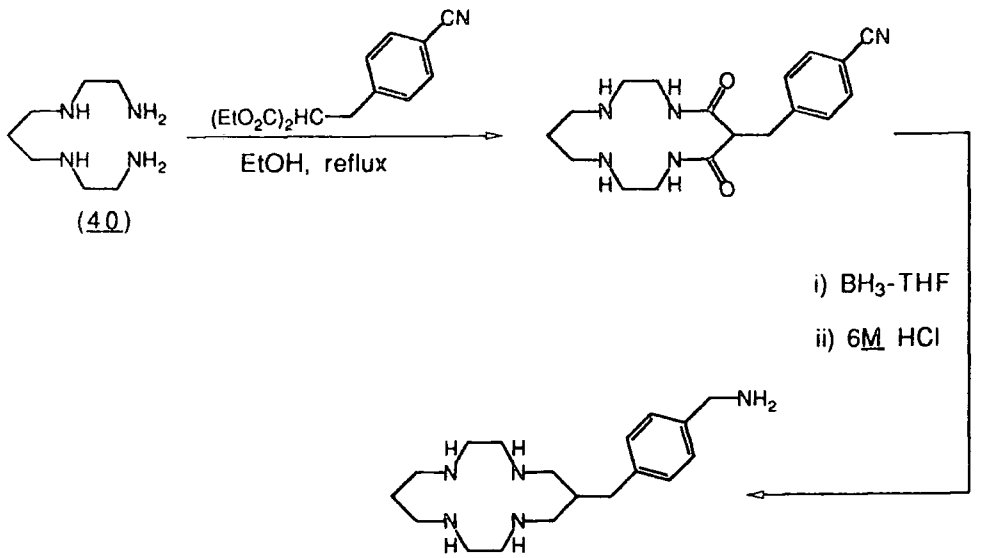


(39)

4.2.2 C-Functionalisation

The metal complexes of C-functionalised PCA derivatives, with all the carboxylates free to bind, are considerably more stable *in vivo* than their N-functionalised analogues as Gansow has demonstrated for the indium(III) complexes of C-functionalised DTPA and EDTA[4] [see also Figure 4.2, but it should be recalled (section 1.4.9) that serum studies are an inadequate assessment of a complex's stability *in vivo*]. C-functionalisation at a carbon adjacent to a binding nitrogen atom is also thought to confer kinetic stability on the complex by sterically hindering the attack of a proton or metal ion at that nitrogen[6].

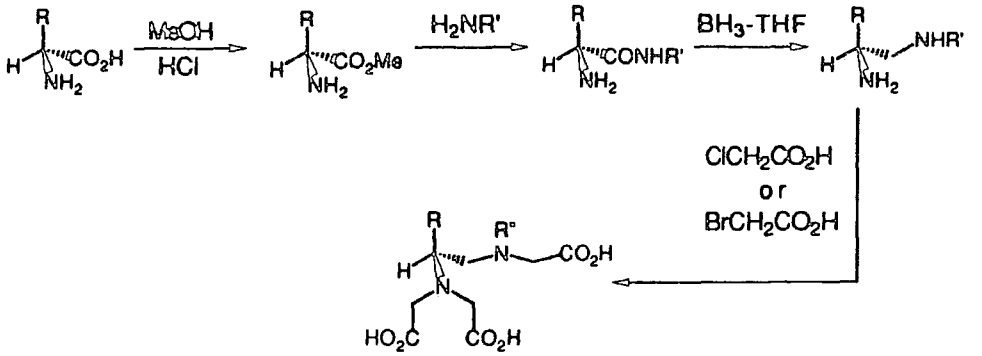
Polyazamacrocycles containing propylene units are relatively easy to C-functionalise. For example, cyclam [*cf.* $Cu(cyclam)^{2+}$ (3), Section 1.4.10] can be C-functionalised by condensation of the acyclic tetraamine (40) with a 2-functionalised diethyl malonate ester followed by borane-THF reduction of the secondary amide groups (Scheme 4.1)[7].



Scheme 4.1: *Synthesis of C-functionalised cyclam.*

The synthesis of a C-functionalised derivative of DOTA (which contains no propylene groups) was less facile. All three of our attempts were based on a general route to C-functionalised polyaminocarboxylate complexing agents, the precursor of which is a (2S)-*α*-amino acid (Scheme 4.2)[6].

The amino acid is first esterified and then amidated. Choice of amine for the amidation can give rise to different complexing agents. If ammonia is used, C-functionalised EDTA results. If ethylenediamine is used, C-functionalised DTPA results. After borane-THF reduction, the carboxylate groups can be introduced using chloro- or bromoacetic acid.



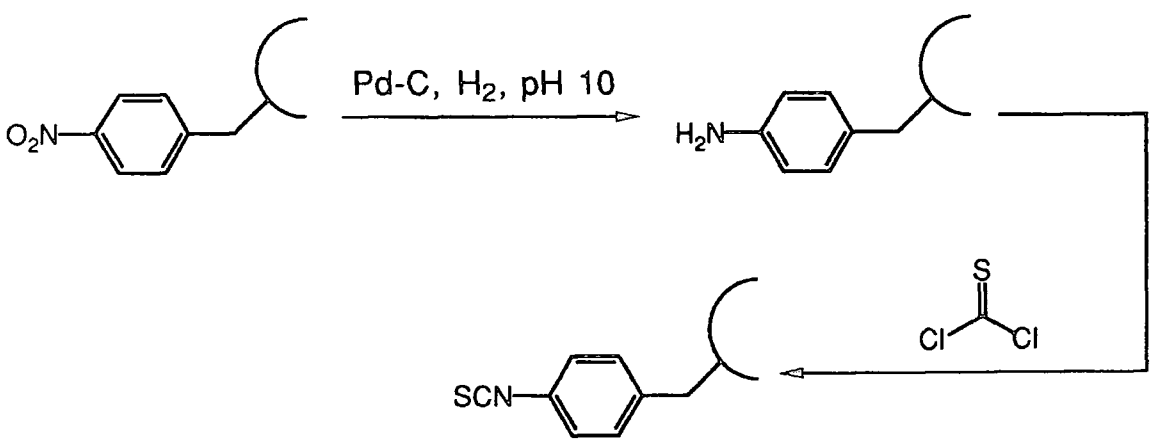
R= amino acid side chain

R'= H, R"=CH_2CO_2H (EDTA)

R'=$CH_2CH_2NH_2$, R"=$CH_2CH_2N(CH_2CO_2H)_2$ (DTPA)

Scheme 4.2: *General route to C-functionalised polyaminocarboxylate complexing agents based on an α-amino acid precursor.*

The amino acid side chain (R) provides the means of attachment to the MoAb. The side chain commonly used is the 4-nitrobenzyl moiety (Scheme 4.3), the nitro group of which acts as a masked primary amine.



Scheme 4.3: *Modification of the 4-nitrobenzyl group of an acyclic complexing agent.*

After attaching the acetic acid groups to the modified amino acid in the final step of Scheme 4.2, the primary amine can be unmasked by catalytic hydrogenation. Reaction with thiophosgene gives the isothiocyanate

group which can be coupled efficiently to a lysine ϵ-amino group on the MoAb[4] (see Figure 1.7). We also, but for slightly different reasons (section 4.4), required a primary amine on the side chain of the amino acid precursor for the synthesis of C-functionalised DOTA.

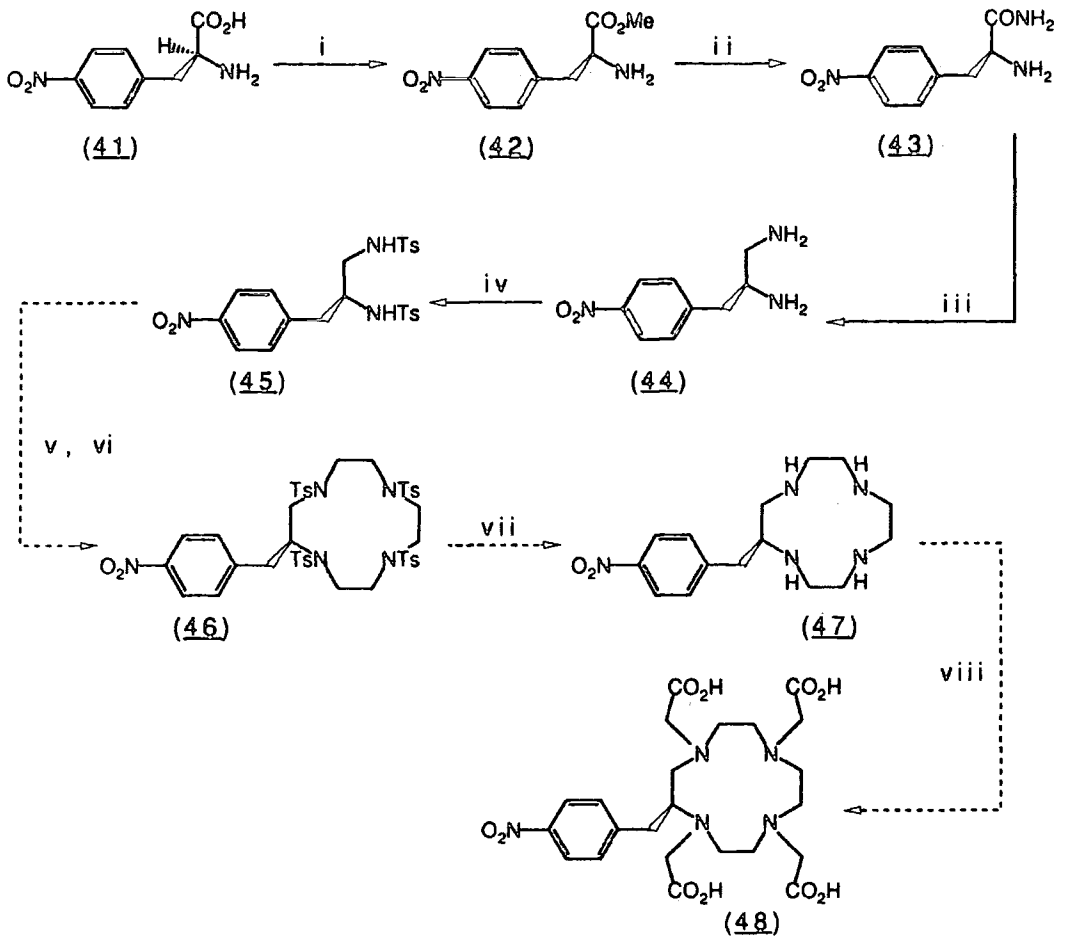
4.3 SYNTHESIS OF C-FUNCTIONALISED DOTA

Our first attempt at synthesizing C-functionalised DOTA employed (+)-(2S)-(4-nitrophenyl)alanine, a commercially available unnatural amino acid, as the precursor. The second and third attempted syntheses used (+)-(2S)-lysine, also commercially available.

4.3.1 Attempted Synthesis of (2S)-(4-Nitrophenyl)DOTA

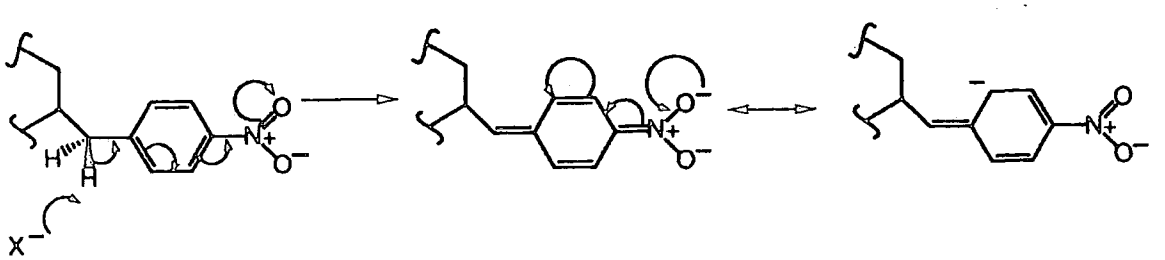
The proposed synthesis of (2S)-2-(4-nitrophenyl)DOTA (46) is given in Scheme 4.4. Successful steps are indicated by solid arrows, unsuccessful steps by broken arrows. Steps i-iii had previously been carried out by Gansow *et al.* in their synthesis of a C-functionalised DTPA derivative[4].

The synthesis failed at the cyclisation stage (step vi). During the course of this step, the colour of the reaction mixture changed from deep red to dark red-brown. Work-up gave an inseparable multi-component mixture as assessed by ^{1}H NMR and mass spectroscopy and from IR and TLC data. The yield of the tetratosylated cycle (46) was less than 5%.



Scheme 4.4: *Reagents: i, MeOH, HCl; ii, NH_3, MeOH; iii, BH_3-THF; iv, TsCl, pyridine, 0°C; v, NaOEt; vi, $TsO(CH_2CH_2NTs)_2$-$(CH_2)_2OTs$, DMF; vii, H_2SO_4; viii, $ClCH_2CO_2H$, NaOH.*

One possible explanation of this result might be that the acidity of the benzylic protons is enhanced by the presence of the nitro group, the negative charge generated by a deprotonation being stabilised within the benzene nucleus to give rise to the fleeting red colouration observed in the reaction mixture. The base in this scheme might be another tosylamide anion of (45) [denoted X^- below]:



The fate of this anion presumably involves more than just reprotonation.

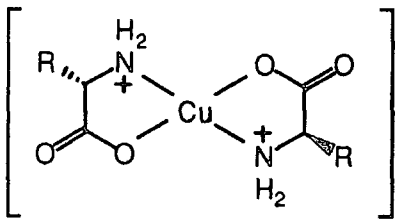
If this explanation is correct then the nitro group does not remain passive during the course of the synthesis. A different amino acid side chain is needed.

4.3.2 Attempted Syntheses of (2S)-(4-Aminobutyl)DOTA (Routes 1 and 2)

(+)-(2S)-Lysine was chosen as the α-amino acid precursor in these routes because the aminobutyl side chain should not, once protected, interfere with the cyclisation reaction.

Route 1[1]

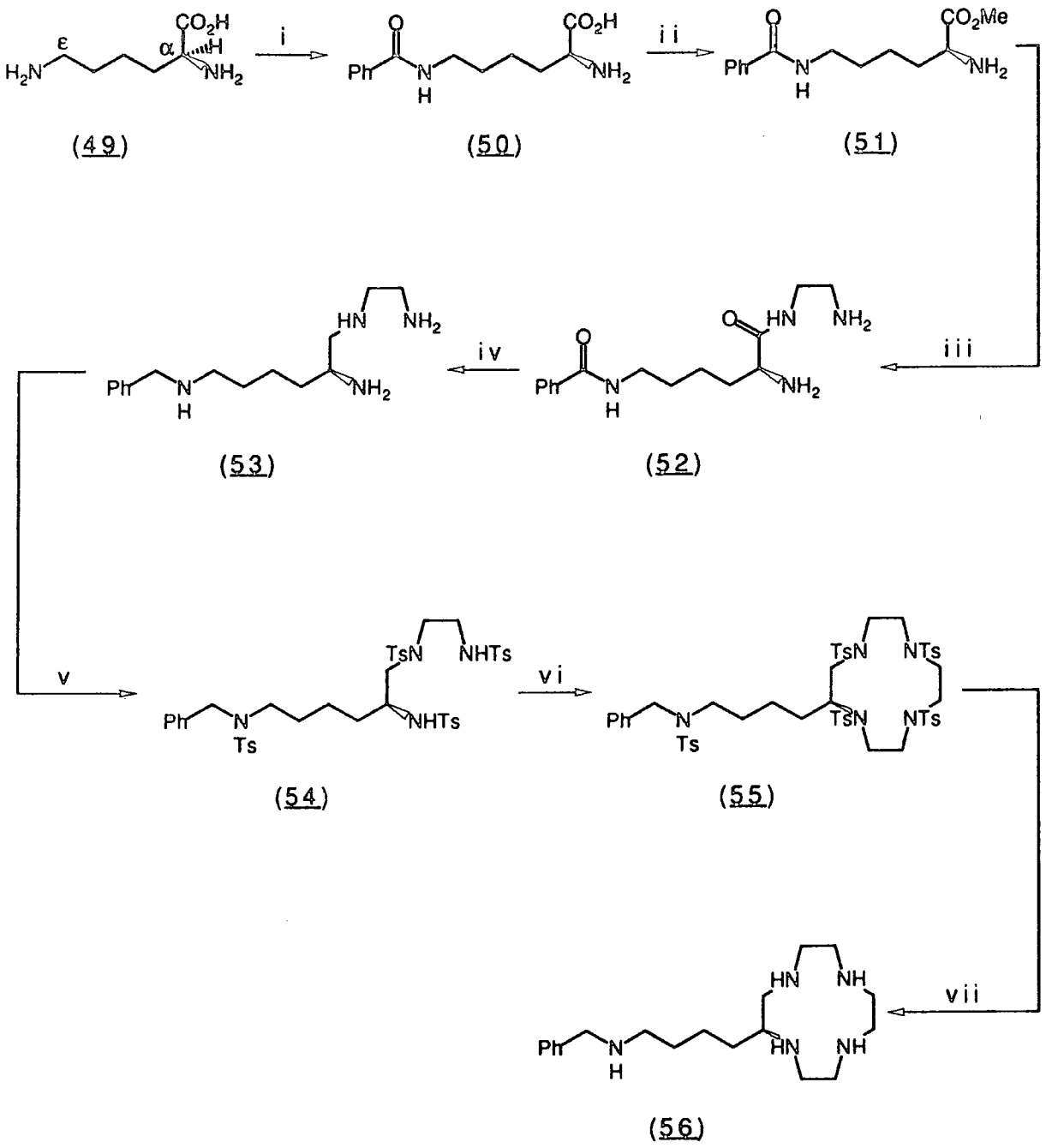
In Route 1 (Scheme 4.5), the protection was effected in the first step by masking the α-amino group with Cu^{2+} and selectively acylating the ϵ-amino group with benzoyl chloride between pH 9 and 10 at $0^{o}C$. Copper(II) masks the α-NH_2 group[8] by forming a square planar complex with the α-NH_2 and carboxylate groups of two lysine molecules which probably adopt the following stereochemistry:



R= $(CH_2)_4NH_2$

Once the acylation was complete, the copper(II) complex was destroyed by bubbling hydrogen sulphide through the aqueous reaction mixture. Esterification followed by amidation with ethylenediamine

[1]Carried out in conjunction with I.M. Helps and K. Jankowski.



