SYNTHETIC STUDIES ON ENHANCERS FOR EUROPIUM LUMINESCENCE

A Thesis submitted for the degree of Doctor of Philosophy

by

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ABSTRACT

Lanthanide luminescence and in particular sensitised emission from both europium (III) and terbium (III) is reviewed. The use of both europium and terbium as biological markers is covered, concentrating on time-resolved fluoroimmunoassays (TR-FIA), and more recently nucleic acid hybridisation assays.

A new approach is described for the design of cooperative ligands that enhance europium luminescence in aqueous solution. This involves a three-component system comprised of the europium ion, a sensitising ligand, 1,10-Phenanthroline-2,9-biscarboxylic acid, and a separate non-sensitising ligand, N-(butylacetamido)-ethylenediamine-N,N,N'-triacetate.

The synthesis of a novel class of europium sensitiser is described 5-(Hexanoylphenanthridinium)-2,9-biscarboxylic acid-1,10-phenanthroline.

A homogeneous method for identifying the presence of a single strand of DNA is described which employs 5-(Hexanoylphenanthridinium)-2,9-biscarboxylic acid-1,10-phenanthroline as a cooperative sensitiser of europium (III) luminescence.

CONTENTS

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TO MUM, DAD, AND ELIZA

I seem to have been only a boy playing on the sea shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

Sir Isaac Newton (1642-1727)

1.1 INTRODUCTION

Advances in molecular biology and biotechnology are creating exciting possibilities for DNA diagnostics[1]-the analysis of disease at the nucleic acid level. These developments will profoundly alter many aspects of modern medicine.

DNA probes are an attractive target for clinical diagnosis. They can be used to detect DNA sequences in a wide range of pathogenic organisms such as viruses (*Hepatitis B*, *Herpes* and HIV), and bacteria (*Chlamydia*)(Table 1.1); the presence of gene sequences associated with genetic diseases (cystic fibrosis, sickle cell anaemia); to establish the identity of a particular person in crime cases and paternity disputes (genetic fingerprinting) and to test for mutation, activation, amplification, and the expression of oncogenes.

In the future, the availability of the complete sequence of the human genome should make it possible to identify all human genes; the accompanying complete physical map of the human genome will serve as the ultimate source of DNA probes for any human gene[2].

Table 1.1 Infectious organisms detected by DNA probes[3]

Viruses	Bacteria	Others
Hepatitis B	*Escherichia coli*	*Trypanosoma*
Hepatitis A	*Shigella*	*Plasmodium sp.*
Cytomegalovirus	*Salmonella*	*Chlamydia*
Adenoviruses	*Legionella*	*Candida*
Herpes simplex	*Campylobacter*	
Measles	*Mobiluncus*	
Epstein-Barr	*Staphylococcus a.*	
Enteroviruses		
Varicella zoster		
Human papilloma		
Rotavirus		
HTLVI		
HIV		
Papoviruses		
Rubella		

Although there are plenty of applications for DNA probe tests the big challenge has been turning the basic concept into a commercial reality, and making the transition from a specialised research technique to a clinical tool. Obtaining suitable complementary DNA sequences and reagents is fairly easy. The DNA sequence for a number of bacteria and viruses have been published and can be used to design an appropriate complementary probe. The specificity of a strand of DNA for a target molecule varies with the length of the strand[4,5]. Most useful probes need only be 20-50 bases long to avoid mismatches and such DNA strands can easily be made with the new generation of DNA synthesisers.

The difficult part has been in finding a sensitive detection method. In the past researchers have generally opted for radioisotopic markers but because of the cost, short half-life and health hazards associated with the commonly used radionuclides, major efforts are under way to develop alternatives based on non-isotopic detection systems. DNA probes can be labelled with fluorescent or chemiluminescent molecules, or attached to an enzyme that can be used to generate a large number of coloured molecules. All of the DNA probe tests currently on the market are based on a heterogeneous assay design and use one or more of the detection labels described above. They are all labour intensive, and not very easy to use. This has hindered their transition from specialised testing to routine clinical use.

The ideal would be completely automated DNA probe tests, which could be used in hospitals where rapid testing of a large number of samples is required, in small clinical laboratories and even the doctor's office. For this to occur there has to be a shift from the two-phase heterogeneous assay system. In heterogeneous assays the nucleic acid material being investigated is immobilised on a solid matrix and then incubated in individual steps (pre-hybridisation, hybridisation with the reagent probe, washing and detection) with various solutions. Homogeneous assay conditions would make the pre-hybridisation and washing steps superfluous and would eliminate the need for a solid support. This appreciable simplification would lend itself to the development of automated procedures.

The development of a single-phase homogeneous DNA assay is thus a real commercial target. However such homogeneous assays have been few and far between. The difficulty has been in determining the difference between single stranded and hybridised probes in solution. This will require the design of a signalling system that will exploit the characteristics of the duplex, to give a measurable signal only after hybrid formation with the complementary sequence has taken place.

Potentially, fluorescence resonance energy transfer (FRET) may provide a practical basis for a homogeneous hybridisation assay[6]. In one format, two probes binding to adjacent sequences on a target nucleic acid contained fluorescein and rhodamine, respectively. When both probes are bound, fluorescein emission was quenched and rhodamine emission enhanced (Figure 1.1)[6,7]. However, this approach suffers from a lack of sensitivity due to high background problem that afflicts most fluorescent labels.

Several approaches have been described that attempt to overcome the background problem with fluorescence detection. The most significant of these are those employing labels which have delayed (long-lived) luminescence, namely lanthanide metal chelates. Over the last ten years lanthanide chelates (europium and terbium) have become the labels of choice in fluorescence immunoassays and recently in DNA hybridisation assays. This has not been by accident but has been as a result of the unique photophysical properties of certain members of the lanthanides. This review will cover the literature of lanthanide chelates as luminescent probes and markers.

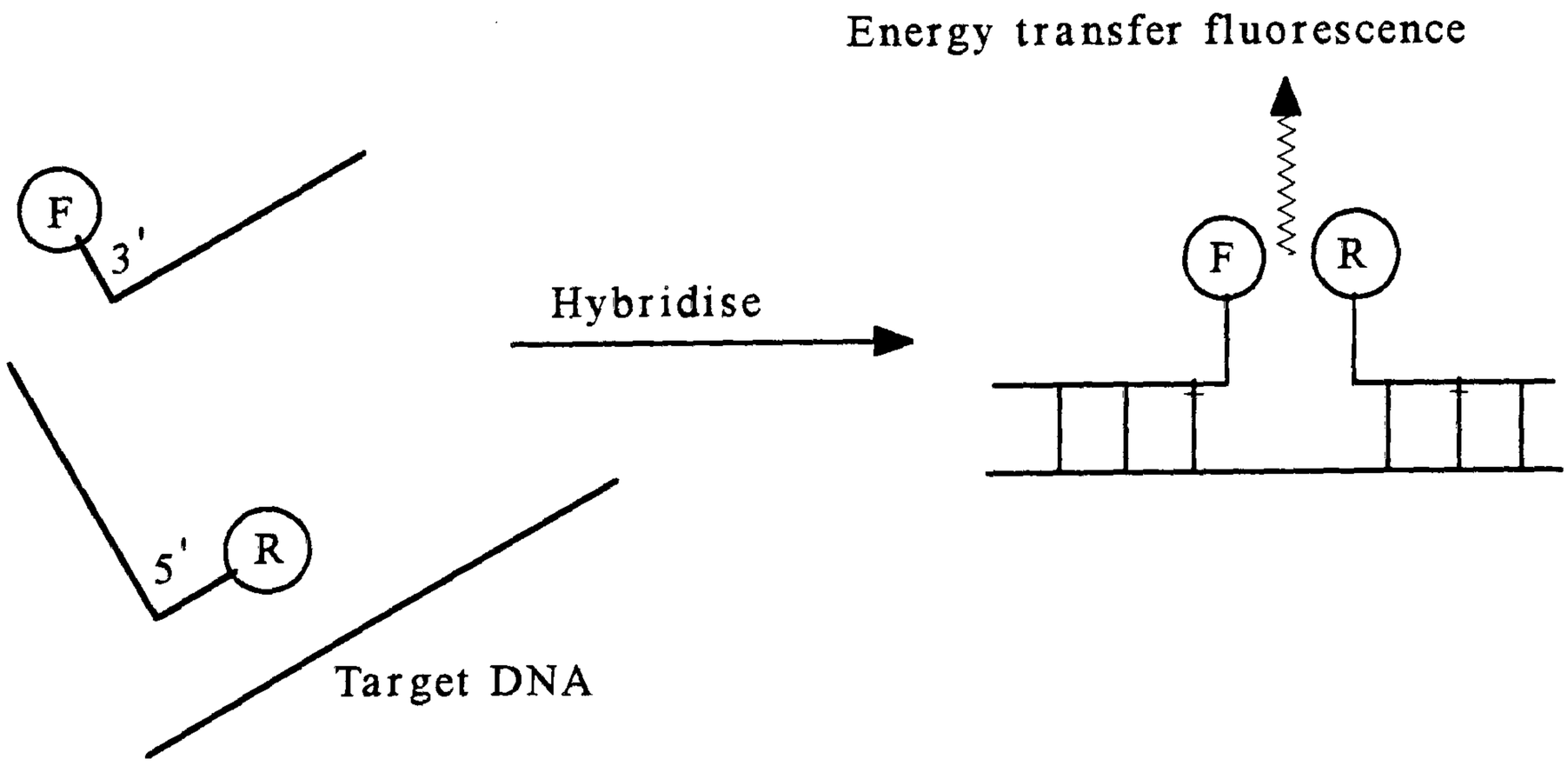


Figure 1.1 A homogeneous DNA assay based on energy transfer

1.2 USE OF LANTHANIDES IN LUMINESCENT PROBES

Lanthanide luminescent probes are presently extensively used to solve a variety of structural and analytical problems. This includes the determination of the local structures in crystalline materials and glasses. Isomorphous substitution of Ca(II) or Zn(II) by lanthanide ions help the investigation of metal-binding sites in biological materials: the number of these sites and their chemical composition as well as the number of metal-bonded water molecules can be assessed by this method. Trivalent lanthanide probes also facilitate the study of the effects of chemical and thermal treatment on catalysts. They are also used in the analysis of inorganic anions and cations and in the development of immunoassays having a sensitivity comparable to that of radioisotopic methods. The various applications of lanthanide luminescent probes are summarised in Table 1.2, together with a list of the relevant review articles.

Table 1.2 Luminescent lanthanide probes: Applications and recent reviews

Applications	Probe	Refs.
Determination of local symmetries in crystalline inorganic materials	Ln^{3+}	8
Determination of local structure in inorganic glasses by laser spectroscopy	Ln^{3+}	9-10
Probing the structure of biological molecules (Ca-binding sites)	Eu^{3+} Tb^{3+}	11-15
Catalysts: coordination environments, changes upon thermal and chemical treatments, oxidation state changes	Ln^{3+}	16
Analysis of inorganic anions and cations by SEPIL (selectively excited probe ion luminescence)	Eu^{3+} Er^{3+} Ho^{3+}	17
Immuno/DNA hybridisation assays using lanthanide probes and time-resolved fluorescence	Eu^{3+} Tb^{3+}	18-20

1.2.1 Lanthanide Complexes and Spectroscopic Properties

Most metal ion complexes absorb visible and/or UV radiation, however, very few re-emit even a small fraction of the absorbed energy in the form of UV or visible photons. This situation is a consequence of the facile non-radiative deexcitation pathways that compete efficiently with radiative modes. The lack of many luminescent complexes of transition metal complexes is a result of the strong coupling of their d-electron excited states with the environment (e.g water molecules) via the ligand field which provides an efficient de-excitation mechanism.

On the other hand, all of the trivalent lanthanide ions, symbolically Ln(III), are known to luminesce, particularly in the solid state under anhydrous conditions. This property is also shown by their complexes and is due to fact that the low energy excited states originate from the $4f^n$ configurations.

The basic excited-state properties of lanthanide ions have been extensively described in many monographs.[21,22,23,24]

All 14 lanthanides have the same number of electrons in their outer shell. Their differences lie deep within their electronic cores, far from the hurly-burly of chemical reactions. (Table 1.3)

Table 1.3 Ground state electronic configurations of the lanthanides

Element	Ln°	Ln^{3+}
Lanthanum (La)	$5d^16s^2$	$4f^0$
Cerium (Ce)	$4f^15d^16s^2$	$4f^1$
Praseodymium (Pr)	$4f^36s^2$	$4f^2$
Neodymium (Nd)	$4f^46s^2$	$4f^3$
Promethium (Pm)	$4f^56s^2$	$4f^4$
Samarium (Sm)	$4f^66s^2$	$4f^5$
Europium (Eu)	$4f^76s^2$	$4f^6$
Gadolinium (Gd)	$4f^75d^16s^2$	$4f^7$
Terbium (Tb)	$4f^96s^2$	$4f^8$
Dysprosium (Dy)	$4f^{10}6s^2$	$4f^9$
Holmium (Ho)	$4f^{11}6s^2$	$4f^{10}$
Erbium (Er)	$4f^{12}6s^2$	$4f^{11}$
Thulium (Tm)	$4f^{13}6s^2$	$4f^{12}$
Ytterbium (Yb)	$4f^{14}6s^2$	$4f^{13}$
Lutetium (Lu)	$4f^{14}5d^16s^2$	$4f^{14}$

The lanthanide elements are associated with the progressive filling of the 4f-electronic sub-shell. The energy of the 4f eigen function drops suddenly at the beginning of the series, which means their maximum lies inside the $5s^2 5p^6$ closed subshells of the xenon structure. The 4f orbitals of a lanthanide cation are thus shielded from the effects of its immediate environment (surrounding anions and dipolar molecules) by the outer s and p orbitals and are minimally involved in bonding. This results in minimal pertubation of electronic transitions between the energy levels of these f orbitals. As a consequence radiationless deactivation processess in Ln(III) are relatively inefficient, and the emission of radiation as luminescence is able to compete in many instances. The important characteristics of lanthanide ion luminescence, namely the long radiative lifetimes and line-like emission bands, in contrast to the broad d-d absorption bands of the transition elements, makes these ions unique among the species that are known to luminesce.

The energy of the $4f^n$ electronic configuration of a lanthanide ion is determined by a combination of inter-electronic repulsion (electrostatic interaction), spin-orbit coupling and, in a coordination environment, the ligand field (Figure 1.2).[25]

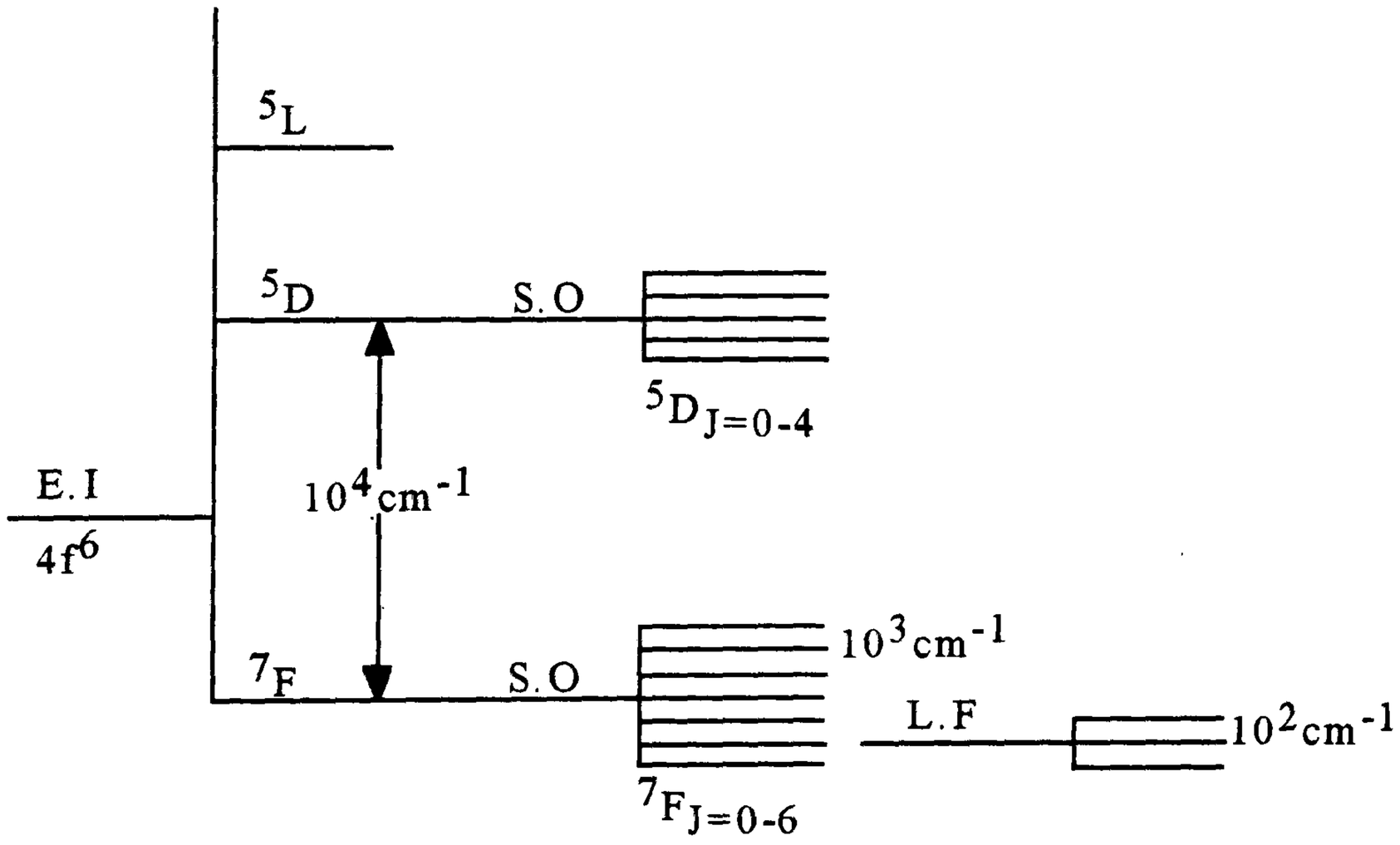


Figure 1.2 Splitting of the $4f^6$ electronic configuration of Eu^{3+} ion owing to electronic interaction, spin-orbit coupling, and ligand field.

The intensities of the f→f absorption spectra can be explained by the Judd-Ofelt theory.[26] Electric dipole transitions between 4f levels, involving no change in parity are forbidden by the Laporte rule and only occur because of interaction with the ligand field causes mixing of electronic states, the so-called J-mixing[27]. The transitions between these modified states are called **forced** (or **induced**) **electric dipole transitions.** Although these admixtures make transitions possible the consequences are f→f transitions which are both weak in absorption and emission. The probability of such transitions is so low that molar extinction coefficients are of the order of 1 (weak absorption bands) and radiative lifetimes of the excited state are in the millisecond timescale.[26] This behaviour contrasts sharply with organic fluorescent molecules where molar extinction coefficients of tens of thousands and radiative lifetimes in the nanosecond range are common.

Basic to the understanding of any Ln(III) luminescence experiment is the appropriate energy level diagram. Figure 1.3[18] shows the energy levels of the five central members: Sm(III), Eu(III), Gd(III), Tb(III) and Dy(III).

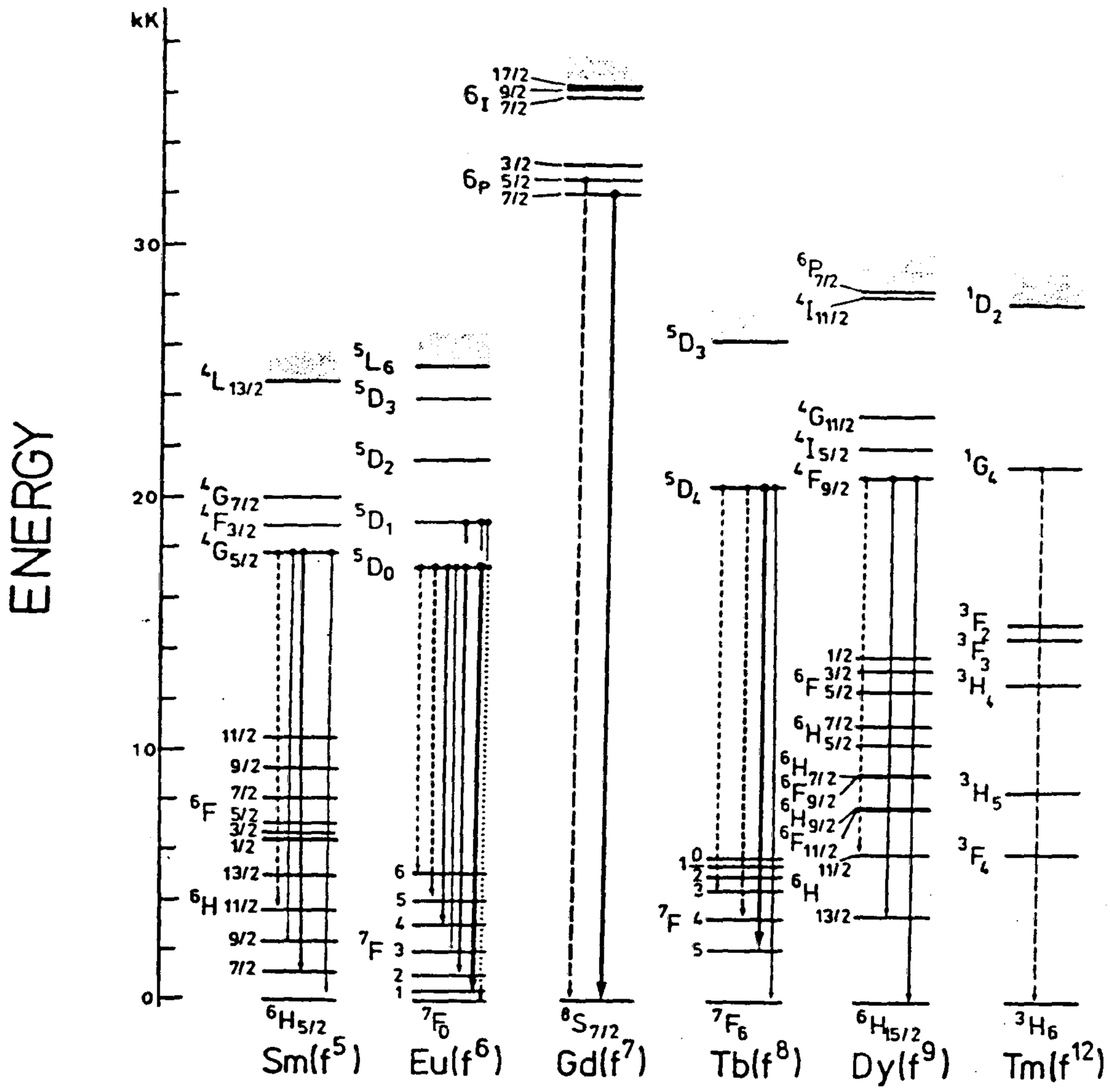


Figure 1.3 Principle emission lines of certain lanthanides.

The remaining members rarely luminesce except in the solid state. The principal reasons for this is that there are no large gaps between potential emissive levels and acceptor levels of the ground manifold. These smaller energy level differences are efficiently bridged by non-radiative processes. The rate of radiationless de-excitation is a strong function of the energy gap. Both Eu(III) and Tb(III) have reasonably large energy gaps and emit relatively strongly in the visible region of the spectrum. The emission of the Eu^{3+} ion consists of transitions from the ^5D manifold (mainly from the $^5D_0$ level) to the ^7F manifold. (See Figure 1.3)

The emissive levels of Eu^{3+} ($^5D_0$) and Tb^{3+} ($^5D_4$) lie 17,250 cm^{-1} (580 nm) and 20,500cm^{-1} (488 nm) above the ground level. For Eu^{3+}, the allowed transitions are $^5D_0 \rightarrow {}^7F^{1,2,4,6}$. The strongest emissions are observed in the $^5D_0 \rightarrow {}^7F_1$ and $^7F_2$ transitions. The relative intensities of these two emissions are very sensitive to the detailed nature of the ligand environment, reflecting the hypersensitive character of $^5D_0 \rightarrow {}^7F_2$. The $^5D_0 \rightarrow {}^7F_{0,3,5}$ transitions are strictly forbidden and emissions resulting from these are either weak or unobservable, see Table 1.4.

Table 1.4 Major characteristics of Tb^{3+} and Eu^{3+} emission intensity spectra for complexes in aqueous solution

Transition	Spectral region, nm	Relative Intensities	Other characteristics
Terbium			
$^5D_4 \rightarrow {}^7F_6$	485-500	med-strong	moderate sensitivity to ligand environment
$\rightarrow {}^7F_5$	540-555	stongest	best probe transition
$\rightarrow {}^7F_4$	580-595	medium	moderate sensitivity to ligand environment
$\rightarrow {}^7F_3$	615-625	med-weak	some structuring under high resolution
$\rightarrow {}^7F_2$	645-655	weak	moderate sensitivity to ligand environment
Europium			
$^5D_0 \rightarrow {}^7F_0$	578-580	weak	nondegenerate transition: appears as a single sharp line
$\rightarrow {}^7F_1$	585-600	strongest	sharp and structured under high resolution
$\rightarrow {}^7F_2$	610-630	strongest	intensitity exibits *hypersensitivity* to ligand environment
$\rightarrow {}^7F_3$	645-660	weak	always very weak
$\rightarrow {}^7F_4$	680-705	weak	intensity and structuring very sensitive to ligand environment

The generally low emission quantum yields[28] and the fact that experimentally measured decay times are one or two orders of magnitude lower than the estimated radiative lifetimes times[26] indicate that the decay of the emitting $^5D_0$ state is mainly governed by non-radiative transitions.

According to the theory of non-radiative transitions in lanthanide complexes[22,23,29] the radiative relaxation between the various j states may occur by interaction of the electronic levels of the lanthanide ion with suitable vibrational modes of the environment. The efficiency of these processes depends on the energy gap between the ground and excited states and the vibrational energy of the oscillators.[22,23,30]

The pronounced hard character of Eu^{3+} means it will have a preferance for 'hard' bases.[31] Water molecules and hydroxide ions (OH^-) are particularly strong ligands for Eu^{3+}, so that water molecules are commonly coordinated to Eu^{3+} ions simultaneously with other ligands. When solvents containing O-H groups are coordinated to lanthanide ions, efficient non-radiative deactivation of the emitting $^5D_0$ level takes place via weak vibronic coupling with the vibrational states of the O-H oscillators.[32,33] The consequences are that lanthanide luminescence is rarely observed in aqueous solution. If the O-H oscillators are replaced by low frequency O-D oscillators, the vibronic deactivation pathway becomes much less efficient[32,33] (Figure 1.4).

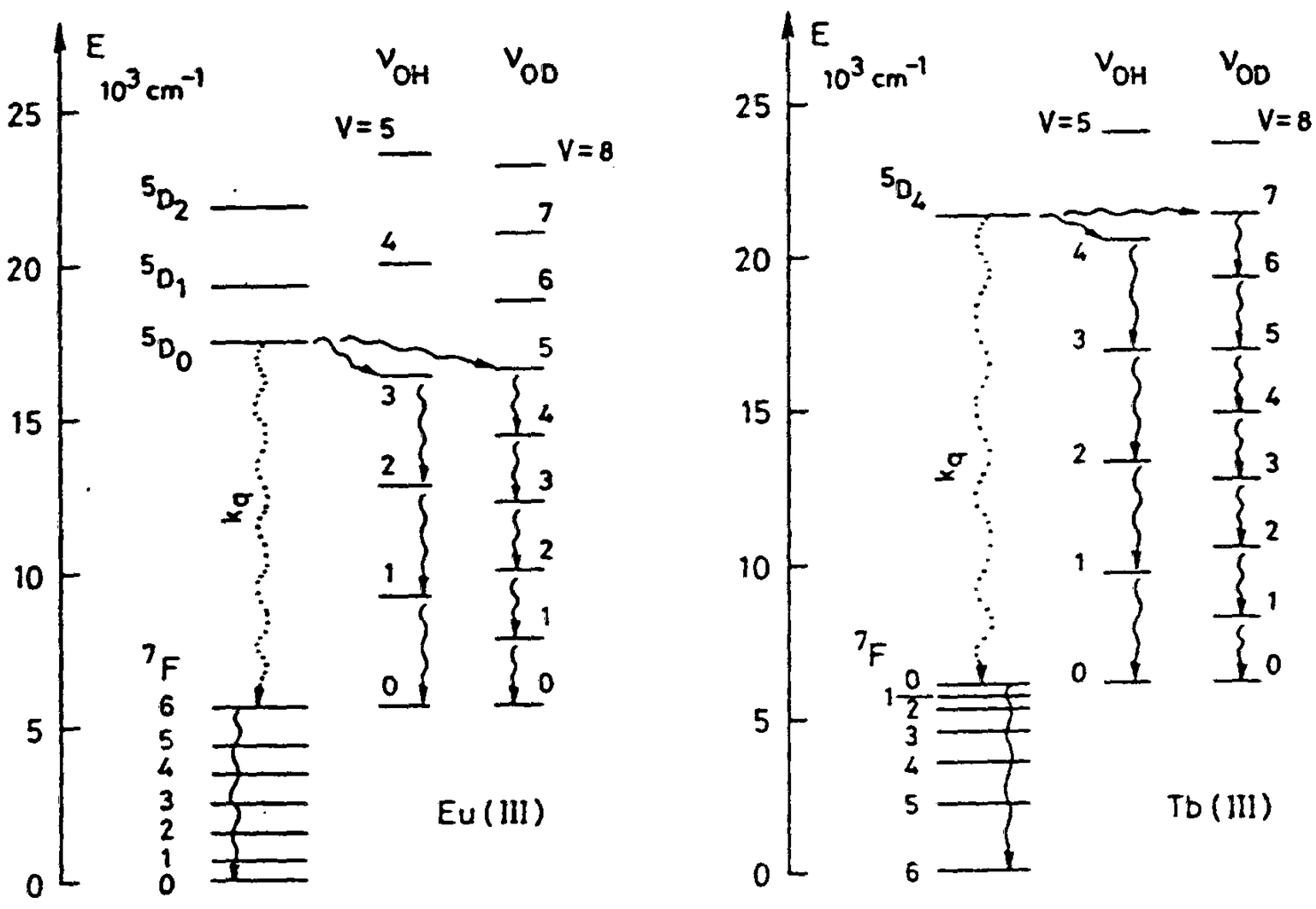


Figure 1.4 Radiationless deexcitation of Eu(III) and Tb(III) ions by OH and OD oscillators.

The OH oscillators act independently and the rate of radiationless de-excitation is therefore directly proportional to the number of OH oscillators in the inner coordination sphere of the metal ion. The differences in the effects of H_2O versus D_2O upon luminescence lifetimes can be exploited in determining the number of water molecules coordinated to Eu^{3+} or Tb^{3+} in the presence of other ligands. Horrocks and Sudwick[11,34] by carrying out experiments in H_2O and D_2O solutions have shown that for Eu^{3+} and Tb^{3+} complexes, the number of coordinated water molecules n, is given with an estimated uncertainty of 0.5 by equation (1), where τ_{H2O} and τ_{D2O} are the experimental excited-state lifetimes (in ms) in H_2O and D_2O solutions and q is 1.05 and 4.2 for Eu^{3+} and Tb^{3+} complexes respectively. The q values show that the vibronic coupling is more effective for the Eu^{3+} ion than for Tb^{3+}, as expected on the basis of the energy gap between the ground and excited states.

$$n = q\left(\frac{1}{\tau_{H_2O}} - \frac{1}{\tau_{D_2O}}\right) \qquad \text{.....(1)}$$

The method has been applied to various aminopolycarboxylate complexes including EDTA, NTA,and HEDTA among others, as well as encapsulating ligands like the fuctionalised polyaza macrocycles (Figure 1.5).[34]

This work is important in designing and evaluating new ligand systems to evaluate how effectively they exclude water from the the coordination sphere of Ln^{3+}, which is necessary for Ln^{3+} luminescence.

We can conclude that the coordination environment effects the luminescence intensity and lifetimes via the presence of (*i*) solvent molecules coordinated to the metal ion and (*ii*) low lying short-lived excited states. Non-radiative losses can therefore be controlled by an appropriate choice of the coordination environment of the lanthanide ion. The luminescence intensity, in addition to depending on the competition between radiative and non-radiative deactivations, is related to the efficiency of light absorption.

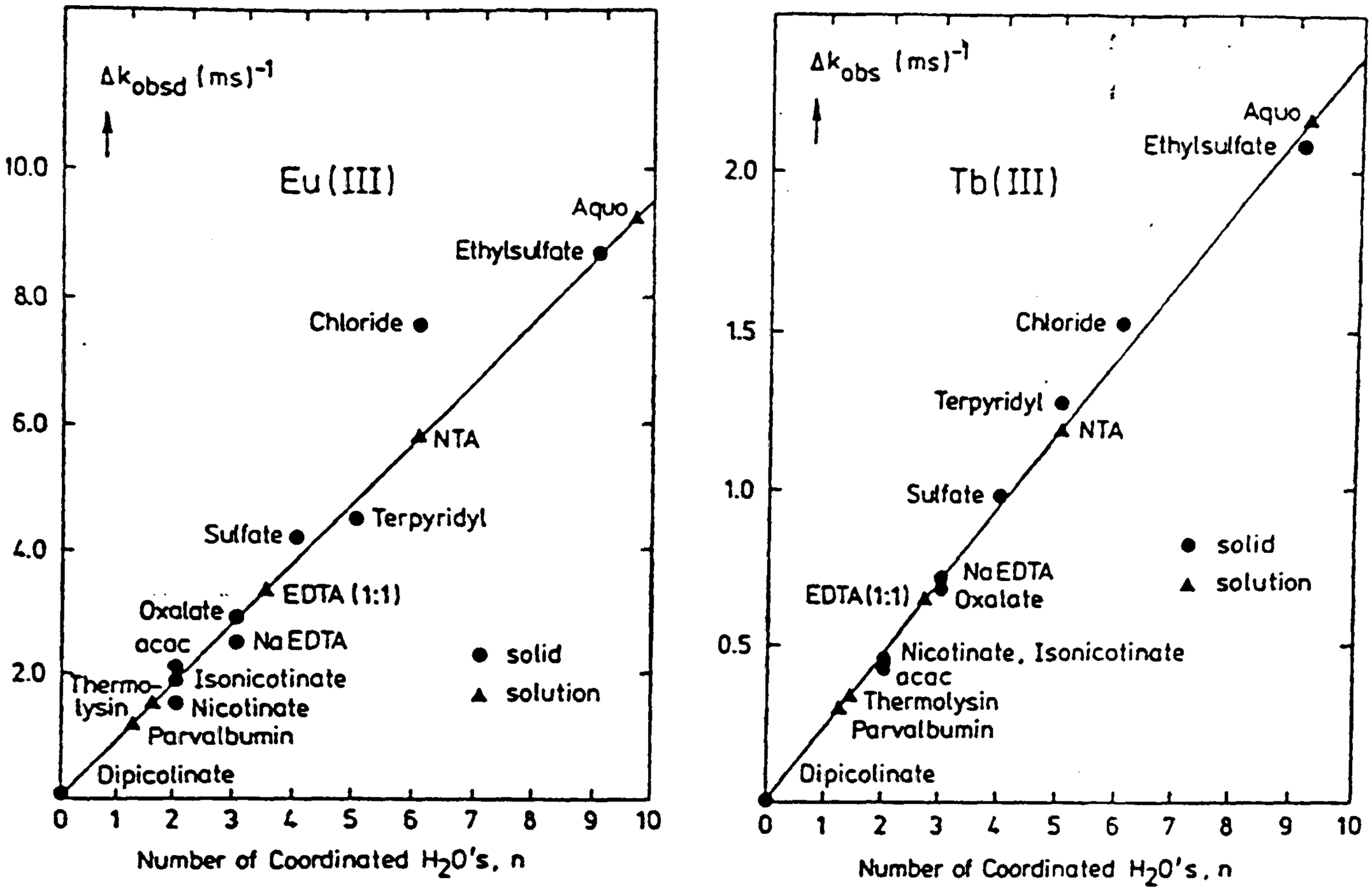


Figure 1.5 Plots of Δk_{obsd} (ms^{-1}) versus the number of coordinated water molecules.
$\Delta k = 1/\tau_{H_2O} - 1/\tau_{D_2O}$

1.2.2 Coordination Properties of the Lanthanides

All of the trivalent lanthanide ions Ln^{3+}, share a number of common coordination properties. They can all be classified as type 'a' cations in the Ahrland, Chatt and Davies classification scheme[35], and as '*hard*' acids in the Pearson classification scheme.[36] The binding preference is O>N>S. It is also generally agreed that Ln^{3+} coordination occurs predominanatly via ionic bonding interactions, leading to a strong preferance for negatively charged donor groups that are also '*hard*' bases. Water molecules and hydroxide ions (OH^-) are particularly strong Ln^{3+} ligands and in neutral to basic aqueous solution, only donor groups having negatively charged oxygens can bind sufficiently strongly to prevent $Ln(OH)_3$ precipitation. Also, in aqueous solution, neutral oxygen or nitrogen ligands are only able to bind when present in multidentate ligands that contain at least one or two other donor groups having negatively charged oxygens. The results are that many lanthanide complexes are highly labile in aqueous solution. However, recent spectroscopic studies on the hexaazamacrocyclic (HAM) complex $Eu(C_{22}H_{26}N_6)^{3+}$ (Figure 1.6) has shown this complex to be remarkably stable in aqueous solution even in the presence of a strong chelating agent diethylenetriaminepentaacetic acid (dtpa) or a competing metal ion (Y^{3+}).[37,38]

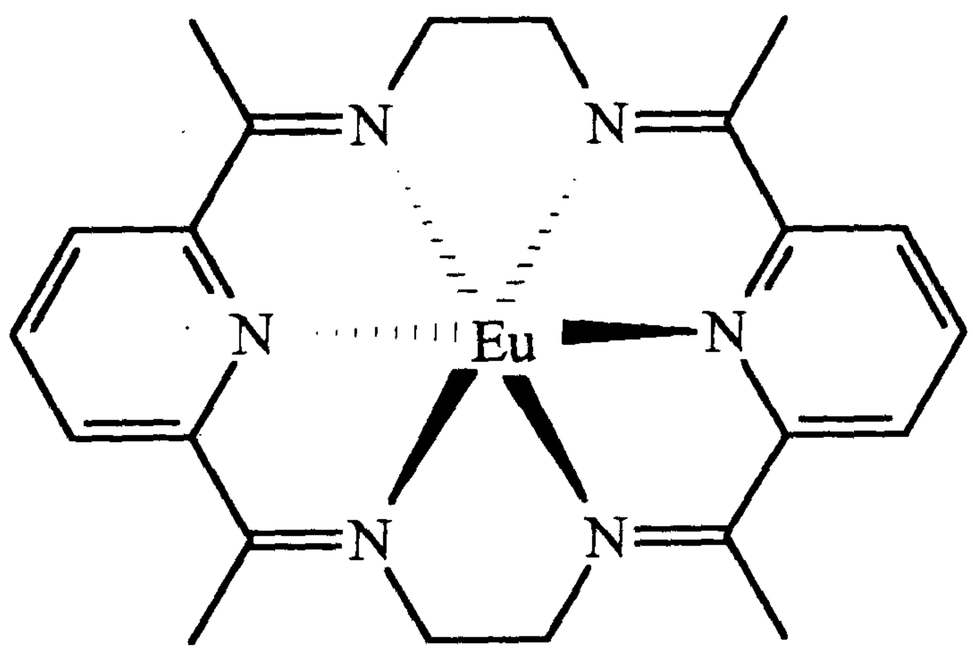


Figure 1.6 Structure of $Eu(HAM)^{3+}$.

The predominantly ionic character of Ln^{3+} interactions and the relative low charge to ionic radius ratios of Ln^{3+} can account, in large part, for several additional aspects of lanthanide coordination chemistry. Firstly, there is little or no '*directionality*' in Ln^{3+}-ligand interactions so that primary coordination numbers and complex geometries are determined almost entirely by ligand characteristics, (conformational properties and the number, size and charged nature of donor groups). The most common coordination numbers exibited by Ln^{3+} are eight and nine.[12,21] In aqueous solution, the '*average*' primary hydration numbers for Eu^{3+} and Tb^{3+} in water are between eight and nine.[12,22,39]

In the lanthanides there are no strong directionality requirements by ligand field effects, the ligands arranging as best they can around the spherical surface of the cation.

1.3 THE ANTENNA EFFECT: SENSITISED EMISSION

Even though both europium and terbium have some unique photophysical properties - strong luminescent and long-lived excited states - they are very poor light absorbers with very small absorption coefficients in the visible and UV spectral regions.[22] The strongest absorption band of $Eu^{3+}aq$ in the near UV and visible region occurs at 393 nm with an extinction coefficient lower than $3M^{-1}cm^{-1}$. Therefore, to make use of the excellent emitting properties of these ions, one has to compensate for the difficulty in populating their excited states, for the lack of absorption bands.

Weissman's 1942 paper[40] effectively launched the area of sensitissed emission from lanthanide chelates. Using the sun as a source of white radiation, he noticed that the absorption of UV light by a variety of salicylaldehyde and β-diketonate ligands resulted in emission lines characteristic of the f-f transitions of Eu^{3+} ion, to which these ligands were coordinated. This has recently been demonstrated as the **antenna effect**.[41,42,43,44] This study and subsequent work demonstrated that sensitised emission from Eu(III) and Tb(III) ions arises from the metal ion from an intermolecular energy transfer process. The transfer process is fast (10^6-10^9 s^{-1}) and takes place between the triplet state of the chelating ligand, populated through an intersystem crossing process to the Ln(III) ion emissive levels. In some cases, Ln(III) levels higher than the emissive levels may be involved, but are depopulated via non-radiative processes. The complete mechanism published by Crosby *et al.*[45] is illustrated in Figure 1.7. In its simplest form, the model for the fluorescence of a rare earth chelate can be described as a sequence of four steps.

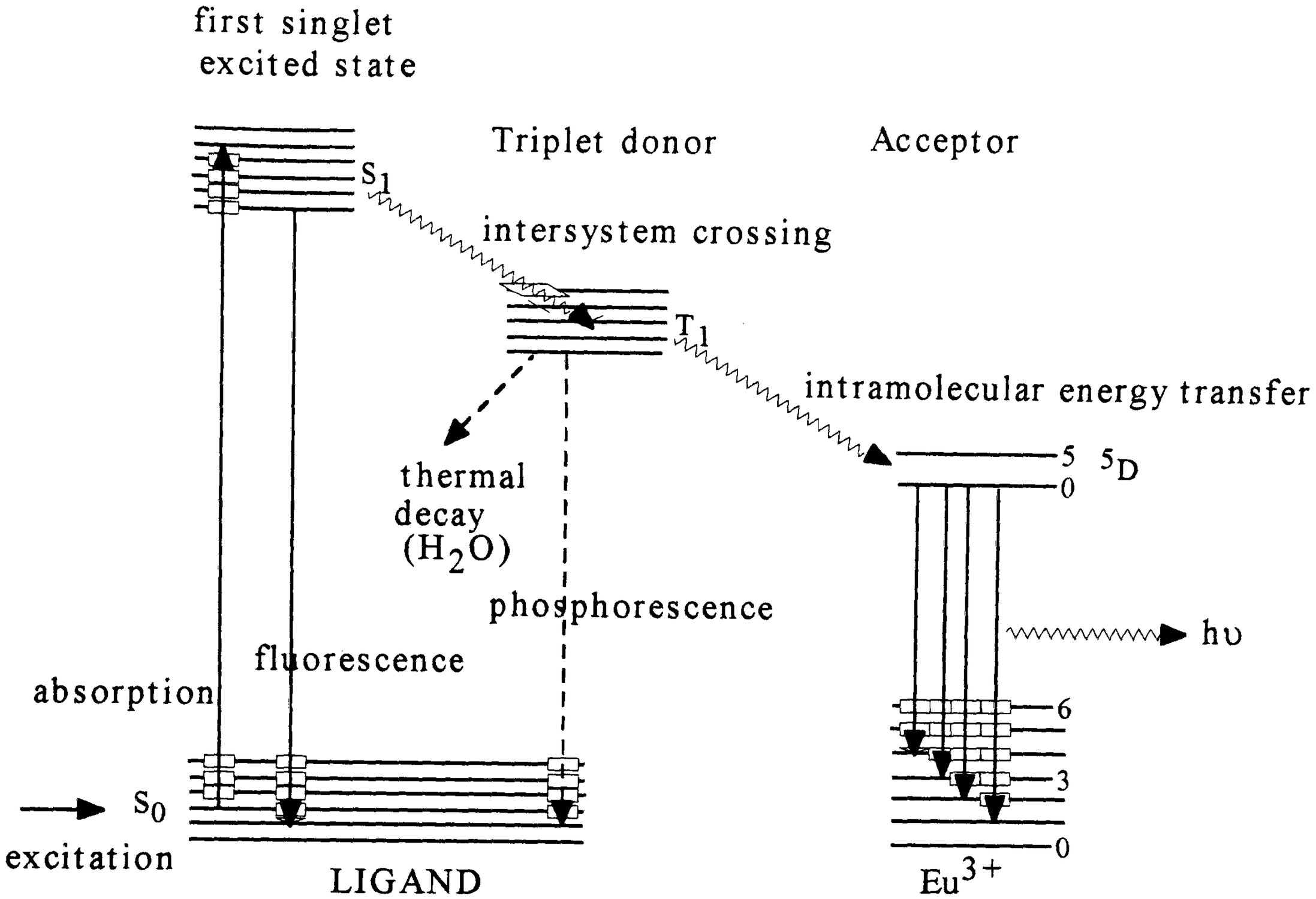


Figure 1.7 Schematic representation of the radiative processes of the chelate leading to Eu^{3+} metal ion luminescence.

(i) Excitation of the organic ligand from the ground singlet state S_o. This excitation brings the molecule to one of the vibrational multiples of the excited state S_1. The molecule rapidly loses its excess vibrational energy through some non-radiative deactivation process and falls to the lowest level of S_1.

(ii) Intersystem crossing within the excited ligand; energy is transferred from the singlet excited state to a triplet excited state of the ligand.

(iii) Intramolecular energy transfer: energy is transferred from the triplet excited state of the ligand to the resonance levels of the Ln(III) metal ion. This process has no parallel in either the fluorescence or phosphorescence of organic molecules.

(iv) Radiative transition resulting in light emission from the metal ion (metal-ion fluorescence).

Not all excited energy levels of the lanthanide ions, however, have this ability. Those that do, and are therefore responsible for the metal-ion fluorescence, are generally designated as 'resonance levels'.

Each route leading to ion fluorescence meets strong competition from parallel radiative and non-radiative deactivating transitions which have to be minimised for efficient ion fluorescence.

Thus for the above series of events to be completed successfully, the following requirements must be fulfilled:

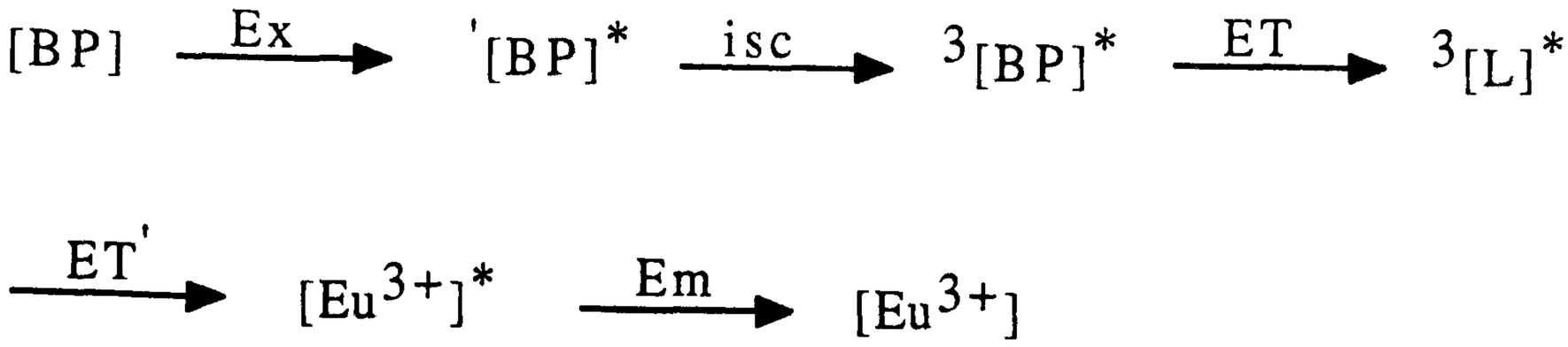
(a) Non-radiative deactivating transitions and $S_1 \rightarrow S_0$ and $T_1 \rightarrow S_0$ radiative transitions (ligand fluorescence and phosphorescence respectively), should be minimal. The excited triplet state of the ligand must have an appropriate lifetime, favouring energy transfer to the metal ion, rather than phosphoresence or thermal decay.

(b) The energy of the ion resonance level should be close to, and preferably just below that of the triplet state of the ligand. In that case the probability of the triplet to resonance level transition,$(T_1 \rightarrow D)$ is high.

(c) Radiationless transitions of the excited metal ion should be low.

From the vast number of studies carried out, there emerges a clear picture of the photophysical processes following ligand singlet excitation in Ln(III) complexes culminating in energy transfer to the emissive levels of the metal ion via a ligand localised triplet state (intra-molecular energy transfer). An alternative view espoused by Kleinerman[46] is that energy transfer from the excited singlet state, S_1, to the metal ion can occur directly with rate constants of 10^{11} sec^{-1} or higher. However the much slower rates of ligand to Ln(III) ion energy transfer observed, of $\sim 10^8$ sec^{-1}, are not consistent with this view and can only be explained by triplet level participation.

If the chelate cannot be efficiently excited by a given wavelength directly, a triplet sensitiser (such as benzophenone) (BP) with a triplet level higher than the triplet level of the ligand and which can be excited by this particular wavelength of light is added to the system; after the sensitiser is excited Eu^{3+} emission appears via intermolecular energy transfer.[47]



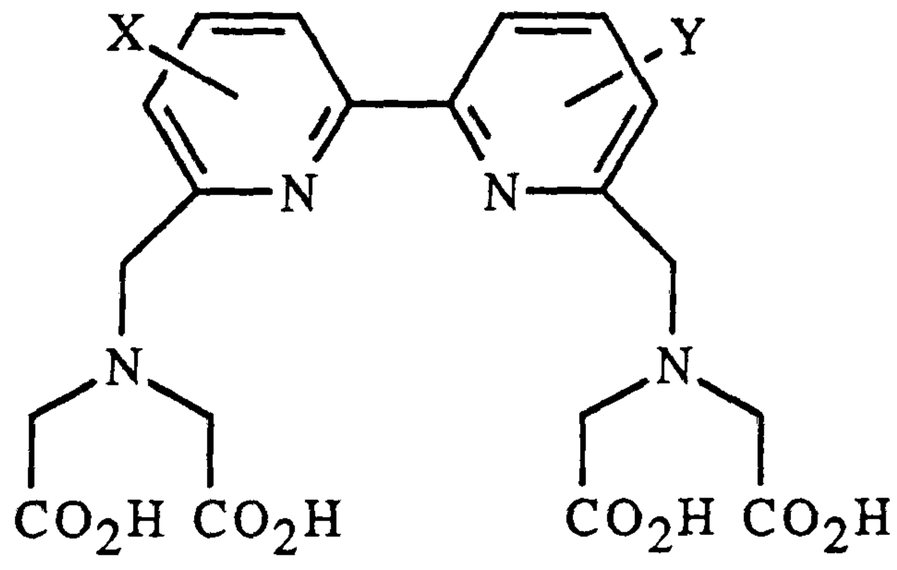
Intermolecular sensitisation of Ln(III)ß-diketonate complexes by 'triplet sensitisers' were first observed by Matowich and Suzuki[48], El-Sayed and Bhaumik[49] in the early 1960's. In later studies it was shown that energy transfer from the triplet state of the sensitiser to the triplet level of the chelate ligand occurs by a collisional process.[49] This transfer, which occurs at a rate greater than $10^6 sec^{-1}$, provides important confirmation of the role of the triplet level in the sensitisation of Ln(III) chelate luminescence, for they show that the triplet level of the ligand is higher than Eu^{3+} ion excited levels, and the triplet level of the sensitiser is higher than the chelate ligands. These are the necessary conditions for energy transfer.

The intensity of lanthanide ion fluorescence is mainly controlled by two important factors: first, the ability of the triplet state of the ligand to receive energy from the excited singlet state and to transfer it to the resonance level of the ion; second, the extent of non-radiative (quenching) of the excited state of the lanthanide chelate. These factors are influenced by the nature of the ion, the ligand, the ion-ligand bond, loosely attached adduct molecules, and the solvent. The influence of these different factors on fluorescence properties has been reviewed by Sinha.[50] The effect of the organic ligand on intramolecular energy transfer in europium and terbium chelates has been studied by Filipescu *et al.*[51] These extensive studies with various ß-diketonate chelates of europium and terbium were an attempt to find some correlation between the structure of the ligand, the position of the triplet level and the intensity of the fluorescence. Although some correlation was found between the structure of the ligand and the position of the triplet level, no systematic trends were observed between these two parameters and the variation in fluorescence intensity.

What is evident from both studies is that the overall ligand-ion energy transfer governs the absorption and emission properties of the ion, and the correct ligand-ion combination has to be found for optimal fluorescence intensity.

In general, it is difficult to predict theoretically which organic ligands will form fluorescent complexes with the lanthanides. Apart from the ß-diketonates, semi-quantitative studies on the fluorescent properties of a number of aromatic complexes with lanthanide ions identified certain classes of ligands as sensitisers of Eu^{3+} and/or Tb^{3+}. These ligands included the phenanthrolines, acetylene derivatives, five-membered heterocyclic rings, benzoic acid derivatives, biphenyl derivatives, pyridine derivatives, pyrimidine and pyrazine derivatives, di- and tripyridyl derivatives and quinoline derivatives.[52]

Recently, Mukhala and Kankare[53] have described the synthesis and luminescent properties of 20 different 2,2'-bipyridine derivatives chelated with Eu^{3+} or Tb^{3+} ions (Figure 1.8) The purpose of the work was to study the influence of substituents on the luminescence properties, with the aim to enhance the relative luminescence yield and to develop lanthanide chelates suitable for immunoassays.



(1) X=X=Y
(2) X=4-NO_2, Y=4'-Me
(3) X=4-NO_2, Y=H
(4) X=4-NO_2, Y=4'-NO_2
(5) X=4-EtO, Y=H
(6) X=4-EtO, Y=4'-EtO
(7) X=4-Br, Y=H
(8) X=4-Br, Y=4'-Br
(9) X=5-Br, Y=H
(10) X=3-COOEt, Y=3'-COOEt
(11) X=4-COOH, Y=4'-COOH
(12) X=3-PhCOOH, Y=3'-PhCOOH
(13) X=4-Ph, Y=4'-Ph
(14) X=4-(4-$MeOC_6H_4$), Y=4'-(4-$MeOC_6H_4$)
(15) X=4-(fur-2-yl), Y=4'-(fur-2-yl)
(16) X=4-(PhCH=CH), Y=4'-(PhCH=CH)
(17) X=Y=H, N,N'-dioxide
(18) X=4-NH_2, Y=H
(19) X=3-COOH, Y=3'-COOH
(20) X=3-OH, Y=3'-OH

Figure 1.8 2,2'-Bipyridine derivatives

The substituents at the bipyridine moiety have a significant effect on the luminescence properties: the best relative luminescence yields R were obtained for ligands with electron-donating substituents (Me, Ph), with electron-withdrawing substituents (NO_2, COOH) having a reverse effect (Table 1.5).

This is in agreement with the general observatons of Sinha and Filipescu. However, no clear correlation between the relative luminescence yields and the substituent parameters could be found.

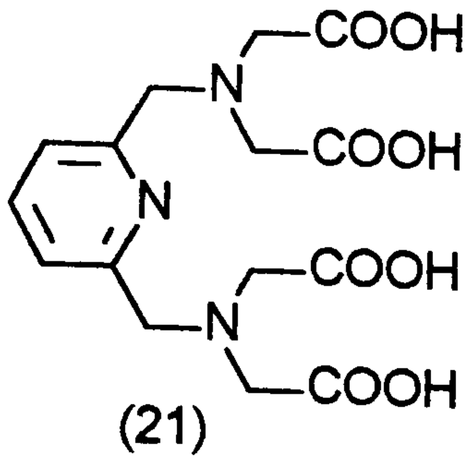
Table 1.5 Relative luminescence yields (logR), excitation maxima (λ_{exc}), and emission decay constants (k_{chel}) of the Europium(III) and Terbium(III) chelates of 2,2',2'',2'''-[(2,2'-Bipyridine-6,6'-diyl)bis-(methylenenitrilo)]tetrakis-(acetic acids) **1-20**

	Substitution at bipyridine	Eu^{3+}			Tb^{3+}	
		LogR	λ_{exc} [nm]	k_{chel} [ms^{-1}]	LogR	k_{chel} [ms^{-1}]
1	unsubstituted	5.50	307	1.70	5.27	0.82
2	4,4'-dimethyl	5.61	310	1.69	5.51	0.68
3	4-nitro	4.12	328	1.87	2.94	0.71
4	4,4'-dinitro	4.14	338	1.87	weak	
5	4-ethoxy	5.33	298	1.72	5.31	0.62
6	4,4'-diethoxy	5.11	290	1.80	5.13	0.67
7	4-bromo	5.36	315	1.76	5.12	0.96
8	4,4'-dibromo	5.31	310	1.86	5.02	1.33
9	5'-bromo	4.16	320	1.77	4.50	1.37
10	3,3'-bis(ethoxycarbonyl)	4.31	275	1.80	weak	
11	4,4'-dicarboxy	4.42	325	1.71	3.54	1.59
12	3,3'-bis(benzyloxy)	5.36	292	1.84	2.38	1.59
13	4,4-diphenyl	5.52	325	1.73	5.18	1.12
14	4,4'-bis(4-methoxyphenyl)	5.57	325	1.78	4.84	3.07
15	4,4'-bis(fur-2-yl)	5.32	330	1.76	2.10	1.50
16	4,4'-distyryl	2.95	315	1.75	2.63	1.11
17	N,N'-dioxide	4.72	280	1.35	3.74	1.11
18	4-amino	4.42	290	1.72	4.62	0.67
19	3,3'-dicarboxy	4.90	283	1.74	2.23	0.63
20	3,3'-dihydroxy	3.91	340	2.62	weak	

Further work by Mukhala *et al.*[54] concentrated on the role of the aromatic ligand in sensitising lanthanide ion luminescence. Twelve different candidates containing six-membered N-heteroaromatic rings as the energy absorbing and transferring moieties were synthesised (Figure 1.9), and the relative luminescence yields, excitation maxima, and emission decay constants of their europium(III) and terbium(III) chelates determined (Table 1.6).

Table 1.6 Relative luminescence yields (logR), excitation maxima (λ_{exc}), and emission decay constant (k_{chel}) of the Europium(III) and Terbium(III) chelates of ligands **21-32**

Ligand	Parent aromatic structure	logR	λ_{exc} [nm]	k_{chel} [ms^{-1}]	LogR	λ_{exc} [nm]	k_{chel} [ms^{-1}]
21	pyridine	4.83	265	2.48	4.87	2.67	0.80
22	isoquinoline	4.44	324	2.63	weak		
23	benz(f)isoquinoline	4.41	355	2.65	2.35	355	
24	acridine	weak			weak		
25	2,2'-bipyridine	5.50	307	1.70	5.27	307	0.82
26	3,3;'biisoquinoline	5.33	330	2.75	weak		
27	di(pyrid-2-yl)ketone	4.61	272	1.02	4.51	272	0.54
28	2,2'-bipyrimidine	5.17	250	1.96	weak		
29	4,4'-bipyrimidine	5.08	290	1.78	2.27	284	1.60
30	1,8-naphthyridine	4.65	312	3.09	4.50	312	1.02
31	1,10-phenanthroline	5.26	272	1.98	4.71	272	1.46
32	2,2':6',2''-terpyridine	5.94	333	0.73	5.64	333	0.76



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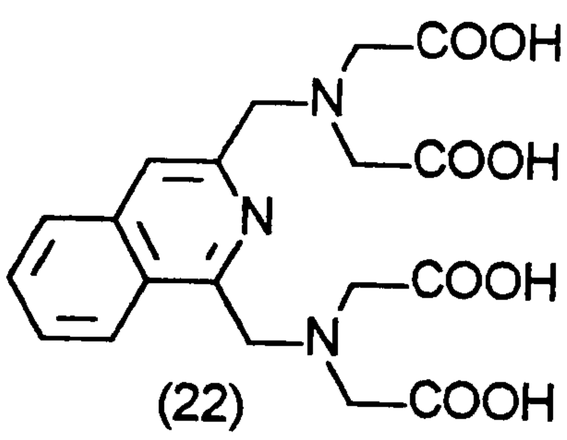

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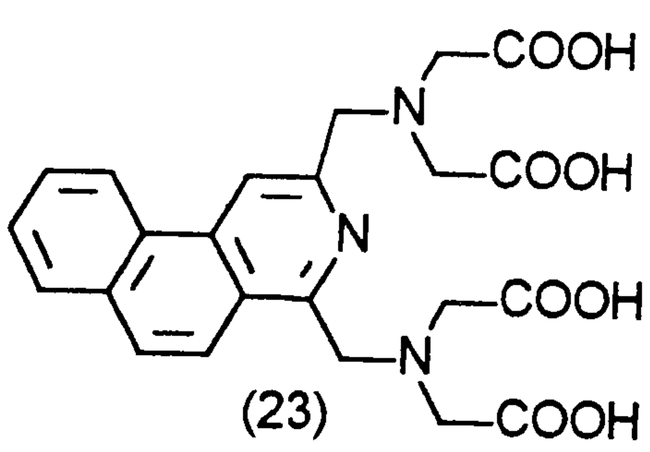

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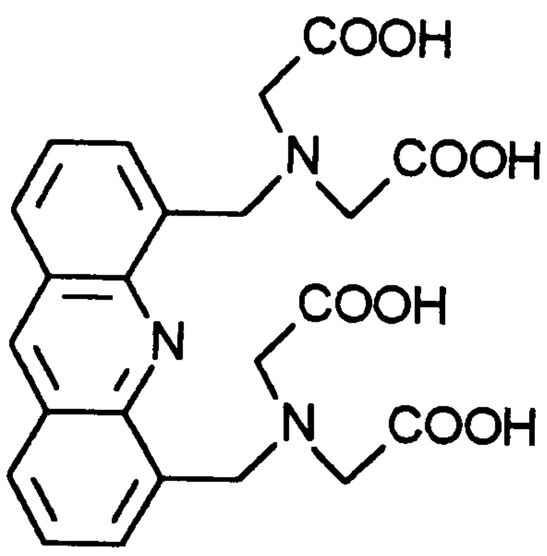

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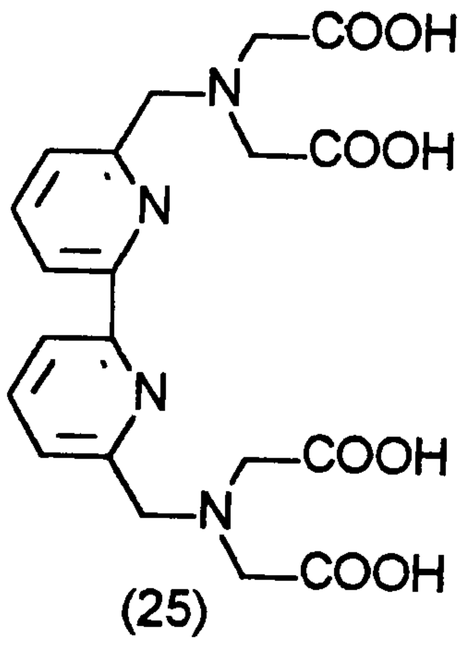

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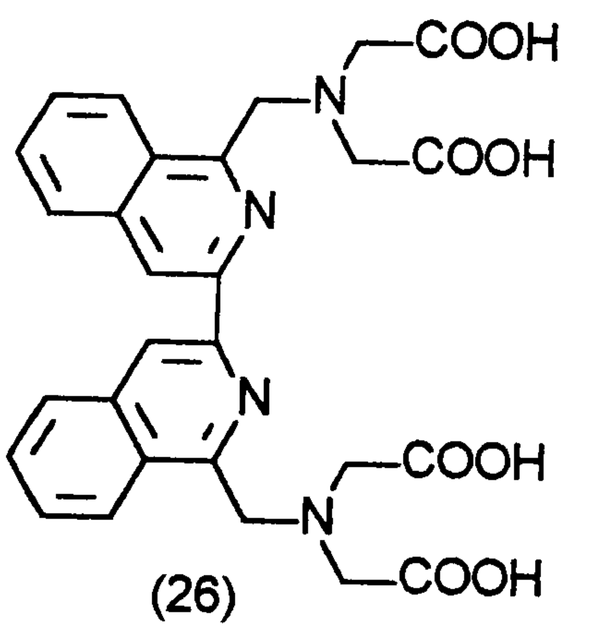

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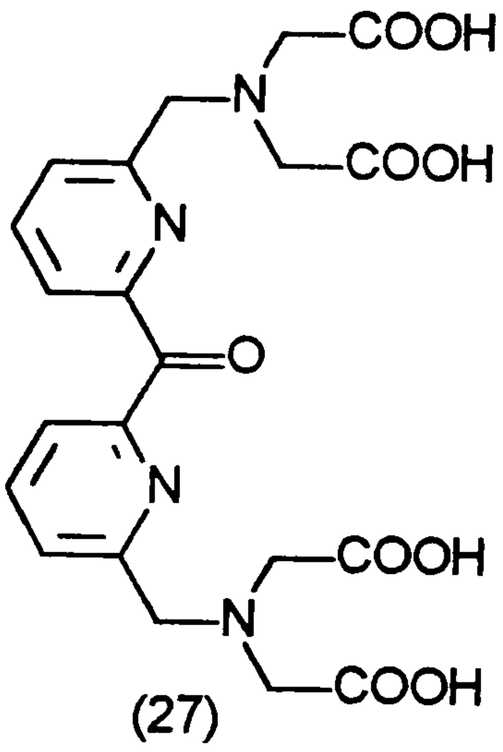

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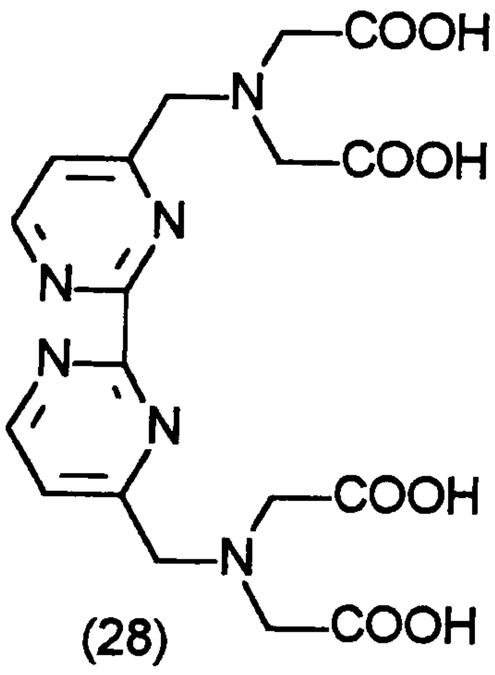

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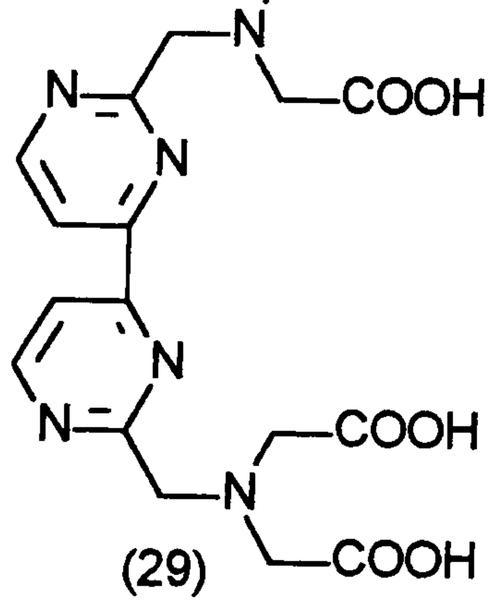

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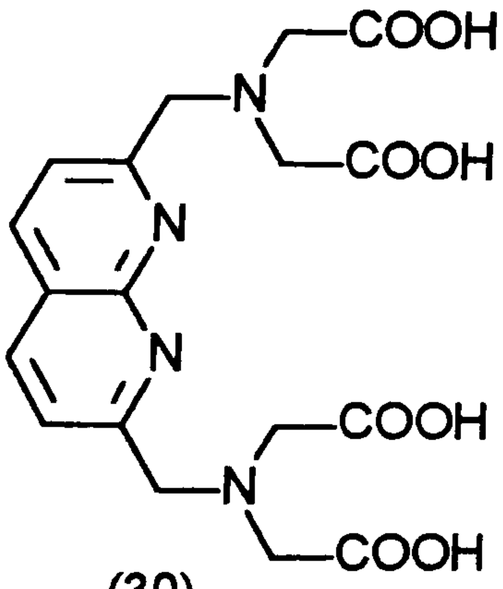

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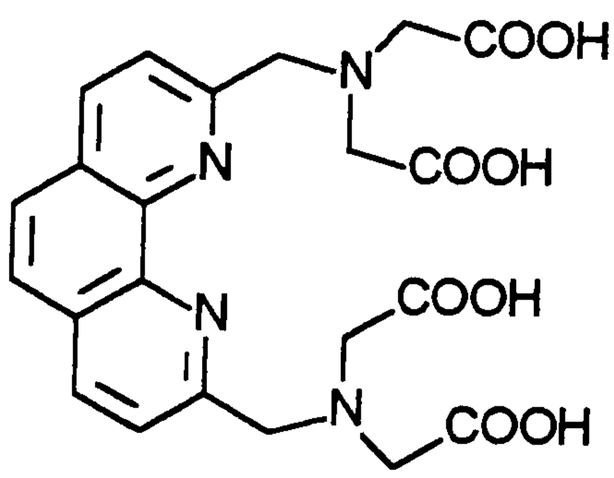

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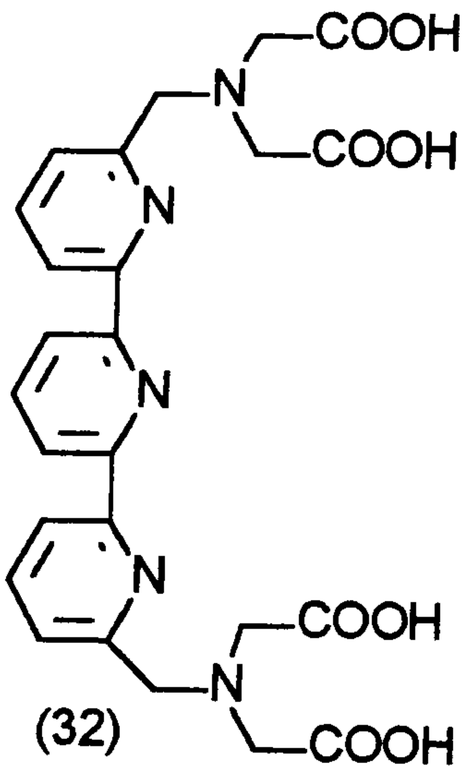


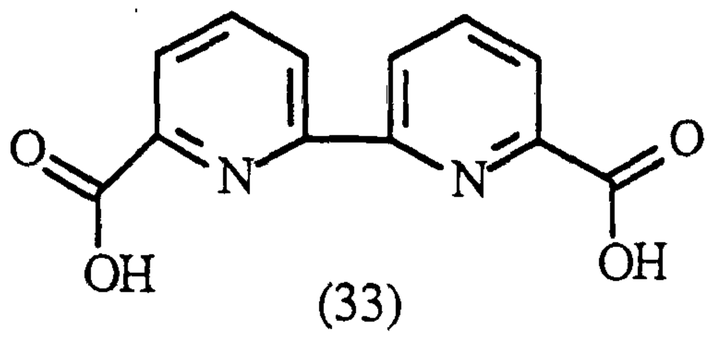
(32)

Figure 1.9

The results show the importance of the ligand having a triplet state of the right energy in relation to the resonance levels of the metal-ion. The triplet state of the ligand must lie above the resonance levels of the lanthanide (III) ion to enable energy transfer to take place. The triplet states of the acridine, isoquinoline, 3,3'-bis-isoquinoline, and 2,2'-bipyrimidine ligands, **22,24,26,28** probably lie below the resonance level of the Tb^{3+} ion. These complexes show very weak luminescence. Also in spite of the higher conjugation of benzyl(f)isoquinoline, compared to isoquinoline, ligand **23** has almost the same relative luminescence yield with Eu^{3+} ion as **22**, while the Eu^{3+} chelate of the 3,3'-bis-isoquinoline derivative **26** luminesces nearly ten times stronger than the Eu^{3+} chelate of ligand **22**. Since k_{chel} of the chelates of **22** and **26** are almost the same, the difference in their relative yields is due to the aromatic part of the complexes. The relative luminescence yields are generally higher for Eu^{3+} than for Tb^{3+} chelates of the ligands **21-32**.

The excitation wavelengths λ_{exc} of chelates is also very important, and should be as high as possible; a high excitation wavelegth makes the selection of materials (strips, lenses, excitation sources, *etc.*) for a bioaffinity assay more flexible.

Continuing their systematic studies, Mukhala *et al.*[55] have investigated the influence of chelating groups on the luminescence properties of a number Eu^{3+} and Tb^{3+} chelates of 2,2'-bipyridine (Figure 2.0). Their results based on relative luminescence yields and emission decay constants identified the (methylenenitrilo)bis(acetic acid) **36** and (methylenenitrilo)bis(methylphosphonic acid) **40** as suitable chelating groups. In fact, ligand **40** appears to offer better protection to the lanthanide ion from the surrounding solvent, with calculations showing there are fewer H_2O molecules coordinated to the lanthanide ion than in the corresponding carboxylate chelate **36**.[55] This might be due to the bulkiness of the phosphonate groups.[56] Ligand **34** had the lowest luminescence both with Eu^{3+} and Tb^{3+} ions, probably due to the low chelating ability of the amide functions and their influence on the triplet state of the ligand. The use of trimethylene bridges between the acetic acid groups in ligand **37** make the chelating parts more rigid compared to the parent ligand **36**. However, this is not reflected in the luminescence parameters.



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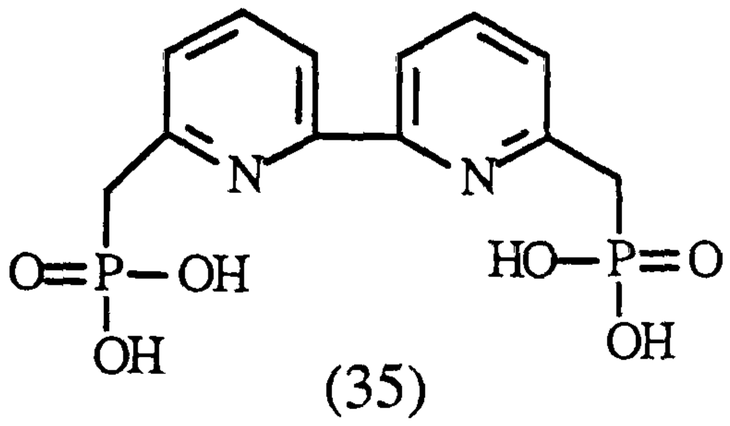

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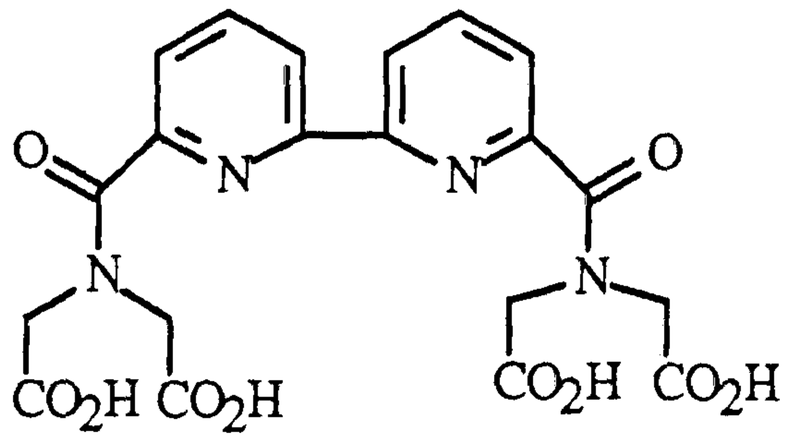

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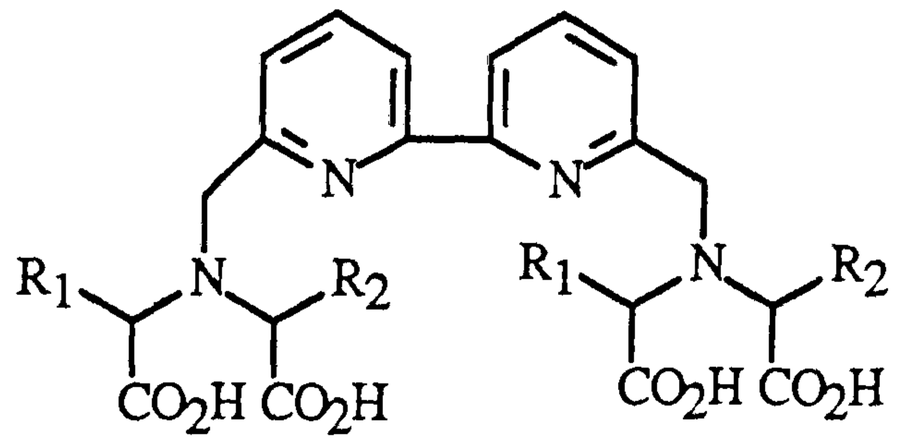

(36) $R_1=R_2=H$
(37) $R_1=R_2=CH_2CH_2CH_3$
(38) $R_1=H$, $R_2=(CH_2)_4OH$
(39) $R_1=H$, $R_2=CH_2COOH$

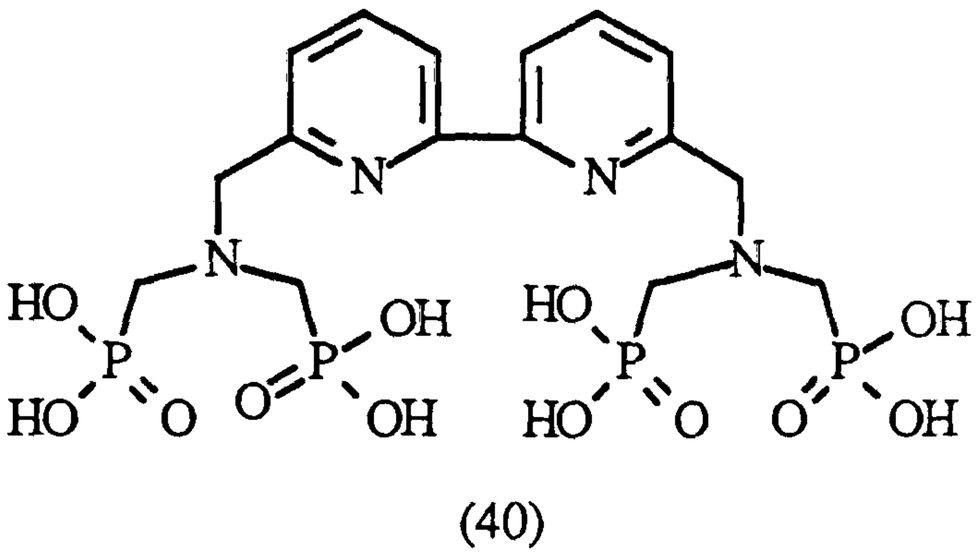

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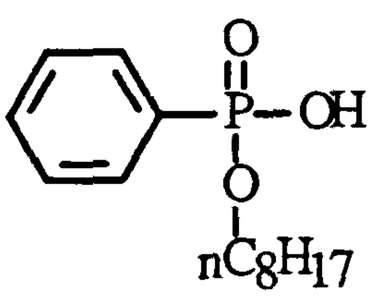
Figure 2.0

In a move away from traditional N-heterocyclic ligands as sensitisers of lanthanide luminescence Shinkai *et al.*[57] report on lipophilic phenylphosphonic acids which are able to form stable complexes with Eu^{3+} and Tb^{3+} ions which show strong luminescence, quantum yields (Φ= 0.25-0.30 for Tb^{3+} and 0.09-0.13 for Eu^{3+}) (Figure 2.1).



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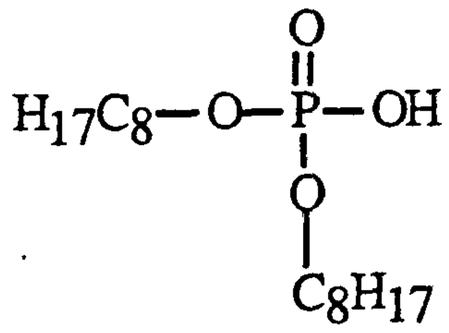

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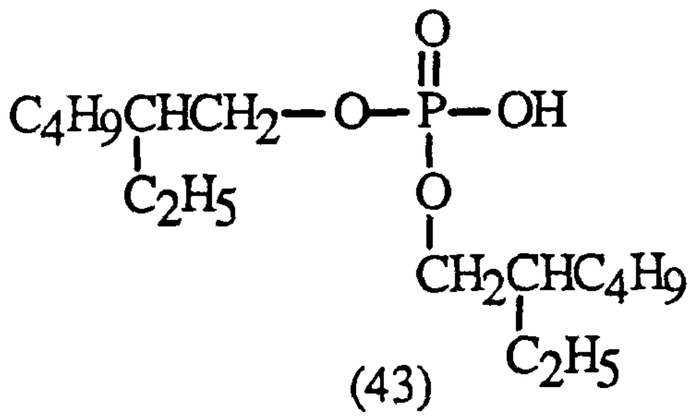

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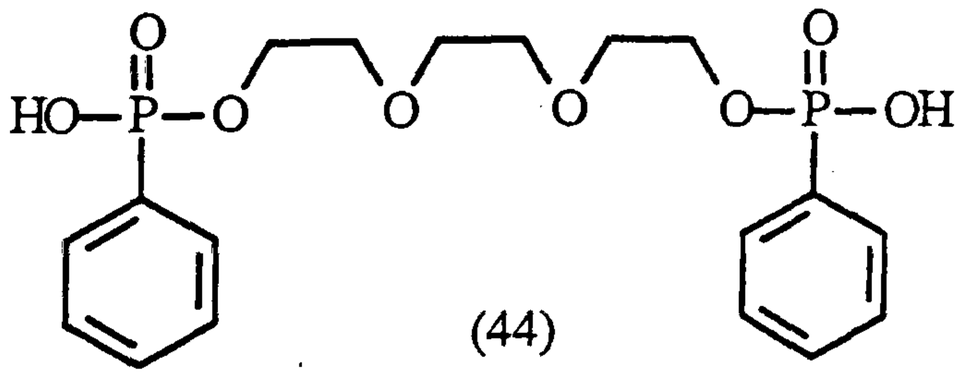

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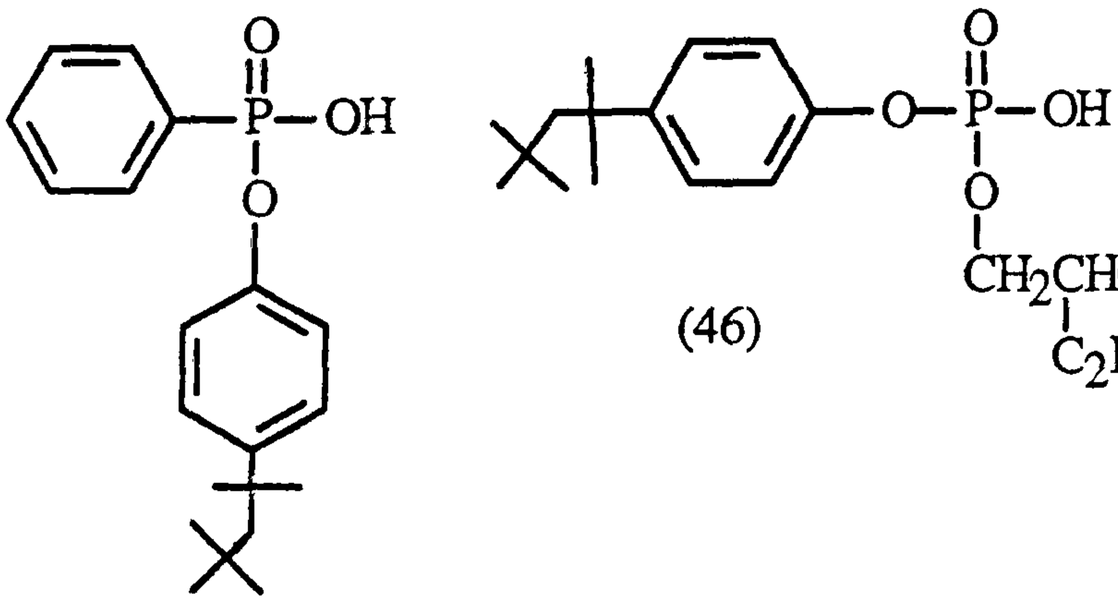

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(46)

Figure 2.1

Results of studies carried out in methanol/acetonitrile between the lanthanide complexes of ligands which have a chromophoric benzene ring **41**, **44**, **45**, **46** and those ligands which have no aryl group **42** and **43** indicates that emission of Tb^{3+} and Eu^{3+} luminescence is induced by an energy-transfer mechanism (sensitisation) (Figure 2.2).[57]

All the ligands form 1:3 metal/ligand complexes which ensures considerable encapsulation of the metal ion. Correlation between the ligand structure and the quantum yield reveals that energy-transfer from from Ar-P is about 12-90 times more efficient than that from ArO-P, implying that the sensitiser should be 'directly' linked to the ligation atom. Compund **44** which has two Ar-P groups within a molecule shows the highest quantum yield, probably a result of the more efficient energy transfer from two Ar-P groups and the podand-like metal encapsulation effect.

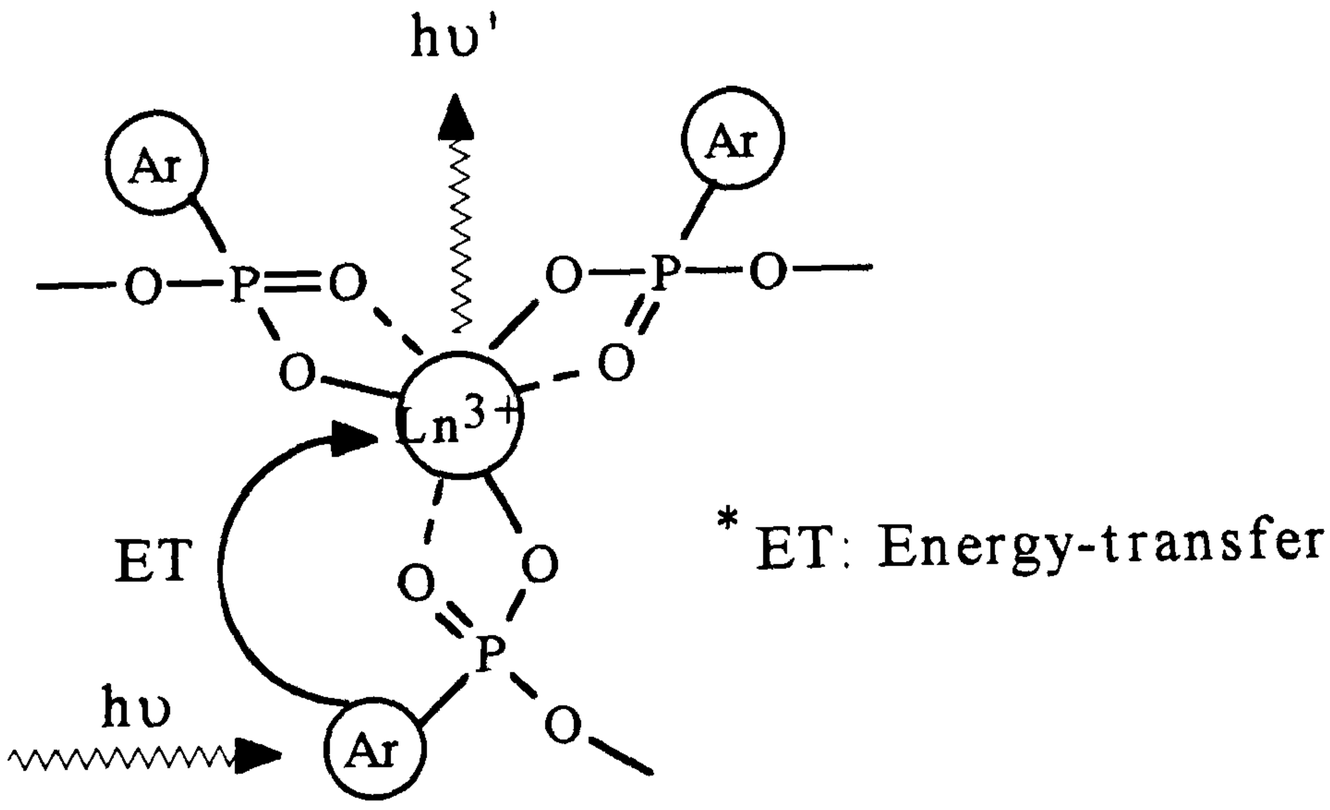
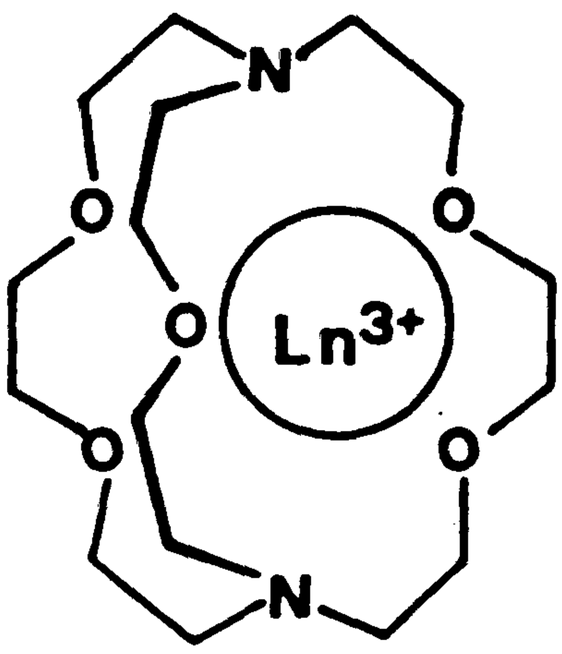


Figure 2.2

1.3.1 Lanthanide cryptates.

The choice of aromatic ligands to play the role of chromophore/sensitiser ligand in any lanthanide complex presents problems. Namely, lanthanide ions do not exibit strong coordinating ability towards such ligands, and in aqueous solution where solvent molecules efficiently compete to occupy coordination sites, the results are quite unstable chelates, with dissociation occurring, particularly at low concentrations. The problem can be overcome by the use of additional chelating groups, an example being the use of two (methylenenitrilo) bis(acetic acid) groups in suitable positions to increase the stability of various aromatic chelates, Mukhala *et al.*[53,54] (Figures 1.6, 1.7).

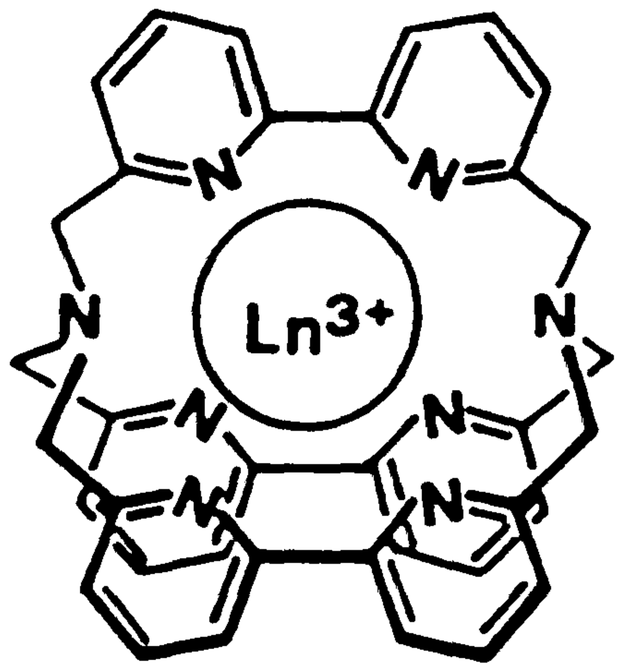
A different approach is based on the use of encapsulating ligands. In this way the metal cannot escape its coordination sphere, and interactions with external species like solvent molecules are prevented or reduced. Lehn and co-workers were the first group to synthesise and study the photophysics of complexes of lanthanide ions with cage-type ligands. These were the Eu^{3+} complexes (cryptands) based on 221[58] and trispyridine[59] ligands, [Eu⊂ 2.2.1]$^{3+}$ **47** and [Eu⊂ bpy.bpy.bpy]$^{3+}$ **48** respectively (Figure 2.3).



