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STUDIES OF THE ANTIVIRAL NATURE OF PYROPHOSPHATE ANALOGUES

by

GRAEME SEMPLE, B.Sc. (Warwick)

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Warwick.

Department of Chemistry

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ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
Ci	Curie
CTP	Cytidine 5'-triphosphate
DEAE	Diethylaminoethyl
DMEM	Dulbecco's modification of Eagle's medium
DNA	Deoxyribonucleic acid
EMEM	Eagle's minimal essential medium
GTP	Guanosine 5'-triphosphate
h	hour
HEPES	N-2-hydroxyethylpiparazine-N'-2-ethane sulphonic acid
ID_{50}	50% inhibitory dose
min.	minute
NCS	Newborn calf serum
NEAA	Non-essential amino acids
PBS	Phosphate buffered saline
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 except where acknowledgement is made to work and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick, between October 1983 and September 1986, and has not been submitted previously for a degree at any institution.

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PUBLICATIONS

Parts of the research described in this thesis have appeared in the scientific literature as follows:

1. The dehalogenation of dihalogenomethylene bispnosphonates.
   Hutchinson, D. W. and Semple, G. (1984)
   Phosphorus and Sulfur, 21, 1-5
2. Fast atom bombardment mass spectrometry of salts of substituted methylene bisphosphonic acids and other analogues of pyrophosphoric acid.
   Hutchinson, D. W. and Semple, G. (1984)
   Org. Mass Spectrom., 20, 143-146
3. Pyrophosphate analogues as inhibitors of viral polymerases.
   Hutchinson, D. W., Naylor, M. A., Semple, G. and Cload, P. A. (1985)
   Biochem. Soc. Trans., 13, 752-753
4. Synthesis of alkylated methylene bisphosphonates *via* organothallium intermediates.
   Hutchinson, D. W. and Semple, G. (1985)
   J. Organometal. Chem., 291, 145-151
5. Inhibition of viral nucleic acid synthesis by analogues of inorganic pyrophosphate.
   Hutchinson, D. W., Naylor, M. A. and Semple, G. (1986)
   Chemica Scripta, 26, 91-95

6 Relative reactivities of tetra-alkyl esters of methylene bisphosphonic acid.
Hutchinson, D. W. and Semple, G. (1986)
J. Organometal. Chem., 309, C7-C10

7 Synthesis and biochemical properties of some pyrophosphate analogues.
Hutchinson, D. W., Semple, G. and Thornton, D. M. (1986) Proc. 2nd Intl. Symp. Phosphorus Chemistry Directed Towards Biology (W. J. Stec, ed.) Elsevier, 1987 in press

ABSTRACT

Several methods for the preparation of halogenated and alkylated methylenebisphosphonates have been examined. C-Alkyl chloromethylenebisphosphonates were prepared by dihalogenation of tetraisopropylmethylene-bisphosphonate with sodium hypochlorite and selective monodehalogenation with methyl lithium, followed by alkylation of the thallium(I) salt and deesterification with bromotrimethylsilane and water. Such compounds were shown to be inhibitors of influenza A/X49 RNA transcriptase activity, and to be significantly better inhibitors of the replication of the virus in cell cultrue than either PAA or any unalkylated bisphosphonates. The C-benzyl analogue was found to show no reduction in affinity for calcified tissue over the parent species.

Using the synthetic methodology developed, an affinity label was prepared for the virus proteins. When this was incubated with the virus and reduced with $[^3H]$-$NaBH_4$, the three virus P proteins were radioactively labelled.

Some simple nucleopeptides were prepared as models of the VPg-pU linkage in poliovirus vRNA. The compounds were best prepared via a phosphorus(III) route in analogy to the phosphite triester method of nucleotide synthesis.

CHAPTER 1

INTRODUCTION

1.0 GENERAL ASPECTS

The effect of viruses as disease agents were well documented long before their actual discovery in the late nineteenth century. The term "virus" was coined by Beijerinck from the Latin phrase "*contagium vivum fluidium*" (living infectious fluid) which he used to describe an infectious, but bacteria free, filtrate of tobacco leaf sap. Similar filtration techniques were subsequently used to prove the viral nature of many of the common diseases of the early twentieth century, such as foot and mouth disease, chicken pox, smallpox and yellow fever.

Today there are more than a dozen different families of viruses known which affect vertebrates, and the advent of modern electron microscopy and molecular biology techniques has given a great deal of information about some of their unique properties.

Viruses, and the related viroids, are the only species known to use RNA molecules as their genetic material although not all do so (Table 1.0). In general, viruses are genetically compact. There are seldom any redundant sequences in the genome, and frequently there are overlapping sequences.

Viruses have a protein coat which can be surrounded by an envelope for further protection of the

Type of nucleic acid	RNA										DNA					
Capsid symmetry	Icosahedral			Helical							Icosahedral					Complex
Envelope	Absent		Present								Absent			Present		
Virus family	Picorna	Reo	Toga	Retro	Orthomyxo	Paramyxo	Bunya	Arena	Corona	Rhabdo	Parvo	Papova	Adeno	Unclassified	Herpes	Pox
Morphology																
Size nm	25	70 – 80	40 – 60	100 – 120	80 – 90	120 – 150	90 – 120	90 – 120	80 – 120	50 × 180	20	45 – 55	70 – 80	40 – 45	150 – 200	[illegible]
Examples of members or group (genus)	Entero (polio, cox-sackie, echo) Hepatitis A Rhino	Rota	Yellow fever RSSE Rubella	Leukaemia Sarcoma	Influenza A,B,C	Mumps Measles Parainfluenza RS				Rabies		Papilloma Polyoma SV40		Hepatitis B	Herpes simplex Varicella zoster Cytomegalo Epstein Barr	Variola Vaccinia Molluscum contagiosum

Table 1.0 Summary of the structural properties of different RNA and DNA viruses.

genome, and they range in size from about 20 nm to 300 nm in diameter. Unlike bacteria which are essentially independent, in that they can prosper in cell free media, viruses require a host cell within which to replicate. Often they do not contain any enzymes within their protein nucleocapsids but take over host cell processes for their own replication. This makes them very difficult to inhibit without interrupting normal cellular functions, but progress is being made in this area and new possibilities identified.

1.1 CONTROL OF VIRAL DISEASE

The arguments for funding of research aimed at controlling viral diseases are obvious. Despite the significant advances in recent years, many millions of people suffer from illnesses which could be prevented. Effective immunisation programs have resulted in the eradication of smallpox, a dramatic reduction in the number of cases of poliomyelitis in the developed world, and the hope that measles, mumps and rubella may soon be under similar control (Assaad and Lednyi, 1984).

Drawbacks have been encountered with the administration of vaccines. Live attenuated vaccines for instance, can produce disease symptoms in immunocompromised patients (Melrick, 1980). A number of viruses are capable of mutation to render any immunoprophylaxis campaign virtually useless. The best known example of such a virus is influenza A which will be discussed later. New viral

diseases have appeared in recent years, such as non A-non B hepatitis and acquired immune deficiency syndrome (AIDS), necessitating lengthy research programs before prophylactic control can be achieved. If, however, a broad spectrum therapeutic antiviral drug were available already, treatment could begin immediately to control the diseases.

1.1.1 Antiviral Chemotherapy

It is now fifty years since the beginning of practical antibacterial chemotherapy, and it is probably true to say that currently, antiviral chemotherapy is at about the same stage. At that time many antibacterial compounds were known, nearly all of them discovered serendipitously, but they were too toxic to human cells to be used in patients (Geddes, 1985). Now the same scenario can be seen again with antivirals, many are known but few have proven effective in humans.

It was originally thought that the intracellular nature of viruses rendered them invulnerable to attack, and it is this attitude that has meant that the development of antiviral compounds has lagged so far behind that of antibacterials. A considerable amount of research into viral specific processes has shown that targets for the rational design of antiviral agents do exist. All the antiviral compounds currently known however, result from chance discoveries or modifications thereof. This study is concerned with methods of enhancing the known activity of one such group of compounds, the pyrophosphate analogues, against influenza virus, and

so it is prudent to discuss the nature of this virus and methods by which it can be combatted therapeutically.

1.2 INFLUENZA VIRUS

Influenza is a global disease causing epidemics in many birds and domestic animals as well as humans. In its simplest form, the disease involves infection of epithelial cells in the upper respiratory tract. An incubation period of one to three days is followed by a fever peak characterised by nausea, headache, chills and cough.

All the influenza viruses belong to the orthomyxovirus family (Melnick, 1974) and are classified into three types, A, B and C. There are some differences between the effects of the three types on humans - types B and C have not been isolated from animals (Air and Compans, 1983) - but it has recently been suggested that these differences are not as great as was first thought (Palese and Young, 1983). Influenza A has been the subject of the greatest research effort, since it has been responsible for each of the worldwide epidemics this century, including the pandemic of 1918-19 which claimed over 20 million lives.

1.2.1 Virion Structure

Influenza is an enveloped RNA virus, the internal nucleocapsid being protected by an envelope consisting of a matrix protein, a lipid bilayer and glycoproteins (Fig.1.2.1). The virions are of non-uniform shape, the

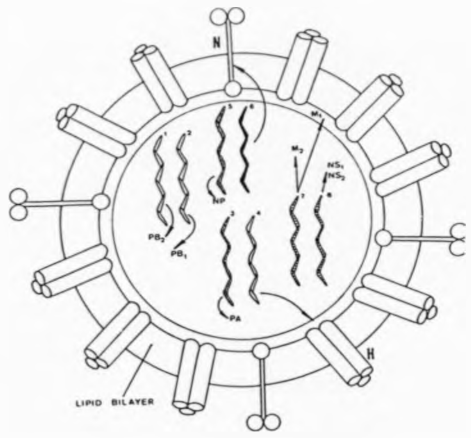

Fig. 1.2.1 Structure of the influenza virion.

the most common being nearly spherical particles of about 100 nm in diameter, but elongated and filamentous particles of the same diameter are known.

The surface glycoprotein spikes are made up of clusters of protein chains and are divided into two types according to their biological properties. The haemagglutinin (HA) proteins appear as three-sided rods whereas the neuraminidases (NA) are mushroom shaped with a head of four spheres (Varghese, *et al.*, 1983).

Inside the virion shell, the genetic material is comprised of eight different molecules of negative strand RNA. This segmented genome must be transcribed to the complementary mRNAs, a process catalysed by an enzyme made up of the four proteins carried within the virion particle. These are the three P proteins (PB_1, PB_2 and PA) and the nucleoprotein (NP), which is closely associated with the genome RNA segments in the virion.

1.2.2 Influenza Virus Replication

1.2.2.1 Initial Stages of Infection

The infectious cycle of influenza viruses is variable and dependent on virus strain and host cell. The latent period is usually from three to six hours and peak infectious virus production can be attained after only eight hours and may continue for days.

The initial interaction between virus and host cell takes place by attachment of haemagglutinin to the cell receptors containing neuraminic acid (Nayak, 1977). Neuraminidase has little effect on this stage since it is only present in one tenth of the amount of haemagglutinin (Kilbourne, *et al.*, 1968). When host cell receptors are in excess, 70-90% of virus becomes cell associated within two hours (White, 1960).

Penetration and uncoating of the virions follow adsorption, although none of these processes are fully understood. Penetration may be by viropexis or by membrane fusion and uncoating could be by release of ribonucleoprotein, activation of transcriptase or a combina-

tion of both. Host nuclei play a significant role at an early stage and DNA-dependent RNA transcription is necessary to produce capped primers for mRNA synthesis.

1.2.2.2 RNA Synthesis

Virus-coded RNA synthesis can be detected twenty minutes after infection and takes place in two phases. Primary transcription is the formation of the mRNAs and secondary transcription, the production of progeny RNA molecules.

Transcription of virus mRNA is begun by cleavage at a purine residue of a capped oligonucleotide of 10-13 bases from cellular mRNA. This oligonucleotide serves as the primer for the initiation of mRNA synthesis by directing the incorporation of a GMP residue complementary to the second base at the 3' end of each viral template (Lamb and Choppin, 1983), followed by elongation according to the template sequence (Fig. 1.2.2.2(i)). All of these processes are carried out by the viral RNA transcriptase complex, a group of proteins of total molecular weight 255 000 (Winter and Fields, 1982) consisting of the three P proteins, PB_1 (the larger basic P protein), PB_2 (the smaller basic P protein) and PA (the acidic P protein), along with the nucleoprotein (NP). The contribution of each of these proteins to the synthesis of the virally-coded mRNA has now been extensively studied.

It has been established that the PB_2 protein recognises the 5'terminal methylated cap structure of the cellular mRNA primer, binds to it, and may be

CLEAVAGE

endonucleolytic cleavage

nucleotides

INITIATION

ELONGATION

Fig. 1.2.2.2(i) Priming and synthesis of influenza viral mRNA (Krug, 1983).

responsible for its cleavage (Krug, 1983). PB_1 appears to initiate transcription (Ulmanen, *et al.*, 1981) and so it was inferred that PA could be involved in the elongation phase of transcription (Lamb and Choppin, 1983). However, it has been demonstrated more recently that PB_1 is most likely to catalyse the addition of each nucleotide to the growing mRNA chain, since it leads the way as the transcriptase complex moves down the nascent chains (Braam, *et al.*, 1983). It was also suggested that PB_2 becomes

dissociated from the primer cap soon after 11-15 nucleotides have been added to the capped fragments, thus enabling all the P proteins to move as a unit (Fig. 1.2.2.2(ii)). Specific roles for PA and NP have not been found and it is possible that their role is structural, allowing such a switch in the protein conformation to occur.

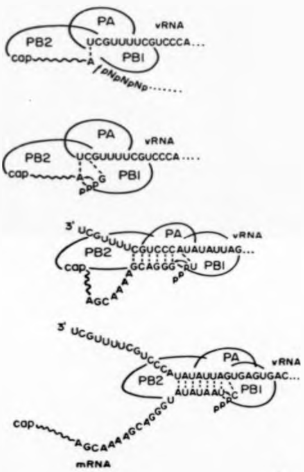

Fig. 1.2.2.2(ii) Movement and interaction of the three P proteins during viral mRNA synthesis (Braam, *et al.*, 1983).

The viral mRNAs have been shown to lack the sequence complementary to the 5'terminal 20-30 nucleotides of the vRNAs (Hay, *et al.*, 1977). Instead there are sequences of poly(A). This is assumed to be due to repetitive copying of a short tract of U residues followed by termination of transcription (Krug, 1983). This effect may be caused by formation of a duplex "panhandle" structure extending up to the U-tract, thus blocking transcription of the last 20-30 5'terminal nucleotides and causing the observed stuttering (Robertson, *et al.*, 1981).

In contrast to virally coded mRNAs, little information has been obtained concerning the synthesis of progeny vRNA. Full length complementary copies of the vRNA segments have been isolated, showing that the termination mechanism described above does not operate in the case of secondary transcription (Hay, *et al.*, 1977). Production of the complementary transcripts is thought to take place without a primer as they have a pppA 5'terminus and their 5'terminal sequences are complementary to the 3'terminal sequences of the vRNA templates (Hay, *et al.*, 1982). These transcripts are thought to function as templates for synthesis of new vRNA (Hay, *et al.*, 1977) and production of both species is probably catalysed by the P proteins. Since capped mRNA primers are not involved, it is more likely that a modified PB_1 protein participates in the initiation of synthesis of full length transcripts rather than PB_2 (Krug, 1983). However, the mechanism of such a modification or of the switch from mRNA to full

length vRNA synthesis are not understood.

1.2.2.3 Organisation of the vRNA Genome and Protein Function

The virion RNA can be separated by polyacrylamide gel electrophoresis into eight segments in the range 890-2341 nucleotides in length (McGeoch, *et al.*, 1976). Only two of these segments code for more than one protein. Segments 1, 2 and 3 code for the P proteins PB_2, PB_1 and PA respectively (Palese, *et al.*, 1977), the functions of which were discussed above.

Segment 4 of the genome codes for the virus haemagglutinin (HA). HA can be cleaved proteolytically into HA_1 and HA_2 (usually held together by disulphide bonds) which greatly increases the infectivity of the virus since HA is involved with virus attachment to cell receptors (Lazarowitz and Choppin, 1975).

Processing of segment 5 results in the nucleocapsid protein (NP) which in combination with the RNA segments, forms the viral ribonucleoprotein (RNP) particles found in virions (Compans, *et al.*, 1972). It is estimated that 20 nucleotides interact with a single protein subunit (Lamb, 1983) and it is supposed that the RNA is bound to the outside of the RNP structure as it is susceptible to ribonuclease digestien (Murti, *et al.*, 1980). It is the antigenicity of NP which is used to distinguish the three influenza virus types A, B and C.

RNA segment 6 codes for the glycoprotein, neuraminidase (NA), which can alter the host range of the virus by removing sialic acid and exposing HA to

cleavage (Schulman and Palese, 1977).

Segments 7 and 8 code for the pairs of proteins M_1 and M_2, and NS_1 and NS_2 respectively using overlapping sequences. M_1, the matrix protein, is the most abundant protein in virions and may help to provide a binding site for RNP during assembly (Choppin, *et al.*, 1972). The remaining three proteins are only found in infected cells and not in the virion, and their functions are unknown, although NS_1 has been implicated in shut off of host cell protein synthesis (Lamb, 1983).

1.2.2.4 Assembly and Release of Virions

The process by which virions are assembled is not well understood. It is known that the process takes place only at the cell membrane by a budding mechanism. The cell membrane must be extensively modified by replacement of host proteins with viral antigens as viral envelopes contain only virally coded proteins (Nayak, 1977). The mechanism of RNP molecule selection for packaging remains unclear, but could take place either by selective processing by linkage via RNA or protein linkers or by random selection, which would account for the appearance of hybrid viruses. How the RNP molecules are attracted to the budding site is unknown.

The final phase of virion release appears to be caused by removal of sialic acid from the budding region by viral neuraminidase (Klenk, 1974). This action would result in there being no site of binding for the haemagglutinin and dissemination from the host cell

would ensue, allowing the new virion to move on to infect other hosts, continuing the infectious cycle.

1.2.3 Variation in Antigenic Proteins

One of the most striking features of the influenza virus is its ability to change its antigenic identity. Thus, specific immunity built up against one particular strain may not protect against new viruses that arise, which may result in epidemic infections (Wilson, *et al.*, 1982). The antigenic change is a result of changes in the two surface proteins which in turn reflect changes in the parent genes.

There are two mechanisms by which these different strains can arise. Antigenic drift involves a series of minor point mutations, which leads to amino acid sequence changes, altering the antigenic sites so that they are no longer recognised by the host's immune system. Antigenic shift results from more drastic changes in which the gene segments for one or both of the surface proteins are shuffled between two viruses by reassortment. There is evidence for such a process occurring between influenza A viruses of man and animals (Webster, *et al.*, 1971) or between two human strains (Bean, *et al.*, 1980) or two animal strains (Gardner and Shortridge, 1979).

Four major antigenic shifts have occurred since the virus was first isolated in 1933, each coinciding with new influenza epidemics. In 1957, the H2N2 subtype (Asian 'flu') replaced the H1N1 subtype. In 1968 the H2N2 (Hong Kong) virus appeared and in

1977 the H1N1 subtype made a reappearance. It is not clear how these different subtypes arose in each case, and most surprising was the re-emergence of the H1N1 strain, but it is probable that genetic reassortment is occurring constantly, resulting in these virulent new strains every 8-15 years (Bean, *et al.*, 1980).

1.2.4 Influenza Vaccines

Due to the frequent changes in the influenza virus antigens, no single vaccine can hope to control the disease in the way that smallpox, polio, or other stable viruses have been controlled in recent years. Influenza vaccines have been prepared though, for the protection of those sections of the populations thought to be most at risk, and two approaches have been used. The first is use of inactivated influenza and two types of formalin-killed vaccines are in general use - intact virus and ether-treated virus vaccines. The protective efficacy is clearly related to the antigenic match of the vaccine with the epidemic virus and doubts have been expressed as to the long-term cummulative efficacy of these types of vaccine (Hoskins, *et al.*, 1979).

Vaccines have also been prepared using live, attenuated virus by various means, but again it has been shown that if there has been only a two-step antigenic drift between virus and vaccine, poor protection from infection is obtained (Wang, 1983). Thus, new vaccines must be constantly prepared to keep up with changes in the virus.

1.3 CHEMOTHERAPY OF INFLUENZA INFECTIONS

Even if the present problems formulating an influenza vaccine can be overcome, vaccination is a primary preventative measure, and gives little hope of treating the disease once contracted. Ideally a range of antiviral compounds would be available which could be used to treat patients at risk from complications to infections. Several compounds are known to show activity against influenza, and some of these are discussed below.

1.3.1 Adamantane Derivatives

Amantadine hydrochloride (1-aminoadamantane hydrochloride, Fig. 1.3.1) has been licensed in the U.S.A.