Scheme 4.5: *Reagents: i, $CuCO_3 \cdot Cu(OH)_2$; PhCOCl, KOH, H_2S; ii, MeOH, HCl; iii, $H_2N(CH_2)_2NH_2$; iv, BH_3-THF; v, TsCl, K_2CO_3, H_2O; vi, $TsN(CH_2CH_2OTs)_2$, Cs_2CO_3, DMF; vii, Li, NH_3, EtOH, THF.*

followed by borane-THF reduction gave the tetraamine (53). Note that the benzamide protecting group was reduced to a benzamine in this last step. Tosylation and cyclisation by the caesium carbonate method (see Section 2.2.1) gave the tosylated cycle (55). Reductive cleavage of the five tosyl groups with lithium in ammonia in the presence of ethanol afforded the corresponding pentaamine (56).

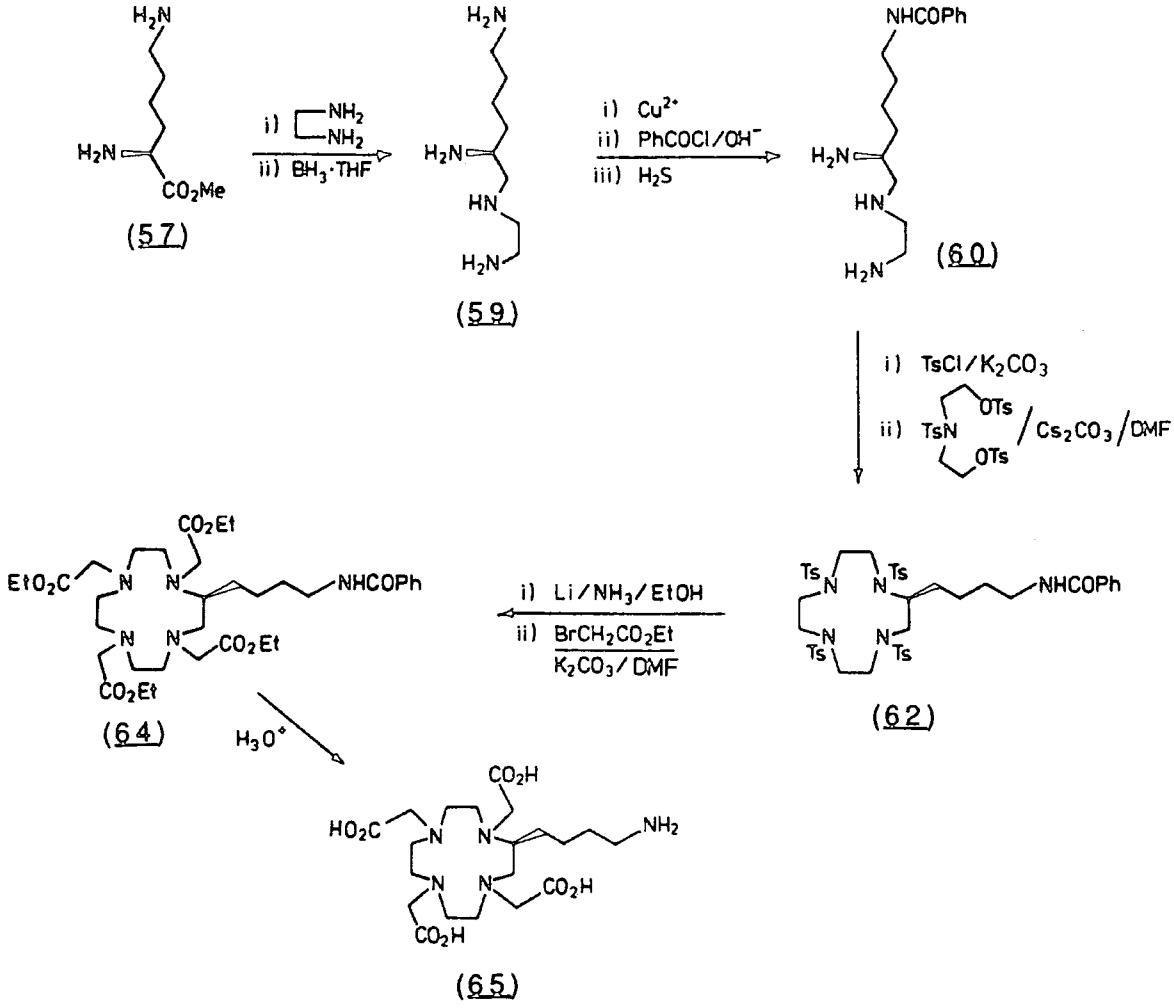
At this point, it was intended to remove the benzyl group by catalytic hydrogenolysis and then reprotect the exocyclic primary amine so generated as a benzamide. However, attempts to remove the benzyl group with two different catalysts, Pearlman's [$Pd(OH)_2$] and palladium black, in a variety of different solvent systems [9:1 formic acid:water; 1:1 formic acid:4-dioxane; 4:1:4 formic acid:methanol:4-dioxane] were unsuccessful. Similarly, attempts to oxidise the the benzylic methylene group of the pentatosylated cycle (55) to a carbonyl, thereby effecting the direct conversion to the benzamide, also failed. Oxidising agents used in these attempts were a cerium(IV) salt [$(NH_4)_2Ce(NO_3)_6$] in aqueous acetic acid and a permanganate salt [$(Bu)_4NMnO_4$] in dichloromethane.

Thus this synthesis failed as a result of the benzamide protecting group being converted into a benzylamine in step iv (Scheme 4.5) which, after cyclisation, subsequently resisted all attempts to remove it.

Route 2[2]

To avoid producing the quiescent benzyl group, a second route was proposed (Scheme 4.6) in which the protection of the ϵ-amino group was delayed until *after* the borane-THF reduction of the amide (see general

[2]The last two steps of this synthesis were performed by B. Boyce and A.T. Millican at Celltech Ltd., Slough.



Scheme 4.6: *Synthesis of (+)-(2S)-(4-aminobutyl)DOTA.*

route to C-functionalised PCA's - Scheme 4.2).

After esterification of (+)-(2S)-lysine (49) and treating the ester (57) with neat ethylenediamine, the resulting amide (58) was reduced with borane-THF to give the tetraamine hydrochloride (59). The ϵ-amino group was then selectively acylated with benzoyl chloride in a fashion similar to that for the acylation of (+)-(2S)-lysine in Route 1. Here, however, the Cu^{2+} protected a diethylenetriamine subunit as opposed to a single α-amino group. It was noted before the synthesis that diethylenetriamine forms a stable 1:1 complex with Cu^{2+} in aqueous solution ($\log K_{ML} = 16.1$).

Subsequent steps were effected according to Route 1. The lithium/-ammonia/ethanol reduction of the tetratosylated cycle (62) gave the desired amine (63) in 57% yield with 11% of the benzylamine compound (56) (see Scheme 4.5) and <10% of the deprotected amine (66) as deduced by ^1H NMR (Figure 4.3) and mass spectroscopy.

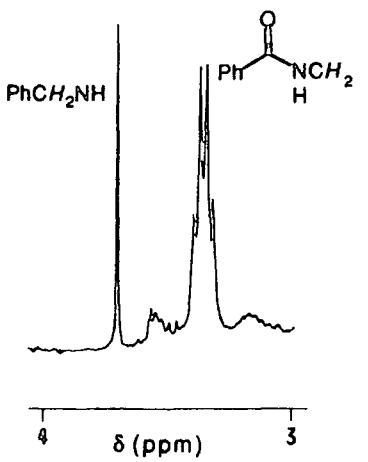
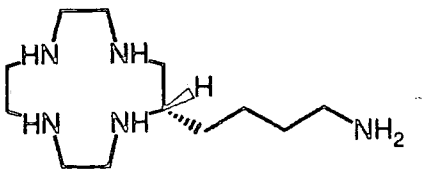
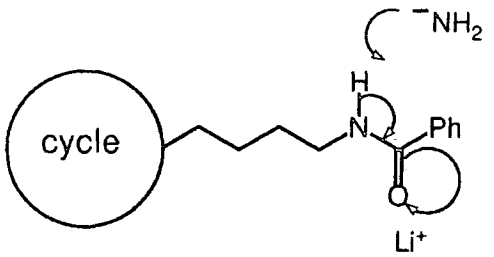


Figure 4.3: *Partial ^1H NMR spectrum (250 MHz) of the N-benzyl and N-benzoyl products of the lithium/ammonia reduction of the tetratosylated macrocycle (62).*



(66)

The low yield of the benzylamine (56) was probably due to protection of the secondary amide as its lithium salt[9] during the course of the reduction:



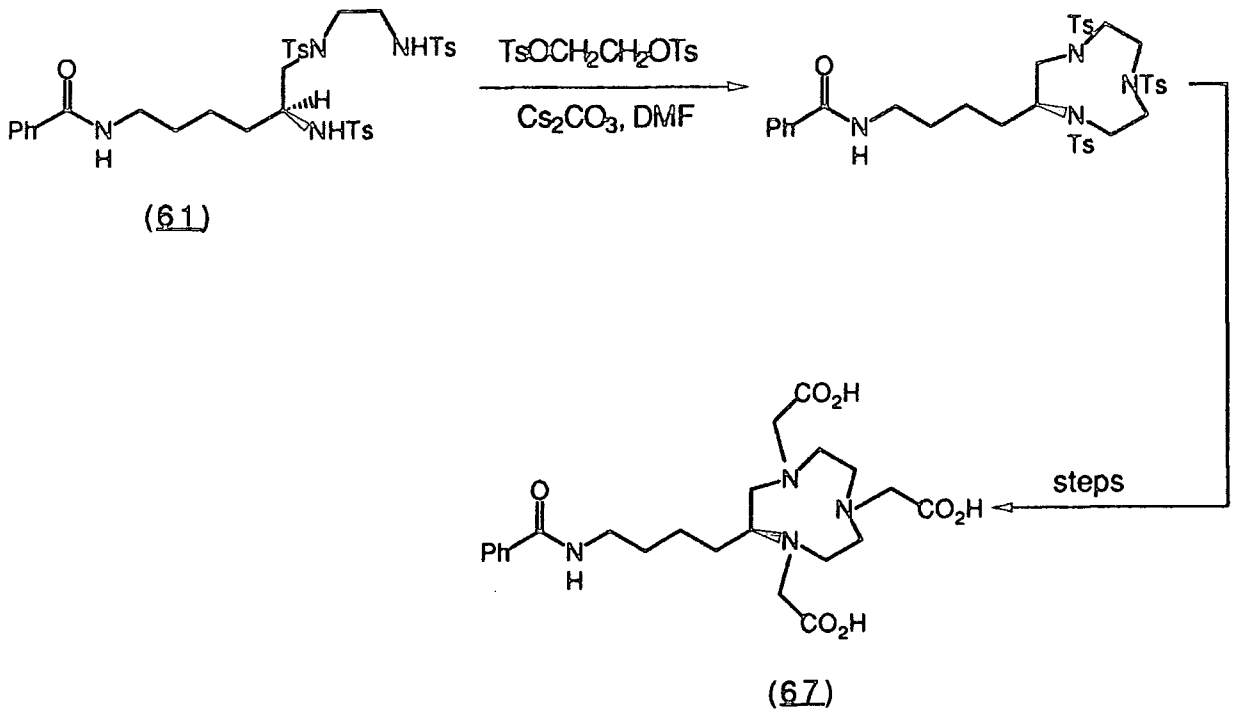
Before this step was attempted, we were aware that aromatic primary and secondary amides, ArCONHR, underwent Birch reduction without attendant amide reduction[10].

Ethoxycarbonylmethylation of the tetraamine (63) gave the tetra-ester (64) which was hydrolysed to afford aminobutyl C-functionalised DOTA (65).

The tetratosylated cycle (62), the tetraamine (63) and (4-amino-butyl)DOTA (65) were all dextrarotatory, $[\alpha]_D^{22}$ = + 14 (c 1.00 in CH_2Cl_2), $[\alpha]_D^{25}$ = + 2.5 (c 0.80 in CH_2Cl_2) and $[\alpha]_D^{22}$ = + 2.7 (c 0.75 in H_2O) respectively, suggesting that the steps in this route are not accompanied by racemisation. The overall yield of this route from the methyl ester (57) is 7%.

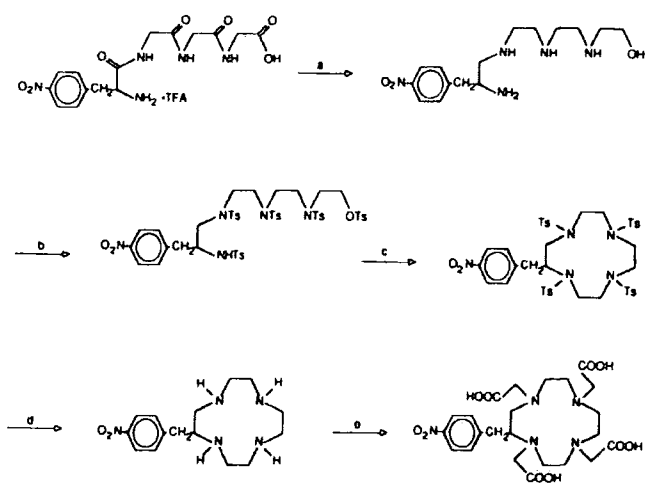
By replacing $TsN(CH_2CH_2OTs)_2$ with $TsOCH_2CH_2OTs$ at the cyclisation stage, Route 2 has also been successfully applied by our group to the synthesis of (2S)-(4-aminobutyl)-1,4,7-triazacyclononane-1,4,7-triacetic

acid [(4-aminobutyl)-NOTA] (67):



(61) (67)

Simultaneous with this work, Meares *et al.* published[3] the synthesis of another C-functionalised DOTA compound, (2S)-(4-nitrophenyl)DOTA (Scheme 4.7), based on a tetrapeptide precursor, NO_2-Phe-Gly-Gly-Gly, and which employed a clever *intra*molecular ring closure.



Scheme 4.7: *Synthesis of (2S)-(4-nitrophenyl)DOTA (reproduced from Ref.3); Reagents: a) BH_3-THF; b) TsCl, NEt_3, MeCN; c) Cs_2CO_3, DMF; d) H_2SO_4, PhOH; e) $BrCH_2CO_2H$, NaOH.*

The overall yield of this route is 13%. This does not take into account the liberation of the amine from the nitro group (but this is usually quantitative)[4] and the initial tetrapeptide synthesis. At first glance, then, this is a comparable synthesis of C-functionalised DOTA to our own. However, it is longer and less versatile - for instance, it appears less amenable to the synthesis of the NOTA analogue (67). Also, the starting material, (+)-(2S)-(4-nitrophenyl)alanine, is 250 times more expensive than (+)-(2S)-lysine. Our route has been scaled up to "Good Laboratory Practice" to produce 2g of aminobutyl C-functionalised DOTA.

PART 2: RADIOLABELLING THE MONOCLONAL ANTIBODY

4.4 SPACER GROUPS

Attaching a macrocyclic (as opposed to an acyclic) complexing agent directly to a monoclonal antibody leads to a poor labelling yield on subsequent addition of the radionuclide[11]. To achieve a good radiolabelling yield, it is necessary to interpose a *spacer group* between antibody and macrocycle, much as a spacer group is required for good yields in solid phase oligonucleotide synthesis[12]. In the absence of the spacer, it has been suggested[11,13] that the macrocycle becomes buried within the antibody structure and that the path the radionuclide must take to become bound by the macrocycle is therefore sterically hindered (this does not explain, however, why the same problem is not encountered with acyclic complexing agents).

Spacer groups are bifunctional, one functionality specific for thiol residues on the MoAb (Section 4.5), the other for the exocyclic amine of the macrocycle. Figure 4.4 shows two typical spacers groups,

the active esters of 2-vinyl-6-(4-carboxy-2-oxabutyl)pyridine (68) and 3-carboxy-4-oxa-4-(2,5-dicarboximidocyclopentane)maleimide (69).

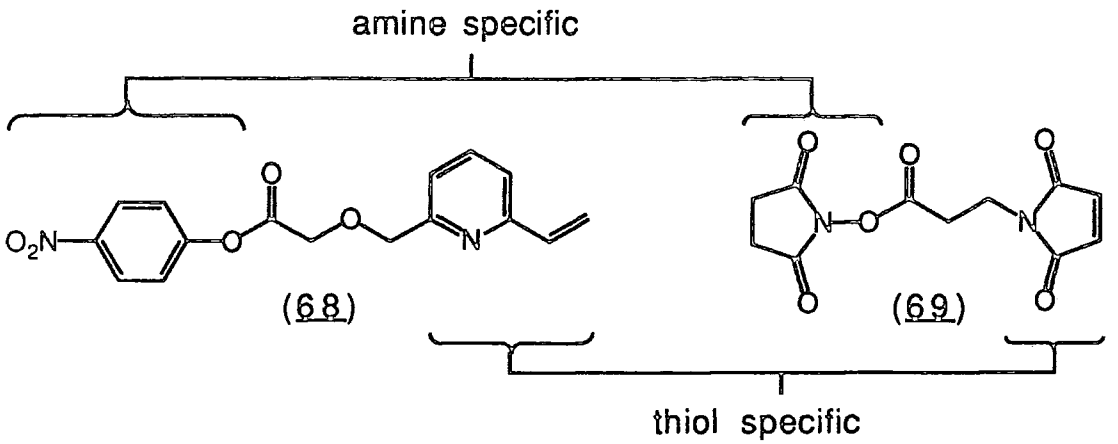
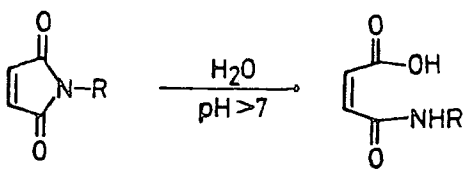
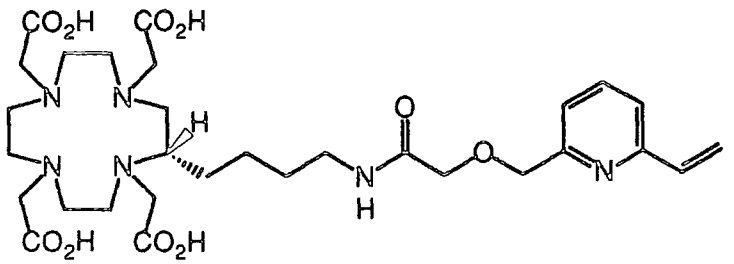


Figure 4.4: *Vinylpyridine (68) and maleimide (69) spacer groups used in the conjugation of a macrocyclic complexing agent to a MoAb.*

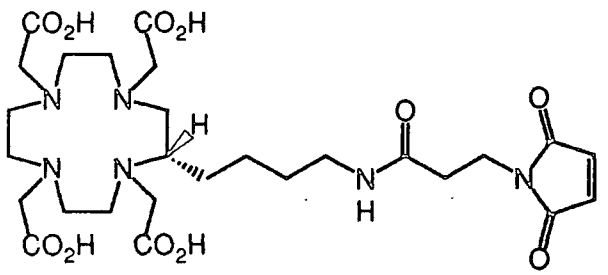
The 2-vinylpyridine unit of (68), with an operating range pH 5 to 9, is extremely thiol-specific (being 300 times more reactive towards thiols than towards amino groups)[13]. The maleimide unit of (69) is less specific, but is more reactive towards thiols. In addition, above pH 7, the maleimide ring is prone to hydrolysis:



The resulting acyclic maleamic acid has less affinity for thiols. However, the maleimide (69) is commercially available - production of the vinylpyridine (68) requires a 6 step synthesis. (2S)-(4-Amino-butyl)DOTA has been coupled to both the vinylpyridine and the maleimide spacer groups to give compounds (70) and (71) respectively:



(70)



(71)

Treating the MoAb with the maleimide derivative (71) resulted in a better coupling efficiency (1-3 molecules per antibody) than with the vinylpyridine derivative (70), which gave less than one macrocycle per antibody. Our work has thus favoured the use of the maleimide-coupled macrocyclic antibody conjugate.

