TITLE THE SYNTHESIS OF NOVEL PYROPHOSPHATE ANALOGUES AND THEIR ANTIVIRAL ACTIVITIES

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THE SYNTHESIS OF NOVEL PYROPHOSPHATE ANALOGUES AND THEIR ANTIVIRAL ACTIVITIES

by

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"Honni soit qui manipule"

CONTENTS

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ATP	Adenosine 5′-triphosphate
Ci	Curie
CTP	Cytidine 5′-triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
GTP	Guanosine 5′-triphosphate
h	hour
HIV	Human immunodeficiency virus
HTLV-III	Human T-lymphotropic virus type III
ID50	50% inhibitory dose
min.	minute
PBS	Phosphate buffered saline
PPTS	Pyridinium paratoluene sulphonate
RNA	Ribonucleic acid
TCA	Trichloroacetic acid
tris	Tris(hydroxymethyl)amino-ethane
UTP	Uridine 5′-triphosphate

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DECLARATION

The work described in this thesis is the original work of the author and was carried out at the University of Warwick between October 1985 and September 1988. Full acknowledgement is made to all work and ideas included in this thesis but previously reported. This work has not previously been submitted for a degree at any institution.

ACKNOWLEDGEMENTS

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I am greatly indebted to Mr. A.S. Carver for his invaluable help in the preparation of influenza virus stocks and to Dr. G. Semple for his initial assistance with this project. I must express my thanks to Dr. S. Masson (Laboratoire de Chimie des Composés Thiorganiques, Université de Caen, 14032, Caen, France) for his expert assistance with the thiophosgene work.

I would like to express my gratitude to all members of the laboratory both past and present for an excellent working atmosphere and also to Mrs. D.M. Hewitt for typing this thesis. Finally I would like to thank my project supervisor, Dr. D.W. Hutchinson, for giving me this opportunity and for his total enthusiasm towards the project.

PUBLICATIONS

Parts of the research described herein have appeared in the chemical literature as follows:

1. Synthesis and biochemical properties of some pyrophosphate analogues.

   Hutchinson, D.W., Semple, G. and Thornton, D.M. (1986) in "Biophosphates and Their Analogues, Synthesis, Structure, Metabolism and Activity" (K.S. Bruzik and W.J. Stec, eds.) Elsevier, Amsterdam, 1987, p. 441-450

2. A simple synthesis of monofluoromethylenebisphosphonic acid.

   Hutchinson, D.W. and Thornton, D.M. (1988), *J. Organometal. Chem.*, 340, 93-99

3. Michael addition reactions of ethenylidenebisphosphonates.

   Hutchinson, D.W. and Thornton, D.M. (1988), *J. Organometal. Chem.*, 346, 341-348

4. The reaction between thiophosgene and trialkyl phosphites.

   Masson, S., Sene, A., Hutchinson, D.W. and Thornton, D.M. (1988), *Phosphorus and Sulphur*, 40, 1-8

ABSTRACT

Several methods for the preparation of novel substituted methylenebisphosphonates were examined. A new synthesis of monofluoromethylenebisphosphonic acid was developed involving the in situ nucleophilic debromination of tetraisopropyl bromofluoromethylenebisphosphonate. The Michael reaction of tetraalkyl ethenylidenebisphosphonates with alkanethiols was shown to be a facile method of preparation of C-(thioalkyl)methyl methylenebisphosphonates. Tetraisopropyl cyclopropane-1,1-bisphosphonate was synthesised via an intramolecular alkylation of a thallium(I) salt. Thallium(I) salts of methylenebisphosphonates were also used in the synthesis of bisphosphonates related to arildone.

The reaction of trialkyl phosphites with thiophosgene was investigated and it was shown that in THF at low temperature thiocarbonylbisphosphonate was not formed but that phosphorus stabilised ylids were the principal products.

The bisphosphonates prepared were tested for inhibitory effect against the RNA polymerase of influenza A/X49; bisphosphonates with electron-withdrawing bridge substituents showing most inhibition. Selected bisphosphonates were found to have slight activity against the reverse transcriptase of HTLV-III; the causative agent of AIDS.

CHAPTER 1

INTRODUCTION

1.0 Background

Until the end of the 19th century most diseases were thought to be bacterial or fungal in origin. In 1898 it was demonstrated that after an infectious solution of foot and mouth disease was passed through a fine filter; capable of withholding the smallest bacteria, the filtrate remained infectious (Loeffler and Frosch, 1898). Thus the viral nature of this disease was shown. Similar experiments were carried out by Beijerinck (1899) who introduced the term "virus" for the causative agent of tobacco mosaic disease which was shown to be viral in the same manner.

In 1917 Twort identified viruses which infect bacteria (bacteriophages) and viruses are now known which infect most forms of life. They have been discovered to infect insects (Bergold, 1958), fungi (Hollings, 1962), algae (Schneider, *et al*, 1964) and protozoa (Diamond, *et al*, 1972). They are now known to be responsible for many diseases of man, e.g. acquired immune deficiency syndrome (AIDS) and influenza, for which little in the way of vaccines or chemotherapy is currently available.

Viral reproduction is governed by the availability of suitable cells which can be attacked. Once this occurs the cells' nucleic acid synthesis mechanisms are diverted to produce viral nucleic acid. New virions are assembled inside the cell and are released by budding through the cell wall. This intimacy of viral and cellular reproduction is responsible for the lack of viable treatments available against viral infection.

1.1 INFLUENZA VIRUS

Influenza virus possesses a most unusual RNA polymerase activity which is primed by a capped oligonucleotide cleaved from cellular mRNA. The virus has been extensively studied in the search for methods of

inhibition and in the viral life-cycle interruption of the RNA polymerase activity is one possibility.

1.1.1 Influenza Virion Structure

Influenza viruses consist of about 70% protein, 25% lipid, 4% carbohydrate and 1% single-stranded RNA. New virions are formed by budding out of an infected cell and are usually spherical with a diameter of about 100nm although other shapes do exist. The RNA is associated with the nucleoprotein (NP) to form an ordered filamentous structure called the ribonucleoprotein (RNP). This is surrounded by a 6nm thick layer of matrix protein, which is almost one third of the mass of the virion (White, *et al*, 1970), and a viral envelope. This is a lipophilic bilayer which gives additional protection to the virion (White, 1973), (Fig. 1.1.1).

From the surface of the viral envelope project several thousand glycoprotein spikes which can be divided into two classes; the haemagglutinins (HA) and the neuraminidases (NA) whose functions are described later (sections 1.1.2.1 and 1.1.2.3). The ratio of these has been found to vary greatly in different strains of influenza (Webster, 1968).

Polyacrylamide gel electrophresis of influenza virus RNA indicates eight fragments in the range 890-2341 nucleotides in length (McGeoch, *et al*, 1976). Segments 1, 2 and 3 code for the proteins PB_1, PB_2 and PA (Palese, *et al*, 1977). Segment 4 codes for the haemagglutinin (HA). Segment 5 codes for the nucleoprotein (NP) which in combination with the RNA forms the ribonucleoprotein (RNP) (Compans, *et al*, 1972). Segment 6 codes for the neuraminidase (NA). Segment 7 codes for two proteins; M_1 (the matrix protein) and M_2 and segment 8 codes for the proteins NS_1 and NS_2. The functions of these final two proteins along with M_2 have not been determined although NS_1 may be involved in inhibiting host cell protein synthesis (Lamb, 1983).

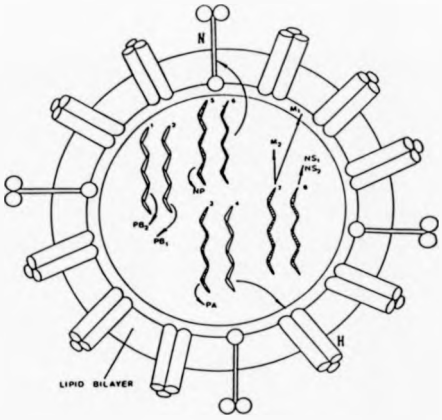


Fig. 1.1.1 The structure of the influenza virion.

1.1.2 Infectious Cycle of Influenza

1.1.2.1 Initial Stages of Infection

The first stage of infection occurs when a virion attaches itself to a suitable receptor site on the surface of the cell plasma membrane. For influenza the receptor site was discovered to be a glycoprotein containing N-acetylneuraminic acid (sialic acid) (Gottschalk, *et al*, 1972). The function of the haemagglutinin spikes is to attach the virion to the N-acetyl neuraminic acid residue at a susceptible site so that infection may be initiated (Mayak, 1977). The viral envelope then

fuses with the cell plasma membrane, the virion uncoats and the nucleocapsid is injected into the cytoplasm. Fusion with the plasma membrane occurs rapidly at 37°C (Morgan and Rose, 1968). These processes are not well understood and it is probable that virions also enter the cell by other processes such as pinocytosis and phagocytosis.

1.1.2.2 RNA Synthesis

Influenza virus expresses an RNA dependent RNA polymerase activity which is retained in purified samples of ribonucleoprotein (RNP) indicating that one or more of the core proteins are responsible (Compans and Caliguri, 1973). This is now known to be the case. The synthesis of viral RNA may be divided into primary and secondary transcription.

1.1.2.2.1 Primary Transcription

This results in the synthesis of viral mRNA and is begun by cleavage, at a purine residue, of a capped oligonucleotide of 10-13 bases in length from cellular mRNA. This oligonucleotide acts as a primer for the initiation of viral mRNA synthesis which is then found to continue according to the template sequence (Lamb and Choppin, 1983) (Fig. 1.1.2.2.1(a)). These processes are carried out by the RNA polymerase complex which consists of PB_1 (the smaller basic protein), PB_2 (the larger basic protein), PA (the acidic protein) and NP (the nucleoprotein) with a total molecular weight of 255,000 (Winter and Fields, 1982) (Fig. 1.1.2.2.1(b)).

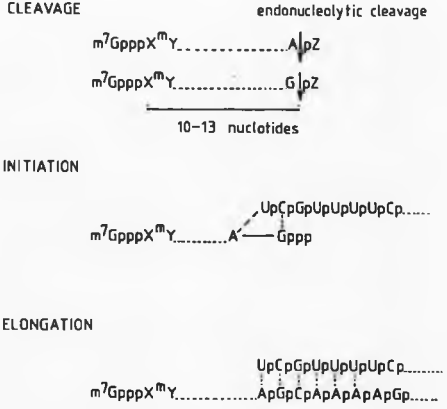


Fig. 1.1.2.2.1(a) Initiation of influenza viral RNA synthesis (Krug, 1983)

It is known that the PB_2 protein recognises the 5′ end of the cleaved oligonucleotide primer and binds to it (Krug, 1983). It is suggested that PB_2 becomes dissociated from the 5′ end of the primer again after the addition 11-15 nucleotides (Braam, *et al*, 1983). The protein PB_1 is involved in initiating transcription and appears to be responsible for directing the addition of each incoming nucleotide to the growing mRNA chain (Braam, *et al*, 1983). Specific roles in the process for the proteins PA and NP have not been determined. When

transcription is complete a complex termination mechanism comes into effect (Robertson, *et al*, 1981).

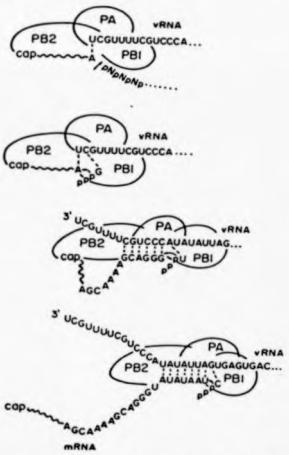


Fig. 1.1.2.2.1(b) The role of the P proteins during viral mRNA synthesis (Braam, *et al*, 1983).

1.1.2.2.2 Secondary Transcription

This has been far less investigated than primary transcription and results in the production of progeny RNA. Viral messenger RNA has been detected after only 15 min from fusion with the cell membrane whereas it is found that secondary transcription dominates after 2-3h (Nayak and Baluda, 1968).

1.1.2.3 Virion Assembly and Release

Maturation of new virions occurs near the plasma membrane and coincides with virion release by budding. A layer of matrix protein is laid down inside the plasma membrane which at the same time develops spikes of haemagglutinin and neuraminidase. Ribonucleoprotein is found

to associate with these areas and a new virion is formed by budding of the plasma membrane (Compans and Dimmock, 1969).

Exactly how the ribonucleoprotein is packaged remains unclear. The probability of selection of the correct eight fragments of RNA by chance is unrealistically small. It has been proposed that the viral RNA is associated into one continuous strand and that the known fragments are due to weak points between the individual sections (Pons, 1970).

Finally the new virion is released from the cell. This is expedited by the neuraminidase spikes. The neuraminidase enzyme hydrolyses the bond between N-acetylneuraminic acid and its adjacent carbohydrate. This removes the N-acetylneuraminic acid from the budding region and so there is no binding site for the haemagglutinin (Klenk, 1974).

1.1.3 Influenza Antigenicity

Human influenza viruses represent an intriguing problem to preventative medicine because their antigenicity is constantly changing. Changes in viral surface proteins can occur such that the virus is no longer recognised by the immune system.

An antigenic shift is where a drastic change in surface proteins occurs. There is evidence that this results from gene exchange when two different strains of influenza infect the same cell. This process has been observed between both animal (Gardner and Shortridge, 1979) and human (Bean, *et al*, 1980) strains of influenza. The most striking example of the results of antigenic shift occurred in 1918-1919 when an influenza epidemic was responsible for the deaths of over twenty million people.

1.1.4 Influenza Vaccines

Incubating influenza virus suspensions in formalin for long periods results in a loss of infectiousness but not of antigenicity (Gard, 1960). This technique has been developed for preparing inactivated virus suspensions for use as vaccines but doubts have been expressed as to the efficacy of this approach if there is a poor antigenic match (Hoskins, *et al*, 1978) with the new strain of influenza.

Attempts have been made to prepare vaccines from live attenuated virus by various means but it has been shown that only small antigenic shifts are required for a great reduction in immunity to occur (Wang, 1983). There is also the risk in live virus preparations of the presence of other viruses causing further infection.

The continually changing antigenicity of influenza means that new vaccines have continually to be developed and thus they have only seen use in select high risk sections of the population. Vaccines can only play a prophylactic role and are of little use once infection has begun.

1.2 Antiviral Chemotherapy

In view of the problems with vaccines and the lack of treatment currently available after infection has started many classes of compounds have been examined for antiviral activity but the number in clinical use is very small. The approach taken has been to try and inhibit virus specific processes such as uncoating or nucleic acid synthesis (Smith, *et al*, 1980).

A good example is provided by 9-(2-hydroxyethoxymethyl)guanine (acyclovir, Fig. 1.2) which is now in clinical use. It is a very effective inhibitor of herpes and other DNA viruses and has low toxicity. Only in herpes infected cells is acyclovir phosphorylated and the triphosphate competes with GTP for the viral DNA polymerase.

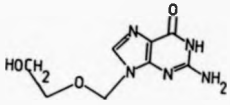
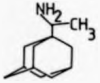


Fig. 1.2 The structure of acyclovir

The very small number of clinical antiviral drugs contrasts starkly with the vast range of antibacterial compounds in clinical use. This is because an easy target is bacterial cell wall biosynthesis. Inhibiting this is the mode of action of the penicillins and cephalosporins (Tomasz, 1979). Some of the more effective compounds against influenza are discussed below.

1.2.1 Amantadine And Rimantadine

(1)



(2)

Fig. 1.2.1 The structures of amantadine (1) and rimantadine (2)

Amantadine and rimantadine (Fig. 1.2.1) are now well established in the treatment of influenza (Oxford and Galbraith, 1980). Amantadine was licensed for use in the USA in 1966 and rimantadine is available clinically in the USSR (Zlyndikov, *et al*, 1981). Their mode of action has been investigated and appears to be involved in viral uncoating (Kato and Eggers, 1969). In the presence of amantadine no transcription of viral RNA can be detected (Tilley and Kramer, 1981).

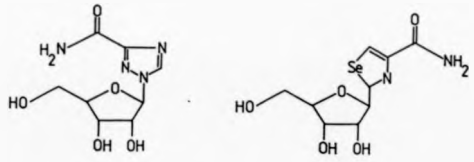
In spite of this the clinical use of amantadine has been restricted for several reasons. It is only really effective against enveloped viruses such as influenza and has side effects such as insomnia and hallucinations. These are indicative of a secondary effect on the central nervous system. Other lipid soluble amines show similar effects to amantadine (Hay and Zambon, 1984). It has also been reported that when amantadine was used against influenza in the lungs of mice, resistant strains of virus were found to develop (Oxford, *et al*, 1970).

Rimantadine has been reported to be more effective than amantadine against influenza (Schulman, 1968) and also demonstrates reduced side effects (Oxford, 1984). This has led to its more widespread use. In prophylactic use both of these are almost totally effective in preventing clinical illness. Although administration within 48h post infection does not halt the infection, fever symptoms can be reduced (Dolin, *et al*, 1982).

1.2.2 Ribavirin and Analogues

Ribavirin (Fig. 1.2.2) expresses a broad spectrum of activity against a range of viruses, including influenza, both in cell culture and animals (Allen, *et al*, 1978; Browne, 1979). It is known to inhibit the synthesis of viral mRNA in influenza where *in vivo* the active species is probably the triphosphate. Ribavirin 5'-triphosphate is an inhibitor of influenza RNA polymerase *in vitro* under conditions where neither ribavirin or ribavirin 5'-monophosphate show any activity (Eriksson, *et al*, 1977). However ribavirin 5'-monophosphate is known to be a potent inhibitor of IMP dehydrogenase which is involved in the biosynthesis of GMP and this may be important in its action against viruses other than influenza (Oxford, 1975).

In animals ribavirin is effective in reducing mortality and fever symptoms even if given as late as 72h after initiation of infection (Chen, *et al*, 1983). However the clinical use is limited as the compound is teratogenic.



(3) (4)

Fig. 1.2.2 The structures of ribavirin (3) and selenazofurin (4)

Various analogues have been examined for activity where other atoms are included in the heterocyclic ring and selenazofurin has been found to be a good inhibitor of influenza (Sidwell, *et al*, 1985) being more effective than ribavirin although it is cytotoxic.

1.2.3 Metal Ion Chelators As Antiviral Agents

1.2.3.1 Involvement of Metal Ions

The presence of metal ions in both RNA and DNA polymerases has been well documented (Mildvan and Loeb, 1979; Perrin and Stünzi, 1981). The effects of these ions have been examined and different metal ions exert both structural and catalytic roles. Many enzymes in both infected and uninfected cells require metal ions for activity (Williams, 1983) and indeed the suggestion has been made that all nucleotidyl transferases are in fact metalloenzymes (Valenzuala, *et al*, 1973). The presence of these metal ions suggests the possibility of inhibiting viral enzymes by the use of chelating agents to coordinate to essential metal ions.

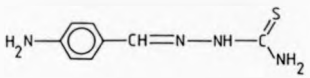
Zinc ions in particular appear to have a catalytic role in some nucleic acid polymerases (Coleman, 1984), having been found to be present in the RNA polymerase of Escherichia coli in 1971 (Scrutton, *et al*, 1971). A role for zinc in nucleic acid synthesis was proposed, as removal of zinc ions from the DNA polymerase of the same organism by the chelating agent 1,10-phenanthroline led to loss of polymerase activity which was restored by the addition of zinc ions to the apoenzyme (Springgate, *et al*, 1973). In the case of the bacteriophage T_7 RNA polymerase the activity of the enzyme was found to correlate approximately with the zinc content (Coleman, 1974).

In the case of influenza virus the RNA polymerase activity is known to be dependent on the presence of zinc ions (Oxford and Perrin, 1977) and a correlation has been found between the ability of pyrophosphate analogues to bind zinc and their *in vitro* antiviral activity (Cload and Hutchinson, 1983).

1.2.3.2 Thiosemicarbazones

The first report of a thiosemicarbazone with antiviral activity was that p-aminobenzaldehyde thiosemicarbazone (Fig. 1.2.3.2 (5)) had activity against vaccina virus (Hamre, *et al*, 1950). The related compound (6) will inhibit the replication of herpes viruses type 1 and 2 *in vitro* (Shipman, *et al*, 1981) and also influenza virus (Oxford and Perrin, 1974).

The mode of action of these compounds probably involves metal ion chelation. Thiosemicarbazones are good chelators of metal ions and in



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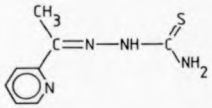


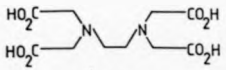
(6)

Fig. 1.2.3.2 Thiosemicarbazones with antiviral activity

the case of compound (6) and its isomers only the 2-substituted isomer shows any activity against influenza. It has been proposed that this may be due to formation of a terdentate complex with an essential zinc ion which is not possible for the 3- and 4-substituted isomers (Oxford and Perrin, 1974).

1.2.3.3 Other Chelating Agents

Several metal chelating compounds which are used industrially in metal ion extraction (Burger, 1973) have been found to possess antiviral activity against influenza *in vitro* (Hutchinson, 1985) but not *in vivo*. This is probably due to their polarity preventing their crossing of cell walls (Oxford and Perrin, 1977).



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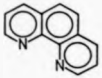

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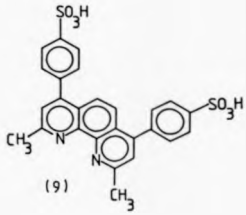


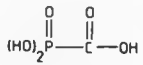
(9)

Fig. 1.2.3.3 The structures of (7) EDTA, (8) 1,10-phenanthroline, (9) bathocuprin disulphonic acid

Ethylenediamine tetraacetic acid (EDTA; Fig. 1.2.3.3 (7)) is found to inhibit the neuraminidase activity of influenza viruses of the N1 serotype. This enzyme has an absolute requirement for calcium ions (Dimmock, 1971) with which EDTA can form strong complexes. 1,10-phenanthroline (Fig.1.2.3.3. (8)) and bathocuprin disulphonic acid (Fig. 1.2.3.3 (9)) inhibit in vitro the RNA polymerase activity of influenza (Oxford and Perrin, 1974) but relatively high concentrations of these

have no detectable effect on the haemagglutinin, neuraminidase or infectivity of influenza and are inactive in animals (Oxford and Perrin, 1977).

1.2.3.4 Pyrophosphate Analogues



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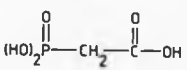

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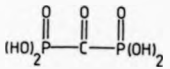


(12)

Fig. 1.2.3.4 (10) Phosphonoformic acid, (11) Phosphonoacetic acid, (12) Carbonylbisphosphonic acid

Interest in this class of compounds was initiated by the report that phosphonoacetic acid (Fig. 1.2.3.4 (11)) was found to inhibit herpes viruses in animals (Shipkowitz, *et al*, 1973); having been discovered in a random screen. It was subsequently demonstrated that phosphonoacetic acid was active against herpes viruses, in tissue culture, under conditions where cell nucleic acid synthesis was little affected (Overby, *et al*, 1974). It was recognised that the activity of phosphonoacetic acid was due to inhibition of a herpes induced DNA polymerase in infected cells (Leinbach, *et al*, 1976).

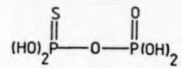
Extensive structure-activity studies have been carried out on phosphonoacetic acid for the inhibition of herpes *in vitro* (Mao, *et al*, 1985) and these demonstrate strict requirements for activity. Monoesterification of the phosphono group results in retention of only slight activity and others (Eriksson, *et al*, 1980) report no significant activity for monoesters of phosphonoacetic acid. Di- and triesterification results in loss of *in vitro* activity although a recent report indicates some *in vivo* activity (Mao, *et al*, 1985).

Insertion of one or more methylene units results in a loss of antiviral activity but removal of the one methylene unit present to give phosphonoformic acid (Fig. 1.2.3.4 (10)) gives a compound of good anti-herpes activity (Eriksson, *et al*, 1982). Phosphonoformic acid is also found to be a good inhibitor of influenza viruses (Stridh, *et al*, 1979) where it is found to inhibit chain elongation in viral RNA synthesis (Stridh and Datema, 1984).

1.3 Mode of Action of Pyrophosphate Analogues Against Influenza

The mode of action of pyrophosphate analogues has been extensively studied and it is now clear that they act without prior metabolism and inhibit the RNA polymerase of influenza, probably via chelation to an essential zinc ion. The influenza RNA polymerase has been shown to be zinc requiring (Oxford and Perrin, 1977) and there is a correlation between the ability of pyrophosphate analogues to bind zinc under physiological conditions and their *in vitro* activities against influenza (Cload, 1983).