(47)

Eu^{3+} or Tb^{3+}

2.2.1 cryptates



(48)

Eu^{3+} or Tb^{3+}

bpy.bpy.bpy cryptates

Figure 2.3

Results obtained for the [Eu⊂ 2.2.1]$^{3+}$ cryptate[42,60,61] showed that, although the cryptand ligand shielded the lanthanide ion from interactions with water molecules (average of 2.5 coordinated water molecules), the absence of a chromophore and the presence of a thermally activated decay pathway involving LMCT (Ligand-to-Metal Charge Transfer) observed as absorption bands in the UV region resulted in complexes displaying poor luminescent properties. Although the energies of the LMCT excited states are not known, from the absorption spectrum of the [Eu⊂2.2.1]$^{3+}$ the lowest LMCT level can be expected to lie between the $^{1}\pi\pi^{*}$ and $^{3}\pi\pi^{*}$ ligand-centred levels.

However, in contrast the cryptand [Eu⊂ bpy.bpy.bpy]$^{3+}$ displays excellent luminescent properties.[41-44,62] This cryptand containing three 2,2'-bipyridine units exibits intense absorbtion bands in the near UV region due to π-π^{*} transitions in the bipyridine units (Figure 2.4).[42] Excitation to the spin-allowed $^{1}\pi\pi^{*}$ ligand centered (LC) level leads to population of the luminescent metal centered (MC) $^{5}D_{o}$ f-f level of Eu^{3+} via intersystem crossing from the higher energy lowest spin-forbidden $^{3}\pi\pi^{*}$ level with reasonably high efficiency (10%). The result is a complex which is an efficient molecular device for the conversion of UV light absorbed by the ligands into visible luminescence emitted by the metal ion. Even in very dilute aqueous solutions (10^{-5}M) this complex is able to convert about 1% of the incident photons into emitted visible photons. Lehn termed these complexes incorporating lanthanides as Light Conversion Molecular Devices (Figure 2.5).[43]

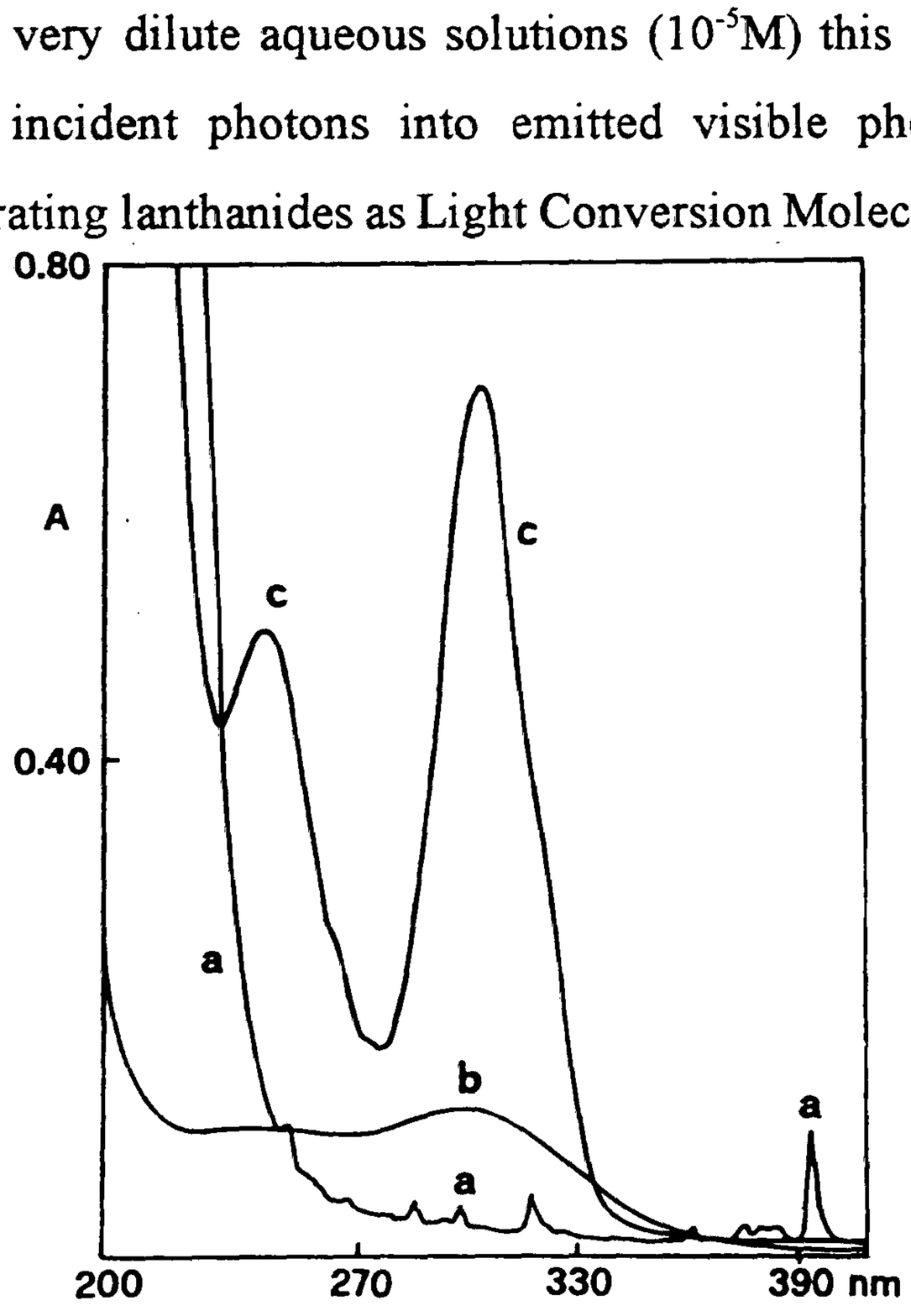


Figure 2.4 Absorption spectra of Eu(aq)$^{3+}$ $5x10^{-2}$M (a), [Eu 2.2.1]$^{3+}$ $1x10^{-3}$M (b), and [Eu bpy.bpy.bpy]$^{3+}$ $3x10^{-5}$M (c). Note the difference in concentations

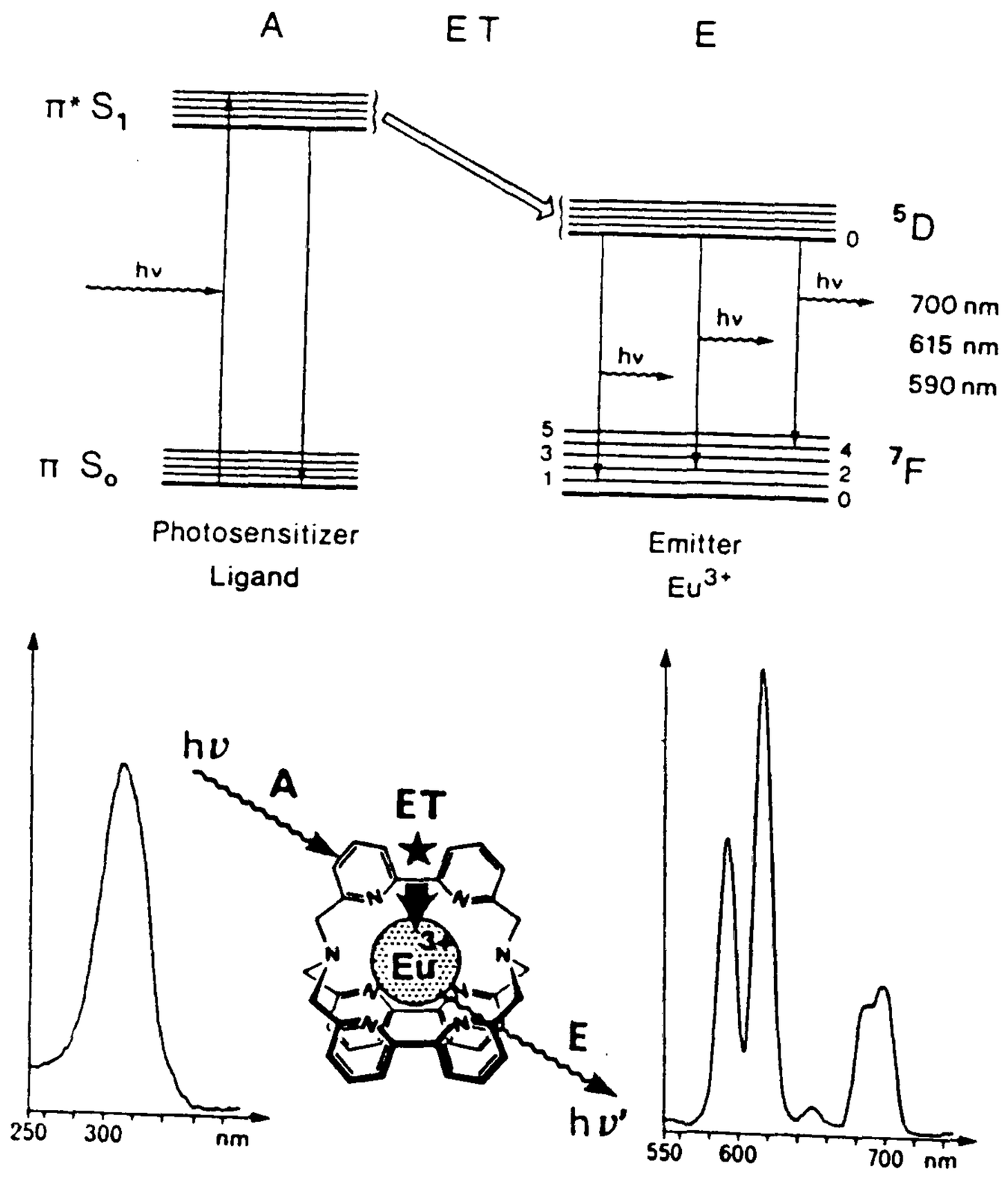
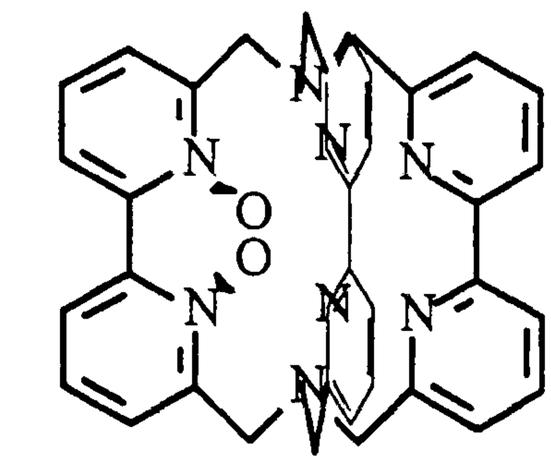


Figure 2. 5 Top: Absorption-Energy Transfer-Emission A-ET-E process illustrated for a photosensitising ligand Eu(III) emitter. Bottom: Light conversion process performed by the cryptate [Eu(III)48]; excitation spectrum (left) and emission spectrum (right).

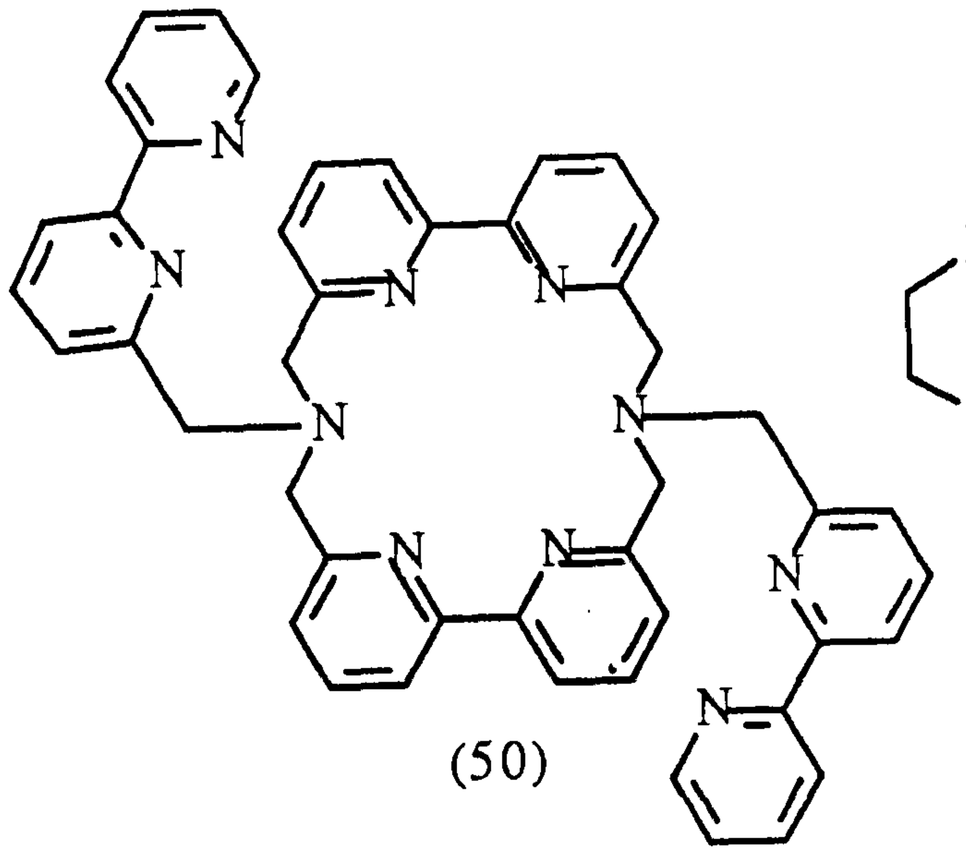
However, for both the $[Eu\subset 48]^{3+}$ and $[Tb\subset 48]^{3+}$ cryptates, comparisons of the lifetimes and quantum yields in H_2O and D_2O solutions indicates that non-radiative deactivation via O-H vibrations take place. It has been estimated that ~2.5 water molecules are still coordinated to the encaged metal ions.

This would explain the low values of the emission quantum yields, which in the case of the Tb^{3+} complex is further aggravated by the presence of a thermally activated decay pathway involving the triplet excited state of the ligand. In an effort to reduce non-radiative loses and increase emission quantum yields, considerable effort has been devoted to the synthesis of a vast array of different encapsulating ligands (Figure 2.6).[63,64,65,66]

The results of the photophysical characteristics of the corresponding Eu^{3+} and Tb^{3+} complexes[63] has shown the difficulty in predicting how even small logical variations in the ligand structure will effect the photophysics of the lanthanide complex.[63,64,65,66]



(49)



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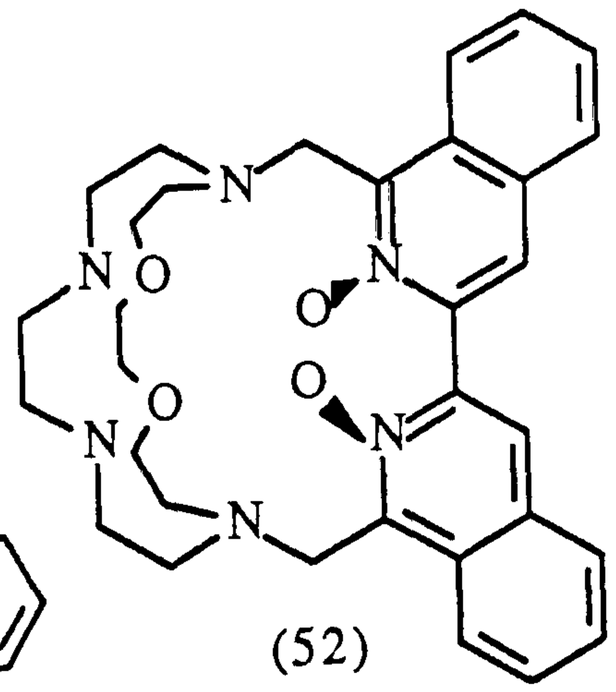
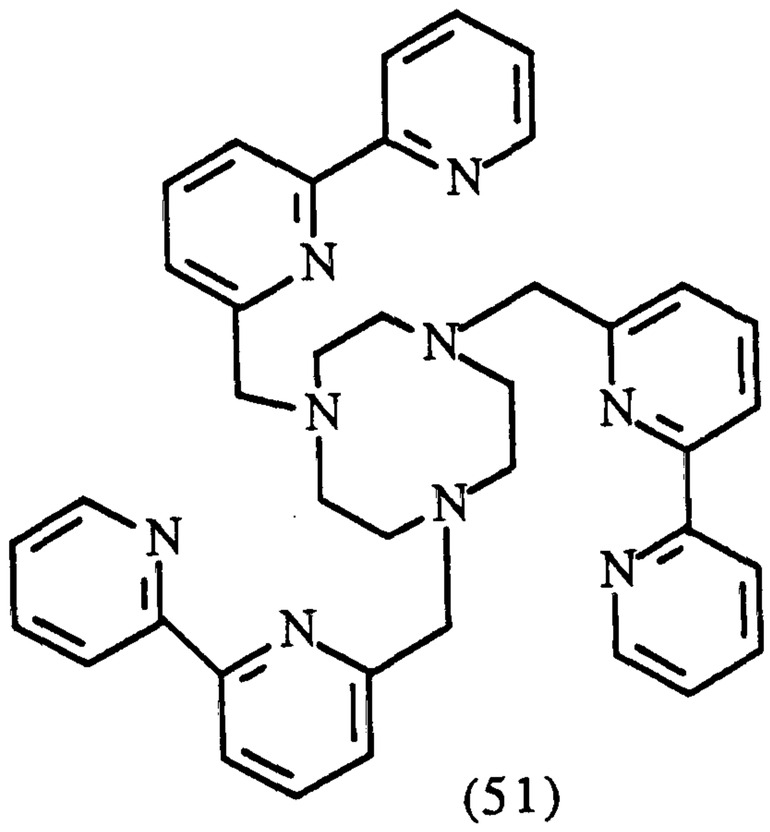
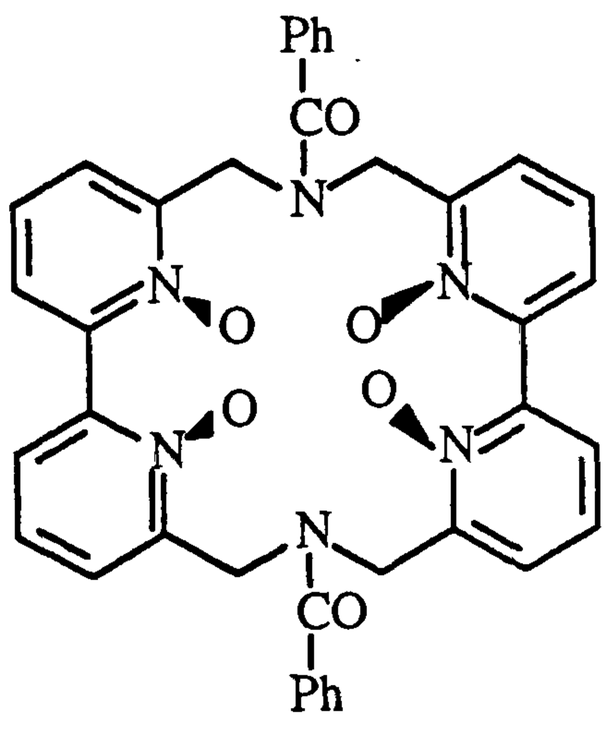


(52)



(51)



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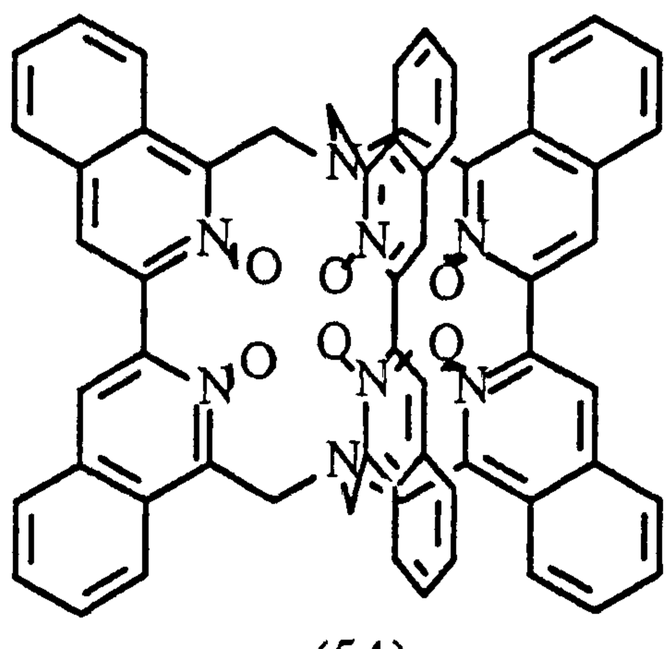


(54)

Figure 2.6

Generally, Eu^{3+} complexes of N-Oxide cryptates **49** and **52** display excellent luminescent properties with quantum yields considerably higher than the corresponding $[Eu \subset 48]^{3+}$ cryptate. This appears to be a result of (i) better protection of the encapsulated metal ion from H_2O molecules and (ii) more efficient ligand-to-metal energy transfer due to a closer approach between the ligand and the metal ion. This, is in spite of the introduction of an additional decay pathway for the ^5D_o Eu^{3+} level (population of LMCT excited states). Infact among the complexes examined the most intense luminescence is shown by the Eu^{3+} cryptate of 3,3'-biisoquinoline-2,2'-dioxide (Φ= 0.17) **52**. Attempts to improve the photophysical properties of ligand **49** by the incorporation of more photoactive units (antenna) gave disappointing results with the $[Eu \subset 54]^{3+}$ showing a dramatic decrease in the quantum yield (Φ= 0.002).[65]

In contrast, although measurements indicate that for both the Eu^{3+} and Tb^{3+} complex of the triazacyclonane ligand **51** there appears to be no water molecules coordinated to the metal ions, the Eu^{3+} complex displays short luminescent lifetimes and low quantum yields where

as the Tb^{3+} complex displays a high quantum yield (Φ= 0.37 in H_2O).[63,66] This difference has been attributed to the presence of low-lying LMCT levels in the Eu^{3+} complex.

The main deactivation processes occurring in Tb^{3+} and Eu^{3+} complexes is illustrated in Figure 2.7.[63] Studies have shown that both thermally activated decay processes involve different mechanisms. In Eu^{3+} complexes this process involves the population of low-lying LMCT states from the $^5D_0$ Eu^{3+} emitting state, followed by efficient non-radiative decay to the ground state. The role played by the LMCT states has been clearly pointed out by the photophysical behaviour of the ion pairs between the $[Eu\subset 2.2.1]^{3+}$ cryptate and fluoride anions. In Tb^{3+} complexes, the Tb^{3+} excited state ($^5D_4$) undergoes back energy transfer to the triplet ligand level before decay to the ground state. This only occurs where the triplet excited state of the ligand lies just above the Tb^{3+} emitting level enabling thermal population at room temperature. On the whole, results have shown that the non-radiative deactivation due to LMCT states in Eu^{3+} complexes are more difficult to suppress than those due to the ligand triplet state in Tb^{3+} complexes.

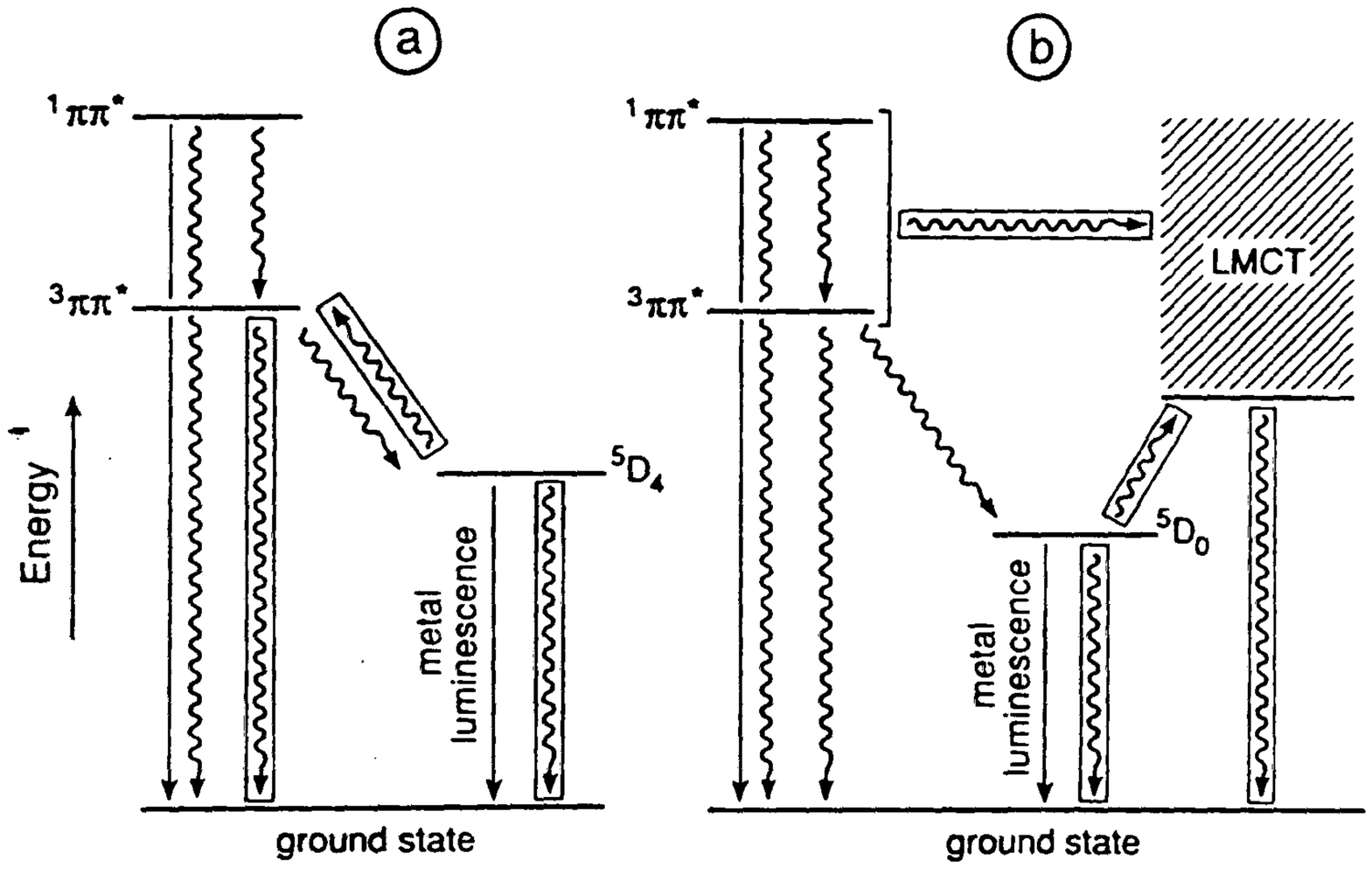


Figure 2.7 Scheme of the deactivation processes involved in the conversion of the light absorbed in the ligand into the light emitted by the metal ion in (a) Tb^{3+} and (b) Eu^{3+} complexes. The $^1\pi\pi^*$ state is the ligand excited state obtained upon excitation and the $^3\pi\pi^*$ state is the ligand excited state involved in the ligand-to-metal energy transfer. The $^5D_4$ and $^5D^0$ excited states are the luminescent states for the Tb^{3+} and Eu^{3+} ions respectively. The LMCT excited states are the ligand-to-metal energy transfer states in the Eu^{3+} complexes.

This is clearly illustrated by the Tb^{3+} and Eu^{3+} complexes of functionalised calixarenes.[67] Both ions form relatively stable water-soluble complexes with p-t-butylcalix[4]arene tetraacetamide **55** (Figure 2.8). But, where as the Tb^{3+} complex exibits a remarkably high

luminescence quantum yield (Φ= 0.2) and a long luminescence lifetime (1.5 ms) which is temperature-independent, indicating the absence of thermally activated decay processes and in the ligand **55** a $^3\pi\pi^*$ level from which efficient energy-transfer to $^5D_3$ or $^5D_4$ can take place. The properties which make **55** an ideal ligand for Tb^{3+} means the corresponding Eu^{3+} complex suffers from a combination of low quantum yield (Φ= $2x10^{-4}$) and short lifetime due to poor energy-transfer between the triplet excited state of the ligand ($^3\pi\pi^*$) and the emitting level of Eu^{3+} (^5D_o) and the presence of LMCT states observed as a shoulder in the absorption spectra, efficiently deactivating the ^5D_o emitting state.

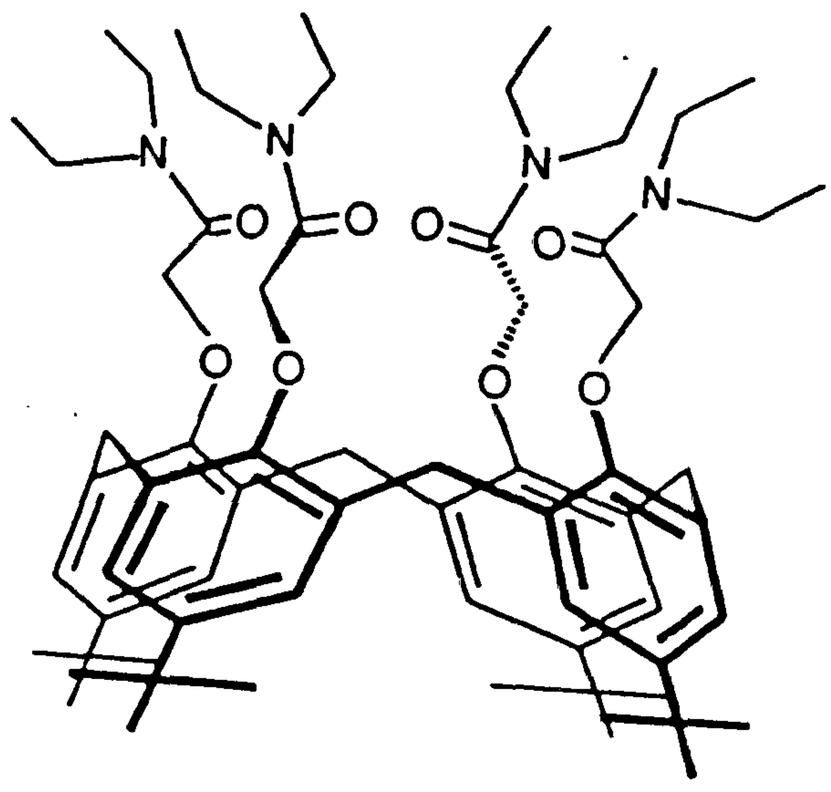
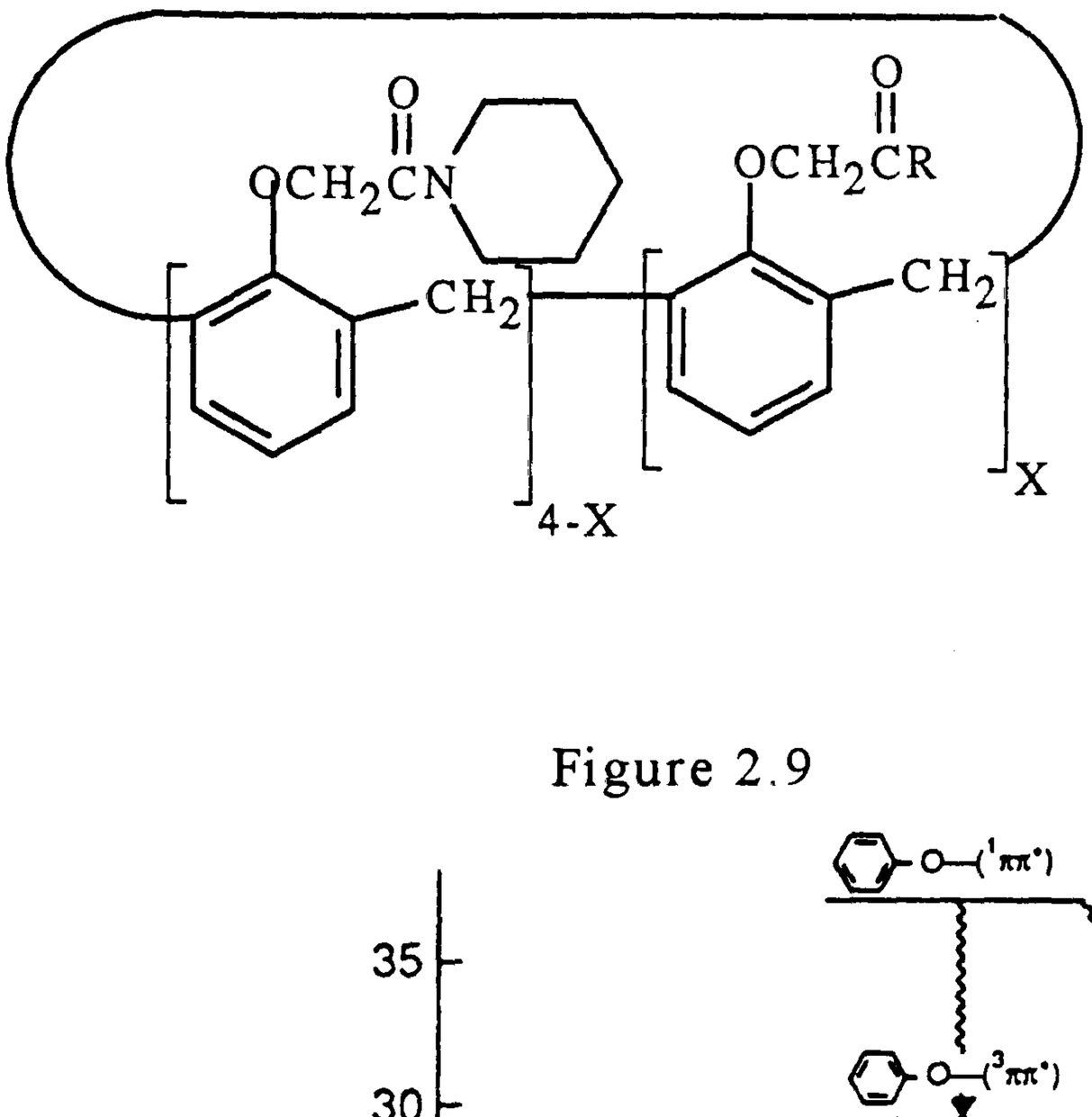


Figure 2.8 (55)

Recently Shinkai *et al.*[68] have shown that by appropriately modifying the ionophoric cavity of a conventional calix[4]arene with a sensitiser, energy-transfer to Eu^{3+} becomes possible. Their modified calix[4]arenes bearing three amide groups (composing the metal-binding site) and one sensitiser group (phenacyl or diphenacylcarbonyl group) (Figure 2.9) exibits a high luminescence quantum yield for Tb^{3+} (Φ= 0.27) and a vastly improved quantum yield for Eu^{3+} (Φ= 0.061). The significantly enhanced quantum yields for Tb^{3+} appears to be a result of energy-transfer from both the phenol units and the phenacyl unit (path A and path B) and just the phenacyl unit in the corresponding Eu^{3+} complexes (Figure 2.9). Their proposed energy diagram is shown in Figure 3.0. The triplet energy levels of the phenol unit and phenacyl unit are high enough to transfer the energy to Tb^{3+} ion levels whereas for the biphenyl-carbonyl group it is not sufficiently high. The energy levels for Eu^{3+} are lower than those for Tb^{3+} enabling energy transfer to take place from the triplet energy level of the biphenylcarbonyl group. The lack of energy transfer from the phenol unit to Eu^{3+} is rationalised by the C=O-to-Eu^{3+} charge-transfer band which efficiently deactivates the excited state of the phenol to the ground state.



(56a) X=0

(56b) X= 1, R= Ph-

(56c) X= 1, R= Ph-Ph-

Figure 2.9

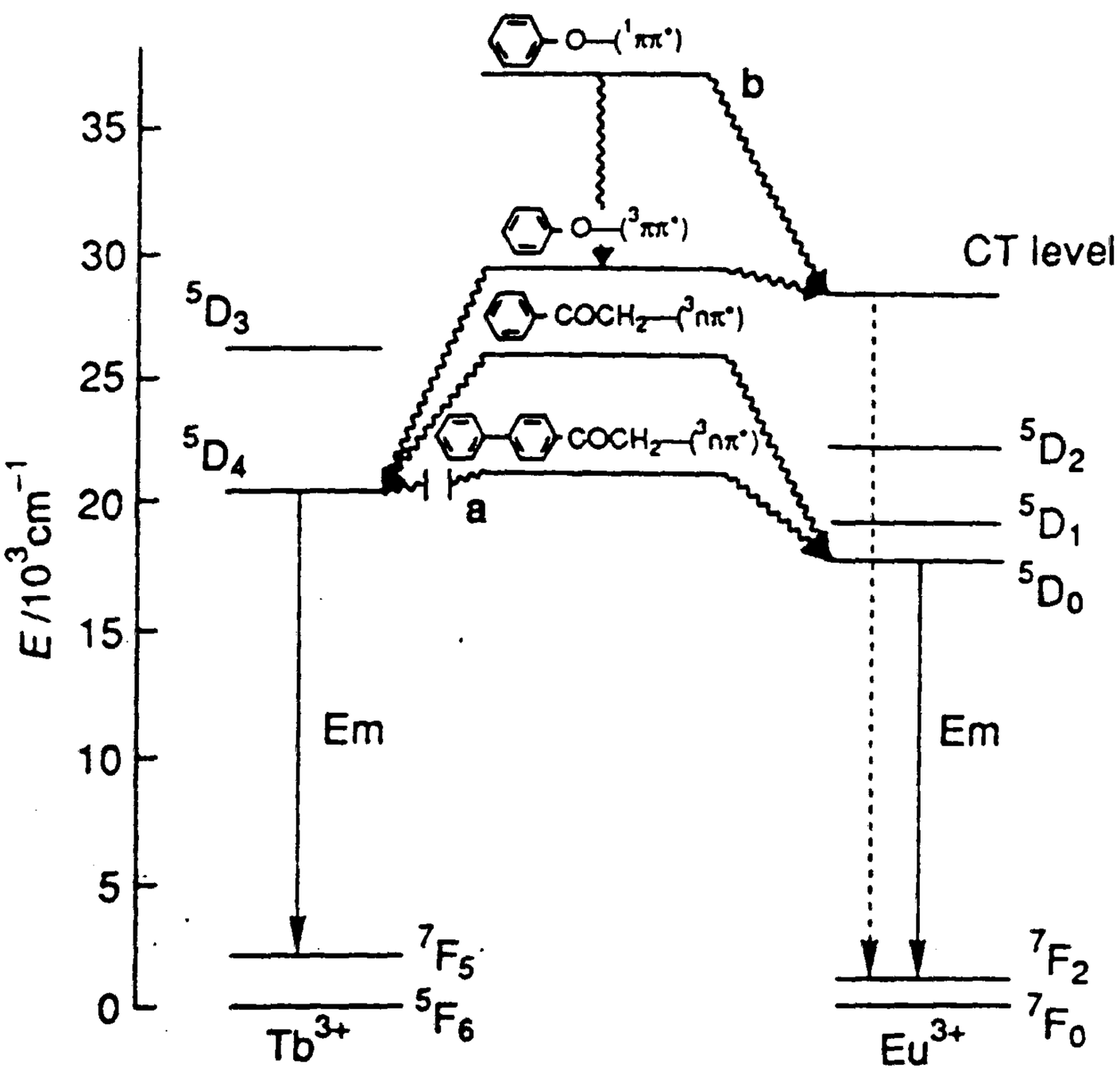


Figure 3.0 Energy transfer diagram for the system: excitation wavelength PhCO- (λ= 270nm), $PhCOCH_2$- (λ= 325). Energy transfer to the Tb^{3+} ion levels from triplet energy levels of the biphenyl carbonyl is not possible (see a in figure). As the C=O to Eu^{3+} charge-transfer band deactivates the excited-state of the phenol units to the ground state one cannot transfer energy to the Tb^{3+} ion levels from the excited energy levels of the phenol units (see b).

1.4 APPLICATIONS.

The considerable interest shown in luminescent lanthanide complexes reflects the view that as labels for both immuno/DNA hybridisation assays, they appear to represent a step forward from traditional organic fluorescent labels offering the kind of sensitivity to rival radioisotopes.

1.4.1 Immunoassays.

Immunoassay is a general analytical technique that relies upon the immunological reaction between the analyte to be measured (the antigen) with a specific antibody, under competitive or noncompetitive conditions. The immunological methods for the determination of biological materials are far superior to almost all other methods as far as sensitivity and specificity are concerned. They are used particularly for clinical investigation of compounds that are in very low concentrations and for which chemical methods are not sufficiently specific. Specifity and strong binding are intrinsic properties of antibodies which are necessary for their function as defence molecules. These same characteristics give specificity and sensitivity to immunoassays. The sensitivity of nucleic acid hybridisation assays arise from the specificity of one nucleotide sequence for its complementary sequence. The sensitivity of both techniques arise from the detection system employed to monitor the reaction, namely radioisotopes. Current state of the art competitive and noncompetitive radioisotopically based immunoassays are capable of measuring in the sub-picomolar range, the limit being ~10^7 molecules/mL, according to Ekins.[69]

Radioimmunoassays (RIA) were introduced about 27 years ago by Yalow and Berson[70] with radionuclides such as ^{125}I,^{31}P,^{3}H and ^{35}S as labels of choice. However in the last 15 years many alternative detection systems (labels) have been explored because of the serious disadvantages of radioactive labels (Table 1.7).[19] These are, the special handling techniques required, the problems of radioactive waste disposal, and the lifetime of an assay kit as determined by the half life of the isotope.

Table 1.7 Advantages and disadvantages of radioactive labels

Advantages	Disadvantages
1. Very high sensitivity 2. Freedom from environmental interference (pH, temp., ionic strength, *etc.* 3. Precise measurement of radioactivity 4. No background signal from samples or reagents	1. Potential health hazzard 2. Require licensing 3. Special disposal 4. Limited shelf life 5. Expensive γ-counters 6. Automation difficult

Among the alternative labels developed to substitute RIA, are enzymes (EIA, ELISA, EMIT), fluorescent labels (FIA) and luminescent labels or combinations (enzymes liberating fluorescent or luminescent products) (Table 1.8).[20] Enzyme Immunoassays (EIA) are the most widely applied having practically the same sensitivity as RIA. The problems with EIA are caused by the bulky, labile label (enzyme), its susceptability to inhibition and denaturation and the additional incubation with a substrate for monitoring the enzyme's activity.

Table 1.8 Nonisotopic labelling systems

Type of label	Example	Measurement principle
Enzymatic	Horseradish peroxidase (HRP)	Colourless substrate releases product absorbing in the UV-vis region
	Alkaline phosphotase (ALP)	Same as above
Fluorometric	Fluorescein, rhodamine	Conventional fluorescence
Chemiluminometric	Luminol, isoluminol	Oxidation by H_2O_2 with light emission
	Acridinium esters	Same as above
Combination	Enhanced luminescence	Oxidation of isoluminol by H_2O_2 in the presence of HRP and an enhancer
	ALP with fluorogenic substrates	ALP releases fluorescent products from nonfluorescing substrates
	ALP with chemiluminogenic substrates	ALP releases chemiluminescent products from nonluminescing substrate
	Replicase enzyme with replicable substrate	Replicase exponentially amplifies the substrate population

A wide range of fluorescent probes have been developed for use as direct labels in FIA (Figure 3.1)[71,72] fluoresceine and rhodamine derivatives being the most frequently used. Although in principle, fluoresceine measurement is very sensitive, giving many detectable events per labelled molecule and a quantum yield approaching unity, in practice the use of fluoresceine and rhodamine as labels has met with limited success with sensitivity being restricted to a concentration range of 10^{-9} - 10^{-10} M[20,73]

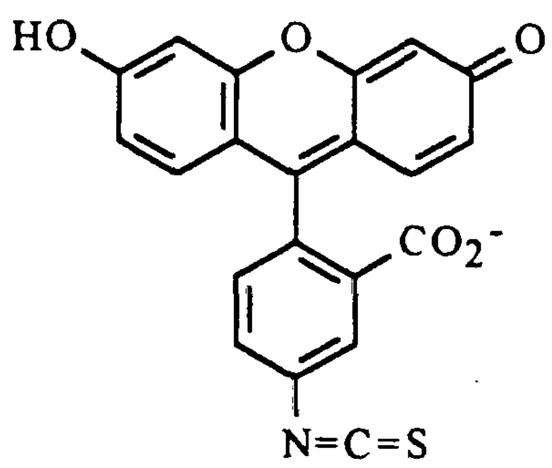
This limited sensitivity of FIA is mainly attributed to high background signals, originating from light scattering, background fluorescence and self-quenching.

Scattering causes very high background values in immunoassays of solutions containing high concentrations of proteins and even small colloidal particles (*e.g.* from serum). The excitation beam can be scattered from either soluble molecules (Rayleigh and Raman scattering), small particles or solid phase material (Tyndall scattering). Rayleigh and Tyndall scattering have the same wavelength as the excitation beam. These scattering interferences are aggravated by the small Stokes shifts (usually 24-50 nm) of conventional organic fluorophores.

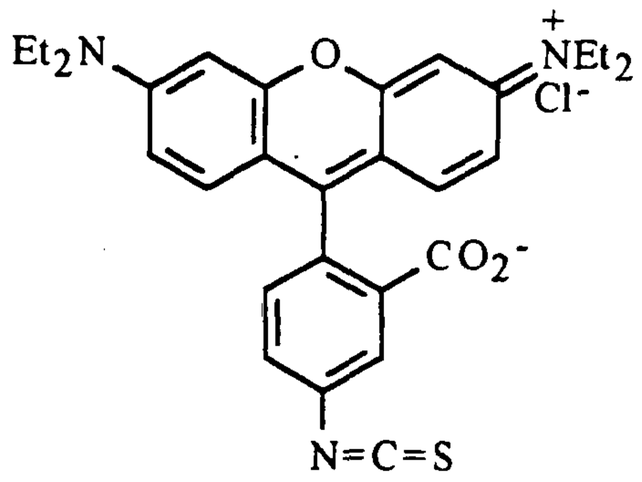
Background fluorescence signals are a major problem when dealing with biological samples like serum. This exibits a very high background fluorescence extending over a wide range of wavelengths (300-600 nm), which overlap extensively with the emission spectra of many organic fluorophores.

A further problem in using conventional organic fluorophores is the so called 'inner filter' effect, which precludes the use of multiple labelling to increase sensitivity. This is caused by the significant overlapping of the excitation and emission spectra of many fluorophores resulting in energy transfer ('self-quenching'). These drawbacks have confined FIA to the nanomolar range with the pico-range not being accessible.

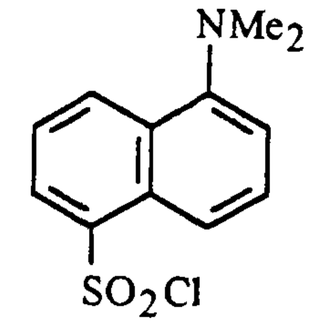
Lanthanide chelates with their unique luminescent properties offer the possibility of very high detection sensitivity, comparable or better than radioisotopes. At a stroke the disadvatages of conventional fluorophores are overcome. These chelates exibit large Stoke shifts (~290nm), with no overlap between the excitation and emission spectra and very narrow (10nm wide band width) emission spectra, away from native serum fluorescence. The chelates of europium and terbium also exibit exceptionally long luminescence life-times (600-1000μs for Eu^{3+}), compared with 5-100ns for conventional fluorophores (Table 1.9).



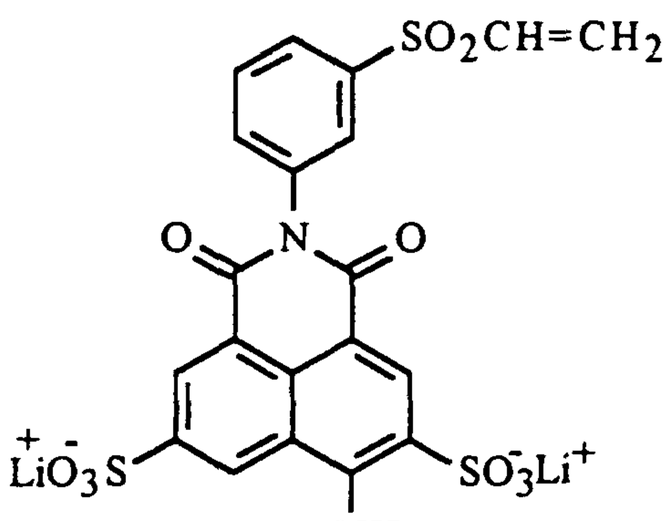
(57) Fluorescein isothiocyanate



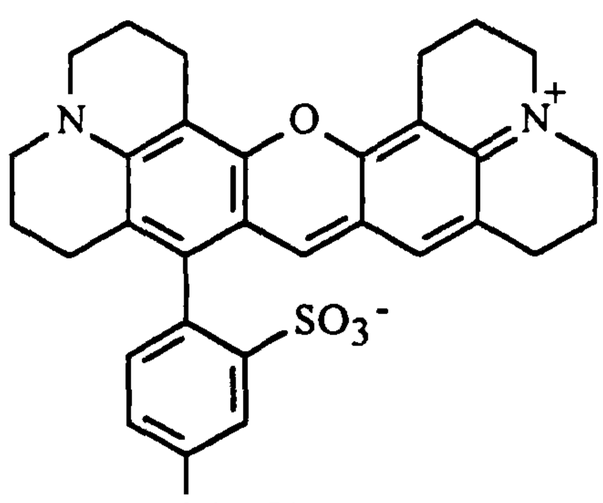
(58) Rhodamine isothiocyanate



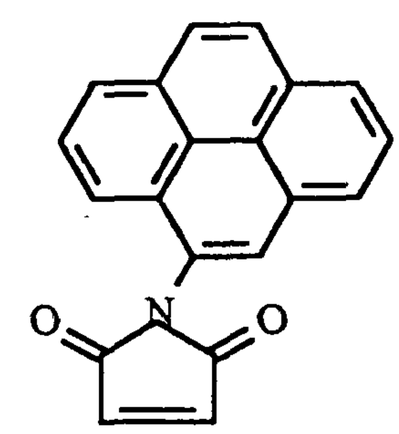
(59) Dansyl chloride



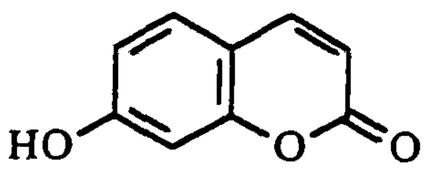
(60) Lucifer yellow



(61) Texas red



(62) Pyrene maleimide



(63) Umbelliferone

Figure 3.1 Fluorescent probes used in fluoroimmunoassays

Table 1.9 Fluorescence decay time of some fluorophores and proteins

Substance	Decay time (ns)
Humanserum albumin	4.1
Cytochrome *c*	3.5
Globin(haemoglobin)	3.0
Fluorescein isothiocyanate	4.5
Dansyl chloride	14
Europium chelates	10^3-10^6

1.4.2 Time-Resolved Fluoroimmunoassays (TR-FIA)

The key elements of time-resolved fluorescence techniques for biospecific assays are (*i*) a time-resolved fluorometer and (*ii*) a long decay time fluorescent label and its conjugation with biomolecules. Each of these two elements were developed separately and the possibility of combining them became obvious in the middle of the 1970s.[74]

The principle of time-resolved fluorescence is illustrated in Figure 3.2. A delay is set between the excitation pulse and the measurement of the resulting luminescence. In this way short lived background fluorescence, which dissipates to zero in <100μs is eliminated, and long-lived luminescence signals can be measured with high sensitivity.

The central problem of the availability of useful long-lived fluorescent probes was not resolved until the late 1970s.

Leif[74] was one of the very first authors who suggested a complete concept of chemistry for TR-FIA, and in 1975 suggested the use of europium complexes of the type Eu(ß-diketonate)$_3$ and Eu(1,10-phenanthroline-x) complexed to antibodies as labels. He assumed that the complex would have the necessary thermodynamic stability and chemical inertness under the desired conditions of solvent, pH and concentration. Although this complex demonstrated long-lived europium luminescence with reasonable intensity, it proved to be unstable in aqueous environments and did not lead to any practical system.

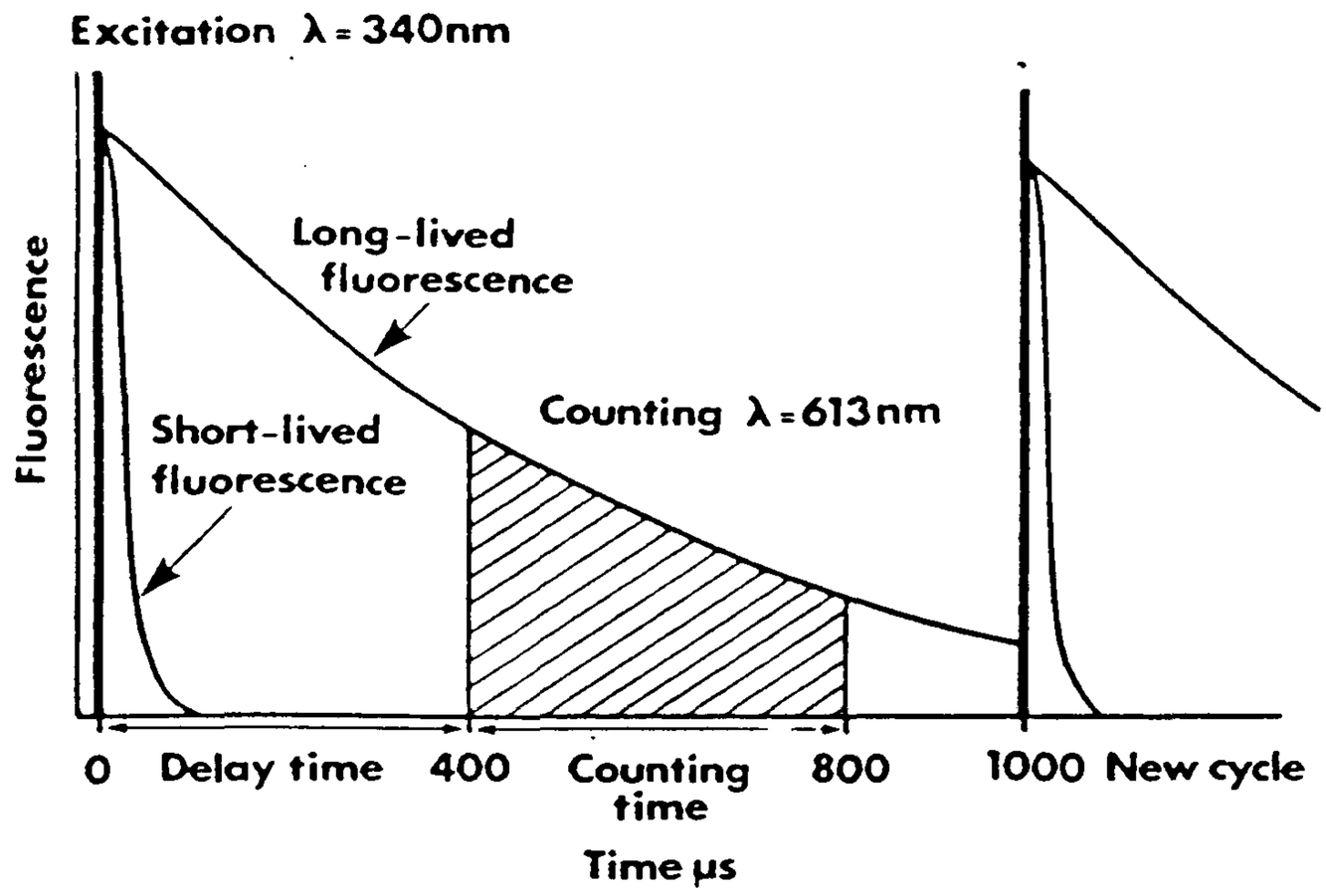


Figure 3.2 Schematic presentation of operation of a pulsed source time-resolved fluorometer.

The use of lanthanide chelates as potential long lived probes suffers from a number of drawbacks.

The lanthanides must be sensitised for effective emission to occur. The sensitiser must be capable of strongly complexing the lanthanide, forming both stable and kinetically inert chelates. Keeping the ion or chelate firmly on the immunoreagent at low concentrations in the vicinity of competing ligands requires the presence of powerful binding groups. Finally in aqueous media lanthanide luminescence is strongly quenched by interactions with water. Now since both Eu^{3+} and Tb^{3+} are nona-hydrates in aqueous media, a great deal of water needs to be removed to ensure effective luminescence.

The drawbacks to the use of lanthanides in fluoroimmunoassays governs the design of effective sensitisers, and to achieve the maximum intensity of luminescence from the lanthanide chelate, the following general requirements must be met:

(1) The sensitiser must bind the lanthanide strongly at low concentrations in water, at or above room temperature.

(2) The sensitiser must have a very high extinction coefficient (> 10,000 $M^{-1}cm^{-1}$) and the quantum efficiency for lanthanide emission to be high (Φ=1).

(3) The sensitiser should absorb above 320nm.

(4) Overall the complex should have good water solubility.

(5) There should be the existence of functional groups for covalent coupling to antigen or antibody.

(6) The coupling process should neither increase nor decrease the immunoreactivity of the labelled substances nor increase its unspecific binding to plastics *etc.*

In spite of the difficulties of accomplishing the above requirements in a given lanthanide host, there have been two commercial TR-FIA avaliable since the early 1980s which have utilised lanthanide luminescence.

1.4.3 The LKB System.

The first systematic studies on the use of europium as a label in immunoassays was started by Soini and Hemmila in the mid 1970s, by the beginning of the 1980s Soini and Hemmila had patented the first commercial heterogeneous time-resolved fluoroimmunoassay.[75] The procedure which has been found to be sufficiently reliable and robust for routine clinical use, was named 'DELFIA'-**D**issociation **E**nhanced **L**anthanide **F**luoro **I**mmuno**A**ssay (LKB-Wallac Oy, Turku, Finland).

The approach taken by Soini and Hemmila to overcome the problems of combining good absorption and energy transfer properties with a strong chelating capacity to the same ligand was to separate these two functions. Although the ß-diketonates chelate Eu^{3+} ions and possess the properties required for sensitisation of lanthanide luminescence, these complexes are not stable enough to be used directly. Their approach was to stick with the ß-diketonates as sensitisers but to separate the europium labelling and measurement procedures. The LKB system is shown diagramatically in Figures 3.3, 3.4.[76]

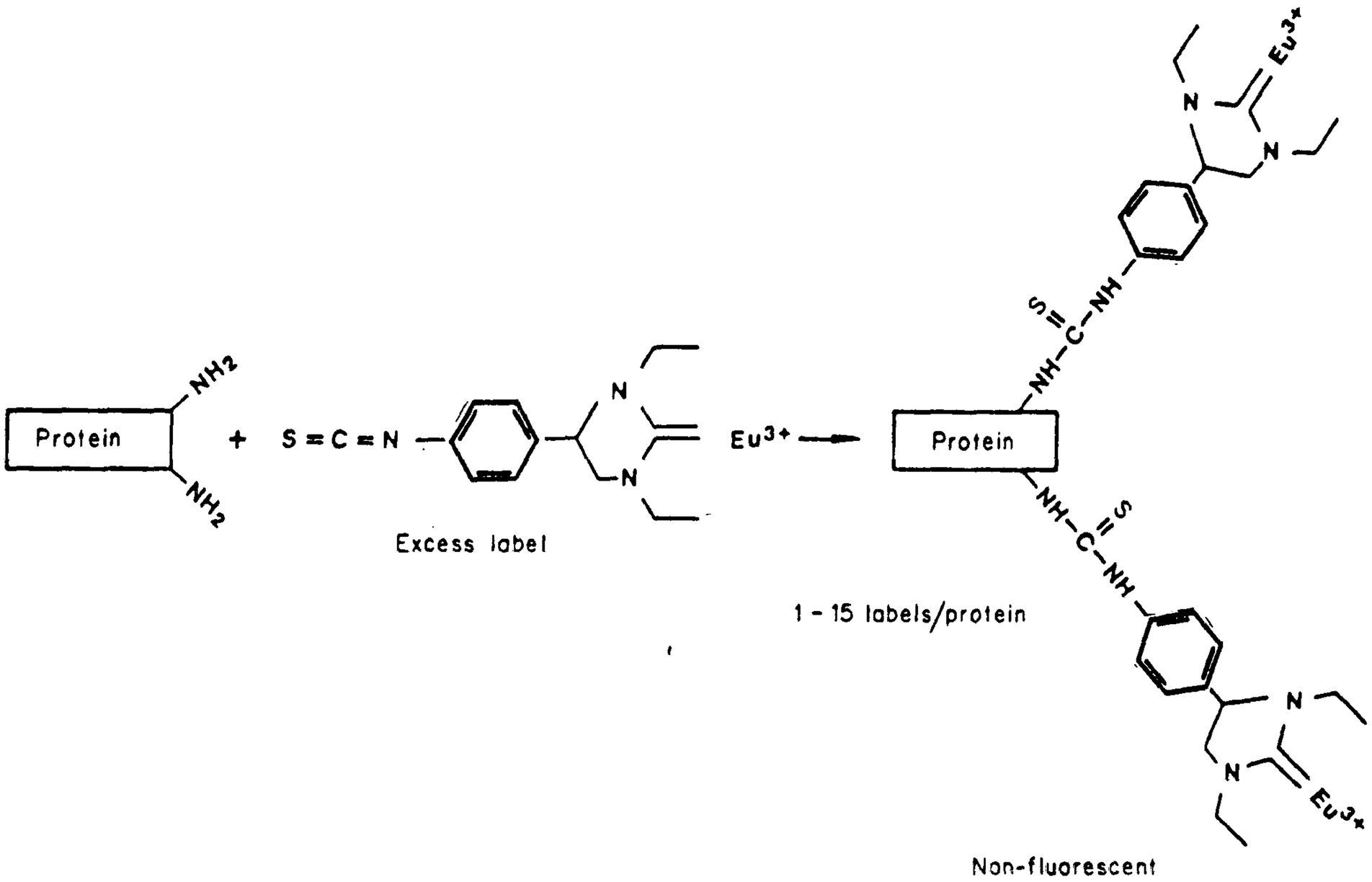


Figure 3.3 Principle of protein labelling with isothiocyanatophenyl-EDTA-Eu^{3+}. The protein is reacted with 60-fold molar excess of the label at pH 9.3 overnight at +4°. The labelled protein is separated from excess reagent by gelfiltration. The conjugation yield is obtained by comparison of the europium fluorescence of the labelled protein with europium standards.

The lanthanide is bound to one of the immuno-components in an essentially non-fluorescent form. The immunoassay is carried out as a non-competitive or competitive reaction in a solid phase system. After the immuno-reaction has been completed, part of the labelled component (antigen or antibody) is bound to the solid phase and the separation of the free and bound fraction is accomplished by extensive washing of the solid phase surface. In the final step, the lanthanide ion is dissociated from the immunocomponent on the solid phase into solution where a highly fluorescent lanthanide chelate is formed before measurement.

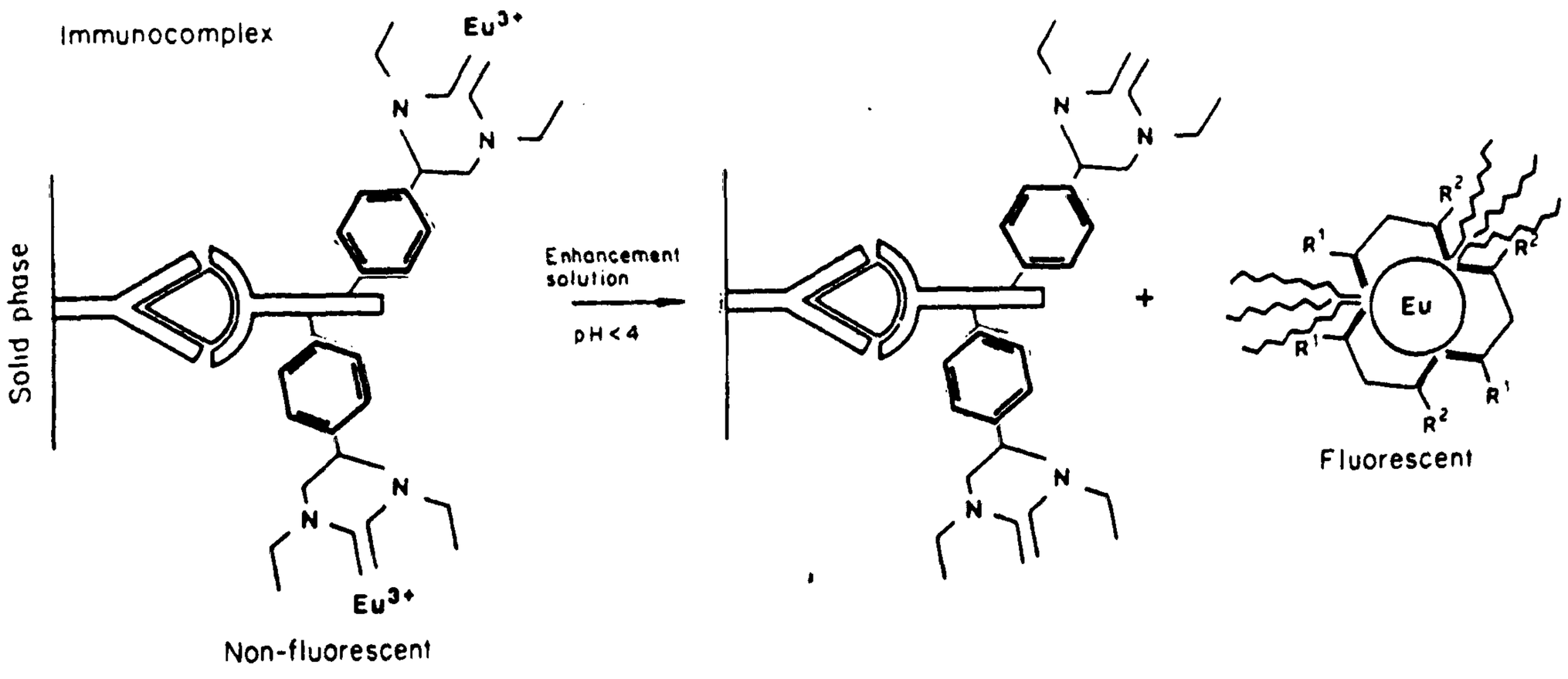


Figure 3.4 Principle for the release after the immunometric assay has been completed. As a final step before fluorescence measurement an enhancement solution is added consisting of 0.1M acetone-potassium hydrogen phthalate, pH 3.2, containing15M 2-naphthoyltrifluoroacetate, 50M tri(n-octyl)phosphine oxide and 0.1% Triton X-100. The europium ion dissociates from the labelled protein and forms a new fluorescent chelate in solution.

In the DELFIA system, the binding of Eu^{3+} to an immunoreactive component is accomplished by using derivatives of strongly chelating compounds such as EDTA and DTPA containing a suitable functional group for covalent coupling, *e.g.* isothiocyanatophenyl-EDTA (Figure 3.3).

Polycarboxylic acids like EDTA, EGTA and DTPA have a very high binding constant for the lanthanides (10^{16}-10^{22}).[77] However, this property is strongly dependant on the pH, and if the pH is lowered the stability of chelate complex decreases and the lanthanide is readily dissociated into solution. This conditional stability is utilised in the quantitation of Eu^{3+}, performed by dissociating it from the immunocomponent after the immunoreaction by adding an enhancement solution which is at pH 3.2. This consists of a ß-diketone (diketone-2-naphthoyltrifluoroacetone), tri-octylphosphine oxide and TritonX-100. The result is a highly fluorescent europium chelate solubilised in the micelles that are formed. The postulated fluorescent product is shown in Figure 3.5.

The non-ionic detergent TritonX-100, dissolves the sparingly soluble organic component in the micellar phase and excludes water from the chelated Eu^{3+}. The insulation from the solvent is further optimised by the addition of a synergistic agent, tri-octylphosphine oxide. The success in insulating the europium chelate from the quenching effect of water, means the system can detect as little as 10^{-13}molL^{-1} Eu^{3+}, the same order of sensitivity as is commonly reported for ^{125}I.

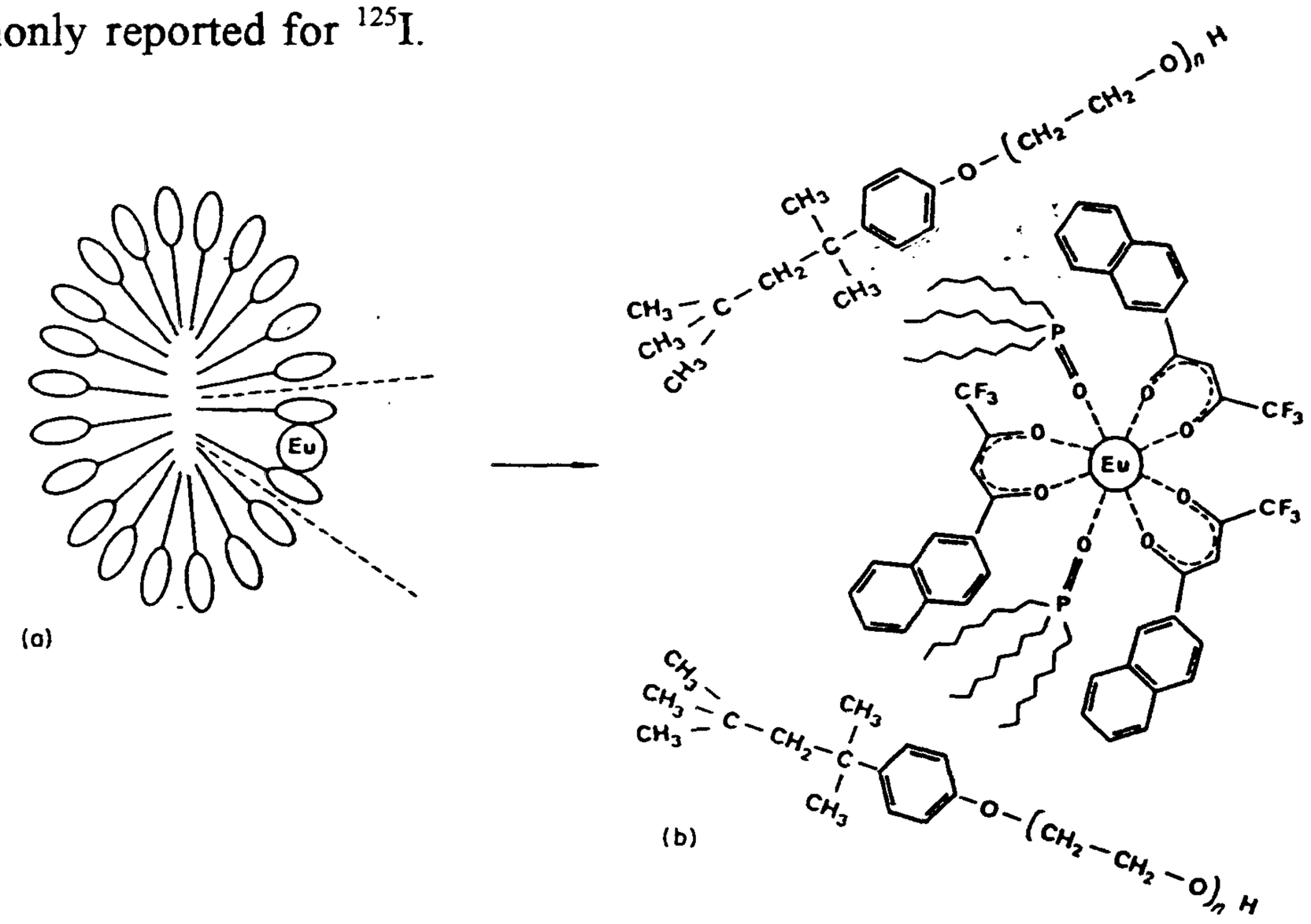


Figure 3.5 (a) micelle consisting of Triton X-100 molecules with an association number of about 140 in which a fluorescent europium chelate is solubilised. (b) A hypothesised form of the europium chelate.

1.4.3.1 Applications of the DELFIA system.

Since the introduction of the immunoassay based on the DELFIA concept, a great variety of assays have been developed. The technique is applicable generally and assay procedures have been successfully worked out for essentially all analytes intended to be measured with the DELFIA system.

A. Protein and Peptide Hormone Assays.

The first assays using the DELFIA system were the noncompetitive immunometric assays of proteins and peptide hormones: Rabbit IgG[78], hCG[76,79,80], hTSH[81,82,83], AFP[84], Phospholipase A_2[85], Insulin[86], C-reactive protein[87], LH[88], and Myelin basic protein[89].

Most of these non-competitive assays are performed using monoclonal antibodies, employing polystyrene microtitration wells as the solid phase. Flow charts for two typical assay protocols (hTSH) and (hCG) are shown in Figure 3.6.[18] All other non-competitive and competitive analyte protocols are essentially similar to those presented in the flow chart.The major benefits of TR-fluorimetry in these non-competitive assays are sensitivity and a wide dynamic range, which are important, for example in assays of serum thyroid stimulating hormone (hTSH), where the determination of low and normal hormone levels contributes to an improved clinical discrimination of patients with thyroid disease.

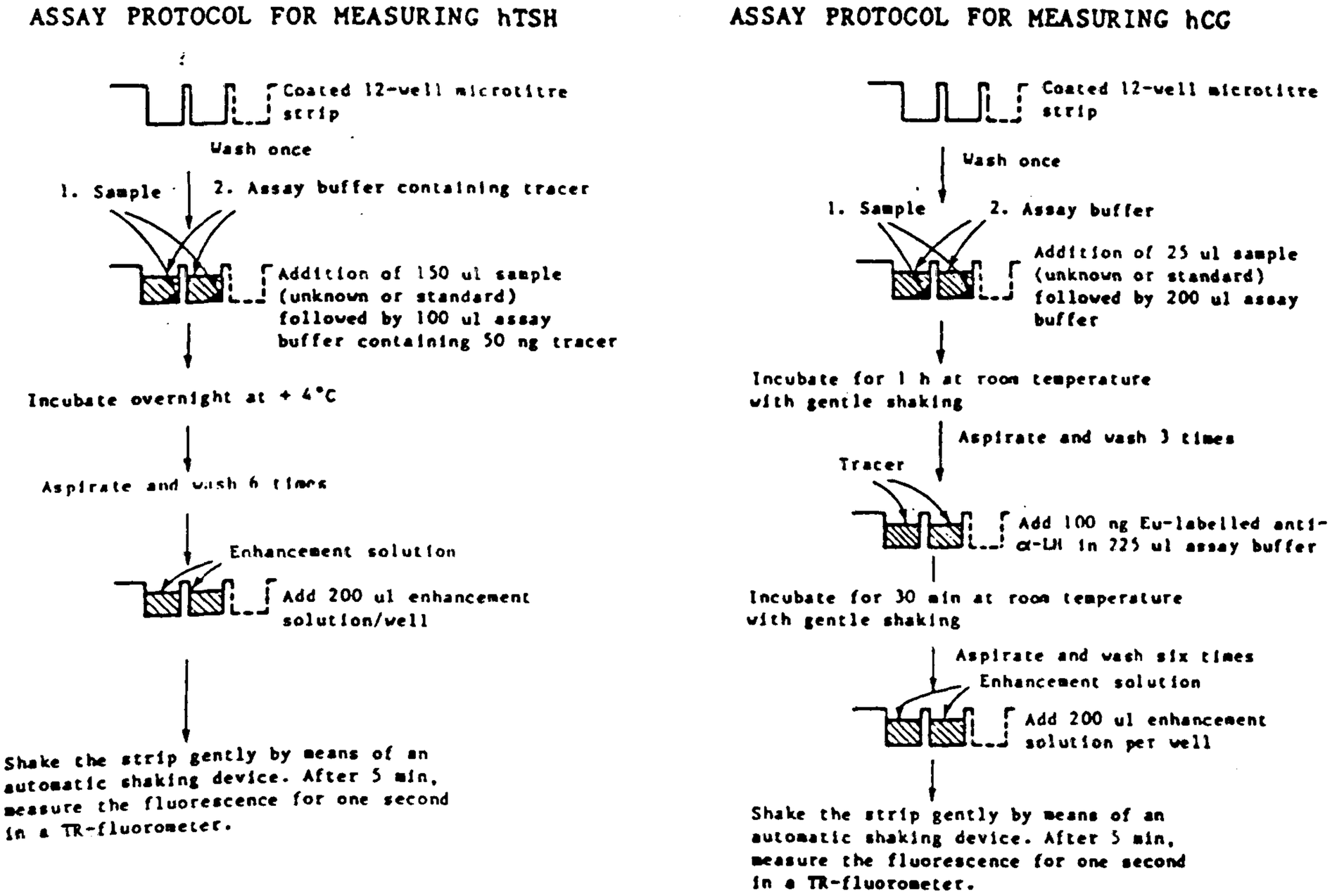


Figure 3.6 Assay protocols for (hTSH) and (hCG).

B. Hapten Assays

The reported competitive hapten assays based on TR-fluorimetry using europium chelates include those for cortisol[81,90], testosterone[81,91], digoxin[87,92] and thyroxine[87]. These assays perform at least as well as corresponding radioimmunoassays.

Competitive assays are carried out either using labelled haptens or labelled antibodies. The assay principle is outlined in Figure 3.7[18]. A constant amount of protein-bound thyroxine is immobilised on the wall of microtitration strip wells and the antibody is labelled with europium. After the immunoreaction has been completed, the label is released into solution by addition of the enhancement solution.

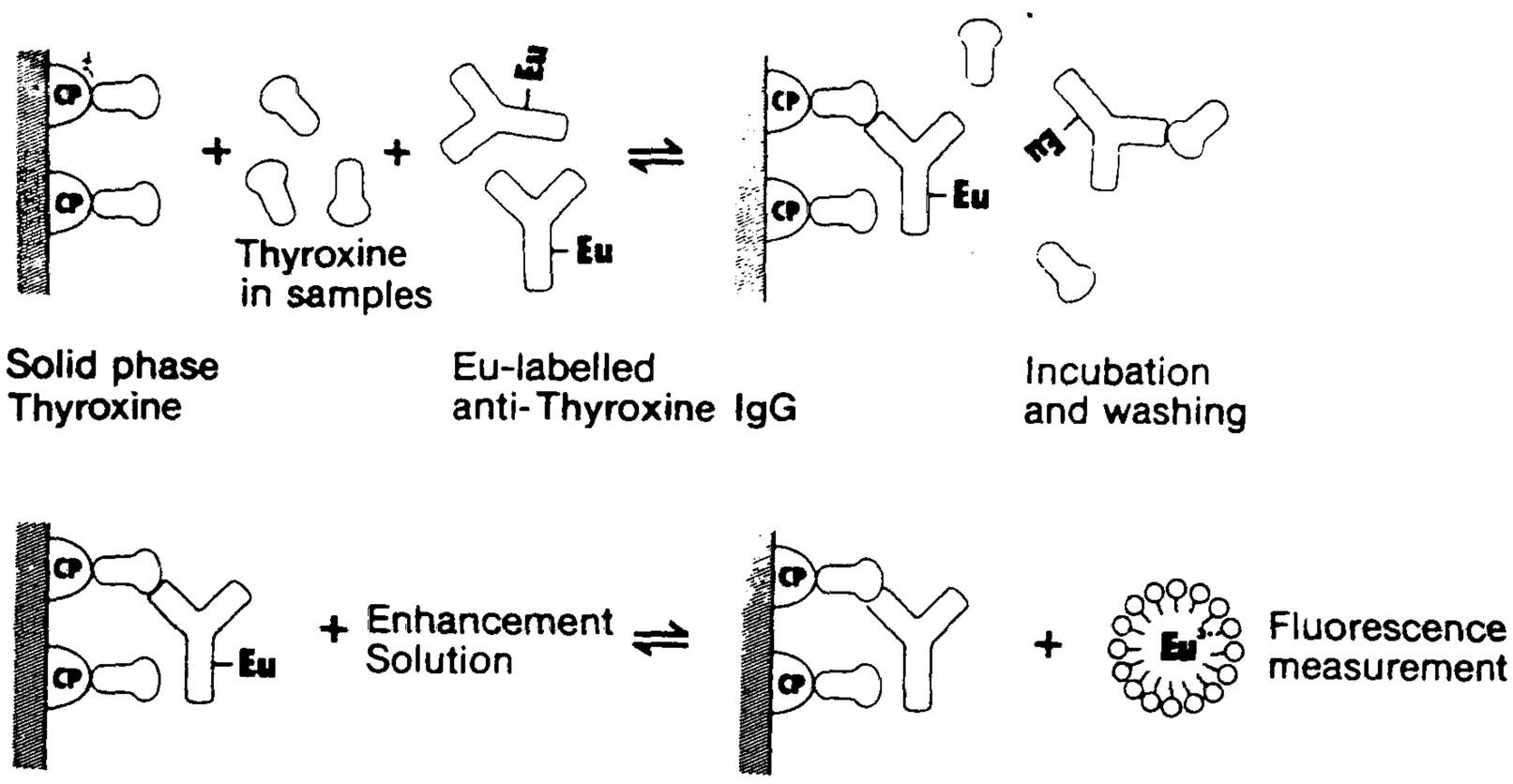


Figure 3.7 The principle of the non-competitive assay of thyroxine. A constant ammount of protein-bound thyroxine is immobilised on the wall of the microtitration strip wells and the antibody is labelled with europium. After immunoreaction is complete, the label is released into solution by the addition of the enhancement solution.

C. Serological Assays

In serological immunoassays labelled antispecies antibodies are widely used in various indirect and direct assays to measure human serum antibody levels. The DELFIA principle has been applied in assays for rubella[93] and tetanus[94] antibodies.

D. Virus Antigen Assays

Originally even with the use of europium chelates and time-resolved fluorescence these assays were laborious and complicated, because indirect assay procedures requiring three different incubation steps were applied.[95]

Recently these tests have been simplified by using a direct assay procedure. Direct one-incubation TR-fluorometric assays of the hepatitis-B surface antigen[96], rotavirus and adenovirus in stool specimens and for influenza A have been developed.[97,98] All the assays were performed using a polyclonal antibody preparation both on the solid phase and as the labelled reagent (Figure 3.8) The sensitivity of these far simpler assays are at least as good as that obtained with the corresponding indirect radio- or enzyme immunoassays.

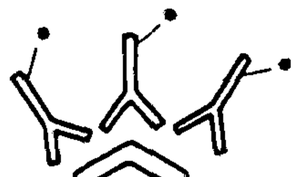
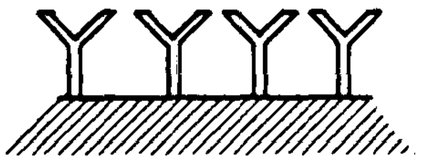
Layer		Immunoreagent		Incubation at 37 °C
		Type	Concentration	
Anti-viral indicator		Eu-chelate-labelled rabbit anti-virus Ig	100 ng per assay	1 h
Specimen		Virus (structural protein)	0.001–1 mg $m\ell^{-1}$	
Catching antibody		Ig fraction of rabbit anti-virus hyperimmune serum	500 ng per tube	
Solid phase		Polystyrene tube		

Figure 3.8

In the LKB system, because of the dissociation of the lanthanide ion from a solid phase, from the labelled compound, followed immediately by the development of a new chelate in the solution, the developing stage is very sensitive to external lanthanide contamination. The result can be an immediate growth of "background" fluorescence.[19] The possibility of contamination is excluded by the use of lanthanide chelates which are both stable and fluorescent.

In a recent patent Kankare[99] reports on a TR-lanthanide chelate assay which attempts to combine the advantages of both the DELFIA and Cyberfluor systems. (Figure 3.9)

The assay is based on the use of a stable fluorescent lanthanide chelate. After the immunochemical reaction of an antibody labelled with a fluorescent europium chelate, namely the isothiocyanate derivative of 4-aminophenyl-ethynyl-2,6-bis (N,N'-bis(carboxymethyl)-aminomethyl)pyridine-Eu, with the antigen, the lanthanide chelate is released into solution before measurement. During the releasing process, the chelate complex between the lanthanide ion and the chelate ligand must remain intact. In this way only the fluorescent lanthanide chelate is released into the solution, and no background induced by the material of the solid phase nor any external contamination can interfere with the result.

To achieve this, the europium chelate is bound to one of the immunochemical components using a single covalently breakable bond: *e.g.* a disulphide (-S-S-). a vicinal diol, an ester bond, a diselenium bond, or a photosensitive bond, or combinations of the above.

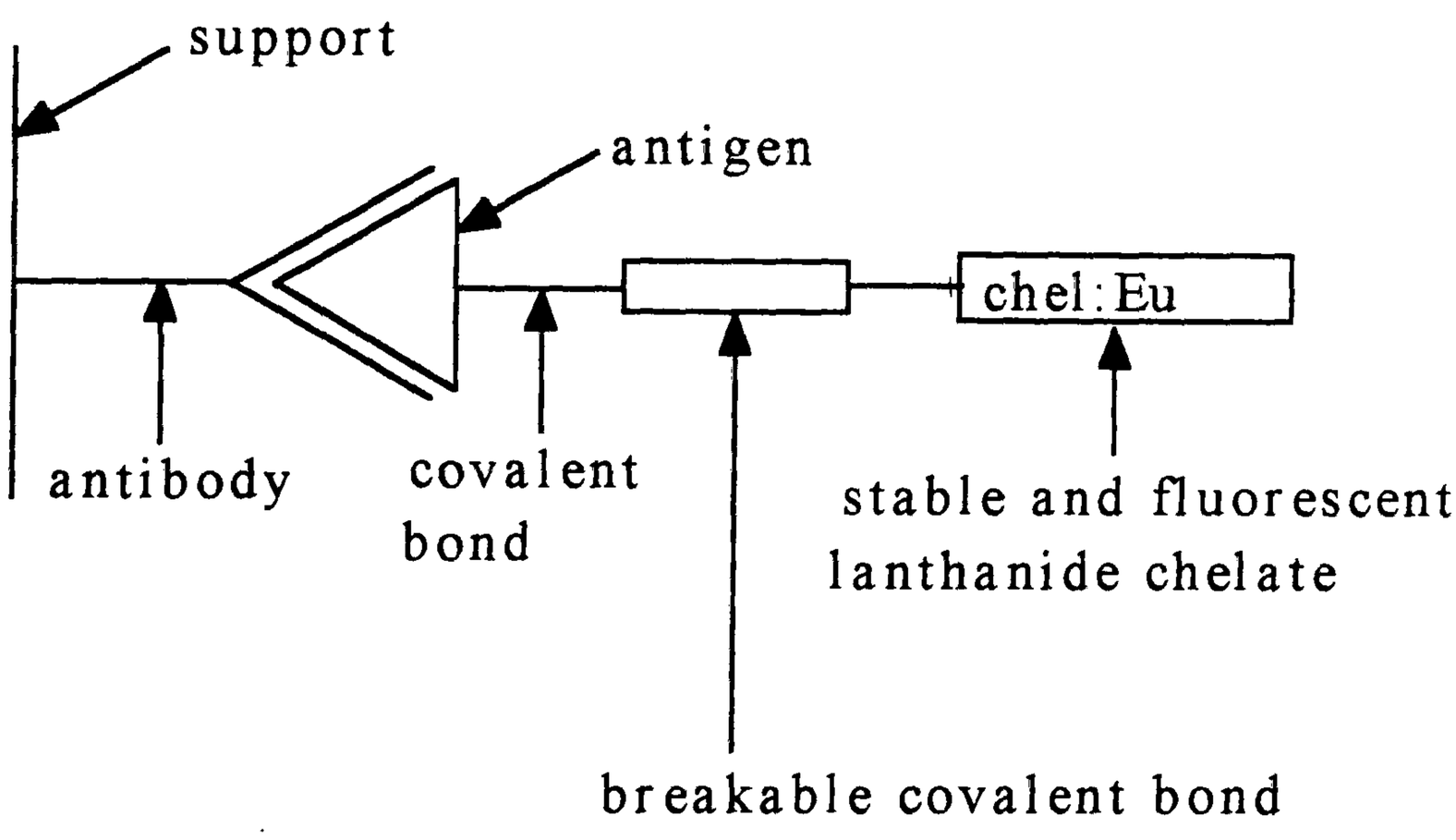


Figure 3.9

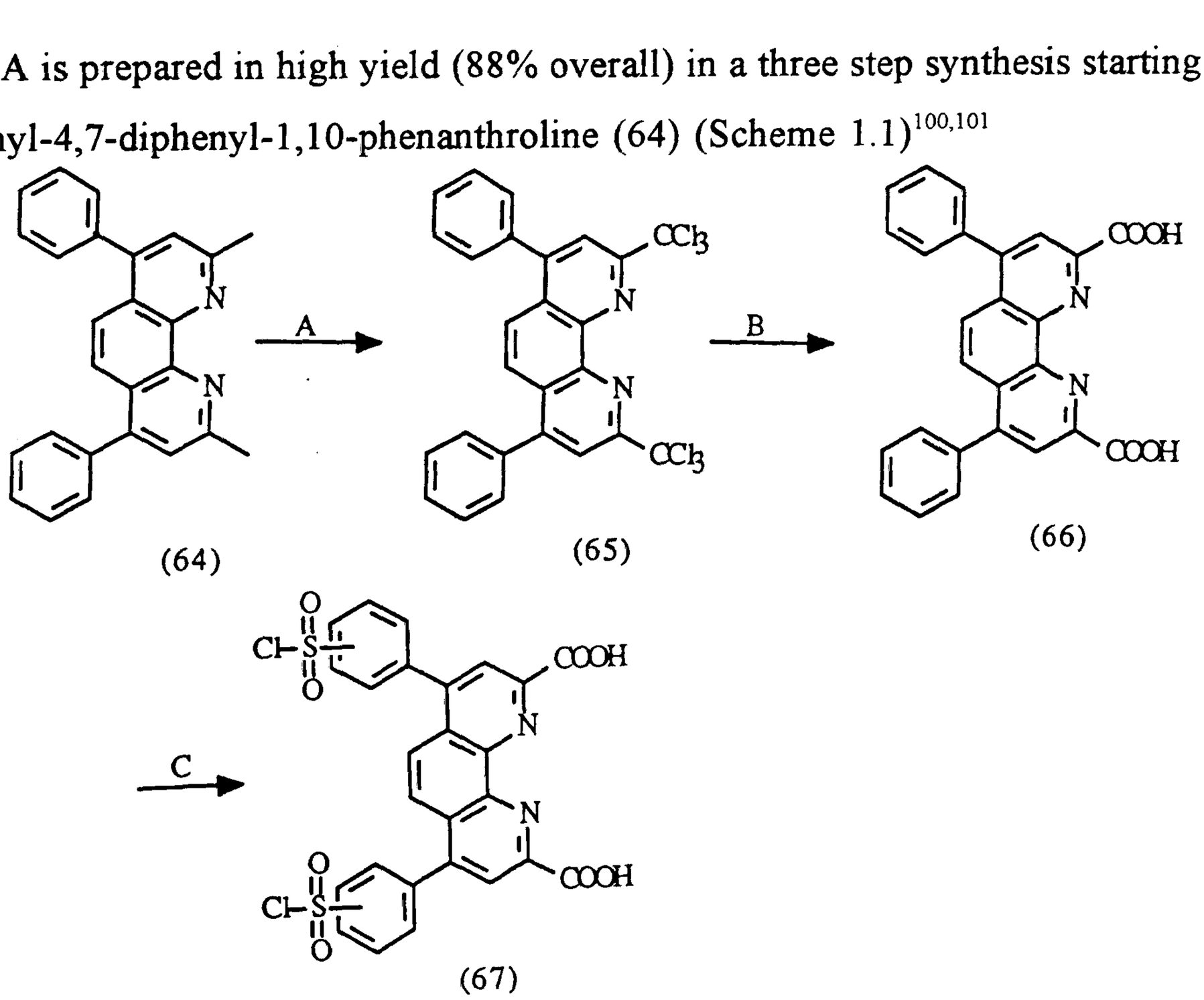
1.4.4 The CYBERFLUOR System.

Instead of using europium as the label for TR-FIA, it is possible to introduce a europiun chelator into the immunoreactants and use an excess of Eu^{3+} to form the fluorescent complex. After the immunoreaction and all the washings have been completed in a competitive or non-competitive immunoassay, the fluorescence of the chelate-europium complex can be measured. The success of this approach depends on finding a suitable europium chelator, which apart from forming stable complexes is also an efficient sensitiser.

The two most general types of chelates associated with the lanthanides, namely the ß-diketonates and the polycarboxylic acid (EDTA) derivatives do not fulfill the two main criteria, of forming a stable Eu^{3+} complex that exibits luminescence.