4.5 GENERATION OF THIOLS ON A MONOCLONAL ANTIBODY

The class of monoclonal antibody used in this work (IgG) does not possess any free thiol residues. Introducing a small number of thiol residues (between 3-5) allows the attachment of an equally small number (1-3) of thiol-specific macrocycles, *ie.* macrocycles which have been previously coupled to spacer groups as outlined in the preceding section. The thiol residues are generated by treating the MoAb with 2-iminothiolane[14] (Traut's reagent), which reacts with lysine ϵ-amino

groups on the MoAb. The thiol-specific macrocycle can then be coupled to the MoAb according to Figure 4.5.

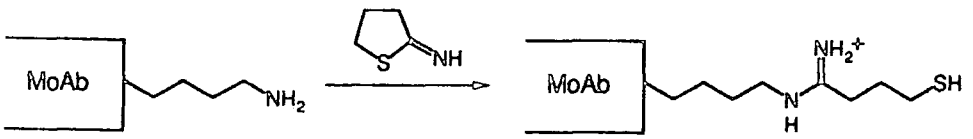


Figure 4.5: *Generation of a thiol on a monoclonal antibody using Traut's reagent.*

The disadvantage of this method is that thiol residues may be created on the two binding sites of the MoAb, impairing its immunoreactivity. It is therefore important to test the latter after radiolabelling a MoAb (Section 4.6).

Another interesting and more recent way of generating free thiols on a MoAb (collaborative work with Celltech) which avoids alteration of the binding sites is as follows. The MoAb is selectively cleaved (pepsin digestion) to give a $F(ab')_2$ section and several smaller fragments (Figure 4.6). The disulphide bridge between the heavy chains of the $F(ab')_2$ section is reduced with 2-aminoethanethiol to give the free thiols. A macrocycle bearing *two* thiol specific spacer groups can then be inserted between these thiols.

An even more recent method of generating a thiol at a specific site on the MoAb has been to replace, by recombinant DNA technology, an amino acid in the constant region of the MoAb with a cysteine residue (cysteine has a free thiol group)[15].

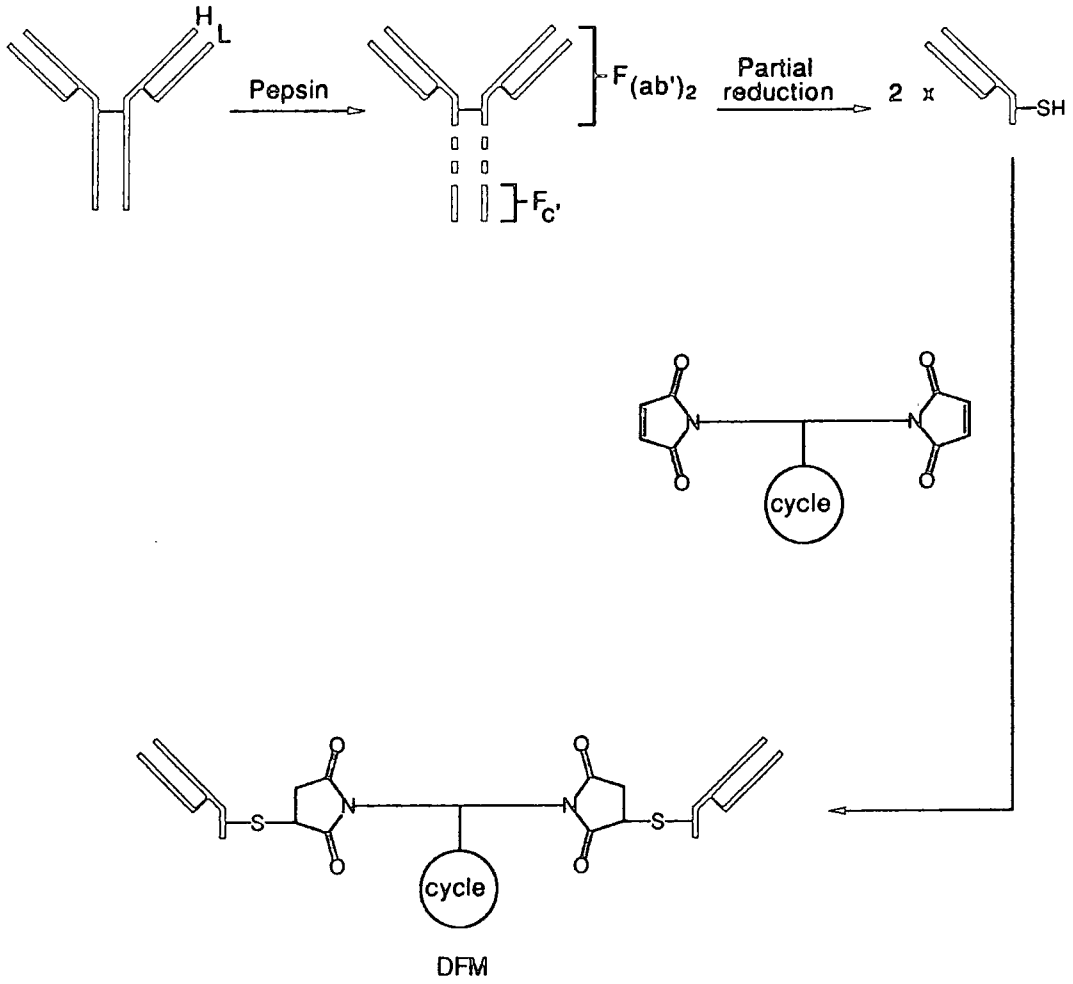


Figure 4.6: *Production of a Di-F(ab')-maleimide MoAb(DFM).*

4.6 RADIOLABELLING PROCEDURE[11]

The monoclonal antibody used for this work was B72.3 which has a high affinity for tumour-associated glycoprotein (TAG-72) found in human colon and breast cancer. TAG-72 has been found in 80% of colon carcinoma[3] studied. B72.3 is unreactive towards normal adult tissue[4].

A general radiolabelling procedure, beginning with the generation of the free thiols, is described schematically in Figure 4.7. The MoAb is treated with 2-iminothiolane, purified by gel-filtration[4] to remove any unreacted 2-iminothiolane and further reacted with a two- to three-fold excess of the maleimide-coupled macrocycle (71). Gel-filtration, capping any unreacted thiol groups with iodoacetamide and further gel-filtration gives the macrocyclic antibody conjugate (IV). These steps are performed in ammonium phosphate buffer, pH 8.0, to minimise metal ion contamination. Metal phosphates tend to be insoluble and are therefore easily removable.

At this stage, it is important to quantify the number of macrocycles bound to the antibody, not only for characterisation purposes, but also to enable an accurate determination of the radiation dose delivered to a patient. The number of macrocycles per antibody is estimated by adding a solution of $^{57}Co^{2+}$ (positron emitter, $T_{\frac{1}{2}}$ = 270 days)[2] of known activity and therefore concentration in ammonium citrate buffer (pH 4.0) and incubating for 20 minutes at 4°C. The antibody-bound macrocycles will complex $^{57}Co^{2+}$. Adding DTPA will complex any

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[3]A carcinoma is a malignant tumour of the epithelium (the outermost layer coating an organ or tissue).

[4]Gel-filtration separates small molecules, *eg.* 2-iminothiolane, from larger ones *eg.* proteins. Separation is achieved by passing the mixture down a column of carbohydrate beads (normally Sephadex G50 or PD-10). The smaller molecules are able to enter the beads, whereas the larger ones cannot. As a consequence of the diminished volume available to them, the larger molecules elute first (Ref.16).

free $^{57}Co^{2+}$. The ^{57}Co-macrocyclic antibody conjugate is separated from ^{57}Co(DTPA) by gel-filtration, and the activity of the two fractions measured. Knowing the concentration of protein in the antibody fraction (from the absorbance at 280 nm), the number of macrocycles per antibody can be calculated.

On addition of yttrium-90 in the final step of the protocol, there is, as well as the desired macrocyclic binding of $^{90}Y^{3+}$, the possibility of $^{90}Y^{3+}$ becoming loosely or *non-specifically* bound to the antibody. Non-specific binding sites include peptide bonds along the protein backbone and the amide and carboxylate groups of amino acid side chains, *eg.* of asparagine and glutamic acid.

In the body the loosely bound radionuclide may be complexed by transferrin and transported to the liver. Ultimately, the ^{90}Y-transferrin complex would be catabolised (broken down) and the free $^{90}Y^{3+}$ would localise in the bone marrow. Direct release of the non-specifically bound $^{90}Y^{3+}$ into the blood would also result in bone marrow localisation (Section 4.8).

To avoid such a hazardous distribution of radionuclide, three precautions are taken when radiolabelling. First, a buffer is used which competes with the non-specific sites on the protein for the metal ion, *eg.* ammonium acetate. Second, radiolabelling is effected at a pH at which the majority of the amino acid side chains will be protonated, *eg.* pH 4-6. Third, after radiolabelling, the ^{90}Y-MoAb conjugate is challenged with another complexing agent such as EDTA or DTPA. Gel-filtration and size exclusion HPLC then remove any small complexes formed.

Before injecting into the body, the radiolabelled MoAb (VI) must be tested against unmodified MoAb (I) for any loss in immunoreactivity. The test is a technique known as affinity chromatography[16,17]:

^{90}Y-B72.3 is incubated with cells bearing the TAG-72 antigen. The mixture is then passed down a column covalently attached to which are groups (X, for example) with a high affinity for *cell-bound* B72.3 (and not free ^{90}Y-B72.3, which will elute rapidly). The cell-bound ^{90}Y-B72.3 is eluted with a high concentration of soluble X. The concentrations of cell-bound and non-cell bound ^{90}Y-B72.3 can then be compared with a similar experiment in which the cells bearing TAG-72 are incubated with unmodified B72.3. Typically, immunoreactivity is lost when B72.3 is linked to more than two macrocycles.

PART 3: BIODISTRIBUTION STUDIES[5]

4.7 INTRODUCTION

As a prerequisite to treating human patients, the radiolabelled MoAb must first undergo trials in animals. Mice were used in our case. It must be emphasised that due to the different constitutions of mice and men, what happens to the radiolabelled MoAb in a mouse is not necessarily related directly to what happens to it in a human being. Animal experiments merely provide a guideline. The trials involve two stages:

(a) Injection of the radiolabelled MoAb into athymic (immuno-suppressed) mice to assess whether it remains stable *in vivo*. Athymic mice will be the type used in the tumour regression studies of stage (b).

(b) If the radiolabelled MoAb maintains its stability, it can be injected into tumour-bearing athymic mice. This is the

[5]Performed by Alice Harrison and Carol Walker at the Medical Research Council Radiobiology Unit, Harwell.

crucial test to determine the effectiveness of the radiolabelled MoAb as a therapeutic agent. Tumour regression can be monitored by histological techniques and by measuring the size of the tumour directly.

At the time of writing, the trials have reached the first stage.

Before embarking on any of these experiments, two important controls were required, namely to determine the fate of the free $^{90}Y^{3+}$ and of ^{90}Y(DOTA) in the mouse.

Figures given in the following sections, unless otherwise stated, refer to the percentage of the injected dose of radioactivity per gramme of tissue - each is the average of data recorded for three or more mice.

4.8 THE FATE OF FREE YTTRIUM(III)

$^{90}Y^{3+}$ was injected into athymic mice as its citrate, acetate and chloride salts [Y(citrate), $Y(acetate)_3$ and YCl_3 respectively] in sodium phosphate buffered saline solution. After 24 hours, the mice were killed and the retention of radioactivity in the various tissues measured (Table 4.1).

TISSUE	YCl_3 %Dose/g	$Y(acetate)_3$ %Dose/g	Y(citrate) %Dose/g
BLOOD	0.06 ± 0.03	0.08 ± 0.05	0.05 ± 0.02
KIDNEYS	0.71 ± 0.06	0.39 ± 0.05	2.11 ± 0.11
LIVER	21.9 ± 2.69	20.8 ± 4.09	1.79 ± 0.04
LUNGS	0.41 ± 0.16	0.37 ± 0.11	0.80 ± 0.17
SPLEEN	3.13 ± 0.08	3.92 ± 0.70	0.25 ± 0.07
BONE	3.44 ± 1.10	3.04 ± 0.95	13.3 ± 4.42
%RET	41	42	7

Table 4.1: *Murine distribution after 24 hours of $^{90}Y(III)$ injected as three different salts; %RET = percentage retention of original dose (whole body retention).*

For the chloride and acetate salts, the highest amount of yttrium was found in the liver, probably as a result of transferrin uptake, with significant amounts in the bone and spleen. In the case of the citrate salt, the highest amount of yttrium was found in the bone, suggesting that a mechanism other than just transferrin uptake determines the fate of the yttrium(III) citrate. No skin measurements were made.

These results are similar to another study[18] in which $^{90}Y(acetate)_3$ was injected into normal and leukaemic mice. In both instances, $^{90}Y^{3+}$ was found to localise in, in decreasing amounts, the bone, skin, liver and kidney.

4.9 THE FATE OF THE YTTRIUM(III)-DOTA COMPLEX

Twenty four hours after injection of $^{90}Y(DOTA)$ into athymic mice, the amount of radioactivity in the bone and bone marrow was too low to measure, while the amounts in the liver, lungs and spleen were measurable but still very low: 0.02, 0.03 and 0.04% respectively (Table 4.2).

TISSUE	%Dose/g	%Dose
BLOOD	0.003	0.01
KIDNEYS	0.22	0.13
LIVER	0.02	0.04
LUNGS	0.03	0.004
SPLEEN	0.04	0.003
STOMACH	--	0.04
SMALL INTESTINE	--	0.12
LARGE INTESTINE	--	0.19
URINE	0.17	0.08

Table 4.2: *Murine distribution of $^{90}Y(III)$ 24 hours after injection of $^{90}Y(DOTA)$.*

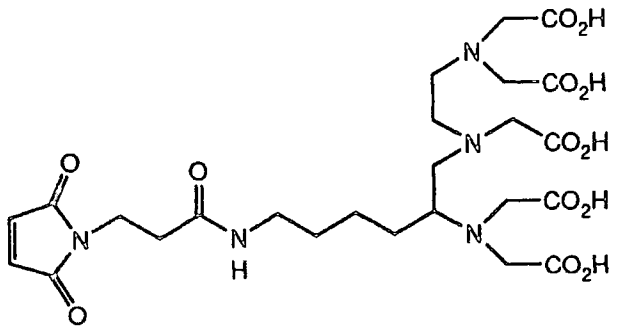
Approximately 0.5% of the original dose was retained after 24 hours, in stark contrast to the much greater retentions of $^{90}Y^{3+}$ when administered as its chloride, acetate and citrate salts (Table 4.1), implying that Y(DOTA) is rapidly cleared from the body and, more importantly, that it remains kinetically stable *in vivo*.

4.10 BIODISTRIBUTION OF THE ^{90}Y-LABELLED DOTA-MONOCLONAL ANTIBODY CONJUGATE AND COMPARISON WITH THE BIODISTRIBUTION OF THE ^{90}Y-LABELLED DTPA-MONOCLONAL ANTIBODY CONJUGATE

Table 4.3 compares, over a 120 hour period, the biodistribution of $^{90}Y^{3+}$ in athymic mice injected with the ^{90}Y-DOTA-[mal]-B72.3 conjugate (mal = the maleimide spacer group) with the biodistribution of $^{90}Y^{3+}$ in mice injected with the ^{90}Y-DTPA-[mal]-B72.3 conjugate [DTPA linked to the MoAb through its maleimide derivative (72)]. Table 4.4 expresses these figures in terms of the tissue to blood ratio.

As B72.3 is unreactive towards normal tissue, the ^{90}Y-labelled MoAb, whether as the DOTA or DTPA conjugate, should remain in the blood and extracellular fluid (with an expected 50% blood level after 24 hours). Any build-up of activity in other tissues will be a result of the dissociation of $^{90}Y^{3+}$ from the MoAb conjugate or the catabolism of the ^{90}Y-labelled MoAb. The tissue to blood ratio is thus an indicator of the *in vivo* stability of the ^{90}Y-labelled MoAb. For a safe therapy, this ratio should not show a substantial increase over a given time, particularly that for a dose-limiting organ such as bone (here represented by the femur).

This criterion is met for both radiolabelled MoAb conjugates, implying that they remain intact *in vivo*. There are small differences, however, which suggest that it would be preferable to use ^{90}Y-DOTA-



(Z2)

TISSUE	^{90}Y-DTPA-[mal]-B72.3			^{90}Y-DOTA-[mal]-B72.3		
	24h	90h	120h	24h	77h	120h
BLOOD	13.1	8.78	4.72	11.9	8.60	5.68
LIVER	4.19	3.53	7.89	4.76	4.27	4.72
KIDNEYS	3.37	2.49	2.39	3.05	2.20	1.78
LUNGS	5.23	2.72	2.51	3.48	2.63	2.23
SPLEEN	2.54	2.52	3.77	3.19	2.76	3.24
FEMUR	1.82	1.71	2.10	1.70	1.43	1.67

Table 4.3: *Murine distribution (%injected dose/g of tissue) of $^{90}Y^{3+}$ at various time intervals after injection of ^{90}Y-DTPA-[mal]-B72.3 and ^{90}Y-DOTA-[mal]-B72.3.*

TISSUE	^{90}Y-DTPA-[mal]-B72.3			^{90}Y-DOTA-[mal]-B72.3		
	24h	90h	120h	24h	77h	120h
LIVER	0.32	0.42	1.53	0.39	0.58	0.97
KIDNEYS	0.26	0.28	0.40	0.27	0.31	0.33
LUNGS	0.43	0.31	0.51	0.30	0.37	0.40
SPLEEN	0.19	0.29	0.65	0.28	0.34	0.57
FEMUR	0.14	0.20	0.44	0.14	0.19	0.29

Table 4.4: *Murine tissue to blood ratio of $^{90}Y^{3+}$ at various time intervals after injection of ^{90}Y-DTPA-[mal]-B72.3 and ^{90}Y-DOTA-[mal]-B72.3.*

[mal]-B72.3 for RIT. In particular, the marginal increase in the tissue to blood ratio seen for all tissues for both radiolabelled MoAb conjugates is more marked in the case of the radiolabelled DTPA conjugate, especially with regard to the liver and femur. Furthermore, considering Table 4.3, there is a small increase in radioactivity in the femur for ^{90}Y-DTPA-[mal]-B72.3 whereas there is a small *decrease* in radioactivity in the femur for ^{90}Y-DOTA-[mal]-B72.3.

