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P A R A M A G N E T I C P R O B E S

F O R

N . M . R . A N D E . S . R .

S T U D Y

A thesis submitted for the degree of Doctor of Philosophy in chemistry at The University of Leicester.

April 1991

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ABSTRACT

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Paramagnetic Probes For N.M.R. And E.S.R. Study

Novel paramagnetic compounds, specifically nitroxyl radicals, were prepared and evaluated as possible contrast agents for Nuclear Magnetic Resonance (N.M.R.) imaging and as probes for Electron Spin Resonance (E.S.R.) spectroscopy. Attempts were made to target a nitroxyl radical to areas of calcification, particularly bone, in order to alter the local N.M.R. image. Several analogues of pyrophosphate are bone selective and have been used to prepare nucleotide analogues with altered phosphate chains.

The novel pyrophosphate analogue N-oxyl-imidobisphosphonate, with a nitroxyl group bridging two phosphate groups, was synthesised. However, this nitroxyl was inherently unstable and could not be investigated further.

The effects of sulphur atoms on bone specificity and the enzyme activity of nucleotide analogues were evaluated. This was achieved by preparing analogues of methylenebisphosphonate (a known bone agent) containing thiophosphonate groups.

Methylenebisthiophosphonate and thiophosphonomethylphosphonate were both found to have poor bone selectivity. An ATP analogue was prepared with a methylenebisthiophosphonate group in the β, γ position of the 5'-phosphate group. This was investigated as a competitive inhibitor of hexokinase with ATP.

The possibility of attaching a nitroxyl radical to a bone specific agent was investigated. To this end a piperidinyl nitroxyl N-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl)-oxo-iminobisphosphonate, containing two phosphonate groups, was synthesised. However, this nitroxyl was not bone specific when administered *in vivo* and is currently awaiting trials as a contrast agent for N.M.R. imaging.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout the thesis.

Ad	=	Adenosine
ADP	=	Adenosine diphosphate
AMP	=	Adenosine monophosphate
ATP	=	Adenosine triphosphate
Ap_4A	=	Bisadenosine tetraphosphate
C.T.	=	X-ray Computed Tomography
DMF	=	N,N-Dimethylformamide
DTPA	=	Diethylenetriaminepentaacetate
EDTA	=	Ethylenediaminetetraacetate
E.S.R.	=	Electron Spin Resonance
F.A.B.	=	Fast Atom Bambardment
I.R.	=	Infra Red
M.R.I.	=	Magnetic Resonance Imaging
NADP	=	Nicotinamide adenine dinucleotide phosphate
N.M.R.	=	Nuclear Magnetic Resonance
P.R.E.	=	Proton Relaxation Enhancement
R.F.	=	Radio Frequency
TEAB	=	Triethylammonium bicarbonate
THF	=	Tetrahydrofuran
T.L.C.	=	Thin Layer Chromatography
Tris	=	Tris(hydroxymethyl)aminomethane
Ts	=	Tosyl
U.V.	=	Ultra Violet

INDEX OF COMPOUNDS SYNTHESISED

C H A P T E R O N E

GENERAL INTRODUCTION

A paramagnetic substance is considered to be one that contains one or more unpaired electrons. The practical applications of such electron paramagnetism was the basis of the following work.

FREE RADICALS

Paramagnetic compounds containing one or more unpaired electrons are termed free radicals. The magnetic properties of these free radicals result from the motion of unpaired electrons. The electrons are affected by the motion of the orbital in which they are contained and by their own spinning motion.

Organic free radicals, which will be investigated later, have very strong magnetic fields associated with their covalent bonds. These fields prevent the orbitals containing the unpaired electrons from moving with respect to the rest of the molecule. The net result is that paramagnetism in organic free radicals is due solely to the spin of the unpaired electrons, since the orbital motion is quenched.

INORGANIC AND ORGANIC RADICALS

Inorganic paramagnetic substances are to be found amongst the transition metals and lanthanides. Generally, as the number of unpaired electrons associated with a metal ion increases, the net spin increases and hence the paramagnetic effect becomes stronger. Some examples of paramagnetic metals are shown in Figure 1.

METAL	UNPAIRED ELECTRONS	OVERALL SPIN
Cu^{2+}	1	1/2
Co^{2+}	3	3/2
Mn^{2+}	5	5/2
Gd^{3+}	7	7/2

FIG 1

Paramagnetic molecules possessing odd numbers of electrons in valence molecular orbitals include nitric oxide and nitrogen dioxide. Oxygen is also paramagnetic because although it possesses an even number of electrons, there are two which occupy separate orbitals and have parallel spins.

Organic molecules can also exhibit paramagnetism although most organic free radicals have very short lifetimes. These radicals exist mainly as transitory intermediates, for example, in photochemical reactions and re-arrangements.

ORGANIC FREE RADICALS

Radicals are generated via homolytic bond cleavage, as opposed to heterolytic cleavage in the case of ion formation, see Figure 2.

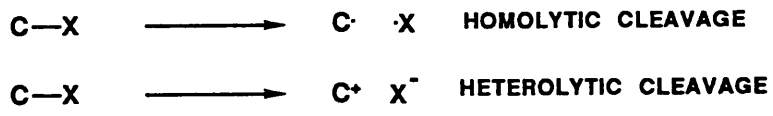


FIG 2

The unpaired electron of a radical is represented as a dot (·). Short-lived organic radicals can undergo a wide range of reactions such as radical transfers and additions. Polymerisation products can result from continued radical additions. Radical-radical reactions such as combination, disproportionation and fragmentation are also possible. These are illustrated in Figure 3.

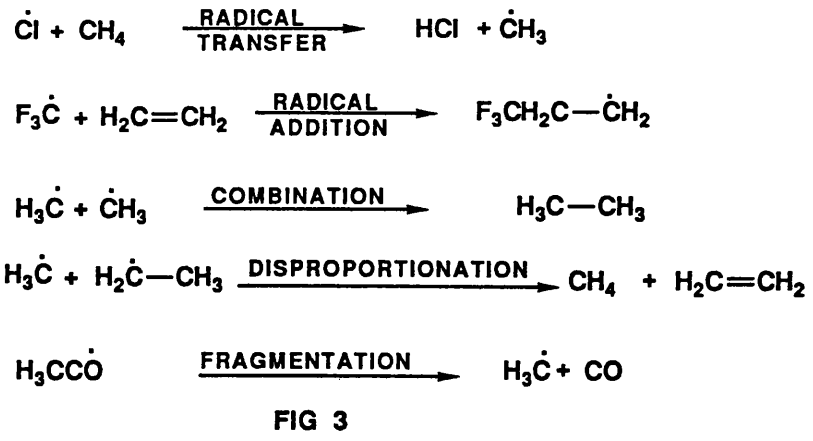


FIG 3

Non-radical products can only be formed from radical-radical reactions. These reactions are in fact, extremely fast but are dependent on the square of the radical concentration and are hence less likely to occur than transfer and addition reactions. High concentrations of radicals are not likely in solution due to the highly reactive nature of most radical species.

STABLE FREE RADICALS

A very important exception to short-lived radicals are persistent radicals, these radicals are stable due to their lack of reactivity towards a solvent or recombination. Two main factors confer stability, or more accurately a lack of reactivity, onto a radical, these are delocalisation and steric hindrance.

Carbonium ions are stabilised by the delocalisation of the charge through the Π orbital system. In a similar manner the unpaired electron of a radical can be delocalised over the whole molecule. However, with radicals steric factors have a greater importance and help prevent recombination and disproportionation.

The first stable organic radical reported was the triphenyl-methyl radical (Gomberg 1900), with stability attributed to both steric hindrance and delocalisation. This contrasts with the triphenylmethyl carbonium ion which is stabilised mainly by the delocalisation of the positive charge over the ortho and para positions of the benzene ring. Some examples of stable (persistent) radicals are shown in Figure 4. Extensive delocalization of the unpaired electron over the aromatic systems can occur, steric hindrance is also important due to the presence of the aryl rings. No real delocalization is feasible over the tri-^{t}butyl radical though, dimerisation and disproportionation being retarded by steric hindrance.

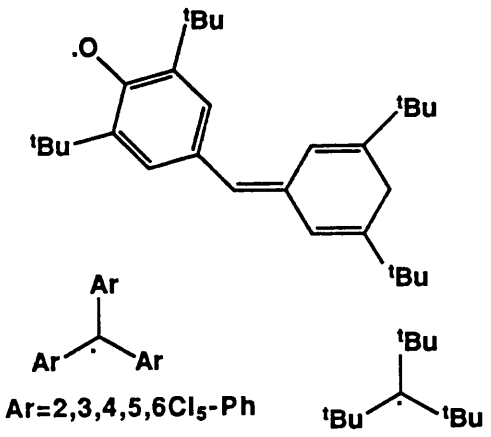


FIG 4

Although persistent, radicals can still be highly reactive towards oxygen. The extended lifetime more reliant on kinetic factors than any inherent stability, making the term persistent more accurate than stable.

To summarise, the terms persistent, long lived or stable radical refer to a molecule containing an unpaired electron. The molecule is resistant to dimerisation, disproportionation or any other routes of decomposition.

NITROXYL RADICALS

A species of persistent radicals central to this work are nitroxyl radicals, sometimes called nitroxides. These radicals are stabilised by three factors: the effective delocalisation of an electron over a nitrogen-oxygen bond; steric hindrance and by a lack of α-hydrogen atoms. Figure 5 illustrates a nitroxyl radical with the paramagnetism arising from the unpaired electron shown. R is an organic fragment containing no α-hydrogen atoms, for example a tertiary butyl group. The presence of α-hydrogen atoms allows disproportionation, a major decomposition pathway for radicals, to occur. The use of tertiary carbons attached to the nitroxyl nitrogen precludes this from occurring. A potential disproportionation pathway is also shown in Figure 5.

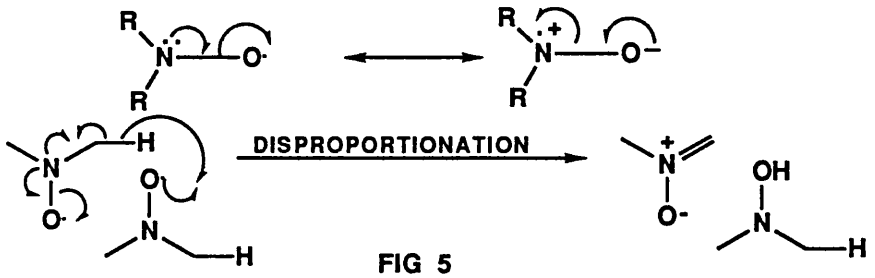


FIG 5

ELECTRON SPIN RESONANCE

Electron Spin Resonance (E.S.R.) is the technique used to study and characterise paramagnetic species. The technique is applicable to both transition metals and organic free radicals. The principles of E.S.R. are very similar to those of Nuclear Magnetic Resonance (N.M.R) which are outlined in the appendix.

In E.S.R. the intrinsic spin and electric charge associated with an unpaired electron (as opposed to that of an unpaired nucleon in N.M.R.) results in electronic precession at a precise frequency when the sample is exposed to an external static magnetic field.

If electromagnetic energy is applied at the required frequency, the electron undergoes transitions between energy states, called spin states. The energy difference between these spin states is given by;

$\Delta E = h\nu = g\mu_B B$

g is a dimensionless proportionality constant

μ_B is the Bohr magneton

and B is the applied magnetic field

The E.S.R. spectrum observed is a measure of the energy absorbed in order to cause a transition. The E.S.R. absorption is monitored by radiating electromagnetic energy at a fixed frequency and varying the magnitude of the applied magnetic field, B. More detailed information can be found by referring to the literature (Symons, Pryor, Nonhebel).

USES OF FREE RADICALS

Radicals have been used to study a wide variety of phenomena, usually in conjunction with E.S.R. spectroscopy (Berliner 1976, 1979). The techniques most often employed are those of spin labelling and probing. Spin labelling can be regarded as the covalent attachment of a paramagnetic compound to the molecule under investigation. If no covalent attachement is involved then the radical is referred to as a spin probe.

The E.S.R. spectrum can provide detailed information about the local environment of the paramagnetic centre, for example an enzyme active site. The extent of hydration can also be determined, as can conformational properties of macromolecules, such as membrane structure and the active site conformation of a protein.

Spin labelling has also been used in pharmacology for spin-assays and spin-membrane immuno assays (Piette 1979; Berliner 1979, Chapter 6). The use of transition metals as probes and nitroxyl radicals as labels has led to the development of a spin label-spin probe method of investigation (Hyde 1979; Berliner 1979, Chapter 2). More general uses of paramagnetic transition metals have involved the binding of copper and manganese ions to biomolecules and porphyrin-metal complexes.

Spin-trapping is an important technique whereby nitroxyl radicals are generated by the reaction of an unstable radical with a "spin trap", for example a nitrone, see figure 6. The relatively stable nitroxyl radical is then investigated and information on the parent radical gleaned from the E.S.R. spectrum.

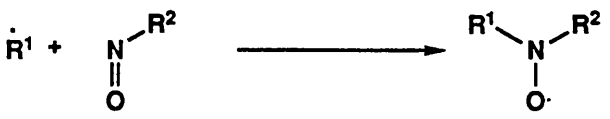


FIG 6

NITROXYL RADICALS

As reported in the previous section, nitroxyl radicals have been widely used in the investigation of biological systems as both spin labels and probes. The majority of structures investigated have been derived from piperidine and pyrollidine (Figure 7).

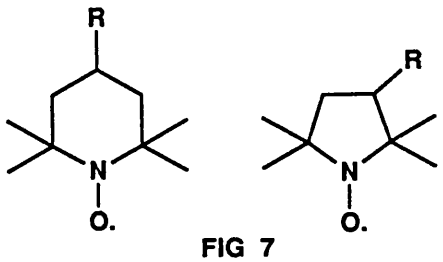


FIG 7

Variation of the R group can lead to a wide range of biological and chemical properties and further potential uses for nitroxyl molecules. It has been found that nitroxyl radicals tend not to perturb the natural behaviour of molecules under investigation (McConnell 1970), thus potentially allowing very sensitive areas and structures to be investigated. The nitroxyl radicals are directed by the nature of the R group, enabling specific regions to be investigated. Nitroxyl spin labels and probes have been used to study nucleic acids, drugs, carbohydrates and polymers.

Due to the fact that nitroxyl radicals are sensitive to molecular motion, orientation and their electric and magnetic environments they can provide varied information. Structural and conformational changes, such as local movement and environmental changes could provide data on the extent of hydration. Since spin labels, specifically nitroxyl radicals, are weakly basic and they do not bind water molecules tightly, they can be used to study non-aqueous regions such as membranes, micelles and proteins.

Many reviews have been published on the properties and uses of nitroxyl radicals, mainly in biological systems (Griffith 1969, McConnell 1970, Rozantsev 1971, Keana 1978, Gnewuch 1986, Berliner 1976, 1979).

N.M.R. AND IMAGING

A further use of paramagnetic substances has been in Nuclear Magnetic Resonance imaging (N.M.R. imaging), also referred to as Magnetic Resonance Imaging (M.R.I.) where they have been used to enhance the natural image contrast. Imaging is a recent medical application of N.M.R., the major difference between imaging and spectroscopy being that N.M.R. imaging is a relaxation as opposed to the absorption phenomenon of conventional N.M.R. spectroscopy. The principles of N.M.R. spectroscopy are covered in the appendix.

NUCLEAR MAGNETIC RESONANCE IMAGING

Conventional N.M.R. spectroscopy utilises a homogeneous magnetic field B_0 (see appendix 1). In N.M.R. imaging, a linear magnetic field gradient is superimposed upon B_0 introducing a spatially dependent variation in resonant frequency across the object. This allows the acquisition of N.M.R. signals which contain spatial information which when decoded, lead to the formation of an image of the object. Simplistically, N.M.R. imaging provides a geographical map of the nucleus under investigation. This contrasts with N.M.R. spectroscopy where differences in the local magnetic field strength and hence resonant frequency, are due to changes in the chemical environment of nuclei and not their spatial position.

In the N.M.R. imaging experiment R.F. excitation is applied at regulated intervals (pulses). The rate of decay of the signal following R.F. excitation is governed by two relaxation processes characterised by the parameters T_1 and T_2. These relaxation parameters correspond to the two possible modes of relaxation available to the nuclei. T_1 corresponds to the interaction of the nucleus with its physical surroundings and is referred to as the spin-lattice relaxation time. The T_2, or spin-spin relaxation results from the interaction of the spinning nucleus with a nucleus of opposite spin.

Together with the relative densities of the nuclei under investigation, these two methods of relaxation enable an image to be constructed *via* a variety of pulse sequences and computer aided techniques (Hoult 1984, Pykett 1982). The image produced provides a spatial distribution of nuclei and hence molecules in the sample.

The images generated in N.M.R. imaging are usually dependent on protons due to their isotopic abundance and high sensitivity. Other biologically important nuclei are insufficiently abundant in biological tissues to enable successful image construction. Such nuclei, for example phosphorus and fluorine, can provide valuable biological spectroscopic data via conventional N.M.R. techniques (Gadian 1982 and references therein).

Soft tissues containing cellular water and lipids provide the most intense signals in N.M.R. imaging. Water accounts for approximately 55% of the human bodyweight and is between 60% and 90% abundant in soft tissues. The image signal is mainly derived from water and to a lesser extent from lipids . The intensity of the image, I, is related to the relaxation times T_1 and T_2 and the proton density P and is given by the equation below.

$$I = Pf(v)\exp(-a/T_2)[1-\exp(-b/T_1)] \text{ ----}$$

a and b are parameters of the technique, f(v) is a function of flow, specifically a function of both the speed with which the protons move through the region being imaged and the fraction of the total number of nuclei that are moving (Brasch 1983 a, Herfkens 1981).

From the above relationship it can be shown that $I \propto P^{T_2/T_1}$.

Solid materials have a much larger T_1 than T_2 resulting in a low N.M.R. signal, whilst fluids give relatively high signals since T_1 and T_2 are more equal. Therefore, in N.M.R. imaging mobile compounds provide more information than static ones, this is directly comparable to conventional N.M.R. spectroscopy. Large, immobile biological structures such as proteins are not observed in N.M.R. imaging due to their lack of mobile protons. Membrane components such as phospholipids are more mobile but do not contribute significantly to the proton image. Small proteins and metabolites present in solutions are not present in a high enough concentration to be observed.

CONTRAST IN N.M.R. IMAGING

The contrast provided by N.M.R. imaging is a consequence of the natural differences in P, T_1 and T_2 between different organs and tissues (Herfkens 1981). Contrast between tissues is enhanced by the dependence of the image intensity on relaxation times, allowing tissues with the same proton density (or water concentration) but different values for T_1 and T_2 to provide image contrast. It has been shown that the T_1 values for protons in malignant tissues are longer than those for normal tissues, thus providing a natural contrast (Damadian 1971). Further experiments have suggested that different relaxation times exist for protons in normal and abnormal tissues of the same organ (Davis 1981, Herfkens 1981).

Obtaining contrast between tissues by virtue of their relaxation times is therefore possible. The experimental and analytical conditions can be varied so as to link the image construction to either P, T_2, or T_1.

SCOPE OF N.M.R. IMAGING

N.M.R. imaging is as far as is known, a safe and non-invasive procedure and does not use potentially harmful ionizing radiation as with X-ray techniques.

Images can be obtained from any orientation that can improve diagnosis such as transverse, coronal, sagittal or oblique slices. The imaging technique can be used for a wide range of medical applications as listed below.

Brain	**(Brasch 1983 b, Carr 1984)**
Spinal Cord	**(Amersham International)**
Heart Function	**(Wesbey 1984, McNamara 1984)**
Liver	**(Davis 1981, Stark 1985)**
Spleen and Pancreas	**(Amersham International)**
Kidney	**(Wolf 1984, Hricak 1983)**
Bone Marrow Diseases	**(Amersham International)**

Some interesting observations on medical diagnosis can be made from the information above, for example, tumours of the lung can be detected by virtue of their increased water content. Differences in the relaxation times of circulating blood and the surrounding tissues highlight the possibility of imaging the vascular system. A low signal is received from bone due to the low concentration of protons in the bone matrix, this allows bone marrow diseases such as leukaemia and aplastic anaemia to be diagnosed.

The potential applications of N.M.R. imaging for detecting areas of disease, such as tumours, lie with the ability to distinguish between different types of tissue.

CONTRAST AGENTS

Comparisons can be drawn between N.M.R. imaging and X-ray computed tomography (C.T.), the diagnostic technique with the closest areas of direct competition. Such comparisons tend to highlight the absence of reliable contrast media for N.M.R. imaging.

C.T. has readily available contrast media, such as barium meals and iodinated contrast agents. In order to make N.M.R. imaging more diagnostically accurate much effort has centred on the development of pharmaceutical contrast agents. The aim has been to improve the contrast between tissues with similar N.M.R. characteristics (Brasch 1983 a, Ogan 1985, Runge 1983 a and b, 1984 c). In fact, a commercial contrast agent "Magnevist" is available for N.M.R. imaging, this agent is based upon a gadolininium complex, Gd DTPA, and is discussed later.

It has been shown that image contrast can be achieved by alterations in proton density (hydrogen concentration). Unfortunately, large changes in hydration are required in order to induce major changes in the proton density and hence enhance the contrast (Hricak 1983). Such changes in hydration are not feasible *in vivo*.

Direct manipulation of physical properties, such as temperature and viscosity can cause changes in T_1. Again, the large changes required would make this an unlikely method of contrast enhancement. A higher signal intensity can be obtained by increasing the proportion of fat within a tissue. This change is brought about by the shorter T_1 and longer T_2 relaxation times of the protons in fat compared to water (Hansen 1980).

PARAMAGNETIC CONTRAST AGENTS

The most powerful and also the most practical method of contrast enhancement in N.M.R. imaging has been provided by paramagnetic species (Runge 1983 a and b, 1984 a, b and c; Brasch 1983 a, b and c). These agents work by altering the local magnetic environments and reducing the T_1 and T_2 relaxation times of the tissue in close proximity to them. Paramagnetic compounds produce their own fluctuating local magnetic fields which shorten the relaxation times of neighbouring protons.

This effect is termed proton relaxation enhancement (P.R.E.) and is proportional to the concentration of the paramagnetic agent and its proximity to the protons. The major pathway of relaxation via P.R.E. is by spin-lattice relaxation and is brought about by interactions with the surrounding magnetic spin lattice. The effect of this process is a reduction in T_1. Paramagnetic compounds also induce fluctuating local magnetic fields, leading to a reduction in T_2.

The rate of relaxation (^{1}/relaxation time) is proportional to the square of the corresponding magnetic moment of the centre inducing relaxation, in this case a paramagnetic centre. The resultant reduction in T_1 by the induced relaxation processes leads to an increase in signal intensity and hence contrast enhancement, since $I \propto {}^{1}/_{T_1}$. However, a reduction in T_2 leads to a reduction in signal intensity, since $I \propto T_2$ (Runge 1983 a, Brasch 1983 a). The result of these effects is that conditions are optimised to create the maximum possible decrease in T_1 and at the same time keep the reduction in T_2 to a minimum. Such optimisation is achieved by alterations in concentration, pulse sequence and the nature of the paramagnetic agent. The image construction can be weighted towards T_1 or T_2 depending upon the situation. More detailed accounts of image construction and the phenomena involved with N.M.R. imaging can be found in the literature (Gadian 1982, Morris 1986).

In the diagnosis of a tumour by N.M.R. imaging normal contrast is provided by a weaker signal intensity from the tumour. This is a consequence of increased T_1 and T_2 with the immobile tumour protons (Carr 1984). Contrast can be enhanced by significantly increasing the signal intensity of the diseased tissue relative to the normal tissue. It has been found that a contrast agent can be incorporated into the tumour or diseased tissue to reduce the T_1 and T_2 values for the tumour protons (McNamara 1984, Wesbey 1984).

Potential contrast agents have been sought from paramagnetic metal ions and nitroxyl radicals. These contrast agents must be highly paramagnetic (resulting in low doses), chemically stable and non reactive in vivo, water soluble, quickly excreted, non toxic and have enough chemical versatility to induce tissue targetting.

PARAMAGNETIC IONS AS CONTRAST AGENTS

Paramagnetic metal ions studied as contrast agents for N.M.R. imaging include manganese (Kang 1984, Runge 1983 b), chromium (Runge 1984 a, b and c, 1983 b), copper (Runge 1983 b), iron (Runge 1983 b), and gadolinium (Wesbey 1984, McNamara 1984, Carr 1984, Runge 1984 b, Wolf 1984).

Several of the metal ions are toxic and have been complexed with ligands such as ethylenediaminetetraacetate (EDTA) to reduce their toxicity. The paramagnetism of these complexes is reduced due to the shielding of the paramagnetic centre by the ligand, preventing the paramagnetic centre from getting as close to the protons as the free metal ion. This reduced paramagnetism can be partially overcome by using higher doses of the complex compared to the free metal ions.

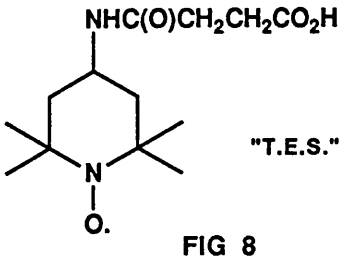
The most impressive results have been obtained using a gadolinium complex, GD DTPA, which is the basis of the only marketed N.M.R. imaging contrast agent, "Magnevist". It has been shown that Gd DTPA can enhance the contrast between normal and infarcted myocardium (Wesbey 1984, McNamara 1984). Initially the contrast agent is incorporated into the normal myocardium and reduces the signal intensity by shortening the T_2 value. After approximately five minutes the contrast agent is washed out of the normal myocardium and excreted. Some of the contrast agent, previously insignificant, accumulates in the infarcted myocardium where it remains after the normal tissue is free of contrast agent. The effect of this is to reduce the T_1 value of the infarcted tissue and leads to an increase in the signal intensity for the infarcted over the normal myocardium.

More generally, Gd DTPA has been found to accumulate in tumours as a result of extra vascular leakage (Carr 1984), this increases the image signal of the tumour by reducing T_1.

This effect differs from an N.M.R. imaging experiment using no contrast agent where the contrast between tumour and normal tissue is a result of increased T_1 and T_2 values for the tumour protons. Hence an image can have contrast provided by either an increased tumour signal intensity, as when a contrast agent is used or by a reduced signal intensity when no agent is used.

NITROXYL RADICALS AS CONTRAST AGENTS

Nitroxyl radicals have been widely used as spin labels and spin probes for biological systems. Their biocompatability make them attractive possibilities as N.M.R. imaging contrast agents. The nitroxyl radicals possess larger magnetic moments than paramagnetic ions and hence, in theory, can induce a larger reduction in T_1 and T_2. Contrast has been observed using a piperidine nitroxyl radical, T.E.S. illustrated in Figure 8 which has been found to cross the blood brain barrier only at sites of disease (Brasch 1983 b and c).



"T.E.S."

FIG 8

A major drawback of nitroxyl radicals is their instability. They can be reduced *in vivo* by ascorbic acid to give the corresponding hydroxylamine (Couet 1985, Perkins 1980). However, *in vitro* they are relatively stable to reduction, for example by lithium aluminium hydride (Keana 1978). The *in vivo* reduction could help to provide useful diagnostic information. Regions of the body containing hypoxic areas would have a lower concentration of any nitroxyl contrast agent due to their reduction. As a result, these areas will have no image enhancement compared to the surrounding non-hypoxic regions, where reduction can be up to thirty times less (Swartz 1987, 1986 a and b, Pals 1987). This phenomenon allows the possibility of some contrast development.

SCOPE OF RESEARCH

The aim of this work was to invent and synthesise novel nitroxyl radicals, using both new and established chemistry. These nitroxyl radicals were considered as potential N.M.R imaging contrast agents.

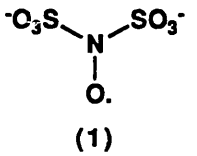
Methods of attaching the nitroxyl radicals to biologically interesting molecules were devised. This allowed the novel molecules to be considered as E.S.R. spin labels and probes, as well as N.M.R. contrast agents. Attempts were made to characterise these species by E.S.R spectroscopy.

C H A P T E R T W O

INTRODUCTION

PHOSPHORUS STABILISED NITROXYL RADICALS

The chemistry of the common nitroxyl radicals has been well documented, (see chapter one). Nitroxyl radicals are stablised most effectively by steric hindrance and an absence of α-hydrogen atoms (Gaffney 1976). Stablisation can also be conferred through electron withdrawing groups as in Fremy's Salt (1) (Fremy 1845), N-trifluoromethylsulphamate N-oxyl (2) (Haszeldine 1974); bis-(trifluoromethyl)-nitroxyl (3) (Blackley 1965) and bis-(triethylmethyltricarboxylate)-nitroxyl (4) (Flesia 1975).



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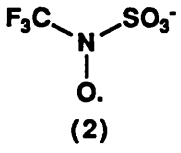

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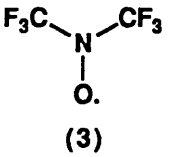
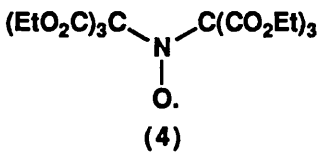
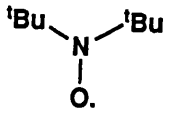


(3)



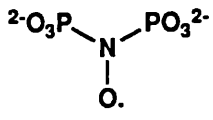
(4)

The substituents in the molecules shown above all confer stability on the nitroxyl centre by allowing delocalisation of the unpaired electron over the whole molecule, for example over the sulphonate groups of (1). This situation contrasts sharply with the stable bis-^{t}butyl nitroxyl radical (5) (Hoffmann 1961) in which stability arises from steric hindrance, with no contributions from electronic effects.



(5)

In the search for novel nitroxyl radicals as potential contrast agents for N.M.R. imaging attention was focussed on nitroxyl radicals stabilised by electron withdrawing groups. A potential analogue of Fremy's Salt (1) can be envisaged whereby the sulphonate groups are replaced by phosphonates to give the unknown molecule (6).



(6)

This novel phosphorus stabilised nitroxyl radical has two areas of potential interest and use.

(i) As a contrast agent for:-

(a) N.M.R. imaging

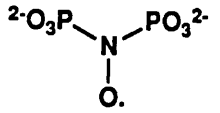
(b) E.S.R. imaging

(ii) As a spin labelled probe of biological systems, for E.S.R. investigations into ligand binding in kinases, nucleotidyl transferases etc.

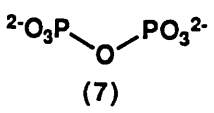
(i) (a)

Since (6) is not only an analogue of (1) but also of pyrophosphate (7), it may possess some interesting biological properties, and was expected to be biologically acceptable in vivo. Analogues of pyrophosphate, following initial work with linear polyphosphates (Subramanian 1972) have been used extensively as in vivo bone imaging agents in radionucleotide chemistry. The majority of work has been carried out on the hydrolytically stable methylenebisphosphonate (8) and its analogues, and has been widely reviewed (see chapter three for a full discussion of methylenebisphosphonates as bone imaging agents). Similarly, the imidobisphosphonate (9) has been investigated as an in vivo bone imaging agent. The novel nitroxyl radical (6), would thus be a good candidate as a contrast agent for N.M.R. imaging as a result of its similarity to (7) and (9), both acceptable agents for in vivo studies.

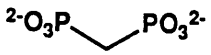
Importantly, this analogy to known bone imaging agents may also confer some selectivity for bone interfaces, providing contrast for areas of bone and calcification in N.M.R. imaging. At the moment N.M.R. imaging does not provide good imaging of bone due to the limited amount of water in the bone matrix (this aspect is developed in chapter four). The targetting of contrast agents to these areas may enhance any albeit low levels of imaging.



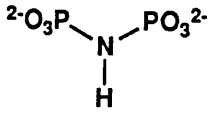
(6)



(7)



(8)



(9)

(i) (b)

The nitroxyl radical (6) would also warrant investigation in the relatively new field of E.S.R. imaging. This technique is based on similar principles to N.M.R. imaging, with the majority of work being carried out using nitroxyl radicals (Berliner 1985-1987). The presumed biological acceptability of (6) due to its similarity to natural systems makes it a potential probe for this technique.

(ii)

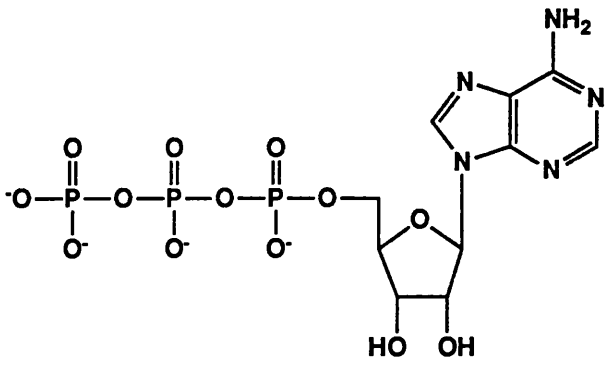
Due to its novelty (6) has potential as an E.S.R. probe, (see chapter one for a description of the uses of radicals in E.S.R.). The major use of (6) in E.S.R. investigations was envisaged as a spin labelling agent for biological systems.

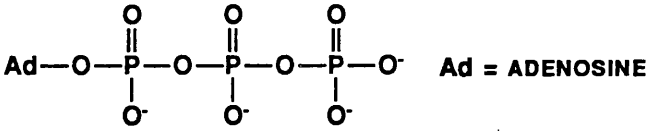
Analogues of pyrophosphate (7), specifically methlenebisphosphonate (8) and imidobisphosphonate (9) have been used to synthesise analogues of nucleotide triphosphates (see chapter three for a full investigation of nucleotide analogues). Thus, (9) has been used to form analogues of adenosine triphosphate (ATP) (10), the most common system studied so far. These analogues, the α, β adenylyl imidobisphosphonate, AMPNPP (11) and β, γ adenylyl imidobisphosphonate, AMPPNP (12) (Yount 1971 a and b, 1975, Kenyon 1983, 1988) have provided valuable information on the binding of ATP, along with the intrinsic enzymic processes in which it is involved (Yount 1975, Kenyon 1988, Tran-Dinh 1977, Taylor 1981, Scheit 1980 and references therein).

By incorporating (6) in place of (9) in nucleotides, it should be possible to synthesise novel spin labelled analogues of nucleotides, for example (13) and (14). Previously nucleotides have only been spin labelled with bulky organic nitroxyl radicals attached to the purine ring, (15) (Atkinson 1969) and (16) (Cooke 1971), or the phosphate chain (17) (Weiner 1969).

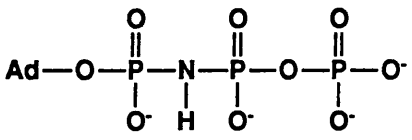
The use of (6) to create the spin labelled analogues (13) and (14) would have the advantage that it introduces only a small pertubation. This may ensure binding by a wide range of nucleotide binding proteins.

The above approach highlights one example where the novel nitroxyl (6) could be employed as a spin label for E.S.R. studies. Such ATP analogues could also be viable contrast agents for N.M.R. imaging as their structural similarity to natural biological systems makes them potentially acceptable for <u>in vivo</u> studies.

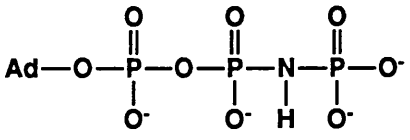




ATP (10)



(11)



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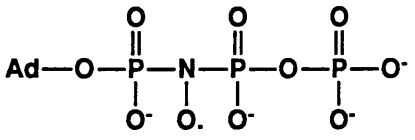

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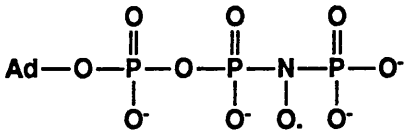

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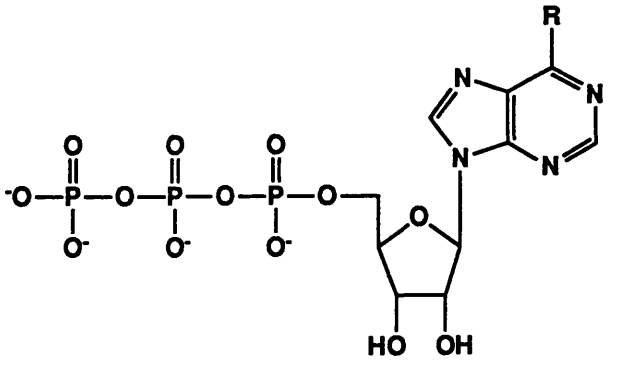
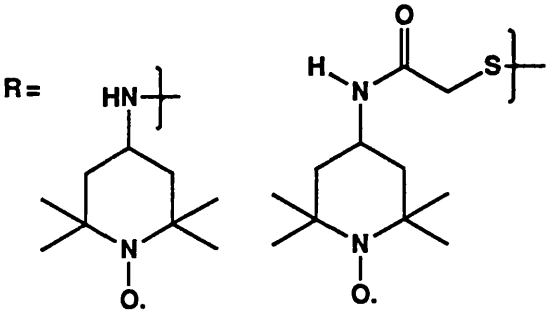
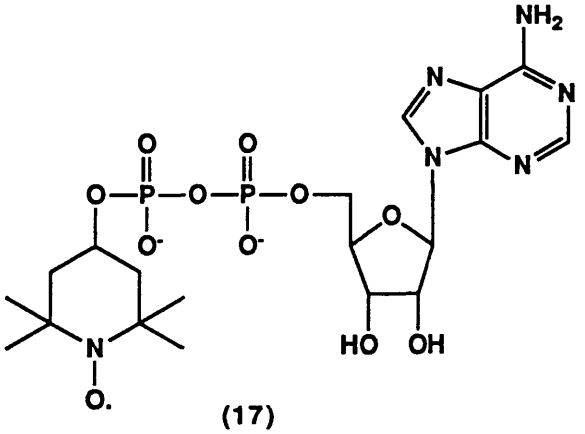




(15) (16)



(17)

PREPARATION OF NITROXYL RADICALS

The most common methods for preparing nitroxyl radicals have involved oxidation of the corresponding amine or hydroxylamine (Rozantsev 1971). Some examples are illustrated in Figure 9.

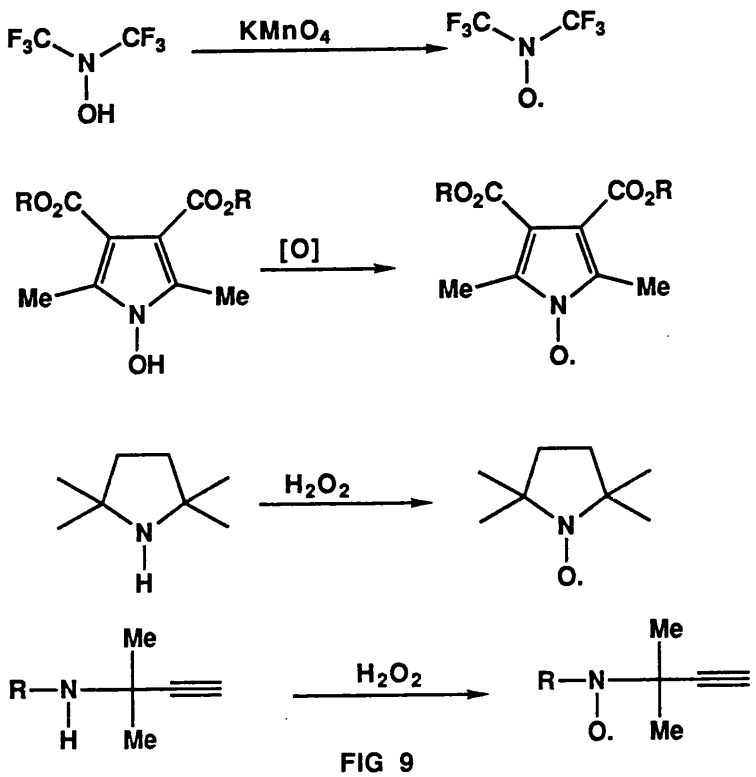
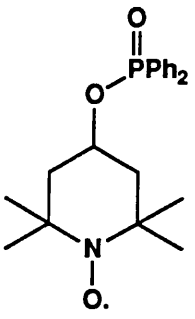


FIG 9

The synthesis of nitroxyl radicals by other methods, such as the reduction of nitro and nitroso compounds and diarylimmonium oxide perchlorates is not as versatile or widely used. Further information concerning the preparation of nitroxyl radicals can be obtained from the reviews cited in chapter one.

PHOSPHORUS-CONTAINING NITROXYL RADICALS

Spin labelled phosphoryl derivatives are of considerable interest due to their high profile in biological systems, which has been reviewed (Konieczny 1981). Compounds synthesised thus far have the radical centre removed from the phosphoryl group and attached *via* an ester linkage, for example (18). There are no derivatives with direct attachment of phosphorus onto the nitroxyl nitrogen as in (6). It was envisaged that any synthesis of (6) would involve known methods for the synthesis of phosphorus-nitrogen bonds, as briefly reviewed below.



(18)

SYNTHESIS OF PHOSPHORAMIDATES

Phosphoramidates (19) can be prepared by two general methods as illustrated in Figure 10. Reaction of a primary amine with a dialkyl phosphite in carbon tetrachloride (Atherton 1945 a) or with a phosphorochloridate (McCombie 1945, Atherton 1945) yields the desired phosphoramidate. The latter route being more attractive due to the availability of dialkyl phosphorochloridates.

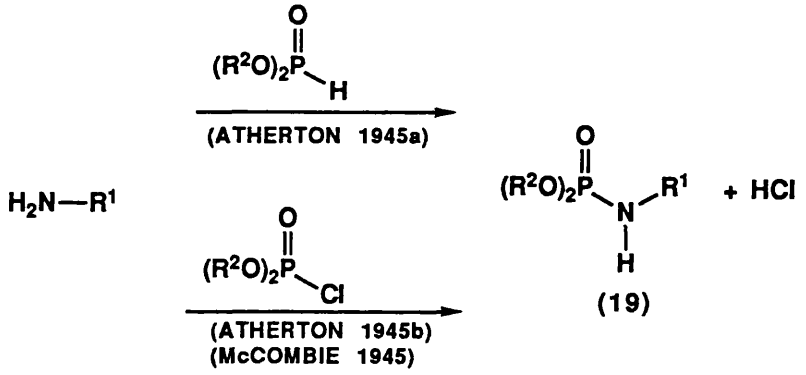


FIG 10

Phosphinamides and phosphoramidates have both found application as amino protecting groups for the synthesis of peptides (Ramage 1976, 1984) and secondary amines (Zwierzak 1977-1982). The respective phosphoramidate (or phosphinamide) anions have been generated using an appropriate base (Wadsworth 1962, Shahak 1976, Corbel 1976, Ramage 1976, Challis 1987) and subsequently alkylated or acylated. Alternatively, a phase-transfer method has also been used for nitrogen alkylation (Zwierzak 1977).

Both methods rely on mild acid treatment to effect deprotection *via* phosphorus-nitrogen bond cleavage. See Figure 11.

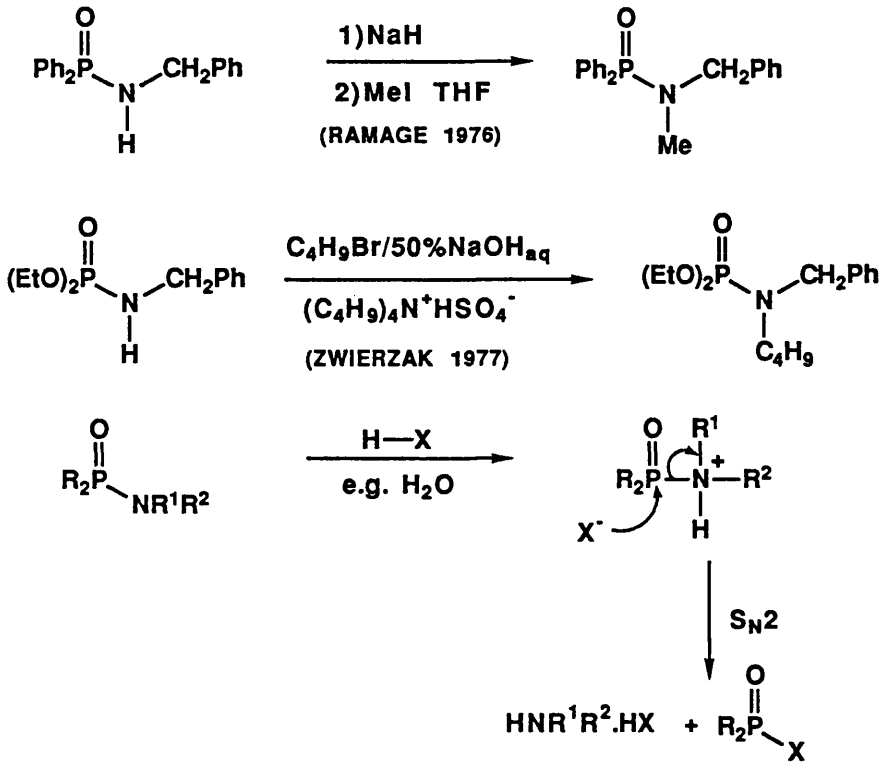
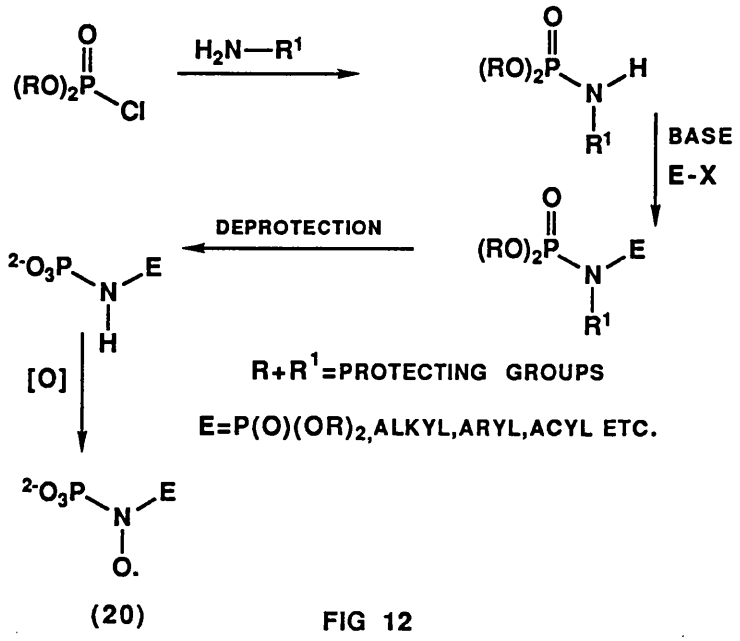


FIG 11

NITROXYL RADICALS VIA PHOSPHORAMIDATES

A potential synthetic route towards the target nitroxyl (6), was envisaged as proceeding *via* the corresponding amine. There is ample precedent for the alkylation and acylation of phosphoramidate anions, (Ramage 1976, Challis 1987) as highlighted earlier.

This provides scope for not only phosphorylating the phosphoramidate anion to develop the nitroxyl (6), but of extending the synthetic route towards nitroxyl radicals of general structure (20). See Figure 12.



(20)

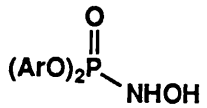
FIG 12

A more efficient alternative to oxidising the amine to give the nitroxyl, may be the introduction of the nitrogen-oxygen bond early in the synthesis.

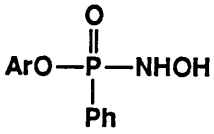
PHOSPHORYLATED HYDROXYLAMINE DERIVATIVES

N-phosphinyl hydroxylamines have been prepared and the chemistry of their O-sulphonyl derivatives investigated (Harger 1983, 1984). The corresponding N-phosphoryl and phosphonyl hydroxylamines (21) and (22) have also been synthesised (Harger 1985).