Against influenza monothiopyrophosphoric acid (Fig. 1.3(a)(13) and dithiopyrophosphoric acid (Fig.1.3.(a) (14)) are found to exhibit a greater activity than their fully oxygenated analogue (Hutchinson, *et al*, 1985). Thiophosphonoacetic acid (Fig. 1.3(a) (15)) shows a greater activity than phosphonoacetic acid (Hutchinson and Masson, 1986).



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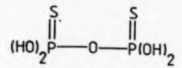

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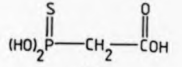


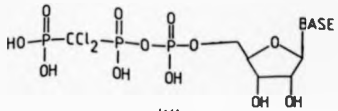
(15)

Fig. 1.3(a) Sulphur containing pyrophosphate analogues (13) Monothiopyrophosphoric acid, (14) Dithiopyrophosphoric acid, (15) Thiophosphonoacetic acid.

The increased activity appears to be related to the increased ability to bind zinc. The greater affinity of sulphur towards zinc under the Pearson rules for hard and soft acids and bases (Pearson, 1966) results in the thiopyrophosphate analogues forming stronger complexes with zinc than their fully oxygenated counterparts (Hutchinson, *et al*, 1985). Addition of metal ions to aqueous solutions of pyrophosphoric acid and bisthiopyrophosphoric acid and examination by ^{31}P NMR spectroscopy indicates that bisthiopyrophosphoric acid binds to "soft" zinc ions through sulphur and "hard" magnesium ions through oxygen (Hutchinson, *et al*, 1985). Nucleoside thiophosphates have been shown to complex to cadmium ions through sulphur (Pillai, *et al*, 1980). These compounds (Fig. 1.3.(a)(13), (14), (15)) are also of interest because of their apparent low toxicity to cells (Hutchinson, *et al*, 1985).

There is also evidence that pyrophosphate analogues act directly on the viral RNA polymerase from enzyme kinetic studies. In the case of the herpes DNA polymerase phosphonoacetic acid and pyrophosphoric acid have been shown to compete for the same binding site (Leinbach, *et al*, 1976). Also in the case of influenza virus phosphonoformic acid is found to have no effect on the initiation of viral mRNA synthesis but that the chain elongation step is prevented (Stridh and Datema, 1984).

In the case of herpes viruses the suggestion has been made that pyrophosphate analogues inhibited the viral DNA polymerase after metabolism to nucleoside triphosphates which then act as competitive inhibitors to the natural substrates (Leinbach,*et al*, 1976). For influenza this does not seem likely for a number of reasons. Ribo- and



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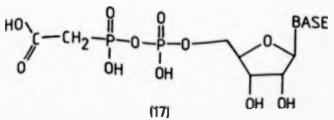


(17)

Fig.1.3(b) Nucleoside triphosphate analogues with the β,γ-residues replaced by (16) dichloromethylenebisphosphonic acid, (17) phosphonoacetic acid

deoxyribonucleoside triphosphate analogues have been synthesised with the β,γ-phosphoryl residues replaced by dichloromethylene-bisphosphonic acid and phosphonoacetic acid (Cload, 1983) (Fig. 1.3(b)). These were found not to interact with the RNA polymerase of influenza virus (Cload and Hutchinson, 1983).

Phosphonoformic acid is a good inhibitor of the influenza RNA polymerase (Cload and Hutchinson, 1983) but nucleotide analogues of phosphonoformic acid cannot be prepared by standard methods and there is evidence that they are very unstable (Hutchinson, *et al*, 1983). Finally when [^{3}H]-labelled phosphonoacetic acid was incubated with detergent disrupted suspensions of influenza, under conditions of limited nucleic acid synthesis, no labelled nucleotide analogues were formed (Cload and Hutchinson, 1983).

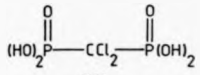
1.4 Clinical Use Of Pyrophosphate Analogues

At present the clinical use of pyrophosphate analogues is very limited. In animal models phosphonoacetic acid has shown toxic effects (Boezi, 1979) and skin irritation (Eriksson and Öberg, 1984). Phosphonoformic acid has undergone clinical trials against a range of viruses and most recently shown benefit in compassionate use for severely immunocompromised AIDS patients (Öberg, *et al*, 1987). The reasons for their limited clinical use are twofold; problems associated with their facile adsorption by bones and teeth, and difficulties in crossing cell membranes. Each of these are discussed below.

1.4.1 Adsorption By Bones

A major problem limiting the clinical use of pyrophosphate analogues is that they have a great affinity for calcified tissue and are readily adsorbed by bones and teeth (Fleisch and Felix, 1979). On administration they are rapidly cleared from the blood and accumulate in calcified tissue. On administration of [^{14}C]-labelled dichloromethylene-

bisphosphonic acid to young rats the measured bone radioactivity reached a maximum in only thirty minutes (Mönkkönen, *et al*, 1987). This is due to the binding of the bisphosphonic acid and not of any metabolites as it is known that dichloromethylenebisphosphonic acid is not metabolised in rats (Fleisch, 1983). Although highly undesirable for an antiviral compound this ability has led to other therapeutic uses.



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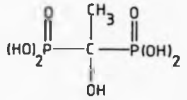
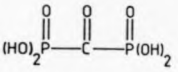


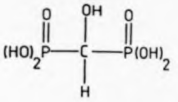
(19)

Fig. 1.4.1(a) Diphosphonates used to treat Paget's disease (18)Dichloromethylene-bisphosphonic acid (19) Ethane-1-hydroxy-1,1-bisphosphonic acid

Dichloromethylenebisphosphonic acid (Fig. 1.4.1(a) (18)) and ethane-1-hydroxy-1,1-bisphosphonic acid (Fig. 1.4.1(a)(19) are found to inhibit the aggregation and dissolution of calcium phosphate crystals and this has led to their use in regulating bone turnover in Paget's disease (Francis and Centner, 1978).

Diphosphonates are commonly complexed with technetium-99 and the resulting complexes are found to be excellent bone imaging agents (eg Wang, *et al*, 1980). These however lack the specificity which might allow treatment of bone tumours (Spencer and Hosain, 1986). Three of the most commonly used diphosphonates are shown below (Fig. 1.4.1(b)).

(20) 

(21) 

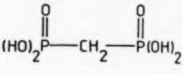
(22) 

Fig. 1.4.1(b) Diphosphonates used in bone imaging (20) Carbonylbisphosphonic acid, (21) Hydroxymethylenebisphonoic acid, (22) Methylenebisphosphonic acid

1.4.2 Crossing Cell Membranes

A second major drawback is that cells do not readily take up small highly charged molecules because of the presence of a lipid cell wall. High plasma concentrations of pyrophosphate analogues are thus often required to be effective in vivo.

Nucleoside esters of phosphonoacetic acid have shown antiviral activity in tissue culture (Heimer and Nussbaum, 1977) and esters of phosphonoformic acid have shown antiherpes activity in tissue culture (Noren, *et al*, 1983). The mode of interference of these compounds is not clear but it is possible that because of their increased lipophilicity they are absorbed more readily and are hydrolysed inside the cell to yield their parent acids. This behaviour as pro-drugs is not unreasonable as the presence of esterases in cells has been well documented (eg Stryer, 1975).

1.5 Outline of Work Undertaken

The aim of the present work was to investigate the preparation of novel substituted methylenebisphosphonates and to examine the activities of a broad range of these compounds against the RNA polymerase of influenza virus A/X49. It was hoped that this would lead to a better understanding of the features required for effective inhibition of the RNA polymerase activity and enable new compounds to be developed which are more active against influenza virus than those currently available.

CHAPTER 2

SYNTHESIS OF FLUORINATED METHYLENEBISPHOSPHONATES

2.1 Introduction

2.1.1 The Arbuzov and Michaelis-Becker Reactions

The Arbuzov reaction has been of enormous synthetic value in organophosphorus chemistry. In its simplest form a trialkyl phosphite reacts with an alkyl halide via two successive S_N2 processes to yield a dialkyl phosphonate (Fig. 2.1.1(a)] (Bhattacharya and Thyagarajan, 1981).

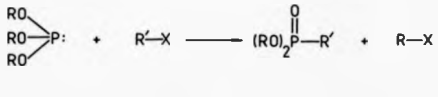


Fig. 2.1.1(a) The Arbuzov reaction

The first step of the reaction is the formation of an intermediate phosphonium salt which can be isolated if the leaving group is a weak nucleophile (Colle and Lewis, 1978). Arbuzov (1910) originally postulated the formation of a pentacoordinate intermediate. Bodkin and Simpson (1972) suggested that a pentacoordinate intermediate was formed in the reaction and studies on the dealkylation step by Michalski (Michalski, *et al*, 1978) give some evidence for an equilibrium between the two species. In the second step of the reaction both S_N2 and S_N1

mechanisms appear possible depending on the alkyl group (Griffiths and Berg, 1962).

The Arbuzov reaction does not normally require catalysis but in special cases catalysis is reported to be effective. Arbuzov reactions of unsaturated alkyl halides are reported to be accelerated by the addition of palladium chloride (Tavs and Korte, 1967) and reactions of halobenzenes may be photocatalysed (eg Kyba, *et al*, 1983; appendix I).

In the synthesis of phosphonates only one of the alkoxy residues of the trialkyl phosphite participates in the reaction, thus the reaction may be used directly in the synthesis of phosphinates and phosphine oxides. These variations of the reaction were developed by Michaelis (1903). Sodium salts of dialkyl phosphites are found to react with alkyl halides to yield the same product and this reaction has become known as the Michaelis-Becker reaction (Michaelis and Becker, 1897) (Fig. 2.1.1(b)).

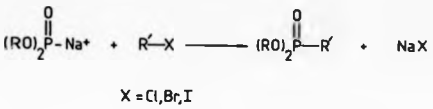


Fig. 2.1.1(b) The Michaelis-Becker reaction

This reaction has also been extensively used in synthesis with the diethyl ester normally being the ester of choice. With halobenzenes the Michaelis-Becker reaction may be photocatalysed (Bunnett and Creary, 1974). The presence of a negative charge on the nucleophile causes an increased reactivity and often alkyl chlorides may be used in cases where the Arbuzov reaction is too slow and the alkyl bromide would be the halide of choice. In this study the increased reactivity of sodium

diisopropyl phosphite over triisopropyl phosphite was exploited in the synthesis of fluorinated methylenebisphosphonates.

2.1.2 Halogenated Methylenebisphosphonic Acids

Methylenebisphosphonic acid is found not to inhibit the RNA polymerase of influenza virus but halogenated methylenebisphosphonic acids do show inhibition (Cload and Hutchinson, 1983). The reason for this is proposed to be the failure of the bridge methylene unit to reflect the electronegativity of the bridge oxygen atom in pyrophosphoric acid (Blackburn, 1981) with the subsequent increase in acid dissociation constants. Simple syntheses of dichloro- and dibromomethylenebisphosphonate are now well known (Quimby, *et al*, 1968) and employ the base catalysed reaction of tetraalkyl methylenebisphosphonate with sodium hypohalite or molecular halogen (Fig. 2.1.2(a)).

Monochlorination and monobromination of tetraalkyl methylenebisphosphonates are difficult to control and lead to mixtures of mono- and dihalogenated species which are difficult to separate (Quimby, *et al*, 1968). This has led to the development of routes for the selective monodehalogenation of dichloro- and dibromomethylenebisphosphonate. This can be accomplished by reaction with butyllithium (Seyferth and Marmor, 1971) or potassium fluoride in the presence of [18,6]crown ether (Hutchinson and Semple, 1984) (Fig. 2.1.2(a)).

Mata has prepared monohalogenated analogues of compounds containing active methylene groups by heating together mixtures of the unhalogenated and dihalogenated species for long periods (Mata, 1965). However this procedure has been found to be unsuccessful in the case of methylenebisphosphonates (Nicholson and Vaughn, 1971); no reaction being detectable by ^{31}P NMR.

The reduction of dichloro- and dibromomethylenebisphosphonates with sodium hydrosulphide has been reported to give good yields of the

corresponding monochalogenated species (Nicholson and Vaughn, 1971) (Fig. 2.1.2(b)). The reaction probably proceeds via nucleophilic dehalogenation followed by reduction. The synthesis is reported to be unreliable (Semple, 1986) with the earlier methods for monodehalogenation being preferable.

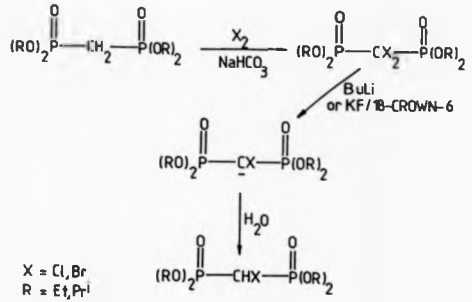


Fig. 2.1.2(a) Syntheses of halogenated methylenebisphosphonates

2.1.3 Fluorinated Methylenebisphosphonic Acids

The anti-influenza activity of halogenated methylenebisphosphonic acids has led to interest in the corresponding fluorinated compounds. The above methods for halogenation of tetraalkyl methylenebisphosphonates cannot readily be adapted for fluorination, which is often difficult to control and unselective. The reaction of tetraalkyl methylenebisphosphonates with solutions of fluorine in toluene (Proctor and Gamble Co, 1966) is reported to be difficult to reproduce (Blackburn, *et al*, 1981). Reaction with fluorine gas, perfluoro-

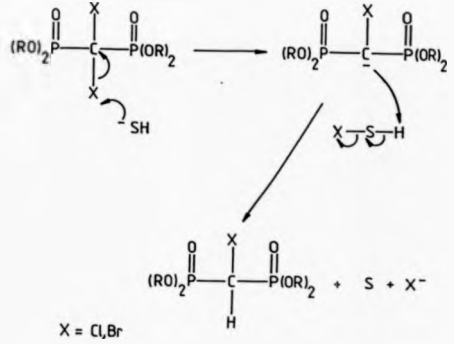


Fig. 2.1.2(b) The reaction of dihalogenomethylenebisphosphonates with sodium hydrosulphide

piperidine and perfluoro-2,6-dimethylpiperidine have all been attempted (Blackburn, *et al*, 1981) but in each case partial fluorine substitution, at every position in tetramethyl methylenebisphosphonate, was observed.

The electrophilic fluorinating agent perchloryl fluoride has been shown to fluorinate the sodium salt of diethyl malonate (Gershon, *et al*, 1966) and this has prompted the use of this reagent for fluorinating methylenebisphosphonates (Blackburn,1981; McKenna and Shen, 1981). However these syntheses suffer from the fact that perchloryl fluoride is hazardous to use and also mixtures of mono- and difluoromethylenebisphosphonates are formed which are difficult to separate. Fluorination of tetraalkyl methylenebisphosphonates is thus not

particularly suited to the synthesis of mono- and difluoromethylene-bisphosphonates. Although α-hydroxybenzylphosphonates have recently been shown to react smoothly with DAST (diethylaminosulphurtrifluoride, Middleton,1975; Markovskij, *et al*, 1973) to yield α-fluorobenzyl-phosphonates (Blackburn and Kent, 1986).

An alternative strategy is to synthesise mono- and difluoromethylenebisphosphonic acids from fluorinated precursors. The first example of a fluorinated phosphonate was reported by Soborovskii and Baina (1959) prepared by the reaction of chlorodifluoromethane and sodium diethyl phosphite (Fig. 2.1.3(a)).

The reaction probably does not proceed via an S_N2 mechanism but via positive chlorine abstraction and reaction of the resulting carbanion with the diethyl phosphorochloridate formed. S_N2 reactions on the α-carbon atom of phosphonates only occur with very good leaving groups such as triflates (Phillion and Andrew, 1986).

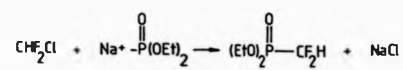


Fig. 2.1.3(a) The reaction of chlorodifluoromethane with sodium diethyl phosphite

The Arbuzov reaction of trialkyl phosphites with dibromodifluoro-methane was reported to yield bromodifluoromethylphosphonates (Burton

and Flynn, 1977) and this synthesis was continued with a Michaelis-Becker reaction to yield the first examples of tetraalkyl difluoromethylenebisphosphonates (Burton and Flynn, 1980) (Fig. 2.1.3(b)). In the application of this route to the synthesis of unsymmetrical difluoromethylenebisphomphonates mixtures of difluorinated methylenebisphosphonates were obtained (Fig. 2.1.3(c)). This was rationalised by the formation of difluorocarbene and competition between the dialkyl phosphite anions to trap the difluorocarbene (Burton, *et al*, 1982). Trapping of difluorocarbene by strong bases in reactions of dibromodifluoromethane is known to occur (Sheppard and Shats, 1969).

In this study a one-step synthesis of tetraisopropyl difluoromethylenebisphosphonate was developed and the understanding gained used to construct a simple synthesis of monofluoromethylenebisphosphonic acid which avoids the use of perchloryl fluoride.

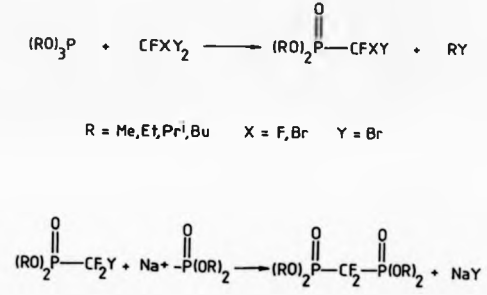


Fig. 2.1.3(b) The synthesis of tetraalkyl difluoromethylenebisphosphonates

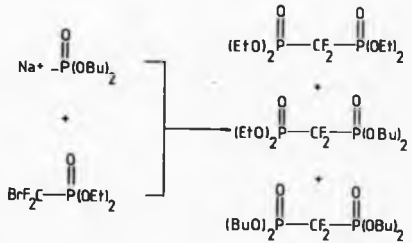


Fig. 2.1.3(c) The reaction between sodium dibutyl phosphite and diethyl bromodifluoromethylphosphonate

2.2 Results And Discussion

2.2.1 Tetraalkyl Difluoromethylenebisphosphonates

The reported Arbuzov reaction between triisopropyl phosphite and dibromodifluoromethane (Burton and Flynn, 1977) was found to be unsuccessful. In refluxing diethyl ether or tetrahydrofuran no reaction was observed and under more forcing conditions, eg refluxing triglyme or in sealed tube reactions, mixtures of products were obtained but by NMR none were found to contain both phosphorus and fluorine. These observations contradict those of Burton and Flynn (1977), although it was found that heating triethyl phosphite with dibromodifluoromethane in a sealed tube, followed by distillation under vacuum yielded diethyl bromodifluoromethylphosphonate in low yield (Fig. 2.2.1(23)).

Bromodifluoromethyl dialkyl phosphonates are known to react with activated zinc dust to form dialkoxyphosphinyl difluoromethylzinc bromides (Burton, *et al*, 1982). When diethyl bromodifluoromethylphosphonate was treated with a slight molar excess of activated zinc dust (prepared as described by Rieke, *et al*, 1981) in anhydrous triglyme the majority of the zinc dust dissolved to leave a colourless solution of diethoxyphosphinyl difluoromethylzinc bromide (Fig. 2.2.1 (24)). Treatment of this solution with diethyl phosphorochloridate did not result in any coupling to form tetraethyl difluoromethylenebisphosphonate. The analagous reaction with acetyl chloride is reported to give a good yield of diethyl 2-oxo-1,1-difluoropropylphosphonate (Fig. 2.2.1(25)) (Burton, *et al*, 1982a).

In view of these difficulties and the report that dibromodifluoromethane would react directly with the sodium salt of diethyl phosphite (Burton and Flynn, 1980) the reaction with the sodium salt of diisopropyl phosphite was investigated. Reaction of dibromodifluoromethane with two equivalents of sodium diisopropyl phosphite in hexane at low temperature followed by flash chromatography on silica gel yielded tetraisopropyl difluoromethylenebisphosphonate in 40% yield. This is comparable to the yields obtained for the tetraethyl and tetrabutyl esters synthesized by the two step route described earlier (Burton and Flynn, 1980). The reaction showed a surprising solvent effect in that if petroleum ether were used as the solvent the yield of product was greatly reduced (11% by ^{31}P NMR).

2.2.2 Tetraisopropyl Monofluoromethylenebisphosphonate

The synthetic approach adopted was to synthesize tetraisopropyl bromofluoromethylenebisphosphonate and debrominate via positive halogen abstraction as described earlier (Seyferth and Marmor, 1973).

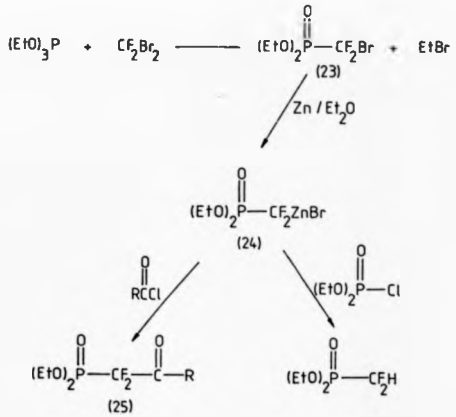


Fig. 2.2.1 The preparation and reactions of diethoxyphosphinyl difluoromethylzinc bromide

2.2.2.1 Diisopropyl Dibromofluoromethylphosphonate

In contrast to the Arbuzov reaction of dibromodifluoromethane treatment of fluorotribromomethane with triisopropyl phosphite in refluxing diethyl ether yielded the desired product diisopropyl dibromofluoromethylphosphonate in good yield (Fig. 2.2.2.1). The

corresponding reaction with the much cheaper Freon fluorotrichloromethane was investigated and was found not to occur in diethyl ether. In a sealed tube reaction between triisopropyl phosphite and fluorotrichloromethane a small qualtity (≈5%) of diisopropyl dichlorofluoromethylphosphonate was detectable by ^{31}P NMR spectroscopy. The lower reactivity of fluorotrichloromethane relative to fluorotribromomethane is not unexpected. In a recent report (Blackburn and Taylor, 1988) the reaction of fluorotrichloromethane with triethyl phosphite is found to require very forcing conditions.

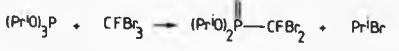


Fig. 2.2.2.1 The reaction of triisopropyl phosphite with fluorotribromomethane

2.2.2.2 Michaelis-Becker Reactions of Diisopropyl Dibromofluoromethylphosphonate

Treatment of diisopropyl dibromofluoromethylphosphonate with one molar equivalent of sodium diisopropyl phosphite in dry hexane at low temperature did not yield any of the desired tetraisopropyl bromofluoromethylenebisphosphonate. A mixture of products was obtained including tetraisopropyl monofluoromethylenebisphosphonate. It appeared that nucleophilic debromination had occurred in situ. After

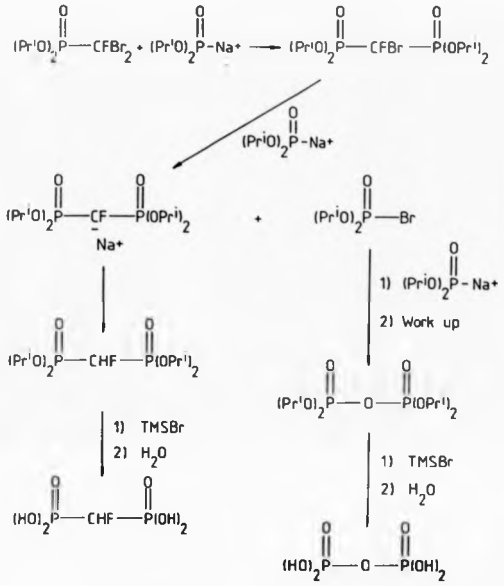


Fig. 2.2.2.2(a) The synthesis of monofluoromethylenebisphosphonic acid

optimisation of the reaction conditions the highest yield of tetraisopropyl monofluoromethylenebisphosphonate was obtained using three equivalents of sodium diisopropyl phosphite.

After flash chromatography of the crude reaction products on silica gel a fraction was obtained which contained tetraisopropyl monofluoromethylenebisphosphonate (65%) and tetraisopropyl pyrophosphate (35%) by ^{31}P NMR spectroscopy. Tetraisopropyl pyrophosphate was identified by synthesis of an authentic sample (Steinberg, 1950). These two materials co-chromatographed on silica gel, however de-esterification of the mixture with bromotrimethylsilane followed by anion exchange chromatography on DE52 cellulose gave bis(triethyl-ammonium) monofluoromethylenebisphosphonic acid. Further cation exchange chromatography on Dowex 50 resin yielded the free acid (Fig. 2.2.2.2(a)).