Fig. 1.3.1 Structures of adamantane derivatives with antiviral activity. (i) Amantadine, (ii) Rimantadine.

for prophylactic treatment of respiratory illnesses due to the A2 strains of influenza virus (Asian 'flu'), for twenty years. However, the precise mode of action for its antiviral effect is still not clearly established. Initial studies suggested that the drug inhibited virus penetration (Davies, *et al.*, 1964) but subsequent research has indicated that it may be some part of the uncoating phase which is affected (Kato and Eggers, 1969). Either way in the presence of amantadine, expression of the virus genome is prevented and no transcription can be detected (Tilley and Kramer, 1981).

The drug has been used sparingly in clinical situations despite it being one of the few available, which may be due to a combination of factors. Firstly, amantadine is not effective against respiratory tract illnesses other than influenza A, and accurate diagnosis is not always possible. Secondly, the drug can cause side effects such as insomnia, nervousness and halucinations, all symptoms of a secondary effect on the central nervous system. It is interesting then, that amantadine and some other close analogues have been used for treatment of Parkinson's disease (Tilley and Kramer, 1981).

Rimantadine (1-(1-aminoethyl)adamantane, Fig. 1.3.1) has also been shown to possess antiviral activity against influenza A and a number of other viruses (Tsunoda, *et al.*, 1966). Its mechanism of action is similar to that of its congener but it is reportedly more effective (Schulman, 1968) and with less drastic side effects (Oxford, 1984). This has led to it being more widely used than amantadine, mainly in the U.S.S.R.

(Zlydnikov, *et al.*, 1981).

When given prophylactically, amantadine and rimantadine are 50% effective in the prevention of infection and virtually 100% effective in prevention of clinical illness. When administered therapeutically within the first 48 hours of illness, the duration of fever can be reduced by one third (Dolin, *et al.*, 1982). Use of these adamantane derivatives can result in the appearance of resistant mutants, and in an attempt to overcome this, simultaneous administration of amantadine and an inactivated vaccine has been used, a combination which has proved an effective prophylactic (Webster, *et al.*, 1985).

Another approach has been to use rimantadine in combination with interferons. This has the twin advantages of overriding the low efficacy and toxic side effects of interferon, and enhancing the antiviral effects of rimantadine (Hayden, *et al.*, 1984).

1.3.2 Norakin

Having noted that the anti-influenzal adamantane derivatives show activity against Parkinson's disease, the reverse approach was tried, and several anticholinergic, antiparkinsonism drugs were tested as inhibitors of influenza in cell culture (Pebster, *et al.*, 1984). Norakin (Fig. 1.3.2) and some close analogues were shown to inhibit influenza A and B viruses. Further studies suggested that these compounds act by preventing a conformational change in the haemagglutinin at acid pH essential

Fig. 1.3.2 Structure of Norakin.

for cell-virus fusion, although other effects at later stages of the virus replicative cycle were not ruled out (Ghendon, *et al.*, 1986).

1.3.3 Ribavirin and Analogues

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Fig. 1.3.3) has been shown to exhibit

(i) (ii)

Fig. 1.3.3 Structures of (i) Ribavirin and (ii) Selenazofurin.

activity against both DNA and RNA viruses (Smith and Kirkpatrick, 1980). Ribavirin 5'-triphosphate is a specific inhibitor of the influenza virus RNA polymerase in a cell free system (Eriksson, *et al.*, 1977). Under the same conditions neither ribavirin nor its 5'-monophosphate showed any activity. Thus, it is probable that the observed activity of ribavirin *in vivo* results from its phosphorylation by cellular enzymes, and subsequent incorporation into viral RNA.

In animals, ribavirin provides reduced mortality from influenza, even if administered as late as 72 hours post-infection (Chen, *et al.*, 1983) and in clinical trials ribavirin shows promise against influenza A and B infections, giving reduction in the duration of fever (Chen, *et al.*, 1983).

Ribavirin analogues with antiviral activity have been described, in which other heteroatoms are introduced into the heterocyclic ring portion of the molecule (Robins, *et al.*, 1983). Selenazafurin (Fig. 1.3.3) has been reported as having greater activity against influenza A and B viruses than ribavirin or amantadine (Sidwell, *et al.*, 1985).

1.3.4 Tilorone

Tilorone hydrochloride (Fig. 1.3.4) is a broad spectrum, orally active antiviral agent when administered prophylactically (Kreuger and Meyer, 1970). In clinical trials against influenza A (H1N1 strain) it has yielded favourable results and no obvious side effects were seen

Fig. 1.3.4 Structure of Tilorone.

(Chen, *et al.*, 1983). The mode of action of tilorone has been studied, and it is claimed that it exerts its broad spectrum activity by inducing an interferon as the active antiviral species (Meyer and Kreuger, 1970).

1.3.5 Metal Chelators

Several metal chelating compounds are known to possess antiviral activity against influenza *in vitro* (Hutchinson, 1985). Ethylenediamine tetraacetic acid (EDTA, Fig. 1.3.5) inhibits the neuraminidase activity of the N1 serotype influenza viruses, probably by forming strong complexes with calcium ions, which the enzyme requires to function normally (Dimmock, 1971).

1,10-Phenanthroline and bathocuprin sulphonic acid (Fig. 1.3.5) inhibit the RNA transcriptase activity of influenza A and B viruses by forming strong complexes with an essential zinc ion (Oxford and Perrin, 1974). However, none of these metal complexing species show

Fig. 1.3.5 Structures of metal chelating antivirals. (i) EDTA, (ii) 1,10-Phenanthroline, (iii) Bathocuprin disulphonic acid.

activity in animal models (Oxford and Perrin, 1977) probably due to their highly polar nature preventing them from entering cells.

1.3.6 Pyrophosphate Analogues

Phosphonoacetic acid (PAA, Fig. 1.3.6), originally synthesised by Nylen in 1924, was the first pyrophosphate analogue discovered to possess antiviral potential. Its activity was revealed by random screening in herpes simplex virus infected cells (Shipkowitz, *et al.*, 1973). PAA was subsequently shown to inhibit nearly all the viruses of the herpes family both in cell culture and in animals (Meyer,

$$(HO)_2\overset{O}{\overset{\|}{P}}CH_2CO_2H$$
(i)

$$(HO)_2\overset{O}{\overset{\|}{P}}CO_2H$$
(ii)

$$(HO)_2\overset{O}{\overset{\|}{P}}-\overset{O}{\overset{\|}{C}}-\overset{O}{\overset{\|}{P}}(OH)_2$$
(iii)

Fig. 1.3.6 Pyrophosphate analogues (i) PAA, (ii) PFA, (iii) Carbonylbisphosphonate.

et al., 1976, Shipkowitz, *et al.*, 1973).

Further *in vivo* studies demonstrated that the normal cell synthetic machinery of uninfected cells was unaffected by PAA whereas synthesis of virus-specific DNA was totally inhibited (Overby, *et al.*, 1974). It was soon shown that PAA owes its activity to inhibition of the HSV-induced DNA polymerase (Mao, *et al.*, 1975).

Despite PAA being a small molecule with little scope for optimisation of activity, a number of analogues have been prepared which demonstrate the features necessary for antiviral action.

In general, if phosphonate or carboxylate esters are introduced, activity against HSV-1 is greatly reduced (Eriksson, *et al.*, 1980) although a recent report claims that such low molecular weight esters are active against HSV-2 in tissue culture (Mao, *et al.*, 1985).

Insertion of one or more methylene groups between the phosphoryl and carboxyl moieties results in

loss of activity. The homologue without a methylene group, phosphonoformic acid (PFA, Fig. 1.3.6), is however, a slightly better inhibitor of DNA polymerases than PAA (Datema, *et al.*, 1986).

Replacement of either functional group of PAA with sulphonyl groups or replacement of the phosphoryl group with a second carboxyl also results in compounds with no antiviral activity (Leinbach, *et al.*, 1976). Replacement of the carboxyl group with a second phosphono group produces methylenebisphosphonate which also lacks significant activity(Eriksson, *et al.*, 1980). However, substitution at the central carbon atom by various electron withdrawing groups, particularly halogens, results in compounds which have no activity against herpes viruses, but which are good inhibitors of influenza A RNA polymerase (Cload and Hutchinson, 1983a).

The only analogues found to inhibit both herpes and influenza viruses are PAA, PFA and carbonyl bisphosphonate (Fig. 1.3.6). These three compounds have also been shown to be inhibitors of the replication of a number of other viruses including most recently, hepatitis B (Oberg, 1983), HTLV-III (Vrang and Oberg, 1986) - the retrovirus responsible for the initial AIDS infection - and avian myoloblastosis virus (AMV) (Eriksson, *et al.*, 1982). In every case PFA is the most active compound.

Structure-activity relationships for pyrophosphate analogues against influenza A virus showed similar requirements as were found for herpes viruses in that esters were not active (Cload, 1983). It has been shown

that halogenated methylene bisphosphonates are excellent isosteres of inorganic pyrophosphate, their acid properties being virtually identical (Blackburn and Kent, 1981), and such compounds have proven to be the best inhibitors of influenza viruses (Cload, 1983). Inorganic pyrophosphate itself is an inhibitor of influenza and superior inhibitors are obtained by replacement of the phosphate groups by thiophosphate, a two-fold increase in activity being observed for each such replacement, both in enzyme assays and cell culture models (Hutchinson, *et al.*, 1985).

Different influenza strains have been shown to exhibit different sensitivities to pyrophosphate analogues in cell culture (Hutchinson and Naylor, 1985). A similar type of strain sensitivity to adamantane derivatives has been assigned to differences in the M2 membrane protein (Hay, *et al.*, 1985) and the observed variation in sensitivity to pyrophosphate analogues may be a result of differences in the proteins making up the transcriptase complex (Naylor, 1985).

1.4 MODE OF ACTION OF PYROPHOSPHATE ANALOGUES AGAINST INFLUENZA

The mode of action of the inhibition of influenza viruses by pyrophosphate analogues has been investigated quite extensively, and it now seems clear that they exert their activity by interacting directly on the RNA transcriptase rather than first being metabolised to some more active form. For example, it

had been suggested that in their inhibition of herpes virus DNA polymerases, pyrophosphate analogues act as substrates for the reverse reaction of polymerisation, and that the resultant triphosphate analogues are the active species, acting as competitive inhibitors for the natural nucleoside triphosphate substrates in the polymerisation reaction (Leinbach, *et al.*, 1976). However, no incorporation of radiolabelled PAA into nucleoside phosphates was observed in DNA or RNA polymerase reactions and such nucleoside triphosphate analogues are neither inhibitors nor substrates for the polymerases of herpes or influenza (Cload, 1983).

In multiple inhibition analyses, pyrophosphate and PAA were found to be mutually exclusive inhibitors, showing that they both bind to the same site in the polymerase (Leinbach, *et al.*, 1976), partially explaining why isosteres of inorganic pyrophosphate make good inhibitors.

It has been shown that influenza viruses require zinc (Oxford and Perrin, 1977) and studies of a range of pyrophosphate analogues which inhibit influenza RNA transcriptase, have shown a good correlation between the inhibitory power of each analogue and its ability to chelate zinc ions under physiological conditions (Cload and Hutchinson, 1983a). The superior inhibition of influenza A by thiopyrophosphate analogues (Hutchinson, *et al.*, 1985) has been proposed to be due to the greater affinity of sulphur for zinc under the Pearson rules for hard and soft acids and bases, resulting in thiopyrophosphate analogues forming stronger zinc complexes than their oxygenated counterparts (Hutchinson, *et al.*, 1985).

Studies on the mode of action of PFA have revealed that in the presence of this inhibitor, initiation of viral mRNA synthesis takes place as normal, but that elongation beyond 12 or 13 bases is prevented (Stridh and Datema, 1984). This point of inhibition appears to coincide with the time that the RNA transcriptase complex becomes dissociated from the primer cap, and begins to move down the nascent RNA chains (Section 1.2.2.2), and so it is possible that a structural change in the proteins at this stage allows interaction of pyrophosphate analogues with the complex through an essential zinc ion, thus blocking further transcription.

1.5 CLINICAL USE OF PYROPHOSPHATE ANALOGUES

Phosphonoacetic acid, when used in animal models shows pronounced dermal toxicity (Boezi, 1979). PFA is less toxic, and is currently undergoing clinical trials after showing promise against cutaneous herpes infections when applied as a 3% (w/w) cream (Oberg, 1983).

One of the problems which must be overcome before pyrophosphate analogues can be used generally in chemotherapy is their great affinity for calcified tissue. Although undesirable for an antiviral, this property has proved useful in other medical fields.

The pyrophosphate analogues ethylidenehydroxy-diphosphonate (EHDP) and dichloromethylenediphosphonate (Cl_2MDP, Fig. 1.5) inhibit resorption of bone and the former also inhibits mineralisation of soft tissue.

$(HO)_2P(O)-C(CH_3)(OH)-P(O)(OH)_2$

(i)

$(HO)_2P(O)-CCl_2-P(O)(OH)_2$

(ii)

Fig. 1.5 Pyrophosphate analogues. (i) EHDP, (ii) Cl_2MDP.

Hence the two compounds can be used to treat abnormalities in bone turnover such as Paget's disease (Francis and Centner, 1978).

Aqueous complexes of these species can be tagged with technetium-99 and used as skeletal imaging agents, demonstrating further their tendancy to accumulate in bones and teeth (Fleisch and Felix, 1979). The main difficulty as far as antiviral usage is concerned is that little is known about the long term effects of these compounds on calcified tissue (Francis and Centner, 1978).

A second major drawback with the systemic use of pyrophosphate analogues is their reluctance to enter cells, as their highly polar nature prevents them from crossing lipid membranes (Cload, 1983).

A solution to both of these problems would be to use pyrophosphate analogues which incorporate a bulky lipophilic group, and the halogen necessary to give the physical properties required to make it a good inhibitor. Such a compound would then be expected to inhibit replication of influenza virus both in enzyme assays, and

with improved efficacy in cell culture. The bulkyness of the lipophilic group may also render the compound incompatible with the hydroxyapatite matrix of calcified tissue for stereochemical reasons, reducing the tendency to accumulate in bones and teeth.

1.6 OUTLINE OF WORK UNDERTAKEN

The aim of the present work was to prepare some lipophilic pyrophosphate analogues, which for the reasons described above might be expected to be improved inhibitors of influenza virus replication in cells.

This required a thorough investigation of the synthetic methods available, preparation of monohalogenated methylenebisphosphonates and their subsequent alkylation *via* metallated intermediates. The compounds would then be assessed as inhibitors in enzyme assay and cell culture systems. It was hoped that the lipophilic analogues would show greater virus inhibition but with less cytotoxicity than has been observed for other halogenated analogues (Cload, 1983) in cell culture.

It was also proposed to use the methodology developed, to prepare an affinity label to identify which of the viral proteins the analogues interact most closely with in exerting their observed antiviral effect.

CHAPTER 2

SYNTHESIS OF HALOGENATED METHYLENEBISPHOSPHONATES

2.1 EARLY WORK

The first reported preparation of a halogenated derivative of a methylenebisphosphonate ester, was the formation of tetraethyl dichloromethylenebisphosphonate as a byproduct of the reaction between bromotrichloromethane and triethylphosphite (Bunyan and Cadogan, 1962). It was proposed that the reaction proceeded *via* a radical chain transfer mechanism (the major product being diethyltrichloromethylphosphonate, Fig. 2.1(i)). If chloroform was used in place of bromotrichloromethane, small amounts of tetraethylmethylenebisphosphonate were isolable (Burn, *et al.*, 1964) with tetraethylchloromethylenebisphosphonate postulated as an intermediate. This method proved difficult to adjust to give high yields of the halogenomethylenebisphosphonate esters (Nicholson and Vaughn, 1971), so other routes have been sought to these halogenated species.

Preparations of methylenebisphosphonate esters have been reported *via* an Arbusov reaction (Fig. 2.1(ii)), with near quantitative yields being claimed for the tetraisopropyl ester (Proctor and Gamble Co., 1966) and more modest yields for other esters (Schwarzenbach and Zurc, 1950). Halogenation of these esters has proven to be the most versatile and widely used route to tetra-

$$2P(OEt)_3 + 2BrCCl_3 \longrightarrow CCl_2[P(O)(OEt)_2]_2 + 2EtBr + CCl_4$$

Fig. 2.1(i) Formation of tetraethyldichloromethylenebisphosphonate (Bunyan and Cadogan, 1962).

$$XCH_2X + P(OR)_3 \longrightarrow (RO)_3\overset{+}{P}{-}CH_2X \; X^- \longrightarrow (RO)_2\overset{O}{\overset{\|}{P}}CH_2X + RX$$

$$(RO)_2\overset{O}{\overset{\|}{P}}CH_2X + P(OR)_3 \longrightarrow (RO)_2\overset{O}{\overset{\|}{P}}CH_2{-}\overset{+}{P}(OR)_3 \; X^- \longrightarrow \text{Prod}$$

Fig. 2.1(ii) Preparation of methylenebisphosphonate by an Arbusov reaction.

alkyl halogenomethylenebisphosphonates. Fluorination to the mono- and difluoro-derivatives is possible with perchlorylfluoride (McKenna and Shen, 1981). A less hazardous route to the difluoro compound though, involves preparation first of dialkyl bromodifluoromethylphosphonate (Burton and Flynn, 1977) followed by a Michaelis-Becker reaction with a dialkyl phosphite to yield the difluorinated bisphosphonate (Blackburn, *et al.*, 1981, Burton, *et al.*, 1982, Fig. 2.1(iii)). However, several methods are known for the preparation of other halogenated derivatives which do proceed *via* tetraalkylmethylenebisphosphonates.

Excellent yields have been claimed for preparation of a variety of dihalogenated methylenebisphosphonates by reaction of the parent compound with alkaline hypohalite (Quimby, *et al.*, 1968), using an excess of either commercially available sodium hypochlorite for chlorination, or the appropriate hypohalite prepared *in situ* for bromination or iodination (Fig. 2.1(iv)). It has been demonstrated (Curry, 1971) that whilst dihalogenation of tetraisopropyl methylenebisphosphonate proceeds smoothly, the analogous ethyl and methyl esters must be well buffered to prevent base-induced cleavage of the P-C bond. Indeed it has been found that for the ethyl ester, the cleavage reaction predominates in the absence of carbonate as a buffer, the product being diethyldichloromethylphosphonate (Seyferth and Marmor, 1973). In this study it was observed that even with buffered mixtures, up to 15% (by ^{31}P n.m.r.) of the products can result from this side reaction. Despite this, good yields of all esters of dichloro- and

$$(RO)_3P + CF_2Br_2 \xrightarrow{\text{Arbusov}} (RO)_2\overset{O}{\overset{\|}{P}}CF_2Br$$

$$\xrightarrow[\text{Michaelis-Becker}]{Na^{+}\ ^{-}\overset{O}{\overset{\|}{P}}(OR)_2} (RO)_2\overset{O}{\overset{\|}{P}}CF_2\overset{O}{\overset{\|}{P}}(OR)_2$$

Fig. 2.1(iii) Two-step preparation of tetraalkyl difluoromethylenebisphosphonate.

$$(RO)_2\overset{O}{\overset{\|}{P}}CH_2\overset{O}{\overset{\|}{P}}(OR)_2 \xrightarrow[NaCO_3]{NaOX} (RO)_2\overset{O}{\overset{\|}{P}}CX_2\overset{O}{\overset{\|}{P}}(OR)_2$$

X = Cl, Br

Fig. 2.1(iv) Reaction of tetraalkyl methylenebisphosphonate with sodium hypohalite.

dibromo methylenebisphosphonates can be prepared in this way.

2.1.1 Preparation of Monohalogenated Species

The method discussed above is not however, readily adapted for the preparation of the analogous monohalo derivatives. It has been shown that using a single equivalent of hypohalite, prepared *in situ*, mixtures of dihalo-, monohalo- and unhalogenated products were formed (Quimby, *et al.*, 1968). Adjustment of the solvent system allowed a maximum yield of 60% (by ^{31}P n.m.r.) tetraisopropyl monobromomethylenebisphosphonate to be prepared, which was not readily separable from the other species present in the mixture.

Direct reaction of one equivalent of molecular halogen with the sodium salt of methylenebisphosphonate esters also does not simply yield the monohalo derivative, but rather mixtures similar to those described above. The monohalo species in this case was never observed as constituting more than 50% of the product mixture (Quimby, *et al.*, 1968).

Hata has prepared monohalogenated derivatives of active methylene compounds (e.g., monobromomalonitrile) by extended heating of the corresponding dihalogenated and unhalogenated species (Hata, 1964), but this method has been shown to be unsuccessful in the case of methylenebisphosphonates (Nicholson and Vaughn, 1971). In the same paper it was noted that the most appropriate method for the preparation of the monohalo derivative, given the difficulties

inherent in other methods, would be to selectively remove a single halogen from the bridge carbon of the more accessible tetraalkyl dihalomethylenebisphosphonates. It was reported that this could be achieved by reacting the dihalo compound with one equivalent of sodium hydrosulphide (Nicholson and Vaughn, 1971). The mechanism for the reaction was unclear but was thought to involve both nucleophilic displacement and reduction. The method proved unreliable in this study, which led to the investigation of other methods by which dihalo species could be dehalogenated, and the mechanism involved in the process.