Compounds of type (21) were of direct interest for the synthesis of (6), as they possessed the correct oxidation level at phosphorus. Derivatisation of the hydroxylamine functionality has not previously been carried out on the nitrogen atom of systems such as (21) only on oxygen, e.g. via sulphonylation (Harger 1985).



(21)



(22)

The synthesis of the nitroxyl radical (6) was intended to proceed via oxidation of the corresponding hydroxylamine, this is in keeping with literature precendents for the preparation of nitroxyls (Rozantsev 1971). It was hoped that the necessary derivatisation of a N-phosphorylated hydroxylamine would be achieved via phosphorylation of the corresponding anion. As mentioned earlier, phosphoramidate anions can be generated using an appropriate base.

Although phosphorus electrophiles have not been used in these reactions, it was intended to use similar methodology to synthesise a suitably protected hydroxylamine (23). See Figure 13 for the proposed route.

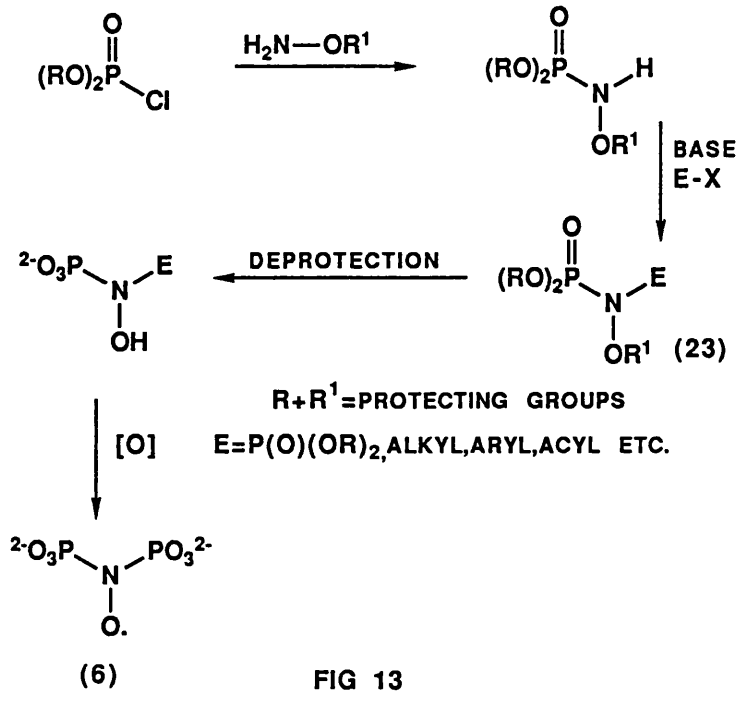


FIG 13

The hydroxylamine was highlighted as a target molecule since oxidation would be expected to be milder than for the corresponding amine. These novel intermediates suggest future possibilities for derivatising hydroxylamines.

RESULTS AND DISCUSSION

Unless otherwise stated ^{31}P N.M.R. spectra are ^{1}H decoupled.

PROPOSED ROUTE

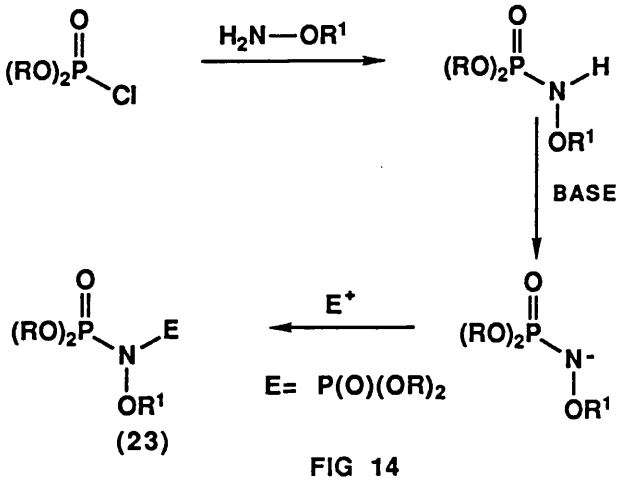


FIG 14

Figure 14 shows the proposed route to the desired N, N bisphosphorylated hydroxylamine (23). Initially, it was hoped to explore the use of simple esters (R = Me, Et, ^{i}Pr) in conjunction with a stable O-protecting group. Since it was necessary to remove the protecting groups R and R^1 in order to form the desired hydroxylamine, a careful choice of R and R^1 was necessary.

Removal of the phosphorus ester group, R, would need to avoid acid conditions since this was expected to lead to cleavage of the phosphorus-nitrogen bond. See Figure 11.

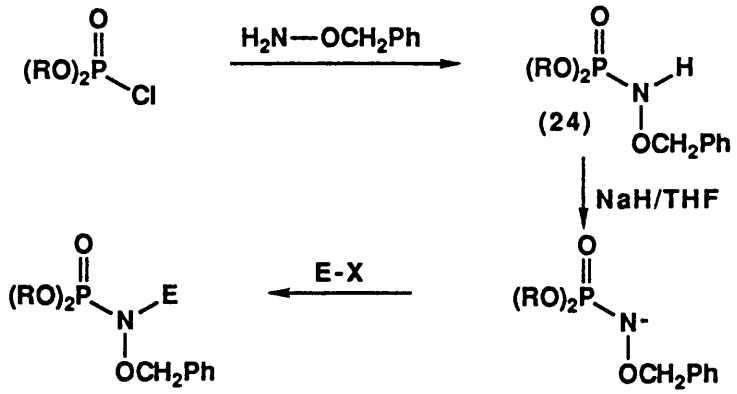
Treatment with a halotrimethylsilane should give the corresponding trimethylsilyl esters, (Rabinowitz 1963, Blackburn 1980) which may be readily hydrolysed to give salts directly. This method is mild and selective for phosphorus esters, removing the need for strong acid treatment. Initially the benzyl group, R^1, was explored as a protecting group for the hydroxylamine function. Previously, N-O-benzyl and N-obenzyl phosphoramidates have been synthesised and derivatised *via* the alkylation and acylation of their corresponding anions (Zwierzak 1977-1982, Challis 1987). The benzyl group can be removed by hydrogenolysis or by other mild methods of reductive cleavage.

O-BENZYLHYDROXYLAMINE DERIVATIVES

The synthetic route undertaken is illustrated in Figure 15. The initial reaction to give (24) proceeded essentially quantitatively as judged from the ^{31}P N.M.R. spectra. ^1H N.M.R. analysis showed that the ethoxy protons had a more complex splitting pattern than expected, with the methyl protons giving a triplet in the low resolution (90 MHz) spectrum and a doublet of triplets in the high resolution (300 MHz) spectrum. This phenomenon was due to long range coupling between the phosphorus nucleus and the methyl protons ($^4J_{PH}$).

The methylene protons gave rise to a quintet in the low resolution spectrum, due to the similarity of the $^{3}J_{HH}$ and $^{3}J_{PH}$ values. The high resolution spectrum yielded a complex splitting pattern which could not easily be resolved. This complexity could arise by virtue of restricted rotation about the phosphorus-nitrogen bond, leading to the methylene protons existing in different environments. These observations on the ^{1}H N.M.R. spectrum of the ethoxy esters were repeated throughout this work and will not be commented upon again.

The phosphorylated hydroxylamine (24) was treated with sodium hydride under anhydrous conditions to generate the corresponding anion, as shown in Figure 15. Before reacting this anion with suitable phosphorylating agents the formation of the anion was confirmed by reaction with a range of electrophiles. The electrophiles explored were methyl iodide, acetyl chloride and diethyl chlorophosphate. The results are tabulated below.



E-X= Me-I,Ac-Cl,P(O)(OEt)$_2$.

FIG 15

	E-X	YIELD
(25)	Me-I	96%
(26)	Ac-Cl	92%
(27)	$(EtO)_2P(O)$-Cl	79%

Compound (25) showed a characteristic doublet in the ^1H N.M.R. ($^3J_{PH}$ = 12Hz) due to the N-methyl protons coupling to the adjacent phosphorus nucleus. All the products gave ^1H and ^{31}P N.M.R. data consistent with the assigned structures and existed as oils. The identity and purity of (27) was confirmed by high resolution mass spectrometry.

After the successful synthesis of (27), the next step in the proposed route was the removal of the protecting group to expose the free hydroxylamine functionality. Hydrogenolysis of (27) according to Figure 16 led to cleavage of the nitrogen oxygen bond to yield the amine (28), rather than the desired hydroxylamine. This was confirmed by comparison with an authentic sample of (28) synthesised independently (see Figure 38).

Addition of authentic (28) to the hydrogenolysis product gave a single peak in the ^{31}P N.M.R. spectrum at δ_P 0.2. Mass spectrometry of the hydrogenolysis product also confirmed the proposed structure (28). In a similar reaction (24) was hydrogenolysed and was found to give diethyl aminophosphonate (29) and not the desired hydroxylamine. This finding confirmed the inappropriate choice of the benzyl protecting group, since hydrogenolysis led to the undesired cleavage of the nitrogen-oxygen bond in preference to the oxygen-carbon bond.

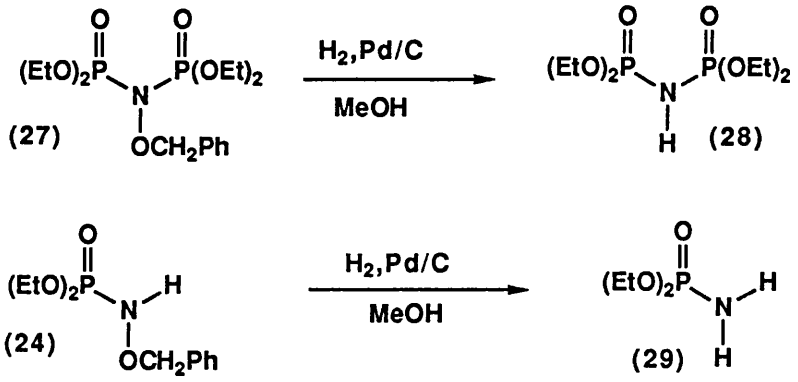


FIG 16

O-TRIMETHYLSILYLHYDROXYLAMINES

An alternative to the benzyl protecting group was the trimethylsilyl group. N-phosphinyl-, N-phosphonyl-, and N-phosphoryl hydroxylamines have been prepared <u>via</u> the reaction of O-trimethylsilylhydroxylamine and the appropriate phosphorus acid chloride (Harger 1983, 1984, 1985) with subsequent desilylation being achieved using methanol.

This mild deprotection procedure and synthetic precedent made this a good alternative protecting group for the proposed synthesis. O-trimethylsilylhydroxylamine was prepared by the reaction of hydroxylamine hydrochloride and hexamethyldisilazane in the presence of tri-^{n}propylamine (Douboudin 1974). The desired phosphoramidate (30) was synthesised by reaction of the protected hydroxylamine with diethyl chlorophosphate using triethylamine as base, according to Figure 17. The product was characterised by ^{31}P and ^{1}H N.M.R., the amino proton giving rise to a doublet due to coupling with the phosphorus nucleus. The ^{1}H N.M.R. signal due to the trimethylsilyl protons integrated for less than nine, which together with the presence of an extra small peak to the upfield side of (30) in the ^{31}P N.M.R., suggested the presence of a small amount of the free hydroxylamine compound (31).

The crude product (30) was stirred in methanol and analysed by ^{31}P N.M.R. The product from this desilylation showed a single peak at δ_P 9.47 corresponding to (31), see Figure 17, having the same chemical shift as the small peak observed in the ^{31}P N.M.R. spectrum of (30). This supports the supposition made earlier of some loss of the silyl group during the isolation of (30). The ^{1}H N.M.R. spectrum of (31) showed no trimethylsilyl protons and a broad signal corresponding to the two hydroxylamine (N-H and O-H) protons. The presence of the free hydroxylamine group was further supported by the infrared spectrum which showed a broad signal at 3,200 cm^{-1}.

The hydroxylamine derivative (31), when mixed with a sample of the amino compound (29), showed two separate signals in the ^{31}P N.M.R. Thus confirming that the removal of the trimethylsilyl group had not led to cleavage of the nitrogen-oxygen bond.

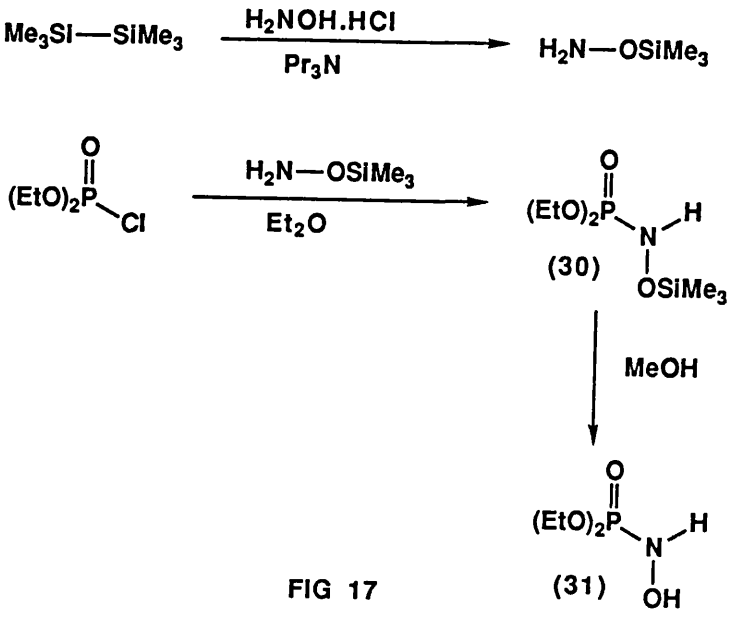


FIG 17

Samples of (31) were found to be rather sensitive and decomposed on standing to give diethyl phosphate and tetraethyl pyrophosphate. The mechanism of this reaction was further investigated.

MECHANISM OF DECOMPOSITION OF (31)

The mechanism of decomposition of (31) was studied using isotopic labelling techniques. On storing, (31) was found to decompose to tetraethyl pyrophosphate δ_P -13, and diethyl phosphate, indicating cleavage of the phosphorus-nitrogen bond. The tetraethyl pyrophosphate presumably arose from the reaction between diethyl phosphate and (31). In a separate experiment (31) was treated with water to yield diethyl phosphate exclusively, see Figure 18.

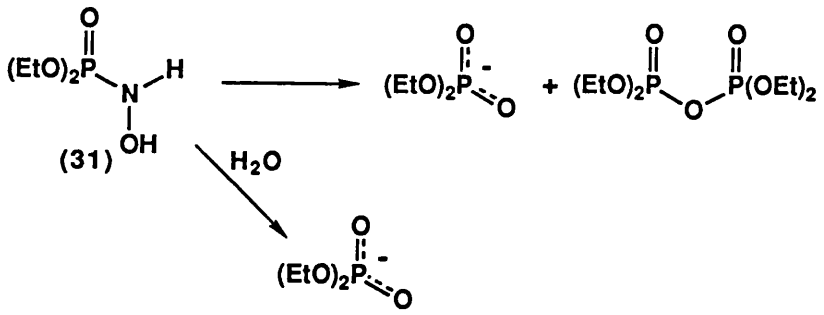


FIG 18

N-phosphinyl hydroxylamine can re-arrange to the corresponding O-phosphinyl derivative in the presence of methoxide (Harger 1983 a, Sturtz 1988). A similar intramolecular re-arrangement which involves the hydroxylamine oxygen of (31) to give (32) could occur. Attack by water at the phosphorus or nitrogen atom of (32) may be in competition with the more straight forward S_N2 attack of water on the phosphorus atom of (31), see Figure 19.

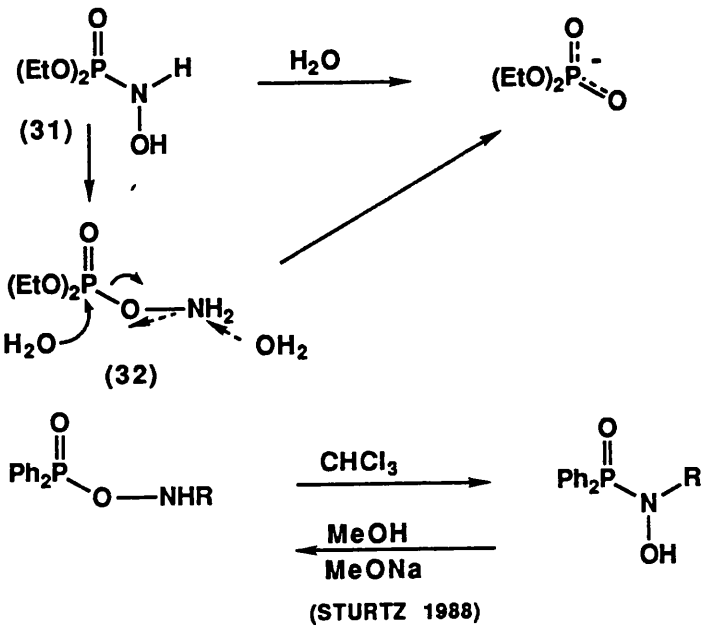


FIG 19

In order to shed light on the exact mechanism of hydrolysis, the origin of the oxygen in the diethyl phosphate was established by isotopic labelling. Use was made of the isotope shifts observed in ^{31}P N.M.R. when a phosphorus is bonded to an ^{18}O atom. The point to be noted in this investigation is that in a highfield ^{31}P N.M.R. spectrum a phosphorus nucleus bonded to an ^{18}O atom has an upfield shift (of the order of 0.02 ppm) relative to the corresponding molecule containing an unlabelled oxygen. The absolute magnitude of the upfield shift is bond order dependent (P=O shift > P-O). For more information the reader is referred to the literature (Lowe 1979).

By determining the level of ^{18}O incorporation in the diethyl phosphate obtained by hydrolysis of (31) in ^{18}O-water, the hydrolysis mechanism should be clearer. Thus re-arrangement of (31) to (32), followed by attack at nitrogen would not lead to any ^{18}O incorporation, whereas the S_N2 pathway or the re-arrangement followed by attack at phosphorus both lead to ^{18}O incorporation.

The free hydroxylamine (31) was treated with a 50% volume by volume mixture of anhydrous dioxane and ^{18}O water (95 atom %) to yield one product in the low field ^{31}P N.M.R. spectrum, identified as diethyl phosphate. Analysis of the highfield ^{31}P N.M.R. spectrum showed the presence of two peaks separated by 3.5 Hz (0.029 ppm). This analysis confirmed the incorporation of ^{18}O into the diethyl phosphate, the highfield signal corresponding to the enriched product. The ^{31}P N.M.R. spectrum is reproduced in Figure 20.

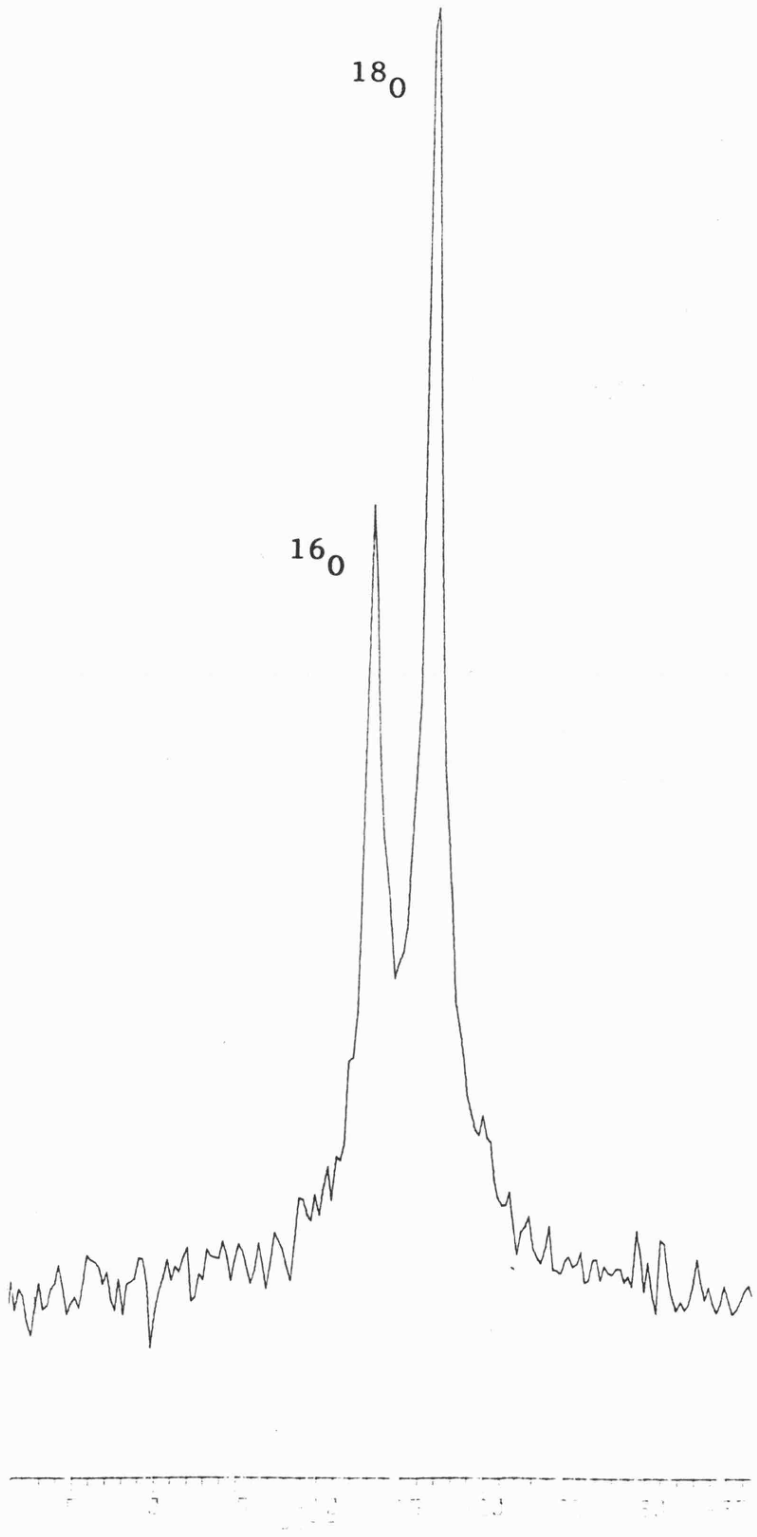


FIG 20-^{31}P N.M.R. SPECTRUM (121 5 MHz) SHOWING ^{18}O INCORPORATION INTO DIETHYL PHOSPHATE.

The presence of a significant amount of the unlabelled product (approximately 30%) suggests that some intramolecular re-arrangement must be occurring, as the only source of unlabelled oxygen is the hydroxylamine oxygen. Attack of ^{18}O water on (31) would lead exclusively to a product containing ^{18}O. The proposed intramolecular re-arrangement would lead to an intermediate susceptible to attack by ^{18}O water at two positions, see Figure 21. The two possible products consist of one with ^{18}O incorporation and one without. The results of this experiment confirm that some decomposition must go *via* the re-arrangement product, that is the O-phosphoryl hydroxylamine derivative (32) as this would be the obvious source of unlabelled oxygen.

However, since direct attack at phosphorus on either the N-phosphoryl (31), or the O-phosphoryl hydroxylamine derivative (32), would lead to incorporation of ^{18}O, it is not possible to quantify the contribution of each of the two pathways. This experiment proves that two hydroysis pathways for (31) do exist, and that at least 30% goes *via* the re-arrangement (B) and hydrolysis step (D).

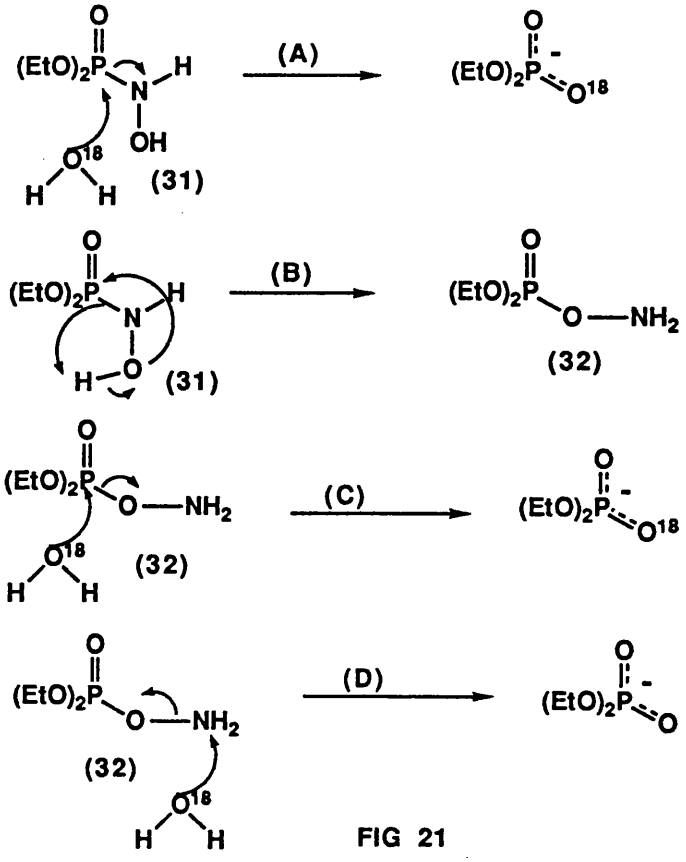


FIG 21

SUBSTITUTED O-TRIMETHYLSILYLHYDROXYLAMINES

In a similar approach to that used for (24), the generation of the anion of (30) was demonstrated using a range of electrophiles. The proposed route is shown in Figure 22 with the target being the phosphorylated derivative (33). After deprotection, the exposed hydroxylamine function could be oxidised, in an attempt to generate the desired nitroxyl radical. The oxidation could be attempted both with and without the phosphorus ester groups present, thus allowing a range of oxidation conditions to be used.

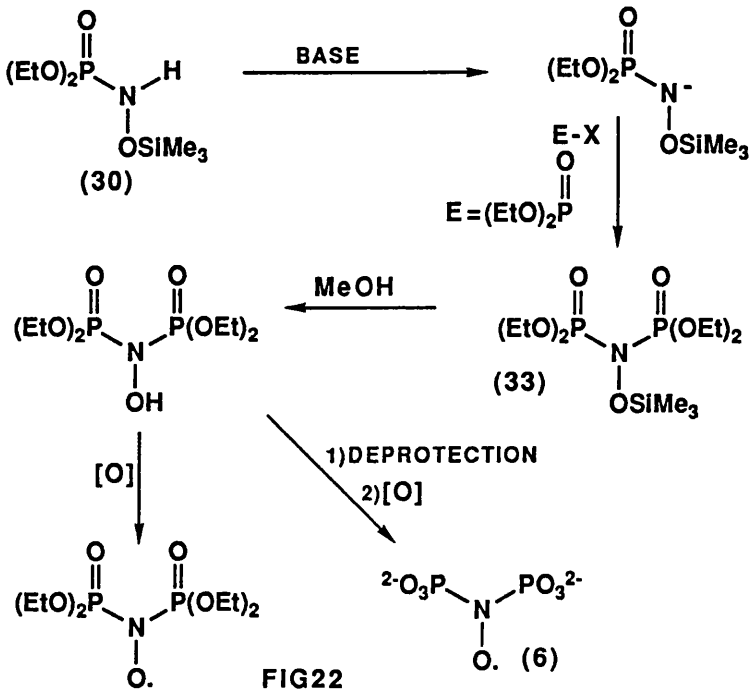


FIG22

ANION GENERATION AND DERIVATISATION

Following the successful generation of the anion of the phosphorylated O-benzylhydroxylamine derivative (24) with sodium hydride, the same conditions were applied to (30). Initially the alkylation of the anionic species was attempted with methyl iodide.

By analogy with the previous alkylations, the desired product (34) would be expected to have a chemical shift in the ^{31}P N.M.R. slightly to the highfield side of the starting material, in this case at approximately δ_P 9.0. However, the reaction was observed to yield products with resonances in the crude ^{31}P N.M.R. spectrum at approximately δ_P 0. This region of the spectrum is indicative of simple phosphate esters, suggesting cleavage of the phosphorus-nitrogen bond. The reaction was repeated at lower temperatures, down to -78°C with the same effect and no evidence for the alkylated product.

The attempted alkylation was repeated using ^{n}butyl lithium as base with methyl iodide as the electrophile. The reaction was observed to give two products in approximately equal quantities, one of which had the expected ^{31}P N.M.R. chemical shift of δ_P 9 and the other δ_P 0.6. On standing, the product characterised by δ_P 0.6 resonance, increased to the detriment of the product at δ_P 9. This suggests that the desired product (34) decomposed to the unidentified product at δ_P 0.6 on standing. The two compounds were separated by silica gel flash chromatography and (34) characterised by ^{31}P and ^{1}H N.M.R. The protons of the methyl group gave rise to a doublet, due to coupling with the phosphorus nucleus ($^{3}J_{PH}$ = 12 Hz). This confirmed the successful anion formation and subsequent alkylation.

On further examination of the ^{1}H N.M.R. spectrum, the integration for the methyl and trimethylsilyl protons was less than that expected, while the ^{31}P N.M.R. contained a small peak to the low field side of (34). These two observations indicate the possible presence of a small amount of the free hydroxylamine derivative (31). On storing in a stoppered vessel at -40°C the product (34) was found to decompose to give signals in the ^{31}P N.M.R. at δ_P 2 and δp 0.

The instability of the product (34) may be attributed in part to the reaction conditions employed. During the reaction the environment was basic, allowing for the possibility of nucleophilic attack leading to the product at δ_P 0.6. On purification, the acidic nature of silica gel could allow protonation of the nitrogen, thus facilitating the observed decomposition. Finally, the deuterated chloroform used could contain traces of hydrogen chloride which could also promote decomposition. These possible decomposition pathways are illustrated in Figure 23. The two likely sources of nucleophilic species present in the reaction media were lithium hydroxide (from the ^{n}butyl lithium) and particularly, iodide ions arising from the methyl iodide.

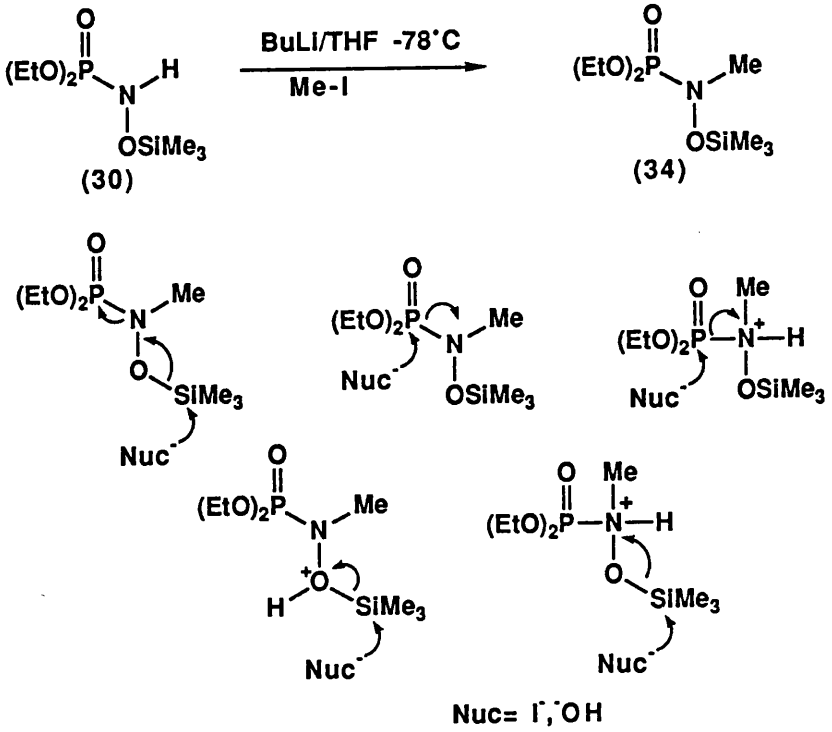


FIG 23

THE ATTEMPTED SYNTHESIS OF THE N,N BISPHOSPHORYLATED HYDROXYLAMINE

The previous reaction confirmed the formation of the desired anion and its subsequent alkylation, even though the product proved to be unstable. In a similar reaction, (30) was again treated with ^{n}butyl lithium at -78°C, followed by diethyl chlorophosphate as the electrophilic species according to Figure 24.

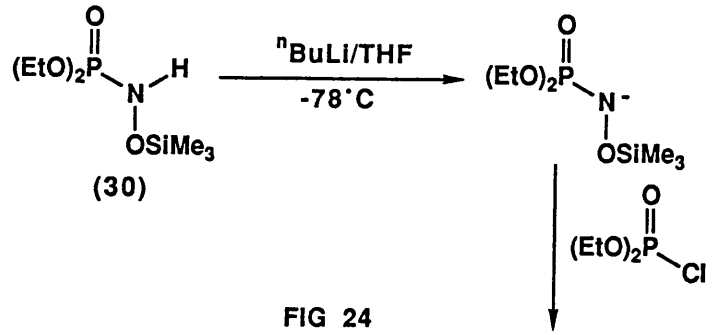


FIG 24

The reaction failed to give the desired bisphosphorylated species under a variety of conditions, such as having the diethyl chlorophosphate present at the start in order to react with the anion generated <u>in situ</u>. The ^{31}P N.M.R. of the crude reaction mixture was complex with the major signals corresponding to unreacted diethyl chlorophospate and tetraethyl pyrophosphate. Further signals present in the ^{31}P N.M.R. may have arisen from decomposition of (30), or may in fact, include the desired product, albeit in very low yield. The identification and characterisation of these products proved to be impossible.

The electrophiles (methyl iodide and diethyl chlorophosphate) have different properties, such as the relative "hard" and "soft" nature of the phosphorus and carbon atoms and their respective leaving groups (chloride and iodide). It has been shown that phosphorus stabilised anions can be derivatised with a wide range of electrophiles. The problem would appear not to lie with the choice of electrophile therefore, but possibly with that of the counterion which could influence the reactivity of the anion.

It was decided to investigate the phosphorylation of (30) with sodium hydride and triethylamine. Firstly, the reaction was carried out using sodium hydride as base, but yielded only unreacted diethyl chlorophosphate, tetraethyl pyrophosphate and an ether insoluble product at δ_P 0. Similarly, reaction using triethylamine as base gave the same result, with a phosphoramidate, presumably unreacted (30), also present. The desired product was expected to have a similar chemical shift in the ^{31}P N.M.R. to that of the O-benzyl derivative (24), that is, at approximately δ_P 0.

The above reactions gave products with signals in the ^{31}P N.M.R. at around δ_P 0 in the crude mixture. On work up these products did not extract into organic solvents, suggesting that they were charged. The possibility does exist that the desired product was formed, but is inherently unstable. It has been shown that related compounds, for example N-phosphoroyl O-trimethylsilylhydroxylamines (Harger 1985) have proved to be unstable unless isolated in crystalline form.

Attempts to remove the silyl group before isolation were also unsuccessful. In a separate reaction, methanol was added to the crude product mixture to remove the trimethylsilyl group in situ. This treatment yielded the free hydroxylamine (31), tetraethyl pyrophosphate and what appeared to be diethyl methylphosphate, with no evidence of the desired product.

The evidence suggesting the identity of the diethyl methylphosphate lies with the presence of a doublet in the ^{1}H N.M.R. indicative of a methyl phosphate ester (J_{PH} = 10 Hz) and a singlet at δ_P 3.7 in the ^{31}P N.M.R. the expected region.

POSSIBLE MECHANISMS FOR DECOMPOSITION OF (30)

The limited evidence available from the attempted phosphorylations of the protected hydroxylamine (30), indicates that one possible decomposition pathway could be *via* the *in situ* generation of the free hydroxylamine (31). Some supportive evidence is available for this hypothesis from two reactions.

After treatment of (30) with triethylamine in diethyl ether for two days there was no sign of decomposition or loss of the trimethylsilyl group. However, under the same conditions the free hydroxylamine (31) decomposed to compounds with chemical shifts of δ_P 0.2, -1.6 and -12.9 in the ^{31}P N.M.R.. The latter signal corresponds to tetraethyl pyrophosphate, the other two having similar chemical shifts to the products generated during the attempted phosphorylations of (30), *via* generation of the corresponding anion. One conclusion is that under the reaction conditions employed (30) was converted to (31), which in turn decomposed.

The trimethylsilyl group could be removed from (30) to give (31) by adventitious water present in the reaction media, although this is unlikely under the anhydrous conditions employed. It is possible that there could be hydrogen chloride present, arising either from decomposition of the diethyl chlorophosphate during the reaction or present in the initial sample used.

A further decomposition pathway could lie with the ability of the phosphoryl oxygen of (30) (or diethyl chlorophosphate) to be silylated by a second molecule of (30) in a manner analogous to that found in reactions with trimethylsilyl halides. See Figure 25 and Figure 39 for a full description of this type of reaction.

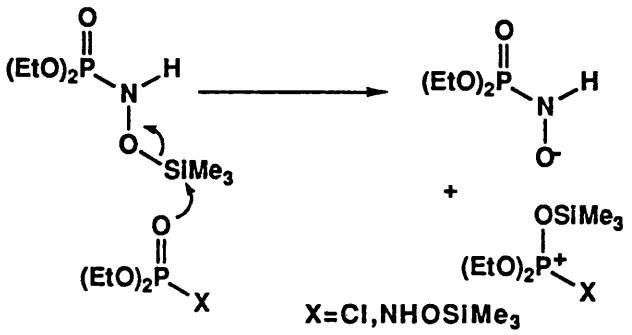


FIG 25

The tetraethyl pyrophosphate observed could also be generated as a result of hydrolysis of the diethyl chlorophosphate or phosphorylated hydroxylamines during the work up procedure.

Treatment of (31) with diethyl chlorophosphate in the presence of triethylamine gave mainly tetraethyl pyrophosphate. A minor product formed in this reaction was later identified as the bisphosphorylated derivative (35), with the second phosphorylation occuring on the hydroxylamine oxygen.

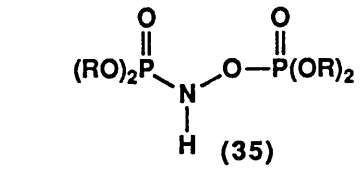
The failure of the phosphorylation attempts using the O-trimethylsilyl derivative (30), were not due to the unsuccessful generation of the anion, since alkylation was possible to yield (34). The possible desilylation of (30) to generate the free hydroxylamine, is one potential decomposition pathway, and is highlighted by the instability of (31). Therefore, the problem seems to arise from the extreme lability of the chosen protecting group under the reaction conditions employed. The actual decomposition pathway remains unclear, due to the various possible sources of diethyl phosphate and tetraethyl pyrophosphate outlined above. There also remains a degree of uncertainty over the stability of the intended product (33).

It was decided to investigate the synthesis of the bisphosphorylated derivative (35), to confirm its production in the previous reaction and also as it would be interesting in its own right.

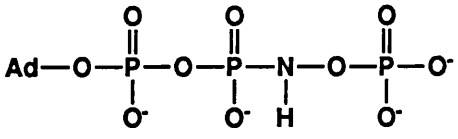
SYNTHETIC ROUTES TOWARDS A NOVEL NITROGEN-OXYGEN LINK IN NUCLEOTIDE ANALOGUES

Following on from the work with protected hydroxylamine derivatives, it was decided to investigate routes towards the bisphosphorylated derivative (35). Such compounds have not previously been reported in the literature, either of the phosphoryl type shown, or of the more widely investigated phosphinyl type. Hydroxylamine has previously been phosphinylated and phosphorylated. The re-arrangements of some phosphinylated derivatives have been studied (Harger 1981, 1983, 1984, 1985, Sturtz 1988).

Once synthesised, the compound (35) would be suitable after removal of the phosphate ester groups for incorporation into nucleotide analogues, such as (36).



(35)



(36)

PROPOSED ROUTES

Two synthetic routes were considered as potentially useful towards the synthesis of (35). Harger (1981) has synthesised compounds of the type (37) from free hydroxylamine and the corresponding phosphinic chloride.

By analogy, the use of a phosphoryl chloride should generate (38), which could then be phosphorylated at the amino group to yield (35). Alternatively, the N-phosphoryl hydroxylamine (31) could be phosphorylated on the oxygen, in an analogous reaction to the sulphonylation of the related compounds (39) (Harger 1983, 1984, 1985). These proposed routes are illustrated in Figure 26.

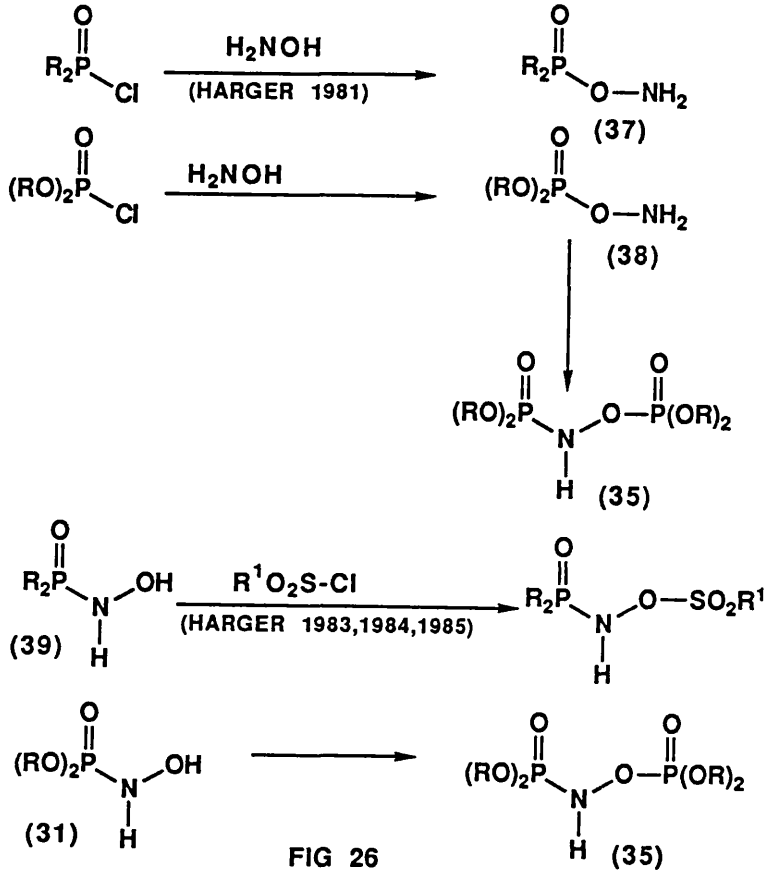


FIG 26

O-PHOSPHORYL HYDROXYLAMINE

Free hydroxylamine was prepared according to the method of Hurd (1945) and treated with diethyl chlorophosphate in an attempt to form the O-diethylphosphoryl hydroxylamine (32).

The reaction afforded only the ammonium salt of diethyl phosphate, confirmed by ^{1}H N.M.R. mass spectrometry and by mixing with an authentic sample to give a single peak by ^{31}P N.M.R. On repeating the reaction in dioxane, the crude reaction mixture initially showed what appeared to be the N-phosphoryl hydroxylamine (31) at δ_P 9 in the ^{31}P N.M.R. Further reaction yielded only tetraethyl pyrophosphate and a phosphate (probably diethyl phosphate) salt. This then did not seem to be a viable route, the results being summarised in Figure 27, with no evidence for the desired O-phosphorylation.

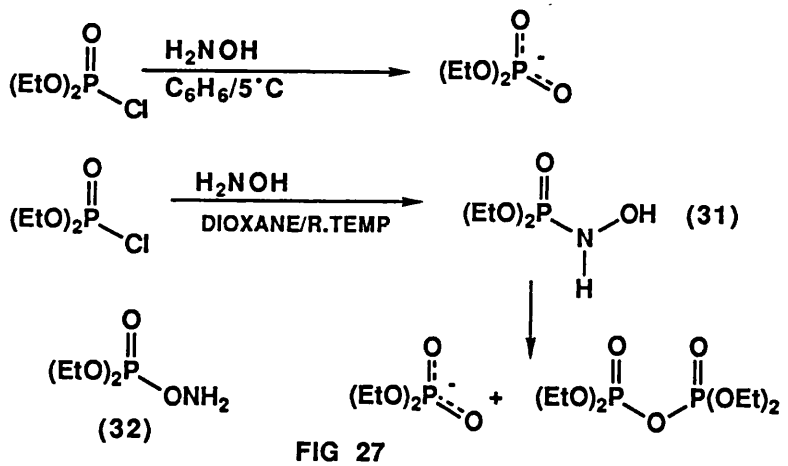


FIG 27

O-PHOSPHORYLATION OF (31)

The N-phosphoryl hydroxylamine (31) was treated with diethyl chlorophosphate in pyridine, but no evidence for the desired product was observed. The only products isolated being diethyl phosphate and tetraethyl pyrophosphate.

In a related piece of work described in this chapter , there was evidence that (31) could be phosphorylated on the hydroxylamine oxygen to give (35). This bisphosphorylated species was a minor product in the reaction illustrated in Figure 28.

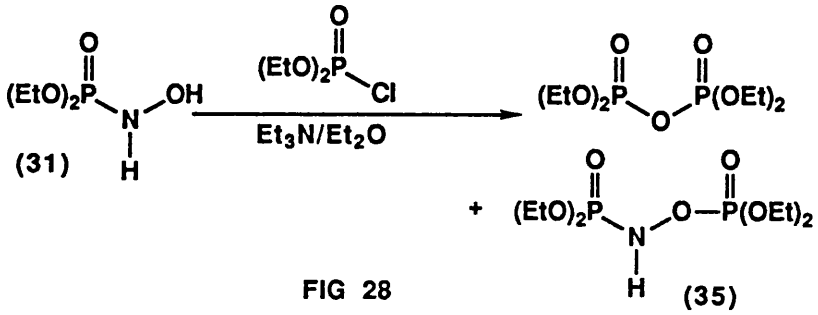


FIG 28

There was, therefore, evidence that the desired molecular framework could be developed, albeit in a low percentage yield. It was decided to attempt a "one-pot" reaction to bisphosphorylate hydroxylamine, since it had proved unfeasible to phosphorylate the oxygen of free hydroxylamine, while subsequent reactions involving (31) were not conclusive. Due to the difficulty involved in synthesising free hydroxylamine, it was decided to use hydroxylamine hydrochloride and to generate the free base in situ.

The reaction was carried out according to Figure 29, using three equivalents of triethylamine, to generate the free hydroxylamine and to remove the two moles of hydrogen chloride generated on reaction with diethyl chlorophosphate. Three products were identified in the crude reaction mixture by ^{31}P N.M.R. the desired product (35), the N-phosphorylated hydroxylamine (31) and tetraethyl pyrophosphate.

During aqueous work up (31) was removed and identified by comparison with an authentic sample. The remaining two products were separated by silica gel flash chromatography,(35) being obtained in a 47% yield. The identity of (35) was confirmed by high resolution mass spectrometry and N.M.R. The ^{31}P N.M.R. gave rise to two doublets arising from the coupling between two non-equivalent phosphorus nuclei ($^{3}J_{PP}$ = 19.5 Hz). In the ^{1}H N.M.R. a doublet for the amino proton coupling to one phosphorus ($^{2}J_{PH}$ = 8 Hz) was observed. The other product formed in the reaction was confirmed as tetraethyl pyrophosphate.

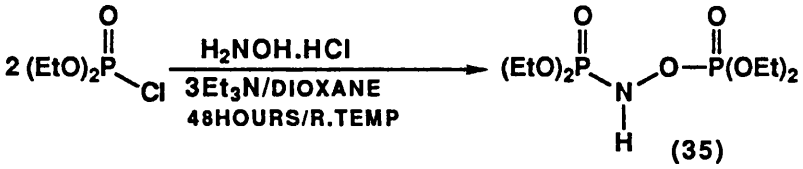


FIG 29

MECHANISM OF FORMATION OF (35)

The mechanism of formation of the novel compound (35), initially involved phosphorylation of the hydroxylamine nitrogen to generate (31), confirmed by its isolation from the reaction mixture. There was no evidence for the analogous phosphorylation at oxygen, unless the signal in the ^{31}P N.M.R. was masked by those of the other materials present in the crude reaction media.