4.11 SUMMARY

The aminobutyl C-functionalised derivative of DOTA was successfully synthesized from the methyl ester of lysine in eight steps with an overall yield of 7%. Linkage of this derivative *via* a maleimide spacer group to the monoclonal antibody B72.3 gave an average of two macrocycles per antibody. The modified MoAb complexed $^{90}Y^{3+}$ efficiently [70%, pH 5.5, 0.1 M NH_4OAc, 310K, 1.5h incubation period, DTPA quench (over 25 min, final [DTPA] = 5 x 10^{-3} M)] and with no loss of immunoreactivity. Preliminary animal experiments have indicated that ^{90}Y-DOTA-[mal]-B72.3 is slightly more stable than ^{90}Y-DTPA-[mal]-B72.3 *in vivo*. Results of tumour regression studies with ^{90}Y-DOTA-[mal]-B72.3 are awaited.

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CHAPTER FIVE

EXPERIMENTAL

5.1 SYNTHETIC EXPERIMENTAL

5.1.1 Reagents and Techniques

All reactions, except those involving water as the solvent and detosylations, were carried out under nitrogen. Water throughout refers to deionised water. Other solvents were distilled prior to use from the appropriate drying agent: diethyl ether (lithium aluminium hydride); tetrahydrofuran (sodium benzophenone); ethanol and methanol (from their respective magnesium alkoxides); toluene (sodium); ethyl acetate (calcium hydride); dichloromethane and chloroform (phosphorus pentoxide); triethylamine (3A molecular sieves). Acetonitrile was Aldrich HPLC grade. DMF and borane-THF (1.0 M) were obtained from Aldrich as Sure-Seal reagents, pyridine from the same company as a Gold Label reagent. Organic extracts were dried over anhydrous potassium carbonate.

Melting points were obtained on a Kofler block and are uncorrected. The optical rotations quoted for compounds (62), (63) and (65) were measured with a Thorn-Automation NPL polarimeter. HPLC, analytical and semi-preparative, was performed on a Varian Vista 5500/Polychrom 9060 system. Columns and gradient elution conditions were as follows:

CATION EXCHANGE: Column: SynChropak CM300

TIME(min)	%A	%B	%C
0	80	0	20
5	60	20	20
10	0	80	20

A = H_2O;
B = Ammonium acetate (1.0 M, pH 5.6);
C = CH_3CN

ANION EXCHANGE: Column: SynChropak AX100

TIME(min)	%A	%B	%C
0	70	10	20
20	0	80	20

A = B = C = Same as for cation exchange (above)

REVERSE PHASE: Column: Spherisorb SODS2

TIME(min)	%A	%B	%C
0	95	0	5
20	5	0	95

A = 0.1% trifluoroacetic acid/H_2O;
C = 0.1% trifluoroacetic acid/CH_3CN

Flow rates were 1.4 ml/min (analytical) and 4.0 ml/min (semi-preparative). Water refers to water purified by the Milli-Q system. Other solvents were Aldrich HPLC grade. The wavelength monitored was 254 nm.

Infrared spectra were recorded as thin films, KBr discs or Nujol mulls on a Perkin-Elmer 577 spectrophotometer.

Proton and carbon NMR spectra were recorded on a Bruker AC 250 spectrometer (250.13 and 62.90 MHz respectively). The only 60 MHz ^1H NMR spectrum was recorded on a Perkin-Elmer R-24B spectrometer. Chemical shifts are quoted in ppm. TMS was the internal standard for $CDCl_3$ solutions. D_2O solutions were externally referenced to TMS or internally referenced to t-butanol. CD_3CN solutions were also internally referenced to TMS. $(CD_3)_2SO$ solutions were externally referenced to TMS.

Mass spectra were obtained on a VG7070E spectrometer and were recorded in the DCI mode unless otherwise stated. In the DCI and CI modes ammonia was employed as the impingent gas. DCI samples were presented as dichloromethane solutions unless otherwise stated. The matrix used for FAB samples was glycerol unless otherwise stated.

Accurate masses were measured in the DCI mode.

Column chromatography refers to flash chromatography using Merck 60 9385 silica.

5.1.2 Synthesis of DOTA (7)

1,4,7,10-Tetrakis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (15). 1,2-Bis(4-toluenesulphonato)ethane (7.28g, 19.7 mmol) in DMF (100 ml) was added dropwise over 3h to 1,8-bis(4-toluenesulphonamido)-3,6-bis(4-toluenesulphonyl)-1,3,6,8-tetraazaoctane (15.0g, 19.7 mmol) and caesium carbonate (13.5g, 41.3 mmol) in DMF (200 ml). The reaction mixture was stirred (20°C, 18h), heated at 60°C (3h) and solvent evaporated. The residue was taken up into hot chloroform (200 ml); this solution was washed with water (2 x 20 ml), dried and evaporated. The residue was taken into hot toluene (100 ml). After stirring (100°C, 1h), the suspension was filtered to afford the *title compound* as a white powder (10.0g, 64%; lit.[1] 80%). MPt. 278-280°C; Analysis Found: C, 55.1; H, 5.6; N, 6.8. Required for $C_{36}H_{44}N_4S_4O_8$: C, 54.8; H, 5.6; N, 7.1%; IR ν_{max} (film): 3060, 1600, 1340 (SO_2) and 1160 (SO_2) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.44 (12H,s,$ArCH_3$), 3.43 (16H,s,NCH_2), 7.33 and 7.69 (16H,AB system,$\underline{J}_{AB}$ 8.1 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.5 (CH_3); 52.3 (NCH_2); 127.7, 129.9; 134.0, 143.9; MS m/z (Intensity%): 789 (30,$\underline{M}^+$+1), 633 (6,$\underline{M}^+$-155), 479 (17,$\underline{M}^+$-309).

1,4,7,10-Tetraazacyclododecane tetrahydrochloride monohydrate (16). Ammonia (300 ml) was condensed into a mixture of the tetratosylated macrocycle (15) (4.33g, 5.49 mmol) in THF (20 ml) and ethanol (10 ml) at -78°C. Lithium (1.93g, 278 mmol) was added in small portions to this mixture and an intense blue colour developed which discharged over 1-10

min. The reaction mixture was allowed to warm to room temperature, water (40 ml) was added and solvents evaporated. The residue was dissolved in hydrochloric acid (20 ml, 6M), washed with diethyl ether (2 x 30 ml) and evaporated. Recrystallisation from hydrochloric acid (6M) gave the *title compound* as a white solid (1.27g, 72%; lit.[1] >90%). Analysis Found: C, 28.5; H, 7.9; N, 16.5. Required for $C_8H_{20}N_4.4HCl.H_2O$: C, 28.6; H, 7.7; N, 16.7%; ^{1}H NMR δ_H (D_2O): 3.25 (s,NCH_2); ^{13}C NMR δ_C (D_2O): 41.3; MS m/z (Intensity%) CI: 173 (100,M^++1).

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid hydrochloride dihydrate (DOTA) (7). Chloroacetic acid (1.47g, 15.5 mmol) in water (5 ml) was neutralised with sodium hydroxide (0.62g, 15.5 mmol) in water (5 ml), keeping the temperature between 0-5^{o}C. 1,4,7,10-Tetraazacyclododecane (0.61g, 3.53 mmol) was added to this solution which was then heated at 80^{o}C (24h), keeping the pH between 9 and 10 with periodic addition of a sodium hydroxide solution (5 ml, 3.1M). The reaction mixture was concentrated under reduced pressure and the pH lowered to 2.5 with hydrochloric acid (6M). After 12h a white microcrystalline solid precipitated and was collected to give the *title compound* (0.84g, 50%; lit.[2] 82%). MPt. 265-267^{o}C; Analysis Found: C, 40.1; H, 6.9; N, 11.8. Required for $C_{16}H_{28}N_4O_8.HCl.2H_2O$: C, 40.3; H, 6.9; N, 11.75%; IR ν_{max} (KBr): 2500br (NH^+), 1690 (CO) and 1620 (CO_2^-) cm^{-1}; ^{1}H NMR δ_H (D_2O, pD 5.0, t-BuOD): 3.25 (16H,s,$N(CH_2)_2N$), 3.61 (8H,s,CH_2CO); ^{13}C NMR δ_C (D_2O): 49.0 (NCH_2), 56.9 ($\underline{C}H_2CO$), 178.6 (CO); MS m/z (Intensity%) (FAB, m-nitrobenzyl alcohol): 405 (3,M^++1).

Yttrium(III) Complex of DOTA (35). DOTA (7) (5.0mg, 0.01 mmol) in water (1 ml) was added to yttrium nitrate hexahydrate (4.7mg, 0.01 mmol) in water (1 ml) and the pH adjusted to 4.0 with sodium hydroxide (1M). The

volume of the solution was reduced to approximately 0.1 ml. Vapour diffusion of propan-2-ol gave colourless tetragonal crystals (3mg, 47%). Analysis Found: C, 31.6; H, 4.1; N, 9.0. Required for $C_{16}H_{24}N_4O_8YNa.5H_2O$: C, 31.9; H, 4.0; N, 9.3%; ^{1}H NMR δ_H (D_2O, pD 5.0, t-BuOD): 2.40 (4H,AB system,J_{AB} 14 Hz,NCH_2), 2.73 (8H,AB system,J_{AB} 7.3 Hz,NCH_2), 3.19 and 3.57 (8H,AB system,J_{AB} 16 Hz,CH_2CO), 3.38 (4H,AB system,NCH_2); MS m/z (Intensity%) FAB: 491 (0.2, M^++2).

5.1.3 Synthesis of TRITA (8)

1,4,7,10-Tetrakis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclotridecane (17). The *title compound* was synthesized according to the method for (15) to give a white solid (70%). MPt. 117-118^{o}C (from toluene); Analysis Found: C, 55.6; H, 6.0; N, 6.8. Required for $C_{37}H_{46}N_4S_4O_8$: C, 55.4; H, 5.7; N, 7.0%; IR ν_{max} (Nujol): 3020, 1600, 1350 and 1160 cm^{-1}; ^{1}H NMR δ_H ($CDCl_3$): 2.05 (2H,br,quin,$NCH_2CH_2CH_2N$), 2.43 (12H,s,$ArCH_3$), 3.16 (4H,br,t,$NCH_2CH_2CH_2N$), 3.29 (4H,s,NCH_2), 3.32-3.38 (8H,m,NCH_2), 7.30-7.35 (8H,m,ArH), 7.65-7.68 (8H,AB system,J_{AB} 8.0 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.6 (CH_3); 47.6, 49.1, 51.7 (NCH_2); 127.6, 127.9, 129.9; 133.7, 134.4, 143.8, 144.1; MS m/z (Intensity%) CI: 803 (46,M^++1), 647 (21,M^+-155).

1,4,7,10-Tetraazacyclotridecane hexahydrate (18). The tetratosylated cycle (17) (6.55g, 8.16 mmol) and phenol (4.55g, 48.4 mmol) were dissolved in 45% w/v hydrobromic acid in glacial acetic acid (80 ml) and the mixture heated at 80^{o}C (1 week) and filtered. Anion exchange chromatography (Amberlite IRA-400) with water elution and evaporation of eluent afforded the *title compound* as a waxy white solid (0.48g, 20%). MPt. 40-42^{o}C (lit.[3] 40-41^{o}C); Analysis Found: C, 55.0; H, 12.4; N, 28.1.

Required for $C_9H_{22}N_4.6H_2O$: C, 54.8; H, 11.9; N, 28.4%; ^{1}H NMR δ_H ($CDCl_3$): 1.69 (2H,quin,J 5.2 Hz,$NCH_2\underline{CH}_2CH_2N$), 2.31 (4H,br,s,NH), 2.64-2.77 (16H,m,NCH_2); ^{13}C NMR δ_C ($CDCl_3$): 28.8 ($NCH_2\underline{C}H_2CH_2N$); 47.2, 47.5, 48.7, 49.8 (NCH_2); MS m/z (Intensity%): 188 (34,$\underline{M}^+$+2), 187 (100,$\underline{M}^+$+1).

1,4,7,10-Tetraazacyclotridecane-1,4,7,10-tetraacetic acid trihydrochloride (TRITA) (8). The *title compound* was synthesized according to the method for (7) to give a glassy solid (0.30g, 60%; lit.[4] 18%). MPt. 184-185^{o}C (dec) [lit.[4] 185^{o}C (dec)]; Analysis Found: C, 38.6; H, 6.4; N, 10.8. Required for $C_{17}H_{30}N_4O_8.3HCl$: C, 38.7; H, 6.3; N, 10.6%; ^{1}H NMR δ_H (D_2O): 2.03 (2H,br,quin,$CH_2\underline{CH}_2CH_2$), 3.24 (4H,br,t,$\underline{CH}_2CH_2\underline{CH}_2$), 3.29 (12H,s,$N(CH_2)_2N$), 3.78 (4H,s,$CH_2CO_2$), 3.84 (4H,s,$CH_2CO_2$); ^{13}C NMR δ_C (D_2O): 19.3 ($CH_2\underline{C}H_2CH_2$); 50.4, 51.2, 52.0, 52.5, 53.7, 54.7; 169.1, 171.7 (CO); MS m/z (Intensity%) FAB: 419 (4,$\underline{M}^+$+1).

5.1.4 Synthesis of TETA (9)

1,4,8,11-Tetraazacyclotetradecane-1,4,8,11-tetraacetic acid dihydrate (TETA) (9). The *title compound* was synthesized according to the method for (7) to give a white crystalline solid (58%; lit.[4] 51%). MPt. 255-257^{o}C (dec); Analysis Found: C, 45.7; H, 8.1; N, 12.0. Required for $C_{18}H_{32}N_4O_8.2H_2O$: C, 46.2; H, 7.7; N, 12.0%; IR ν_{max} (KBr): 2900br (OH), 2500br (NH^+), 1720 (CO) and 1630 (CO_2^-) cm^{-1}; ^{1}H NMR δ_H (D_2O): 1.85 (4H, quin,J 6.6 Hz,$NCH_2\underline{CH}_2CH_2N$), 3.07 (8H,t,J 6.6 Hz,$NCH_2CH_2\underline{CH}_2N$), 3.14 (8H, s,$N(CH_2)_2N$), 3.51 (8H,s,$CH_2CO$); ^{13}C NMR δ_C (D_2O): 22.5 ($NCH_2\underline{C}H_2CH_2N$), 51.5 ($N\underline{C}H_2CH_2\underline{C}H_2N$,$N(CH_2)_2N$), 56.1 ($\underline{C}H_2CO$), 175.5 (CO); MS m/z (Intensity%) FAB: 429 (0.3,$\underline{M}^+$-3).

5.1.5 Synthesis of DOTA-BMA (10)

1,4,7-Tris(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (19). 1,4,7,10-tetraazacyclododecane (0.15g, 0.89 mmol) in dichloromethane (5 ml) was added dropwise over 10 min to 4-toluenesulphonyl chloride (0.36g, 1.87 mmol) and triethylamine (0.26 ml, 1.87 mmol) in dichloromethane at 0°C. Stirring was continued (0°C, 2h) and solvent evaporated. Column chromatography (1% methanol in dichloromethane) gave the *title compound* as a glassy oil (0.06g, 11%). IR ν_{max} (film): 3310 (NH), 1600, 1340 and 1160 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 1.50 (1H,br,s,NH), 2.43 (6H,s, $ArCH_3$), 2.46 (3H,s,$ArCH_3$), 2.91-2.96 (4H,m,NCH_2), 3.09-3.11 (4H,m,NCH_2), 3.32 (4H,t,J 5.7 Hz,$NTsCH_2CH_2NTs$), 3.54 (4H,t,J 5.7 Hz,$NTsCH_2CH_2NTs$), 7.26-7.36 (6H,m,ArH), 7.63-7.67 (4H,AB system,J_{AB} 8.1 Hz,ArH), 7.75-7.78 (2H,AB system,J_{AB} 8.1 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 22.9 (CH_3); 49.7, 50.6, 50.8 (NCH_2); 127.4, 127.8, 129.8; 135.1, 135.6, 141.4, 143.5; MS m/z (Intensity%): 635 (100,M^++1), 479 (15,M^+-155), 325 (13,M^+-309).

A second compound, *1,7-bis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane*, was also isolated as a glassy oil (0.06g, 10%). ^1H NMR δ_H ($CDCl_3$): 2.44 (6H,s,$ArCH_3$), 3.16-3.19 (8H,m,$NHCH_2CH_2N$), 3.40-3.41 (8H,m, $NHCH_2CH_2N$), 7.36 and 7.68 (8H,AB system,J_{AB} 8.0 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.5 (CH_3); 49.2, 49.5 (NCH_2); 127.3, 130.2; 134.1, 144.5; MS m/z (Intensity%): 481 (44,M^++1).

10-(N,N-Dimethylcarboxamidomethyl)-1,4,7-tris(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (20). 2-Bromo-N,N-dimethylethanamide (0.08g, 0.48 mmol) was added to the tritosylated macrocycle (19) (0.20g, 0.32 mmol) and potassium carbonate (0.07g, 0.48 mmol) in acetonitrile (10 ml) at 80°C. The reaction mixture was refluxed (5h) and more 2-

bromo-N,N-dimethylethanamide added (0.04g, 0.24 mmol). Further reflux (15h) and evaporation gave a residue which was column chromatographed (1% methanol in dichloromethane) to give the *title compound* as a colourless oil (0.21g, 90%). IR ν_{max} (film): 3020, 1650 (CO), 1600, 1340 and 1160 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.43 (6H,s,$ArCH_3$), 2.46 (3H,s, $ArCH_3$), 2.89 and 2.98 (6H,2xs,NCH_3), 3.09-3.12 (8H,m,NCH_2), 3.32-3.34 (4H,m,NCH_2), 3.52-3.55 (6H,m,NCH_2,NCH_2CO), 7.27-7.36 (6H,m,ArH), 7.60-7.63 (4H,AB system,J_{AB} 8.1 Hz,ArH), 7.71-7.74 (2H,AB system,J_{AB} 8.1 Hz, ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.5 (CH_3); 35.2, 36.7 (NCH_3); 48.8, 50.2, 50.9, 52.6, 54.3 (NCH_2); 127.5, 127.6, 129.7; 134.5, 135.8; 170.2 (CO); MS m/z (Intensity%): 720 (100,M^++1), 564 (18,M^+-155).