The success of the Cyberfluor is due to a new type of europium chelator: 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA) (67) which does fulfill the above criteria.[100,101]

BCPDA is prepared in high yield (88% overall) in a three step synthesis starting from 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (64) (Scheme 1.1)[100,101]



Scheme 1.1 Synthesis of BCPDA; A. NCS, Benzoyl peroxide, CCl_4
B. c. H_2SO_4, 90°C; C. chlorosulphonic acid, 80°C.

The chelating site consists of two heteroaromatic nitrogens and importantly, two carboxyl groups, enabling BCPDA to form a luminescent complex with Eu^{3+} both in aqueous solution and when absorbed to a solid phase, in a heterogeneous immunoassay configuration. The molecule has two sulphonyl chloride groups available for reaction under mild conditions with amino groups of proteins, making the labelling of proteins a simple procedure.

Diamandis and co-workers have developed a universal detection system based on labelling Streptavidin with BCPDA.[102]

The noncovalent, specific and very strong binding of the water soluble vitamin biotin to the tetrameric protein avidin (or streptavidin) has become one of the most useful tools in the field of immuno and DNA hybridisation assays. The affinity (formation) constant for the biotin-avidin interaction is amongst the highest reported at $10^{15}M^{-1}$ (compared to the antibody-antigen interactions of 10^{11}-$10^{12}M^{-1}$).

Biotin covalently bound to an antibody or DNA probe is still available for high-affinity interactions with avidin. If the avidin is labelled with a suitable reporter molecule (*e.g.* an enzyme or fluorescent compound), the avidin-biotin complex can be used for the quantification of the biotinylated molecule.

The biotin-streptavidin system offers some distinct advantages:

(1) It is a universal detection system; one streptavidin based reagent can be applied to any immunoassay or DNA hybridisation assay using biotnylated reactants,

(2) Antibodies and DNA sequences can easily be biotinylated without the loss of bilogical activity,

(3) The biotinylation process introduces many biotin molecules per antibody which inturn bind to more than one labelled streptavidin, leading to a significant amplification factor,

(4) Streptavidin is very stable and usually not deactivated on labelling.

A typical non-competitive assay based on biotin-streptavidin labelled with BCPDA is shown in Figure 4.0.

All reactions are carried out in white microtitration strip wells and luminescence is measured on the dry solid phase.

This system has been successfully applied in the non-competitive assay of alpha fetoprotein[103] choriogonadotropin[104], and the competitive assay of cortisol in serum[105].

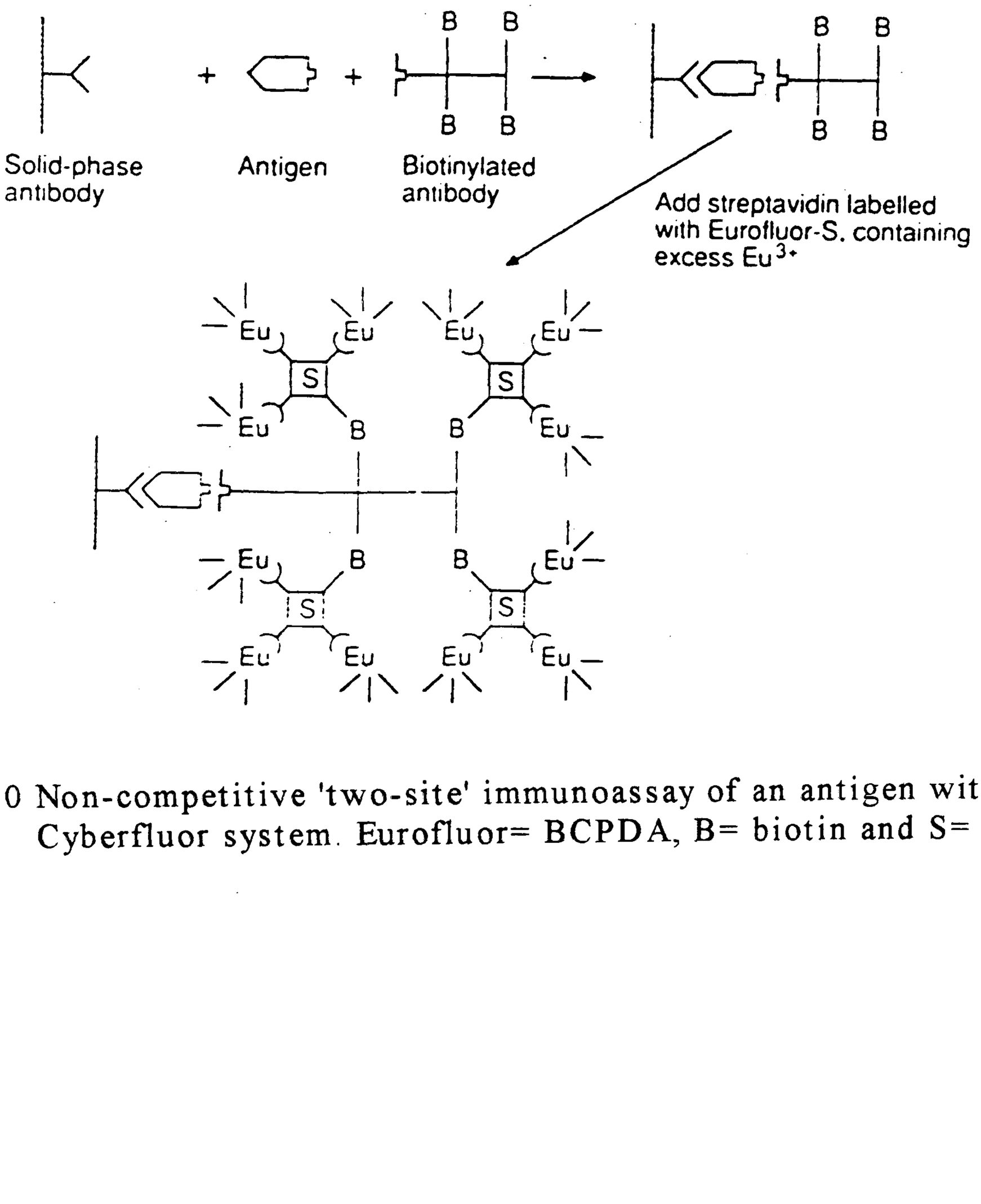


Figure 4.0 Non-competitive 'two-site' immunoassay of an antigen with the Cyberfluor system. Eurofluor= BCPDA, B= biotin and S= streptavidin

The Cyberfluor system does not suffer from the external lanthanide contamination problems that can afflict the DELFIA assay, since an excess of europium is used in the developing stage. The use of excess europium is necessary because the stability of BCPDA chelates with Eu^{3+} is relatively low (~10^5-10^6). Also a 1:1 Eu^{3+}:BCPDA complex is formed, which allows the coordination of a number of water molecules to the lanthanide ion. This means that before any measurement of the luminescence can take place, the solid phase must be dried.

Overall, the Cyberfluor assay system is less sensitive in comparison with DELFIA. Efforts to increase the sensitivity has concentrated on multiple fluorescent labelling with europium chelators. Diamandis and co-workers found that by labelling a streptavidin-thyroglobulin conjugate with BCPDA instead of directly labelling streptavidin they were able to incorporate approximately 150 BCPDA molecules per streptavidin[106] as compared to 14 incorporated by direct labelling. No quenching (inner filter) effects are observed, presumably due to the large Stokes shift of the label (BCPDA-Eu^{3+} complex) and the lack of overlap between the excitation and emission spectrum. By using the universal detection reagent based on the streptavidin-thyroglobulin conjugate (Figure 4.1), and excess Eu^{3+} they were able to improve the detection limit of a model α-fetoprotein assay by a factor of 25-fold or 5-fold in comparison to BCPDA labelled antibody or BCPDA labelled streptavidin, respectively[106]

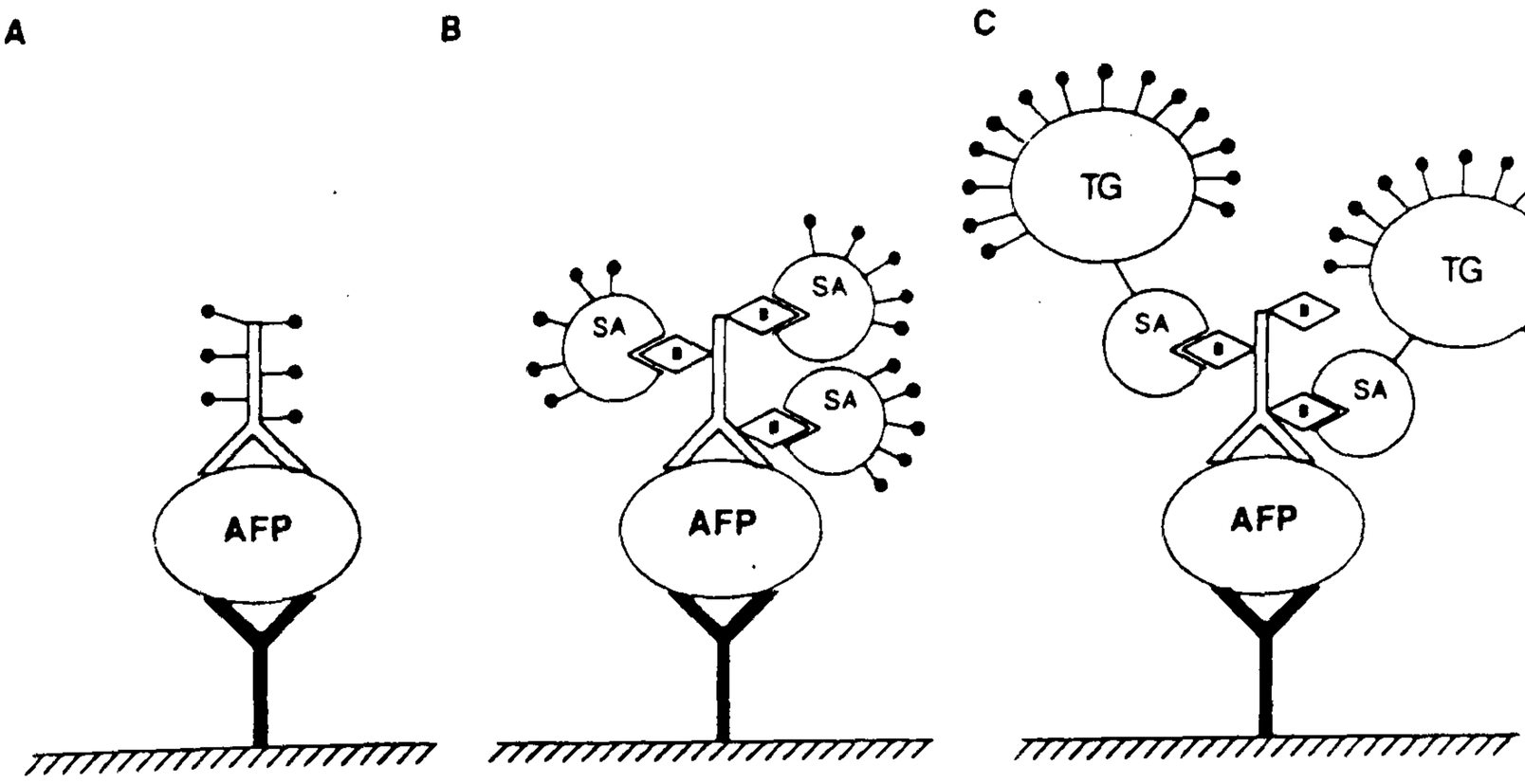


Figure 4.1 Schematic representation of the BCPDA labelled reagents and their utilisation in the AFP assay. The solid phase is indicated by the horizontal line with hatched lines. The solid ball and stick represents BCPDA. AFP= α-fetoprotein, B= biotin, SA= streptavidin and TG= thyroglobulin.

In a further development Diamandis *et al.*[107] found that by incubating SA-TG-$(BCPDA)_{150}$ (conjugate of streptavidin (SA) with thyroglobulin (TG), which is labelled with BCPDA), with BCPDA-labelled thyroglobulin [TG-$(BCPDA)_{150}$], in the presence of a suitable amount of Eu^{3+}, a new macromolecular complex is formed with a molecular weight of 3x 10^6 (Figure 4.2). This macromolecular complex was found to be stable enough to be used as a sreptavidin based universal detection sysytem for TR-FIA. Detection limits of seven immunofluorometric assays showed an 8-26 fold improvement in comparison to the reagent SA-TG$(BCPDA)_{150}$ described earlier[106].

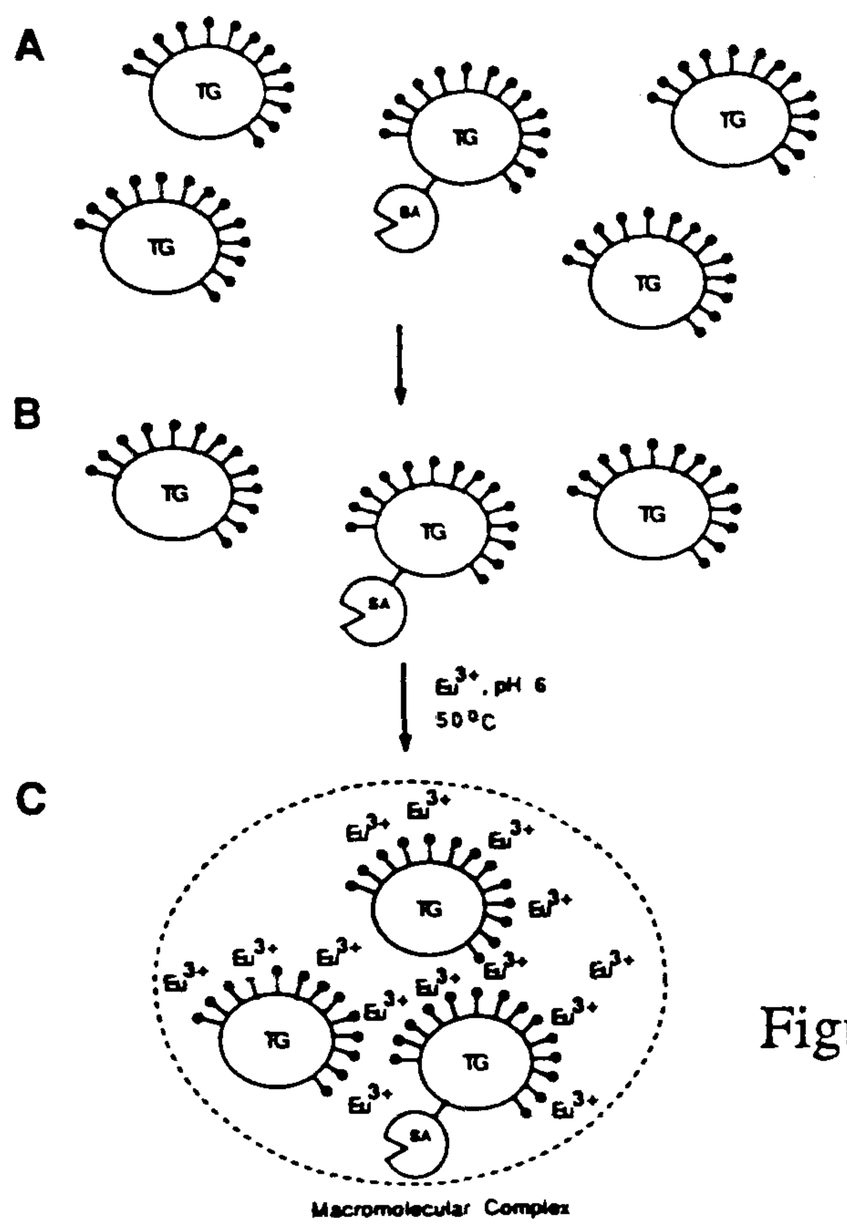


Figure 4.2 Schematic representation of the proposed mechanism of the formation of the macromolecular complex.

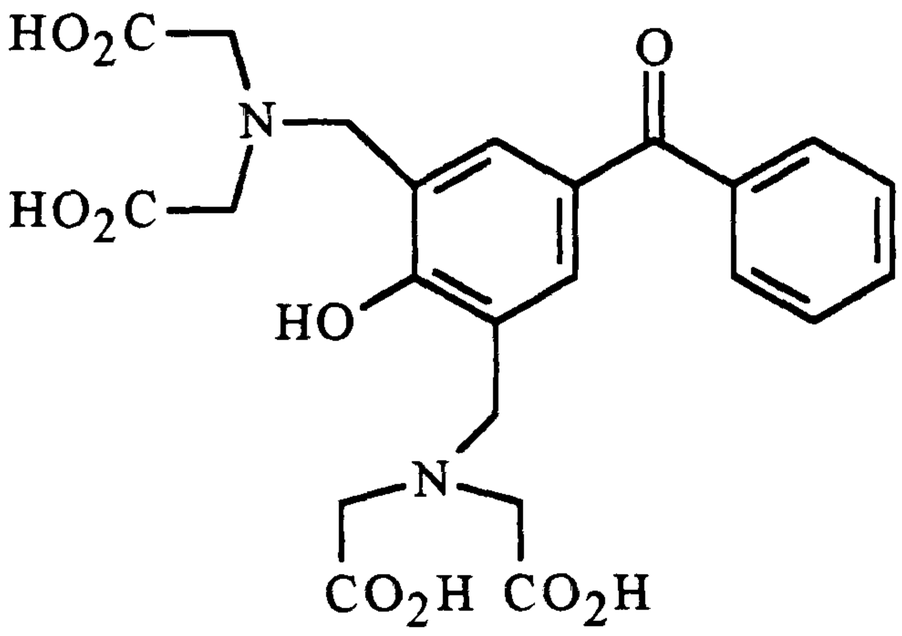
In order to overcome some of the limitations of the Cyberfluor system, Kropf and co-workers have reported[108] a modification of the measurement stage. The amount of bound europium is quantified, but the luminescence is measured in solution after dissociation of the solid phase complex, analogous to the DELFIA system. BCPDA labelled solid phase complexes obtained by conventional immunoassay procedures are transferred into solution using urea/sodium dodecyl sulphate/Eu^{3+} as the dissociating and fluorescent lanthanide ion reagent. A "sandwich-type" assay based on this modification and using BCPDA labelled streptavidin was used in the determination of small amounts of fibronectin in biological fluids.

The main problem with this variant is that, one has to assume the dissociating solution is in fact leading to complete dissociation of the BCPDA:Eu^{3+} complexes attached to the solid phase.

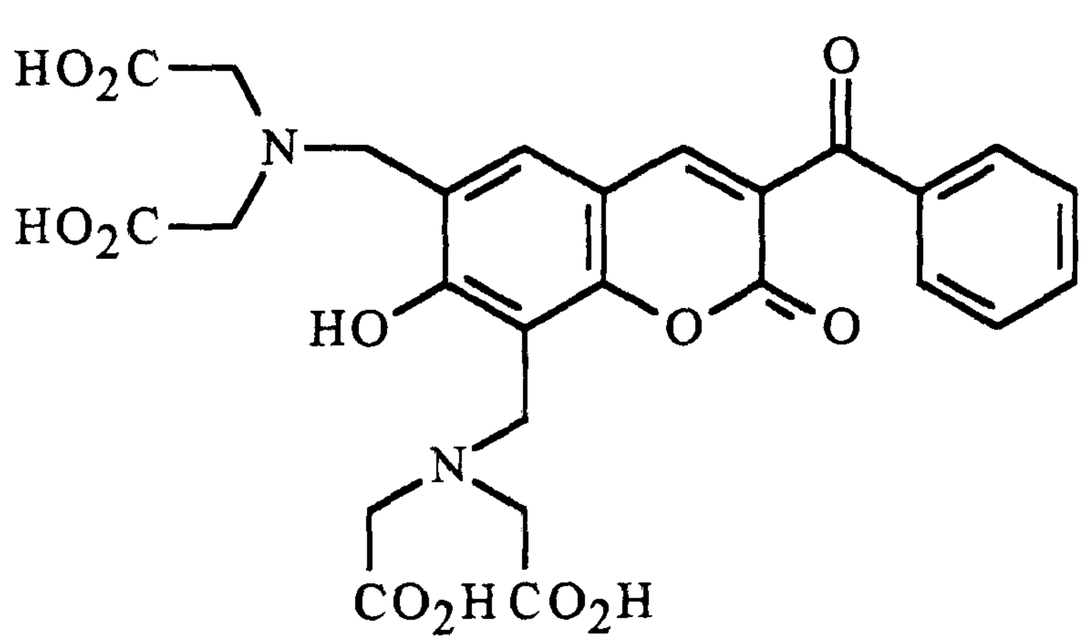
Others have tried to tackle the problem of sensitivity associated with the Cyberfluor system by looking at alternative sensitising/strong binding ligands.

Toner and Hinshaw at Eastman Kodak[109,110,111,112,113] have over the last six years synthesised a whole series of potential ligands capable of strongly binding both Eu^{3+} and Tb^{3+} and sensitising lanthanide luminescence in water. Their approach involved incorporation of powerful binding groups, usually iminodiacetates or carboxylates on suitable lanthanide sensitisers.

One of the first class of sensitiser they examined were the benzophenones, which have excellent intersystem crossing efficiences (Φ_{isc}=1.0 for benzophenone). (Figure 4.3)



(68)



(69)

Figure 4.3

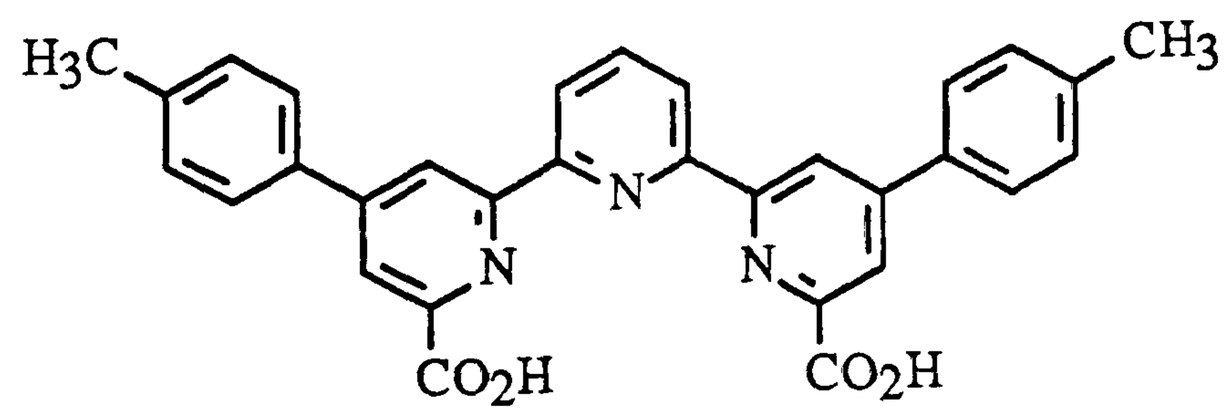
However, the Eu^{3+} chelate of ligand **68** shows weak luminescence with low quantum efficiency (Φ_{Eu}=0.03). This is probably a result of poor energy transfer between the triplet level of the sensitiser and emitting level of the metal ion.

The quantum efficiency is dramatically increased with the Eu^{3+} chelate of coumarin **69**, indicative of a better match between the sensitiser triplet level and the lanthanide ion. Normally, coumarins are highly fluorescent which renders them inappropriate as lanthanide sensitisers. However, Farid and co-workers, demonstrated that the incorporation of a keto group in the 3-position of the coumarin nucleus converts the molecule to an excellent triplet sensitiser, with Φ_{isc} approaching unity[114]. For all this, the Eu^{3+} chelate of **69** still exibits poor luminescence in aqueous environments, showing the difficulty in designing ligands that fulfil all the criterea.

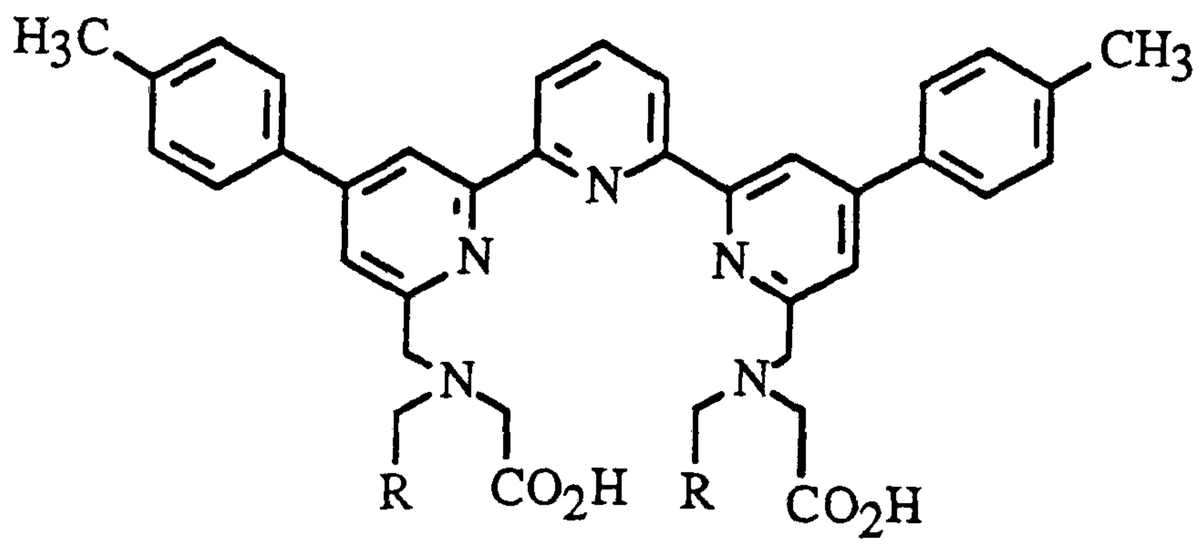
Better results, in terms of luminescence intensity, are achieved with substituted oligopyridines and phenanthrolines as sensitisers. Toner *et al.* synthesised a large number of these hosts in a systematic study (Figure 4.4), observing an increase in luminescence lifetimes and intensity with an increase in the number of chelation sites[111,112,113]. This probably reflects greater shielding of the metal ion from the quenching effect of the solvent.

Attempts by Toner to develop a competitive TR-FIA for thyroxin, by attaching thyroxin to one of the above chelators, gave poor results[113].

Recently more impressive results have been obtained by Toner *et al* using the bifunctional terpyridine chelate 4'-(3-amino-4-methoxyphenyl)-6,6'-bis[N,N-bis(carboxymethyl) aminomethyl-2,2':6',2''-terpyridine] (TMT) **73**.[115] This ligand is able to form a nine-coordinate complex with Eu^{3+}, occupying all Eu^{3+} coordination sites. The resultant TMT-Eu^{3+} complex appears to have excellent photophysical properties illustrated by its excitation and emission spectra. (Figure 4.5) By labelling a monoclonal antibody molecule with TMT, Toner reports detection limits of 6×10^{-16} mol for the antibody.

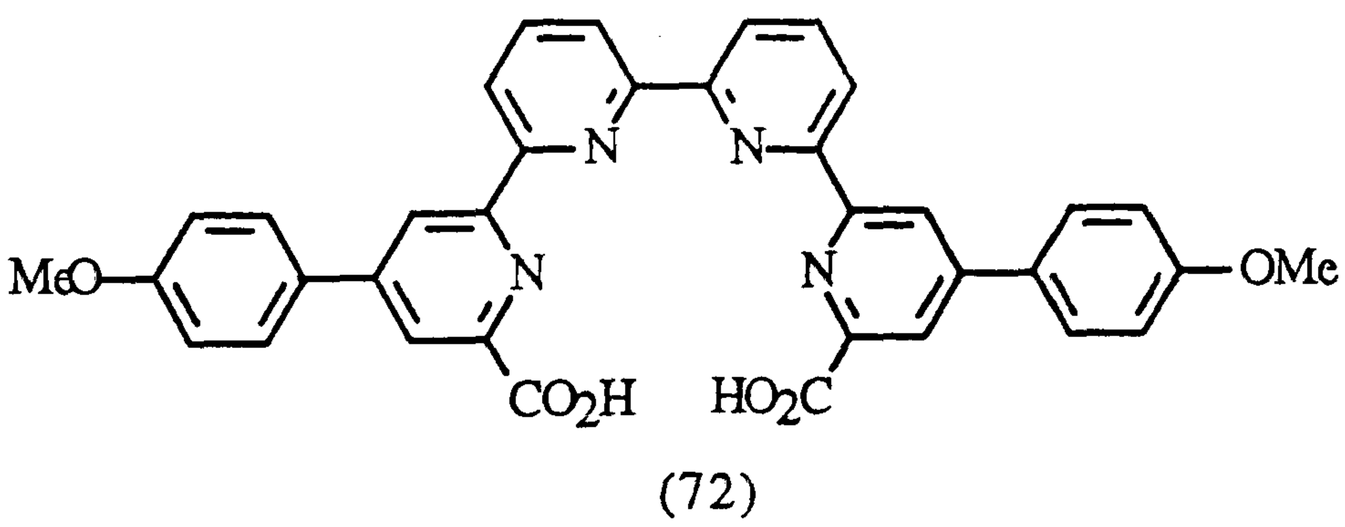


(70)



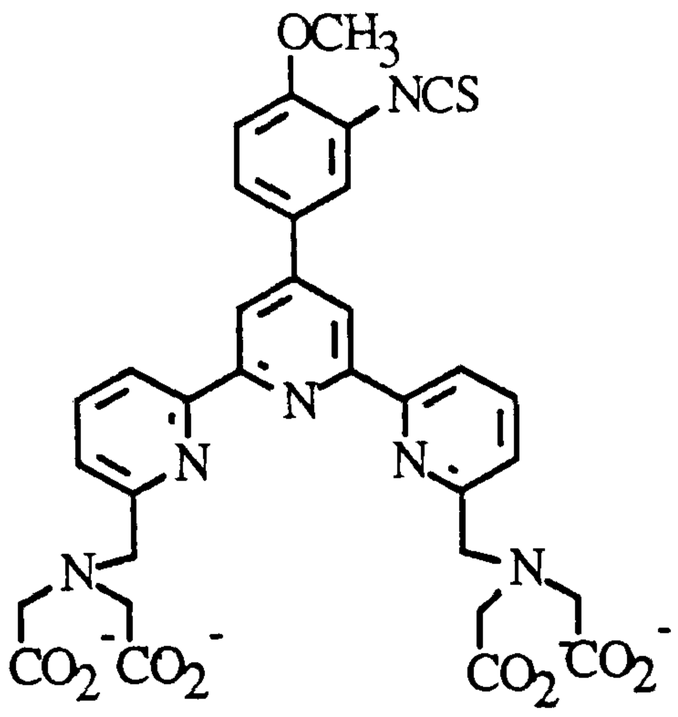
R= CO_2H

(71)



(72)

Figure 4.4



TMT (73)

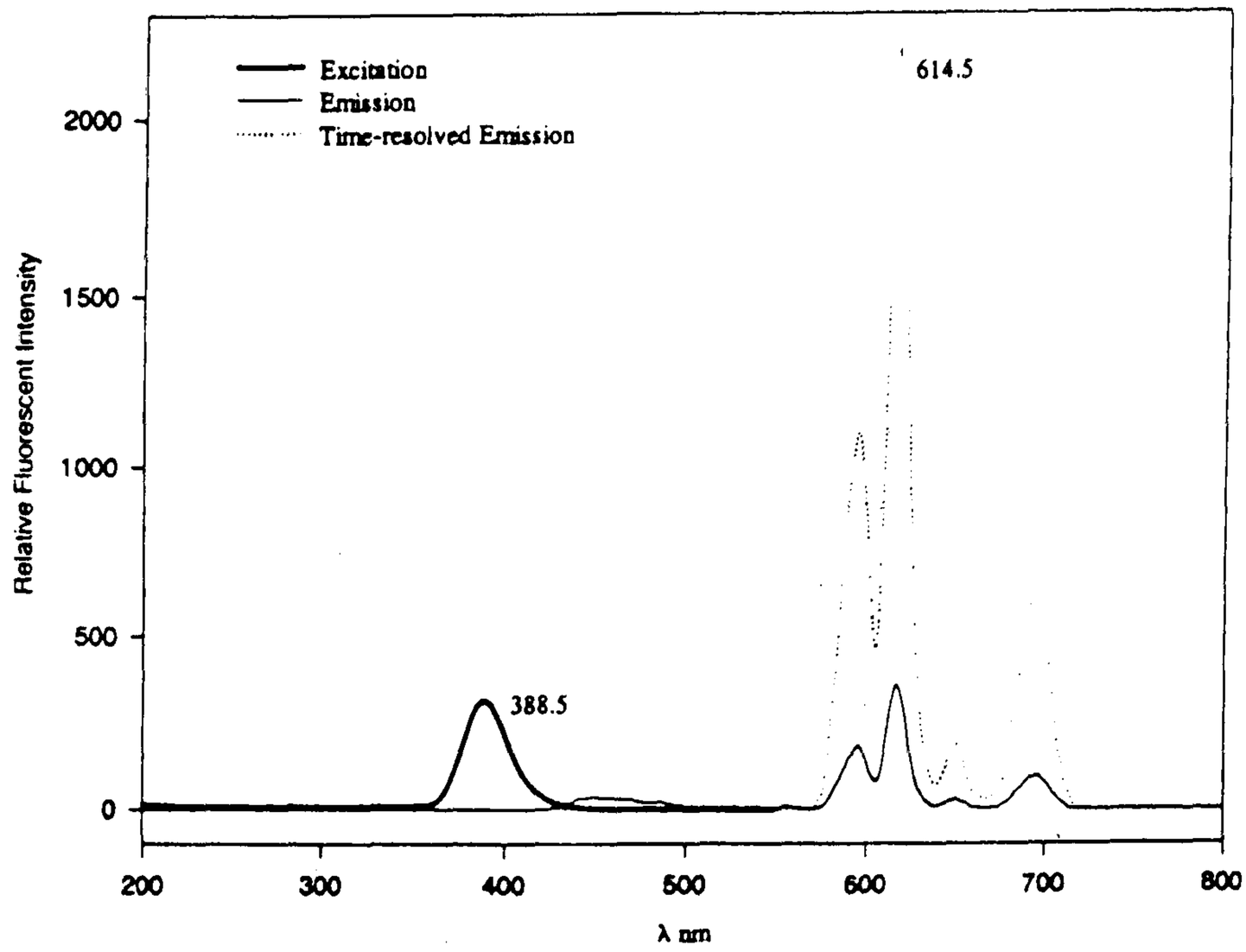
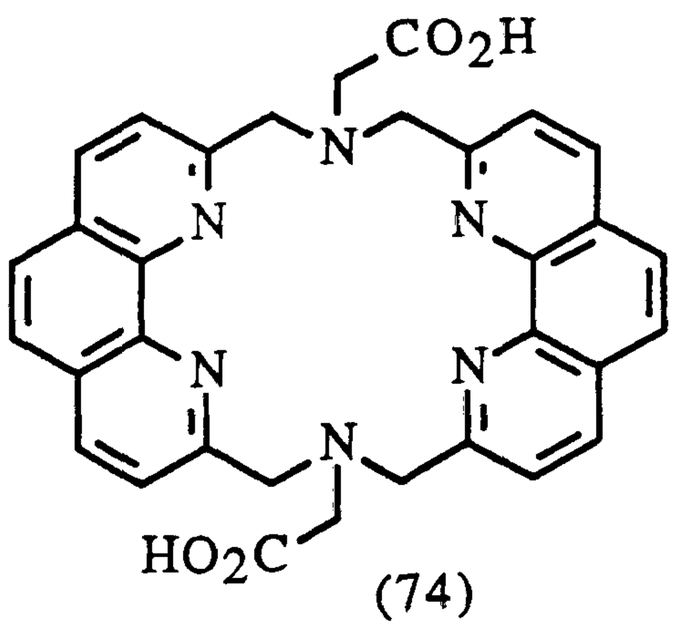


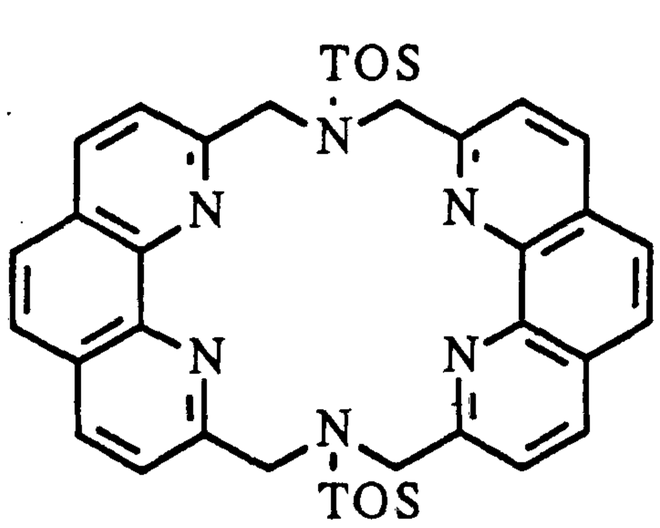
Figure 4.5 Excitation and emission spectra of TMT-Eu^{3+}

Sasamoto *et al.*[116] have recently developed a series of ligands which are able to form stable Eu^{3+} complexes in water, which also exibit fairly intense luminescence.

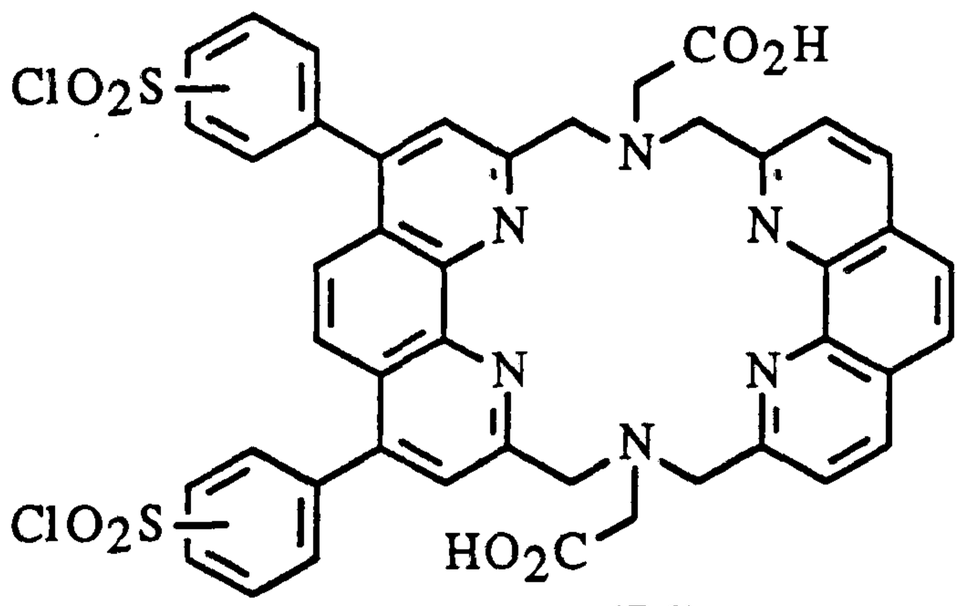
Their approach has been to incorporate two phenanthroline moities (as sensitisers) into an aza-crown framework containing two carboxylate groups **74**. Crystallographic evidence of the N-tosyl derivative **75**[117] , indicates that the ring is not planar but bent. Thus what is postulated by Sasamoto is a europium complex of **74**, where the lanthanide ion is coordinated three dimensionally, almost resembling a cryptand. This brings about improved stability of the complex and better shielding from solvent molecules (Figure 4.6)



(74)



(75) TOS= $-SO_2C_6H_4CH_3$



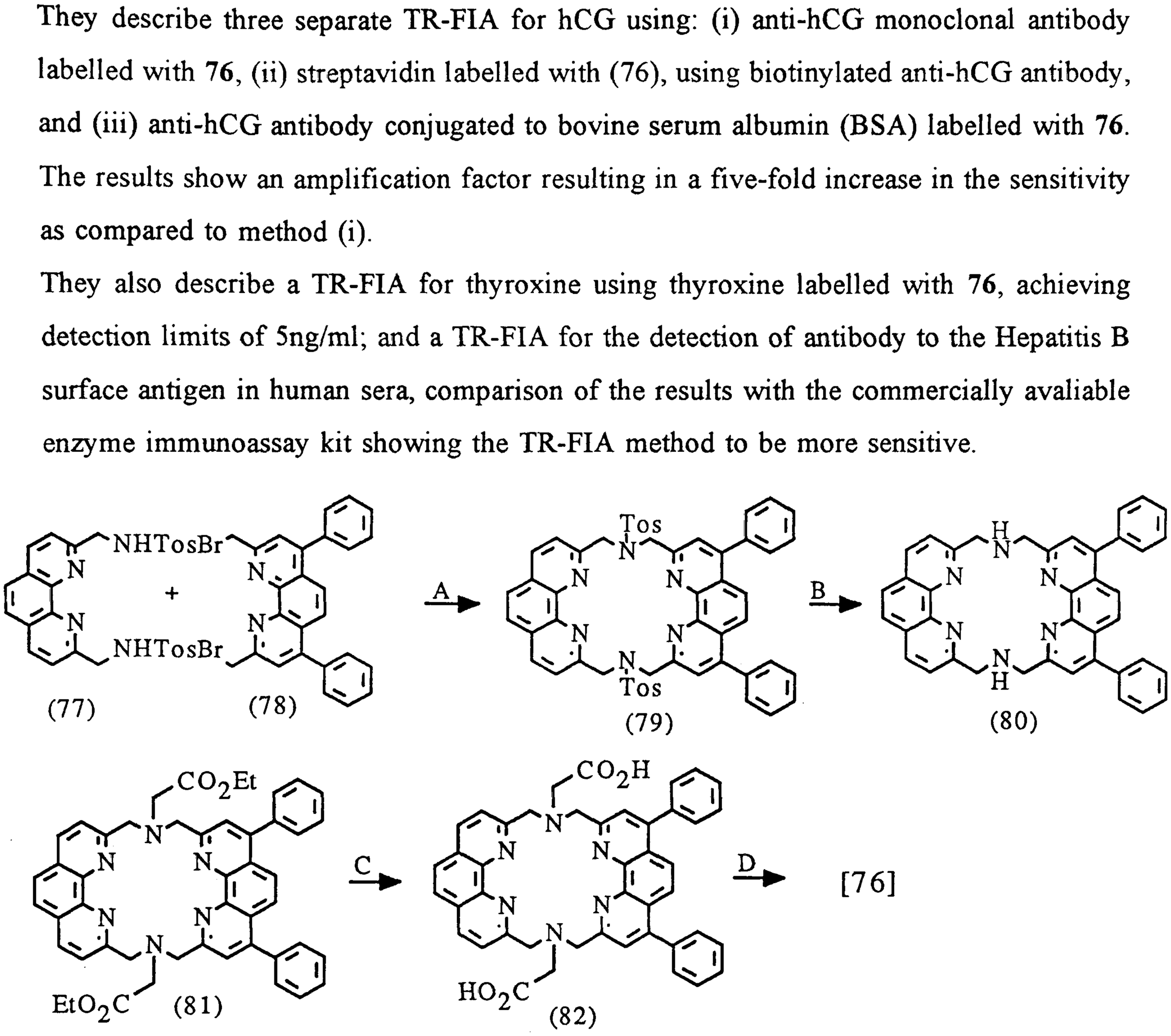
(76)

Figure 4.6

By synthesising the BCPDA analogue **76** (Scheme 1.2), they were able to tap into the immunoassay technology developed by Diamandis and co-workers.

They describe three separate TR-FIA for hCG using: (i) anti-hCG monoclonal antibody labelled with **76**, (ii) streptavidin labelled with (76), using biotinylated anti-hCG antibody, and (iii) anti-hCG antibody conjugated to bovine serum albumin (BSA) labelled with **76**. The results show an amplification factor resulting in a five-fold increase in the sensitivity as compared to method (i).

They also describe a TR-FIA for thyroxine using thyroxine labelled with **76**, achieving detection limits of 5ng/ml; and a TR-FIA for the detection of antibody to the Hepatitis B surface antigen in human sera, comparison of the results with the commercially avaliable enzyme immunoassay kit showing the TR-FIA method to be more sensitive.



Scheme 1.2 A. K_2CO_3, DMF; B. H_2SO_4AcOH; C. KOH, MeOH; D. HSO_3Cl.

The relentless search for new and better ligands to prepare luminescent lanthanide (III) complexes goes on unabated with the latest additions being the polyacid chelates derived from 2,6-bis(N-pyrazoyl)pyridine **83**[118] and the tetraza phosphinates **84**[119] (Figure 4.7)

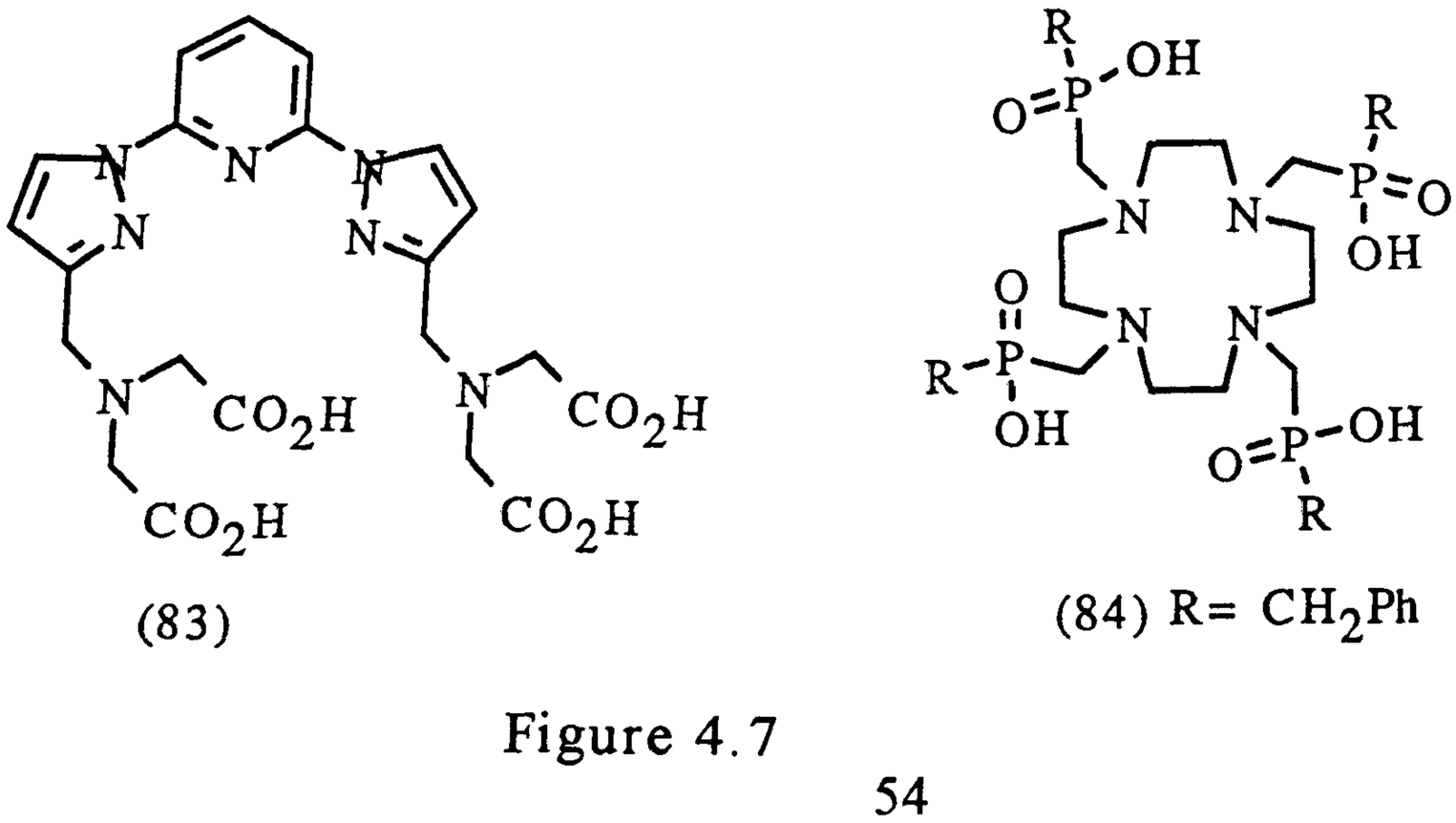


Figure 4.7

1.5 ENZYME-AMPLIFIED LANTHANIDE LUMINESCENCE.

Enzyme-amplified lanthanide luminescence (EALL) is an attempt to combine the capabilities of time-resolved fluorescence detection of lanthanide chelates with the amplification provided by the use of an enzyme as label. Signal generation is performed by enzymatically transforming a substrate which does not form a luminescent chelate with either Tb^{3+} or Eu^{3+} into a product which does form such a chelate. Examples of such transformations include aromatic compounds which initially do not form highly luminescent complexes with the lanthanides, but contain groups which can be removed by enzymatic hydrolysis such as a phosphate, sulphate, acyl, or glycoside. Upon hydrolysis the product is a compound capable of forming a luminescent lanthanide chelate. One such example is the EALL detection of alkaline phosphatase using substituted salicylphosphates. (Figure 4.8)[120]

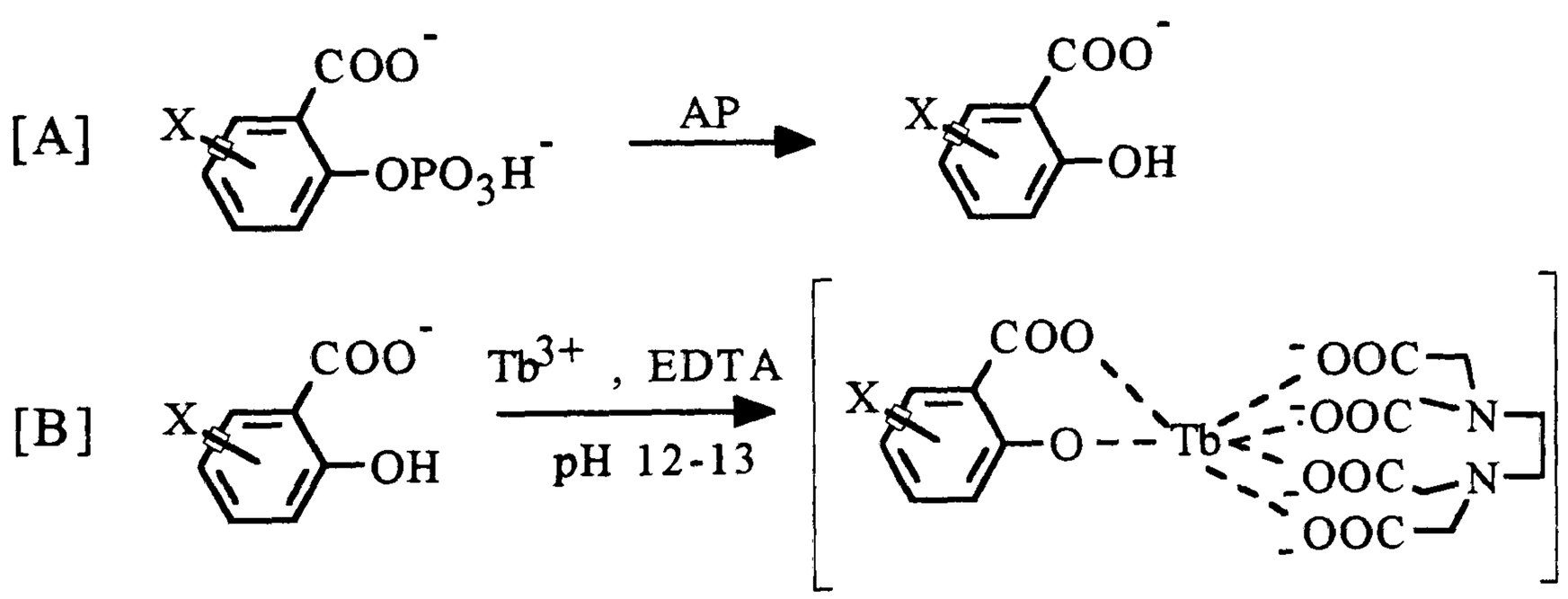


Figure 4.8 EALL system for detection of AP using substituted salicyl phosphates; [A] dephosphorylation of XASP is followed by [B] formation of the luminescent ternary XAS:Tb:EDTA complex at elevated pH.

Enzymes can also be used to promote a hydrolytic or oxidative reaction upon chromophores containing poorly coordinating groups which can be either hydrolysed by a hydrolytic enzyme in the presence of water or oxidised by an oxoreductase enzyme in the presence of a suitable oxidising agent to the more strongly coordinating carboxylic acid anion $-COO^-$. Examples of such groups are esters, amides, aldehydes, and cyano groups. The detection of xanthine oxidase is based on the conversion of salicyladehyde to salicylic acid by xanthine oxidase in the presence of O_2 and H_2O. (Figure 4.9)[120]

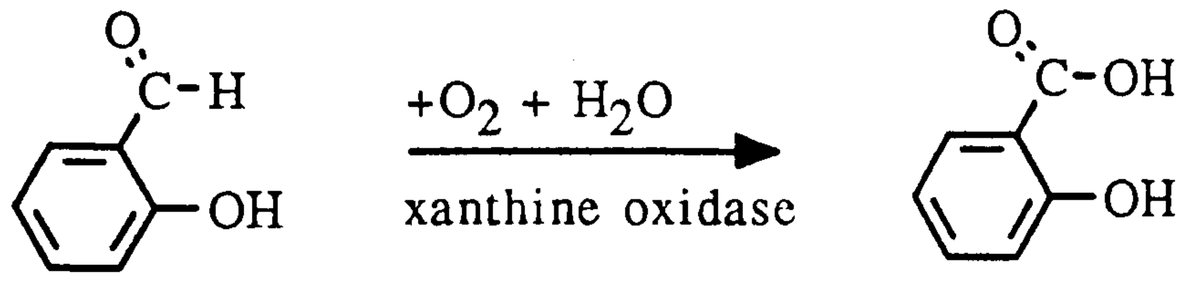
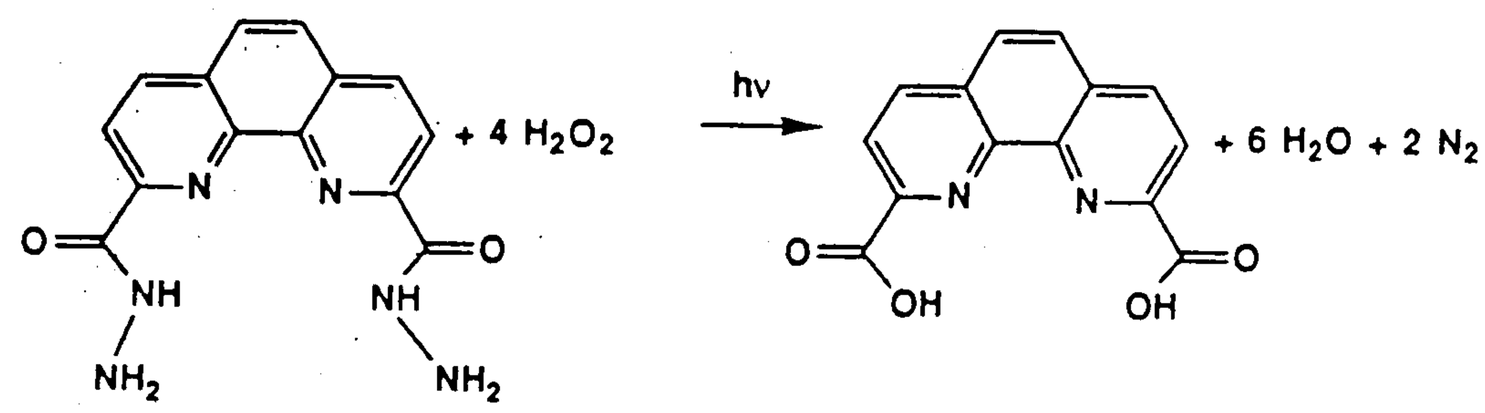


Figure 4.9 Conversion of salicylaldehyde to salicylic acid by xanthine oxidase. Luminescence detection of SA is performed as for Figure 4.8

The detection of glucose oxidase (GOD) involves a more sophisticated coupled or two-step detection system. Here the photoassisted oxidation of 1,10-phenanthroline-2,9-dicarboxylic acid dihydrazide (PDACdh) to 1,10-phenanthroline dicarboxylic acid (PDCA) by H_2O_2 generates a compound able to form highly luminescent complexes with Eu^{3+}. ß-D-GOD is then detected using glucose as a substrate via the reactions illustrated in Scheme 1.3[120], carried out in the presence of PDCAdh, Eu^{3+}, and UV irradiation.



Scheme 1.3 Coupled EALL detection system for glucose oxidase using photoassisted oxidation of PDAdh by H_2O_2 to form luminescent Eu^{3+} complexes.

The principles of EALL have been translated into a model assay of α-fetoprotein (AFP) by Diamandis *et al.*[121]. In their sensitive time-resolved immunoassay (Figure 5.0), the primary immunological label is alkaline phosphatase (ALP); its substrate is the phosphate ester of 5-fluorosalicylic acid (FSAP). Enzymatic hydrolysis of FSAP produces 5-fluorosalicylic acid (FSA). The non-hyrolysed ester (FSAP) and the hydrolysed ester (FSA) have different behaviour in Tb^{3+}-EDTA solutions. The free fluorosalicylic acid (FSA) is able to form a highly fluorescent ternary complex [FSA-Tb^{3+}-EDTA] on addition of the Tb^{3+}-EDTA solution, which FSAP is unable to do. An intact hydroxyl group on the FSA molecule is essential to form these highly fluorescent complexes. The detection limit obtained corresponds to 1.5x 10^5 molecules of AFP in a sample volume of 100μL. The

sensitivity achieved is a result of the enzymatic amplification introduced by ALP and the quantification by laser-induced time-resolved fluorometry.

Diamandis[122] has embarked on a deliberate search of fluorogenic chelators of Eu^{3+} and/or Tb^{3+} where the lanthanide binding site consists of at least one carboxyl group and a hydroxyl group. The hydroxyl group is needed for conversion to either a phosphate ester or a galactosidase, to act as substrates for ALP or ß-galactosidase respectively. One of the compounds identified by Diamandis, 4-methylumbelliferylphosphate **85** forms a fluorescent, long-lived chelate with Eu^{3+}, however, enzymatic cleavage of the phosphate group by ALP to form 4-methylumbelliferone **86**, results in a compound which is unable to form fluorescent complexes with Eu^{3+}. This pair of compunds which work in the opposite way in comparison with the reported FSAP-FSA-Tb^{3+} system, have been used to develop two model time-resolved fluoriometric immunoassays for TSH and T_4 in serum.[122]

(Figure 5.1)

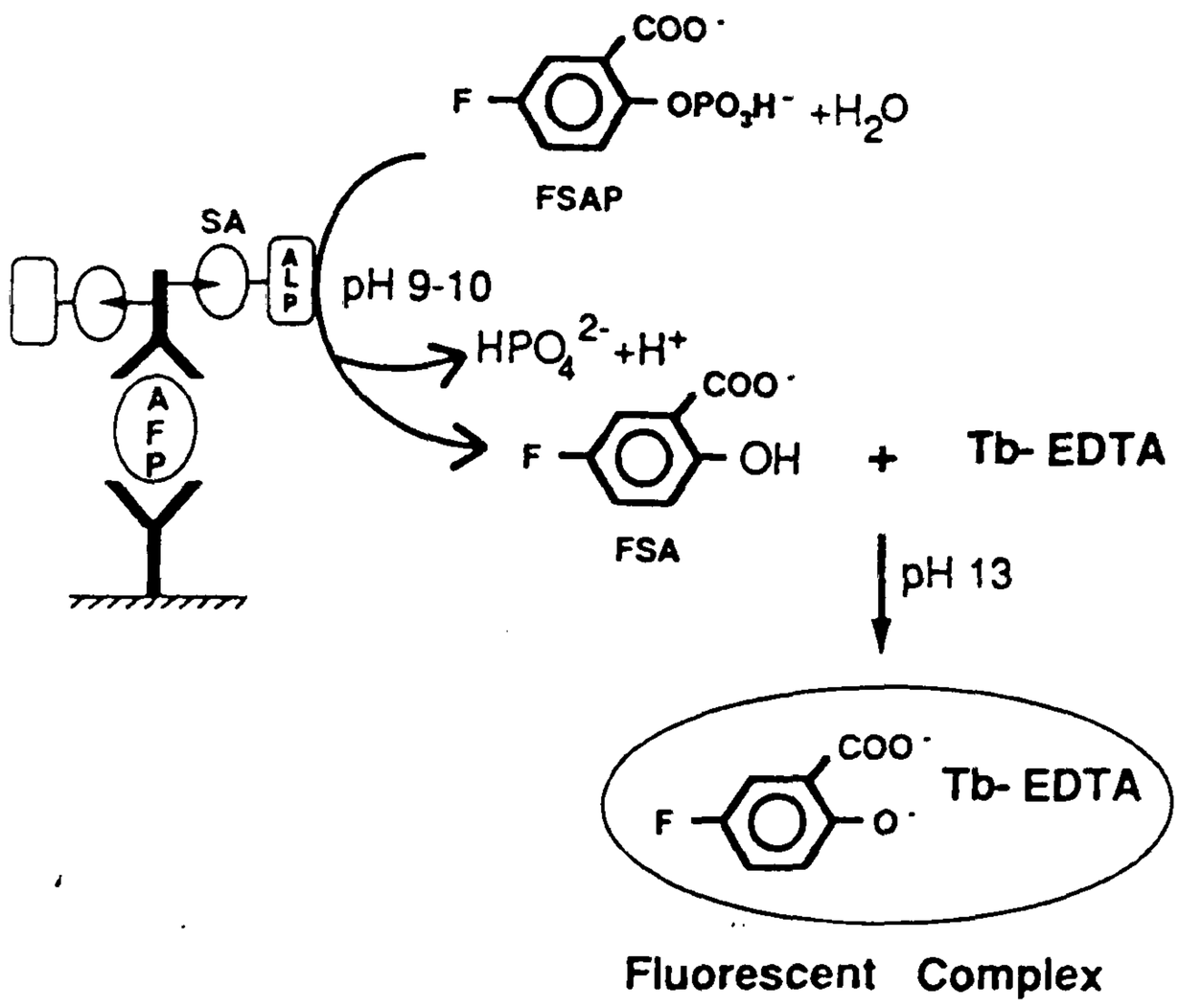
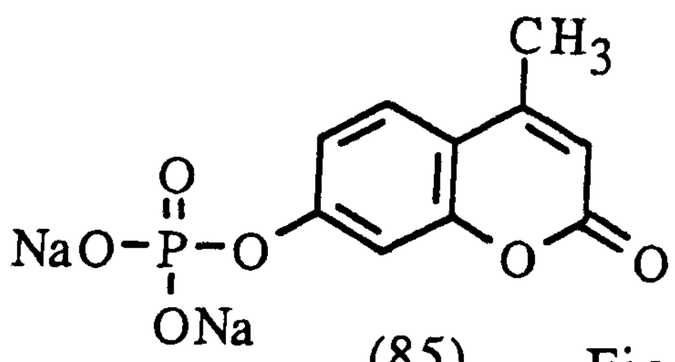
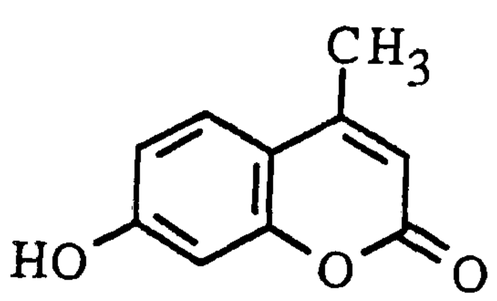


Figure 5.0 The analyte is captured by the coating antibody. A 'sandwich' is formed by adding a biotinylated detection antibody, followed by the addition of alkaline phosphotase (ALP)-labelled streptavidin (SA). ALP hydrolyses the acid (FSA) which is then quantitated by adding Tb^{3+}-EDTA solution and measuring the luminescence of terbium.



(85)

Figure 5.1



(86)

1.6 NUCLEIC ACID HYBRIDISATION ASSAYS.

The sensitivity and specificity that can be achieved by nucleic acid hybridisation probes has made them irreplaceable in molecular biology for the detection of specific, complementary nucleic acid sequences. However, the use of isotopic labels has limited the acceptance of nucleic acid methodology in routine diagnosis. Today, a DNA detection method must fulfill the following criteria: safety, speed, specificity, sensitivity, reproducebility, and the possibility of automation.

Various substances have been either used or advocated as labels for DNA probes, including radioisotopes, fluorescent markers, and enzymes. Most attention has focused on alternatives to radioisotopic labels because of the associated problems of safety, stability, and waste disposal. From the vast array of non-isotopic labels, no single label has yet emerged as an ideal replacement for radioisotopes.

An ideal label for a DNA probe would have the following properties:

(a) be easily attached to DNA,

(b) be detectable at very low concentrations using simple instrumentation,

(c) produce a signal which is modulated when labelled DNA probe is hybridised to its complementary DNA sequence (thus facillitating the development of nonseparation (homogeneous) DNA probe assays, and

(d) be stable at the elevated temperatures sometimes used in hybridisations.

1.6.1 Labelling of Nucleic Acid Probes.

Two main types of labelling strategy have evolved: direct and indirect labelling, both of which have been used in several different DNA hybridisation assay designs. (Figure 5.2)[20]

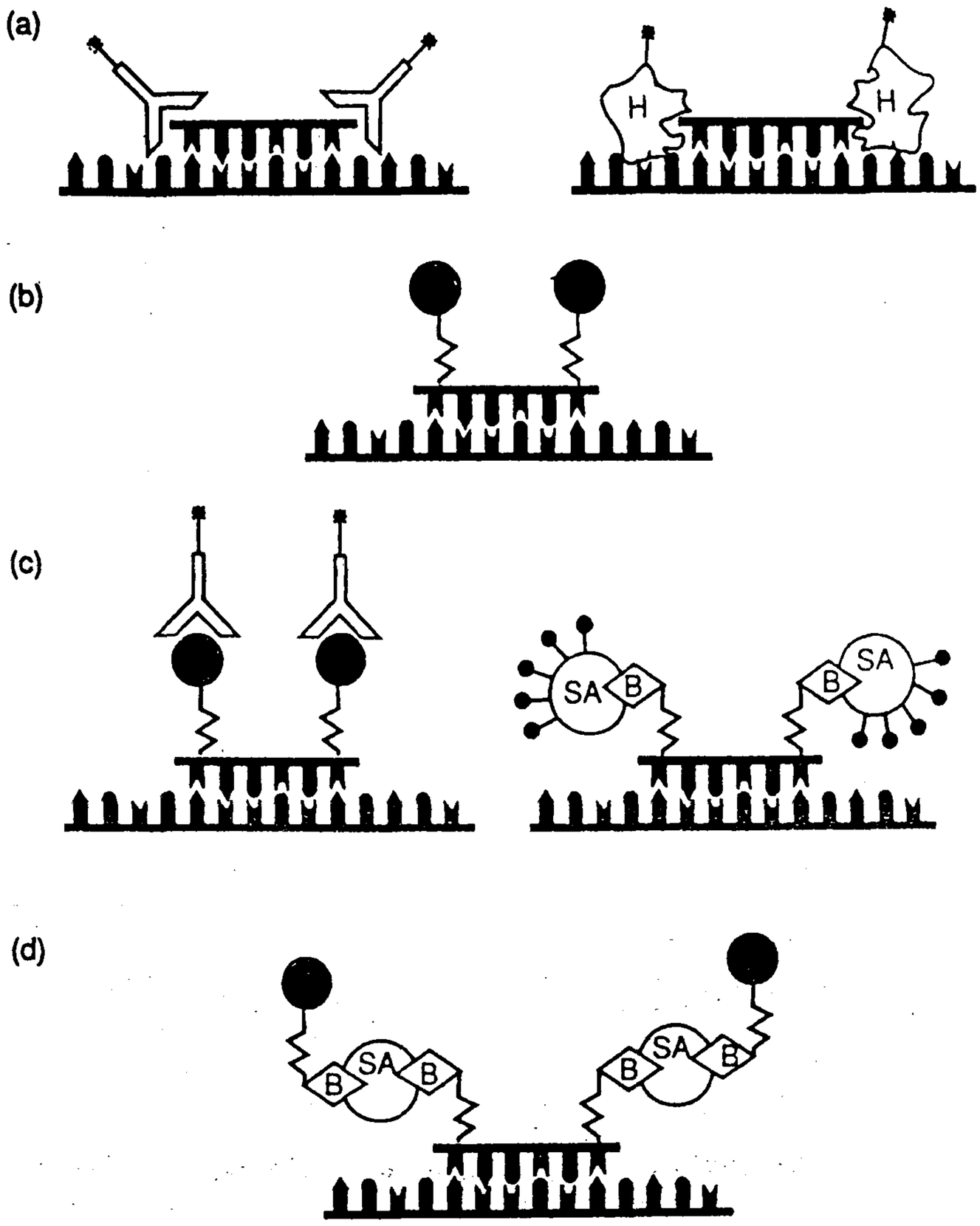


Figure 5.2 Assay designs in DNA hybridisation assays: (a) The DNA probe is used unlabelled and the target-probe hybrid is detected by using either labelled antibodies againts the duplex or histones (H); (b) the DNA probe is directly labelled with a reporter molecule (●); and (c) the DNA probe is conjugated to a hapten molecule, and antihapten labelled antibodies are used to detect the hybrid. In a variation of this technique, the probe is biotinylated (B) and labelled streptavidin (SA) is used as the detection reagent; (d) unlabelled SA is used to bridge a biotinylated probe with a biotinylated reporter molecule.

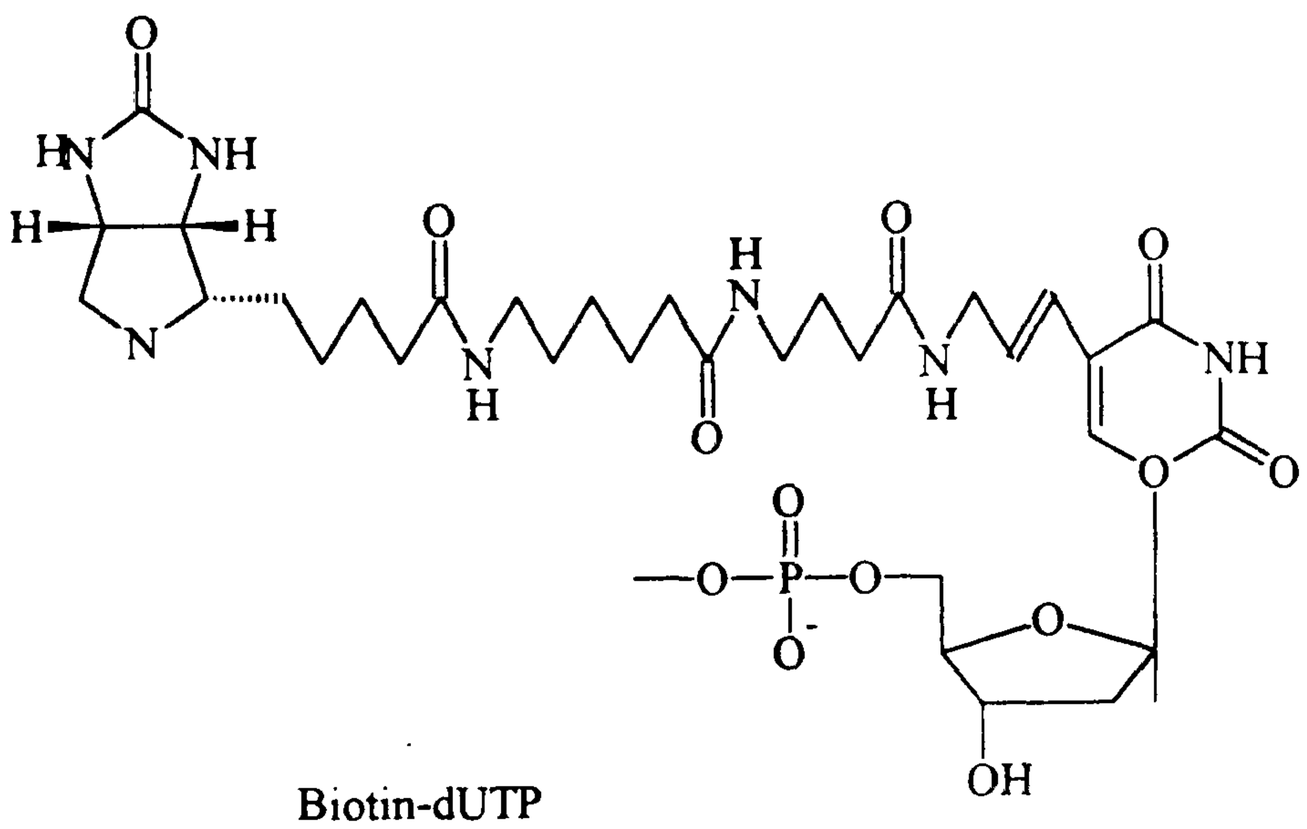
(a) Indirect Labelling

Indirect labelling requires the DNA probe to contain a hapten which is detected by using a labelled binding protein with specificity for the hapten. Among the indirectly detectable markers, biotin has gained the widest use. Biotin can be introduced into DNA enzymatically or chemically, it is detected by labelled avidin (or streptavidin) (Figure 5.3).[123] The most common signal-generating labels used in indirect detection are enzymes such as alkaline phosphatase and horse radish peroxidase. The enzymatic reaction resulting in a coloured, chemiluminescent, or fluorescent end product.

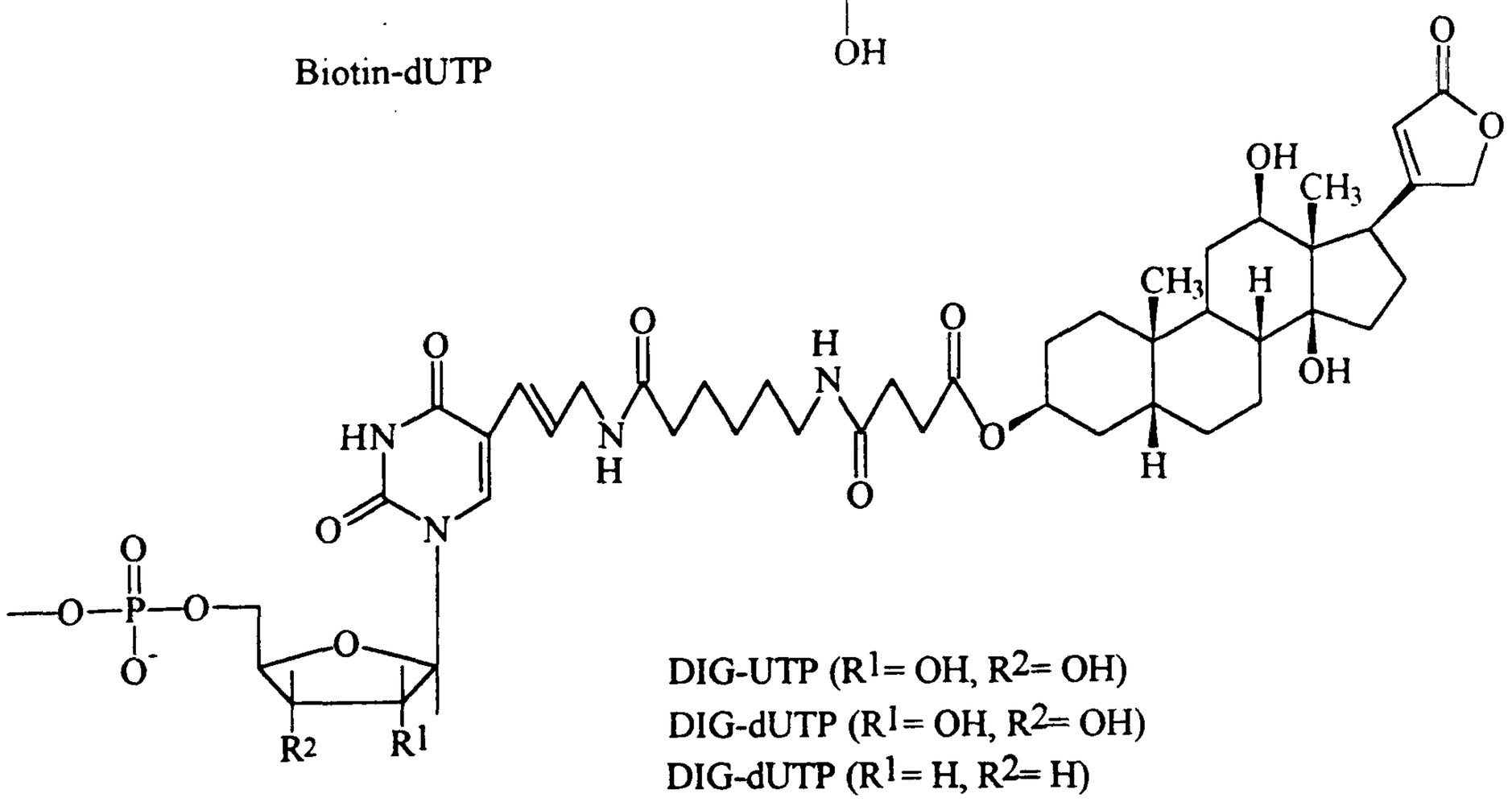
Many fluorescent dyes such as rhodamine and fluoresceine are also avalible as fluorometric reporter groups attached to avidin or streptavidin. But they suffer from low sensitivity due to high background fluorescence.

Similar to the biotin-avidin system, Boehringer Mannheim introduced the Digoxigenin (DIG) labelling towards the end of the 1980s (Figure 5.3)[5,124]. The method is based on a steroid isolated from the digitalis plants (Digitalis purpurea and Digitalis lanata). These plants are the only natural source of digoxigenin, no binding of anti-DIG antibody in other biological materials occurs. Detection of hybridised DIG probes is mediated by high affinity anti-DIG antibodies conjugated to enzymes or fluorescent dyes.

An advantage of indirect procedures is the ability to build amplification steps into the assay. For example, if biotin is the primary label, it can be bound to one of four binding sites on avidin and the three remaining sites can be filled with biotin, thus producing a threefold amplification. The use of enzyme-labelled biotin introduces a further amplification factor.



Biotin-dUTP



DIG-UTP (R^1= OH, R^2= OH)
DIG-dUTP (R^1= OH, R^2= OH)
DIG-dUTP (R^1= H, R^2= H)

Figure 5.3

(b) Direct Labelling

In direct labelling, the detectable molecule (reporter) is bound directly to the nucleic acid probe so that detection can be achieved immediately after hybridisation of the probe with the target nucleic acid. For such methods it is essential that the probe-reporter bond survives the rather harsh hybridisation and washing conditions. More importantly the reporter molecule should not interfere with the hybridisation reaction.

The most common direct nucleic acid labels are radioisotopes, primarily ^{32}P and ^{35}S, which are easily introduced by a variety of enzymatic techniques. They offer the possibility of incorporating many labels per probe, thus increasing the sensitivity of detection, in terms of which they remain the "gold standard".

Enzymes such as alkaline phosphatase and horseradish peroxidase can also be used as direct labels, as enzyme labelled DNA probes are stable during hybridisation. Although enzyme catalysed methods are sensitive, measurements can only be performed once and the enzyme activity has to be monitored.

DNA probes can also be labelled directly with classic fluorochromes, such as fluorescein and rhodamine, but this approach suffers from low sensitivity. Recently new directly labelled nucleotides have become available, containing resorufin (red fluorescence), and hydroxycoumarin (blue fluorescence)[124].

Fluorescein which can be incorporated enzymatically into nucleic acids can also be used as an indirect label being detected by anti-fluorescein antibody coupled to an enzyme or an unconjugated antibody which can be detected by a secondary antibody labelled with fluorescein isothiocyanate[124].

By using combinations of digoxigenin-, biotin-, and fluorescence-labelled probes, multiple simultaneous hybridisations can be performed to localise different chromosal regions in one operation. The availability of three different fluorescent dyes coupled to antibodies makes this possible.

1.6.2 Nucleic Acid Labelling with Lanthanide Chelates.

It comes as no suprise that DNA hybridisation assays using the principles of the immunological versions of both the DELFIA and Cyberfluor systems have been described.

Dahlen *et al.* have described the detection of biotinylated DNA probes using europium-labelled streptavidin, both in dot blot and sandwich hybridisation assays[125] (Figure 5.4)

Viral DNA (adenovirus type 2) was detected by attaching the target DNA onto microtitration wells before the hybridisation reaction. The detection limit was 10pg of target DNA added to the well. The target DNA must be extensively purified before immobilisation onto the solid support (overnight procedure). The limitations inherent in direct hybridisation formats on plastic surfaces are omitted in sandwich hybridisation. The ß-lactamase gene was detected by this assay, with a detection limit of 1.9pg of target DNA.

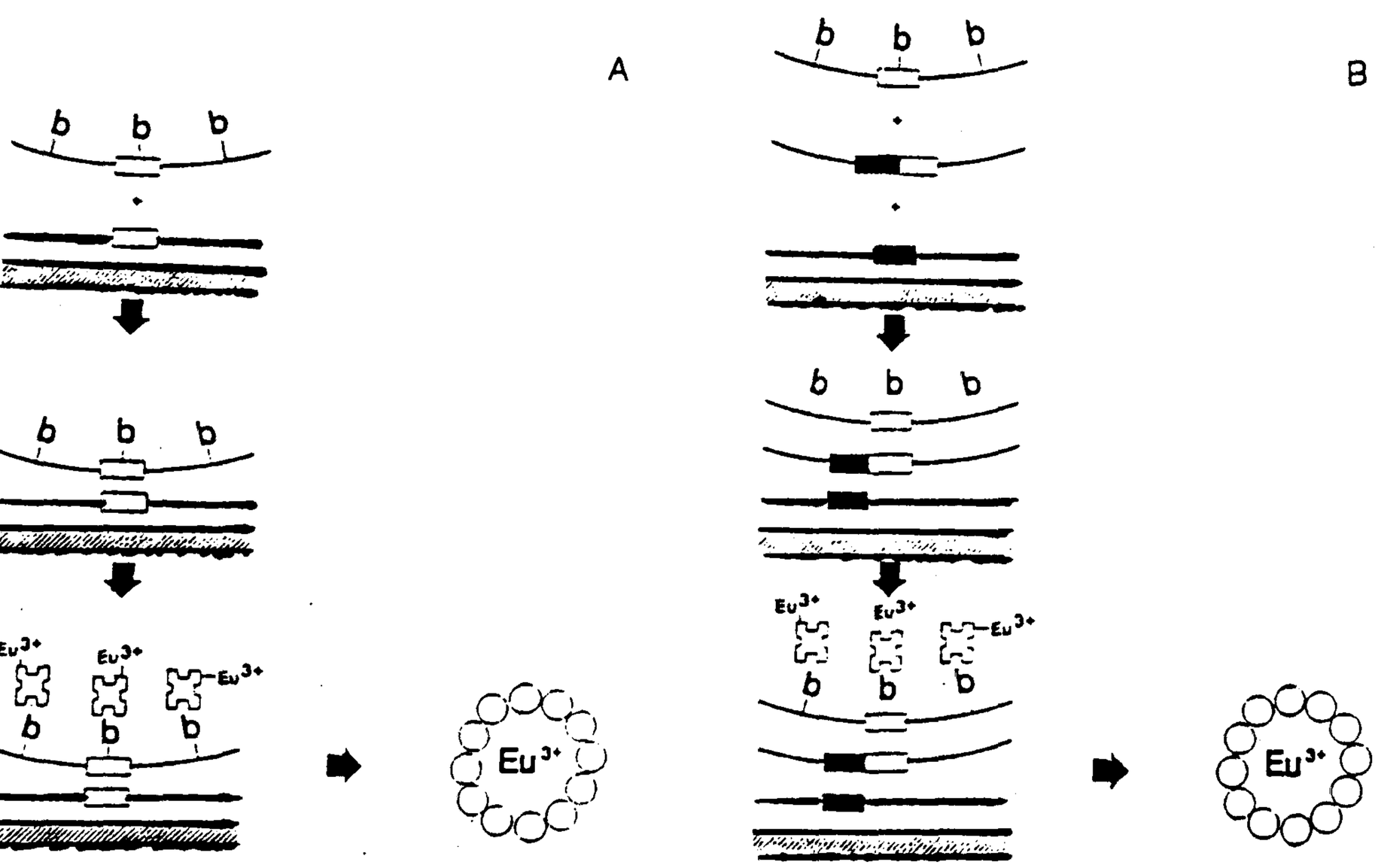


Figure 5.4 (A) Dot blot hybridisation. The sample DNA is immobilised on a solid support. A biotinylated DNA probe is allowed to hybridise to its target sequences. The bound biotin-probe is detected by Eu-labelledstreptavidin. The Eu-fluorescence is developed by adding enhancement solution. (B) Sandwich hybridisation. The sample is added simultaneously with the biotinylated DNA probe to the hybridisation reaction. The captive probe, which has been attached to the solid support before the assay, and the biotinylated probe will hybridise to the target DNA. The bound biotin-probe is detected by using Eu-labelled streptavidin.

However, the DELFIA assay system is restricted to dot-blot hybridisations, and even here it ends up complicating the assay procedure. The essentially non-fluorescent europium chelate and the procedure of dissociation and enhancement is not suitable for use in Southern, Northern and *in situ* hybridisations, where the label needs to be fluorescent.

Recently Diamandis and co-workers have described a DNA hybridisation assay (dot-blot and Southern blot), based on the detection of biotinylated DNA with a highly fluorescent streptavidin based reagent labelled with the europium chelate of BCPDA[126]. This streptavidin based macromolecular complex (SBMC), has already been used as a multiple fluorescent label in time-resolved fluoroimmunoassays (TR-FIA).

In comparison to other isotopic and nonisotopic methodologies, the system lacks sensitivity (detection limit of 4ng of biotinylated DNA on Southern blot). However the SBMC reagent is stable for over a year, and signals on nitrocellulose are stable for months and are visible on UV illuminators with the possibility of instant visualisation and photography. Also, unlike the DELFIA system, the label has the potential to be adapted for both Northern and Western blots.

Mathis *et al.* have recently reported on the TR-fluorescent detection of DNA hybrids on dot-blots[127]. The process utilises DNA or oligonucleotide probes labelled with biotin, followed by recognition by streptavidin conjugated to a europium (III) cryptate as the fluorescent label. The structure of the molecule is shown in Figure 5.5.

One of the main advantages of the europium cryptate is its exceptional thermodynamic and kinetic stability compared with conventional europium chelates, and the shielding provided from the solvent molecules means that unlike both the DELFIA and Cyberfluor systems, the extra step for the formation of the fluorescent complex is eliminated as the europium cryptate is permanently fluorescent. This property of the cryptate allows the direct measurement of fluorescence in the medium and solid supports (dot-blots). Measurements can be performed several times, the sensitivity reaching 2amol of target DNA.

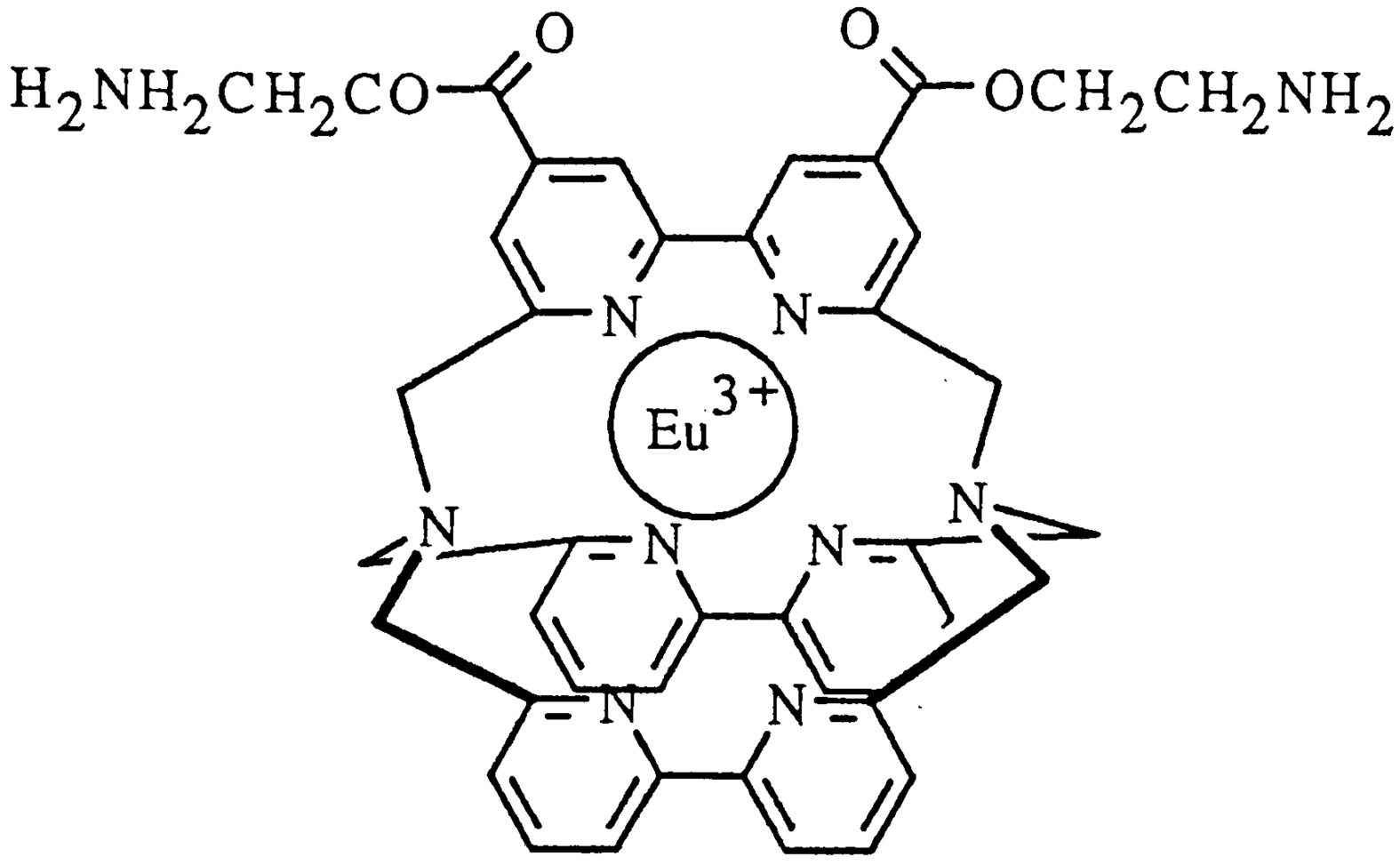
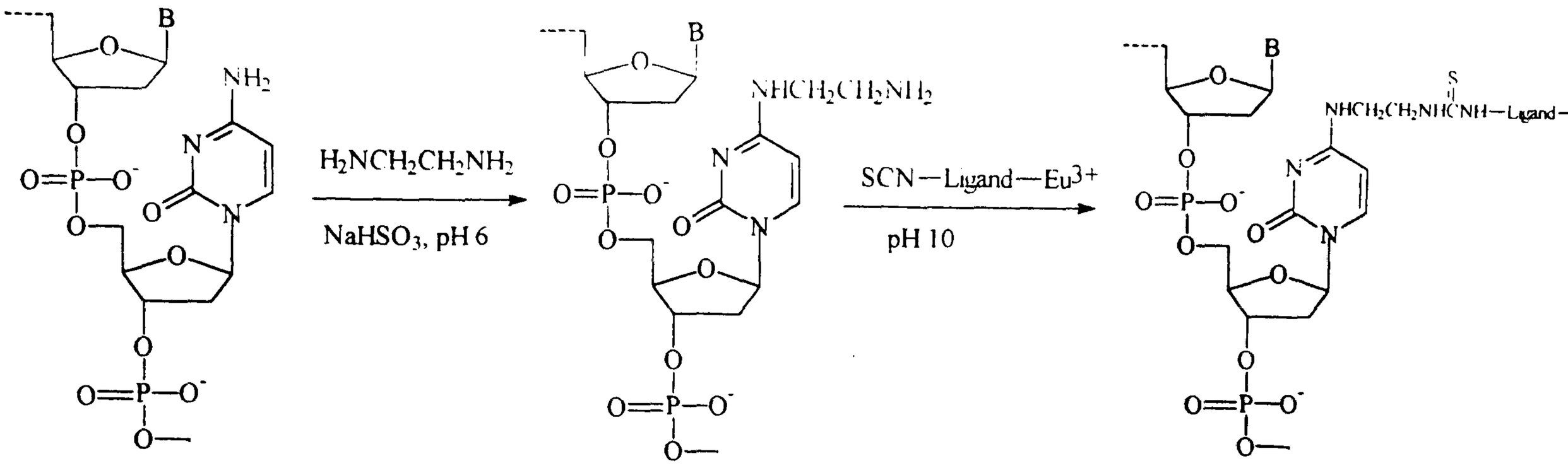


Figure 5.5 Europium(III) trisbipyridine cryptate

Because of the complexity of indirect hybridisation assays like those described above, direct hybridisation assays are preferred. Direct labelling of nucleic acid probes with europium chelates, apart fron simplifying the assay procedure offers the possibility of greater sensitivity. However, direct labelling brings about its own problems besides the labelling chemistry, namely the stability of the europium chelate under the demanding conditions used in hybridisations.