The next step in the reaction was a second phosphorylation, this time on the hydroxylamine oxygen of (31) to yield (35). This proposal is supported by the production of (35) when (31) was treated with diethyl chlorophosphate in a separate reaction. See Figure 28.

The problem in earlier reactions was in achieving phosphorylation at the oxygen of the hydroxylamine, this was overcome to a large extent in this reaction. This was therefore, a straightforward, "one-pot" synthesis of a new molecular framework, giving a good yield (47%) of the desired product. It should be applicable to other phosphorus halides and provide interesting and novel structures.

DE-ESTERIFICATION VIA THE USE OF HALOTRIMETHYLSILANES

Several attempts at the de-esterification of (35) were undertaken using halotrimethylsilanes to generate the phosphate. Treatment with iodotrimethylsilane or bromotrimethylsilane led to the decomposition of the molecular framework, even at low temperatures (down to -78°C). The ^{31}P N.M.R. evidence indicated that the phosphorus-nitrogen bond was being cleaved to give a phosphate and a phosphoramidate. The separate use of chlorotrimethylsilane also resulted in the decomposition of (35). No products could be isolated from these reactions.

Taking into account the limited evidence obtained from the ^{31}P N.M.R. spectra of the de-esterification attempts, there appears to be two reasonable decomposition pathways. After the initial silylation of the nitrogen, there is the possibility of cleavage of the phosphorus-nitrogen bond as illustrated in Figure 30. It is highly likely that de-esterification of the phosphorus esters could be occurring concurrently with the decomposition.

A further mechanism for the decomposition of (35), or its partially de-esterified derivative, was suggested by work in the following section, possibly involving the generation of a phosphorus-nitrene and a phosphate, see Figure 32. The actual mechanism of decomposition is however still unclear.

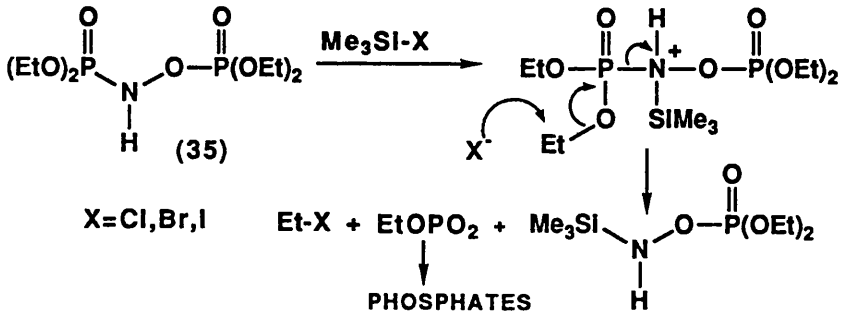


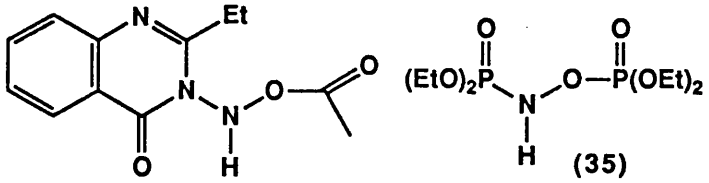
FIG 30

There exists the possibility for using alternative ester groups and that any subsequent de-esterification steps may be more amenable to the system shown. However, this seems unlikely given the accepted mildness of the halotrimethylsilane hydrolysis treatment (Blackburn 1980). Acidolysis of the ester groups would be expected to lead to cleavage of the phosphorus-nitrogen bond.

Other possibilities might include the use of methyl or aryl esters and to effect the de-esterification with thiophenolate or hydroxide ions. Due to this inability to easily remove the ester groups, the potential to use (35) for developing nucleotide analogues remains untested.

STUDY OF POSSIBLE BREAKDOWN OF (35)

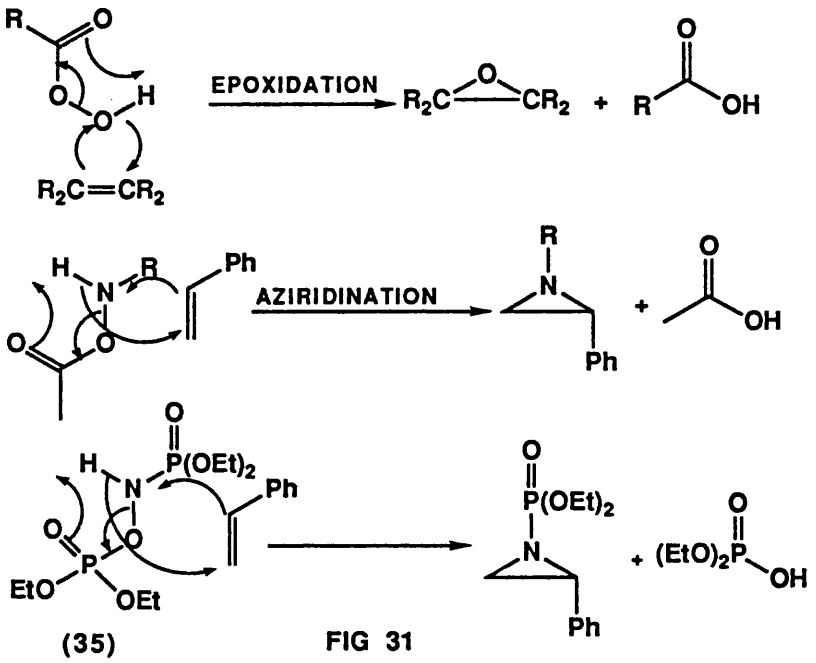
In an attempt to study the possible mode of decomposition or breakdown of (35), attention was focussed on some recent work by Atkinson (1987, 1989) with N-acetoxy quinazolones. Similarities between (35) and the quinazolone (40) can be drawn in that both structures have attached to the protonated nitrogen a leaving group, an electron withdrawing group and a heteroatom.



(40) (35)

Systems such as (40) have been shown to be the reactive species involved in the aziridination of olefins. These reactions formerly were believed to proceed <u>via</u> an N-nitrene.

The reaction mechanism in Figure 31 can be seen to be completely analogous to the well known peracid epoxidation mechanism. The possibility that (35) might undergo a similar breakdown was investigated.



(35)

FIG 31

The reaction of (35) with styrene under varying conditions of temperature and concentration was carried out in a solvent of benzene. No change was observed in the ^{31}P N.M.R. of the crude reaction mixture. These preliminary results suggested that (35) did not breakdown to give diethyl phosphoric acid in the presence of styrene.

A possible reason for this would be the relative acid strengths of diethyl phosphoric acid and acetic acid (pKa ≃ 2 and pKa = 4.75 respectively). This would indicate that the phosphate oxygen is not basic enough to assist in the required deprotonation.

This area of investigation does however highlight a possible mechanism for the decomposition of (35) with trimethylsilyl halides. In this case the monoanion of the generated phosphate would be more basic and may provide the kinetic assistance required for the reaction. The species (41) shown in Figure 32 is formalised as the P-nitrene although this may not, in fact, be a liberated free intermediate.

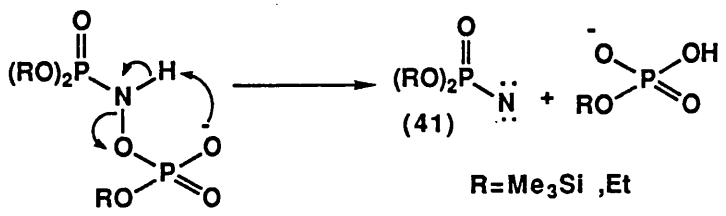


FIG 32

As an aside it was decided to attempt the acetylation of (31), see Figure 33, with a view to undertaking a reaction similar to that in Figure 31, for the quinazolone derivative reported by Atkinson.

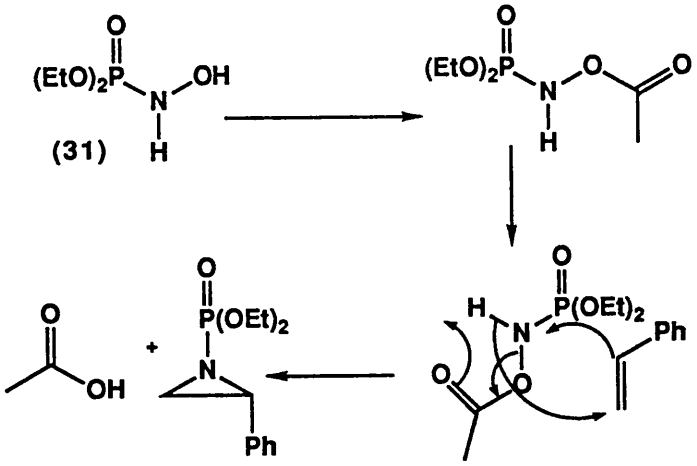


FIG 33

The attempted O-acetylation of (31) with acetyl chloride or acetic anhydride gave rise to several signals in the ^{31}P N.M.R. spectra of the respective reactions. The "one-pot" reaction of (31) with acetic anhydride and styrene was attempted. The intention was to form the apparently unstable O-acetyl derivative and react this <u>in situ</u> with styrene. The only observable product in the ^{31}P N.M.R. was however, tetraethyl pyrophosphate.

THE USE OF PHOSPHORAMIDATES IN THE SYNTHESIS OF A BISPHOSPHORYLATED NITROXYL

The synthetic routes attempted aimed at developing the desired nitroxyl radical (6) proved to be unsuccessful. The work thus far, had centred on the overall bisphosphorylation of the nitrogen of hydroxylamine.

The use of protecting groups on oxygen, failed to allow the necessary derivatisation of the appropriate nitrogen anion and regeneration of the free hydroxylamine function. Investigations with free hydroxylamine itself again failed to realise the desired bisphosphorylated derivative, although the novel structure (35) was generated.

Due to this failure to develop a viable synthetic route towards molecules of the general type (23), and the corresponding nitroxyl radicals (20), attention was switched to the analogous amine compounds (19) in Figure 34. Literature precedents are available for syntheses of this type (see introduction).

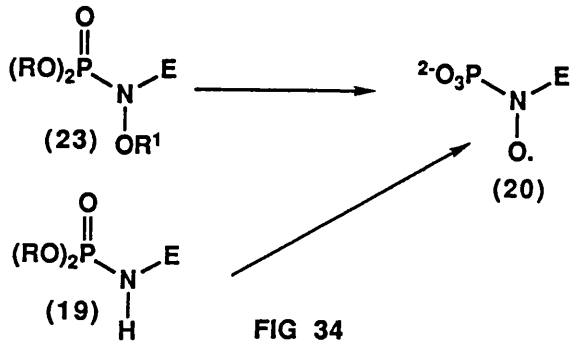


FIG 34

PROPOSED ROUTE

The proposed synthetic route towards molecules of type (20) is illustrated in Figure 35 and was essentially the same as that undertaken with the hydroxylamine system. Again the use of appropriate phosphoryl and nitrogen protecting groups was required, with a range of electrophiles being used to derivatise the anion. In this manner it was hoped to create a general route towards the corresponding nitroxyl radicals.

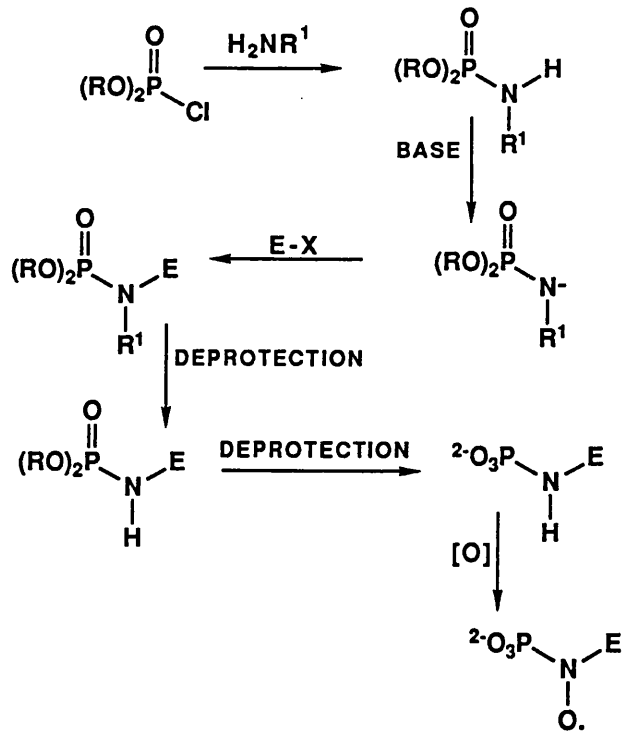


FIG 35 (20)

N-BENZYLAMINE DERIVATIVES

It was decided to undertake the synthetic route using a benzyl protecting group for the amine function. The reasons for this choice of protecting group were twofold. Firstly, there is literature precedent for the generation of the required anion (see the section on phosphoramidates in the introduction to this chapter), and the benzyl group can be removed by reductive cleavage, e.g. hydrogenolysis.

The actual synthesis undertaken is shown in Figure 36. The initial reaction to give (42) proceeded smoothly in a near quantitative yield. Characterisation was made by ^{1}H N.M.R. and ^{31}P N.M.R., the methylene protons of the N-benzyl group giving rise to a doublet in the ^{1}H N.M.R. due to coupling with the phosphorus nucleus ($^{3}J_{PH}$ = 12 Hz). The corresponding anion of (42) was generated with sodium hydride and its formation confirmed by reaction with a range of electrophiles. This had the dual purpose of providing a synthetic route towards the compounds of general formula (20), as well as finding suitable conditions for the desired phosphorylation. The range of electrophiles explored was the same as that used with the hydroxylamine derivatives, namely methyl iodide, acetyl chloride and diethyl chlorophosphate. The results are shown in the table below.

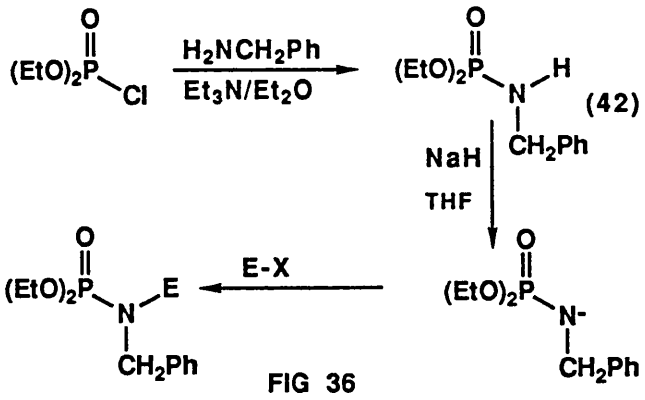


FIG 36

	E-X	YIELD
(43)	Me-I	92%
(44)	Ac-Cl	28%
(45)	$(EtO)_2P(O)$-Cl	16%

The compounds were all characterised by ^{31}P and ^{1}H N.M.R. (43) showed a doublet in the ^{1}H N.M.R. from coupling of the methyl group with the phosphorus nucleus. The bisphosphorylated derivative (45) gave rise to a triplet in the ^{1}H N.M.R., corresponding to the benzylic methylene protons coupling to two equivalent phosphorus nuclei ($^{3}J_{PH}$ = 12 Hz).

The anion derived from (42) showed satisfactory reactivity with this range of electrophiles, although the isolated yields for (44) and (45) were relatively poor. The poor yields observed were mainly attributable to difficulties in purifying the desired products by silica gel column chromatography. There was difficulty in separating the product from the appropriate starting materials and in the case of (45) from tetraethyl pyrophosphate. The ^{31}P N.M.R. spectra of the two crude reaction mixtures showed significantly higher relative yields than those isolated, showing that anion generation and subsequent derivatisation are viable.

The tetraethyl pyrophosphate formed as a by product to give (45) was generated, either by the hydrolysis of unreacted diethyl chlorophosphate during work up, or by the presence of adventitious water in the reaction mixture. The mechanism of formation is shown in Figure 37.

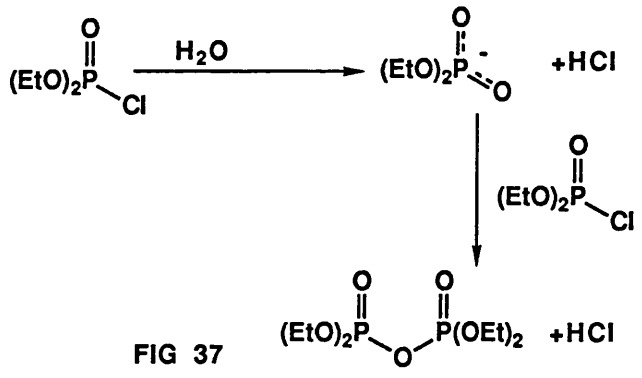


FIG 37

DE-BENZYLATION TO GENERATE THE FREE AMINE GROUP

In order to remove the benzyl protecting group (45) was hydrogenolysed in a similar manner to that attempted with the O-benzylhydroxylamine derivative (27). The bisphosphorylated amine (28) was characterised and found to be identical to that obtained by hydrogenolysis of (27). The reactions are shown in Figure 38. Logistically it was more convenient to synthesise (28) from the available O-benzylhydroxylamine derivative (27), due to the poor yield obtained in the synthesis of (45). Nevertheless, the use of the N-benzyl protecting group met the requirements of allowing derivatisation of the generated anion and of being easily removed.

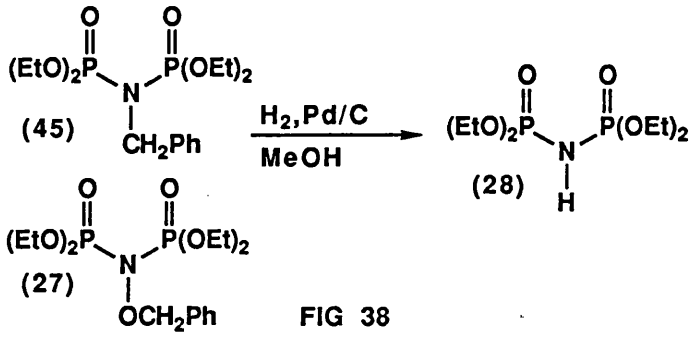


FIG 38

DE-ESTERIFICATION BY THE USE OF HALOTRIMETHYLSILANES

The next step towards the target molecule, and a general synthetic route, was the removal of the phosphorus ester groups. It was decided to remove the ester groups prior to oxidation of the amine to the nitroxyl radical, since the radical centre may not be stable to the de-esterification conditions employed. Halotrimethylsilanes have been used to mildly and selectively remove phosphorus esters following the mechanism shown in Figure 39 (Blackburn 1980). The driving force for these reactions is the formation of the strong oxygen-silicon bond between the phosphoryl oxygen and the trimethylsilyl group.

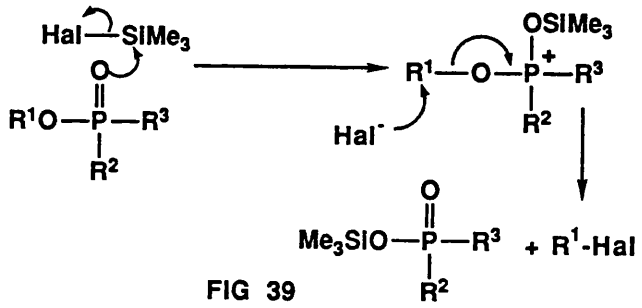


FIG 39

Accordingly, (28) was treated with bromotrimethylsilane (an additional equivalent was used to counter any N-silylation) in a solvent of benzene at 50°C. The reaction was followed by ^{31}P N.M.R. and allowed to continue until no further change was observed.

The desired product was characterised by a large upfield shift in the ^{31}P N.M.R., culminating in a singlet at δ_P -19. The intermediate partially silylated compounds were characterised by doublets in the ^{31}P N.M.R., due to the non-equivalence of the phosphorus nuclei. The trimethylsilyl esters were removed by hydrolysis in aqueous sodium bicarbonate to give the corresponding sodium salt. See Figure 40.

The successful generation of the product (9), showed that deprotection of both the amine and the phosphoryl group was possible. This route should then be applicable to other derivatised phosphoramidates.

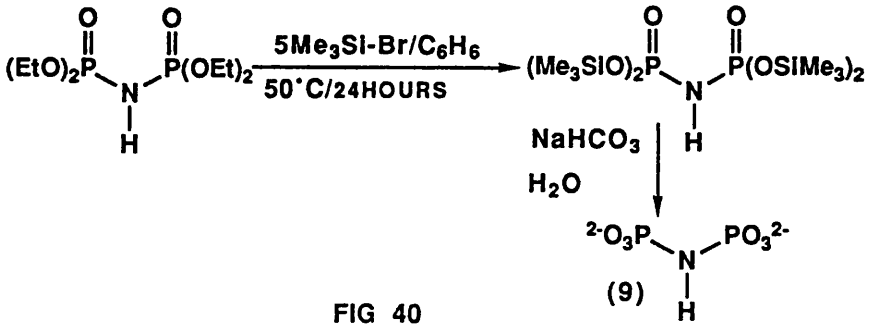


FIG 40

OXIDATION OF IMIDOBISPHOSPHONATE (9)

Preliminary attempts at oxidising (9) proved to be fruitless. Initially, alkaline conditions were employed to prevent acidic cleavage of the phosphorus-nitrogen bond. Accordingly, alkaline solutions of lead dioxide and potassium permanganate were used to effect the desired oxidation. The desired nitroxyl radical (6) should not be observed in the ^{31}P N.M.R. due to the fact that the electron on the nitroxyl group and the phosphorus nuclei would experience a large coupling. This large coupling would result in the relaxation of the nuclear spin and cause a severe broadening of the ^{31}P N.M.R. signal. In effect the radical centre would act as a relaxation centre for any N.M.R. active nuclei, in this case the phosphorus. However, under the above reaction conditions three products were observed in the ^{31}P N.M.R. spectrum. These products appeared to be unreacted (9), a phosphate and a phosphoramidate. This result indicated that some phosphorus-nitrogen bond cleavage was occuring.

It has been shown in the literature (Holden 1982) that the oxidation of dimethyl phosphoramides with meta-chloroperbenzoic acid proceeds via nitrogen oxidation, with subsequent re-arrangement to eliminate formaldehyde. Repeated oxidations cause decomposition of the molecule. see Figure 41. A similar mechanism might be envisaged for the oxidation and subsequent decomposition of (9).

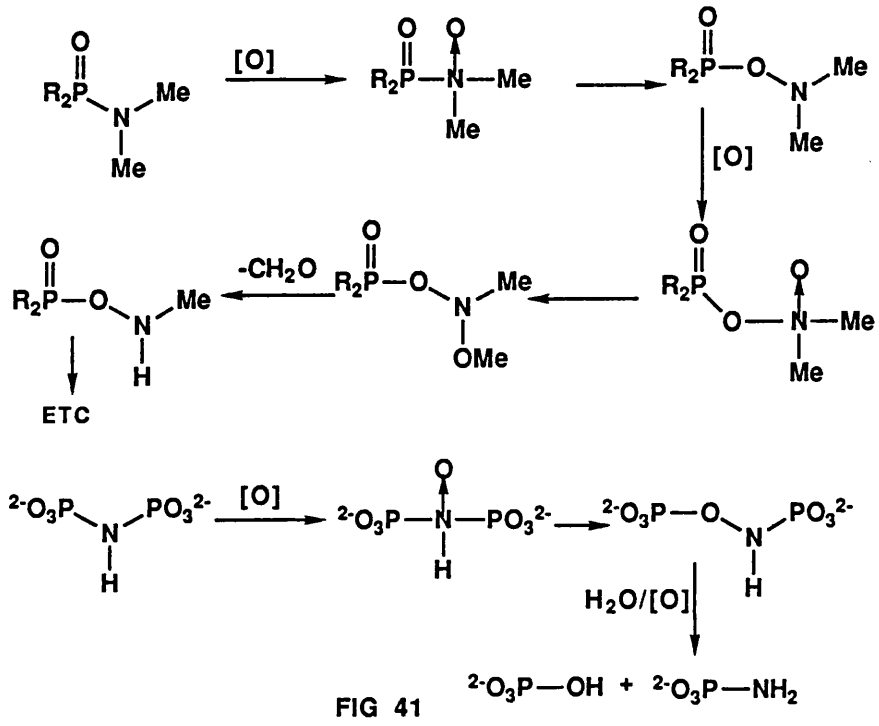


FIG 41

There was no evidence observed in the ^{31}P N.M.R. for the production of the proposed re-arrangement product. However, previous investigations using this type of system have shown it to be rather unstable and not suitable for ester hydrolysis e.g. with (35).

Although it was expected that the radical (6) would be coloured, this was not easily detectable. Since lead dioxide and potassium permanganate are themselves coloured and it was not possible to monitor the oxidation by changes in the U.V. or visible spectra.

The oxidation of (9) was also attempted using hydrogen peroxide in the presence of sodium tungstate. The reaction was carried out using conditions outlined in Figure 42 and followed by ^{31}P N.M.R. The signal due to (9) remained throughout the reaction, but as the desired radical should not be observed by N.M.R. it would appear that at best the reaction had not gone to completion. Initially, the reaction took on a yellow/orange colouration which disappeared within twenty four hours.

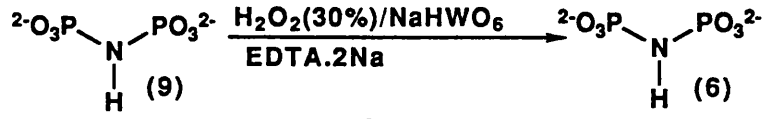


FIG 42

The oxidation of (9) with hydrogen peroxide and sodium tungstate was repeated and followed by E.S.R. spectroscopy. The E.S.R. spectrum of the crude reaction mixture, immediately after mixing, showed a triplet of triplets. This arose from the coupling of the unpaired electron to two equivalent phosphorus nuclei (A = 26 G) and then to the nitrogen nucleus A = 20 G), see Figure 43.

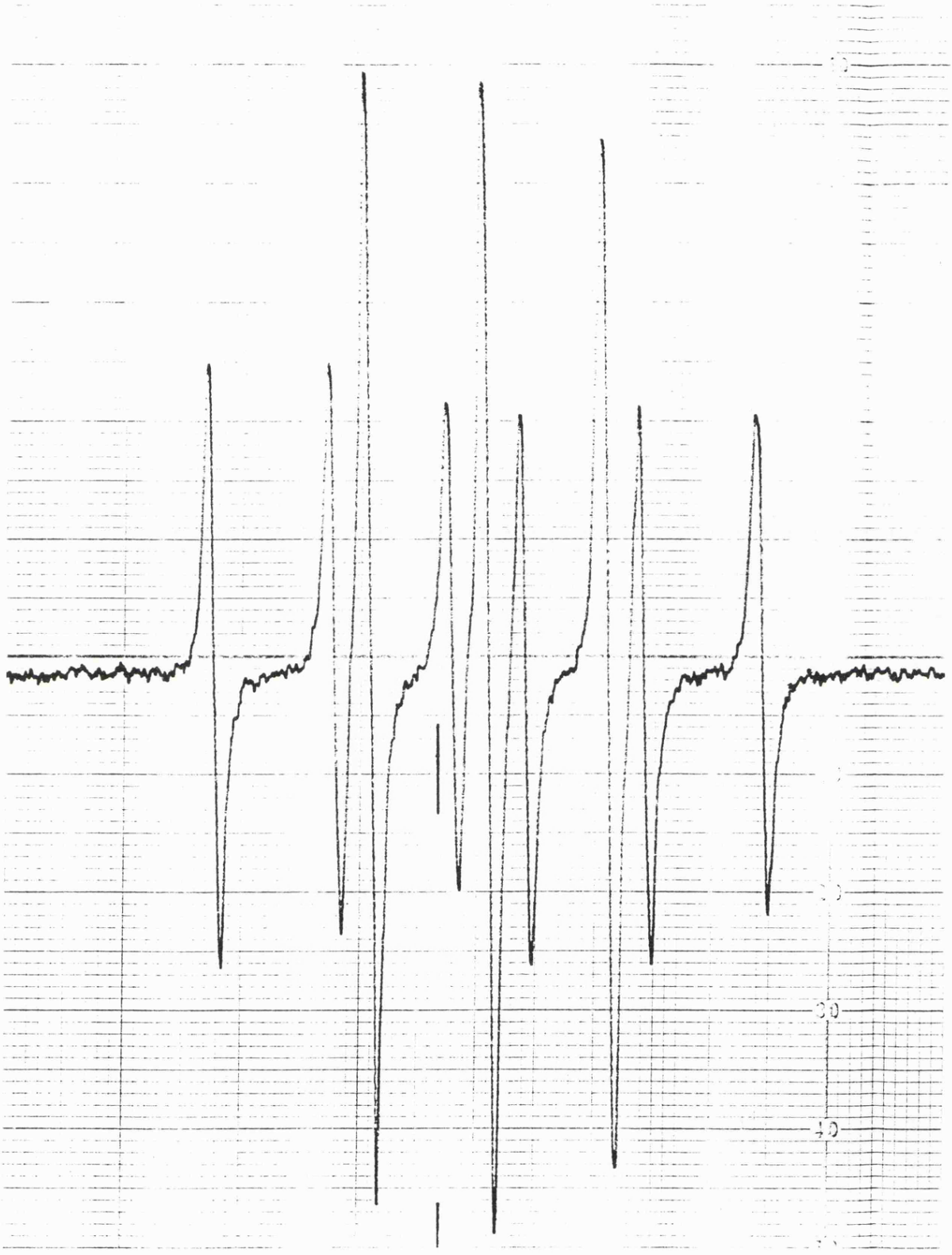


FIG 43-OBSERVED E.S.R. SPECTRUM OF (6).

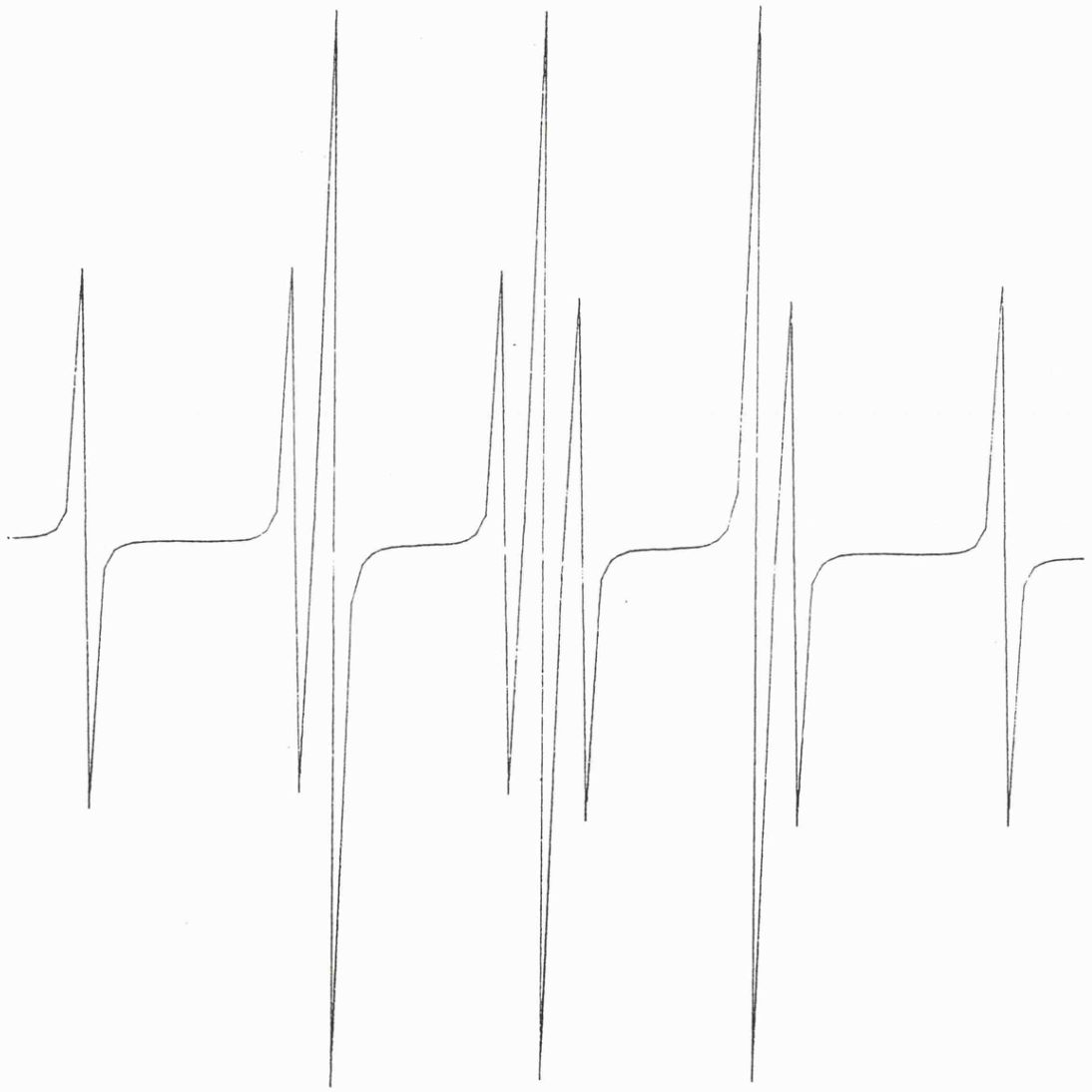


FIG 44-SIMULATED E.S.R. SPECTRUM OF (6).

The simulated spectrum shown in Figure 44 was in good agreement with that observed. Double integration of the E.S.R. spectrum provided an estimate of the concentration of the radical. Since this was approximately ≤1% of the concentration of the imidobisphosphonate,it was concluded that the desired radical was only present at a low steady state concentration.

This low level was not observed to build up even with excess oxidising reagent. The concentration of the radical remained constant during its lifetime as observed by E.S.R. spectroscopy, and for extended periods in the dark, but decayed after twenty four hours in daylight.

No changes were observed in the ^{31}P N.M.R. of the reaction mixture during the lifetime of the radical. The use of a bicarbonate/carbonate buffered aqueous solution led to no increase in the observed radical concentration. The radical (6) appeared to be present at a steady state concentration which is given by K_1 and K_2 in Figure 45.

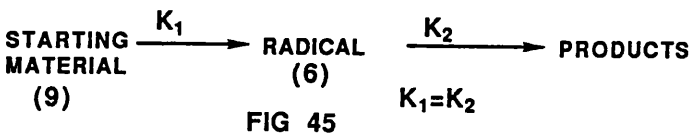


FIG 45

After the radical centre had decayed the ^{31}P N.M.R. signal was observed to shift downfield from δ_P 1.6 to δ_P 2. There are at least three possibilities for this. Any residual radical species (paramagnetic) may act as an N.M.R. shift agent, resulting in a downfield signal shift for (9) in the reaction media. The radical species could also have been oxidised to the N-oxide or dimerised. Any dimerisation might be expected to take the same form as that exhibited by Fremy's salt, to give a species such as (47). See Figure 46.

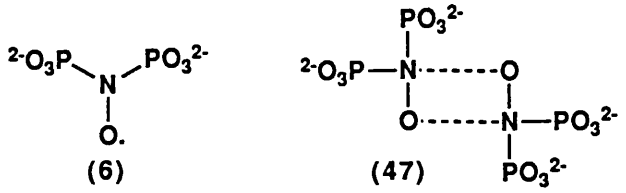


FIG 46

The desired radical (6), thus appeared to be an intermediate in the oxidation of the imidobisphosphonate (9), but never accumulated in sufficient amounts to allow attempts at isolation.

These observations also suggest that (6) is rather unstable, making it unsuitable as a contrast agent for N.M.R. and E.S.R. imaging or for incorporation into a nucleotide analogue.

OVERALL CONCLUSIONS

A general synthetic route to nitroxyl radicals stabilised by one or more phosphonate groups has been established. The synthesis of the radical (6) overcame problems in the choice of an appropriate N-protecting group. However, the radical was unstable and present in too low a concentration to allow further work to be carried out. The phosphoramidate intermediates could be derivatised *via* their respective anions with a wide range of electrophiles. Therefore, a variety of N-substituents could be incorporated into a phosphonate stabilised nitroxyl radical.

The observed re-arrangement of the hydroxylamine (31) during hydrolysis supplements the information previously obtained on similar systems. The novel bisphosphorylation of hydroxylamine to give (35) should allow the future development of novel nucleotide analogues.

C H A P T E R T H R E E

INTRODUCTION

PHOSPHONATES AS ANALOGUES OF NATURAL SYSTEMS

The importance of phosphates in numerous biological systems has led to an investigation of their analogues for the potential modification and control of biological activity. Work has focused on the replacement of a phosphate oxygen with either nitrogen, sulphur or a methylene group (Blackburn 1981). The resistance of the carbon-phosphorus bond to hydrolytic cleavage, has led to the extensive use of phosphonates as stable analogues of natural phosphates and has been the subject of much work and a review (Engel 1977, Blackburn 1981).

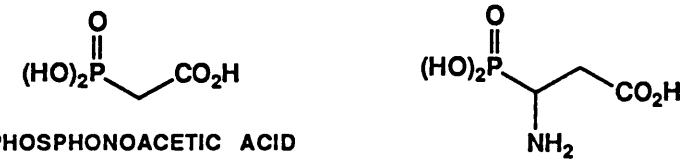
There are three potential uses for such analogues (Engel 1977):-

(i) As a phosphonic acid, to enhance the lifetime of a metabolite or regulator in which the natural system has a hydrolysable phosphate distant from the site of action.

(ii) As a phosphonic acid or phosphonate ester, which could inhibit or perturb a natural system by non-participation in a normal phosphate cleavage pathway.

(iii) A phosphonic acid could lead to either specific or non-specific inhibition of an enzymic process.

Two examples which illustrate the potential biological activity of phosphonates are shown in Figure 47.



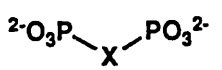
PHOSPHONOACETIC ACID
- ACTIVE VS HERPES TYPE II VIRUS

GLYPHOSATE
- HERBICIDE

FIG 47

BISPHOSPHONATES

The bisphosphonates with general structure (48), which are analogues of inorganic pyrophospate (7), have been used as bone imaging agents in nuclear medicine and for the development of nucleotide analogues. Their development followed on from work with (7), which binds to calcium phosphate to inhibit its formation and dissolution in vitro. It was envisaged that (7) could regulate the formation and dissolution of hard tissues in vivo and protect soft tissues from calcification. However, (7) is hydrolysed rapidly by endogenous enzymes in vivo. Therefore, more stable analogues have been investigated as potential therapeutic agents, the greatest attention being focused on the bisphosphonates with general structure (48).

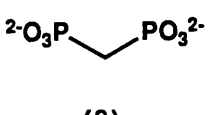


X=O (7)
X=CR_2 (48)

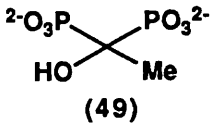
Bisphosphonates have been found to act in a manner similar to pyrophosphate (7), and are extremely active at inhibiting bone resorption. Intraveneously administered bisphosphonates are rapidly cleared from the bloodstream and accumulate exclusively in calcified rather than soft tissues. The low toxicity of bisphosphonates has led to a range of medical applications, for example; with heterotopic calcification caused by spinal cord injury, or after total hip replacement. Bisphosphonates are also extremely effective against the bone destroying Paget's disease.

One of the major applications of pyrophosphate analogues has been in the field of nuclear medicine. Thus technecium-99m (a γ-emitting isotope), when complexed with linear polyphosphates (Subramanian 1972) has been exploited for skeletal imaging. However, because of their greater in vivo stability, and the molecular variability of polyphosphates, the bisphosphonates have been introduced as alternative complexing ligands (Tofe 1972, Wellman 1972).

The bisphosphonates, once complexed with technecium-99m, accumulate in calcified tissues whilst being rapidly cleared from the bloodstream. A gamma camera is then used to determine the spatial distribution of the γ-emitting isotope. Given the selective accumulation of such bisphosphonate complexes in areas of calcification, this technique is particularly powerful for detecting bone tumours. Previous studies (Engel 1977, Amersham International) have been carried out with methylenebisphosphonic acid (8), 1-hydroxyethylidenebisphosphonic acid (49), dichloromethylenebisphosphonic acid (50), hydroxymethylene-bisphosphonic acid (51), 2,3-dicarboxypropanebisphosphonic acid (52) and a nitrogen analogue, imidobisphosphonic acid (9).



(8)



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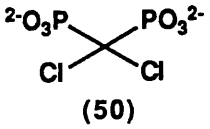
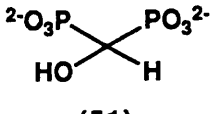


(50)



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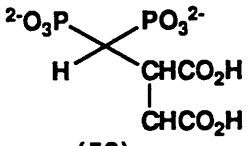
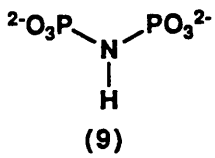


(52)

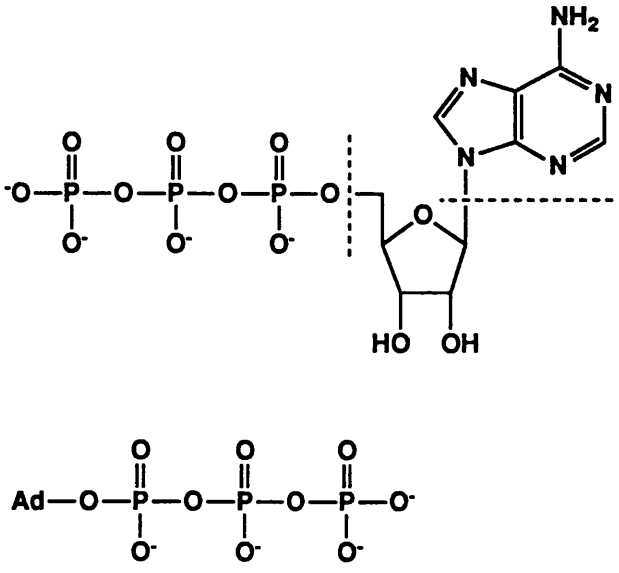


(9)

In addition to investigating pyrophosphate analogues, this chapter describes the synthesis of nucleotides incorporating such modified pyrophosphates.

NUCLEOTIDE ANALOGUES

Many nucleotide analogues have been reported (Yount 1975, Scheit 1980) with modifications at all of the possible sites : the triphosphate chain, the sugar or the heterocyclic base. See Figure 48.



ATP (10)

FIG 48

The modification of either the ribose or the base can lead to potential drug preparation. The triphosphate chain is the site of most enzymic action, and many analogues have been synthesised. Investigation of the literature highlights a wide range of nucleotide analogues, derived from imidobisphosphonates, bisphosphonates and thiophosphates. These analogues are reviewed below, although confined to ATP, the principles can be applied to other nucleotides.

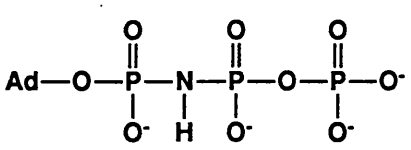
IMIDOBISPHOSPHONATE NUCLEOTIDE ANALOGUES

The imidobisphosphonate analogues of ATP have been introduced in Chapter Two. These analogues, (11) and (12), have been used in a range of experiments designed to gain information on the role of ATP in biological systems (Yount 1975).

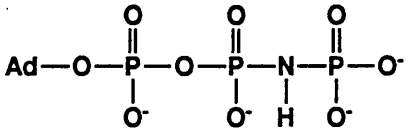
Enzymes which cleave ATP have been investigated using these imidobisphosphonate analogues however, enzymic cleavage of the analogue (11) has not been reported, an earlier reported α-phosphate cleavage (Kenyon 1988) by T7RNA polymerase has since been retracted.

The γ-phosphate group of (12) was cleaved by alkaline phosphatase (E.Coli) (Yount 1971 a) and sarcoplasmic recticulum ATPase (Taylor 1981), although in general this analogue is stable to cleavage by enzymes at the γ position.

Enzymic cleavage is known to occur at the α position, for example with snake venom phosphodiesterase (Papas 1970). Much information has been derived about the binding of ATP to enzymes and proteins by the use of the analogue (12), for example with myosin (Yount 1971 b), actin and muscle fibres (Yount 1975).



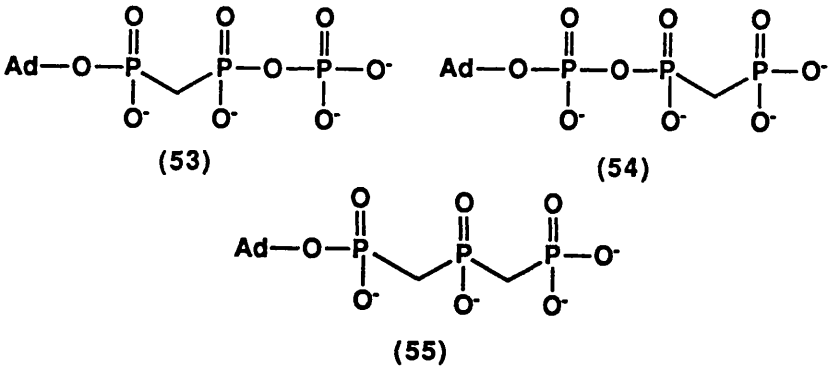
(11)



(12)

BISPHOSPHONATE NUCLEOTIDE ANALOGUES

The bisphosphonate ATP analogues studied the most (Yount 1975, Scheit 1980) have been those where the bridged oxygen atom in the α, β (53) or β, γ (54) position has been replaced by a methylene group (Myers 1963, 1965), while the bismethylene analogue (55) has also been prepared (Kenyon 1972). The synthetic methods have been reviewed by Scheit (1980), with the optimum conditions involving reaction between a nucleophilic bisphosphonate and either an activated adenosine monophosphate (AMP) or 5'-O-tosyladenosine. The latter reaction has provided adenosine diphosphate (ADP) analogues. See Figure 49.



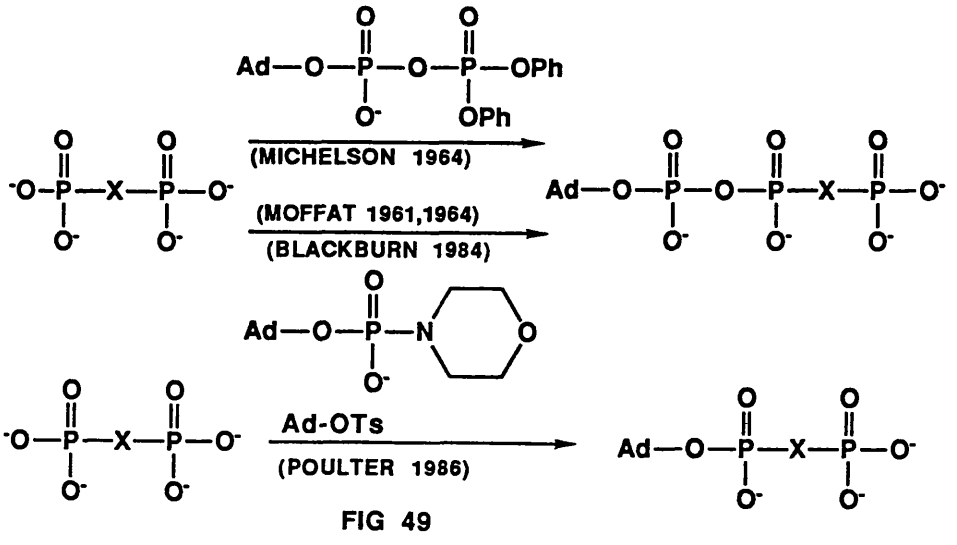


FIG 49

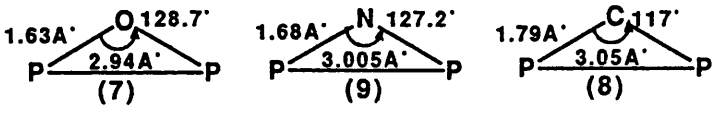
These methylenebisphosphonate analogues incorporating stable carbon-phosphorus bonds are resistant to enzymic cleavage at these positions.