2.2 RESULTS AND DISCUSSION

2.2.1 Nucleophilic Displacements of Halogen from Dihalogenomethylenebisphosphonates

In the present study, it was noticed that reaction of tetraisopropyldichloromethylenebisphosphonate with an excess of potassium fluoride in acetonitrile, with [18,6]crown ether as catalyst, resulted in complete dehalogenation to yield primarily (> 60% by ^{31}P n.m.r.) the corresponding ester of methylenebisphosphonate. It was thought that the mechanism may be similar to the aforementioned speculative mechanism of Nicholson, and if so the method may be adaptable to give good yields of the monochlorinated analogue.

It was found that reaction of a single equivalent of potassium fluoride under the same conditions yielded the desired tetraisopropyl chloromethylene bisphosphonate as 70% of the phosphorus-containing material (55% isolated

yield) after 7 days at elevated temperature. No fluorine-containing products could be detected at any stage either by ^{19}F n.m.r. or by ^{31}P-^{19}F coupling in the ^{31}P n.m.r. spectrum, showing that no nucleophilic displacement had occurred at the central carbon, nor had any P-F bonds formed by direct attack at phosphorus. A byproduct of the reaction (^{31}P n.m.r., δ 8.5 p.p.m.) could be diisopropylchloromethyl phosphonate resulting from P-C bond cleavage (Fig. 2.2(i)). This type of cleavage was subsequently observed, also as a side reaction, when the anions $[(RO)_2P(O)]_2CCl^-$ (R = Me, Et) were formed using a strong base. Hence it was thought that the reaction could proceed *via* a carbanion intermediate, produced by positive halogen abstraction from the bridge carbon by fluoride, which then becomes reprotonated under the reaction conditions (Fig. 2.2.1(i)). Positive halogen

Fig. 2.2.1(i) Mechanism of the reaction between tetraisopropyldichloromethylenebisphosphonate and naked fluoride ion.

abstraction has been reported previously as a mechanism in reactions of halogenated phosphonates (Burton and Flynn, 1980) and stable carbanions of the type proposed have been observed both by others (Seyferth and Marmor, 1973, Quimby, *et al.*, 1968) and in this study.

Addition of one equivalent of cyclohexene, which might be expected to react with any chlorine monofluoride produced to accelerate the reaction, increased the reaction rate so that no starting material was present in the mixture after 2 days, but resulted in an increased proportion of side products.

The same reaction using a sodium fluoride/[18,6]crown system produced no chloromethylenebisphosphonate esters. This is because the sodium ion does not make such good complexes with [18,6]crown ethers as potassium ion due to the cavity size of the ether. This is estimated at 2.6-3.2 Å (Pederson and Frensdorff, 1972) and as such is more suitable for potassium ion (ionic diameter, 2.66 Å) than sodium ion (ionic diameter, 1.90, Whaley, 1973). Thus, "naked" fluoride ion is not available using sodium fluoride and so the displacement does not occur.

When potassium fluoride/[18,6]crown ether was reacted with tetraisopropyl dibromomethylenebisphosphonate under the conditions described, positive halogen abstraction was again observed and tetraisopropylbromomethylenebisphosphonate was produced as 70% of the reaction mixture (by ^{31}P n.m.r., 50% isolated).

Fluoride ion as the naked anion in acetonitrile is the smallest and most nucleophilic of the halide ions

(Liottov, *et al.*, 1975). Furthermore, chlorine monofluoride has the highest bond energy of any of the interhalogen compounds containing chlorine (Downs and Adams, 1973a), which may explain why analogous reactions to those described, were not seen when naked chloride, bromide, iodide or cyanide ions were used in place of fluoride ion in the reaction mixture.

Other nucleophiles were shown to react in the same way as fluoride. Hydroxide ion gave tetraisopropyl-chloromethylenebisphosphonate as 40% of the product mixture from the dichloro analogue under similar conditions to those described, but this was also accompanied by partial de-esterification. Hydride ion reacted more slowly than fluoride and hydroxide to yield a mixture of the mono-chloro (30%) and the fully dehalogenated (10%) derivatives after 7 days.

Bulkier nucleophiles, such as 2-mercaptopyridine, triphenyl phosphine and dithionite ion showed no reaction under similar conditions over a period of 3 days.

Thus, it seems that only small, highly nucleophilic species are capable of removing a chlorine from the bridge carbon by positive halogen abstraction due to the highly constrained nature of the reaction site. It appears likely that the reaction described by Nicholson proceeds via a similar route, with sodium ion stabilisation of the intermediate anion, followed by breakdown of the products into sodium chloride and elemental sulphur (Fig. 2.2.1(ii)).

Fig. 2.2.1(ii) Mechanism of the reaction between tetraisopropyl-dichloromethylenebisphosphonate and sodium hydro-sulphide.

2.2.2 Metal-Halogen Exchange

Metal-halogen exchange between organic halides and metal alkyls is well documented (e.g., Wardell, 1982) and the reaction has been shown to be well suited to the dichloromethylenebisphosphonate ethyl ester (Seyferth and Marmor, 1973) and can be adapted to give good yields of the monohalogenated analogue by aqueous quenching of the intermediate anion (Fig. 2.2.2). The reaction is cleaner than any of the nucleophilic displacements described in the previous section, which is due to the greatly increased stability of the intermediate anion when generated in the presence of an available metal counter-ion. This stabilising effect is so great that metal salts of the unhalogenated analogue have been observed as stable species

Fig. 2.2.2 Preparation of tetraalkyl chloromethylenebisphosphonate by metal/halogen exchange.

in solution by ^{31}P and ^{1}H n.m.r. (Quimby, *et al.*, 1968) and air stable compounds prepared and analysed (Baldeschweiler, *et al.*, 1962). In this study such salts of esters of chloromethylenebisphosphonate have been studied by ^{31}P and ^{13}C n.m.r. spectroscopy. No analogous stable anions could be observed in the course of any of the experiments described in the previous section.

The increase in selectivity and speed of this method over the fluoride reaction, means it has proven to be the most efficient preparation of tetraalkylchloromethylenebisphosphonates in this study. The method described by Seyferth (Seyferth and Marmor, 1973) has proved widely applicable in this present work if the

alkyl lithium is chosen so as to match the reactivity of the dichloromethylenebisphosphonate ester. The original reaction of 1 equivalent of n-butyl lithium with the tetraethyl ester, was found to give a quantitative yield of the corresponding anion (Table 2.2.2 for ^{31}P n.m.r.) which when quenched with water and the product purified gave the desired tetraethylchloromethylenebisphosphonate in 75% isolated yield.

Reaction of a single equivalent of n-butyl lithium with the tetraisopropyl ester of dichloromethylenebisphosphonate was found to only provide 80% conversion to the intermediate anion, and an excess of n-butyllithium was required to give quantitative conversion. Reaction of one equivalent of methyl lithium however, with the same dichloro species under identical conditions was observed to give 100% conversion to the anion. Quenching of this intermediate and work up gave tetraisopropylchloromethylenebisphosphonate in 82% isolated yield.

Reaction of n-butyllithium with the tetramethyl ester of dichloromethylenebisphosphonate at $-78^{o}C$ resulted in formation of the corresponding intermediate lithium salt in only 24% yield by ^{31}P n.m.r. This salt was accompanied by several species which can be postulated as being dimethylhalomethylphosphonates due to base induced C-P bond cleavage (^{31}P n.m.r. δ 11.5, 10.9 and 1.3 p.p.m.). However, when the tetramethyl ester of the dichloro compound was reacted under the same conditions with tert-butyl lithium, no cleavage was observed and the only species seen by ^{31}P n.m.r. was the desired lithium salt.

TABLE 2.2.2 ^{31}P N.m.r. Chemical Shifts (δ, p.p.m.) of Esters of Methylene Bisphosphonate and their Halogenated Derivatives

Compound	R = Me	R = Et	R = ^{i}Pr
$[(RO)_2P(O)]_2CH_2$	23.0	19.3	17.4
$[(RO)_2P(O)]_2CHCl$	15.5	13.2	11.5
$[(RO)_2P(O)]_2CCl_2$	10.2	8.3	6.5
$[(RO)_2P(O)]_2CH^-Na^+$	45.5[a]	41.5[a]	40.5[a]
$[(RO)_2P(O)]_2CCl^-Li^+$	36.8	34.7	32.3
$[(RO)_2P(O)]_2CHBr$	-	-	11.5
$[(RO)_2P(O)]_2CBr_2$	-	-	6.4

[a]Quimby, *et al.*, 1968

Quenching and distillation yielded the corresponding monochloro species in 78% isolated yield.

Hence a clean, rapid method for preparation of several esters of chloromethylenebisphosphonate is now available by dichlorination of the parent methylenebisphosphonate ester followed by metal-halogen exchange with a suitable alkyl lithium. The good yields available and the ease of purification of the products make this the route of choice to the monohalogenated compounds.

2.3 MATERIALS AND METHODS

2.3.1 Materials

(i) All compounds were commercially available and were used as received unless otherwise stated (Appendix I).

(ii) All reagents were of analytical grade or were purified prior to use.

2.3.2 General Chemical Procedures

(i) ^{1}H N.m.r. spectra were recorded at 220 MHz using a Perkin-Elmer R34 spectrometer. Chemical shifts are quoted with either tetramethylsilane (TMS) (in $CDCl_3$, d_4-methanol or d_6-benzene) or sodium 3-(trimethylsilyl)-1-propane sulphonate (TSS) (in D_2O) as internal standards. ^{31}P N.m.r. were recorded at either 36.44 MHz using a Bruker WH90 spectrometer or at 162.0 MHz using a Bruker WH400 spectrometer and were proton decoupled. ^{31}P N.m.r. shifts are quoted relative to an external standard (85% H_3PO_4) with downfield shifts positive. ^{13}C N.m.r.

were recorded at 100.62 MHz on a Bruker WH400 instrument with TMS as internal standard.

(ii) Mass spectra were recorded using a Kratos MS80 instrument, and the method of Cload and Hutchinson (1983b) for chemical ionisation spectra (CI), or the method described in Appendix II for fast atom bombardment (FAB) spectra.

(iii) Flash column chromatography was carried out according to the method of Still (1978) using the solvent systems described in the text.

(iv) Elemental analyses were carried out by Elemental Micro-Analysis Ltd., Okehampton, Devon, U.K. or Butterworth Laboratories Ltd., Teddington, Middlesex, U.K.

2.4 EXPERIMENTAL

2.4.1 Preparation of Tetraisopropyl Methylenebisphosphonate

Triisopropyl phosphite and dibromomethane were combined in the molar ratio 3:1 and the mixture heated under vigorous reflux for 48 h. Tetraisopropyl methylenebisphosphonate was isolated by vacuum distillation (b.p. 118-120°C @ 0.08 mmHg). Yield 42% [^{31}P n.m.r. ($CDCl_3$) δ 17.4 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 1.35 (24 H, d, J = 7.5 Hz), 2.38 (2H, t, J = 20 Hz), 4.7 (4H, m) p.p.m.; ^{13}C n.m.r. (C_6D_6) δ 23.8, 24.0, 28.5 (t, J = 136.8 Hz), 70.6, 70.8 p.p.m.; Accurate Mass $(M + H)^{+}$ Calc. 345.1596 Found 345.1594]

2.4.2 Preparation of Tetraethyl methylenebisphosphonate

Elemental sodium (10 g, 0.435 mol) was added in small pieces, with stirring and occasional cooling, to diethyl phosphite (125 g, 0.905 mol) at 40°C, under dry nitrogen, in a 500 ml 3-necked flask equipped with a thermometer and reflux condenser, over a period of 2 h. Dichloromethane (25 g, 0.294 mol) was then added and the mixture stirred at 40°C for 3 h. A further portion of CH_2Cl_2 (15 g, 0.177 mol) was then added and stirring continued at 40°C overnight. After cooling to room temperature, toluene/petroleum ether b.p. 60-80°C, 1/1 (v/v) 150 ml) was added and the mixture stirred for a further ½ h. The precipitated sodium chloride was then removed by filtration, washed with petroleum ether b.p. 60-80°C (50 ml) and the combined filtrates dried over $MgSO_4$ and evaporated. The title compound was isolated by vacuum distillation (b.p. 120-122°C @ 0.1 mmHg). Yield = 35.8 g, 57%. [^{31}P n.m.r. ($CDCl_3$) δ 19.3 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 1.35 (12H, t, J = 7 Hz), 2.45 (2H, t, J = 19 Hz), 4.2 (8H, m) p.p.m.; ^{13}C n.m.r. (C_6D_6) δ 16.3, 26.1 (t, J = 135.3 Hz), 62.2 p.p.m.; Accurate mass ($M^{+\cdot}$) calcd. 288.0892, Found 288.0886]

2.4.3 Preparation of Tetraisopropyl dichloromethylenebisphosphonate

Tetraisopropyl dichloromethylenebisphosphonate was prepared by the method of Quimby (Quimby, *et al.*, 1968) in 91% yield after distillation (b.p. 120°C @ 0.05 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 6.4 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$)

δ 1.4 (24H, d, J = 8 Hz) 4.9 (4H, m) p.p.m.].

2.4.4 Preparation of Tetraethyl dichloromethylenebisphosphonate

Tetraethyl dichloromethylenebisphosphonate was prepared as described by Curry (1971). ^{31}P N.m.r. analysis of the residue showed it to be a mixture of the title compound (88%) and $CHCl_2P(O)(OEt)_2$ (12%) (δ 10.6 p.p.m.). Distillation yielded the pure title compound (76%, b.p. 118-120°C @ 0.05 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 8.3 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 1.42 (12H, t, J = 7 Hz) 4.48 (8H, m); Accurate mass calc. (for $^{35}Cl_2$) 356.0112, Found 356.0110].

2.4.5 Preparation of Tetramethyl dichloromethylenebisphosphonate

Tetramethyl dichloromethylenebisphosphonate was prepared as described by Curry (1971) and the pure product isolated by distillation (60%, b.p. 92-94°C @ 0.04 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 10.2 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 4.05 (d, J = 11 Hz) p.p.m.; Accurate mass calcd. (for $^{35}Cl_2$) 299.9486, Found 299.9519].

2.4.6 Preparation of Tetraisopropyl chloromethylenebisphosphonate

Tetraisopropyl chloromethylenebisphosphonate was prepared by the method of Seyferth and Marmor (1973) using a solution of methyl lithium in place of n-butyllithium. The product was isolated pure by distillation (82%, b.p. 110-112°C @ 0.05 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 11.4 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 1.35 (24H, d, J = 8 Hz) 3.9 (1H, t, J = 20 Hz), 4.9 (4H, m) p.p.m.; Analysis found: C, 40.9;

H, 7.35; Cl, 9.7%; calc. for $C_{13}H_{29}ClP_2O_6$ C, 41.2; H, 7.72; Cl, 9.4%].

2.4.7 Preparation of Tetraethyl chloromethylenebisphosphonate

Tetraethyl chloromethylenebisphosphonate was prepared as described by Seyferth and Marmor (1973), the product being isolated by vacuum distillation (75%, b.p. 126-128°C @ 0.38 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 13.2 p.p.m.(s); ^{1}H n.m.r. ($CDCl_3$) δ 1.4 (12H, t, J = 7Hz), 4.05 (1H, t, J = 18.5 Hz), 4.35 (8H, m) p.p.m.; Analysis found: C, 33.4; H, 6.65; Cl, 11.5%; Calc. for $C_9H_{21}ClP_2O_6$: C, 33.5; H, 6.56; Cl, 11.0%].

2.4.8 Preparation of Tetramethyl chloromethylenebisphosphonate

Tetramethyl chloromethylenebisphosphonate was prepared by the method of Seyferth and Marmor (1973), except that a solution of tert-butyllithium was used in place of n-butyllithium. The product was isolated by distillation (78%, b.p. 90-91°C @ 0.05 mmHg). [^{31}P n.m.r. ($CDCl_3$) δ 15.4 p.p.m.(s); ^{1}H n.m.r. δ 3.75 (12H, m), 4.0 (1H, t, J = 18 Hz) p.p.m.; Analysis found: C, 22.36; H, 5.29%; Calc. for $C_5H_{13}ClP_2O_6$; C, 22.53; H, 4.91%].

2.4.9 Preparation of Tetraisopropyl dibromomethylenebisphosphonate

Tetraisopropyl dibromomethylenebisphosphonate was prepared by hypohalogenation of tetraisopropyl methylenebisphosphonate. Tetraisopropyl methylenebisphosphonate (10 g, 0.029 mol) was stirred vigorously in a solution of

K_2CO_3 (55 g) in water (180 ml) at 40°C. Bromine (20 g, 0.125 mol) in heptane (20 ml) was added dropwise over 2 h with stirring. The reaction mixture was cooled to room temperature and extracted with chloroform (2 x 150 ml). The combined extracts were dried over $MgSO_4$, evaporated and the residue distilled *in vacuo* to yield the pure title compound (12.7 g, 87%, b.p. 124-125°C @ 0.08 mmHg) [^{31}P n.m.r. ($CDCl_3$) δ 6.4 p.p.m.(s); ^1H n.m.r. ($CDCl_3$) δ 1.35 (24H, d, J = 8 Hz), 4.8 (4H, m) p.p.m.; CIMS $(M + H)^+$ 505, 503, 501 (1:2:1); Accurate mass $(M + H)^+$, ($^{79}Br_2$), calc.: 500.9805; Found: 500.9785].

2.4.10 Preparation of Tetraisopropyl bromomethylenebisphosphonate by Monohalogenation

Tetraisopropyl methylenebisphosphonate (11.5 g, 0.037 mol) was reacted as described in Section 2.4.9 with one equivalent of bromine (0.85 ml, 0.019 mol) in heptane (4.5 ml). The product was isolated by flash column chromatography eluting with 10/1 (v/v) petroleum ether b.p. 40-60°C/acetone (3.24 g, 30%). [^{31}P n.m.r. ($CDCl_3$) δ 11.5 p.p.m.(s); ^1H n.m.r. ($CDCl_3$) δ 1.35 (24H, d, J = 8 Hz), 3.75 (1H, t, J = 20 Hz), 4.9 4H, m) p.p.m.; Analysis found: C, 36.83; H, 7.22%; calc. for $C_{13}H_{29}BrP_2O_6$: C, 36.9; H, 6.91%].

2.4.11 Preparation of Tetraisopropyl bromomethylenebisphosphonate by Selective Dehalogenation

Tetraisopropyl dibromomethylenebisphosphonate was dehalogenated by metal/halogen exchange as described in Section 2.4.6. The product was isolated by flash

column chromatography and distillation (58%, b.p. 118-120°C @ 0.08 mmHg).

2.4.12 Nucleophilic Displacements from Tetraisopropyl dichloromethylenebisphosphonate

All reactions described in Section 2.2.1 were prepared similarly to the following.

[18,6]Crown ether (0.1 g) was dissolved in acetonitrile (5 ml) with stirring and dry KF (0.17 g, 2.9 mmol) added slowly over 30 min. to ensure complete solvation. Tetraisopropyl dichloromethylenebisphosphonate (1.0 g, 2.4 mmol) was added and the mixture maintained at 55-60°C with stirring. The reaction was followed by extracting small aliquots, quenching them with water and recording the ^{31}P n.m.r. spectrum. This showed that there was no more starting material present after 7 days. The reaction was quenched by cooling to room temperature and addition of water (20 ml). The aqueous layer was extracted with chloroform (2 x 40 ml), the extracts washed with water (3 x 25 ml) dried over $MgSO_4$ and the solvent removed *in vacuo*. The major product was tetraisopropyl chloromethylenebisphosphonate which was isolated by flash column chromatography eluting with petroleum ether b.p. 40-60°C/chloroform 2/1 (v/v) (0.51 g, 55%).

CHAPTER 3

ALKYLATIONS OF METHYLENEBISPHOSPHONATES

3.1 BACKGROUND

Alkylations of tetraalkyl methylenebisphosphonates have been known for some time. Low yields have been reported for the monoalkylation of tetraethyl methylenebisphosphonate *via* both the potassium (Kosolopoff, 1953) and sodium salts (Cotton and Schunn, 1963). In more extensive studies it was shown that alkylations of the tetraisopropyl ester in the same manner gives only moderately improved yields (Hays and Logan, 1966, Quimby, *et al.*, 1968). *In situ* alkylation of the lithium salt of tetraethyl chloromethylenebisphosphonate (generated as described in Section 2.2.2 by metal/halogen exchange between n-butyllithium and tetraethyl dichloromethylenebisphosphonate) has also been reported (Seyferth and Marmor, 1973). In a limited study, good yields were only observed for methylation with significantly reduced yields for alkylation with more complex groups.

In this study these methods have all been assessed with a view to finding the best method available for alkylation, particularly of monohalogenated methylenebisphosphonates.

One of the major problems with the alkylation methods described above is one of selectivity. It was observed that for sodium salts of tetraisopropyl methylenebisphosphonate, monohalogenation did not occur exclusively

(Quimby, *et al.*, 1968). ^{31}P N.m.r. analysis confirmed that mixtures of mono- and dialkylated products were obtained along with unalkylated starting material. The monoalkylated product predominated to a varying degree depending on the compound, but isolated yields suffered for each due to the difficulty of separating these mixtures as a result of the very similar properties of the species. The formation of the dialkyl derivative has been attributed to an exchange reaction between the monoalkylated species and the metal salt (Quimby, *et al.*, 1968).