4-(N,N-Dimethylcarboxamidomethyl)-1-(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane trihydrobromide (21). The tritosylated macrocycle (20) (0.21g, 0.29 mmol) was dissolved in 45% w/v hydrobromic acid in glacial acetic acid (10 ml) and to this solution was added phenol (0.25g, 2.60 mmol). The mixture was heated at 80°C (2 weeks) and filtered to give the *title compound* as a cream solid (0.09g, 48%). ^1H NMR δ_H (D_2O): 2.34 (3H,s,$ArCH_3$), 2.92 (6H,s,NCH_3), 3.08-3.25 (14H,m,NCH_2), 3.61 (2H,s, NCH_2CO), 3.93-4.02 (2H,m,$NTsCH_2CH_2NCH_2CO$), 7.40 and 7.70 (4H,AB system, J_{AB} 8.1 Hz,ArH); MS m/z (Intensity%) CI: 412 (100,M^++1).

1-(N-Benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraazacyclododecane (22). 2-Bromo-N-benzyl-N-methylethanamide (113mg, 0.47 mmol) in DMF (3 ml) was added dropwise over 45 min to 1,4,7,10-tetraazacyclododecane (100mg, 0.58 mmol) and potassium carbonate (80mg, 0.58 mmol) in DMF (5 ml) at room temperature. The temperature was raised to 50°C and stirring continued (24h). Solvent was evaporated and the residue taken into dichloromethane (2 ml) and filtered. Evaporation of the filtrate

and preparative HPLC (cation exchange) gave the *title compound* as a colourless oil (58mg, 30%). $\underline{R}_t$ 8.1 min; ^{1}H NMR δ_H ($CDCl_3$): 2.84 and 2.93 (3H,2xs,CH_3), 2.96 (12H,br,s,NHC$\underline{H}_2$), 3.05 (4H,br,s,NCH_2), 3.57 and 3.64 (2H,2xs,CH_2Ph), 4.47 and 4.57 (2H,2xs,CH_2CO), 7.20-7.40 (5H,m,ArH); MS m/z (Intensity%): 335 (7,$\underline{M}^+$+2), 334 (30,$\underline{M}^+$+1).

The following compound was also isolated: *1,4-bis(N-benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraazacyclododecane* or *1,7-bis(N-benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraazacyclododecane* as a colourless oil (31mg, 29%). $\underline{R}_t$ 7.6 min; ^{1}H NMR δ_H ($CDCl_3$): 2.77-3.07 (22H,m,NHC$\underline{H}_2$, NCH_2), 3.44 and 3.47 (2H,2xs,CH_2Ph), 3.56 and 3.61 (2H,2xs,CH_2Ph), 4.46 and 4.51 (2H,2xs,CH_2CO), 4.55 (2H,s,CH_2CO), 7.11-7.36 (10H,m,ArH); MS m/z (Intensity%): 495 (36,$\underline{M}^+$+1).

1-(N-Benzyl-N-methylcarboxamidomethyl)-4,7,10-tris(ethyoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (23). Ethyl bromoacetate (68μl, 0.61 mmol) was added dropwise over 10 min to the macrocyclic amide (22) (58mg, 0.17 mmol) and potassium carbonate (84mg, 0.61 mmol) in DMF (2 ml) and the mixture heated at 60^{o}C (10h). Solvent was removed under reduced pressure and the residue taken into dichloromethane (2 ml) and filtered. Evaporation of the filtrate and preparative HPLC (cation exchange) gave the *title compound* as a colourless oil (16mg, 16%). $\underline{R}_t$ 8.8 min; Found M^++1: 592.3855. Required for $C_{30}H_{50}N_5O_7$: 592.3710; ^{1}H NMR δ_H ($CDCl_3$): 1.24-1.31 (9H,m,$CH_2C\underline{H}_3$), 2.36 (16H,br,s,$N(CH_2)_2N$), 2.90 and 2.91 (3H,2xs,NCH_3), 3.33 (8H,br,s,NCH_2CO), 4.19 (6H,q,$\underline{J}$ 7.1Hz, $C\underline{H}_2CH_3$), 4.52 (2H,s,CH_2Ph), 7.21-7.33 (5H,m,ArH); MS m/z (Intensity%): 593 (21,$\underline{M}^+$+2), 592 (59,$\underline{M}^+$+1), 333 (8,$\underline{M}^+$+3-3CH_2CO_2Et).

4-(N-Benzyl-N-methylcarboxamidomethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DOTA-BMA) (10). The triester (23) (16mg, 0.03 mmol) and tetramethylammonium hydroxide (30mg, 0.17 mmol) were added to a solution of water (0.5 ml) and methanol (0.5 ml). The reaction mixture was heated at 100°C (6h), solvents removed under reduced pressure and the residue purified by preparative HPLC (anion exchange) to give the *title compound* as a glassy solid (2mg, 15%). R_t 7.9 min; ^{1}H NMR δ_H (D_2O, pD 5.0, t-BuOD): 2.93 and 2.99 (3H,2xs,CH_3), 3.11 (8H,br,s,NCH_2CH_2N), 3.31-3.44 (10H,br,m,NCH_2CH_2N,NCH_2CO), 3.63 and 3.65 (2H,2xs,CH_2Ph), 3.70, 3.76 and 3.81 (4H,3xs,NCH_2CO), 4.54 and 4.55 (2H,2xs,NCH_2CON), 7.21-7.41 (5H,m,ArH); MS m/z (Intensity%) FAB: 508 (0.4,M^++1).

5.1.6 Synthesis of DDOTRA (11)

4,7,10-Tris(4-toluenesulphonyl)-1-oxa-4,7,10-triazacyclododecane (24). 1,5-Bis(4-toluenesulphonato)-3-oxapentane (3.66g, 8.85 mmol) in DMF (50 ml) was added dropwise over 3h to a vigorously stirred solution of 1,5-bis(4-toluenesulphonamido)-3-(4-toluenesulphonyl)-3-azapentane (5g, 8.85 mmol) and caesium carbonate (6.06g, 18.6 mmol) in DMF (150 ml) at RT. The reaction was allowed to stir for a further 18h and then heated at 60°C for 3h. Solvent was removed under reduced pressure and the pale yellow residue taken up into dichloromethane (100 ml); this solution was washed with water (2 x 20 ml), dried and solvent evaporated. The residue was recrystallised from dichloromethane-hexane to give the *title compound* as a white solid (4.26g, 76%; lit.[5] 52%). MPt. 190-192°C; Analysis Found: C, 55.5; H, 6.0; N, 6.3. Required for $C_{29}H_{37}N_3O_7S_3$: C, 55.8; H, 5.8; N, 6.6%; IR ν_{max} (film): 3040, 1600, 1350 (SO_2) and 1165 (SO_2) cm^{-1}; ^{1}H NMR δ_H ($CDCl_3$): 2.48 (6H,s,$ArCH_3$), 2.51 (3H,s, $ArCH_3$), 3.20-3.27 (8H,m,NCH_2), 3.57 (4H,t,J 6.3 Hz,NCH_2), 3.70 (4H,t,J

3.9 Hz,$NCH_2\underline{CH_2}O$), 7.36 and 7.68 (8H,AB system,$\underline{J}_{AB}$ 6.7 Hz,ArH), 7.40 and 7.86 (4H,AB system,$\underline{J}_{AB}$ 8.1 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.5 (CH_3); 47.9, 50.6 (NCH_2); 72.0 (OCH_2); 127.3, 127.6, 129.8; 135.0, 136.7, 143.4, 143.7; MS m/z (Intensity%): 636 (8,$\underline{M}^+$+1), 480 (2,$\underline{M}^+$-155), 327 (3,$\underline{M}^+$-308).

1-Oxa-4,7,10-triazacyclododecane (25). The tritosylated macrocycle (24) (2.00g, 3.15 mmol) was dissolved in 45% w/v hydrobromic acid in glacial acetic acid (30 ml) and to this solution was added phenol (2.67g, 28.4 mmol). The mixture was heated at 80°C for 3h and filtered. Anion exchange chromatography (Amberlite IRA-400 converted to the hydroxide form with tetramethylammonium hydroxide) with water elution on the solid collected and evaporation of the eluent afforded the *title compound* as a waxy white solid (311mg, 57%). MPt. 87-89°C; Analysis Found: C, 55.1; H, 11.25; N, 24.0. Required for $C_8H_{19}N_3O$: C, 55.5; H, 11.0; N, 24.3%; IR ν_{max} (Nujol): 3295 (NH) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.33 (3H,br,s,NH), 2.59-2.64 (4H,m,NCH_2), 2.76-2.83 (8H,m,NCH_2), 3.57-3.62 (4H,m,OCH_2); ^{13}C NMR δ_C ($CDCl_3$): 46.3, 46.4 (NCH_2); 66.5 (OCH_2); MS m/z (Intensity%) FAB: 174 (100,$\underline{M}^+$+1).

1-Oxa-4,7,10-triazacyclododecane-4,7,10-triacetic acid hydrochloride monohydrate (ODOTRA) (11). Chloroacetic acid (206mg, 2.18 mmol) in water (1 ml) was neutralised with sodium hydroxide (87mg, 2.18 mmol) in water (1 ml), keeping the temperature between 0-5°C. The triamine (25) (111mg, 0.64 mmol) was added and the temperature raised to 80°C for 24h keeping the pH above 9 with periodic addition of a sodium hydroxide solution (0.5 ml, 4.35 M). The reaction mixture was concentrated under reduced pressure and the pH lowered to 2.5 with hydrochloric acid (6 M). Liquid diffusion of methanol gave the *title compound* as colourless

crystals (145mg, 65%; lit.[5] 70%). MPt. 134-136°C (lit.[5] 132-134°C); Analysis Found: C, 38.2; H, 7.0; N, 9.4. Required for $C_{14}H_{25}N_3O_7.HCl.H_2O$: C, 38.4; H, 6.6; N, 9.6%; IR ν_{max} (KBr): 3000br (OH) and 1690vs (CO) cm^{-1}; ^1H NMR δ_H (D_2O, pD 5.0, t-BuOD): 3.05 (4H,t,J 5.2 Hz,NCH_2), 3.34 (2H,s,CH_2CO_2), 3.49 (4H,br,s,NCH_2), 3.59 (4H,t,J 4.6 Hz,NCH_2CH_2O), 3.83 (4H,s,CH_2CO_2), 3.91 (4H,t,J 4.7 Hz,NCH_2CH_2O); MS m/z (Intensity%) FAB: 348 (9,M^++1).

5.1.7 Synthesis of DTCTA (12)

1,11-Bis(4-toluenesulphonato)-6-(4-toluenesulphonyl)-3,9-dioxa-6-azaundecane (27). 4-Toluenesulphonyl chloride (38.1g, 200 mmol) was added in small batches over 1h to a solution of 3,9-dioxa-6-azaundecan-1,11-diol (26) (9.65g, 50.0 mmol) in pyridine (150 ml) at 0°C. After stirring for 30 min the reaction mixture was left at -10°C for 18h. The solution was poured onto crushed ice, stirring continuously until the ice melted. The water layer was decanted leaving an oily residue which was dissolved in dichloromethane (50 ml) and combined with the subsequent dichloromethane extracts (3 x 50 ml) of the water layer. Washing with hydrochloric acid (1 M, 3 x 50 ml) and water (2 x 50 ml) followed by drying and solvent evaporation gave an oil which was column chromatographed (0.5% methanol in dichloromethane) to give the *title compound* as a colourless oil (15.1g, 46%). Found M^++1: 656.0848. Required for $C_{29}H_{38}NO_{10}S_3$: 656.1659; ^1H NMR δ_H ($CDCl_3$): 2.39 (3H,s,$ArCH_3$), 2.41 (6H, s,$ArCH_3$), 3.28 (4H,t,J 5.7 Hz,NCH_2), 3.50-3.57 (8H,m,OCH_2), 4.08 (4H,t, J 4.5 Hz,$TsOCH_2$), 7.29 and 7.67 (4H,AB system,J_{AB} 8.3 Hz,ArH), 7.34 and 7.77 (8H,AB system,J_{AB} 8.2 Hz,ArH); MS m/z (Intensity%): 656 (2,M^++1).

1,11-Bis(1,2-benzenedicarboximido)-6-(4-toluenesulphonyl)-3,9-dioxa-6-azaundecane (28). The tritosylated compound (27) (1.36g, 2.08 mmol) in DMF (20 ml) was added dropwise over 30 min to a solution of potassium phthalimide (0.77g, 4.14 mmol) in DMF (10 ml) and the mixture heated (90°C, 20h). The solution was poured onto crushed ice with stirring. A white oil separated which, after decanting the aqueous phase, was dissolved in dichloromethane (10 ml). The aqueous phase was extracted with dichloromethane (4 x 25 ml) and the combined dichloromethane solutions dried and evaporated. Recrystallisation from ethyl acetate gave the *title compound* as a white solid (0.77g, 61%). MPt. 78-80°C; Analysis Found: C, 61.7; H, 5.0; N, 6.8. Required for $C_{31}H_{31}N_3O_8S$: C, 61.5; H, 5.1; N, 6.9%; IR ν_{max} (film): 3060, 1770 (imide), 1710 (imide), 1610 and 1595 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.39 (3H,s,$ArCH_3$), 3.29 (4H,t,J 5.8 Hz,$TsNCH_2$), 3.52-3.64 (8H,m,OCH_2CH_2phthal), 3.82 (4H,t,J 5.7 Hz, OCH_2), 7.22 and 7.62 (4H,AB system,J_{AB} 10 Hz,Ar(Ts)H), 7.70 and 7.76 (4H,AB system,J_{AB} 5.7 Hz,ArH), 7.72 and 7.84 (4H,AB system,J_{AB} 5.5 Hz, ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.4 (CH_3); 37.3, 48.4 (NCH_2); 67.8, 69.7 (OCH_2); 123.2, 127.1, 129.5, 133.9; 132.1, 138.4, 144.9; 168.1 (CO); MS m/z (Intensity%) EI: 147 (31,phthalimide).

6-(4-Toluenesulphonyl)-3,9-dioxa-6-aza-1,9-undecanediamine (29). Hydrazine monohydrate (0.12g, 2.44 mmol) was added to a solution of the diphthalimide compound (28) (0.74g, 1.22 mmol) in ethanol (50 ml) at 60°C. After refluxing for 18h, concentrated hydrochloric acid (7.5 ml) was added dropwise to the cooled reaction mixture which was then refluxed for a further 30 min. A white solid precipitated and was removed by filtration. The filtrate was concentrated to a white solid which was suspended in water (5 ml) and filtered. The filtrate was basified with potassium hydroxide pellets, extracted with chloroform

(4 x 5 ml), dried and evaporated to give the *title compound* as a colourless oil (0.37g, 87%). Found M^++1: 346.1242. Required for $C_{15}H_{28}N_3O_4S$: 346.1801; IR ν_{max} (film): 3200br (NH), 1595, 1330 (SO_2) and 1160 (SO_2) cm^{-1}; ^1H NMR δ_H (60 MHz,$CDCl_3$): 1.3 (4H,s,NH_2), 2.3 (3H,s,$ArCH_3$), 2.7 (4H,t,J 5 Hz,NCH_2), 3.2-3.6 (12H,m,OCH_2,NCH_2), 7.3 and 7.5 (4H,AB system,J_{AB} 8 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 20.7 (CH_3); 41.0, 47.9 (NCH_2); 68.9, 72.5 (OCH_2); 126.3, 128.9; 136.1, 142.5; MS m/z (Intensity%): 347 (9,M^++2), 346 (34,M^++1).

1,11-Bis(4-toluenesulphonamido)-6-(4-toluenesulphonyl)-3,9-dioxa-6-azaundecane (30). 4-Toluenesulphonyl chloride (0.52g, 2.75 mmol) was added in small batches over 30 min to a stirred solution of the diamine compound (29) (0.37g, 1.06 mmol) and potassium carbonate (0.38g, 2.75 mmol) in water (30 ml) at 60°C. The temperature was raised to 80°C and stirring was continued for 18h. The cooled solution was decanted leaving a white oily residue which was taken into dichloromethane (10 ml) and combined with the subsequent dichloromethane extracts (4 x 10 ml) of the aqueous phase. Drying and evaporation of the organic phase gave a residue which was column chromatographed (0.5% methanol in dichloromethane) to give the *title compound* as a colourless oil (0.41g, 60%). IR ν_{max} (film): 3290 (NH), 1600, 1330 (SO_2) and 1160 (SO_2) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.35 (9H,s,$ArCH_3$), 3.03 (4H,dt,J 2x5.0 Hz,CH_2NHTs), 3.21 (4H,t,J 5.0 Hz,$TsNCH_2$), 3.39 (4H,t,J 4.7 Hz,OCH_2CH_2NH), 3.46 (4H,t, J 5.0 Hz,$TsNCH_2CH_2O$), 5.82 (2H,t,J 5.9 Hz,NH), 7.25 and 7.71 (8H,AB system,J_{AB} 8.1 Hz,ArH), 7.24 and 7.64 (4H,AB system,J_{AB} 8.1 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.3 (CH_3); 42.7, 48.8 (NCH_2); 69.0, 69.4 (OCH_2); 126.8, 127.0, 129.6; 135.8, 136.9, 143.0, 143.4; MS m/z (Intensity%): 656 (21,M^++3), 655 (21,M^++2), 654 (100,M^++1), 498 (8,M^+-155).