The first step of the reaction sequence is the debromination of diisopropyl dibromofluoromethylphosphonate by the phosphite anion. This process is analagous to that described by Burton and Flynn (1980) for the treatment of diethyl bromodifluoromethylphosphonate with sodium diethyl phosphite (Fig. 2.2.2.2(b)). In the crude reaction mixture small quantities of diisopropyl bromofluoromethylphosphonate (8% by ^{31}P NMR) were detected, having arisen from protonation of the anion.

The second step in the sequence is the reaction of the diisopropyl bromofluoromethylphosphonate anion with diisopropyl phosphorobromidate to yield tetraisopropyl bromofluoromethylenebisphosphonate. The isolation of tetraisopropyl fluoromethylenebisphosphonate can be ascribed to the ready nucleophilic debromination by the diisopropyl phosphite anion. This yields the sodium salt of tetraisopropyl monofluoromethylenebisphosphonate which would be expected to be very stable (the sodium salt of tetraisopropyl methylenebisphosphonate is a stable solid (Quimby, *et al*, 1968) (Fig. 2.2.2.2(c)).

This was confirmed by the synthesis of an authentic sample of tetraisopropyl bromofluoromethylenebisphosphonate via the bromination procedure of Quimby (1968) and treatment with the sodium salt of diisopropyl phosphite in hexane at low temperature. The crude reaction products consisted of tetraisopropyl monofluoromethylenebisphosphonate (62%) and tetraisopropyl pyrophosphate (31%) by ^{31}P NMR (Fig. 2.2.2.2(c)), indicating that nucleophilic debromination does occur under the reaction conditions.

The formation of tetraisopropyl pyrophosphate as a by-product in Michaelis-Becker reactions of both diisopropyl dibromofluoromethylphosphonate and tetraisopropyl bromofluoromethylenebisphosphonate was unexpected. This appears to arise from the reaction of sodium

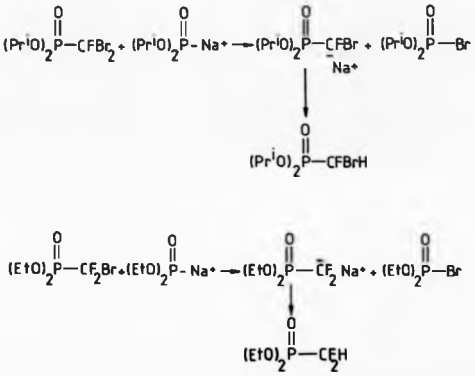


Fig. 2.2.2.2(b) Debromination of bromodifluoromethyl- and dibromofluoromethylphosphonates

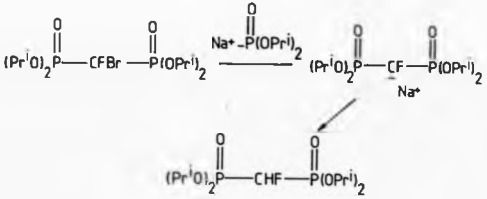


Fig. 2.2.2.2(c) Debromination of tetraisopropyl bromofluoromethylene-bisphosphonate

diisopropyl phosphite with diisopropyl phosphorobromidate. This process is akin to that described by earlier authors (Steinberg, 1950; Atherton and Todd, 1947) for the base catalysed reaction of dialkyl phosphites with carbon tetrachloride.

It has been known since 1930 that the reaction between the sodium salt of diethyl phosphite and diethyl phosphorochloridate yielded tetraethyl pyrophosphate (Nylen, 1930); a close mimic of the observed reaction. Finally when the sodium salt of diisopropyl phosphite in hexane at low temperature was treated with an authentic sample of diisopropyl phosphorobromidate (Goldwhite and Saunders, 1955) a mixture of products were obtained including tetraisopropyl pyrophosphate (40% by ^{31}P NMR).

It has recently been reported that tetraethyl monofluoromethylene-bisphosphonate can be synthesised from diethyl dichlorofluoromethyl-phosphonate and sodium diethyl phosphite (Blackburn and Taylor, 1988) (Fig. 2.2.2.2(d)). The mechanism is reported to be exactly analogous to that above.

A question arises as to the source of the bridge proton. In the Blackburn synthesis the final product is the sodium salt of tetraethyl monofluoromethylenebisphosphonate which is quenched on work up. However in the preparation from diisopropyl dibromofluoromethylphosphonate quenching the reaction with D_2O did not yield any deuterium incorporation in the product, indicating no carbanions present at the end of the reaction.

It was reported recently that on forming the sodium salt of dimethyl phosphite the initially formed anion was quantitatively alkylated by the remaining neutral ester; on quenching with D_2O no phosphorus-centred anion was found to be present (Spears, *et al*, 1987)

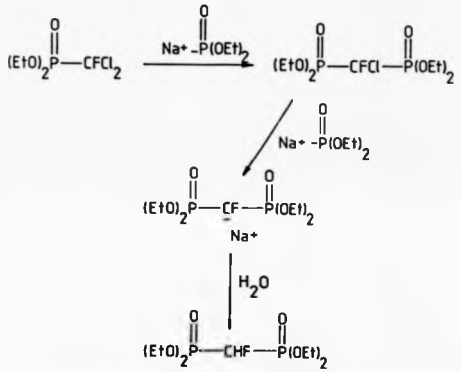


Fig. 2.2.2.2(d) The synthesis of tetraethyl monofluoromethylenebisphosphonate (Blackburn and Taylor, 1988)

(Fig. 2.2.2.2(e)). Repeating the experiment with diisopropyl phosphite in hexane it was found that on quenching with D_2O only 50% of the diisopropyl phosphite had been converted into its carbanion (Fig.

2.2.2.2(e)]. The remainder had been alkylated to yield diisopropyl isopropylphosphonate. It is possible that the proton α to the phosphorus atom in this phosphonate is the source of the bridge proton in the final product. This side reaction is reported to be limited to the use of sodium hydride as the base in THF (Spears, *et al*, 1987); quantitative for the methyl ester, less important for the ethyl ester, but from the result above it is clear that with sodium metal used directly it also occurs to a significant extent with the isopropyl ester.

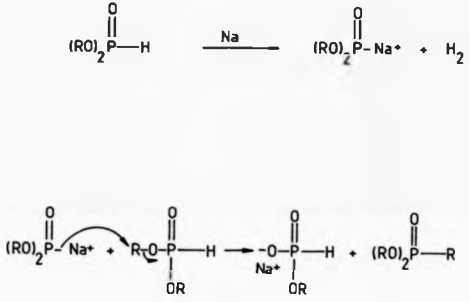


Fig. 2.2.2.2(e) A side reaction involved in forming the carbanions of dialkyl phosphites

Table 2.2.3 Michaelis-Becker reactions leading to fluorinated methylene-bisphosphonates

REACTANTS O R = -P(OPr^n)$_2$	PRINCIPAL PRODUCTS	^{31}P NMR ($COCl_3$)	Yield by ^{31}P NMR	^{19}F NMR ($CDCl_3$)	Yield by ^{19}F NMR	J_{PF}	J_{FH}
R—$CFBr_2$	R—CHF—R	9·4(d)	52(42)[a]	-227(td)	80	65	46
•	R—CFBrH	5·6(d)	8	-166(dd)	15	74	44
3(Na$^+$ R-)	R—O—R	-15·2	20	-	-	-	-
$CFBr_3$	R—CHF—R	9·4(d)	22	-227(td)	65	65	46
•	R—CFBrH	5·6(d)	5	-166(dd)	20	74	44
3(Na$^+$ R-)	R—O—R	-15·2	13	-	-	-	-
$CHFBr_2$	R—CHF—R	9·4(d)	50	-	-	65	46
•	R—H	5·9	8	-	-	-	-
2(Na$^+$ R-)	R—O—R	-15·2	8	-	-	-	-

CF_2Br_2	$R—CF_2—R$	2·1(t)	65(40)[a]	-123(t)	70	88	-
+	$R—CF_2H$	2·9(t)	16	-136(dd)	25	92	49
3(Na+ R-)	R—O—R	-15·2	8	-	-	-	-
$CFCl_3$	R—CHF—R	9·4(d)	30	-227(td)	60	65	46
	R—CFCl—R	4·9(d)	12	-145(t)	2	78	-
+	$R—CFCl_2$	2·3(d)	12	-74(d)	17	89	-
	R—CFClH	5·6(d)	11	-161(dd)	20	77	44
3(Na+ R-)	R—O—R	-15·2	7	-	-	-	-

a) Isolated yield

2.2.3 Michaelis-Becker Reactions of Other Fluorohalomethanes

In view of the results obtained above attempts were made to synthesise tetraisopropyl monofluoromethylenebisphosphonate directly from fluorohalomethanes. The reactions of fluorotribromomethane, fluorotrichloromethane and fluorodibromomethane with sodium diisopropyl phosphite in hexane at low temperature were all investigated (Table 2.2.3). Fluorodibromomethane gave the best yield (50% by ^{31}P NMR) but a complex mixture of products. Fluorotribromomethane gave a very complex product mixture with a low yield (22% by ^{31}P NMR) of tetraisopropyl monofluoromethylenebisphosphonate and the reaction of fluorotrichloromethane was only slightly better with a 30% yield (by ^{31}P NMR) of the desired product. These reactions were considered to hold no advantage over the above two step synthesis because of the increased difficulty in purifying the product.

The reaction of the sodium salt of diisopropyl phosphite with fluorotrichloromethane contrasts with that of the sodium salt of diethyl phosphite (Blackburn, *et al*, 1986). The differences appear to be related to the stability of the intermediate chlorofluoromethanephosphonate carbanion. This is unstable at 0°C (Blackburn, *et al*, 1985), but at -78°C the diisopropyl chlorofluoromethanephosphonate carbanion persists long enough to be phosphorylated to yield tetraisopropyl chlorofluoromethylenebisphosphonate.

2.3 Hydrolysis of Methylenebisphosphonates

Conversion of methylenebisphosphonic acid tetraesters to the corresponding free acids can be effected with concentrated hydrochloric acid at high temperature (Kosolapoff, 1950) but this often results in low yields. The need for these harsh conditions had led to the development of a range of trialkylhalogenosilanes for the transesterification of phosphate and phosphonate esters.

Iodotrimethylsilane has been reported to be very effective for the dealkylation of phosphate and phosphonate esters (Blackburn, 1980). The reaction involves extremely mild conditions and has been used to dealkylate the normally very reactive dialkyl phosphorobromidates (Chojnowski, *et al*, 1978). Bromotrimethylsilane gives virtually quantitative yields of the corresponding trimethylsilyl esters when reacted with phosphate esters at room temperature (Lalinde, *et al*, 1983). This reagent is reported to give a cleaner reaction than iodotrimethylsilane (Hutchinson and Semple, 1985) which has to be used in the dark at low temperature. Bromotrimethylsilane was the reagent of choice for all deesterifications performed in this study (Fig. 2.3).

Methylenebisphosphonic acids produced by reaction with bromotrimethylsilane and hydrolysis were obtained as viscous oils and were usually converted into their tris(cyclohexylammonium) salts. This procedure was widely used in this study and the deesterification of tetraisopropyl difluoromethylenebisphosphonate (Section 2.5.7) may be considered typical.

2.4 Conclusions

Michaelis-Becker reactions of the appropriate fluorobromomethane may be used to synthesise fluorinated methylenebisphosphonates directly or they can be prepared by reaction of a precursor fluorobromomethyl-phosphonate. In the case of tetraisopropyl difluoromethylene-bisphosphonate the first approach is the method of choice but for tetraisopropyl monofluoromethylenebisphosphonate the second approach is preferred as the yield is higher and the reaction mixture less complex. Both these methods appear more suited to the syntheses than fluorination of tetraalkyl methylenebisphosphonates.

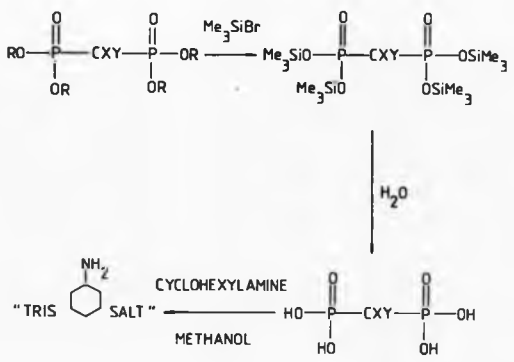


Fig. 2.3 The dealkylation of methylenebisphosphonates with bromotrimethylsilane

2.5 Materials and Methods

2.5.1 Materials

i) Starting materials were either commercially available or synthesized as described in the text. [^{3}H]-UTP was obtained from Amersham (Amersham International PLC, Amersham, Buckinghamshire, U.K.).

ii) All solvents were dried and distilled before use.

2.5.2 General Chemical Procedures

i) ^1H NMR spectra were recorded at 220 MHz on a Perkin-Elmer R34 spectrometer or at 400 MHz on a Bruker WH400 spectrometer, with either tetramethylsilane (TMS) or sodium 3-(trimethylsilyl)-1-propane sulphonate (TSS) as internal standards. ^{31}P NMR spectra were recorded at 36.43 MHz on a Bruker WH90 spectrometer or at 162.0 MHz on a Bruker WH400 spectrometer. ^{31}P NMR shifts are quoted relative to an external standard of 85% H_3PO_4 with shifts to higher frequency expressed as positive.

^{19}F NMR spectra were recorded at 84.67 MHz or at 75.69 MHz on Bruker WH90 and Bruker WP80 spectrometers respectively. Where appropriate hexafluorobenzene, 1,2-difluorotetrachloroethane, fluorotrichloromethane and trifluoroacetic acid were used as internal standards with shifts to increasing frequency given a positive sign. All ^{19}F NMR shifts are quoted relative to fluorotrichloromethane ($CFCl_3$, $\delta = 0$ p.p.m.). ^{13}C NMR spectra were recorded at 100.62 MHz on a Bruker WH400 spectrometer or at 22.63 MHz on a Bruker WP80 spectrometer with TMS as an external reference.

ii) Mass spectra were recorded using a Kratos MS80 instrument fitted with a DS55 data system. Electron impact (EI) spectra were recorded at 70eV and for chemical ionisation (CI) spectra ammonia was used as the reagent gas (Cload and Hutchinson, 1983a).

iii) Microanalyses were carried out by Butterworth Laboratories Ltd., Teddington, Middlesex, U.K.

iv) Compounds were usually purified by flash chromatography on silica gel (Still, *et al*, 1978).

v) In listing NMR data the following abbreviations are used:

s	singlet
d	doublet
t	triplet
q	quartet
quint	quintet
m	multiplet
M	series of multiplets

2.6 Experimental

Full NMR data for the fluorinated phosphonates and bisphosphonates synthesised are listed in table 2.6(a). NMR data for the bisphosphonic acids synthesised are listed in table 2.6(b).

2.6.1 Preparation of Diethyl Bromodifluoromethylphosphonate

A mixture of dibromodifluoromethane (5.9cm³, 64 mmol) and triethyl phosphite (10.4cm³,60 mmol) was heated in a sealed glass tube for 24h at 100°C. The crude product was distilled under vacuum to yield the title compound (90-92°C/10mmHg) as a colourless oil (3.0g, 19%). [Mass spectrum (NH_3CI) $(M+H)^+$ 266,268].

2.6.2 Preparation of Diisopropyl Dibromofluoromethylphosphonate

This was prepared from triisopropyl phosphite and fluorotribromomethane essentially as described (Burton and Flynn, 1977). Purification by flash chromatography, elution with acetone/petroleum ether (1:20°/v), yieldsd the title compound as a colourless oil (19.4g, 78%). [Analysis C, 24.10; H, 4.12; P, 8.55; F, 4.86%. $C_7H_{14}Br_2FO_3P$ requires C,23.62; H, 3.96; P, 8.70; F, 5.34%; Mass spectrum (NH_3CI) $(M + H)^+$ 355,357,359].

2.6.3 Preparation of Tetraisopropyl Difluoromethylenebisphosphonate

A solution in dry hexane ($10cm^3$) under nitrogen of sodium diisopropyl phosphite was prepared from diisopropyl phosphite (8.0g, 48 mmol) and sodium metal (1.25g, 54 mmol). After cooling to -78°C a solution of dibromodifluoromethane ($2.20cm^3$, 24 mmol) in dry hexane ($10cm^3$) was added dropwise over 30 min. The solution was stirred for 3h at -78°C and was then filtered through a plug of celite. Evaporation of the filtrate yielded a colourless oil which was distilled under vacuum. The fraction distilling in the range 75-85°C/0.8 mmHg was collected. Flash chro- matography on silica gel, elution with acetone/petroleum ether bpt 40-60°C (1:15°/v) yielded the title compound as a colourless oil (3.66g, 40%). [Analysis C, 40.15; H, 7.51; P, 16.25; F, 7.95%. $C_{13}H_{28}F_2O_6P_2$ requires C, 41.06; H, 7.42; P, 16.29; F, 9.99%; Mass spectrum (NH_3CI) $(M + H)^+$ 381.1427 ($C_{13}H_{28}F_2O_6P_2$ calc. 381.1425)].

2.6.4 Preparation of Tetraisopropyl Bromofluoromethylenebisphosphonate

This was prepared by reaction of a mixture of tetraisopropyl monofluoromethylenebisphosphonate and tetraisopropyl pyrophosphate (1.0g, 1:1 by ^{31}P NMR, obtained during the preparation of tetraisopropyl monofluoromethylenebisphosphonic acid) with bromine essentially as described (Quimby, *et al*, 1968). Flash chromatography on silica gel, elution with acetone/petroleum ether (1:5 °/v), yielded the title compound as a colourless oil (0.55g, 90%). [Analysis C, 35.12; H, 6.42; P, 14.20; F, 3.98%. $C_{13}H_{28}BrFO_6P_2$ requires C, 35.39; H, 6.40; P, 14.04; F, 4.31%; Mass spectrum (NH_3CI) $(M + H)^+$ 441,443].

Table 2.6(a) NMR shifts of fluorinated phosphonates and bisphosphonates

COMPOUND	^{1}H NMR($CDCl_3$)	^{13}C NMR($CDCl_3$)	^{31}P NMR($CDCl_3$)	^{19}F NMR($CDCl_3$)
$(Pr^iO)_2P(O)-CFBr_2$	δ 1.45(12H,dd,J=6.7), 5.02(2H,m,J=6.7)p.p.m.	δ 23.1(d,J=5.9), 23.3(d,J=2.7), 75.6(d,J=214), 88.9(dd,J=203, J=333)p.p.m.	δ -0.6(d,J=7.7) p.p.m.	δ -77.2(d,J=7.7) p.p.m.
$(Pr^iO)_2P(O)-CF_2-P(O)Pr^i_2$	δ 1.42(24H,dd,J=7.2,J=2.0), 4.95(4H,m)p.p.m.	δ 23.3(s),23.9(s),74.2(t,J=3.7) 115.3(t,J=199.,J=279)p.p.m.	δ 2.05(t,J=8.8) p.p.m.	δ -122.5(t,J=8.8) p.p.m.
$(Pr^iO)_2P(O)-CFBr-P(O)Pr^i_2$	δ 1.35(24H,dd,J=7.2,J=6.0), 5.01(4H,m)p.p.m.	δ 23.4(s),24.1(s),74.5(s),74.8 (s),95.2(dt,J=166.,J=277)p.p.m.	δ 5.35(d,J=7.4) p.p.m.	δ -155.0(t,J=7.4) p.p.m.
$(EtO)_2P(O)-CF_2Br$	δ 1.45(6H,t,J=7.2),4.40(4H, quint,J=7.2)p.p.m.	—	δ -0.8(t,J=9.3) p.p.m.	δ -62.0(d,J=9.3) p.p.m.

Table 2.6(b) NMR shifts of fluorinated methylenebisphosphonic acids

COMPOUND	^{1}H NMR(D_2O)	^{13}C NMR(D_2O)	^{31}P NMR(D_2O)	^{19}F NMR(D_2O)
	δ 5·02(1H,dt,J=13,J=47) p.p.m. δ 5·45(1H,dt,J=14,J=46)[b] p.p.m.	δ 35·3(dt,J=150,J=181)p.p.m.	δ 9·65(d,J=66) p.p.m.	δ -149·5(td,J=46, J=65)p.p.m.
	δ 1·1-1·5(15H,m),1·65(3H,m), 1·80(6H,m),2·00(6H,m),3·15 (3H,m)p.p.m.	—	δ 5·20(t,J=80) p.p.m.	—
	δ 1·1-1·5(15H,m),1·65(3H,m), 1·80(6H,m),2·00(6H,m),3·15 (3H,m)p.p.m.	—	δ 8·0(d,J=70) p.p.m.	—

a) TRIS  SALT

b) ^{1}H NMR spectrum recorded in D_2O(80%)/DCl(20%)

2.6.5 Preparation of Tetraisopropyl Pyrophosphate

This was prepared exactly as described (Steinberg, 1950) to yield the title compound, distilling at 101-102°C/0.1 mmHg (lit 94-99°C/0.01-0.02 mmHg), as a colourless oil (11.1g, 71%). [^{1}H NMR ($CDCl_3$) δ 1.38 (24H, d, J=6.2Hz), 4.80 (4H,m) p.p.m.; ^{31}P NMR ($CDCl_3$) δ -15.1 p.p.m.(s); ^{13}C NMR ($CDCl_3$) δ 23.4 (q, J=2.9 Hz), 73.9 (t, J=2.9Hz) p.p.m.; Analysis C, 41.92; H, 8.34; P, 16.88%. $C_{12}H_{28}O_7P_2$ requires C, 41.62; H, 8.15; P, 17.89%; Mass spectrum (NH_3CI) (M + H)$^+$ 347].

2.6.6 Preparation of Monofluoromethylenebisphosphonic Acid

To a solution in dry hexane (15cm^3) of sodium diisopropyl phosphite (prepared from diisopropyl phosphite (1.50cm^3, 9mmol) and sodium (0.27g, 12 mmol) stirred at -78°C under nitrogen was added a solution in dry hexane (5cm^3) of diisopropyl dibromofluoromethylphosphonate (1.07g, 3.0 mmol). After stirring at -78°C for 2h the solution was allowed to warm to room temperature; hexane (20cm^3) was added and the solution filtered through celite. The filtrate was stripped in vacuo to yield a colourless oil.

Flash chromatography on silica gel, eluting with acetone/petroleum either (1:10°/$_v$), gave a major fraction which contained tetraisopropyl monofluoromethylenebisphosphonate (65%) and tetraisopropyl pyrophosphate (35%) by ^{31}P NMR. This fraction was treated with bromotrimethylsilane (6.0cm^3, 45 mmol) for 48h, lyophilised, shaken with water (10cm^3) and again lyophilised to yield a colourless oil.

Anion exchange chromatography on a DE52 cellulose column (3 x 33 cm, HCO_3^- form) with elution by a 0.1-0.5M linear gradient of triethylammonium bicarbonate (pH 7.5) yielded, after freeze drying, bis(triethylammonium) monofluoromethylenebisphosphonate as a white solid. Acid containing aliquots were detected by spotting onto chromatography paper and spraying with a phosphate detecting spray (Bockner, *et al*, 1981). Further ion exchange chromatography on a short

column of Dowex 50 (H^+ form) yielded the title compound as a colourless oil (0.25g, 42%). [Analysis C, 6.17; H, 2.76; P, 30.67%. $CH_5FO_6P_2$ requires C, 6.19; H, 2.58; P, 31.93%].

2.6.7 Preparation of Tris(cyclohexylammonium) Difluoromethylene-bisphosphonate

Tetraisopropyl difluoromethylenebisphosphonate (0.50g, 1.3 mmol) was treated with bromotrimethylsilane (1.72cm^3, 13 mmol) under nitrogen for 48h. The product was lyophilised, shaken with water (10 cm^3) and again lyophilised to yield a colourless oil. Treatment with methanol (5cm^3) and cyclohexylamine (excess) yielded the crude title compound. Recrystallisation from methanol/diethyl ether yielded the title compound as a white crystalline solid (0.44g, 66%). [Analysis C, 44.76; H, 8.46; N, 7.97; F, 6.88%. $C_{19}H_{42}F_2N_3O_6P_2$ requires C, 44.79; H, 8.51; N, 8.25; F, 7.46%].

2.6.8 Preparation of Tris(cyclohexylammonium) Bromofluoromethylene-bisphosphonate

This was prepared from tetraisopropyl bromofluoromethylene bisphosphonate (0.25g, 0.57mmol) in a manner analagous to that above. Recrystallisation from methanol/diethyl ether yielded the title compound as a white solid (0.24g, 73%). [Analysis C, 39.99; H, 7.54; N, 7.24; F, 2.97; P, 10.36%. $C_{19}H_{42}BrFN_3O_6P_2$ requires C, 40.01; H, 7.60; N, 7.37; F, 3.33; P, 10.86%].