3.2 RESULTS AND DISCUSSION

3.2.1 Alkylations of Tetraisopropyl chloromethylenebisphosphonate

3.2.1.1 Sodium and Lithium Salt Intermediates

Preparation of the sodium salt of tetraisopropyl chloromethylenebisphosphonate, by treatment of the species with sodium hydride, and alkylating *in situ* with primary alkyl iodides resulted in the yields listed in Table 3.2.1.1.

As can be seen, a good yield of the methylated derivative can be obtained but for alkylation with larger groups, yields deteriorate rapidly with increasing chain length and for reaction of the salt with hexyl iodide, the monoalkylated species is no longer the predominant species.

On alkylation of the lithium salt of tetraisopropyl chloromethylenebisphosphonate good yields were again only observed for reaction with iodomethane. As previously

	Alkylating Agent					
	MeI		EtI		BuI	
	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)
Starting material	2	11.4	15	11.4	19	11.4
Monoalkylated product	89	14.8	44	14.7	39	14.7
Dialkylated product	9	25.6	30	25.3	26	25.3
Others	-	-	11	two peaks	16	three peaks

Table 3.2.1.1 Yields and chemical shifts of the products from the alkylation of tetraisopropyl sodiochloromethylenebisphosphonate.

observed (Seyferth and Marmor, 1973) this method could not readily be used to obtain good yields for reaction with larger primary alkyl iodides. For reactions *via* both the sodium and lithium salts, several side products were observed which can be rationalised in terms of a metal/halogen exchange process (Fig. 3.2.1.1). The monoalkylated product and the unreacted metal salt can undergo exchange to furnish directly the dihalogenated derivative observed in product mixtures. This also results in a new anion which on subsequent reaction yields the dialkylated derivative. Other side products can be seen which are assignable to cleavage processes *via* the intermediate salt. Thus, slower quenching of the intermediate anion by

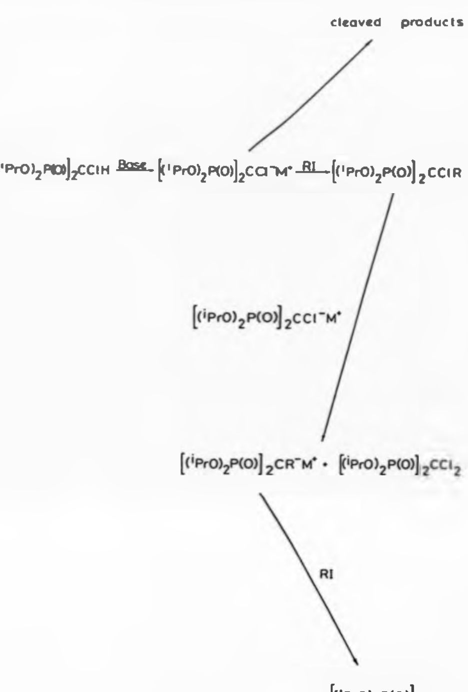

Fig. 3.2.1.1 Metal/halogen exchange between the initial product and intermediate during alkylation of lithio- or sodio chloromethylenebisphosphonate, to give all observed products.

an alkyl iodide, due to its increased bulkyness, can result in all these side reactions becoming more significant.

3.2.1.2 Thallium Salt Intermediates

Similar side reactions to those described above are known to accompany the alkylation of sodium or potassium enolates of β-diketones (i.e., dialkylation, β-diketone cleavage as well as O-alkylation, Claisen condensation, etc.). These competitive reactions have been shown to be avoidable if the crystalline thallium(I) salt of the requisite enolate is prepared and then reacted with an alkyl iodide (Taylor, *et al.*, 1968). Due to the similarity of the β-diketone and methylenebisphosphonate systems, it was thought that the same approach may prove similarly successful for monoalkylation of the latter.

Treatment of tetraisopropylchloromethylenebisphosphonate with one equivalent of thallium(I) ethoxide in tetrahydrofuran at room temperature immediately resulted in formation of a cloudy white solution. Reaction of this thallium(I) salt intermediate with primary alkyl iodides produced excellent yields of the monoalkylated products (Table 3.2.1.2). The thallium intermediate was found to be more versatile than either the sodium or lithium salts in that side product formation was not important, even with alkyl iodides of up to six carbon atoms in length.

Reaction of the thallium(I) salt with secondary alkyl iodides (2-iodopropane and 2-iodobutane) was seen to be far less selective. No reaction was observed using the standard procedure developed, and only under forcing conditions (elevated temperature and prolonged reaction times) could the monoalkylated product be observed by

TABLE 3.2.1.2 Characteristics of alkylated methylenebisphosphonates, $[(RO)_2P(O)]_2CR^1R^2$

Compound		Tetraisopropyl Esters (R = ^iPr)				Tetrasodium Salts (R = Na)			
R^1	R^2	% Alkylation by ^{31}P N.M.R.	% Isolated Yield	^{31}P N.M.R. Shift ($CDCl_3$) δ (p.p.m.)	Parent Ion(s) in CIMS	^{31}P N.M.R. Shift (D_2O) δ (p.p.m.)	Analysis (Found (calcd.)%) C	H	P	^1H N.M.R. in D_2O δ (p.p.m.)
H	H	-	-	17.2	345	18.0	4.73 (4.55)	0.81 (0.76)	23.5 (23.5)	2.40 (2H, t, J = 21 Hz)
H	Me	72	47	22.0	359	23.2	8.67 (8.63)	1.27 (1.44)	20.8 (22.3)	1.44 (3H, t(d), J = 18, 8 Hz), 2.25 (1H, t(q), J = 23, 7.5 Hz)
H	Et	68	45	21.9	373	22.7	12.96 (12.33)	2.74 (2.05)	22.15 (21.23)	1.10 (3H, t, J = 8 Hz), 1.7-2.0 (3H, m)
H	nBu	74	49	22.0	401	22.7	18.64 (18.75)	2.70 (2.50)	19.52 (19.38)	0.90 (3H, t, J = 7 Hz), 1.35 (2H, sextet, J = 7 Hz), 1.55 (2H, quintet, J = 7 Hz) 1.75-2.0 (2H, m), 2.55 (1H, t(t), J = 23, 7.5 Hz)
Cl	H	-	-	11.4	379,381	13.2	3.84 (4.02)	0.76 (0.34)	19.30 (20.77)	3.90 (1H, t, J = 20 Hz)
Cl	Me	98	80	14.8	393,395	17.8	7.75 (7.70)	1.22 (0.96)	20.40 (19.84)	1.85 (3H, t, J = 15 Hz)
Cl	Et	97	81	14.75	407,409	17.2	11.5 (11.03)	2.19 (1.53)	18.07 (18.99)	1.17 (3H, t, J = 7.5 Hz), 2.25 (2H, m)
Cl	n-Pr	97	78	14.8	421,423	17.3	14.37 (14.10)	2.66 (2.06)	19.61 (18.21)	0.87 (3H, t, J = 7.5 Hz), 1.65 (2H, sextet, J = 7.5 Hz), 2.10 (2H, m)
Cl	n-Bu	96	78	14.7	435,437	17.3	16.81 (16.93)	2.90 (2.54)	16.70 (17.50)	0.86 (3H, t, J = 7.5 Hz), 1.32 (2H, sextet, J = 7 Hz), 1.62 (2H, quintet, J = 7.5 Hz), 2.15 (2H, m)
Cl	n-Hex	90	70	14.75	463,465	17.3	21.40 (21.96)	3.89 (3.40)	16.37 (16.21)	0.86 (3H, t, J = 7.5 Hz), 1.30 (6H, m), 1.75 (2H, quintet, J = 7.5 Hz), 2.15 (2H, m)
Cl	CH_2Ph	99	82	13.7	469,471	16.9	23.11 (24.70)	2.39 (1.80)	16.75 (15.96)	3.40 (2H, t, J = 13 Hz), 7.25 (3H, m), 7.40 (2H, m)
Cl	^iPr	15	-	14.8	421,423	17.2	- -	- -	- -	-
Br	H	-	-	11.4	423,425	12.9	3.80 (3.50)	0.95 (0.30)	17.60 (18.10)	3.75 (1H, t, J = 20 Hz)
Br	Me	93	70	14.9	437,438	17.8	6.09 (6.70)	0.72 (0.84)	- -	2.02 (3H, t, J = 15 Hz)
Br	Et	87	63	14.7	451,453	17.5	9.70 (9.70)	1.51 (1.35)	17.01 (16.70)	1.14 (3H, t, J = 8 Hz), 2.18 (2H, m)

^{31}P n.m.r. as constituting about 15% of a complex product mixture. The main compound identified in this mixture was the unalkylated (i.e., reprotonated) species, but several other peaks could be seen which were attributed to P-C bond cleavage reactions. In this case the competing reactions occur because of the reluctance of the secondary alkyl iodide to react, presumably due to the effect of steric hindrance. As expected, primary alkyl bromides reacted more slowly than their corresponding iodide. Primary alkyl iodides of six or more carbons in length also react rather sluggishly under the conditions described so that slightly reduced yields are isolable for hexylation and even smaller yields are seen for longer or more complex iodides. For example, reaction of the thallium(I) salt of tetraisopropyl chloromethylenebisphosphonate with 1-iodohexadecane results in 72% monoalkylation by ^{31}P n.m.r. Reaction of the same salt with 1-(2-chloro-4-methoxy-phenoxy)-6-iodohexane overnight, resulted in an isolated yield of 62% of the bisphosphonate ester analogue of arildone (Diana, *et al.*, 1977). These decreased yields can be attributed to the reduced mobility of the larger iodides under the reaction conditions, causing the desired reaction to proceed more slowly and allowing competing reactions to occur.

3.2.2 Alkylation of Tetraisopropyl bromomethylenebisphosphonate

The method described above was also extended to provide modest isolated yields of monoalkylated derivatives of tetraisopropyl bromomethylenebisphosphonate.

It was found though, that the competing reactions were more predominant than for the chloro-analogue. The side products could be minimised by forming the thallium salt intermediate at low temperature, followed by a short period of low temperature alkylation and then proceeding in the usual manner. Again such side reactions become more important with increasing size of the alkylating group, although reasonable yields could be isolated for primary alkyl groups of up to four carbon atoms in length (Table 3.2.1.2).

3.2.3 Alkylation of Tetraisopropyl methylenebisphosphonate

Tetraisopropyl methylenebisphosphonate was less reactive towards thallium ethoxide than its halogenated derivatives. Reaction of the ester with one equivalent of the base, followed by alkylation with ethyl iodide yielded only 51% of the monoalkylated derivative by ^{31}P n.m.r. For optimum yields of the monoalkylated product, 1.4 equivalents of base were required to form the anion and in this way up to 75% of the product mixture comprised the monoalkylated derivative (Table 3.2.1.2).

Some exchange occurred to give the dialkylated compound and hence it was concluded that this method held no advantage over the method of Quimby, *et al.* (1968) for alkylation of tetraisopropyl methylenebisphosphonate, despite being superior for monoalkylation of the halogenated derivatives. Improved isolated yields were obtainable by separating the products by flash column chromatography (Still, *et al.*, 1978) rather than by fractional distillation.

3.2.4 Relative Reactivities of Derivatives of Tetraisopropyl methylenebisphosphonate

The differences in the reactions of the three methylenebisphosphonate tetraisopropyl ester species can be rationalised in terms of their reactivity towards thallium(I)ethoxide and the stability of their thallium salts once formed. The salt of tetraisopropyl methylene-bisphosphonate is the least easily formed of the three. This is probably because there is no electron withdrawing group on the bridge which would give extra stability to the intermediate anion, as is the case for the two halogenated analogues. Since 1.4 equivalents of thallous ethoxide are required to give the best yields of monoalkylated product, the opportunity exists for dialkylation by both exchange between the monoalkylated species and the intermediate, or reaction between the monoalkylated species and a second molecule of base, each followed by a second alkylation.

For the chloro- and bromo-derivatives, quantitative yields of the anion intermediate appear to be formed with a single equivalent of thallium(I)ethoxide. Hence, the only opportunities for competing reactions are through metal/halogen exchange or P–C bond cleavage reactions. The bromo-compound is much more prone to metal/halogen exchange, and this property can be equated with the fact that the first ionisation potential of bromine is less than that of chlorine (Downs and Adams, 1973b), since the halogen must be abstracted as a positively charged entity.

P-C bond cleavage is not important for the reaction of the chloro-species, but is more prevalent for the bromo-analogue, which may mean that the heavier halogen fails to stabilise the intermediate anion to the same extent as chlorine, rendering the thallium salt of the bromo-analogue more susceptible to cleavage.

3.2.5 Differing reactivities of Different Esters of Methylenebisphosphonate and Derivatives to Alkylation

It was anticipated that alkylation of the tetraethyl or tetramethyl esters of all the compounds examined, by the same route, would produce similar or even improved yields in cases, due to the reduction in steric hindrance around the carbanion reaction centre. These primary esters would also be more rapidly hydrolysed, *via* the tetra(trimethylsilyl)ester (McKenna and Schmidhauser, 1979), than the isopropyl ester, which could prove invaluable for preparation of more sensitive species. However, reduced yields for the alkylation of tetraethyl esters were observed and for the tetramethyl esters the P-C bond cleavage reaction was the predominant reaction, making them useless for the preparation of monoalkylated products. These observations required further investigation.

3.2.5.1 ^{31}P N.m.r. Examination of the Relative Rates of Reaction of Esters of Chloromethylenebisphosphonate

The relative rates of alkylation of the tetraisopropyl and tetraethyl esters of chloromethylenebisphosphonate were examined qualitatively by ^{31}P n.m.r. A sample of the lithium salt of each ester was prepared

and its ^{31}P n.m.r. spectrum recorded, a primary alkyl iodide was added and further spectra recorded at suitable intervals. The relative rates of alkylation can be seen from the proportions of the peaks due to the anion (δ 32.3 (^{i}Pr) and 34.7 (Et) p.p.m.) and the monoalkylated product (δ 14.7 (^{i}Pr) and 16.4 (Et) p.p.m.) (Fig. 3.2.5.1). It can be estimated that the alkylation of the lithium salt is nearly twice as fast (at room temperature) for the tetra-isopropyl ester than for the tetraethyl ester. Side reactions however, proceed at a comparable rate for both esters so that the slower production of a monoalkylated product with the tetraethyl ester, means that it accounts for a smaller proportion of the final product distribution.

These observations rationalise the behaviour seen both in this study and with previous work, in that alkylations of tetraisopropyl methylenebisphosphonate (Quimby, *et al.*, 1968) give better isolated yields than the corresponding reaction with the tetraethyl ester (Kosolopoff, 1953).

Similar relative rates of reaction were seen for the alkylation of thallium salts, the tetraisopropyl esters again reacting more rapidly. However, this reaction was less satisfactorily followed by n.m.r., since the anion intermediate is only very sparingly soluble in organic solvents. Quenching of aliquots of the reaction mixture with water at intervals and equating the proportion of the reformed chloromethylenebisphosphonate ester with that of the anion in solution, was the method used to follow these reactions. This method assumes

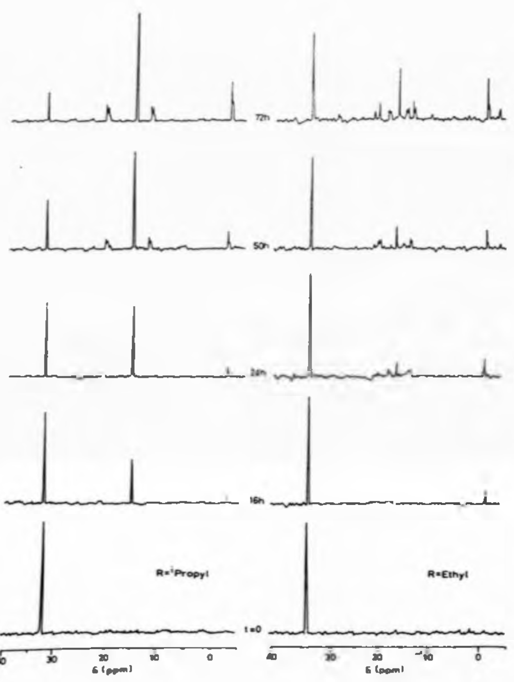

Fig. 3.2.5.1 Time-course of the alkylation of tetraisopropyl and tetraethyl lithiochloromethylenebisphosphonate.

that the chloromethylenebisphosphonate esters do not exist in the reaction mixture, which is almost certainly not the case.

The observation that the alkylation of the tetraisopropyl ester proceeds more rapidly than that of the tetraethyl ester was the opposite of what might be expected on purely steric grounds. Thus, the anion intermediates must differ either electronically or energetically to account for the behaviour described.

3.2.5.2 ^{13}C N.m.r. Study of Methylenebisphosphonate Esters and their Anions

The electronic nature of the central carbon atom in the methylene bisphosphonate esters and their anions were investigated by ^{13}C n.m.r. (Table 3.2.5.2). The first feature of the results to be noticed is that there is an increase in the $^{31}P-^{13}C$ one bond coupling constants for all the phosphorus containing species, on moving from the parent compound to the anion. This is consistent with a change in hybridisation of the central carbon atom, on removal of the bridge proton, from sp^3 to sp^2 (Stothers, 1972). The ^{13}C chemical shifts, however, would seem to indicate that this can only be the case if the anions are not appreciably delocalised. The signals for the anions are shifted 12-17 p.p.m. upfield from those of the corresponding parent species. These changes in chemical shift can be compared with those seen for the carbonyl analogues where the negative charge would be epxected to be delocalised to a greater extent.

Formation of the anion of triethylphosphonoacetate, results in a downfield shift of 5.6 p.p.m. for

TABLE 3.2.5.2 ^{13}C N.m.r. data for the central carbon atom in methylenebisphosphonates, triethylphosphonacetate, pentane-2,4-dione and their anions

Compound	δ (p.p.m.)	$J_{^{13}C-^{31}P}$ (Hz)
$[(MeO)_2P(O)]_2CHCl$	42.1	141.9
$[(MeO)_2P(O)]_2CCl^{(-)}Na^{(+)}$	30.2	236.9
$[(EtO)_2P(O)]_2CHCl$	43.9	141.9
$[(EtO)_2P(O)]_2Cl^{(-)}Na^{(+)}$	31.1	236.4
$[(i\text{-}Pro)_2P(O)]_2CHCl$	45.5	143.4
$[(i\text{-}PrO)_2P(O)]_2CCl^{(-)}Na^{(+)}$	33.8	234.3
$[(MeO)_2P(O)]_2CH_2$	24.0	135.3
$[(MeO)_2P(O)]_2CH^{(-)}Na^{(+)}$	7.3	217.5
$[(EtO)_2P(O)]_2CH_2$	26.1	135.3
$[(EtO)_2P(O)]_2CH^{(-)}Na^{(+)}$	8.6	217.7
$[(i\text{-}PrO)_2P(O)]_2CH_2$	28.5	136.8
$[(i\text{-}Pro)_2P(O)]_2CH^{(-)}Na^{(+)}$	11.1	214.7
$(EtO)_2P(O)CH_2COOEt$	34.7	133.8
$(EtO)_2P(O)CH^{(-)}COOEtNa^{(+)}$	40.3	219.1
*$MeCOCH_2COMe$	52.8	-
*$MeCOCH^{(-)}COMeNa^{(+)}$	96.9	

*Wilkie and Haworth, 1978

the signal due to the central carbon atom, relative to the protonated parent species, and it has been reported for pentane-2,4-dione that the analagous transformation results in a downfield shift of 44 p.p.m. in the ^{13}C n.m.r. for the central carbon (Wilkie and Haworth, 1978). So the species with the greatest delocalisation have the greatest downfield shift on removal of a proton by sodium hydride.

A similar pattern for the relative amounts of delocalisation for these species has been suggested from infrared evidence (Cotton and Schunn, 1963), so it can be concluded that there is negligible delocalisation of negative charge through the phosphate bonds in methylenebisphosphonates, and furthermore that there is little difference in the electronic properties of the anions regardless of which ester is being examined.

Thus the difference in reactivity of the different esters can be ascribed to the differing stabilities of their anions. It can be seen that the anions of tetraisopropyl ester species would be the most subject to steric destabilisation, on account of the bulkyness of the isopropyl groups, and as the least stable anion it might be expected to react most rapidly. The more stable anions, those of the ethyl and methyl ester species react more sluggishly with alkyl iodides and so become susceptible to side reactions, rendering them less useful for the preparation of monoalkylated methylenebisphosphonates.

3.2.6 Hydrolysis of Methylenebisphosphonates

Hydrolysis of esters of methylenebisphosphonates

by treatment with refluxing hydrochloric acid is the traditional method of preparation of the corresponding free acid (Kosolopoff, 1950). Yields are not high, however, (Hays and Logan, 1966), and so alternative procedures have been sought to facilitate deesterification under milder conditions.