Hurskainen *et al.* have described a simple chemical method for direct labelling of DNA with a europium chelate[125,128]. The sensitivity of Eu-labelled DNA probes depends on the labelling degree and the hybridisation efficiency of the labelled probe. Thus Eu-labelling chemistry should allow the incorporation of large numbers of Eu-chelates into DNA without sacrificing its hybridisation efficieny.

Hurskainen's approach was to introduce primary aliphatic amino groups into DNA using a transamination reaction. Cytosine bases undergo transamination reaction in the presence of sodium bisulphite and an amine. By using ethylene diamine, they were able to incorporate free aliphatic amino groups into single stranded DNA, which were subsequently reacted with the isothiocyanate derivative of the europium chelate. (Scheme 1.4)
Although the whole process is lengthy, it allows the labelling of large amounts of DNA.



Scheme 1.4 Chemical labelling of DNA with a europium chelate. In the first reaction the cytosine bases in DNA are transaminated in the presence of sodium bisulphite and ethylenediamine. In the second reaction the modified DNA is reacted with an isothiocyanate derivative of the europium chelate.

However, europium labelling affects DNA by lowering the thermal stability of the duplex and the hybridisation efficiency and although the transamination reaction is a very efficient method of modifying DNA, allowing the Eu-labelling of practically all deoxycytosine residues, it needs to be carefully controlled to minimise the extent of labelling. They describe the incorporation of 4 to 8 Eu-chelates per 100 bases as the optimum degree of labelling that makes sensitive and specific hybridisation assays possible[128].

These Eu-labelled probes have been used in the detection of adenovirus in clinical specimens, fixed onto nitrocellulose and hybridised against Eu-labelled adenovirus DNA. Simple and sensitive sandwich and solution hybridisation assays with Eu-labelled DNA probes have also been made in microtitration wells for the detection of cytomegalovirus and human papillomavirus, detection limit being $5x10^5$ molecules of target DNA. A comparison between the sensitivities of Eu- and ^{32}P-labelled DNA probes showed only minor differences.

Europium labelling of DNA can also be achieved enzymatically by nick translation. Here in the presence of all four deoxynucleotide triphosphates, one or more of which carry a label, the enzymes DNA polymerase and deoxyribonuclease incorporate the labelled nucleotide into double-stranded DNA. An alternative method introduced in 1983 by Feinberg and Vogelstein is random priming.[125,129]

The properties and use of enzymatically Eu-labelled DNA probes are identical to those of chemically labelled ones.

1.6.3 Europium Labelled Oligonucleotides.

Nowadays, there is a tendency to employ short oligonucleotides instead of longer poly-DNA fragments as hybridisation probes. This has come about from the development of the polymerase chain reaction (PCR), which by amplifying target sequences by up to 10^8 times before hybridisation has overcome the lack of sensitivity associated with the use of synthetic oligonucleotides.

The advantages of oligonucleotides are that they can be synthesised in large quantities even by non-chemists using modern oligonucleotide synthesisers. They are characterised by rapid hybridisation kinetics, allowing the assay to proceed at low temperatures and in a much shorter time period, high specificity and the denaturation step is eliminated.

Modern oligonucleotide synthesisers have also meant that the preferred method of labelling of oligonucleotides with Eu-chelates is to incorporate primary amino groups into the oligonucleotide during synthesis (Figure 5.6).

By using an amino-modified building block such as diaminohexane modified deoxycytidine phosphoramidite, the fuctionalised base can introduced at any position during the synthesis. Although it is possible to intoduce up to 50 modified bases to the 5'-end of the oligonucleotide, a tail of 15 to 25 modified nucleotides is generally used. After deprotection and purification of the oligonucleotide containing the modified bases, the Eu-chelate containing an isothiocyanate is reacted with the primary amino groups avaliable. Hurskainen *et al.* have successfully labelled a single oligonucleotide with 35 Eu-chelates, although 15-20 Eu-chelates is sufficient for very good sensitivity.[125,130]

Unlike direct labelling, the melting temperature (T_m) and the hybridisation kinetics do not seem to be significantly affected by the labelling procedure.

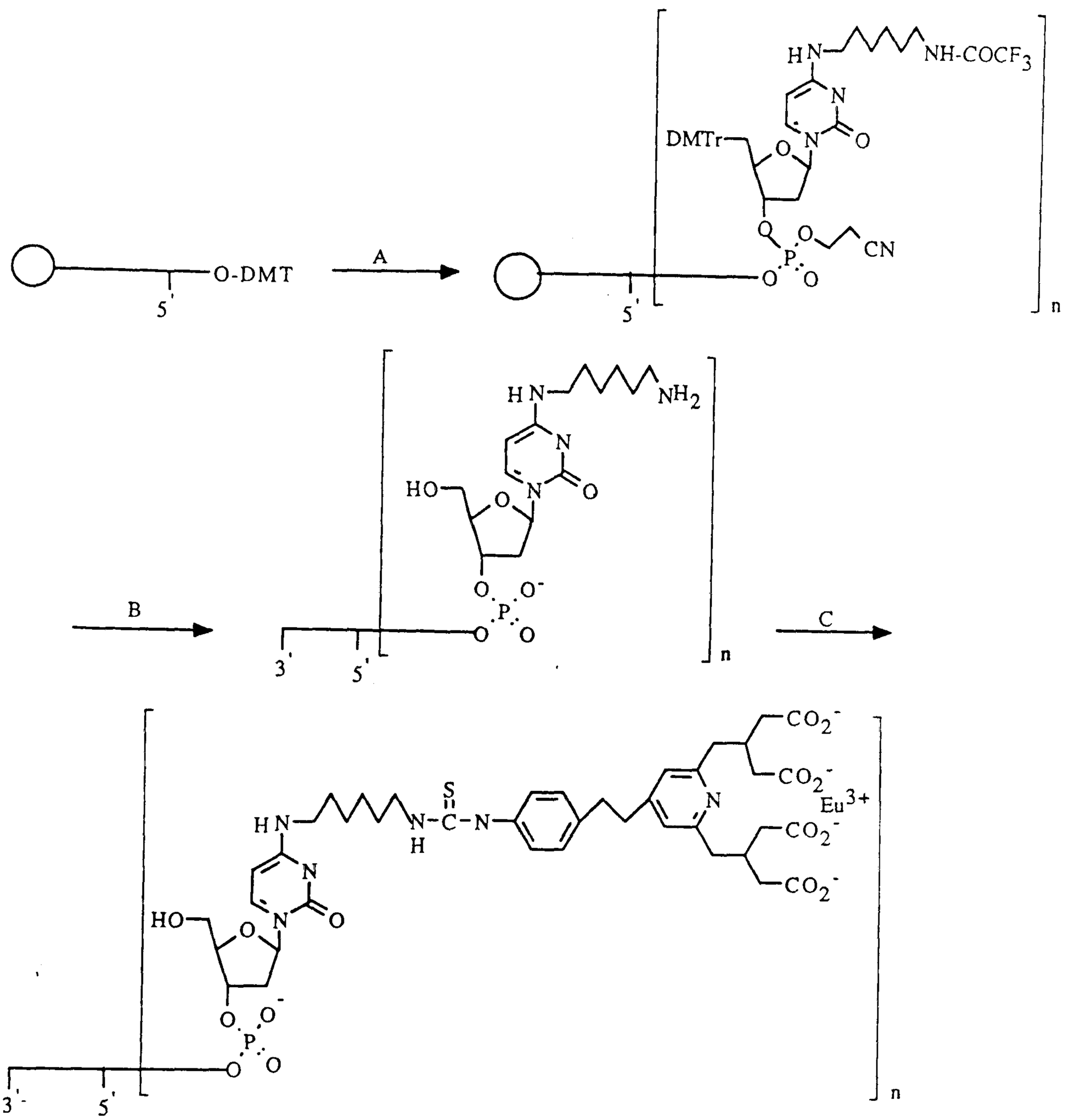


Figure 5.6 Synthesis of Eu-labelled oligonucleotides. **A.** The fully protected diaminohexane-modified cytidine is incorporated at the 5' end of the oligonucleotide during synthesis. **B.** The deprotection and purification is carried out standard procedures. **C.** The isothiocyanate analog of the Eu-chelate is reacted to the amino groups avaliable on the modified oligonucleotide.

By using europium labelled oligonucleotides as hybridisation probes, Sund *et al.* were able to achieve a detection sensitivity of $4x10^6$ target molecules (200pg), in a direct hybridisation format.[130] Dahlen *et al.* have developed a rapid in solution hybridisation assay for detecting *in vitro* amplified nucleic acid sequences (Figure 5.7)[130,131].

Two probes complementary to the amplified DNA are allowed to hybridise simultaneously with the amplified target sequence. One of the probes is labelled with Eu-chelates, and the other is labelled with biotin. The formed hybrid is collected onto streptavidin coated strips employing affinity based collection. The bound europium is dissociated with the enhancement solution before measurement of the luminescence.

This methodology has been successfully applied to the detection of the human immunodefficiency virus (HIV)-1, with a sensitivity of 10^7 target molecules.[131]

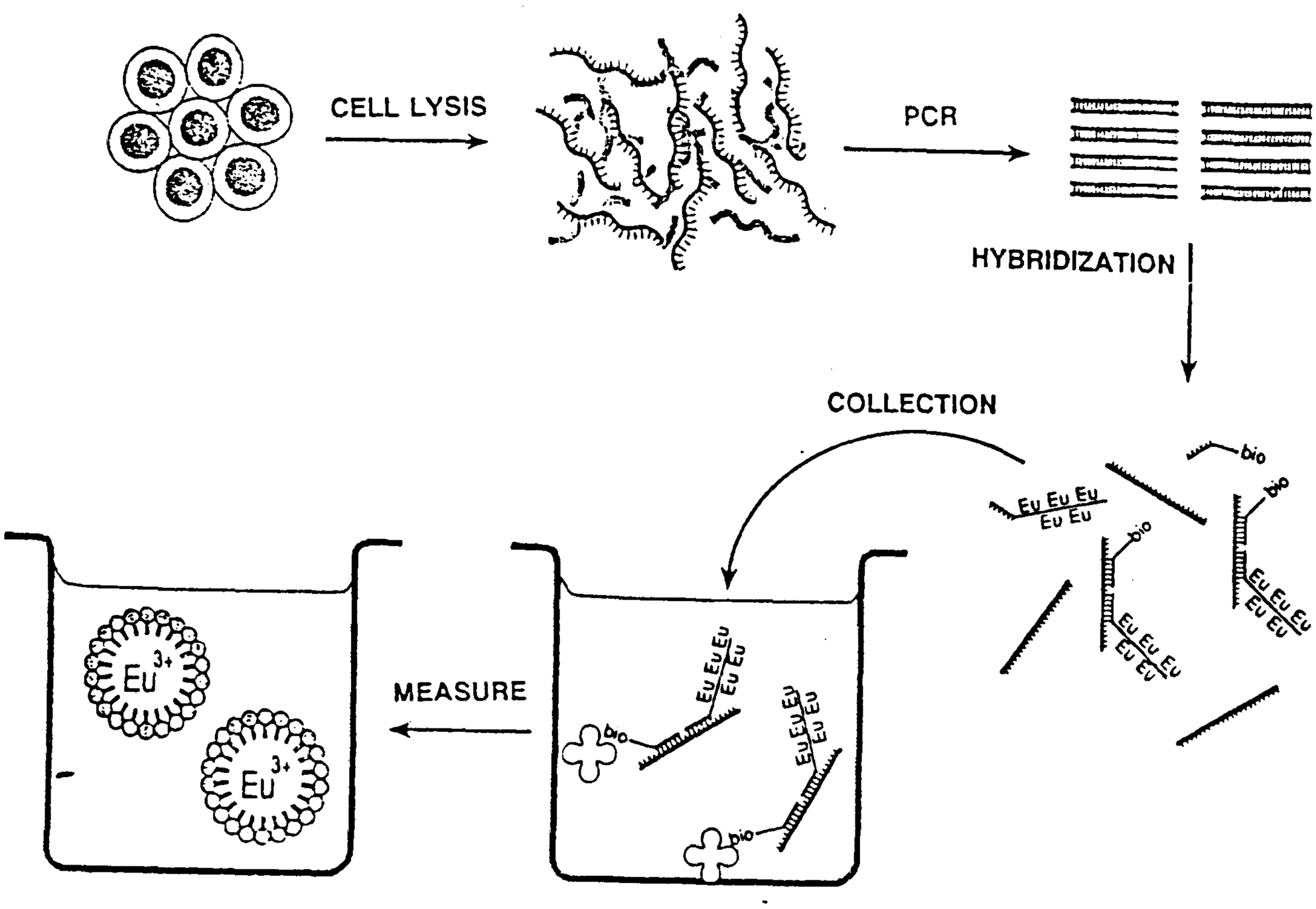


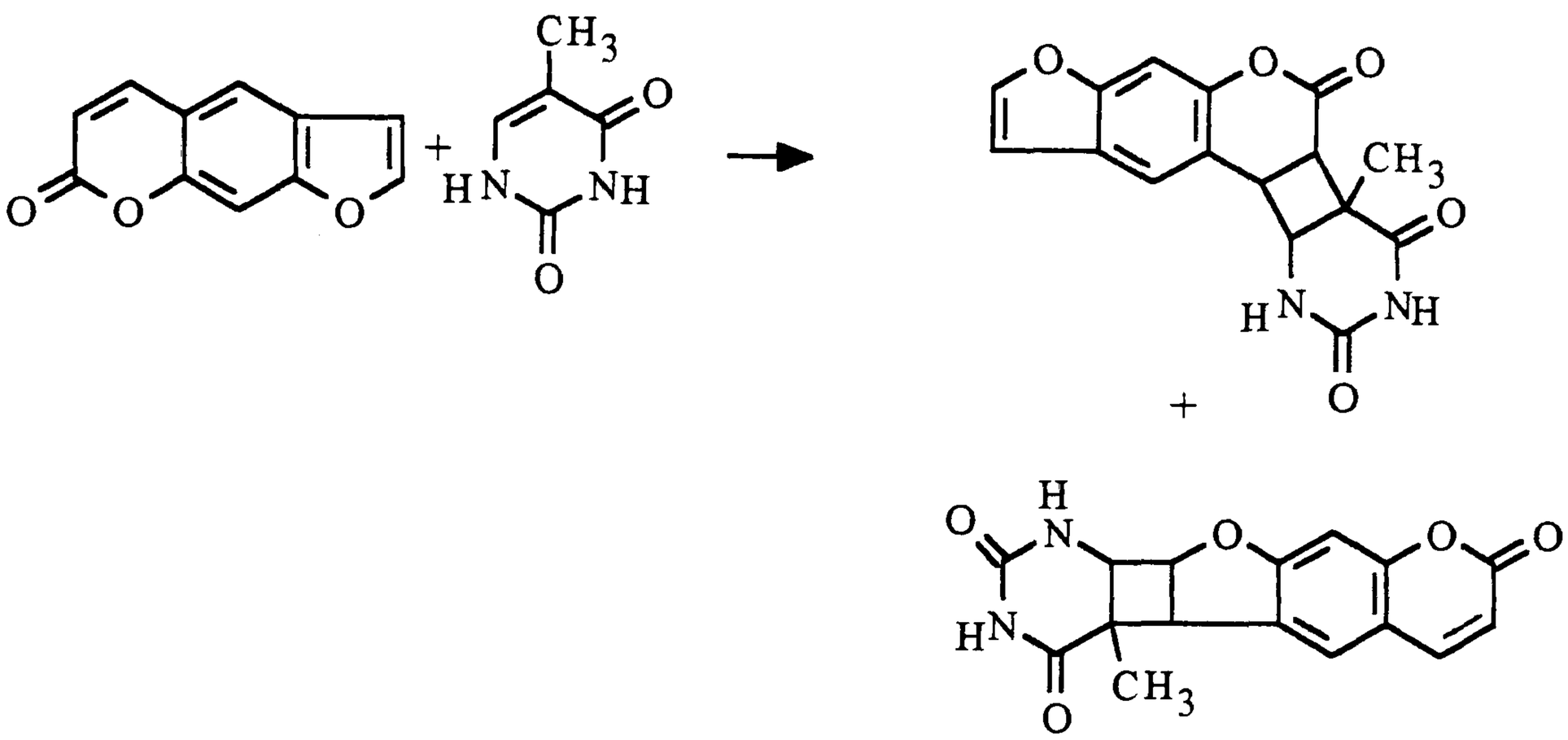
Figure 5.7 Schematic presentation of PCR amplification and a solution hybridisation assay employing labelled oligonucleotides. The cells are lysed, and the PCR performed on the crude sample. Two probes, one biotinylated and the other labelled with Eu complementary to the amplified fragment, are hybridised to the same target. The hybrids are subsequently collected onto streptavidin-coated microtitration wells. The bound europium is dissociated with an enhancement solution before measurememt of fluorescence.

Europium-labelled allele-specific oligonucleotide probe has been used for the detection of a single base change in the human genome.[125] The Eu-labelled oligonucleotide probe is designed in such a way that the point mutation site is in the middle of the hybridisation sequence. By washing under stringent conditions only the perfectly matched probes will remain hybridised.

Work has also started by the Wallac-Oy group on the application of Eu-labelled oligonucleotides in the diagnosis of genetic disease and oncogene detection.

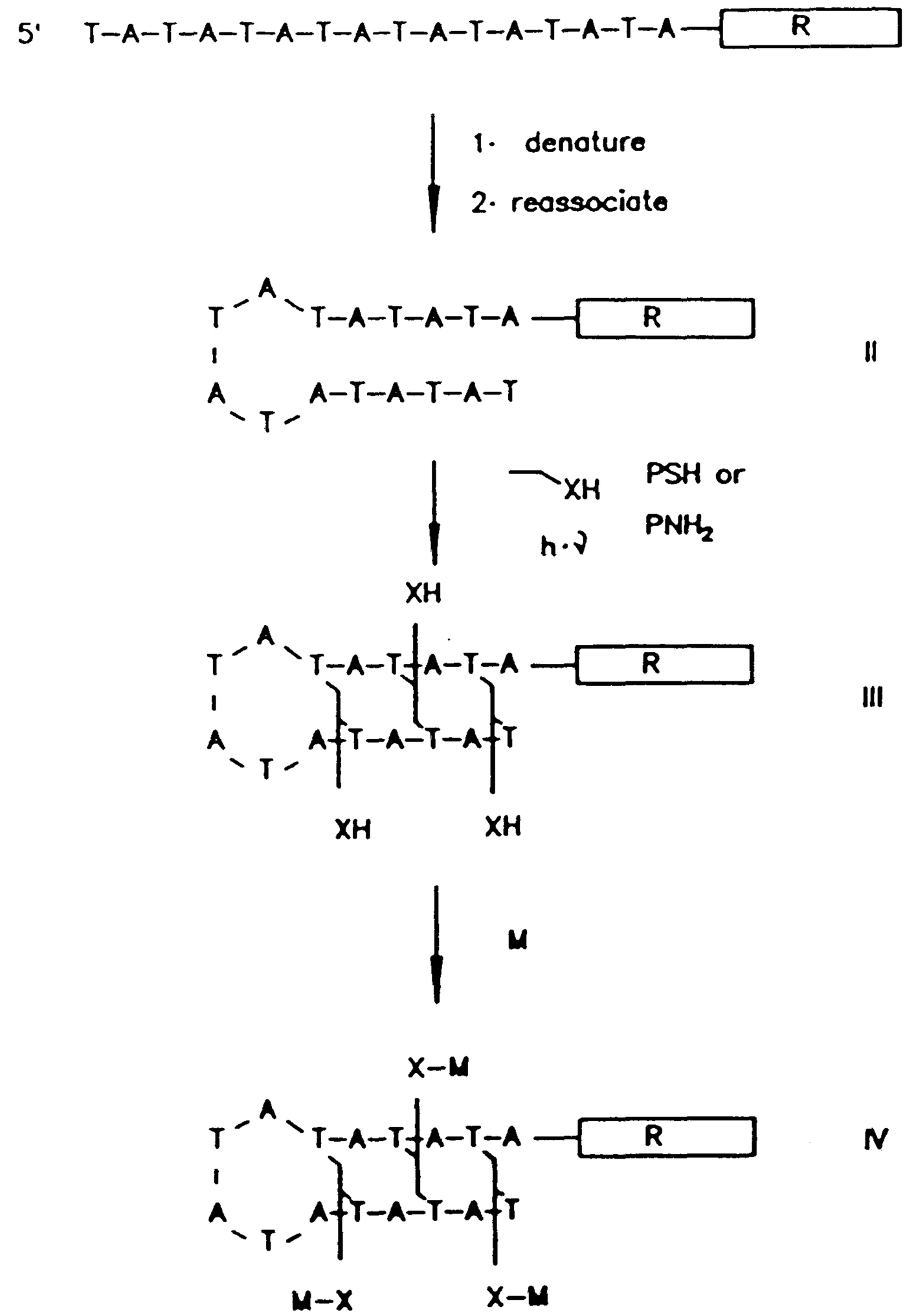
An earlier approach by Valet *et al.* to the multiple labelling of oligonucleotides with lanthanide chelates involved the use of psoralen derivatives.[132,133] This chemistry has already been used for the photochemical labelling of DNA with biotin and Terbium chelates.

Psoralens undergo photochemical cycloaddition reaction with pyrimidine bases (Thymidine in DNA and Uridine in RNA). The reaction proceeds in two steps: in the first, the psoralen forms an intercalation complex with the duplex, and in the second, light induces the cycloaddition reaction between the pyrimidine bases. (Scheme 1.5)[3]



Scheme 1.5 Photocycloaddition of psoralen to thymine

In Valet's approach (Scheme 1.6), a double stranded poly(A-T) tail is attached to the 5'- or 3'-end of the synthetic oligonucleotide to be labelled. After denaturation, this poly(A-T) tail folds back under reannealing conditions to form a partly duplex oligonucleotide (step 2). The duplex poly(A-T) tail is then covalently crosslinked by psoralen molecules containing reactive thiol or amino groups for the attachment of labels. In Valet's case, the label consists of a terbium chelate of DTPA-pAS, which is attached to the intercalated psoralen molecule. The assumed structure of this terbium chelate bound to the psoralen moiety is shown in Figure 5.8.



Scheme 1.6 The preparation of end-labelled oligonucleotides via functionalised psoralen derivatives. R is the deprotected oligonucleotide of ant sequence of interest. P is the psoralen moiety, X is the reactive group (either S or NH), and M is the label. The structure in step II ensures the most stable loop size of four nucleotides with maximum base pairing.

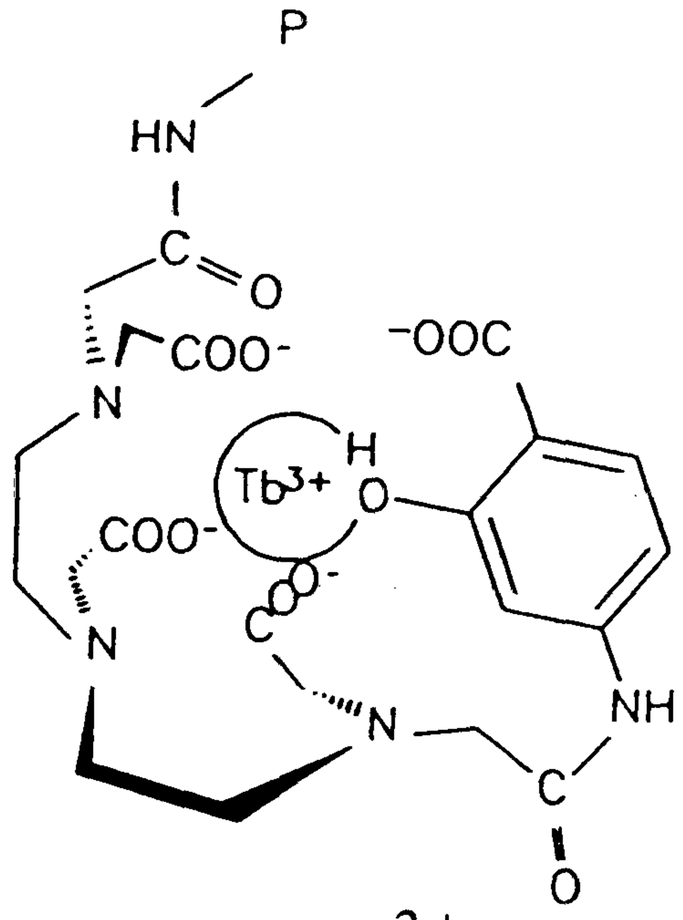


Figure 5.8 Proposed structure of Tb^{3+}-DTPA-pAS attached to the primary amino group of one psoralen moiety in an oligonucleotide (X-M in Scheme 1.6).

Two very recent papers have reported the use of europium chelates as labels for the TR-fluorescence detection of polymerase chain reaction (PCR) products.

In the first, Diamandis and co-workers describe a new method for the quantitative assesment of PCR products directly on agarose gels.[134] The method involves the labelling the 5'-end of one PCR primer with the europium chelator 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (BCPDA). After PCR the products are separated by agarose gel electrophoresis. The gel is then immersed in to an aqueous Eu^{3+} solution. During soaking, Eu^{3+} diffuses into the gel and associates with BCPDA to form a long lived fluorescent complex. The complex is then quantified in the gel with scanning TR-fluorometry. A feature of this method is that neither BCPDA nor Eu^{3+} are fluorescent by themselves, and thus background signals are very low. The detection limit was about 5ng of cytomegalovirus DNA.[134]

They also showed that by immobilising BCPDA labelled PCR products on to nylon membranes by blotting, these could be detected using an anti-BCPDA antibody with an alkaline phosphatase-based detection system. BCPDA may be a useful hapten for DNA labelling and may find applications similar to those of digoxigenin.

In a more sophisticated approach, Mathis *et al.* report the detection of DNA PCR products by europium (III) tris-bipyridine cryptate.[135] This is illustrated in Figure 5.9. The procedure is a standard PCR amplification with outer primers (a,b), after the PCR products are diluted a second PCR amplification is carried out with inner (modified) primers (c,d). The DNA hybrids bearing biotin (●) and 2,4-Dinitrophenol (◆) moieties are then affinity collected on a streptavidin(SA) coated microtitre plate by their biotin end. Fluorescence detection is then carried out at the DNP labelled end by using anti-DNP anti-body labelled with TPB(Eu^{3+}) cryptate (K).

The procedure was used in the detection of human papillomavirus type 16 DNA in clinical cervical smears.

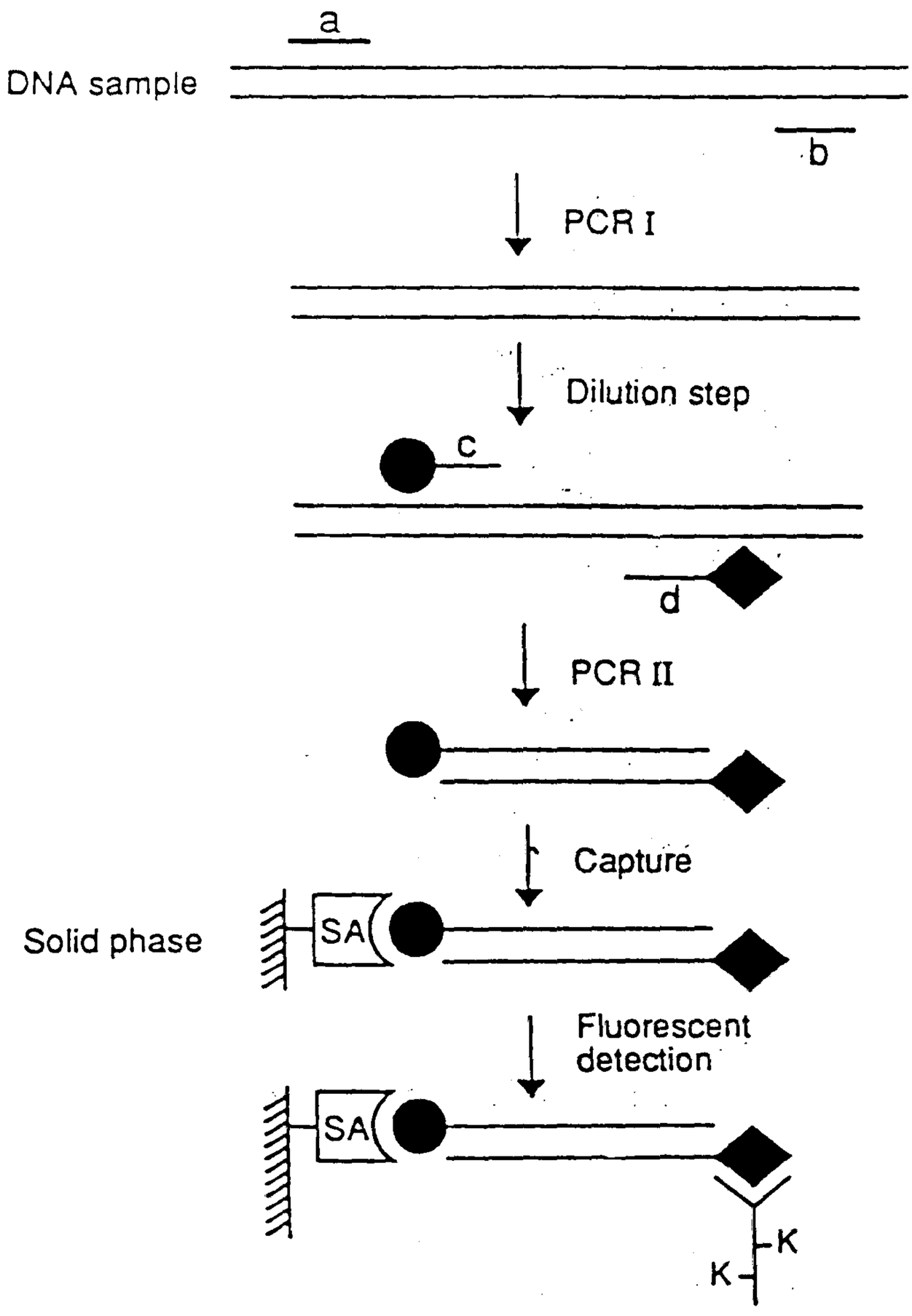


Figure 5.9 Detection of DNA PCR products by europium(III) trisbipyridine cryptate.

After standard amplification with the outer primers (a, b), the PCR products were diluted and a second amplification with the inner (modified) primers(c,d) was performed. The DNA duplexes obtained were immobilised by their biotin end (●) onto streptavidin (SA)-coated microtiter plate. The detection was carried out by the DNP (♦)-labelled end using an anti-DNP antibody coupled to europium cryptate (K).

1.7 HOMOGENEOUS (NON-SEPARATION) DNA ASSAYS.

The majority of DNA assay formats are heterogeneous, in that they rely upon binding the polynucleotide analyte to an insoluble support material. This facilitates the separation of hybridised and unhybridised polynucleotide probes so that only labelled probe hybridised to the polynucleotide target is measured. Less common are homogeneous or solution-phase hybridisation assays. These assays rely upon a labelling strategy in which the measurable charecteristic of the label is altered by hybridisation between the target and the probe.

Potentially fluorescence resonance energy transfer (FRET), sometimes called Förster energy transfer offers the basis for detecting nucleic acid hybridisation under homogeneous conditions. Förster exchange of energy occurs by a dipole-dipole induced mechanism and does not require orbital overlap (direct chemical bonding). The efficiency of energy transfer is given by equation 2:

$$E = d^{-6}/(d^{-6} + R_o^{-6}) \quad \text{.....(2)}$$

where d is the distance between the centres of the donor and acceptor molecules and R_o is a variable depending on factors such as the spectral overlap integral.[136,137] Forster energy transfer can occur over relatively long distances, up to 50 or even 100Å, and this means that, provided the local concentation is such that the two tags (energy donor and energy acceptor) are within such a distance of each other, energy transfer will occur, creating a distinct signal.

One of the first groups to exploit the FRET principle in the detection of nucleic acid hybridisation were Zamecnik *et al.* They described three approaches to using the FRET principle. (Figure 6.0)[6]

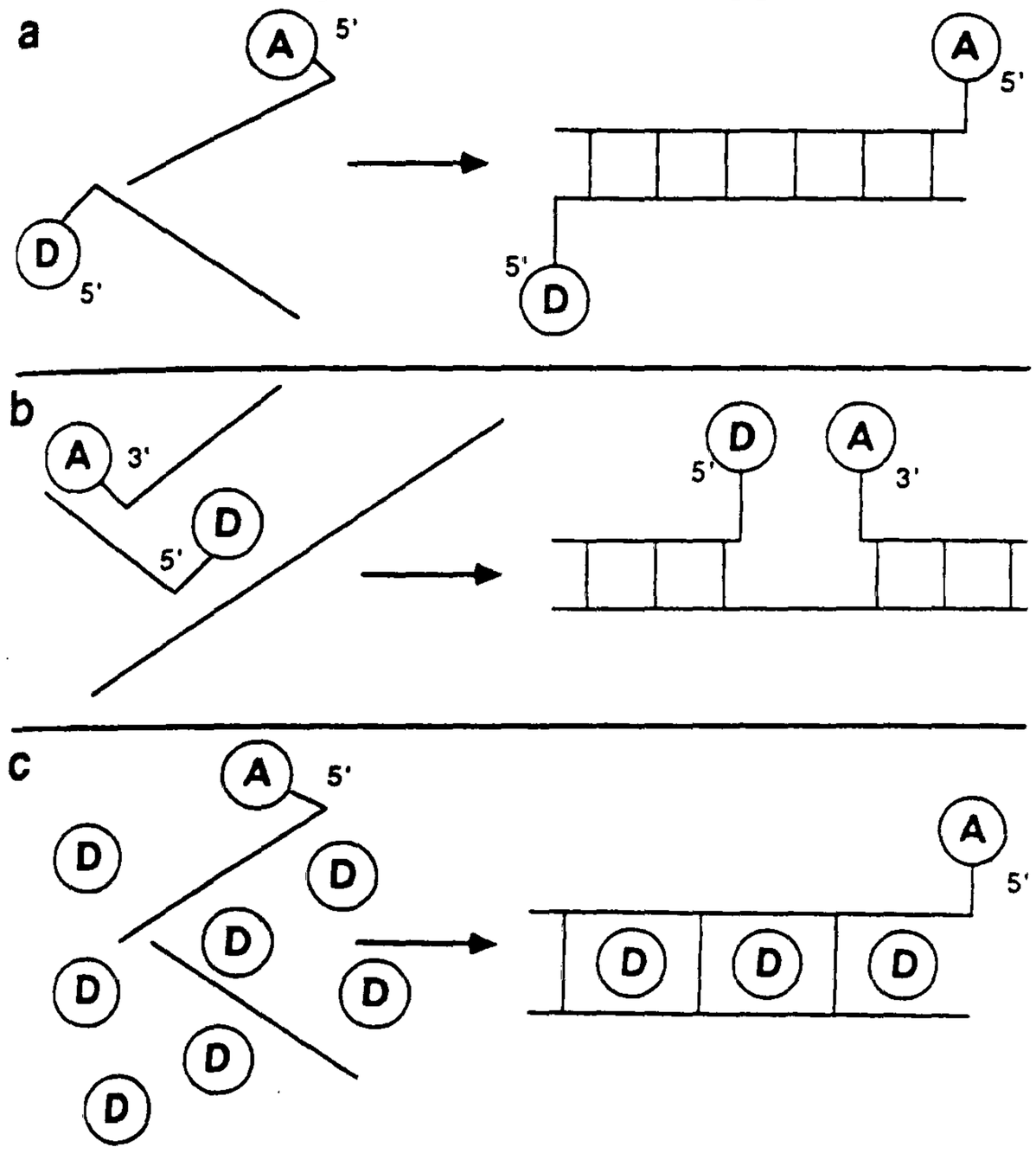


Figure 6.0 Strategies for determining nucleic acid hybridisation by FRET. (a) Fluorescent probes are covalently attached to the 5' ends of complementary nucleic acids, allowing energy transfer to occur between a donor (D) and acceptor (A) fluorophore over the length of the hybridised complex. (b) Fluorescent molecules are covalently attached to two nucleic acids, one at the 3' end and the other at the 5' end. The fluorophore-labelled nucleic acids are complementary to disticnt but closely spaced sequences of longer, unlabelled nucleic acid. (c) An intercalating dye is used as a donor for an acceptor fluorophore covalently attached at the 5' end of one of the nucleic aicds.

In the first and second approach, fluorescein (donor) and rhodamine (acceptor) were covalently attached either the 5'-ends of two probes or the 5'-end of probe and 3'-end of a second probe. Upon hybridisation to the target, where both probes are bound, the two labels are brought within short proximity of each other, and on irradiation the excited-state energy of the donor fluorophore is transferred by Förster transfer to the neighbouring acceptor fluorophore. The results are a decrease (or quenching) of the fluorescein intensity and an enhancement of the rhodamine emission. In the third design, an intercalating dye such as acridine orange is the donor fluorophore to a single rhodamine covalently attached to the 5'-end of one probe. This is a very interesting idea, for only upon hybridisation to form the duplex will acridine orange be able to intercalate and thus be nera enough to the acceptor foe energy transfer to occur.

The findings of Zamecnik *et al.* have been used in several homogeneous assay designs, employing different combinations of energy donors and acceptors.

Morrison *et al.* describe a rather complicated competitive hybridisation assay using 5'-fluorescein labels whose fluorescence is quenched by either 3'-pyrenebutyrate or 3'-sulphorhodamine labels. Probes prepared in this manner were able to detect unlabelled target DNA by competitive hybridisation producing fluorescence signals which increased with increasing target DNA concentration.[7,138]

In another approach, a luminol-labelled probe is hybridised to the target in the presence of ethidium bromide. The chemiluminescent emission spectra from luminol overlaps the absorption spectrum of ethidium bromide, leading to a fluorescent signal.[3]
However, all of the above methods by using traditional organic fluorophores suffer from low sensitivity of detection due to high background fluorescence.

It comes as no surprise that efforts to improve the sensitivity of such homogeneous assays has concentrated on the use lanthanide chelates .

In a very recent patent, the ideas of Zamecnik *et al.* for the development of homogeneous nucleic acid assays has taken a step forward in terms of the sensitivity of detection.

Wetmur and co-workers[139] by using a terbium chelate (energy acceptor, E_2) attached to the probe oligonucleotide were able to sensitise long lived Tb^{3+} fluorescence through fluorescence resonance energy transfer from an intercalating dye as the energy donor (E_1). This process can only occur once probe and target have hybridised to form the duplex. The assay they describe is essentially homogeneous, for intercalation is a characteristic property of the duplex and thus, there is no need to remove unbound probe molecules. This is dealt in more detail in chapter 2.

A slightly different approach is taken by Oser and Valet but still using terbium as the label. They describe a system involving suitable pairs of oligonucleotides as probes, chemically modified to form a ternary Tb^{3+} complex upon hybridisation with a target nucleic acid. (Figure 6.1)[140] Upon photoexcitation, the salicylate group of one oligonucleotide, serving as a sensitiser ligand is able to transfer the excitation energy to the lanthanide ion of the second oligonucleotide, resulting in Tb^{3+} specific, long-lived fluorescence emission.

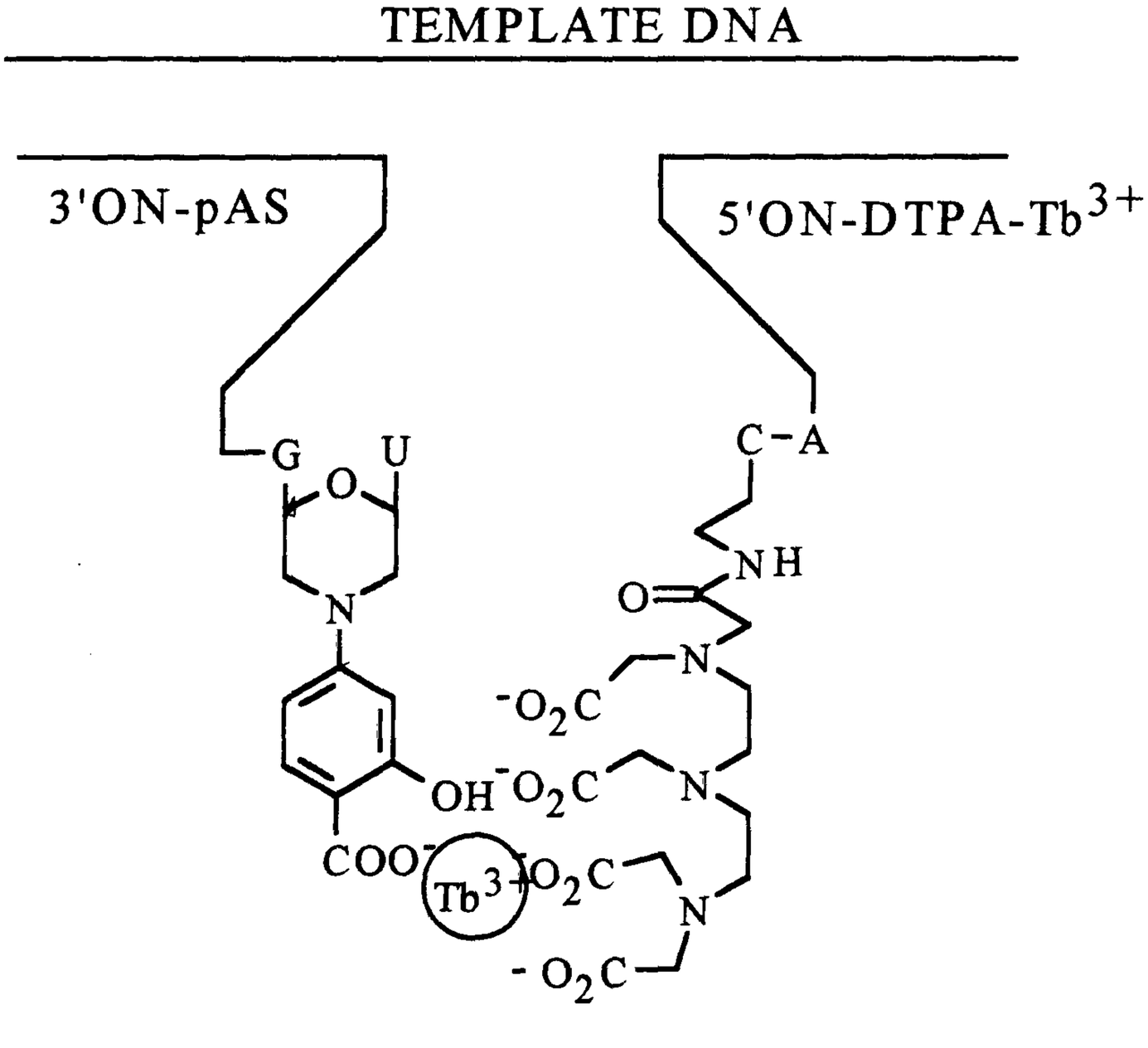


Figure 6.1

The approach of Oser and Valet, although showing only modest differences in fluorescence between the hybridised and unhybridised probes, throws up many interesting ideas. The oligonucleotide sequences are chosen in such away that after hybridisation to the complementary DNA strand, the energy donor (pAS) and energy acceptor (DTPA-Tb^{3+}) are positioned in direct proximity. Compared with ternary complex formation in free solution, the formation of a complex between DTPA-Tb^{3+} on the one hand and salicylate on the other is strongly favoured on the DNA template.

1.8 RUTHENIUM AS NON-RADIOACTIVE LABELS.

The considerable problems in using the lanthanides and their complexes as reporter molecules in both immuno and DNA assays has forced some people to look at ruthenium (II) complexes as potential non-radioactive labels of biolgical molecules.

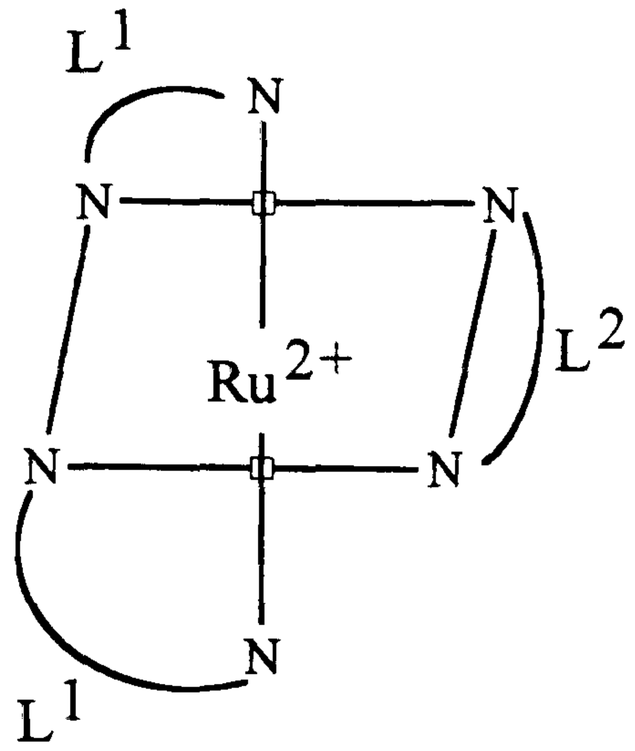
One of the biggest advantages of utilising ruthenium (II) is the fact that its chelation chemistry is less demanding than those of either europium or terbium, with nitrogen containing ligands able to form strong and stable complexes.

Banwarth *et al.* have shown that Bathophenanthroline-ruthenium (II) complexes (87) show a number of properties which make them ideal non-radioactive labels to the already well established Eu^{3+} complexes.[141,142] (Figure 6.2). These complexes are thermodynamically very stable, and therefore do not dissociate at low concentrations. They are chemically very inert and show strong long-lasting fluorescence, allowing detection by time-resolved techniques.

Where L^2 bore a linker arm with a terminal alkyl carboxy group (88), the corresponding Ru^{II}(bathophenanthroline) complexes were coupled to 5'-NH_2-modified oligonucleotide probes through a carboxamide bond.[141]The end-specific attachment, coupled with a spacer molecule, ensured minimal interference of the complexes during hybridisation processes to target complementary DNA sequence, during which no quenching of the ruthenium luminescence was observed. The detection limit corresponded to 10^{-18}mol or $6x10^5$ molecules of target.

In more recent work Banwarth and Schmidt have been able to couple Ru^{II}-

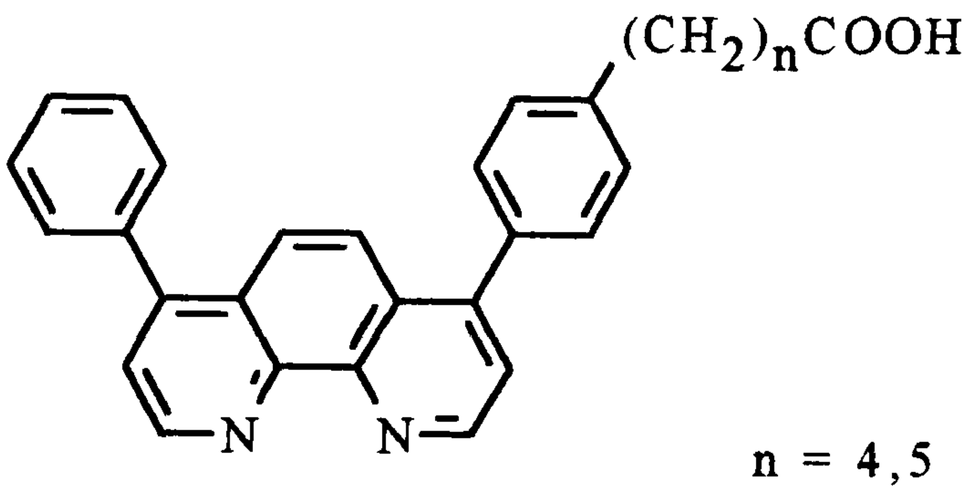
bathophenanthroline complexes directly to oligonucleotides in the course of their synthesis on a solid support by the phosphoramidite procedure.[142] This is made possible by L^2 bearing a hydroxy function (89).



L^1= 1,10-phenanthroline

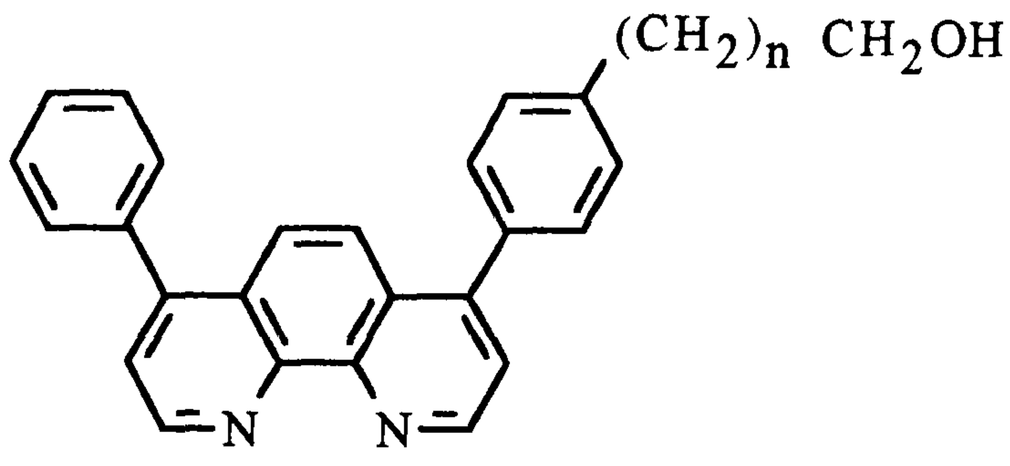
L^2= (88), (89)

(87)



n = 4,5

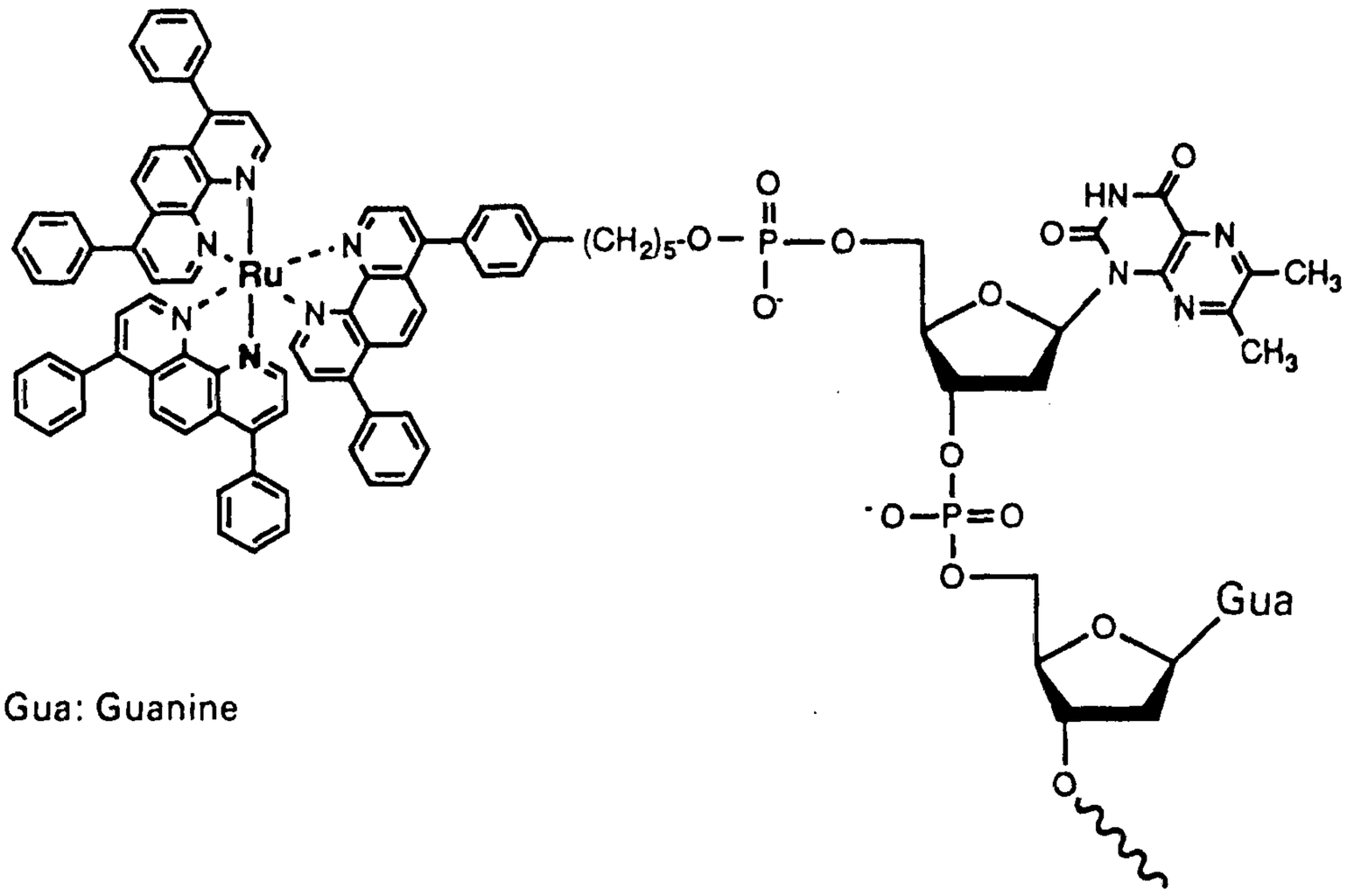
(88)



(89)

Figure 6.2

By choosing a suitable transmitter molecule as an energy donor, which can be incorporated into an oligonucleotide already labelled with the Ru^{II}-complex, Banwarth *et al.* were able to create a fluoresecence energy transfer system.[143] They showed that the chromophoric system 6,7-dimethyl lumazine (6,7-dimethylpteridine-2,4(1H,3H)-dione) (90) is able to act as an energy donor, transfering absorbed energy onto bathophenanthroline-Ru(II) complexes acting as energy acceptors via the long-wavelength metal-to-ligand charge transfer (MLCT) band. (Figure 6.3)

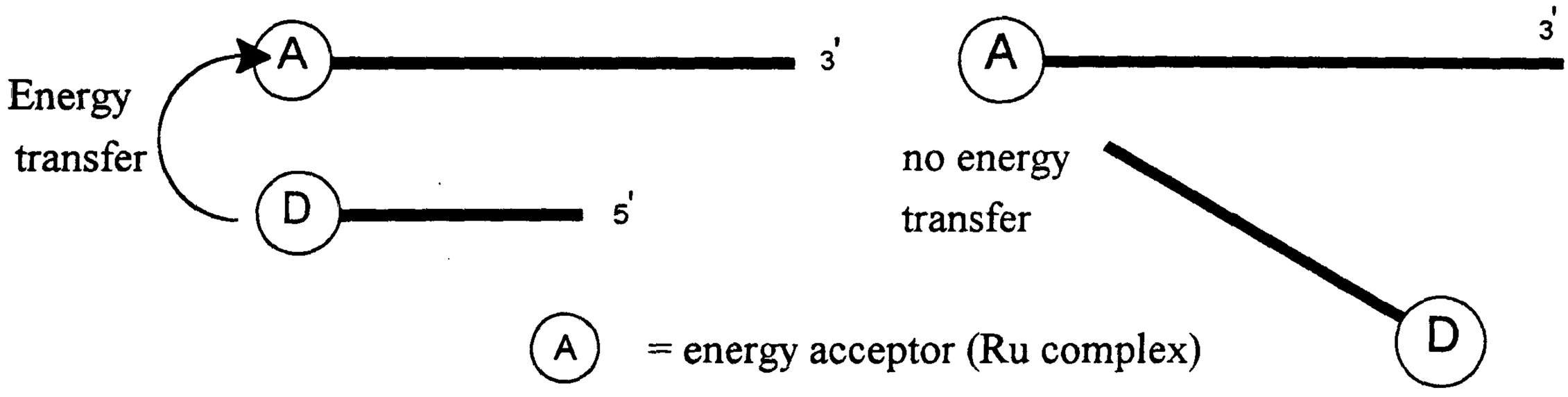


Gua: Guanine

Figure 6.3

By incorporating 6,7-dimethyl lumazine into the 3'-end of one oligonucleotide probe and Ru(II)-bathophenanthroline at the 5'-end of a second oligonucleotide probe by standard solid phase DNA technology, they were able to show by energy transfer measurements that a clear differentiation is possible between the hybridised and unhybridised state of two complementary and non-complementary oligonucleotide strands.[144] (Scheme 1.7)

A doubling of the fluorescence intensity was observed in cases where hybridisation-mediated energy transfer was possible.

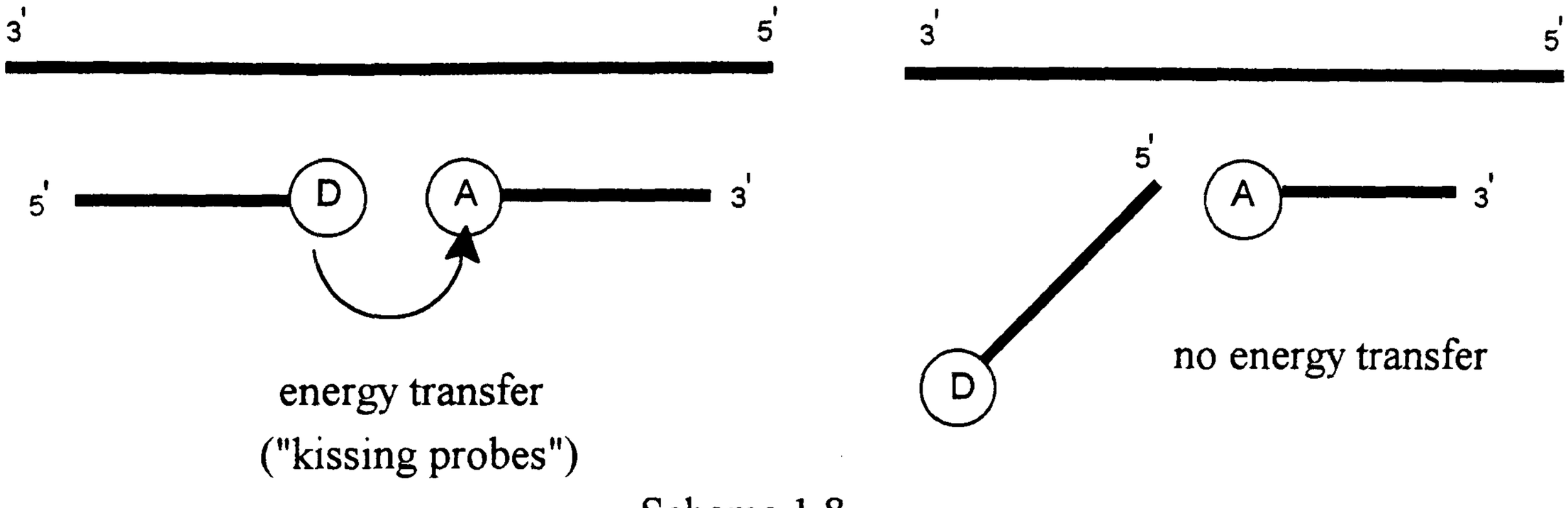


Scheme 1.7

They were also able to demonstrate that an arrangement in which two oligomers hybridise side by side to a single stranded complementary DNA target ("kissing probes"), resulted in energy transfer of the same order of magnitude as observed above. In the absence of a complementary DNA target, where the side by side arrangement is not possible, no energy

transfer is observed.[144] (Scheme 1.8)

This method, therefore lends itself to the development of homogeneous DNA assays.



Scheme 1.8

Barton *et al* have developed $Ru(phen)_3^{2+}$ (phen= 1,10-phenanthroline) and its derivatives as spectroscopic probes of DNA structure[145], based on the well characterised photophysical properties of Ru(II)-polypyridyl complexes.[146] These complexes are intensely coloured owing to the well-characterised localised metal-to-ligand charge transfer (MLCT) transition. Importantly this transition is perturbed on binding to DNA. Extensive photophysical studies have indicated that $Ru(phen)^{2+}_3$ bound to DNA displays an increase in luminescence owing to intercalation. Using chiral complexes of $Ru(phen)_3^{2+}$ and $Ru(dpp)_3^{2+}$ (dpp= 4,7-diphenyl-1,10-phenanthroline) they were able to distinguish between right- and left-handed DNA helices by observing enantiomeric selectivity in binding to right-handed DNA.[145,147] (Figure 6.4)

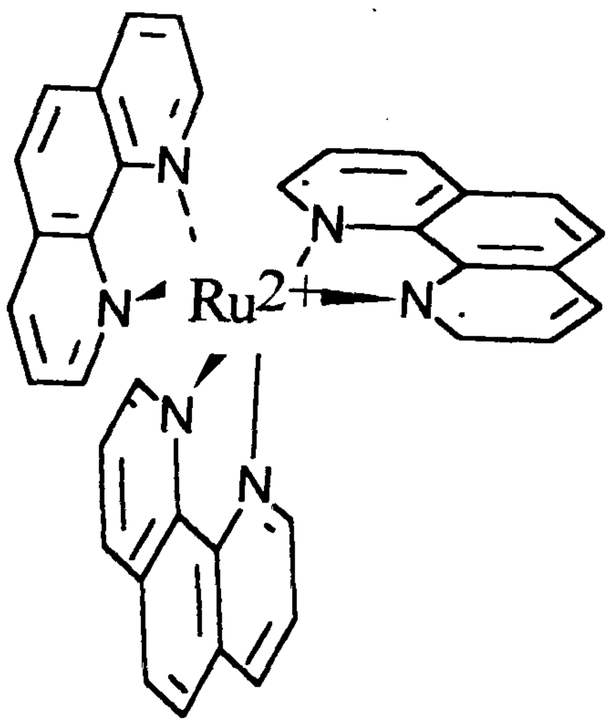


Figure 6.4 $Ru(phen)_3^{3+}$

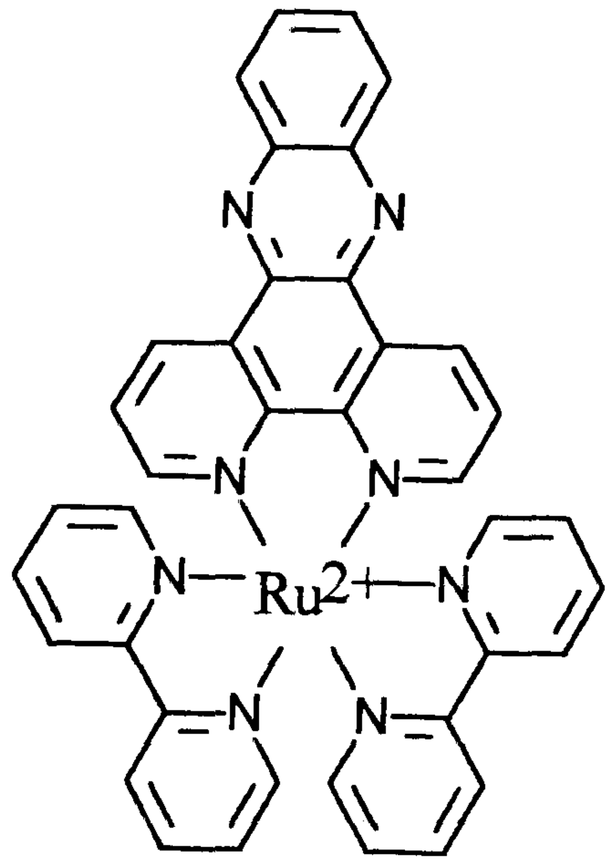


Figure 6.5 $[Ru(bpy)_2DPPZ]^{2+}$

More recently, the complex $[Ru(bpy)_2DPPZ]^{2+}$ (bpy= 2,2'-bipyridine, DPPZ= dipyrido[3,2-a:2',3'-c]phenazine **91** was shown to be a remarkable luminescent reporter of DNA structure.[148] (Figure 6.5) The probe is described as a molecular "light switch" for DNA, and shown no photoluminescence in aqueous solution at ambient temperatures , but displays intense photoluminescence in the presence of double-helical DNA, to which it binds avidly by intercalation. By intercalation into the hydrophobic environment of the helix protection is afforded to the phenazine ring from quenching by interaction with water molecules.

By tethering a dppz complex of Ru(II) to an oligonucleotide probe, a sequence-specific molecular light switch to target single-stranded DNA is obtained. (Scheme 1.9)[149]
Little luminescence is apparent for the single-stranded complex. Addition of the complementary strand, however yields intense luminescence. In contrast, the addition of a noncomplementary strand produces no detectable emission. (Figure 6.6)

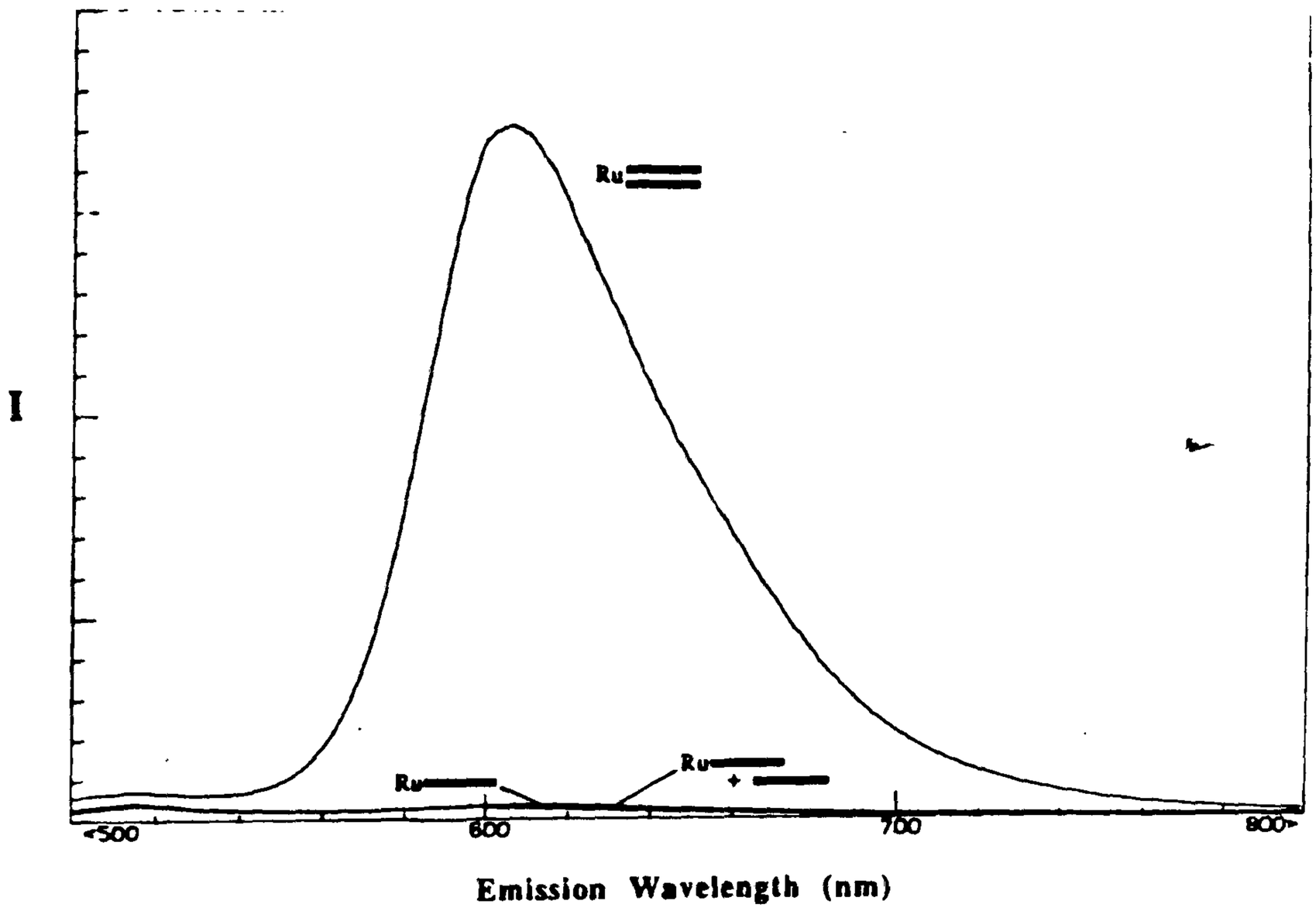
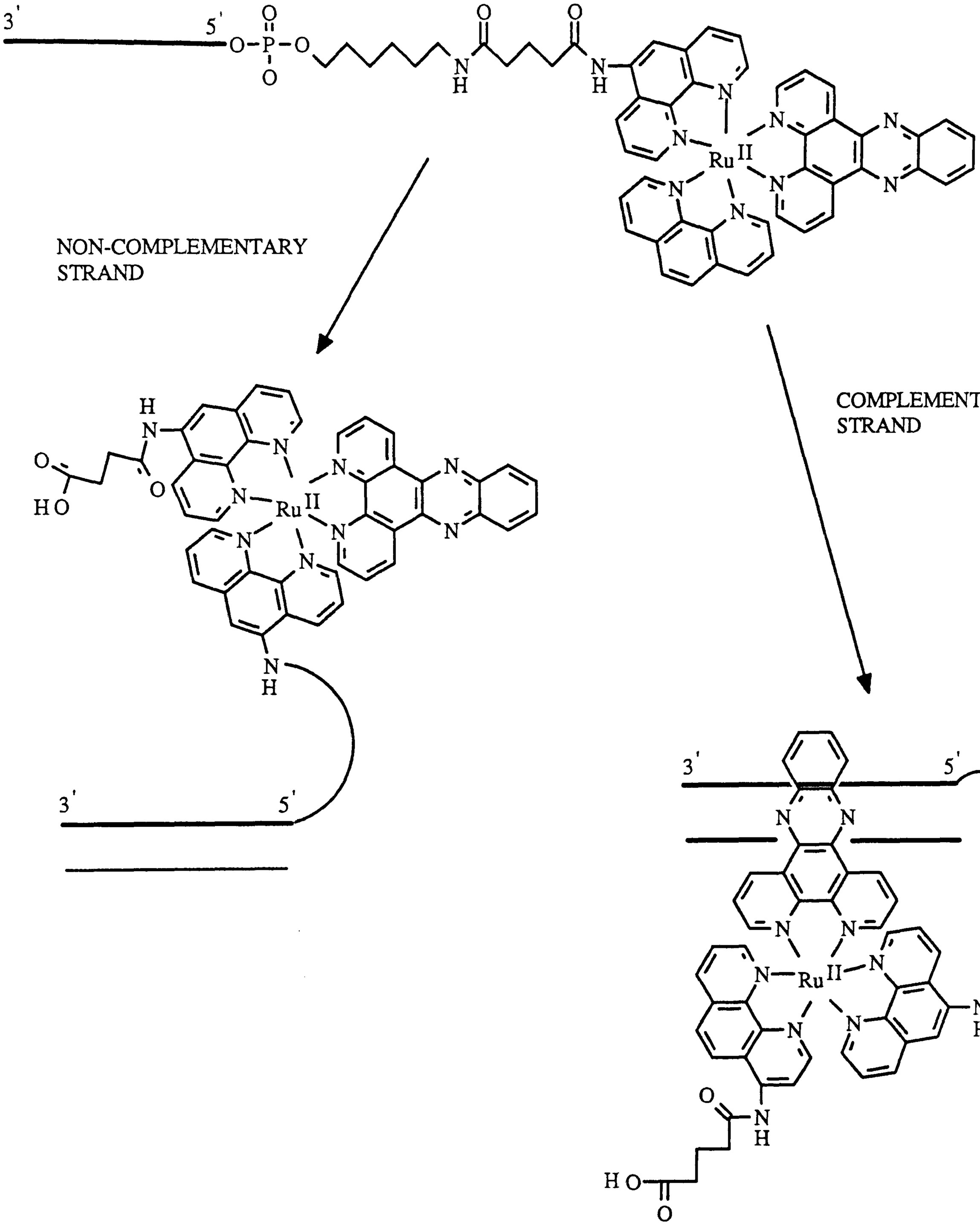


Figure 6.6 Emission spectra of the single stranded metallated complex in the presence of its complementary strand (Ru═), in the absence of added oligonucleotide (Ru—), and in the presence of a noncomplementary strand (Ru—+—). Excitation is at 440 nm. Intense luminescence is observed only in the presence of the complementary strand. For Ru═ $\Phi = 0.0071$, while $Ru(phen)_2dppz^{2+}$ noncovalently bound to oligomer gives $\Phi = 0.0063$.



Scheme 1.9 A sequence specific molecular light switch.

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2.1 INTRODUCTION.

Nucleic acid hybridisation with a labelled probe is the only practical way to detect the presence of a complementary target in a complex nucleic acid mixture. The aim of this work was to develop a new and sensitive detection system for the identification of a target DNA sequence in a strand of DNA.

The range of hybridisation assays are many and varied and the number of such assays currently in use has grown tremendously over the last few years. The majority of assay formats are heterogeneous (two phase) in that they rely upon binding the polynucleotide target to an insoluble support material. This facilitates the separation of hybridised and unhybridised polynucleotide probes (usually in excess) so that only the probe hybridised to the target polynucleotide is measured. We aimed to develop a homogeneous or (single phase) assay method where we eliminate the need to separate target DNA and probe system from any excess of reagents.

Homogeneous hybridisation assays have been few and far between. The problem with developing an homogeneous assay has been to find a signalling system where the measurable characteristic of the label is altered by hybridisation between probe and target, without the generation of signals by the reagents or excess of reagents. The approach chosen was to exploit the characteristic of DNA strands to hybridise with complementary strands to form a duplex, so that a signal would only be generated when this process had occurred. The advantage of this approach is the assurance that the assay system would have the appropriate selectivity.

The rules for complementarity in the hybridisation of DNA strands are reasonably well known and the chances of mismatches can be estimated and, by the use of long enough probe strands, minimised[1]. Calculations have shown that the minimum length a synthetic oligonucleotide needs to have to recognise a single specific sequence is 11-15 nucleotides. Thus short synthetic oligonucleotides can be designed to recognise selectively a target nucleic acid. This is a fundamental prerequisite for the design of DNA assays.[2]

For the reasons outlined in section 1.2 the luminescent properties of europium provided a suitable approach for the design of a signalling system. In particular the known propensity of europium ions only to luminesce when certain conditions are fulfilled, including:-

(I) the exclusion of most of the solvent water from the chelating shell,

(II) the need for a ligand to act as a photosensitiser to excite the ion,

(III) the delayed luminescent properties of the excited europium ions enabling the use of time delayed luminescence (gating) techniques, thus minimising the problems caused by short-lived background luminescence, and

(IV) the fact that europium emits in the red region, an area where photodetectors are most sensitive.

Two commercial heterogeneous assay methods utilising europium luminescence have been on the market for a number of years, the **DELFIA**[3] and **CYBERFLUOR**[4] systems. Since their arrival on the market many procedures for assaying a wide variety of biological molecules based on either method have been patented. All of these are essentially heterogeneous assays. For a homogeneous assay the envisaged key components for obtaining a luminescent signal are summarised in figure 2.1. Only when the europium ion (Eu^{3+}), the sensitiser (S) and a shielding ligand (L) come together can light be used to generate the characteristic europium emission.

$$Eu^{3+} + L \rightleftharpoons [\, Eu^{3+}.\, L\,] + S \rightleftharpoons [\, Eu^{3+}.\, L\, .\, S\,]$$

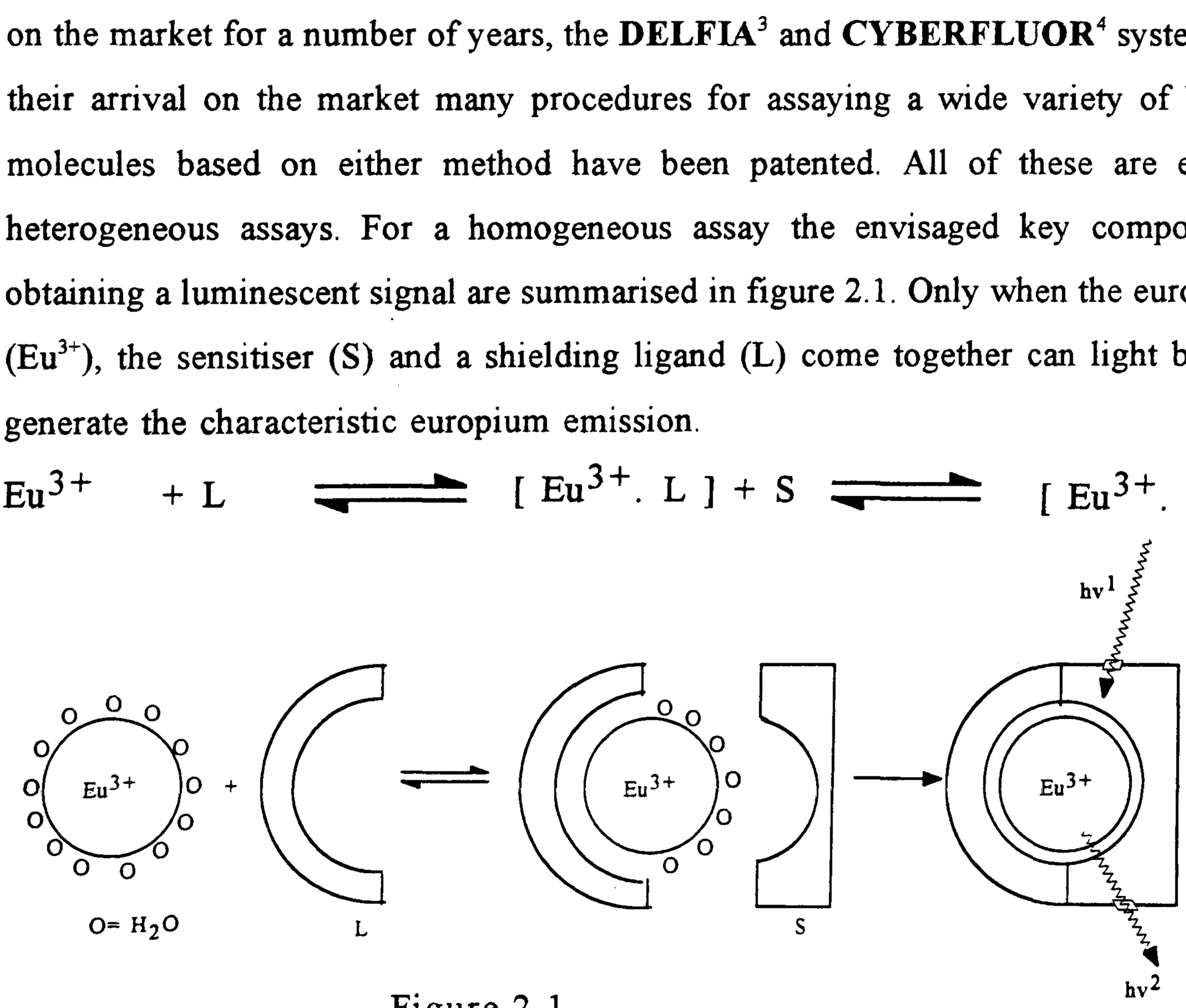


Figure 2.1

2.2 APPROACHES CONSIDERED.

Several schemata were envisaged and are outlined in figures 2.2 and 2.3

(A) Direct binding of europium to functional groups on the bases. (Figure 2.2)

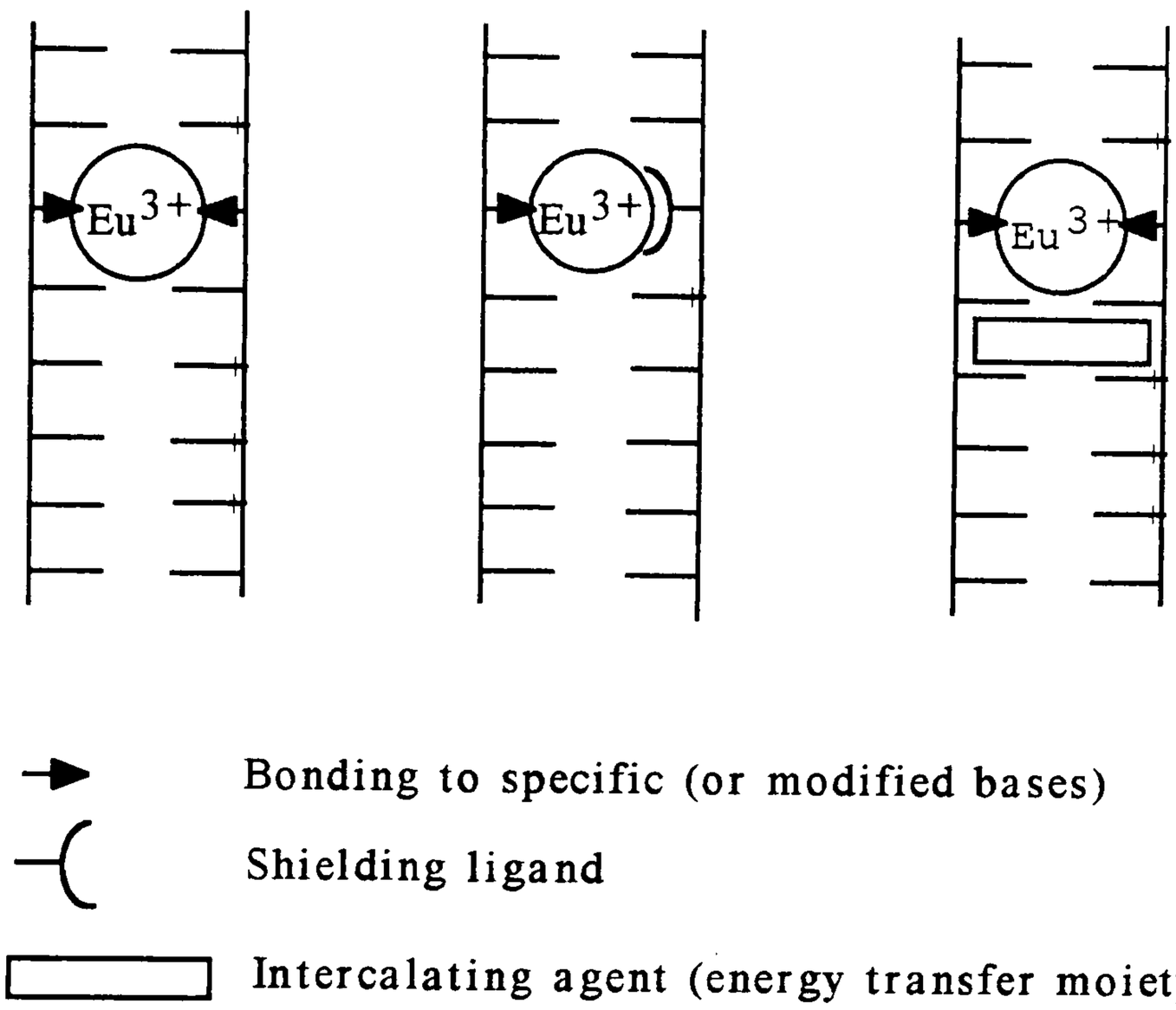
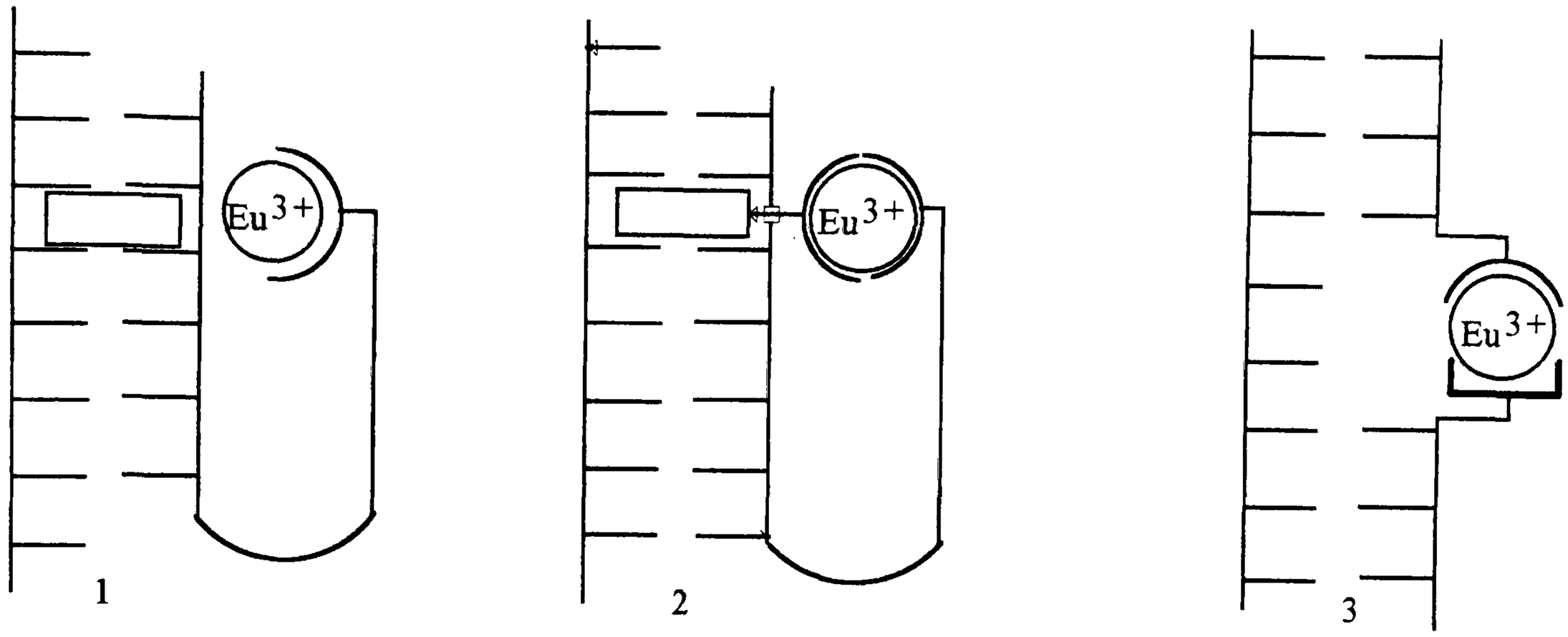


Figure 2.2 Interaction of europium with DNA-modified bases

The problem with all three approaches is that one has no or little control on the photophysical properties of natural bases, which would not be expected to sensitise europium luminescence. Also any approach at synthesising chemically modified bases that would be capable of strongly binding europium might negatively affect the ability of the polynucleotide probe to hybridise with its target strand. Considering the homogeneous nature of the assay that is envisaged, together with the concentration ranges that we hope to work in, the possible effects of the bulky labels in preventing hybridisation of the bases adjacent to these labels would severely limit the sensitivity and specificity of this approach.

Figure 2.2 (3) illustrates the use of a secondary sensitiser molecule bound as an intercalator. This is an interesting approach and will be discussed in the next section.

(B) Use of tethered chelates of Europium. (Figure 2.3)



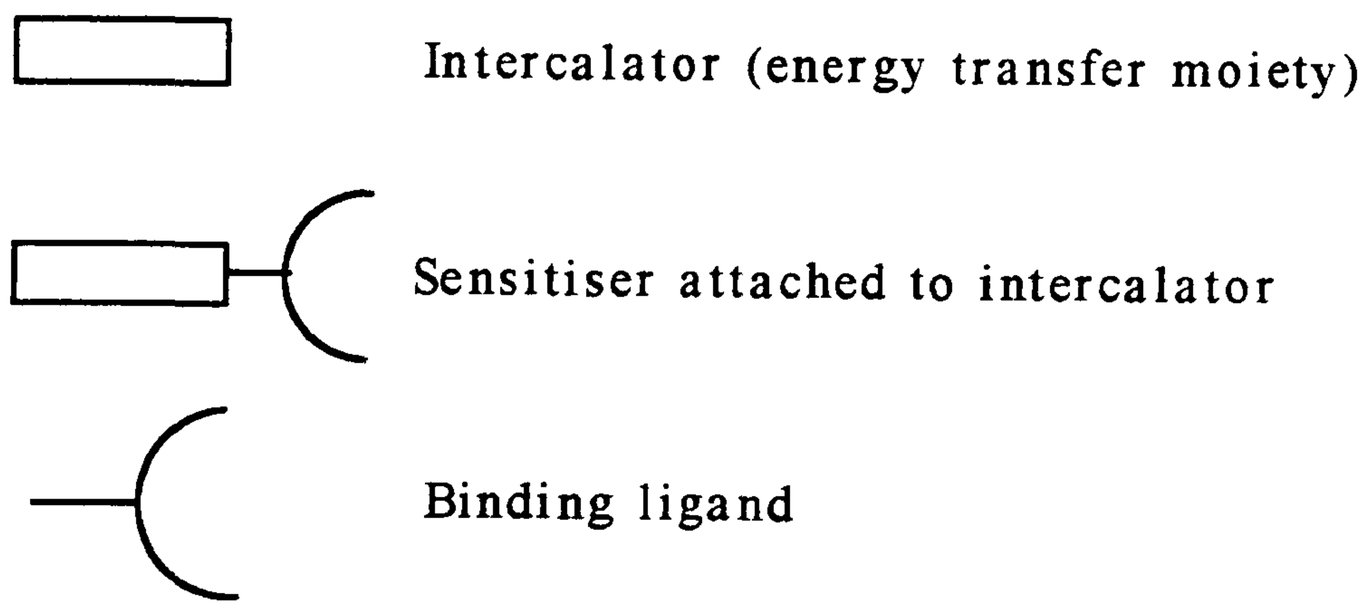


Figure 2.3 Tethered chelates of europium

This approach proved to be the most useful and of the three schemes proposed those illustrated by Figure 2.3 (2) and (3) were the most attractive. The approach illustrated by Figure 2.3 (1) was dismissed at an early stage because the requirements for europium sensitisation appear to be very specific and at the time this work was started no confirmed example of the "through space" (Fluorescence Resonance Energy Transfer, FRET) sensitisation of europium luminescence in aqueous solution had been reported; all examples involve sensitisation by attached ligands.

The ideas embodied in Figure 2.3 (1) using FRET was first explored by Zamecnik *et al*[5] (Figure 6.0, Chapter 1), with a practical system appearing in a European Patent[6] towards the end of our work.

In their patent Abrahams *et al*[6] describe a type Figure 2.3 (1) system for the detection of a polynucleotide analyte by means of energy transfer. The presence of the target polynucleotide is thus determined by hybridisation with a modified polynucleotide probe comprising of bound terbium as the energy acceptor (E2) attached via a short linker arm to the probe. The energy transfer moiety (E1) is an intercalating agent such as an aromatic dye whose emission spectra ovelaps with the absorbtion spectra of terbium. As the intercalation process proceeds along the hybrid, the energy acceptor and emitter fall within the Förster radius resulting in energy transfer and subsequent delayed fluorescence from terbium. However, there are no figures as to the sensitivity of the assay or any description of using europium as the energy acceptor, although there are claims within the patent covering the application of europium.

In approach Figure 2.3 (2), it was envisaged that a cooperative ligand system was required. One in which two different ligands could surround the europium ion once hybridisation between probe and target had taken place, where one of the ligands was an appropriate sensitiser attached to an intercalating agent, and one was a tight europium binder attached by a short flexible chain to the probe strand. On hybridisation with the target the europium chelate would be held near the duplex, which would now be capable of intercalating with the appropriate sensitiser ligands. The consequence of this overall cooperativity is that the local concentration of the components is dramatically increased at the target and hence the signal is generated only at this site. The steps leading to the final ternary complex around the europium ion, once hybridisation has taken place is shown schematically in Figure 2.4.