CHAPTER 3

MICHAEL REACTIONS OF TETRAALKYL ETHENYLIDENEBISPHOSPHONATES

3.1 Introduction

Alkylated methylenebisphosphonates are of interest as potential antiviral agents as modification of the alkyl substituent might enable bisphosphonates to be developed which were more readily taken up by cells and less readily absorbed by bones. Alkylated methylenebisphosphonates may be synthesised by reaction of the sodium, lithium or thallium(I) salts of methylenebisphosphonates (Quimby, *et al*, 1968; Seyferth and Marmor, 1973; Hutchinson and Semple, 1985) with alkyl iodides. However these methods do not readily lend themselves to the preparation of tetraalkyl methylenebisphosphonates with labile groups attached either to the bridge carbon atom or to the alkyl substituent.

The possibility exists however to synthesise novel substituted methylenebisphosphonates via reactions of the double bonds in tetraalkyl alkylidenebisphosphonates. These may be synthesised by the Knoevenagel condensation of tetraalkyl methylenebisphosphonates with aldehydes and ketones (Lehnert, 1974). The parent compounds, tetraalkyl ethenylidenebisphosphonates, may be synthesised via the thermal dehydration of tetrasodium 1-hydroxyethylidenebisphosphonate (Carroll, 1972) followed by treatment with triethylorthoformate (Nicholson, *et al*, 1970). A much improved preparation has recently been reported (Degenhardt and Burdsall, 1986) which involves the base catalysed condensation of tetraalkyl methylenebisphosphonate with formaldehyde (Fig. 3.1).

Although the conjugate addition of nucleophiles to α,β- unsaturated carbonyl compounds is well documented (eg Bergmann, *et al*, 1959), little has been reported on the analogous reaction with α,β-unsaturated phosphonates. Diethyl ethenylphosphonate (Kosolapoff, 1948)

is known to undergo a Michael-type reaction with organocuprate reagents (Nicotra, *et al*, 1984) and 1-(functionally) substituted vinylphosphonates have been reported to undero Michael additions with various organometallic reagents (Barbot, *et al*, 1984), but no analogous reaction with tetraethyl ethenylidenebisphosphonate has been described.

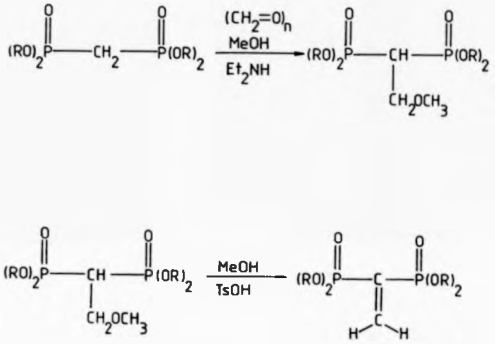


Fig. 3.1 Syntheses of tetraalkyl ethenylidenebisphosphonates

In this study the Michael-type reactions of tetraalkyl ethenylidenebisphosphonates were investigated with a view to increasing

the range of substituted methylenebisphosphonates which could be assayed against the RNA polymerase of influenza.

3.2 Results and Discussion

When tetraethyl or tetraisopropyl ethenylidenebisphosphonate was treated with diethylamine in chloroform a Michael-type addition was observed across the carbon-carbon double bond. Similar additions were observed with other amines, thiols and also dialkyl phosphites when the reaction was catalysed by base (diisopropylamine or 2,2,6,6-tetramethylpiperidine) (Table 3.2(a)). The reactions presumably proceed via formation of an intermediate zwitterion with the negative charge on the bridge carbon atom stabilised by the two phosphorus atoms; intramolecular proton transfer yielding the reaction product (Fig. 3.2(a)). On a small scale (50-100 mg) the reactions were observed to be quantitative by ^{1}H and ^{31}P NMR spectroscopy.

Diethyl and diisopropyl phosphite took part in the reaction only when diisopropylamine or 2,2,6,6-tetramethylpiperidine was added as a catalyst. In these reactions only carbon-phosphorus bond formation was observed. There was no evidence (by ^{31}P NMR spectroscopy) for carbon-oxygen bond formation.

Oxygen nucleophiles did not take part in the reaction. Treatment of tetraethyl ethenylidenebisphosphonate with water, methanol, phenol or acetic acid did not result in any reaction over 72h. More potent nucleophiles such as ethoxide or hydroxide ions led to hydrolysis of the bisphosphonate before any Michael addition was observed.

Carbon nucleophiles did not give a Michael reaction. Tetraisopropyl and tetraethyl methylenebisphosphonates (both in the presence and absence of added diisopropylamine) failed to react in 72h. Addition of butyllithium or methyllithium at low temperature yielded a complex mixture of products none of which had the characteristics of a Michael adduct.

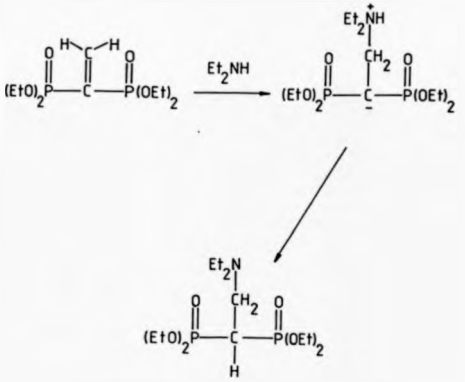


Fig. 3.2(a) The Michael addition of diethylamine to tetraethyl ethenylidene-bisphosphonate

When the Michael reactions were carried out on a larger scale (2g) the products were still obtained in high yield. For example treatment of tetraethyl ethenylidenebisphosphonate with a slight molar excess of thiophenol for 24h at 45°C yielded C-(thiophenyl)methyl methylene-bisphosphonate as the dominant product (97%) by ^{31}P NMR (Table 3.2(b)).

The Michael reaction is, in principle, reversible (March, 1977) and in the adducts it was found that when X was a good leaving group (X-H having undergone reaction to yield the adduct) the adduct was unstable to elimination. Only the products formed from reactions of thiols were stable enough to be isolated and transesterified with bromotrimethylsilane. For example flash chromatography of crude

Table 3.2(a) Reactions of tetraalkyl ethenylidenebisphosphonates with nucleophiles

REACTANT[d]	Reaction time (min)	^{31}P NMR[a] of adduct (p.p.m)	NH_3Cl (M+H)+
TETRAETHYL ESTER			
Nitrogen nucleophiles			
Et_2NH	< 5	23·8	374
	< 5	22·4	388
	< 5	19·6	369
	< 5	22·9	400
	12h	22·0	394
$H_2NCH_2CH_2NH_2$[b]	< 5	22·6	661
	-	-	-
Pr^i_2NH[a]	-	-	-
Sulphur nucleophiles			
	60	21·2	411
$HOCH_2CH_2SH$	720	21·7	379
EtSH	60	21·6	363
PrSH	70	21·7	377
$C_{12}H_{25}SH$	150	21·7	504

Phosphorus nucleophiles			
$(EtO)_2\overset{O}{\overset{\|}{P}}-H$[e]	-	-	-
$(EtO)_2\overset{O}{\overset{\|}{P}}-H + Pr^i_2NH$[c]	180	23·0 (d, J = 269) 28·7 (t, J = 26·9)	439
$(Pr^iO)_2\overset{O}{\overset{\|}{P}}-H$[e]	-	-	-
$(Pr^iO)_2\overset{O}{\overset{\|}{P}}-H + Pr^i_2NH$[c]	36h	25·9 (dd, J = 30, J = 24), 22·3 (d, J = 24), 22·3 (d, J = 30)	467
TETRAISOPROPYL ESTER			
Nitrogen nucleophiles			
Et_2NH	< 5	21·2	430
	< 5	20·5	444
	< 5	17·5	425
	< 5	20·9	457
	17h	20·0	450
Sulphur nucleophiles			
	240	19·2	468
$HOCH_2CH_2SH$	40h	19·7	435

a) In $CDCl_3$ b) Reaction at both $-NH_2$ groups c) One equivalent

d) Reaction in $CDCl_3$ as described e) No reaction

Table 3.2(b) Preparative scale Michael reactions of tetraethyl ethenylidene-bisphosphonate

REACTANT	Temperature (°C)	Starting Material[a] (%)	Michael Adduct[a] (%)
Et_2NH	20	3	94
-SH	45	2	97(91)[b]
$C_{12}H_{25}SH$	45	3	97(92)[b]
$(EtO)_2\overset{O}{\overset{\|}{P}}-H$	45	3	92

a) By ^{31}P NMR spectroscopy

b) Isolated yield

C-(diethylamino)methyl methylenebisphosphonate on silica gel and evaporation of the eluate fractions gave an almost quantitative yield of tetraethyl ethenylidenebisphosphonate. Elimination of diethylamine had occurred.

Differences in the stabilities of the Michael adducts could readily be demonstrated by ^1H NMR spectroscopy. Dissolving C-(thiophenyl)methyl methylenebisphosphonate in d_4-methanol resulted in no change in the ^1H NMR spectrum over 24h. On the other hand, dissolving C-(diethylamino)methyl methylenebisphosphonate in d_4-methanol resulted in rapid deuterium exchange of the bridge proton and this was confirmed by mass spectrometric analysis of the residue after evaporation of the solvent (Fig. 3.2(b)).

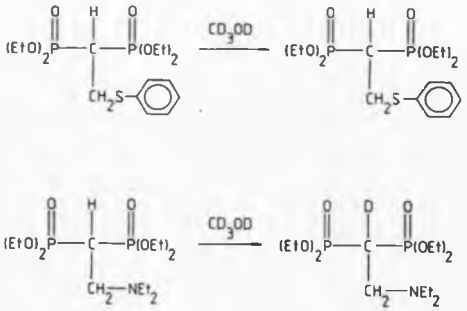
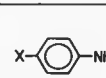


Fig. 3.2(b) Deuterium exchange on dissolving C-substituted methylene-bisphosphonates in methanol-d_4

Changing from a non-polar solvent ($CHCl_3$) to a polar protic solvent (MeOH) would be expected to increase the rate of Michael reaction via stabilisation of the zwitterionic intermediate and providing an additional proton source to neutralise the negative charge on the bridge carbon atom. This was found to be the case; in chloroform, aniline required 400 min for complete reaction at 45°C but under identical conditions in d_4-methanol the reaction was complete in under 30 min (Table 3.2(c)).

Table 3.2(c) Reaction of 4-substituted anilines with tetraethyl ethenylidene-bisphosphonate

	pKa (of conjugate acid)	SOLVENT	Temperature (°C)	Time for reaction
X= -OMe	5·3	CD_3OD	45	<5min
X= -H	4·3	CD_3OD	45	27min
X= -H	4·3	$CDCl_3$	45	400min
X= -Br	3·8	CD_3OD	45	220min
X= $-NO_2$	1·0	CD_3OD	45	90h

The Michael reaction of tetraethyl ethenylidenebisphosphonate with a series of 4-substituted anilines was investigated (Table 3.2(c)). It was observed that strongly basic anilines which had a high pK_a for their conjugate acid (eg 4-methoxyaniline) reacted rapidly but less basic anilines reacted more slowly. The presence of a high electron density on the attacking nucleophile appears to be important in these reactions.

Tetraisopropyl ethenylidenebisphosphonate was found to undergo Michael additions more slowly (Table 3.2(a)) as would be expected on purely steric grounds. However on examination of the ^{13}C NMR chemical shifts of methylenebisphosphonates and their anions it is found that the $^1J^{13}C-^{31}P$ coupling constant is increased upon anion formation consistent with a change in hybridisation from sp^3 to sp^2 (Strothers,

1974) and that the chemical shift moves upfield (Semple, 1986) indicating negligible delocalisation of the negative charge (Gray, 1973). Thus in forming the intermediate zwitterion it appears that the phosphate ester groups may be moved slightly apart as the PCP bond angle increases upon change in hybridisation. This would relieve some of the steric crowding in tetraisopropyl ethenylidenebisphosphonate and might be expected to facilitate the reaction.

3.3 Conclusions

Tetraalkyl ethenylidenebisphosphonates can undergo a facile Michael type reaction with soft nucleophiles to yield C-substituted methylenebisphosphonates. In the case of sulphur nucleophiles the products are stable and can be readily deesterified with bromotrimethylsilane to yield C-substituted methylenebisphosphonic acids. The reaction is high yielding and with sulphur nucleophiles the products are readily isolated by flash chromatography on silica gel. This procedure is useful for the preparation of lipophilic methylenebisphosphonates as even dodecyl-1-thiol reacts to give a Michael adduct in high yield.

3.4 Experimental

3.4.1 Preparation of Tetraethyl Ethenylidenebisphosphonate

This was prepared as described (Degenhardt and Burdsall, 1986) and purified by distillation under vacuum at 120-121°C/0.2 mmHg (lit 115-116°C/0.05 mmHg) to yield the title compound as a colourless oil (13.6g, 65%). [^{1}H NMR ($CDCl_3$) δ 1.32 (12H, t, J=7.1 Hz), 4.12 (8H,m), 6.97 (2H, distorted dd, J=33.8 Hz, J=37.8 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 13.0(s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 148.5 (s,broad), 131.5 (t,J=166.6Hz), 62.0 (t,J=2.8 Hz), 15.7 (t,J= 3.3 Hz) p.p.m.; Analysis C, 39.98; H, 7.67%. $C_{10}H_{22}O_6P_2$ requires C, 40.01; H, 7.39%; Mass spectrum (NH_3CI) $(M+H)^+$ 301.0970 ($C_{10}H_{22}O_6P_2$ calc. 301.0970)].

3.4.2 Preparation of Tetraisopropyl Ethenylidenebisphosphonate

This was synthesised in an analogous manner to the tetraethyl ester described above except that in the first stage of the preparation the reaction mixture was heated for eleven days. Distillation under vacuum yielded the title compound (114-115°C/0.15 mmHg) as a colourless oil (13.5g, 33%). [^{1}H NMR ($CDCl_3$) δ 1.35 (24H, dd, J=7.2Hz, J=6.1Hz), 4.75 (4H,m), 6.96 (2H, distorted dd, J=34.4Hz, J=38.3Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 11.3(s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 147.2 (m), 134.7 (t,J=169Hz), 71.2 (t,J=3.2Hz), 23.8 (dt, J=2.5 Hz, J=22 Hz) p.p.m.; Analysis C, 46.81; H, 8.62; P, 17.51%. $C_{14}H_{32}O_6P_2$ requires C, 47.20; H, 8.49; P, 17.38%; Mass spectrum (NH_3CI) $(M+H)^+$ 357.1637 ($c_{14}H_{31}O_6P_2$ calc. 357.1641)].

3.4.3 Michael Addition Reactions

3.4.3.1 NMR Scale Experiments

To tetraethyl ethenylidenebisphosphonate (75mg, 0.25 mmol) in $CDCl_3$ (0.25cm^3) was added a solution of thiophenol (50 mg, 0.45 mmol) in $CDCl_3$ (0.25 cm^3). After rapid mixing the solution was maintained at 45°C and the reaction followed by ^{1}H NMR spectroscopy. When no further change in the ^{1}H NMR spectrum occurred the ^{31}P NMR spectrum was recorded. The solvent was removed by blowing nitrogen through the solution and a mass spectrum recorded of the residue. For other Michael additions a similar procedure was employed except that when dialkyl phosphites were used as the nucleophiles one equivalent of diisopropylamine was also added.

Table 3.4.3.2 NMR characteristics of tetraethyl C-(thioalkyl)methyl methylenebisphosphonates

$(EtO)_2P(O)-CH(CH_2SR)-P(O)(OEt)_2$	^1H NMR ($CDCl_3$)	^{13}C NMR ($CDCl_3$)	^{31}P NMR ($CDCl_3$)	ANALYSIS FOUND (EXPECTED) C	H	P	S	NH_3 CI $(M+H)^+$
R = Et	δ 1·30(3H,t,J=7·8), 1·40(12H,t,J=7·2),2·63(2H,q,J=7·1),2·64(1H,tt,J=5·8,J=27·8),3·11(2H,td,J=5·8,J=17·2),4·27(8H,m,J=7·2) p.p.m.	δ 14·6(s),16·6(d,J=6·1),26·7(s),27·1(t,J=4·6),38·9(t,J=131·6),62·6(dd,J=6·6,J=14·6) p.p.m.	21·65	39·51 (39·78)	7·93 (7·79)	15·09 (17·10)	8·53 (8·85)	363
R = Pr	δ 1·00(3H,t,J=7·8), 1·38(12H,t,J=7·5),1·64(2H,m,J=7·8),2·56(2H,t,J=7·8),2·57(1H,tt,J=6·4,J=25·6),3·08(2H,td,J=6·4,J=16·7),4·23(8H,m,J=7·5)p.p.m.	δ 13·2(s),16·2(d,J=5·8),22·5(s),27·5(t,J=4·4),34·9(s),38·9(t,J=134),62·6(dd,J=6·5,J=14·8)p.p.m.	21·70	40·83 (41·48)	8·13 (8·03)	15·04 (16·46)	7·95 (8·52)	377

R = Ph	δ 1·35 (12H, td, J=7·5, J=2·8), 2·67 (1H, tt, J=5·8, J=24·4), 3·48 (2H, td, J=5·8, J=15·8), 4·22 (8H, m, J=7·5), 7·20–7·50 (5H, m) p.p.m.	δ 15·9 (d, J=6·1), 29·8 (t, J=4·6), 37·6 (t, J=131·2), 126·2 (s), 128·6 (s), 129·6 (s), 135·2 (s) p.p.m.	21·2	46·54 (46·83)	7·02 (6·88)	14·10 (15·09)	7·29 (7·81)	4·11
R = $C_{12}H_{25}$ (dodecyl)	δ 0·90 (3H, t, J=6·4), 1·28 (18H, m), 1·46 (12H, t, J=7·2), 1·60 (2H, m), 2·57 (2H, t, J=7·2), 2·60 (1H, tt, J=6·4, J=24·4), 3·06 (2H, dt, J=6·4, J=16·7), 4·22 (8H, m, J=7·2) p.p.m.	–	21·7	52·35 (52·58)	9·63 (9·63)	11·86 (12·32)	6·24 (6·38)	5·04

Table 3.4.4 NMR characteristics of tris(cyclohexylammonium) C-(thioalkyl)methyl methylenebisphosphonates

	^{1}H NMR (D_2O)	^{31}P NMR (D_2O)	ANALYSIS Found (Expected)				
			C	H	N	P	S
R = Et	δ 1.25 (3H, t, J = 7.4), 2.08 (1H, t, J = 7.5, J = 2 Hz), 2.60 (2H, q, J = 7.4), 2.90 (2H, Nd, J = 7.8, J = 17.2 Hz, p.p.m.	18.0	47.54 (48.25)	8.98 (9.39)	7.72 (7.67)	11.68 (11.39)	5.49 (5.85)
R = Pr	δ 0.98 (3H, t, J = 7.5), 1.65 (2H, m, J = 7.5, J = 7.8), 2.06 (1H, t, J = 7.8, J = 2.2 Hz), 2.60 (2H, t, J = 7.8), 3.00 (2H, Nd, J = 7.8, J = 16.0 Hz, p.p.m.	18.0	49.96 (49.18)	9.23 (9.50)	7.61 (7.48)	10.63 (11.03)	5.60 (5.71)

R =	δ 2·16 (1H, t t, J = 7·2, J = 20·0), 3·44 (2H, t d, J = 7·2, J = 15·6), 7·25-7·55 (5H, m) p.p.m.	18·0	52·26 (53·36)	8·67 (8·46)	6·78 (6·91)	9·96 (10·19)	5·41 (5·28)
R = $C_{12}H_{25}^{b}$ (dodecyl)	δ 0·90 (3H, t, J = 6·9), 1·30 (18H, m), 1·65 (2H, m), 2·58 (1H, t t, J = 6·7, J = 23·3), 2·63 (2H, t, J = 6·9), 3·06 (2H, t d, J = 6·7, J = 16·7) p.p.m.	19·6 (broad)	55·58 (55·87)	10·16 (10·40)	6·17 (6·11)	8·93 (9·00)	4·43 (4·66)

a) In addition all ^{1}H NMR spectra contain peaks due to the cyclohexylammonium residues at δ 1·1-1·5 (15H, m), 1·65 (3H, m), 1·80 (6H, m), 2·00 (6H, m), 3·15 (3H, m) p.p.m.

b) NMR spectra recorded in CD_3OD (80%)/D_2O (16%)/DCl (4%)

3.4.3.2 Preparative Scale Reactions - Synthesis of Tetraethyl C-(Thioalkyl)methyl Methylenebisphosphonates

To a solution of tetraethyl ethenylidenebisphosphonate (2.0g, 6.67 mmol) in chloroform (15cm³) was added the alkylthiol (13.3 mmol) via syringe. After stirring at 40°C for 2h (after which time no starting material was detectable by TLC on silica gel) the solvent was stripped in vacuo to yield an oil. Flash chromatography on silica gel, elution with methanol/diethyl ether (1:20 v/v) yielded the Michael adduct (≈90%). In the case of ethane-1-thiol 8 equivalents were added. Data for all C-(thioalkyl)methyl methylenebisphosphonates prepared via this route are listed in table 3.4.3.2.

3.4.4 Preparation of Tris(cyclohexylammonium) C-(Thioalkyl)methyl Methylenebisphosphonates

The bisphosphonate esters (0.5g) were deesterified with bromotrimethylsilane via the usual procedure (section 2.5.7). The crude salts were recrystallised from methanol-water (5:1)/diethyl ether to yield the title compounds as white solids (≈70%). Data for all compounds synthesised via this route are listed in table 3.4.4.

3.4.5 Preparation of Tris(cyclohexylammonium) Ethenylidene-bisphosphonate

Tetraethyl ethenylidenebisphosphonate (4.00g, 13.3 mmol) was deesterified as described above. The crude product was recrystallised from methanol-water (10:1)/diethyl ether to yield the title compound as a white solid (4.23g, 67%). [^{1}H NMR (D_2O) δ 1.1-1.5(15H,m), 1.65 (3H,m), 1.80 (6H,m), 2.0 (6H,m), 3.15 (3H,m), 6.22 (2H,dd,J=35.6Hz, J=33.3Hz) p.p.m.; ^{31}P NMR (D_2O) δ 11.7 (s) p.p.m.; [Analysis C, 49.37; H, 9.12; N, 8.00; P, 11.90%. $C_{26}H_{44}N_3O_6P_2$ requires C, 49.57; H, 9.15; N, 8.67; P, 12.79%].

3.4.6 Preparation of Ethenylidenebisphosphonic Acid

The above salt was passed down a short column (2 x 15cm) of Dowex 50 (H^+ form) ion exchange resin, eluting with water. Lyophilisation of the eluate yielded the title compound as a hygroscopic white solid (1.52g, 92%). [^1H NMR (D_2O) δ 6.67 (2H, dd, J=35.6Hz, J=37.8Hz) p.p.m.; ^{31}P NMR (D_2O) δ 12.5(s) p.p.m.; ^{13}C NMR (D_2O) δ 144.8(s), 136.7 (t, J=161 Hz) p.p.m.; Analysis C, 12.59; H, 3.44%. $C_2H_6O_6P_2$ requires C, 12.78; H, 3.22%].

CHAPTER 4

CYCLOPROPYL DERIVATIVES OF PYROPHOSPHATE ANALOGUES

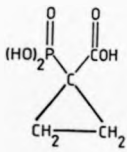
4.1 Background

Previously the cyclopropyl derivatives of phosphonoacetic acid and methylenebisphosphonic acid have not been assayed for antiviral activity and no synthesis of cyclopropane-1,1-bisphosphonic acid exists in the literature. In cyclopropane itself (Jones and Stoicheff, 1964) the carbon-carbon bond lengths (0.1514nm) are reduced relative to propane (0.1526nm) (Lide, 1960) and the HCH bond angles are increased from 107° to 116.5°. Analogous changes would be expected to occur on ring formation at the bridge carbon atom in methylenebisphosphonic acid and phosphonoacetic acid. For example the PCP bond angle in cyclopropane-1,1-bisphosphonic acid (27) would be expected to be larger, and the carbon-phosphorus bond length shorter, than in methylenebisphosphonic acid. These changes might have significant effects on the ability of these compounds to chelate zinc. In this study 1-phosphonocyclopropane carboxylic acid (Fig. 4.1(26)) and cyclopropane-1,1-bisphosphonic acid (Fig. 4.1(27)) were synthesised and their activities against the RNA polymerase of influenza examined.