4,10,13-Tris(4-toluenesulphonyl)-1,7-dioxa-4,10,13-triazacyclopentadecane (31). 1,2-Bis(4-toluenesulphonato)ethane (1.75g, 4.73 mmol) in DMF (50 ml) was added dropwise over 3h to a vigorously stirred solution of the tritosylated compound (30) (3.09g, 4.73 mmol) and caesium carbonate (3.24g, 9.93 mmol) in DMF (150 ml) at room temperature. Stirring was continued for a further 18h, whereupon the reaction mixture was heated at 60°C for 3h. Solvent was removed under reduced pressure and the residue taken into dichloromethane; the organic layer was washed (2 x 20 ml), dried and evaporated. Column chromatography (gradient eluent 0.5-1.5% methanol in dichloromethane) afforded the *title compound* as a colourless oil (0.14g, 24%). IR ν_{max} (film): 1600, 1330 and 1165 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.42 (9H,s,ArH), 3.27 (4H,t,J 4.3 Hz,NCH_2CH_2O), 3.40 (4H,s,$N(CH_2)_2N$), 3.48-3.53(12H,m,NCH_2,OCH_2), 7.28 and 7.64 (4H,AB system,J_{AB} 8.1 Hz,ArH), 7.32 and 7.74 (8H,AB system,J_{AB} 8.0 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.4 (CH_3); 42.7, 48.9 (NCH_2); 69.1, 69.5 (OCH_2); 126.9, 127.1, 129.6; 135.8, 137.0, 143.1, 143.4; MS m/z (Intensity%): 682 (3, M^++3), 681 (5,M^++2), 680 (13,M^++1), 526 (17,M^+-153), 370 (23,M^+-309).

1,7-Dioxa-4,10,13-triazacyclopentadecane (32). The tritosylated macrocycle (31) (0.38g, 0.56 mmol) and phenol (0.48g, 5.08 mmol) were dissolved in 45% w/v hydrobromic acid in glacial acetic acid (10 ml). The mixture was heated (80°C,12h) and filtered to give the trihydrobromide salt of the *title compound* as a yellow crystalline solid (0.20g, 79%). Found M^++1: 218.1775. Required for $C_{10}H_{24}N_3O_2$: 218.1869; ^1H NMR δ_H (D_2O): 3.40 (4H,t,J 4.8 Hz,NCH_2), 3.46 (4H,t,J 4.7 Hz,NCH_2), 3.58 (4H,s, $N(CH_2)_2N$), 3.84-3.92 (8H,m,OCH_2); ^{13}C NMR δ_C (D_2O): 41.8 ($N(CH_2)_2N$); 45.1, 46.3 (NCH_2); 64.6, 65.5 (OCH_2); MS m/z (Intensity%) CI: 219 (2, M^++2), 218 (13,M^++1).

The trihydrobromide salt of the *title compound* (0.20g, 0.43 mmol) was dissolved in water (1 ml) and basified (pH 14) by the addition of tetramethylammonium hydroxide. The aqueous layer was extracted with chloroform (10 x 1 ml) and the combined chloroform extracts dried and evaporated to give the *title compound* as a colourless oil (0.09g, 96%). ^{1}H NMR δ_H ($CDCl_3$): 2.40 (3H,br,s,NH), 2.76-2.85 (12H,m,NCH_2), 3.56-3.64 (8H,m,OCH_2).

4,10,13-Tris(benzoxycarbonylmethyl)-1,7-dioxa-4,10,13-triazacyclopentadecane (33). Caesium carbonate (68mg, 0.21 mmol) was added to the triamine (32) (13mg, 0.06 mmol) and 2-bromobenzylacetate (48mg, 0.21 mmol) dissolved in DMF (3 ml). The suspension was stirred (60^{o}C, 36h), monitoring the reaction by analytical HPLC (cation exchange). Solvent was evaporated, the residue taken into dichloromethane (2 ml), filtered and the filtrate evaporated to give an orange-brown residue. Preparative HPLC (cation exchange) gave the *title compound* as a colourless oil (7mg, 18%). R_t 8.8 min; ^{1}H NMR δ_H ($CDCl_3$): 2.84 (4H,s,$N(CH_2)_2N$), 2.87 (4H,t,J 5.0 Hz,NCH_2), 2.94 (4H,t,J 5.0 Hz,NCH_2), 3.47 (6H,s,NCH_2CO), 3.50-3.56 (8H,m,OCH_2), 5.12 (6H,s,CO_2CH_2), 7.34 (15H,s,ArH); MS m/z (Intensity%): 662 (3,M^++1), 661 (6,M^+), 571(3,M^+-C_7H_6), 514 (4,M^++2-CH_2CO_2Bz).

Also isolated was the mixed *dibenzyl methyl ester derivative of (33), (34)* as a colourless oil (6mg, 17%). R_t 6.9 min; ^{1}H NMR δ_H ($CDCl_3$): 2.83-2.93 (12H,m,NCH_2), 3.47-3.58 (14H,m,OCH_2,CH_2CO), 3.67 (3H,s,CH_3), 5.13 (4H,s,CH_2Ph), 7.35 (10H,s,ArH); MS m/z (Intensity%): 586 (13,M^++1), 438 (4,M^++2-CH_2CO_2Bz).

1,7-Dioxa-4,10,13-triazacyclopentadecane-4,10,13-triacetic acid trihydrochloride (DTCTA) (12). All glassware for this step was steeped in hydrochloric acid (6 M) for 18h prior to use. The dibenzyl methyl ester (34) (6mg, 0.01 mmol) was dissolved in hydrochloric acid (1 ml, 6 M) and heated (110°C, 18h). The aqueous layer was washed with diethyl ether (3 x 1 ml) and deuterated chloroform (1 ml) and subsequently evaporated to give the *title compound* as a glassy white solid (5mg, 97%). ^{1}H NMR δ_H (D_2O, pD 4.5, t-BuOD): 3.33 (4H,t,J 4.5 Hz,NCH_2CH_2O), 3.44 (4H,s,$N(CH_2)_2N$ or CH_2CO), 3.57-3.60 (4H,m,NCH_2CH_2O), 3.63 (4H,s, $N(CH_2)_2N$ or CH_2CO), 3.80 (8H,t,J 4.7 Hz,NCH_2CH_2O), 3.83 (2H,s,CH_2CO); MS m/z (Intensity%) FAB: 392 (13,M^++2).

5.1.8 Synthesis of (+)-(2S)-(4-Aminobutyl)DOTA

(2S)-Methyl-2,6-diaminohexanoate dihydrochloride (57). (+)-(2S)-Lysine hydrochloride (49) (16.0g, 87.6 mmol) was dissolved in methanol (150 ml) and acetyl chloride (15 ml) added cautiously. The reaction mixture was refluxed (18h) and partially evaporated. A white crystalline solid precipitated which was collected and dried to give the *title compound* (15.4g, 75%). MPt. 214-216°C (dec); Analysis Found: C, 35.8; H, 7.7; N, 14.0. Required for $C_7H_{16}N_2O_2.2HCl$: C, 36.1; H, 8.1; N, 14.3%; IR ν_{max} (Nujol): 3000br (NH_3^+), 2000br (NH_3^+), 1745 (CO) and 1605 (NH_3^+) cm^{-1}; ^{1}H NMR δ_H (D_2O): 1.44-1.62 (2H,m,CH_2), 1.74 (2H,quin,J 7.5 Hz,$CH_2CH_2NH_2$), 1.89-2.11 (2H,m,CH_2), 3.03 (2H,t,J 7.7 Hz,CH_2NH_2), 3.85 (3H,s,CH_3), 4.19 (1H,t,J 6.4 Hz,CH); ^{13}C NMR δ_C (D_2O): 45.6, 50.3, 53.3 (CH_2); 63.1 (NCH_2); 76.7, 77.7 (CH_3,CH); 194.7 (CO); MS m/z (Intensity%) CI: 161 (23,M^++1), 144 (9,M^+-NH_2).

(2S)-N-(2-Aminoethyl)-2,6-diaminohexanamide (58). The methyl ester (57) (7.09g, 30.4 mmol) was added in small batches over 1h to ethylenediamine (100 ml) at 90°C, stirring continuously. The reaction mixture was refluxed (6h) and the ethylenediamine removed by distillation. The residue was taken into sodium hydroxide solution (25 ml, 4 M), evaporated, dissolved in methanol (30 ml), filtered, the filtrate evaporated to leave a residue and the process repeated from the methanol stage. The subsequent residue was dissolved in dichloromethane (100 ml), filtered, and the filtrate evaporated to give a clear brown oil (5.32g, 93%) of the *title compound.* Found M^+: 188.1640. Required for $C_8H_{20}N_4O$: 188.1637; IR ν_{max} (film): 3340 (NH), 1650 (CO) and 1570 (NH) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 1.37-1.60 (13H,m,NH_2,CH_2), 1.82-1.91 (1H,m,CH_2), 2.71 (2H,t,J 6.5 Hz,NCH_2), 2.83 (2H,t,J 6.0 Hz,NCH_2), 3.38 (1H,t,J 3.8 Hz, CH), 7.56 (1H,br,s,CONH); ^{13}C NMR δ_C (D_2O): 22.2, 31.4, 34.0 (CH_2); 39.6, 40.2, 41.4, 42.7 (NCH_2); 54.6 (CH); 178.0 (CO); MS m/z (Intensity%): 189 (9,M^++1), 171 (4,M^+-NH_3).

(5S)-5-Amino-3-aza-1,9-nonanediamine (59). The amide (58) (3.75g, 20.0 mmol) was refluxed in borane-THF (130 ml, 130 mmol) for 20h. Excess borane was quenched with methanol (100 ml) and solvents were evaporated to leave a residue which was refluxed in hydrochloric acid (160 ml, 6 M) for 3h. Evaporation of solvent, entraining in methanol (2 x 20 ml) and further evaporation gave the *title compound* as a hygroscopic white solid (6.28g, 98%). Found M^+: 174.1850. Required for $C_8H_{22}N_4$: 174.1844; IR ν_{max} (film): 3300br (NH,NH_2) cm^{-1}; ^1H NMR δ_H (D_2O): 1.46-1.58 (2H,m, $CH_2C\underline{H}_2CH_2$), 1.66-1.92 (4H,m,$C\underline{H}_2CH_2C\underline{H}_2$), 3.01 (2H,t,J 7.5 Hz,$NCH_2$), 2.70-2.79 (1H,m,NC<u>H</u>H), 3.38-3.53 (5H,m,NC<u>H</u>H,NCH_2), 3.71 (1H,quin,J 6.4 Hz, CH); ^{13}C NMR δ_C (D_2O): 21.1, 26.1, 29.5 (CH_2); 35.2, 38.7, 44.8, 48.4, 48.9 (CH,NCH_2); MS m/z (Intensity%): 175 (21,M^++1), 157 (12,M^+-NH_3).

(5S)-5-Amino-9-benzamido-3-azanonylamine (60). The tetraamine tetrahydrochloride (59) (13.8g, 43.0 mmol) was dissolved in water (100 ml) and converted to the free tetraamine by adding potassium hydroxide (9.65g, 172 mmol). Addition of copper carbonate (5.71g, 25.8 mmol) to the stirred solution at 50°C gave an intense blue colour. After continued stirring (30 min, 50°C), benzoyl chloride (6.5 ml, 55.9 mmol) was added over 1h to the cooled solution (0°C), maintaining the pH above 9 with periodic addition of potassium hydroxide pellets. The solution was allowed to achieve room temperature and stirring was continued for 1h. The brown-black solution was filtered and the filtrate treated with hydrogen sulphide over 30 min. Filtration followed by partial evaporation and exhaustive extraction with chloroform, with subsequent drying and evaporation of the combined extracts, gave the *title compound* as a pale yellow oil (6.40g, 54%). Found M^++1: 279.2010. Required for $C_{15}H_{27}N_4O$: 279.2027; IR ν_{max} (film): 3300br (NH,NH_2,CONH), 3060, 1640 (CO) and 1600 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 1.35 (7H,br,s,NH,NH_2,CH_2), 1.58 (4H,br,s,CH_2), 2.25-2.33 (1H,dd,J 12 Hz,8.7 Hz,CH), 2.51-2.74 (6H,m, NCH_2), 3.37 (2H,dt,J 2x6.5 Hz,CONHCH_2), 6.38 (1H,br,t,CONH), 7.39-7.50 (3H,m,ArH), 7.75-7.78 (2H,AB system,J_{AB} 6.6 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 23.4, 29.5, 35.6 (CH_2); 39.7, 41.6, 50.8, 52.4, 56.4 (CH,NCH_2); 126.8, 128.3, 131.1; 134.7; 167.5 (CO); MS m/z (Intensity%): 280 (23,M^++2), 279 (100,M^++1).

(5S)-9-Benzamido-1,5-bis(4-toluenesulphonamido)-3-(4-toluenesulphonyl)-3-azanonane (61). 4-Toluenesulphonyl chloride (2.70g, 14.2 mmol) was added in small batches over 30 min to a stirred solution of the benzamide (60) (1.05g, 3.78 mmol) and potassium carbonate (1.96g, 14.2 mmol) in water (40 ml) at 50°C. The temperature was raised to 80°C and stirring continued (18h). The water was decanted leaving a white solid

which was dried *in vacuo*, crushed to a powder and suspended in hot dichloromethane. Filtration gave the *title compound* as a white powder (1.29g, 46%). MPt. 144-146°C; Analysis Found: C, 58.0; H, 6.1; N, 7.4. Required for $C_{36}H_{44}N_4O_7S_3$: C, 58.4; H, 6.0; N, 7.6%; IR ν_{max} (KBr): 3290 (NH), 3060, 3030, 1640 (CO), 1600, 1580, 1530 (NH), 1320 (SO_2) and 1160 (SO_2) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 0.84-0.88 (1H,m,CH_2), 1.03-1.12 (2H,m, CH_2), 1.43-1.50 (2H,m,CH_2), 1.69-1.75 (1H,m,CH_2), 2.31 (3H,s,$ArCH_3$), 2.43 (6H,s,$ArCH_3$), 3.14-3.19 (5H,m,NCH_2,CH), 3.25-3.32 (2H,m,NCH_2), 3.35-3.41 (2H,m,NCH_2), 5.30 (1H,d,J 6.3 Hz,CHNHTs), 5.35 (1H,br,t,NHTs), 6.49 (1H,br,t,NHCO), 7.18 and 7.60 (4H,AB system,J_{AB} 8.1 Hz,Ar(Ts)H), 7.32 and 7.72 (8H,AB system,J_{AB} 8.7 Hz,Ar(Ts)H), 7.37-7.49 (3H,m, Ar(Ph)H), 7.81-7.84 (2H,AB system,J_{AB} 8.8 Hz,Ar(Ph)H); MS m/z (Intensity%) FAB: 743 (2,M^++3), 430 (1,M^+-310).

(+)-(2S)-2-(4-Benzamidobutyl)-1,4,7,10-tetrakis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (62). 1,5-Bis(4-toluenesulphonato)-3-(4-toluenesulphonyl)-3-azapentane (2.79g, 4.93 mmol) in DMF (50 ml) was added dropwise over 3h to the tritosylated compound (61) (3.65g, 4.93 mmol) and caesium carbonate (4.01g, 12.3 mmol) in DMF (200 ml) at room temperature. After stirring at room temperature for 18h, the reaction mixture was heated at 60°C (72h). A further batch (0.42g, 7.39 mmol) of 1,5-bis(4-toluenesulphonato)-3-(4-toluenesulphonyl)-3-azapentane was added in one lot, and stirring continued (12h, 60°C). Solvent was removed under reduced pressure and the residue dissolved in dichloromethane (100 ml). After washing with water (2 x 30 ml) and drying, the organic layer was evaporated. Column chromatography (gradient eluent 0.5-1.5% methanol in dichloromethane) of the residue afforded the *title compound* as a glassy solid (3.41g, 72%). MPt. 108-110°C; $[\alpha]_D^{22}$ +14.0° (c 1.00 in CH_2Cl_2); R_t 2.9 min (reverse phase); Analysis Found: C, 58.2;

H, 5.6; N, 7.0. Required for $C_{47}H_{57}N_5O_9S_4$: C, 58.6; H, 5.9; N, 7.3%; IR ν_{max} (film): 3400 and 3290 (NH), 3040, 1650 (CO), 1600, 1580, 1530 (NH), 1340 and 1160 cm^{-1}; ^1H NMR δ_H (CD_3CN,333K): 0.67 (2H,br,s,CH_2), 1.09-1.14 (2H,m,CH_2), 1.27-1.45 (2H,m,CH_2), 2.35, 2.41, 2.46 and 2.53 (12H, 4xs,$ArCH_3$), 2.57-4.22 (17H,7xm,NCH_2,NCH,$NHC\underline{H}_2$), 6.86 (1H,br,s,NH), 7.16-7.27 (4H,AB system,ArH), 7.37-7.51 (9H,AB system,ArH), 7.63-7.83 (8H,AB system,ArH); ^{13}C NMR δ_C ($CDCl_3$): 21.4 (CH_3); 28.7, 39.4, 49.4, 51.9, 55.4, 65.9 (NCH_2,NCH,CH_2,all br); 126.8, 127.1, 128.2, 129.8; 131.0, 134.5, 135.6, 143.4, 143.9, 144.5, 167.3 (CO); MS m/z (Intensity%): 968 (5,$\underline{M}^+$+5), 967 (14,$\underline{M}^+$+4), 966 (36,$\underline{M}^+$+3), 965 (56,$\underline{M}^+$+2), 964(100,$\underline{M}^+$+1), 809 (21,$\underline{M}^+$-154).

(+)-(2S)-2-(4-Benzamidobutyl)-1,4,7,10-tetraazacyclododecane (63).
Ammonia (100 ml) was condensed into a mixture of the tetratosylated macrocycle (62) (0.50g, 0.52 mmol) in THF (20 ml) and ethanol (2 ml) at -78°C. Lithium (0.18g, 25.9 mmol) was added in small portions to this mixture and an intense blue colour developed which discharged over 1-10 min. The reaction mixture was allowed to warm to room temperature, water (20 ml) was added and solvents evaporated. The residue was dissolved in hydrochloric acid (20 ml, 6 M), washed with diethyl ether (3 x 10 ml), evaporated, redissolved in potassium hydroxide solution (10 ml, 6 M) and extracted with dichloromethane (4 x 5 ml). The combined extracts were evaporated to give a colourless oil. Preparative HPLC (cation exchange) afforded the *title compound* as a colourless oil (0.10g, 57%). $[\alpha]_D^{25}$ +2.5° (c 0.80 in CH_2Cl_2); R_t 7.3 min; Found M^++1: 348.2616. Required for $C_{19}H_{34}N_5O$: 348.2763; ^1H NMR δ_H ($CDCl_3$): 1.41-1.48 (4H,m,CH_2), 1.58-1.66 (2H,m,CH_2), 2.38 (4H,br,s,NH), 2.46 (1H,dd,J 12 Hz,7.7Hz,$NHCH\underline{H}$), 2.67 (14H,s,NCH_2), 3.46 (2H,dt,J 2x6.4 Hz,$CONHC\underline{H}_2$), 6.58 (1H,br,t,CONH), 7.39-7.49 (3H,m,ArH), 7.76-7.80 (2H,AB system,J_{AB}

9.3 Hz,ArH); MS m/z (Intensity%): 348 (100,$\underline{M}^+$+1).

(+)-(2S)-2-(4-Aminobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid trihydrochloride (65). Ethyl bromoacetate (190mg, 1.24 mmol) was added to the macrocyclic amine (63) (75mg, 0.22 mmol) and potassium carbonate (176mg, 1.28 mmol) in DMF (3 ml) and the mixture heated (90°C, 18h). Evaporation of solvent and preparative HPLC (cation exchange) gave the tetraester (64) as a colourless oil, R_t 10.7 min. The tetraester (64) was hydrolysed according to the method for (12) to give the *title compound* as a white gum (97mg, 77%). $[\alpha]_D^{22}$ +2.7° (c 0.75 in H_2O); Analysis Found C, 41.3; H, 5.9; N, 11.75. Required for $C_{20}H_{37}N_5O_8.3HCl$: C, 41.1; H, 6.3; N, 12.0%; ^{1}H NMR δ_H (D_2O): 1.47 (2H, br,s,CH_2), 1.74 (2H,br,s,CH_2), 1.95 (2H,br,s,CH_2), 3.05, 3.47 and 4.00 (25H,3x vbrs,CH,NCH_2,CH_2NH_2); MS m/z (Intensity%) FAB: 476 (0.6,$\underline{M}^+$+1).