These analogues, (53) and (54) have been shown to be relatively poor substrates for enzymes utilising ATP and disappointing as inhibitors (Yount 1975, Milner-White 1983). This ineffectiveness as substrates and inhibitors can be attributed to two factors:-

i Bond angles and lengths

ii PKa values.

i Considering the relative bond lengths and angles in pyrophosphate (7), imidobisphosphonate (9) and methylenebisphosphonate (8) (see Figure 50), it is apparent that the bond angles in (7) and (9) are similar but differ from that in (8). However, due to the phosphorus-carbon bond length, the distance between phosphorus atoms in (8), is essentially the same as in (7). Enzymic studies tend to support the closer similarity between analogues based on (9) rather than (8).



(SCHEIT 1980)

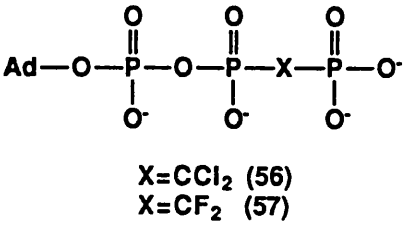
FIG 50

ii The lower electronegativity of the methylene group in (54) compared to that of the bridging oxygen in ATP, causes an increase in the basicity and nucleophilicity of the phosphonate anions compared to that of the phosphate equivalent. That is, the stronger electronegativity of the bridging oxygen, leads to the attached phosphate group being a stronger acid than in the methylene analogue (as expected the imidobisphosphonate lies in between the two). This difference is illustrated by differences between the pKa of the last hydrogen of β, γ-methylene ATP (54), β, γ-imido ATP (12) and ATP which are 8.4, 7.7, and 7.1 respectively (Scheit 1980). Therefore the net charge on the analogues (53) and (54) at neutral pH may be different from that on ATP, due in this case to the lower acidity of the last phosphonate proton.

ATP and its analogues are known to bind divalent metals ions (Yount 1975, Burt 1982, Bridger 1982) to form a metal chelate in enzymic actions, therefore in the presence of divalent metal ions, the net charge on the analogues should be the same as for ATP. The observed lack of suitability of the methylenebisphosphonate analogues is not due to the lack of metal binding, but may be due to the formation of a metal chelate of the wrong structure.

A further possibility worth mentioning is that the bridged oxygen in ATP may be directly involved in binding through hydrogen-bonding, which the imidobisphosphonate but not the methylene-bisphosphonate analogues can mimic.

The problem of charge and acidity of the ATP methylenebisphosphonate analogues (53) and (54),has previously been addressed (Blackburn 1981, 1984, 1986, 1987). Initially, the acidity of methylenebisphosphonate (8) was increased by the introduction of halogens to yield halomethylenephosphonates and phosphonic acids (Blackburn 1981). By introducing dichloro or difluoro methylene groups into the ATP analogues, the pKa values for (56) and (57) respectively were found to be much closer to those of ATP. See Figure 51.



	PKa[4]
(56)	7.0
(57)	6.7
ATP(10)	7.1
(54)	8.4

FIG 51

The use of these halogenated analogues has led to some improvement in the binding of bisphosphonate ATP analogues as illustrated in the table below.

Nucleotide	Inhibition	Ki (mM)
ATP	Substrate	0.25 (Km)
(57)	Competitive	0.9
(56)	Competitive	1.1
(54)	Competitive	2.5

Enzyme activity with hexokinase (Blackburn 1986)

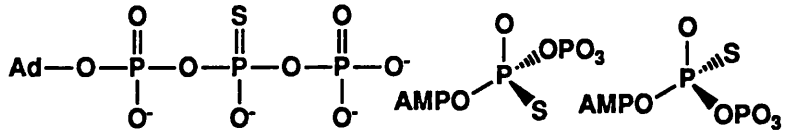
The level of inhibition obtained with (57) was the same as that obtained with the imidobisphosphonate analogue (12), showing that electronic requirements are at least as important as steric ones.

THIOPHOSPHATE NUCLEOTIDE ANALOGUES

A lot of work has been undertaken with thiophosphate nucleotide analogues (Yount 1975, Engel 1977, Scheit 1980, Eckstein 1985). The substitution of a non-bridged oxygen for a sulphur, yields derivatives containing the same overall charge of the parent compound and which may be more resistant to enzymic cleavage (with, for example, phosphatases and phosphodiesterases) (Yount 1975).

Interestingly, the incorporation of a sulphur atom lowers the pKa values of the thiophosphate protons (Neumann 1965), indicating that the analogue may be more negatively charged than ATP

Since thiophosphate analogues can mimic the parent nucleotide, important information may be obtained regarding the specificity of enzymes and the binding properties of the analogues (Cohn 1978). Much use has been made of the fact that the α and β thio substituted ATP analogues are chiral and exist as pairs of diastereoisomers. This is illustrated for adenosine 5'-(O-2-thiotriphosphate) (58) in Figure 52. Some enzymes have been shown to act preferentially on one or other of the diastereoisomers, and have been used to determine the stereochemical course of nucleotidyl transfer (Eckstein 1976).

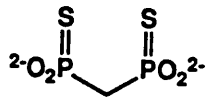


(58)

FIG 52

PROPOSED AREAS OF STUDY - BISTHIOPHOSPHONATES IN NUCLEOTIDE ANALOGUES AND BONE IMAGING STUDIES

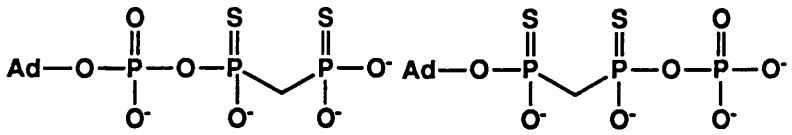
For the purpose of investigating novel bisphosphonates as potential bone imaging agents and ATP analogues, it was intended to synthesise methylenebisthiophosphonate (or the corresponding acid) (59).



(59)

Oxygen and sulphur, by virtue of their respectively hard and soft nature, bind preferentially to different metal ions. Therefore, an alteration in the chelating properties of a bisphosphonate by the introduction of thio groups will alter and may improve its ability to chelate Technecium and hence deliver it to areas of calcification. The thiophosphonate, by virtue of its altered charge (more negative due to the lowering of pKa values) may also have a greater specificity for areas of calcification than the parent phosphonate. In a previous study (Cullis-unpublished data) dithiopyrophosphate proved to be too hydrolytically unstable to be tested as a bone imaging agent. The stable methylene analogue should provide the necessary data on the potential for thiophosphate and thiophosphonate bone agents.

It was felt that (59) would prove to be an excellent choice to incorporate into an ATP analogue. The presence of the sulphur atom should lower the pKa of the last proton in a similar way to that observed with the halomethylene analogues (56) and (57) (Blackburn 1986, 1987). This analogue would hopefully be a good competitive inhibitor of ATP, for example with hexokinase. There is the further possibility that once (59) was incorporated into an ATP analogue, the resulting two diastereoisomers would show different modes of interaction with ATP binding proteins. There are several methods available for incorporating (59) into an ATP or ADP analogue (See Figure 49), both the analogues (60) and (61) could then be synthesised.



(60) (61)

RESULTS AND DISCUSSION

PROPOSED ROUTE

The proposed synthetic route towards methylenebisthiophosphonate (59) and the intended nucleotide analogues is shown in Figure 53. Literature precedent is available for the synthesis of (63) (R = Et) (Czekanski 1982), which after removal of the alkyl ester groups would allow the generation of the novel nucleotide analogue (60). See the introduction to this chapter for general methods of nucleotide synthesis. (Figure 49).

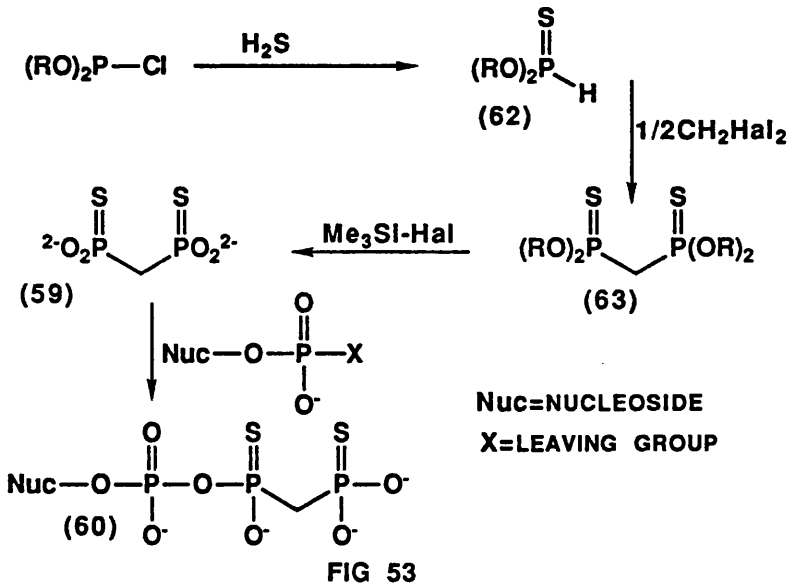


FIG 53

SYNTHESIS OF BISTHIOPHOSPHONATE PRECURSOR

Diethyl thiophosphite (62) was synthesised according to Figure 54 (Krawiecki 1960) and characterised by ^{31}P and ^{1}H N.M.R. The phosphite proton gave rise to a large doublet from coupling to the phosphorus ($^{1}J_{PH}$ = 647 Hz). A U.V. induced Michaelis-Becker reaction between (62) and dibromomethane afforded the desired tetraethyl methylenebisthiophosphonate (63) (Czekanski 1982), in a yield of 31%, see Figure 54. The ^{1}H N.M.R. spectrum of (63) showed a triplet for the methylene protons coupling to two equivalent phosphorus nuclei ($^{2}J_{PH}$ = 18 Hz).

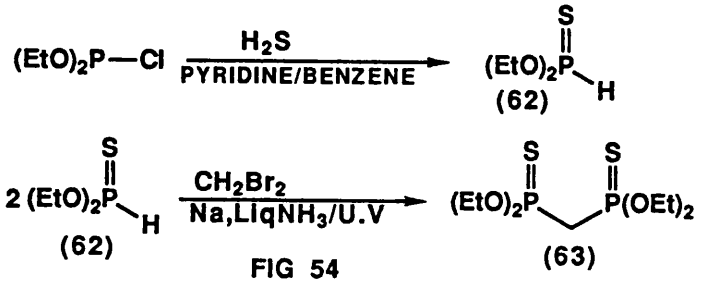


FIG 54

Following the literature precedent (Hutchinson 1986), (63) was treated with iodotrimethylsilane according to Figure 55. However, this gave rise to three broad signals in the ^{31}P N.M.R. spectrum of the crude reaction mixture, at δ_P 1.8, 49.2 and 56.8. After treatment with aqueous sodium bicarbonate solution, the ^{31}P N.M.R. spectrum showed a mixture of two compounds.

A singlet at δ_P 49.2 corresponded to methylenebisthiophosphonate (59) and two doublets, δ_P 16.6 and 49.5, to thiophosphonomethylphosphonate (64). Sulphur loss occurred during the reaction of (63) with iodotrimethylsilane, to give a mixture of the trimethylsilyl esters of (59) and (64). Since iodine could be generated during the reaction, this could presumably be responsible for the oxidative loss of sulphur. The mixture of (59) and (64) was purified and the two products isolated as their triethylammonium salts, after ion exchange chromatography, on DEAE Sephadex.

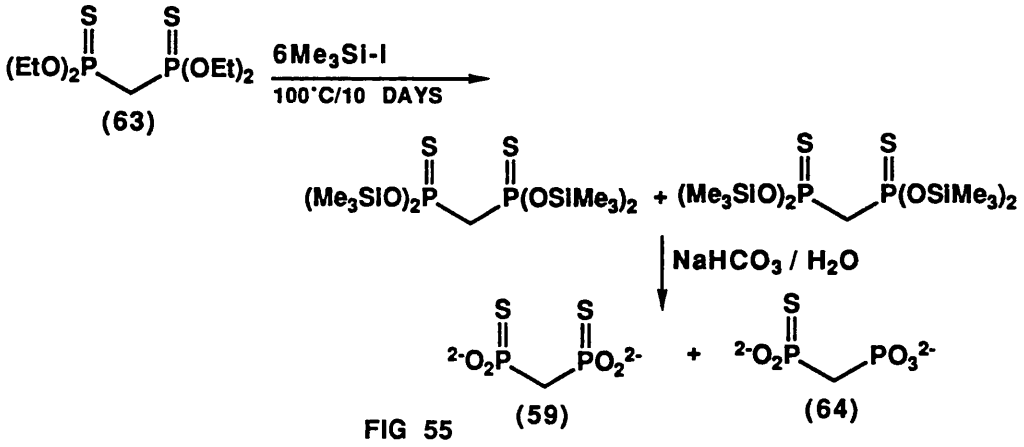


FIG 55

MECHANISM OF DE-ESTERIFICATION

The removal of phosphorothioic alkyl esters by halotrimethylsilanes has been investigated (Chojnowski and Michalski 1979).

The overall reaction was dramatically slower than the corresponding reaction with phosphate esters. This was thought to be due to the low nucleophilicity of sulphur, compared to that of oxygen, with the silicon electrophile. The reaction is illustrated in Figure 56 for a trialkyl thiophosphate (A) and involves the intermediate (B).

High temperatures and extended reaction times were required due to the slow reaction between the thiophosphate sulphur and the silicon electrophile. At elevated temperatures, the generated alkyl halides catalyse Pishchimuka re-arrangements to give highly reactive S-alkyl thiolates (C). A shorter reaction route was then available *via* (D) and (E).

These conditions could contribute to the oxidative sulphur loss observed with the related reaction of (63) (Figure 55).

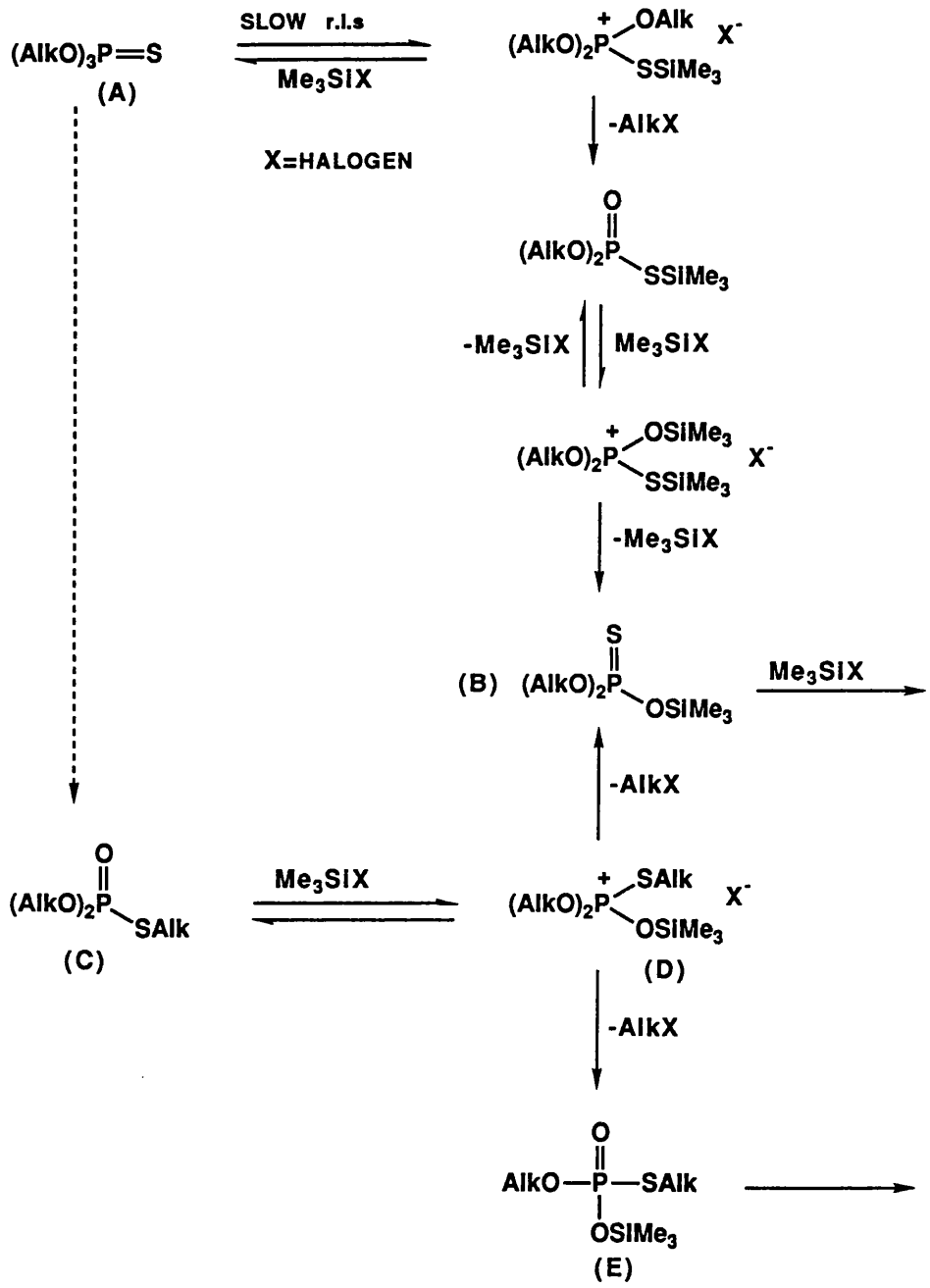

SLOW r.l.s
Me3SiX
X=HALOGEN
-AlkX
-Me3SiX
Me3SiX
-Me3SiX
-AlkX
-AlkX
(A)
(B)
(C)
(D)
(E)

FIG 56

IMPROVED DE-ESTERIFICATION PROCEDURE

The reaction of (63) with iodotrimethylsilane was repeated in the presence of an alkyl halide (methyl iodide) to reduce the reaction time and prevent sulphur loss. The methyl iodide should catalyse the Pishchimuka re-arrangement to give the S-methyl intermediate shown in Figure 57. This would then be expected to undergo rapid reaction with a halotrimethylsilane.

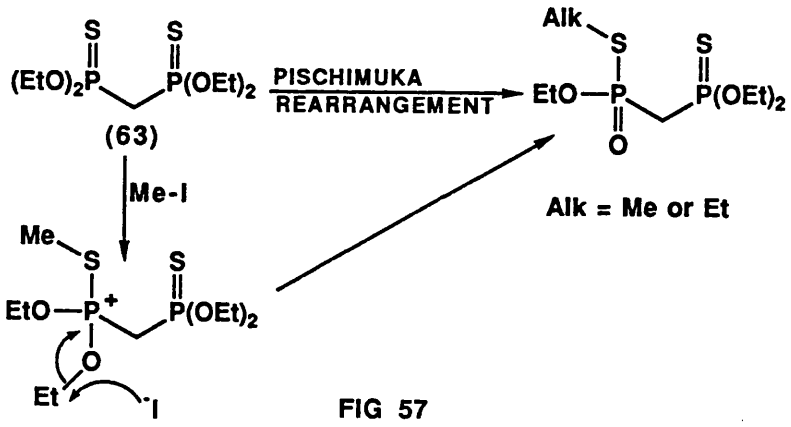


FIG 57

The de-esterification of (63) was repeated according to Figure 58. The reaction was monitored by ^{31}P N.M.R., no sulphur loss being detected, giving a single peak at δ_P 57. Treatment with aqueous sodium bicarbonate solution and purification by ion exchange chromatography yielded (59) as its triethylammonium salt.

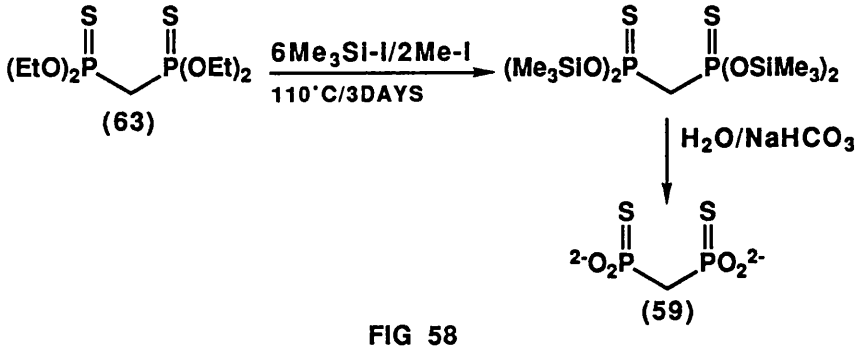


FIG 58

By consideration of the reaction mechanism, the loss of sulphur was prevented and the reaction time shortened, thus improving the synthetic route to (59). However, the loss of sulphur did present a further compound, namely (64), which was investigated along with (59) as a potential bone imaging agent.

EVALUATION OF (59) AND (64) AS BONE IMAGING AGENTS

The following work was carried out at Amersham International while on industrial placement. Both methylenebisthiophosphonate (59) and thiophosphonomethylenephosphonate (64) were tested as potential bone imaging agents for nuclear medicine .

In a typical test, approximately 12.5 mg of the potential bone agent was mixed with 2 cm^3 of a $Tc^{99m}O_4^-Na^+$ (sodium pertechnetate) saline solution, in the presence of 1 mg of stannous fluoride (SnF_2). The stannous reduced the oxidation state of the technecium from +7 to +4, thus allowing it to complex with the potential bone agent (ligand). Analysis of the mixture in a T.L.C. system indicated the presence of uncomplexed (or unreduced) sodium pertechnetate or any colloid formation. A successful ligand (in vitro) should not contain either of these.

In separate reactions, the complexes derived from (59) and (64) were administered intraveneously to a laboratory rat which was imaged with a γ-camera, photographs being taken at ten minute intervals after injections, see Figures 59 and 60.

Analysis of the rat organs provided a measure of the areas of ligand uptake. The results obtained with (59) and (64) were compared to a standard, methylenebisphosphonate (8). The results tabulated below show that the two compounds have a comparatively low selectivity for bone uptake with respect to muscle compared to (8). High selectivity for bone is obviously a key requirement for a good bone imaging agent. The two ligands (59) and (64) were found to complex well to technecium in vitro, but the in vivo distribution of the complex was not bone specific.

The results with (64) were slightly better than with (59), suggesting that the introduction of sulphur ligands does not improve the performance of bone imaging agents. The reasons for this may be due to the different charge distribution over the thio analogues, thus removing the bone specificity present in the oxygenated species.

	%BONE	BONE / MUSCLE	BONE / BLOOD	BONE / LIVER+SPLEEN
(8)	50	280	90	120
(59)	13.5	12.6	1.9	4.6
(64)	23.6	42.1	11.4	21.8

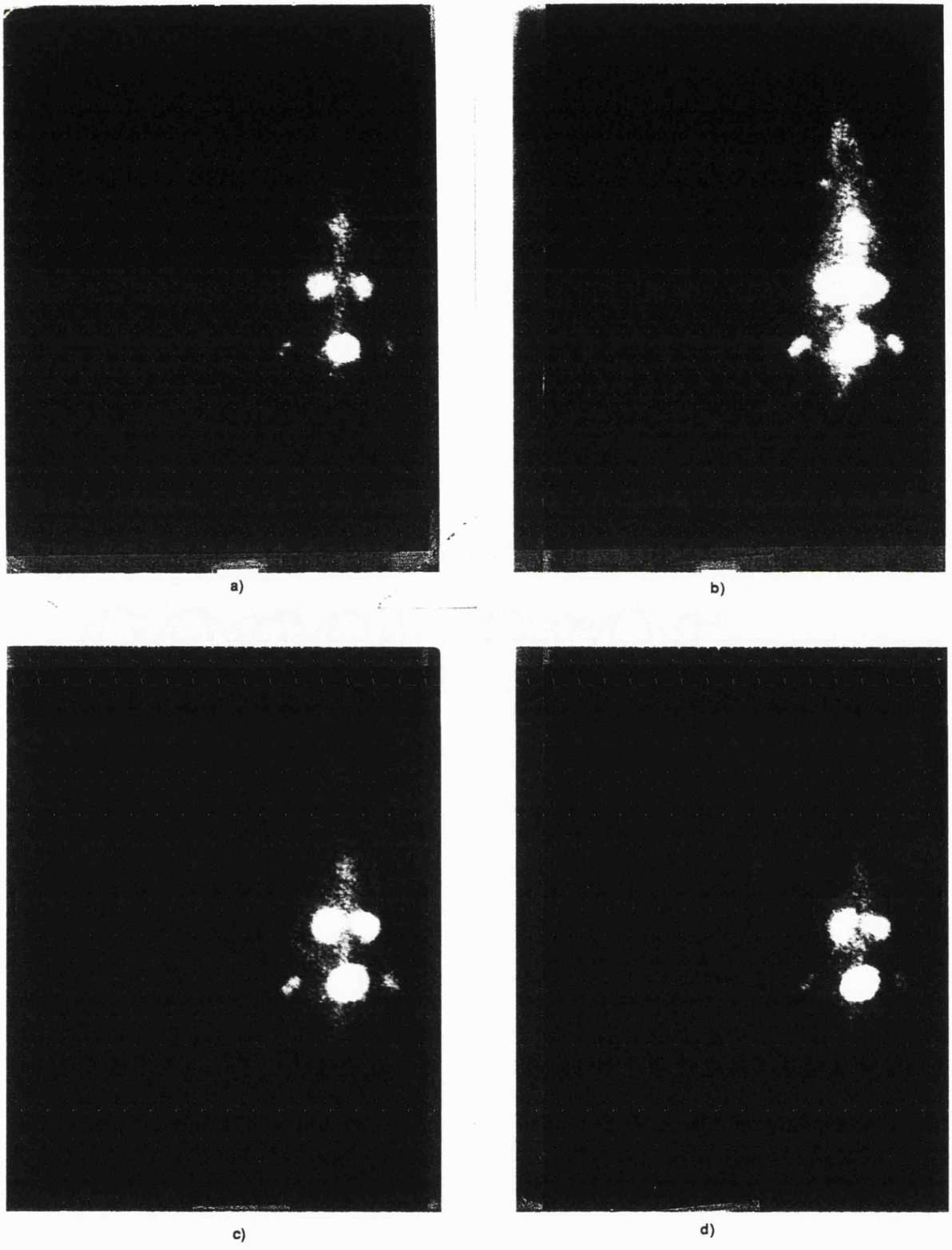


FIG 59-PHOTOGRAPHS TAKEN WITH A GAMMA CAMERA -SHOWING THE UPTAKE OF THE TECHNECIUM COMPLEX OF (59) IN A RAT AFTER;

a) 10 MINS
b) 20 MINS
c) 30 MINS
d) 60 MINS

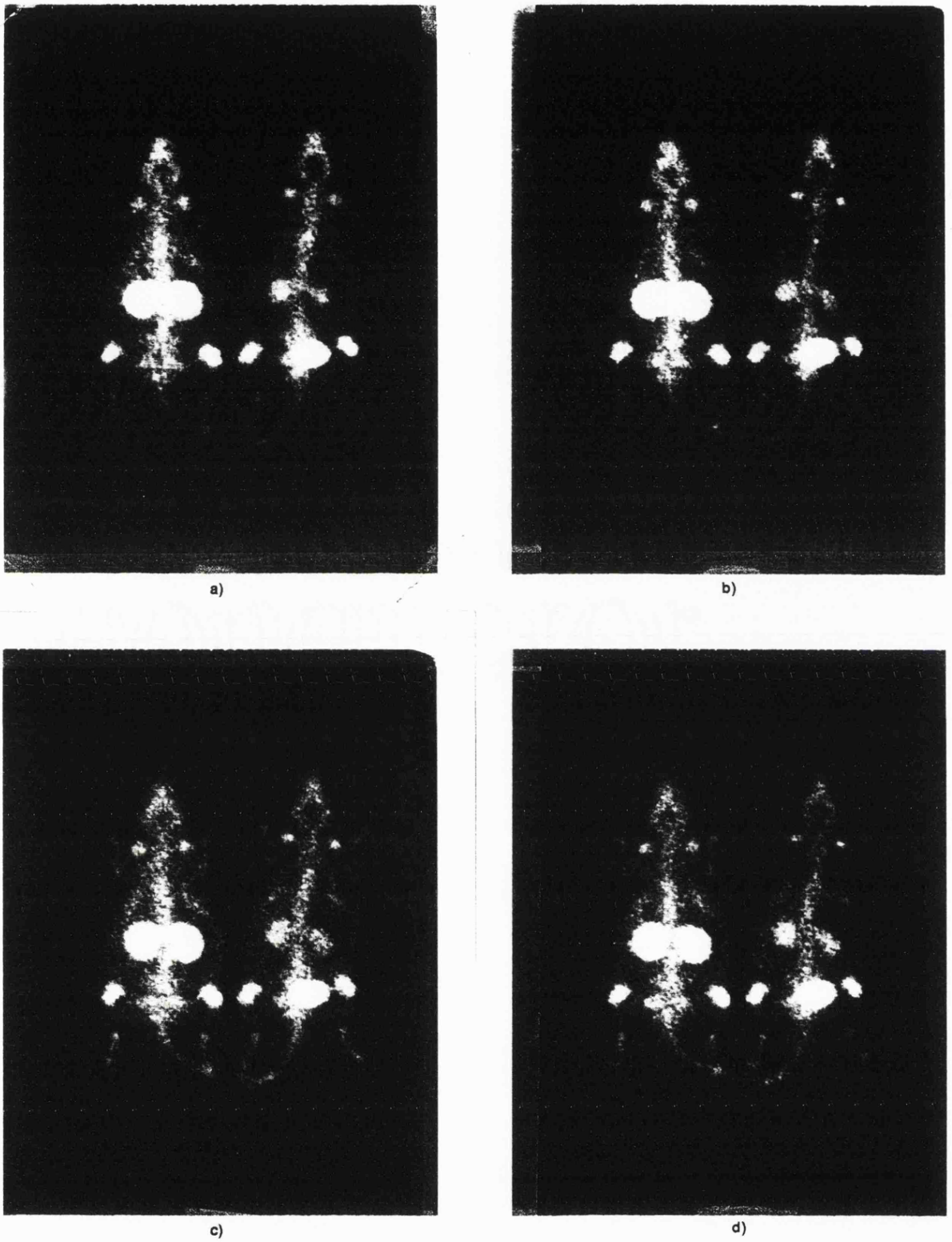


FIG 60-PHOTOGRAPHS TAKEN WITH A GAMMA CAMERA -SHOWING THE UPTAKE OF THE TECHNECIUM COMPLEX OF (64) IN A RAT AFTER;

a) 10 MINS
b) 20 MINS
c) 30 MINS
d) 60 MINS

ROUTES TOWARDS NUCLEOTIDE ANALOGUES

Routes to the methylenebisthiophosphono ATP analogues, (60) and (61), were explored using the electrophiles shown in Figure 61. The synthesis of (61) was expected to proceed via the ADP analogue (65).

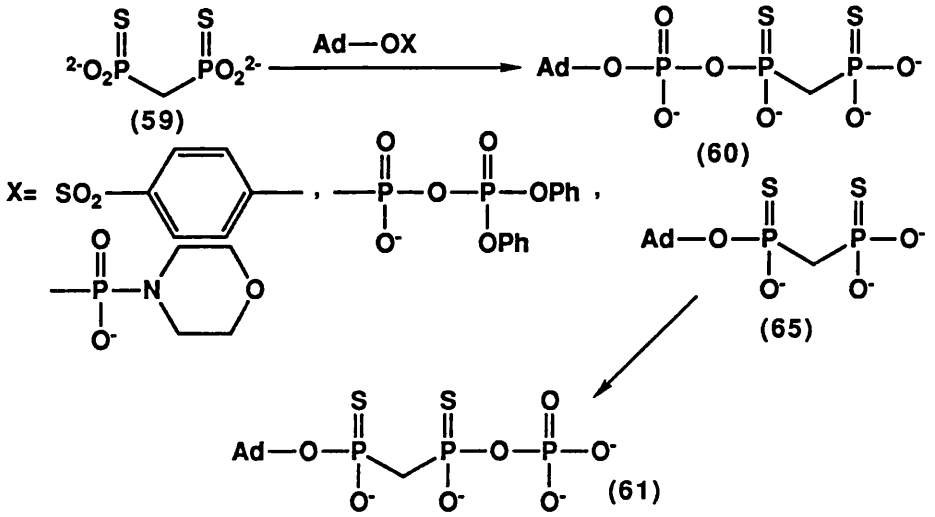


FIG 61

INCORPORATION OF BISTHIOPHOSPHONATE INTO NUCLEOTIDE ANALOGUES

The synthesis of (60) was attempted by treating adenosine 5'-phosphoromorpholidate with the bis tri-^{n}butylammonium salt of (59) according to Figure 62. However, little or no (60) was formed despite changes to the reaction conditions.

For example, N,N-dimethylformamide (D.M.F.) was used as the solvent in place of pyridine, with tri-^{n}butylamine as the base and reaction times of several days were employed. The products obtained after ion exchange chromatography, were unreacted adenosine 5'-phosphoromorpholidate, P^1-adenosine P^2-adenosine pyrophosphate (66) and what appeared to be decomposition products of (59). The formation of (66) was presumably brought about by the hydrolysis shown in Figure 62. Monitoring of the reaction mixture by ^{31}P N.M.R. showed no change after several days, resulting in increased reaction times and decomposition of (59), apparently involving loss of sulphur.

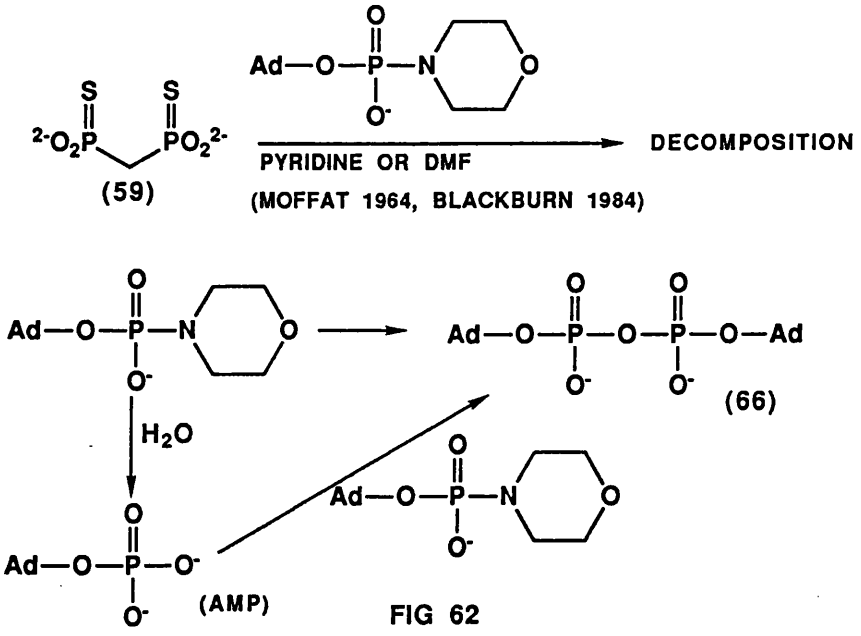


FIG 62

The reduction in the reactivity of (59) compared to methylenebisphosphonate (8),which is known to undergo this reaction is a consequence of the reduced nucleophilicity of the thiophosphonate oxygens. Thiophosphates and thiophosphonates have lower pKa's than the corresponding phosphates and phosphonates, implying that the thio anions are more stable and hence less nucleophilic. It is thought that the negative charge is localised on sulphur. However, no evidence for nucleophilic attack by the sulphur ligand was observed. This can be rationalised in terms of the concepts of hard and soft centres, whereas on a thiophosphate (or thiophosphonate) a sulphur anion is selectively alkylated, both acylation and phosphorylation occurs selectively on oxygen. There have been few reports in the literature of compounds containing a phosphorus-sulphur-phosphorus bridge, one example being the symmetrical monothiopyrophosphate reported by Loewus (1983). They appear to readily re-arrange where possible to the thermodynamically more stable oxygen bridged analogues (Yount 1975, Rogers 1968).

β, γ DITHIO ATP ANALOGUE (60)

AMP was treated with diphenyl phosphorochloridate to yield P^1-adenosine P^2-diphenyl pyrophosphate (67) according to Figure 63. After washing with diethyl ether and drying <u>in vacuo</u>, (67) was treated with (59) in pyridine to give (60).

The product (60) was purified by ion exchange chromatography and its identity confirmed by negative ion Fast Atom Bombardment (F.A.B.). The low field ^{31}P N.M.R. spectrum (24.4 MHz) showed doublets for Pα and Pγ and a doublet of doublets for Pβ.

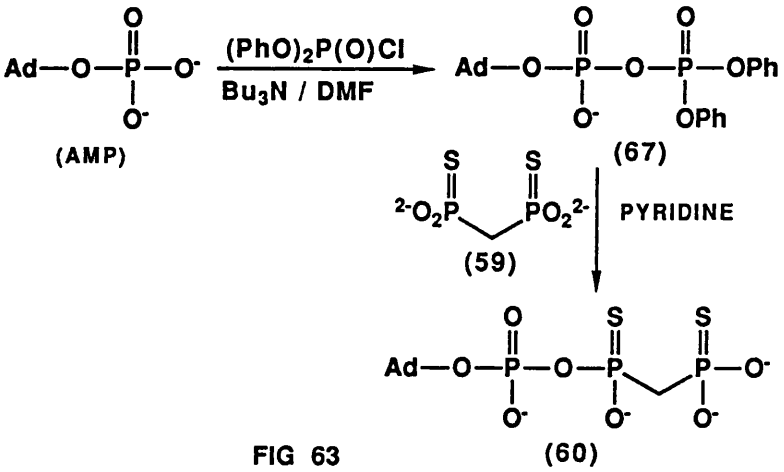


FIG 63

The new chiral centre produced at Pβ caused (60) to exist as a mixture of two diastereoisomers. The high field ^{31}P N.M.R. spectrum (121.5 MHz) showed two doublets of doublets for Pβ, confirming the presence of a pair of diastereoisomers. The resonances for Pα and Pγ were not fully resolved. See Figure 64 for the highfield ^{31}P N.M.R. spectrum.

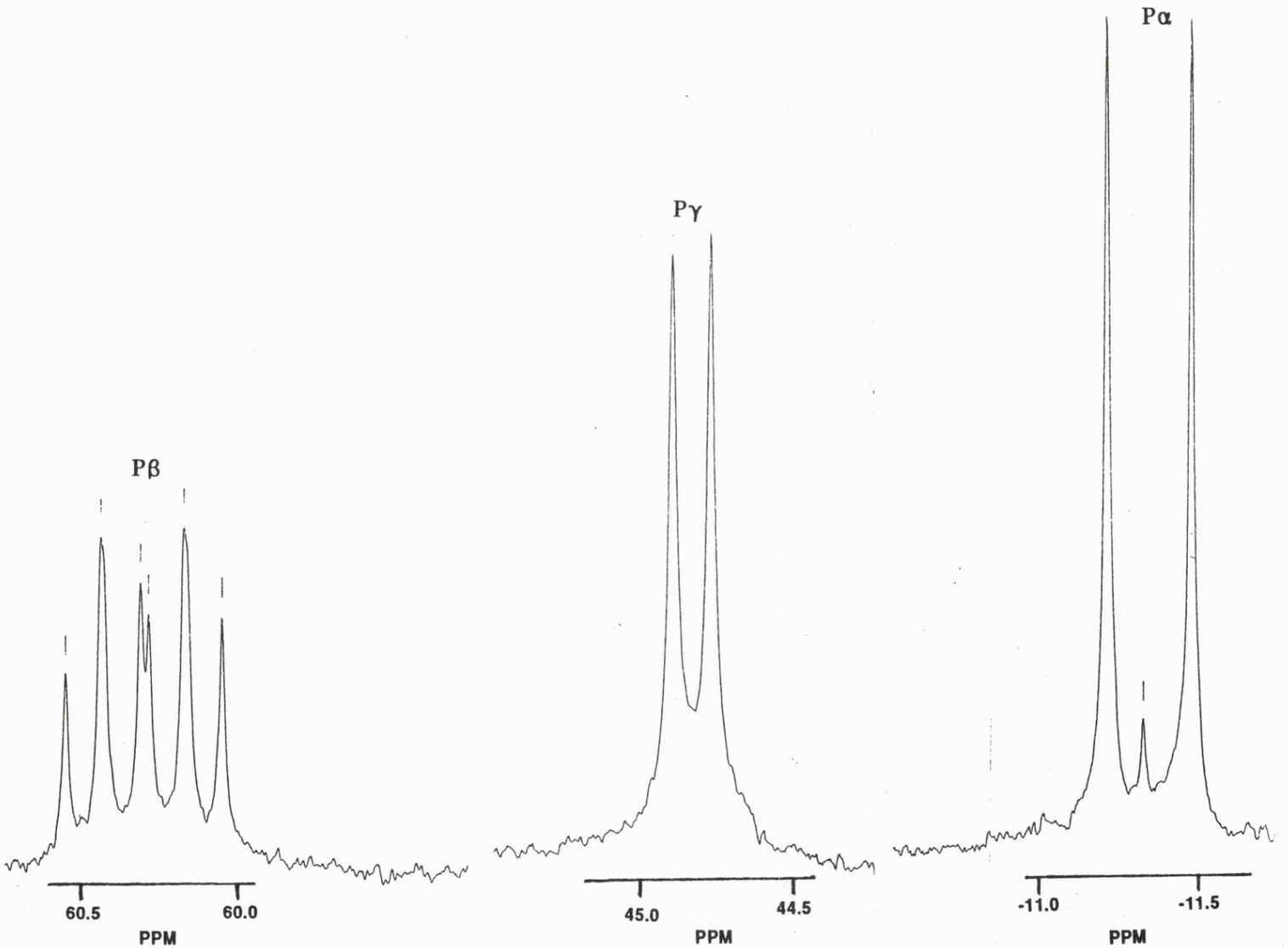


FIG 64-^{31}P N.M.R. SPECTRUM (121.5 MHz) OF (60).

BISNUCLEOTIDE DERIVATIVES

The previous reaction (Figure 63) was repeated with an excess of (67) (three equivalents) in order to attempt the synthesis of the bisnucleotide analogue (68). This compound would be an analogue of bisadenosine tetraphosphate, AP_4A (69). Similar analogues have been synthesised and used to probe the effects of AP_4A <u>in vivo</u> (Blackburn 1987, 1990 and references therein). See Figure 65.

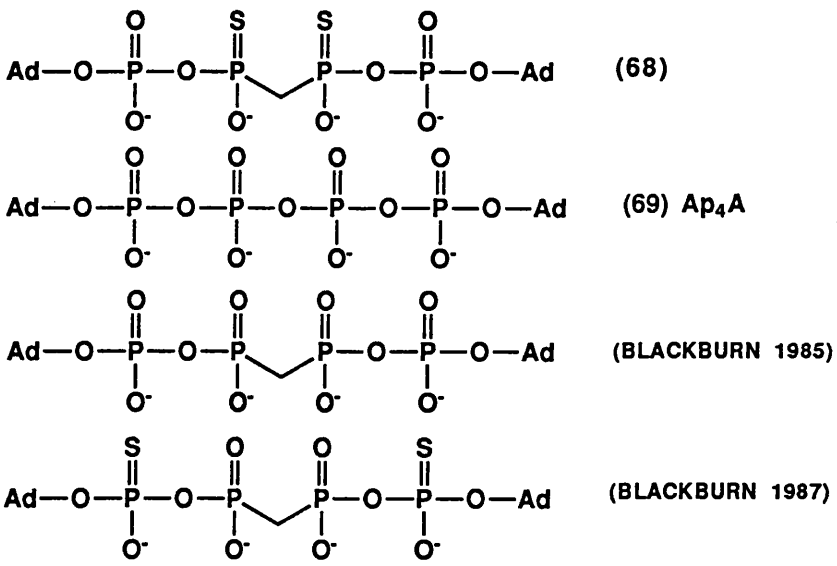


FIG 65

The ^{31}P N.M.R. spectrum of the purified reaction mixture gave no evidence for the desired product (68). By comparison with similar analogues,one would have expected to observe doublets at approximately δ_P-11 and 50. The products isolated, after ion exchange chromatography were (60) and (66), the latter resulting from hydrolysis of unreacted (67). The reaction was repeated under varying conditions, i.e. amount of pyridine, order of mixing (addition) and length of reaction, but no (68) was detected.

The incorporation of a nucleotide moiety into the methylenebisthiophosphonate framework reduced the nucleophilicity of the γ thiophosphate oxygens.

MECHANISM OF ANALOGUE SYNTHESIS

The synthesis of (60) (see Figure 63) proceeded smoothly in the presence of pyridine, which is presumed to act as a nucleophilic catalyst (Frey 1983). The pyridine displaces the diphenyl phosphate group from (67) to form the more reactive intermediate (70), Figure 66. The positively charged pyridinium group on (70) is then displaced by the nucleophile and the pyridine regenerated.

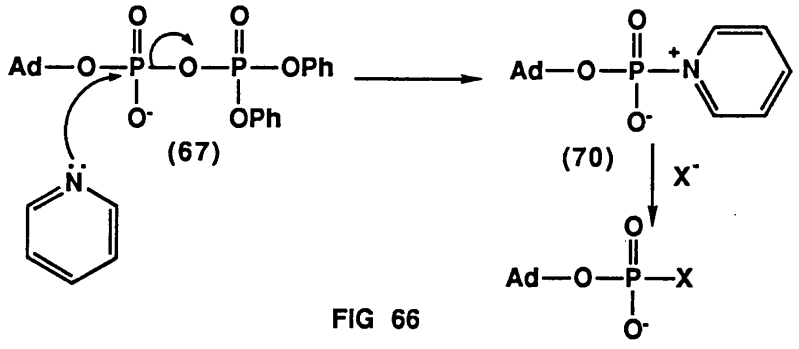


FIG 66

The synthesis of (60) was attempted in the absence of pyridine, by using DMF as the solvent and tri-^{n}butylamine as the base. Longer reaction times were required, compared to Figure 63, to observe any reaction by ^{31}P N.M.R., providing circumstantial evidence for the involvement of (70) in the synthesis of (60). The ^{31}P N.M.R. of the purified reaction mixture consisted of a doublet at δ_P -11, a doublet at δ_P 40 and a multiplet at δ_P 60. However, a coeluting product was present with ^{31}P N.M.R. resonances at δ_P 12 and δ_P 8, suggesting loss of sulphur.

The methylenebisthiophosphonate (59) appears to be less nucleophilic than methylenebisphosphonate (8), as evidenced by its lack of reactivity with adenosine 5'-phosphoromorpholidate. The use of the more reactive electrophile, (67), or more likely (70), allowed the reaction to proceed. However, even these electrophiles failed to react with (60), precluding the synthesis of the AP_4A analogue (68).

α, β DITHIO ATP ANALOGUES

The introduction of a methylenebisphosphonate residue into the α, β position of nucleotide analogues has previously been achieved by the reaction of the bisphosphonate with 5'-O-tosyladenosine (Poulter 1984, 1987) in a solvent of hot acetonitrile. However, due to the poor solubility of the 5'-O-tosyladenosine in acetonitrile and the wish to avoid heating (so as to curtail sulphur loss), the reaction was carried out in DMF. See Figure 67. The minimum of DMF was used to increase the relative concentrations of reactants and improve the rate of reaction.

Accordingly, (59) was treated with 5'-O-tosyladenosine to give a mixture of two products, purified by ion exchange chromatography and isolated in almost equal quantities. The bisnucleotide (72) was eluted from the column first due to its lower charge. Both compounds, (71) and (72), failed to give accurate molecular ions by negative ion F.A.B. analysis.