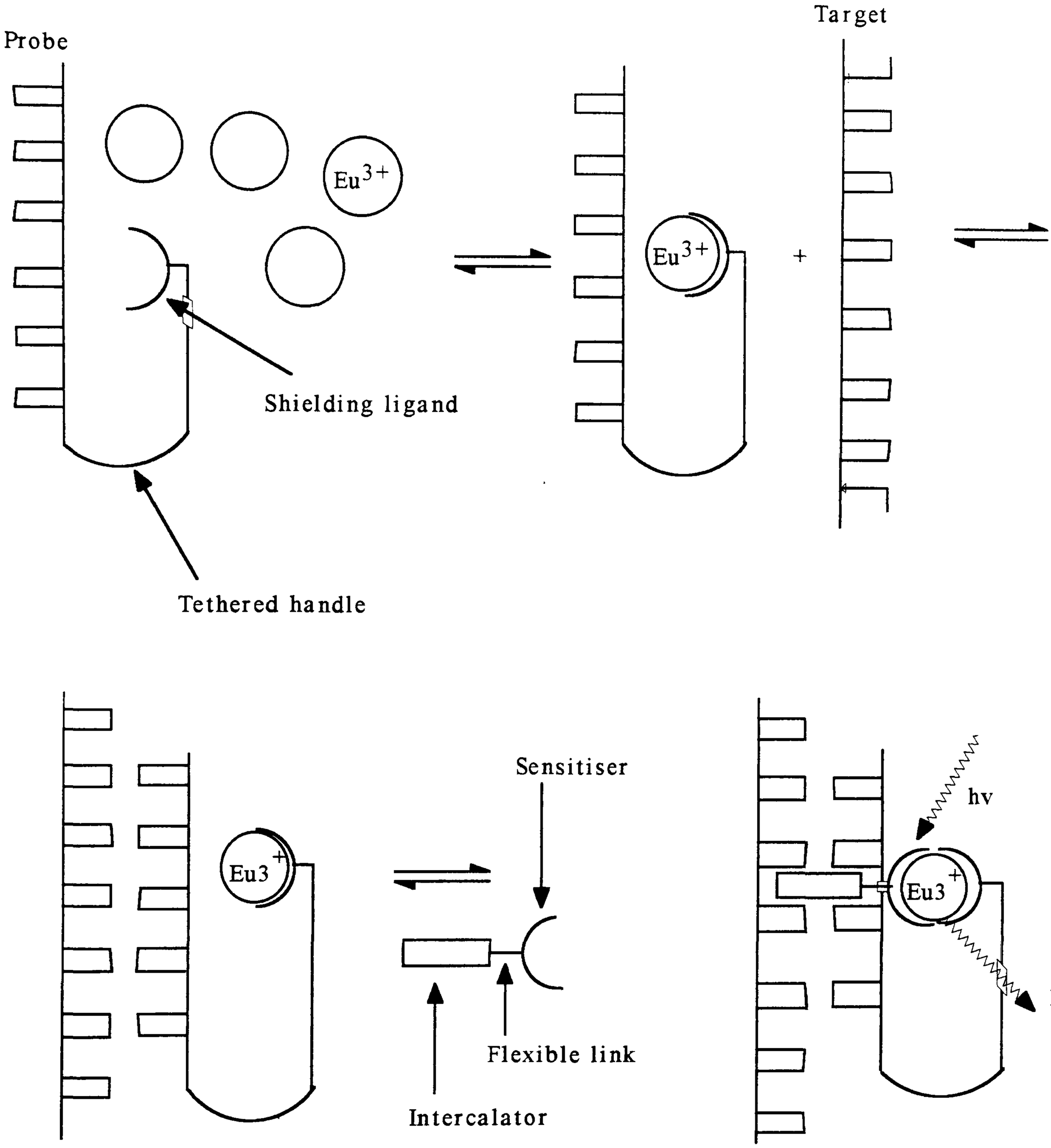


Figure 2.4 Schematic representation of 'our' approach

The ideas represented in Figure 2.4 were the results of numerous experiments with various shielding and sensitising ligands over along period of time, culminating in the design of two different, but cooperating ligands for europium. Each ligand with its own predetermined function would, in aqueous solution, encapsulate the metal ion, replacing its hydration sphere, and thus prevent the deactivation pathway of the excited state, resulting in europim luminescence: **in effect an observable switch.**

The work can be divided into two different topics:

A. SYNTHETIC WORK. (Section 2.3)

(a) A search for non-sensitising or shielding ligands that can be easily attached to a polynucleotide chain by variable chain lengths and which can shield the europium ions from the ingress of water but not inhibit the approach of a sensitising ligand.

(b) A search for a range of ligands capable of sensitising europium luminescence.

(c) The attachment of the photosensitising unit to the intercalating agent and the attachment of the shielding ligands to the polynucleotide strands. These represent the components of the actual probe system.

B. LUMINESCENCE STUDIES. (Section 2.4)

(a) Studies on the cooperativity of the sensitiser and neutral shielding ligands and the efficiency of luminescence of the ternary system. Modulation of europium luminescence by interaction of the non-photosensitising (shielding ligand), the sensitising ligand and the europium ion was an essential prerequisite for further work.

(b) DNA studies. Studies with oligonucleotides comprising an actual assay system.

2.3 SYNTHETIC WORK.

(a) Shielding ligands. (Non-sensitising)

A fundamental requirement of the basic scheme is the need to select two ligands that by acting cooperatively can surround the positively charged europium ion, forming a cage in solution. In their complexes the lanthanide cations behave as typical 'hard' acids and interact preferentially with hard bases such as fluoride and oxygen rather than with softer bases such as nitrogen, sulphur, phosphorus etc. Lanthanide interactions with the softer donors can be achieved in organic solvents of low solvating power; however in aqueous solutions, the soft base sites can rarely compete with water. Interaction between Ln^{3+} and nitrogen donor sites occurs in aqueous solutions for ligands such as the aminopolycarboxylates. In these complexes, it is likely that the Ln^{3+}-carboxylate interactions produce sufficiently strong bonds with the lanthanide ions and that the nitrogen ligands are forced into juxtoposition with the ion, with the consequent displacement of water molecules. Many polydentate inorganic anions such as NO_3^- or SO_4^{2} form stable lanthanide species and can prevent the formation of lanthanide complexes with poorly coordinating ligands. The hard acid character of the lanthanides is evidence that their complexation in aqueous solution can be interpreted in terms of an electrostatic model. The variety of coordination numbers and geometric structures of lanthanide complexes is further evidence that there is no limitation due to the necessity to maximise orbital overlap between metal and ligand as in the d-transition metal complexes.

By assuming that purely electrostatic attractive forces will be responsible for the final organisation of the two ligands around the positively charged metal ion, we were able to refine our design criteria for the shielding ligand.

From the approach outlined in Figure 2.4 the shielding ligand L needs to have certain properties. Ideally it should have a very high binding constant for the europium ion such that, at the concentrations of the assay (at anything down to 10^{-15}M) the bound state is maintained. In order to ensure no exchange of the europium ion with other metals occurs, a slow rate of chelation and dissociation is desirable. If this situation can be realised, the europium ion being essentially irreversibly bound to ligand L, the rest of the

design parameters become relatively easy to explore since one can consider the Eu^{3+}.L complex as a single entity.

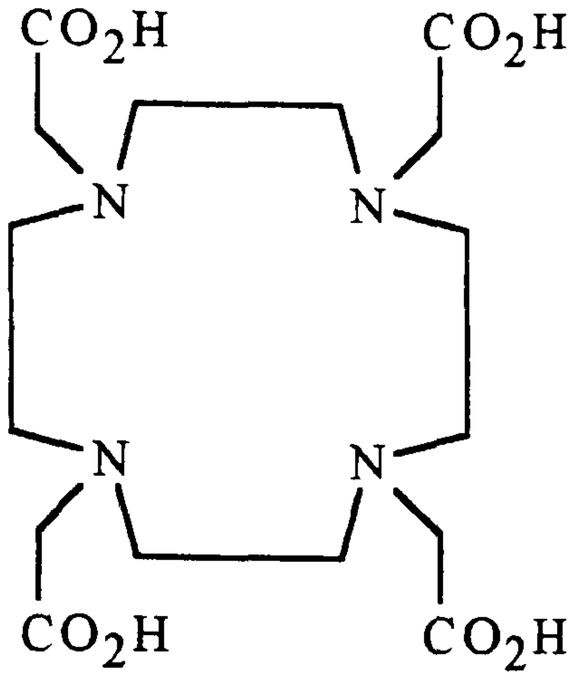
2.3.1 Polyazapolycarboxylic Acids.

Related to the linear aminopolycarboxylic acids as complexing ligands are the macrocyclic polyazapolycarboxylic acids. In these ligands, amine groups are associated in a cyclic alkyl system with pendant carboxylate groups attached to the amines. They have large stability constants for complexation with rare-earth cations and very slow dissociation kinetics. Because of these characteristics, their lanthanide complexes are of interest as *in vivo* NMR imaging contrast agents, where the chelates must be exceedingly stable in order to reduce the toxicity of the metal ion.[7,8] Kinetic inertness results from shielding the metal ion from attack by water as hydration is usually necessary to cause decomplexation. This protection from hydration is closely related to the cavity size and rigidity of the aza ring. One of the first group of ligands that we explored as a possible shielding ligand was the derivatives of 'cyclen' (5), namely its tetraacid derivative, 1,4,6,10-tetrazacyclododecane-N,N',N'',N'''-tetracetate (DOTA) (1). (Figure 2.5)

The relative stability constants of the lanthanide complexes of DOTA, TETA (2),NOTA (3) and EDTA are:

$$\text{DOTA} > \text{EDTA} > \text{TETA} > \text{NOTA}$$

DOTA is known to bind europium at neutral pH with a stability constant K_s in the range 10^{25}-10^{26}, this drops to 10^{15} for calcium which being a common biological ion would be in competition for any potential binding site.[9,10]



(1)

Figure 2.5 DOTA

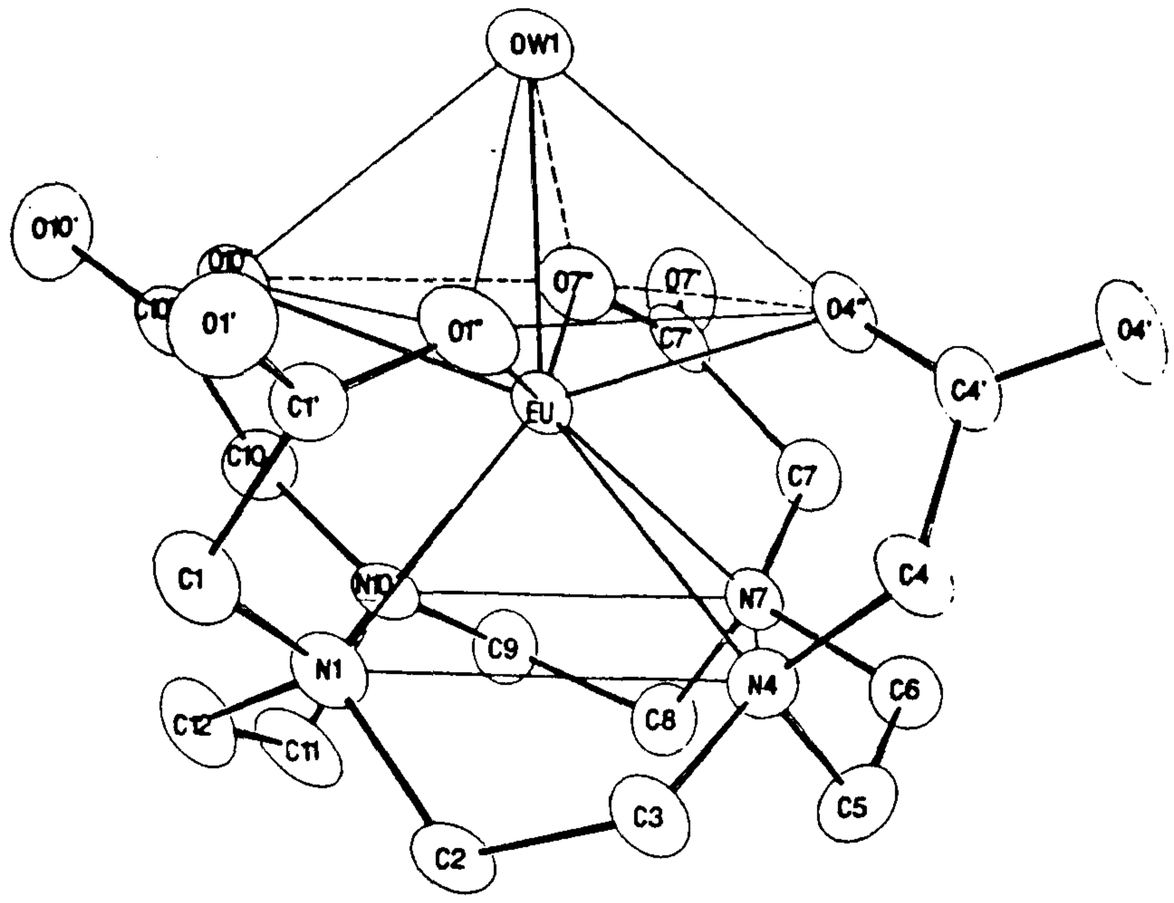
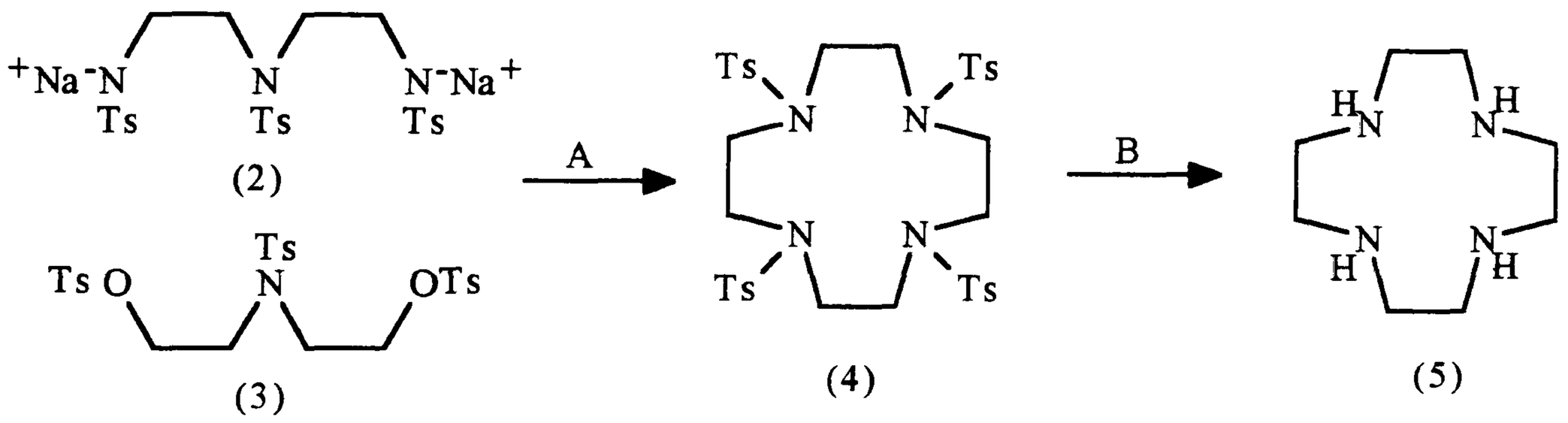


Figure 2.6 The coordination polyhedron of the $(Eu\ DOTA)^-$ complex.

The extent of DOTA's encapsulation of the Eu^{3+} ion is clearly shown by the crystal structure of the NaEuDOTA complex. (Figure 2.6)[11] This shows 9-coordination, the 8 donor atoms (four oxygen and four nitrogen) in DOTA in a square antiprism geometry about the Eu^{3+} cation with the aza ring as one square face and one water ligand associated with Eu^{3+}; Eu^{3+} can accommodate up to 10 solvent molecules.



(2) (3) (4) (5)

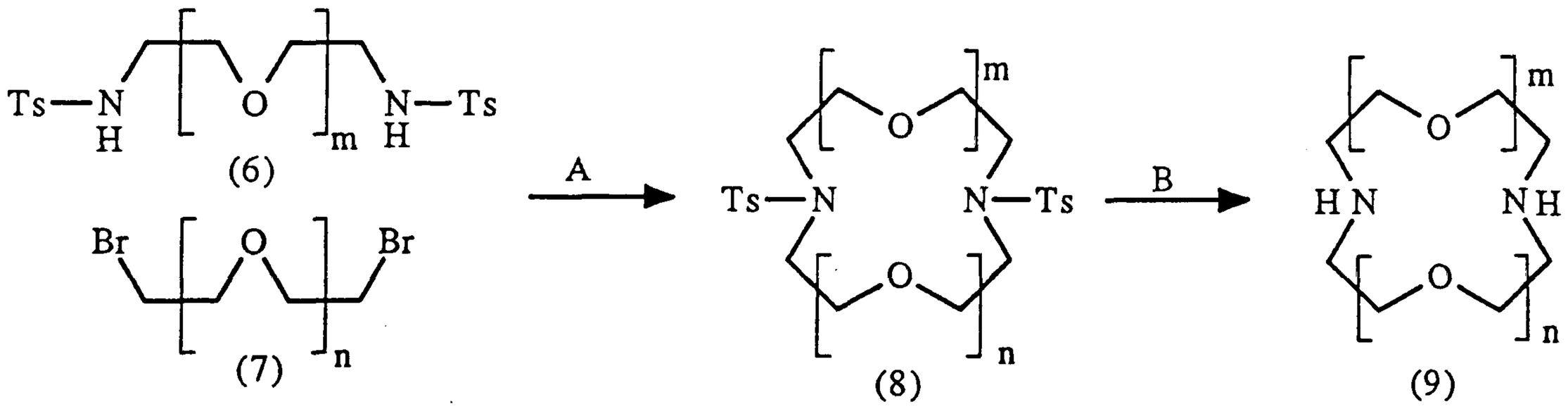
Scheme 2.1 A. DMF, 110^o; B. c. H_2SO_4, 90^o

The standard literature synthesis of DOTA involves the reaction of the parent aza-crown cyclen (5) with chloroacetic acid under conditions where the pH of the system is kept at around pH 10 throughout the reaction.The initial synthesis of cyclen (5) was attempted using pre-tosylated reactants (Rickman and Atkins).[12] (Scheme 2.1)

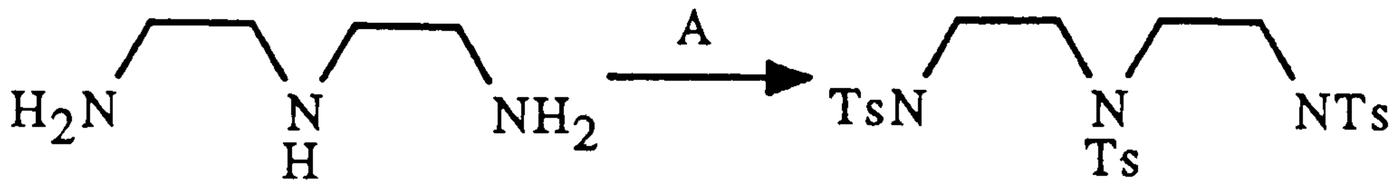
Although this is meant to be a useful and direct method of obtaining tosylated macrocycles with yields of 50% or better, it was found that the reaction which is carried out in dry DMF is very sensitive to moisture with yields depending on the anhydrous nature of the solvent. In our hands 23% for the tosylated cyclen(7) was the best yield obtained.

A number of procedures exist for the detosylation of these products to give the parent free amine crowns; concentated sulphuric acid for several days at 80°,[12] a mixture of phenol and 32% hydrobromic acid/acetic acid at reflux[13], LiAlH[14] or sodium amalgam.[15] However, it was obvious that a higher yielding reaction was necessary to obtain the tosylated macrocycle, for detosylation accounts for a large decrease in the molecular weight of the desired product and thus the yields obtained.

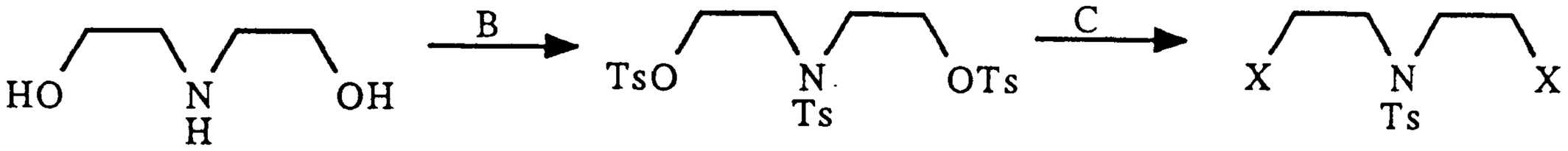
Although there are a number of well established procedures for the synthesis of aza-crown ethers, there has been a distinct lack of new methodologies for the synthesis of polyaza-crowns. One such procedure developed by Bogatsky and co-workers used phase-transfer conditions to obtain a number of macrocyclic poly-N-tosylaza-crowns ethers.[16] (Scheme 2.2). However, because of the need for the protection and subsequent deprotection of the nitrogens, this reaction to form aza-crown ethers has largely been ignored and surpassed by simpler procedures. These are the reaction of diacid chlorides with diamines under high dilution conditions, successfully used by Lehn and co-workers to synthesise a variety of aza-crowns ethers and cryptands.[17]



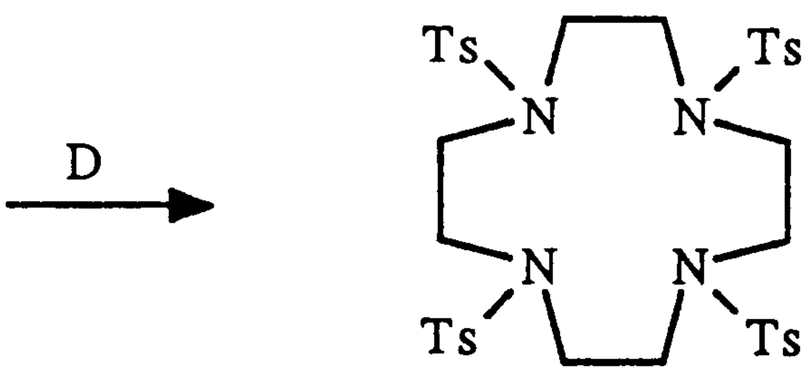
Scheme 2.2 A. $(nC_4H_9)_4N^+Br^-$/NaOH/PhH; B. HBr/AcOH/PhOH



(10) (12)



(11) (3) (13) X= Br, I

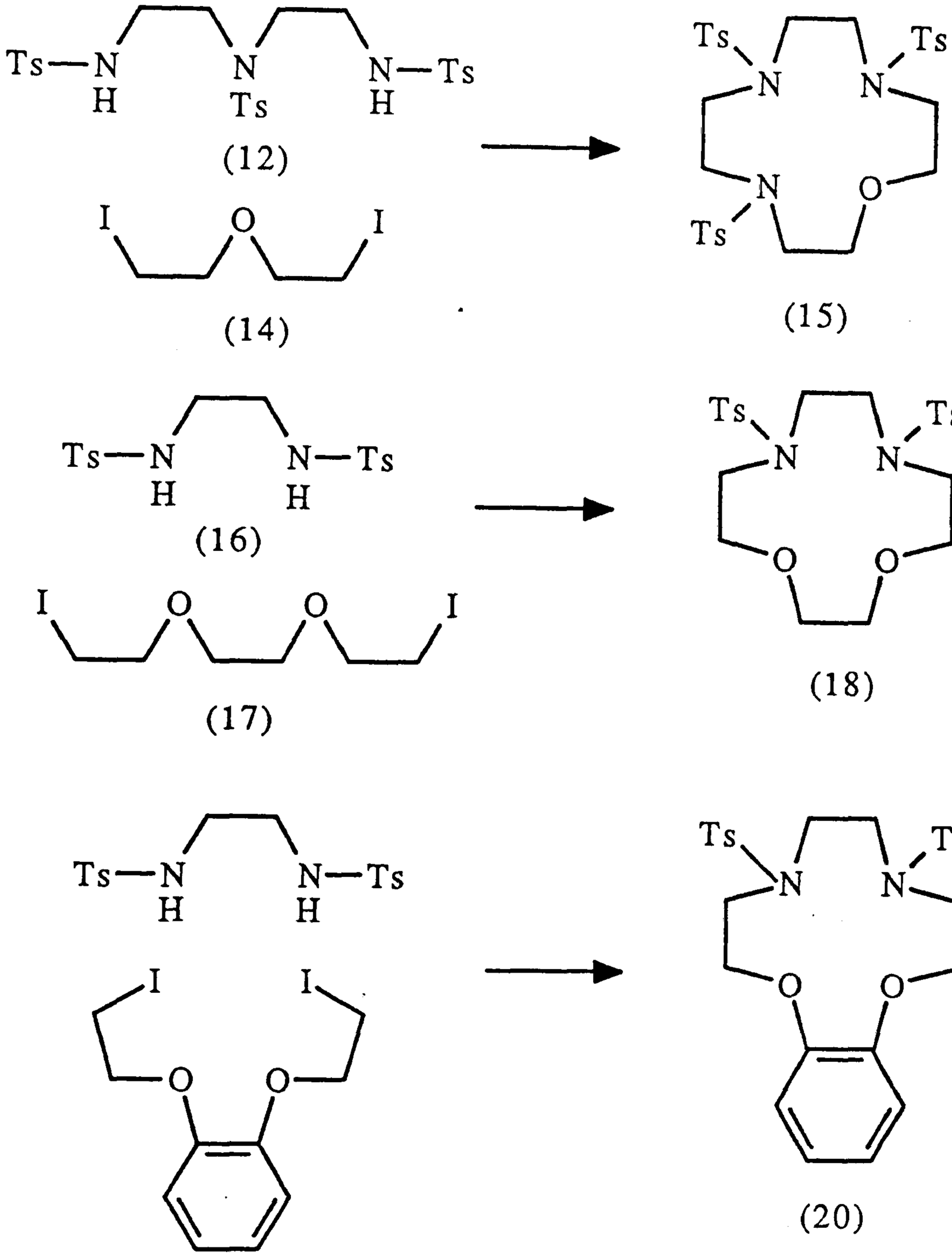


(4)

Scheme 2.3 A. p-toluene sulphonyl chloride, NaOH (aq), 0-5^{o}C;
B. p-toluene sulphonyl chloride, $(Et)_3N$, Et_2O;
C. NaX, acetone; D, $(nC_4H_9)_4N^+Br^-$, NaOH, PhH.

The methodology of the phase-transfer approach was successfully applied to the synthesis of tetra-N-tosylated cyclen (4). This was obtained in high yields by using the appropriate fragments, namely tri-N-tosyl diethylene triamine (12) and the nitrogen mustard N-tosyl-bis(2-Iodoethyl amine) (13). (Scheme 2.3) The reaction, carried out in one-pot, was over in two days. The pure tosylated product (4) was simply isolated by separating the benzene or toluene layer, drying it over sodium sulphate and removal of the solvent giving a sticky residue which was triturated overnight in methanol, to give a white solid, collected by filtration in yields approaching 70%, in an analytically pure state, with no chromatography being required. The tosylated N-mustards are all solids which makes their handling relatively easy and safe.

The versatility of the phase-transfer reaction for ring formation has been applied to the synthesis of a number of other N-tosylaza macrocycles which are often difficult to obtain through other routes. (Scheme 2.4)



(12) (14) (15) (16) (17) (18) (19) (20)

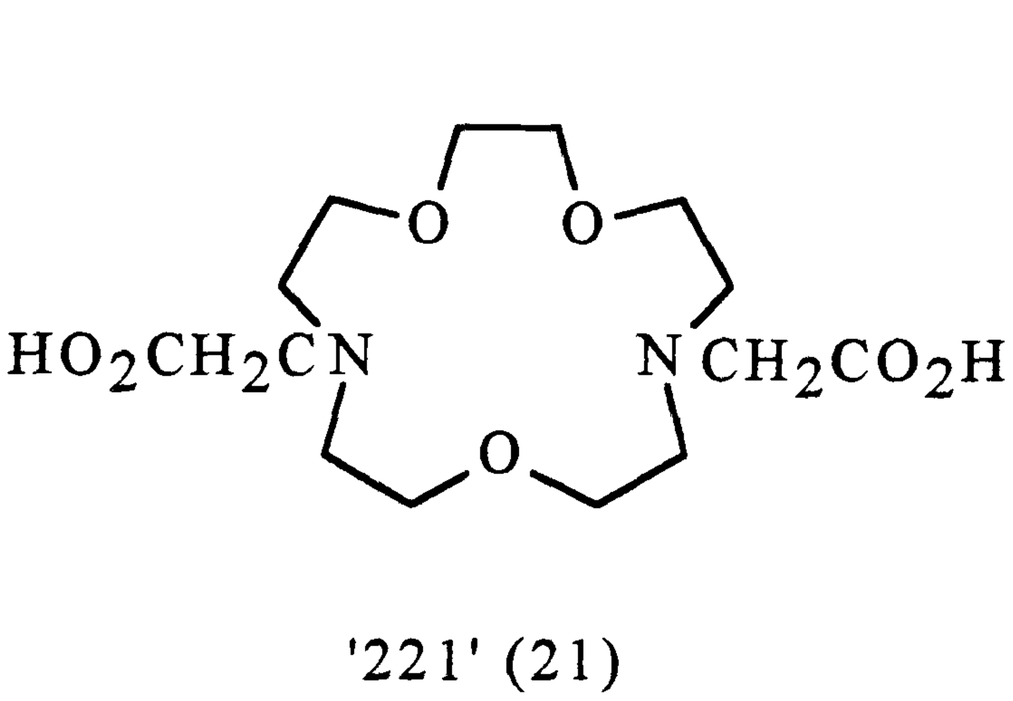
Scheme 2.4

However it was soon obvious that from a purely electrostatic approach, our design of sensitiser ligands with two carboxylate binding sites would be unable to approach the coordination sphere of a Eu^{3+} ion held tightly in a complex which had an overall net negative charge. Also as the crystal structure of EuDOTA complex shows, only one water ligand is able to penetrate the cage of 4 carboxylate groups encapsulating the europium ion. The approach of a bulky organic ligand as sensitiser would almost certainly be hindered.

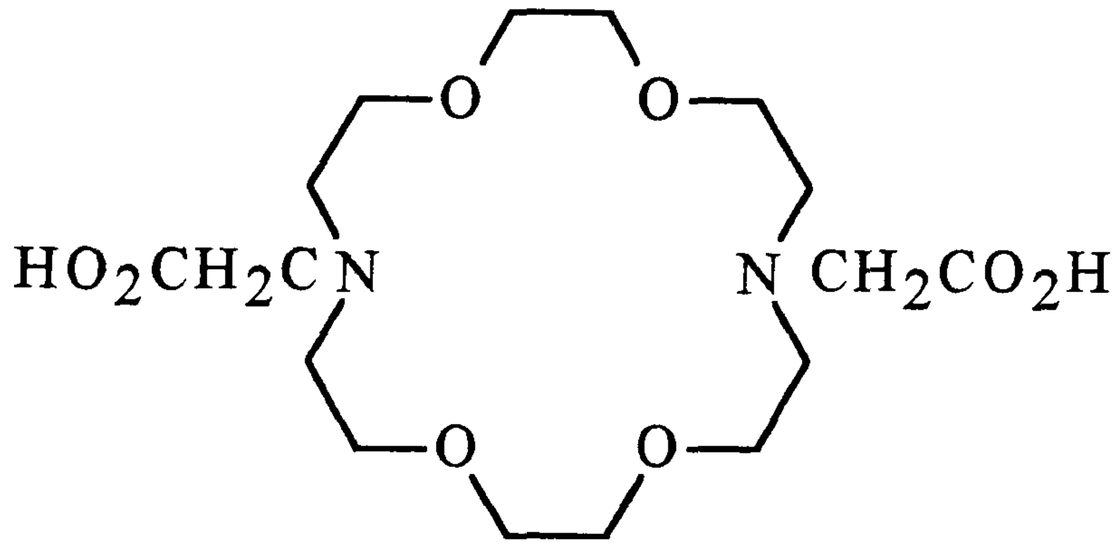
2.3.2 Diazacrown Dicarboxylic Acids.

A search of the literature revealed a class of macrocyclic aminocarboxylic acids that have binding selectivity towards the lanthanide ions in general and within this series, towards individual lanthanide ions.

We initialy focused on 7,13-diaza-1,4,10-trioxacyclopentadecane-N,N'-diacetic acid (21) (loosely called '221') (Figure 2.7). Studies by Chang *et al*[18] on the stability constants of complexes of 221 with the lanthanides showed that overall the binding constants are high and reach a maximum at Eu^{3+} (binding constant 10^{12}). This is in contrast with results obtained for a similar ligand of larger cavity size, 1,10-diaza-4,7,13,16-tetraoxacyclo-octadecane-N,N'-diacetic acid (22) ('222') (Figure 2.7), where the highest binding constants are observed with those lanthanides with the largest ionic radii.[18] These results show the importance of achieving an appropriate match between a metal ion and the cavity size.



'221' (21)



'222' (22)

Figure 2.7

Recent studies using a combination of laser induced europium ion luminescence and NMR spectroscopy have provided detailed analysis of the solution structure of the Eu-221 complex.[19] These studies show that 221 forms a 1:1 metal-ligand complex, with complete coordination of all macrocyclic ligating atoms and two coordinated water molecules. A proposed possible structure of the complex is shown in Figure 2.8.
What this shows is that only one of these ligands can surround the europium ion at any one time. This leaves a cleft around the europium ion which is able to accomodate a second (photosenstising) ligand. The approach of a second chelating ligand is made possible by the overall net positive charge of the Eu-221 complex.

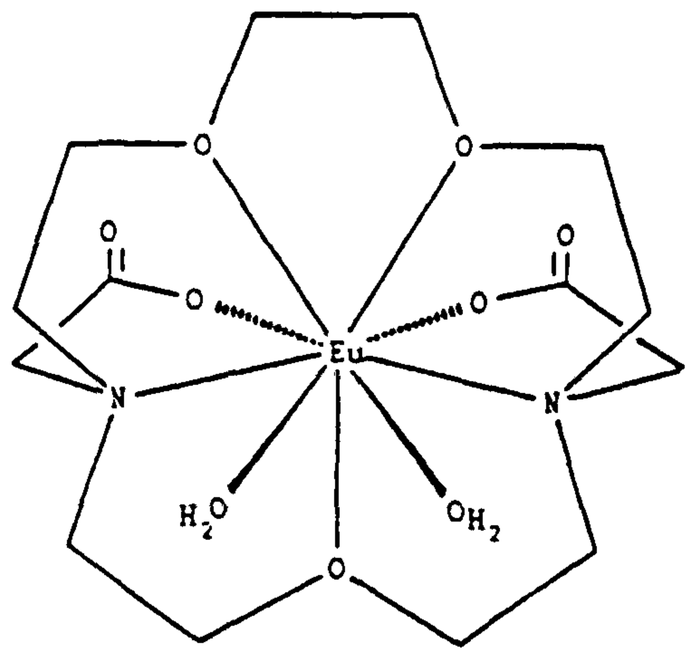
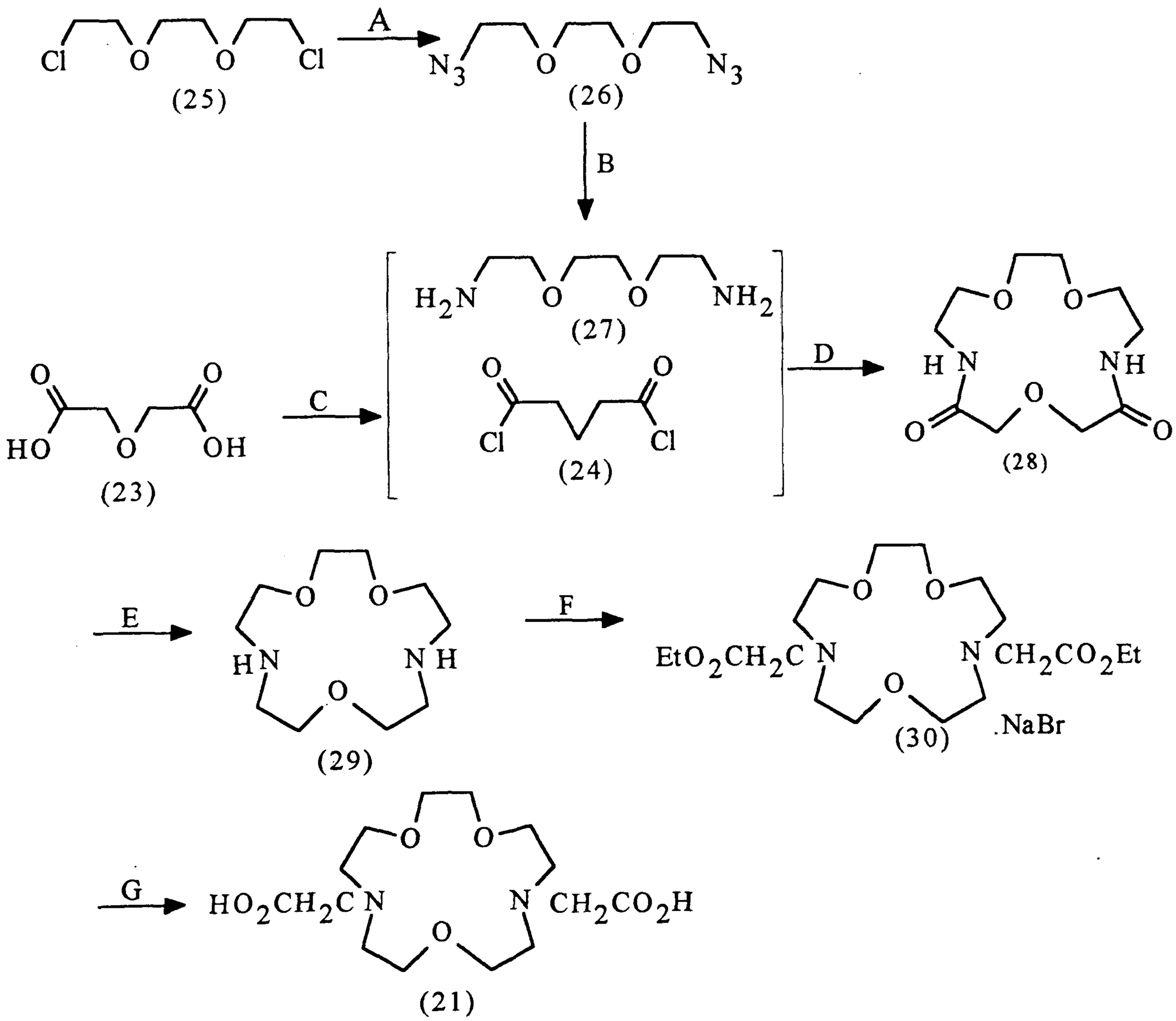


Figure 2.8 Possible structure of (Eu 2.2.1) complex.

The synthesis of '221' (21) was by using literature methods. (Scheme 2.5)[17,20,21]



Scheme 2.5 A. NaN_3, DMSO; B. $LiAlH_4$, THF; C. PCl_5, $CHCl_3$;
D. EtN_3, $PhCH_3$, 0-5° 36h (high dilution);
E. $LiAH_4$, THF; F. $BrCH_2CO_2Et$, Na_2CO_3, MeCN;
G. H_2O, 100°C

The diglycollic acid (23) was converted to the diacid chloride (24) by reaction with PCl_5 in chloroform, and in two simple steps the 1,2-bis(2-chloroethoxy)ethane (25) was converted into the bis-amino ethylether (27). The coupling of these two reactive fragments were carried out under high dilution conditions, developed by Lehn.[17] This involves the slow addition of solutions (in dry benzene or toluene) of both fragments independently over 8-12h to a large vigorously stirred volume of either benzene or toluene at 0-5° containing an excess of dry triethylamine, with the whole system under dry nitrogen. The high dilution conditions are necessary to prevent polymer formation, and the triethylamine is present to 'mop up' HCl that is formed as the cyclisation reaction proceeds, to prevent protonation of the amine.

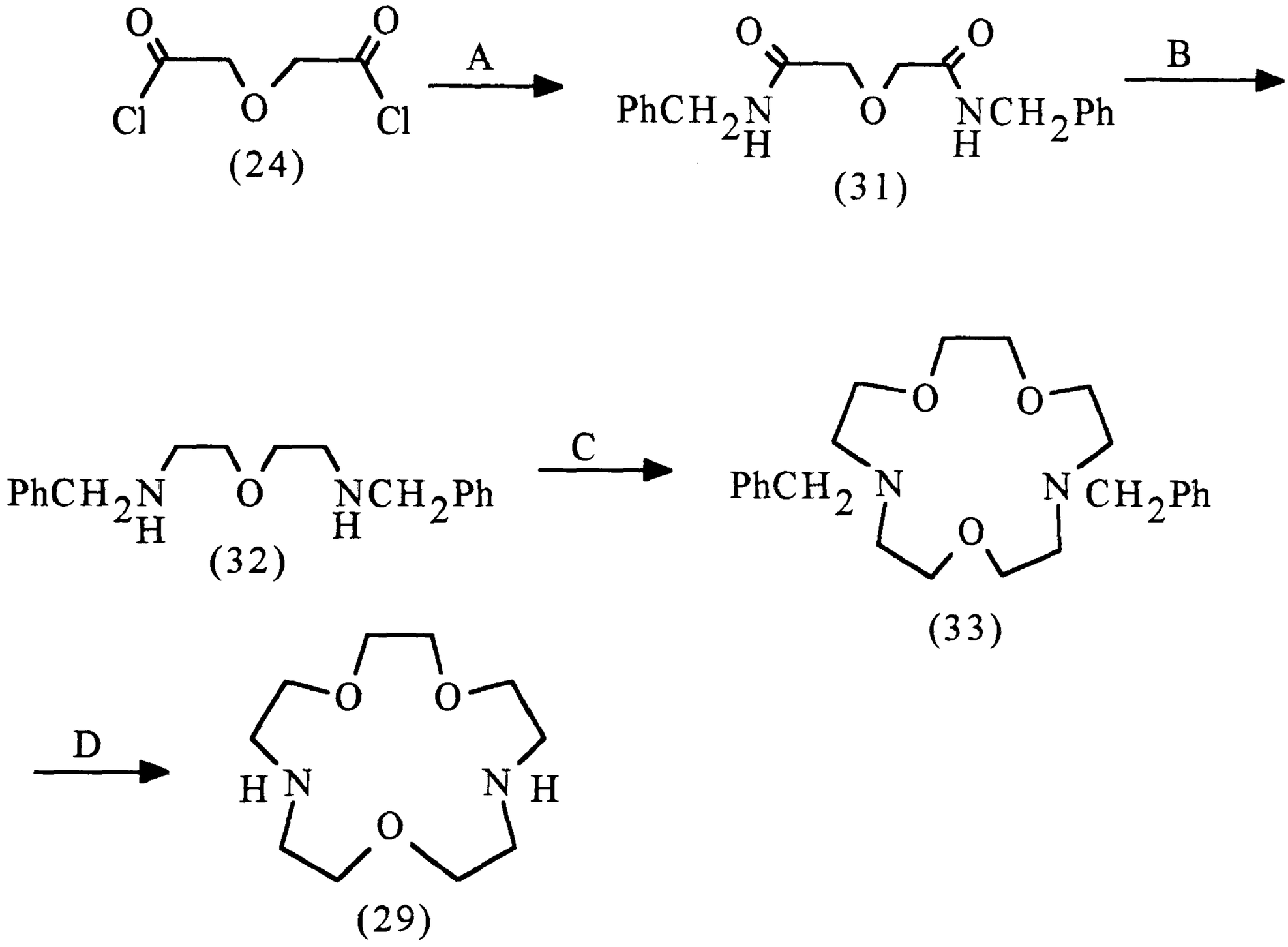
The desired macrocyclic diamide (28) was obtained in reasonable yields as a white solid from the reaction of diacid chloride (24) and diamine (27) under the said high dilution conditions.

The amide (28) was smoothly converted into the parent macrocyclic free amine (29) by LAH reduction in THF, which was purified by Kugelrohr distillation to give the amine as a white hygroscopic crystalline solid. Alkylation with ethyl bromoacetate and sodium carbonate gave the diester as the sodium bromide complex (30). This 'complex' (the cryptand replacing the hydration sphere of the sodium bromide) is decomposed by Kugelrohr distillation to give the metal free diester (30)[21]. This was hydrolysed by simply stirring in hot water overnight to give '221' (21) as a hygroscopic crystalline solid.

As luminescent studies later showed, '221' successfully fulfilled its designed role as the non-sensitising ligand of a ternary complex; binding Eu^{3+} tightly to prevent dissociation, but still allowing the approach of a sensitising ligand. The result is a ternary complex in aqueous solution where the central Eu^{3+} ion is imprisoned in a cage. The formation of the ternary complex results in the displacement of coordinated water molecules and leads to the first reports of efficient sensitisation of europium luminescence in aqueous solution. The luminescence data will be discussed in section 2.4.3.

Other synthetic routes to diaza-crowns were also examined, as the nature of high dilution cyclisations renders them unsuitable for large scale preparations. These fall into the category of methods involving a protection/deptotection sequence, for example using either the tosyl or benzyl groups to protect primary amines.

Synthetic work focused on the use of the benzyl protecting group as used by Gokel *et al.* (Scheme 2.6)[21]



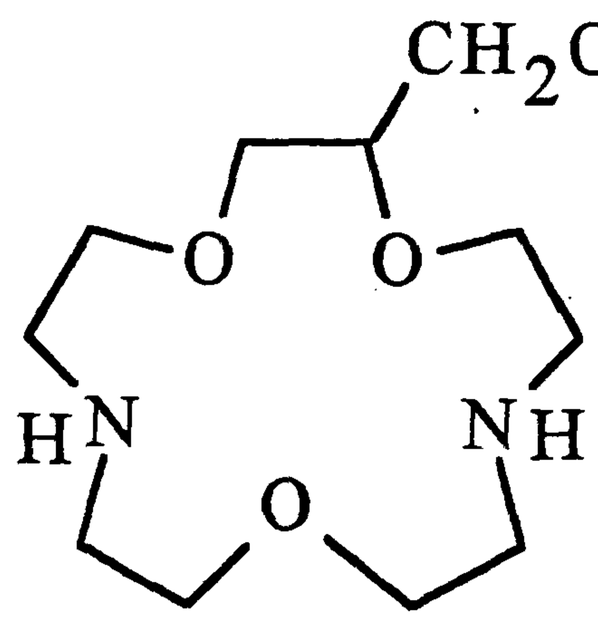
Scheme 2.6 A. $PhCH_2NH_2$, Et_3N, PhH, 0^o; B. $LiAlH_4$, THF;
C. 1,2-Bis(2-chloroethoxy)ethane, Na_2CO_3, NaI, MeCN;
D, $Na/NH_3(liq)$, THF

The reaction of the diacid chloride (24) with benzylamine proceeds in very high yields, to give the desired diamide (31) as a white crystalline solid. This was converted again in high yields to the corresponding secondary amine (32) by reduction using LAH in THF. The cyclisation of this amine fragment with bis-iodoethoxy ether (17) is carried out in acetonitrile using sodium carbonate, using conditions which were not high dilution to give the cyclised N,N'-dibenzyl-15-crown-5 (33) as a clear oil. The high yields are due to the template effect of the sodium carbonate. This effect has been studied and proved for a large number of macrocyclic systems of differing ring size.[22]

Although debenzylation of the aza crown (33) by catalytic hydrogenolysis under pressure is claimed to work efficiently,[21] this requires access to high pressure hydrogenators. Attempts to deprotect the nitrogens using conventional atmospheric hydrogenolysis or the use of catalysed hydrogen transfer methods failed to give clean deprotection, reactions being slow, only partially complete and with some degradation of the crown system. Others methods like the use of sodium in liquid ammonia were studied. Using a solution of sodium in liquid ammonia with the absence or addition of small quantities of a proton source, such as ethanol, gave poor results. This was found to be due to the virtual insolubility of the aza-crown (33) in the solvent! During the reaction the aza-crown tended to settle out as a viscous mass on the sides of reaction vessel. This problem was readily overcome by using an appropriate mixed solvent system, in this case adding a solution of the aza-crown in anhydrous THF to a 2:1 mixture of liquid ammonia and THF containing the dissolved sodium. Under these conditions a smooth deprotection resulted and work-up involving Kugelrohr distillation gave the free 4,10-diaza-15-crown-5 (29) in high yields. This methodology has been successfully used deprotect large quantities > 50g of numerous dibenzyl diaza-crowns.

The initial results from the luminescence studies with '221' (21) encouraged us try and attach potential handles to the aza-crown frame work, enabling attachment to biological molecules.

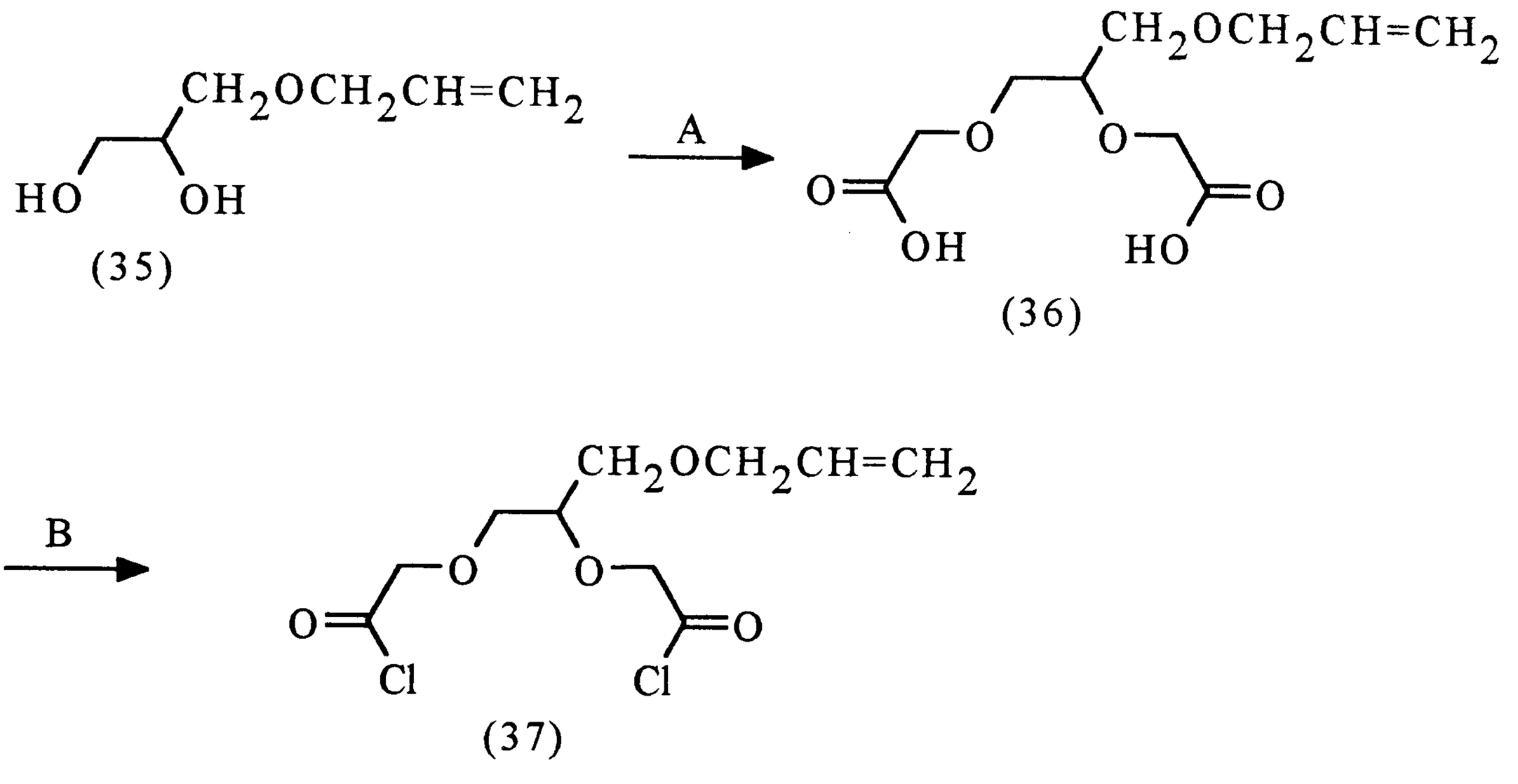
One of the first compounds targetted was the hydroxymethyl substituted diaza-15-crown-5 (34) (Figure 2.9). The key intermediate in its synthesis is the diacid chloride (37). The synthetic route to (37) is shown in Scheme 2.7[23]



(34)

Figure 2.9

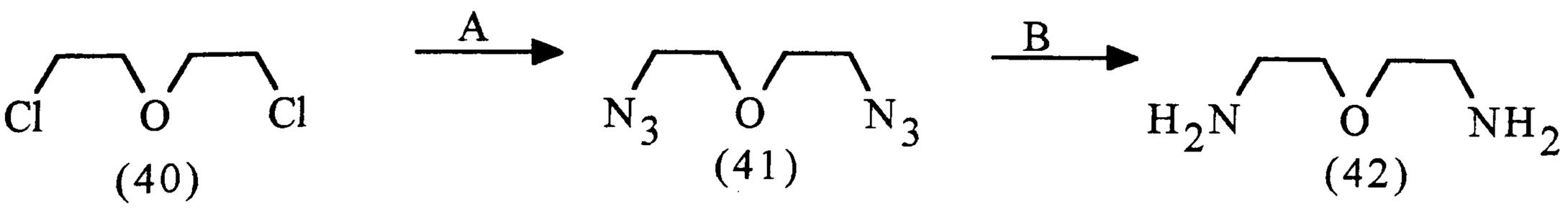
Readily available allyl protected glycerol (35) (Aldrich), was the starting point for the preparation of diacid chloride (37). Reaction of the diol (35) with potassium t-butoxide and chloroacetic acid in t-butyl alcohol (Williamson Ether synthesis), followed by careful workup and the use of high vacuum distillation at low temperatures to prevent acid-catalysed polymerisation gave the diacid (36) in good yield. Treatment of (36) with oxalyl chloride gave a quantitative yield of the desired diacid chloride (37).



(35) (36) (37)

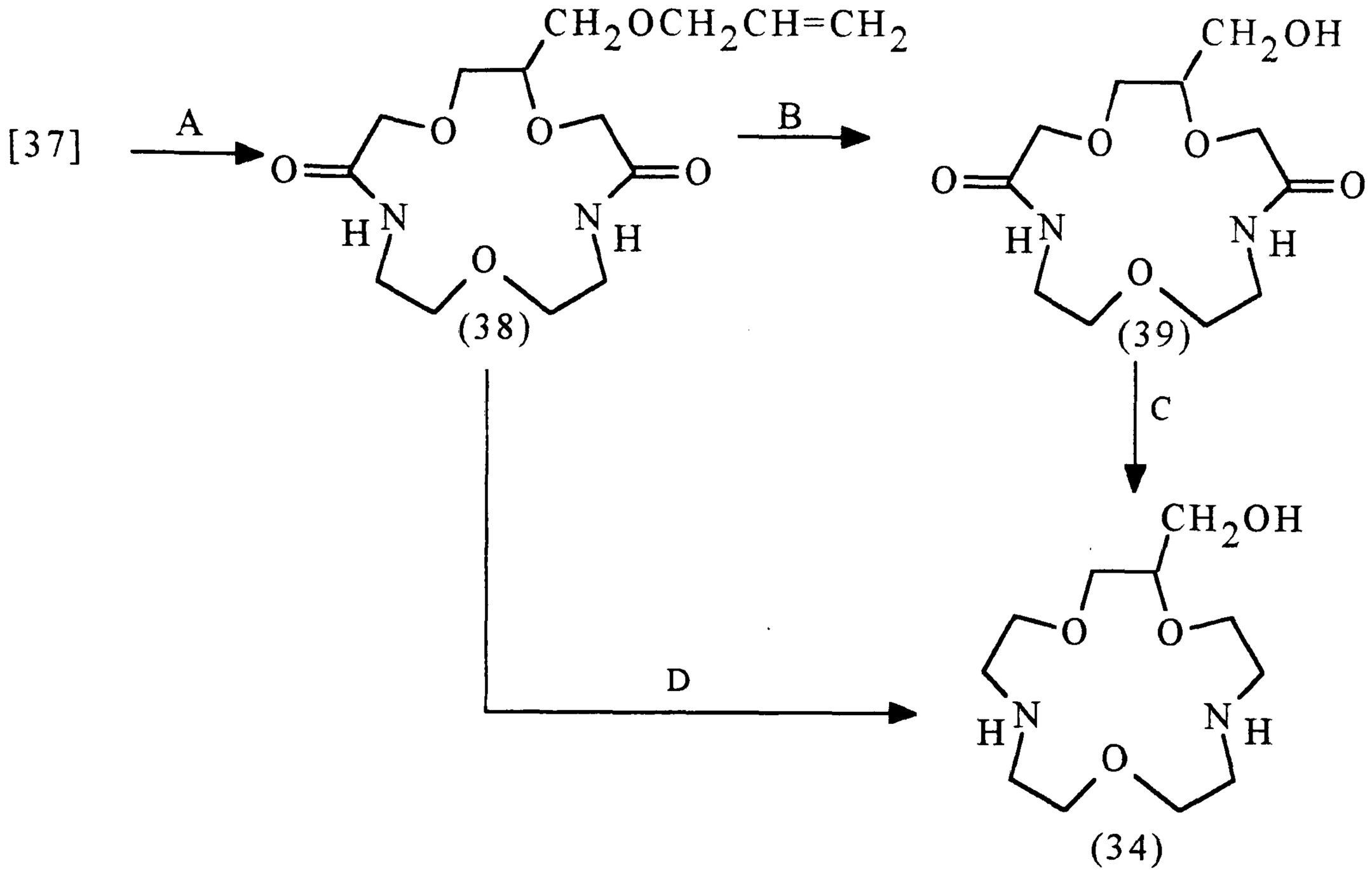
Scheme 2.7 A. $ClCH_2CO_2H$, K^tBUO, ^tBUOH; B. ClCOCOCl

The synthetic route to the hydroxymethyl diaza-crown (34) is depicted in Scheme 2.9. Diacid dichloride (37) was reacted with diamine (42), synthesised by standard literature procedures (Scheme 2.8)[21], under high dilution conditions in the presence of triethylamine to form the (allyloxy)-methyl substituted cyclic diamide (38) in moderate yields. Deprotection of (38) by isomerisation of the allyl group with palladium on carbon followed by acid catalysed cleavage gave the hydroxymethyl substituted cyclic diamide (39). The hydroxymethyl diaza-15-crown-5 (34) was obtained by reduction of (39) with LAH in THF in good yields. Deprotection and reduction was also achieved in a single step by the use of a large excess of LAH in THF.



(40) (41) (42)

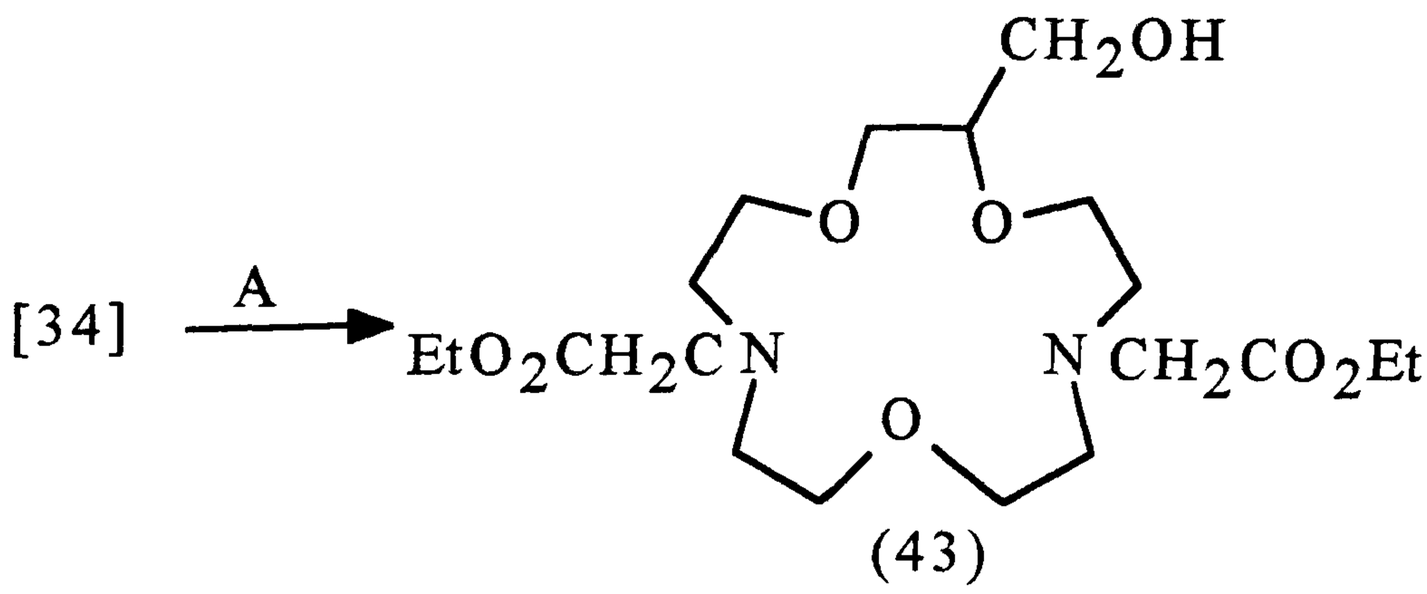
Scheme 2.8 A. NaN_3, DMSO; B. $LiAlH_4$, THF



Scheme 2.9 A. [42], Et_3N, $PhCH_3$, 0^o, (high dilution);
B. Pd/C, $HClO_4$, $EtOH/H_2O$; C. $LiAlH_4$, THF
D. $LiAlH_4$ (large excess), THF

Initial attempts at dialkylation of hydroxymethyl diaza-15-crown-5 (34) with 2.1 equivalents of ethylbromoacetate in acetonitrile in the presence of anhydrous sodium carbonate resulted in alkylation at the ring nitrogens (as expected), and an analytical sample of the metal free ligand (43) was obtained after repeated Kugelrohr distillation. (Scheme 3.0)

After successfuly obtaining (43), work was at hand to start functionalisation at the hydroxy methyl position in (43), by initially trying to form the active ester, but before serious practical work could begin an important discovery was made about ethylenediaminetetracetic acid (EDTA).

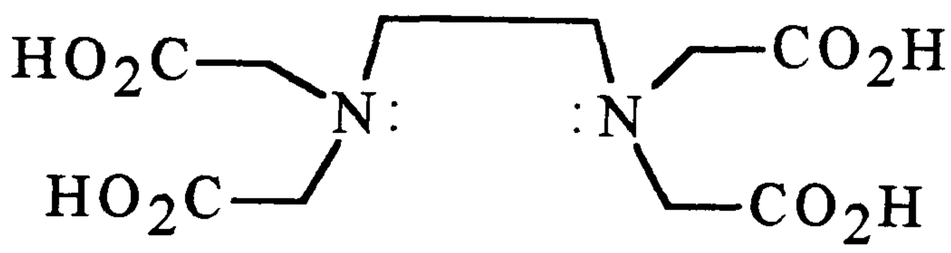


Scheme 3.0 A. $BrCH_2CO_2Et$, Na_2CO_3, MeCN

2.3.3 Linear Aminopolycarboxylic Acids

Aminopolycarboxylic acids like EDTA (44) (Figure 3.0) form very stable multidentate complexes with the lanthanides.[24,25] Typically, these ligands have at least one secondary or tertiary amino function and at least two carboxylates. Because of the weak basicity of the carboxylate groups in these compounds , the lanthanide aminopolycarboxylates must owe their unusually high stability to chelation involving the nitrogen donor sites. The involvement of nitrogen in the coordination results in the formation of stable five-membered chelate rings.

The actual structure in solution of a metal-aminopolycarboxylate chelate will depend on the metal ion as well as on structural and thermodynamic constraints of the ligand. As coordination numbers of 8 or 9 are common for the lanthanide cations (Ln^{3+}), few ligands fill the coordination sphere in 1:1 complexes and water molecules are always present. For example, $LnEDTA^{-1}$ complexes have 3 to 4 hydrate waters in the primary coordination sphere.



EDTA (44)

Figure 3.0

Although EDTA has a very high binding constant for Eu^{3+} ions, initial luminescence studies (at pH 8.5) showed little enhancement in europium luminescence on addition of the sensitiser (2,9-biscarboxylic acid-1,10-phenanthroline) to Eu-EDTA solution. We initially considered that this was because the ion is effectively encapsulated by the ligand thus preventing the approach of the sensitiser.

When EDTA is dissolved in water, it behaves like an amino acid, in this case forming a double zwitterion structure (H_4Y) at low pH and the fully deprotonated species Y^{4-} at high pH (Figure 3.1A-E). The dissociation constants for the deprotonation of H_4Y are pK (ca. 2.0, 2.67, 6.16 and 10.26). The relative amounts of each of these species as a function of pH is illustrated in Figure 3.2[26]

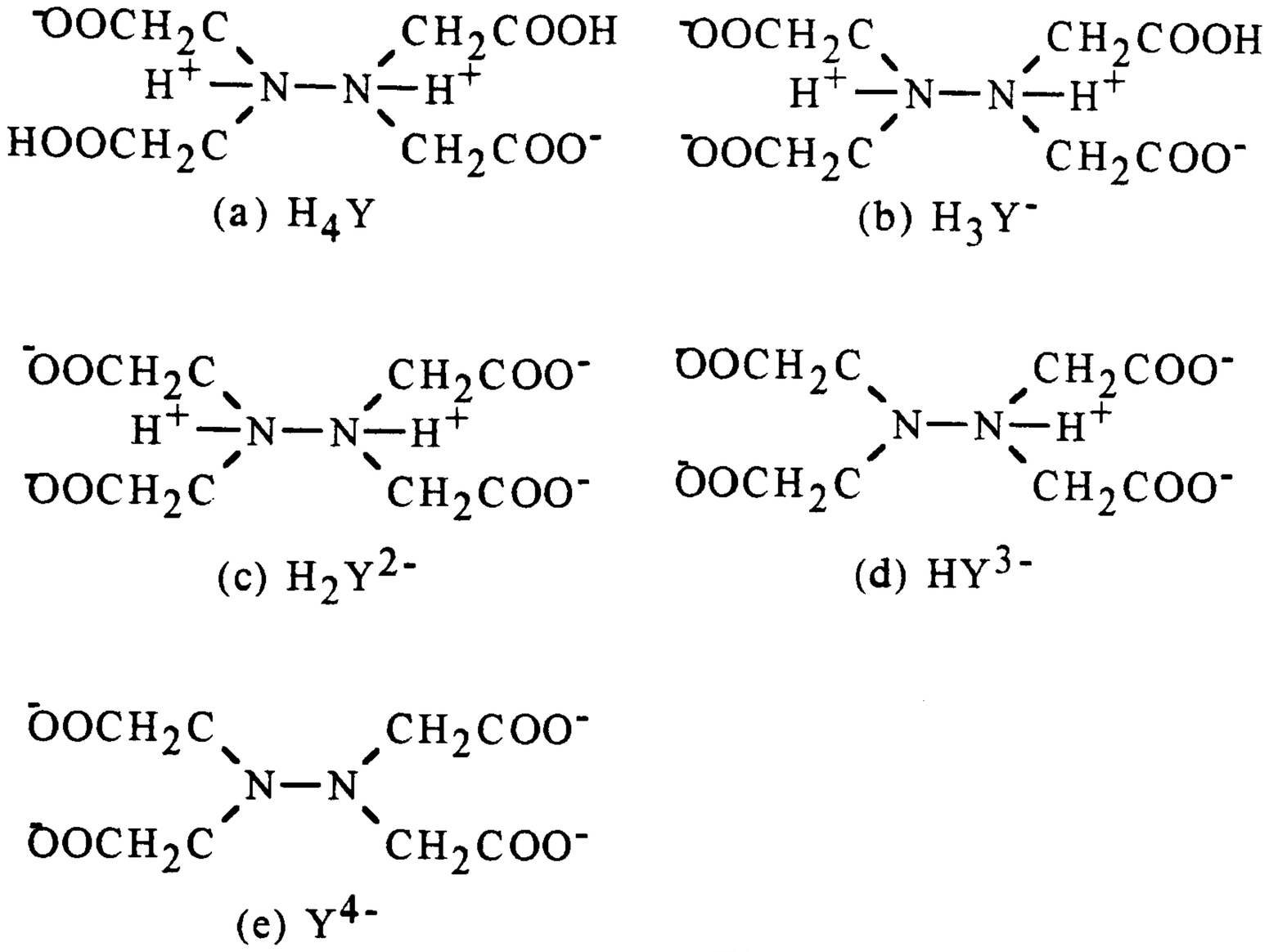


Figure 3.1

When studies with the EDTA/Eu^{3+}/PDCA system was repeated, but this time varying the pH of the solution, to our suprise a significant enhancement in the luminescence was observed with the pH/I_{max} locus occurring at (5.5, 308). Although it is difficult to predict the effects on both binding and dissociation constants for individual ligands involved in ternary complexes, it is reasonable to assume that enhancement we are observing is consistent with a definite role for probably two perhaps three of the EDTA carboxylates being involved in chelation. To test this idea, a couple of EDTA derivatives were synthesised. (Scheme 3.1)

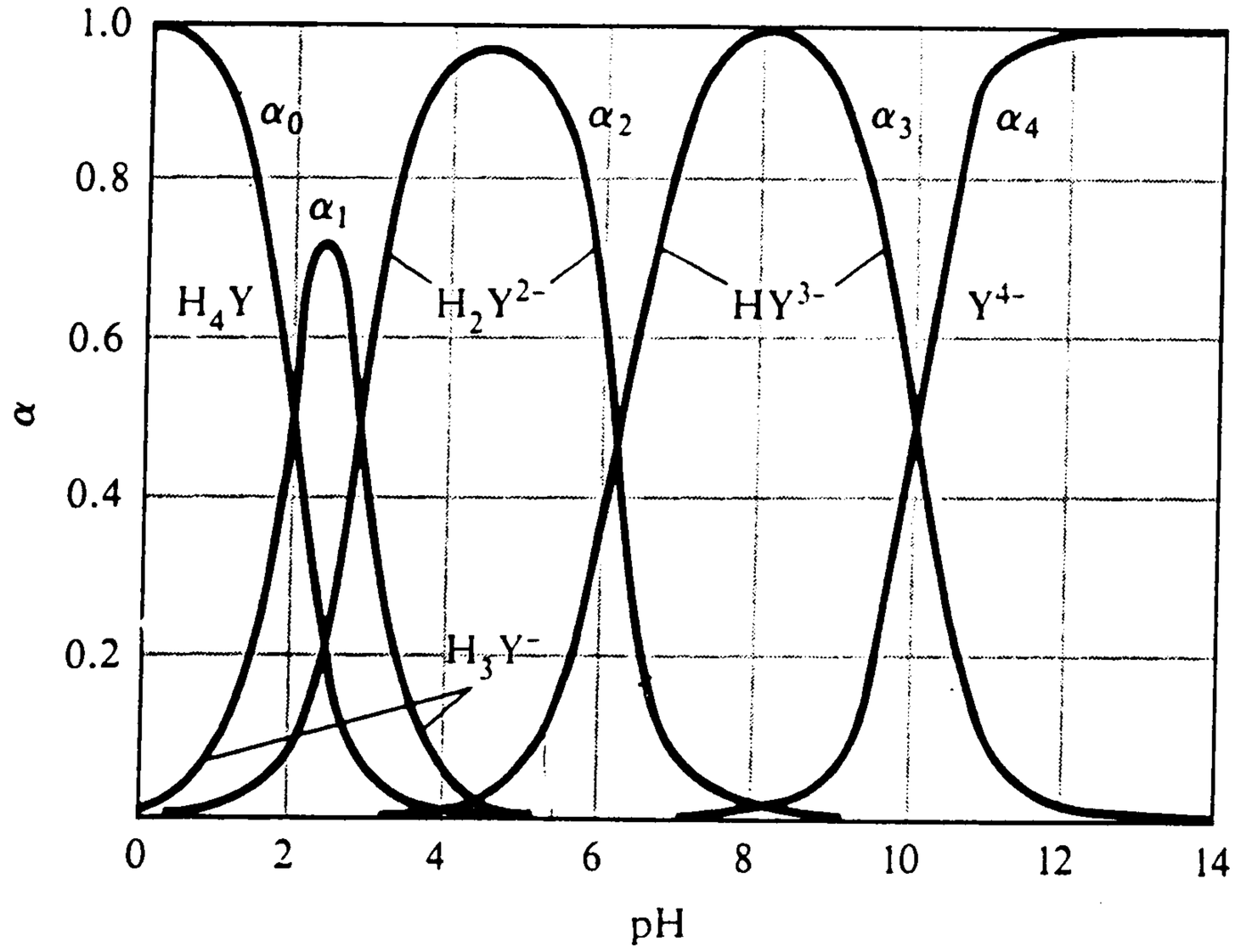
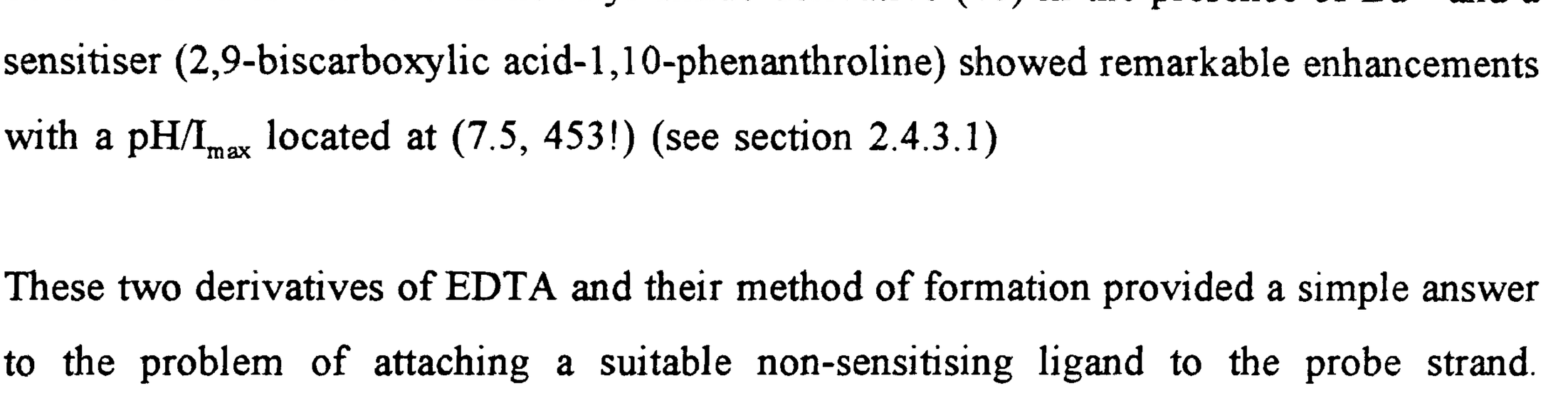


Figure 3.2 Composition of EDTA solutions as a function of pH.

These EDTA derivatives were simply made by reacting the appropriate amine with commercially available EDTA-bisanhydride (45) in dry THF at room temperature. Purification of these compounds involved making the mono-or-disodium salt of the appropriate carboxylic acid *in situ* and extracting with ether to remove any unreacted amine. The desired ligands were obtained as their sodium salts on evaporation and these were further purified by crystallisation from methanol.

Solution studies with the monobutyl amide derivative (46) in the presence of Eu^{3+} and a sensitiser (2,9-biscarboxylic acid-1,10-phenanthroline) showed remarkable enhancements with a pH/I_{max} located at (7.5, 453!) (see section 2.4.3.1)

These two derivatives of EDTA and their method of formation provided a simple answer to the problem of attaching a suitable non-sensitising ligand to the probe strand. Oligonucleotide probes were supplied by Cellmark Diagnostics functionalised with a hexyl-amino group at the 5'-end which was simply reacted with EDTA-bisanhydride. (see section 2.5.2)



Scheme 3.1 A. $R\text{-}NH_2$, THF, r.t

(b) Sensitising Ligands.

So far we have dealt with the photophysically inert part of the generalised assay system as illustrated in Scheme 2.4

By the choice of suitable ligands like the EDTA-derivatives which are able to bind Eu^{3+} tightly, we can ensure that the lanthanide ion will remain be bound at the biological target at low concentrations. However, to observe lanthanide ion luminescence we have to find away of channeling energy in to the europium ion.

Unfortunately, as explained earlier the lanthanide ions are very poor light absorbers (lack of absorption bands), although direct excitation of these ions using powerful lasers is one option, used by Horrocks to study metal ion binding sites in biological materials.[27,28] A more practical approach is the use of a photosensitising or `antenna` ligand, as an indirect way of obtaining the excited metal state which precedes luminescence from the metal ion. This process of sensitisation occurs as a result of intramolecular energy transfer from the triplet excited state of the ligand to the emitting levels of the metal ion (see section 1.3, Chapter 1).

In general it is difficult to predict which organic ligands will make effective sensitisers, although one essential property is to have a triplet state higher in energy than the emitting state of the lanthanide ion. Also apart from having the right photophysical properties, the sensitiser must be capable of complexing the lanthanide ion (lanthanide binding site), with water acting as a competitive ligand. These lanthanide binding sites normally incorporate powerful binding groups usually iminodiacetates or carboxylates. However, a complication as a result of our approach/design is the need that any potential sensitiser has to be able to approach and bind Eu^{3+} which is already bound in a complex.

Balzani and Lehn chose the bipyridines and phenanthrolines as their lanthanide sensitisers incorporating them into cryptands.[29,30] The encapsulation approach was needed to prevent dissociation of the lanthanide ion (due to lack of powerful binding groups on either ligand). However this approach by its nature does not allow the approach of a second binding ligand and would be useless in our cooperative design.

2.3.4 Simple 1,10-phenanthroline derivatives

Our initial approach therefore was to find a suitable lanthanide sensitiser based on simple ligands. We focussed on 1,10-phenanthroline and its analogues, namely 1,10-phenanthroline-2,9-biscarboxylic acid (PDCA) (48), a derivative of which (49) is the photosensitising component of the Cyberfluor system.[4] (Figure 3.4)

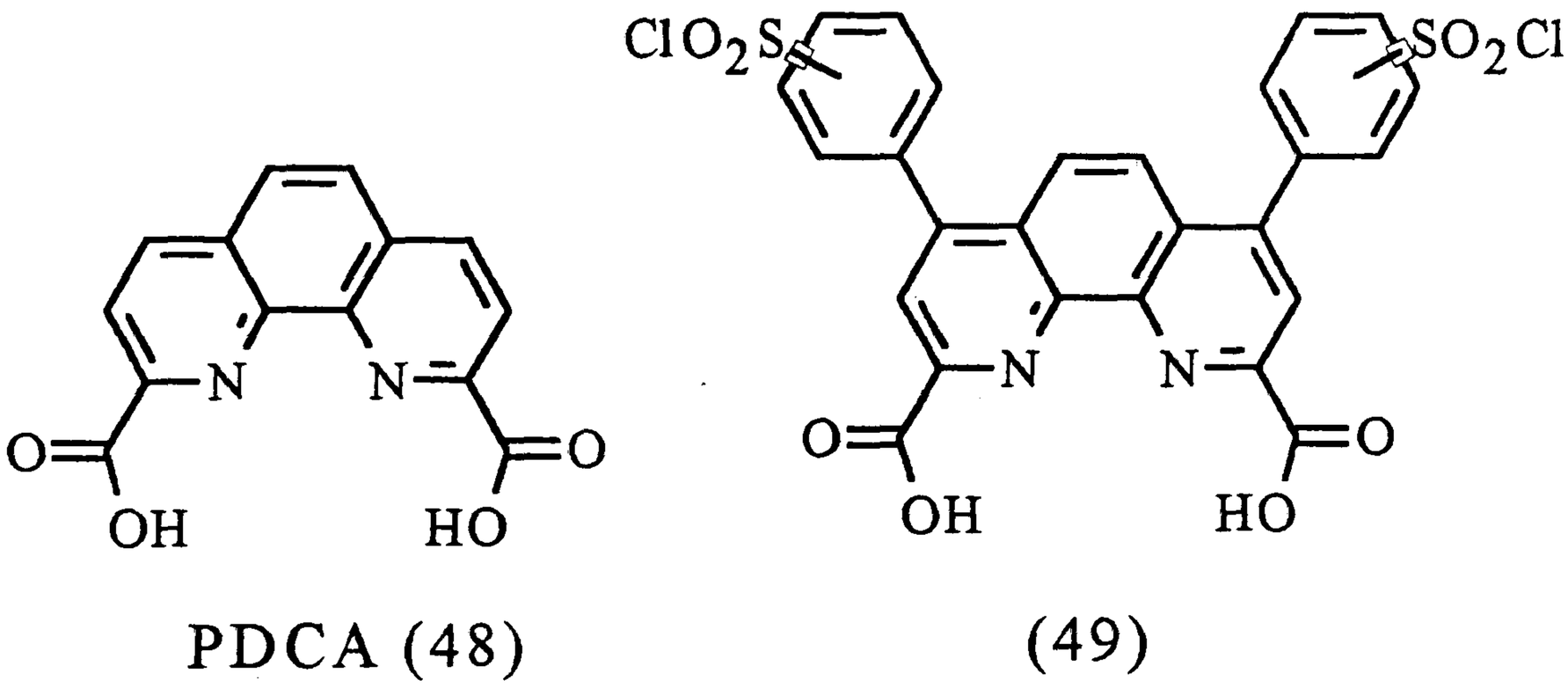
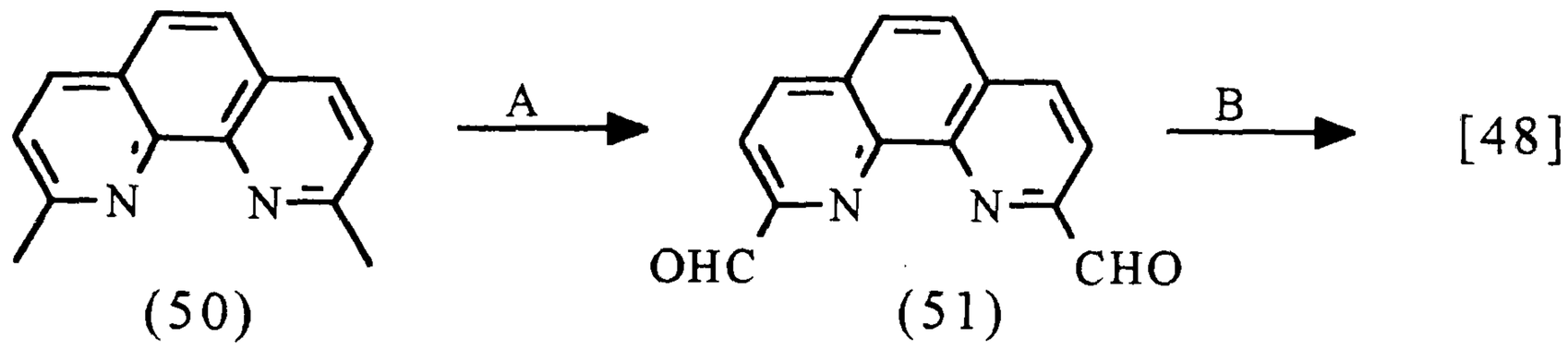


Figure 3.4

Initial efforts concentrated on the synthesis of 1,10-phenanthroline 2,9-biscarboxylic acid, PDCA (48), and its simple analogues. The synthesis of PDCA can be achieved through two distinct literature routes, both starting with commercially avaliable 2,9-dimethyl-1,10-phenanthroline (Neocuproine) (50). Inital efforts concentrated on the Chandler route. (Scheme 3.2)[31]

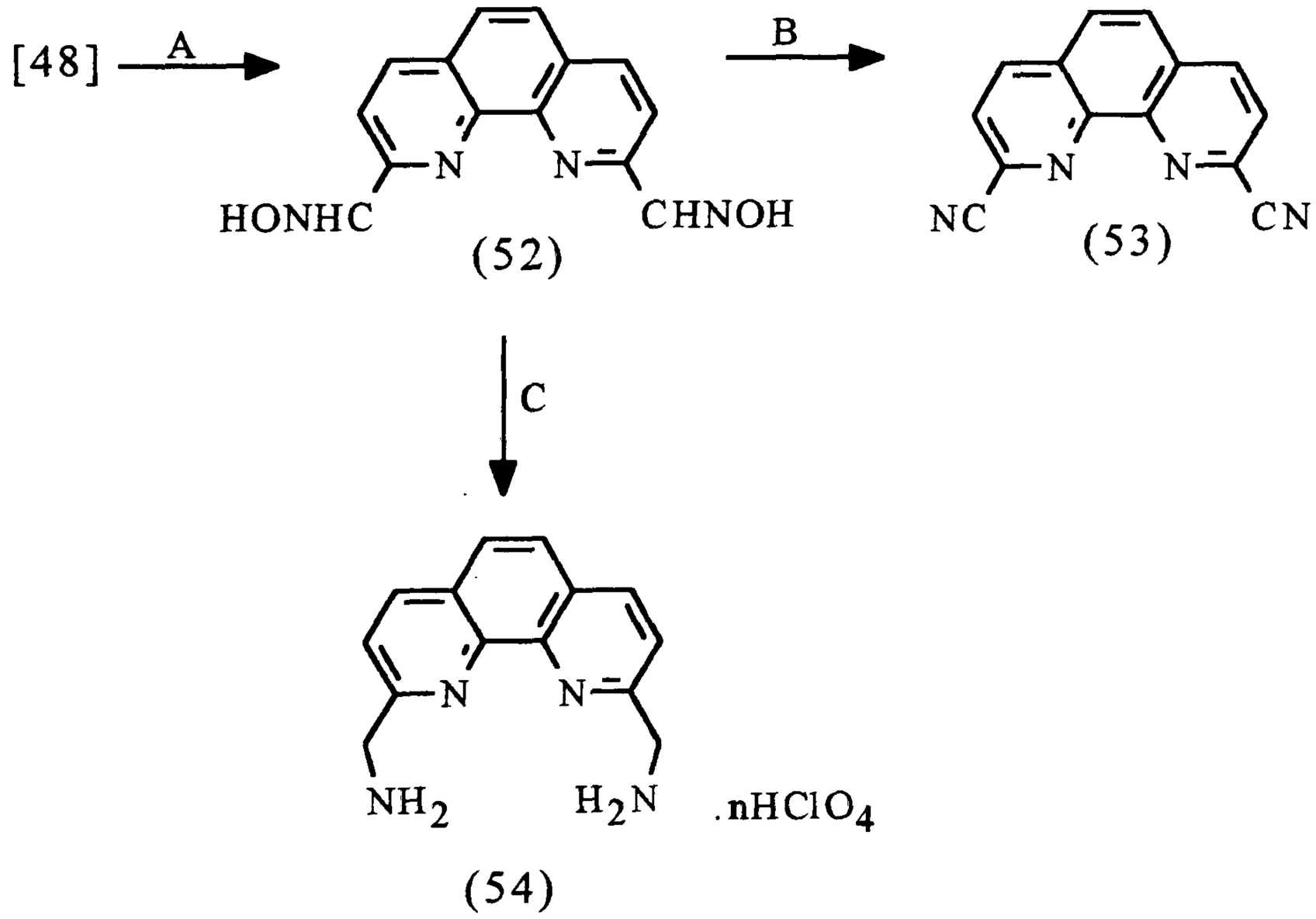
Neocuproine (50) was oxidised with selenium dioxide to the corresponding dialdehyde (51) in high yields. Further oxidation of the dialdehyde (51) with nitric acid gives the desired dicarboxylic acid (48) obtained as the monohydrate after recrystallisation from THF/H_2O.



(50) (51) [48]

Scheme 3.2 A. SeO_2, Dioxan; B. c. HNO_3

Although the dialdehyde (51) is a useful intermediate in the synthesis of a number of important 2,9-disubtituted phenanthroline derivatives like the dicarbonitrile (53) and the bisaminomethyl (54) (Scheme 3.3)[31], residual metallic selenium contamination was a problem since it made the purification of the dialdehyde very difficult, and was carried forward to contaminate any subsequent derivative. Thus traces of red metallic selenium was observed in the diacid (48) even after several recrystallisations.

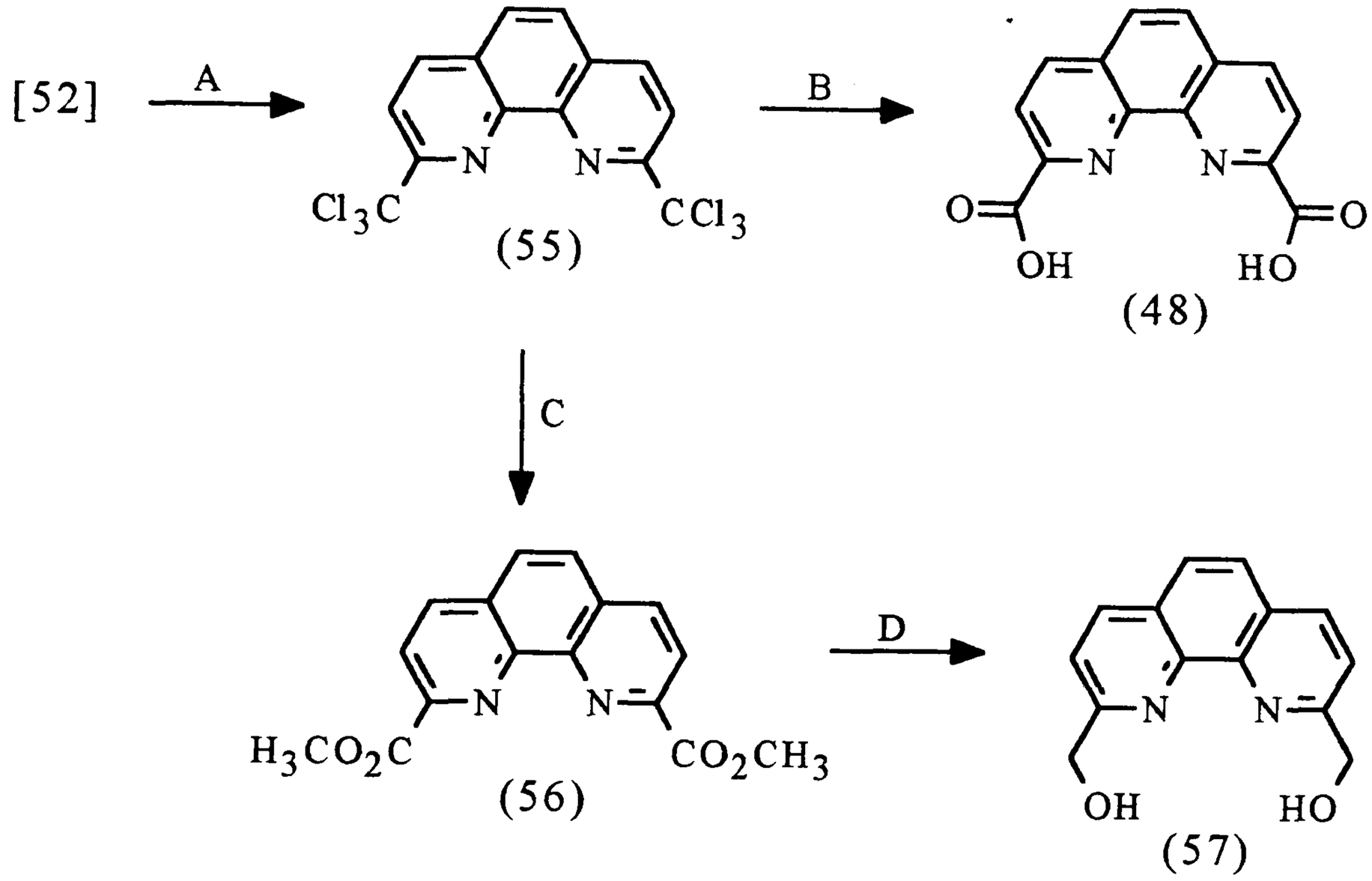


(52) (53)

(54)

Scheme 3.3 A. $H_2NOH.HCl$, pyridine, EtOH; B. Acetic anhydride; C. 10%Pd/C/H_2, 2% $HClO_4$, EtOH

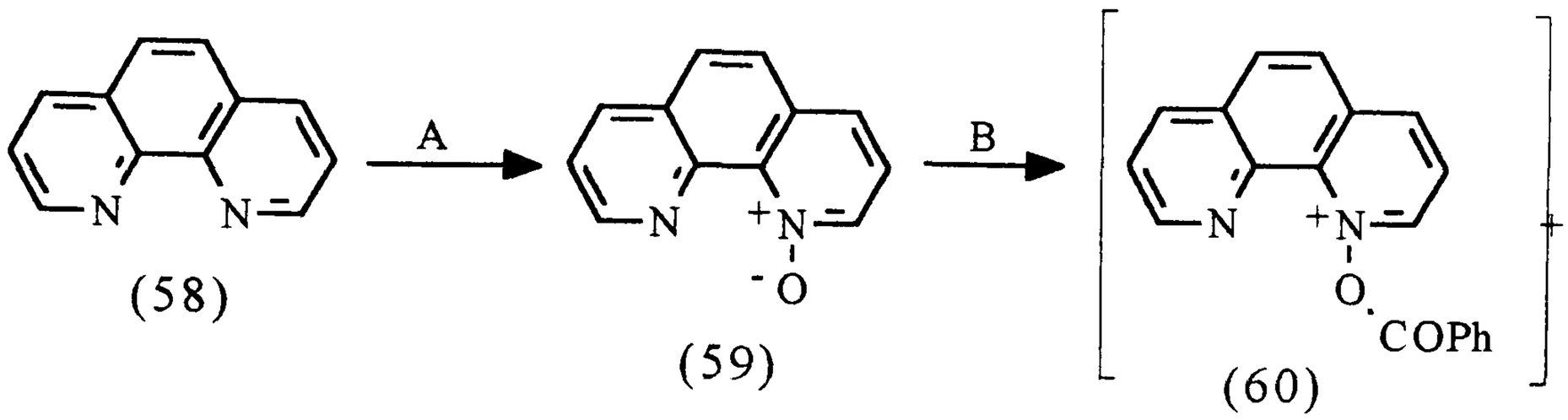
An alternative route to PDCA (48) reported by Newkome *et al*[32], involved the bis(trichloromethyl) derivative (55). This was readily synthesised by free-radical chlorination of neocuproine (50) with excess N-chlorosuccinimide in CCl_4 using benzoyl peroxide as initiator. Hydrolysis of (55) with concentrated sulphuric acid converted the bis(trichloromethyl) derivative to the corresponding acid (48), obtained by quenching the reaction with water. (Scheme 3.4)



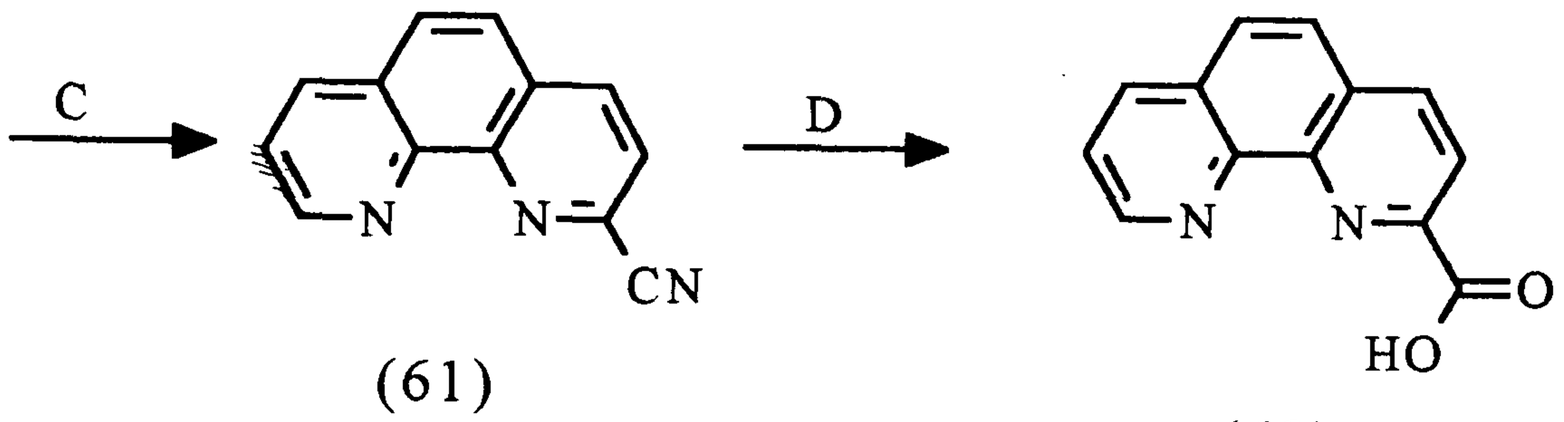
Scheme 3.4 A. NCS, CCl_4, mCPBA; B. c.H_2SO_4, 90°;
C. c.H_2SO_4, MeOH, reflux; D. $NaBH_4$, EtOH

After the acid hydrolysis of (55) if solvolysis with dry methanol was carried out instead of quenching with water, the bis(methoxycarbonyl) derivative (56) was obtained in high yields. This is a useful intermediate, for it is readily reduced with sodium borohydride in ethanol to afford the bis(hydroxymethyl) derivative (57), which can be futher derivatised by halogenation.