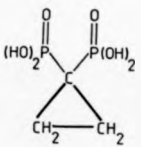
The synthesis of 1-phosphonocyclopropanecarboxylic acid has been described in the literature (Erion and Walsh, 1987) and the preparation of cyclopropane-1,1-bisphosphonic acid is discussed below.

4.2 Introduction - Cyclopropane-1,1-bisphosphonate

The recent improvement in the synthesis of tetraalkyl ethenylidene-bisphosphonates (Degenhardt and Burdsall, 1986) suggests the possibility of synthesising tetraalkyl cyclopropane-1,1-bisphosphonate; hence the corresponding bisphosphonic acid, via a classical Simmons-Smith reaction



(26)



(27)

Fig. 4.1(26) 1-Phosphonocyclopropanecarboxylic acid
(27) Cyclopropane-1,1-bisphosphonic acid

(Simmons and Smith, 1959). This reaction has been modified by later authors e.g. use of diethylzinc (Furukawa, *et al*, 1968, 1969) and has enabled a wide range of cyclopropyl derivatives to be produced from carbon-carbon double bonds.

However these reactions proceed via an electrophilic attack on the double bond which in the case of tetraalkyl ethenylidenebisphosphonate is unlikely to be successful as the double bond in this compound is extremely polar and prefers to react with nucleophiles (Chapter 3). Tetraethyl ethenylidenebisphosphonate resisted epoxidation with MCPBA under forcing conditions or reaction with trifluoroperacetic acid which can form epoxides with electron poor double bonds (Emmons and Pagano, 1955).

Grieco has described the construction of the cyclopropane unit via the reaction of alkylidene phosphoranes with a series of α,β-unsaturated esters (Grieco and Finkelhor, 1972). This reaction closely resembles the Michael reactions of tetraalkyl ethenylidine-bisphosphonates (Chapter 3) but is specific for the preparation of the gemdimethylcyclopropyl functionality (Fig. 4.2(a)).

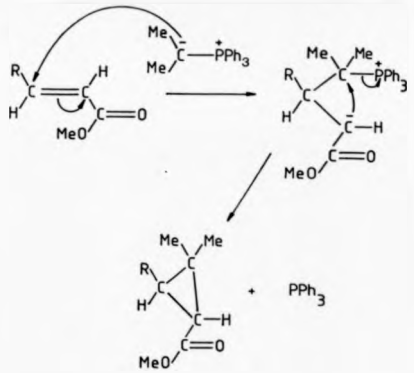


Fig. 4.2(a) The reaction of triphenylphosphonium isopropylide with α,β-unsaturated esters

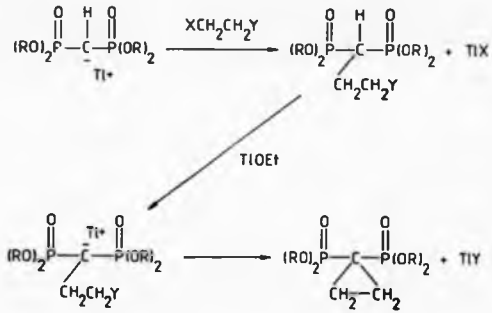


Fig. 4.2(b) The synthetic approach adopted for the synthesis of tetraalkyl cyclopropane-1,1-bisphosphonate.

An alternative approach to the synthesis of tetraalkyl cyclopropane-1,1-bisphosphonate is to prepare a suitable precursor which can by cyclised via an intramolecular reaction. The synthetic approach adopted is shown in Fig. 4.2(b)].

This synthesis requires the alkylation of tetraalkyl methylene-bisphosphonate; a process which has been known for some time. The monoalkylation of both the potassium (Kosolopoff, 1953] and sodium (Cotton and Schunn, 1963) salts of tetraethyl methylenebisphosphonate occurs in low yield. Alkylation of the sodium salt of tetraisopropyl methylenebisphonate gives a slightly improved yield (Quimby, *et al*, 1968). In situ alkylation of the lithium salt of tetraethyl chloromethylenebisphosphonate has also been reported (Seyferth and Marmor, 1973).

Later authors (Hutchinson and Semple, 1985) report poor yields of alkylation of tetraisopropyl methylene- and halogenomethylene-bisphosphonates when sodium, sodium hydride or butyllithium were used to form the reactive anions. Exclusive monoalkylation did not occur but dialkylation and cleavage reactions were found to interfere. Similar results were observed by Quimby et al (1968) (Fig. 4.2(c)).

In the same publication good yields of monoalkylation were observed when the thallium(I) salt of tetraisopropyl methylene- or halogenomethylenebisphosphonate was treated with excess of a primary alkyl iodide (Hutchinson and Semple, 1985). The use of other alkyl halides results in much lower yields of alkylation. Similar results have been reported for the alkylation of β-diketones via thallium(I) intermediates (Taylor, *et al*, 1968).

In view of these results 3-bis(diisopropylphosphono)-1-iodopropane was selected as a target molecule (Fig. 4.2(d)). In this study this compound was prepared and ring-closed via an intramolecular alkylation of the thallium(I) intermediate.

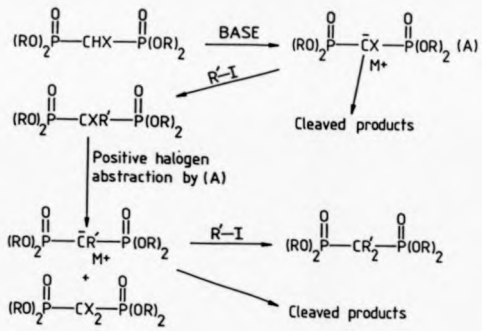


Fig. 4.2(c) Side-product formation during the alkylation of the sodium or lithium salts of tetraalkyl methylenebisphosphonates

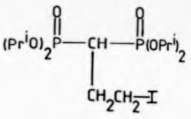


Fig. 4.2(d) 3-Bis(diisopropylphosphono)-1-iodopropane

4.3 Results and Discussion

4.3.1 Synthesis of 1-Phosphonocyclopropanecarboxylic Acid

t-Butyl diethyl phosphonoacetate was synthesised in good yield by the Arbuzov reaction of triethyl phosphite with t-butyl bromoacetate (Erion and Walsh, 1987; Fig. 4.3.1). Treatment of the resulting ester

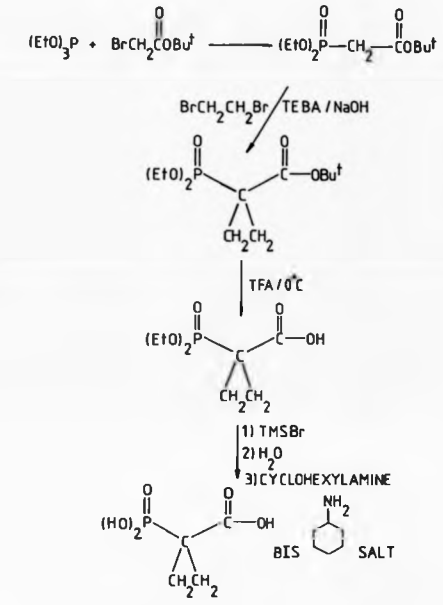


Fig. 4.3.1 The synthesis of bis(cyclohexylammonium) 1-phosphonocyclopropane-carboxylate

with 1,2-dibromoethane, sodium hydroxide and TEBA (triethylbenzylammonium chloride) yielded t-butyl diethyl 1-phosphonocyclopropanecarboxylate (Erion and Walsh, 1987; Fig. 4.3.1). A longer reaction time than reported was used to totally remove any t-butyl diethyl phosphonoacetate that might remain at the end of the reaction as this was found to co-chromatograph with the desired product. The use of benzyltrimethylammonium chloride as a phase transfer catalyst was found not to be effective in the reaction, presumably due to less favourable solubility properties.

When the reaction was attempted with a commercially available alternative starting product; triethyl phosphonoacetate, no alkylation was detected but a rapid and total hydrolysis to diethyl phosphonoacetic acid was observed. The hydrolysis of triethyl phosphonoacetate is reported to be rapid when treated with 1M sodium hydroxide solution at room temperature (Clayton, *et al*, 1979). On reducing the concentration of sodium hydroxide to 5% total hydrolysis was still observed.

Hydrolysis of the t-butyl ester was readily accomplished by treatment with cold TFA (Erion and Walsh, 1987). Residual TFA was removed by washing a solution of the crude product in dichloromethane with brine. Drying and evaporation of the organic layer yielded diethyl 1-phosphonocarboxylic acid of sufficient purity to be used without further purification (Fig. 4.3.1).

Dealkylation of the phosphonate ester groups with bromotrimethylsilane via the usual procedure (section 2.5.7) did not yield 1-phosphonocyclopropanecarboxylic acid as the sole product. It was accompanied by 20% (by ^{1}H NMR) of products arising from opening of the cyclopropane ring. Purification of the bromotrimethylsilane had no significant effect on this. The title compound was obtained pure as its bis(cyclohexylammonium) salt by treatment of the crude acid with excess cyclohexylamine and fractional crystallisation. Thus the target molecule was prepared in an overall yield of 31% (4 steps).

4.3.2 Synthesis of Cyclopropane-1,1-bisphosphonic Acid

Tetraisopropyl methylenebisphosphonate; synthesised as described (Roy, 1966), was readily converted into its thallium(I) salt by treatment with thallium(I) ethoxide (Hutchinson and Semple, 1985). However when reacted with excess 1,2-diiodoethane little alkylation (<5% by ^{31}P NMR) was observed. The primary alkyl iodide was found to be very unstable under the reaction conditions. Thus 3-bis(diisopropylphosphono)-1-iodopropane (Fig. 4.2(d)) could not be prepared via a direct alkylation with 1,2-diiodoethane. It was thus found necessary to prepare a primary alkyl iodide with a stable substituent at the 2-position that could be developed into an iodo-substituent after alkylation.

4.3.2.1 Synthesis of 3-Bis(diisopropylphosphono)propan-1-ol

Alkylation of the thallium(I) salt of tetraisopropyl methylenebisphosphonate with 6-iodohexyltetrahydropyranyl ether is known to occur in good yield (Semple, 1986), hence the analogous reaction with 2-iodoethyltetrahydropyranyl ether was expected to be equally as facile as the reaction proceeds more readily with smaller alkyl iodides (Hutchinson and Semple, 1985). After alkylation, removal of the ether group by acid hydrolysis would yield an alcohol functionality which could readily be developed to a primary iodide.

The conversion of alcohols into their tetrahydropyranyl ethers is recognised as a good method of protecting them (Greene, 1981). Treatment of 2-iodoethanol with excess dihydropyran in dichloromethane in the presence of PPTS (pyridinium p-toluene sulphonate, a mild acid catalyst; Miyashita, *et al*, 1977) yielded, after vacuum distillation, 2-iodoethyltetrahydropyranyl ether in 94% yield (Fig. 4.3.2.1(a)).

Treatment of the thallium(I) salt of tetraisopropyl methylene-bisphosphonate with excess 2-iodoethyltetrahydropyranyl ether gave only a poor yield (24% by ^{31}P NMR) of alkylation. (Fig. 4.3.2.1(b)). Under identical conditions iodoethane yielded 75% (by ^{31}P NMR) of mono-alkylation and an isolated yield of 45% is reported (Hutchinson and

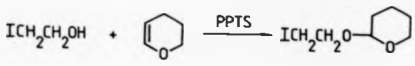


Fig. 4.3.2.1(a) The protection of 2-iodoethanol as its tetrahydropyranyl ether

Semple, 1985) for the reaction with iodoethane. Few cleavage products were detected but attack at phosphorus by the excess thallium(I) ethoxide; needed for complete formation of the salt, was observed.

The anion prepared from tetraisopropyl chloromethylene-bisphosphonate is more stable and usually gives higher yields of alkylation (Hutchinson and Semple, 1985). However with 2-iodoethyltetrahydropyranyl ether this was found not to be the case. Only 20% (by ^{31}P NMR) of monoalkylation was observed and also some positive chlorine abstraction occurred yielding a more complex product mixture. These difficulties in alkylation can be rationalised by steric hindrance between the bulky nucleophile and the tetrahydropyranyl ring of the protected iodoalcohol (Fig. 4.3.2.3(b)). The tetrahydropyranyl ring and the iodine atom are probably in a trans orientation in the conformational ground state of the molecule. Alkylation of thallium(I) salts of tetraisopropyl methylenebisphosphonates is very dependent on steric effects; giving much reduced yields with secondary alkyl iodides (Hutchinson and Semple, 1985).

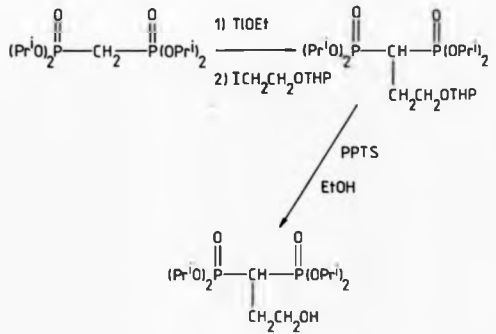


Fig. 4.3.2.1(b) The preparation of 3-bis(diisopropylphosphono)propan-1-ol

The 3-bis(diisopropylphosphono)propyltetrahydropyranyl ether co-chromatographed with tetraisopropyl methylenebisphosphonate. However after deprotection of the alcohol functionality with PPTS in ethanol (Miyashita, *et al*, 1977), the presence of the hydroxyl group caused a much greater affinity for silica gel, hence easy separation, to yield pure 3-bis(diisopropylphosphono)propan-1-ol as a colourless oil (Fig. 4.3.2.1(b)).

4.3.2.2 Synthesis of 3-Bis(diisopropylphosphono)-1-iodopropane

The preparation of the title compound required conversion of the above alcohol (Fig. 4.3.2.1(b)) into the corresponding iodide. Primary alcohols iodinate readily with phosphorus and iodine (eg Streitwieser

and Heathcock, 1981) but this procedure would cause reaction of the bridge proton to yield an unstable C-alkyl iodomethylenebisphosphonate. More recently iodination of primary and secondary hydroxyl groups has been reported to occur in good yield (Verheyden and Moffatt, 1970, 1972). However the method chosen was the classical route via formation of an intermediate tosylate followed by displacement with iodide.

Treatment of 3-bis(diisopropylphosphono)propan-1-ol with p-toluene sulphonyl chloride in pyridine (as used by McMurry, *et al*, 1979) caused an almost quantitative reaction of the hydroxyl group. Flash chromatography yielded 3-bis(diisopropylphosphono)propan-1-ol tosylate in 72% yield (Fig. 4.3.2.2).

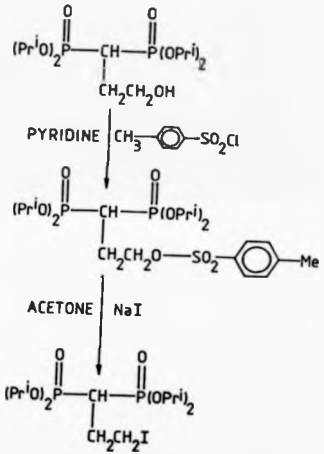


Fig. 4.3.2.2 Preparation of 3-bis(diisopropylphosphono)-1-iodopropane

A clean displacement of the tosyl group was effected by refluxing in acetone with excess sodium iodide. After flash chromatography the title compound was obtained as a colourless oil (Fig. 4.3.2.2).

4.3.2.3 Cylisation of 3-Bis(diisopropylphosphono)-1-iodopropane

Treatment of the title compound with one equivalent of thallium (I) ethoxide in THF at room temperature caused the formation of an instantaneous precipitate of thallium(I) iodide. A ^{31}P NMR spectrum indicated >95% of tetraisopropyl cyclopropane-1,1-bisphosphonate to be present in the crude reaction mixture. Work up as described (section 4.6.10) yielded the desired tetraisopropyl cyclopropane-1,1-bisphosphonate as a colourless oil in 70% yield (Fig. 4.3.2.3(a)).

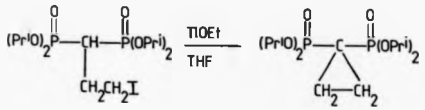


Fig. 4.3.2.3(a) Cyclisation of 3-bis(diisopropylphosphono)-1-iodopropane

Alkylation of the thallium(I) salts of methylenebisphosphonates (Hutchinson and Semple, 1985) and of β-dicarbonyl compounds (Taylor, *et al*, 1968) usually require reaction times of 4-24h. The above reaction is considerably faster than this as it is an intramolecular rather than an intermolecular reaction, hence there is a far greater probability of close proximity of the two reacting centers. The lowest energy rotational conformation about the C_1-C_2 bond in the title compound (Fig. 4.3.2.3(b)) probably places the bis(diisopropylphosphono)

methyl group and the iodine atom in a trans orientation. After deprotonation the carbanion centre is in an ideal position for attack on C_1 which is required for displacement of the iodine atom as iodide.

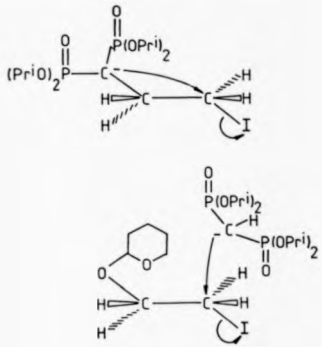


Fig. 4.3.2.3(b) Intramolecular and intermolecular alkylations of the thallium(I) salts of methylenebisphosphonates

4.3.2.4 Dealkylation of Tetraisopropyl Cyclopropane-1,1-bisphosphonate

The cyclopropane ring was found to be very stable under the standard reaction conditions used for dealkylation (section 2.5.7). Upon treatment with bromotrimethylsilane and aqueous hydrolysis no ring opening or side reactions were detectable by ^1H NMR spectroscopy. Cyclopropane rings exhibit considerable ring strain (Liebman and Greenberg, 1987) and in particular rings activated by electron withdrawing groups readily undergo reactions with nucleophiles leading to ring opening (Danishefsky, 1978). This was found to be a problem

during the dealkylation of diethyl 1-phosphonocyclopropanecarboxylic acid (section 4.3.2). Reactions of cyclopropanes can mimic alkenes (Meijere, 1979) and during the dealkylation of tetraethyl ethenylidene-bisphosphonate reactions of the double bond were observed. The stability of the cyclopropane ring under the reaction conditions used for dealkylation was thus unexpected. Cyclopropane-1,1-bisphosphonic acid was readily isolated as its tris(cyclohexylammonium) salt (Fig. 4.3.2.4).

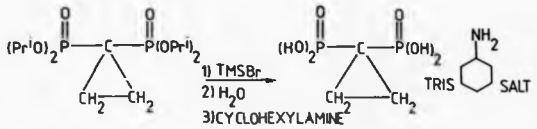
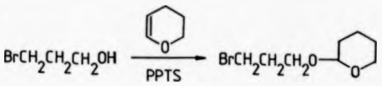


Fig. 4.3.2.4 Preparation of tris(cyclohexylammonium) cyclopropane-1, 1-bisphosphonate

4.4 Preparation of Larger Ring Sizes

It was hoped that the route described above would be readily adaptable for the synthesis of cycloalkyl-1,1-bisphosphonates of other ring sizes. This requires the syntheses of the appropriate precursor iodoalcohols; whose preparation may be accomplished by cleavage of cyclic ethers with Lewis acids in the presence of iodide ion. Tetrahydrofuran may be cleaved to 4-iodobutan-1-ol upon treatment with boron trifluoride-etherate and sodium iodide (Mandal, *et al*, 1985). The same reaction may be accomplished with sodium borohydride and molecular iodine (Long and Freeguard, 1965) but these methods appear to be restricted to tetrahydrofuran (Semple, 1985). However the combination of diborane and iodine (Long and Freeguard, 1964) is applicable to a wide range of cyclic ethers (Semple, 1985).

Using this combination of reagents 4-iodobutan-1-ol was readily prepared from tetrahydrofuran but protection of the alcohol group as its tetrahydropyranyl ether was impossible. The 4-iodobutan-1-ol rapidly cyclised to yield tetrahydrofuran even in the dark at -20°C. It was considered that this would be a problem for iodoalcohols containing three or more carbon atoms. Thus for larger rings it is necessary to include the iodide functionality after the protection of the hydroxyl group so as to prevent cyclisation.



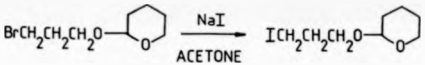


Fig. 4.4 Preparation of 3-iodopropyltetrahydropyranyl ether

3-Bromopropan-1-ol was readily reacted with dihydropyran (Miyashita, *et al*, 1977) and the resulting 3-bromopropyltetrahydropyranyl ether obtained as a colourless oil. This alkyl bromide was converted into the corresponding alkyl iodide, by treatment with sodium iodide in acetone at reflux (Diana, *et al*, 1977), in good yield (Fig. 4.4). Thus this route appears to be more suitable for the preparation

of the protected alkyl iodides than direct reaction of the appropriate iodoalcohol with dihydropyran.

The prerequisite bromoalcohols may be prepared by the cleavage of cyclic ethers, using the method of Long and Freeguard (1964), using bromine instead of iodine. Tetrahydrofuran yielded 4-bromobutan-1-ol in good yield which did not cyclise readily at room temperature.

4.5 Conclusions

Intramolecular alkylation of the thallium(I) salt of 3-bis (diisopropylphosphono)-1-iodopropane occurs very rapidly to yield tetraisopropyl cyclopropane-1,1-bisphosphonate. The precursor to cyclisation can be prepared via alkylation of the thallium(I) salt of tetraisopropyl methylenebisphosphonate with 2-iodoethyltetrahydropyranyl ether followed by development of the protected hydroxyl group into an iodide functionality. This procedure should be readily adapted to the synthesis of cycloalkyl-1,1-bisphosphonates of other ring sizes.

4.6 Experimental

4.6.1 Preparation of t-Butyl Diethyl Phosphonacetate

This was prepared as described (Erion and Walsh, 1987) and was purified by distillation under vacuum at 82-83°C/0.25 mmHg (lit 115°C/2 mmHg) to yield the title compound as a colourless oil (43.1g, 94%).

[^{1}H NMR ($CDCl_3$) δ 1.38(6H,t,J=6.8Hz),1.50(9H,s), 2.92 (2H,d,J = 21.7 Hz), 4.92 (4H,dq,J = 7Hz, J=7.8 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 24.1(s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 172(s), 81.8(s), 62.3 (d,J = 6.1 Hz), 35.4 (d,J = 133 Hz), 27.7(s), 16.2 (d,J = 6.1 Hz) p.p.m.; Analysis C, 47.58; H, 8.61; P, 11.56%. $C_{14}H_{31}O_5P$ requires C, 47.62; H, 8.39; P, 12.28%; Mass spectrum (NH_3CI) $(M+H)^+$ 253].

4.6.2 Preparation of t-Butyl Diethyl 1-Phosphonocyclopropane Carboxylate

This was prepared essentially as described (Erion and Walsh, 1987), via a phase transfer reaction of the above, except that a longer reaction time (24h) was found necessary. Flash chromatography on silica gel, eluting with chloroform, yielded the title compound as a colourless oil (4.9g, 59%).

[^{1}H NMR ($CDCl_3$) δ 4.26 (4H, quint, J=7.5 Hz), 1.56 (9H,s), 1.42(6H,t,J = 7.5Hz), 1.5-1.3 (4H,m) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 25.1 (s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 170 (s), 81.7(s), 62.1 (d,J = 6.0Hz), 27.8(s), 19.7 (d,J = 197 Hz), 16.2 (d,J = 6.0 Hz), 14.4(s) p.p.m.; Analysis C, 51.56; H, 8.46; P, 9.96%. $C_{12}H_{23}O_5P$ requires C, 51.79; H, 8.33; P.11.13%; Mass spectrum (NH_3CI) $(M+H)^+$ 279].

4.6.3 Preparation of Diethyl 1-Phosphonocyclopropanecarboxylic Acid

This was prepared as described (Erion and Walsh, 1987) by the hydrolysis of the above with cold (0°C) TFA to yield the title compound as a colourless oil (3.16g, 94%). This crystallised on standing; mpt 61-63°C (lit 65-67°C).

[^{1}H NMR ($CDCl_3$) δ 4.24 (4H, quint, J = 7.4 Hz), 1.55 (2H,m,broad), 1.50 (2H,m), 1.35 (6H,t,J = 7.4 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 25.0(s) p.p.m.; Analysis C, 42.26; H, 6.63; P, 12.13%. $C_8H_{15}O_5P$ requires C, 43.25; H, 6.81; P, 13.94%; Mass spectrum (NH_3CI) $(M+H)^+$ 223].