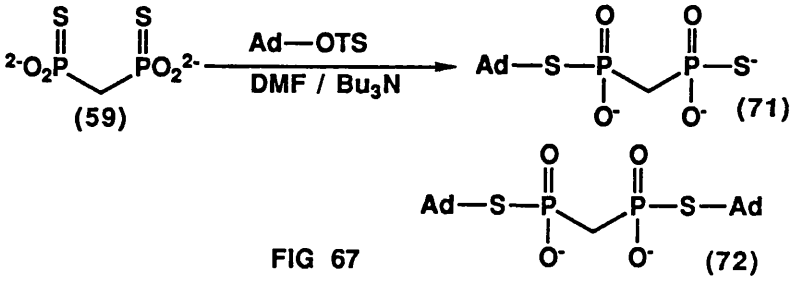


FIG 67

^{31}P N.M.R. CHEMICAL SHIFTS OF THIOPHOSPHATES AND THIOPHOSPHONATES

The ^{31}P N.M.R. chemical shift of thiophosphates and thiophosphonates depend on the phosphorus-sulphur bond order. Generally, a higher bond order would result in a lower field resonance. Of relevance to this work is the fact that changing from a phosphorus-sulphur double bond to a single bond results in an upfield shift. Some examples from the literature are shown in Figure 68 together with their ^{31}P N.M.R. chemical shifts.

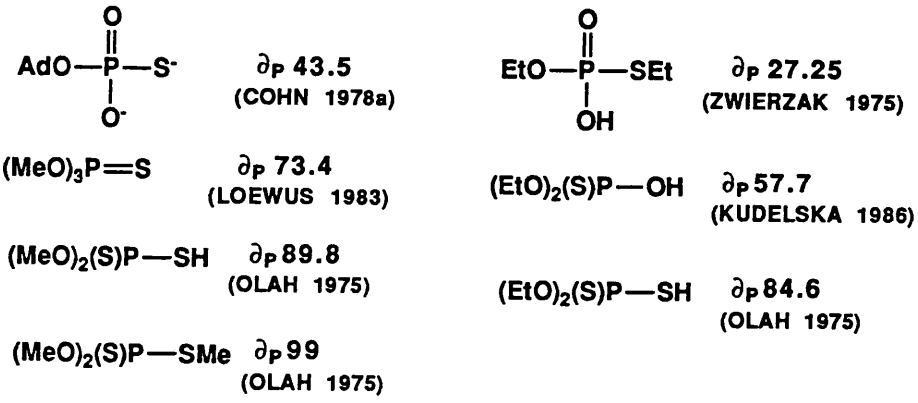
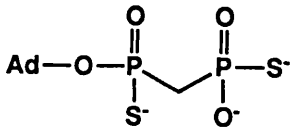


FIG 68

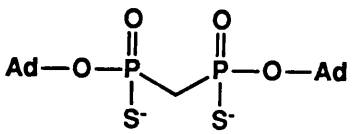
The ADP analogue (71) showed two doublets, in the ^{31}P N.M.R. at δ_P 44.4 and δ_P 34.8 ($^2J_{PP}$ = 16.7 Hz). The lower field doublet at δ_P 44.4 was assigned to the β phosphorus on the basis of the higher phosphorus-sulphur bond order. The presence of the nucleoside moiety in (71) caused the β phosphorus to resonate at a higher field than in (59). For the α phosphorus, the phosphorus-sulphur single bond, shifted the ^{31}P N.M.R. resonance to highfield as expected (see above).

The bisnucleotide (72) gave rise to a singlet in the ^{31}P N.M.R. spectrum at δ_P 31.2, indicative of a compound with phosphorus-sulphur single bond character (an upfield shift of 26.3 ppm from (59)). With (72) the presence of two nucleosides and the symmetry of the molecule resulted in phosphorus resonances at a higher field than the α-phosphorus of (71).

The possibility that the products could have resulted from attack of an oxygen to give compounds such as (65) or (73) can be discounted on the basis of the ^{31}P N.M.R. chemical shifts.



(65)



(73)

The negative charge appears to reside mainly on the sulphur atoms of (59), making the oxygen atoms less nucleophilic and the synthesis of (60) more difficult. However, in the reaction in Figure 67, it was possible for the sulphur anion to act as a nucleophilic species and displace the tosyl group from the 5'-O-tosyladenosine.

It is a consequence of the hard or soft nature of the nucleophile which, in this case, allowed the sulphur to be alkylated. It has been shown (Scheit 1980) that 5'-iodo 5'-deoxynucleosides alkylate thiophosphates at sulphur, see Figure 69. This provides a good precedent for the observed reaction to give (71) and (72).

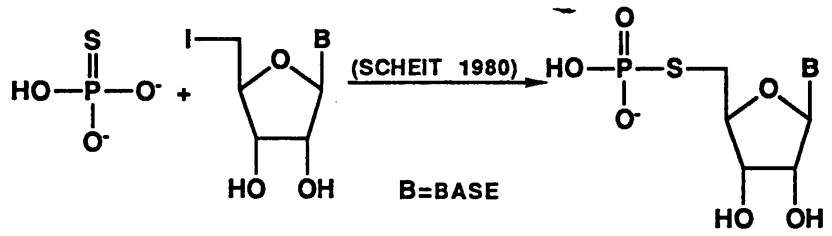


FIG 69

ENZYME STUDIES

The β, γ methylenebisthiophosphonate ATP analogue (60) was investigated as a competitive inhibitor of yeast hexokinase with ATP. Hexokinase activity was assayed by the coupled assay with glucose-6-phosphate dehydrogenase. (Fromm 1962), monitoring the conversion of $NADP^+$ to NADPH by U.V. absorption. The assays were carried out at constant concentrations of ATP with varying concentrations of the potential inhibitor (60). The velocity of the reaction was obtained from a time plot of U.V. absorbance at 340 nm. A plot of $^{1}/_{\text{velocity}}$ versus inhibitor concentration, at various ATP concentrations, gave a measure of the inhibition constant, K_I for the ATP analogue (60). See Figure 70.

[ATP] mM	[I] mM	1/V ($m^{-1}s$)
0.6	0.6	1.18
0.6	0.28	1.00
0.6	0.06	0.9
0.6	0.00	0.87
0.28	0.28	1.04
0.28	0.06	0.95
0.28	0.01	0.83
0.28	0.20	0.75
0.1	0.1	1.51
0.1	0.06	1.35
0.1	0.00	1.10
0.06	0.18	2.42
0.06	0.06	1.7
0.06	0.02	1.75
0.06	0.00	1.4

ENZYME ACTIVITY WITH (60) AS A POTENTIAL INHIBITOR OF HEXOKINASE.

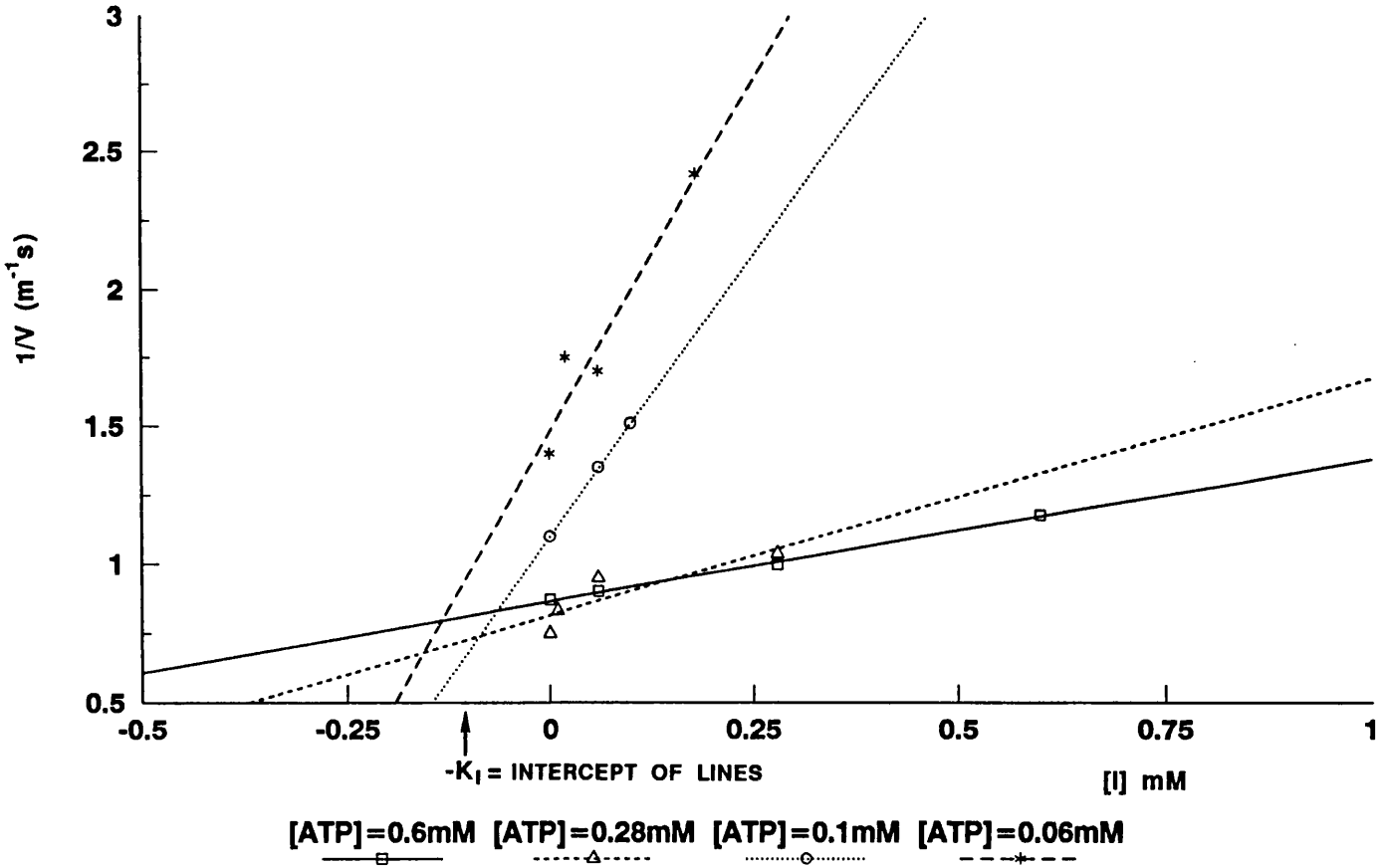


FIG 70-GRAPH OF 1/V VERSUS CONCENTRATION OF (60), SHOWING INHIBITION OF THE ENZYME ACTIVITY OF HEXOKINASE WITH ATP.

The results suggested that (60) may be a competitive inhibitor of hexokinase, having a K_I of between 0.07 and 0.16 mM. This value compares to a K_I value of 2.5 mM for the methylenebisphosphonate analogue (54), suggesting that (60) may be a better inhibitor.

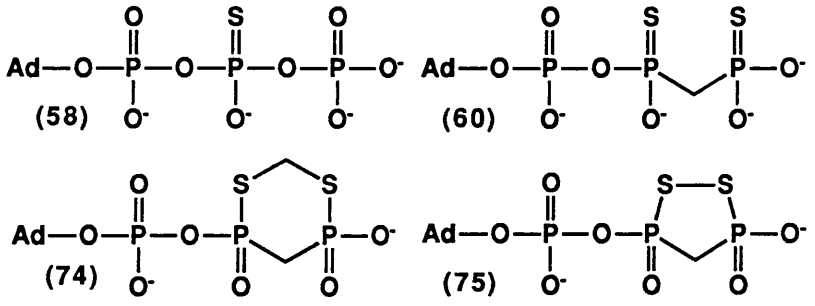
The lack of a clear intercept in Figure 70 and a single value for the K_I may be because (60) exists as a pair of diastereoisomers. The two diastereoisomers may have different K_I values, possibly with only one being an inhibitor of hexokinase. Therefore, it was not possible to draw any firm conclusions from these results.

It has been shown that ATP and its analogues form complexes of a defined stereochemistry with metal ions (Scheit 1980). Further analogues were developed from (60) in an attempt to mimic the metal complex of the active stereoisomer.

LIPOPHILIC ANALOGUES AND METAL COMPLEX MIMICS

The divalent metal complexes and resultant properties of (58) have been studied with magnesium, cadmium, zinc and cobalt (Cohn 1978 a and b). The absolute stereochemistry of the metal complex, since (58) exists as diastereoisomers, determines the resultant enzymic activity. An inversion of stereospecificity for enzyme reactions occurs when cadmium and magnesium are substituted for each other. This is a direct result of their preferred chelation to sulphur and oxygen respectively.

The analogue (60) would be expected to form similar metal complexes to (58) and ATP. The analogues (74) and (75) were synthesised as potential mimics of the metal complexes of (60), (58) and ATP and as competitive inhibitors of ATP.



These analogues, (74) and (75), would be less charged than other ATP analogues and as such can be regarded as lipophilic ATP analogues. Other lipophilic analogues have been reported in the literature, for example the non-phosphate analogue (76) (Wilcox 1988). This structure was derived from that of the magnesium complex of ATP. See Figure 71.

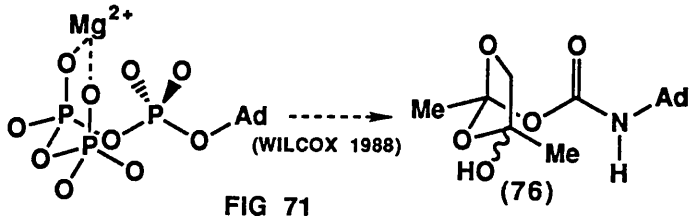
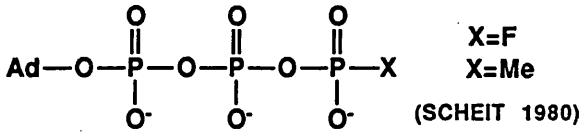


FIG 71

Two further ATP analogues, which may be considered as lipophilic have been reported in the literature. They contained either a γ-fluoro or -methyl group, both proving to be poor inhibitors of ATP utilising enzymes (Haley 1972, Scheit 1980).



Literature precedent is available for the synthesis of (74) and (75) via the alkylation or oxidation of (60). A similar cyclic esterification of a bisphosphonate has also been reported, see Figure 72 for these literature reactions.

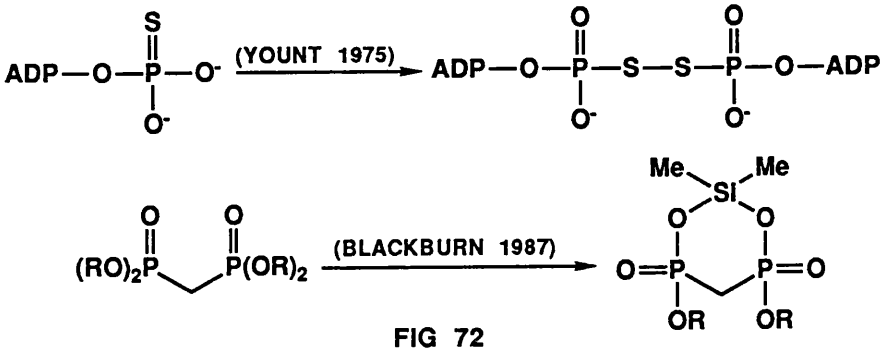


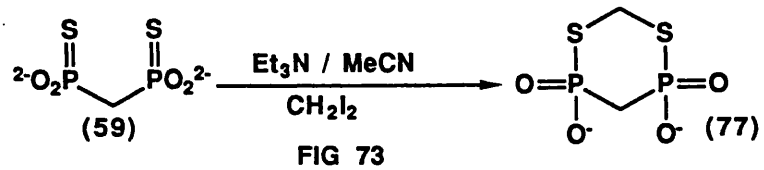
FIG 72

NOVEL RING COMPOUNDS AND ATP ANALOGUES - DITHIOMETHYLENE BRIDGED DERIVATIVES

In order to determine the viability of synthesising ATP analogues such as (74), that have geometrical similarities to the ATP-metal complex structure, the reaction of (59) with diiodomethane was investigated. Methylenebisthiophosphonate (59) was treated according to Figure 73 to give (77), the same product being formed under a variety of conditions.

The concentrations of reactants and the order of mixing were varied to eliminate the possibility of intermolecular reactions. The reaction gave a single peak in the ^{31}P N.M.R. spectrum at δ_P 32.8, indicative of phosphorus-sulphur single bonds.

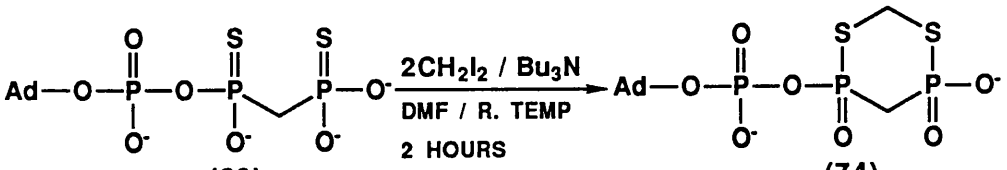
The product (77) was purified by ion exchange chromatography. The ^{31}P N.M.R. proton coupled spectrum showed a quintet ($^2J_{PH}$ = $^3J_{PH}$ = 18 Hz) indicating very similar or identical coupling between the phosphorus nuclei and the two different methylene groups. The ^1H N.M.R. showed two triplets at δ_H 2.3 and δ_H 4 (J_{PH} = 18 Hz for both triplets) for the two methylene groups.



(59) (77)

FIG 73

The reaction of (60) with diiodomethane was undertaken according to Figure 74. After purification by ion exchange chromatography the product (74) was found to be stable in a Tris/EDTA buffered solution at pH 4, both at room temperature and when stored as a frozen solution. However, in the absence of buffer, (74) decomposed to give (77) and AMP, confirmed by their ^{31}P N.M.R. resonances. As a result of this instability (74) could not be subjected to a molecular ion determination, such as negative ion F.A.B.



(60) (74)

FIG 74

The lipophilic analogue (74) contains a chiral centre at Pβ, leading to the formation of diastereoisomers. The low field ^{31}P N.M.R. data, gave no indication of diastereoisomers, showing the coupling expected of a single compound. Investigation of the highfield spectrum, Figure 75, showed Pα and Pγ giving rise to two doublets, confirming the presence of diastereoisomers. The resonances for the diastereoisomers at Pβ were not resolved. The relative highfield resonances for Pβ and Pγ with respect to (60) were brought about by the loss of phosphorus-sulphur double bond character.

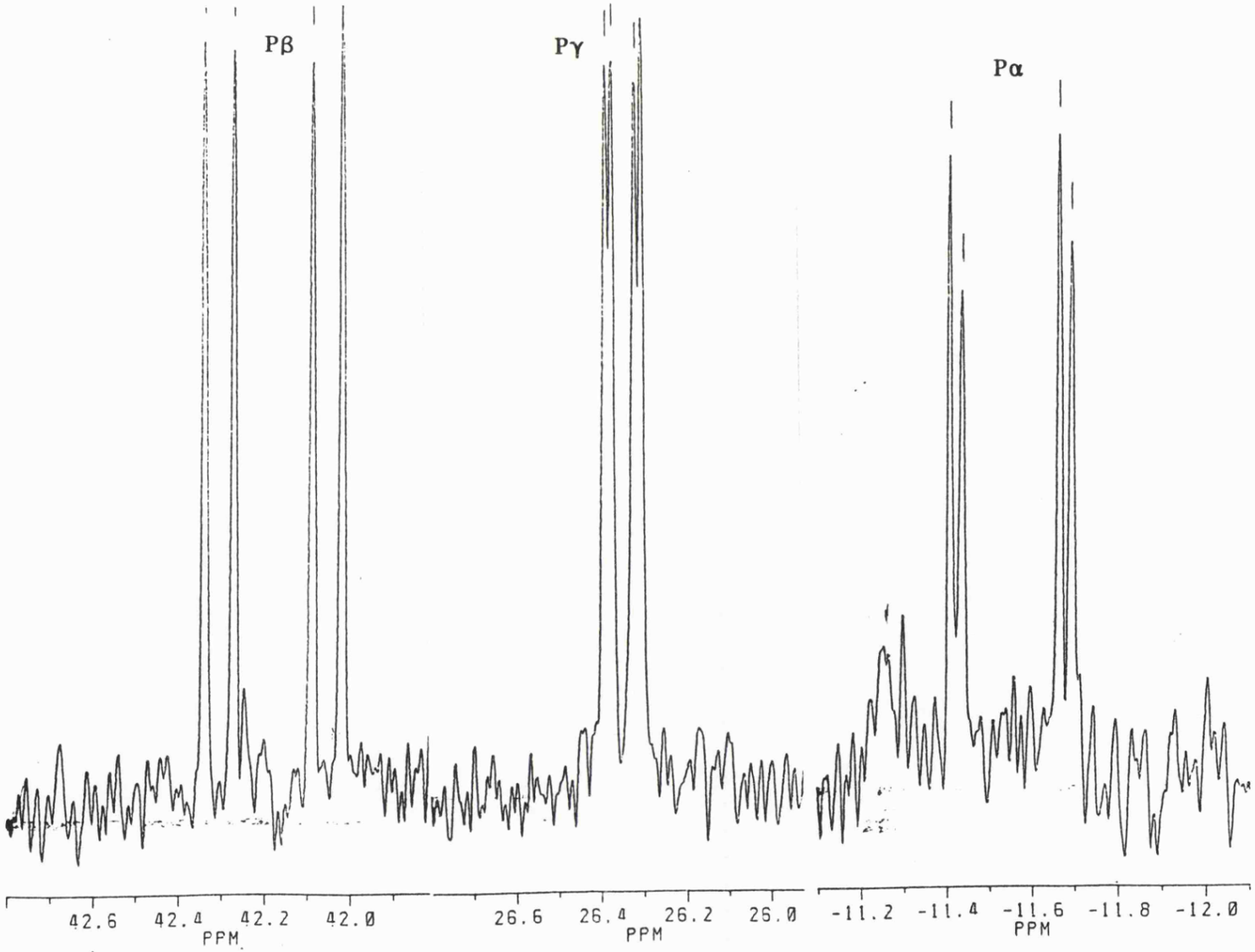


FIG 75-^{31}P N.M.R. SPECTRUM (121.5 MHz) OF (74).

The characterisation of (74) rests with the ^{31}P N.M.R. data and its comparison with that of (60), the presence of diastereoisomers and the identification of an adenosine moiety by U.V. absorbance. Further supportive evidence is supplied by its place of elution from the ion exchange column and hence its overall charge and by the fact that it decomposes to (77).

DISULPHIDE BRIDGED DERIVATIVES

In an attempt to synthesise a further lipophilic analogue of ATP, disulphide formation on the ATP analogue (60) was investigated. Initially, the oxidation of (59) was undertaken according to Figure 76, to give a single product at δ_P 31 in the ^{31}P N.M.R. Treatment of this product (78) with mercaptoethanol reversed the reaction to give (59) at δ_P 60.

The addition of an authentic sample of (59) gave only one peak at δ_P 60, confirming its identity. The loss of phosphorus-sulphur double bond character from (59) results in an upfield shift of the phosphorus resonance for (78). The possibility of dimer or polymer formation cannot be ruled out, however, since only one sharp resonance was observed in the ^{31}P N.M.R. spectrum this seems unlikely.

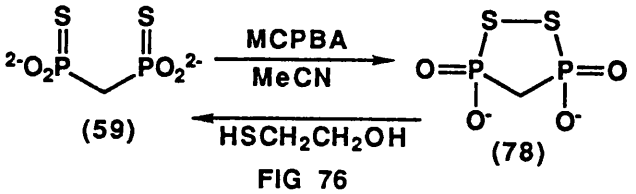


FIG 76

Preliminary results with the oxidation of (60), indicated the successful formation of the disulphide (75) according to Figure 77. The low field ^{31}P N.M.R. spectrum (36 MHz) showed a doublet for both Pα and Pγ and a doublet of doublets for Pβ. There was the suggestion of further peaks being present for Pβ, due to diastereoisomer formation. No further work was carried out with this analogue.

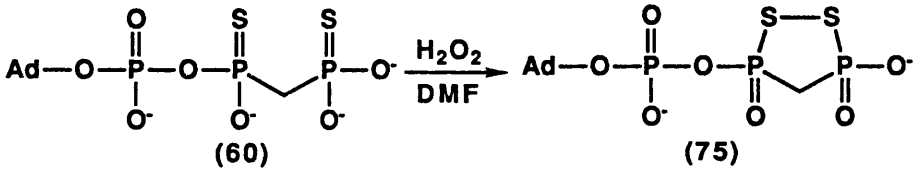


FIG 77

ENZYME STUDIES

The ATP analogue (74) was tested as a competitive inhibitor of yeast hexokinase in a similar assay experiment to that involving (60). See Figure 78. The assay gave a K_I value of between 0.29 and 0.46 mM, suggesting that (74) may be a better inhibitor than the β, γ methylenebisphosphonate ATP analogue (54) (K_I = 2.5 mM). As with (60), the results are complicated by the potential inhibitor existing as a pair of diastereoisomers.

[ATP] mM	[I] mM	1/V ($m^{-1}s$)
0.28	0.28	0.66
0.28	0.1	0.47
0.28	0.06	0.5
0.28	0.00	0.48
0.1	0.28	0.625
0.1	0.1	0.52
0.1	0.00	0.5
0.06	0.28	0.80
0.06	0.1	0.73
0.06	0.06	0.636
0.06	0.02	0.75
0.06	0.00	0.696
0.02	0.28	2.08
0.02	0.1	1.47
0.02	0.00	1.39

ENZYME ACTIVITY WITH (74) AS A POTENTIAL INHIBITOR OF HEXOKINASE.

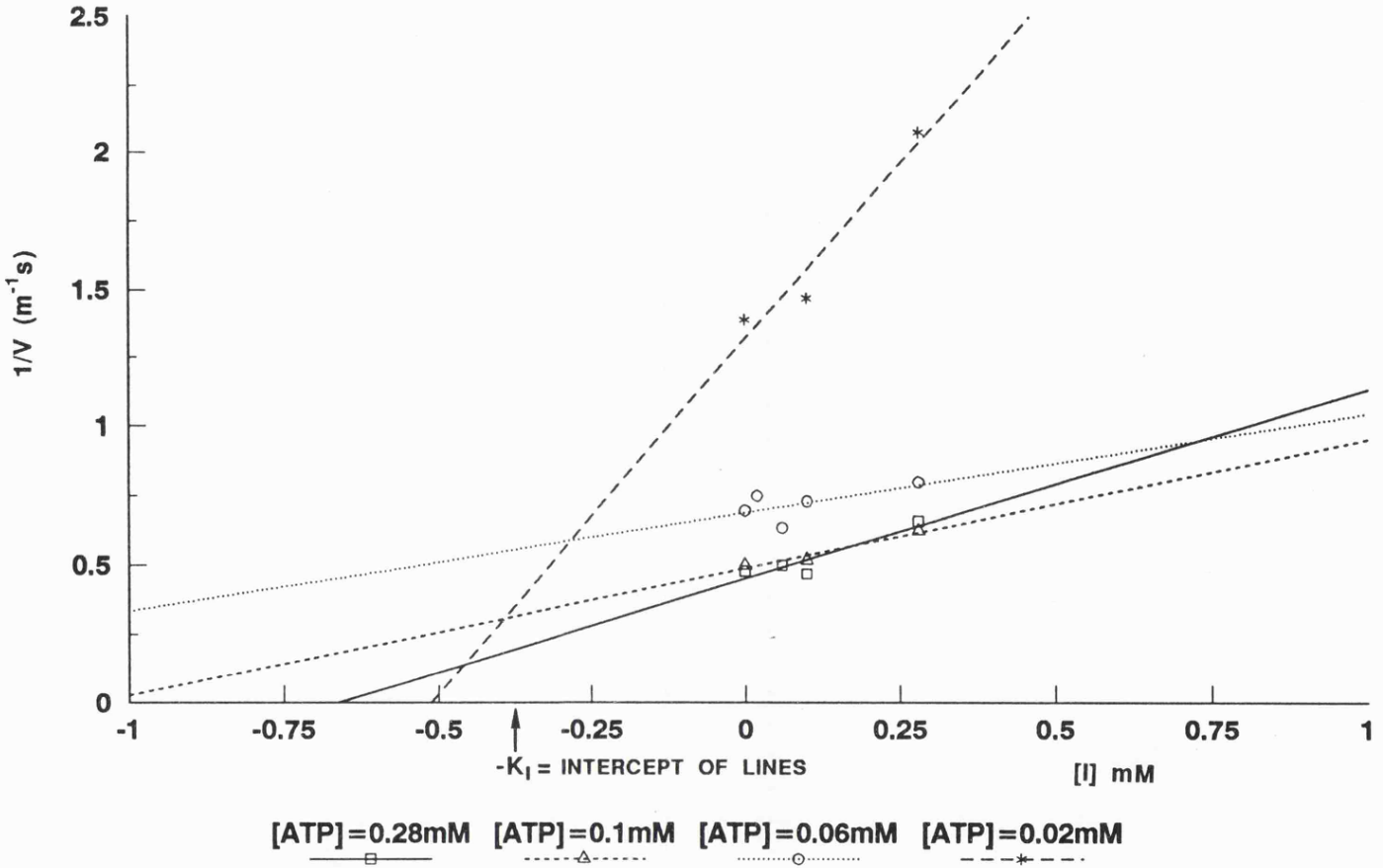


FIG 78-GRAPH OF 1/V VERSUS CONCENTRATION OF (74), SHOWING INHIBITION OF THE ENZYME ACTIVITY OF HEXOKINASE WITH ATP.

This very preliminary work carried out with lipophilic ATP analogues shows some of their potential. Altering the electronic and steric properties, leading to possible changes in binding and hydration, by going from (60) to (74) is acceptable, giving a possible competitive inhibitor of hexokinase. The potential of (74) as a metal complex mimic of the active diastereoisomer of (60) or other systems was not investigated further.

OVERALL CONCLUSIONS

The aim of the work in this chapter was to alter the electronic and binding properties of methylenebisphosphonate and ATP analogues derived from it. This was achieved by the use of thiophosphonate analogues, with lower pKa values than the parent phosphonates.

The compounds tested as potential bone agents, (59) and (64), were not bone specific in their <u>in vivo</u> distribution. The dual requirements of chelation to technecium and interaction with the bone matrix were not achieved, indicating that thiophosphonates are not viable bone imaging agents.

The importance of electronic requirements when designing analogues of natural systems was highlighted by the enzyme assays suggesting that (60) and (74) may act as competitive inhibitors of hexokinase. The nucleophilicity of the thiophosphonate anions of both (59) and (60) was restricted, contributing to the production of the S-adenosine compounds (71) and (72). The area of lipophilic ATP analogues briefly investigated, opens up the possibility of future chemistry.

C H A P T E R F O U R

INTRODUCTION

POTENTIAL N.M.R. IMAGING OF BONE AND AREAS OF CALCIFICATION

Bone and certain types of bone tumour do not readily contribute to an N.M.R. image due to the low concentration of water present in the bone matrix (see Chapter One for a description of the N.M.R. imaging technique).

This can be advantageous when studying bone marrow diseases such as leukemia and tissues close to bone. Diseased soft tissue cells can irreversibly precipitate calcium ions into phosphate-rich regions (Cheung 1986). Such an increase in calcium content can be seen in certain diseases such as cerebral and myocardial infarcts and tumours (Adzamli 1989). Although providing some image contrast, clearly defined boundaries showing the extent of pathology are not readily observed in these calcified areas.

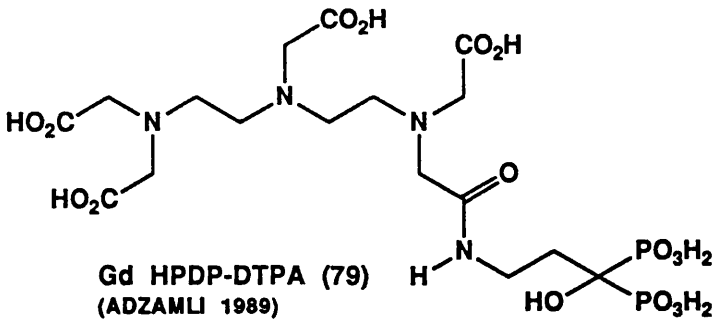
The aim of the work in this chapter was to develop novel ligands that may facilitate imaging of bone, bone tumours and other areas of calcification. Encouragement for this study was derived from the observation that bisphosphonates of general structure (48) have been used to target areas of calcification, specifically bone. This ability has been utilised in nuclear medicine, where bisphosphonates have been complexed with technecium (a γ-emitting nucleus).

The resulting complex, once administered, collects in areas of calcification and is detected with a γ-camera (see Chapter Three for more detail).

It has been shown that paramagnetic species (specifically nitroxyl radicals for the purpose of this investigation) can enhance contrast in N.M.R. images (Chapter One). The targetting of a nitroxyl containing moiety to areas of calcification would represent a potentially important extension to the current applications of N.M.R. imaging.

During the course of this study a related piece of work was published in which gadolinium chelates were modified with phosphonate groups (Adzamli 1989). These workers attempted to direct the paramagnetic gadolinium towards areas of calcification, specifically diseased cells, with a view to altering the local N.M.R. signal. This work utilised the ability of phosphonates and bisphosphonates to target areas of calcification. Gadolinium chelates such as (79) were initially investigated in order to measure their ability to target bone in vivo. Bone in this case was used as a model system, with any bone specific chelates being investigated for N.M.R. imaging of calcified soft tissues.

The validity of our approach was enhanced by this preliminary investigation. Our aim was to attempt to image bone and bone tumours by the use of related chelates incorporating a nitroxyl radical.



Gd HPDP-DTPA (79)
(ADZAMLI 1989)

Nitroxyl radicals have previously been shown to be directed *in vivo* by the nature of their substituents (see Chapter One). By incorporating a nitroxyl radical into the structure of a bone specific agent, it was anticipated that the required targetting would be achieved. An example of such a compound would be the bisphosphonate (80). Approaches towards synthesising a bisphosphonate containing a nitroxyl moiety were considered.

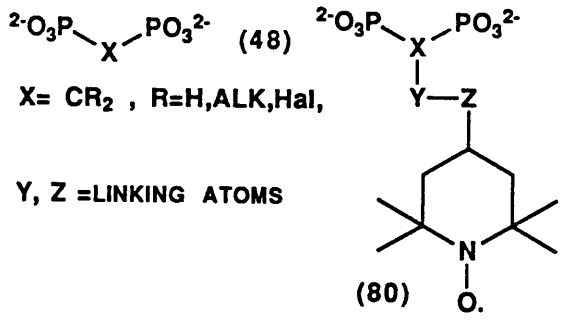


FIG 79

NITROXYL-CONTAINING PHOSPHONATES AND BISPHOSPHONATES AND THEIR POTENTIAL TARGETTING TOWARDS AREAS OF CALCIFICATION

Spin labelled phosphoryl compounds have previously been reviewed (Konieczny 1981). However, the majority of compounds reported had a nitroxyl moiety attached to the phosphorus via a phosphate or phosphonate ester group, see Figure 80. Such compounds were of limited use for this work, as a phosphoric or phosphonic acid (or salt thereof) is required for bone targetting. The acids presented, such as (81) also used a phosphate ester to link the nitroxyl to the phosphorus atom. Whilst this compound was closer to our requirements, a phosphate ester is less hydrolytically stable than a phosphonate group. Also, a bisphosphate or preferably a bisphosphonate is more closely related to known bone agents, and therefore more likely to succeed.

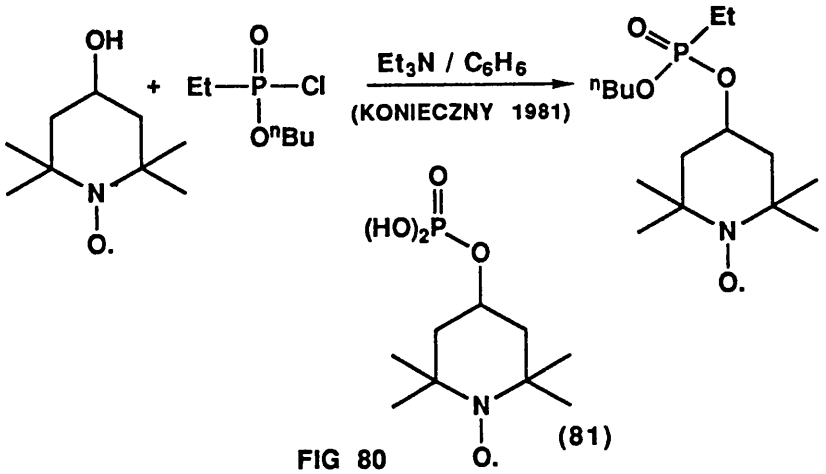


FIG 80

Due to the lack of relevant compounds in the literature, the synthesis of novel bisphosphonates containing a nitroxyl group attached to the methylene group was investigated.

POTENTIAL ROUTES TOWARDS TARGET MOLECULES - METHYLENEBISPHOSPHONATE BASED STRUCTURES

The incorporation of a suitable functional group onto the methylene bridge of a bisphosphonate, would provide a potential site for the incorporation of a nitroxyl radical. A good method of alkylating methylenebisphosphonates involved the use of thallium (I) ethoxide and primary alkyl halides (Hutchinson 1985). Extension of this chemistry by the use of functionalised halides, should provide a route to the desired molecular framework, see Figure 81.

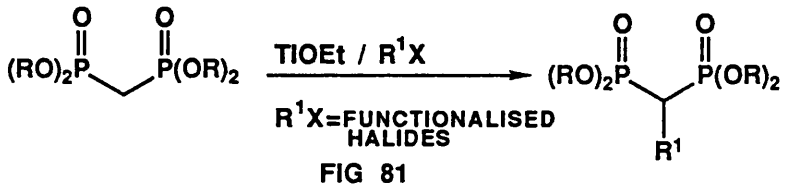


FIG 81

The reaction between sodium methylenebisphosphonate and ^{i}propyl chloroformate has been reported to give penta-^{i}propyl (carboxymethylene)bisphosphonate in low yield (25%).

While the attempted acid hydrolysis of the ester groups resulted in decomposition, an alternative route to this structure was envisaged as proceeding via the phosphorylation of triethyl phosphonoacetate. Removal of the phosphorus ester groups should give (82), which could then be coupled to a nitroxyl radical via the ester functionality.

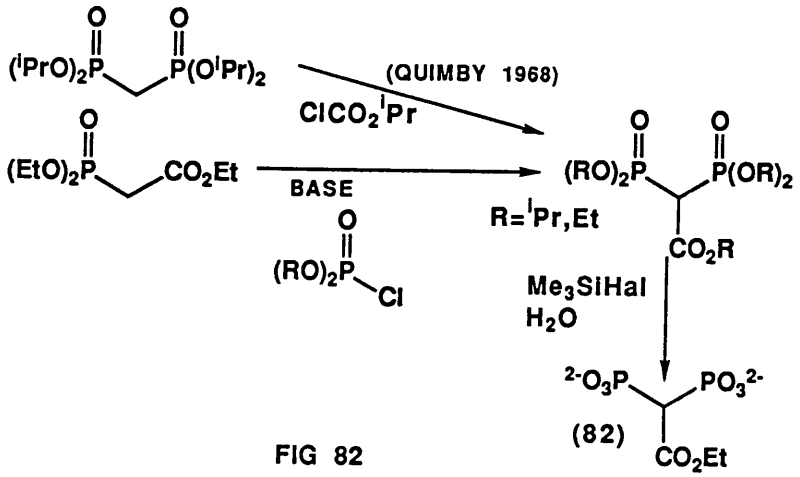


FIG 82

Phosphonoacetic acid and its derivatives possess antiviral activity and bone affinity (Lambert 1989), thus making structures derived from phosphonoacetate potentially useful in a wider field. Phosphonate carbanions have been treated with sulphur to yield the corresponding thiols (Mikolajczyk 1979). The similar treatment of the phosphonoacetate anion to give (83), would allow for the introduction of a nitroxyl radical via alkylation of the thiol with, for example (84), see Figure 83. The mixed phosphonic and carboxylic binding capabilities of (83) may provide bone specificity, also making it an interesting compound for investigation.

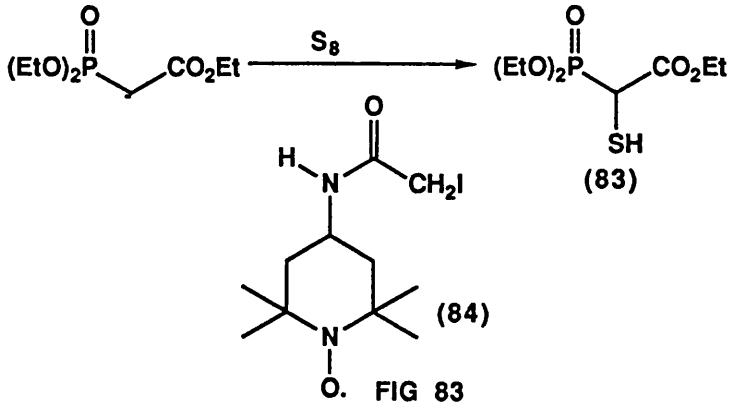
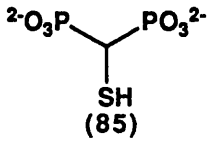


FIG 83

The synthesis of mercaptomethylenebisphosphonate (85) has been reported (Griffith 1987) and provided a further functionalised bisphosphonate with potential for the introduction of a nitroxyl radical, such as (84).



(85)

INCORPORATION OF A NITROXYL RADICAL INTO AN AMINOALKYLBISPHOSPHONIC ACID

Aminoalkylphosphonic acids (86), because of their inherent biological activity, have been the subject of much work and a review (Moedritzer 1966, Fields 1952, Redmore 1976, Dhawan 1987).

They have been synthesised via the reaction of an amine (primary or secondary) with an aldehyde or ketone and a phosphite. The intermediate imine has been isolated prior to reaction with the phosphite, or alternatively the reaction undertaken in "one-pot". The phosphites used have usually be dialkyl phosphites, which necessitated de-esterification, or phosphorous acid which gave the aminoalkylphosphonic acid directly, see Figure 84.

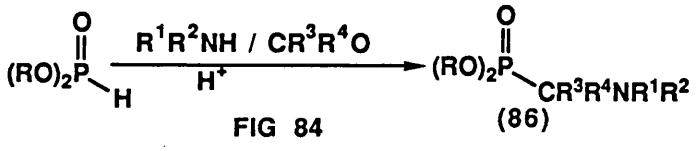
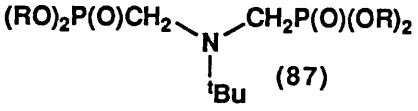


FIG 84

(86)

The corresponding iminobismethylenebisphosphonate derivatives have also been synthesised by an extension of the above routes. This approach has been shown to give tetraethyl ^{t}butyliminobisphosphonate (87) (Moedritzer 1973). Therefore, by the use of a suitable amine, it appears that a nitroxyl moiety could be incorporated into an aminoalkylbisphosphonate. A related piece of chemistry has been reported (Skowronski 1987), leading to the synthesis of (88). The use of similar chemistry was expected to lead to the desired structure, such as (89) in Figure 85.



(87)

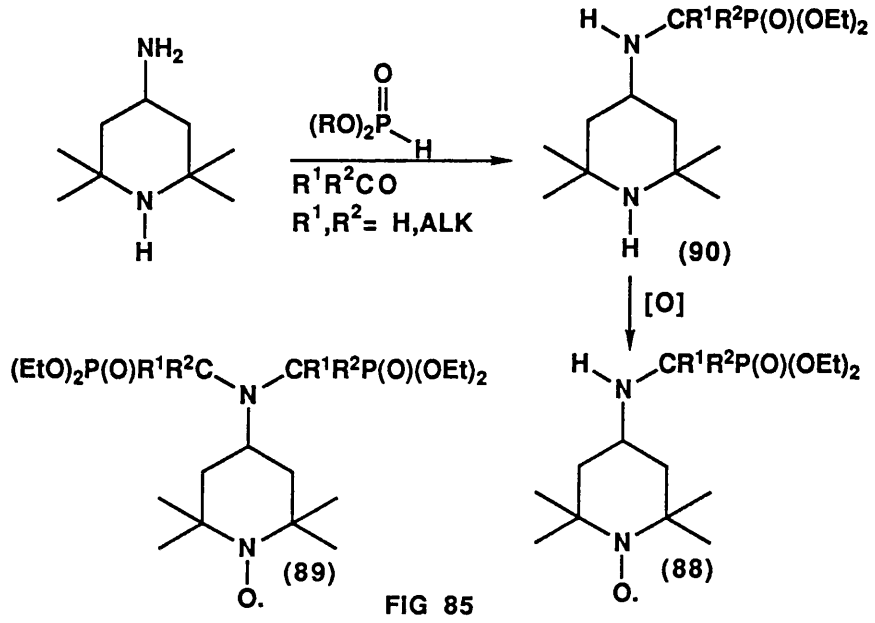


FIG 85

Two routes to (89) were therefore considered, either <u>via</u> treatment of the intermediate (90) with diethyl phosphite and an aldehyde (or ketone) followed by de-esterification and subsequent oxidation. Alternatively phosphorous acid could be used, removing the need for a de-esterification step. The work of Moedritzer (1966) provided precedent for this "one-pot" synthesis of (89), where R would be a tetramethylpiperidine moiety, see Figure 86.

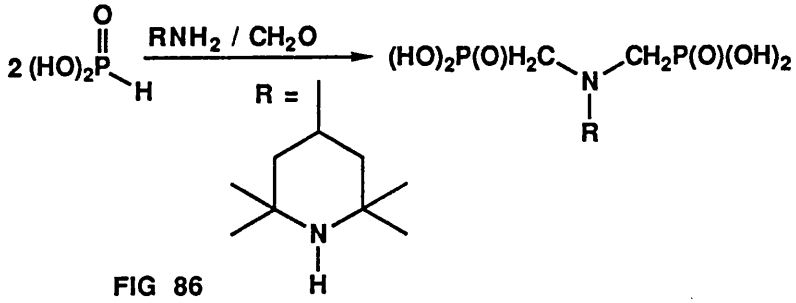
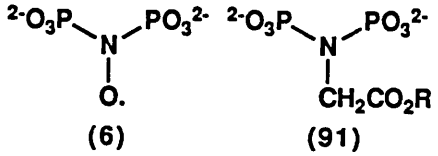


FIG 86

IMIDOBISPHOSPHONATES WITH POTENTIAL SITES OF NITROXYL INCORPORATION

The novel nitroxyl radical (6), presented in Chapter Two, would be a suitable molecule to consider as a bone imaging agent. However, the low yield and instability of this nitroxyl radical made its use impractical.

The general route developed for synthesising bisphosphorylated amines could be adapted and extended to functionalised amines. A good example of such a target would be (91) which has the potential for attaching a nitroxyl radical to the carboxyl group. The synthesis of novel compounds such as (91) would create a precedent for the incorporation of amino acids into similar imidobisphosphonates. In addition to creating potentially new N.M.R. imaging contrast agents, intermediates such as (91) would be worthy of investigation as bone agents in nuclear medicine.



(6) (91)

The various synthetic routes outlined above have a reasonable amount of precedent. The intention therefore, was to synthesise the desired product in reasonably large amounts to enable extensive *in vitro* and *in vivo* studies to be undertaken.

Therefore, an efficient route needed to be developed that would afford a reasonable yield of the desired compound. Of the routes outlined, the most practical were attempted in order to determine the viability of imaging areas of calcification, specifically bone by N.M.R.

RESULTS AND DISCUSSION

The proposed routes for synthesising a bone specific agent, from either the methylenebisphosphonate or phosphonacetate parent structures, are shown in Figure 87. Both routes have as the key step, the introduction of a suitable functionalised group, R^1, which would allow for the incorporation of a nitroxyl radical group. The most appropriate functional groups were thought to be alcohols, esters, carboxylic acids, halides, or thiols, the latter allowing for the reaction with the known radical (84).