Other simple 1,10-phenanthroline derivatives synthesised included the monocarboxylic acid (62) and the bis(butylamide) (64). The 2-carboxy-1,10-phenanthroline (62) was synthesised by the method of Corey.[33] (Scheme 3.5)



(58) (59) (60)



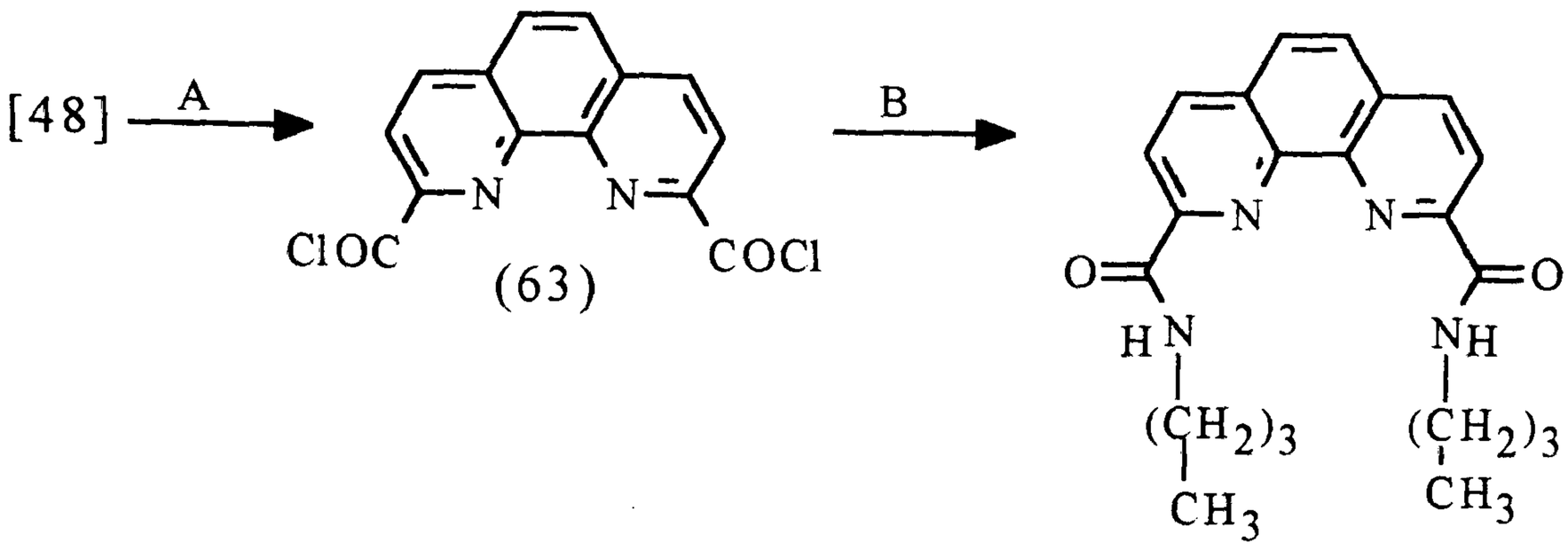
(61) (62)

Scheme 3.5 A. $AcOH/H_2O_2$; B. PhCOCl; C. KCN; D. $NaOH/H_2O$

1,10-Phenanthroline-1-oxide (59) was prepared by the method of Engbersen[34], in a slightly modified procedure to that reported by Corey[33], resulting in improved yields (>80%) and purity. Treatment of the 1-oxide (59) with benzoyl chloride and potassium cyanide at room temperature afforded 2-cyano-1,10-phenanthroline (61) in excellent yields. It is interesting to note that the substitution reaction to form (61) takes precedence over the Reissert at C-9, N-10. This would require the attachment of benzoyl chloride to N-10 which is sterically unfavourable. The parent 1,10-phenanthroline has been found not to undergo the Reissert reaction under the conditions useful for quinolines.[33,35,36]

Hydrolysis of 2-cyano-1,10-phenanthroline (61) to 2-carboxy-1,10-phenanthroline (62) was achieved under basic conditions using aqueous sodium hydroxide. This hydrolysis reaction is strongly promoted by metal ions such as Cu^{2+} and Ni^{2+}. The mechanism is thought to involve attack of hydroxide ions on the metal complexed substrate.[36,37]

The starting point for the synthesis of 2,9-bis(butylamide)-1,10-phenanthroline (64) was PDCA (48), which was converted to the diacid choride (63) with thionyl chloride at reflux. This was used without further purification, and reacted with two equivalents of butylamide in dry $CHCl_3$ to afford the desired compound (64) in high yields. (Scheme 3.6)



Scheme 3.6 A. $SOCl_2$; B. $CH_3(CH_2)_3NH_2$, $CHCl_3$, r.t

Results from the luminescence studies confirmed PDCA (48), as the sensitiser with the necessary physical and photophysical properties required for its designed role in the final ternary complex as an efficient sensitiser of Eu^{3+}.

From the general scheme 2.4, we envisage the potential sensitiser being attached by a short flexible link to an intercalator. (Figure 3.5)

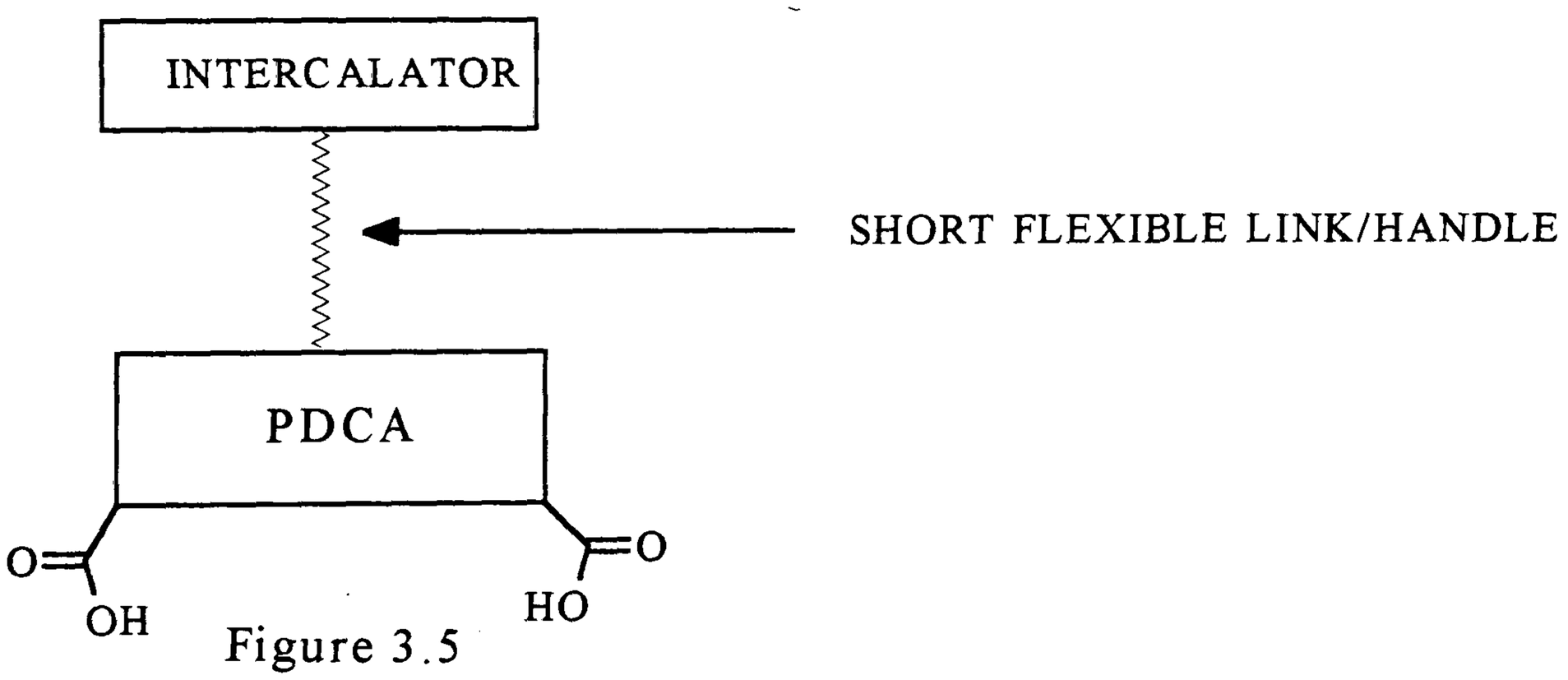


Figure 3.5

2.4 INTERACTION OF NUCLEIC ACIDS WITH SMALL MOLECULES.

Intercalation and the effects of intercalation has been designed in to the assay as a means of:

(i) discriminating between the hybridised and unhybridised probe and

(ii) providing a local concentration effect of the sensitiser near the reporter molecule (bound Eu^{3+}) over bulk solution once hybridisation between probe and target strand has taken place.

Molecules and ions interact with duplex nucleic acids in three primary ways which are significantly different:

(a) binding along the exterior of the helix through interactions which are generally non-specific and primarily **electrostatic** in origin;

(b) **groove-binding** interactions which involve direct interactions of the bound molecule with the edges of base-pairs in either of the (major or minor) grooves of nucleic acids; and

(c) **intercalation** of planar or approximately planar aromatic ring systems between base-pairs (Figure 3.6)[38]

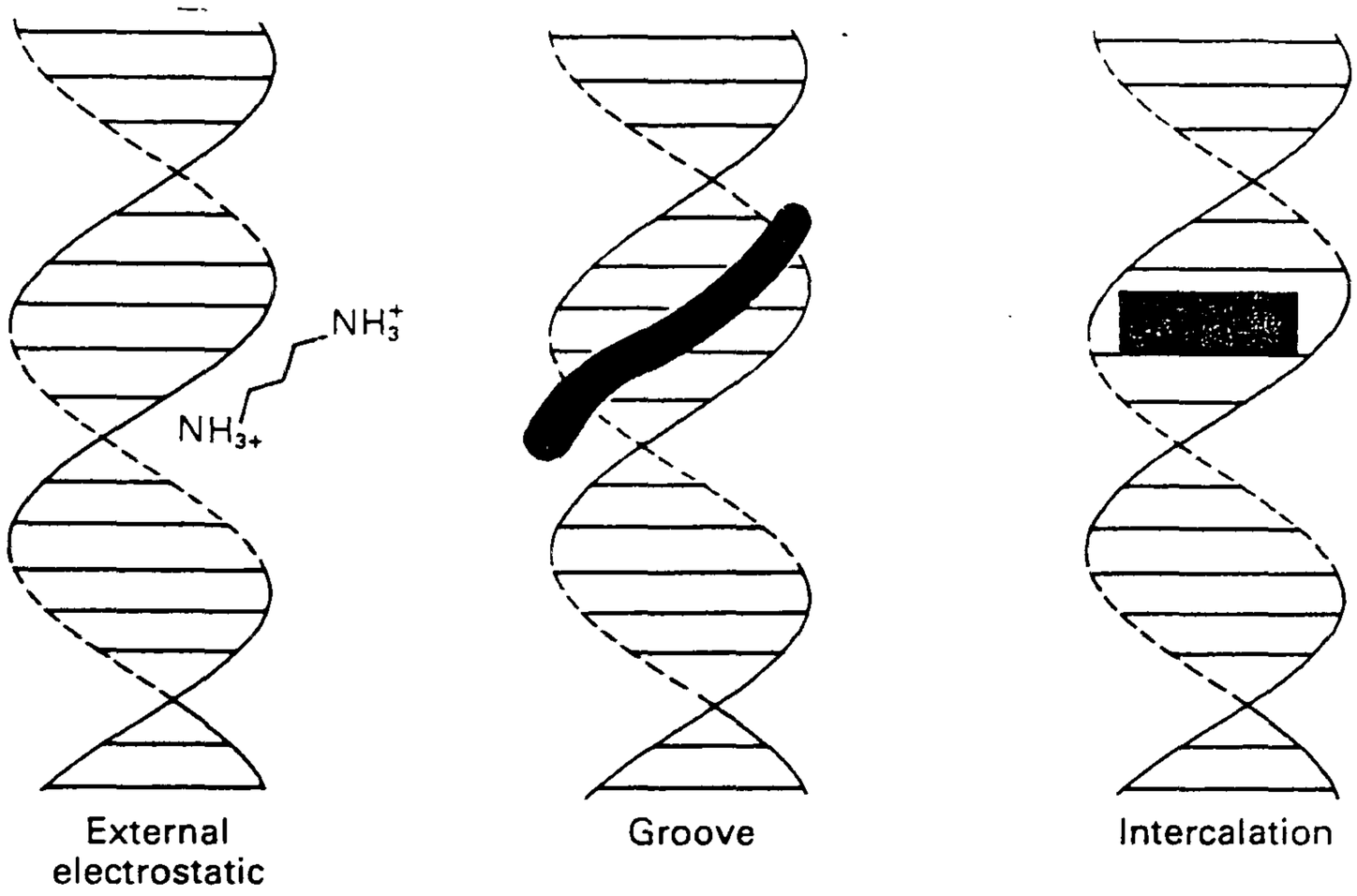
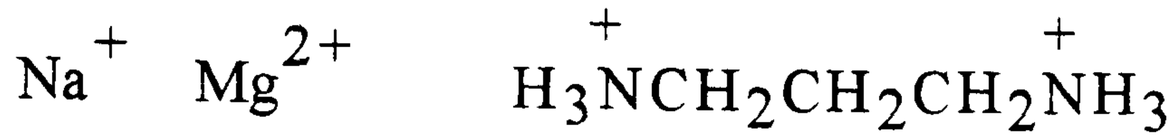


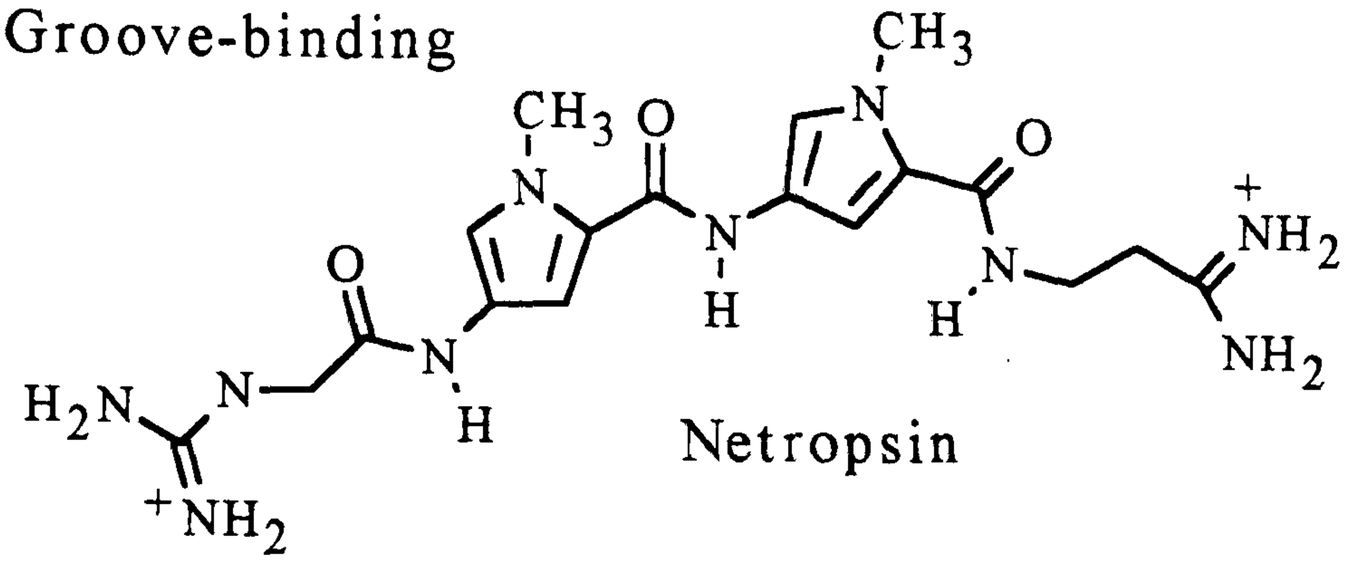
Figure 3.6 The three primary binding modes of B-DNA.

The first two binding modes do not require a nucleic acid conformational change but may induce structural transitions on complex formation. Intercalation requires a change in the sugar-phosphate chain torsional angles for separation of adjacent base-pairs by a distance (typically 3.4Å) sufficient to allow insertion of the intercalating ring system. This can be accompanied by other changes in the helical parameters such as unwinding, bending, etc. Examples of the types of molecules and ions that bind to nucleic acids by three different modes are shown in Figure 3.7.

A. External electrostatic interactions

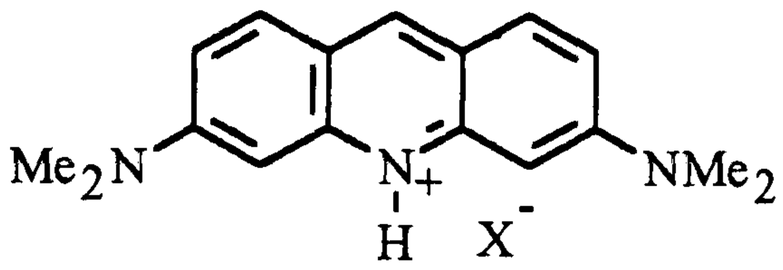


B. Groove-binding



Netropsin

C. Intercalation



Acridine orange

Figure 3.7 Examples of cations which bind by the three primary modes.

2.4.1 Structure of DNA

Since the double helical structure of DNA was first described by Watson and Crick[39] in 1953, it has been clear that double stranded polynucleotides ought to be able to adopt a wide family of conformations. These helical conformations can now be classified into three general families; the A, B, and Z forms, with the A and B forms being right handed and the Z form left handed.[38,40,41] Although each conformation involves a helix made up of two anti-parallel polymer strands with the bases paired through W-C hydrogen bonding, the overall shapes of the helices are quite different. (Figure 3.8)[42]

The predominant B-form is a regular right handed helix, with the base pairs oriented essentially perpendicular to the helix. It has well defined major and minor grooves, both approximately equally deep but with very different widths, the minor groove being narrow, with the major groove being much more open. A-DNA on the other hand has a deep yet narrow major groove which is virtually inaccessible to binding by other molecules and a shallow but wide minor groove. The base pairs are tilted and pulled away from the helix axis, so that the polymer has a cylindrical hole running down its middle. The overall effect is that of a ribbon-like structure wrapped around a pole. The Z-form is a left handed helix with a zig-zag sugar phosphate back bone, which is adopted mostly

by alternating cytosine/guanine sequences. It has a very deep and narrow minor groove with an almost non-existent major groove, making it impossible for intercalating agents to bind to Z-DNA.

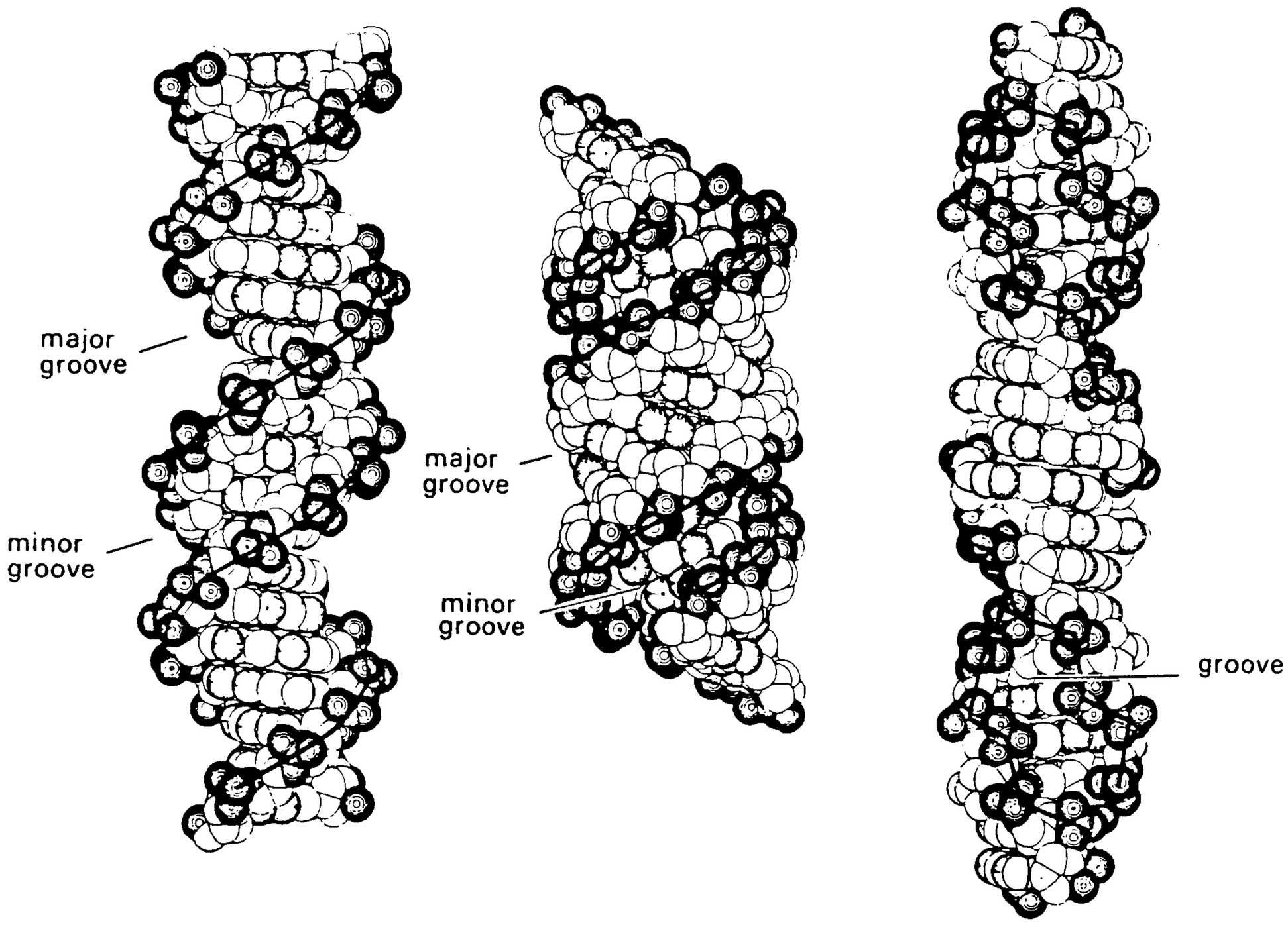


Figure 3.8 Space filling (van der Waals) representations of B, A, and Z DNA's

2.4.2 External electrostatic interactions

Nucleic acids are highly charged polyelectrolytes whose anionic phosphate groups strongly affect their structure and interactions.[43] It is a polyanion, with one negative charge for each nucleotide or a charge of -2 for each step along the helix. The helical column is therefore awash in negative charge, and it must 'condense' a significant number of cations from solution to exist in stable conformations. Manning[44] has shown that simple ions such as the alkali metals associate with nucleic acids largely as a function of the polymer charge density. This association of ions with the polyelectrolyte is called counter-ion condensation and causes an unfavourable entropy term in the overall polymer conformational free energy summation. This unfavourable term is more than outweighed by the numerous favouable interactions in the folded polymer (such as the DNA double helix). Partial release of these cations on denaturation or on binding of cationic ligands to specific nucleic acid sites accounts for the strong dependence of these processes on salt

concentration. Multiply charged simple cations such as Mg^{2+} and cations of simple organic amines such as 1,3-diaminopropane interact with DNA more strongly than mono-valent cations (Na^+, K^+ etc.) and displace these mono-cations from DNA.[45]

Water is also bound along the exterior of nucleic acid structures. Its specific interactions with polar groups on bases and sugars as well as with the charged phosphate groups are essential for the stability of nucleic acid conformations.

2.4.3 Groove-binding molecules

The major and minor grooves tend to differ significantly in electrostatic potential, hydrogen-bonding characteristics, steric effects, and hydration. Many protein and oligonucleotide molecules exibit binding specificity primarily through major groove interactions while small, groove-binding molecules in general prefer the minor groove. Typically, minor groove-binding molecules share many structural similarities having several simple aromatic rings such as pyrrole, furan, or benzene connected by bonds with torsional freedom. This creates crescent shaped compounds which, with the appropriate twist, can fit into the helical curve of the minor groove with displacement of water from the groove. The interior of the crescent shape tend to contain molecules with hydrogen bonding N-H groups. These N-H groups form hydrogen bonding with A:T base pairs in the minor groove but are excluded from similar interactions with G:C base-pairs by the amino group of guanine.[38]

2.4.4 Intercalation

The foundations of the intercalative mode of binding were laid by Lerman as a result of his X-ray diffraction and hydrodynamic studies on the proflavine-DNA complex.[46] He proposed a model of binding in which the planar proflavine molecule (65) becomes inserted between the base pairs of duplex DNA. (Figure 3.9)

The following changes occur upon intercalation[47,48]:

(a) Unwinding and lengthening of the DNA helix.

Intercalation produces an extension (by 3.4Å) , unwinding, and stiffening of the DNA helix. These structural distortions are a consequence of the untwisting of the base pairs and helical backbone needed to accomodate the intercalator.

(b) Electronic interaction of the intercalator within the helix.

Intercalation results in an ordered stacking of the bound species between the base pairs at 3.4Å separation. The intercalating surface is sandwiched tightly between the aromatic, heterocyclic base pairs and stabilised electronically in the helix by π-π stacking and dipole-dipole interactions.

(c) Rigidity and orientation of the intercalator within the DNA helix.

Upon intercalation at the unwound site, there is substantial structural overlap between the base pairs and the intercalator. The intercalator becomes rigidly held and oriented with the planar moiety perpendicular to the helical axis.

The above physical and electronic changes form the basis of experimental methods used to establish intercalation:

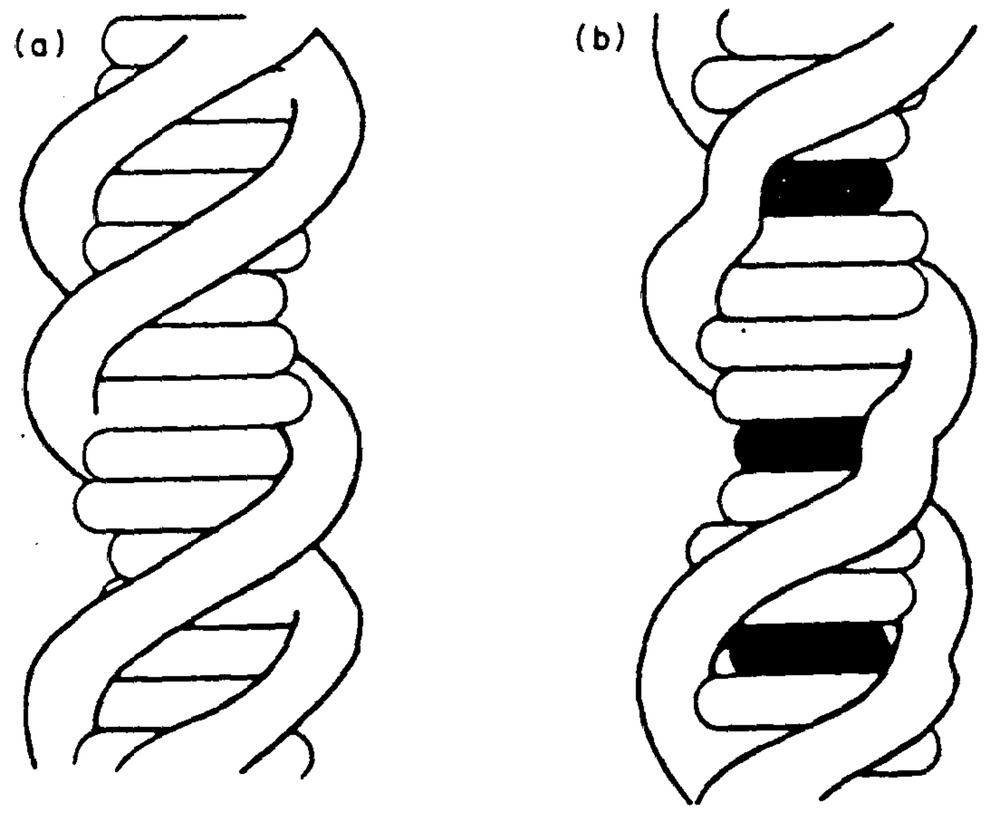


Figure 3.9 The intercalation model: (a) is a representation of B-DNA; (b) shows the same molecule containing intercalated drug molecules.

This mode of binding has been established for a large number of planar polycyclic aromatic cations. These in general contain a planar chromophore with two to four rings with an optimum of three, groups avaliable for hydrogen bonding and a positive charge.[47] (Figure 4.0) Typical binding constants for these complexes are K ~10^5 to 10^6 lM^{-1}.[47,49]

However, the description of intercalative binding is clouded by the fact that many intercalators have also been found to be groove-binders.[38,50] This is not suprising looking at the physical make-up of many intercalators (Figure 4.0). What is not clear are the factors that determine which process predominates at the surface of the helix or whether surface binding is the first step towards intercalation. Temperature-jump relaxation measurements have shown that proflavine intercalates *via* a mechanism involving initial fast bimolecular attachment to the surface of the helix, followed by one or more slower insertion steps.[47,51] It appears that the intercalation event does not require preopening of the duplex, and at equilibrium a proportion of the ligand remains externally bound. However, similar studies with ethidium have revealed quite a different mechanism involving direct intercalation with no externally bound intermediates.[52]

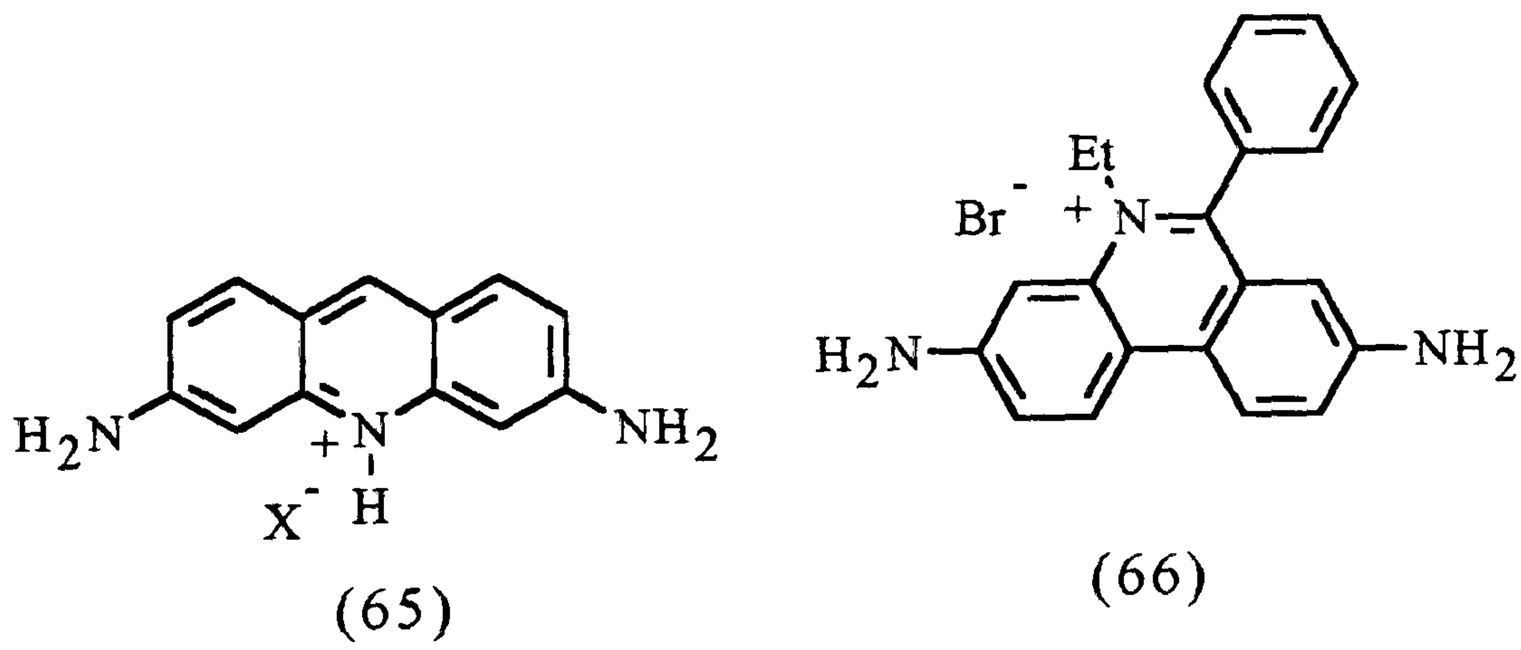
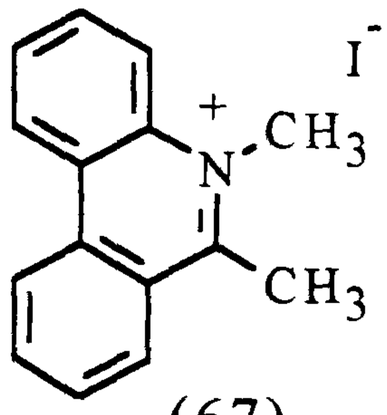


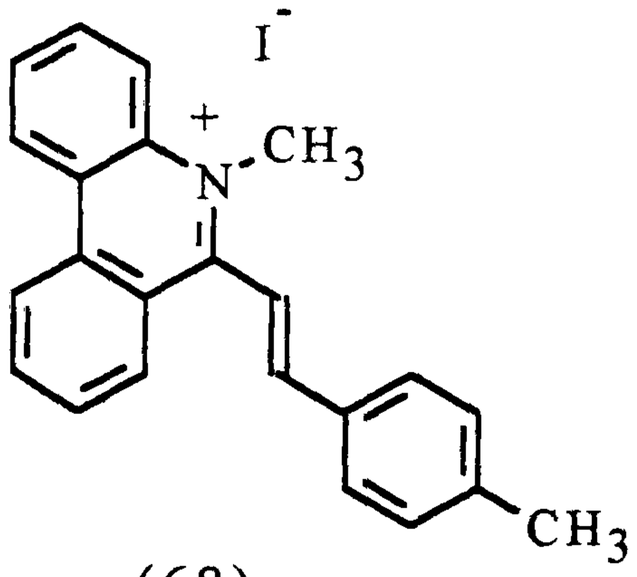
Figure 4.0

Recent studies by Lowe *et al.* using a newly developed indirect assay for unwinding of linear DNA have provided further strong evidence of intercalative binding by a range of simple quaternised phenanthridines and acridines.[53,54] (Figure 4.1)

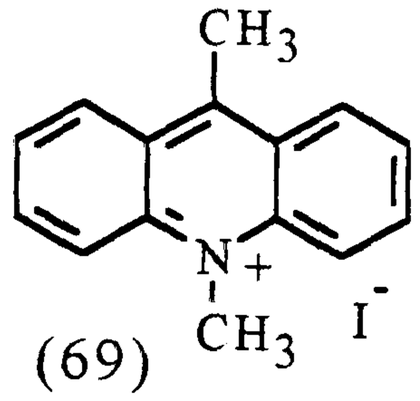
In terms of the intercalating component of our assay design, it was decided to employ either unsubstituted phenanthridine or acridine, with the sensitiser being attached through a quaternised link to the ring nitrogen of either intercalator. This would provide the neccessary positive charge on the intercalator. The absence of substituents on either intercalator also meant we avoided lengthy protection/deprotection steps before and after quaternisation.



(67)



(68)



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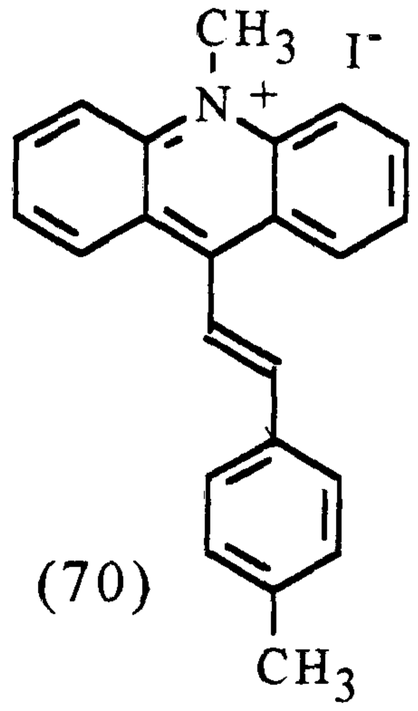


(70)

Figure 4.1

2.5 MULTIFUNCTIONAL 1,10-PHENANTHROLINES.

The phenanthrolines have been known for over one hundred years, with the parent 1,10-phenanthroline being synthesised by Blau[55] in 1898. Their discovery as metal complexing agents and subsequent use in many analytical applications provided the impetus for an extensive study of procedures for their synthesis.[35] The last ten years has seen a resurgence in the chemistry of phenanthrolines, particularly the parent 1,10-phenanthroline and its derivatives. This has followed closely the rapid development of macrocyclic chemistry, with the phenanthrolines as metal recognition centres in a host of macrocyclic frameworks.[56,57,58] (Figure 4.2) This latter period of research has seen the synthesis of a whole host of 1,10-phenanthroline derivatives, particularly those functionalised at the C-2,9 position, like PDCA, where the two carboxylates provide additional chelation sites. However, little new work appears to have been carried out on the synthesis of multifunctional phenanthrolines.

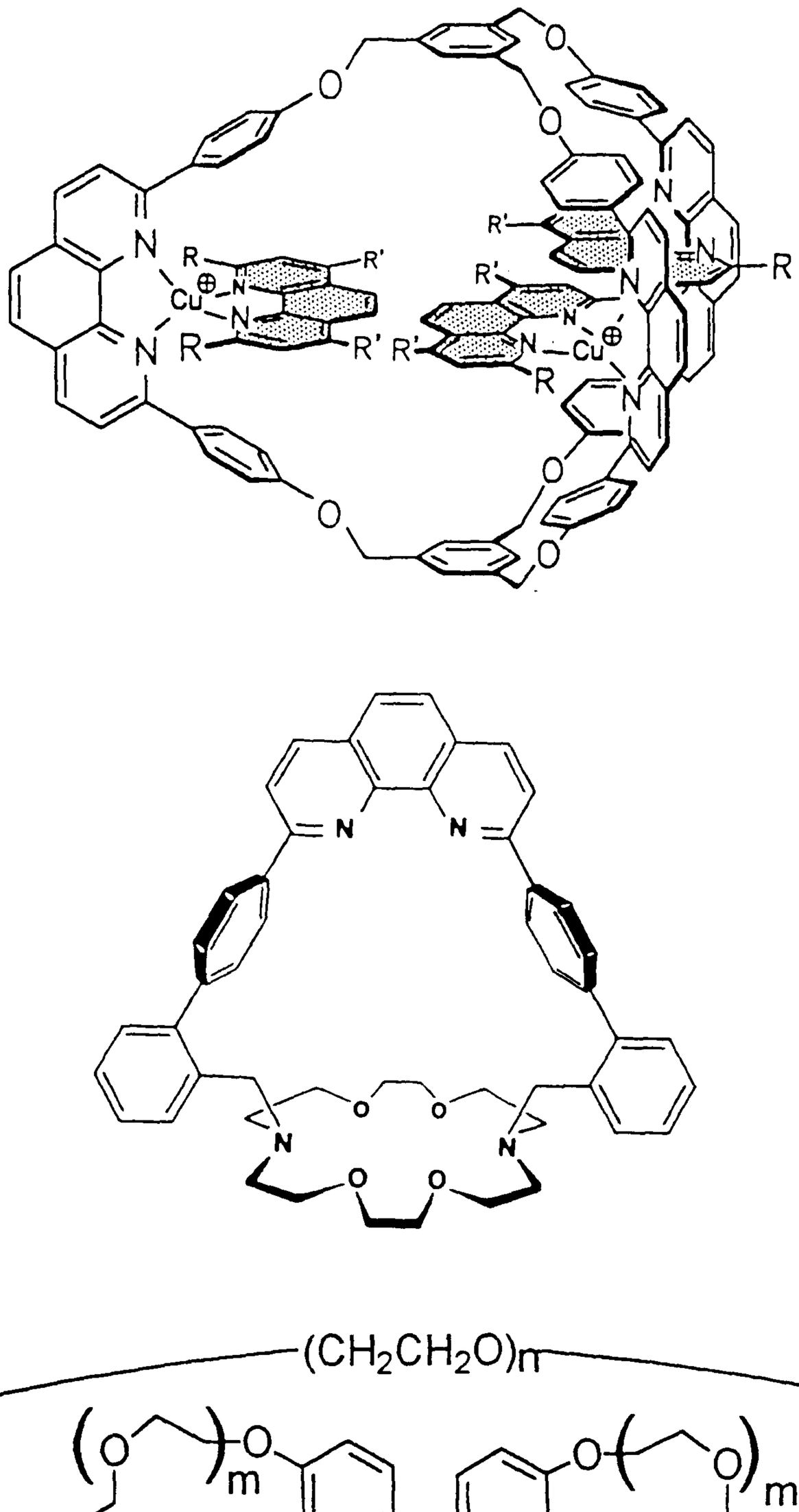


Figure 4.2

Thus a synthetic scheme to the 1,10-phenanthroline framework was required which would provide substituents next to the heteroatoms (at C2 and C9) for conversion into the carboxylic acid, and a functionality to allow attachment of potential "handles". The synthetic scheme needed to be flexible enough to allow easy variations in the chain length of the handle, which inturn needed to possess some funtionality (an alkyl halide or tosylate) which could be used for attachment to different intercalators through quaternisation.

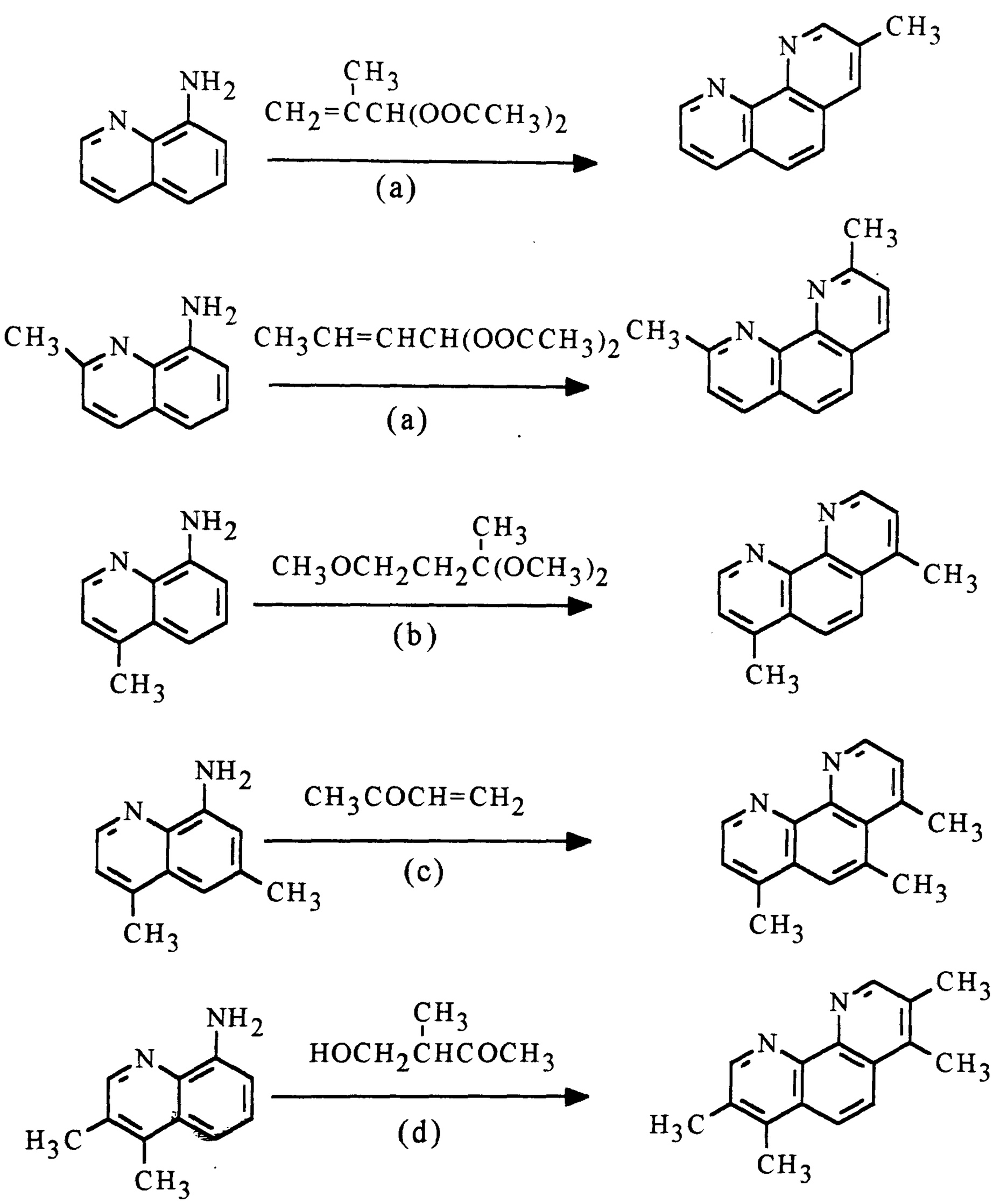
The original synthesis of 1,10-phenanthroline by Blau was by a double Skraup reaction on o-phenylenediamine using glycerol and sulphuric acid in the presence of an oxidising agent[55]. This quickly evolved into the single step Skraup ring closure reaction starting from 8-aminoquinoline, which proceeds in higher yields. To this day 8-aminoquinolines remain the starting materials of choice for the synthesis of 1,10-phenanthrolines. Case and co-workers were able to prepare numerous methyl and polymethyl derivatives of 1,10-phenanthrolines by treatment of various substituted 8-aminoquinolines under Skraup conditions[59,60,61,62] (Scheme 3.7) using:

(a) α,ß-unsaturated aldehydediacetates, intoducing methyl groups into the α- and ß-positions in the ring formed.

(b) Ketals, e.g 1,3,3-trimethoxybutane, introducing a methyl group in to the γ-position of the ring formed.

(c) α,ß-unsaturated ketones, e.g methylvinyl ketone, again introducing a methyl group into the γ-position of the ring formed.

(d) Oxoalcohols, e.g 4-hydroxy-3-methyl-2-butanone, enabling the intoduction of two methyl groups (ß and γ) into the ring formed.

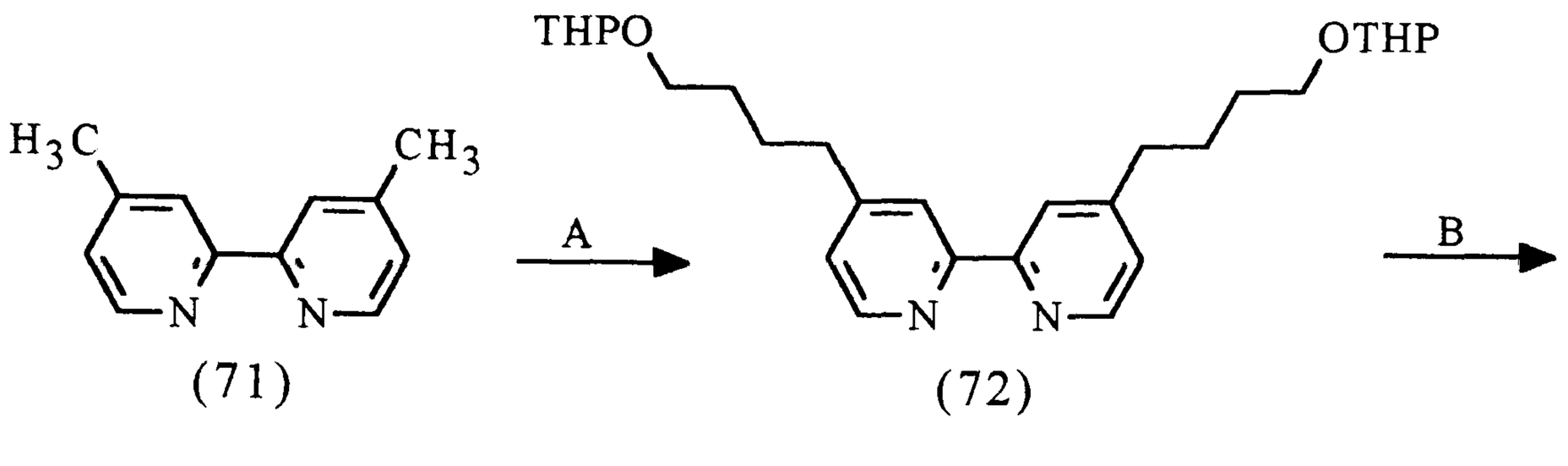


Scheme 3.7 (a-d)

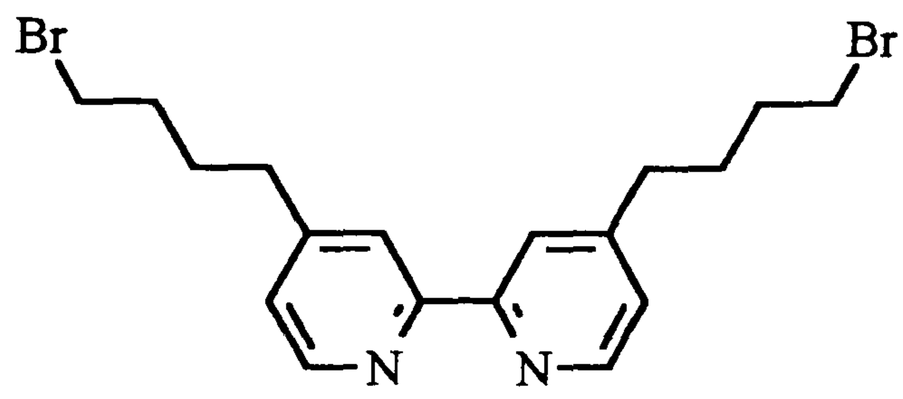
By choosing appropriate reactants we would end up with methyl groups at the desired positions in the ring (C-2,9), and a third methyl group somewhere on the phenanthroline backbone as the functional group anchor for the attachment of potential handles.

The methodology of alkylating methyl groups has already been successfully applied in the corresponding 2,2'-bipyridine series by Sauvage *et al.* in their synthesis of 4,4'-bis(4-bromobutyl)-2,2'-biyridine (73) (Scheme 3.8).[63] The treatment of 4,4'-dimethyl-2,2'-biyridine (71) with lithium diisopropylamide (LDA) followed by quenching of the

resulting dianion with THP-protected bromopropanol gave the desired alkylated bipyridine (72). Deprotection followed by bromination of the resulting alcohol gave the corresponding alkyl halide (73) as a very usefull reactive fuctionality.



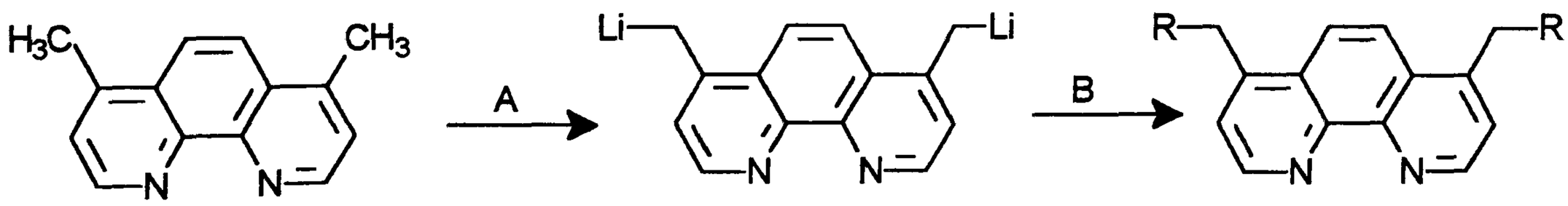
(71) (72)



(73)

Scheme 3.8 A. (i) LDA/THF, 0^{o} (ii) $Br(CH_2)_3OTHP$;
B. (i) p-TsOH, EtOH, (ii) HBr (48%), H_2SO_4

The viability of this approach has recently been demonstrated by the synthesis of 4,7-dialkylated-1,10-phenanthroline (75), by the lithiation of commercially available 4,7-dimethyl-1,10-phenanthroline (74) using LDA followed by alkylation of the dilithiated intermediate.[64] (Scheme 3.9)

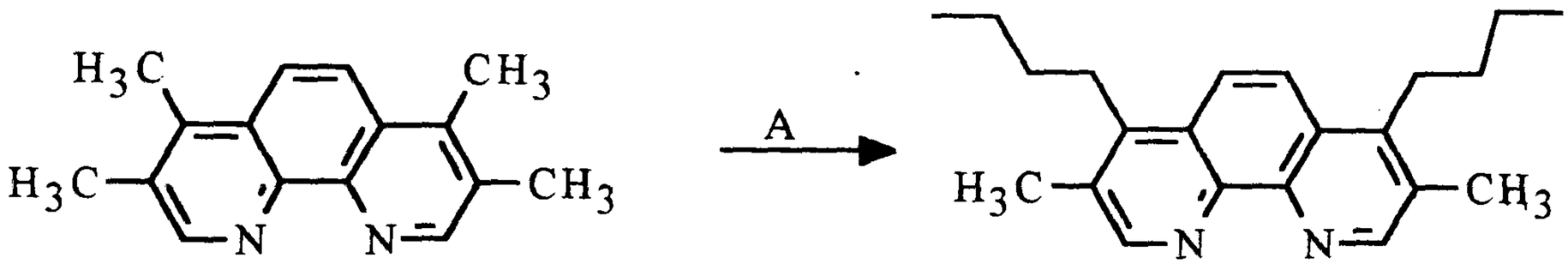


(74) (75)

R= Et, i-Bu, i-Pr
$PhCH_2$, $C_{12}H_{25}$

Scheme 3.9 A. LDA/THF, -78°C; B. RI, r.t

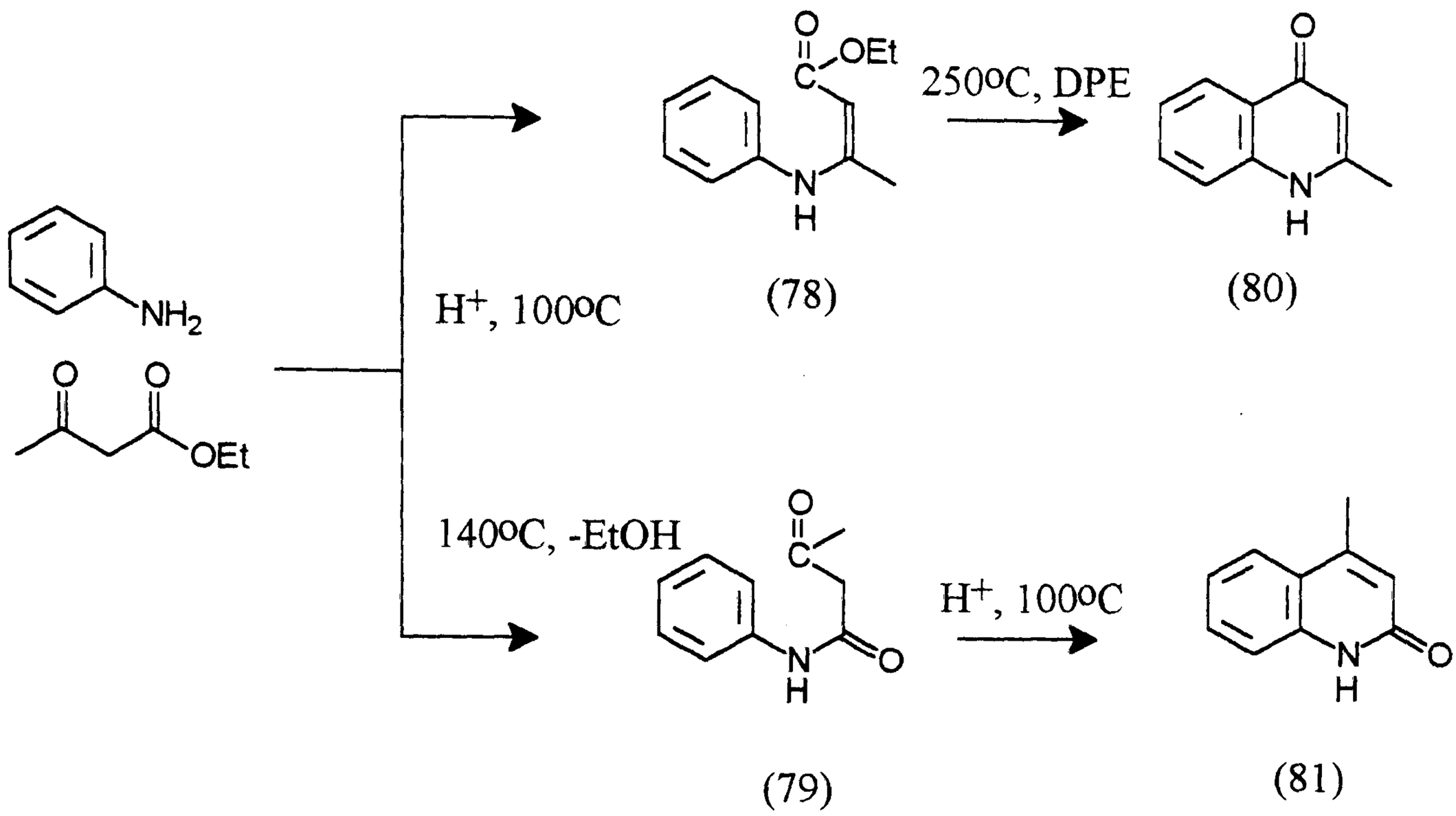
However, the choice of polymethyl phenanthrolines with a methyl group anchor were ruled out at an early stage due to the lack of discrimination, in terms of reactivity that exists between methyl groups in the ring. The 2,4-position of 1,10-phenanthroline are the most reactive and with little or no difference in reactivity followed by the other positions which are relatively unreactive. This difference in reactivity was clearly demonstrated by Katritzky *et al.* in their alkylation of 3,4,7,8-tetramethyl-1,10-phenanthroline (76) using LDA to give exclusively the 4,7-dialkylated phenanthroline (77).[64] (Scheme 4.0)



(76) (77)

Scheme 4.0 A. (i) LDA/THF, -78°, (ii) $CH_3(CH_2)_3I$

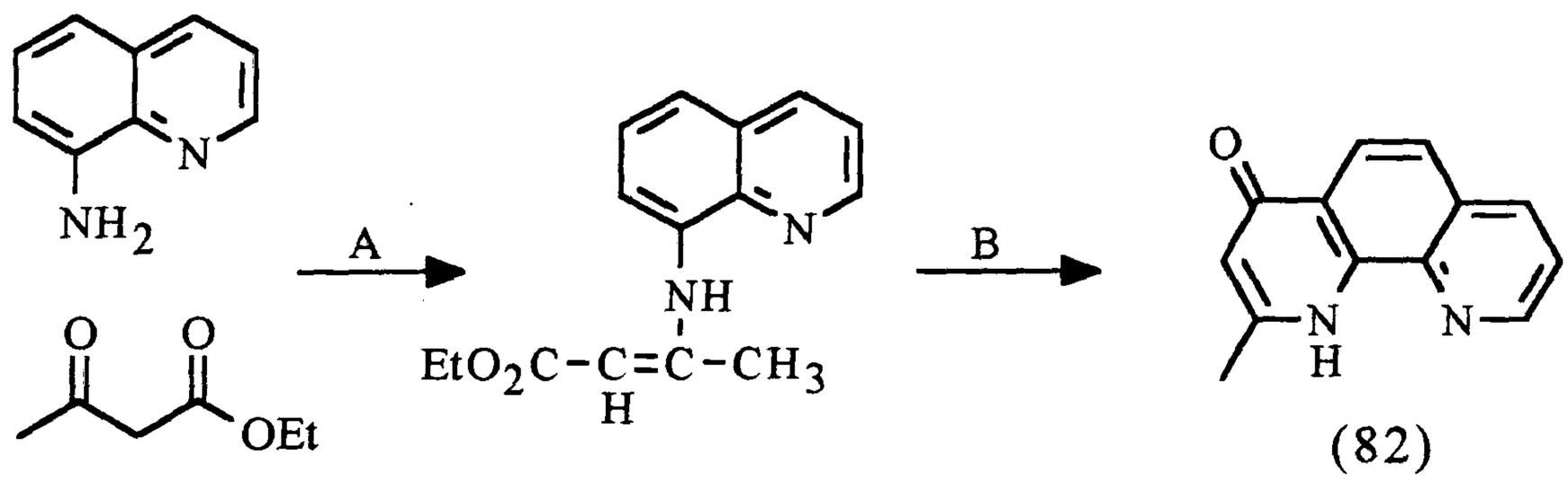
A different and successful approach was provided by the Conrad-Limpach synthesis of quinolines.[65] β-Keto esters react with primary aromatic amines in two ways: at low temperatures under acid catalysed conditions, reaction occurs at the keto group to give the imine (or enamine) (78), at high temperatures reaction occurs at the ester group to give an amide (79) (The Knorr quinoline synthesis).[65] The resulting compounds can each be cyclised to quinolines, with the acid catalysed cyclisation of the amide (79) with H_2SO_4 at 100° yielding 2-hydroxyquinoline (81) where as the imine (78) is converted into 4-hydroxyquinoline (80). (Scheme 4.1)



Scheme 4.1 (DPE= diphenyl ether)

By reacting 8-aminoquinoline with ethylacetoacetate, followed by cyclisation in paraffin oil at 270°C, Hazelwood *et al.* were able to obtain 4-hydroxy-2-methyl-1,10-phenanthroline (82). (Scheme 4.2)[66]

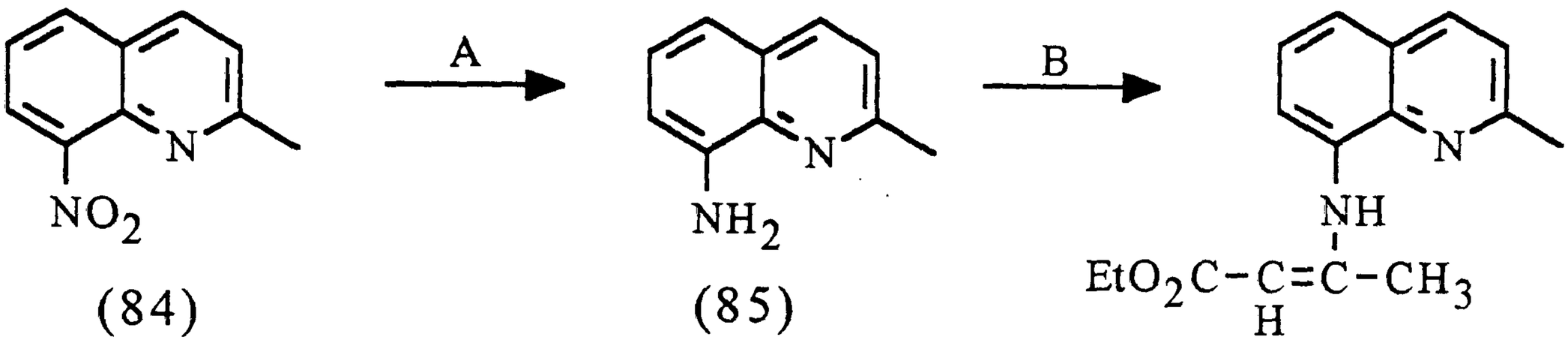
The yield of the thermal cyclisation was later improved by substituting boiling diphenyl ether for the hot parafin oil.



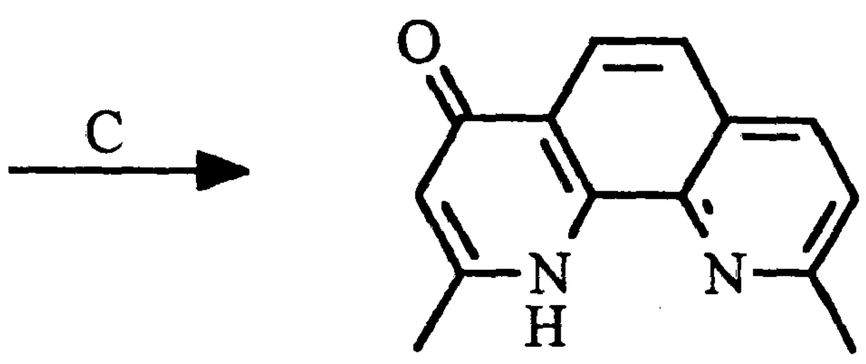
Scheme 4.2 A. dil. HCl, 100°; B. Parafin oil, 270°

From the work of Hazelwood, 4-hydroxy-2,9-dimethyl-1,10-phenanthroline (83) was identified as potentially possessing the correct functional groups to enable the synthesis of a new class of Eu^{3+} sensitiser which could function under our homogeneous assay conditions according to the general scheme 2.4.

This initial target (83) was synthesised according to the method of Case[67] by a Conrad-Limpach reaction of ethyl acetoacetate with 2-methyl-8-aminoquinoline (85). (Scheme 4.3)



(84) (85) (86)



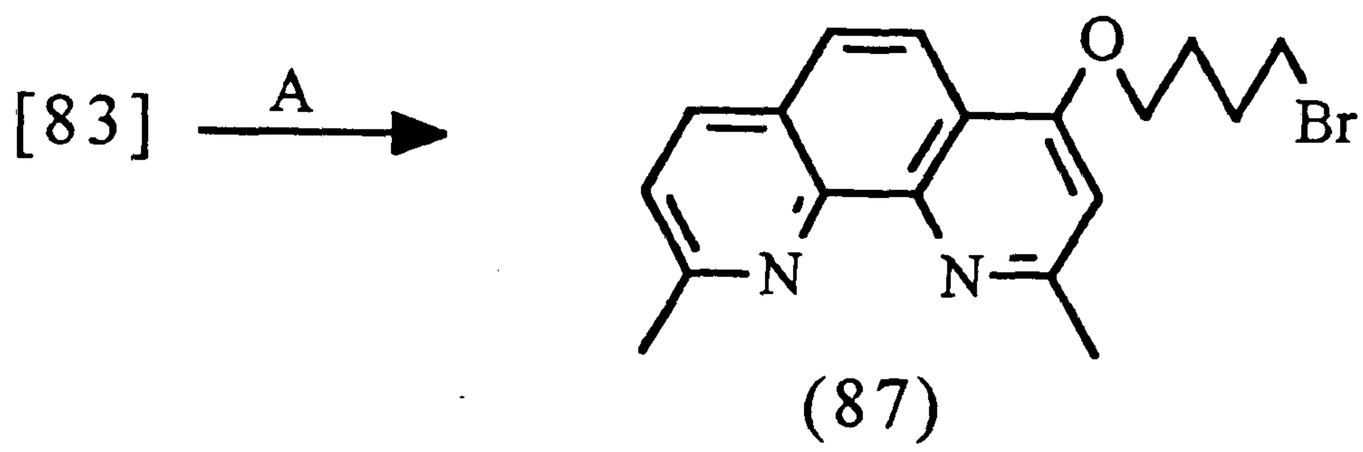
(83)

Scheme 4.3 A. Hydrazine hydrate, 10%Pd/C, MeOH;
B. Ethylacetoacetate, H^+, PhH; C. Diphenyl ether, 255°

2-Methyl-8-aminoquinoline (85) was obtained in almost quantitative yield by reduction of 2-methyl-8-nitroquinoline (84) by catalytic transfer hydrogenation using hydrazine hydrate and 10% Pd/C in methanol. This was then reacted with ethylacetoacetate in refluxing dry benzene with a trace of acetic acid as catalyst, removing the water as formed. No attempt was made to purify the intermediate enamine (86), obtained as a brown solid following the removal of excess benzene. Thermal cyclisation of the intermediate enamine (86) was carried out in boiling diphenyl ether at 255°C. The desired product, 4-Hydroxy-2,9-dimethyl-1,10-phenanthroline (83), was obtained as an off-white powder by precipitation from the cooled diphenyl ether solution on addition of a large volume of 100-120 Petroleum Ether. The last traces of diphenyl ether could be removed from the product by repeated boiling in petroleum ether. Spectroscopic analysis of the pure product obtained by recrystallisation from water clearly showed this compound to exist mainly in the tautomeric pyridone form, the NH group being strongly hydrogen bonded to the heterocyclic ring nitrogen. Although this was expected, there was concern

that attempts to attach a handle to the phenanthroline framework at the 4-OH position via O-alkylation, using a simple alkyl dihalide would be complicated as the N-alkylation of pyridones is much more favoured.

However, our fears proved to be groundless, and selective O-alkylation of (83) with 1,4-dibromobutane in acetonitrile using potassium carbonate proceeded smoothly, giving the alkyl ether (88) in over 70% yield after chromatography. (Scheme 4.4)
Yield of the O-alkylation was further increased to above 80% by using caesium carbonate as the base.

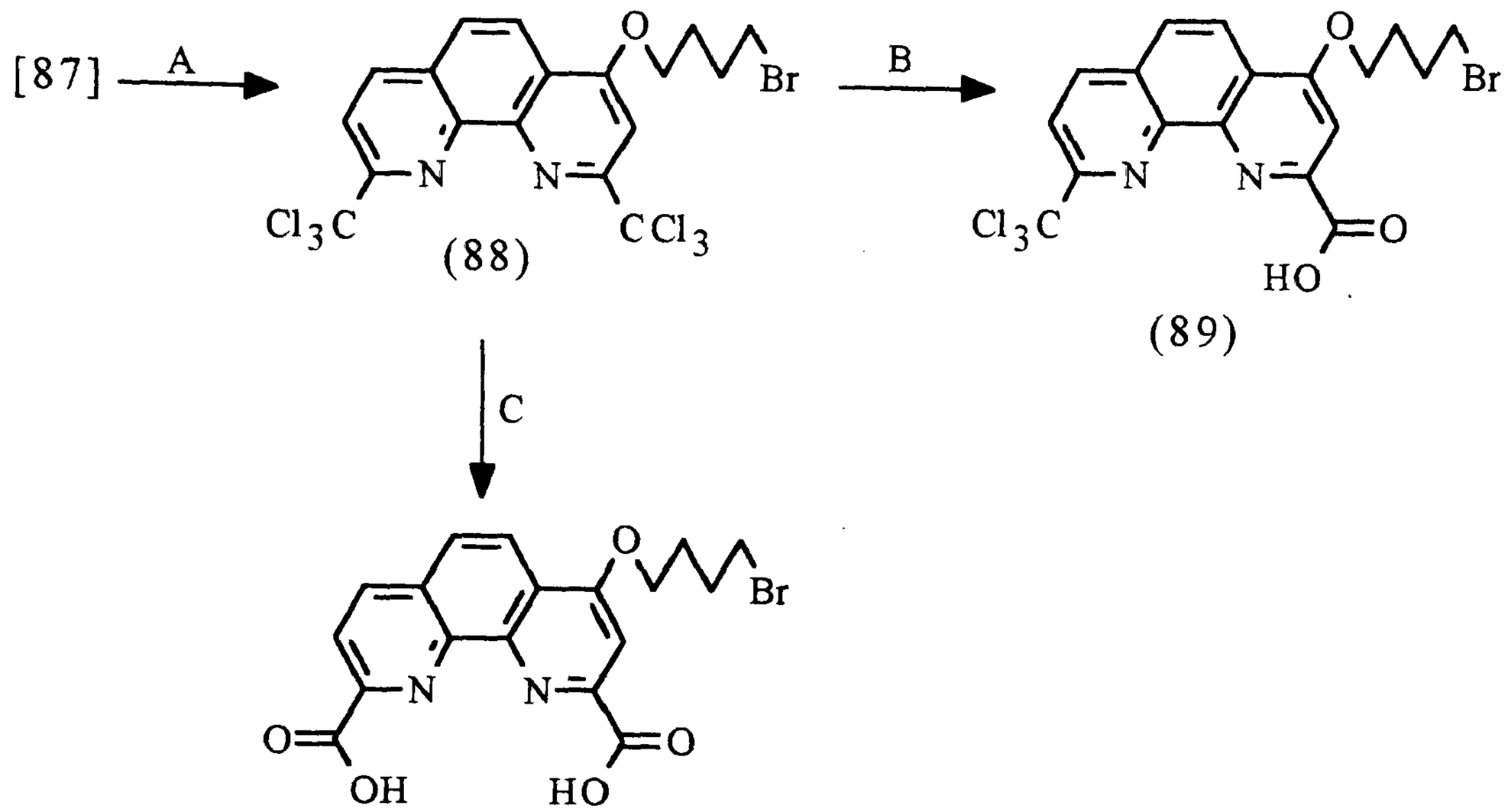


(87)

Scheme 4.4 A. $Br(CH_2)_4Br$, Cs_2CO_3, MeCN

The two stage route to the diacid (90) (Scheme 4.5), involved hydrolysis of the bis-trichloromethyl derivative (88), which was obtained in excellent yields by the hexachlorination of the dimethyl derivative (83), using N-chlorosuccinimide in dry CCl_4 with m-CPBA as initiator. However, the second stage involving the hydrolysis to the diacid (90) caused many problems. Hydrolysis of tri-chloromethyl derivatives to the corresponding acids is normally achieved by heating in concentrated sulphuric acid (see Scheme 3.4). This was not successful with the acid sensitive alkyloxy side chain present, hydrolysis being accompanied by degradation of the side chain.
Attempts at reducing the reaction time of the hydrolysis reaction to less than 1h was only partially successful with the monoacid-monotrichloromethyl derivative (89), with its alkyloxy side chain intact, isolated by chloroform extraction as a mustard coloured fine powder in 50% yield. The problem appeared to be the HCl that is produced during the course of the hydrolysis of the trichloromethyl groups. After several attempts we found that hydrolysis of (88) could be achieved in buffered acetic acid (glacial acetic acid/sodium acetate), to give the required diacid (90) as an off-white solid.

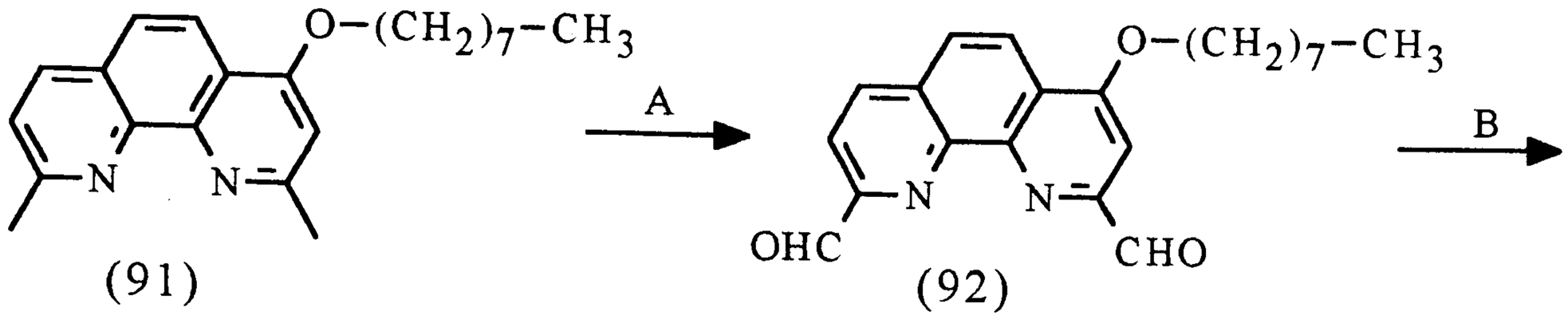
However, the yield of the pure diacid (90) after co-solvent recrystalisation from THF/H_2O was very dissapointing.

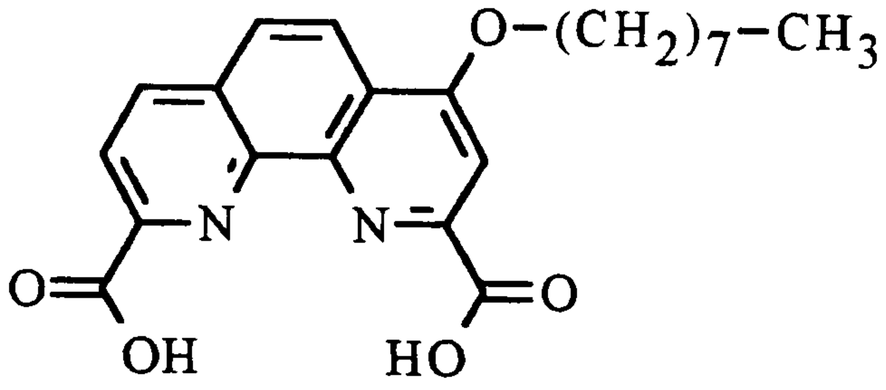


Scheme 4.5 A. NCS, CCl_4, m-CPBA; B. c.H_2SO_4, 1h;
C. AcOH/NaOAC

It was heartening to discover towards the end of our work that the problem of synthesising the 1,10-phenanthroline nucleus containing both the alkoxy link and the bis-2,9-carboxcylic acid funtionality had posed similar problems to others.

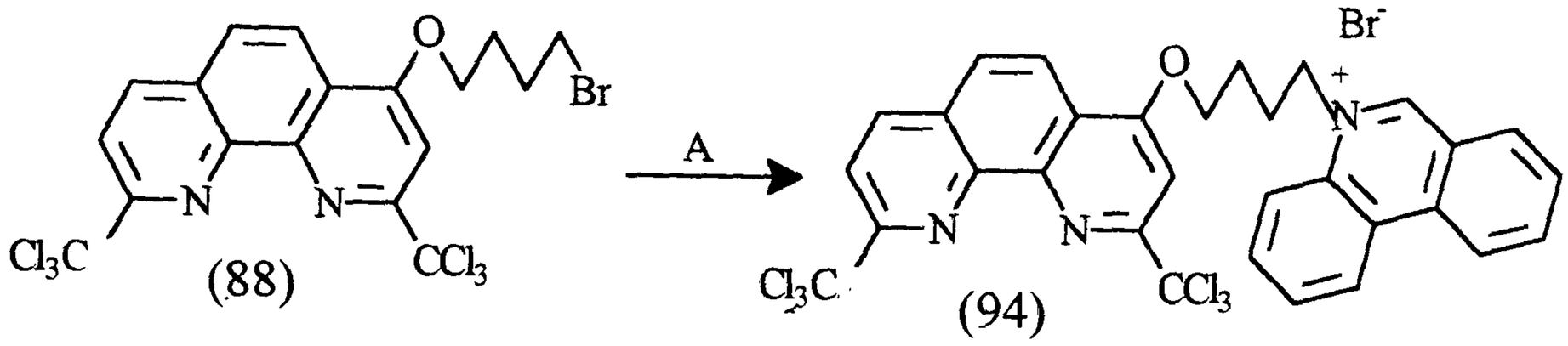
In their synthesis of potentiometric sensors based on 4-octyloxy-1,10-phenanthroline-2,9-dicarboxcylic acid (93), Deady and co-workers[68] initial approach to the acid (93) was through a standard two-stage oxidation by way of the aldehyde (92). But the second stage involving the oxidation of the aldehyde (92) to the acid (93) using concentrated nitric acid was not successful with the alkoxy link present. The oxidation was finally performed at room temperature in high yields by using the mild oxidant sodium chlorite in aqueous *tert*-butyl alcohol with 2-methyl-2-butene as a chlorine scavenger. (Scheme 4.6)





Scheme 4.6 A. SeO_2, Dioxan; B. $NaClO_2$, ^tBuOH, isobutene

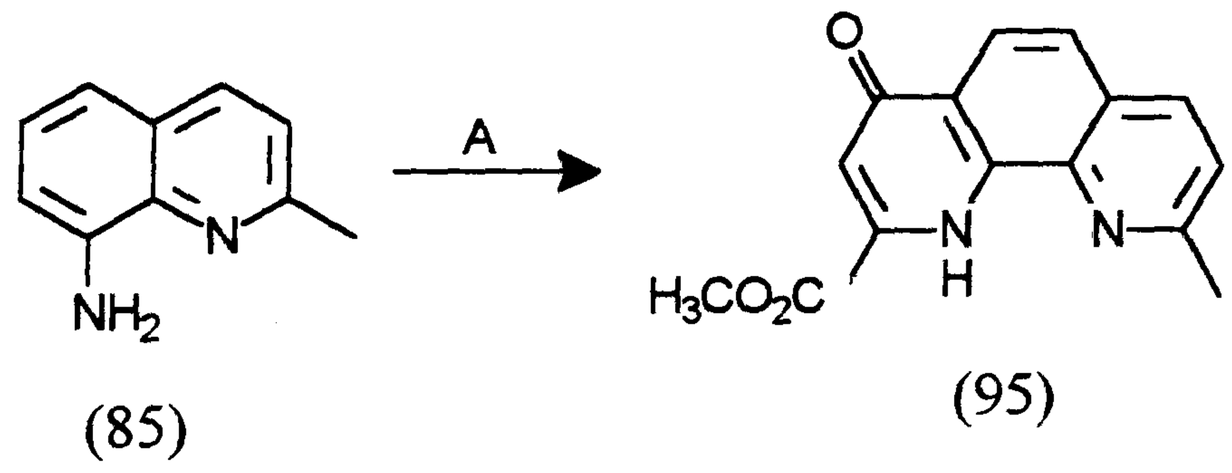
An initial attempt at attaching the intercalator to the sensitiser was carried out with 4-bromobutyloxy-2,9-bistrichloromethyl-1,10-phenanthroline (88). Quaternisation reactions are normally carried out in solvents like nitrobenzene or where the alkylating agent also acts as the solvent. Attempts to react phenanthridine and (88) in nitrobenzene at 140°C failed with no sign of reaction. The N-alkylation of phenanthridine was eventually achieved in good yields by carrying out the reaction as a neat melt, using excess phenanthridine as the reaction medium at 115°, to which the alkylated phenanthroline (88) was added portionwise with stirring. (Scheme 4.7) The reaction is over within two hours and once allowed to cool, it sets into a very hard mass which is initially stirred with diethyl ether and filtered to give a fine light-brown powder. Final purification is achieved by dissolving the crude quaternised material (94) in $CHCl_3$ and adding it dropwise to stirred diethyl ether, precipitating the product as a pale yellow solid. Attempts at alkylating phenanthridine with the biscarboxylic acid derivative (90) failed giving an intractable material which could not be identified.



Scheme 4.7 A. Phenanthridine, 115°C

Although, by the use of glacial acetic acid/sodium acetate modification we were able to effect the hydrolysis of the bis(trichloromethyl) derivative (88) to the corresponding bis(carboxylic acid) (90), the persistent low yields combined with failures in the quaternisation reaction convinced us of the need for a better synthetic approach to our desired target.

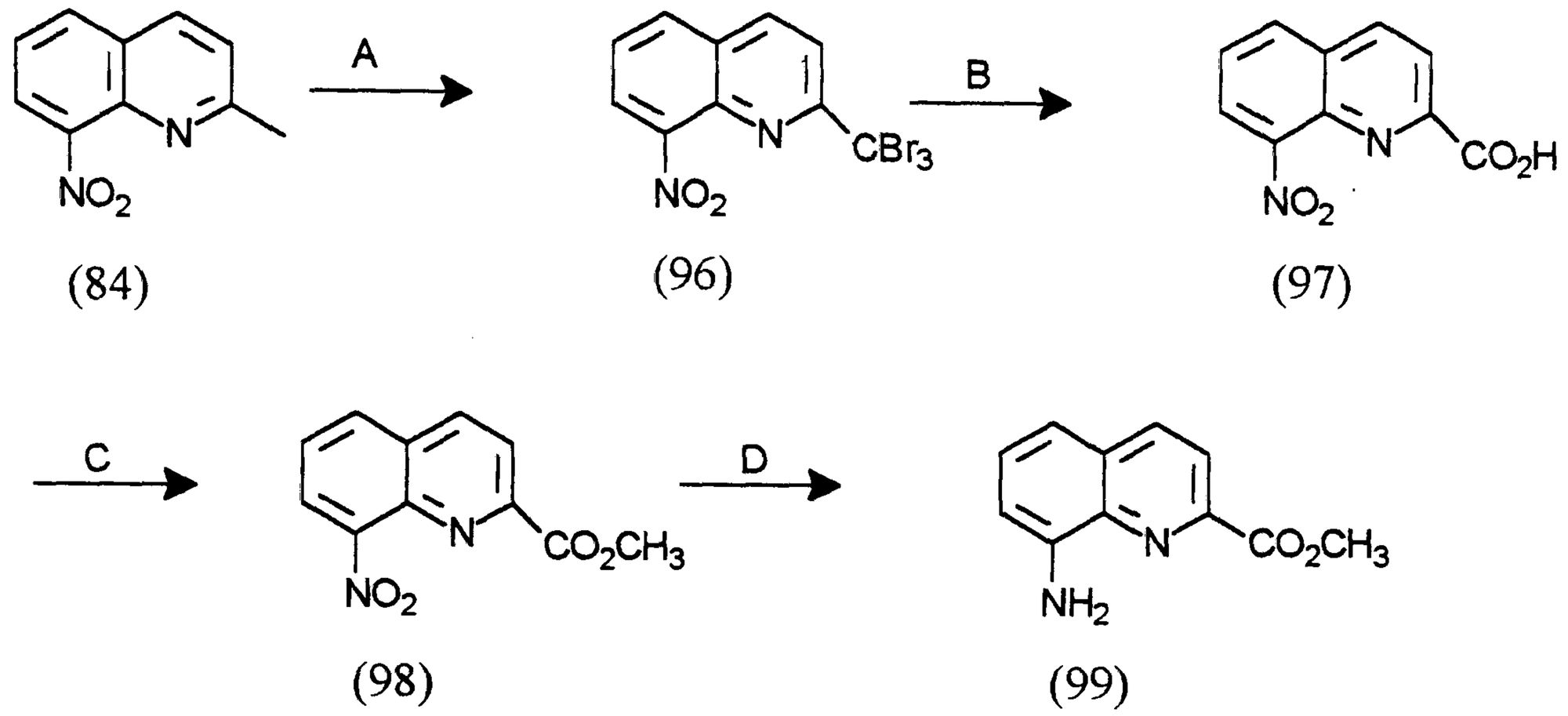
In amongst a rather long winded synthetic approach to 2-methyl-1,10-phenanthroline, Heindel *et al.* reported the synthesis of 2-carboxymethyl-4-hydroxy-9-methyl-1,10-phenanthroline[69] (95). This compound was obtained by a modified Conrad-Limpach procedure employing 8-amino-2-methylquinoline (85) and dimethyl acetylenedicarboxylate. (Scheme 4.8)



Scheme 4.8 A. Dimethylacetylenedicarboxylate, MeOH

It became obvious that by reacting an appropriately functionalised aminoquinoline, namely 8-amino-2-carboxymethylquinoline (99) with dimethyl acetylenedicarboxylate we should obtain 4-hydroxy-2,9-bis(carboxymethyl)-1,10-phenanthroline (101), following the thermal cyclisation of the corresponding enamine derivative (101).

The synthesis of 8-amino-2-carboxymethylquinoline (99) was reported in the early 1950s by Roth and Erlenmeyer[70]. Their synthesis starts from commercially avaliable 8-nitro-2-methylquinoline (84). (Scheme 4.9)



(84) (96) (97)

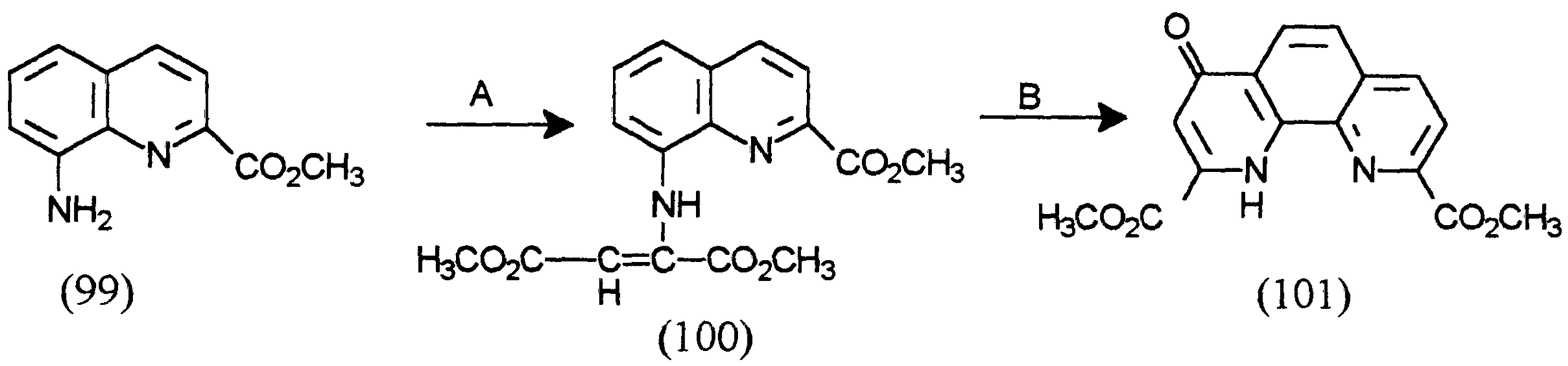
(98) (99)

Scheme 4.9 A. AcOH, NaOAC, Br_2; B, 20% H_2SO_4, 80°C;
C. MeOH, H^+; D. Cyclohexene, 10%Pd/C, MeOH

The tribromomethyl derivative (96) was obtained in excellent yields by the bromination of 8-nitro-2-methylquinoline using acetic acid, sodium acetate and bromine. Hydrolysis to the corresponding monocarboxylic acid (97) was achieved using 2M sulphuric acid at reflux, the acid being obtained as a white solid in high yields. Suprisingly, the use of standard hydrolysis conditions of concentrated sulphuric acid at 90°C failed completely with the starter being recovered unchanged.