4.6.4 Preparation of 1-Phosphonocyclopropanecarboxylic Acid Bis(cyclohexylammonium) Salt

The diethyl ester above was dealkylated via the usual procedure (section 2.5.7) and the title compound was obtained pure by twice recrystallising from acetone-methanol (10:1)/diethyl ether and dried in vacuo over phosphorus pentoxide to yield a white solid (1.45g, 59%).

[^{1}H NMR (D_2O) δ 3.16 (2H,m), 1.98 (4H,m), 1.80 (4H,m), 1.65 (2H,m), 1.2-1.5 (10H,m), 1.6-1.5 (4H,m) p.p.m.; ^{31}P NMR (D_2O) δ 21.9(m) p.p.m.; ^{1}H coupled δ 21.9 (tt,J = 8.6 Hz, J = 9.1 Hz) p.p.m.; Analysis C, 51.69; H, 9.15; N, 7.36; P, 7.96%. $C_{14}H_{32}N_2O_5P$ requires C, 52.73; H, 9.13; N, 7.69; P, 8.50%].

4.6.5 Preparation of 2-Iodoethyltetrahydropyranyl Ether

This was prepared by coupling 2-iodoethanol with dihydropyran in the presence of PPTS as described (Miyashita, *et al*, 1977). The product was purified by distillation under vacuum at 49-50°C/0.1 mmHg to yield the title compound as a colourless oil (31.2g, 94%).

[^{1}H NMR ($CDCl_3$) δ 4.75 (1H,t,J = 3.4 Hz), 4.1 - 3.8 (2H,m), 3.80 (1H,dt, J = 5.7 Hz, J = 6.9 Hz), 3.60 (1H,dt,J = 5.7 Hz, J = 6.9 Hz), 3.35 (2H,t,J = 6.7 Hz), 2.0-1.5 (6H,m) p.p.m.; Analysis C, 32.44; H,5.14; I, 47.73%. $C_7H_{13}IO_2$ requires C, 32.83; H, 5.12; I, 49.56%; Mass spectrum (NH_3CI) $(M+H)^+$ 257].

Note

PPTS was prepared according to the method of Miyashita, *et al*, (1977).

4.6.6 Preparation of 3-Bis(diisopropylphosphono)propyltetrahydropyranyl Ether

Alkylation of tetraisopropyl methylenebisphosphonate with excess of the alkyl iodide synthesised above was carried out as described (Hutchinson and Semple, 1985). Chromatography on silica gel, eluting with acetone/petroleum ether (1:14%/v), followed by rechromatography, eluting with methanol/diethyl ether (1:50%/v) returned a mixture of the title compound and tetraisopropyl methylenebisphosphonate (≈1:1 by ^{1}H NMR) as a colourless oil (3.21g). A small sample was rechromatographed for NMR analysis but the remainder was used in the next stage without further purification.

[^{1}H NMR ($CDCl_3$) δ 4.80 (4H,m), 4.65 (1H,t,J = 3.3Hz), 3.90 (2H,m), 3.50 (2H,m), 2.42 (1H,tt,J = 6.1 Hz, J = 24.4 Hz), 2.20 (2H,m,J = 6.1Hz, J = 16.4 Hz), 1.9 - 1.5 (6H,m), 1.38 (24H,dd,J = 6.1Hz, J = 2.8 Hz) p.p.m.].

4.6.7 Preparation of 3-Bis(diisopropylphosphono)propan-1-ol

The residue from above (1.6g) was treated with PPTS in ethanol as described (Miyashita, *et al*, 1977). Flash chromatography on silica gel, elution with methanol/diethyl ether (1:30^v/$_v$), yielded the title compound as a colourless oil (0.48g, 66%).

[^{1}H NMR ($CDCl_3$) δ 4.73 (4H,m,J = 7.0 Hz, J = 7.4 Hz), 3.76 (2H,t,J = 6.1 Hz), 2.36 (1H,tt,J = 6.1 Hz, J = 25 Hz), 2.12 (2H,tq, J = 6.1 Hz, J = 16.8 Hz), 1.33 (12H,dd,J = 7.0 Hz, J = 2.0 Hz), 1.31 (12H,d,J = 7.0 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 22.2(s) p.p.m.; Analysis C, 46.34; H, 8.82%. $C_{18}H_{34}O_7P_2$ requires C, 46.39; H, 8.82%; Mass spectrum (NH_3CI) $(M+H)^+$ 389].

4.6.8 Preparation of 3-Bis(diisopropylphosphono)propyl Tosylate

The above alcohol (0.44g, 1.1mmol) was dissolved in chloroform (5cm^3). Pyridine (0.72cm^3, 9 mmol) and p-toluene sulphonyl chloride (0.43g, 2.2 mmol) were added. After 24h the mixture was dropped into aqueous hydrochloric acid (5%^{v}/$_v$) and extracted with chloroform (2 x 25 cm^3). The solution was dried, filtered and evaporated to yield a colourless oil. Flash chromatography on silica gel, elution with methanol/diethyl ether (1:40^v/$_v$), yielded the title compound as a colourless oil (0.44g, 72%).

[^{1}H NMR ($CDCl_3$) δ 7.77 (2H,d,J = 8.4 Hz), 7.31(2H,d,J = 8.4 Hz), 4.72 (4H,m,J = 5.7 Hz), 4.23 (2H,t,J = 6.6 Hz), 2.42 (3H,s), 2.24 (1H,tt,J = 6.2 Hz, J = 24 Hz), 2.20 (2H,m,J = 6.2 Hz, J = 6.6 Hz, J = 16 Hz), 1.29 (24H,dd,J = 5.7 Hz, J = 1.0 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ

20.2(m) p.p.m.; Analysis C, 50.73; H, 7.83%. $C_{22}H_{44}O_6P_2S$ requires C, 48.70; H, 7.43%; Mass spectrum (NH_3CI) $(M+H)^+$ 543].

4.6.9 Preparation of 1-Bis(diisopropylphosphono)-1-iodopropane

The above ester (0.41g, 0.75mmol) was dissolved in dry acetone ($10cm^3$). Anhydrous sodium iodide (0.56g, 3.8mmol) was added and the mixture refluxed under nitrogen for 1h. The mixture was filtered through celite and the filtrate stripped in vacuo. Flash chromatography of the residue on silica gel, eluting with methanol/diethyl ether (1:40 v/v), yielded the title compound as a colourless oil (0.27g, 73%).

[^1H NMR ($CDCl_3$) δ 4.77 (4H,m,J = 6.4 Hz, J = 6.5 Hz), 3.46 (2H,t,J = 7.2 Hz), 2.35 (2H,m,J = 6.2Hz, J = 7.2 Hz), 2.32 (1H,tt,J = 6.2 Hz, J = 28 Hz), 1.34 (24H,td,J = 6.4 Hz, J = 1.7 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 22.2(m) p.p.m.; Analysis C, 36.81; H, 6.89%. $C_{15}H_{33}IO_6P_2$ requires C, 36.16; H, 6.68%; Mass spectrum (NH_3CI) $(M+H)^+$ 499].

4.6.10 Preparation of Tetraisopropyl Cyclopropane-1,1-bisphosphonate

The above compound (0.25g, 0.5 mmol) in dry THF ($10cm^3$) under nitrogen was treated with a solution of thallium(I) ethoxide in THF (500 μl, 0.5 mmol of thallium(I) ethoxide) dropwise over 2 min. After stirring for 2h the reaction mixture was passed down a florosil column (2 x 10 cm) eluting with acetone/petroleum ether (1:1 v/v, $150cm^3$). Evaporation of the eluate and flash chromatography of the residue on silica gel, eluting with methanol/diethyl ether (1:40 v/v), yielded the title compound as a colourless oil (0.13g, 70%).

[^1H NMR ($CDCl_3$) δ 4.68 (4H,m,J = 6.2 Hz, J = 7.7 Hz), 1.32 (24H,dd,J = 6.2 Hz, J = 4.1 Hz), 1.31 (4H,t,J = 13.9 Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 23.5(m) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 70.9(m), 24.0(m), 11.6 (t,J = 181 Hz), 10.8 (m) p.p.m.; Analysis C, 47.79; H, 8.20%; $C_{15}H_{32}O_6P_2$ requires C, 48.64; H, 8.71%; Mass spectrum (NH_3CI) $(M+H)^+$ 371.1707 ($C_{15}H_{33}O_6P_2$ calc. 371.17 53)].

4.6.11 Preparation of Tris(cyclohexylammonium) Cyclopropane-1,1-bisphosphonate

The above ester was dealkylated via reaction with bromotrimethylsilane and the product converted into its tris(cyclohexylammonium) salt as described (section 2.5.7). Recrystallisation from methanol/diethyl ether yielded the pure title compound as a white solid (83mg, 59%).

[^{1}H NMR (D_2O) δ 3.18 (3H,m), 2.0 (6H,m), 1.8 (6H,m), 1.65 (3H,m), 1.1-1.5 (15H,m), 0.95 (4H,t,J = 14.2 Hz) p.p.m.; ^{31}P NMR (D_2O) δ 21.4(s) p.p.m.; (^{1}H coupled) δ 21.4 (quint,J = 14.2 Hz) p.p.m.; Analysis C, 49.81; H, 9.20%. $C_{21}H_{47}N_3O_6P_2$ requires C, 50.49; H, 9.48%].

4.6.12 Preparation of 3-Bromopropyltetrahydropyranyl Ether

This was prepared by coupling 3-bromopropan-1-ol with dihydropyran in the presence of PPTS essentially as described (Miyashita, *et al*, 1977). The product was purified by distillation under vacuum (60-62°C/0.3 mmHg) to yield the title compound as a colourless oil (66.2g, 83%).

[^{1}H NMR ($CDCl_3$) δ 4.66 (1H,t,J = 7.8 Hz), 3.91 (2H,dt,J = 6.8 Hz, J = 10.6 Hz), 3.68 (2H,t,J = 6.8 Hz), 3.66 (2H,dt,J = 6.0 Hz, J = 10.0 Hz), 2.16 (2H,quint,J = 6.8 Hz), 2.0-1.5 (6H,m) p.p.m.; Mass spectrum (EI) (M)$^+$ 222,224; Analysis C, 44.19; H, 7.17%. $C_8H_{15}BrO_2$ requires C, 43.07; H, 6.78%].

4.6.13 Preparation of 3-Iodopropyltetrahydropyranyl Ether

A mixture of the above compound (66.2g, 0.3 mol) and anhydrous sodium iodide (60.0g, 0.4 mol) in dry chloroform (400 cm^3) under nitrogen was refluxed for 2h. After filtering through celite the solvent was stripped in vacuo and the residue partitioned between chloroform (500 cm^3) and water (250 cm^3). The chloroform layer was

dried, filtered and the solvent evaporated. Distillation under vacuum (60-61°C/0.01mmHg) yielded the title compound as a colourless oil (67.5g, 84%].

[^{1}H NMR ($CDCl_3$) δ 4.65 (1H,t,J = 3.6Hz), 3.85 (2H,m,J = 6.0Hz, J = 10.0 Hz), 3.5 (2H,m,J = 6.5 Hz, J = 10.5 Hz), 3.32 (2H,t,J = 6.5 Hz), 2.12 (2H,quint,J = 6.5Hz), 2.0-1.5 (6H,m) p.p.m.; Analysis C, 35.27; H, 5.77; I, 46.81%. $C_8H_{15}IO_2$ requires C, 35.57; H, 5.60; I, 46.98%; Mass spectrum (EI) (M)$^+$ 270].

CHAPTER 5

THE REACTION OF TRIALKYL PHOSPHITES WITH THIOPHOSGENE

5.1 Introduction

Carbonylbisphosphonic acid expresses significant in vitro anti-influenza activity at concentrations lower than most pyrophosphate analogues (Cload and Hutchinson, 1983). This compound is also found to be an inhibitor of a number of other viruses such as hepatitis B, herpes virus, human cytomegalovirus (Öberg, 1983), avian myoblastosis virus (Eriksson, *et al*, 1982a) and most recently human T-lymphotropic virus type III (HTLV-III) (Vrang and Öberg, 1986); the causative agent of AIDS. Methods for the preparation of carbonylbisphosphonic acid and analogues are thus of interest.

Only one literature synthesis of a tetraester of carbonyl-bisphosphonic acid has been reported (Kabachnik and Rossiiskaya, 1957), claiming that tetramethyl carbonylbisphosphonate was prepared from trimethyl phosphite and phosgene. However, this publication was retracted in the following year (Kabachnik and Rossiiskaya, 1958). The expected Arbuzov reaction (section 2.1.1) does not take place but diethyl phosphorochloridate is the reaction product (Fig. 5.1(a)). This was confirmed experimentally.

Tetrasodium carbonylbisphosphonate may be prepared by the hydrolysis, in strong alkalai, of dichloromethylenebisphosphonate or dibromethylenebisphosphonate (Quimby, *et al*, 1967) (Fig. 5.1(b)).

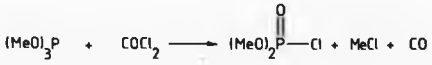


Fig. 5.1(a) The reaction of trimethyl phosphite with phosgene

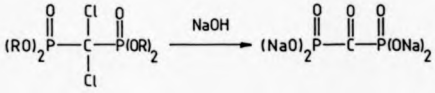


Fig. 5.1(b) Preparation of tetrasodium carbonylbisphosphonate

This small molecule has little scope for modification apart from exchanging the carbonyl oxygen atom for other functionalities. Replacement by a methylene unit yields ethenylidenebisphosphonic acid which is found not to be an inhibitor of influenza (table 7.5) and only a poor inhibitor of other viruses (table AIII). Thus the electron withdrawing nature of the carbonyl oxygen atom appears to be important for antiviral activity.

Another possibility is replacement of the carbonyl oxygen atom by sulphur. The object of this study was to investigate the synthesis and possible antiviral activity of thiocarbonylbisphosphonic acid (Fig. 5.1(c)). Thiocarbonylbisphosphonic acid tetraesters have only been mentioned once in the literature (Brokke and Stoffey, 1966) as the product of the reaction between triethyl phosphite and thiophosgene.

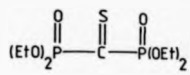


Fig. 5.1(c) Tetraethyl thiocarbonylbisphosphonate

However, no experimental details for the preparation were given and no references cited. In this study the reaction of trialkyl phosphites with thiophosgene was investigated in an attempt to synthesise this compound.

5.2 Results

5.2.1 Intermediates In The Reaction of Trialkyl Phosphites With Thiophosgene

The reaction between triethyl phosphite and thiophosgene was found to be very exothermic and, unless performed at low temperature, a large mixture of species resulted. A wide range of molar ratios of trialkyl phosphite to thiophosgene were examined (2:1, 3:1, 4:1 or 5:1) but in no case could O,O-dialkyl thiocarbonylbisphosphonate be detected by ^{31}P NMR or mass spectroscopy. In a typical experiment trimethyl, triethyl or triisopropyl phosphite was added at -78°C to a solution of thiophosgene in THF and the reaction mixture allowed slowly to warm to room

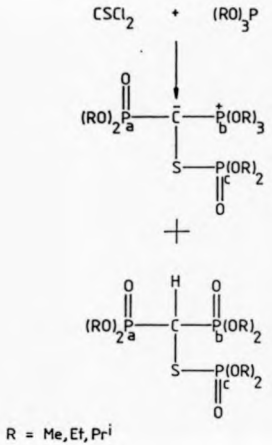
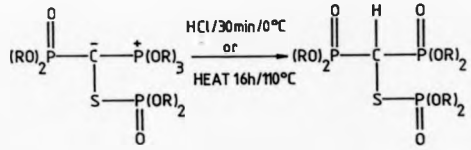


Fig. 5.2.1(a) The reaction of trialkyl phosphites with thiophosgene

temperature. Under these conditions phosphorus stabilised ylids were the predominate reaction products together with the corresponding fully esterified phosphorothio-substituted methanebisphosphonates (7-27% by ^{31}P NMR) (Fig. 5.2.1(a)).



R = Me, Et, Pr^{i}

Fig. 5.2.1(b) Preparation of 0,0-dialkyl S-[bis(dialkoxyphosphinyl)-methyl phosphorothioates

The ylids appear to arise from one thiophilic addition and two carbophilic additions to thiophosgene. Both thiophilic additions (eg Corey and Winter, 1963; Corey, *et al.* 1965) and carbophilic additions (eg Yoneda, *et al.* 1977) to thiocarbonyl groups are well documented. When the reaction mixtures were treated with gaseous hydrogen chloride or heated 0,0-dialkyl S-[bis-(dialkoxyphosphinyl)methyl] phosphorothioates were the only products detectable by ^{31}P NMR spectroscopy (Fig. 5.2.1(b)).

Even employing doubly distilled thiophosgene it was impossible to obtain the ylids in a pure state. Some of the 0,0-dialkyl S-[bis-(dialkoxyphosphinyl)methyl] phosphorothioates were always present along with small amounts of dialkyl phosphites (2-5% by ^{31}P NMR) and trialkyl

phosphates (4-12% by ^{31}P NMR). These arise from the difficulty in removing all traces of water and hydrogen chloride from the thiophosgene. When the reaction was carried out deliberately using a sample of partially hydrolysed thiophosgene O,O-dialkyl S-[bis-(dialkoxyphosphinyl)methyl] phosphorothioates were obtained directly but there was also an increase in the yields of dialkyl phosphite and trialkyl phosphate by-products.

5.2.2 NMR Studies of Ylids and Phosphorothioates

5.2.2.1 Ylid Structure

The ^{31}P NMR spectra of the ylids formed were ABX systems with a large J(AB) (^2J 120Hz) coupling and small J(AX), J(BX) ($^2J \simeq 3.5$-6Hz) couplings. For these compounds the signals centred at δ (P_A) $\simeq$ 28,27.5 and 26 p.p.m. can be attributed to the phosphonate residues; the signals centred at δ (P_B) $\simeq$ 55,49.5 and 46.5 p.p.m. can be attributed to the trialkoxyphosphonium residues (a higher shift as expected (Burgada, *et al*, 1980)) and finally the signals centred at $\delta(P_C) \simeq 30.5$, 28.5 and 27 p.p.m. are consistent with the phosphorothioate residues present (Zimin, *et al*, 1983).

In the ^{13}C NMR spectra of the ylids the bridge carbon atom resonated as a triplet of doublets with $^1J(CP_A) \simeq {}^1J(CP_B) \simeq 220$Hz, $^2J(CP_C) \simeq 4$Hz and shifts in the range 9-14 p.p.m. These high field shifts and large coupling constants are consistent with a high degree of localisation of the negative charge on the bridge carbon atom (Gray, 1973; Bottin-Strzalko, *et al*, 1978).

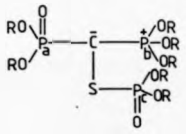
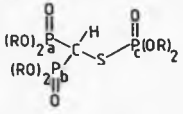
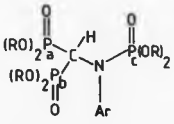


Fig. 5.2.2.1 Structure of ylids formed in the reaction of trialkyl phosphites with thiophosgene

5.2.2.2 Phosphorothioate Structure



R = Me, Et, Pr^{i}



$^{2}J(HP_a) = {}^{2}J(HP_b) \simeq 26Hz$

$^{3}J(HP_c) = 11 - 14.5Hz$

Fig. 5.2.2.2(a) Structures of O,O-dialkyl S-[bis-(dialkoxyphosphinyl)-methyl] phosphorothioates and nitrogen analogues

The ^{31}P NMR spectra of the phosphorothioates were A_2X systems. The doublets centred at $\delta(P_A) = \delta(P_B) \simeq 19, 16.5$ and 15.5 p.p.m. are attributable to the phosphonate residues and the triplets centred at $\delta(P_C) \simeq 28, 24$ and 22.5 p.p.m. are consistent with the phosphorothioate residues ($^{3}J(PP) \simeq 12Hz$). In the ^{13}C NMR spectra of the

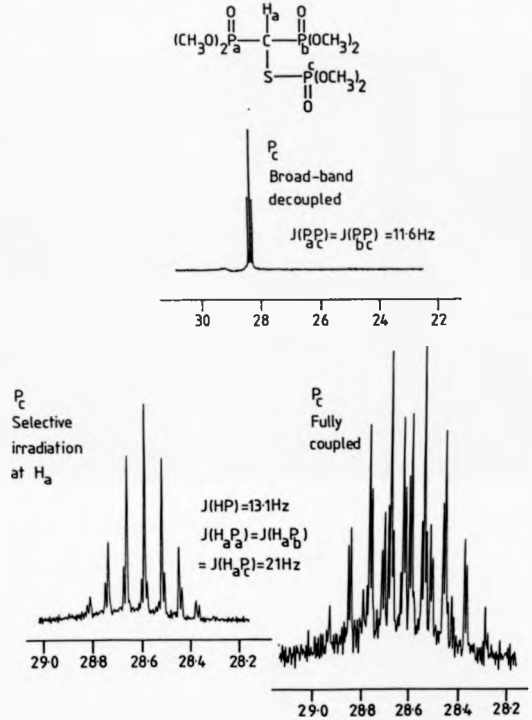


Fig. 5.2.2.2(b) Fully coupled, broad band decoupled and selectively decoupled ^{31}P NMR spectra of O,O-dimethyl S-[bis(dimethoxy-phosphinyl)methyl] phosphorothioate

phosphorothioates the bridge carbon atom in each case resonated as a triplet of doublets with $^1J(CP_A) = {}^1J(CP_B) \simeq 140Hz$, $^2J(CP_C) \simeq 4Hz$ with shifts in the range 35-39 p.p.m. The increased shift and lower coupling constant, relative to their respective ylids, being consistent with a loss of negative charge and change in hybridisation.

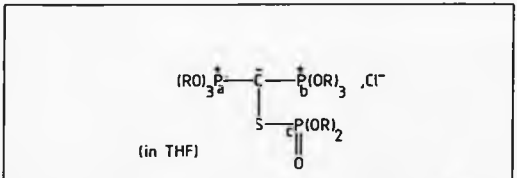
In the ^1H NMR of the phosphorothioates the bridge proton was found in each case to resonate as a quartet, implying an equivalence of the $^2J(HP)$ and $^2J(HP)$ couplings ($\simeq$ 22Hz). Simplification of the fully coupled ^{31}P NMR spectrum of Pc occurred upon selective irradiation of the bridge proton in O,O-dimethyl S-[bis-(dimethoxyphosphinyl)-methyl] phosphorothioate indicating the $^2J(HPc)$ coupling to be 21Hz; a typical $^2J(HP)$ value. (Fig. 5.2.2.2(b)).

During selective irradiation of the isopropyl methine protons in O,O-diisopropyl S-[bis-(diisopropyloxyphosphinyl)-methyl] phosphorothioate the ^{31}P NMR resonance of Pc appeared as a doublet of triplets indicating $^2J(HPc)$ to be 20Hz (slightly attenuated by an off-resonance effect). In nitrogen analogues of these compounds $^2J(HP)$ couplings are smaller (11-14.5Hz) and in no reported examples are these equal to the $^2J(HP)$ couplings (Gross, *et al*, 1972) (Fig. 5.2.2.2(a)).

5.3 Reaction Mechanism

To ascertain the mechanism of the reaction leading to the ylids the reaction was followed by ^{31}P NMR spectroscopy from -80°C to room temperature. The reactions of thiophosgene with triethyl phosphite and triisopropyl phosphite commenced at -80°C and the reaction with trimethyl phosphite commenced at -60°C. The intensity of the trialkyl phosphite resonance diminished until at -40°C (with an excess of thiophosgene) all the phosphite was consumed and species (29) (Fig. 5.3) were the main reaction products. ^{31}P NMR data for these species are listed in table 5.3.

Table 5.3 ^{31}P NMR data of intermediates detected in the reaction of trialkyl phosphites with thiophosgene



R	Me	Et	Pr^i
$\delta P_a = \delta P_b$ (d)	46·26	42·50	37·69
δP_c (t)	27·75	24·38	24·76
$^3J(P_aP_c) = {}^3J(P_bP_c)$	5·3	5·5	5·6

Above -40°C, for the reactions involving trimethyl and triethyl phosphite, the resonances due to species 29a and 29b decreased with the appearance of signals due to the ylids 30a and 30b (the production of traces of the corresponding phosphorothioates was detectable at the same time). In the case of the isopropyl ester the intermediate 29c was more stable and was dealkylated to the ylid 30c only at room temperature.