5.1.9 Attempted Synthesis of (2S)-(4-Nitrophenyl)DOTA

(2S)-Methyl-2-amino-3-(4-nitrophenyl)propanoate hydrochloride (42). (2S)-(4-Nitrophenyl)alanine (41) (7.51g, 36.8 mmol) was dissolved in methanol (25 ml) and acetyl chloride (2.5 ml) added cautiously. The reaction mixture was refluxed (18h) and partially evaporated. A white crystalline solid precipitated which was collected and dried to give the *title compound* (6.11g, 66%; lit.[6] 88%). ^{1}H NMR δ_H (D_2O): 3.41 (1H,dd,J 15 Hz,7.4Hz,CH_2), 3.51 (1H,dd,J 15 Hz,6.4Hz,CH_2), 3.86 (3H,s,CH_3), 4.56 (1H,t,J 6.9 Hz,CH), 7.55 and 8.27 (4H,AB system,J_{AB} 6.8 Hz,ArH); ^{13}C NMR δ_C (D_2O): 36.2 (CH_2); 54.4 (CH,CH_3); 125.0, 131.3; 142.7, 148.0; 170.4 (CO); MS m/z (Intensity%) CI: 225 (46,$\underline{M}^+$+1), 165 (10,$\underline{M}^+$-CO_2Me).

(2S)-2-Amino-3-(4-nitrophenyl)propanamide (43). Triethylamine (3.6 ml, 25.9 mmol) in diethyl ether (100 ml) was added to the ester (42) (6.11g, 23.5 mmol) in methanol (3 ml). The solution was filtered and the filtrate concentrated to a limpid yellow oil. Ammonia was bubbled through a solution of the oil in methanol (100 ml) for 1h. The reaction vessel was tightly stoppered and allowed to stand (24h). Evaporation of solvent gave the *title compound* as a red-brown oil (3.61g, 74%; lit.[6] 92%). IR ν_{max} (film): 3400 (NH), 1680 (CO) and 1610 (NH) cm^{-1}; ^1H NMR δ_H $[(CD_3)_2SO]$: 2.04 (2H,br,s,NH_2), 3.03 (1H,dd,J 12 Hz,8.4Hz,CH_2), 3.31 (1H,dd,J 12 Hz,4.8Hz,CH_2), 3.66-3.71 (1H,m,CH), 7.31 (1H,s,NH_2), 7.66 (1H,s,NH_2), 7.79 and 8.41 (4H,AB system,J_{AB} 8.5 Hz,ArH); ^{13}C NMR δ_C $[(CD_3)_2SO]$: 41.6 (CH_2); 56.8 (CH); 123.9, 131.4; 146.8, 148.5; 177.1 (CO); MS m/z (Intensity%): 210 (30,M^++1).

(2S)-2-Amino-(4-nitrophenyl)propylamine dihydrochloride (44). The amide (43) (3.61g, 17.3 mmol) was refluxed in borane-THF (80 ml, 80 mmol) for 20h. Excess borane was quenched with methanol (25 ml) and solvents were evaporated to leave a residue which was refluxed in hydrochloric acid (100 ml, 6M) for 3h. Evaporation of solvent, entraining in methanol (2 x 20 ml) and further evaporation gave the *title compound* as a yellow solid (1.95g, 42%; lit.[6] 85%). Analysis Found: C, 39.8; H, 5.2; N, 15.7. Required for $C_9H_{13}N_3O_2.2HCl$: C, 40.3; H, 4.85; N, 15.7%; IR ν_{max} (Nujol): 3360 (NH) and 1590 cm^{-1}; ^1H NMR δ_H (D_2O): 3.36 (1H,dd,J 12 Hz, 8.6Hz,CH_2), 3.49-3.67 (3H,m,CH_2), 4.16-4.27 (1H,m,CH), 7.77 and 8.47 (4H,AB system,J_{AB} 8.7 Hz,ArH); ^{13}C NMR δ_C (D_2O): 36.0 (NCH_2); 40.7 (CH_2); 50.2 (CH); 124.3, 130.6; 141.9, 147.3; MS m/z (Intensity%) CI: 272 (3,M^++1+2[$H^{37}Cl$]), 267 (2,M^++2[$H^{35}Cl$]).

(2S)-3-(4-Nitrophenyl)-1,2-bis(4-toluenesulphonamido)propane (45). 4-Toluenesulphonyl chloride (3.56g, 18.7 mmol) was added in small batches over 1h to the diamine dihydrochloride (44) (2.00g, 7.46 mmol) in pyridine (100 ml) at 0°C. The vessel was tightly stoppered and left (0°C, 24h). The solution was poured onto crushed ice and the oil which separated taken into dichloromethane (100 ml), washed with hydrochloric acid (2 x 50 ml, 0.1 M), dried and evaporated. Recrystallisation from ethanol gave the *title compound* as a pale yellow solid (0.89g, 24%). Mpt. 135-137°C; Analysis Found: C, 54.7; H, 5.0; N, 8.4. Required for $C_{23}H_{25}N_3O_6S_2$: C, 54.9; H, 5.0; N, 8.3%; IR ν_{max} (film): 3280 (NH), 3020, 1600, 1520 (NO_2), 1350 (NO_2,SO_2) and 1160 (SO_2) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 2.33 (3H,s,$ArCH_3$), 2.39 (3H,s,$ArCH_3$), 2.70 (1H,dd,J 15 Hz,9.4Hz, $ArCH_2CH$), 2.91 (1H,dd,J 12 Hz,5.1Hz,$ArCH_2CH$), 3.07 (2H,br,s,$CHCH_2NH$), 3.47 (1H,br,s,CH), 5.24 (1H,br,s,NH), 5.40 (1H,br,s,NH), 6.99-7.05 (4H,AB system,ArH), 7.25-7.28 (2H,AB system,J_{AB} 8.1 Hz,ArH), 7.36-7.39 (2H,AB system,J_{AB} 8.2 Hz,ArH), 7.68-7.71 (2H,AB system,J_{AB} 8.2 Hz,ArH), 7.80-7.84 (2H,AB system,J_{AB} 8.4 Hz, ArH); MS m/z (Intensity%): 504 (100,M^++1).

(2S)-2-(4-Nitrobenzyl)-1,4,7,10-tetrakis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (46). The ditosylated compound (45) (0.89g, 1.77 mmol) was added to a solution of sodium (0.08g, 3.54 mmol) in ethanol (50 ml) and refluxed (2h). Solvent was evaporated and the residue taken into DMF (200 ml). 1,8-Bis(4-toluenesulphonato)-3,6-bis(4-toluenesulphonyl)-3,6-diazaoctane (1.35g, 1.77 mmol) in DMF (100 ml) was added dropwise over 3h to this solution at 60°C. Stirring was continued (90°C, 24h). Water (100 ml) was added and the solution extracted with dichloromethane (4 x 50 ml), washed with hydrochloric acid (2 x 50 ml, 1 M), dried and solvent evaporated to give a dark red-brown oil.

5.1.10 Attempted Synthesis of (2S)-(4-Aminobutyl)DOTA

(2S)-2-Amino-6-benzamidohexanoic acid (50). The *title compound* was synthesized according to the method for (60). After treatment with hydrogen sulphide and filtration, the filtrate was adjusted to pH 4 by adding concentrated hydrochloric acid. A white solid precipitated which was collected and suspended in methanol. Filtration gave the *title compound* as a white powder (91%). MPt. 264-266°C (lit.[7] 267-269°C); Analysis Found: C, 62.2; H, 6.8; N, 10.75. Required for $C_{13}H_{18}N_2O_3$: C, 62.4; H, 7.2; N, 11.2%; IR ν_{max} (KBr): 3320 (NH), 3040 (OH), 1640 (PhCO, NH_3^+), 1580 (CO_2^-) and 1540 (CO_2^-) cm^{-1}; ^1H NMR δ_H (D_2O, pD 11.0): 1.25-1.37 (2H,m,CH_2), 1.48-1.59 (4H,m,CH_2), 3.15 (1H,t,J 6.4Hz,CH), 3.29 (2H,t,J 6.9Hz,NCH_2), 7.39-7.55 (3H,m,ArH), 7.62-7.66 (2H,AB system,J_{AB} 8.7 Hz,ArH); MS m/z (Intensity%) FAB: 250 (83,M^+).

(2S)-Methyl-2-amino-6-benzamidohexanoate (51). The *title compound* was synthesized according to the method for (57). Work-up involved evaporation of the reaction mixture; the residue was taken into dichloromethane, washed with aqueous sodium carbonate solution, dried and evaporated to give the *title compound* as a colourless oil (42%). IR ν_{max} (film) 3300 (NH), 3060, 1740 (ester CO), 1640 (amide CO), 1600 and 1540 (amide NH) cm^{-1}; ^1H NMR δ_H (D_2O): 1.61-1.72 (2H,m,CH_2), 1.77-1.85 (2H,m, CH_2), 2.08-2.17 (2H,m,CH_2), 3.55 (2H,t,J 6.3Hz,NCH_2), 3.91 (3H,s,CH_3), 4.32 (1H,t,J 6.4Hz,CH), 7.64-7.79 (3H,m,ArH), 7.88-7.91 (2H,AB system, J_{AB} 6.8 Hz,ArH); MS m/z (Intensity%): 265 (100,M^++1), 188 (10, M^++1-Ph).

(2S)-2-Amino-N-(2-aminoethyl)-6-benzamidohexanamide (52). The *title compound* was synthesized according to the method for (58). After refluxing (3h), the ethylenediamine was removed by distillation to give

the *title compound* as a brown oil (88%). IR ν_{max} (film): 3280 (NH), 3060, 1640 (CO), 1605 and 1540 (amide NH) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 1.43-1.52 (2H,m,CH_2), 1.62 (6H,br,s,NH_2,CH_2), 1.80-1.86 (2H,m,CH_2), 2.80 (2H, t,J 6.0Hz,$CH_2\underline{CH_2}NH_2$), 3.28 (2H,dt,J 2x6.0Hz,$NHC\underline{H_2}CH_2NH_2$), 3.36-3.41 (1H, m,CH), 3.46 (2H,dt,J 6.6 Hz,6.3 Hz,$PhCONHC\underline{H_2}$), 6.54 (1H,br,t,CHCONH), 7.39-7.49 (3H,m,ArH), 7.62 (1H,br,t,PhCONH), 7.77-7.80 (2H,AB system,J_{AB} 7.3 Hz,ArH); ^{13}C NMR δ_C ($CDCl_3$): 22.8, 29.2, 34.5 (CH_2); 39.5, 41.6, 41.8, 55.1 ($NHCH_2$, CH_2NH_2,CH); 126.9, 128.5, 131.4; 134.7; 167.6 (PhCO), 175.4 ($CH\underline{C}ONH$); MS m/z (Intensity%): 293 (100,$\underline{M}^+$+1), 275 (95,$\underline{M}^+$-NH_3).

(5S)-5-Amino-11-phenyl-3,10-diazaundecylamine (53). The *title compound* was synthesized according to the method for (59). After refluxing (3h) in hydrochloric acid (6 M) and evaporation of solvent, the residue was taken into a small volume of potassium hydroxide solution (6 M) and extracted with dichloromethane (x4). Drying and evaporation of the organic layer gave the *title compound* as a colourless oil (66%). IR ν_{max} (film): 3260br (NH), 3020 and 1570 (NH) cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 1.44 (12H,br,s,NH_2,NH,CH_2), 2.36 (1H,dd,J 12Hz,8.8Hz,NCH_2), 2.61-2.69 (7H,m, NCH_2), 2.78-2.82 (1H,m,NCH_2), 3.78 (2H,s,$PhCH_2$), 7.24-7.33 (5H,m,ArH); MS m/z (Intensity%): 265 (100,$\underline{M}^+$+1), 174 (43, $\underline{M}^+$+1-CH_2Ph).

(5S)-1,5-Bis(4-toluenesulphonamido)-11-phenyl-3,10-bis(4-toluene-sulphonyl)-3,10-diazaundecane (54). The *title compound* was synthesized according to the method for (61). After stirring (80°C,18h) and decanting the water layer, the oily residue was taken into dichloromethane. The water layer was extracted with dichloromethane (x4). The dichloromethane solutions were combined, dried and evaporated. Column chromatography (1.5% methanol in dichloromethane) afforded the *title compound* as a colourless oil (77%). IR ν_{max} (film): 3280br (NH), 3020,

1600, 1330 and 1160 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 0.85-0.90 (2H,m,CH_2), 1.06-1.20 (2H,m,CH_2), 1.37-1.49 (2H,m,CH_2), 2.41, 2.42, 2.43 and 2.44 (12H, 4xs,$ArCH_3$), 2.84-2.90 (1H,m,$CHCH_2NTs$), 3.00-3.26 (6H,m,$NTsCH_2$), 3.71 (2H,s,CH_2Ph), 4.20 (2H,dt,J 2x15Hz,$NHTsCH_2$), 5.16 (1H,d,J 5.9Hz,CHNHTs), 5.27 (2H,t,J 5.6Hz,$NHTsCH_2$), 7.17-7.34 (13H,m,Ar(Ts)H,Ar(Ph)H), 7.58-7.61 (2H,AB system,J_{AB} 8.2Hz,Ar(Ts)H), 7.68-7.83 (6H,m,Ar(Ts)H,Ar(Ph)H); ^{13}C NMR δ_C ($CDCl_3$): 21.4 (CH_2); 21.5 (CH_3); 27.3, 31.3 (CH_2); 42.5, 47.8, 50.8, 52.5, 53.1, 55.2 (NCH_2); 127.2, 127.4, 127.8, 128.3, 128.6, 129.8, 130.0; 134.6, 136.6, 136.9, 137.2, 143.4, 144.1; MS m/z (Intensity%): 880 (0.4,M^+), 727 (0.9, M^++2-Ts).

(2S)-2-(N-Benzyl-4-aminobutyl)-1,4,7,10-tetrakis(4-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (55). The *title compound* was synthesized according to the method for (62). After work-up, recrystallisation from methanol gave the *title compound* as a white solid (34%). MPt. 218-219°C; Analysis Found: C, 59.0; H, 6.0; N, 6.3. Required for $C_{54}H_{65}N_5O_{10}S_5$: C, 58.7; H, 5.9; N, 6.3%; IR ν_{max} (film): 3020, 1600, 1340 and 1160 cm^{-1}; ^1H NMR δ_H ($CDCl_3$): 0.94 (2H,br,s,CH_2), 1.09 (2H,br,s,CH_2), 1.18-1.25 (2H,m,CH_2), 2.39 (3H,s,$ArCH_3$), 2.44 (12H,s,$ArCH_3$), 2.86, 3.32, 3.59, 3.94, 4.23 (19H,5xvbrm,$NTsCH_2$,NTsCH,$NTsCH_2Ph$), 7.21-7.35 (15H,m, Ar(Ts)H,Ar(Ph)H), 7.60-7.74 (10H,m,Ar(Ts)H); ^{13}C NMR δ_C ($CDCl_3$): 14.2 (CH_2); 21.5 (CH_3); 22.7, 27.4, 29.7, 31.9, 45.1, 47.8, 50.5, 52.0, 63.2 (NCH_2,NCH,CH_2, all br); 127.1, 127.3, 127.6, 127.8, 128.3, 128.6, 129.9; 136.2, 143.4, 143.9, 144.6; MS m/z (Intensity%): 1104 (2,M^++1), 949 (12, M^++1-Ts).

(2S)-2-(N-Benzyl-4-aminobutyl)-1,4,7,10-tetraazacyclododecane (56). The *title compound* was synthesized according to the method for (63) to give a pale yellow oil (78%). ^1H NMR δ_H ($CDCl_3$): 1.25-1.38 (4H,m,CH_2), 1.46-

1.54 (2H,m,CH_2), 2.11 (5H,br,s,NH), 2.38-2.47 (1H,m,$NC\underline{H}_2CH$), 2.56-2.71 (16H,m,NCH_2,NCH), 3.77 (2H,s,$NHC\underline{H}_2Ph$), 7.20-7.30 (5H,m,ArH); MS m/z (Intensity%): 334 (4,$\underline{M}^+$+1), 174 (3,$\underline{M}^+$+3-$(CH_2)_4NHBz$).

5.2 KINETIC EXPERIMENTS

5.2.1 ^{1}H NMR Experiments

Spectra were recorded on a Bruker AC 250 spectrometer (250.13 MHz) at 293K. The pD was kept constant at 5.0 by a d_3-sodium acetate (0.14 M)-d_4-acetic acid (0.06 M) buffer in D_2O. Ligand and metal ion concentrations were both 0.028 M. t-Butanol was the internal standard.

Yttrium(III) was added to the ligand in solution as $Y(NO_3)_3.6D_2O$, the D_2O salt obtained by adding D_2O to $Y(NO_3)_3.6H_2O$, evaporating and repeating this process twice.

Where irradiation of an AB "doublet" was required during the course of spin-spin decoupling experiments, the decoupling power was applied to the more intense of the two peaks.

5.2.2 ^{13}C NMR Experiments

The 1:1 Y(DOTA) complex (0.012 M) was prepared by mixing a solution of $Y(NO_3)_3.6H_2O$ in D_2O (0.5 ml, 4.8×10^{-2} M) with a solution of the ^{13}C tetracarbonyl-labelled analogue of DOTA (37) in D_2O (0.5 ml, 4.8×10^{-2} M). The combined solution was adjusted to pH 4.5 with sodium hydroxide solution (2.5 M). After leaving to stand at room temperature for 10 min and checking the ^{13}C NMR spectrum (Bruker AC 250, 62.9 MHz, 200 scans, 0.27s acquisition time, 2.00s relaxation delay, 8K data storage, 293K) the solution was adjusted to the required pH by adding concentrated

nitric acid and made up to 2 ml with deionised water. pH measurements above 0 were made with a Jenway 3020 pH meter.