Following esterification of the acid (97) in methanol, alternative methods of reduction to that reported by Roth and Erlenmeyer of the corresponding nitroester (98) to 8-amino-2-carboxymethylquinoline (99) were investigated. The best of these was again catalytic transfer hydrogenation with 10% Pd/C in methanol, this time using cyclohexene as the hydrogen source; under these conditions it is converted to benzene, thus providing the hydrogen source necessary to effect the reduction[71]. Under these conditions the reduction of the nitro group proceeds smoothly and in very high yields. Isolation is simply by hot filtration over celite, followed by removal of the solvent *in vacuo*.

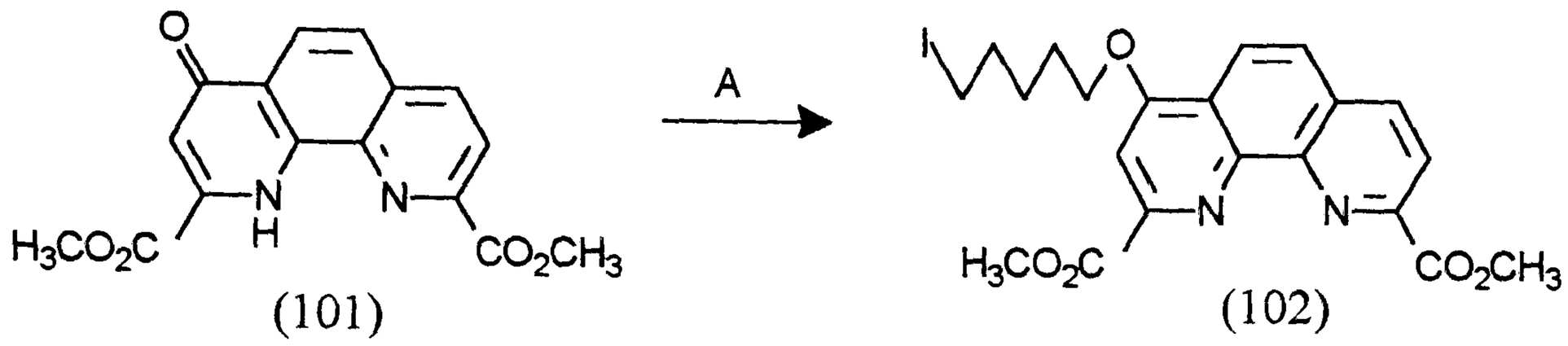
By reacting 2-carboxymethyl-8-aminoquinoline (100) with dimethyl acetylenedicarboxylate under reflux in dry methanol, the intermediate enamine derivative (100) was obtained as a bright yellow solid after removal of solvent. This was used without further purification. The thermal cyclisation was carried out in boiling diphenyl ether at 255° , with the enamine derivative (100), being added portion wise over 15 minutes to the boiling diphenyl ether solution. After approximately 30 minutes of reaction time, the solution was allowed to cool before the addition of a large volume of 100-120° Petroleum ether resulting in the precipitation of 4-hydroxy-2,9-bis(carboxymethyl)-1,10-phenanthroline (101), isolated by filtration in 60% yield. (Scheme 5.0)



Scheme 5.0 A. Dimethylacetylenedicarboxylate, MeOH;
B. DPE, 255°C

Attempted O-alkylation of (101) under conditions used previously for the O-alkylation of 4-hydroxy-2,9-dimethyl-1,10-phenanthroline (83), namely caesium carbonate in acetonitrile gave very poor results with tlc [silica gel: 5%MeOH/$CHCl_3$ or neutral alumina: $CHCl_3$ showing a multitude of fluorescent spots. Studies by Hopkins *et al* on the alkylation of 4-hydroxypyridones and the factors in determining the proportion of either N-or O-alkylation identified the effects of the nature of the alkylating agent, the solvent and the base employed[72]. By variation of the solvent and metal ion, virtually exclusive nitrogen or oxygen alkylation could be obtained.

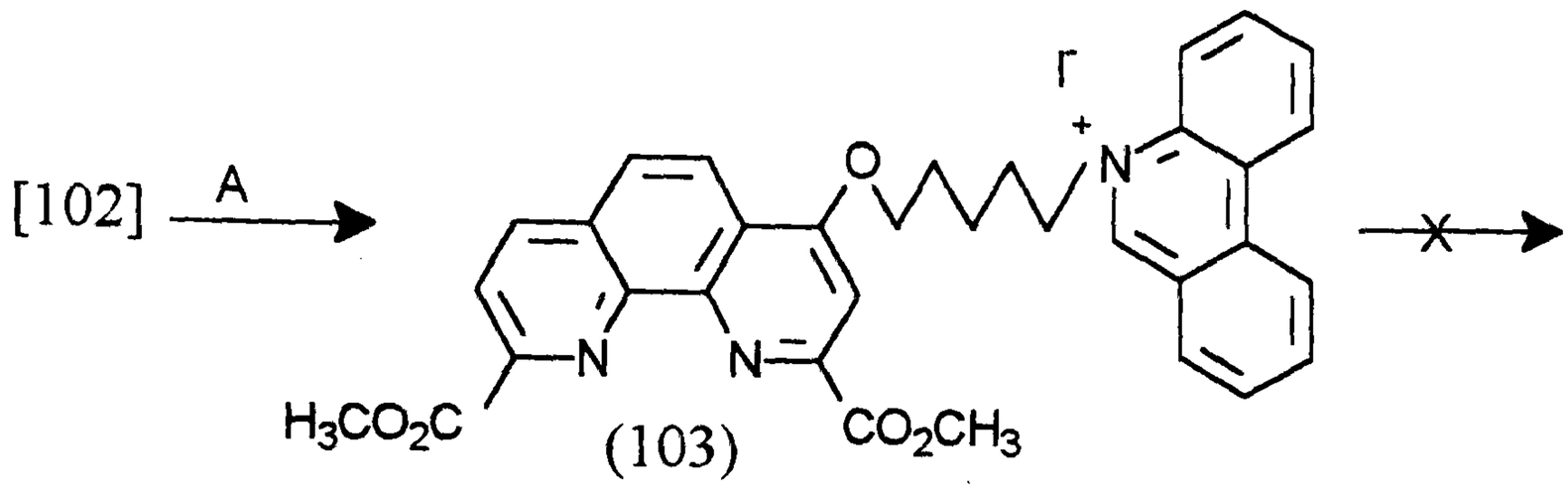
In the end O-alkylation to give 4-(5-iodopentyloxy)-2,9-biscarboxymethyl-1,10-phenanthroline (102) was achieved by the use of anhydrous silver carbonate in benzene with 1,5-diiodopentane as the alkylating agent, the reaction mixture being protected from light during the course of the reaction. (Scheme 5.1)



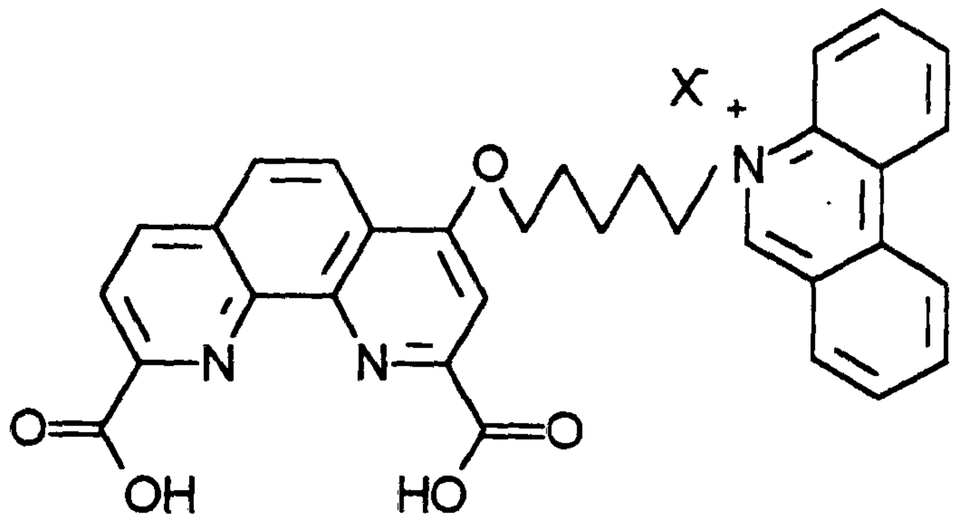
(101) (102)

Scheme 5.1 A. $I(CH_2)_5I$, $AgCO_3$, PhH

Isolation and purification of the O-alkylated phenanthroline (102) proved to be a major problem with extensive degradation being observed on attempts at column chromatography using either silica or neutral alumina. However, we were able to isolate some of the O-alkylated product (102) (≈28% yield), although an analytically pure sample was never obtained. This material was subsequently used for the quaternisation of phenanthridine, the reaction being carried out as a neat melt with excess phenanthridine as the reaction medium. (Scheme 5.2) The desired quaternised phenanthridine (103) was obtained in an analytically pure state as pale yellow microcrystals by slow precipitation from a solution in chloroform by the controlled addition of diethyl ether. However, the overall yield turned out to be very poor (<15%), and this coupled with the problems we encountered in the final step involving hydrolysis of the ester functions to give the desired biscarboxcylic acid as our target compound (104), forced us to search for other approaches prefeably those involving a more stable link between the sensitiser and the intercalator.



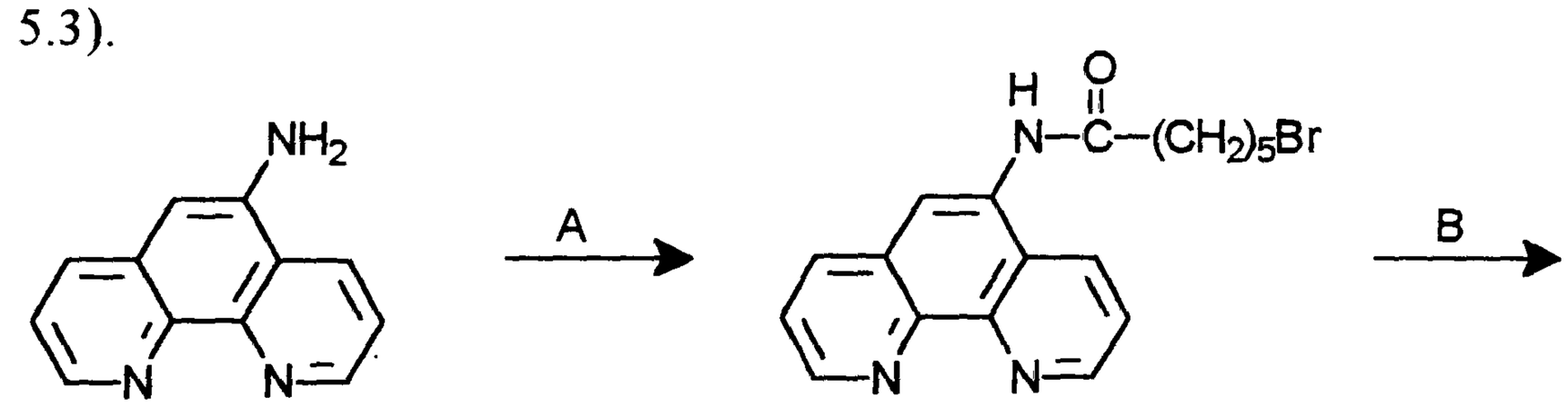
(103)



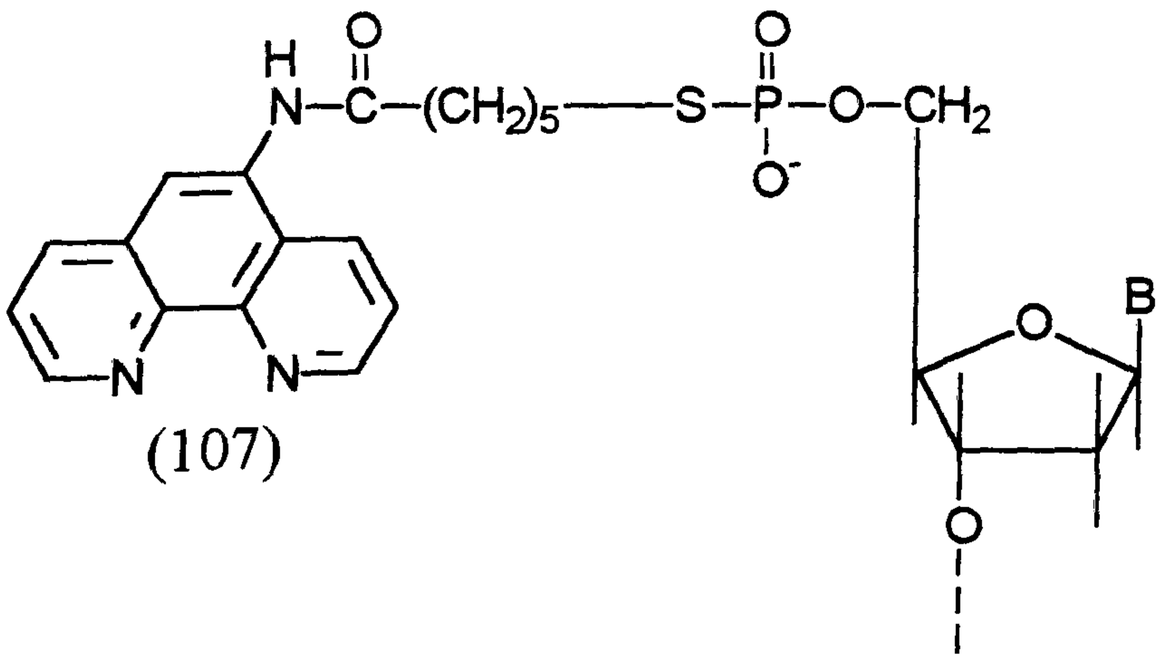
(104)

Scheme 5.2 A. Phenanthridine, 115°C

In recent years oligonucleotides carrying reactive groups have been developed to direct specific reactions at preselected sequences on single-stranded nucleic acids. One such group are the Cu-phenanthroline chelates, recently reported by Helene *et al.*[73,74] (Scheme 5.3).



(105) (106)



(107)

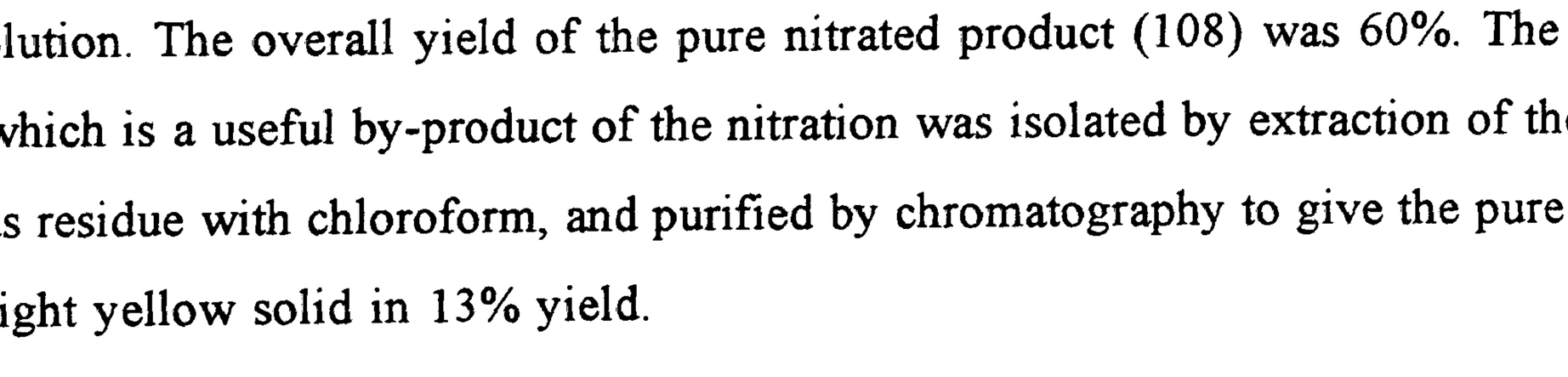
Scheme 5.3 A. $Br(CH_2)_5COCl$, $(iPr)_2NEt$, MeCN;
B. Pharmacia automatic synthesiser

Covalent linkage of the phenanthroline derivative to the 5'-end of the oligodeoxynucleotide was achieved via reaction of the thiophosphate group with 5-(halogenoalkylamido)-1,10-phenanthroline (106). This derivative (106) is easily prepared from 5-amino-1,10-phenanthroline (105) by reaction with the appropriate acid halide. Thus, the 5-amino functionality represents, potentialy a very convenient anchor with which to attach alkylhalide handles for subsequent reaction with potential intercalators via quaternisation.

Our initial target was the synthesis of 5-nitro-2,9-dimethyl-1,10-phenanthroline (108). Electrophilic substitution of the electron-deficient phenanthrolines requires drastic conditions and occurs preferentially in the benzene ring.

The nitration of 2,9-dimethyl-1,10-phenanthroline (neocuproine) (50) was achieved using fuming nitric and sulphuric acid at 140°C. (Scheme 5.4) The desired 5-nitro-2,9-dimethyl-1,10-phenanthroline (108) was isolated and purified by first neutralisation of the reaction mixture by the addition of solid sodium carbonate, resulting in the precipitation of the crude nitrated product, and subsequent purification by reprecipitation from dilute sulphuric acid solution. The overall yield of the pure nitrated product (108) was 60%. The dione (109) which is a useful by-product of the nitration was isolated by extraction of the total aqueous residue with chloroform, and purified by chromatography to give the pure dione as a bright yellow solid in 13% yield.

Attempts at nitration of neocuproine using concentrated nitric and sulphuric acid failed with the starter being recovered.



(50) (108) (109)

Scheme 5.4 A. c. HNO_3 c. H_2SO_4

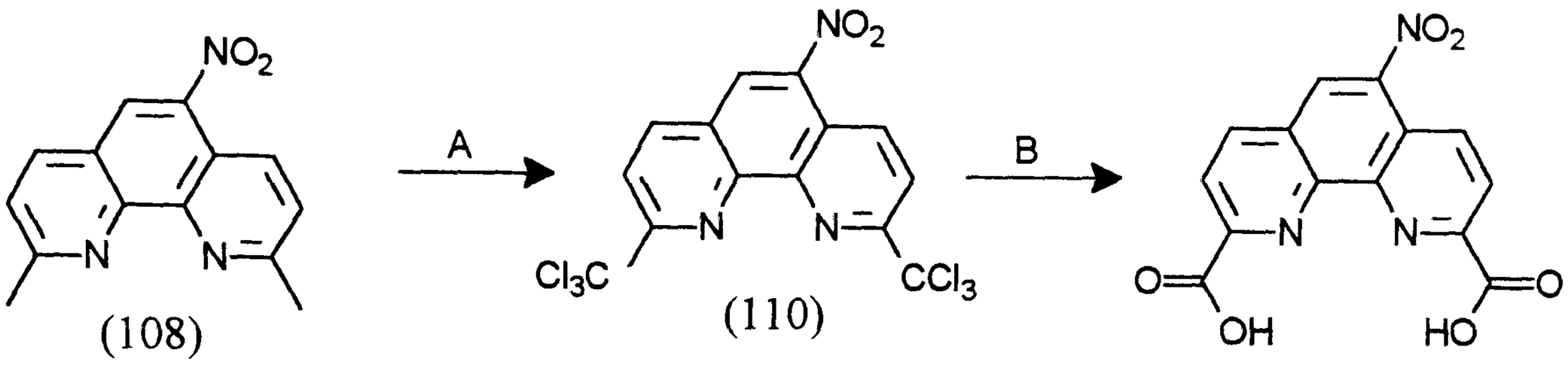
Our next envisaged target was 5-amino-2,9-bis(carboxymethyl)-1,10-phenanthroline (113). The synthetic route to this derivative is outlined in Scheme 5.5, and contains many of the synthetic transformations already carried out on earlier phenanthroline systems containing the 2,9-dimethyl functionality.

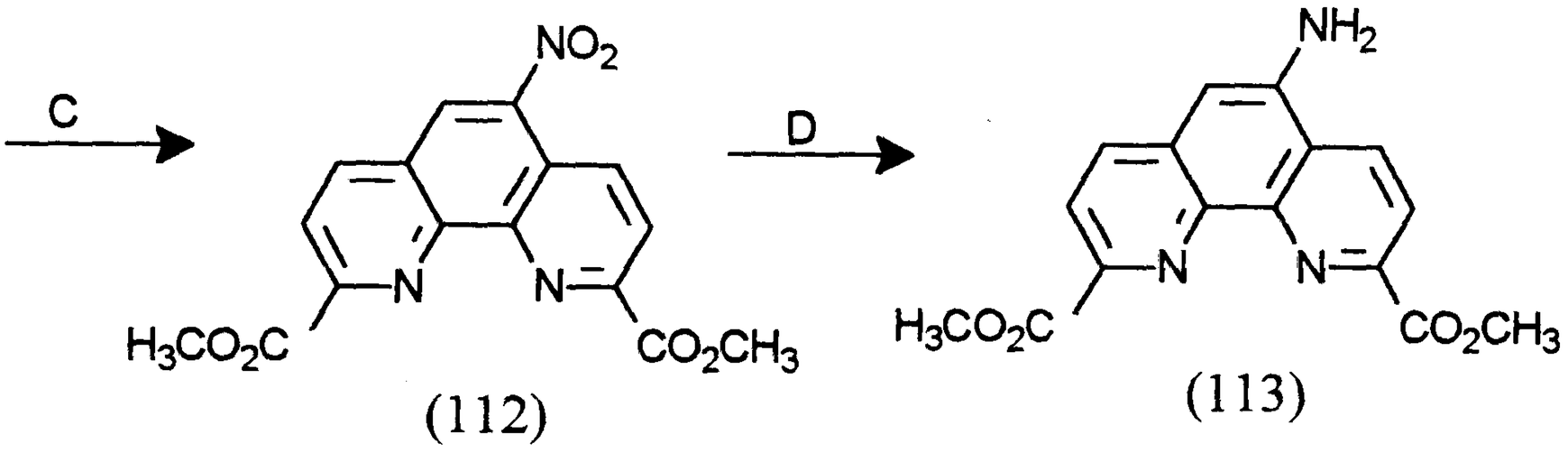
Hexachlorination of 5-nitro-2,9-dimethyl-1,10-phenanthroline (108) to 5-nitro-2,9-bis(trichloromethyl)-1,10-phenanthroline (110) was readily achieved by reaction with excess N-chlorosuccinimide (7 equivalents) in carbontetrachloride/chloroform solution with 3-chloroperoxybenzoic acid as initiator. The chloroform was neccessary as the nitrated material (108) had limited solubility in carbontetrachloride. The crude yields were excellent (>95%) and purification was readily achieved by column chromatography using chloroform as the eluent.

Hexachlorination is in fact a convenient way to separate the crude products of the nitration of neocuproine, and both the nitro-hexachloromethyl derivative (110) and the 5,6-dione-2,9-bis(trichloromethyl)-1,10-phenanthroline derivative, can be isolated in high yields by column chromatography.

Conversion of the hexachloride (110) to the corresponding 5-nitro-2,9-dicarboxylic acid (111) was again carried out in concentrated sulphuric acid at 90°C for 5 hours. The crude product was obtained as a yellow solid after quenching by addition of the reaction mixture to crushed ice. The pure diacid was obtained in high yields after recrystallisation from hot water/tetrahydrofuran as a pale yellow solid.

Fluorometric studies with this compound in the presence of Eu^{3+} and an EDTA derivative indicated that the electron withdrawing nitro group has a detrimental effect on the luminescence properties of the ligand. These results back the general findings of Mukhala *et al.* that the best relative luminescence yields are obtained for sensitising ligands with electron donating substituents (Me, Ph), electron withdrawing subtituents (NO_2, COOH) having a reverse effect (Chapter 1, Table 1.5).





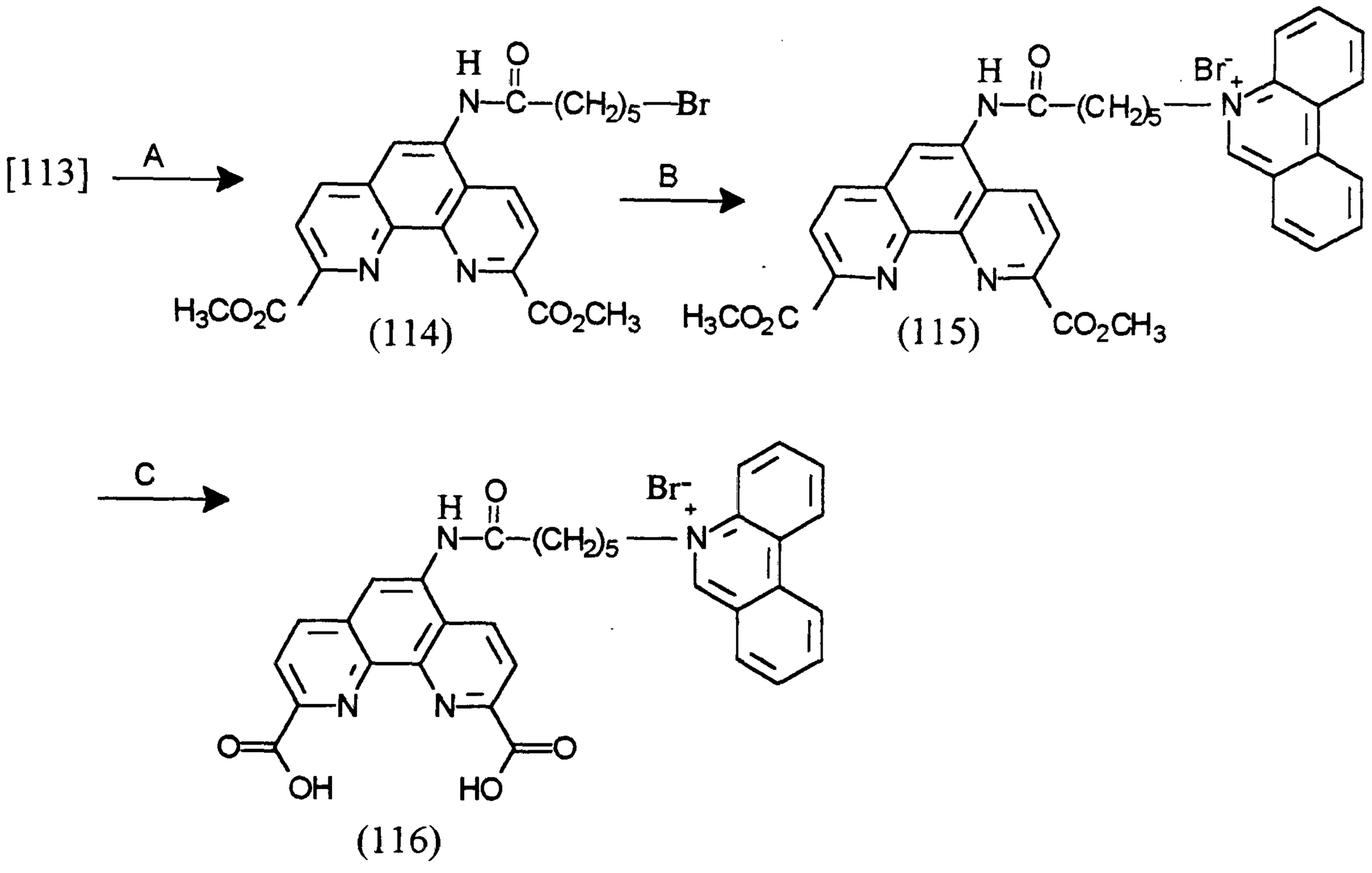
Scheme 5.5 A. NCS, $CCl_4/CHCl_3$, m-CPBA; B. c. H_2SO_4, 90°C;
C. MeOH, H^+; D, Cyclohexene, 10%Pd/C, MeOH

By quenching the hydrolysis reaction of the hexachloride derivative (110) with dry methanol instead of water 5-nitro-2,9-bis(carboxymethyl)-1,10-phenanthroline (112) is obtained. The yield of the pure product after recrystallisation from hot methanol/chloroform is >80%.

Reduction of the nitrodimethyl ester (112) to the corresponding amine, 5-amino-2,9-bis(carboxymethyl)-1,10-phenanthroline (113), was again performed using catalytic transfer hydrogenation with 10% Pd/Carbon as catalyst and cyclohexene as the hydrogen source. The reaction was carried out in methanol and was over within 3 hours. The crude product was obtained as a bright yellow solid after hot filtration over celite and removal of solvent. This compond is amenable to column chromatography, and purification was readily achieved by silica gel chromatography using 10% methanol in chloroform as the eluant.

In the initial reduction attempt using 10% Pd/Carbon and cyclohexene, ethanol was the used as the solvent. This resulted in complete trans-esterification and the 5-amino-2,9-bis(carboxyethyl)-1,10-phenanthroline derivative is obtained.

The final set of reactions to the desired target compound (116) is outlined in Scheme 5.6).



Scheme 5.6 A. $Br(CH_2)_5COCl$, $(iPr)_2NEt$, MeCN;
B. Phenathridine, 115°C; C. H_2O/HBr, pH 4

The amine diester (113) was reacted with commercially available 6-bromohexanoyl chloride in dry chloroform at room temperature. The reaction mixture also contained an excess of diisopropylethylamine, added as a base to scavenge the HCl liberated during the reaction. The reaction, which was easily monitored by tlc (silica gel: 15% MeOH/$CHCl_3$) was over within the hour and after work-up, the crude product was purified by column chromatography (silica gel: 10% MeOH/$CHCl_3$). The desired alkylated product (114) was isolated as a viscous oil, and the pure product was only obtained as a solid in 80% yield after trituration with diethyl ether.

The proton-nmr of compound (114) (Figure 4.3) clearly shows the all the peaks that we would expect; the methylene protons next to the bromine appear as a triplet at 3.46ppm while the protons next to the amide link appear as a triplet upfield from TMS at 2.6ppm.

In between these two signals reside the signal due to hydrate water at 3.21ppm. This is present even after drying in a vacuum oven, and confirms that the product exists as the hydrate. The six protons of the alkyl chain appear as a complex set of overlapping signals between 2.1-1.55ppm. Downfield from TMS the singlet associated with C-6 of the phenanthroline ring at 7.0 ppm (in the amine diester) (113) appears to have shifted into the group of signals associated with the ring protons around 8.0ppm. Also visible in the proton-nmr is the amide proton, a broad signal at 9.2ppm which is exchangeable with D_2O.

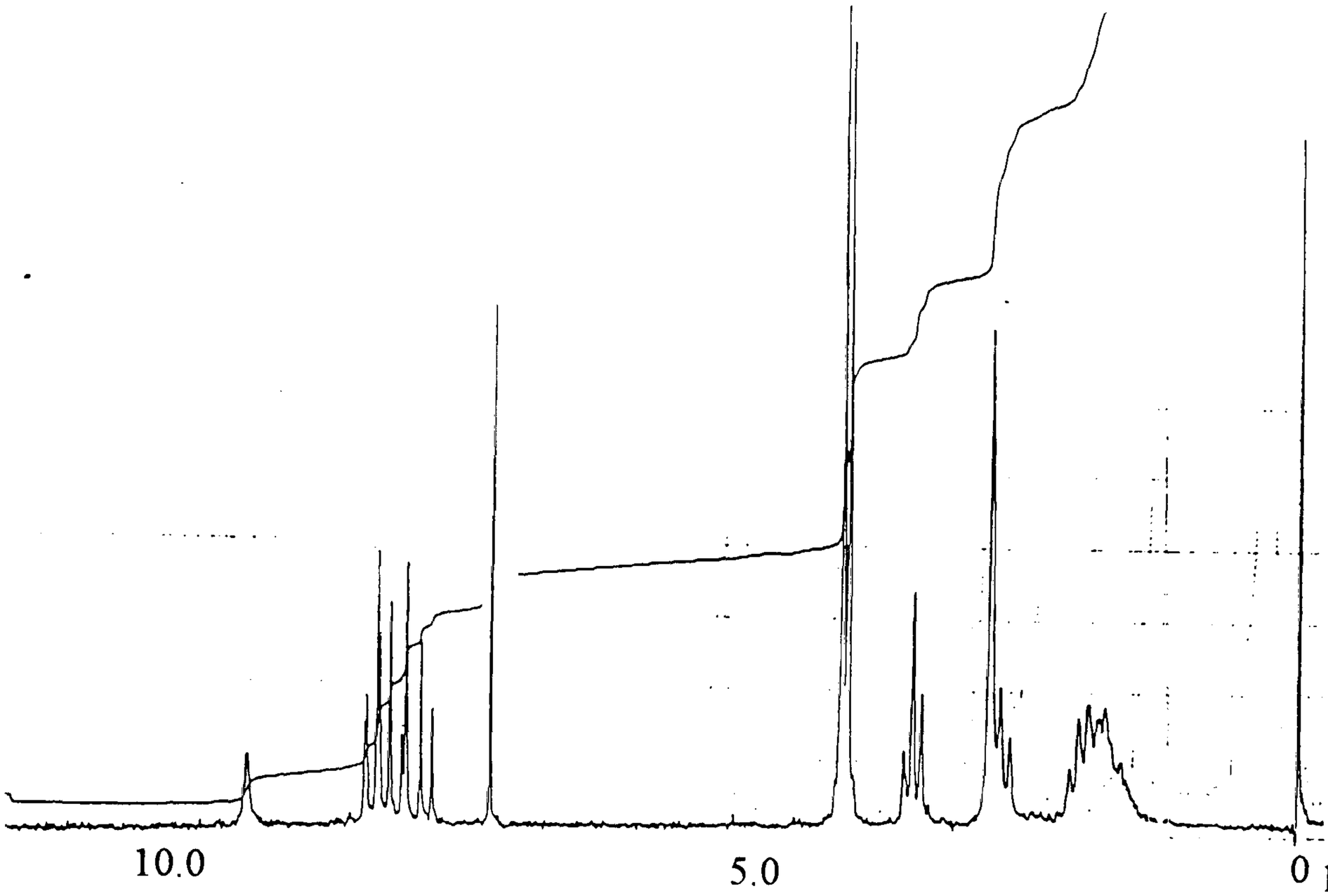


Figure 4.3 The proton nmr of (114) (200MHz; $CDCl_3$).

The penultimate step involved a quaternisation reaction between the alkyl bromide handle of (114) and the ring nitrogen of our heterocyclic intercalator, phenanthridine, to give the corresponding phenanthridinium compound (115), as the bromide salt. Like previous quaternisations, the reaction was carried out as a neat melt, using an excess of phenanthridine. A large excess of phenanthridine ensures that the reaction mixture does not become too viscous to stir before the reaction has proceeded to completion. The alkyl bromide phenanthroline (114) was added portionwise to liquid phenanthridine stirred at 115°, the quaternisation reaction is over within 1.5 hours.

After cooling, the reaction mixture sets into a hard red/brown solid which is stirred with diethyl ether and filtered to give exclusively the desired product (115), which unlike

recrystallisation from hot water but the recovery was very poor (<10%). On exposure to air it tended to become sticky.

The proton-nmr of the phenanthridinium salt (115) is shown in Figure 4.4. Although we appear to have lost some of the fine structure, probably due to the presence of charged species, the signals are still clearly visible and discernable.

What we were looking for in the proton-nmr was evidence of quaternisation. This is clearly shown by the shift in the signal due to the terminal methylene group of the alkyl chain from 3.45ppm in (114) to 5.2ppm in the phenanthridinium species (115), as a result of replacing the bromine with the electron-withdrawing quaternary nitrogen. Further evidence is provided by the large shift down-field from TMS of the proton on C-6 of the phenanthridine ring (next to N^+), from around 9.0ppm to over 11.0ppm in the phenanthridinium salt (115).

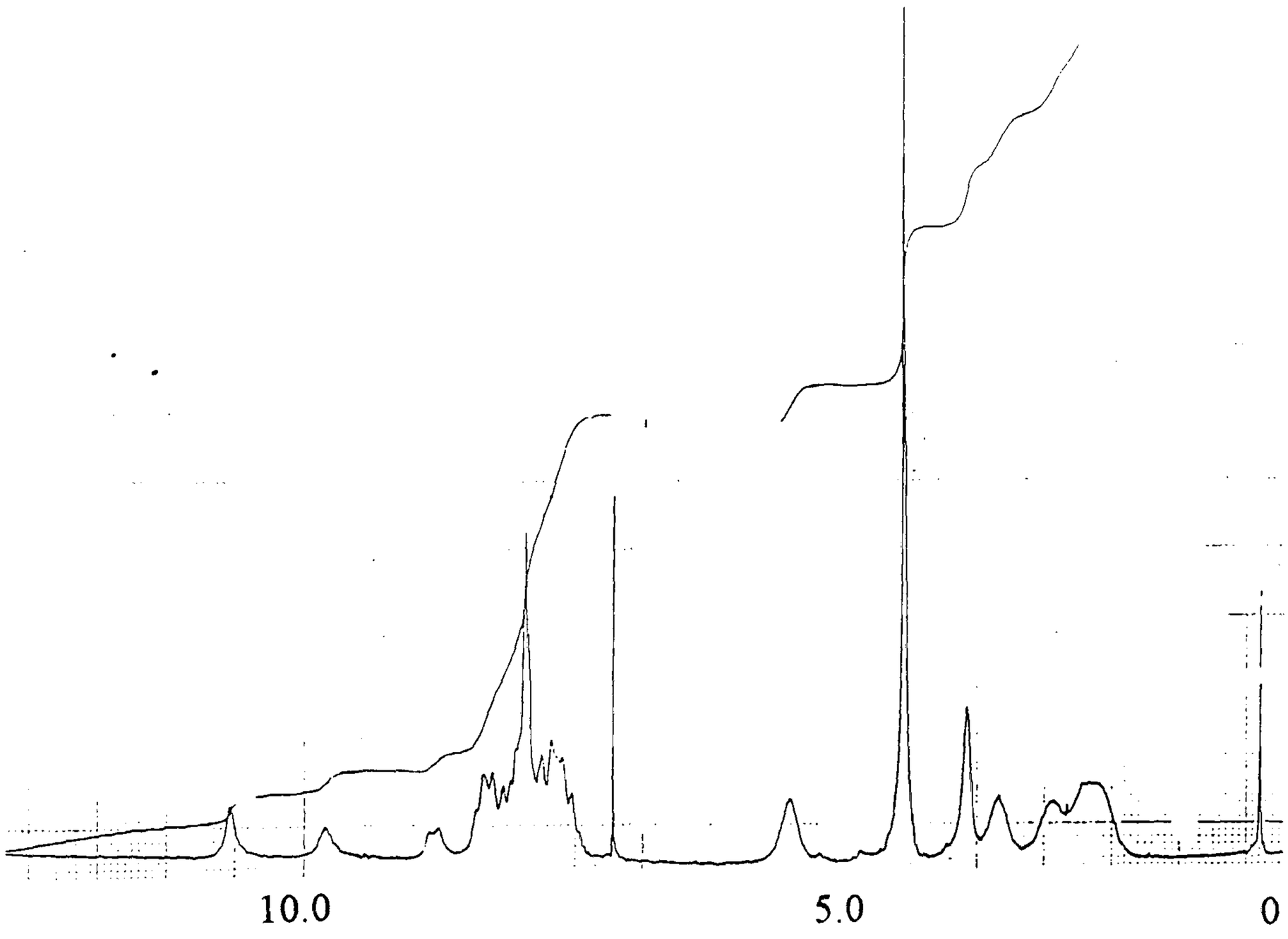


Figure 4.4 The proton nmr of (115) (200MHz; $CDCl_3$).

The final synthetic step was the hydrolysis of the two methyl ester groups of (115), to give the desired target molecule as the dicarboxylic acid (116). Base catalysed hydrolysis was ruled out as the phenanthridinium system is sensitive to nucleophilic attack at the

carbon attached directly to the electron-withdrawing quaternary nitrogen. However, these quaternised phenanthridines have been shown to be very stable to even very strong acids at reflux.

Initial attempts centred on hydrolysis in water at pH7 at 100°C, however, after 16 hours there was no indication of any significant reaction that could be observed by tlc (alumina: 10% MeOH/$CHCl_3$). By dropping the pH of the solution to pH 1 by the addition of concentrated HBr gave reaction overnight; indicated by the disappearance of the diester and appearance of a baseline spot by tlc.

However, analysis of the bright orange solid isolated by proton-nmr indicated that some degradation of the product at the alkyl chain had taken place. Hydrolysis at pH 4 gave much better results, and a bright orange solid was isolated by simply removing the solvent under high vacuum. Analysis by proton-nmr showed the characteristic signals of the phenanthridinium group were still present at 10.7 and 5.2ppm, and that the two singlets due to the methyl ester groups at around 4.0ppm had virtually disappeared. Attempts at obtaining an analytically pure sample of (116) by recrystallisation have been unsuccessful, and analysis by tlc on both normal and reverse phase systems have failed been unsuccessful. However, the match of the molecular ion peak was well within the required 5ppm limits for calculated mass.

2.6 LUMINESCENCE STUDIES

The key to our approach and design for a homogeneous DNA assay was whether:

(i) we could enhance europium ion luminescence in aqueous solution using cooperative ligands *viz.* a three componenet system; the 1:1:1 ternary complex composed of the europium ion, a sensitising ligand and a separate non-sensitising ligand,

(ii) the same ternary complex could be made to assemble once hybridisation between probe and target strand to form the duplex had taken place.

Our initial efforts concentrated on trying to understand how to prepare such ternary complexes in solution, and the effects of pH and concentration on the relative luminesecence intensities.

The photoactive component of the ternary complex is the sensitiser, and initial studies (binary solution studies) concentrated on finding the best overall sensitiser from our the simple 1,10-phenanthroline derivatives synthesised and the optimum conditions for maximum energy transfer to the europium ion. (section 2.4.2)

Studies involving the ternary sysytem were initially carried out with '221' as the non-sensitising ligand and phenanthroline diol (57), monocarboxcylic acid (62) and PDCA (48) as sensitisers. From these studies PDCA was identified as the sensitiser with the best 'cooperative properties' resulting in large enhancements in the luminescence. When studies were repeated, but this time using the mono-and-bis butylyamide derivatives of EDTA with PDA as sensitiser, the highest enhancements were observed at a pH ideal for subsequent biological studies. (see sections 2.4.3 and 2.4.3.1)

2.6.1 General Procedures

Luminescence spectra were determined on a Perkin Elmer LS50 spectrometer fitted with a red-sensitive photomultiplier. The solvent of choice for spectroscopic studies was high grade water (Elgastat). Solutions were buffered using 5mM tris(hydroxyethyl)aminomethane (Gold label, Aldrich) ('tris') in the presence of 50mM sodium chloride (Analar, BDH). Preliminary studies showed that the luminescence of the europium ion was not affected by the presence of air; solutions were not routinely degassed.

The protocol for cleaning cuvettes was as follows: At the start of a run they were washed with sulphuric acid/hydrogen peroxide (1:1 v/v, Care!) mixture, allowing them to stand for full 30 min.; rinsed with high grade water until the washings were neutral; rinsed with five lots of Analar methanol and air dried.
After each measurement, the cuvettes were washed with three lots of 3M hydrochloric acid, four lots of water, and, finally, five lots of methanol before air drying.

The procedure for adjusting the pH was as follows: a 30 ml solution of the concentration to be studied was prepared using the relevant stock solutions and 5mM tris and 50mM NaCl buffer. The solution was then adjusted in pH using concentrated Analar HCl and/or KOH and the solutions left for 15 min. to stabilise. Luminescence measurements were made on 2 ml samples; each being returned to stock before further manipulation. Total changes in concentration were <10%.

2.6.2 Solution Studies - Binary Systems

Direct studies in aqueous solution proved to be a quick method for screening luminescence. These solution studies were conducted varying two main parameters, either the pH or the concentration. The pH used in these studies is critical because europium solutions are extremely pH sensitive. At high pH (>6) hydroxide ions can form and these bind more strongly to the metal ion than water, eventually leading to precipitation of the insoluble hydroxide. This tendency to bind to water and hydroxide ion is also reflected in the speciation behaviour of this element; europium behaves some what like aluminium in its

chemistry and increasing concentrations leads to the formation of aggregates of the ions bound by either water or hydroxyl bridges.[75] Such aggregation could impair the luminescence behaviour leading to non-linear responses with concentration changes.

The buffer chosen for the solution studies tris [2-amino-2-(hydroxymethyl)propane-1,3-diol] was found the prevent the formation of europium-hydroxy species in the pH range 7-8; such solutions were stable for days, whereas, in the absence of this buffer, precipitates started to form within a few hours. Tris is commonly used as a buffer in DNA studies at pH 7-8, since it buffers around this important range. Control studies showed that the addition of tris to solutions of photosensitising ligands and europium gave no luminescence enhancements, indicating that it does not interfere. These same conditions (5mM tris and 50mM NaCl) are also used by other groups, such as Barton's[76], when studying metallo-complex - DNA interactions.

Studies on luminescence versus pH are shown for the phenanthroline diol (57), monocarboxyclic acid (62) and dicarboxcyclic acid (48) in the presence of excess europium; with no sensitiser no luminescence is observed (Figures 4.5 - 4.7).

These results show that as expected the diol (57) shows the lowest enhancement in luminescecnce and the dicarboxylic acid (48) the most. As expected the diol shows the lowest variation with pH, the broad hump showing a maximum at pH 6.7 (23.4 intensity units). This suggests a relatively weak affinity of the diol for europium; that some luminescence is observed does confirm that some interaction does occur between the ligand and metal.

The results for the monocarboxylic acid (62) and dicarboxylic acid (48) are more instructive. The enhancements both show a strong pH dependence. At low pH the carboxyl group will be protonated and coordination of this group will have to compete with solvent water. As the pH is increased the carboxylate anion is generated and this appears to coordinate strongly with the metal. However, as the pH is further increased the intensity rapidly falls away and this is explained by competition with hydroxyl ions. Finally, at pH's above 10.5, the carboxylate group can no longer compete for binding sites and formation of europium oxide occurs. The dicarboxylate shows both higher enhancements

than the monocarboxylate (72.1 versus 42.8) and peaks at a higher pH (9.0 versus 8.7); a higher pH allows both carboxylate groups to ionise and hence compete more effectively againts the ingress of hydroxide ions. The presence of the two, carboxylate groups has the effect of holding the europium closer to the sensitiser with the corresponding increase in the efficiency of energy transfer.

The binding constant for the Eu^{3+}:dicarboxylic acid (48) was determined to be $\approx 10^6$ by luminescence versus concentration studies. However, the low quantum efficiency of luminescence of these binary systems made it very difficult to carry out these studies on a conventional fluorometer, and more detailed studies are needed.

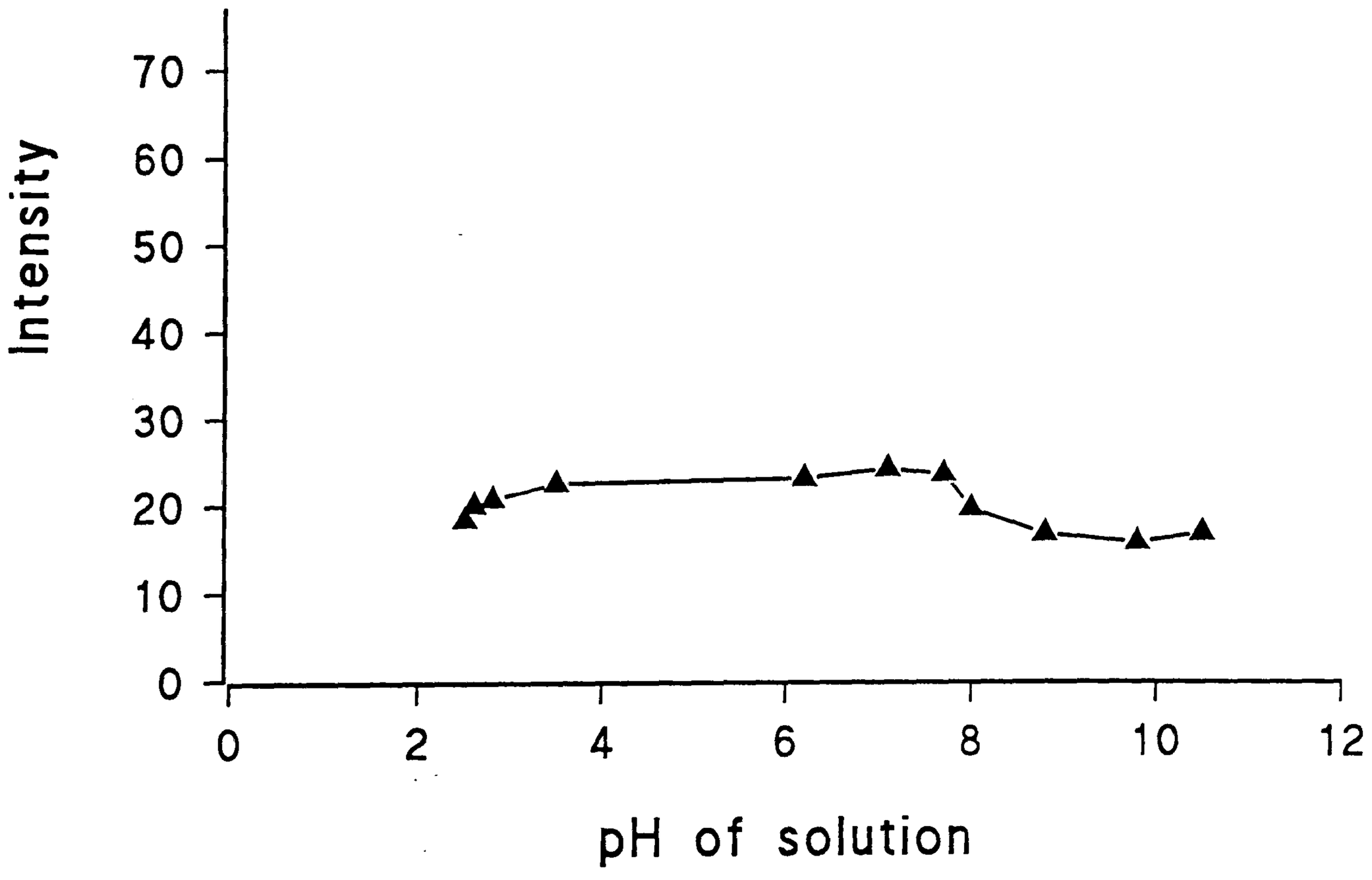


Figure 4.5 Variation of luminescence with changing pH. Eu [10^{-4}M] : phen. diol (57) [10^{-6}M].

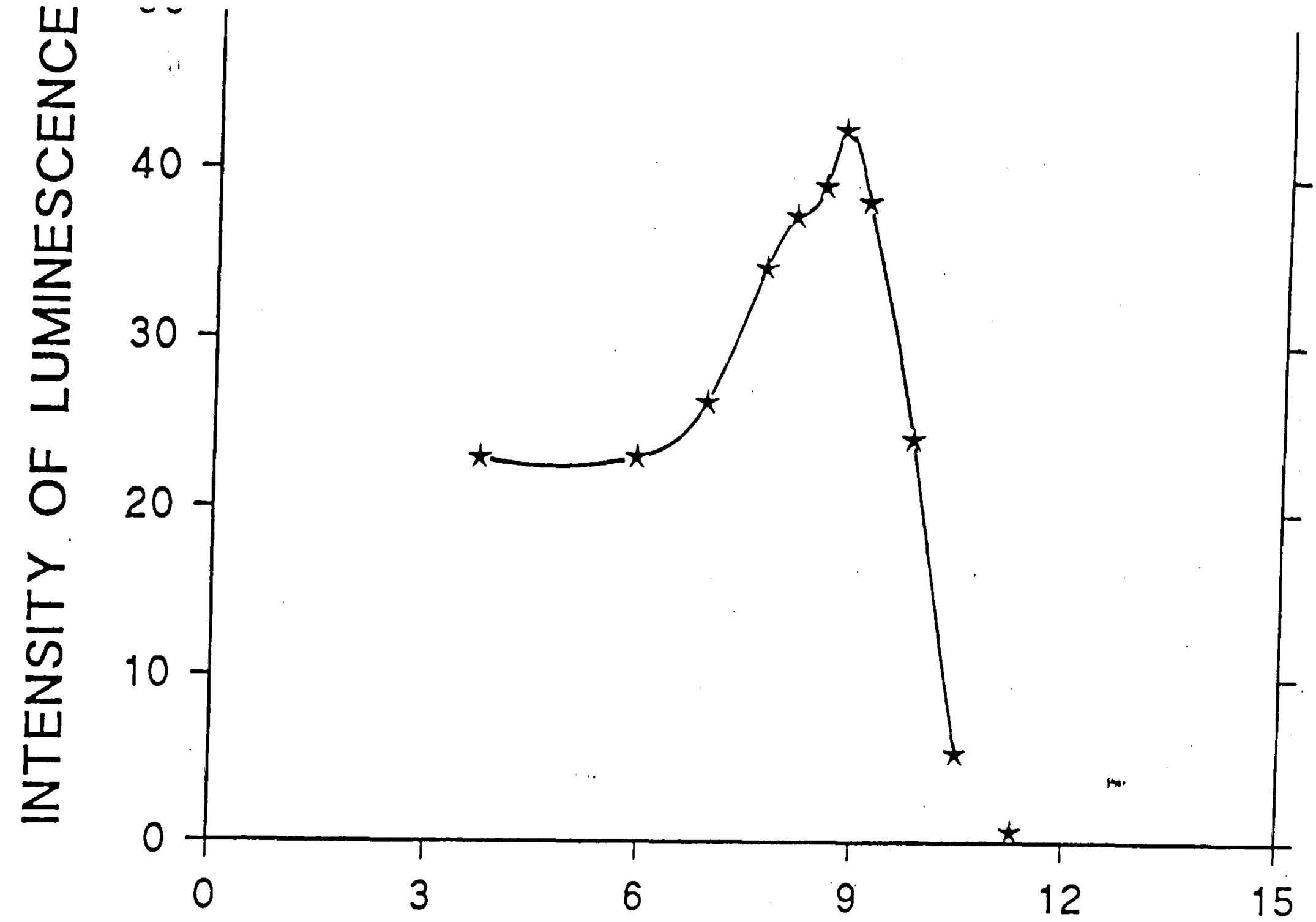


Figure 4.6 Variation of luminescence with changing pH. Eu [10^{-4}M] : phen. mca (62) [10^{-6}M].

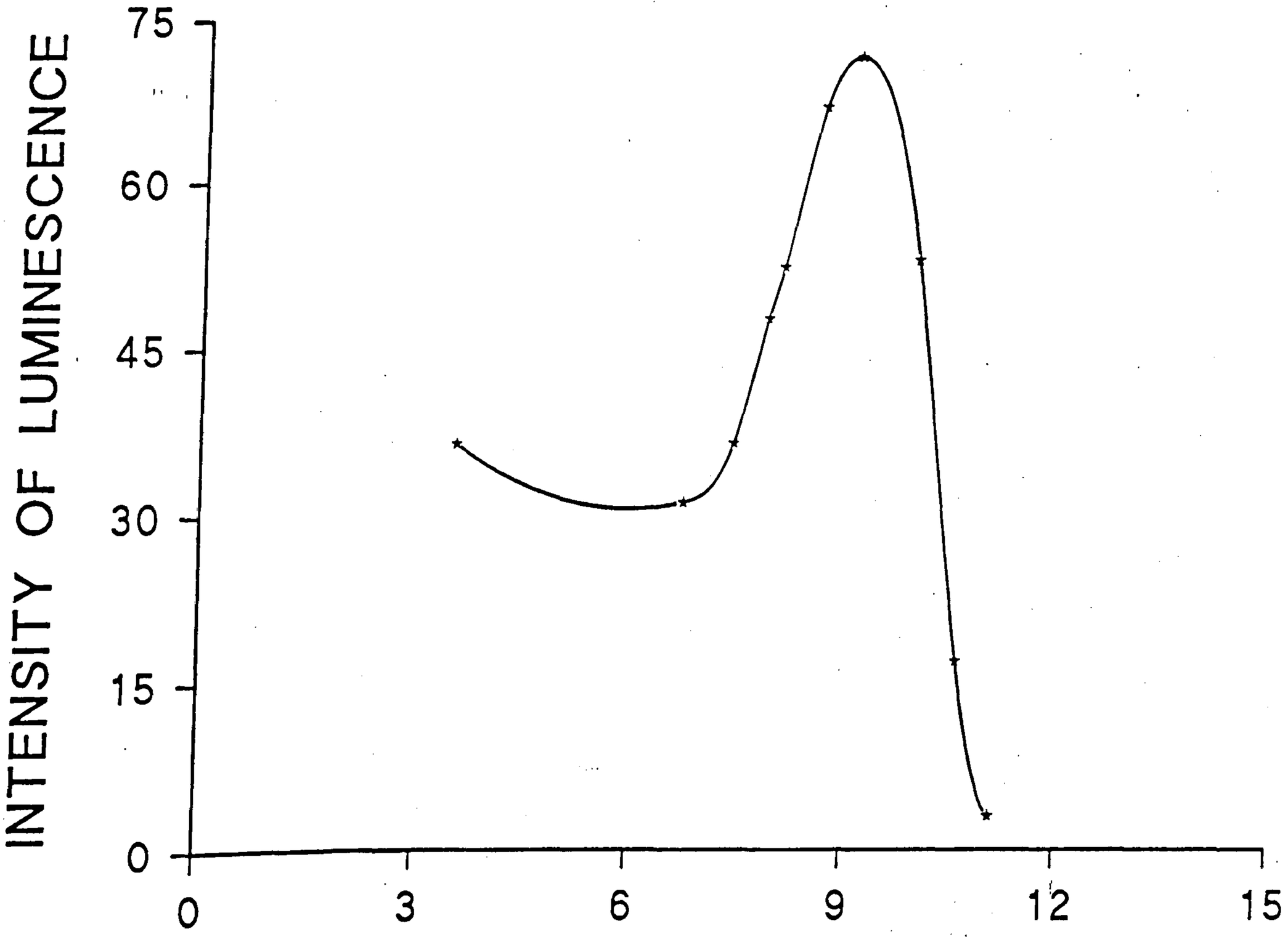


Figure 4.7 Variation of luminescence with changing pH. Eu [10^{-4}M] : PDCA (48) [10^{-6}M].

2.6.3 Ternary Systems using [2.2.1] (21).

Since the europium ion behaves as a highly charged sphere, our approach to forming ternary complexes in solution was to try and exploit electrostatic field effects. Thus, choosing ligands that do not completely saturate the positive charge on an europium ion allows the initial 1:1 complex to retain a positive charge so that a second negatively charged ligand can be attracted into its coordination sphere.

Luminescence studies were repeated in the presence of a third component, the non-photosensitising ligands, based on [2.2.1] (21) and EDTA and its derivatives.

Earlier we mentioned that the bis-acetic acid derivative of 1,7-diaza-4,10,13-trioxacyclopentadecane [2.2.1] (21) forms tight complexes with europium ions ($K_{ass} > 10^{11}$) and molecular models have shown that this is achieved whilst leaving a cleft suitable for occupation by water molecules or *another ligand*, which in our case will be the photosensitiser.

The luminescence results for the diol (57), monocarboxylic acid (62) and the diacid (48) are shown in Table 2.1, additionally luminescence results for the dicarboxylic acid (48) are shown graphically in Figure 4.8.

Equimolar quantities of Eu^{3+} to [2.2.1] were used , the ratios being kept constant to ensure a 1:1 complex and avoid 2:1 complexes involving these species. The concentrations used of both Eu^{3+} and [2.2.1] were a 100-fold excess to the sensitiser concentration (10^{-6}M). Since the [2.2.1] in the pH range 7-9 has an association constant for $Eu^{3+} > 10^{11}$, no free europium should exist in solution under these conditions.

The most exciting results were obtained for the diacid (48). As expected , the Eu^{3+}.[2.2.1] complex shows no luminescence when irradiated at 300 nm [Figure 4.8(a)]. However, as soon as the diacid (48) was added a large enhancement of the luminescence was observed [Figure 4.8 (c)]. As predicted, in the absence of the shielding [2.2.1] ligand, the diacid.Eu^{3+} complex (K_{ass} *ca.* 10^6) only showed a weak luminescence [Figure 4.8 (b)].

Table 2.1 Ternary system enhancements[a] with [2.2.1] (21).

LIGAND	Without [2.2.1]		With [2.2.1]	
	I_{max}	pH_{max}	I_{max}	pH_{max}
Diol (59	22	(4-8)	95	≈10
Monoacid (64)	43	9.0	46	7.5
Diacid (50)	70	8.7	257	8.0

[a]Concentration of ligands: [(59)], [(64)], [(50)]= 10^{-6}M
[(21)]= 10^{-4}M
[Eu^{3+}]M
λ_{ex} at maximum in the region 280 - 290nm.
λ_{em} 615nm.

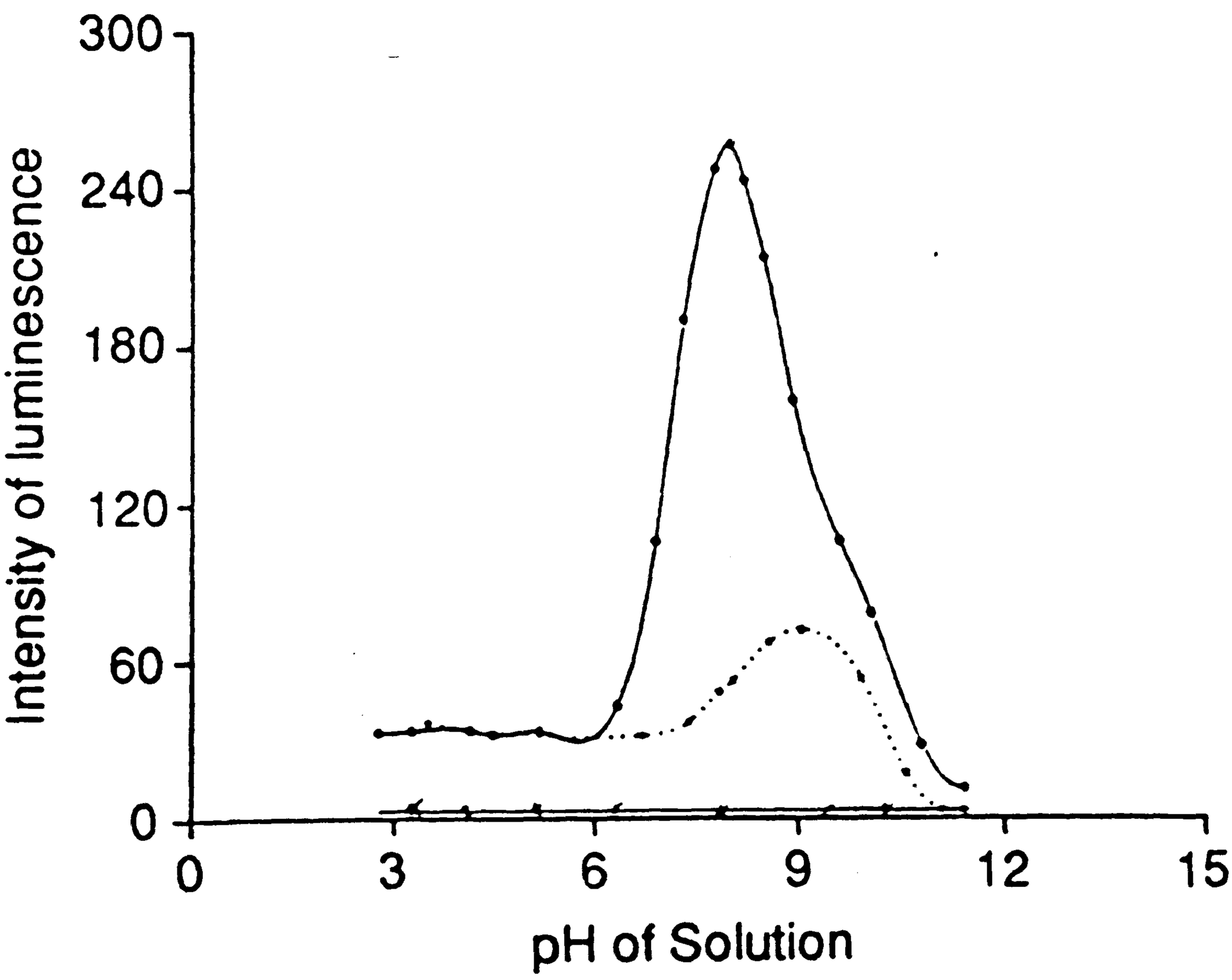


Figure 4.8 Variation of luminescence with changing pH (*a*) —×— Eu^{3+} (10^{-4}), [2.2.1] (10^{-4}); (*b*) ···×·· Eu^{3+} (10^{-4}), PDA (10^{-6}); (*c*) —●— Eu^{3+} (10^{-4}), [2.2.1] (10^{-4}), PDA (10^{-6} mol dm^{-3}). Aqueous solutions; pH measured immediately prior to luminescence measurements; graphs are average of triplicates; λ_{em} 615 nm.

The diol (57) was also shown to enhance luminescence, although not to the same extent as the diacid, but to our initial surprise the mono carboxylic acid showed little increase in luminescence.

The luminescence curves for the diacid.Eu^{3+}.[2.2.1] complex show an interesting pH dependence. At high pH the europium complexes decompose by hydroxide attack, formation of europium oxides being observed. At lower pH protonation of the carboxylate groups occurs, thus lowering their affinity for the metal ion. The luminescence peak for the ternary complex {Figure 4.8 (c)] occurs at pH 8.0 as compared to that of the binary complex (pH 9.2): over a sixfold enhancement in luminescence is observed at pH 8.0.

These results were very heartening as it demonstrated one of our key postulates, that a ternary complex can be made to assemble in solution around a europium ion resulting in large modulations of the metal ion luminescence.

The results also suggested that to achieve maximum 'ligand cooperativity' at least two 'hard' centres are required for tight binding of the sensitiser molecule to the [2.2.1].Eu^{3+} complex. Thus, the monocarboxylic acid is only able to bind weakly to the europium complex, probably via the single carboxylate group, the nitrogen centres being, in themselves, rather weak ligands to the europium ion compared to water and, and as a consequence, are unable replace solvent molecules from the coordination sphere of the metal. Although the hydroxy groups of the diol are able to compete with solvent water molecules for coordination sites, this only occurs at high pH (I_{max} maximum at pH 10), when they are fully ionised . However, since this pH was outside the range we were aiming for , no further work with the diol was carried out.

The formation of both the 1:1 diacid.Eu^{3+} complex and the 1:1:1 ternary complex occurs rapidly upon the addition of the diacid (48) to the other components, as reflected by the rapid onset of the observed luminescence. In contrast, addition of [2.2.1] to the diacid.Eu^{3+} complex results only in a slow increase in the luminescence to the full value (half-life *ca.* 3h. We were able to explain this by the fact that the organisation of the more mobile [2.2.1] ligand about the europium ions is a far more demanding process, in terms of the

conformational reorganisation and displacement of water,[77] than that of, the diacid with europium. However, the reverse is also true and, once formed, unravelling of the [2.2.1] ligand (total dissociation) from the europium ion is also expected to be slow.

2.6.3.1 Ternary systems using EDTA and its Derivatives.

Although we were aware that EDTA has a very high binding constant for Eu^{3+} ions. An initial screen (at pH 8.5) showed little enhancement and we initially considered that this was due to over zealous encapsulation of the ion by the ligand, making it hard for the sensitiser to approach the bound metal ion. However, further consideration of the pK values of EDTA (*ca.* 2.0, 2.67, 6.16 and 10.26) suggested that, at lower pH (pH< 6.16) only 2 carboxylates groups should be present and, at this point EDTA may mimic [2.2.1]. This prediction was demonstrated by the enhancement of luminescence observed with the dicarboxylic acid (48), with the maximum observed luminescence (I_{max} 308) occurring between pH 5.5-6.0 (Table 2.2). The increased I_{max} is explained by a combination of less steric crowding in the complex allowing closer approach of the phenanthroline dicarboxylate resulting in tighter coordination.

By repeating these experiments with the monobutylamide derivative of EDTA (46) (Figure 4.9), a very high increase in the luminescence (I_{max} 540) was observed over a broad pH range (pH 7-8), ideal for assays of biological materials. Under these conditions we may assume that at least one of the two EDTA carboxylates is ionised (maybe, both) and at least one of those from the phenanthroline groups (again maybe both) so that very tight coordination occurs around the metal ion.

Luminescence half-time measurements have shown that in this ternary complex only one water molecule is able to coordinate to the encapsulated europium ion, (τ_{H2O} *ca.* 0.75 msecs.)

It is worth noting that in our ternary systems the problem we face is the fact that the binding constants of the various species present will be interdependent. For example, the binding constant of the photosensitiser PDCA for europium in aqueous solution would be different from the association constant of PDCA for the europium-EDTA complex

Eu^{3+}.EDTA and, also, these association constants will vary with pH. Thus, we would expect the presence of the EDTA ligand to result in a decrease in the binding constant of the phenanthroline dicarboxylic acid (48). In order to work out respective binding constants detailed concentration-fluorescence studies would be needed.

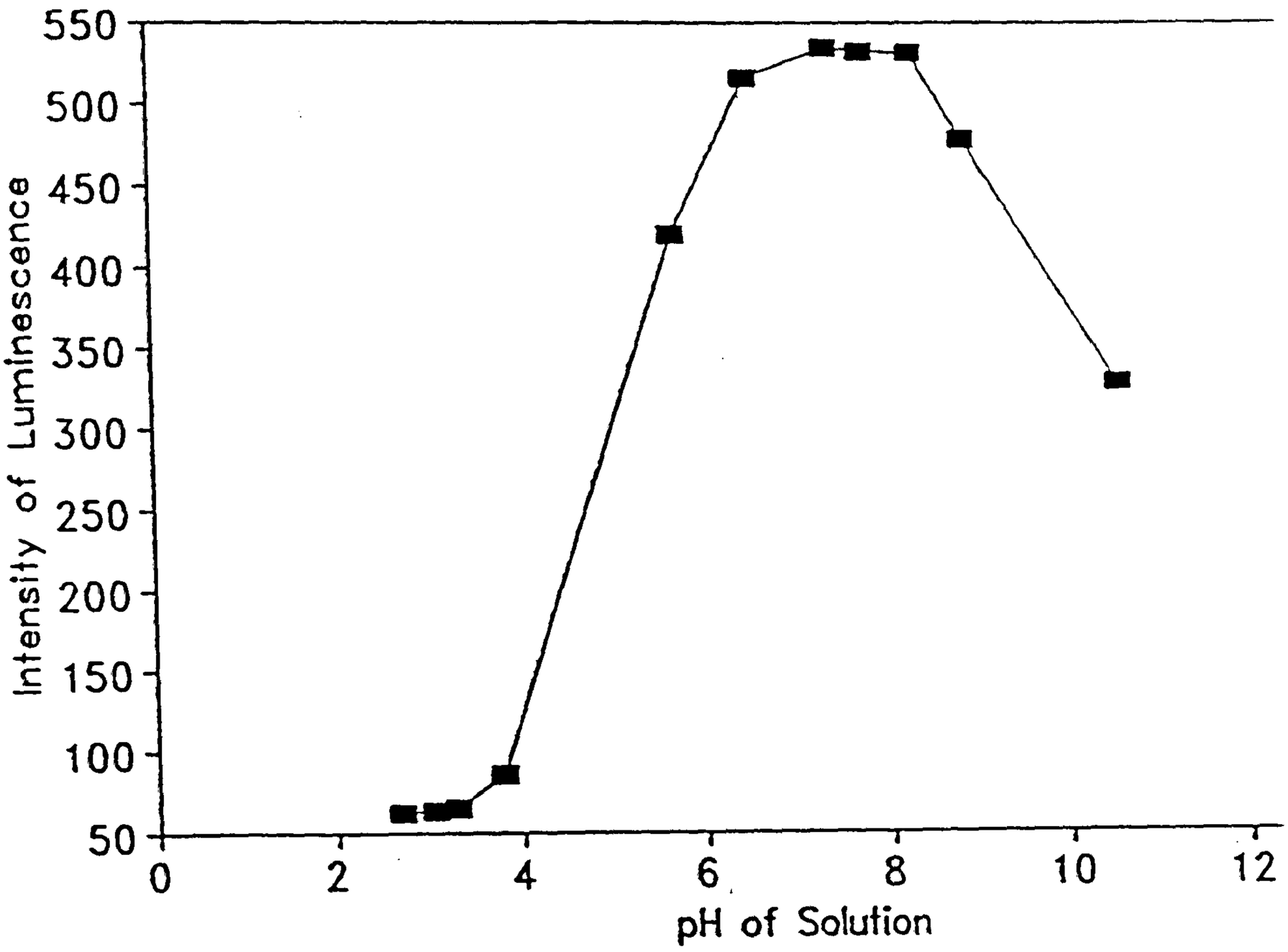


Figure 4.9 Variation of luminescence with changing pH. Eu [10^{-4}M] : EDTABuAmide (46) [10^{-4}M] : PDCA (48) [10^{-6}M].

2.7 STUDIES WITH SYNTHETIC OLIGONUCLEOTIDES.

Our approach to a direct *in situ* (homogeneous) identification of target DNA strands is illustrated in Figure 2.1 and Scheme 2.4. The probe is linked to a chelator to which is strongly coordinated a europium ion. The probe is then hybridised to the target DNA to form a double stranded segment of DNA. In this situation, where there is no sensitiser present neither the probe nor its duplex with the target gives a signal under irradiation with light. The europium ion has a very weak absorption coefficient since the excitation process is formally spin forbidden[78].

The initial excited state of europium is only efficiently reached by a ligand-to-metal sensitised energy transfer process involving a triplet sensitiser like PDCA (48). Such energy transfer requires very close contact between sensitiser and the metal ion. We have shown[77] that under certain conditions, chelates of europium can accept a second ligand, such as 1,10-phenanthroline-2,9-dicarboxylic acid (48), which acts both as a sensitiser and shielding agent to displace solvated water molecules; solvated water inhibits europium luminescence.

This is a key feature of our assay design; the need for cooperation between the europium ion and two different ligands to form a ternary complex (a three component cooperative system) in which the sensitiser is able to both approach and bind tightly to the already coordinated metal ion. This assembly process occuring once probe and target have hybridised to form the duplex.

As mentioned earlier the non-sensitising ligand L needs to have a very high binding constant for the europium ion ($K_{ass} > 10^{12}$) so that once attached to the oligonucleotide probe the bound state will be maintained and we can consider the euopium.ligand complex [Eu^{3+}.L] as a single stable entity.

In contrast, one does not want a very high binding constant of the photosensitiser S for the Eu^{3+}.L entity; as this would result in the components coming together in bulk solution rather than at the target DNA site, leading to high background counts. The binding constant of the sensitising ligand for the chelated europium is in the range K_{ass} 10^6 - 10^7 and thus,

at concentrations of the two components at < 10^{-7} M little association between the reagents occurs. This ensures the background signal for europium luminescence is small and is expected to fall away rapidly upon dilution. The success of our system relies upon an enhancement of the signal for europium luminescence once the probe strand has met the target strand of DNA. This is achieved by making use of intercalation using a positively charged aromatic species such as the phenanthridinium group linked to the sensitiser. Intercalation is a characteristic property of the duplex and the binding constant of such species for double stranded DNA is in the order of K_{ass} 10^6. Intercalation has the effect of increasing the local concentration of the sensitiser and this works **in cooperation with** the binding costant of the sensitiser to the metal ion. As a consequence the overall binding constant of the sensitiser to the metal ion is increased and, results in the enhancement of the europium signal. Since, in the presence of the target strand the signal is generated in a cooperative, intramolecular manner, the relative strength of the signal is expected to fall away less rapidly upon dilution.

2.7.1 Oligonucleotide Test System.[79]

The only way to test our approach was to actually perform a DNA assay under homogeneous conditions. This was carried out using an oligonucleotide probe (24mer) and two strands of target oligonucleotide; one complementary to the probe (R) and the other a complete mismatch (Q). (Figure 4.3)

PROBE: H_2N-$(CH_2)_6$-O-**AA.GAT.GAT.ATT.TTC.TTT.AAT.GGT.G**

(5') (3')

TARGET R (MATCH): **TT.CTA.CTA.TAA.AAG.TTA.CCA.C**

(3') (5')

TARGET Q

(MISMATCH): **GTC.CAT.TAA.CAT.CTG.TCT.TCC.ACT.AAG.GGG**

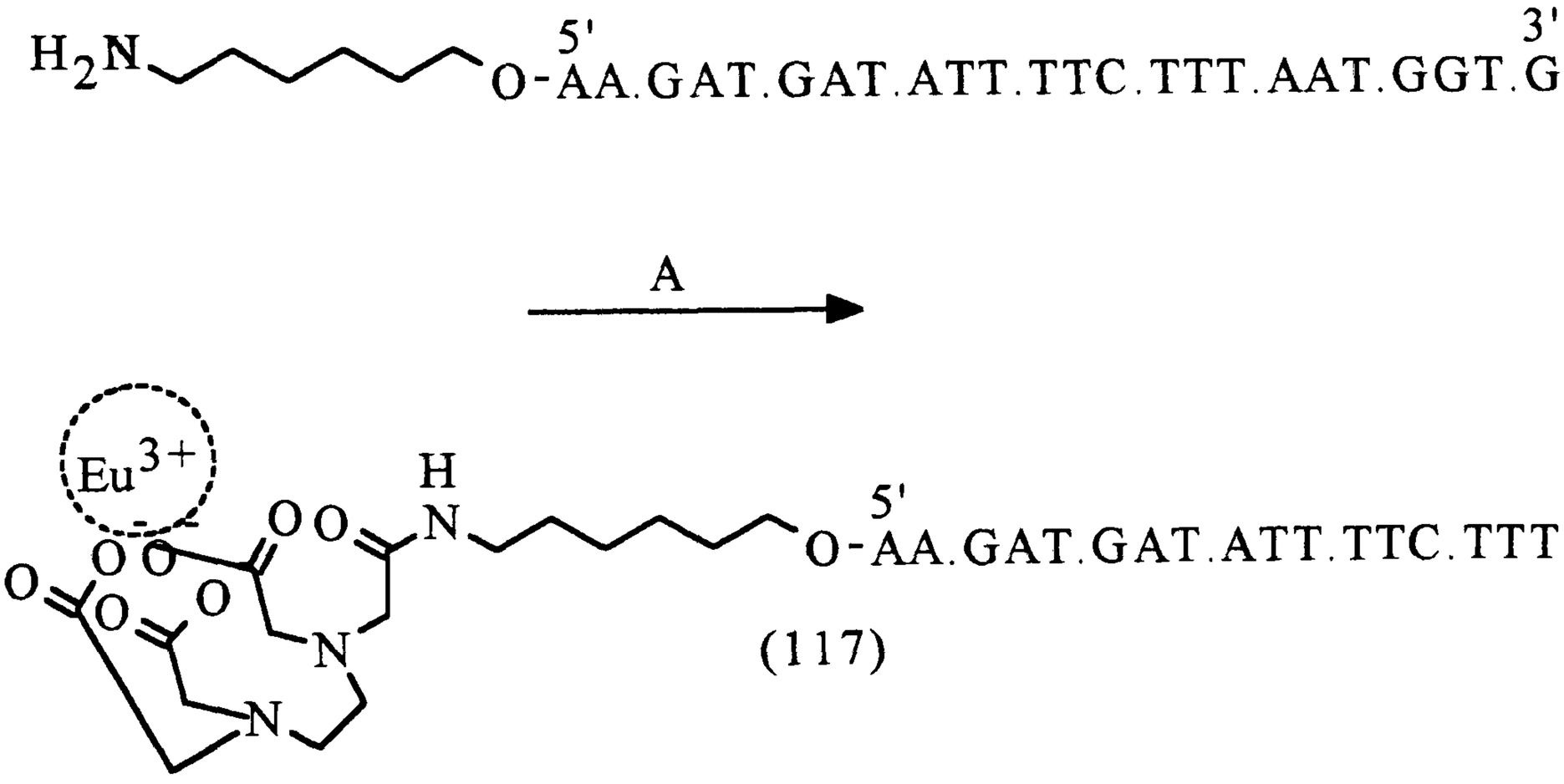
(3') (5')

Figure 5.0

2.7.2 Preparation of the EDTA adduct of the 24mer.

The 5'-aminohexyl group of the oligonucleotide probe was reacted at room temperature with 25 fold excess of the anhydride of ethylenediaminetetraacetic acid in 10 mM TRIS buffer at pH 9.0, followed by saturation with europium (III) chloride overnight to give the chelate derivative (117) (Scheme 5.7). The chelate was adequately purified by passing the whole solution through a short Sephadex G25 column (NAP 5) using 10mmol TRIS/HCl at pH 7.5 as the eluent, taking *ca.* 20µl fractions. All the collected fractions were subjected to UV analysis at 260nm, with those fractions showing a strong absorption at λ_{max} 260 nm being combined. The presence of tagged europium could be checked by stimulating europium luminescence using a solution of PDCA as the sensitiser.

The final concentration of the tagged oligonucleotide probe (117) in the combined fractions was determined by O.D. measurements using a Pharmacia Gene-Quant automated analyser.



Scheme 5.7 A. (i) EDTA bisanhydride, THF, NaOH
(ii) $EuCl_3$

2.7.3 Hybridisation Experiments.

Hybridisation experiments were carried out with Mr J. Coates (Brunel University) using the general procedures of Lövgren *et al.*[80], 150µl of target oligo was made up to the approximate concentration of the probe ($1x10^{-5}$M) diluting with TRIS/HCl buffer. Hybridisation of target oligo's with the tagged probe (117) were carried out in a mixture of Denharts solution (25µl), and 600µl of buffer (0.1M Tween 20, 1M NaCl, 0.1M $MgCl_2$ and 10mM HEPES) at 42°C for 3 hours, giving a concentration of $5x10^{-6}$M for the duplex.

After hybridisation was complete, solutions of the sensitiser (116) ($5x10^{-6}$M) from a stock solution ($1x\ 10^{-5}$M) of the sensitiser (116) made up in TRIS/HCl buffer were added, and the solutions left to equilibrate. The luminescence of each solution was then measured exciting at 298nm. Then a series of dilutions were performed on both targets (Q and R), ending up with a final concentration of $2.6x10^{-8}$M (112x dilution), the results are illustrated in Table 2.3 and luminescence spectra of both the matched and un-matched in Figure 5.1.

Table 2.2 Luminescence results from the hybridisation of both matched and mismatched oligonucleotide targets with the labelled probe.

Concentration (M)	OLIGO R/INTENSITY[a]	OLIGO Q/INTENSITY
$3.0x10^{-6}$	140	60.5
$1.5x10^{-6}$	86	39
$3.8x10^{-7}$	30	15
$1.3x10^{-7}$	9	3
$5.4x10^{-8}$	3	0.4
$2.6x10^{-8}$	1.12	0.05

[a] λ_{ex}= 298nm, λ_{em}= 615nm

From these results we can clearly see that as predicted, there is a distinct difference in the luminescence intensities between R (matched) and Q (mismatched), with a stronger signal being observed in the presence of the matching target R as compared to that observed with the mismatch target Q. Also, upon dilution, a more rapid decrease in the intermolecular (background) signal is observed than for the cooperative (intramolecular) signal observed in the presence of the matching target. All the results were reproducible.

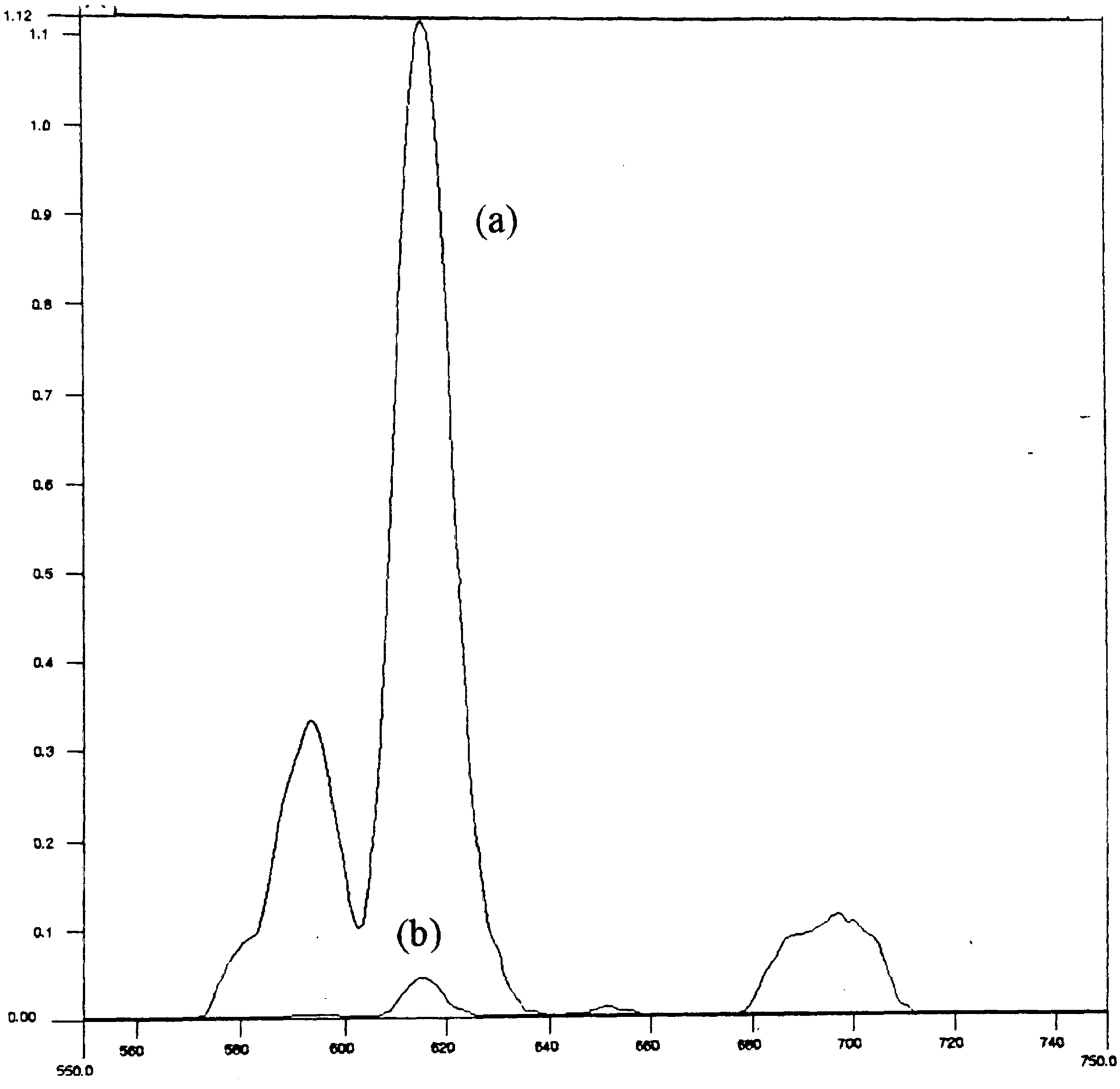


Figure 5.1 Emission in range 550 - 750 nm observed on mixing reagents **117** and **116** with (a) target R (match) and (b) Target Q (mismatch). Buffer as in text; initial concentrations on mixing 5 x 10^{-6}M **117**, 5 x 10^{-6}M **116** and 5 x 10^{-6}M Target, followed by x112 dilution. Delay time 0.1 ms, slit widths 10 nm.

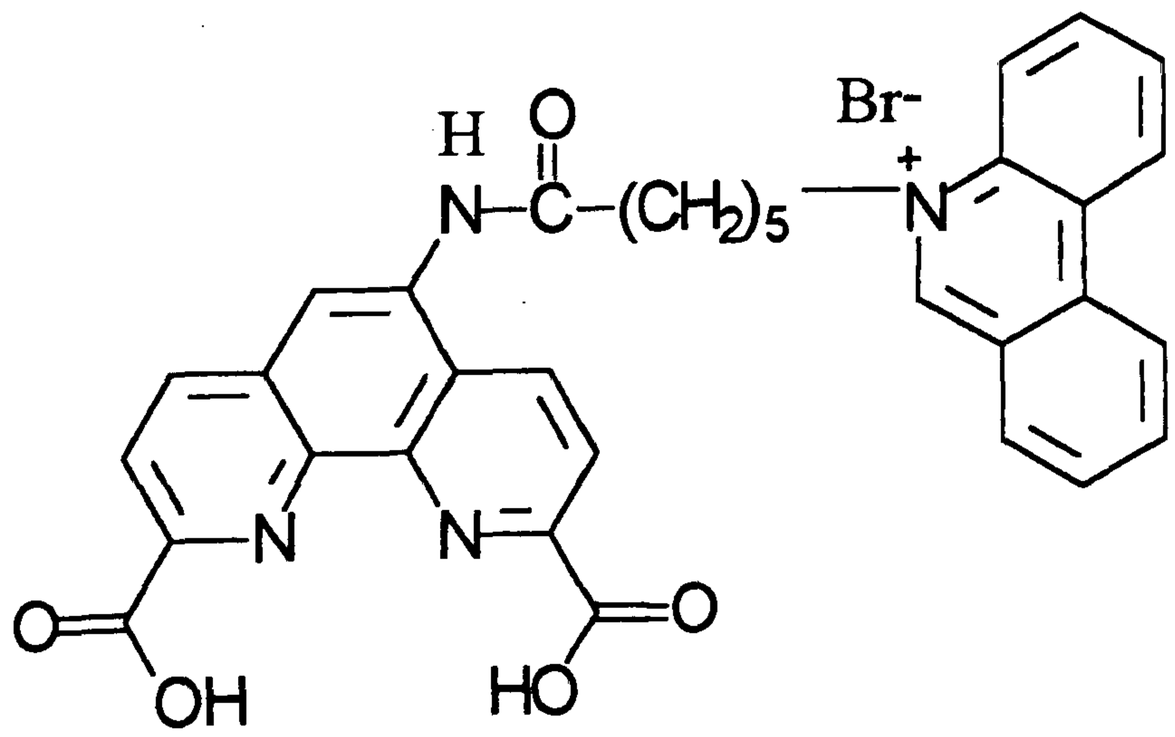
2.8 CONCLUSIONS.

With the aim of developing new homogeneous assays for DNA, various strategies have been listed, all making use of the characteristic property of the lanthanide ion, europium, to luminesce when certain conditions have been satisfied. The most important of these being the need for a suitable sensitiser which possess: (i) the correct photophysical properties to enable it to excite the europium ion by ligand-to-metal triplet energy transfer, and (ii) 'hard' binding sites to enable tight binding of the europium ion; and also a way of preventing or limiting the coordination of water molecules to the europium ion, which have been identified as the main culprits in deactivating the excited state of the europium ion.

We have found and demonstrated a new method for the cooperative binding of ligands to Eu^{3+} ions which works like a switch turning on the luminescence of the Eu^{3+} ion in aqueous solution.

The main components of this cooperative binding have been identified as 1,10-phenanthroline-2,9-biscarboxylic acid (PDCA) (48) as the most efficient sensitiser of europium luminescence and both linear and cyclic derivatives of EDTA as ligands with the necessary high binding constants for Eu^{3+}, (termed non-sensitising ligands).

Our approach, making use of the cooperative binding ('ternary complex') principle has involved the successful synthesis of a new class of phenanthroline sensitiser (116).



(116)

We have been able to tag appropriate DNA probe strands with an EDTA derivative and initial assay results using a tagged probe strand of oligonucleotide and two target strands; one complementary (matched) and the other a complete mismatch under **homogeneous** (non-separating) conditions has given very encouraging results, with clear discrimination in the modulated luminescence where a match has occured between target strand of DNA and it's complementary probe strand and a complete mismatch.

3.1 EXPERIMENTAL

The following abbreviations for reagents have been used in the experimental section: AcOH (acetic acid); *t*-BuOH (tertiary butanol); $CDCl_3$ (deuteriated chloroform); $CHCl_3$ (chloroform); DCM (dichloromethane); DMF (dimethyl sulphate); DMSO (dimethyl sulphoxide); DMSO-d_6 (deuteriated dimethyl sulphoxide); D_2O (deuteriated water); EDTA (ethylenediaminetetraacetic acid); EtOH (ethanol); HCl (hydrogen chloride); HNO_3 (nitric acid); H_2SO_4 (sulphuric acid); K_2CO_3 (potassium carbonate); $LiAlH_4$ (lithium aluminium hydride); $MgSO_4$ ((magnesium sulphate); MeCN (acetonitrile); MeOH (methanol); NaCl (sodium chloride); Na_2CO_3 (sodium carbonate); $NaHCO_3$ (sodium hydrogen carbonate); Na_2SO_4 (sodium sulphate); THF (tetrahydrofuran).

Purifications by column chromatography were performed on either silica gel (Sorbsil C60) or aluminium oxide (neutral; Brockmann grade 1), using redistilled solvents. Thin layer chromatography (tlc) was performed on either Whatman 2.5 x 7.5 cm glass-backed plates with a 0.25 mm layer of silica gel 60 F_{254}, or on Merck E-type aluminium backed sheets with a 0.2 mm layer of aluminium oxide 60 F_{254} (neutral).