The initial use of ester protecting groups for the phosphoryl moiety would necessitate their removal in order to generate the desired free acid (or a suitable phosphonate salt). There was the possibility to remove these ester groups either early in synthesis or after the introduction of the R^1 group, depending on the nature of this group and the stability of any intermediates.

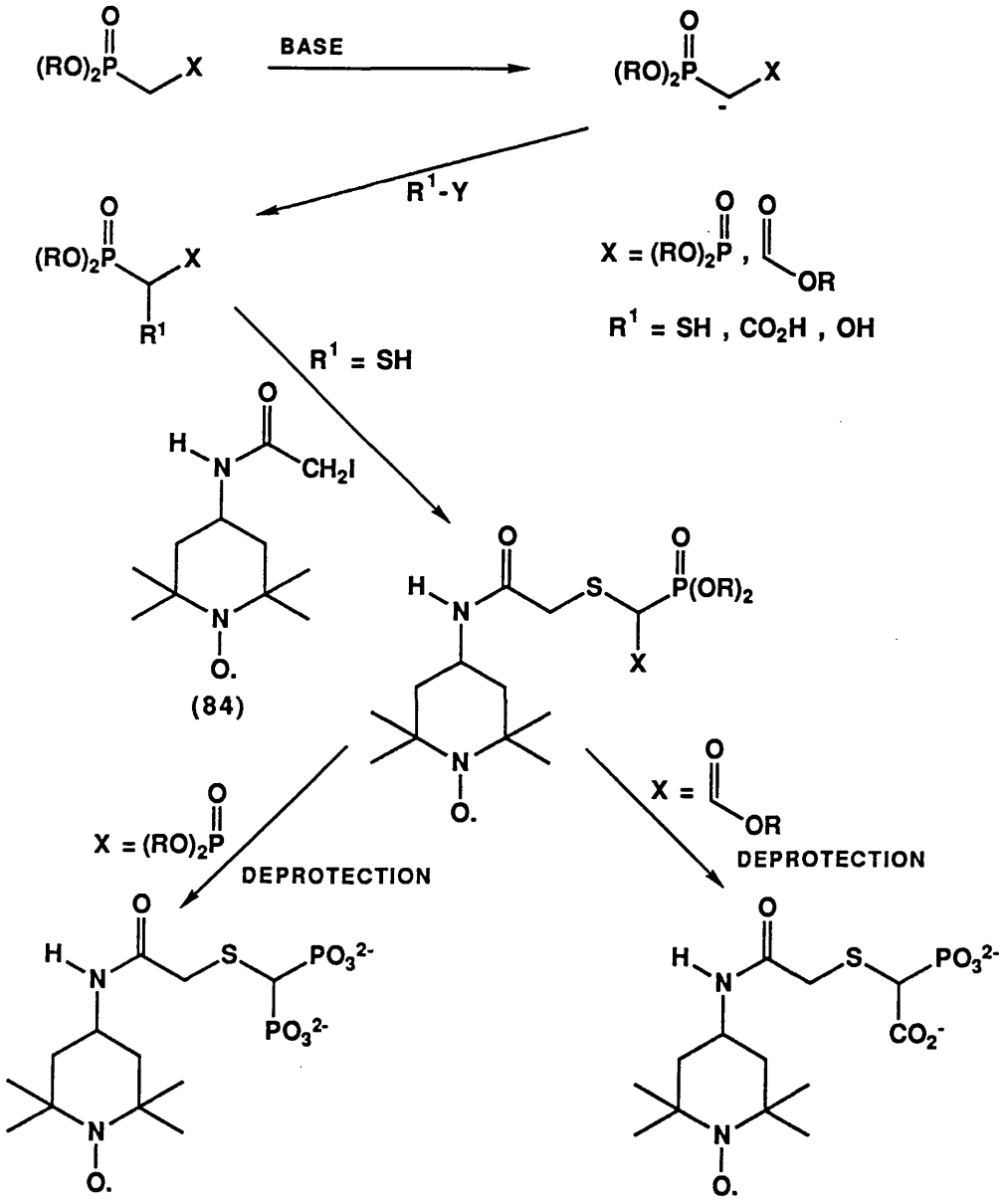

BASE
R1-Y
X = (RO)2P , OR
R1 = SH , CO2H , OH
R1 = SH
(84)
X = (RO)2P
DEPROTECTION
X = OR
DEPROTECTION

FIG 87

FUNCTIONALISED METHYLENEBISPHOSPHONATES AND PHOSPHONOACETATES

Initially, commercial tetra-^{i}propyl methylenebisphosphonate was alkylated with methyl iodide, as shown in Figure 88. The product (92) was characterised by ^{31}P N.M.R., giving a singlet at δ_P 21.9, with a small amount of the bismethylated product being observed at δ_P 25.4.

However, on undertaking similar reactions with the functionalised halides shown in Figure 88, only the starting bisphosphonate was recovered. The two reactions using the halides containing acidic protons, that is the alcohol and carboxylic acid, were repeated using a further equivalent of base, again giving only the starting bisphosphonate. Thallium residues were removed from the reaction mixture, the possibility of also removing the desired products as thallium salts was considered unlikely.

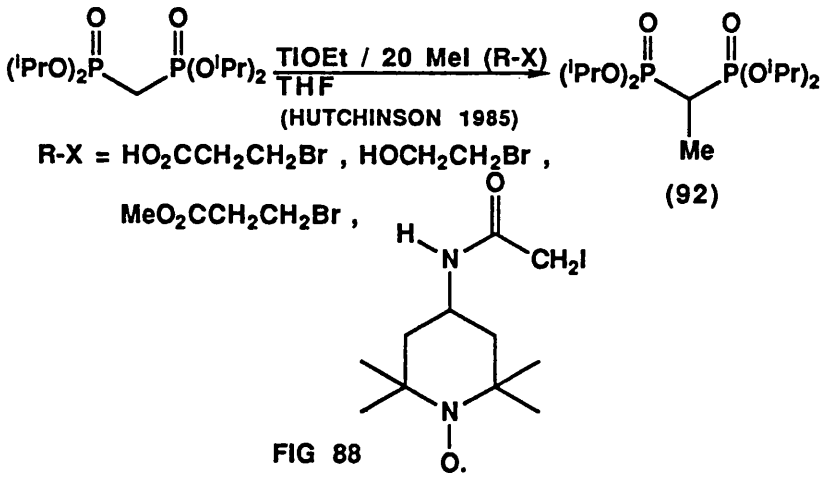


FIG 88

The lack of reactivity of the functionalised halides investigated and problems encountered in removing the thallium residues made this synthetic route unviable. Therefore, attention was focussed on the phosphonoacetate derived targets.

The phosphorylation of triethyl phosphonoacetate, was undertaken with sodium hydride and diethyl chlorophosphate according to Figure 89. The product formed was not the desired bisphosphonate (93), but the olefinic compound (94). This product was purified by silica gel flash chromatography. The ^{31}P N.M.R. spectrum of (94) showed a doublet of doublets, resulting from the coupling between two non-equivalent phosphorus nuclei ($^{4}J_{PP}$ = 3.6 Hz), the phosphate nucleus resonating at the higher field. The observed ^{1}H N.M.R. spectrum was complex due to the presence of five ethoxy groups, the identity of the product being supported by mass spectrometry.

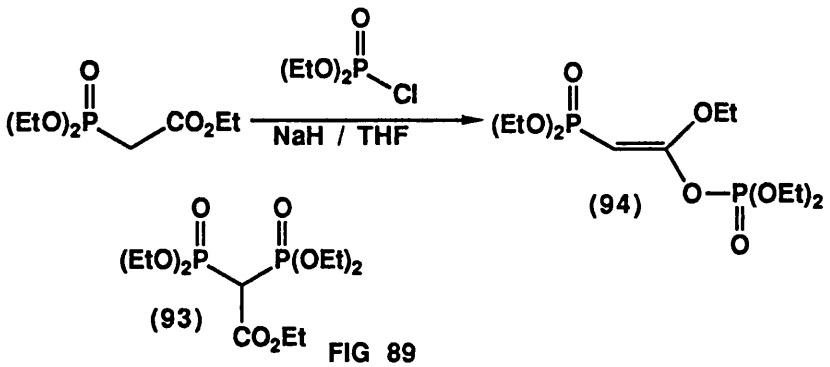


FIG 89

MECHANISM OF OLEFIN FORMATION

The observed reactivity of the triethyl phosphonoacetate anion can be rationalised in terms of the hard and soft concept of nucleophiles and electrophiles. The anion was resonance stabilised by the ester groups, with two possible nucleophilic sites at the oxygen or methylene anions, see Figure 90. The hard nature of the phosphorus electrophile favoured phosphorylation of the oxyanion. In contrast, treatment of the anion with methyl iodide led to alkylation on the methylene group to give (95).

The ^{31}P N.M.R. spectrum of (95) showed a singlet at δ_P 23.3 with the ^{1}H N.M.R. showing the expected doublet from the coupling of the methylene proton with the phosphorus nucleus. The structure was further confirmed by mass spectrometry.

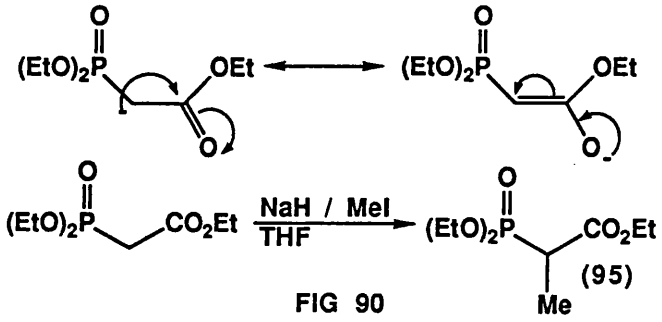
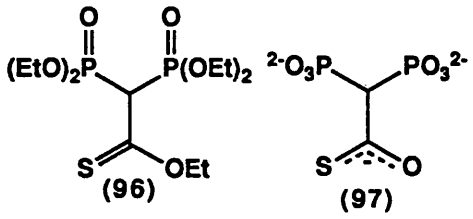


FIG 90

(95)

CHANGING THE SITE OF NUCLEOPHILICITY BY USE OF A SULPHUR ANALOGUE

The triethyl phosphonoacetate anion reacted with the phosphorus electrophile at the "hard" oxygen anion. The use of the corresponding thioacetate was expected to alter the site of attack onto the "soft" methylene anion. This was expected to give the bisphosphonate (96), with the possibility of incorporating a thio specific spin label into the corresponding free acid (97).



O-ethyl(diethylphosphono)thioacetate (98) was prepared as outlined in Figure 91. The ^{1}H N.M.R. spectrum showed the methylene protons giving rise to a doublet from coupling to the phosphorus nucleus ($^{2}J_{PH}$ = 21 Hz).

The anion derived from (98) was then reacted with diethyl chlorophosphate. Three compounds were observed in the ^{31}P N.M.R. spectrum of the crude reaction mixture.

These compounds were separated by silica gel flash chromatography and identified as (98) (δ_P 17.7), tetraethyl pyrophosphate (δ_P -13.1) and triethyl phosphonoacetate (δ_P 19.3). The latter two resulted from hydrolysis of the diethyl chlorophosphate and the loss of sulphur from (98) respectively. The identity of these compounds was confirmed after comparison with authentic samples. No evidence was obtained for the formation of any of the desired phosphorylated product under these reaction conditions.

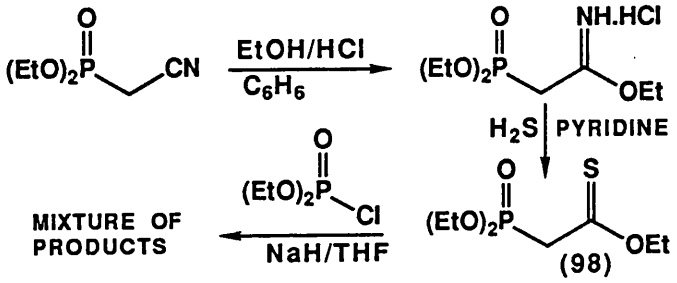


FIG 91

The introduction of a thiol group at the methylene group of the phosphonoacetate framework was attempted next. The desired product (99) was considered to be suitable for the incorporation of a thiol specific spin label. The reaction of the triethyl phosphonoacetate anion with elemental sulphur was undertaken according to Figure 92.

The ^{31}P N.M.R. spectrum of the crude reaction mixture showed the starting material to be the major component, with a further signal at δ_P 14.1.

Purification of the mixture by silica gel flash chromatography yielded coeluting products with two resonances in the ^{31}P N.M.R., at δ_P 14.9 and δ_P 14.1. Mass spectrometry confirmed the presence of the expected molecular ion for (99). The two ^{31}P N.M.R. resonances were thought to correspond to a mixture of (99) and the corresponding disulphide.

The ^{1}H N.M.R. spectrum offered little help, a complex splitting pattern being observed due to the close proximity of the resonant frequencies. The low yield and uncertainty over the identity of the product rendered this synthesis route unsatisfactory.

Attempts were also made to react the anion with dimethyl and di-^{t}butyl disulphide, however the anion proved to be unreactive with disulphide electrophiles in our hands.

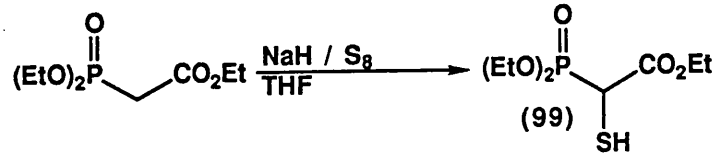


FIG 92

The lack of success with the above chemistry led to the investigation of the known mercaptomethylenebisphosphonate.

SYNTHESIS OF A METHYLENEBISPHOSPHONATE CONTAINING A NITROXYL MOIETY

Mercaptomethylenebisphosphonate (85) was prepared according to the method of Griffith (1987), as illustrated in Figure 93. Tetra-^{i}propyl methylenebisphosphonate was treated sequentially with sodium hydride and sulphur to yield the disulphide (100). No evidence was observed for the production of the corresponding thiol, presumably due to its <u>in situ</u> oxidation to (100).

The disulphide (100) was characterised by ^{31}P N.M.R., showing a singlet at δ_P 15.3. This signal collapsed to a doublet in the proton coupled spectrum ($^{2}J_{PH}$ = 20 Hz), a result of coupling to a single methylene proton. The methylene protons were observed in the ^{1}H N.M.R. as a triplet ($^{2}J_{PH}$ = 20 Hz) due to coupling to two equivalent phosphorus nuclei. The product (100) could be purified by silica gel flash chromatography, but was found to precipitate in sufficient purity from the crude reaction residue.

The reaction of (100) with bromotrimethylsilane gave the corresponding trimethylsilyl derivative (101), the observed ^{31}P N.M.R. signal at δ_P 0.4 was indicative of such an ester exchange. The proton coupled spectrum again showed the expected doublet ($^{2}J_{PH}$ = 20 Hz). The treatment of (101) with water gave the phosphonic acid (102), with a ^{31}P N.M.R. chemical shift of δ_P 14.1.

The next step in the synthesis was the cleavage of the disulphide bond to generate the thiol, the intended site of spin label attachment. Initially, cleavage of the disulphide bond was attempted using mercaptoethanol. However, no reaction occurred as judged by ^{31}P N.M.R. analysis of the reaction mixture. The desired reduction was achieved using sodium borohydride, to give (85). The ^{31}P N.M.R. spectrum of (85) gave rise to a singlet at δ_P 15.7, with the expected doublet present in the proton coupled spectrum. The thiol (85) was isolated from an aqueous solution by precipitation with acetone, yielding the sodium salt of the phosphonate thiol.

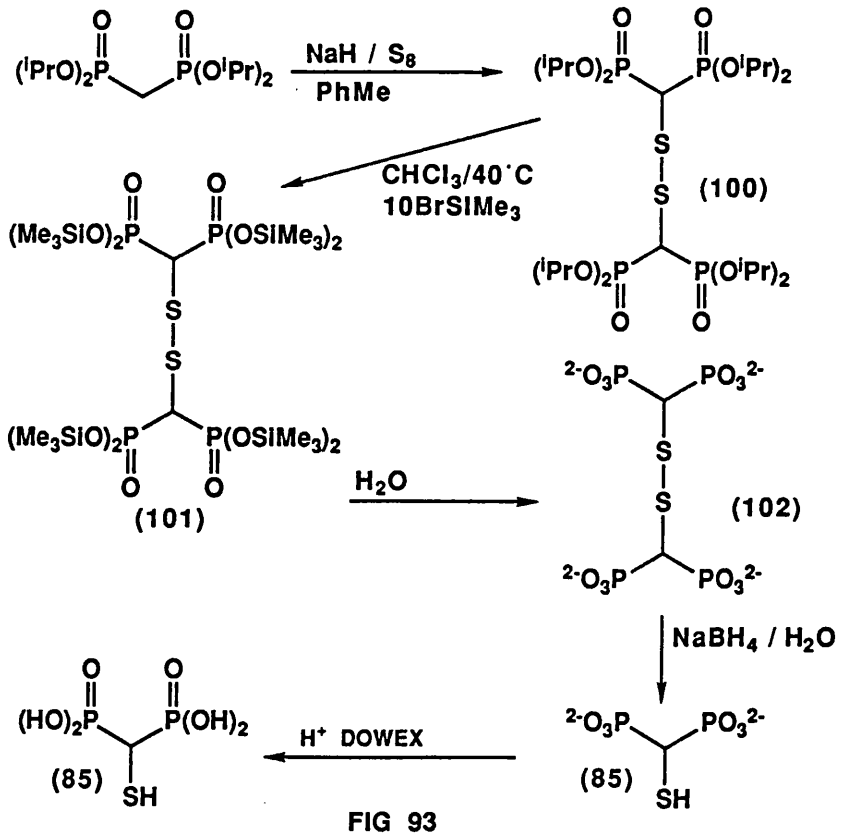


FIG 93

The initial attempt to alkylate (85) with the iodoacetamide spin label (84) failed to give the desired product (103). Purification of the reaction mixture gave (84), methylenebisphosphonate (8), and the disulphide (102).

The free acid was obtained by treating (85) with H^+ dowex, resulting in a ^{31}P N.M.R. resonance at δ_P 13.3. The removal of the sodium and borate salts from (85) increased the nucleophilicity of the thiol group, which then successfully reacted with (84) as shown in Figure 94. Ion exchange purification gave (103) in a 10% to 20% yield by E.S.R. analysis. The E.S.R. spectrum of (103) is reproduced in Figure 94A and is typical of a solid solution spectrum for a nitroxyl radical (Symons 1978). It eluted from an ion exchange column, at a position on the gradient consistent with a compound containing four negative charges and with a resonance at δ_P 12.5 in the ^{31}P N.M.R.

The final step in the synthesis was successful, but only in a 20% yield at best. This coupled to the low yields obtained for (84), 20%, and (85) made this a poor synthetic route to (103). Therefore, although successful this route was not amenable to a large scale synthesis of a potential bone agent. Attention was then focussed on the aminoalkylbisphosphonates.

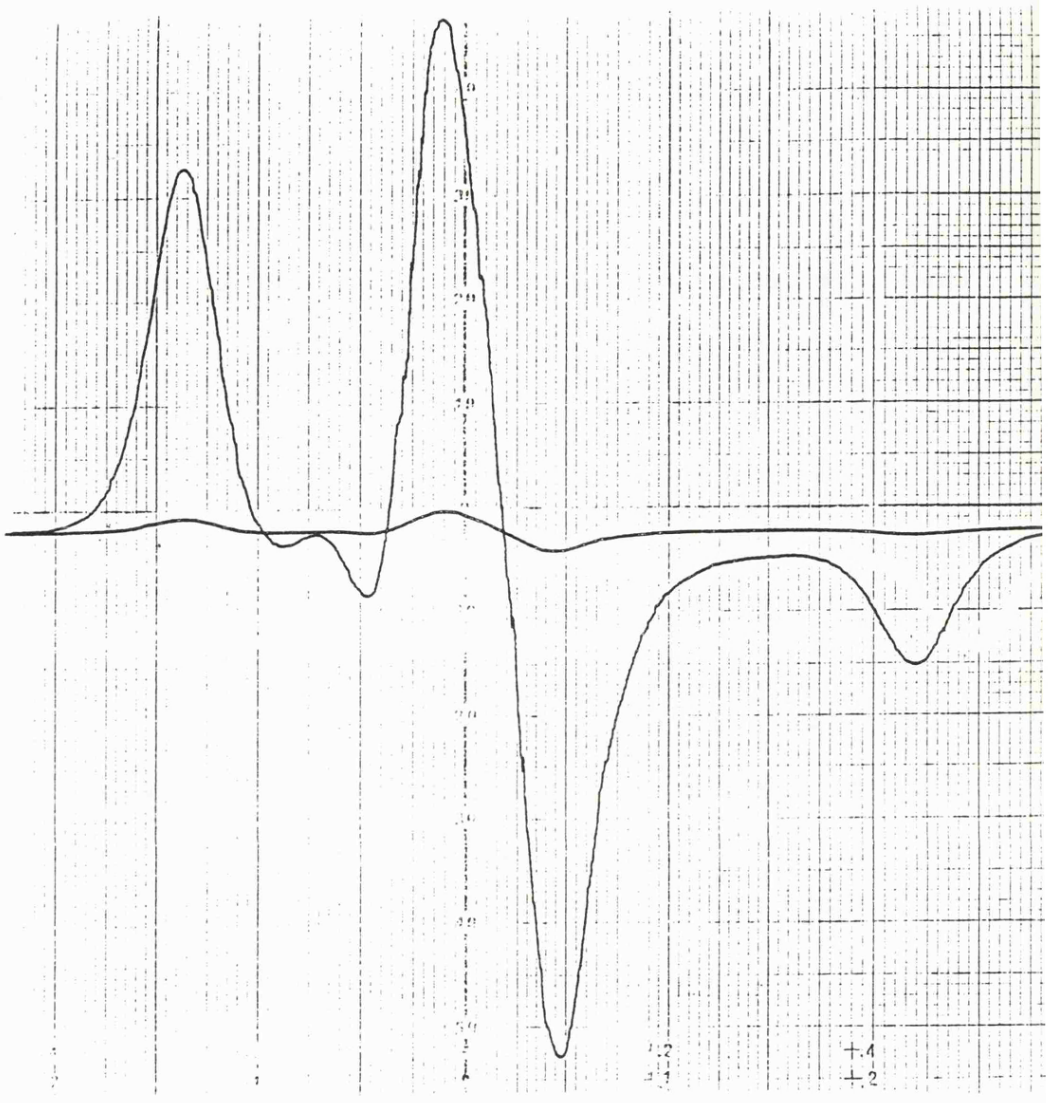


FIG 94A -SOLID SOLUTION E.S.R. SPECTRUM OF (103).

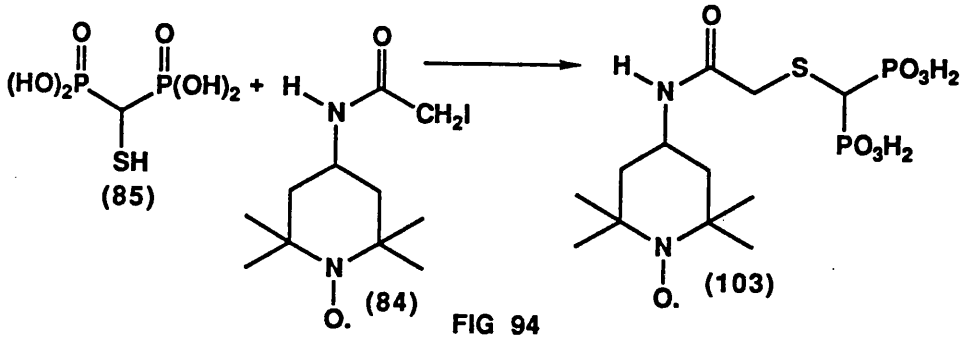


FIG 94

NITROXYL AMINOALKYLBISPHOSPHONATES

The proposed synthetic route to a nitroxyl containing aminoalkylbisphosphonate is illustrated in Figure 95. The 4-amino tetramethylpiperidine (104) was used because of the relative ease of its oxidation to the corresponding nitroxyl radical (Chapter One). Initially, formaldehyde was used, with the possibility for extending the range of analogues by the use of other aldehydes or ketones. An alternative synthetic route was also considered using phosphorous acid instead of a dialkyl phosphite, thus avoiding the need for the de-esterification step.

PROPOSED ROUTE

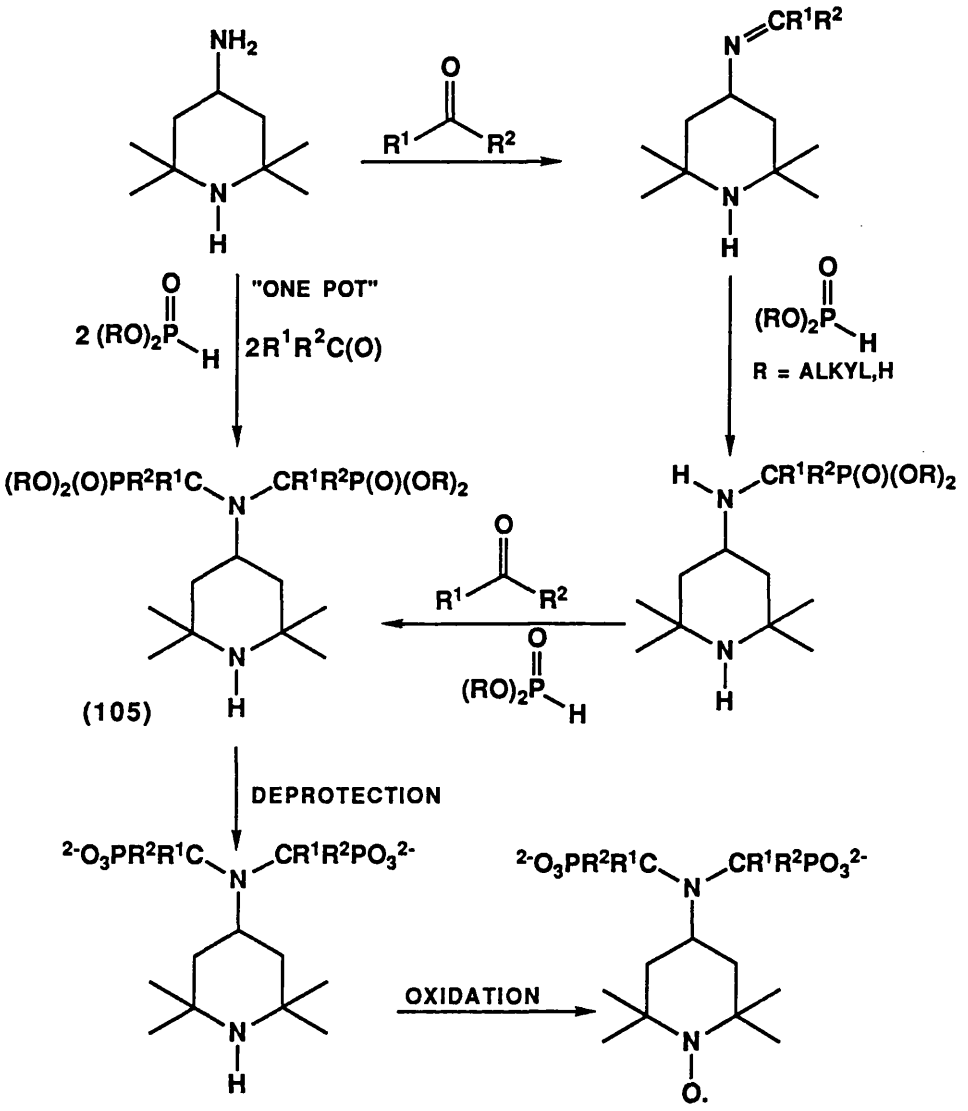


FIG 95

Prior to synthesising the piperidine aminoalkylbisphosphonate (105), tetraethyl ^{t}butylaminomethylphosphonate (106) was prepared, with the aim of using it to develop a general route to analogues of (105).

The synthesis of (106) was carried out according to the method of Fields (1952), Figure 96. The ^{31}P N.M.R. spectrum gave the expected singlet at δ_P 27.8. The ^{1}H N.M.R. showed a quintet for the ethoxy methylene protons, a result of coupling with the three methyl protons and the phosphorus nucleus ($^{3}J_{PH}$ = $^{2}J_{HH}$ = 7 Hz). The phosphonate methylene protons were observed as a doublet due to coupling with the phosphorus ($^{2}J_{PH}$ = 15 Hz). Similarly, the amino proton coupled with the phosphorus to also give a doublet ($^{3}J_{PH}$ = 9 Hz). The identity of (106) was confirmed by high resolution mass spectrometry.

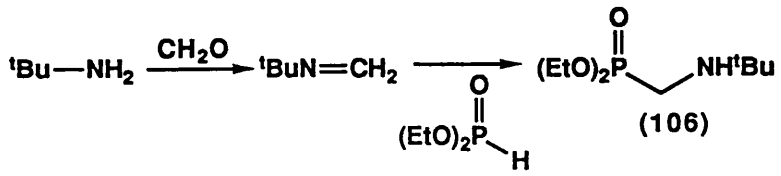


FIG 96

The reaction of (106) with formaldehyde and diethyl phosphite gave the iminobismethylenebisphosphonate (87). The physical data obtained was consistent with that provided by Moedritzer (1973), with a singlet in the ^{31}P N.M.R. at δ_P 27.2.

The ^{1}H N.M.R. showed a doublet for the phosphonate methylene protons, the observed coupling to the phosphorus nucleus ($^{2}J_{PH}$ = 6.5 Hz) was significantly less than that in (106) (15 Hz). This difference was possibly a consequence of the symmetry of the molecule.

The literature method for the synethisis of (87) (Moedritzer 1973) involved the treament of the imine (107) with formladehyde and diethyl phosphite.

This route was thought to involve (106) as an intermediate, as proposed in Figure 97. Another possible route could involve the alcohol (108), potentially formed from diethyl phosphite and formaldehyde. This alcohol could in turn, react with the imine (106) to give (87). However, the reaction to give (108) was found not to occur, see Figure 98.

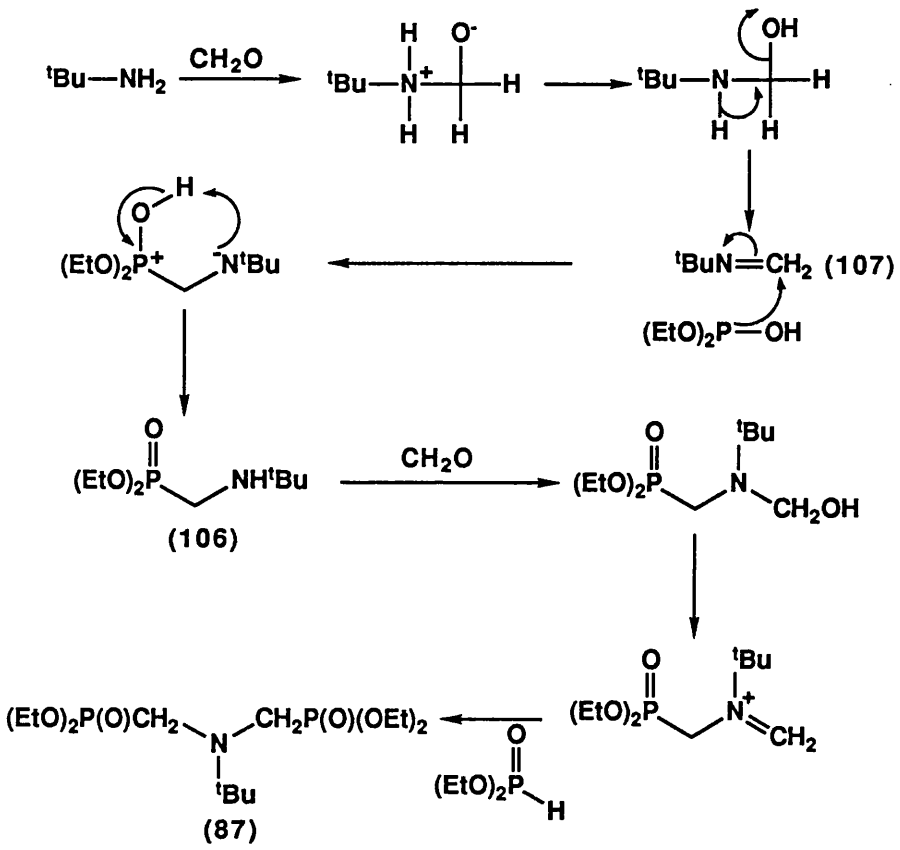


FIG 97

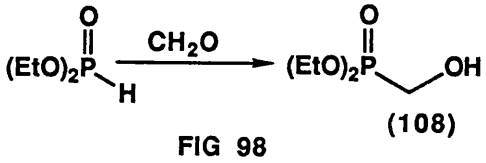


FIG 98

The phosphorylation of (106), either at the amino or methylene group, was attempted in separate reactions with diethyl chlorophosphate and diethyl iodophosphonate. These reactions could have potentially given four different products, depending on the preferred nucleophilic site on (106).

These four potential products are illustrated in Figure 99. Of these (109) and (110) contain only phosphonate groups, whereas (111) also has a phosphoramidate.

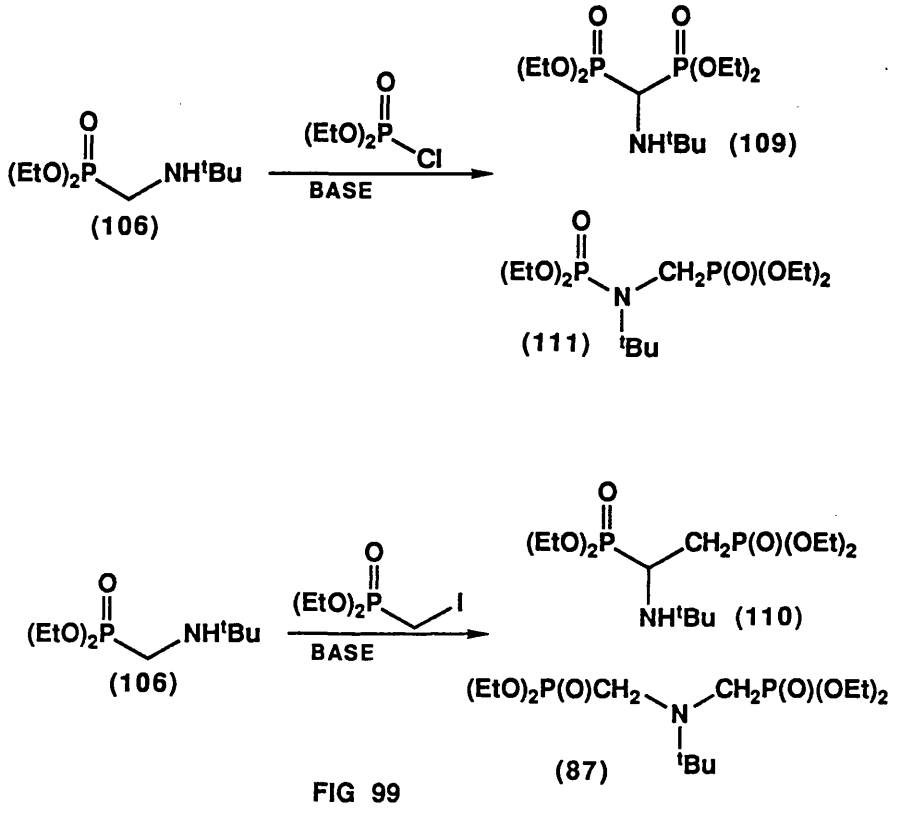


FIG 99

The reaction of the sodium salt of (106) with either diethyl chlorophosphate or diethyl iodophosphonate resulted only in the isolation of (106), with decomposition of the electrophiles occurring during the aqueous work up. The sodium salt was thus considered to be unreactive with the electrophiles employed.

The use of ^{n}butyl lithium as base in the reaction between (106) and diethyl chlorophosphate led to the decomposition of both starting materials. The similar reaction with diethyl iodophosphonate yielded a complex ^{31}P N.M.R. spectrum of unidentified products, with no ^{t}butyl group being observed in the ^{1}H N.M.R. spectrum.

A potential decomposition pathway for the anion of (106), based on similar mechanisms proposed by Redmore (1975) is illustrated in Figure 100.

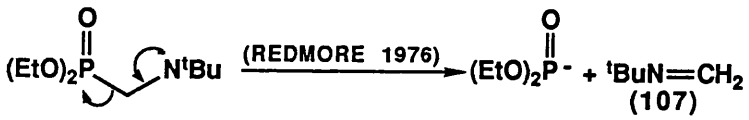


FIG 100

Therefore, modifications of the model structure did not appear to be feasible along the lines explored above.

PIPERIDINE NITROXYL DERIVATIVE

The synthesis of a piperidine based aminoalkylbisphosphonate was attempted *via* a similar route to that used for (87). The imine (112) was formed by reacting 2,2,6,6-tetramethyl-4-aminopyridine and acetaldehyde according to Figure 101. The ^{1}H N.M.R. spectrum showed coupling between the imino proton and methyl group, giving rise respectively to a quartet and a doublet ($^{3}J_{HH}$ = 5 Hz).

The reaction between (112) and diethyl phosphite yielded the aminoalkylphosphonate (113). The low field ^{1}H N.M.R. (90 MHz) of (113) showed two signals for the piperidine methyl groups, due to the different axial and equatorial positions. The ethoxy protons gave rise to a triplet for the methyl protons and a quintet for the methylene protons. This methylene quintet arose from the similar coupling experienced with the phosphorus nucleus and the methyl protons ($^{3}J_{PH} = {}^{3}J_{HH}$ = 7 Hz). The aminoalkyl proton was observed as an unresolved multiplet, a result of coupling to the aminoalkyl methyl group and the adjacent phosphorus.

The highfield ^{1}H N.M.R. spectrum of (113) proved to be complex, possibly a consequence of the unresolved chiral centre at the aminoalkyl carbon. There may also have been restricted rotation about some of the aminoalkylphosphonate bonds.

Less likely reasons may be the protonation of one of the amino nitrogens in the N.M.R. solvent, or the slow inversion of the 4-amino group on the N.M.R. time scale. The molecular weight of (113) was confirmed by mass spectrometry.

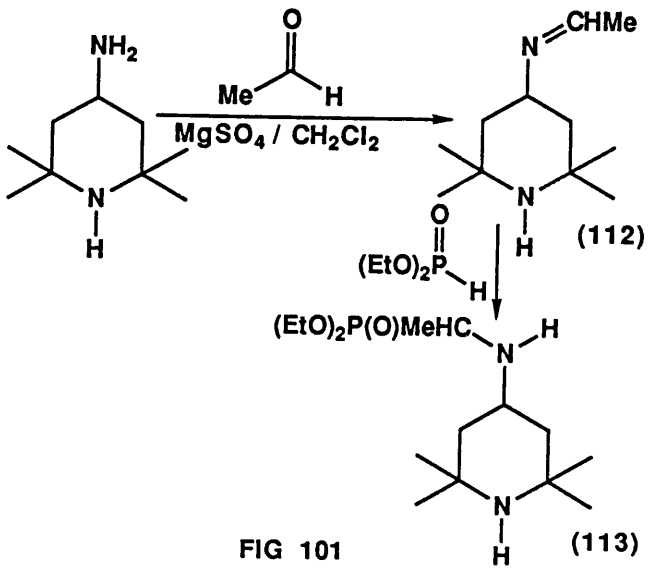


FIG 101

The treatment of (113) with bromotrimethylsilane, in order to remove the phosphorus ester groups, yielded a compound having ^{31}P N.M.R. data consistent with (114). The signal for (114) was in the expected region, to the highfield side of that due to (113), at δ_P -10. The hydrolysis of the reaction mixture with aqueous sodium bicarbonate gave two compounds, with signals at δ_P 25 and δ_P 12 in the ^{31}P N.M.R. The former was consistent with the desired structure (115), see Figure 102.

By analogy with the de-esterifications of similar phosphonates, the resultant salts have ^{31}P N.M.R. chemical shifts close to those of the parent alkyl esters.

The crude reaction mixture was oxidised with aqueous hydrogen peroxide and the presence of a nitroxyl radical confirmed by E.S.R. analysis. The attempted purification of the reaction mixture by ion exchange chromatography yielded two products, neither consistent with the desired product (116). The first fraction isolated contained a nitroxyl radical, identified by E.S.R., but no phosphorus, as judged by N.M.R. The second, higher charged fraction, contained a phosphorus centre, having a resonance in the ^{31}P N.M.R. at δ_P 23, but no nitroxyl moiety. The evidence indicated that the structure had decomposed, either during the oxidation step, or during the reaction with bromotrimethylsilane.

It has been reported that (113) can be oxidised to the corresponding nitroxyl radical (Skowronski 1987), which suggests that in the above case the decomposition occurred during the de-esterification procedure. This route was clearly not practical for the synthesis of (116), with further work being required to determine the actual point of decomposition.

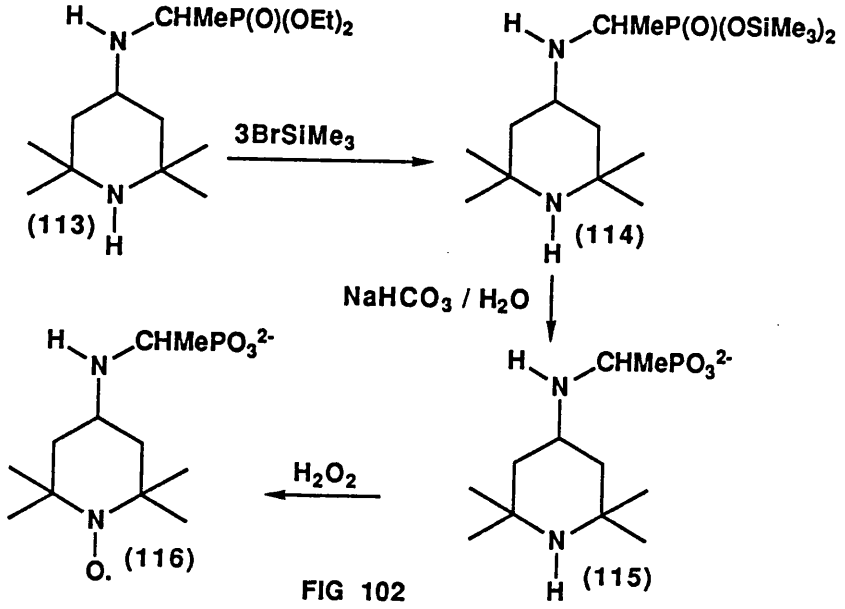


FIG 102

In light of the problem experienced above with the de-esterification procedure, the possibility of using phosphorous acid in place of a dialkyl phosphite was investigated.

A FACILE SYNTHESIS OF THE DESIRED NITROXYL FRAMEWORK

Following a similar method to that used by Moedritzer (1966) for the direct synthesis of α-aminomethylphosphonic acids, 2,2,6,6-tetramethyl-4-aminopiperidine was treated with formaldehyde and phosphorous acid to give (117).

The ^{31}P N.M.R. spectrum showed a singlet with a pH dependant chemical shift at δ_P 9. This singlet was observed to shift to δ_P 17 after the addition of four molar equivalents of sodium hydroxide.

By analogy with the work of Moedritzer, (117) was thought to exist in an aqueous solution as (118). The addition of four equivalents of base led to the neutralisation of the nitrogen. The removal of the quaternary nature of the nitrogen altered the shielding of the phosphorus nuclei and results in a change in the chemical shift. The addition of more sodium hydroxide did not result in any further change in the chemical shift, since the now trivalent nitrogen could undergo no further alteration in its electronic structure, see Figure 103.

The proton coupled ^{31}P N.M.R. spectra of (117) and (118) showed a triplet from the coupling of the phosphorus nuclei with the aminomethyl protons ($^2J_{PH}$ = 15Hz).

The ^1H N.M.R. spectrum of the sodium salt of (117) showed the expected signals for the piperidine protons, with the aminomethyl protons coupling to the phosphorus to give a doublet ($^2J_{PH}$ = 15 Hz).

A crude sample of (117) was oxidised to form the corresponding nitroxyl radical (119). Two distinct signals were observed to form in the ^{31}P N.M.R. spectrum of the reaction mixture at δ_P 6 and δ_P 3.2.

After three hours, the reaction was terminated and yielded a mixture of the two phosphorus containing products. The chemical shifts (δ_P 6 and 3.2) were not in the expected region of the spectrum, i.e. close to that observed for (117), at approximately δ_P 17.

Purification of the reaction mixture by ion exchange chromatography gave three phosphorus containing products. The first fraction isolated was orange coloured, contained a nitroxyl centre and had a chemical shift of δ_P 6.2 in the ^{31}P N.M.R. This minor fraction was initially thought to be the phosphonate (120), although no direct evidence was obtained. The second, colourless fraction contained no nitroxyl centre and had a ^{31}P N.M.R. chemical shift of δ_P 1.4, possibly corresponding to inorganic phosphate.

The third and major fraction had an orange colour, a nitroxyl centre and a broad signal in the ^{31}P N.M.R. at δ_P 3.8. This higher charged fraction was analysed by ^{1}H N.M.R., but gave rise to broad indiscernable signals. The evidence indicated this third fraction to be (119), with (120) and inorganic phosphate resulting from its partial decomposition. However, mass spectral analysis by negative ion F.A.B. gave a molecular ion consistent with the structure (121), presumably a result of the over-oxidation of (119), see Figure 103.

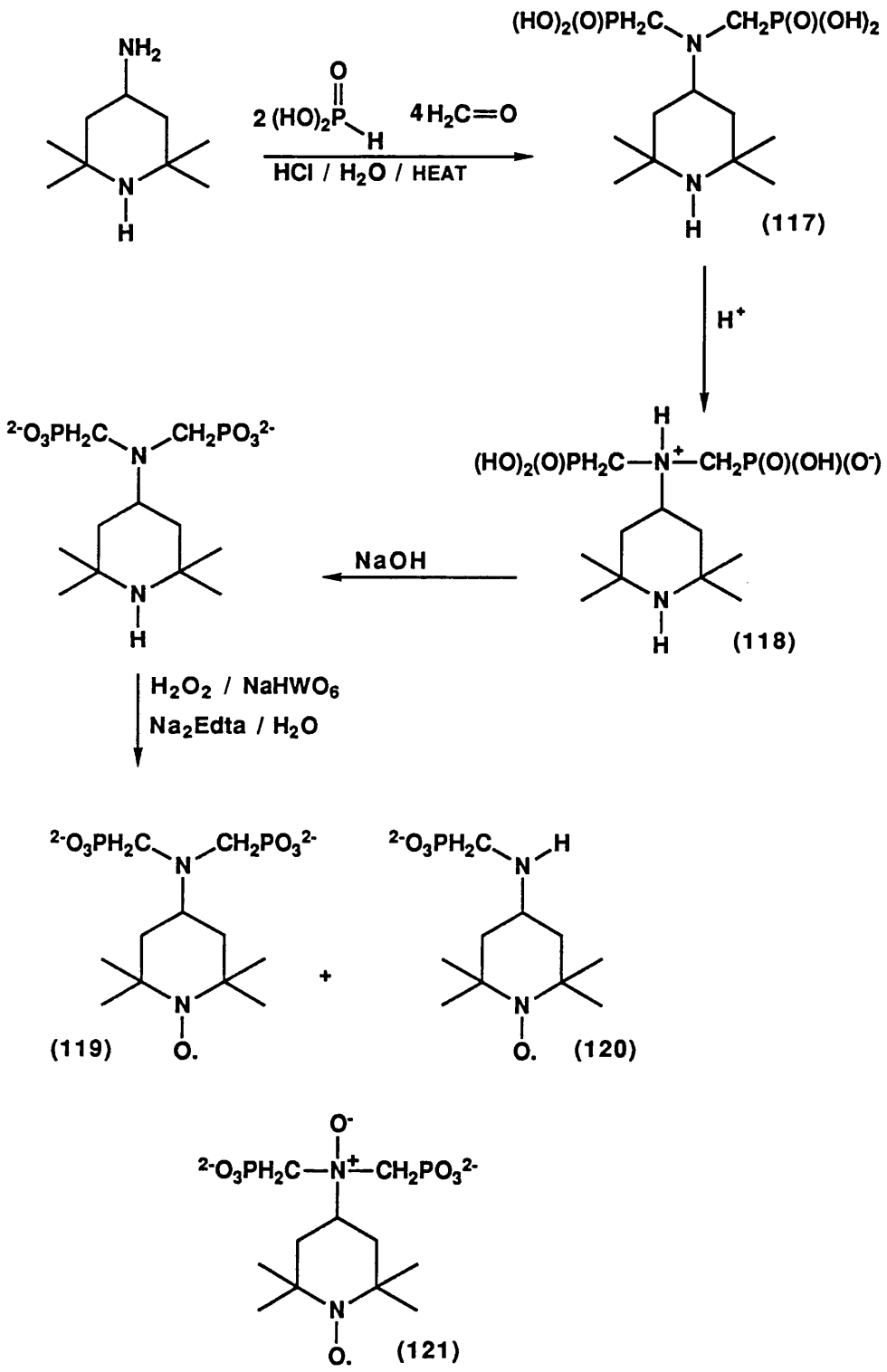

NH2
2 (HO)2P(O)H
4 H2C=O
HCl / H2O / HEAT
(HO)2(O)PH2C
CH2P(O)(OH)2
(117)
H+
(HO)2(O)PH2C—N+—CH2P(O)(OH)(O-)
(118)
NaOH
2-O3PH2C
CH2PO32-
H2O2 / NaHWO6
Na2Edta / H2O
(119)
(120)
(121)

FIG103

Although not of the intended structure, the compound (121) did contain the required nitroxyl centre and phosphonate groups. A practical advantage of (121) was that it could be synthesised in a good overall yield (40%) and on a reasonable scale. As a result, (121) was investigated as a bone agent for nuclear medicine.