The production of the intermediates 29 arises from a mono-dealkylation of their probable precursors 28. In the ^{13}C NMR spectrum of a mixture of triethyl phosphite and thiophosgene, maintained at

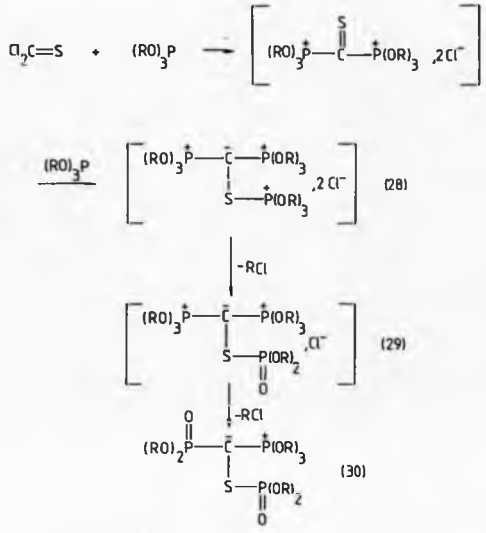


Fig. 5.1 The mechanism of reaction of trialkyl phosphites with thiophosgene

- 60°C, resonances due to the carbon atoms of ethyl chloride were observed. This dealkylation occurs exclusively at the trialkylphosphonium residue α to the sulphur. No dealkylation of a trialkylphosphonium residue β to the sulphur was detectable by ^{31}P NMR spectroscopy. Electronegative substituents attached to the phosphorus facilitate this reaction enabling it to occur at low temperature. This reaction is analagous to that of the salts $[(RO)_3P-S-Cl]^+Cl^-$ which can eliminate RCl at -30°C (Michalski, *et al*, 1978a).

The interaction of the thiocarbonyl group with trialkyl phosphites has been extensively studied and has been used synthetically in the synthesis of alkenes (eg Corey and Winter, 1963) and tetrathiofulvalenes (eg Krief, 1972). Thiophilic addition has also been reported to generate phosphorus ylids (Corey and Markl, 1967). All these reactions arise from a thiophilic addition of trialkyl phosphite to the thiocarbonyl group and ylid formation implies reaction with a second equivalent of trialkyl phosphite leading to an O,O',O''-trialkyl thiophosphate. In the reaction of trialkyl phosphites with thiophosgene O,O',O''-trialkyl thiophosphates were never detected by ^{31}P NMR spectroscopy; stable ylids being the result of nucleophilic attack, by chloride ions, on the alkoxy substituents at phosphorus. Analogous ylids are formed without desulphuration from the reaction of aryl chlorodithioformates with trialkyl phosphites (Birum, 1963) (Fig. 5.4).

5.4 The Reaction Of Phenyl Chlorodithioformate with Trimethyl Phosphite

The patent described above (Birum, 1963) did not report NMR data for the ylid 31 (Fig. 5.4), thus the reaction between phenyl chlorodithioformate and trimethyl phosphite was investigated by ^{31}P NMR spectroscopy to compare the NMR of 31 with those of the ylids 30a—c (Fig. 5.3).

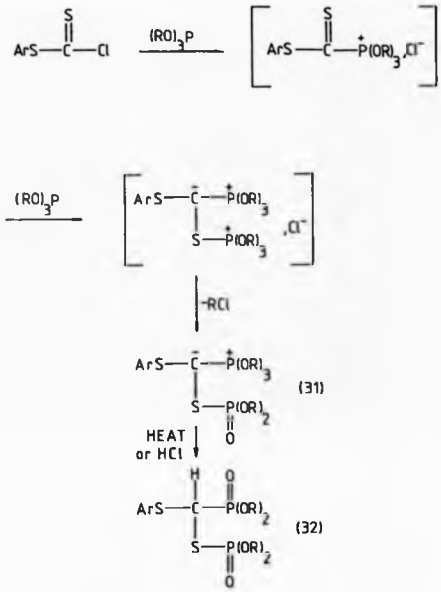


Fig. 5.4 The mechanism of reaction of phenyl chlorodithioformate with trimethyl phosphite

The reaction commenced slowly at -20°C with, in the ^{31}P NMR spectrum, a pair of doublets at δ 48 p.p.m. ($(MeO)_3P$.) and at δ 31 p.p.m. ($(MeO)_2P(O)-S-$), consistent with the ylid 31, becoming apparent. The reaction was complete after 24h at room temperature when in addition to species 31, 32 could be detected by ^{31}P NMR (7%) along with dimethyl phosphite (2%) and trimethyl phosphate (4%).

Warming the ylid 31 or treatment with gaseous hydrogen chloride in dichloromethane resulted in complete conversion to species 32. Thus the ylid 31 behaves in an analogous manner to those formed from trialkyl phosphites and thiophosgene in that a protonation-dealkylation reaction occurs rather than migration of an alkyl group from the trialkoxyphosphonium residue to the ylid carbon, which has previously been observed (Middleton and Sharkey, 1964; Corey and Markl, 1967).

5.5 Conclusions

The reaction between trialkyl phosphites and thiophosgene, even when carried out at low temperature, does not lead to the expected esters of thiocarbonylbisphosphonic acid. The products of the reaction are stable ylids formed by two carbophilic additions and one thiophilic addition of the trialkyl phosphite to thiophosgene. No desulfuration of the thiocarbonyl group was observed.

Phosphorus ylids have seen extensive use in the Wittig reaction (Gosney, 1979) and phosphorus stabilised carbanions are employed in the Wadsworth-Emmons reaction (Wadsworth, 1977). The ylids formed from the reaction of trialkyl phosphites with thiophosgene do not have much scope for use in the Wittig reaction due to their specialised nature.

Upon heating or treatment with dry hydrogen chloride the ylids are found to undergo protonation-dealkylation reactions to yield phosphorothio-substituted methane bisphosphonates. The mechanism for the thermal protonation-dealkylation reaction is unclear as the reaction occurred as

readily for the methyl ester as for the ethyl and isopropyl esters. This mechanism has previously been discussed (Ogata, *et al*, 1974) but not elucidated.

5.6 Experimental

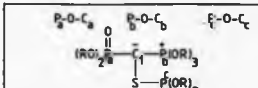
5.6.1 Preparation of O,O-Dialkyl S-[(dialkoxyphosphinyl) (trialkoxyphosphoranylidene) methyl] Phosphorothioates - General Procedure

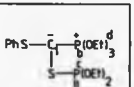
To a solution of thiophosgene (15 mmol) in dry THF (30cm³) maintained at -78°C under nitrogen was added trialkyl phosphite (30 mmol) dropwise over 10 min. The solution was allowed to warm to room temperature over 16h. The solvent and excess thiophosgene were stripped in vacuo to yield a crude sample of the title compound as an orange oil. Full data for all phosphorus ylids prepared in this manner are listed in table 5.6.1. The ylids were always obtained with a small amount (7-27% by ^{31}P NMR) of the corresponding O,O-dialkyl S-[bis-(dialkoxyphosphinyl)-methyl] phosphorothioates.

5.6.2 Preparation of O,O-Dialkyl S-[bis-(dialkoxyphosphinyl)-methyl] Phosphorothioates - General Procedure

The crude ylid from above was dissolved in dry dichloromethane (20cm³) and the solution cooled to 0°C and saturated with hydrogen chloride gas for 20 min. The solvent was stripped in vacuo and the title compound isolated in crude form as an orange oil (83-95% by ^{31}P NMR). The phosphorothioates prepared in this manner could be obtained pure by flash chromatography on silica gel, eluting with acetone, in $\approx$ 50% yield. Full NMR data for phosphorothioates prepared by this procedure are listed below.

Table 5.6.1 NMR characteristics of O,O-dialkyl S-[(dialkoxyphosphinyl) (trialkoxyphosphoranylidene) methyl] phosphorothioates





	^{31}P NMR($CDCl_3$)			^{31}P NMR(CCl_4)
	Me	Et	Pr^i	
δP_a(dd)	28·15	27·63	25·90	-
δP_b(dd)	55·05	49·69	46·69	48·3(d)
δP_c(dd)	30·68	28·70	27·03	30·8(d)
$^3J(P_aP_b)$	12·4	12·0	13·0	-
$^3J(P_aP_c)$	5·5	5·6	6·0	-
$^3J(P_bP_c)$	3·5	5·3	6·0	3·3
	^{13}C NMR($CDCl_3$)			^{13}C NMR(CCl_4)
δC_1(td)	9·60	11·60	13·70	10·27(dd)
δC_a(d)	55·7(J=6·7)	64·6(J=6·7)	67·7(J=6·0)	-
δC_b(d)	51·8(J=5·1)	60·2(J=5·2)	73·6(J=7·0)	-
δC_c(d)	53·4(J=5·9)	62·4(J=6·2)	70·1(J=7·0)	-
	C_1 coupling constants			
	Me	Et	Pr^i	
J_{CP_a} J_{CP_b}	220	222	223	268
J_{CP_c}	4	4	4	2·5
	EI (M)$^{+\cdot}$			
	386	484	582	-

d) Prepared as described (Birum,1963)

5.6.2.1. 0,O-Dimethyl S-[bis-(dimethoxyphosphinyl)-methyl] Phosphorothioate

[^{1}H NMR (C_6D_6) δ 3.2-3.9 (18H,m), 4.25 (1H,q,J = 22Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 19.1 (d,J = 12Hz), 20.7 (t,J = 12Hz) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 35.3 (td,J = 139Hz, J = 4.3Hz), 54.4(m) p.p.m.; Analysis S, 8.62%. $C_7H_{16}O_6PS$ requires S,8.72%; Mass spectrum (EI) M$^+$ 371.9927 ($C_7H_{16}O_6PS$ calc. 371.9962)].

5.6.2.2 0,O-Diethyl S-[bis-(diethoxyphosphinyl)-methyl] Phosphorothioate

[^{1}H NMR ($CDCl_3$) δ 1.38 (18H,t,J = 7Hz), 3.94 (1H,q,J = 22Hz), 4.13 (4H,quint,J = 7Hz), 4.28 (8H,m,J = 7Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 16.7 (d,J = 12Hz), 24.3 (t,J = 12Hz) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 16.4(m), 36.6 (td,J = 139Hz, J = 4.4Hz), 63.8(m) p.p.m.; Mass spectrum (EI) (M)$^+$: 456.0906 ($C_{13}H_{31}O_6P_2S$ calc. 456.0901)].

5.6.2.3 0,O-Diisopropyl S-[bis-(diisopropoxyphosphinyl)-methyl] Phosphorothioate

[^{1}H NMR ($CDCl_3$) δ 1.40 (36H,d,J = 7Hz), 3.9 (1H,q,J = 22Hz), 4.5-5.2 (6H,m) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 15.6 (d,J = 12Hz), 22.5 (t,J = 12Hz) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 24.0(m), 39.0 (td,J = 140Hz, J = 4Hz), 72.5(m) p.p.m.; Mass spectrum (EI) (M)$^+$: 540.1807 ($C_{19}H_{42}O_6P_2S$ calc. 540.1804)].

5.6.3 Preparation of 0,O-Diethyl S-[(diethoxyphosphinyl) (thiophenyl) methyl] Phosphorothioate

This was prepared essentially as described (Birum, 1963) from phenyl chlorodithioformate and triethyl phosphite and treatment of the resulting phosphorus ylid with dry hydrogen chloride in dichloromethane

at 0°C for 20 min. Removal of the solvent in vacuo yielded crude title compound as an orange oil.

[^{1}H NMR ($CDCl_3$) δ 4.80 (1H,t,J = 16Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 20.8 (d,J = 18Hz), 22.5 (d,J = 18Hz) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 48.2 (dd,J = 155Hz, J = 4.3Hz) p.p.m.]

CHAPTER 6

SYNTHESIS OF BISPHOSPHONATE ANALOGUES OF ARILDONE

6.1 Introduction

Through random screening studies β-diketones have been found to possess antiviral activity. These compounds are good chelators of metal ions; being used industrially in metal ion extraction from aqueous media (Burger, 1973). Lipophilic β-diketones and in particular arildone (4-[6-(2-chloro-4-methoxy)phenoxy]hexyl-3,5-heptanedione; Fig. 6.1(a)) possess in vivo activity against a range of viruses (McSharry and Pancic, 1982).

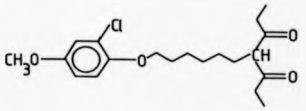
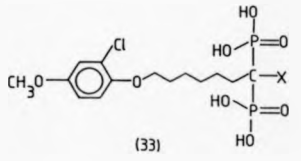


Fig. 6.1(a) The structure of arildone

The mode of action of arildone remains unclear but metal ion chelation may be involved (Hutchinson, 1985). β-diketones are known to react with arginine groups in proteins (Kabayo and Hutchinson, 1977) which could result in inactivation of an essential viral protein, however it has been reported that in cell culture assays with arildone washing the cells readily reverses any inhibitory effect (Kim, *et al*, 1980), so this seems an unlikely mode of action. It was also reported

that, for herpes virus in cell culture, addition of arildone was only effective in reducing virus titre if the addition was carried out less than 6h post-infection (McSharry and Pancic, 1982). After this period of incubation addition of arildone had no effect. Arildone thus appeared to inhibit an early process in the infectious cycle such as cell penetration or viral uncoating. The extreme lipophilicity of arildone means that when administered in vivo it concentrates in the lipophilic bilayer of the cell wall where viral uncoating occurs. For general chemotherapeutic use arildone is insufficiently soluble in water. Its use is thus limited to cutaneous infections where it is usually applied topically as a cream in DMSO.



(33)

X=H,Cl

Fig. 6.1(b) Bisphosphonate analogues of arildone

The objective of this study was to prepare bisphosphonates resembling arildone and examine any antiviral activity (Fig. 6.1(b)). Compounds such as 33 may be regarded as both an analogue of arildone and also an analogue of inorganic pyrophosphoric acid. Substituted methylenebisphosphonates which retain a halogen substituent on the bridge carbon atom possess *in vitro* activity against influenza virus and the addition of a large lipophilic side chain has been found to increase their activity in cell culture (Semple, 1986). This is because they are

more readily taken up by cells than the parent bisphosphonic acids. Thus compounds such as 33 might be expected to express a range of activity by inhibiting RNA synthesis in viruses such as influenza and viral penetration or uncoating processes in viruses such as herpes and polio.

6.2 Synthesis of Bisphosphonate Analogues of Arildone

6.2.1 Synthetic Approach

The approach adopted is shown in Fig. 6.2.1. The preparation of the side chain has been described by Diana (Diana, *et al*, 1977), thus the synthesis is reduced to the alkylation and halogenation of tetraisopropyl methylenebisphosphonate. Alkylation of the sodium salt of tetraisopropyl methylenebisphosphonate (Seyferth and Marmor, 1973) followed by reaction with sodium hypohalite (Curry, 1971) is one possibility to prepare the target molecule. However it is more effective to invert the two steps. Monohalogenation of tetraisopropyl methylenebisphosphonate cannot be accomplished in good yield but dichloromethylenebisphosphonate can be easily prepared (Quimby, *et al*, 1968) and monodehalogenated by metal-halogen exchange (Seyferth and Marmor, 1973). Alkylation of the thallium(I) salt may then be accomplished in good yield (Hutchinson and Semple, 1985).

6.2.2 Results and Discussion

The procedures for dichlorination of tetraisopropyl methylene-bisphosphonate (Quimby, *et al*, 1968) and monodehalogenation via metal halogen exchange (Seyferth and Marmor, 1973) both proceeded in good yield. The alkyl side chain was obtained as a 1:4 mixture of the corresponding bromide/iodide as described (Diana, *et al*, 1977) and could be used directly in alkylation experiments or could be further purified as described (section 6.4.6).

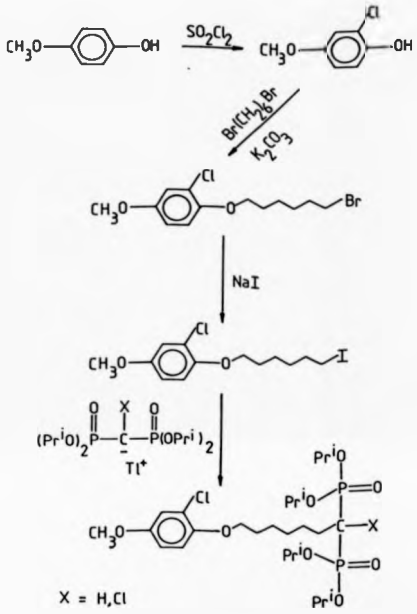


Fig. 6.2.1 The synthesis of bisphosphonates related to arildone

Treatment of a solution of tetraisopropyl chloromethylene-bisphosphonate in THF with thallium(I) ethoxide yielded a white suspension of the thallium(I) salt as described (Hutchinson and Semple, 1985). However treatment with a 10 molar excess of 1-(2-chloro-4-methoxyphenoxy)-6-iodohexane only resulted in a low (22%) yield of isolated product. In repeat experiments this yield could not be improved. Analysis of both the crude reaction product by ^{31}P NMR spectroscopy and fractions obtained after flash chromatography indicated that alkylation of the thallium(I) intermediate was occurring but that this was followed by metal-chlorine exchange leading to cleavage products (Fig. 4.2(c)).

With tetraisopropyl bromomethylenebisphosphonate the thallium(I) salt could again readily be formed but none of the desired product could be obtained. Positive bromine abstraction and carbon-phosphorus bond cleavage were found to dominate the reaction. These increased problems with the thallium(I) salt of tetraisopropyl bromomethylene-bisphosphonate can be equated with the fact that the carbon-bromine bond is weaker than the carbon-chlorine bond (Johnson, 1982) and that the first ionisation potential of bromine is less than that of chlorine (Downs and Adams, 1973).

With the thallium(I) salt of tetraisopropyl methylenebisphosphon-ate a 23% yield of the desired product was isolated. The absence of a bridge halogen atom resulted in less cleavage occurring but the excess thallium(I) ethoxide needed for complete anion formation was found to attack at phosphorus and exchange one of the ester groups of tetraiso-propyl methylenebisphosphonate to ethyl. This was observed previously (section 4.3.2.1).

The problems encountered can be attributed to the reduced mobility of the large alkyl iodide under the reaction conditions. Thus the desired reaction proceeds more slowly and side reactions occur. The two substituted methylenebisphosphonates obtained were readily deesterified

deesterified with bromotrimethylsilane and isolated as sodium salts via cation exchange chromatography (sections 6.4.9, 6.4.10).

6.3 Conclusions

C-Substituted methylenebisphosphonates which are analogues of arildone may be synthesised via alkylation of thallium(I) salts (Hutchinson and Semple, 1985) of tetraisopropyl methylene and chloromethylenebisphosphonate but only in low yield. Despite this problem the products are readily isolated from the crude reaction mixtures by flash chromatography and deesterified by reaction with bromotrimethylsilane and this remains the method of choice for their preparation.

6.4 Experimental

6.4.1 Preparation of Tetraisopropyl Methylenebisphosphonate

This was prepared essentially as described (Roy, 1966). The title compound was obtained pure by distillation under vacuum (100-103°C/0.25 mmHg) as a colourless oil (81.0g, 47%).

[^{1}H NMR ($CDCl_3$) δ 4.80 (4H,m,J = 6Hz), 2.40 (2H,t,J = 22Hz), 1.36 (24H,dd,J = 6Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 18.1(s) p.p.m.; Analysis C, 44.15; H, 8.84%. $C_{13}H_{30}O_6P_2$ requires C, 45.35; H, 8.78%; Mass spectrum (NH_3CI) $(M+H)^+$ 345].

6.4.2 Preparation of Tetraisopropyl Dichloromethylenebisphosphonate

This was prepared essentially as described (Quimby, *et al*, 1968). The title compound was obtained pure by distillation under vacuum (112-115°C/0.1 mmHg) as a colourless oil (18.9g, 72%).

[^{1}H NMR ($CDCl_3$) δ 4.88 (4H,m), 1.41 (24H,d,J = 7.8Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 6.7(s) p.p.m.; Mass spectrum (NH_3CI) $(M+H)^+$ 413,415,417].

6.4.3 Preparation of Tetraisopropyl Chloromethylenebisphosphonate

This was prepared by the method of Seyferth and Marmor (1973) but using methyllithium in the place of butyllithium. The title compound was obtained pure by distillation under vacuum (109-111°C/0.1mmHg) as a colourless oil (11.9g, 69%).

[^{1}H NMR ($CDCl_3$) δ 4.86 (4H,m), 3.92 (1H,t,J = 20Hz), 1.36 (24H,d,J = 8.0Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 11.5(s) p.p.m.; Mass spectrum (NH_3CI) (M+H)$^+$ 379,381].

6.4.4 Preparation of 2-Chloro-4-methoxyphenol

This was prepared as described (Brown, 1955). After distillation under vacuum (118-128°C/15 mmHg) the distillate solidified and the title compound obtained pure, by recrystallisation from petroleum ether (bpt 40-60°C), as colourless crystals (30.4g, 79%), mpt 44-46°C (lit. 46-47°C).

[^{1}H NMR ($CDCl_3$) δ 7.1-6.7 (3H,m), 4.55 (1H,s,broad), 3.80 (3H,s) p.p.m.; Mass spectrum (EI) (M)$^+$ 158,160].

6.4.5 Preparation of 1-(2-Chloro-4-methoxyphenoxy)-6-bromohexane

This was prepared essentially as described (Diana, *et al*, 1977). The title compound was obtained pure by distillation under vacuum (148-152°C/0.2-0.3 mmHg) as a colourless oil (43.4g, 76%).

[^{1}H NMR ($CDCl_3$) δ 7.0 (1H,d,J = 2.5Hz), 6.92 (1H,d,J = 9.8Hz), 6.80 (1H,dd,J = 2.5Hz, J = 9.8 Hz), 4.05 (2H,t,J = 6.5Hz), 1.80 (3H,s), 3.50 (2H,t,J = 7Hz), 1.90 (4H,m), 1.60 (4H,m) p.p.m.; Mass spectrum (EI) (M)$^+$ 320,322,324].

6.4.6 Preparation of 1-(2-Chloro-4-methoxyphenoxy)-6-iodohexane

This was obtained as a 4:1 mixture (by ^{1}H NMR) with the above bromide as described (Diana, *et al*, 1977). The title compound was obtained in sufficient purity for the next step by distilling the alkyl

bromide out of the mixture. ^{1}H NMR analysis of the residue indicated >95% of the title compound (≃ 30g, ≃ 60%). This was used without further purification.

[^{1}H NMR ($CDCl_3$) δ 6.98 (1H,d,J = 2.5Hz), 6.90 (1H,d,J = 9Hz), 6.75 (1H,dd,J = 2.5Hz, J = 9Hz), 4.0 (2H,t,J = 7Hz), 3.80 (3H,s), 3.24 (2H,t,J = 7Hz), 1.85 (4H,m), 1.55 (4H,m) p.p.m.; Mass spectrum (EI) (M)$^+$: 368,370].

6.4.7 Preparation of Tetraisopropyl (2-Chloro-4-methoxyphenoxyhexyl) chloromethylenebisphosphonate

Alkylation of the thallium(I) salt of tetraisopropyl chloromethylenebisphosphonate was carried out essentially as described (Hutchinson and Semple, 1985). The title compound was obtained pure by flash chromatography on silica gel, eluting with acetone/petroleum ether (bpt 40-60°C) (1:12 v/v changing to 1:2 v/v after elution of the excess alkyl iodide), and recrystallisation from petroleum ether (bpt 40-60°C) as a white solid (0.71g, 22%), mpt 68°C.

[^{1}H NMR ($CDCl_3$) δ 6.92 (1H,d,J = 3Hz), 6.85 (1H,d,J = 9Hz), 6.73 (1H,dd,J = 9Hz, J = 3Hz), 4.88 (4H,m), 3.95 (2H,t,J = 7Hz), 3.75 (3H,s), 2.2 (2H,m), 1.80 (2H,quint,J = 7Hz), 1.72 (2H,quint,J = 7Hz), 1.50 (4H,m), 1.35 (2H,d] p.p.m.; ^{31}P NMR ($CDCl_3$) δ 14.8(s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 153.8(s), 148.8(s), 123.7(s), 115.8(s), 115.1(s), 112.8(s) 72.8(d,J = 21.4Hz), 70.0(s), 62.9 (t,J = 145Hz), 55.7(s), 36.0(s), 29.5(s), 29.1(s), 25.6(s), 24.4(s), 23.8(dd,J = 16Hz, J = 69Hz) p.p.m.; Analysis C, 50.49; H, 7.22%. $C_{24}H_{44}Cl_2O_8P_2$ requires C, 50.41; H, 7.48%; Mass spectrum (EI) (M)$^+$: 618,620,622].

6.4.8 Preparation of Tetraisopropyl (2-Chloro-4-methoxyphenoxyhexyl) methylenebisphosphonate

Alkylation of the thallium(I) salt of tetraisopropyl methylenebisphosphonate was carried out as described (Hutchinson and Semple,

1985). The title compound was obtained pure by flash chromatography on silica gel, eluting with acetone/petroleum ether (bpt 40-60°C) 1:15 v/v changing to 1:5 v/v after elution of the excess alkyl iodide), as a colourless oil (0.79g, 23%).