5.2.3 Kinetic Experiments Involving Ni(II)

Spectra were recorded on a Uvikon 930 spectrophotometer; kinetic measurements were made on the same machine and on a Perkin-Elmer Lambda 3 spectrophotometer. Yttrium(III)-ligand solutions (except that of Y(DOTA), see Section 3.3) were allowed to stand at 298K in buffer at the relevant pH for 20 min before adding a solution of $Ni(NO_3)_2$. The pH of the solution was measured both before and after Ni^{2+} addition with a Jenway 3020 pH meter. All measurements were made at 298K.

5.2.4 Reagents

$Y(NO_3)_2.6H_2O$ was obtained from Alfa; $Ni(NO_3)_2.6H_2O$, calcium chloride (as a 1M solution) and sodium hydroxide (as pellets) from BDH; concentrated nitric acid and hydrochloric acid, 15M and 12M respectively, from May and Baker together with glacial acetic acid; glycine was obtained from Sigma, DTPA from W.R. Grace and sodium acetate and sodium perchlorate from Aldrich.

5.3 STABILITY CONSTANT MEASUREMENTS[8]

Potentiometric titrations were carried out at 298 ± 0.1K. The molarities of tetramethylammonium hydroxide (Sigma) titrant solutions were corrected for carbonate contamination by previous titration against dilute hydrochloric acid (0.1 M, BDH). Ionic strength was kept constant with tetramethylammonium nitrate (0.1 M, Sigma).

The titration system consisted of a double-walled glass cell thermostatted at 298 ± 0.1 K containing the ligand solution coupled to a Mettler DV401 automatic burette containing the titrant. The pH was monitored with a Corning 001854 combination microelectrode. Titrations were controlled by a BBC microcomputer which also handled data storage. Data were transferred to an MTS mainframe computer by the program KERMIT and analysed using the program SUPERQUAD.

5.4 REFERENCES

1. J.E. Richman and T.J. Atkins, *J.Am.Chem.Soc.*, 1974, 96, 2268.
2. J.F. Desreux, *Inorg.Chem.*, 1980, 19(5), 1319.
3. L.Y. Martin, L.J. Dettayes, L.J. Zompa and D.H. Busch, *J.Am.Chem. Soc.*, 1974, 96(12), 4046.
4. H. Stetter and W. Frank, *Angew.Chem.,Int.Ed.Engl.*, 1976, 15, 686.
5. M.T.S. Amorim, R. Delgado, J.J.R. Fraústo da Silva, M.C.T.A. Vaz and M.F. Vilhena, *Talanta*, 1988, 35(9), 741.
6. M.W. Brechbiel, O.A. Gansow, R.W. Atcher, J. Schlom, J. Esteban, D.E. Simpson and D. Colcher, *Inorg.Chem.*, 1986, 25, 2772.
7. A.C. Kurtz, *J.Biol.Chem.*, 1949, 180, 1253.
8. Performed by R. Kataky.

APPENDICES

COLLOQUIA, CONFERENCES AND PUBLICATIONS

RESEARCH COLLOQUIA, SEMINARS, LECTURES AND CONFERENCES ORGANISED BY THE DEPARTMENT OF CHEMISTRY DURING THE PERIOD: 1986-1987

* ALLEN, Prof. Sir G. (Unilever Research) Biotechnology and the Future of the Chemical Industry	13th November 1986
BARTSCH, Dr. B. (University of Sussex) Low Co-ordinated Phosphorus Compounds	6th May 1987
BLACKBURN, Dr. M. (University of Sheffield) Phosphonates as Analogues of Biological Phosphate Esters	27th May 1987
* BORDWELL, Prof. F.G.(Northeastern University,USA) Carbon Anions, Radicals, Radical Anions and Radical Cations	9th March 1987
CANNING, Dr. N.D.S. (University of Durham) Surface Adsorption Studies of Relevance to Heterogeneous Ammonia Synthesis	26th November 1986
CANNON, Dr. R.D. (University of East Anglia) Electron Transfer in Polynuclear Complexes	11th March 1987
* CLEGG, Dr. W. (University of Newcastle-upon-Tyne) Carboxylate Complexes of Zinc; Charting a Structural Jungle	28th January 1987
* DÖPP, Prof. D. (University of Duisburg) Cyclo-additions and Cyclo-reversions Involving Captodative Alkenes	5th November 1986
DORFMÜLLER, Prof. T. (University of Bielefeld) Rotational Dynamics in Liquids and Polymers	8th December 1986
GOODGER, Dr. E.M. (Cranfield Inst.Technology) Alternative Fuels for Transport	12th March 1987
* GREENWOOD, Prof. N.N. (University of Leeds) Glorious Gaffes in Chemistry	16th October 1986
HARMER, Dr. M. (I.C.I. Chemicals & Polymer Group) The Role of Organometallics in Advanced Materials	7th May 1987
* HUBBERSTEY, Dr. P. (University of Nottingham) Demonstration Lecture on Various Aspects of Alkali Metal Chemistry	5th February 1987
HUDSON, Prof. R.F. (University of Kent) Aspects of Organophosphorus Chemistry	17th March 1987
HUDSON, Prof. R.F. (University of Kent) Homolytic Rearrangements of Free Radical Stability	18th March 1987

*	JARMAN, Dr. M. (Institute of Cancer Research) The Design of Anti Cancer Drugs	19th February 1987
	KRESPAN, Dr. C. (E.I. Dupont de Nemours) Nickel(0) and Iron(0) as Reagents in Organofluorine Chemistry	26th June 1987
*	KROTO, Prof. H.W. (University of Sussex) Chemistry in Stars, between Stars and in the Laboratory	23rd October 1986
*	LEY, Prof. S.V. (Imperial College) Fact and Fantasy in Organic Synthesis	5th March 1987
*	MILLER, Dr. J. (Dupont Central Research, USA) Molecular Ferromagnets; Chemistry and Physical Properties	3rd December 1986
	MILNE/CHRISTIE, Dr.A./Mr.S.(International Paints) Chemical Serendipity: A Real Life Case Study	20th November 1986
	NEWMAN, Dr. R. (University of Oxford) Change and Decay: A Carbon-13 CP/MAS NMR Study of humification and Coalification Processes	4th March 1987
	OTTEWILL, Prof. R.H. (University of Bristol) Colloid Science a Challenging Subject	22nd January 1987
	PASYNKIEWICZ, Prof. S. (Technical Univ.,Warsaw) Thermal Decomposition of Methyl Copper and its Reactions with Trialkylaluminium	11th May 1987
*	ROBERTS, Prof. S.M. (University of Exeter) Synthesis of Novel Antiviral Agents	24th June 1987
	RODGERS, Dr. P.J. (I.C.I. Billingham) Industrial Polymers from Bacteria	12th February 1987
*	SCROWSTON, Dr. R.M. (University of Hull) From Myth and Magic to Modern Medicine	6th November 1986
	SHEPHERD, Dr. T. (University of Durham) Pteridine Natural Products; Synthesis and Use in Chemotherapy	11th February 1987
*	THOMSON, Prof. A. (University of East Anglia) Metalloproteins and Magneto-optics	4th February 1987
*	WILLIAMS, Prof. R.L. (Metropolitan Police Forensic Science) Science and Crime	27th November 1987
	WONG, Prof.E.H. (University of New Hampshire,USA) Coordination Chemistry of P-O-P Ligands	29th October 1986

WONG, Prof.E.H. (University of New Hampshire,USA) 17th February 1987
Symmetrical Shapes from Molecules to Art and Nature

DURING THE PERIOD: 1987-1988

* BAILEY, Dr. P.D. (University of York) November 1987
Oncogenes

* BIRCHALL, Prof. D. (I.C.I. Advanced Materials) 25th April 1988
Environment Chemistry of Aluminium

* BORER, Dr. K. (University of Durham Industrial Research Laboratories) 18th February 1988
The Brighton Bomb - (A Forensic Science View)

BOSSONS, L. (Durham Chemistry Teachers' Centre) 16th March 1988
GCSE Practical Assessment

* BUTLER, Dr. A.R. (University of St.Andrews) 5th November 1987
Chinese Alchemy

CAIRNS-SMITH, Dr. A. (Glasgow University) 28th January 1988
Clay Minerals and the Origin of Life

* DAVIDSON, Dr. J. (Herriot-Watt University) November 1987
Metal Promoted Oligomerisation Reactions of Alkynes

* GRADUATE CHEMISTS (Northeast Polytechnics and Universities) 19th April 1988
R.S.C. Graduate Symposium

* GRAHAM, Prof. W.A.G. (University of Alberta, Canada) 3rd March 1988
Rhodium and Iridium Complexes in the Activation of Carbon-Hydrogen Bonds

* GRAY, Prof. G.W. (University of Hull) 22nd October 1987
Liquid Crystals and their Applications

HARTSHORN, Prof. M.P. (University of Canterbury, New Zealand) 7th April 1988
Aspects of Ipso-Nitration

HOWARD, Dr. J. (I.C.I. Wilton) 3rd December 1987
Chemistry of Non-Equilibrium Processes

JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) 29th June 1988
GCSE Chemistry Post-mortem

JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) 6th July 1988
GCE Chemistry A-Level Post-mortem

KOCH, Prof. H.F. (Ithaca College, U.S.A.) Does the E2 Mechanism Occur in Solution ?	7th March 1988
LACEY, Mr. (Durham Chemistry Teacher's Centre) Double Award Science	9th February 1988
LUDMAN, Dr. C.J. (Durham University) Explosives	10th December 1987
McDONALD, Dr. W.A. (I.C.I. Wilton) Liquid Crystal Polymers	11th May 1988
MAJORAL, Prof. J.-P. (Université Paul Sabatier) Stabilisation by Complexation of Short-Lived Phosphorus Species	8th June 1988
MAPLETOFT, Mrs. M. (Durham Chemistry Teachers' Centre) Salters' Chemistry	4th November 1987
NIETO DE CASTRO, Prof. C.A. (University of Lisbon and Imperial College) Transport Properties of Non-Polar Fluids	18th April 1988
* OLAH, Prof. G.A. (University of Southern California) New Aspects of Hydrocarbon Chemistry	29th June 1988
PALMER, Dr. F. (University of Nottingham) Luminescence (Demonstration Lecture)	21st January 1988
* PINES, Prof. A. (University of California, Berkeley, U.S.A.) Some Magnetic Moments	28th April 1988
RICHARDSON, Dr. R. (University of Bristol) X-Ray Diffraction from Spread Monolayers	27th April 1988
ROBERTS, Mrs. E. (SATRO Officer for Sunderland) Talk - Durham Chemistry Teachers' Centre - "Links between Industry and Schools"	13th April 1988
* ROBINSON, Dr. J.A. (University of Southampton) Aspects of Antibiotic Biosynthesis	27th April 1988
ROSE, van Mrs. S. (Geological Museum) Chemistry of Volcanoes	29th October 1987
SAMMES, Prof. P.G. (Smith, Kline and French) Chemical Aspects of Drug Development	19th December 1987
* SEEBACH, Prof. D. (E.T.H. Zurich) From Synthetic Methods to Mechanistic Insight	12th November 1987
SODEAU, Dr. J. (University of East Anglia) Durham Chemistry Teachers's Centre: "Spray Cans, Smog and Society"	11th May 1988

SWART, Mr. R.M. (I.C.I.) — 16th December 1987
The Interaction of Chemicals with Lipid Bilayers

TURNER, Prof. J.J. (University of Nottingham) — 11th February 1988
Catching Organometallic Intermediates

UNDERHILL, Prof. A. (University of Bangor) — 25th February 1988
Molecular Electronics

* WILLIAMS, Dr. D.H. (University of Cambridge) — 26th November 1987
Molecular Recognition

* WINTER, Dr. M.J. (University of Sheffield) — 15th October 1987
Pyrotechnics (Demonstration Lecture)

DURING THE PERIOD: 1988-1989

ASHMAN, Mr. A. (Durham Chemistry Teachers' Centre) — 3rd May 1989
The Chemical Aspects of the National Curriculum

AVEYARD, Dr. R. (University of Hull) — 15th March 1989
Surfactants at your Surface

AYLETT, Prof. B.J. (Queen Mary College, London) — 16th February 1989
Silicon-Based Chips: The Chemists Contribution

* BALDWIN, Prof. J.E. (Oxford University) — 9th February 1989
Recent Advances in the Bioorganic Chemistry of Penicillin Biosynthesis

* BALDWIN & WALKER, Drs. R.R. and R.W. (Hull University) — 24th November 1988
Combustion: Some Burning Problems

BOLLEN, Mr. F. (Durham Chemistry Teachers' Centre) — 18th October 1988
Lecture about the use of SATIS in the classroom

* BUTLER, Dr. A.R. (St. Andrews University) — 15th February 1989
Cancer in Linxiam: The Chemical Dimension

* CADOGAN, Prof. J.I.G., (British Petroleum) — 10th November 1988
From Pure Science to Profit

CASEY, Dr. M. (University of Salford) — 20th April 1989
Sulphoxides in Stereoselective Synthesis

WALTERS & CRESSEY, Mr. D. and T. (Durham Chemistry Teachers' Centre) — 1st February 1989
GCSA Chemistry 1988: "A Coroner's Report"

* CRICH, Dr. D. (University College London) Some Novel Uses of Free Radicals in Organic Synthesis	27th April 1989
* DINGWALL, Dr. J. (Ciba Geigy) Phosphorus-containing Amino Acids: Biologically Active Natural and Unnatural Products	18th October 1988
ERRINGTON, Dr. R.J. (University of Newcastle-upon-Tyne) Polymetalate Assembly in Organic Solvents	1st March 1989
FREY, Dr. J. (Southampton University) Spectroscopy of the Reaction Path: Photodissociation Raman Spectra of NOCl	11th May 1989
* HALL, Prof. L.D. (Addenbrooke's Hospital, Cambridge) NMR - A Window to the Human Body	2nd February 1989
HARDGROVE, Dr. G. (St. Olaf College, U.S.A.) Polymers in the Physical Chemistry Laboratory	December 1988
* HARWOOD, Dr. L. (Oxford University) Synthetic Approaches to Phorbols Via Intramolecular Furan Diels-Alder Reactions: Chemistry under Pressure	25th January 1988
JÄGER, Dr. C. (Friedrich-Schiller University GDR) NMR Investigations of Fast Ion Conductors of the NASICOM Type	9th December 1988
JENNINGS, Prof. R.R. (Warwick University) Chemistry of the Masses	26th January 1989
JOHNSON, Dr. B.F.G. (Cambridge University) The Binary Carbonyls	23rd February 1989
JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) Discussion Session on the National Curriculum	14th June 1989
JONES, Dr. M.E. (Durham Chemistry Teachers' Centre) GCSE and A Level Chemistry 1989	28th June 1989
LUDMAN, Dr. C.J. (Durham University) The Energetics of Explosives	18th October 1988
MACDOUGALL, Dr. G. (Edinburgh University) Vibrational Spectroscopy of Model Catalytic Systems	22nd February 1989
MARKO, Dr. I. (Sheffield University) Catalytic Asymmetric Osmylation of Olefins	9th March 1989

McLAUCHLAN, Dr. K.A. (University of Oxford) — 16th November 1988
The Effect of Magnetic Fields on Chemical Reactions

* MOODY, Dr. C.J. (Imperial College) — 17th May 1989
Reactive Intermediates in Heterocyclic Synthesis

MORTIMER, Dr. C. (Durham Chemistry Teachers' Centre) — 14th December 1989
The Hindenberg Disaster - an Excuse for Some Experiments

NICHOLLS, Dr. D. (Durham Chemistry Teachers' Centre) — 11th July 1989
Demo: "Liquid Air"

PAETZOLD, Prof. P. (Aachen) — 23rd May 1989
Iminoboranes XB≡NR: Inorganic Acetylenes ?

PAGE, Dr. P.C.B. (University of Liverpool) — 3rd May 1989
Stereocontrol of Organic Reactions Using 1,3-dithiane-1-oxides

POLA, Prof. J. (Czechoslovak Academy of Sciences) — 15th June 1989
Carbon Dioxide Laser Induced Chemical Reactions - New Pathways in Gas-Phase Chemistry

REES, Prof. C.W. (Imperial College London) — 27th October 1988
Some Very Heterocyclic Compounds

REVELL, Mr. P. (Durham Chemistry Teachers' Centre) — 14th March 1989
Implementing Broad and Balanced Science 11-16

SCHMUTZLER, Prof. R. (Technische Universitat Braunschweig) — 6th October 1988
Fluorophosphines Revisited - New Contributions to an Old Theme

* SCHROCK, Prof. R.R. (M.I.T.) — 13th February 1989
Recent Advances in Living Metathesis

SINGH, Dr. G. (Teeside Polytechnic) — 9th November 1988
Towards Third Generation Anti-Leukaemics

* SNAITH, Dr. R. (Cambridge University) — 1st December 1988
Egyptian Mummies: What, Where, Why and How ?

STIBR, Dr. R. (Czechoslovak Academy of Sciences) — 16th May 1989
Recent Developments in the Chemistry of Intermediate-Sited Carboranes

* VON RAGUE SCHLEYER, Prof. P. (Universitat Erlangen Nurnberg) — 21st October 1988
The Fruitful Interplay Between Calculational and Experimental Chemistry

WELLS, Prof. P.B. (Hull University) 10th May, 1989
Catalyst Characterisation and Activity

* - Indicates Colloquia attended by the author.

CONFERENCES ATTENDED

1. Royal Society of Chemistry, Perkin Division
North East Regional Meeting
UNIVERSITY OF NEWCASTLE UPON TYNE 15th September 1987

2. Royal Society of Chemistry, Annual Chemical Congress.
UNIVERSITY OF KENT, CANTERBURY 12th - 15th April 1988

3. Royal Society of Chemistry, Perkin Division
North East Regional Meeting
UNIVERSITY OF YORK 16th December 1988

4. UK Macrocyclic Group Annual Meeting
UNIVERSITY OF DURHAM 6th January 1989

PUBLICATIONS

1. J.P.L. Cox, K.J. Jankowski, R. Kataky, D. Parker, N.R.A. Beeley, B.A. Boyce, M.A.W. Eaton, K. Millar, A.T. Millican, U.T. Cobley, A. Harrison and C. Walker. *J.Chem.Soc. Chem Commun.*, 1989, 797.

2. D. Parker, J.R. Morphy, K.J. Jankowski and J.P.L. Cox, *Pure Appl. Chem.*, 1989, 61(9), 1637.