It has been demonstrated that halogenotrialkylsilanes interact with alkyl-phosphates and phosphinates to give the corresponding trialkylsilyl derivatives (Schwartz and Schoeller, 1958). This observation has become the basis of the most commonly used hydrolysis procedure, as these silylesters are readily hydrolysed under neutral conditions. Several reagents have been used to effect this transformation reaction which have a range of reactivities so that selectivity is possible between, for example, primary and secondary esters or alkyl and aryl esters.

Chlorotrimethylsilane is the least reactive of the species used. If used in combination with sodium iodide, efficient dealkylation is claimed (Morita, *et al.*, 1979) but formation of halogenated side products has been observed (McKenna and Schmidhauser, 1979). Use of iodotrimethylsilane greatly reduces the amounts of these side products (Blackburn and Ingleson, 1980), and this reagent rapidly and selectively transesterifies phosphate ester functions.

A slightly less reactive homologous reagent, bromotrimethylsilane, has also been shown to effect selective transesterifications of phosphate esters (e.g.,

$$[(RO)_2P(O)]_2CR^1R^2 \xrightarrow{BrTMS} [(Me_3SiO)_2P(O)]_2CR^1R^2$$

$$\xrightarrow{H_2O\ /\ MeOH} [(HO)_2P(O)]_2CR^1R^2$$

Mechanism

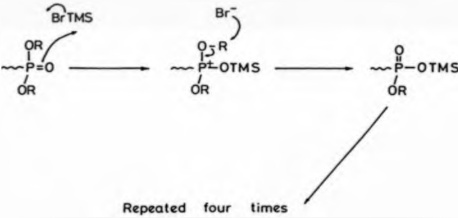

Fig. 3.2.6 Hydrolysis of phosphonate esters using bromotrimethylsilane.

Hata, *et al.*, 1979, McKenna, *et al.*, 1977). This reagent proved in this study to be sufficiently reactive to be used for clean, quantitative hydrolysis of tetraisopropyl esters (Fig. 3.2.6) at room temperature under nitrogen. This was considered more convenient than using iodotrimethyl silane, which although more reactive, had to be used at low temperature in the dark to prevent discolouration of reaction mixtures.

3.2.7 Conclusions on the Synthesis of C-Alkyl Halogenomethylene bisphosphonates

The most efficient preparation of C-alkyl halogenomethylene bisphosphonic acids is by dihalogenation of the isopropyl ester, selective monodehalogenation, alkylation *via* the thallium(I) salt and finally deesterification *via* the tetra-(trimethylsilyl) ester.

It has been demonstrated that yields for the halogenation and alkylation reactions are smaller if tetraethyl or tetramethyl esters are used. Reduced yields are also obtained if any other of the metals examined is used as the counterion for the alkylation intermediate. Finally, the most efficient hydrolysis if affected by treatment with bromotrimethylsilane followed by hydrolysis of the silylated intermediate with aqueous methanol.

3.3 EXPERIMENTAL

3.3.1 Alkylation of Tetraisopropyl sodiochloromethylenebisphosphonate

Alkylation of the sodium salt of tetraisopropyl chloromethylenebisphosphonate was carried out essentially as described for the unhalogenated analogue (Quimby, *et al.*, 1968). Yields are listed in Table 3.2.1.1.

3.3.2 Alkylation of Tetraisopropyl lithiochloromethylenebisphosphonate

The lithium salt of tetraisopropylchloromethylene bisphosphonate was prepared as described in Section 2.4.6. The salt was alkylated by addition of an alkyl iodide and the mixture heated under reflux for 2 h. The products were isolated as described by Seyferth and Marmor (1973) as oils. ^{31}P N.m.r. analysis of the product mixtures showed for the methylation reaction, the tetraisopropyl esters of methylchloromethylenebisphosphonate (65%), chloromethylenebisphosphonate (20%) and dichloromethylenebisphosphonate (15%). For the ethylation reaction, the tetraisopropyl esters of ethylchloromethylenebisphosphonate (36%), chloromethylenebisphosphonate (12%) and dichloromethylenebisphosphonate (25%) as well as traces of four other products.

3.3.3 Preparation of Tetraisopropyl C-Alkylchloromethylenebisphosphonates *via* Thallium(I) Salt Intermediates

Preparation of all the alkylated chloromethylenebisphosphonate esters was effected using the same general

procedure. A typical example is given below.

Tetraisopropyl chloromethylenebisphosphonate (0.5 g, 1.3 mmol) was dissolved in dry, freshly distilled THF (10 ml) and the mixture stirred at room temperature under an atmosphere of dry nitrogen. Thallium(I)ethoxide (95 µl, 1.3 mmol) was added all at once and stirring continued at room temperature for 40 min. during which time the mixture was seen to turn cloudy. Iodoethane (5 ml) was added and the mixture heated under reflux for 2 h. The solution was then cooled and the orange thallium iodide precipitate removed by percolation through a short florisil column, eluting with petroleum ether b.p. 40-60°C/acetone, 1/1 (v/v, 120 ml). The residue was concentrated *in vacuo* to an oil, which was shown by ^{31}P n.m.r. analysis to contain the monoethylated product as 97% of the product mixture. This was purified by flash column chromatography, eluting with petroleum ether b.p. 40-60°C/acetone, 6/1 (v/v), to give the pure product (yield 0.44 g, 81%).

During the preparations of the n-propylated and n-butylated analogues, the reaction vessel was kept dark, since the respective alkyl iodides are light sensitive.

For the preparation of the benzylated compound, the thallium intermediate was alkylated by treatment with an excess of a mixture of benzyl bromide and benzyl iodide prepared, in an efficient fume hood, as follows. Benzyl bromide (5 g, 0.029 mol) was added to a solution of sodium iodide (4.4 g, 0.03 mol) in acetone (25 ml). The mixture was stirred at room temperature for 10 min., filtered and

then concentrated *in vacuo*. This mixture (^1H n.m.r (CCl_4)$PhC\underline{H}_2Br$ δ 4.41 p.p.m.; $PhC\underline{H}_2I$ δ 4.48 p.p.m.; ratio 1:4) was used immediately in the alkylation step.

All data for the compounds prepared are listed in Table 3.2.1.2.

3.3.4 Preparation of Tetraisopropyl C-Alkylbromomethylenebisphosphonates

Alkylation of tetraisopropyl bromomethylenebisphosphonate was by the same general procedure described above for the chloro analogue, except that formation of the anion intermediate was carried out at -20°C and the subsequent alkylation was performed by stirring with the alkylating agent, initially at -20°C for 1 h and then under reflux for 1½ h. The data for the compounds prepared are listed in Table 3.2.1.2.

3.3.5 Preparation of Tetraisopropyl C-Alkylmethylenebisphosphonate

Preparation of tetraisopropyl C-alkylmethylenebisphosphonate was effected as described above, except that 1.4 equivalents of thallous ethoxide were used to form the anion intermediate, which was then alkylated as normal. Purification of the product mixtures was by flash column chromatography eluting with petroleum ether b.p. 40-60°/acetone, 5/2 (v/v). The data for the compounds prepared in this way is given in Table 3.2.1.2.

3.3.6 Analysis of the Time-Course of Reactions by ^{31}P N.M.R.

The required methylenebisphosphonate ester (100 mg) was dissolved in C_6D_6 (2 ml) and the anion formed by addition of a single equivalent of the requisite lithium alkyl. The ^{31}P n.m.r. spectrum (36.4 MHz) was recorded. The alkylating agent was added and the subsequent reaction followed by recording the ^{31}P n.m.r. at suitable intervals.

3.3.7 Analysis of Anions and Parent Species by ^{13}C N.M.R.

The anion was formed as described above in C_6D_6 by addition of one equivalent of sodium hydride. The ^{13}C n.m.r. spectrum (22.62 MHz or 100.62 MHz) was recorded and compared to that of the starting material. Formation of the anion of the methyl ester was accompanied by some cleavage of the P-C bridge bond.

3.3.8 Hydrolysis of Tetraisopropyl Esters of Methylenebisphosphonates

Hydrolyses were performed using a standard procedure. A typical example is described below. Tetraisopropyl(ethylchloromethylene)bisphosphonate (400 mg, 0.96 mmol) was dissolved in CCl_4 (1 ml) under an atmosphere of dry nitrogen. Bromotrimethylsilane (1.3 ml, 10 mmol, 2.5 fold excess) was added and the mixture stirred at room temperature for 20 h. The solution was then lyophilised (^1H n.m.r. analysis showed there to be no isopropyl groups present) and hydrolysed by repeated addition of aqueous methanol and lyophilisation. The

residue was dissolved in a little water and isolated as the tetrasodium salt by ion exchange on Dowex 50 (Na^+ form) (Yield, 0.29 g, 92%). Analytical data for all the compounds isolated in this way is listed in Table 3.2.1.2.

CHAPTER 4

ACTIVITY OF METHYLENEBISPHOSPHONATES AGAINST INFLUENZA VIRUSES

4.1 METHODS

4.1.1 Preparation of Virus Stocks

The influenza A/X49 strain (a cross between A/England/864/75 and A/Puerto Rico/8/34 with the H3N2 surface antigens of the A/England strain) was used for preparation of the influenza RNA transcriptase enzyme assay stocks, and was grown in the allantoic sacs of fertile hens eggs essentially as described by Kelly and Dimmock (1974).

Eleven day old embryonated hen's eggs were innoculated with infected allantoic fluid (0.1 ml of a 10^{-4} dilution in PBS). The eggs were incubated (33°C/48 h) and then chilled (-20°C/2 h). The allantoic fluid was collected and clarified by centrifugation (3000 r.p.m./20 min.); from this point onwards all procedures were carried out at 0-4°C. The supernatant was collected and the virus pelletted by centrifugation (21,000 r.p.m./90 min., 6 x 300 ml rotor). The supernatant was discarded and the virus pellet allowed to soak overnight in PBS. The virus pellet was resuspended in PBS (10 ml) and clarified (2500 r.p.m./ 10 min.) before application onto a velocity gradient of 15-45% (w/v) sucrose in buffer (60 ml, 10 mM *tris*-HCl, 150 mM NaCl, pH 7.4) and centrifuged (22,000 r.p.m./1 h,

3 x 65 ml swing-out rotor). The diffuse virus band was collected by bottom puncture and the sucrose diluted with PBS and layered onto an equilibrium gradient of 30-70% (w/v) sucrose in buffer (50 ml, 10 mM *tris*-HCl, 150 mM NaCl, pH 7.4) and centrifuged (20,000 r.p.m./overnight, 3 x 65 ml swing-out rotor). The virus band was collected as before, diluted with PBS and the virus pelleted by centrifugation (30,000 r.p.m., 2 h, 8 x 50 ml rotor). The supernatant was discarded and the pellet allowed to soak overnight in PBS before resuspending the virus in buffer (0.5 ml, 400 μM *tris*-HCl, pH 8.0) and frozen in aliquots at -70°C, thawing once prior to use.

4.1.2 <u>Influenza RNA Transcriptase Assay</u>

RNA dependent RNA polymerase (transcriptase) activity was assayed in a 200 μl reaction mixture containing 50 mM *tris*-HCl, pH 8.0, 5 mM $Mg(CH_3CO_2)_2$, 150 mM KCl, 5 mM dithiothreitol, 0.4 mM adenylyl-(3'-5')-guanosine(ApG), 0.25% (v/v) Nonidet P-40, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM GTP, $[^3H]$-UTP (5 μCi) and purified virus 10 μl. Mixtures were kept at 4°C until addition of virus and then incubated for 1 h at 30°C. Reactions were terminated by addition of 200 μl of ice cold saturated sodium pyrophosphate followed by cold TCA (2 ml, 10% w/v) and the mixtures kept on ice for 2 h after agitation. Precipitated material was collected on Whatman GF/C discs which were wetted with cold 10% (w/v) TCA, washed with 2 x 10 ml cold 10% (w/v) TCA, 10 ml ethanol and 5 ml ether, then oven dried before

counting in a toluene based scintillant. Each concentration of inhibitor was tested in duplicate and semi-logarithmic dose-response curves drawn for each compound, from which the concentration required to inhibit the enzyme by 50% was determined (ID_{50}).

4.1.3 Plaque Reduction Assay

Madin-Darby canine kidney (MDCK) cells were seeded onto 5 cm plastic culture dishes (in duplicate) at 3 x 10^6 cells/plate. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in air until confluent in maintenance medium (DMEM supplemented with 10% NCS, 4 mM L-glutamine and penicillin/streptomycin, 50 units/ml). The confluent monolayers were washed with PBS and aspirated to dryness. Virus suspension 100 µl, 60 p.f.u.) was added to the cell sheets and left for 15 min. at room temperature, the inhibitor added in PBS (300 µl) and the cell sheets left for a further 45 min. at room temperature. Cells were then overlaid with an agar medium (3.6 ml) containing 0.1% BSA, 2.5 µg/ml crystalline trypsin and 0.1% DEAE Dextran. The plates were incubated at 33°C for 4-6 days, stained with neutral red and the plaques counted. The percentage of plaque inhibition relative to the infected control, was determined for each inhibitor at a concentration of 500 µM.

4.2 EXPERIMENTAL AND RESULTS

4.2.1 Effect of Halogenated and Alkylated Methylenebisphosphonates on Influenza RNA Transcriptase Activity

Table 4.2 shows the ID_{50} values of the compounds tested and their zinc stability constants (Section 4.2.3). All the alkylated analogues showed similar inhibition against the enzyme, regardless of the length of the alkyl group involved. A typical dose-response curve is shown in Fig. 4.2.1).

4.2.2 Effect of Alkylchloromethylenebisphosphonates on Influenza A/X49 Replication in MDCK Cells

Several alkylchloromethylenebisphosphonates were evaluated for anti-influenza activity in MDCK cells by a simple plaque reduction assay, the results of which are shown in Table 4.2. All alkylated analogues were superior inhibitors of the virus in cell culture than PAA or the parent chloromethylenebisphosphonic acid. An increase in activity was seen with increasing lipophilicity of the alkyl side chain. No cell damage could be observed at concentrations of up to eight times those required for activity.

4.2.3 Determination of Zinc Binding Ability

Zinc binding was assessed by determination of the metal complex stability constant, $pK_{d'}$, by the gel filtration method of Hummel and Dreyer (1962). The details of the technique have been described by Yoza (1977).

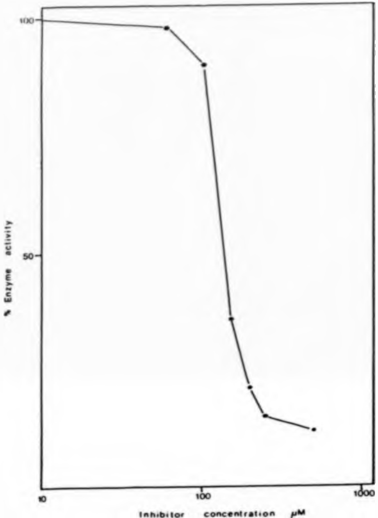

Fig. 4.2.1 A typical dose-response curve, showing the activity of C-benzylchloromethylenebisphosphonate on influenza A/X49 transcriptase at various concentrations.

Table 4.2 Biological Activity of Methylenebisphosphonates, $[(HO)_2P(O)]_2CR^1R^2$, and PAA

R^1	R^2	pK_d' (Zn^{2+})	ID_{50} (μM)	PR 500%
H	H	5.3	> 500	n.d.
H	Cl	> 6	85	22
Cl	Cl	> 6	75	n.d.
Me	Cl	> 6	200	n.d.
Et	Cl	n.d.	190	n.d.
n-Pr	Cl	n.d.	195	34
n-Bu	Cl	> 6	190	37
n-Hex	Cl	> 6	230	83
CH_2Ph	Cl	> 6	130	60
H	Br	> 6	80	n.d.
Br	Br	> 6	40	n.d.
Me	Br	n.d.	160	n.d.
Et	Br	> 6	180	n.d.
(PAA)		(5.5)	(275)	(0)

PR_{500} = %reduction of p.f.u. (virus control = 7.7×10^8) caused by analogue at a concentration of 500 μM.

4.2.4 Assessment of C-Benzylchloromethylenebisphosphonate as a Bone Imaging Agent

C-Benzylchloromethylenebisphosphonic acid was bound to Tc-99m in the presence of $SnCl_2$ as a reducing agent (pH ~ 6). The material chromatographed as one species and when injected intravenously into 3 mice with sacrifice 1 hour later, distribution was similar to other good Tc-99m bone imaging agents. That is, uptake in the femur was quite high, while the blood "background" was slightly higher than with some other agents.

4.3 DISCUSSION

Detergent-disrupted virus preparations, as used in this study, make available an RNA transcriptase activity which can be assayed in the presence of ApG as primer (Plotch and Krug, 1977) and using endogenous RNA as template. In this study the ID_{50} values refer to the inhibition of the whole of the transcriptase activity by 50%, since specific stages of the polymerisation process have not been fully characterised and the transcriptase enzyme kinetics have been found to be complex (Cload, 1983).

Dibromomethylenebisphosphonic acid was the most effective inhibitor of influenza A/X49 tested. The concentration required to inhibit the transcriptase activity by 50% was found to be 40 μM as compared to the published value of 10 μM (Cload and Hutchinson, 1983a). All of the halo- and dihalo-derivatives were found to be effective inhibitors of the enzyme as in other studies (e.g., Cload, 1983) but on substitution with an alkyl

group the concentration required to produce 50% enzyme inhibition increased roughly two-fold (Table 4.2). Within the limits of error of the enzyme assay procedure, it can only be concluded that in cell-free systems the length of the alkyl chain attached to the bridge does not significantly alter the ID_{50}. This is perhaps slightly surprising as it means that the enzyme active site can accommodate a large range of sizes of inhibitor. There would, however, be very little difference in the effect of the various alkyl groups on the acid properties of the compounds and this appears to be the important factor in cell-free systems.

Magnesium ions are essential for the transcriptase activity and one possibility is that pyrophosphate analogues complex with magnesium ions to remove them from solution and hence disrupt viral processes. However, this seems unlikely as magnesium is present in the polymerase reactions at 5 mM whereas the compounds studied here are active at about one twenty-fifth of this concentration.

All of the alkylated analogues assessed for zinc binding ability were found to have pK_d' values of greater than 6 (Table 4.2). It has been shown previously that compounds which bind zinc this effectively, make good inhibitors of influenza RNA transcriptase (Cload, 1983; Naylor, 1985). This lends credence to the proposal that analogues of inorganic pyrophosphate owe their activity to binding tightly at an essential zinc ion at or adjacent to the transcriptase active site.

In the plaque reduction assay the lipophilicity of the side chain has a significant effect. It can be seen from Table 4.2 that all the alkylated analogues tested in cell culture are superior inhibitors to the parent chloromethylenebisphosphonic acid, and that all of the compounds examined were more active than PAA. Furthermore, the compounds with the largest, and hence most lipophilic, side chains are the most effective against influenza in cells. This can only be due to the increased lipophilicity enabling the compounds to cross the cell wall more readily to reach the active site, since all the inhibitors are of comparable efficacy in enzyme assays. Even a small alkyl group such as n-propyl is sufficiently lipophilic to enhance the activity of halogenated methylenebisphosphonates in cell culture. Of the compounds screened, only PAA showed any significant cellular cytotoxicity at a concentration of 8 mM, much greater than that required for activity.

In an attempt to qualitatively assess whether the compounds shown to be improved inhibitors of influenza in cell culture would show any reduced affinity for calcified tissue, C-benzylchloromethylenebisphosphonic acid was examined as a potential bone imaging agent. As described in Section 4.2.4, distribution of the radioactively tagged compound in mice was similar to that observed for other good bone imaging agents. Thus, it could not be said that the benzyl side chain group significantly affected the affinity of halogenated methylenebisphosphonates for calcified tissue. Perhaps a much larger and more bulky

group is needed to make such compounds incompatible with the hydroxyapatite matrix of bones and teeth.

It can be concluded that lipophilic side chains increase the anti-influenza activity of halogenated pyrophosphate analogues in cell culture but not in cell-free systems, and that the small alkyl groups used in this study have little effect on the affinity of such species for calcified tissue.

CHAPTER 5

PREPARATION AND TESTING OF AN AFFINITY LABEL FOR INFLUENZA RNA TRANSCRIPTASE

5.1 INTRODUCTION

As described in Section 1.2.2.2, synthesis of virally-coded mRNA can be divided into three distinct phases. Initiation begins by cleavage of a capped oligonucleotide from cellular RNA and then incorporation of the first residue. Secondly, elongation occurs for 12-14 bases at which time the transcriptase complex begins to move down the nascent chain. Finally, a complex termination mechanism comes into effect.

Phosphonoformic acid has been shown to prevent elongation beyond 12-13 bases (Stridh and Datema, 1984) and thus its activity appears to coincide with the point at which the transcriptase complex begins to move. The question still remains unanswered, however, as to which of the viral proteins which make up the complex, pyrophosphate analogues interact with to exert their observed antiviral activity. It is claimed that pyrophosphate analogues bind at the pyrophosphate/nucleotidetriphosphate binding site of DNA polymerases (Leinbach, *et al.*, 1976), and such a site in the RNA transcriptase complex of fowl plague virus (FPV) has recently been shown to be located in the PB_1 protein (Romanos and Hay, 1984). This would imply that pyrophosphate analogues interact with this protein.