[^{1}H NMR ($CDCl_3$) δ 6.95 (1H,d,J = 2.5Hz), 6.85 (1H,d,J = 9.5Hz), 6.75 (1H,dd,J = 9.5Hz, J = 2.5 Hz), 4.80 (4H,m), 3.96 (2H,t,J = 6Hz), 3.76 (3H,s), 2.1-1.4 (10H,M), 1.35 (24H,d,J = 7.5Hz) p.p.m.; ^{31}P NMR ($CDCl_3$) δ 22.0(s) p.p.m.; ^{13}C NMR ($CDCl_3$) δ 153.6(s), 148.6(s), 123.5(s), 115.6(s), 114.9(s), 112.5(s), 70.5(dd,J = 7Hz, J = 15Hz), 69.7(s), 55.4(s), 38.1(t,J = 135 Hz), 28.9(s), 28.8(s), 25.5(s), 25.3(s), 23.9(s), 23.7(dt,J = 29Hz, J = 4Hz) p.p.m.; Analysis C, 51.49; H, 8.04; P, 9.94%. $C_{24}H_{47}ClO_8P_2$ requires C, 53.38; H, 8.10; P, 10.59%; Mass spectrum (NH_3CI) $(M+H)^+$ 585,587].

6.4.9 Preparation of Tetrasodium (2-Chloro-4-methoxyphenoxyhexyl)-chloromethylenebisphosphonate

The tetraisopropyl ester was deesterified essentially as described (section 2.5.7) except that the crude acid was passed down a short column of dowex 50 cation exchange resin (Na^+ form). Lyophilization of the eluate yielded the title compound as a white solid (0.39g, 68%).

[^{1}H NMR (D_2O/D_5-pyridine, 1:1 v/v) δ 7.1 (3H,m), 4.05 (2H,t,J = 7Hz), 3.95 (3H,m), 2.9 (2H,m), 2.3 (2H,m), 1.85 (2H,m), 1.6 (4H,m) p.p.m.; ^{31}P NMR (D_2O/D_5-pyridine, 1:1 v/v) δ 16.1(s) p.p.m.; Analysis C, 31.24; H, 4.46; P, 11.75%. $C_{14}H_{18}Cl_2O_8P_2Na_4$ requires C, 31.19; H, 4.37; P, 11.49%].

6.4.10 Preparation Of Disodium (2-Chloro-4-methoxyphenoxyhexyl)-methylenebisphosphonate

The tetraisopropyl ester was deesterified as above to yield the title compound as a white solid (0.37g, 76%).

[^{1}H NMR (D_2O/D_8 - pyridine 1:1$^v/_v$) δ 6.55 (3H,m), 3.58 (2H,t,J = 7Hz), 3.45 (3H,m), 2.0 (1H,td,J = 20Hz, J = 10Hz), 1.95 (2H,m), 1.60 (2H,m), 1.40 (2H,m), 1.20 (4H,m) p.p.m.; ^{31}P NMR (D_2O/D_8 - pyridine, 1:1$^v/_v$) δ 21.0(s) p.p.m.; Analysis C, 36.44; H, 4.65%. $C_{14}H_{21}ClO_8P_2Na_2$ requires C, 36.50; H, 4.16%].

CHAPTER 7

THE ACTIVITY OF METHYLENEBISPHOSPHONATES AGAINST INFLUENZA VIRUS

7.1 Introduction

The ability to disrupt the viral protein envelope of influenza with detergent without interruption of the RNA polymerase activity allows this activity to be examined. Under suitable conditions a detergent disrupted suspension of influenza will synthesise viral RNA. The extent of production of RNA can be measured by recording the extent of incorporation of [^{3}H]-UTP into acid-precipitable material. Assaying for polymerase activity both in the presence and absence of potential inhibitors allows ID_{50} values for the compounds to be determined.

In infecting a cell influenza uses a cleaved section of cellular RNA to prime viral mRNA synthesis. Under cell-free conditions this is not possible hence it is necessary to add ApG to the assay solutions to act as a primer. ApG is known to be an effective primer for influenza (Kawakami, *et al*, 1981) and it is noticeable that the coupling of adenosine (A) to guanosine 5'-triphosphate (GTP) yielding the ApG linkage and pyrophosphoric acid is the first reaction of the chain elongation phase of viral mRNA synthesis (Fig. 1.1.2.2.1(a)).

In this study the bisphosphonates synthesised were assayed against the RNA polymerase of influenza A/X49 via a cell free assay in the presence of ApG to act as a primer.

7.2 Preparation of Influenza Virus Suspension

The virus used for determining the inhibitory abilities of the pyrophosphate analogues synthesised was influenza A/X49 and was grown in the chorioallantoic membrane of fertile hens eggs essentially as described (Kelly and Dimmock, 1974).

Eleven day old fertile hens eggs (64) were each innoculated with infected allantoic fluid (100 µl of a 1 x 10^{-3} dilution in PBS) and incubated (33°C/48h). The eggs were then chilled (-20°/2h) and the allantoic fluid was collected (all further procedures were carried out at 0-4°C). Unwanted egg membranes were removed by centrifugation (3,000 r.p.m./20 min.) and the crude virus pelleted by centrifugation (21,000 r.p.m./90 min/6 x 300cm^3 rotor). The supernatant was discarded and the pellet soaked in PBS (10cm^3/18h).

The pellet was suspended in PBS (10 cm^3) (the presence of virus checked by a haemagglutinin assay (Dimmock, 1987)) and the solution applied onto a velocity gradient of 15-45% (%/v) sucrose in buffer (60cm^3, 10 mM tris-HCl, pH 7.4) and centrifuged (22,000 r.p.m./1h/3 x 65cm^3 swing out rotor). The diffuse virus band was collected by bottom puncture and diluted with PBS to a final volume of 30cm^3.

The solution was applied onto an equilibrium gradient of 30-70% (%/v) sucrose in buffer (50cm^3, 10mM tris-HCl, 150 mM NaCl, pH 7.4) and centrifuged (20,000 r.p.m./18h/3 x 65cm^3 swing out rotor). The virus band was again collected, diluted with PBS, and the virus pelleted by centrifugation (30,000 r.p.m./2h/8 x 50cm^3 rotor). The supernatant was then discarded and the virus pellet soaked in PBS for 18h.

The virus pellet was resuspended in buffer (400 µl, 400uM tris-HCl, pH 8.0) and frozen in aliquots (30 µl) at -70°C. Each aliquot was thawed immediately before use.

7.3 Influenza Virus Polymerase Assays

These were carried out essentially as described (Cload and Hutchinson, 1983). RNA polymerase activity was assayed in a standard reaction mixture (200 µl) containing 50mM tris-HCl buffer, pH 8.0; 5mM magnesium acetate, 150mM potassium chloride; 5mM dithiothreitol; 0.4mM

adenylyl ($3'-5'$)-guanosine (ApG), 0.25% (v/v) Nonidet-P40, 0.4mM each of ATP, CTP and GTP and [^{3}H]-UTP (5 μCi, ^{3}H incorporatd at the 5-position) and purified virus suspension (30μl).

The mixtures were maintained at 0-4°C until RNA synthesis was initiated by the addition of virus suspension. The mixtures were incubated during which time the incorporation of tritium into acid-precipitable material increased linearly. Polymerisation was stopped by the addition of cold (0°C) saturated tetrasodium pyrophosphate solution (200μl) and the mixtures added to cold (0°C) TCA (2cm^3, 10% v/v). After thorough agitation the solutions were maintained at 0-4°C for >12h.

Precipitated material was collected on Whatman GF/C glass fibre discs, prewetted with cold (0°C) TCA (10% v/v), which were washed with cold TCA (10% v/v, 3 x 5cm^3), ethanol (5cm^3) and diethyl ether (2 x 5cm^3). After thorough drying the radioactivity of each disc was determined by scintillation counting using a xylene-based scintillant.

The pyrophosphate analogues under test were added to the reaction mixtures before the addition of virus suspension and all reactions were performed in duplicate. For each analogue tested a semilogarithmic dose-response curve was drawn and the concentration of the analogue which inhibited by 50% the incorporation of [^{3}H]-UTP into acid-precipitable material was determined (ID_{50}).

7.4 Determination of Zinc Binding Constants

In view of the report (Cload and Hutchinson, 1983) that a correlation exists between the ability of pyrophosphate analogues to bind zinc and their inhibition of influenza under cell free conditions, the abilities of selected compounds to bind zinc were examined.

The zinc binding constants were determined by the method of Hummel and Dreyer (1962) using a column of Sephadex G-10 (1.6 x 96cm) which was equilibriated with zinc chloride (10 μM) in triethanolamine-HCl buffer (0.1M, pH 8.0; the same pH as the assay solutions). The analogues

(100-200 nmole) were loaded onto column in triethanolamine/zinc buffer ($2cm^3$) and the column eluted with the same buffer. The zinc content of individual fractions was determined by atomic absorption spectrometry and the zinc binding constants determined. Full details of this technique have been described by Yoza (1977).

7.5 Results

7.5.1 Inhibitory Effects Against Influenza RNA Polymerase

Table 7.5 shows the ID_{50} values of all the methylene-bisphosphonates tested. A typical dose-response curve, for the inhibition of incorporation of [^{3}H]-UTP into acid-precipitable material during influenza RNA synthesis, is shown in Fig. 7.5.1.

7.5.2 Zinc Binding Constants

All the zinc binding constants determined as described (section 7.4) are listed in Table 7.5.

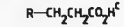
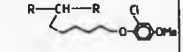
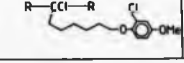
7.6 Discussion

In this study the ID_{50} values reported relate to the inhibition of the total of the RNA polymerase activity by 50%. The kinetics of the inhibition of the RNA polymerase activity of influenza are very complex (Cload, 1983) and the individual stages of the process have not been fully characterised.

Monofluoromethylenebisphosphonic acid expressed the greatest activity of the halogenated methylenebisphosphonic acids studied with difluoromethylenebisphosphonic acid and fluorobromomethylenebisphosphonic acid also showing good activity. It is noticeable that all of the halogeno- and dihalogenomethylenebisphosphonates so far examined in this study and previously (Cload, 1983; Semple, 1986) exhibit activity against the RNA polymerase of influenza except the parent compound; methylenebisphosphonic acid.

Table 7.5 Activity of methylenebisphosphonates and related compounds against influenza RNA polymerase

COMPOUND TRIS [] SALT R=-P(O)(OH)$_2$	ID_{50} (μM)	pKd' (Zn^{2+})
1) R—CF_2—R	130	> 6
(Tetrasodium salt)	(120)	> 6
2) R—CHF—R[a]	85	> 6
3) R—CFBr—R	115	> 6
4) R—C(=CH_2)—R	470(500)[h]	-
5) R—C(=O)—R[b,c]	20	5·4
6) R—CH(CH_2SEt)—R	> 500	4·9
7) R—CH(CH_2SPr)—R	> 500	-
8) R—C(CH_2S-Ph)—R	390	-
9) R—C(cyclopropyl)—R	> 500	-
10) R—CH_2—R[c]	> 500	5·3
11) R—C(cyclopropyl)—CO_2H[d]	320	-
12) R—CH_2—CO_2H[c]	275	5·5

13]		> 500	-
14]		> 500	4
15]		> 500	59
16]		170	-
17]		60	-
18]		> 500	-
19]		450	58

a] Assay performed on free acid

b] Assay performed on tetrasodium salt

c] Cload and Hutchinson,(1983)

d] Assay performed on bis(cyclohexylammonium) salt

e] Prepared as described in appendix 1

f] Kindly donated by Dr S.Masson, I.S.M.R.A.,Université de Caen

g] Assay performed on tetratrimethylsilyl ester

h] Repeat assay

It has been argued by Blackburn (1981) that in the use of bisphosphonates as analogues of inorganic pyrophosphoric acid emphasis should be placed as much on their isopolar properties as their isosteric properties. Methylenebisphosphonic acid is a good isostere of pyrophosphoric acid; the distance between the two phosphorus atoms in pyrophosphate (0.294nm) and methylenebisphosphonate (0.305 nm) differing by less than 4% (Larsen, *et al*, 1969); but shows no activity.

Substituting the bridge carbon atom with electron withdrawing halogen atoms reduces the dissociation constants of the methylenebisphosphonic acid such that it more closely mimics the behaviour of inorganic pyrophosphoric acid in solution. Fluorination of the methylene bridge has a large effect on the dissociation constants. The acid dissociation constants of difluoromethylenebisphosphonic acid have been accurately determined and mimic closely those of inorganic pyrophosphoric acid (Table 7.6). It thus appears that the activity of the halogenated methylenebisphosphonic acids is related to the fact that they are both isopolar and isosteric with pyrophosphoric acid.

Methylenebisphosphonates lacking electron attracting bridge substituents (eg 6-10, 18) showed little or no activity against the influenza RNA polymerase as would be expected for the reason discussed above. The sulphone (17) was more active than its deoxygenated analogue (16) but this compound is unstable in solution (decomposing over several days). It was added to the assay solutions directly as its tetratrimethylsilyl ester (hydrolysis in situ yielding the free acid) and interference in the assay by the other product of hydrolysis, tetratrimethylsilyl hydroxide, has not been ruled out.

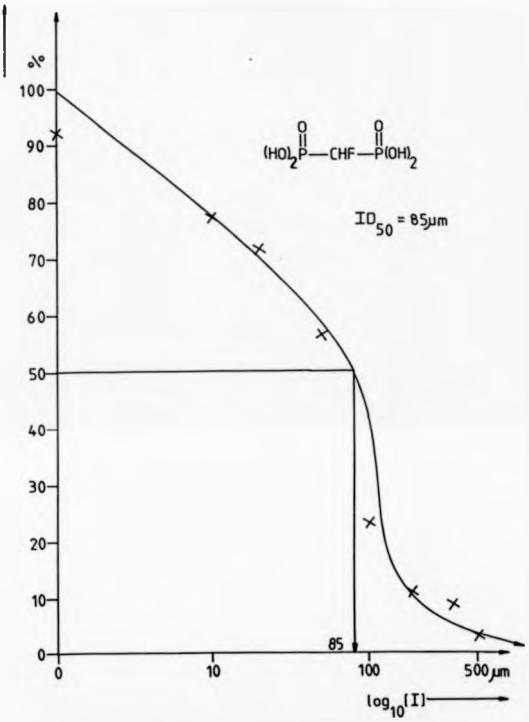


Fig. 7.5.1 The inhibition of influenza A/X49 RNA polymerase by monofluoromethylenebisphosphonic acid.

Table 7.6 Acid dissociation constants of inorganic pyrophosphoric acid and related compounds

COMPOUND R = $-P(OH)_2$ (O)		pK_1	pK_2	pK_3	pK_4
R—O—R	a	-	2·36	5·77	8·22
R—CH_2—R	a	-	2·87	7·45	10·96
R—CHF—R	a	-	<2·7	6·15	9·35
	b	-	2·78	6·62	10·1
R—CF_2—R	c	1·44	2·11	5·66	7·63
	d	1·46	2·14	5·78	8·16
R—CCl_2—R	a	-	<2·6	5·80	8·00
	a	-	-	6·11	9·78
	e	-	-	6·1	9·8
R—CHBr—R	e	-	2·20	6·6	10·2

a) Blackburn,et al(1986a)
b) McKenna and Shen(1981)
c) Burton, et al(1982b)
d) Fonong, et al(1983)
e) Grabenstetter, et al(1967)

The arildone analogue (19) showed considerably less activity than monochloromethylenebisphosphonic acid (ID_{50} = 85 μm, Semple, 1986). This contrasts with the situation in cell culture where halogenated methylenebisphosphonic acids bearing lipophilic side chains are more active than those without (Semple, 1986).

Increasing the rigidity of the analogue, by enclosing the bridge carbon atom in a cyclopropane ring, had no significant effect on the activity of the compound. The cyclopropyl analogue (9) of methylenebisphosphonic acid (10) had no activity and the cyclopropyl analogue (11) of phosphonoacetic acid (12) retained most of the activity of phosphonoacetic acid.

The three fluorinated compounds assayed (1-3), which were all good inhibitors of influenza, were found to have zinc binding constants of

>6. It has been shown previously that bisphosphonates that bind zinc this effectively are good inhibitors of influenza (Cload and Hutchinson, 1983) and this lends weight to the possibility that their mode of action is via chelation to an essential zinc ion present in the RNA polymerase. This enzyme is also known to be dependent on the presence of magnesium ions but it is unlikely that the compounds exhibit their activity by removing essential magnesium ions from solution. In the assay solutions magnesium ions are present at a concentration of 5mM but some of the compounds assayed were active at concentrations less than 100μM and dibromomethylenebisphosphonic acid is reported to be active at 10μM (Cload and Hutchinson, 1983).

7.7 Conclusions

It is concluded that C-substituted methylenebisphosphonic acids are effective inhibitors of the RNA polymerase of influenza virus only when substituted with one or more electron withdrawing groups. In these cases their behaviour in solution more closely mimics that of inorganic pyrophosphoric acid. Isopolar considerations appear to be equally, if not more, important than isosteric ones and that the inhibitory effect appears to be related to the ability to bind zinc under physiological conditions.

APPENDIX I

SYNTHESIS OF BENZENE-1,2-BISPHOSPHONIC ACID

AI.1 Preparation of 1,2-Bis(dimethoxyphosphoryl)benzene

This was prepared by a modification of the procedure described by Kyba (1983). A mixture of trimethyl phosphite (263g, 2.12 mol) and 1,2-dichlorobenzene (94g, 0.64 mol) was photolysed with a mercury emission lamp (50W, λ = 254nm) in a quartz apparatus for 130h. Lyophilisation and cooling (-20°C/2h) yielded a white precipitate which was collected by filtration. Recrystallisation from acetone yielded the title compound as a white crystalline solid (7.5g, 3.4%).

[^{1}H NMR ($CDCl_3$) δ 8.18 (2H,m), 7.66 (2H,m), 3.85 (2H,d,J = 11Hz] p.p.m.; ^{31}P NMR ($CDCl_3$) δ 18.3(s) p.p.m.; Mass spectrum (NH_3CI) $(M+H)^+$ 295; mpt 75-78°C (lit 79-81°C].

AI.2 Preparation of Benzene-1,2-bisphosphonic Acid

The above tetramethyl ester (2.0g, 6.8 mmol) was refluxed in concentrated hydrochloric acid (25cm^3] for 48h. After freeze drying the residue was recrystallised from chloroform to yield the title compound as a white crystalline solid (1.3g, 81%).

[^{1}H NMR (D_2O) δ 8.05 (2H,m), 7.71 (2H,m) p.p.m.; ^{31}P NMR (D_2O) δ 19.1(s) p.p.m.; Mass spectrum (NH_3CI) $(M+H)^+$ 239; Analysis C, 30.02; H, 3.11; P, 24.93%. $C_6H_8O_6P_2$ requires C, 30.27; H, 3.39; P, 26.02%].

APPENDIX II

INHIBITION OF HIV/HTLV-III BY PYROPHOSPHATE ANALOGUES

AII.1 Introduction

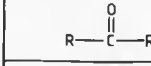
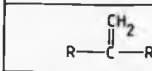
The rapid world-wide increase in cases of acquired immune deficiency syndrome (AIDS) has greatly increased the need for compounds with broad spectrum antiviral activity. The causative agent, human T-lymphotropic virus type III (HTLV-III), or as also known: human immunodeficiency virus (HIV) has an affinity for human T-cells whose destruction causes immunocompromisation. This often results in adventicious infection by other viruses.

HTLV-III possesses a reverse transcriptase activity and this has been found to be inhibited by both carbonylbisphosphonic acid and phosphonoformic acid (Vrang and Öberg, 1986; Sandström, *et al*, 1985). The mode of action of these compounds is unknown but in view of their probable mechanisms of action against influenza and herpes it is possible that they inhibit the reverse transcriptase by binding to an essential metal ion.

In vitro phosphonoformic acid has been reported to be active in concentrations as low as 0.5 μM (Vrang and Öberg, 1986). This has prompted compassionate use of this compound in severely immunocompromised patients (Öberg, *et al*, 1987). *In vivo* activity was indeed observed but the very high doses required (median 6-10g/day) caused undesirable side effects, although these must be weighed against the unfavourable prognosis of these patients.

In view of these results selected compounds were assayed against the reverse transcriptase of HTLV-III and the results are listed in table AII.

Table AII. The Inhibition of HTLV-III by pyrophosphate analogues

Compound O R = -P(OH)$_2$	Inhibition of HTLV-III (μM)	Cell Toxicity (μM)
	<30	>1000
	<30	>300
	<30	>1000
	>100	—
	>100	—

AII.2 Discussion

All of the compounds tested were considerably less active, against the reverse transcriptase of HTLV-III, than phosphonoformic acid and do not show much promise for chemotherapeutic use. Phosphonoformic acid is readily taken up by bones and this small polar molecule does not readily cross cell walls; requiring incubation times of the order of 4h to achieve maximum cellular concentration (Stenberg, *et al*, 1985). This is why a relatively high plasma concentration is needed for *in vivo* activity.

Carbonylbisphosphonic acid and ethenylidenebisphosphonic acid exhibit some activity but show cell toxicity in concentrations only 10-30 times that of their ID_{50} values. They also suffer from similar

problems to phosphonoformic acid in that they are readily absorbed by bones (section 1.4.1) and have difficulty crossing cell walls (section 1.4.2).

Benzene-1,2-bisphosphonic acid is the most promising of the compounds tested because although this molecule itself is no more effective than carbonylbisphosphonic acid or ethenylidenebisphosphonic acid, the possibility exists for preparing a wide range ofsubstituted analogues. A lipophilic side chain could result in an increase in ease of cell uptake and reduced affinity for bones and teeth on steric grounds. More work needs to be done on this class of compound.

At present the most promising compound tested against the reverse transcriptase of HTLV-III is 3'-azido-3'-deoxythymidine (AZT; Fig. AII.2).

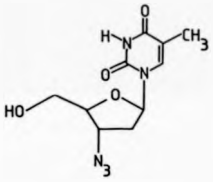


Fig. AII.2 The structure of 3'-azido-3'-deoxythymidine

Under the conditions of the reverse transcriptase assay, AZT was active at concentrations as low as 0.03 μM and AZT is currently undergoing clinical trials (Mitsuta, *et al*, 1985). It expresses low animal toxicity and can be given in relatively large doses.

It can be concluded that pyrophosphate analogues show little promise for the chemotherapy of AIDS relative to AZT but might still be useful in the chemotherapy of adventicious viral infections in these immunocompromised patients.

Note

The HTLV-III assays were kindly performed by Dr. Derek Kinchington (St. Mary's Hospital, London) to whom I must express my gratitude.

APPENDIX III

BROAD SPECTRUM SCREEN OF TRIS(CYCLOHEXYLAMMONIUM) ETHENYLIDENE-BISPHOSPHONATE

The title compound was submitted to the USAMRIID (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, 21701-5011, U.S.A.) screening program for evaluation of activity against a range of viruses. The results are listed in table AIII.

Table AIII Biological activity of tris(cyclohexylammonium) ethenylidenebisphosphonate against a range of viruses

VIRUS	GENUS	ID_{50} (ug/cm^3)	LD^a_{50} (ug/cm^3)	THERAPUTIC INDEX
Vesicular stomatitis	Rhabdovirus	-	100	0
Vaccina	Poxvirus	505	320	63
Adenovirus (Type 2)	Adenovirus	-	320	0
Venezuelan equine encephalomyelitis	Alphavirus	-	320	0
Sandfly fever (Sicilian)	Phlebovirus	755	100	13

a) Minimum concentration resulting in a 50% reduction in viable cells

AIII.1 Discussion

Tris(cyclohexylammonium) ethenylidenebisphosphonate appears to show little antiviral activity. This is in stark contrast to the activity of the similar carbonylbisphosphonic acid which is known to inhibit influenza (Cload and Hutchinson, 1983), herpes, hepatitis B

(Öberg, 1983), HTLV-III (Vrang and Öberg, 1986) and avian myoloblastosis virus (AMV) (Eriksson, *et al*, 1982). Replacement of the electron withdrawing carbonyl group by a carbon-carbon double bond is also found to cause a loss of activity against influenza (table 7.6) where it has been found that substituted methylenebisphosphonates bearing electron withdrawing groups are more active than those without (section 7.6). It is possible that the effectiveness of substituted methylene-bisphosphonates in mimicking inorganic pyrophosphoric acid is important for other classes of viruses.

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