BONE SPECIFITY OF (121)

In a similar experiment to those undertaken with (59) and (64), (121) was tested as a potential bone imaging agent. However, (121) was considered not to be a potential bone agent due to its poor selectivity for bone in vivo, having high kidney retention and low urinary excretion. The nitroxyl (121) was found to complex well to technecium in vitro, but the complex was not bone specific in vivo. The results are summarised and compared to methylenebisphosphonate in the table below, with a photographic representation in Figure 104.

	%BONE	BONE / MUSCLE	BONE / BLOOD	BONE / LIVER+SPLEEN
(8)	50	280	90	120
(121)	8.2	16.7	1.5	2.6

The nitroxyl aminoalkylbisphosphonate is currently awaiting tests as a potential contrast agent for N.M.R. imaging studies.

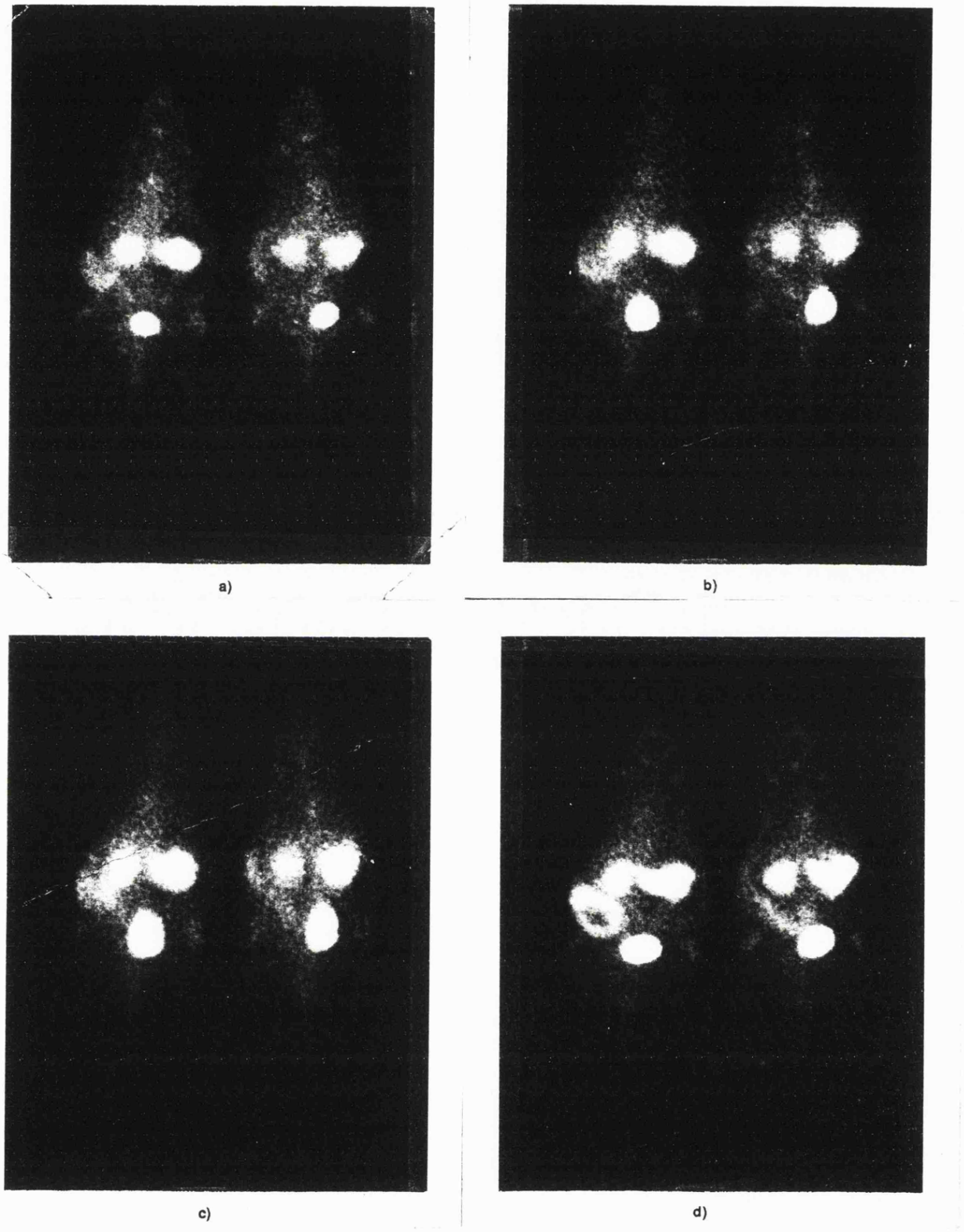


FIG 104-PHOTOGRAPHS SHOWING UPTAKE OF (121) AFTER;
a) 10 MINS
b) 20 MINS
c) 30 MINS
d) 60 MINS

IMIDOBISPHOSPHONATE ANALOGUES OF AMINO ACIDS AS POTENTIAL BONE AGENTS

The proposed route for the synthesis of imidobisphosphonates from functionalised amines, specifically from amino acids, is shown in Figure 105. Glycine, with the carboxyl group protected as an ester, was used as a model for other amino acids.

It was envisaged that a halotrimethylsilane would selectively remove the phosphorus esters and not the carboxyl ester. The possibility of incorporating nitroxyl radicals into the proposed structures was considered most likely to be achieved at the free acid group of the amino acid moiety.

The saponification of N-phosphoroamino acid esters has been achieved under basic conditions (Zervas 1955). Therefore, it was intended to remove the carboxyl ester from the imidobisphosphonate product under similar conditions.

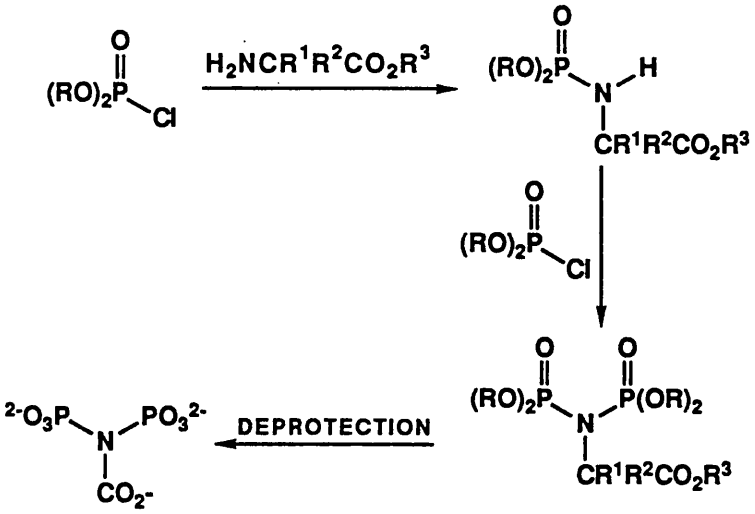


FIG 105

The reaction of diethyl chlorophosphate with methyl gylcine was found to give the phosphoramidate (122). The product isolated from the reaction mixture was a single entity in the ^{31}P N.M.R. at δ_P 8.5 and as a result was not purified further. The ^{1}H N.M.R. showed that the methylene protons coupled to the phosphorus to give a doublet ($^{3}J_{PH}$ = 9 Hz). The ethoxy ester methylene protons were observed as a quintet, from the equivalent coupling experienced with the methyl protons and the phosphorus ($^{3}J_{PH}$ = $^{3}J_{HH}$ = 7 Hz).

The product (122) was then treated with sodium hydride and diethyl chlorophosphate, in an analogous reaction to those carried out in Chapter One, see Figure 106. The imidobisphosphonate (123) was formed together with tetraethyl pyrophosphate and an unidentified product at δ_P 0.2 in the ^{31}P N.M.R.

The desired product (123) was purified by silica gel flash chromatography showing a singlet at δ_P 3 in the ^{31}P N.M.R. spectrum. Infrared analysis of (123) showed a carbonyl stretching band at 1750 cm^{-1}, compared to 1740 cm^{-1} for (122) and 1730 cm^{-1} for the glycine methyl ester. The presence of the phosphorus group had the effect of weakening the carbonyl bond by withdrawing electron density, resulting in a higher wavenumber for the stretching band. The ^{1}H N.M.R. spectrum for (123) showed coupling between the glycine methylene group and the two equivalent phosphorus nuclei, giving rise to a triplet ($^{3}J_{PH}$ = 13.5 Hz). The ethoxy methylene protons produced a complex spin pattern which was not resolved. The identity of (123) was confirmed by high resolution mass spectrometry.

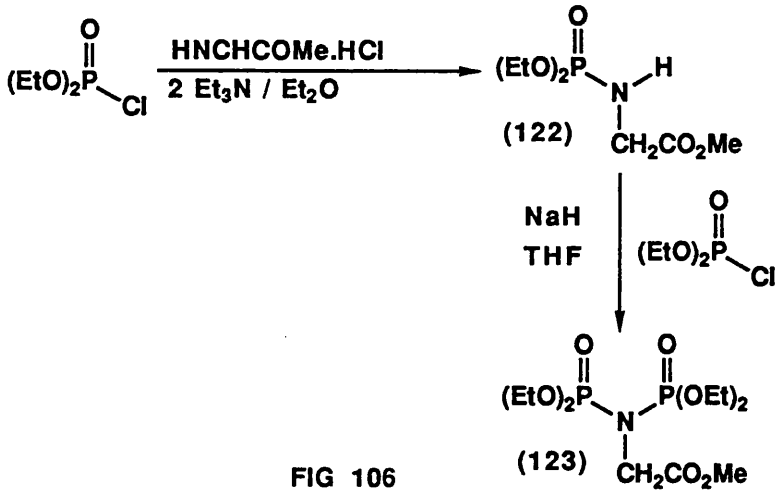


FIG 106

DE-ESTERIFICATION OF (122) AND (123)

The phosphoramidate (122) was treated with bromotrimethylsilane to give the corresponding trimethylsilyl esters (124), see Figure 107. The reaction was monitored by ^{31}P N.M.R. and was observed to give a singlet at δ_P -10.8. The proton coupled spectrum gave a triplet ($^3J_{PH}$ = 16 Hz) due to the coupling experienced with the two methylene protons.

The hydrolysis of the trimethylsilyl esters was carried out in an aqueous sodium bicarbonate solution. This resulted in a signal being observed at δ_P 8.3 in the ^{31}P N.M.R. spectrum, the proton coupled spectrum again showed a triplet. This product (125) was observed to decompose to a single compound, at δ_P 3, after standing in an aqueous solution at room temperature for twelve hours.

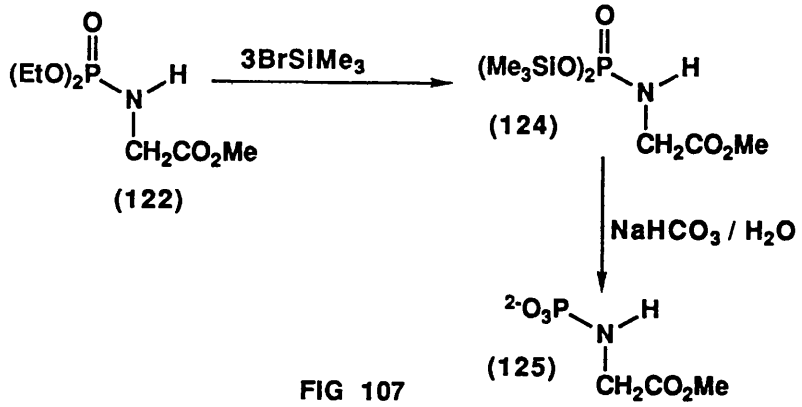


FIG 107

The imidobisphosphonate (123) was similarly treated with bromotrimethylsilane to give (126), characterised by a singlet at δ_P -14.7 in the ^{31}P N.M.R. with the expected triplet in the proton coupled spectrum ($^3J_{PH}$ = 12 Hz). The hydrolysis of (126) in aqueous sodium bicarbonate yielded a mixture of three products with signals in the ^{31}P N.M.R. at δ_P 2.8, δ_P 5 and δ_P 8. The major signal at δ_P 5 corresponded to (127), with a triplet in the proton coupled spectrum ($^3J_{PH}$ = 15 Hz) showing the presence of the glycine moiety, see Figure 108. The variation in the size of the phosphorus-hydrogen coupling constant in (123), (126) and (127) reflects the differing electronic distribution around the phosphorus nuclei with the different substituents, i.e., alkyl ester, silyl ester and free acid/salt.

On standing in an aqueous solution at pH 9 for twelve hours, the only product observed was one at δ_P 2.8. The decomposition of (127) presumably involved (125) (δ_P 8) as an intermediate to give a common product at approximately δ_P 3. Purification was attempted by ion exchange chromatography, in order to remove any impurities and hopefully confer some stability. Because of the known acid lability of phosphorus-nitrogen bonds, (127) was expected to be more stable under the neutral or slightly basic conditions used in the ion exchange buffer solution.

However, a mixture of products with chemical shifts in the range δ_P 0 to δ_P 3 was observed in the ^{31}P N.M.R. of the fraction isolated from the ion exchange column. Therefore, the purification and isolation of (127) did not appear feasible.

At more alkaline pH's (pH 12-13), (127) appeared to be more stable, however, such high pH's could not be conveniently used for ion exchange chromatography. Furthermore, such pH sensitive structures would rapidly decompose <u>in vivo</u> and are unlikely to be useful as bone agents.

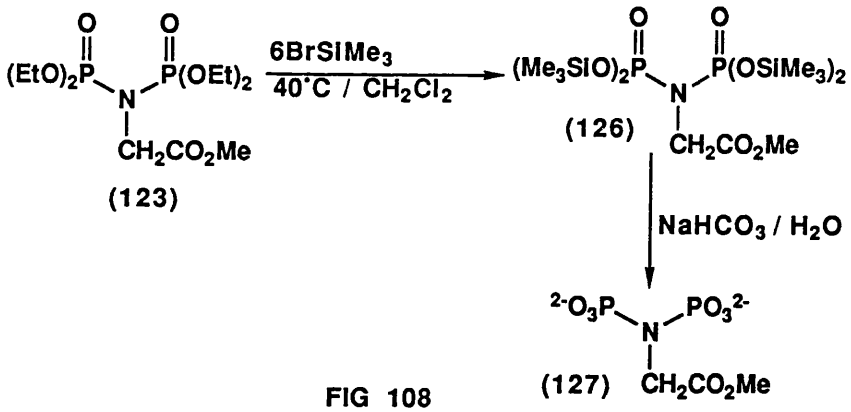


FIG 108

POSSIBLE MECHANISM FOR DECOMPOSITION OF GLYCINE DERIVATIVES

The ^{31}P N.M.R. data obtained suggested that both (125) and (127) decomposed to inorganic phosphate, with a single resonance at δ_P 3. It has been shown that N-phosphoroglycine (128) was susceptible to hydrolysis, especially in acidic solutions (Clark 1966, Winnick 1947, Chanley 1963, Zervas 1955). A mechanism for the decomposition of (128) has been proposed (Clark 1966) and is illustrated in Figure 109.

Similar pathways could be applied to the decomposition of both (125) and (127). The decomposition of (125) and (127) apparently occurs at a higher pH, pH 9, compared to that for (128), however the mechanisms by which they decompose are presumably analogous.

The alkaline hydrolysis of the trimethylsilyl ester could have also led to the hydrolysis of the methyl carboxyl ester giving (129) and (130). An investigation of the ^{1}H N.M.R. of the bisphosphorylated derivative (127) or (130) showed no signal indicative of a methyl ester, thus supporting this rationale. Therefore, the free carboxylic groups present in (129) and (130) would provide increased nucleophilicity compared to (128). The observed decomposition is thus more likely to occur. The different properties shown by (128), (129) and (130) render the phosphorylated glycine derivatives unstable, possibly decomposing via similar mechanisms, as shown in Figure 109.

As a result of the observed instability of these phosphorylated glycine analogues, no further work was attempted in this area.

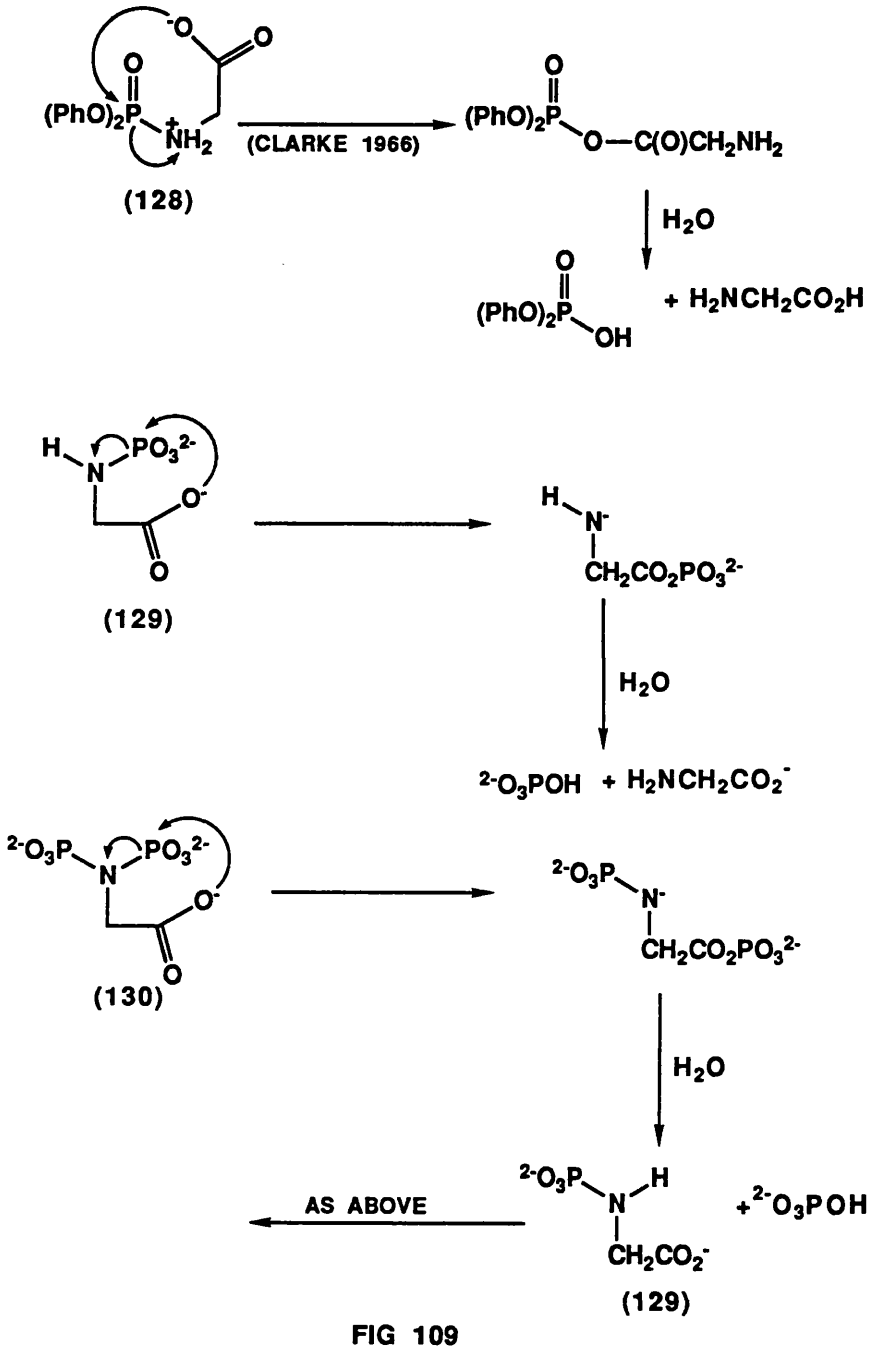


FIG 109

OVERALL CONCLUSIONS

Of the molecules investigated, only (121) was tested as a potential bone agent, but was found to have poor bone selectivity *in vivo*. Since bone selectivity is only one area for contrast enhancement, (121) is awaiting trials as a potential contrast agent for N.M.R. imaging.

The other compounds investigated either did not provide suitable routes for larger scale syntheses, as in the case of (103) or could not be synthesised at all. The glycine derivative (127), although synthesised, was unstable at pH values acceptable for *in vivo* experiments.

The relatively tight constraints of yield and scale imposed on the syntheses undertaken negated the fuller investigation of some interesting chemistry. For example, the attempted alteration of the site of electrophilic attack on the phosphonoacetate and thioacetate structures, i.e. at the phosphonate oxygen or methylene carbon, would appear to be worthy of further investigation.

C H A P T E R F I V E

EXPERIMENTAL

Methods

^{1}H N.M.R. were recorded on a Varian EM390 spectrometer at 90 MHz. Routine ^{31}P N.M.R. spectra were recorded on a Jeol FX60 spectrometer at 24.15 MHz and highfield ^{31}P N.M.R. spectra were recorded on a Bruker AM300 spectrometer at 121.5 MHz. Chemical shifts are reported in parts per million (δ) and are positive for resonances downfield of the reference = tetramethylsilane for ^{1}H N.M.R. and external 85% H_3PO_4 for ^{31}P N.M.R. ^{31}P N.M.R. spectra are proton decoupled unless otherwise noted. Ultraviolet and visible spectrophotometric measurements and assays were performed on a Shimadzu UV-240 Graphicord spectrophotometer with a Shimadzu PR-1 graphic printer, the U.V. cell jackets were temperature equilibrated by a Lauda thermocoupled water bath. Infrared spectra were recorded on a Perkin Elmer 298 instrument. Mass spectra were obtained on either a VG Micromass 168 or a high resolution ZAB-E (SERC Mass Spectrometry Centre, Swansea) instrument. Melting points were measured on a Kofler Hotstage apparatus.

Thin-layer chromatography was performed on Merck 5554 plates and flash column chromatography carried out with Merck Silica gel 60. Ion exchange chromatography was carried out using either DEAE sephadex (A-25) or Dowex 1 x 8-200 resin.

Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling gaseous CO_2 through the appropriate triethylamine and water mixture until the solution was at the required pH. Ion exchange resins were equilibrated with the lowest buffer concentration employed in the respective linear gradient before use. The products were detected by their U.V. chromophores. Pooled fractions were evaporated *in vacuo* (2-5 mmHg) on a Buchi rotary evaporator. Fractions were desalted of excess TEAB by the repeated addition and evaporation of dry methanol.

Materials

^{18}O water was obtained from Amersham International. All other chemicals, enzymes and cofactors were from Aldrich, Sigma or BDH and were used as received unless otherwise noted. All solvents were distilled (under nitrogen where appropriate) before use and stored over activated 4A molecular sieves. Benzene, diethyl ether, dioxane, and tetrahydrofuran were distilled from sodium, pyridine, dimethylsulphoxide and acetonitrile from calcium hydride. N,N-dimethylformamide was stirred over activated magnesium sulphate and decanted before distillation. Methanol and ethanol were dried and distilled from their respective magnesium alkoxides prepared *in situ*. Tri-^{n}butylamine, triethylamine, tri-^{n}octylamine were distilled from calcium hydride and passed through alumina prior to use. Distilled water was purified by a Milli-Q micropore system.

N-(Diethoxyphosphinoyl)-O-benzylhydroxylamine (24)

O-Benzylhydroxylamine hydrochloride (0.45 g, 2.8 mmol) and anhydrous triethylamine (0.8 ml, 5.7 mmol) were stirred in dioxane (10 ml) under a nitrogen atmosphere and at room temperature. Diethyl chlorophosphate (0.49 ml, 3.4 mmol) in dioxane (5 ml) was added dropwise *via* a pressure equalized dropping funnel. The reaction was stirred until no further change was observed by ^{31}P N.M.R. analysis. The reaction mixture was poured into water (100 ml) and the product extracted with chloroform. The organic extracts were dried over magnesium sulphate, filtered and the solvent removed *in vacuo* to yield the crude product as a colourless oil. The product was purified by silica gel flash chromatography, eluting with ethyl acetate (R.f. 0.38).

Yield = 0.63 g (87%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.35 (6H, t J_{HH} = 7 Hz), 4.1 (4H, quin, J_{HH} = 7 Hz), 4.75 (2H, s), 7.35 (5H, m, aromatic)

^{1}H N.M.R.(300 MHz): $\delta(CDCl_3)$: 1.31 (6H, dt, J_{HH} = 7 Hz, J_{PH} = 0.77 Hz), 4.1 (4H, m, unresolved), 4.76 (2H, s), 7.33 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CDCl_3)$:7.46 (s)

I.R. ν (cm^{-1}) 3180, 3150 (NH), 2980, 2900, 1435, 1385, 1365, 1235 (P=O), 1160, 1020, 965

The following procedure was followed for the preparation of compounds (25), (26), and (27).

N-(Diethoxyphosphinoyl)-N-methyl-O-benzylhydroxylamine (25)

Sodium hydride (60% dispersion in oil, 0.015 g, 0.36 mmol) was stirred in dioxane (20 ml) under a nitrogen atmosphere at room temperature. N-(Diethoxyphosphinoyl)-O-benzylhydroxylamine (24) (0.06 g, 0.25 mmol) in dioxane (5 ml) was added dropwise and stirring continued for 90 minutes. Methyl iodide (17 µls, 0.275 mmol) was then added and the reaction mixture stirred for a further 90 minutes. The reaction mixture was poured into water (100 ml) and the product extracted with chloroform. The organic extracts were dried over magnesium sulphate, filtered and the solvent removed <u>in vacuo</u> to give a brown oil. The product was purified by silica gel flash chromatography, eluting with chloroform.

Yield = 0.05 g (74%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 2.8 (3H, d, J_{PH} = 12 Hz), 4.1 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 5 (2H, s), 7.2 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CDCl_3)$: 8.8 (s)

N-(Diethoxyphosphinoyl)-N-acetyl-O-benzylhydroxylamine (26)

Anion formation on N-(Diethoxyphosphinoyl)-O-benzylhydroxylamine (24) (0.31 g, 1.17 mmol) was carried out using sodium hydride (60%) (0.06 g, 1.6 mmol) in THF at 0°C for 90 minutes. Acetyl chloride (80 µl, 1.17 mmol) was added and the reaction mixture allowed to warm to room temperature. Stirring was continued for 90 minutes, followed by work up.

Yield = 0.32 g (91%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 2.2 (3H, s), 4.15 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 4.9 (2H, s), 7.3 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CDCl_3)$: -3.2 (s)

M/Z (low resolution), 301 (m^+)

M/Z (high resolution), 301.1068 (m^+) (found) 301.1078 (m^+) (expected) 259 (16%) 91 (100%)

I.R. ν (cm^{-1}) 2970, 2915, 2900, 1695 (C=O), 1450, 1360, 1275, 1265 (P=O), 1255, 1240, 1155, 1020

Tetraethyl N-benzoxyimidobisphosphonate (27)

The synthesis was carried out using sodium hydride (60%) (0.19 g, 4.8 mmol), N-(Diethoxyphosphinoyl)-O-benzylhydroxylamine (24) (0.83 g, 3.2 mmol) and diethyl chlorophosphate (0.5 ml, 3.2 mmol). The crude product was purified by silica gel flash chromatography, eluting with ethyl acetate, (R.f. = 0.25) to remove the principle by-product, tetraethyl pyrophosphate (δ_P-13.1).

Yield = 0.97 g (77%)

^1H N.M.R.: $\delta(CDCl_3)$: 1.3 (12H, t, J_{HH} = 7 Hz), 4.2 (8H, dq, $J_{HH} = J_{PH}$ = 7Hz), 5 (2H, s), 7.2 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CDCl_3)$: 0.2 (s)

M/Z (low resolution), 395 (m^+) (found), 395 (expected) 259 (100%) 180 (50%)

I.R. ν (cm^{-1}) 3520, 3480, 2980, 2930, 1475, 1440, 1390, 1370, 1275 (P=O), 1160, 1020

Tetraethyl imidobisphosphonate (28)

Tetraethyl N-benzoxyimidobisphosphonate (27) (0.36 g, 0.97 mmol) and palladium on charcoal (0.1 g) were stirred in methanol under an atmosphere of hydrogen. The reaction was monitored by T.L.C. on silica gel, eluting with ethyl acetate, (R.f. (27) = 0.25, R.f. product = 0 (stained in iodine)). The reaction mixture was filtered to remove the catalyst and the solid washed with methanol. The filtrate was evaporated <u>in vacuo</u> to give the desired product as a brown oil. The similar hydrogenolysis of tetraethyl N-benzylimidobisphosphonate (45) gave the same product.

Yield = 0.23 g (82%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (12H, t, J_{HH} = 7 Hz), 4.15 (8H, dq, $J_{HH} = J_{PH}$ = 7 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 0.2 (s)

M/Z (low resolution) 289 (m^+)

M/Z (high resolution) 289.0843 (m^+) (found), 289.0844 (m^+) (expected) 262 (72%), 234 (52%), 206 (28%), 98 (28%), 80 (62%)

I.R. ν (cm^{-1}) 3460 (NH), 2980, 2900, 2720, 1630, 1470, 1440, 1380, 1240 (P=O), 1160, 1020

Diethyl aminophosphonate (29)

The same procedure as described for the preparation of tetraethyl imidobisphosphonate (28) was followed in order to hydrogenolyse N-(Diethoxyphosphinoyl)-O-benzylhydroxylamine (24) (0.2 g, 0.79 mmol).

Yield = 0.11 g (90%)

m.p. 48-50°C (literature 50-51°C)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 3.75 (2H, br d), 4.1 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 10.5 (s)

O-Trimethylsilylhydroxylamine

Prepared according to the method of Douboudin et al (1974).

Hydroxylamine hydrochloride (25.1 g, 0.36 mol) (dried over phosphorus pentoxide) was added to a stirred solution of tripropylamine (70.2 ml, 0.37 mol) and hexamethyldisilizane (36.8 ml, 0.175 mol) with the exclusion of moisture. The reaction mixture was stirred at room temperature for 24 hours. The solid was filtered off with suction under a steady stream of nitrogen and washed with a small volume of xylene.

The product was distilled in a Spaltrohr distillation apparatus and stored in a stoppered vessel under nitrogen.

Yield = 21 g (55%)

b.p. 50°C (100 mmHg), literature 60°C (160 mmHg)

^{1}H N.M.R.: $\delta(CDCl_3)$: 0 (9H, s) 5 (2H, s)

I.R. ν (cm^{-1}) 3320 (NH), 3260, 2960, 2900, 1590, 1250, 1185, 1060, 1050,

N-(Diethoxyphosphinoyl)-O-trimethylsilylhydroxylamine (30)

O-trimethylsilylhydroxylamine (1.5 g, 14.3 mmol) and triethylamine (2.2 ml, 15.7 mmol) were stirred in diethyl ether (50 ml) under a nitrogen atmosphere at room temperature. Diethyl chlorophosphate (2 ml, 14.3 mmol) in diethyl ether (10 ml) was added dropwise and stirring continued for 48 hours. The reaction mixture was filtered under a stream of nitrogen and the solid triethylammonium hydrochloride washed with diethyl ether (20 ml). The solvent was removed <u>in vacuo</u> to give a colourless oil. Further purification was not feasible due to the lability of the trimethylsilyl protecting group, resulting in production of the free hydroxylamine derivative.

The product was stored in a sealed vessel under nitrogen and used without further purification.

Yield = 2.94 g (85%)

^1H N.M.R.: $\delta(CDCl_3)$: 0.2 (9H, s), 1.3 (6H, t, J_{HH} = 7 Hz), 4.1 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 6 (1H, d, J_{PH} = 9Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 10 (s)

N-(Diethoxyphosphinoyl)hydroxylamine (31)

N-(Diethoxyphosphinoyl)-O-trimethylsilylhydroxylamine (30) was stirred in methanol at room temperature for 3 hours, after which no further change was observed in the ^{31}P N.M.R. spectrum. The methanol was removed <u>in vacuo</u> to give the desired product.

^1H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 4.1 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 6.2 (2H, br d)

^{31}P N.M.R.: $\delta(Et_2O)$: 9.47 (s)

I.R. ν (cm^{-1}) 3200 (NH, OH), 2980, 2900, 1465, 1455, 1440, 1390, 1365, 1230, 1220 (P=O)

N-(Diethoxyphosphinoyl)-N-methyl-O-trimethylsilylhydroxylamine (34)

N-(Diethoxylphosphinoyl)-O-trimethylsilylhydroxylamine (30) (0.28 g, 1.19 mmol) was stirred in THF (2 ml) under a nitrogen atmosphere at -78°C. ^{n}Butyl lithium (2.6 M, 0.46 ml, 1.19 mmol) was added and stirring continued at -78°C for 25 minutes. Methyl iodide (0.09 ml, 1.4 mmol) was then added and the reaction vessel allowed to warm to room temperature. The solvent was then removed in vacuo to give a brown oil. The product was purified by silica gel flash chromatography, eluting with ethyl acetate, (R.f. = 0.7).

^{1}H N.M.R.: $\delta(CDCl_3)$: 0 (9H, s), 1.2 (6H, t, J_{HH} = 7 Hz), 2.8 (3H, d, J_{PH} = 12 Hz), 4.8 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 9.4 (s)

Diethyl phosphate

N-(Diethoxyphosphinoyl)hydroxylamine (31) was treated with water and was observed to give diethylphosphate by ^{31}P N.M.R. analysis, the identity of the product being confirmed by comparison with an authentic sample.

^{31}P N.M.R.: $\delta(H_2O)$: 0.8 (s)

^{18}O-labelled hydrolysis experiment

N-(Diethoxyphosphinoyl)hydroxylamine (31) (28.4 mg 0.168 mmol) was treated with dioxane (0.2 ml) and ^{18}O labelled water (96%, 0.2 ml), yielding diethyl phosphate after 3 days.

The product was purified by ion exchange column chromatography on DEAE Sephadex, eluting with a linear salt gradient (0-150 mM triethylammonium bicarbonate) and isolated as the triethylammonium salt. The degree of ^{18}O incorporation was determined by ^{31}P N.M.R. analysis at 121.5 MHz (300 MHz for ^{1}H).

^{31}P N.M.R.: $\delta(H_2O)$: 0.8 (s)

^{31}P N.M.R. (121.5 MHz): $\delta(H_2O)$: -0.1073 (P(O)) (30%) -0.1363 (P(^{18}O) (70%)

Hydroxylamine

Prepared according to the method of Hurd (1939).

Sodium butoxide was prepared by dissolving sodium (5.05 g, 0.219 mol) in ^{n}butyl alcohol (66 ml).

Hydroxylamine hydrochloride (14.936 g, 0.215 mol) and solid phenolphthalein (3 mg) were stirred with ^{n}butyl alcohol (25 ml) under an atmosphere of nitrogen at room temperature. After 10 minutes, the butoxide solution was introduced at such a rate that the colour of the indicator never predominated. A pressure equalised dropping funnel was used to introduce the butoxide solution, heating being required to prevent solidification of the butoxide. After 3 hours, a small quantity of hydroxylamine hydrochloride was introduced in the reaction mixture and stirring continued until the solution became colourless.

The solid sodium chloride was collected by filtration, washed with ^{n}butyl alcohol (5 ml) and then with diethyl ether (4 x 3 ml). The filtrate was placed in a tightly stoppered flask, and cooled to -10°C when crystals of the product were produced. The product was collected quickly by filtration, washed with cold diethyl ether and dried in a desiccator. The product was stored in a stoppered vessel at -40°C prior to use.

Attempted phosphorylation of hydroxylamine

Free hydroxylamine (0.36 g, 11 mmol) was stirred in benzene (5 ml) under an atmosphere of nitrogen at 5°C.

Diethyl chlorophosphate (0.71 ml, 5.5 mmol) in benzene (2 ml) was added dropwise over 20 minutes to the stirred reaction mixture. The reaction was allowed to warm to room temperature and stirred for a further 1 hour. The reaction mixture was filtered and a brown solid collected which was washed into a separate vessel with water. The benzene solution contained unreacted diethyl chlorophosphate and tetraethyl pyrophosphate, the aqueous layer containing diethyl phosphate. The identity of these products was confirmed by comparison of their observed ^{31}P N.M.R. shifts with those of authentic samples.

N-(Diethoxyphosphinoyl)-O-(diethoxyphosphinoyl)hydroxylamine (35)

Hydroxylamine hydrochloride (2.18 g 31.4 mmol) and triethylamine (13.6 ml, 97.4 mmol) were stirred under a nitrogen atmosphere in dioxane (60 ml) at room temperature. Diethyl chlorophosphate (9.07 ml, 62.8 mmol) was added dropwise and the reaction stirred at room temperature for 48 hours. The reaction mixture was poured into water (200 ml) and neutralised with a small amount of dilute acid, and the products were extracted with ethyl acetate. The organic extracts were dried over magnesium sulphate, filtered and the solvent removed in vacuo. The product was purified by silica gel flash chromatography eluting with ethyl acetate (R.f. = 0.25). The major contaminant was tetraethyl pyrophosphate.

Yield = 4.5 g (46.9%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.35 (12H,t, J_{HH} = 7 Hz), 4.2 (8H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 8.25 (1H, d, J_{PH} = 8 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 6.6 (d, J_{PP} = 19.5 Hz, (P-N)), 1.8 (d, J_{PP} = 19.5 Hz, (P-O))

M/Z (low resolution) 305 (m^+)

M/Z 305.0751 (found), 305.0793 (expected), 161 (22%), 126 (84%), 98 (98%), 80 (100%)

I.R. ν (cm^{-1}) 3480 (NH), 3120, 2980, 2900, 2860, 1470, 1440, 1385, 1365, 1250 (P=O), 1150, 1040, 1020

Diethyl N-benzylphosphoramidate (42)

Benzylamine (10 g, 93 mmol) was stirred in diethyl ether (50 ml) under a nitrogen atmosphere at room temperature. Diethyl chlorophosphate (6.7 ml, 46 mmol) in diethyl ether (15 ml) was added dropwise with stirring. After 12 hours the reaction mixture was poured into water (250 ml) and the product extracted with diethyl ether. The organic extracts were dried over magnesium sulphate, filtered and the solvent and excess benzylamine were removed <u>in vacuo</u> to give a light brown oil.

Yield = 10.69 g (95%)

^{1}H N.M.R.: δ($CDCl_3$): 1.25 (6H, t, J_{HH} = 7 Hz)3.6-4.2 (7H; 4H, dq, J_{HH} = J_{PH} = 7 Hz, 2H, d , J_{PH} = 12 Hz, 1H, dt, partially resolved), 7.2 (5H, m, aromatic)

^{1}H N.M.R. (300 MHz): δ($CDCl_3$): 1.24 (6H, dt, J_{HH} = 6.5 Hz, J_{PH} = 0.7 Hz), 3.99 (7H, m, unresolved), 7.3 (5H, m, aromatic)

^{31}P N.M.R.: δ(Et_2O): 9.48 (s)

I.R. ν (cm^{-1}) 3220 (NH), 2980, 2900, 1625, 1600, 1435, 1390, 1360, 1220 (P=O), 1030, 950

The procedure described for the synthesis of N-(Diethoxylphosphinoyl)-N-methyl-O-benzylhydroxylamine (25) was followed for the preparation of (43), (44) and (45).

Diethyl N-methyl-N-benzyphosphoramidate (43)

Diethyl N-benzylphosphoramidate (42) (0.5 g, 2.05 mmol), sodium hydride (0.1 g, 2.5 mmol) and methyl iodide (0.13 ml, 2.05 mmol) were used in a solvent of THF. The isolated product was a brown oil.

Yield = 0.49 g (92%)

^1H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 2.5 (3H, d, J_{PH} = 9 Hz), 4 (6H; 4H, dq, $J_{PH} = J_{HH}$ = 7 Hz, 2H, d, J_{PH} = 12 Hz) 7.25 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CHCl_3)$: 10.48 (s)

Diethyl N-acetyl-N-benzylphosphoramidate (44)

Diethyl N-benzylphosphoramidate (42) (0.51 g, 2.1 mmol), sodium hydride (0.12 g, 2.9 mmol) and acetyl chloride (0.19 ml, 2.1 mmol) were used. The crude product was purified by flash chromatography on silica gel, eluting with ethyl acetate, (R.f. = 0.75). The by-products were unreacted (42) and tetraethyl pyrophosphate (δ_P -13).

Yield = 0.168 g (28%)

^1H N.M.R.: $\delta(CDCl_3)$: 1.2 (6H, t, J_{HH} = 7 Hz), 2.3 (3H, s), 4 (4H, dq, 4.75 (2H, d, J_{PH} = 12 Hz), 7.2 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(Et_2O)$: 1.02 (s)

m/z (low resolution) 243 (m-42) (found), 285 (expected), 214 (92%), 186 (52%), 106 (100%)

The highest peak corresponds to the loss of an acetyl group from the product (44), followed by protonation.

I.R. ν (cm^{-1}) 3500, 2990, 1775, 1690 (C=O), 1495, 1435, 1375, 1270 (P=O), 1165, 1100, 1020, 980, 940, 880

Tetraethyl N-benzylimidobisphosphonate (45)

Diethyl N-benzylphosphoramidate (42) (0.45 g, 1.8 mmol), sodium hydride (0.1 g, 2.5 mmol) and diethyl chlorophosphate (0.29 ml, 1.85 mmol) in THF were used. Purification was attempted by silica gel flash chromatography, eluting with ethyl acetate, (R.f. product = 0.1).

Separation of (45) from the by-products tetraethyl pyrophosphate (R.f. = 0.4) and (42) (R.f. = 0.2) proved difficult. The majority of product isolated was contaminated by the starting material (42).

Yield = 0.124 g (16%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.2 (12H, t, J_{HH} = 7 Hz), 4 (8H, dq), 4.5 (2H, t, J_{PH} = 12 Hz), 7.3 (5H, m, aromatic)

^{31}P N.M.R.: $\delta(CDCl_3)$: 4.2 (s)

Tetrakis-(trimethylsilyl)imidobisphosphonate (46)

Bromotrimethylsilane (0.17 ml, 1.25 mmol) and tetraethyl N-benzylimidobisphosphonate (72 mg, 0.25 mmol) were stirred under a nitrogen atmosphere in benzene (1 ml) for 24 hours at 50°C. The solvent was removed in vacuo to give the desired product.

^{31}P N.M.R.: $\delta(C_6H_6)$: -19.1 (s)

Tetrasodium imidobisphosphonate (9)

Sodium bicarbonate (0.1 g, 1.25 mmol) and water (5 ml) were added to tetrakis-(trimethylsilyl)imidobisphosphonate (46) (0.25 mmol) and the mixture allowed to stand at room temperature for 30 minutes. The removal of the water and trimethylsilyl alcohol in vacuo gave the desired product, confirmed by comparison with an authentic sample.

^{31}P N.M.R.: $\delta(H_2O)$: 2.2 (s)

N-Oxyl-imidobisphosphonate (6)

Sodium imidobisphosphonate (9) (0.09 g, 0.33 mmol) was dissolved in water (2 ml). Hydrogen peroxide (0.2 ml, 30% solution 2 mmol), disodium EDTA (0.01 g 0.026 mmol) and sodium tungstate (0.01 g) were added and the reaction mixture stirred until no solid material remained, giving a 150 mM solution. The reaction was monitored by E.S.R. Spectroscopy and ^{31}P N.M.R. Spectroscopy. The E.S.R. spectrum showed a triplet of triplets, arising from the coupling of the unpaired electron to two equivalent phosphorus nuclei (A=26G) and then to the nitrogen nucleus (A=20G). The E.S.R. active species had a lifetime of 30 hours in the light (longer lived in the dark). The ^{31}P N.M.R. spectrum showed the starting material at δ_P 1.7 shift to a peak at δ_P 2.6, both peaks being present in the intermediate stage. This indicated either the production of the corresponding hydroxylamine derivative (via reduction of the radical), oxidation to an N-oxide, or addition of two identical radicals to give a dimer.

Diethyl thiophosphite (62)

Prepared according to the method of Krawiecki and Michabski (1960). Diethyl chlorophosphite (16 ml, 0.1 mol) and pyridine (8 ml, 0.1 mol) were stirred in benzene whilst hydrogen sulphide was bubbled slowly through the solution for $4\frac{1}{2}$ hours, maintaining the temperature at 5°C.

The reaction mixture was filtered to remove the pyridinium chloride and the filtrate washed with ice-cold water (2 x 25 ml). The filtrate was dried, evaporated *in vacuo* and the pure product was obtained by distillation.

Yield = 9.7 g (63%)

b.p. 72°C-75°C (15 mmHg)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 4.1 (4.5H, 4H, dq J_{HH} = 7 Hz, J_{PH} = 11 Hz + 0.5H, d), 11.4 (0.5H, d, J_{PH} = 647 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 69 (s)

O,O,O',O'-Tetraethyl methylenebisthiophosphonate (63)

Prepared according to the method of Czekanski (1982).

Diethyl thiophosphite (62) and sodium wire (1.15 g, 50 mmol) were stirred together in liquid ammonia under a nitrogen atmosphere. The temperature was maintained at between -35°C and -70°C. After 10 minutes, dibromomethane (1.76 ml, 25 mmol) was added and the stirring continued under U.V. light for 3 hours.

The reaction vessel was then warmed to room temperature and the ammonia allowed to evaporate. Diethyl ether (250 ml) was added and the solid removed by filtration. The filtrate was evaporated in vacuo and the residue distilled to give a colourless oil.

Yield = 2.48 g (31%)

b.p. 110°C-115°C (0.03 mmHg)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (12H, t, J_{HH} = 7 Hz), 2.9 (2H, t, J_{PH} = 18 Hz), 4.1 (8H, dq, J_{HH} = 7 Hz, J_{PH} = 10 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 82.69 (s)

O,O,O',O'-Tetrakis-(trimethylsilyl)methylenebisthiophosphonate (63A)

O,O,O',O'-Tetraethyl methylenebisthiophosphonate (63) (0.68 g, 2.14 mmol), iodotrimethylsilane (1.8 ml, 12.8 mmol) and methyl iodide (0.27 ml, 4.3 mmol) were heated in a sealed vessel at 110°C for 3-4 days. The crude reaction mixture contained one peak in the ^{31}P N.M.R. spectrum.