Melting points were measured on a Reichert-Jung Thermovar hot-stage microscope melting point apparatus and are uncorrected.

Bulb-to-bulb distillations were conducted in a Kugelrohr apparatus and the temperatures quoted refer to the oven temperature.

Microanalysis were performed by MEDAC Ltd., Brunel University, on a Carlo Erba 1106 elemental analyser (dynamic combustion system) or a Control Equipment Corporation Model 240 XA (static combustion system).

Proton nuclear magnetic resonance (nmr) spectra were recorded on a Varian CFT-20 (90 MHz), or a Bruker AMX 200 (200 MHz) nmr spectrometer. Resonances are reported as (frequency; solvent); δ = shift in ppm from tetramethylsilane at 0 ppm and the multiplicity of signals as s, siglet; d, doublet; dd, double doublet; q, quartet; t, triplet; m, multiplet; br, broad; *J* values are in Hz)

Mass spectra were recorded either on an AEI MS902 spectrometer or at the SERC facility, Department of Chemistry, Swansea College, University of Wales for fast atom bombardment (FAB) and accurate mass measurements.

Infrared (ir) spectra were measured on a Perkin Elmer 1420 Ratio Recording spectrophotometer as KBr discs unless otherwise stated.

3.2 Luminescence measurements

Phosphoresence excitation and emission were recorded on a Perkin Elmer LS50B Luminescence spectrometer at room temperature with the following parameters;

Excitation spectra: Slit width 10.0 nm
Gate time 1.0 ms

Excitation spectra were determined by exciting from 200-450 nm whilst monitoring at 615 nm.

Emission spectra: Slit width 10.0 nm
Gate time 1.0 ms
Delay 0.1 ms

Emission spectra were run from 550-800 nm.

All luminescence measurements were run using 2ml quartz cells having a 1cm path length.

Excited state lifetimes were determined in both H_2O and D_2O by utilising the Short-Term Phosphoresence Decay program on the LS 50B spectrometer.

The number of coordinated water molecules was determined by the method of Horrocks and Sudwick (Chapter 1, p. 10), using experimentally determined excited-state lifetimes.

N,O,O'-Tris(p-toluenesulphonyl)-bis-(2-hydroxyethyl)amine (3)[12,81]

An ether solution of tosyl chloride (28.6g, o.15 mole) in ether (150 ml) was added slowly to a stirred solution of bis(2-hydroxyethyl)amine (5.3g, 0.05 mole) in triethylamine (52 ml). The reaction was carried out in an open beaker; after stirring for 1h, water was added to dissolve unreacted materials, and the reaction mixture left standing overnight. A white solid was isolated by filtration which was then purified by recrystallisation from hot absolute ethanol to give **3** as a white solid (16.7g, 58%), m.p. 66-67°C (lit.,[12] 65-67°C) (Found: C, 52.89; H, 5.43; N, 2.72. Calc. for C_{25} H_{29} N O_8 S_3: C, 52.89; H 5.15; N, 2.47%); δ_H (90 MHz; $CDCl_3$) 2.43 (9H, s, Ar-**CH_3**), 3.35 (4H, t, *J*5.8, N-**CH_2**), 4.09 (4H, t, *J*5.8, O-**CH_2**), 7.18-7.76 (12H, m, Aromatic-**H**).

N,N',N''-Tris(p-toluenesulphonyl)diethylenetriamine (12)[12,82]

To a cold vigorously stirred solution of 3-aza-hexane-1,3-diamine (3.2g, 0.013 mole) and sodium hydroxide (3.7g, 0.092 mole) in water (30 ml), a solution of toluene-p-sulphonyl chloride (17.5g, 0.092 mole) in ether (150 ml) was added dropwise over a period of 4h, followed by an additional 1h stirring. A white solid was collected by filtration, washed well with water and dried. The crude product **12** was purified by dissolving it in the minimum of hot DMF and precipitating it out by the addition of a large volume of water. The desired product **12** was isolated as a white solid by filtration, washed well with cold water and dried (11.4g, 41%), m.p. 174-175°C (lit.,[82] 172-174°C) (Found C, 53.14; H, 5.57; N, 7.42. Calc. for C_{25} H_{31} N_3 O_6 S_3; C, 53.08; H, 5.52; N, 7.42%); δ_H (90 MHz; DMSO-d_6) 2.38 (9H, s, Ar-**CH_3**), 2.89 (10H, br s, N-**CH_2** and **NH**), 7.50 (6H, d, *J*8.0, 2,6-Ar**H**), 7.68 (6H, d, *J*8.0, 3,5-Ar**H**).

The disodium salt of N,N',N''-Tris(toluene-p-sulphonyl)diethylene triamine (2)[12,82]

To a stirred refluxing suspension of **12** (10g, 0.018 mole) in absolute ethanol (700 ml), freshly prepared sodium ethoxide (2.72g, 0.04 mole) was added in one go. The mixture was refluxed for 24h, after which the excess ethanol was removed by evaporation *in vacuo* to give an off-white sticky solid. This was stirred with dry ether and filtered to give the desired product **2** as a very fine crystalline solid (10.0g, 91%) (Found C, 49.03; H, 5.08; N, 6.64. Calc. for C_{25} H_{29} N_3 Na_2 O_6 S_3: C, 49.25; H, 4.79; N, 6.89%). This salt was used immediately in the preparation of **4**.

1,4,7,10-Tetrakis-(p-toluenesulphonyl)-1,4,7,10-tetraazacyclododecane (4)[12]

To a stirred solution of **2** (10.0g, 0.016mole) in DMF (200 ml) at 100°, a solution of the tosylated diol **3** (9.1g, 0.016 mole) in DMF (100 ml) was added drop wise over 2h. The solution was then cooled to room temperature at which point a large volume of water was added and the resultant white precipitate collected by fitration. This was dissolved in boiling benzene (900 ml), and the resultant solution washed well with water, dried ($MgSO_4$), filtered and the filtrate reduced in volume to approximately 100 ml before the addition of absolute ethanol (200 ml). The solution was allowed to stand overnight in a refigerator resulting in crystallisation of the desired macrocycle **4** as a white solid (2.7g, 23%), m.p. 292-293°C (lit.,[83] 292°C) (Found: C, 54.76; H, 5.66; N, 7.00. Calc. for C_{36} H_{44} N_4)$_8$ S_4. C, 54.80; H, 5.62; N, 7.10%); δ_H (90 MHz; $CDCl_3$) 2.35 (12H, s, Ar-**C**$\mathbf{H_3}$), 3.32 (16H, s, N-**C**$\mathbf{H_2}$), 7.20 (8H, d, *J*9.0, 2,6-Ar**H**), 7.63 (8H, d, *J*9.0, 3,5-Ar-**H**).

N-(p-toluenesulphonyl)-bis-(2-iodoethyl)amine (13)[12]

To a solution of **3** (5.0g, 0.082 mole) in acetone 150 ml) sodium iodide (3.3g, 0.022 mole) was added, and the mixture refluxed for 8h. Solid material was removed by filtration and the filtrate evaporated to give an oil which was dissolved in ethyl acetate (100 ml) and washed with brine (2x 100 ml), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a clear oil which immediately solidified on standing to give the bisiodo derivative **13** (3.5g, 83%), δ_H (200 MHz; $CDCl_3$) 2.42 (3H, s, Ar-**C**$\mathbf{H_3}$), 3.27 (4H, t, *J*7.0, N-**C**$\mathbf{H_2}$), 3.50 (4H, t, *J*7.0, I-**C**$\mathbf{H_2}$), 7.33 (2H, d, *J*8.3, 2,6-Ar**H**), 7.69 (2H, d, *J*8.3, 3,5-Ar**H**); m/z 479 (M^+, 3.9%), 352 (M^+ - I, 7%), 324 (M^+ - Ts, 2%), 155 (Ts, 62%), 91 ($ArCH_3$, 100%).

1,4,7,10-Tetrakis-(toluene-p-sulphonyl)-1,4,7,10-tetraazacyclododecane (4)

To a vigorously stirred refluxing mixture of tetra-n-butyl ammonium iodide (0.60g, 0.0016 mole (25% mole)) in an aqueous solution of sodium hydroxide (5% w/v, 34 ml) and benzene (34 ml), a solution of the tritosylated ethylenediamine **12** (3.7g, 6.5 mmole) and the bisbromo derivative **13** (2.5g, 6.5 mmole) in benzene (194 ml) was added quickly through an addition funnel. The mixture was refluxed with stirring for 2 days after which it was allowed to cool, the benzene layer separated and the aqueous layer reextracted with

a single portion of benzene (50 ml), the combined organic phases was washed with water, dried ($MgSO_4$), filtered and evaporated to give a sticky residue which was triturated overnight with methanol and filtered to give the desired macrocycle **4** as a white powder (3.55g, 69.5%), m.p. 292-293°C (lit.,[83] 292°C) (Found: C, 54.89; H, 5.66; N, 7.00. Calc. for C_{36} H_{44} N_4 O_8 S_8: C, 54.80; H, 5.62; N, 7.10%); δ_H (90 MHz; $CDCl_3$) 2.42 (12H, s, Ar**CH**$_3$), 3.40 (16H, s, N-**CH**$_2$), 7.26 (8H, d, *J*9.0, 2,6-Ar**H**), 7.64 (8H, d, *J*9.0, 3,5-Ar**H**); m/z FAB 789 (M+H).

Bis-(2-iodoethyl)ether (14)[20]

Bis-(2-chloroethyl)ether (7.0g, 0.05 mole) and sodium iodide (16.5g, 0.11 mole) in acetone (50 ml) was refluxed for 24h and the inorganics filtered. The filtrate was evaporated and the residue dissolved in ether, washed with water then with a dilute solution of sodium thiosulphate (3M) to remove iodine. The ether phase was then dried ($MgSO_4$), filtered, evaporated and the crude residue distilled at reduced pressure (123-124°C @ 10mm Hg) to give **14** as a colourless liquid (13.3g, 86%), (lit.,[20] b.p. 123.5-124°C @ 10 mm Hg); δ_H (90 MHz; $CDCl_3$) 3.23 (4H, t, *J*7.0, I-**CH**$_2$), 3.45 (4H, t, *J*7.0, **O-CH**$_2$); m/z 326 (M^+, 0.2%), 199 (M^+ - I, 0.5%), 171 (M^+ - C_2H_4I, 12%), 155 (M^+ - OC_2H_4I, 32%), 155 (C_2H_4I, 100%).

1,4,7-Tri-(p-toluenesulphonyl)-1,4,7-triaza-10-oxa-cyclododecane (15).

To a vigorously stirred refluxing mixture of tetra-n-butyl ammonium iodide (0.19g, 0.5 mmole (25% mole)) in an aqueous solution of sodium hydroxide (5% w/v, 11 ml) in benzene (11 ml), a solution of the tritosylated ethylene diamine **12** (1.2g, 2 mmole) and bis-(2-iodoethyl)ether **14** (0.62g, 2 mmole) in benzene (70 ml) was added quickly through an addition funnel. The mixture was refluxed with stirring for 24h after which it was allowed to cool, the benzene layer separated and the aqueous layer reextracted with a single portion of benzene (50 ml), the combined organic phases was washed with water, dried ($MgSO_4$), filtered and evaporated to give a sticky white residue which was triturated overnight with methanol and filtered to give the desired macrocycle **15** as a fine white solid (0.83g, 65%), m.p. 203-204°C (lit.,[84] 200-204°C) (Found: C, 54.64; H, 5.81; N, 6.09. Calc.

for C_{29} H_{37} N_3 O_7 S_3: C, 54.78; H, 5.86; N, 6.60%); δ_H (90 MHz; $CDCl_3$) 2.41 (9H, s, -**CH_3**), 3.16-3.68 (16H, m, N-**CH_2**, O-**CH_2**), 7.2-7.82 (12H, m, Ar**H**); m/z FAB 636 (M+H).

N,N'-(p-toluenesulphonyl)ethylenediamine (16)[82]

To a cold vigorously stirred solution of ethylene diamine (3.0g, 0.05 mole) and sodium hydroxide (4.0g, 0.1 mole) in water (30 ml), a solution of p-toluenesulphonyl chloride (19.7g, 0.1 mole) in ether (100 ml) was added drop wise over 2h, followed by an additional 1h stirring. A white solid collected by filtration was purified by dissolving it in the minimum of hot DMF and precipitating the product by the addition of a large volume of water. The desired product **16** isolated as a white solid by filtration was washed well with water and dried (10.3g, 56%), m.p. 163-164°C (lit.,[82] 163-164°C); δ_H (90 MHz; DMSO-d_6) 2.36 (6H, s, Ar-**CH_3**), 2.77 (4H, s, N-**CH_2**), 7.35 (4H, d, *J*8.0, 2,6-Ar**H**), 7.64 (4H, d, *J*8.0, 3,5-Ar**H**), 7.69 (2H, br s, **NH**); m/z 368 (M^+, 0.2%), 213 (M^+ - Ts, 11%), 155 (Ts, 100%).

1,2-Bis-(2-iodoethoxy)ether (17)[20]

A mixture of 1,2-bis-(2-chloroethoxy)ethane (20.0g, 0.11 mole) and sodium iodide (36.0g, 0.24 mole) in acetone (100 ml) was refluxed with stirring for 48h. The solution was then filtered to remove inorganics, the filtrate evaporated and the residue dissolved in ether, this was then washed with water then with an aqueous solution of sodium thiosulphate to remove iodine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a brown oil. This was distilled at reduced pressure (126°C @ 0.5 mm Hg) to give the product **17** as a clear oil (25.5g, 63%), (lit.,[20] b.p. 126-129°C @ 0.5 mm Hg); δ_H (90 MHz; $CDCl_3$) 3.21 (4H, t, *J*7.0, I-**CH_2**), 3.61 (4H, s, I-CH_2**CH_2**), 3.72 (4H, t, *J*7.0, O-**CH_2**); m/z 370 (M^+, 0.3%), 199 (M^+ - OCH_2CH_2I, 18%), 155 (M^+ - $(OCH_2CH_2)_2I$, 100%).

1,4-Di-(p-toluenesulphonyl)-1,4-diaza-7,10-dioxa-cyclododecane (18).

To a vigorously stirred mixture of tetra-n-butyl ammonium iodide (0.37g, 1 mmole (25% mole)) in an aqueous solution of sodium hydroxide (5% w/v, 13.7 ml) in benzene (13.7 ml), a solution of the tosylated ethylene diamine **16** (1.5g, 4 mmole) and 1,2-bis-(2-iodoethoxy)ethane **17** (1.5g, 4 mmole) in benzene (115 ml) was added quickly through an addition funnel. The mixture was refluxed for 24h, allowed to cool, the benzene layer separated and the aqueous phase reextracted with a single portion of benzene (50 ml), the combined organic phases washed with water, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a white sticky solid. This was triturated overnight with MeOH and filtered to give the ditosylated macrocycle **18** as white solid (1.5, 65%), m.p. 224-226°C (lit.,[85] 220-226°C) (Found: C, 54.69; H, 6.19; N, 5.90. Calc. for C_{22} H_{30} N_2 O_6 S_2: C, 54.75; H, 6.26; N, 5.80%); δ_H (90 MHz; $CDCl_3$) 2.41 (6H, s, Ar**CH$_3$**), 3.25 (4H, m, N-**CH$_2$**), 3.50 (8H, s, N-**CH$_2$**, O-**CH$_2$**), 3.65 (4H, m, O-**CH$_2$**); m/z FAB 483 (M+H).

1,2-Bis-(2-iodoethoxy)benzene (19)

To a solution of 1,2-bis-(2-O,O'-p-toluenesulphonylethoxy)benzene (2.0g, 3.9 mmole) in acetone (100 ml), sodium iodide (1.48g, 9.88 mmole) was added and the mixture refluxed for 12h. The mixture was filtered and the filtrate evaporated *in vacuo* to give a dark brown residue which was dissolved in ether and then washed with water then with a dilute solution of sodium thiosulphate (3M) to remove iodine. The ether phase was then dried ($MgSO_4$), filtered and the filtrate evaporated *in vacuo* to give an off-white solid (1.32g, 80%), m.p. 60-61°C; δ_H (90 MHz; $CDCl_3$) 3.41 (4H, t, *J*7.0, I-**CH$_2$**), 4.28 (4H, t, *J*7.0, O-**CH$_2$**), 6.90 (4H, s, Ar**H**); m/z 419 (M^++1, 3.9%), 418 (M^+, 38%), 262 (M^+ - C_2H_4I, 48%), 155 (C_2H_4I, 100%).

1,4-Di-(p-toluenesulphonyl)-1,4-diaza-7,10-dioxa-(8,9-benzene)-cyclododecane (20).

To a vigorously stirred refluxing mixture of tetra-n-butyl ammonium iodide (0.37g, 1 mmole (25% mole) in an aqueous solution of NaOH (5% w/v, 13.7 ml) in benzene (13. 7 ml), a solution of the tosylated ethylene diamine **16** (1.5g, 4 mmole) and **19** (1.67g, 4 mmole) in benzene (115 ml) was added quickly through an addition funnel. The mixture

was refluxed for 24h, allowed to cool, the benzene layer separated and the aqueous phase reextracted with a single portion of benzene (50 ml). The combined organic phases was washed with water, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a sticky white solid. This was triturated overnight with MeOH, filtered and dried to give the macrocycle **20** as a white solid (1.25g, 59%), m.p. 181-182°C (Found: C, 58.77; H, 5.61; N, 5.19. Calc. for $C_{26}H_{20}N_2O_6S_2$: C, 58.85; H, 5.69; N, 5.28%); δ_H (90 MHz; $CDCl_3$) 2.40 (6H, **CH_3**), 3.39-3.5 (8H, m, N-**CH_2**), 4.24 (4H, t, *J*4.1, O-**CH_2**), 6.83 (4H, s, Ar**H**), 7.31 (4H, d, *J*8.4, Ts-2,6**H**), 7.68 (4H, d, *J*8.4, Ts-3,5**H**); m/z 531 (M+H).

1,2-Bis(2-azidoethoxy)ethane (26)[20,21]

1,2-Bis(2-chloroethoxy)ethane **25** (20.0 g, 0.107 mol) and sodium azide (13.9 g, 0.214 mol) in DMSO (100 ml) were reacted and worked up as for **41** to give the desired bisazide **26** as a clear yellow oil (16.4 g, 77%), $\upsilon_{max}cm^{-1}$ 2930-2880 (C-H), 2110vs (N_3), 1150 (C-O-C); δ_H (90 MHz; $CDCl_3$) 3.31 (4H, t, *J*5.0, N_3-**CH_2**), 3.50 (4H, s, N_3-CH_2**CH_2**), 3.55 (4H, t, *J*5.0, O-**CH_2**); m/z 200 (M^+, 2.3%), 116 (M^+ - $3N_3$, 14.7%), 74 (116 - OC_2H_4, 63%).

1,8-Diamino-3,6-dioxooctane (27)[20,21]

The reduction of 1,2-Bis(2-azidoethoxy)ethane **26** (10.0 g, 0.05 mol) with LAH (5.0 g, 0.13 mol) to the corresponding bisamino compound **27** was carried out as for **42**, to give the desired compound as a clear yellow oil (7.2 g, 97%); $\upsilon_{max}cm^{-1}$ 3420, 3340 (NH_2), 3000, 2920 (C-H), 1150 (C-O-C); δ_H (90 MHz; $CDCl_3$) 1.70 (4H, br s, D_2O exchangeable **NH_2**), 2.84 (4H, t, *J*5.0, N-**CH_2**), 3.50 (4H, t, *J*5.0, O-**CH_2**), 3.61 (4H, s, N-CH_2**CH_2**); m/z 147 (M^+, 1.5%), 117 (M^+ - CH_2NH_2, 65%), 87 (M^+ - $OCH_2CH_2NH_2$, 38%), 44 (CH_2NH_2,100%).

Diglycollic acid dichloride (24)[17]

To a stirred mixture of PCl_5 (91.5, 0.44 mole) in $CHCl_3$ (300 ml) at room temperature under nitrogen, diglycollic acid (26.8g, 0.2 mole) was added in small portions. This resulted in the formation of a slurry, however, the addition was continued over 1 hour. After addition of diglycollic acid the slurry was kept stirring overnight resulting in an almost clear solution being formed. The remaining insoluble material was removed by filtration, and the filtrate evaporated *in vacuo* to give the crude diglycollic acid dichloride as a dark oil. This was purified by fractional distillation (64°C @ 2.0 mm Hg), to give the

pure diacid chloride **24** as a clear oil (24.3 g, 71%); $\upsilon_{max}cm^{-1}$ 2980-2900 (C-H), 1800vs (C=O), 1150 (C-O-C); δ_H (90 MHz; $CDCl_3$) 4.65 (4H, s, O-**CH_2**-CO).

5,9-Dioxa-1,7,13-trioxo-4,10-diazacyclopentadecane (28)[17]

To a 2L three-necked round-bottom flask equiped with a mechanical stirrer, toluene (800 ml) was added and the flask and its contents cooled to 0-5°. Under a nitrogen atmosphere, the diamine **27** (7.0g, 0.047 mole) and triethylamine (10.4g, 0.1 mole) in toluene (200 ml) (**solution A**) and the diglycollic acid dichloride **24** (8.2g, 0.048 mole) in toluene (200 ml) (**solution B**) were added dropwise simultaneously to the vigorously stirred toluene solution over a period of 8h. Once addition was complete, the resulting mixture was stirred at room temperature overnight and filtered to remove salts. The filtrate was evaporated to give a white sticky solid, this was taken up in chloroform/benzene (50:50 v/v) and the resulting solution passed through a short column of neutral alumina eluting with the same solvent mixture. After evaporation of the solvent the macrocyclic diamide **28** was obtained as a white solid (6.3g, 54%), m.p. 149-150°C (lit.,[17] 149-150°C); $\upsilon_{max}cm^{-1}$ 3420 (NH), 1690s (C=O), 1115 (C-O); δ_H (90 MHz; $CDCl_3$) 3.54 (8H, m, N-**CH_2CH_2**-O), 3.62 (4H, s, O-**CH_2CH_2**-O); 4.01 (4H, s, CO-**CH_2**-O); 7.15 (2H, br s, **NH**); m/z 247 (M+1), 246 (M^+, 3%), 216 (M^+ - NH_2=CH_2, 5%), 30 (NH_2=CH_2, 100%).

1,7,13-Trioxo-4,10-diazacyclopentadecane (29)[17]

To a cooled suspension of lithium aluminium hydride (5.0g, 0.13 mole) in dry THF (200 ml) under nitrogen, the diamide **28** (5.0g, 0.02 mole) was added portionwise in solid form over 30 minutes (vigorous reaction!). After addition was complete the mixture was refluxed for 12h, allowed to cool when excess LAH was destroyed by the dropwise addition of 5% sodium hydroxide solution. The resulting white solid of hydroxide salts was filtered and the filtrate evaporated *in vacuo* to give an off-white solid. This was recrstallised from light petroleum ether, to give the title compound **29** as a white hygroscopic solid (3.6, 82%), m.p. 89-90°C (lit.,[17] 89-90°C); δ_H (90 MHz; $CDCl_3$) 2.30 (2H, br s, D_2O exchangeable **NH**), 2.76 (8H, t, *J*4.8, N-**CH_2**), 3.53-3.66 (12H, m, O-**CH_2**); m/z 217 (M^+, 2.3%), 173 (16.9%), 150 (80%), 132 (89.7%), 83 (50.3%), 44 (26.9%), 18 (100%).

Ethyl-(1,7,13-trioxo-4,10-diazacyclopentadecane)-N,N'-diacetate (30)[21]

To trioxodiazacyclopentadecane **29** (1.0g, 4.6 mmole) in anhydrous acetonitrile (50 ml) was added sodium carbonate (5.0g, 0.046 mole) and ethylbromoacetate (1.7g, 0.01 mole) . The reaction mixture was refluxed under nitrogen for 18h, cooled and filtered. The filtrate was stripped of solvent to give **30** as its NaBr conplex. The title compound **30** was isolated from the resulting solid complex by bulb-to-bulb distillation (150° @ 10^{-3} mmHg), giving **30** as a clear oil (0.91g, 51%); $\upsilon_{max}cm^{-1}$ 3000-2940 (C-H), 1740s (C=O), 1190-1120 (C-O); δ_H (90 MHz; $CDCl_3$) 1.24 (6H, t, *J*7.1, -**CH_3**), 2.93 (8H, m, N-**CH_2**), 3.60 (16H, m, O-**CH_2**, N-**CH_2**-CO_2), 4.12 (4H, q, *J*7.2, CO_2-**CH_2**); m/z 390 (M^+, 12%), 359 (M^+ - CH_3O, 21%), 317 (M^+ - $CO_2CH_2CH_3$, 100%), 303 (317 - CH_2, 19%), 230 (303 - $CO_2CH_2CH_3$, 8%), 216 (230 - CH_2, 17.2%).

1,7,13-trioxo-4,10-diazacyclopentadecane-N,N'-diacetic acid (21)[20]

To ultra pure water (25 ml), the diester **30** (0.15g, 0.38 mmole) was added and the mixture refluxed for 16h. The water was evaporated under high vacuum and the resulting residue recrystallised from 80% ethanol to give the title diacid **21** as white crystals (77mg, 60%) (Found: C, 49.96; H, 8.05; N, 8.07. Calc. for $C_{14}H_{26}N_2O_7$: C, 50.29; H, 8.05; N, 8.07%); δ_H (90 MHz; D_2O/TSP) 3.57 (8H, m, N-**CH_2**), 3.81 (16H, m, N-**CH_2**-CO_2, O-**CH_2**); m/z 290 (M^+ - CO_2, 6%), 246 (M^+ - $2CO_2$, 32%), 44 (CO_2, 100%).

1,7-Dibenzyl-2,6-dioxo-4-oxa-1,7-diazaheptane (31)[21]

A solution of benzylamine (19.3g, 0.18 mole) and triethylamine (18.2g, 0.18 mole) in benzene (100 ml) was slowly added to a stirred solution of diglycollic acid dichloride **24** (13.0g, 0.076 mole) in benzene (100 ml). The temperature of the reaction was kept at 0-8° during the addition with the aid of an ice/water bath. After addition the reaction mixture was allowed to warm up to room temperature where it was kept for 2h, before being concentrated by evaporation. The residue was dissolved in chloroform (200 ml) and consecutively washed with 3M HCl (20 ml), 1M NaOH (20 ml), and water (100 ml). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo* to give the crude product as an off-white solid. Recystallisation fron benzene gave pure **31** as a white

crstalline solid (13.9g, 51%), m.p. 124-125°C (lit.,[21] 124-125°C); $\upsilon_{max}cm^{-1}$ 3380 (NH), 1650s (C=O), 1560, 1530, 1465 (C=C), 1150 (C-O-C), 710 (NH); δ_H (90 MHz; $CDCl_3$) 4.05 (4H, s, O-**CH_2**-CO), 4.44 (4H, d, *J*5.9, Ph**CH_2**), 7.16 (2H, br s, **NH**), 7.24 (10H, s, Ph**H**); m/z 312 (M^+, 91.7%), 294 (30%), 205 (35.8%), 149 (38.6%), 106 (92.9), 91 ($PhCH_2$,100%).

1,7-Dibenzyl-4-oxo-1,7-diazaheptane (32)[21]

A solution of **31** (10.0g, 0.028 mole) in dry THF (50 ml) was slowly added to a stirred slurry of lithium aluminium hydride (8.0g, 0.21 mole) in dry THF (200 ml) at 0°. After addition was complete, the reaction was stirred at reflux for 21h. The reaction was cooled to 0°, and excess LAH destroyed by the consecutive addition of water (6 ml), 15% NaOH (6 ml), and water (18 ml). The white precipitate was removed by filtration, the filtrate evaporated *in vacuo*, and the residue dissolved in chloroform (200 ml), washed with brine (30 ml) and water (30 ml). The organic phase was dried (Na_2SO_4), filtered and evaporated *in vacuo* to give **32** as a clear yellow oil (6.1g, 77%); $\upsilon_{max}cm^{-1}$ 3320 (NH), 2890s (C-H), 1510, 1450 (C=C), 1120s (C-O-C); δ_H (90 MHz; $CDCl_3$) 1.83 (2H, br s, D_2O exchangeable **NH**), 2.75 (4H, t, *J*5.4, N-**CH_2**), 3.53 (4H, t, *J*5.4, O-**CH_2**), 3.75 (4H, s, Ph**CH_2**), 7.23 (10H, s, Ph**H**); m/z 284 (M^+, 2%), 193 (M^+ - $PhCH_2$, 18%), 91 ($PhCH_2$, 100%).

N,N'-Dibenzyl-1,7,13-trioxo-4,10-diazacyclopentadecane (33)[21]

A solution of 1,7-dibenzyl-4-oxa-1,7-diazaheptane **32** (4.3g, 0.015 mole), 1,2-bis-(2-iodoethoxy)ethane **17** (7.4g, 0.02 mole), sodium carbonate (10.6g, 0.1 mole) and sodium iodide (1.5g, 0.01 mole) in dry acetonitrile (400 ml) was heated at reflux under nitrogen for 24h. The reaction mixture was cooled, filtered to remove inorganics and evaporated. The residue was dissolved in chloroform (100 ml) and extracted with 6M HCl (2 x 50 ml). The combined aqueous phases were then adjusted to pH 8-10 with the addition of solid sodium carbonate, before being extracted with chloroform (2 x 100 ml). The combined organic phases was evaporated *in vacuo* to give a clear red/brown oil, which was purified by column chromatography [neutral alumina: 10% ethylacetate/hexanes], to give the title compound **33** as a clear oil (3.9g, 65%); δ_H (90 MHz; $CDCl_3$) 2.80 (8H, t, *J*5.6, N-**CH_2**),

3.53-3.67 (16H, m, O-$\mathbf{CH_2}$ and Ph$\mathbf{CH_2}$), 7.24 (10H, s, Ph**H**); m/z 398 (M^+, 2.3%), 307 (M^+ - $PhCH_2$, 14%), 176 (16%), 164 (32%), 91 ($PhCH_2$, 100%).

1,7,13-Trioxa-4,10-diazacyclopentadecane (29)

To a stirred solution of liquid ammonia (100 ml) kept at reflux at atmospheric pressure, dry THF (50 ml) was added followed by freshly cut sodium (4.14g, 0.18 mole). To this blue solution , a solution of **33** (3.0g, 7.54 mmole) in dry THF (50 ml) was added dropwise. Stirring was continued for a further 2h, after which the reaction was quenched by the addition of absolute EtOH (10 ml). The reaction mixture was left overnight at room temperature (to evaporate off the ammonia), and filtered to remove inorganic materials. The filtate was evaporated *in vacuo* and the residue dissolved in water (40 ml), extracted with $CHCl_3$ (2 x 50 ml). The combined organic extracts were dried (Na_2SO_4) and the solvent removed *in vacuo* to yield a red/brown oil. This was purified by bulb-to-bulb distillation (110-120° @ 10^{-3} mmHg) to give **29** as a waxy solid (1.3g, 79%), m.p. 88-89°C (lit.,[17] 89-90°C); δ_H (90 MHz; $CDCl_3$) 2.24 (2H, s, D_2O exchangeable **NH**), 2.76 (8H, t, *J*4.8, N-$\mathbf{CH_2}$), 3.54-3.66 (12H, m, O-$\mathbf{CH_2}$).

3,6-Dioxa-4-(allyl)oxymethyl-1,8-octanedioic acid (36).[23]

To a solution of potassium t-butoxide (34.3 g, 0.036 mole) in t-butyl alcohol (230 ml) under a blanket of nitrogen was added 3-(allyl)oxy-1,2-propane diol (3.10 g, 0.076 mole). After stirring at room temperature for 1 h, a solution of chloroacetic acid (14.4 g, 0.152 mol) in t-butyl alcohol (58 ml) was added dropwise over 1 h at reflux. The mixture was stirred and refluxed for 18 h and the solvent removed *in vacuo*. The solid off-white residue obtained was dissolved in the minimum of water and the aqueous solution extracted with diethyl ether (2x 100 ml) and acidified with 6M hydrochloric acid. This was then extracted with ethyl acetate (5x 100 ml) saturating the aqueous layer with solid sodium chloride between extractions. The combined organic extracts were washed with brine (100 ml), dried ($MgSO_4$) and the solvent removed *in vacuo* to give the title compound **36** as a pale yellow

oil (13.0 g, 69%); δ_H(90 MHz; $CDCl_3$) 3.5-4.7 (11H, m, OCH_2), 5.1-5.4 (2H, m, $C=CH_2$), 5.6-6.1 (1H, m, CH=C), 11.2 (2H, s, COOH); ν_{max}/cm^{-1} (film) 3700-2300 (COOH), 1760 (C=O), 1130 (C-O).

3,6-Dioxa-4-[(allyloxy)methyl]-1,8-octanedioic acid dichloride (37).[23]

A solution of the diacid (7.0 g, 2.8 mol) in oxaly choride (35 ml) was refluxed with stirring under nitrogen for 1.5 hours, after which excess oxaly chloride was removed by evaporation to give **37** a light yellow oil (8.0 g ,100%); δ_H(90 MHz; $CDCl_3$) 3.5-4.0 (11H, m, OCH_2), 5.0-5.6 (2H, m, $C=CH_2$), 5.7-6.3 (1H, m, CH=C); ν_{max}/cm^{-1} (film) 1810 (C=O), 1160 (C-O). This was used without further purification.

2,13-Dioxa-1,7,13-trioxo-15-[(allyloxy)methyl]-4,10-diazacyclopentadecane (38).[23]

Bis-1,2-(aminoethyl)ether **42** (1.56 g, 15 mmol) and triethyl amine (4.13 g, 41 mmol) in toluene (100 ml) (**Solution A**), and the diacid dichloride **37** (4.28 g, 15 mmol) in toluene (100 ml) (**Solution B**), were added simultaneously to a vigorously-stirred toluene (500 ml) at 0-5°C over 8 hours under nitrogen. After the addition was complete, the mixture was stirred overnight at room temperature. The mixture was filtered to remove insoluble material, and the filtrate evacuated *in vacuo* to give a light brown oil which was purified by column chromatography on neutral alumina with $CHCl_3$ as eluant to afford pure **38** as a colorless hygroscopic oil (1.9 g, 40%); δ_H(90 MHz; $CDCl_3$) 3.1-4.6 (19H, m, O-CH_2, N-CH_2), 5.1-5.4 (2H, m, $C=CH_2$), 5.6-6.1 (1H, m, CH=C), 7.1 (2H, br s NH); ν_{max}/cm^{-1} (film) 3422 (NH), 1685 (C=O), 1120 (C-O).

2,13-Dioxa-1,7,13-trioxo-15-(hydroxymethyl)-4,10-diazacyclopentadecane (39).[23]

To a solution of the (allyoxy)methyl diamide **38** (100 mg, $3.2x10^{-4}$ mmol) in a 1:1 (v/v) mixture of water and ethanol (1 ml) was added 5% Pd/C (9 mg) and perchloric acid (0.007 ml). The mixture was heated with stirring at 80° for 24 hours. The catalyst was removed by filtration and the filtrate made basic by the addition of aqueous ammonium hydroxide. The solvent was removed in vacuo without heating (CARE! Perchlorates), to leave a sticky

oil. Purification by column chromatography [silica: 10% MeOH/$CHCl_3$] gave the desired product **39** as a white hygroscopic solid (61 mg, 69%), m.p. 110-111°C (lit.,[23] 110-111°C); δ_H(90 MHz; $CDCl_3$) 2.9-4.5 (18H, m, O-CH_2, N-CH_2), 7.1 (2H, br s, NH); ν_{max}/cm^{-1} (KBr) 3400 (NH, OH), 1670 (C=O), 1125 (C-O).

1,7,13-Trioxo-15-(hydroxymethyl)-4,10-diazacyclopentadecane (34).[23]

To a cooled suspension of lithium aluminium hydride (0.11g, 2.79 mmol) in THF (2 ml) under nitrogen, a slurry of the hydroxmethyl diamide **39** (0.1 g, $3.64x10^{-4}$ mol) was added and the mixture was refluxed for 24 hours. After cooling, excess LAH was destroyed by the addition of 5% NaOH solution. Solid material was filtered and washed several times with hot THF, and the combined filtrate and washings evaporated *in vacuo* and the residue purified by column chromatography on neutral alumina with chloroform-ethanol (25:1) to give the title compound **40** as an off-white hygroscopic solid (65 mg ,73%), m.p. 47-49°C (lit.,[23] 47-49°C); δ_H(90 MHz; $CDCl_3$) 2.4 (2H, br s, D_2O exchangeable NH), 2.6-2.9 (8H, m, NCH_2), 3.2-4.0 (13H, m, OCH_2); ν_{max}/cm^{-1} (film) 3310, 3180 (NH, OH), 1112 (C-O); m/z 217 (M^+ - CH_2OH, 22%), 180 (46.5%), 162 (100%), 100 (49.3%), 88 (65%), 85 (58.5%), 83 (82%), 44 (43.5%).

1,7,13-Trioxo-15-(hydroxymethyl)-4,10-diazacyclopentadecane (34).

A solution of the (allyloxy)methyl diaza-15-crown-5-diamide **38** (1.5 g, 4.8 mmol) in THF (40 ml) was slowly added to a stirred solution of LAH (6.1 g, 0.16 mol) at 0-5° under nitrogen. The mixture was refluxed for 36 hours after which tlc [silica: 10% MeOH/$CHCl_3$] showed the disappearance of starter. The mixture was allowed to cool and excess LAH quenched by the addition of water (5 ml), 3M NaOH (5 ml) and water (5 ml). Work-up as for **34** and chromatography using neutral alumina with chloroform-ethanol (25:1) gave the desired product **34** as an off-white hygroscopic solid 0.8 g, 67%), m.p. 47-49°C. Other physical properties were identical to those already reported.

Ethyl-(1,7,13-trioxo-15-(hydroxymethyl)-4,10-diazacyclopentadecane-N,N;-diacetate (35).

To a solution of hydroxymethyl-diaza-15-crown-5 **34** (0.08g, 0.32 mmole) in MeCN (16 ml), anhydrous Na_2CO_3 (0.07g, 0.65 mmole) was added followed by ethylbromoacetate (0.11g, 0.65 mmole). The mixture was refluxed under nitrogen for 24h, allowed to coool to room temperature, filtered and the filtrate evaporated *in vacuo* to give **35** as its NaBr complex. The title compound was isolated from the resulting complex by bulb-to-bulb distillation (140°C @ 10^{-3} mmHg), giving **35** as a clear hygroscopic oil (83 mg, 59%) (Found: C, 53.13; H, 8.68; N, 6.52. Calc. for $C_{19}H_{36}N_2O_8.0.5H_2O$: C, 52.92; H, 8.59; N, 6.58%); δ_H (90 MHz; $CDCl_3$) 1.25 (6H, t, *J*7.2, -**CH_3**), 2.17 (2H, s, H_2O, CH_2**OH**), 2.86-2.96 (8H, m, N-**CH_2**), 3.45-3.89 (17H, m, N-**CH_2**CO, O-**CH_2**), 4.1 (4H, q, *J*7.2, CO_2**CH_2**-); m/z FAB 421 (M+H).

Bis-1,2-(azidoethyl)ether (41).[21]

To a stirred solution of sodium azide (18.8 g, 0.28 mol) in DMSO (100 ml) at 90°, bis-1,2-(chloroethyl)ether (20.0 g, 0.14 mol) was added dropwise under nitrogen. The mixture was refluxed at 90°C for 4 hours, allowed to cool before being added to a large volume of water. This was then extracted with diethyl ether (5x 100 ml), dried and evaporated *in vacuo* to give **41** as a clear yellow oil (18.8 g, 85%), ν_{max}/cm^{-1} (film) 2950-2890 (C-H), 2100vs (N_3), 1120 (C-O-C); δ_H (90 MHz; $CDCl_3$) 3.36 (4H, t, *J*5.0, N_3-**CH_2**), 3.68 (4H, t, *J*5.0, O-**CH_2**); m/z 157 ($M^+ + 1$, 0.96%), 72 ($M^+ - 3N_2$, 8%), 44 (72 - C_2H_4, 23.6%), 28 (72 - OC_2H_4, 54.5%).

Bis-1,2-(aminoethyl)ether (42).[21]

To a cooled suspension of lithium aluminium hydride (7.2 g, 0.189 mol) in THF (300 ml) under nitrogen, a solution of the bis azide **41** (10.0 g, 0.071 mol) in THF (30 ml) was added dropwise over 1 hour (vigorous reaction!). After addition the mixture was refluxed for a further 18 hours, allowed to cool when excess LAH was quenched by the addition of 5% NaOH. The resulting white solid was filtered and the filtrate evaporated *in vacuo* to give the title compound as an almost colourless oil (5.38 g, 73%); $\upsilon_{max}cm^{-1}$ (film) 3420-

3340 (NH_2), 2990-2920 (C-H), 1650 (N-H), 1150 (C-N); δ_H (90 MHz; $CDCl_3$) 1.64 (4H, br s, D_2O exchangeable **NH_2**), 2.84 (4H, t, *J*5.5, N-**CH_2**), 3.47 (4H, t, *J*5.5, O-**CH_2**); m/z 103 (M^+, 0.5%), 74 (M^+ - CH_2NH_2, 72.8%), 44 (CH_2NH_2, 100%).

N-(butylacetamido)-ethylenediamine-N,N,N'-triacetate (sodium salt) (46).

To a slurry of ethylenediaminetetraacetic acid dianhydride (2.0g, 7.81 mmole) in dry THF (40 ml), n-butylamine (0.57g, 7.81 mmole) was added and the reaction mixture stirred at room temperature under nitrogen for 24h. The solvent was removed *in vacuo* to give a white solid residue, which was dissolved in aqueous NaOH (0.94g, 0.024 mole) (15 ml), and extracted with diethyl ether (2 x 15 ml). The aqueous layer was evaporated to give a sticky residue which was dried under high vacuum, and recrystallised from hot methanol to give the monobutylamide **46** as a white solid (2.6g, 79%), m.p. >300°C dec. (Found: C, 40.40; H, 5.72; N, 10.05. Calc. for C_{14} H_{22} N_3 Na_3 O_7 .H_2O: C, 40.49; H, 5.82; N, 10.11%); δ_H (200MHz; D_2O/TSP) 0.95 (3H, t, *J*7.3, **CH_3**), 1.34-1.49 (4H, m, **-CH_2-**), 2.52 (1H, s, N-**CH_2**), 2.69 (3H, s, N-**CH_2**), 3.2 (10H, m, N-**CH_2**CO); m/z FAB 414 (M + H).

N,N'-bis-(butylacetamido)-ethylenediamine-N,N'-diacetate (sodium salt) (47).

To a slurry of ethylenediaminetetraacetic acid dianhydride (2.0g, 7.81 mmole) in dry THF (40 ml), n-butylamine (1.14g, 0.016 mole) was added and the reaction mixture stirred at room temperature under nitrogen for 24h. The work up was identical to that used for **46** apart from the residue was dissolved in two equivalents of NaOH (0.63g, 0.016 mole) in water (15 ml). Recrystallisation from hot methanol gave the bis-butylamide **47** as a white solid (2.4g, 69%), m.p. 173-174°C (Found: C, 46.41; H, 7.23; N, 11.85. Calc. for C_{18} H_{32} N_4 Na_2 O_6 .H_2O: C, 46.55; H, 7.38; N, 12.06%); δ_H (200 MHz: D_2O/TSP) 0.97 (6H, t, *J*7.3, **CH_3**), 1.30-1.48 (8H, m, **-CH_2-**), 2.7 (4H, s, N-**CH_2**), 3.18 (4H, s, N-**CH_2**CO), 3.23 (8H, m, N-**CH_2**CO); m/z FAB 447 (M + H).

1,10-Phenanthroline-2,9-dicarboxaldehyde (51)[31]

To a solution of 2,9-dimethyl-1,10-phenanthroline **50** (6.0g, 0.03 mole) in dioxan containing 4% water (200 ml) was added selenium dioxide (15.0g, 0.14 mole). The solution was heated under reflux for 4h and then filtered hot through celite. The crude dialdehyde **53** separated from the cold filtrate as yellow crystals. Further purification was achieved by recrystallisation from THF/water to give **51** as an off-white crystalline solid (5.2g,73%), m.p. 231-232°C (lit.,[31] 231-232°C); υ_{max}/cm^{-1} 2853 (aldehydic C-H), 1709 (C=O), 1595, 1552 (C=C), 869, 815 (C-H); δ_H (90 MHz; DMSO-d_6) 8.17 (2H, s, 5,6**H-phen**), 8.26 (2H, d, *J*9, 3,8**H-phen**), 8.82 (2H, d, *J*9, 4,7**H-phen**), 10.31 (1H, s, C**H**O); m/z 237 (M^+, 19%), 236 (M^+ - 1, 66%), 208 (236 - CO, 74%), 180 (208 - CO, 100%).

1,10-Phenanthroline-2,9-dicarboxylic acid (48)[31]

The dialdehyde **51** (0.5g, 2.1 mmole) was added to 80% nitric acid (10 ml) and the resulting solution heated under reflux for 3h. The solution was cooled, poured onto ice and the precipitated solid recrystallised from methanol and dried to give the diacid **48** as the monohydrate (0.30g, 55%), m.p. 237°C dec. (lit.,[31] 238°C dec.); υ_{max}/cm^{-1} 3600-2800 (COOH), 1735 (C=O), 880 (O-H); δ_H (90 MHz; DMSO-d_6) 8.20 (2H, s, 5,6**H-phen**), 8.40 (2H, d, *J*8.3, 3,8**H-phen**), 8.73 (2H, d, *J*8.3, 4,7**H-phen**); m/z 224 (M^+ - CO_2, 4%), 180 (M^+ -2CO_2, 55%), 44 (CO_2, 100%).

2,9-Bis-(trichloromethyl)-1,10-phenanthroline (55)[32]

To a solution of 2,9-dimethyl-1,10-phenanthroline **50** (5.0g, 0.024 mole) in carbon tetrachloride (200 ml), N-chlorosuccinimide (19.2g, 0.144 mole) was added followed by a catalytic ammount of 3-chloroperoxybenzoic acid to initiate the reaction. The reaction was heated at reflux for 12h, cooled to room temperature when precipitated succinimide was filtered off and the filtrate concentrated *in vacuo* to give a yellow solid. The solid was dissolved in chloroform (150 ml), this was then washed with a saturated solution of Na_2CO_3, dried (Na_2SO_4), filtered and evaporated to give crude **55** a pale yellow solid. This was purified by column chromatography [silica gel: benzene/acetone (9:1)] to give **55** as a white solid (8.57g, 86%), m.p. 212-214°C (lit.,[32] 212-214°C); δ_H (90 MHz; $CDCl_3$) 7.90

(2H, s, 5,6**H-phen**), 8.25 (2H, d, *J*8.6, 3,8**H-phen**), 8.40 (2H, d, *J*8.6, 4,7**H-phen**); m/z 414 (M^+, isotopic cluster), followed by a complex isotopic pattern in accord with the sequential loss of chlorines; 380 (M^+ - Cl, isotopic cluster), 345 (380 - Cl, isotopic cluster), 308 (345 - Cl, isotopic cluster), 273 (308 - Cl, isotopic cluster), 237 (273 - Cl, isotopic cluster).

1,10-Phenanthroline-2,9-dicarboxylic acid (48)[31,32]

A solution of the hexachloride **55** (2.0g,4.82 mmole) in 98% sulphuric acid (10 ml) was heated at 80-90°C under nitrogen for 6h (evolution of HCl gas!). The resulting dark-brown solution was allowed to cool to room temperature and the viscous solution was then slowly poured over crushed ice/water. The crude diacid **48** precipitated as an off-white solid, it was collected, washed well with cold water and recrystallised from hot water/THF to give the desired pure diacid **48** as a creamy white solid (1.18g, 91%), m.p. 238°C (lit.,[31] 238°C) (Found: C, 58.55; H, 3.46; N, 9.82. Calc. for $C_{14}H_8N_2O_4.H_2O$: C 58.55; H 3.36; N 9.82%); υ_{max}/cm^{-1} 3600-2600 (COOH), 1720 (C=O), 1290-1200 (C-O), 870 (O-H); δ_H (90 MHz; DMSO-d_6) 3.17 (s, $\mathbf{H_2O}$) 8.21 (2H, s, 5,6**H-phen**), 8.41 (2H, d, *J*8.4, 3,8**H-phen**), 8.74 (2H, d, *J*8.4, 4,7**H-phen**); m/z 224 ($M^+ - CO_2$, 7%), 180 ($M^+ - 2CO_2$, 52%), 44 (CO_2, 100%).

2,9-Bis(methoxycarbonyl)-1,10-phenanthroline (56)[32]

A solution of 2,9-bis(trichloromethyl)-1,10-phenantholine **55** (2.0g, 4.82 mmole) in conc. sulphuric acid (5 ml) was heated at 90° under nitrogen for 2h. During the course of the reaction HCl gas is liberated. After cooling the mixture (ice/water), methanol (5.0 ml) was cautiously added with rapid stirring, and the solution refluxed for a further 1h. The solution was cooled to room temperature, and poured over crushed ice. The precipitated solid was collected by filtration, washed several times with ice-cold water, then with cold methanol and dried. Recrystallisation from MeOH/$CHCl_3$ gave **56** as a white solid (1.18g, 83%), m.p. 213-214°C (lit.,[32] 213-214°C); υ_{max}/cm^{-1} 1730 (C=O), 1280 (C-O), 1020 (O-CH_3); δ_H (90 MHz; DMSO-d_6) 4.01 (6H, s, $CO_2\mathbf{CH_3}$), 8.19 (2H, s, 5,6**H-phen**), 3.39 (2H, d, *J*8.3, 3,8**H-phen**), 8.74 (2H, d, *J*8.3, 4,7**H-phen**); m/z 296 (M^+, 7%), 265 ($M^+ - CH_3O$, 7%), 237 ($M^+ - COOCH_3$, 100%), 178 ($M^+ - 2COOCH_3$).

2,9-Bis(hydroxymethyl)-1,10-phenanthroline (57)[32]

To a stirred solution of the diester **56** (2.8g, 9.5 mmole) in absolute EtOH (300 ml), sodium borohydride (1.4g, 0.039 mole) was slowly added portionwise under nitrogen. The solution was refluxed for 3h, cooled, and concentrated *in vacuo* to give a solid which was stirred with water (100 ml), before being filtered to give a solid. This was recrystallised from water to give the dialcohol **57** as light yellow needles (1.82g, 79%), m.p. 197-198°C dec., (lit.,[31]197-198°C dec.) (Found C, 64.91; H, 5.22; N, 10.64. Calc. for $C_{14}H_{12}N_2.H_2O$: C, 65.10; H, 5.46; N, 10.84%); $\upsilon_{max}cm^{-1}$ 3600-3100 (O-H), 1190 (O-H), 880 (C-O); δ_H (90 MHz; DMSO-d_6) 3.2 (H_2O), 5.05 (4H, d, *J*7.1, CH_2-OH), 5.69 (2H, t, **OH**), 7.95 (4H, m, 3,8,5,6**H-phen**), 8.50 (2H, d, *J*8.6, 4,7**H-phen**); m/z 240 (M^+, 17%), 239 (M^+ - 1, 23%), 223 (M^+ - OH, 100%), 209 (M^+ - CH_3O, 25%), 194 (223 - COH, 94%).

1,10-Phenanthroline -1-oxide (59)[33,34]

To a stirred solution of 1,10-phenanthroline **58** (4,7g, 0.026 mole) in glacial acetic acid (30 ml), hydrogen peroxide (30% solution in water) (3 ml) was added and the temperature raised to 72°C were it was carefully maintained for 2.5h, after which an additional ammount of hydrogen peroxide (3 ml) was added. Heating at 72°C was continued for 3h, after which the mixture was allowed to cool when a final portion of hydrogen peroxide was added (2 ml), and the mixture kept at room temperature overnight. The solution was concentrated *in vacuo* to ≈15 ml, when water (45 ml) was added and evaporation continued to a volume of ≈15 ml. To this remaining dark-brown oil, solid Na_2CO_3 (≈125g) was added and the mass extracted with hot chloroform (Soxhlet). The chloroform solution was stirred for 1h with decolourising charcoal and $MgSO_4$, filtered and evaporated *in vacuo* to give **59** as an off-white solid which was recrstallised from chlorobenzene to give pure **59** as needle-like crystals (2.9g, 57%), m.p. 181-182°C (lit.,[34] 180-181°C) (Found: C, 67.11; H, 4.60; N, 12.91. Calc. for $C_{12}H_8N_2O.H_2O$: C, 67.28; H, 4.70, N, 13.08%).

2-Cyano-1,10-phenanthroline (61)[33]

A solution of **59** (0.98g, 5 mmole) and potassiun cyanide (1.0g, 0.015 mole) in water (20 ml) was stirred at room temperature while benzoyl chloride (1.0 ml, 8.6 mmole) was added

dropwise over 10 minutes. The reaction mixture was stirred for a further 30 minutes and the precipitated solid collected by filtration, washed with water and dried to give a tan coloured solid. This was recrystallised from EtOH to give **61** as a light brown fluffy solid (0.52g, 54%), m.p. 238-239°C (lit.,[33] 237-238°C) (Found: C, 75.86; H, 3.45; N, 20.18. Calc. for $C_{13}H_7N_3$: C, 76.09; H, 3.41; N, 20.48%); $\upsilon_{max}cm^{-1}$ 2230 (C≡N); δ_H (90 MHz; $CDCl_3$) 7.60-7.75 (1H, m, 8**H-phen**), 7.85 (2H, d, *J*3.2, 5,6**H-phen**), 7.91 (1H, d, *J*8, 3**H-phen**), 8.26 (1H, dd, *J*1.7 and 4.4, 7**H-phen**), 8.35 (1H, d, *J*8, 4**H-phen**), 9.22 (1H, dd, *J*1.7 and 4.4, 9**H-phen**); m/z 205 (M^+, 100%).

1,10-Phenanthroline-2-carboxylic acid (62)[33]

To a solution of **61** (0.5g, 2.5 mmole) in absolute ethanol (10 ml), an aqueous solution of sodium hydroxide (0.4g, 0.01 mole) (5 ml) was added and the mixture refluxed for 2h. The solution was cooled and acidified to pH ≈1 with the dropwise addition of conc. HCl, and the precipitated product isolated by filtration, washed well with cold ethanol and dried to to give **62** as the hydrochloride salt of the acid as a white solid (0.45g, 80%), m.p. 263-265°C (lit.,[33] 262-265°C) *(Found: C, 57.20; H, 3.62; N, 10.25. Calc. for $C_{13}H_9N_2O_2Cl$ .0.70 H_2O: C, 57.12; H, 3.80; N, 10.25%); m/z 224 (MH^+ (free acid), 17%), 180 (MH^+ - CO_2, 100%);

*The characterisation as a hydrate is consistent with previous work[86] which report the hygroscopic nature of 1,10-phenanthroline-2-carboxylic acid.

1,10-Phenanthroline-2,9-dicarbonyl chloride (63)[87]

1,10-Phenanthroline-2,9-dicarboxylic acid **48** (0.6g, 2.2 mmole) in redistilled thionyl chloride (15 ml) was refluxed with stirring under nitrogen until dissolution occurred, ≈2h. The clear yellow solution was evaporated *in vacuo* to give the diacid chloride **63** as a yellow solid (0.63g, 94%), m.p. 226-228°C dec. (lit.,[87] 227-228°C); $\upsilon_{max}cm^{-1}$ 1770 (C=O); δ_H (90 MHz; $CDCl_3$) 8.02 (2H, s, 5,6**H-phen**), 8.39 (4H, s, 3,4,7,8**H-phen**); m/z 305 (M^+, iostopic cluster, 25%), 178 (100%). This compound **63** was used without further purification.

1,10-Phenanthroline-2,9-bis(butylamide) (64)

To a stirred solution of n-butylamine (0.38g, 5.2 mmole) in dry $CHCl_3$ (50 ml), asolution of **63** (0.4g, 1.3 mmole) in $CHCl_3$ (10 ml) was added dropwise and the reaction stirred at room temperature, under nitrogen for 1h. The chloroform solution was washed with water,then with a saturated solution of Na_2CO_3, dried, (Na_2SO_4), evaporated *in vacuo* and the residue purified by column chromatography [silica gel: 5% MeOH/$CHCl_3$], to give the title compound **64** as an off-white solid (0.38g, 75%), m.p. 179-180°C (Found: C, 69.71; H, 6.80; N, 14.76. Calc. for $C_{22}H_{26}N_4O_2$: C, 69.82; H, 6.92; N, 14.80%); δ_H (90 MHz; $CDCl_3$) 0.91-1.91 (14H, m, -**$(CH_2)_2$-CH_3**), 3.60 (4H, m, N-**CH_2**), 7.87 (2H, s, 5,6**H-phen**), 8.32-8.61 (6H, m, 3,4,7,8**H-phen** and **NH**); m/z 379 (M^+ + 1, 16.2%), 378 (M^+, 59.5%), 335 (M^+ - C_3H_8, 100%), 279 (72%), 179 (77.9%).

2-methyl-8-aminoquinoline (85)

To a solution of 2-methyl-8-nitroquinoline (10.0 g, 0.053 mol) dissolved in boiling methanol (200 ml), hydrazine hydrate (15.9 g, 0.138 mol) was added followed by 10% Pd/C (200 mg). The mixture was refluxed for 1 hour or until effevescence had ceased, at which point a further amount of 10% Pd/C (200 mg) was added and the mixture refluxed. This procedure was repeated once more, to give a total reaction time of 3 hours. After hot filtration through celite, the solution was concentrated under reduced pressure and 3M hydrochloric acid (100 ml) added. The resulting orange precipitate was made basic by the addition of 3M NaOH with cooling to give a light-brown solid which was collected by filtration and washed well with water. The crude product was recrystallised from ligroin to give pure **85** as pale yellow crystals (8.0 g, 95%). m.p. 56-57°C; δ_H(90 MHz; $CDCl_3$) 2.67 (3H, s, **CH_3**), 4.0-5.5 (2H, br s, D_2O exchangeable **NH_2**), 6.7-8.0 (5H, m, 3,4,5,6,7**H-quinoline**); m/z 158 (M^+, 100%), 157 (M^+ - 1, 20%), 130 (M^+ - HCN, 17%).

4-Hydroxy-2,9-dimethyl-1,10-phenanthroline (83).[67]

A mixture of 2-methyl-8-aminoquinoline **85** (4.0 g, 0.025 mol), ethylacetoacetate (3.6 g, 0.028 mol) and glacial acetic acid (0.6 ml) in benzene (60 ml) was refluxed using a Dean-Stark apparatus for 12 hours. Excess benzene was taken off and the resultant brown oil dried in vacuo to give the enamine **86** as a light-brown solid (6.7 g, 99%). This was used without further purification in the thermal cyclisation step.

The enamine **86** (6.75 g, 0.025 mol) was added portion-wise to a solution of boiling diphenyl ether (240 g) at 255°C this was followed by an additional 30 minutes at reflux. The mixture was then allowed to cool resulting in some precipitation which was enhanced by the addition of a large volume of 100-120° Petroleum ether. The desired product was isolated by filtration as a dark-brown powder. At this point the product contained a large amount of diphenyl ether, this was subsequently removed by repeated washing of the solid with hot 100-120° Petroleum ether to give the crude product as a light-brown solid. Recrystallisation from water gave the desired product **83** as the hydrate as a white solid (4.3 g, 77%), m.p. 237-238°C (lit.,[67] 237-238°C) (Found: C, 67.93; H, 5.73;,N, 11.27. Calc. for C_{14} H_{12} N_2 .1.25H_2O: C, 68.14; H, 5.72; N, 11.35%); δ_H (90 MHz; $CDCl_3$) 1.98 (H_2O), 2.52 (3H, s, 9-**CH_3**), 2.76 (3H, s, 2-**CH_3**), 6.30 (1H, s, 3**H-phen**), 7.31-7.49 (2H, dd, *J*8.9, 6,8**H-phen**), 8.04-8.23 (2H, dd, *J*8.9, 5,7**H-phen**), 9.81 (1H, br s, **NH**); m/z 224 (M^+, 100%).

4-(4-Bromobutyl)oxy-2,9-dimethyl-1,10-phenanthroline (87)

To a solution of 4-Hydroxy-2,9-dimethyl-1,10-phenanthroline **83** (0.25 g, 1.12 mmol) in acetonitrile (50 ml), caesium carbonate (1.1 g, 3.35 mmol) was added and the mixture stirred at room temperature before the addition of 1,4-dibromobutane (0.72 g, 3.35 mmol). The mixture was refluxed under nitrogen for 5 hours, cooled, filtered and the filtrate evaporated *in vacuo* to give a brown solid. The solid was taken up into chloroform and this was washed with water, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by column chromatography [silica gel: benzene/acetone (1:1)], to give the alkylated phenanthroline **87** as a white solid 0.28 g (70%), m.p. 100-102°C dec. (Found: C, 55.74; H, 5.55; N, 7.25. Calc. for C_{18} H_{19} N_2 O Br .1.5H_2O: C, 55.97; H, 5.74; N, 7.25%); δ_H (90

MHz; $CDCl_3$) 1.73 (3H, H_2O), 2.15 (4H, m, **CH_2-CH_2**), 2.87 (3H, s, 9-**CH_3**), 2.91 (3H, s, 2-**CH_3**), 3.54 (2H, t, *J*6, Br-**CH_2**), 4.24 (2H, t, *J*6, O-**CH_2**), 6.82 (1H, s, 3**H-phen**), 7.42-7.61, dd, *J*9.1, *6,8**H-phen**), 8.01-8.12 (2H, dd, *J*9, *5,7**H-phen**); m/z 360 (M + 2, 4.8%), 358 (M^+, 4.7%), 278 (M^+ - Br, 20%), 224 (278 - C_4H_6, 100%), 196 (224 - CO, 33%).

*The absolute assignment of these signals are not possible without detailed nmr studies and have been based on previous work by Deady *et. al.*[68]. Their assignment of the position of H5,7 as compared to H6,8 have been based on simple electronic arguments; (i) H5 as compared to H6 is closer to the electronegative oxygen and is therefore slightly more deshielded and (ii) positions 2,9,4,7-on the phenanthroline ring tend to be more deshielded as compared to positions 3,8.

4-(Bromobutyl)oxy-2,9-bistrichoromethly-1,10-phenanthroline (88)

To a refluxing solution of 4-(4-bromobutyl)oxy-2,9-dimethyl-1,10-phenanthroline **87** (0.4 g, 1.11 mmol) in carbon tetrachloride, N-chlorosuccinimide (0.98 g, 7.33 mmol) was added followed by a catalytic ammount of 3-chloroperoxybenzoic acid as initiator. The solution turns a clear yellow immediately the initiator is added. The solution was refluxed for 5 hours, after which it was allowed to cool to room temperature, resulting in the precipitation of succinimide which was removed by filtration. The filtrate was evaporated under reduced pressure to give a yellow solid. This was purified by column chromatography [silica: benzene/petroleum ether (6:4)], to give the title **88** compound as a white solid (0.41 g, 65%), m.p. 171-172°C (Found: C, 38.38; H, 2.53; N, 4.86. Calc.for C_{18} H_{13} N_2 Br Cl_6 O: C, 38.20; H, 2.32; N, 4.95%); δ_H (90 MHz; $CDCl_3$) 2.19 (4H, m, -**CH_2-CH_2**-), 3.52 (2H, m, Br-**CH_2**), 4.37 (2H, m, O-**CH_2**), 7.57 (1H, s, 3**H-phen**), 7.85 (1H, d, *J*7.0, 8-**phen**), 8.33 (3H, m, 5,6,7**H-phen**); m/z 566 (M^+; isotopic cluster, 1%), 530 (M^+ - Cl, isotopic cluster, 5%), 485 (M^+ - Br, 1.9%), 450 (485 - Cl, isotopic cluster, 1.7%), 395 (485 - C_4H_6 and Cl, isotopic cluster, 1.4%), 359 (385 - Cl, isotopic cluster, 2.8%), 36 (Cl, 100%).

4-(4-bromobutyl)oxy-2-carboxylic acid-9-trichloromethyl-1,10-phenanthroline (89)

To a stirred solution of concentrated sulphuric acid (1.0 ml) under nitrogen, the bis(trichloromethyl derivative **88** (0.3 g, $5.3x10^{-4}$ mol) was added portion-wise. After addition the temperature was raised to 90°, where it was kept for 1 hour. On cooling, the dark solution was added drop-wise to crushed ice, resulting in copious precipitation. The precipitate was collected by filtration and dissolved in $CHCl_3$ which was then washed with water (2x), dried over anhydrous sodium sulphate, filtered, and the resultant filtrate evaporated *in vacuo* to give a mustard coloured powder (0.1 g, 50%), m.p. 173°C dec. (Found: C, 43.64; H, 2.85; N, 5.54. Calc. for C_{18} H_{14} Br Cl_3 N_2 O_3: C, 43.89; H, 2.86; N, 5.68%); $\upsilon_{max}cm^{-1}$ 3600-2900 (COOH), 1730 (C=O), 1150 (C-O-C), 860 (OH); δ_H (90 MHz; $CDCl_3$) 2.22 (4H, m, -$\mathbf{CH_2CH_2}$-), 3.48 (2H, m, Br-$\mathbf{CH_2}$-), 4.41 (2H, m, O-$\mathbf{CH_2}$-), 7.72 (1H, s, 3**H-phen**), 7.89 (1H, d, *J*9.0, 8**H-phen**), 8.32 (1H, d, *J*9.0, 7**H-phen**), 8.47 (2H, s, 5,6**H-phen**); m/z 449 (M^+ - CO_2, 1.9%), 311 (449 - C_4H_9Br, 29%, isotopic cluster), 278 (311-Cl, 20.4%, isotopic cluster), 91 (100%), 44 (CO_2,42.5%)

4-(4-bromobutyl)oxy-2,9-dicarboxylic acid-1,10-phenanthroline (90).

To an acetic acid/water (4:1) mixture (50 ml) was added the bis(trichloromethyl derivative **89** (0.4 g, 0.7 mmol) and the mixture brought to reflux. At intervals of 1/2 hour, anhydrous sodium acetate (0.35 g, 4.24 mmol) was added in three equal portions. Hydrolysis was continued for a further 2 hours after which no front running material could be detected by tlc [silica: toluene/acetonitrile/formic acid (5.0/4.5/0.5)]. The solvent was removed by high vacuum and the residue recrystallised from water/THF mixture to give a mustard coloured powder (80 mg, 27%), m.p. >250°C dec. (Found: C, 49.31; H, 3.68; N, 6.93. Calc. for C_{18} H_{15} Br N_2 O_5 .H_2O: C, 49.45;, H, 3.92; N, 6.91%); $\upsilon_{max}cm^{-1}$ 3600-2900 (COOH), 1720s (C=O), 1290-1220 (C-O), 1130 (C-O-C); δ_H (90 MHz; DMSO-d_6) 2.28 (4H, m, -$\mathbf{CH_2CH_2}$), 3.49 (2H, m, Br-$\mathbf{CH_2}$-), 4.38 (2H, m, O-$\mathbf{CH_2}$), 8.21-8.36 (3H, m, 3,6,8-**H-phen**), 8.66 (2H, d, *J*8.4, 5,7**H-phen**); m/z 331 (M^+ - $2CO_2$, 44%), 251 (331 - Br, 12%), 44 (CO_2, 100%).

4-(Phenanthridinium)oxy-2,9-bis-trichloromethyl-1,10-phenanthroline (94).

Phenanthridine (1.0g, 5.59 mmole) was heated to 115°C in a pear-shaped flask, under nitrogen. To this melt the bis(trichloromethyl) derivative **88** (0.25g, 0.44 mmole) was added portionwise over 10 minutes. The reaction mixture immediately turns dark red/brown in colour and becomes very viscous and difficult to stir. The reaction was monitored by tlc [silica gel: benzene/ 40-60° Petroleum ether (6:4)] and was complete after 3h, at which point it was allowed to cool to room temperature. The reaction mixture which had now set solid was dissolved in $CHCl_3$ (8 ml), and this solution was added dropwise to a stirred diethyl ether, precipitating the product as a pale yellow solid. This was collected by filtration and further purified by repeating the reprecipitation step with fresh solvents; the desired quaternised product **94** as an off-white solid (0.23, 69%), m.p. 180°C dec. (Found: M^++H, 746.1625. C_{31} H_{23} Br Cl_6 N_3 O requires 746.1642); δ_H (90 MHz; $CDCl_3$) 2.07 (4H, m, -$\mathbf{CH_2CH_2}$-), 4.45 (2H, m, **O-$\mathbf{CH_2}$**), 5.70 (2H, m, N^+-$\mathbf{CH_2}$), 7.53-8.67 (13H, m, 3,4,5,6,7,8,9,10**H-phenanthridine** and 3,5,6,7,8**H-phenanthroline**), 12.04 (1H, s, 2**H-phenanthridine**); m/z (FAB) 746 (M+H).

2-Tribromomethyl-8-nitroquinoline (96)[70]

To a solution of 2-methyl-8-nitroquinoline **84** (5.0 g, 0.027 mol) in glacial acetic acid (100 ml), sodium acetate was added until a saturated solution was formed. To this stirred suspension, bromine (5 ml) in glacial acetic acid (35 ml) was added dropwise, after addition the mixture was refluxed for 1 hour, allowed to cool and then added to a large volume of ice/water with stirring. This results in the immediate precipitation of a solid, which is collected by filtration, washed well with water and dried to give the title compound as a yellow solid (10.6 g, 92%), m.p. 130-131° (lit.,[70] 130-131°C); δ_H (90 MHz; $CDCl_3$) 7.7-8.11 (3H, m, 3,6,7**H-quinoline**), 8.31 (2H, s, 4,5**H-quinoline**); m/z 425 (M^+, 1.85%), 378 (M^+ - NO_2, 1.2%), 355 (M^+ - Br, 100%), 265 (355 - Br, 58.5%).

2-Carboxylic acid-8-nitroquinoline (97)[70]

A slurry of the 2-tribromomethyl-8-nitroquinoline **96** (7.0 g, 0.16 mol) in 20% sulphuric acid solution (250 ml) was refluxed for 48 hours, or until very little suspended material remained. The dark red solution was then filtered and its pH adjusted to pH 3 by the addition of 3M NaOH solution. This results in the precipitation of a solid which is collected by filtration washed well with water and dried to give the title compound **97** as a light brown solid (1.5 g, 42%), m.p. 180-182°C (lit.,[70] 181-182°C); δ_H (90 MHz; DMSO-d_6) 7.90 (1H, d, *J*7.8, 3**H-quinoline**), 8.35 (3H, m, 4,5,6**H-quinoline**), 8.76 (1H, d, *J*8.4, 7**H-quinoline**); m/z 218 (M^+, 34.6%), 174 (M^+ - CO_2, 100%), 127 (174 - NO_2, 36%), 44 (13%, CO_2).

2-Carboxymethyl-8-nitroquinoline (98)[70]

A slurry of the acid **97** (2.0 g, 9.17 mmol) in methanol (100 ml) was heated to reflux at which point concentrated sulphuric acid (1.0 ml) was added dropwise, resulting in instant solubilisation. Mixture was refluxed overnight, allowed to cool and excess MeOH evaporated under vacuum. The remaining residue was dissolved in DCM and washed well with sodium bicarbonate solution (2x), followed by water (1x) and finally dried over sodium sulphate before filtration and evaporation to give the desired compound **98** as an off-white solid (1.65 g, 79%), m.p. 125-126°C (lit.,[70] 125-126°C); δ_H (90 MHz; $CDCl_3$) 4.02 (3H, s, $CO_2\mathbf{CH_3}$), 7.57-8.33 (5H, m, 3,4,5,6,7**H-quinoline**); m/z 232 (M^+, 19.1%), 202 (M^+ - CH_3O, 49.3%), 174 (M^+ - CO_2CH_3, 100%), 127 (174 - NO_2, 25.7%)

2-Carboxymethyl-8-aminoquinoline (99)[70]

To a stirred solution of 2-carboxymethyl-8-nitroquinoline **98** (2.0g, 8.6 mmole) in ethanol (100 ml) cyclohexene (4.2g, 0.052 mole) was added followed by 10% Pd/C (0.2g) and the solution brought to reflux under nitrogen. The mixture was refluxed for 24 hours with monitoring by tlc [silica: DCM/5%EtOAc] during which time a further 0.2g portion of 10%Pd/C was added. On the dissapearance of the starting material the mixture was filtered hot through celite and the filtrate run down to give the desired product **99** as a orange/yellow solid solid (1.67g, 98%), m.p. 98-99°C (lit.,[70] 97-99°C); δ_H (90 MHz; $CDCl_3$)

4.0 (3H, s, $CO_2\mathbf{CH_3}$), 4.99 (2H, s, D_2O exchangeable $\mathbf{NH_2}$), 6.86-7.38 (4H, m, 3,5,6,7**H-quinoline**), 8.08 (1H, s, 4**H-quinoline**); m/z 202 (M^+, 4.9%), 171 (M^+ - CH_3O, 51%), 143 (M^+ - CO_2CH_3, 100%).

4-Hydroxy-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (101).

To a solution of 2-carboxymethyl-8-aminoquinoline **99** (1.4g, 6.93 mmole) in dry methanol dimethylacetylenedicarboxylate (1.03g, 7.28 mmole) was added in one go and the mixture refluxed under nitrogen for 12 hours. Evaporation of the solvent under vacuum gave the enamine **100** as a bright yellow solid (2.4g, 100%). This was used without further purification in the thermal cyclisation.

The enamine **100** (2.0g, 5.81 mmole) was added portionwise to a solution of refluxing diphenyl ether (150g) at 250°C. After addition was complete the mixture was refluxed for a further 30 min., then allowed to cool to room temperature before the addition of a large volume of 60-80° Petroleum ether to precipitate the desired product as a fine light-brown powder. The product was collected by filtration and washed several times with hot petroleum ether to remove last traces of diphenyl ether and dried to give **101** a light-brown powder (1.08g, 60%). An analytically pure sample was obtained as the hydrate by recrytallisation from hot water, m.p. 220°C dec. (Found: C, 60.57; H, 3.81; N, 8.53. Calc. for $C_{16}\ H_{12}\ N_2\ O_5\ .0.25H_2O$: C, 60:66; H, 8.82; N, 8.84%); $\upsilon_{max}cm^{-1}$ 3350 (NH), 1760 (C=O), 1690 (C=O), 1170 (C-O-C); δ_H (90 MHz; $CDCl_3$) 1.82 (H_2O), 4.08 (6H, s, $CO_2\mathbf{CH_3}$), 7.18 (1H, s, 3**H-phen**), 7.66 (1H, d, *J*9.0, 8**H-phen**), 8.34 (2H, s, 5,6**H-phen**), 8.41 (1H, d, *J*9.0, 7**H-phen**); m/z 313 (M+1, 13%), 312 (M^+, 100%), 252 (M^+ - CO_2CH_3), 36%), 194 (253 - CO_2CH_3, 17%).

4-(5-Iodopentyl)oxy-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (102).

To a stirred solution of **101** (0.2g, 0.64 mmole) in benzene (50 ml), silver carbonate (0.088g, 0.32 mmole) was added followed by 1,5-diiodopentane (0.42g, 1.28 mmole). The reaction mixture which was protected from the light, was refluxed under nitrogen for 36h, whilst being monitored by tlc [neutral alumina: $CHCl_3$]. It was allowed to cool to room

temperature, filtered, the filtrate evaporated *in vacuo* to give a brown oil which was triturated overnight with 60-80 Petroleum Ether to give a light-brown solid. This was purified by column chromatography to give **102** as a light mustard coloured solid (92 mg, 28%); $\upsilon_{max}cm^{-1}$ 2990-2920 (CH_2), 1720 (C=O), 1280 (CO), 1025 (O-CH_3); δ_H (90 MHz; $CDCl_3$) 1.91 (6H, m, -**CH_2**-), 2.06 (H_2O), 3.26 (2H, t, *J*6.8, I-**CH_2**), 4.08 (3H, s, CO_2**CH_3**), 4.11 (3H, s, CO_2**CH_3**), 4.35 (2H, m, O-**CH_2**), 7.78 (1H, s, 3**H-phen**), 7.92 (1H, d, *J*9.0, 8-**phen**), 8.35 (1H, d, *J*9.0, 7**H-phen**), 8.36 (2H, s, 5,6**H-phen**); m/z 380 (M^+ - I, 3.9%), 322 (380 - CO_2CH_3, 60.5%), 263 (322 - CO_2CH_3, 8%), 44 (100%).

4-(5-Phenanthridinium)oxy-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (103).

Phenanthridine (0.18g, 0.98 mmole) was heated to 115°C in a pear-shaped flask, under nitrogen to melt it. To this melt **102** (0.05g, 9.8×10^{-5} mole) was added portionwise over 5 minutes, the reaction mixture immediately turned a dark red/brown colour and became very viscous. The mixture was heated for 1h and allowed to cool to room temperature. Once cold the reaction mixture set solid, this was stirred with benzene (10 ml) and filterd to give a light-brown solid; this procedure was repeated twice with fresh solvents to remove last traces of both phenanthridine and **103**. Final purification was achieved by dissolving the crude material in $CHCl_3$ and adding the minimum volume of diethyl ether to slowly precipitate the desired quaternised material **103** as straw coloured microcrystals (12 mg, 15%), m.p.203°C dec. (Found: M - I, 560.6180. C_{34} H_{30} N_3 O_5 requires M, 560.6281); δ_H (200 MHz; $CDCl_3$) 1.96 (6H, m, -**CH_2**-), 2.14 (H_2O), 4.08 (6H, s, CO_2**CH_3**), 4.34 (2H, m, O-**CH_2**), 5.23 (2H, m, N^+-**CH_2**), 9.12-7.3 (13H, m, 3,4,5,6,7,8,9,10**H-phenanthridine** and 3,5,6,7,8**H-phenanthroline**), 11.61 (1H, s, 2**H-phenanthridine**); m/z FAB 560 (M - I).

5-Nitro-2,9-dimethyl-1,10-phenanthroline (108).

Neocuproine **50** (10g, 0.048 mole), was added portionwise to cold fuming sulphuric acid (45 ml) after which fuming nitric acid (50 ml) was added, and the solution heated at 140°C under nitrogen for 1 hr. The reaction mixture was then allowed to cool to room temperature and then poured over crushed ice/water (CARE!). The resulting solution was then

neutralised to pH 6 by the addition of solid sodium carbonate and the yellow solid produced collected by filtration and purified by reprecipitation from 3M sulphuric acid solution by neutralising with NaOH, to give the desired nitrated product **109** as a mustard coloured solid (6.6g, 50%), m.p. 177-180°C dec. (Found: C, 62.05; H, 4.59; N, 14.99. Calc. for C_{14} H_{11} N_3 O_2 .H_2O: C, 61.99; H, 4.83; N, 15.48%); $\upsilon_{max}cm^{-1}$ 3500-2900 (OH), 1520 ($ArNO_2$), 1350 ($ArNO_2$); δ_H (90 MHz; $CDCl_3$) 2.13 (H_2O), 2.96 (3H, s, 9-**CH_3**), 2.98 (3H, s, 2-**CH_3**), 7.57 (1H, d, *J*8.3, 8**H-phen**), 7.65 (1H, d, *J*8.7, 3**H-phen**), 8.3 (1H, d, *J*8.3, 7**H-phen**), 8. 61 (1H, s, 6**H-phen**), 8.89 (1H, d, *J*8.7, 4**H-phen**); m/z 253 (M^+, 39%), 223 (M^+ - NO, 7%), 207 (M^+ - NO_2, 57%).

2,9-Dimethyl-1,10-phenanthroline-5,6-dione (109).

The combined aqueous filtrates from the nitration reaction was then extracted with chloroform and evaporated to give the crude dione **109** as a by-product of the nitration. This was purified by column chromatography [silica gel: 2% methanol/chloroform] to give the dione **109** as a bright yellow solid (1.8g, 16%), m.p. 185-186°C; $\upsilon_{max}cm^{-1}$ 1690vs (C=O), 1580vs, 1370vs, 1140s (C-CO-C); δ_H (200 MHz, $CDCl_3$) 2.83 (6H, s, 2,9-Ar**CH_3**), 7.43 (2H, d, *J*8.2, 3,8**H-phen**), 8.38 (2H, d, *J*8.2, 4,7**H-phen**); m/z 238 (M^+, 8.3%), 210 (M^+ - CO, 100%).

5-Nitro-2,9-bis-(trichloromethyl)-1,10-phenanthroline (110)

The nitrophenanthroline **108** (2.0g, 7.9 mmole) was dissolved in carbon tetrachloride (150 ml) with the aid of chloroform (20 ml). To this solution N-chlorosuccinimide (6.7g, 0.05 mole) was added followed by a catalytic amount of 3-chloroperoxybenzoic acid to initiate the reaction. The mixture was refluxed for 24h, then cooled to room temperature, and filtered to remove solid succinimide. The filtrate was washed with several portions of 10% sodium carbonate solution, dried (Na_2SO_4), filtered and evaporated to give the crude hexachlorinated product **110**. This was purified by column chromatography [silica gel: chloroform] to give the title compound **110** as a light-yellow solid (2.9g, 81%), m.p. 228-230°C. Found: C, 36.37; H, 0.90; N, 8.88. Calc. for C_{14} H_5 N_2 Cl_6: C, 36.56; H, 1.10; N,

9.14%); $\upsilon_{max}cm^{-1}$ 1510 ($ArNO_2$), 1330 ($ArNO_2$), 820-740 (C-Cl); δ_H (90 MHz; $CDCl_3$) 8.39 (1H, d, *J*8.4, 8**H-phen**), 8.44 (1H, d, *J*9.1, 3**H-phen**), 8.58 (1H, d, *J*8.4, 7**H-phen**), 8.78 (1H, s, 6**H-phen**), 9.19 (1H, d, *J*9.1, 4**H-phen**); m/z 459 (M^+, 5%), 424 (M^+ - Cl, 100%, isotopic cluster), 389 (M^+ - 2Cl, 20%, isotopic cluster), 354 (M^+ - 3Cl, 8%, isotopic cluster), 308 (354 - NO_2, 3%), 273 (308 - Cl, 2%, isotopic cluster), 237 (273 - Cl, 1.7%, isotopic cluster), 202 (237 - Cl, 1.4%, isotopic cluster).

5-Nitro-1,10-phenanthroline-2,6-dicarboxylic acid (111)

The hexachloride **112** (1.0g, 2.17 mmole) was added portionwise to 98% sulphuric acid (6 ml), and the mixture heated at 80-90° under nitrogen for 6h. The dark-brown solution was allowed to cool after which the viscous solution was slowly poured over crushed ice/water resulting in the precipitation of the crude diacid **111** as a fine yellow solid. The precipitated solid was collected by filtration, washed with cold water and recrystallised from hot water/THF to give the desired pure diacid **111** as a pale yellow solid (0.59g, 85%), m.p. 218-220°C. (Found: C, 50.77; H, 2.68; N, 12.42. Calc. for $C_{14}H_7N_3O_6.H_2O$: C, 50.76; H, 2.74; N, 12.68%); $\upsilon_{max}cm^{-1}$ 3500-2900 (COOH), 1735 (C=O), 1540 ($ArNO_2$), 1340 ($ArNO_2$), 1150 (C-O); δ_H (90 MHz; DMSO-d_6) 2.65 (H_2O), 8.44 (1H, d, *J*8.2, 8**H-phen**), 8.48 (1H, d, *J*8.8, 3**H-phen**), 8.90 (1H, d, *J*8.2, 7**H-phen**), 9.03 (1H, d, *J*8.8, 4**H-phen**), 9.13 (1H, s, 6**H-phen**); m/z 269 (M^+ - CO_2, 42%), 225 (M^+ - $2CO_2$, 68%), 44 (CO_2, 100%).

5-nitro-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (112)

The hexachloride **110** (2.5g, 5.43 mmole) was added portionwise to 98% sulphuric acid (8 ml), and the mixture heated at 80-90° for 6h. The mixture was then cooled in ice/water, then methanol (15 ml) was added with caution and the resulting mixture refluxed for a further 2h. After cooling, excess methanol was removed by evaporation and the residue neutralised to pH 6-7 by the addition of saturated aqueous sodium carbonate solution, resulting in precipitation. The crude product was collected by filtration, dried and recrystallised from methanol/chloroform to give the pure diester **112** as a white solid (1.59g, 86%), m.p. 258-260°C dec. (Found: C, 55.39; H, 3.19; N, 11.88. Calc. for $C_{16}H_{11}N_3O_6.0.25H_2O$: C, 55.57; H, 3.21; N, 12.15%); $\upsilon_{max}cm^{-1}$ 3500-3200 (O-H), 1730 (C=O),

1540 ($ArNO_2$), 1340 ($ArNO_2$), 1210 (C-O); δ_H (90 MHz; $CDCl_3$) 3.12 (H_2O), 4.13 (6H, s, $CO_2\mathbf{CH_3}$), 8.57 (2H, s, 3,8**H-phen**), 8.59 (1H, d, *J*8.8, 7**H-phen**), 8.77 (1H, s, 6**H-phen**), 9.15 (1H, d, *J*8.8, 4**H-phen**); m/z 341 (M^+, 2%), 311 (M^+ - NO, 7%), 283 (M^+ - $COOCH_3$, 100%), 225 (M^+ - $2COOCH_3$, 19%).

5-Amino-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (113)

The nitro ester **112** (0.5g, 1.5 mmole) was mixed with methanol (50 ml) and refluxed to dissolve the solid starter. The resulting solution was allowed to cool before the addition of cyclohexene (0.72g, 8.8 mmole) and 10% palladium on carbon (≈0.1g), and the mixture was heated at reflux under nitrogen for a further 2h. The mixture was filtered hot through celite, and the residue washed well with fresh hot methanol, the combined methanolic washings was then evaporated *in vacuo* to give the amino diester **113** as a bright yellow solid. Further purification by column chromatography [silica gel: 10%MeOH/$CHCl_3$] gave **113** in an analytically pure state (0.38g, 83%), m.p. 239-240°C. (Found: 58.06; H, 4.28; N, 12.56. Calc. for $C_{16}H_{13}N_3O_4.H_2O$: C, 58.31; H, 4.53; N, 12.76%); $\upsilon_{max}cm^{-1}$ 3320-3200 ($ArNH_2$), 1730 (C=O), 1510 (N-H), 1300 (C-N), 1150 (C-O); δ_H (90 MHz; DMSO-d_6) 3.91 (3H, s, $CO_2\mathbf{CH_3}$), 3.97 (3H, s, $CO_2\mathbf{CH_3}$), 4.88 (4H, br s, D_2O exchangeable, $\mathbf{NH_2}$, $\mathbf{H_2O}$), 7.00 (1H, s, 6**H-phen**), 8.16 (2H, s, 3,8-**phen**), 8.35 (1H, d, *J*8.8, 4**H-phen**), 8.94 (1H, d, *J*8.8, 8**H-phen**); m/z 311 (M^+, 6%), 253 (M^+ - $COOCH_3$, 43%), 195 (M^+ - $2COOCH_3$, 100%).

5-(Bromohexanoylamino))-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline (114).

To a solution of 5-amino-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline **113** (0.1g, 0.32 mmole) and an excess of diisopropylethylamine (50mg, 0.39 mmole) in anhydrous acetonitrile (10 ml) was added 6-bromohexanoyl cholride (60mg, 0.32 mmole). The reaction was stirred at room temperature under nitrogen for 2h, whilst being monitored by tlc [silica gel: 10%MeOH/$CHCl_3$]. The reaction mixture was quenched by the addition of water (10 ml), and then extracted with $CHCl_3$ (2 x 25 ml), the combined chloroform extracts was then washed with saturated $NaHCO_3$ solution (2 x 20 ml), and brine (2 x 20 ml). The organic solution was then dried (Na_2SO_4), filtered and evaporated *in vacuo* to give

a red/brown sticky oil. This was purified by column chromatography [silica gel: 5%MeOH/$CHCl_3$] to give the desired product **114** as a mustard coloured solid after trituration with diethyl ether (100mg, 66%), m.p. 129-131°C. (Found: C, 51.94; H, 4.64; N, 8.22; Br, 15.91. Calc. for C_{22} H_{22} N_3 Br O_5 .H_2O: C, 52.18; H, 4.77; N, 8.30; Br, 15.78%); $\upsilon_{max}cm^{-1}$ 3460 (NH, OH), 3000-2960 (C-H), 1750s (C=O), 1560 (NH, secondary amide), 1280s (C-O); δ_H (200 MHz; $CDCl_3$) 2.0-1.6 (6H, m, -**CH_2**-), 2.66 (2H, t, *J*7.3, CO-**CH_2**), 3.13 (2H, H_2O), 3.43 (2H, t, *J*6.7, Br-**CH_2**), 4.02 (3H, s, CO_2**CH_3**), 4.08 (3H, s, CO_2**CH_3**), 7.75 (1H, d, *J*8.5, 3**H-phen**), 7.95 (1H, s, 6**H-phen**), 8.1 (1H, d, *J*8.5, 8**H-phen**), 8.30 (1H, d, *J*8.5, 7**H-phen**), 8.34 (1H, d, *J*8.5, 4**H-phen**), 9.49 (1H, s, D_2O exchangeable **NH**); m/z FAB 488 (M + H).

5-(Hexanoylphenanthridinium)-2,9-bis-(methoxycarbonyl)-1,10-phenanthroline(115). Phenanthridine (0.26g, 1.48 mmole) was heated to 115° in a pear-shaped flask, under nitrogen, to melt it. To this melt **114** (0.24g, 0.49 mmole) was added portionwise over 10 minutes. The reaction mixture turned dark red/brown in colour and became very viscous. The reaction was monitored by tlc [silica gel: 15%MeOH/$CHCl_3$] and was complete after 2h, at which point it was allowed to cool to room temperature. Once cold the reaction mixture set solid, this was dissolved in chloroform (5 ml), and this solution was added dropwise to stirred diethyl ether, precipitating the product as a pale yellow solid. This was collected by filtration and further purified by repeating the reprecipitation step with fresh solvents; the desired quaternised product **115** being obtained as an off-white solid (250mg, 73%). The best attempt at obtaining **115** in an analytically pure state was by recrystallisation from hot water, however the recovered yield was always very poor (≈10%), m.p. 158°C dec. (Found: C, 61.45; H, 4.66; N, 8.23. Calc. for C_{34} H_{31} Br N_4 O_5 . H_2O: C, 61.32; H, 4.85; N, 8.17%); $\upsilon_{max}cm^{-1}$ 3500 (NH, OH), 1740s (C=O), 1630 (C=O, secondary amide); δ_H (200 MHz; $CDCl_3$) 2.55-1.63 (6H, m, -**CH_2**-), 2.9 (2H, m, CO-**CH_2**), 3.31 (2H, H_2O), 4.01 (6H, s, CO_2**CH_3**), 5.2 (2H, m, N^+-**CH_2**), 9.35-7.4 (13H, m, 3,4,5,6,7,8,9,10**H-phenanthridine** and 3,4,6,7,8**H-phenanthroline**), 10.4 (1H, br s, D_2O exchangeable **NH**), 11.42 (1H, s, 2**H-phenanthridine**); m/z FAB 587 (M - Br).

5-(Hexanoylphenanthridinium)-2,9-bis-(carboxylic acid)-1,10-phenanthroline (116). The quaternised dimethyl ester **116** (100mg, 015 mmole) was added to distilled water (10 ml), the pH of which had been adjusted to ≈pH4 with dilute hydrobromic acid. The resulting mixture was then heated at 100°, whereupon the solid dissolved to give a pale yellow solution. The reaction was monitored by tlc [neutral alumina: 15%MeOH/$CHCl_3$], and over time there was a gradual dissappearance of the starting material, and appearance of a baseline spot. The reaction appeared complete after 24h- the solution was now a deep yellow colour, with some brownish material around the inside of the flask. The solution was decanted off, frozen using an acetone/cardice bath, then freeze-dried on a high-vacuum pump. The desired probe **117** was isolated as a bright orange solid (47mg, 49%). Attemps to recrystallise this material have been unsuccessful, and chromatography by both normal and reverse phase systems have failed to give anything other than baseline spots, m.p. 173°C dec. (Found: M-Br, 559.1973. C_{33} H_{27} N_4 O_5 requires M, 559.1981); δ_H (200 MHz; DMSO-d_6) 1.62-2.18 (4H, m, -$\mathbf{CH_2CH_2}$), 2.24 (2H, m, -$\mathbf{CH_2}$-), 4.01 (2H, m, CO-$\mathbf{CH_2}$), 5.18 (2H, m, N^+-$\mathbf{CH_2}$), 8.03-9.19 (13H, m, 3,4,5,6,7,8,9,10**H-phenanthridine** and 3,4,5,7,8**H-phenanthroline**), 10.22 (1H, br s, CO**NH**), 10.45 (1H, s, 2**H-phenanthridine**); m/z FAB 559 (M - Br).

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