Alternatively, the experiments with PFA indicate that they could interact with PB_2, to prevent its dissociation from the cap of the growing nucleotide chain.

It was decided to attempt to resolve this problem by preparation of an affinity label for the transcriptase.

5.1.1 Design of an Affinity Label

The affinity label chosen, was based on the alkylated derivatives already shown to be inhibitors of the enzyme, and which must interact with it. The molecule shown in Fig. 5.1.1 has the two phosphonate groups to bind with the metal ion proposed to be at the active site, and the halogen on the bridge to give the correct acid properties necessary for good antiviral activity. The long chain terminates in an aldehyde group which might be expected

Fig. 5.1.1 Structure of the affinity label.

to react with any amino acid residues with nucleophilic side chain functions, close to the active site. A shorter chain may result in the aldehyde group being too far removed from potential sites of reaction and hence being unable to interact with them. The molecule would thus become irreversibly bound to the protein containing the active site. Radioactive reduction of the complex formed would enable identification of this protein by gel electrophoresis and fluorography.

5.2 SYNTHESIS OF THE AFFINITY LABEL

The synthetic strategy adopted for the preparation of the affinity label described above, was to utilise the methods already developed for chlorination and alkylation of tetraisopropyl methylenebisphosphonate. This then, required the preparation of a straight chain iodide, with a group at the opposite end of the molecule which could be developed into an aldehyde functionality, with which to alkylate tetraisopropyl chloromethylenebisphosphonate. It was assumed that the alkylation reaction would affect an aldehyde group if it were already present in the alkylating species.

Two of the most widely used methods of masking a carbonyl group, involve the disguise of the reactive functionality as either a 1,3-dithiane (Seebach and Corey, 1975) or as an oxazine (Meyers, *et al.*, 1969a) which are then developed into a later stage. However, preparation of species with these groups at one end of an alkyl chain and a halogen at the other is not possible

as cyclisation reactions occur in each case (Seebach and Corey, 1965; Meyers, *et al.*, 1969b) (Fig. 5.2(i)).

Fig. 5.2(i) Cyclised structures of (a) bromoalkyldithianes and (b) bromoalkyloxazines.

Preparation of aldehydes is also possible by hydridic reduction of nitriles and quenching with acid (Brown and Garg, 1964), but reaction of iodopropionitrile with the thallium salt of tetraisopropylchloromethylene-bisphosphonate under the standard conditions developed previously (Chapter 3), failed to result in any alkylation of the type required, precluding this as a possible route to the affinity label.

It was decided to approach the aldehyde by way of the corresponding alcohol, and subsequent oxidation. Hence a straight chain iodo-alcohol was the first synthetic objective.

Halo-alcohols can be prepared by cleavage of cyclic ethers by Lewis acids in the presence of halide ion. Several methods are known for cleavage of tetrahydro-

furan using various boron compounds. 4-Iodobutan-1-ol is produced by cleaving THF with borohydride in the presence of molecular iodine (Long and Freeguard, 1965) or boron trifluoride etherate in the presence of iodide ion (Mandal, *et al.*, 1985). Each of these methods was investigated but it was found that they were restricted to the five-membered cyclic ether, and larger ethers were not cleaved. A third method in which diborane is used for cleavage in the presence of molecular iodine (Long and Freeguard, 1964) proved more versatile in that five-, six- and seven-membered cyclic ethers were all cleaved to their respective iodo-alcohols. 6-Iodohexan-1-ol (2) was prepared in this way from oxepane (1) in good yield (Fig. 5.2(ii)), and was reacted with the thallium(I) salt of tetraisopropylchloromethylene bisphosphonate under the standard conditions described (Section 3.3.3). This, however, failed to give the desired derivative, which was thought to be due to the alcohol function interfering with the alkylation reaction. Hence it was decided that protection of the hydroxyl function of 6-iodohexan-1-ol was required to permit a clean alkylation. Protection of the alcohol as an ester was discounted as the electrophilic carbonyl group would probably react with the thallium salt intermediate, so an ether protecting group appeared to be the best option. Thus 6-iodohexan-1-ol was protected as its tetrahydropyranyl ether (3) by reaction with dihydropyran and pyridinium para-toluene sulphonate (PPTS), a mild acid catalyst (Miyashita, *et al.*, 1977) (Fig. 5.2(ii)),

(1) $\xrightarrow{B_2H_6/I_2}$ $HO(CH_2)_6I$ (2) $\xrightarrow{\text{, PPTS}}$ $O(CH_2)_6I$ (3)

Fig. 5.2(ii) Synthesis of 6-iodohexyltetrahydropyranyl ether.

$[(^iPrO)_2P(O)]_2CHCl$ (4) $\xrightarrow[\text{ii) 3}]{\text{i) TlOEt}}$ $[(^iPrO)_2P(O)]_2CCl(CH_2)_6OTHP$ (5)

PPTS, EtOH

$[(^iPrO)_2P(O)]_2CCl(CH_2)_5CHO$ (7) $\xleftarrow[\text{DMP}]{\text{PCC or}}$ $[(^iPrO)_2P(O)]_2CCl(CH_2)_6OH$ (6)

Fig. 5.2(iii) Synthesis of 7-chloro-7,7-bis(diisopropylphosphonato)-heptanal.

and an overall yield of 70% for the cleavage and protection steps was realised. When a large excess of this iodo-ether was used to alkylate the thallium salt of tetraisopropylchloromethylenebisphosphonate in the usual manner, the mono alkylation product (5) was observed by ^{31}P n.m.r., to account for 78% of the phosphorus containing material.

The alkylated material was separated from the unreacted 6-iodohexyltetrahydropyranyl ether by column chromatography. Complete purification was not effected at this stage, but rather the partially purified product was first hydrolysed by mild acid catalysed transesterification (Fig. 5.2(iii)), Miyashita, *et al.*, 1977). The new product (6) was more readily purified since the free hydroxyl group ensured that the affinity of this molecule for silica was very different to the unreacted tetraisopropylchloromethylenebisphosphonate (4) and the ether (5), allowing good separation by flash column chromatography.

Having achieved the preparation of 7-chloro-7,7-bis(diisopropylphosphonato)heptan-1-ol in 40% yield for the two step process, the next steps required were the oxidation of the alcohol to an aldehyde and the removal of the phosphate ester groups.

A number of reagents are known for the transformation of alcohols to the aldehyde level of oxidation which use chromium(VI) as the oxidising species, with pyridinium chlorochromate (PCC) (Corey and Suggs, 1975) perhaps the most widely used. This reagent was used to oxidise the phosphonate alcohol (6) to the corresponding aldehyde (7) in 42% (Fig. 5.2(iii)). This disappointing

return was ascribed to the bisphosphonate moiety forming chelate complexes with a chromium metal ion species (Cotton and Schunn, 1963) and hence being removed from the mixture in the work up. Thus, a non-metallic oxidising agent was sought which would not exhibit such undesirable properties and consequently give improved yields. 1,1,1-Triacetoxy-2,1-benzoxiodol-3(3H)one (Dess-Martin Periodinane; DMP) is a novel reagent which satisfies these conditions (Fig. 5.2(iv)); Dess and Martin, 1983). It has several advantages over some chromium based reagents in that oxidation stops at the aldehyde level, it is soluble in a variety of organic solvents and the work up procedure is clean and rapid. When this reagent was used to oxidise the alcohol (6), the corresponding aldehyde was isolated in 93% yield (Fig. 5.2(iii)).

Fig. 5.2(iv) Structure of Dess-Martin periodinane.

The aldehyde (7) was then treated with bromotrimethylsilane (BrTMS) in an attempt to remove the isopropyl phosphonate ester groups. A clean reaction was not observed. Several phosphorus containing species were seen by ^{31}P n.m.r. and more than one brominated phosphonate seen by fast atom bombardment mass spectrometry (Appendix II). This suggests that several side reactions are taking place at the aldehyde group. It is known that iodotrimethyl silane (ITMS) reacts with aldehydes to give α-iodotrimethylsilyl ethers which in the presence of excess ITMS generally give further reaction such as diiodination (Jung, *et al.*, 1978). It seems that similar side reactions are occurring in this case and so some method had to be found to avoid the use of trimethylsilyl reagents in the presence of the aldehyde function.

One solution to avoid side reactions would be to protect the aldehyde group prior to removal of the phosphonate ester groups. However, most of the widely used cyclic acetal or thioacetal groups can be adversely affected by trimethylsilyl iodide or bromide (e.g., Bryant, *et al.*, 1979) resulting in cleavage and halogenation. Aldehydes can be protected as imidazolidines (Wanzlick and Löchel, 1953) which might be expected to be less reactive than acetals and thioacetals to trimethylsilyl reagents, but no reaction of the aldehyde (7) could be observed with N,N'-diphenylethylenediamine to form such a species.

Thus it was decided to carry out the deesterification of the phosphonate ester groups on the alcohol (6) and

then oxidise the resultant phosphonic acid - alcohol to the desired aldehyde. Alcohols are known, however, to react slowly with bromotrimethylsilane to give the corresponding bromide (Jung and Hartfield, 1978), so direct reaction is not possible in this case, but if the alcohol is converted to its trimethylsilyl ether, no such reaction occurs (Jung and Hartfield, 1978). Thus, when the alcohol (6) was converted to its trimethylsilyl ether with chlorotrimethylsilane (ClTMS), hexamethyldisilazane (HMDS) and pyridine (Sweeley, *et al.*, 1963), followed by treatment with BrTMS and hydrolysis in the usual way, the pure free phosphonic acid-alcohol (8) was obtained in 93% yield (Fig. 5.2(v)). The intermediate trimethylsilyl-

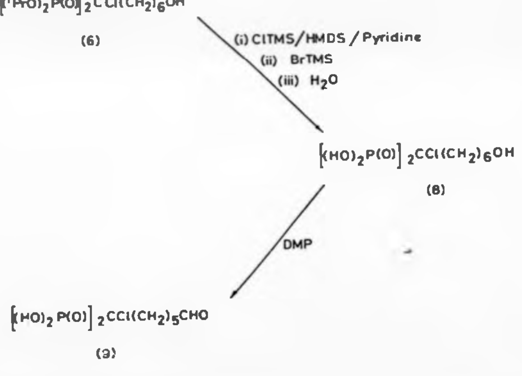

Fig. 5.2(v) Synthesis of 7-chloro-7,7-bisphosphonatoheptanal.

ether was observed by ^{1}H n.m.r. spectroscopy and mass spectrometry as the only species present.

Oxidation of the alcohol (8) presented a problem, in that the compound was insoluble in organic solvents and so none of the conventional methods could be used to perform the transformation. One alternative was to use the Swern technique of DMSO oxidation (Mancuso and Swern, 1981), but this requires the use of anhydrides to activate the DMSO which would probably also catalyse coupling of the phosphonates. The periodinane oxidant used previously is so versatile it was decided to develop a system so that it could be employed in this case to perform the desired oxidation. The periodinane is soluble in acetonitrile and oxidation reactions are unaffected by the presence of pyridine (Dess and Martin, 1983). The alcohol (8) was partially dissolved in a mixture of these two solvents and oxidised with the periodinane reagent. Purification of the product by ion exchange chromatography gave the pure aldehyde (9) in 40% yield (Fig. 5.2(v)) and so the target molecule was isolated in 16% yield overall.

5.3 ASSESSMENT OF THE BIOLOGICAL ACTIVITY OF 7-CHLORO-7,7-BISPHOSPHONATOHEPTANAL

The aldehyde (9) was tested as an inhibitor of influenza RNA transcriptase in the manner described in Section 4.2.1 and was found to cause 50% inhibition of transcription when present at a concentration of 260 μM. This is about the same as was found for 1-chloroheptylidene-1,1-bisphosphonic acid. So as expected,

the aldehyde function has no effect on the inhibitory power of the compound, and thus the species prepared is suitable for use as an affinity label.

5.4 AFFINITY LABELLING OF INFLUENZA RNA TRANSCRIPTASE

Influenza A/X49 virus was incubated with the affinity label and the complex reduced with tritiated sodium borohydride, essentially as described by Romanos and Hay (1984). The proteins were separated by polyacrylamide gel electrophoresis and fluorographed to see which protein contained the radioactive label. It can be seen from Fig. 5.4 that no single protein has been labelled specifically but that the majority of the radioactivity has become incorporated into the three P proteins, two of which run virtually together under the conditions used. No other proteins such as HA or NA are labelled in this experiment. It can not be said, however, which of the P proteins contains the site of activity for pyrophosphate analogues from this experiment. The fact that the affinity label becomes associated with the proteins making up the transcriptase complex though, supports the proposal that such analogues exert their antiviral activity by acting directly on the transcriptase.

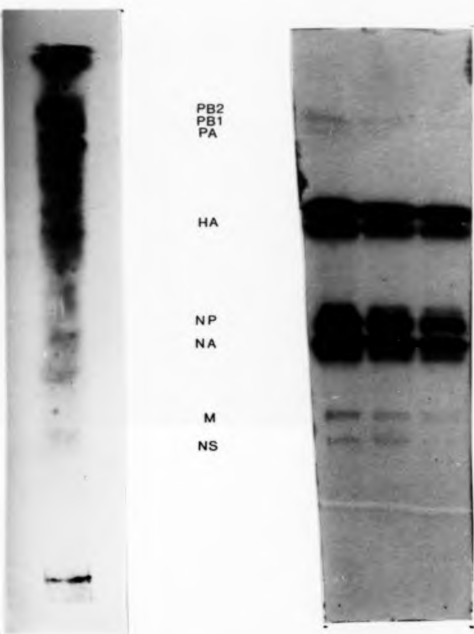

Fig. 5.4 Fluorographed and Coomasie Blue stained gels of influenza A/X49 virus proteins, showing radio-labelling of P proteins.

5.5 EXPERIMENTAL

5.5.1 Preparation of 6-Iodohexyltetrahydropyranyl Ether

Oxepane (14 ml, 0.125 mol) was mixed with iodine (12.65 g, 0.05 mol) in a three-necked flask fitted with a gas inlet, a condenser and a thermometer. The mixture was reacted with diborane by bubbling a large excess of the gas through the reaction vessel maintained at 45°C. The diborane was formed in a gas generator flask by the action of $NaBH_4$ (7 g, 0.185 mol) on a vigorously stirred solution of $BF_3.Et_2O$ (37 ml, 0.226 mol) in diglyme (150 ml) over 3 h (Zweifel and Brown, 1963). During the reaction the dark iodine colour was seen to fade to leave a pale amber solution. This borate ester was then hydrolysed by addition of saturated NaCl solution (30 ml) and stirring for 10 min. The mixture was then extracted with ether (2 x 75 ml) and the organic extracts washed with saturated NaCl solution (50 ml) and water (2 x 50 ml). The organic solution was dried over $MgSO_4$ and the solvent removed *in vacuo* below 45°C to yield a thick yellow oil (18.2 g) which was 6-iodohexan-1-ol [^1H n.m.r. ($CDCl_3$) δ 1.35-1.55 (6H, m), 1.85 (2H, m), 3.15 (2H, t, J = 8 Hz) 3.38 (1H,s), 3.52 (2H, t, J = 8 Hz) p.p.m.]. This was converted immediately to the tetrahydropyranyl ether by dissolving the residue in CH_2Cl_2 (40 ml), adding dihydropyran (14 ml, 0.18 mol) and PPTS (2.5 g, 0.01 mol, prepared as described by Miyashita, *et al.* (1977)) and stirring at room temperature for 3 hours. Diethyl ether (100 ml) was then added and the mixture washed with saturated

NaCl soln. (100 ml) and water (2 x 75 ml). The organic portion was dried over $MgSO_4$ and the solvents removed *in vacuo*, to leave an amber oil. This was purified by flash column chromatography (eluting with petroleum ether b.p. 40-60°C/acetone, 15/1, v/v) to yield a colourless oil (Distillation results in decomposition) (21.7 g, 70%). ^{1}H n.m.r. (CCl_4) δ 1.35-1.7 (10H, m), 1.75-1.95 (4H, m), 3.18 (2H, t, J = 8 Hz), 3.25-3.5 (2H, m), 3.6-3.8 (2H, m), 4.5 (1H, t, J = 2 Hz) p.p.m.; ^{13}C n.m.r. ($CDCl_3$) δ 6.4, 19.3, 24.9, 25.2, 29.2, 30.0, 30.4, 33.1, 61.8, 66.9, 98.4 p.p.m.; Analysis C, 42.0; H, 6.86; I, 40.1%; $C_{11}H_{21}IO_2$ requires C, 42.32; H, 6.78; I, 40.65.

5.5.2 Preparation of 7-Chloro-7,7-bis(diisopropylphosphonato)heptyltetrahydropyranyl Ether

Tetraisopropyl chloromethylenebisphosphonate (5 g, 0.0132 mol) was dissolved in dry THF (15 ml) under nitrogen. Alkylation with 6-iodohexyltetrahydropyranyl ether (25 g, excess) was carried out as described in Section 3.3.3. After the usual work-up the product was partially purified by column chromatography (elution, petroleum ether b.p. 40-60°/acetone, 12/1, v/v). This returned the unreacted iodide, and a mixture of the iodide and the title compound (ratio about 1:12) as an oil (3.85 g). A small sample was rechromatographed for n.m.r. analysis, but the bulk of the product was reacted in the next stage without further purification. [^{1}H N.m.r. ($CDCl_3$) δ 1.32 (24H, m), 1.45-1.7 (14H, m), 1.7-1.9 (2H, m), 2.0-2.15

(2H, m) 3.35-3.55 (1H, m), 3.95-4.15 (2H, m), 4.85 (4H, m) p.p.m.]

5.5.3 Preparation of 7-Chloro-7,7-bis(diisopropylphosphonato)heptan-1-ol

The residue from above was dissolved in ethanol (120 ml) and transesterified using PPTS at elevated temperature as described (Miyashita, *et al.*, 1977). Purification of the product by flash column chromatography (eluting first with petroleum ether b.p. 40-60°/acetone, 10/1, v/v changing to 3/1, v/v after removal of the starting materials) yielded the pure title compound (2.55 g, 40.3% from tetraisopropyl chloromethylenebisphosphonate). [^{1}H N.m.r. ($CDCl_3$) δ 1.2-1.8 (32H, m), 2.0-2.2 (2H, m), 3.62 (2H, t, J = 8 Hz), 4.75-4.9 (4H, m) p.p.m.; ^{31}P n.m.r. ($CDCl_3$) δ 14.75(s); ^{13}C n.m.r. ($CDCl_3$) δ 23.0, 24.0, 24.1 (t, J = 6 Hz), 25.1, 29.3, 34.2, 35.6, 61.9, 62.5 (t, J = 145 Hz), 72.9, 73.1 p.p.m.; mass spec (CI) m/z 479, 481 (3:1)]

5.5.4 Preparation of 7-Chloro-7,7-bis(diisopropylphosphonato)heptanal

The alcohol prepared above (480 mg, 1 mmol) was oxidised with DMP as described (Dess and Martin, 1983) to yield the pure title compound (445 mg, 93%). [^{1}H N.m.r. ($CDCl_3$) δ 1.3-1.45 (26H, m), 1.55-1.75 (4H, m), 2.0-2.2 (2H, m), 2.4 (2H, t, J = 8 Hz), 4.8-4.95 (4H, m), 9.75 (1H, t, J = 1.5 Hz) p.p.m.; ^{31}P n.m.r. ($CDCl_3$) δ 14.75(s); ^{13}C n.m.r. ($CDCl_3$) δ 21.6, 23.4, 24.1 (t, J = 6 Hz), 24.3, 29.2, 35.7 (t, J = 6 Hz), 43.5, 62.5 (t, J = 14.5 Hz) 72.8,

73.0, 202.3 p.p.m.; mass spec. (CI) m/z 477, 479 (3:1)].

5.5.5 Preparation of 7-Chloro-7,7-bisphosphonato-heptan-1-ol

The alcohol prepared in Section 5.5.3 (1.5 g, 3.13 mmol) was dissolved in pyridine (9 ml) and HMDS (1.3 ml, 6.2 mmol) and ClTMS (0.55 ml, 4.3 mmol) added in a dry nitrogen atmosphere. The mixture was stirred at room temperature for 15 min., and the resultant white precipitate removed by filtration. The filtrate was lyophilised to leave the trimethylsilyl ether [^{1}H n.m.r. ($CDCl_3$) $-CH_2OSiMe_3$ δ 3.54 (t, J = 8 Hz) p.p.m.; mass spec. (CI) m/z 551, 553 (3:1)]. The ether was redissolved in CCl_4 (2 ml), BrTMS (6 ml, 45 mmol) added and the mixture stirred at room temperature for 20 h. The usual work up (Section 3.3.8) provided the fully hydrolysed alcohol (0.905 g, 93%) [^{1}H n.m.r. (D_2O) δ 1.2-1.4 (4H, m), 1.4-1.6 (4H, m), 2.0-2.2 (2H, m), 3.55 (2H, t, J = 8 Hz) p.p.m.; ^{31}P n.m.r. (D_2O) 17.3 p.p.m.(s)].