^{31}P N.M.R.: δ(neat): 57 (s)

Methylenebisthiophosphonate (59)

The crude 0,0,0',0'-tetrakis-(trimethylsilyl)methylenebisthiophosphonate was treated with a solution of sodium bicarbonate (1.44 g, 17.1 mmol), water (5 ml) and a few drops of mercaptoethanol (to prevent disulphide formation). The resultant mixture was allowed to stand for 10 minutes and the water and trimethylsilyl alcohol removed <u>in vacuo</u> to give the desired product.

^{31}P N.M.R.: $\delta(H_2O)$: 48.2 (s)

The product was purified by ion exchange column chromatography on a DEAE sephadex column, with a linear salt gradient of 50-750 mM triethylammonium bicarbonate. The product was identified by U.V. absorbance (220 nm), the fractions containing the product were pooled and the buffer removed <u>in vacuo</u> to give the pure product.

Yield = 0.6 g (46%) (assuming the product existed as the tetra triethylammonium salt).

^{31}P N.M.R.: $\delta(MEOH/Et_3N)$: 57.5 (s)

Bis-(trimethylsilyl)[0,0-bis-(trimethylsilyl)thiophosphono]-methylphosphonate (63B)

0,0,0',0'-Tetraethyl methylenebisthiophosphonate (63) was reacted under the conditions used by Hutchinson (1986) for the synthesis of methylenebisthiophosphonate. Compound (63) (0.63 g, 2 mmol) and iodotrimethylsilane (1.7 ml, 12 mmol) were heated together at 100°C in a sealed vessel for 10 days. The crude reaction mixture contained 3 sets of peaks in the ^{31}P N.M.R. spectrum. This indicated a mixture of 0,0,0',0'-tetrakis-(trimethylsilyl)methylenebisthiophosphonate and bis(trimethylsilyl)[0,0,bis(trimethylsilyl)thiophosphono]methylphosphonate.

^{31}P N.M.R.: $\delta(CHCl_3)$: 56.8 (s), 49.2 (br d), 1.8 (br d)

Thiophosphonomethylphosphonate (64)

The crude mixture of 0,0,0',0'-tetrakis(trimethylsilyl)-methylenebisthiophosphonate and bis(trimethylsilyl)[0,0,bis (trimethylsilyl)thiophosphono]methylphosphonate was treated with a solution of sodium bicarbonate (1 g, 12 mmol), water (5 ml) and a few drops of mercaptoethanol to give the corresponding sodium salts. The products were separated and purified by ion exchange chromatography, as previously described for methylenebisthiophosphonate

The first product isolated was thiophosphonomethylphosphonate (64) and the second, methylenebisthiophosphonate (59), apparently in equal quantities from the U.V. trace.

^{31}P N.M.R.: δ(MEOH/Et_3N): 51.2 (d, J_{PP} = 8.55 Hz, P, (S)), δ:15.3 (d, J_{PP} = 8.55 Hz, P(O))

Compound (59); ^{31}P N.M.R.: δ(MEOH/Et_3N): 55.8 (s)

Nucleotide Analogues

Methylenebisthiophosphonate was dried prior to use by repeated evaporation of DMF in vacuo.

The bis tri-^{n}butylammonium salt of methylenebisthiophosphonate was prepared by stirring the triethylammonium salt in methanol with two equivalents of tri-^{n}butylamine and dried as above from DMF.

The bis tri-^{n}octylammonium salt of adenosine 5'-monophosphate was prepared by stirring the free acid in methanol with two equivalents of tri-^{n}octylamine until all the solid material dissolved. The methanol was then removed in vacuo and the residue dried from DMF, as above.

5'-O-tosyladenosine was also dried from DMF prior to its use.

5'-Adenylyl β, γ, methylenebisthiophosphonate (60)

The bis tri-^{n}octylamine salt of adenosine 5'-monophosphate (0.18 g, 0.5 mmol), diphenyl phosphorochloridate (0.1 ml, 0.5 mmol) and tri-^{n}butylamine (0.12 ml, 0.5 mmol) were dissolved in DMF (2 ml) and stirred for 10 minutes in a sealed vessel. The solvent was removed in vacuo, diethyl ether (20 ml) added and the reaction vessel sealed. The resultant mixture was stirred and allowed to stand at ice bath temperature for 30 minutes. The diethyl ether was decanted off and the product dried by repeated evaporation of DMF in vacuo. The product, P^{1}-adenosyl P^{2}-diphenyl pyrophosphate, was stirred with tri-^{n}butylamine (0.07 ml, 0.3 mmol) in pyridine (2 ml) and the bis tri-^{n}butylammonium salt of methylenebisthiophosphonate (0.3 mmol) in pyridine (2 ml) added dropwise.

The resultant solution was allowed to stand for 30 minutes at room temperature in a sealed vessel. The pyridine was then removed in vacuo and the residue dissolved in water. The product was purified by ion exchange chromatography on a DEAE sephadex column using a linear salt gradient of 50-900 mM triethylammonium bicarbonate at pH8. The product was detected by U.V. absorbance (259 nm) and isolated as the triethylammonium salt, by evaporation of the triethylammonium bicarbonate buffer.

Yield = 0.190 mmol (67%) (assuming the product existed as the tetra triethylammonium salt)

^{31}P N.M.R. (121.5 MHz): δ(Tris/EDTA): -11.354 (d, J_{PP} = 33.45 Hz, Pα), 44.834 (d, J_{PP} = 15.799 Hz, Pγ), 60.234 (dd, J_{PP} = 33.4, 15.5 Hz, Pβ), 60.354 (dd, J_{PP} = 33.45, 15.5 Hz, Pβ)

F.A.B. (-ve ion) .536 $(M-H)^-$ (found), 536 (expected)

5'-Thioadenylyl methylenebisthiophosphonate (71) and P^1,P^2-Bis (5'-thioadenosyl)methylenebisphosphonate (72)

The triethylammonium salt of methylenebisthiophosphonate (0.15 mmol), 5'-O-tosyladenosine (63.2 mg, 0.15 mmol) and tri-^{n}butylamine (0.035 ml, 0.15 mmol) were dissolved in the minimum amount of DMF and sealed in a vessel at room temperature for 7-10 days. The reaction was monitored at intervals by ^{31}P N.M.R. until no further change was observed. The mixture of products was purified by ion exchange chromatography as described previously.

The first product isolated was P^1,P^2-bis(5'-thioadenosyl)methylenebisphosphonate (72).

Yield = 20 μmol

^{31}P N.M.R.: δ(Tris/EDTA): 31.2 (s)

The second product was 5'-thioadenylyl methylenebisthiophosphonate (71).

Yield = 22 μmol

^{31}P N.M.R.: δ(Tris/EDTA): 44.37 (d, J_{PP} = 16.7 Hz, P=S), 34.78 (d, J_{PP} = 16.7 Hz, P-S-Ad)

Total Yield (determined by U.V. absorbance at 320 nm) = 0.42 μmol (30%)

Methylene(P^1,P^2,bisthiomethylene)bisthiophosphonate (77)

The triethylammonium salt of methylenebisthiophosphonate (0.075 mmol) was dissolved in acetonitrile (50 μl). Triethylamine (50 μl, 0.375 mmol) and diiodomethane (30 μls, 0.375 mmol) were added and the reaction mixture left in a sealed vessel for 2 hours, after which time no change was observed in the ^{31}P N.M.R. spectrum. After removing the solvent in vacuo, the crude product was dissolved in aqueous sodium bicarbonate solution and washed with diethyl ether. The aqueous extract was absorbed onto a DEAE sephadex column and the product purified by ion exchange chromatography using a linear buffer gradient (20-200 mM triethylammonium bicarbonate at pH 8).

^{31}P N.M.R.: $\delta(D_2O)$: 32.8 (s)

^{31}P N.M.R. (^{1}H coupled): $\delta(D_2O)$: 32.8 (dt, J_{PH} = 18 Hz)

^{1}H N.M.R.: $\delta(D_2O)$ δ:2.3 (2H, t, J_{PH} = 18 Hz), 4 (2H, t, J_{PH} = 18 Hz)

5'-Adenylyl(Pβ,Pγ,bisthiomethylene)β,γ-methylenebisthiophosphonate (74)

5'-Adenyl β,γ-methylenebisthiophosphonate (60) (0.05 mmol) was dried from DMF, dissolved in DMF (0.5 ml) and placed in an N.M.R. tube , tri-^{n}butylamine (24 µl, 0.1 mmol) and diiodomethane (4 µl, 0.05 mmol) were added and the N.M.R. tube sealed. The reaction was followed by ^{31}P N.M.R., when no further change was observed (1½-2 hours) the reaction mixture was dissolved in Tris buffer (pH 7) and washed with diethyl ether. The aqueous layer was then applied to a DEAE sephadex column and purified by ion exchange chromatography using a linear salt gradient (0-250 mM triethylammonium bicarbonate).

The product was stored frozen in Tris buffer, as it was found to be unstable when stored as a solid at room temperature. The product was observed to decompose to methylene(P^1,P^2, bisthiomethylene)bisthiophosphonate (77) and adenosine 5'-monophosphate.

Yield = 15 μmol (30.6%)

^{31}P N.M.R. (121.5 MHz): δ(Tris/EDTA): -11.535, (d, J_{PP} = 30.76 Hz,Pα), -11.563 (d, J_{PP} = 30.497 Hz, Pα), 26.343 (d, J_{PP} = 8.352 Hz, Pγ), 26.357 (d, J_{PP} = 8.294 Hz, Pγ), 42.18 (dd, J_{PP} = 30.698, 8.358 Hz, Pβ)

Methylene(P^1,P^2,disulphide)bisthiophosphonate (78)

Methylenebisthiophosphonate (59) (75 μmol) was dissolved in acetonitrile (0.5 ml) and placed in an N.M.R. tube, 3-chloroperbenzoic acid (0.035 g, 0.2 mmol) was added and the reaction monitored by ^{31}P N.M.R. When no further change was observed in the ^{31}P N.M.R. spectrum, the disulphide bond was cleaved with mercaptoethanol to produce (59). This was confirmed by the addition of authentic (59) to give a single peak in the ^{31}P N.M.R. spectrum.

^{31}P N.M.R.: δ(MeCN): 31.4 (s)

5'-Adenylyl(Pβ,Pγ,disulphide)β,γ-methylenebisthiophosphonate (75)

5'-Adenylyl β,γ-methylenebisphosphonate (60) (40 μmol) was dissolved in water (0.5 ml) and placed in an N.M.R. tube, hydrogen peroxide (30% solution, 0.1 ml) was added and the reaction monitored by ^{31}P N.M.R. spectroscopy.

After 5 minutes the reaction was found to have gone to completion, no purification being undertaken.

^{31}P N.M.R.: $\delta(H_2O)$: -10.768 (d, J_{PP} = 24.41 Hz, Pα), 9.1195 (dd, J_{PP} = 7.32, 24.41 Hz, Pβ), 14.773 (d, J_{PP} = 7.33 Hz, Pγ)

Tetra-^{i}propyl C-methyl methylenebisphosphonate (92)

Prepared according to the method of Hutchinson (1985).

Tetra-^{i}propyl methylenebisphosphonate (0.5 g, 1.45 mmol) in THF (20 ml) was stirred under a nitrogen atmosphere at room temperature. Thallium ethoxide (0.111 ml, 1.6 mmol) was added and stirring continued for 45 minutes. Methyl iodide (1.8 ml, 29 mmol) was then added and the reaction heated under reflux for 2 hours. The reaction vessel was cooled and the reaction mixture passed through a short silica column containing a layer of celite (to remove thallium(I)iodide), eluting with petroleum ether (b.p. = 40°C-60°C)/acetone. The solvent was removed <u>in vacuo</u> to give an oil containing two products by ^{31}P N.M.R., tetra-^{i}propyl C-methyl methylenebisphosphonate (92) and the bismethylated derivative (δ_P 25.4)

Crude Yield = 0.47 g (90%)

^{31}P N.M.R.: $\delta(CHCl_3)$: 21.9 (s)

1-Diethyl phosphono(2-ethoxy,2-O-diethyl phosphono)ethylene (94)

Sodium hydride (60%, 0.168 g, 4.2 mmol) was stirred in THF (20 ml) under a nitrogen atmosphere at 0°C, triethyl phosphonoacetate (0.59 ml, 3 mmol) was added and stirring continued at 0°C. After 20 minutes diethyl chlorophosphate (0.47 ml, 3.3 mmol) was added and the reaction allowed to warm up to room temperature. Stirring was continued until no further change was observed in the ^{31}P N.M.R. spectrum, i.e. until the triethyl phosphonoacetate anion, δ_P 40, had completely reacted. The reaction mixture was poured into water (100 ml) and extracted with chloroform. The products were purified by silica gel flash chromatography, eluting with chloroform (80)/methanol (20). The by-product isolated was tetraethyl pyrophosphate (δ_P -13), with some unreacted starting triethyl phosphonoacetate also present (δ_P 20.1). The fractions containing the pure product were pooled, other fractions containing the product were contaminated with starting material and tetraethyl pyrophosphate.

Yield = 1 g (66%)

^{31}P N.M.R.: $\delta(CDCl_3)$: -9 (d, J_{PP} = 3.6 Hz), 18.3 (d, J_{PP} = 3.6 Hz)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz + 6H, t, J_{HH} = 7 Hz + 3H, t, J_{HH} = 7 Hz) (overlapping), 4 (4H, dq, + 4H, dq + 2H, q, J_{HH} = 7 Hz + 1H d) (overlapping) (Due to the overlapping signals, the spectrum was not fully resolved)

M/Z (low resolution) 360 (m^+) (found), 360 (expected), 331 (11%), 197 (26%), 183 (17%), 179 (65%), 155 (100%), 151 (46%), 127 (39%), 123 (56%), 122 (40%),

Triethyl C-methyl phosphonoacetate (95)

Triethyl phosphonoacetate (0.595 ml, 3 mmol) was stirred in THF (20 ml) at 0°C, under an atmosphere of nitrogen. Sodium hydride (60%, 0.12 g, 3 mmol) was added portionwise to form the carbanion (δ_P 40). Stirring was continued for 30 minutes at 0°C, after which time methyl iodide (0.19 ml, 3 mmol) was added. The reaction was allowed to warm to room temperature and stirring was continued for 3 hours. The reaction mixture was poured into water (50 ml) and extracted with chloroform to give a mixture of three products by ^{31}P N.M.R., at δ_P 26.8, δ_P 23.3, and δ_P 19.5. The first signal corresponded to the bismethylated material and the third to the starting triethyl phosphonoacetate. Purification by silica gel flash chromatography, eluting with ethyl acetate proved to be unsuccessful, with little or no separation being achieved.

Crude Yield = 0.6 g (84%)

^{31}P N.M.R.: $\delta(CDCl_3)$: 23.3 (s)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (3H, t, J_{HH} = 7 Hz + 6H, t J_{HH} = 7 Hz), 1.4 (3H, d, J_{PH} = 7 Hz), 2.9 (1H, d, J_{PH} = 21 Hz), 4.1 (4H, dq + 2H dq)

M/Z (low resolution) 239 $(m + 1)^+$, 238 (m^+) (found), 238 (expected), 193 (23%), 165 (27%), 155 (26%), 137 (30%), 109 (50%)

O-Ethyl(diethyl phosphono)thioacetate (98)

Diethyl cyanomethylphosphonate (5 g, 0.028 mol) and ethanol (3.31 ml, 0.056 mol) were dissolved in benzene (60 ml). The solution was cooled to 5°C, gaseous HCl was bubbled through the solution for 15 minutes and the reaction stirred for 16 hours. The benzene was removed in vacuo and the residue taken up in pyridine (70 ml). The solution was cooled to 0°C and hydrogen sulphide bubbled through for 2 hours. The reaction was stirred for 16 hours and then poured into an aqueous solution of 20% hydrochloric acid (350 ml), extracted with diethyl ether and washed several times with hydrochloric acid solution, water and brine. The organic layer was dried and the solvent removed in vacuo. The residue was distilled to give the desired compound as a pale yellow liquid.

Yield = 4.35 g (64%)

b.p. 90°C-91°C (0.08 mmHg)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.4 (6H, t, J_{HH} = 7 Hz + 3H, t, J_{HH} = 7 Hz), 3.5 (2H, d, J_{PH} = 21 Hz) 4.1 (4H, dq, $J_{PH} = J_{HH}$ = 7 Hz), 4.5 (2H, q, J_{HH} = 7 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 18.8 (s)

Attempted phosphorylation of O-Ethyl(diethyl phosphono)-thioacetate (98)

Sodium hydride (60%, 0.14 g, 3.43 mmol) was stirred in THF (20 ml) at 0°C under an atmosphere of nitrogen. O-Ethyl(diethyl phosphono)thioacetate (98) (0.686 g, 2.86 mmol) in THF (5 ml) was added dropwise to the reaction mixture. Stirring was continued at 0°C until the ^{31}P N.M.R. showed complete generation of the anion (δ_P 34.3). Diethyl chlorophosphate (0.41 ml, 2.86 mmol) in THF (5 ml) was added to the reaction and the vessel allowed to warm to room temperature. Stirring was continued until the ^{31}P N.M.R. showed complete reaction of the anion. The reaction was quenched with dilute hydrochloric acid and extracted with chloroform. The organic layer was dried over magnesium sulphate, filtered and the solvent removed <u>in vacuo</u> to give signals in the ^{31}P N.M.R. at δ_P 19.3, δ_P 17.7 and δ_P -13.1.

The products were separated by silica gel chromatography on a chromatotron, eluting with chloroform and identified as tetraethyl pyrophosphate (δ_P -13.1), triethyl phosphonoacetate (δ_P 19.3) and (98) (δ_P 17.7).

Triethyl C-mercapto phosphonoacetate (99)

The same procedure as described for the preparation of triethyl C-methyl phosphonoacetate (95) was followed. Sulphur (96 mg, 3 mmol) was used instead of iodomethane and the reaction stirred for 24 hours at room temperature. The reaction was quenched with diluted hydrochloric acid and extracted with chloroform. The product was separated from the starting triethyl phosphonoacetate by silica gel flash chromatography, eluting with ethylacetate (90)/methanol (10). The purified product consisted of two co-eluting compounds, possibly a mixture of the desired compound and the corresponding disulphide.

Yield = 0.1 g (13%)

^{31}P N.M.R.: $\delta(CHCl_3)$: 14.9 (s) (57%), 14.1 (s) (43%)

M/Z (low resolution) 256 (m^+) (17%) (found), 256 (expected), 210 (11%), 182 (11%), 154 (16%), 137 (12%), 109 (25%)

Di(tetra-^{i}propyl mercaptomethylenebisphosphonate)disulphide (100)

Prepared according to the method of Griffith (1987)

Sodium hydride (60%, 0.9 g, 22 mmol) was stirred in toluene (100 ml) at -20°C under a nitrogen atmosphere. Tetra-^{i}propyl methylenebisphosphonate (6.9 g, 20 mmol) was dissolved in toluene (10 ml) was and added dropwise. The reaction mixture was warmed to room temperature, sulphur (0.65 g, 20 mmol) added and stirring continued at room temperature for 24 hours. The reaction was quenched with dilute hydrochloric acid, until the intense colour was removed. The toluene layer was separated and the aqueous layer washed with chloroform. The organic extracts were combined, dried over magnesium sulphate, filtered and the solvents removed <u>in vacuo</u>. Unreacted sulphur was removed by filtration and the crude product allowed to stand exposed to the atmosphere for several days. The disulphide precipitated from the residue and was collected by filtration, washed with petroleum ether (40°C-60°C) and dried <u>in vacuo</u>.

Yield = 3 g (40%) (after repeated precipitations)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (24H, s), 4 (2H, t, J_{PH} = 20 Hz), 4.9 (8H, m)

^{31}P N.M.R.: $\delta(CDCl_3)$: 15.3 (s)

^{31}P N.M.R. (^{1}H coupled): $\delta(CDCl_3)$: 15.3 (d, J_{PH} = 20 Hz)

Di(mercaptomethylenebisphosphonate)disulphide (102)

Prepared according to the method of Griffith (1987)

Di(tetra-^{i}propyl mercaptomethylenebisphosphonate)disulphide (100) (0.22g, 0.29 mmol) was dissolved in chloroform (1 ml) and placed in an N.M.R. tube, bromotrimethylsilane (0.4 ml, 2.9 mmol) was added and the N.M.R. tube warmed to 40°C. The production of the trimethylsilyl esters was monitored by ^{31}P N.M.R. (δ_P 0.4). On completion of the reaction the solvent and excess bromotrimethylsilane were removed <u>in vacuo</u>. The residue was treated with water (3 ml) and the mixture allowed to stand at room temperature until all the material had dissolved. The water and trimethylsilyl alcohol were removed <u>in vacuo</u> to give the crude product.

Yield = 0.1 g (85%)

^{31}P N.M.R.: $\delta(H_2O)$: 14.1 (s)

^{31}P N.M.R. (^{1}H coupled): $\delta(H_2O)$: 14.1 (d, J_{PH} = 20 Hz)

Mercaptomethylenebisphosphonate (85)

Prepared according to the method of Griffith (1987).

The crude Di(mercaptomethylenebisphosphonate)disulphide (102) (0.1 g, 2.4 mmol) was taken up in water and sodium borohydride added until reduction was complete, as indicated by a lower field resonance in the ^{31}P N.M.R. at δ_P 15.7, consistent with the data of Griffith (1987). The aqueous solution was filtered and the product isolated by precipitation with acetone. Treating the product with H^+ dowex for 15 minutes, filtering and the subsequent removal of the water yielded the free acid of the desired compound.

Yield = 0.06 g (64%)

^{31}P N.M.R.: δ(TEAB/EDTA): 13.3 (s)

^{31}P N.M.R. (^{1}H coupled): δ(TEAB/EDTA): 13.3 (d, J_{PH} = 24 Hz)

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (84)

Prepared according to the method of McConnell (1969). Chloroacetyl chloride (1.6 ml, 0.02 mol) was added dropwise to a stirred solution of 2,2,6,6-tetramethyl-4-aminopiperidine (3.4 ml, 0.02 mol) in diethyl ether (20 ml). After 2 hours, the reaction mixture was extracted with dilute hydrochloric acid and the ether layer discarded.

Proportions of dilute sodium hydroxide were added until a pH of 9.5-10 was achieved and a white solid was observed to precipitate out of the solution.

The white solid was collected by filtration and suspended in water (120 ml). Sodium tungstate (0.6 g), disodium EDTA (0.6 g) and hydrogen peroxide (30% aqueous solution, 12 ml) were added and the solution stirred until all the solid material dissolved, the resultant solution was then allowed to stand at room temperature for 2-3 hours.

The orange solution which formed was saturated with sodium chloride and extracted with diethyl ether. The organic layer was washed with water and dried over magnesium sulphate. (N.B. re-extraction of the aqueous layers yielded further product.) The solvent was removed <u>in vacuo</u> to give an orange solid. This solid was dissolved in acetone and treated with sodium iodide (3 g, 0.02 mol) in acetone.

The mixture was left for 24 hours at room temperature and a further portion of sodium iodide (c.f. 0.5 g) in acetone was added. After a further 24 hours, the acetone was removed <u>in vacuo</u> and the dark oil dissolved in hot toluene (40 ml). After 3 days the product crystallized as dark orange needles, occasionally the product was deposited from solution as an oil, this necessitated a further recrystallization from toluene.

Yield = 1.36 g (20%)

m.p. 120-122°C (Literature m.p. 121-123°C)

M/Z (low resolution) 339 (m^+) (found), 339 (expected)

Mercapto[N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)]-acetamido-methylenebisphosphonate (103)

Mercaptomethylenebisphosphonate (85) (0.02 g, 0.1 mmol) was dissolved in triethylammonium acetate buffer (pH6-pH6.5) and treated with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-iodoacetamide (84) (0.05 g, 0.15 mmol) dissolved in methanol.

The reaction was monitored by ^{31}P N.M.R., when no further change was observed the solvents were removed <u>in vacuo</u>. The residue was washed with ethyl acetate to remove excess (84), monitoring the extraction by T.L.C. on silica gel plates, eluting with ethylacetate (R.f. product = 0, R.f. (84) = 0.8). The product was purified by ion exchange chromatography and the presence of the radical confirmed by E.S.R.

Yield = 0.01-0.02 mmol (by E.S.R.) (10%-20%)

^{31}P N.M.R.: δ(MEOH/Et_3N): 12.5 (s)

^{31}P N.M.R. (^1H coupled): δ(MEOH/Et_3N): 12.5 (d, J_{PH} = 24 Hz)

N-Methylene-^{t}butylamine (107)

Prepared according to the method of Fields (1952)

^{t}Butylamine (5.52 mls, 50 mmol) was refluxed with formaldehyde (40% aqueous solution, 3.75 ml, 50 mmol) and potassium carbonate (0.5 g, 3.6 mmol) for 4 hours. The two layers were separated and the organic layer dried over magnesium sulphate to give the desired product.

Crude Yield = 1.1 g (26%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.2 (9H, s), 3.5 (2H, s)

Diethyl ^{t}butylaminomethylphosphonate (106)

Prepared according to the method of Fields (1952).

Diethyl phosphite (1.7 ml, 13 mmol) was added to the crude compound (1.1 g, 13 mmol) and the reaction mixture allowed to stand at room temperature for 30 minutes. The reaction mixture was distilled under vacuum to give the desired product.

Yield = 2 g (69%)

b.p. 112-114°C (2.5 mmHg)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.1 (9H, s), 1.35 (6H, t, J_{HH} = 7 Hz), 2.9 (2H, d, J_{PH} = 15 Hz), 3.75 (1H, d, J_{PH} = 9 Hz), 4.25 (4H, dq, J_{PH} = J_{HH} = 7 Hz)

^{31}P N.M.R.: $\delta(CDCl_3)$: 27.8 (s)

M/Z (low resolution) 223 (m^+)

M/Z (high resolution) 223.1333 (m^+) (found), 223.1337 (m^+) (expected), 208 (60%), 86 (100%), 70 (74%)

I.R. ν (cm^{-1}), 3490, 3425 (NH), 3300, 2960, 2900, 2860, 2810, 1630, 1470, 1440, 1380, 1360, 1290, 1230 (P=O), 1060, 1020, 960

The attempted phosphorylation of Diethyl ^{t}butylaminomethyl-phosphonate (106)

Diethyl ^{t}butylaminomethylphosphonate (106) (0.211 g, 0.95 mol) was dissolved in THF (10 ml) under a nitrogen atmosphere at 0°C, sodium hydride (60% dispersion in oil, 0.042 g, 1.05 mmol) was added and the reaction stirred at 0°C for 45 minutes. Diethyl chlorophosphate (0.14 ml, 0.95 mmol) was added dropwise and the reaction allowed to warm to room temperature. Stirring was continued for 3 hours, the reaction mixture poured into water (100 ml) and extracted with chloroform. The only product observed was the starting material (106).

^{31}P N.M.R.: $\delta(CDCl_3)$: 27.4 (s)

A similar reaction using diethyl iodophosphonate instead of diethyl chlorophosphate gave unreacted (106) and approximately 30% of the diethyl iodophosphonate used, the remainder decomposed and was extracted into the aqueous layer.

(106) (56 mg, 0.25 mmol) was dissolved in THF (2 mls) and stirred under a nitrogen atmosphere at -78°C. ^{n}butyl lithium (2.5 M, 0.1 ml, 0.25 mmol) was added and stirring continued at -78°C for 1 hour. Diethyl iodophosphonate (0.077 g, 0.275 mmol) was added and the reaction stirred at room temperature for 3 hours. The reaction was quenched with saturated ammonium chloride and extracted with ether to give 4 unidentified signals in the ^{31}P N.M.R., the ^{1}H N.M.R. showing no ^{t}butyl signal.

^{31}P N.M.R.: $\delta(CDCl_3)$: 29.8 (s), 20.1 (s), 19.5 (s), 0.8 (s)

A similar reaction using 2 equivalents of ^{n}butyl lithium and diethyl chlorophosphate gave signals in the ^{31}P N.M.R. indicative of diethyl phosphate and tetraethyl pyrophosphate.

4-N-Ethylidene(2,2,6,6-tetramethyl-4-aminopiperidine) (112)

2,2,6,6-tetramethyl-4-aminopiperidine (0.9 ml,5 mmol) and acetaldehyde (0.3 ml, 5.5 mmol) were stirred in dichloromethane (10 mls) in the presence of magnesium sulphate (0.6 g, 4.15 mmol). The reaction was monitored by T.L.C. on silica gel plates, eluting with methanol (R.f. product = 0.5, R.f. starting material = 0). On completion of the reaction, the mixture was filtered and the solvent removed <u>in vacuo</u> to give a brown oil.

Crude Yield = 1.16 g (127%)

^1H N.M.R.: $\delta(CDCl_3)$: 1.2 (6H, s), 1.3 (6H, s), 1.6 (4H, dd, J_{HH} = 12, 4 Hz), 2 (3H, d, J = 5 Hz), 3.45 (1H, m), 7.8 (1H, J_{HH} = 5 Hz)

The ^1H N.M.R. also indicated the presence of some unreacted amine.

Diethyl N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (113)

Diethyl phosphite (0.27 ml, 2.15 mmol) was added to the crude 4-N-ethylidene(2,2,6,6-tetramethyl-4-aminopiperidine) (112) (0.36 g, 1.95 mmol) and the resultant mixture allowed to stand for 24 hours at room temperature in a sealed vessel.

The crude product was distilled under vacuum to give a brown oil.

Yield = 0.498 g (79%)

b.p. 130°C (0.1 mmHg)

^1H N.M.R.: $\delta(CDCl_3)$: 1.1 (6H, s), 1.2 (6H, s), 1.35 (6H, t, J_{HH} = 7 Hz), 1.4 (3H, d, J_{HH} = 7 Hz), 1.8 (4H, dd, J_{HH} = 12, 6Hz), 3.15 (2H, m, unresolved), 4.15 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz)

^1H N.M.R. (300 MHz): $\delta(CDCl_3)$: 1.13 (6H, 2s), 1.2015 (3H, s), 1.2075 (3H, s), 1.285 (3H, d, J_{HH} = 7 Hz), 1.34 (6H, t, J_{HH} = 7 Hz), 4.15 (4H, m, unresolved)

^{31}P N.M.R.: $\delta(CDCl_3)$: 2 (s)

M/Z (low resolution) 320(m^+)

M/Z (high resolution) 320.2225 (m^+) (found) 320.2228 (expected), 183 (78%), 155 (46%), 124 (100%)

I.R. ν (cm^{-1}) 3500-3300 (NH), 2960, 2900, 1445, 1360, 1230 (P=O), 1040, 1020, 955

Bis(trimethylsilyl) N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (114)

Diethyl N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (113) (0.17 g, 0.54 mmol) was dissolved in dichloromethane and placed in an N.M.R. tube. Bromotrimethylsilane (0.22 ml, 1.62 mmol) was added and the N.M.R. tube sealed. The reaction mixture was left at room temperature and monitored by ^{31}P N.M.R.. On production of the trimethylsilyl esters, the solvent and excess bromotrimethylsilane were removed <u>in vacuo</u> to give the crude product.

Yield = 0.2 g (92%)

^{31}P N.M.R.: $\delta(CH_2Cl_2)$: 10 (s)

N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (115)

The crude bis(trimethylsilyl) N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (114) (0.2 g, 0.49 mmol) was treated with a solution of sodium bicarbonate (0.14 g, 1.7 mmol) in water (8 ml) and the resultant solution allowed to stand at room temperature for 15 minutes. The water and trimethylsilyl alcohol were removed <u>in vacuo</u> to give a mixture of two compounds by ^{31}P N.M.R.

The product at δ_P 25 was assumed to be the desired product, no attempt was made to identify the product at δ_P 12.

Crude Yield = 0.1 g (79%)

^{31}P N.M.R.: $\delta(H_2O)$: 25 (s), 12 (s)

Attempted oxidation to give N-(1-Oxyl-2,2,6,6-tetramethyl--4-piperidinyl)-aminoethylphosphonate (116)

The crude reaction mixture, assumed to contain N-(2,2,6,6-tetramethyl-4-piperidinyl)-aminoethylphosphonate (115) (0.1 g, 0.38 mmol) was dissolved in water (5 ml). This was then treated with a solution of hydrogen peroxide (0.2 ml, 30% solution, 2 mmol), disodium EDTA (0.01 g, 0.03 mmol) and sodium tungstate (0.01 g, 0.03 mmol). The resultant solution was allowed to stand at room temperature for 3 hours. Removal of the water <u>in vacuo</u> gave a yellow/orange solid which was soluble in methanol. The presence of a nitroxyl radical was confirmed by E.S.R. spectroscopy. The crude product was washed with ethyl acetate prior to purification, thus removing the radical, leaving a white solid behind. The ethyl acetate solution contained no phosphorus nuclei by ^{31}P N.M.R. spectroscopy. The white solid dissolved in water to give a phosphorus signal in the ^{31}P N.M.R. spectrum, but was found to contain no radical.

N-(2,2,6,6-tetramethyl-4-piperidinyl)aminobismethylenebisphosphonate (117)

2,2,6,6-tetramethyl-4-aminopiperidine (1.7 ml, 10 mmol), phosphorous acid (1.64 g, 20 mmol), concentrated hydrochloric acid (2 ml) and water (2 ml) were heated together under reflux. Aqueous formaldehyde (40%) 3.2 ml, 40 mmol) was added dropwise over 1 hour, reflux being maintained. The reaction mixture was heated under reflux for a further 2 hours, the ^{31}P N.M.R. spectrum of the crude reaction mixture showing a signal at δ_P 9, corresponding to the quaternised 4-amino product. The reaction mixture was made basic by the addition of dilute sodium hydroxide and the water removed <u>in vacuo</u> to give the corresponding sodium salt of the product.

Yield = assumed to be quantitative

^1H N.M.R.: $\delta(D_2O)$: 1.4 (6H, s), 1.45 (6H, s), 2 (4H, dd, unresolved), 2.7 (1H, m, unresolved), 3.1 (4H, d, J_{PH} = 15 Hz)

^{31}P N.M.R.: $\delta(H_2O)$: 17 (s)

^{31}P N.M.R. (^1H coupled): $\delta(H_2O)$: 17 (t, J_{PH} = 15 Hz)

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-oxoiminobisphosphonate (121)

The crude N-(2,2,6,6-tetramethyl-4-piperidinyl)iminobismethylbisphosphonate (117) was treated with sodium tungstate (0.3 g), disodium EDTA (0.3 g), hydrogen peroxide (30% solution, 6 ml) and water (60 ml). The resultant mixture was stirred until no solid material remained, and the solution left at room temperature for 2-3 hours. The resulting orange solution showed two resonances in the ^{31}P N.M.R. spectrum at δ_P 3.2, corresponding to the product and an unidentified compound at δ_P 6. The reaction mixture was purified by ion exchange chromatography (0-600 mM triethylammonium bicarbonate), the fractions identified by U.V. absorbance at 220 nm. The unidentifed product eluted first from the column and contained a nitroxyl radical centre, as judged by E.S.R. analysis. The second, product containing fraction, eluted from the column consistent with a compound containing four negative charges. The presence of the nitroxyl group was confimred by E.S.R. spectroscopic analysis.

Yield = 2.2 g (40%)

^{31}P N.M.R.: δ(MeOH): 3.8 (s)

^{1}H N.M.R.: $\delta(CD_3OD)$: Broad signals

F.A.B. (-ve ion) 521 (found), 521 (expected)

N-diethylphosphoroglycine methyl ester (122)

Methyl glycine hydrochloride (1.95 g, 15.5 mmol) and triethylamine (4.8 ml, 34.1 mmol) in diethyl ether (30 ml) were stirred together under a nitrogen atmosphere at room temperature. Diethyl chlorophosphate (2.24 ml, 15.5 mmol) in diethyl ether (10 ml) was added dropwise and stirring was continued for 24 hours. The reaction mixture was filtered to remove the triethylammonium hydrochloride and the solid washed with a further portion of diethyl ether. The filtrate was dried and the solvent evaporated in vacuo to give a brown oil, an unidentified solid was also deposited (δ_P 0). Filtration gave the product, which was essentially pure by ^{31}P N.M.R. spectroscopy.

Crude Yield = 2.4 g (69%)

^1H N.M.R.: $\delta(CDCl_3)$: 1.3 (6H, t, J_{HH} = 7 Hz), 3.6 (2H, d, J_{PH} = 9 Hz), 3.7 (3H, s), 4.1 (4H, dq, $J_{HH} = J_{PH}$ = 7 Hz), 4.4 (1H, br d)

^{31}P N.M.R.: $\delta(CDCl_3)$: 8.47 (s)

I.R. ν 3200 (NH), 2980, 2900, 1740 (C=O), 1430, 1390, 1360, 1210 (P=O), 1150, 1055, 1045, 960, 870

N-bis(trimethylsilyl)phosphoroglycine methyl ester (124)

N-diethylphosphoroglycine methyl ester (122) (0.13 g, 0.57 mmol) was treated with bromotrimethylsilane (0.3 ml, 2.3 mmol) in an N.M.R. tube at room temperature. When no further change was observed in the ^{31}P N.M.R. spectrum, excess bromotrimethylsilane was removed <u>in vacuo</u> to give the crude product.

^{31}P N.M.R.: δ(neat): -10.8 (s)

^{31}P N.M.R. (^{1}H coupled): δ(neat): -10.8 (t, J_{PH} = 16 Hz)

N-Phosphoroglycine methyl ester (125)

The crude N-bis(trimethylsilyl)phosphoroglycine methyl ester (124) was treated with sodium bicarbonate (0.19 g, 2.3 mmol) and water (5 ml). The resulting solution was allowed to stand for 15 minutes, after which the water and trimethylsilyl alcohol were removed <u>in vacuo</u>. The residue was dissolved in water and the stability of the product monitored by ^{31}P N.M.R. N-Phosphoroglycine methyl ester (125) had a chemical shift of δ_P 8.26 and decomposed to a product with δ_P 3.22 after 12 hours.

^{31}P N.M.R.: $\delta(H_2O)$: 8.26 (s)

^{31}P N.M.R. (^{1}H coupled): $\delta(H_2O)$: 8.26 (t, J_{PH} = 8.8 Hz)

Tetraethyl N-methyl glycine imidobisphosphonate (123)

The procedure described for N-(diethoxyphosphinoyl)-N-methyl-benzylhydroxylamine (25) was followed. Sodium hydride (60%, 0.29 g, 7.14 mmol), N-diethylphosphoroglycine methyl ester (122) (1.15 g, 5.13 mmol) and diethyl chlorophosphate (0.74 ml, 5.13 mmol) being used. The reaction mixture was stirred for 24 hours at room temperature. The product was purified by silica gel flash chromatography, eluting with chloroform (98)/MeOH (2) (R.f. = 0.5) to remove tetraethyl pyrophosphate (δ_P -13) and an unidentified product (δ_P 0).

Yield = 0.67 g (37%)

^{1}H N.M.R.: $\delta(CDCl_3)$: 1.3 (12H, t, J_{HH} = 7 Hz), 3.7 (3H, s), 4.2 (10H, m, unresolved)

^{1}H N.M.R. (300 MHz): $\delta(CDCl_3)$: 1.35 (12H, dt, J_{HH} = 7 Hz, J_{PH} = 0.5 Hz), 3.75 (3H, s), 4.08 (2H, t, J_{PH} = 13.5 Hz), 4.21 (10H, m, unresolved)

^{31}P N.M.R.: $\delta(CDCl_3)$: 3 (s)

M/Z (low resolution) 361 (m^+)

M/Z (high resolution) 361.0748 (found), 361.0729 (expected), 302 (100%), 166 (26%), 110 (14%)

I.R. ν (cm^{-1}) 3520, 3480, 2980, 2950, 2900, 1750 (C=O), 1440, 1390, 1365, 1255 (P=O), 1215, 1160, 1125, 1025, 975, 900

Tetrakis-(trimethylsilyl)N-methyl glycine imidobisphosphonate (126)

Tetraethyl N-methyl glycine imidobisphosphonate (123) (0.1 g, 3 mmol) was stirred with bromotrimethylsilane (0.24 ml, 18 mmol) in dichloromethane (1 ml) under a nitrogen atmosphere for 48 hours at 40°C. The solvent and excess bromotrimethylsilane were removed <u>in vacuo</u> to give the crude product.

^{31}P N.M.R.: $\delta(CH_2Cl_2)$: -14.7

^{31}P N.M.R. (^1H coupled): $\delta(CH_2Cl_2)$: -14.7 (t, J_{PH} = 12 Hz)

N-(methyl glycine) imidobisphosphonate (127) or N-glycine imidobisphosphonate (129)

Sodium bicarbonate (0.15 g, 18 mmol) was dissolved in water (5 ml) to give a solution of pH 9. The solution was mixed with crude tetrakis(trimethylsilyl)N-methyl glycine imidobisphosphonate and allowed to stand at room temperature for 10 minutes. Water and the trimethylsilyl alcohol were removed <u>in vacuo</u> to give a white solid. The ^{31}P N.M.R. spectrum of the crude product contained three compounds at δ_P 2.8 (26%), δ_P 5 (57%) and δ_P 8 (13%). The major product was the desired N-methyl glycine imidobisphosphonate (127). On standing in water (pH9) the product was observed to decompose to the compound at δ_P 2.8, this being the only product observed after 12 hours. The attempted purification by ion exchange chromatography yielded what appeared to be decomposition products at δ_P 0-3. Stability seemed to be conferred, by ^{31}P N.M.R. analysis, at higher pH values, i.e. pH 12-13.

^{31}P N.M.R.: $\delta(H_2O)$: 5 (s)

^{31}P N.M.R. (^{1}H decoupled): $\delta(H_2O)$: 5 (t, J_{PH} = 15 Hz)

The ^{1}H N.M.R. gave no indication of the methyl ester being present, suggesting that the product was N-glycine imidobisphosphonate (129).

Inhibitor properties of (60) and (74) with yeast hexokinase

In separate reactions the compounds (60) and (74) were tested as competitive inhibitors of hexokinase with ATP.

The hexokinase activity was assayed by the coupled enzyme method of Fromm (1962), measuring the ΔA_{340} plotted as a function of time. The assay solution for a single determination of initial velocity at 25°C contained the following final concentrations in 3 ml of solution at pH 7.6 contained in a 1 cm pathlength cuvette:

50 mM Tris.HCl; 20 mM glucose; 6.2 mM $MgCl_2$; 0.05 mM dithiothreitol; 0.1 mM EDTA; 0.2 mM NADP; ca 1.5 units glucose-6-phosphate dehydrogenase; ca 0.05 units hexokinase; 0.06-0.6 mM ATP; 0-0.6 mM (60) or (74).

A P P E N D I X

PRINCIPLES OF N.M.R.

The principles of N.M.R. are well established and documented (Andrew 1984, Pykett 1982, Gadian 1982). N.M.R. experiments are carried out on nuclei which contain a net nuclear spin, defined by the spin quantum number I. I has either integral or half integral values.

The nuclear spin can be regarded as the spinning motion of the nucleus about its own axis, it thus possesses angular momentum. Since the nucleus has an electrical charge distribution and angular momentum, it has an associated magnetic moment. The angular momentum is quantized and is determined by I. The magnitude, P, of the nuclear angular momentum is given by equation (1).

$$P = \not{h}(I(I+1))^{\frac{1}{2}} \text{----(1)} \quad \not{h} = h/2\Pi$$

h = Planck Constant

I has integral values for nuclei with an even mass number and half integral for nuclei with odd mass numbers. In the case of nuclei with even numbers of both neutrons and protons then I is zero.

Individual spins are cancelled by the formation of pairs of neutrons or protons. Hence nuclei with I=0 are not suitable for N.M.R. as they have no overall nuclear angular momentum.

Angular momentum is a vector quantity and in N.M.R. requires a directional quantum number, M, to give it certain discrete orientations. The component of angular momentum parallel to the axis of spin is given by equation (2), where M can have any of the 2I + 1 values.

$$P = Mh \quad \text{---- (2)}$$

The magnetic moment of the nucleus is parallel to the angular momentum and has magnitude μ, equation (3).

$$\mu = \gamma P \quad \text{---- (3)}$$

γ = magnetogyric (or gyromagnetic) ratio of the nucleus.

During a conventional N.M.R. experiment a uniform magnetic field, B_0, is applied to the sample under investigation. The nuclei possessing magnetic moments take up a number of discrete orientations determined by I. The interaction of the field and the magnetic moment results in the nucleus acquiring energy, E, given by equation (4).

$$E = \mu B_0 = -\gamma h M B_0 \quad \text{---- (4)}$$

Since M can have 2I + 1 values, the nuclear energy levels are split into 2I + 1 states . The energy difference between adjacent states being given by equation (5).

$$\Delta E = \gamma h B_0 \quad \text{---- (5)}$$

The above relationship (equation (5)) shows that the energy difference between the possible spin orientations for the nucleus is proportional to the magnitude of the applied field.

The equilibrium difference in populations of the energy levels is determined by the Boltzmann distribution. That is the difference in population in favour of the lower level is given by equation (6).

$$\frac{N\Delta E}{2KT} \quad \text{---- (6)}$$

Where T is temperature, K the Boltzmann constant and N the number of nuclei.

The transitions between adjacent states required to give rise to an N.M.R. signal are induced by an oscillating magnetic field B_1, perpendicular to B_0, of frequency ν_o. This field leads to an absorption of energy and the transition of nuclei from a lower energy level to a higher one.

The subsequent emission of the absorbed energy by the relaxation of the nuclei from the upper to the lower level gives rise to the N.M.R. signal.

The field B_1 oscillates with a frequency ν_o which is given by equation (7).

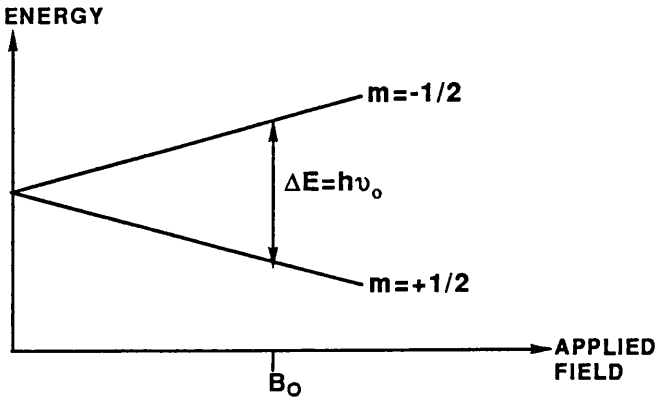
$$\Delta E = h\nu_o \text{ ---- (7)}$$

$$\text{and therefore, } \nu_o = \frac{\Delta E}{h} = h\gamma B_0/h = \gamma B_0/2\Pi \text{ ---- (8)}$$

Equation (8) is the resonance condition for N.M.R. experiments. The only allowable transitions are those between adjacent states (or levels).

As an example, consider a nucleus of spin, $I = \frac{1}{2}$ (eg ^1H), the possible values for m are $+\frac{1}{2}$ and $-\frac{1}{2}$. Hence the nuclear energy levels can be split into two states, representing the two possible spin orientations.

Transitions between the two states are induced in the N.M.R. experiments and are illustrated below.



The magnetogyric ratio, γ is unique for every nuclear isotope thus causing different nuclear species to resonate at different frequencies in the field B_0 (from equation (8)). The oscillating field B_1 is referred to as the radiofrequency (r.f.) field.

The different chemical environments experienced by the species under consideration lead to variations in the local magnetic fields. Shielding effects due to different electronic environments induced by electrons in the molecule are the prime factor behind these variations. The changes in the magnetic field experienced by nuclei lead to a variation in their resonant frequency and give rise to unique N.M.R. signals.

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