5.5.6 Preparation of 7-Chloro-7,7-bisphosphonato-heptanal

The fully hydrolysed alcohol from above (800 mg, 2.58 mmol) was suspended in pyridine (6 ml) and acetonitrile (12 ml). DMP (1.3 g, 3.06 mmol) was added and the mixture stirred at room temperature under nitrogen for 18 h. The solvents were removed *in vacuo* and water (5 ml) added to the dried mixture, and the insoluble matter removed by filtration. Triethylammonium bicarbonate (1 M, 10 ml) was added to the filtrate and the mixture lyophilised. The residue was purified by ion exchange chromatography on a DEAE Sephadex-A25 column (50 g, dry support, eluting with

$Et_3NH^+HCO_3^-$, pH 8.4, 0-1 M gradient) to yield the pure aldehyde as the bis(triethylammonium) salt (622 mg, 40%). [^1H N.m.r. (D_2O) δ 1.2 (20H, m), 1.4-1.7 (4H, m), 1.95-2.15 (4H, m), 3.12 (12H, q, J = 8 Hz), 9.65 (1H, t, J = 1.5 Hz) p.p.m.; ^{31}P n.m.r. (D_2O) δ 15.0 p.p.m.(s); Analysis C, 44.75; H, 9.37; N, 6.0%; $C_{19}H_{44}ClN_2O_7P_2$ requires C, 44.75; H, 8.8; N, 5.5%].

5.5.7 Affinity Labelling of Influenza Virus

Influenza A/X49 virus, prepared as described in Section 4.1.1, was incubated at 30°C with the affinity label (2 mM) as described by Romanos and Hay (1984). The complex formed was reduced with [^3H]$NaBH_4$ (3 mCi), and the proteins isolated by sedimentation through a 10% sucrose cushion (w/v in 10 mM *tris*-HCl, pH 7.4) (40,000 r.p.m., 6 x 20 angle rotor). The proteins were separated by polyacrylamide gel electrophoresis on a 17% discontinuous gel, and the gel fluorographed by soaking in commercially available fluor (Amplify) for 30 min., drying and exposing to Fuji X-ray film for 11 days at -70°C.

CHAPTER 6

POLIO AND OTHER PICORNAVIRUSES

6.1 INTRODUCTION

Poliomyelitis has been all but eradicated throughout most of the developed world by the introduction of effective vaccines (e.g., Sabin, 1959) as a result of intensive research since the 1930's. Despite this the virus is still widely investigated in molecular biology, as the simplicity of its structure makes it an attractive species through which to study complex molecular processes, many of which are common to both viruses and animal cells. There are also several other related viruses which are not under the same control.

Polioviruses are members of the family of picornaviridae (a compound word made up of Pico/RNA/viridae, i.e., small RNA viruses) and the genus enteroviruses. Other genera in the family include rhinoviruses (responsible for the common cold), cardioviruses (causing infections often localised in the heart muscle) and foot and mouth disease viruses (affecting livestock) (Newman, *et al.*, 1973). All would appear to be evolved from a common ancestor, since their biology is remarkably similar, with mutations occurring due to external influences (such as pH) allowing growth of the viruses in a greater variety of cells.

6.2 STRUCTURE OF POLIOVIRUS

Picornaviruses are probably the simplest known animal viruses. The polio virion consists of a non-enveloped icosahedral protein nucleocapsid of about 22 nm in diameter, containing one molecule of positive single stranded RNA. The virion has a weight of about 6.2×10^6, of which around 30% is the weight of the RNA. The protein coat is formed from 60 molecules of each of four proteins (Rueckert, 1971) known as virion proteins (VP) 1, 2, 3 and 4, of molecular weights 37,000, 31,000, 26,000 and 8,000 respectively. Because the virion has no lipid envelope, it is able to withstand attack from both water-immiscible solvents, and anionic detergents such as sodium dodecyl sulphate (SDS). Indeed treatment with SDS forms the basis of the purification of virions and their isolation from cell debris.

Polio virion RNA has been shown to be infectious (Baltimore, 1969) and has a molecular weight of about 2.6×10^6, corresponding to a length of around 7,500 bases. The 3'-terminus consist of polyadenylic acid (Yugo and Wimmer, 1972) with an estimated length of 75 nucleotides (Spector and Baltimore, 1975). Removal of this poly(A) results in greatly reduced infectivity of the remaining RNA (Spector and Baltimore, 1974).

The 5'-terminus of vRNA has received much attention in recent years and although it was first suggested that it terminated with pAp (Wimmer, 1972) it has since been shown that the 5'-terminus is pUp,

covalently linked through its phosphate to the tyrosine hydroxyl of a short peptide called VPg (Lee, *et al.*, 1977). This 5'-terminal is not required for infectivity and is cleaved from the vRNA prior to protein synthesis, so that most of the intracellular RNA lacks VPg (Lee, *et al.*, 1977).

6.3 REPLICATION OF POLIOVIRUS

6.3.1 Initial Stages of Infection

Polioviruses only infect human or simian cells, but the isolated vRNA can be used to infect non-susceptible cells (Baltimore, 1969), probably because it can bypass the specific cell receptors responsible for binding the intact virion. For other picornaviruses it has been demonstrated that the protein VP4 is involved in the adsorption process, and that if the virion lacks VP4 it is not adsorbed (Crowell and Phillipson, 1971).

The ensuing events are not fully understood, but the attached virus somehow becomes uncoated at the membrane receptor and the vRNA enters the cytoplasm. Between three and four hours after infection host cell protein and RNA synthesis is completely inhibited (Baltimore, 1969) and it has been suggested that virally coded proteins may be responsible (Wright and Cooper, 1974; Baltimore, 1969). It has been shown by pulse labelling experiments that viral synthesis occurs only in the cytoplasm (Baltimore, 1969) and enucleated cells are capable of carrying out the entire virus life cycle (Pollack and Goldman, 1973).

6.3.2 Translation of Virus mRNA

Translation of infecting RNA must begin prior to viral RNA synthesis since virus specific proteins are required for the latter. Many virally coded proteins have been isolated from infected cells and the sum of their molecular weights greatly exceeds the coding capacity of the mRNA, resulting in the proposal that extensive secondary processing occurs, i.e., the final gene products are derived from larger initial translation products (Summers and Maizel, 1968).

The degradation pathway has been partially elucidated (Fig. 6.3.2) by the use of pulse-chase labelling

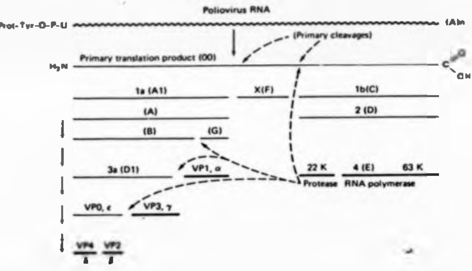

Fig. 6.3.2 Processing of poliovirus proteins.

experiments, as well as tryptic digest fingerprinting with amino acid analogue inhibitors of protolytic cleavage (Baltimore, 1971).

The polyprotein NØØ is the primary translation product if all proteolytic activity is inhibited. It has a molecular weight of > 200,000 and as such represents the total translatable information carried by the poliovirus RNA (Jacobson, *et al.*, 1970). In the normal course of events, this polyprotein is subject to nascent cleavage to give the primary digestion products N1a and N1b. A premature termination mechanism for formation of these proteins can be discounted since they are formed at the same stage (along with NX) and only a single initiation site has been identified (Jacobson and Baltimore, 1968). Such nascent cleavages have been shown to occur in cell free systems, but subsequent protein processing does not occur (Villa-Komoroff, *et al.*, 1975) showing that further cleavages take place in the cytoplasm. It is here that N1b is processed in two further steps to produce the protein N4 and the virally coded protease of molecular weight 22,000. It is uncertain whether this protease is responsible for the nascent cleavages (i.e., that it is enzymically active whilst still part of the polyprotein) or whether host cell enzymes exact this primary step, but the viral protease is known to perform all the secondary cleavages to produce the final gene products (Fraenkel-Conrat, *et al.*, 1982).

The N4 protein is a component of the RNA polymerase. In combination with a host cell protein, it has been shown to be capable of performing both steps

of the RNA replication cycle (Baltimore, 1984).

Protein N1a is processed into the structural proteins of the procapsid-VPO, 1 and 3 - and VPO is further cleaved into VP2 and VP4 during virion morphogenesis (Jacobson and Baltimore, 1968a). Other transitory products shown (Fig. 6.3.2) may be responsible for the previously mentioned supression of host cell processes, or in a viral release mechanism.

6.3.3 Transcription of Viral RNA

Kinetic studies have revealed that RNA replication begins within half an hour of infection and increases exponentially until a maximum is reached after about four hours, after which it is linear until ceasing about eight hours post-infection (Baltimore, 1969). Three types of intracellular RNA have been identified and implicated in poliovirus replication. The first, intracellular single stranded mRNA, differs from vRNA only in that it lacks the 5'-terminal protein VPg (Lee, *et al.*, 1977), while their nucleotide sequences are identical (Baltimore, 1984). A double stranded RNA containing the plus strand and a complete complementary minus strand of RNA has also been isolated and is thought to be a byproduct of the replication process (Baltimore, 1984). The third type of RNA is the replicative intermediate (RI) RNA which also contains a minus strand which serves as the template for plus strand synthesis (the opposite sense RI has never been detected but is presumed to exist). Because both minus and plus strands are synthesised, two

initiation processes are required and one model has the same initiator involved in both cases.

The location of poly(A) at the 3'-end of viral RNA and the fact that it is not involved in translation (Spector, *et al.*, 1975) led to speculation that it might be implicated in initiation of minus strand synthesis (which must commence at the 3'-terminus). This was reinforced when it was shown that infected cells contain a poly(A) dependent poly(U) polymerase activity (Flanegan and Baltimore, 1977). This enzyme was used to copy viral RNA (Dasgupta, *et al.*, 1979) and in combination with a host cell enzyme forms the poliovirus RNA replicase (Baron and Baltimore, 1982).

Use of anti-protein antibodies on the replicase reactions has indicated that the virally coded protein, VPg, must be involved in the initiation reaction and this, along with the discovery of VPgpUpU in infected cells (Crawford and Baltimore, 1983) suggested the replication model shown in Fig. 6.3.3(i).

It is thought that VPg or a precursor of VPg is a substrate for enzymatic uridylation at the tyrosine hydroxyl. The resultant molecule then binds to the replicase and plus strand RNA by hybridisation to the 3'-poly(A) and is then elongated to give the minus strand. The minus strand is then copied by hybridisation of VPgpUpU to the two 5'-As of the minus strand followed by elongation by the replicase (Baltimore, 1984).

A recent extension to this mechanism has been proposed that has the plus and minus strands synthesised

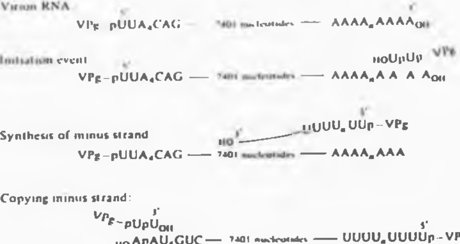

Fig. 6.3.3(i) Model of poliovirus replication in which VPgpUpU is involved in initiation of synthesis of both plus and minus strands.

by different means (Fig. 6.3.3.(ii)). It is suggested that synthesis of minus strands on a plus strand template is actually initiated by terminal uridyl transferase which adds a short chain of uridyl residues onto the poly(A) end of the poliovirion RNA molecule. This oligo(U) chain soon hydrogen bonds to the poly(A), and further elongation gives a double stranded RNA structure which, it is hypothesised, is cut by a pre-VPg on a cellular

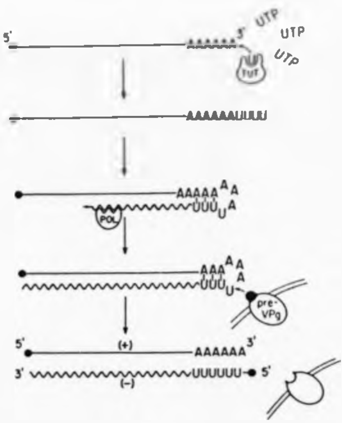

Fig. 6.3.3(ii) Model for the role of terminal uridyl transferase in replication of poliovirus RNA.

membrane, liberating two strands (Andrews, *et al.*, 1985).

It is possible that VPg-pUpU is involved in initiation of plus strand synthesis by this mechanism, but there is no direct evidence of this from labelling studies (Andrews, *et al.*, 1985).

6.3.4 Assembly and Release

Assembly of polioviruses has been widely

studied and there is good evidence for the model shown in Fig. 6.3.4. Five N1a protein molecules appear to

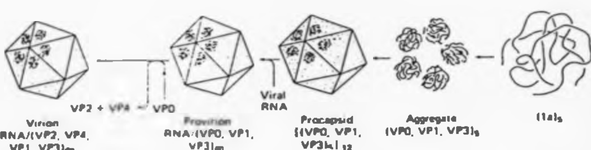

Fig. 6.3.4 Assembly of the poliovirion.

aggregate and then cleave to the constituent proteins VPO, 1 and 3, which further aggregate to form the procapsid (Jacobson and Baltimore, 1968a). The next stage is association with vRNA to form the provirion (Fernandez-Thomas and Baltimore, 1973). The vRNA of the provirion is insensitive to RNAase digestion implying that it is inside the particle (Rekosh, 1977). The final stage is proteolysis of VPO to VP2 and VP4, resulting in the formation of the virion particle (Jacobson and Baltimore, 1968a) although some VPO remains uncleaved. Virus release is much less well studied and the control mechanism is not understood, although complete virions leave the cytoplasm after cell rupture and some virus-specific protein may be involved (Rekosh, 1977).

6.4 TARGETS FOR INHIBITION OF REPLICATION

Despite the highly successful vaccines for poliovirus cases still occur, and other picornaviruses, which have very similar biological properties, are widespread amongst humans and livestock. Thus, with the current advanced state of knowledge of picornavirus replicative processes *in vivo*, potential targets for antiviral chemotherapy can be proposed and examined more closely.

Since many of the infections caused by picornaviruses are relatively mild, it is especially important in their case to ensure that uninfected cells are not disrupted by chemotherapy. Thus, only virus specific events or proteins must be selected as targets for inhibition. Clearly from the preceding discussion, there are several poliovirus specific processes which represent potential candidates for the inhibition of viral replication.

6.4.1 Inhibition of Initial Stages of Infection

Since the viral protein VP4 is required for attachment and penetration (Crowell and Phillipson, 1971), it represents a possible line of attack. Myxoviruses have been inhibited by oligopeptides of similar sequence to the virus fusion protein (F protein) (Choppin, *et al.*, 1983) which is thought to perform a similar function to VP4. However, a greater understanding of the attachment and penetration phases of the infection is required

before an inhibitor of these processes can be designed rationally.

6.4.2 Inhibition of Intracellular Events

As mentioned in Section 6.3.3, poliovirus has a virally induced RNA dependent RNA polymerase activity, and such virus specific enzymes provide good targets for antiviral agents since they are absent from uninfected cells. This enzyme may be the site of action of the poliovirus inhibitor, guanidine hydrochloride (Baltimore, 1969).

Another essential virus specific protein is the virally coded protease (Section 6.3.2), and a specific inhibitor of this enzyme would shut down the virus secondary protein processing and prevent formation of the structural proteins. This protease is probably responsible for the proteolytic cleavage of VPg from the vRNA to form the intracellular mRNA (Fig. 6.4.2) since it can be seen from comparison of the nucleotide sequences that an RNAase cannot be responsible for the cleavage (Lee, *et al.*, 1977). Thus, inhibition of the protease may also interfere with formation of primary gene products by preventing release of mRNA.

The unmasking of vRNA to mRNA could also be prevented by modification of groups around the nucleopeptide linkage so as to make the bonds chemically inert to hydrolysis. If any method of blocking the removal of VPg were found, the modified 5'-terminus could be incorporated into the vRNA by using a suitable precursor as

(i) VPg-O-p-UUAAACAG......

(ii) p-UUAA ACAG......

(iii) GAYTGLPNKKPNVPTIRTAKVQ
  |
  O~~~

Fig. 6.4.2 Poliovirus RNAs (i) mRNA, (ii) vRNA, (iii) sequence of VPg (one letter amino acid codes).

a primer for vRNA synthesis according to the model described (Section 6.3.3).

For this preliminary study it was decided to focus on synthetic procedures for the preparation of simple nucleopeptide model compounds of the poliovirus vRNA.

CHAPTER 7

SYNTHESIS OF NUCLEOPEPTIDE FRAGMENTS

7.1 INTRODUCTION

Several routes to simple nucleopeptides can be envisaged. Phosphorylation of one fragment followed by reaction with the second fragment, is one conceivable method. Phosphorylations of both tyrosine (Alewood, *et al.*, 1983) and tyrosine containing peptides (Ferrell, *et al.*, 1948) are known and uridine 5'-monophosphate is commercially available. Many reagents are known for coupling of such species to alcohols via mixed anhydrides (e.g., Reese,1978). Such a stepwise method would be wasteful, since low yields are observed for phosphorylation and coupling, and time consuming as several steps are required. Hence the most attractive route would be a 'one-pot' synthesis of the type used for coupling of nucleotides through a 3'→5' phosphate linkage.

The synthetic strategy adopted then, was to prepare small protected peptides, corresponding to portions of the sequence of VPg. The free hydroxyl of the tyrosyl residue would then be coupled to a protected uridine using one of the so-called 'bifunctional' phosphorylating agents in analogy to the phosphotriester approach to nucleotide synthesis (Reese, 1978). The resulting fully blocked nucleopeptide would then be deprotected to give the desired peptidyluridylphosphodiester.

7.2 RESULTS AND DISCUSSION

7.2.1 Protection and Coupling of Amino Acids

The first phase of the synthetic route was the preparation of the protected building blocks.

Amino acids were protected using well established methods. The benzyloxycarbonyl group introduced by Bergmann and Zervas (1932) was used to block the amino group of the N-terminal amino acid. The next amino acid in the sequence was protected as its ethyl ester hydrochloride by the method of Curtius (Greenstein and Winitz, 1961) before being coupled to the protected N-terminal residue.

Two methods were used for the coupling reactions. The first involved use of phosphorus oxychloride (Wieland and Heinke, 1956) which activates the carboxylic acid reagent by formation of a mixed carboxylic-dichlorophosphoric acid anhydride which then reacts with the amine reactant (Fig. 7.2.1(i)) leading to the desired product. The simple isolation of the pure product from the reaction was the major advantage of this method, however a serious disadvantage was found in that the t-butyl ether group used to protect the hydroxyl side chain of threonine was labile in the presence of phosphorus oxychloride. Thus a second coupling method was required to which this protecting group would be stable. Dicyclohexylcarbodiimide (DCC) was selected as a non-acidic, well established coupling reagent (Khorana, 1952). The mechanism of formation of the peptide again involves intermediacy of

Fig. 7.2.1(i) Mechanism of $POCl_3$ activated amino acid coupling.

Fig. 7.2.1(ii) Mechanism of DCC activated amino acid coupling.

TABLE 7.2.1 Preparations of Protected Peptides

Protected Peptide	Method	Yield	^{1}H n.m.r., p.p.m. (Solvent)
Z-Ala-Tyr-OEt	$POCl_3$	72	1.1-1.3 (6H, m), 3.0 (2H, m), 4.15 (3H, m) 4.6 (1H, t, J = 7 Hz), 5.1 (2H, s), 6.7 (2H, d, J = 8 Hz), 7.0 (2H, d, J = 8 Hz), 7.35 (5H, m). (CD_3OD)
Z-GlyAla-OEt	$POCl_3$	69	1.3 (3H, t, J = 8 Hz), 1.42 (3H, d, J = 7 Hz), 3.9 (2H, br.m), 4.2 (2H, q, J = 8 Hz), 4.6 (1H, m), 5.18 (2H, s), 7.38 (5H, m). (CD_3OD)
Z-Tyr-Thr(O^{t}Bu)OEt	DCC	80	1.1 (12H, m), 1.3 (3H, t, J = 8 Hz), 3.05 (2H, m), 4.0-4.3 (5H, m), 4.5 (2H, m), 5.15 (2H, s), 6.7 (2H, d, J = 8 Hz), 7.05 (2H, m), 7.35 (5H, m). ($CDCl_3$)
Z-Thr(O^{t}Bu)GlyOEt	DCC	76	1.1-1.3 (15H, m), 4.0 (2H, m), 4.15 (3H, m), 4.35 (1H, m), 5.12 (2H, s), 7.35 (5H, m). ($CDCl_3$)
Z-Tyr-Thr(O^{t}Bu)GlyOEt	DCC/$POCl_3$	22	1.1-1.3 (15H, m), 3.0-3.1 (2H, m), 4.0 (2H, m), 4.1-4.4 (5H, m), 5.1 (2H, s), 6.7 (2H, d, J = 8 Hz), 7.1 (2H, m), 7.35 (5H, m). (CD_3OD)
Z-GlyAlaTyr-OEt	$POCl_3$	34	1.0 (3H, t, J = 8 Hz), 1.4 (3H, d, J = 7 Hz), 3.15 (2H, m), 3.95-4.25 (4H, m), 5.0 (4H, m), 7.0-7.3 (9H, m). (d^5-pyridine)
Z-AlaTyrThr(O^{t}Bu)OEt	DCC	49	1.1 (12H, m), 1.28 (6H, m), 2.9-3.25 (2H, m), 4.1-4.3 3H, m), 4.5 (1H, d, J = 3 Hz), 4.65-4.8 (2H, m), 5.15 (2H, s), 6.7 (2H, m), 7.1 (2H, m), 7.35 (5H, m). (CD_3OD)

an activated species, this time formed initially between the carboxylic acid reagent and the diimide, followed by reaction of the active anhydride with the amine reagent (Fig. 7.2.1(ii)). Under these conditions the t-butyl ether remained intact, but the work-up procedures were more laborious due to the dicyclohexyl urea residue present in the product and the reluctance of the t-butyl ether compounds to recrystallise.

If a tripeptide were to be prepared, the C-terminal ethyl ester protecting group of the dipeptide was removed by saponification and the product coupled with a further carboxy-protected amino acid (Fig. 7.2.1(iii)) to give the desired sequence tripeptide (Table 7.2.1).

7.2.2 Protection of Uridine

The uridine, to which the fully blocked peptides would be coupled, was protected as the well known 2',3'-isopropylidene ketal, by reaction with acetone and an acid catalyst (Fig. 7.2.2), in good yield (Tipson, 1968).

7.2.3 Coupling of Uridine to Peptides through a Phosphate

A method was required for coupling of the two protected fragments, each with a single free hydroxyl, through a phosphate linkage. In the field of nucleotide synthesis, 3'→5' phosphate linkages can be introduced by sequential addition of a single equivalent of each of the properly protected nucleotides to a phosphodichloridate modified by reaction with a triazole (Broka, *et al.*, 1980) or hydroxybenzotriazole (van der Marel, *et al.*, 1981).

Fig. 7.2.1(iii) Synthesis of protected peptides.

Fig. 7.2.2 Preparation of 2',3'-O-isopropylidene uridine.

Such an approach has been used to prepare nucleopeptides of uridine by reaction of the hydroxybenztriazole derivative of 2-chloro phenylphosphodichloridate first with a protected uridine and then with a tyrosine containing peptide derivative (Fig. 7.2.3(i); Schattenkerk, *et al.*, 1984). However, in this study this work was not reproducible. Although the uridine residue reacted smoothly under the conditions described, no aryl species could be induced to react with the intermediate to form the triester directly. This failure to react was assumed to be due to the lack of nucleophilicity of the aryl hydroxyl, and efforts to improve the reactivity by addition of tertiary bases such as pyridine and 1-methylimidazole were also unsuccessful. Hence, another coupling method was required if this route was to prove fruitful. Again, a method widely used in nucleotide synthesis was explored. Letsinger, *et al.* (1975) introduced the phosphite method for preparation of 3'→5' nucleotide phosphate linkages, in which the two nucleotides are added stepwise to a dichlorophosphite and the P(III) product oxidised to the P(V) species with iodine and water.

The phosphite species used is considerably more reactive than the phosphate employed in the previous method, and the reaction is complete in one hour even at -78°C. In this study, methyl dichlorophosphite proved sufficiently reactive to allow reaction, firstly with the hydroxy group of a tyrosine containing protected peptide, and secondly with a protected uridine, at low temperature. Subsequent room temperature oxidation to the P(V) product and

Fig. 7.2.3(i) Preparation of protected nucleopeptides by the phosphate route.

Fig. 7.2.3(ii) Preparation of protected nucleopeptides by the phosphite route.

work-up gave the fully protected nucleopeptide as a fluffy white solid (Fig. 7.2.3(ii)). Modest yields were obtained in this manner (Table 7.2.3) but sufficient material was isolated to proceed with the deprotection phase. Improved yields may be obtainable by modification of the dichlorophosphite with triazole or tetrazole, which it is claimed can give a more selective 'bifunctional' species for nucleotide synthesis (Fourrey and Shire, 1981).

Subsequent to this work, dialkyl phosphochloridites have been used to phosphorylate N-protected hydroxy containing amino acids in good yield (Perich, *et al.*, 1986) again showing the increased reactivity of the P(III) species.

7.2.4 Deprotection of nucleopeptides

Having prepared the fully blocked species, deprotection to the free acid nucleopeptide was required. The major advantage of the protecting groups used was their compatibility, that is they could be removed selectively if desired. Benzyloxycarbonyl groups can be readily removed by hydrogen donor hydrogenation (Anantharamaiah, *et al.*, 1977), ethyl esters of peptides by saponification (Bodansky and Bodansky, 1984) and t-butyl ethers by treatment with cold trifluoroacetic acid (Beyerman and Bontekoe, 1961). The 2',3'-isopropylidene ketal of uridine is readily hydrolysed under mild acid conditions, and the phosphate methyl ester can be selectively removed by treatment with thiophenol and triethylamine (Daub and

Table 7.2.3 Protected uridine nucleopeptides prepared by the phosphite route

Protected Peptide	Yield (%)	^{31}P n.m.r. (p.p.m.)
N-Ac-Tyr-OEt	57	-5.10
Z-Ala-Tyr-OEt	40	-4.85, -4.97
Z-Tyr-Thr(O^{t}Bu)OEt	45	-4.79, -4.91

van Tamelen, 1977).

Initially, however, a single step procedure for removal of all protecting groups was sought for brevity. Boron tribromide is known to remove all the amino acid protecting gorups used in this study but does not affect peptide bonds (Felix, 1974). When the fully protected nucleopeptide was treated with this reagent, it was found that the isopropylidene group was easily hydrolysed (which might be expected in view of the highly acidic nature of the reagent) and the phosphate methyl ester group was also removed to the extent of about 60% (by ^{1}H n.m.r.) during the reaction time, along with the expected deblocking of all the amino acid functional groups. Thus, purification of the product mixture by ion exchange on DEAE Sephadex and Dowex 50, afforded the pure products as a white fluffy solid in yields of between 10 and 15%. The low yield was due primarily to the incomplete hydrolysis of the methyl ester group, which could, if improved yields were required, be completed by a brief treatment with thiophenol and triethylamine, and secondly to a side reaction of cleavage of the base-sugar bond of the uridine to give uracil. This could be observed as occurring in 8-10% of the product by ^{1}H n.m.r. Boron tribromide has been used to cleave benzyl groups from dibenzyl uracil at elevated temperature and prolonged reaction times (Kundu, *et al.*, 1980) and clearly the more fragile sugar-base bond is susceptible to such vigorous acid hydrolysis even under the much milder conditions employed here.

Thus, it has been demonstrated that nucleopeptide

fragments of the type found in poliovirus vRNA can be readily synthesised. The next stage would be to examine the hydrolysis of such species by the poliovirus coded protease and to design an inhibitor of that protease based on the observations made.

7.3 EXPERIMENTAL

7.3.1 Preparation of Amino Acid Ethyl Ester Hydrochlorides

The required amino acid (20 g) was suspended in ethanol (300 ml) and dry HCl gas bubbled through until the amino acid had dissolved. The mixture was then heated under reflux for 3 h during which time the stream of gas was maintained. The mixture was cooled and evaporated to dryness twice from ethanol. The resultant solid was dried *in vacuo* over NaOH and recrystallised from ethyl acetate [TyrOEt.HCl, yield 19.53 g, 72%; ^{1}H n.m.r. (D_2O) δ 1.25 (3H, t, J = 7 Hz), 3.2 (2H, m), 4.2-4.4 (3H, m), 6.8-7.2 (4H, ABm) p.p.m.; AlaOEt.HCl, yield 29.3 g, 85%; ^{1}H n.m.r. (D_2O) δ 1.25 (3H, t, J = 7 Hz), 1.5 (2H, d, J = 8 Hz), 4.1-4.3 (3H, m) p.p.m.; GlyOEt.HCl yield 29.0 g, 78%; ^{1}H n.m.r. (D_2O) δ 1.25 (3H, t, J = 7 Hz), 3.95 (2H, s), 4.3 (2H, quart., J = 7 Hz)p.p.m.]

7.3.2 Preparation of Amino Acid Benzyl Carbamates

Benzyloxycarbonylalanine, benzyloxycarbonyl-tyrosine and benzyloxycarbonyl glycine were purchased from Lancaster Synthesis Ltd. (Morecombe, Lancs.). Benzyloxy-carbonyl threonine was prepared as described (Bodansky

and Bodansky, 1984) in 66% yield.

7.3.3 Preparation of O-t-Butylthreonine Ethyl Ester

Benzyloxycarbonyl threonine (10 g, 39.5 mmol) was reacted with ethyl iodide as described by Bocchi, *et al.*, (1979) to give the corresponding ethyl ester which was purified by column chromatography (elution, petroleum ether b.p. 40-60°/acetone, 6/1, v/v). [Yield, 9.92 g, 34.8 mmol, 88%; ^{1}H n.m.r. ($CDCl_3$) δ 1.2 (6H, m), 2.4 (1H, br.s), 4.1-4.3 (4H, m), 5.1 (2H, s) 5.65 (1H, m) 7.3 (5H, m) p.p.m.]

Benzyloxycarbonylthreonine ethyl ester (3.9 g, 13.67 mmol) was dissolved in CH_2Cl_2 (30 ml) and cooled to 0°C. 2-Methylpropene (36 ml) and conc. H_2SO_4 (0.4 ml) were added and the mixture stoppered and left at room temperature for 4 days. The mixture was then cooled to 0°C and washed with cold 5% (w/v) $NaHCO_3$ (3 x 40 ml). The washings were re-extracted with CH_2Cl_2 (2 x 40 ml) and the combined organic portions washed with water (50 ml), dried over $MgSO_4$ and the solvent removed *in vacuo*. The product was purified by column chromatography (elution, petroleum ether b.p. 40-60°/acetone, 20/1, v/v). [Yield 3.40 g, 73%; ^{1}H n.m.r. ($CDCl_3$) δ 1.1 (9H, s), 1.2-1.3 (6H, m), 4.0-4.25 (4H, m), 5.1 (2H, s) 5.5 (1H, m), 7.35 (5H, m) p.p.m.]

The benzyl carbamate was then removed by hydrogenolysis as described (Anantharamaiah, *et al.*, 1977) in 78% yield [^{1}H n.m.r. ($CDCl_3$) δ 1.1 (9H, s), 1.25-1.4 (6H, m), 3.3 (1H, d, J = 2 Hz) 4.0-4.4 (4H, m) p.p.m.]

7.3.4 Preparation of Peptides

7.3.4.1 Phosphorus Oxychloride Method

The appropriately protected amino acids or peptides were reacted as described by Greenstein and Winitz (1961) and the resultant protected peptides recrystallised from methanol/water (yields and analytical data in Table 7.2.1).

7.3.4.2 Dicyclohexylcarbodiimide (DCC) Method

The protected amino acids or peptides containing a t-butyl ether group were coupled using DCC as described by Bodansky and Bodansky (1984) and the resultant protected peptides purified by column chromatography (yields and analytical data in Table 7.2.1).

7.3.5 Saponification of Dipeptide Ethyl Esters

Saponification of dipeptide ethyl esters was carried out as described (Bodansky and Bodansky, 1984) (yields and analytical data in Table 7.2.1).

7.3.6 Preparation of 2',3'-O-Isopropylidene Uridine

Uridine (10 g, 0.041 mol) was reacted with acetone as described (Tipson, 1968) and the product (8.73 g, 75%) recrystallised from methanol [^{1}H N.m.r. (D_2O) δ 1.4 (3H, s), 1.6 (3H, s), 3.8 (2H, m), 4.35 (1H, m), 4.9 (1H, m), 5.1 (1H, m), 5.85 (2H, m), 7.75 (1H, d, J = 8 Hz) p.p.m.]

7.3.7 Preparation of Methyl dichlorophosphite

Methyl dichlorophosphite was prepared from PCl_3 and methanol as described (Martin and Pizzolato, 1950) and the product distilled twice prior to use (b.p. 92-93°C).

7.3.8 Preparation of Protected Uridyl-Peptidyl-Phosphates

Methyl dichlorophosphite (100 µl, 1.1 mmol) in THF/pyridine (4/1, v/v, 8 ml) was cooled to -78°C. The protected peptide (1 mmol) was dried by coevaporation with pyridine and added as a solution in THF (1 ml) and the mixture stirred for 15 min. at -78°C. Isopropylidene uridine (225 mg, 0.8 mmol) was dried by coevaporation with pyridine and added to the mixture as a solution in THF (1 ml) and stirring continued at -78°C for a further 30 min. The temperature was then allowed to rise to -30°C, when a mixture of iodine (500 mg) in water/THF (1/2, v/v 6 ml) was added and stirring continued for 10 min. at room temperature. The mixture was then evaporated to dryness and dissolved in $CHCl_3$ (30 ml). The organic solution was washed with 5% (w/v) NaS_2O_3 (10 ml) and water (10 ml), dried over $MgSO_4$ and evaporated to leave a white solid. The product was purified by column chromatography (elution, petroleum ether b.p. 40-60°C/ acetone, 1/1, v/v). Yields and analytical data are shown in Table 7.2.3).

7.3.9 Preparation of N-Acetyltyrosyluridylphosphate

The fully protected tyrosyluridylphosphate prepared as described above (270 mg, 0.44 mmol) was

dissolved in methanol (2 ml) and treated with 2 M NaOH (0.25 ml, 0.5 mmol) and stirred at room temperature for 1½ h. The mixture was neutralised by dropwise addition of conc. HCl. The residue was treated with Dowex 50 (H^+ form, 2 ml beads) at 70°C for 2 min. and passed down a short Dowex column. The resulting product was dissolved in a mixture of dioxan (7 ml), triethylamine (3 ml) and thiophenol (2.5 ml) and the mixture stirred at room temperature for 4 h. After this time, the solvents were removed *in vacuo* and the residue partitioned between water and ethyl acetate. The water layer was lyophilised and the product isolated by ion exchange chromatography on DEAE Sephadex A-25, eluting with a 0-0.5 M gradient of $HNEt_3^+HCO_3^-$, pH 8.3. The product was converted to the free acid by ion exchange on Dowex 50 (H^+ form) (30 mg, 13%). [^1H N.m.r. (400 MHz, D_2O) δ 1.83 (3H, s) 2.85-3.1 (2H, m), 4.05-4.1 (1H, m), 4.15 (3H, m), 4.5 (1H, dd, J_1 = 5.5 Hz, J_2 = 4.5 Hz), 5.6 (1H, d, J = 8 Hz), 5.82 (1H, d, J = 4.5 Hz), 7.0-7.1 (4H, m), 7.6 (1H, d, J = 8 Hz) p.p.m.; ^{31}P n.m.r. (162 MHz, D_2O) δ - 4.1 p.p.m.(s); Analysis C, 43.94; H, 4.55%; Calc. for $C_{18}H_{22}N_3O_{11}P$, C, 44.26; 4.51%.]

7.3.10 Preparation of Other Peptidyluridylphosphates

The protected nucleopeptide prepared as described above (250 mg) was dissolved in CH_2Cl_2 (25 ml) under nitrogen at -10°C. Boron tribromide (5 ml of 1 M solution in CH_2Cl_2) was added dropwise with stirring at -10°C. Stirring was continued for 2 h at 0°C and then for 1 h

at room temperature. Water (40 ml) was then added and the organic layer separated and extracted with water (3 x 30 ml). The combined aqueous portions were evaporated to dryness and the product isolated by ion exchange chromatography on DEAE Sephadex A-25 ($HNEt_3^+HCO_3^-$, pH 8.3, 0-0.5 M gradient) followed by conversion to the free acid by ion exchange on Dowex 50 (H^+ form).

[AlaTyr-p-Urd: Yield, 24.8 mg, 14.3%; ^1H n.m.r. (400 MHz, D_2O) 1.4 (3H, dd, J_1 = 7 Hz, J_2 = 3.5 Hz), 2.8-3.2 (2H, m), 4.1 (1H, m), 4.15-4.2 (7H, m) 5.6 (1H, d, J = 8 Hz), 5.85 (1H, d, J = 4.5 Hz), 7.0-7.1 (4H, m), 7.6 (1H, d, J = 8 Hz) p.p.m.; ^{31}P n.m.r. (162 MHz, D_2O) δ -4.1 p.p.m.(s); Urd-p-TyrThr: Yield, 27.5 mg, 16.1%; ^1H n.m.r. (400 MHz, D_2O) δ 1.0 (3H, d, J = 6.5 Hz), 2.95-3.05 (2H, m), 4.0 (1H, m), 4.05-4.15 (4H, m), 4.18 (1H, t, J = 7 Hz), 4.26 (1H, d, J = 4 Hz), 5.5 (1H, d, J = 8 Hz), 5.75 (1H, d, J = 4 Hz), 6.95-7.1 (4H, m) 7.55 (1H, d, J = 8 Hz) p.p.m.; ^{31}P n.m.r. (162 MHz, D_2O) δ -4.1 p.p.m.(s).]

APPENDIX I

SOURCES OF MATERIALS

Adenosine-(3'-5')guanosine

Sigma (London) Chemical Co.,
Poole, Dorset, U.K.

Amplify

Amersham International PLC,
Amersham, Bucks., U.K.

Bromotrimethylsilane

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

[18,6]-Crown ether

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

DEAE Sephadex A-25

Pharmacia Fine Chemicals,
Uppsala, Sweden

Deuterated Solvents

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

D.M.E.M. (X 10)

Flow Laboratories Ltd.,
Irvine, Ayrshire, U.K.

D.M.P.

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

Dowex 50 (H^+)

B.D.H. Chemicals Ltd.,
Poole, Dorset, U.K.

GF/C Discs

Whatman Ltd.,
Maidstone, Kent, U.K.

1-Hydroxyethanebisphosphonic Acid

Albright and Wilson Ltd.,
Warley, West Midlands, U.K.

Iodotrimethylsilane

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

Lithium Alkyls

Aldrich Chemical Co. Ltd.,
Gillingham, Dorset, U.K.

MDCK Cells

Flow Laboratories Ltd.,
Irvine, Ayrshire, U.K.

[^{3}H]$NaBH_4$

Amersham International PLC,
Amersham, Bucks., U.K.

NEAA

Flow Laboratories Ltd.,
Irvine, Ayrshire, U.K.

Nucleotide triphosphates

Sigma (London) Chemical Co.,
Poole, Dorset, U.K.

Oxepane

Aldrich Chemical Co.,
Gillingham, Dorset, U.K.

Penicillin/Streptomycin

Flow Laboratories Ltd.,
Irvine, Ayrshire, U.K.

PAA

Sigma (London) Chemical Co.,
Poole, Dorset, U.K.

PFA

Sigma (London) Chemical Co.,
Poole, Dorset, U.K.

3-Phosphonopropionic Acid

Fluorochem Ltd.,
Glossop, Derbyshire, U.K.

Sephadex G-10

Pharmacia Fine Chemicals,
Uppsala, Sweden

Silica Gel 60 (230-400 Mesh)

E. Merck, G.F.R.

Thallium(I)ethoxide

B.D.H. Chemical Ltd.,
Poole, Dorset, U.K.

[5-H3]Uridine Triphosphate

Amersham International PLC,
Amersham, Bucks., U.K.

Zinc Chloride (Spectrosol)

B.D.H. Chemicals Ltd.,
Poole, Dorset, U.K.

APPENDIX II

FAST ATOM BOMBARDMENT MASS SPECTROMETRY OF SALTS OF PYROPHOSPHATE ANALOGUES

AII.1 INTRODUCTION

Esters of pyrophosphate analogues are readily analysed by chemical ionisation mass spectrometry (CIMS) with ammonia as the reagent gas of choice (Cload and Hutchinson, 1983b). However, the active antiviral species, the free acids and salts, are not sufficiently volatile for analysis by this technique or by conventional electron impact mass spectrometry (EIMS).

Several methods of ionisation have appeared in recent years aimed at overcoming these types of volatility problems, such as field desorption (FD) (Bubzikiewicz and Linschied, 1977) and secondary ion mass spectrometry (SIMS) (Liu, *et al.*, 1981), but perhaps the most versatile of these newer methods is fast atom bombardment mass spectrometry (FABMS) (Barber, *et al.*, 1981).

As the name suggests this technique involves bombarding a solvated sample with an energetic beam of either xenon or argon atoms (accelerated to 4-8 k eV as ions and then charge exchanged without significant loss of momentum). Particles are sputtered from the sample surface, some of which are charged and consequently can be detected in a conventional mass spectrometer.

The majority of FABMS has been carried out on samples suspended in matrices of low vapour pressure, viscous solvents, such as glycerol. This means that the surfactant properties of the compound and its solubility are important factors in the quality of the spectra obtained, since replenishment of the sample at the surface is necessary so that it can be continuously ejected from that surface. It is this fact that makes highly polar and charged species the most amenable to study by FABMS, since they are most likely to have the required properties. Thus, FABMS is a complementary technique to CIMS and EIMS in that different and previously inaccessible information is obtained.

The ionic species observed by FABMS also differ from those seen conventionally. No radical ions are obtained, instead ionisation is by addition or loss of a proton to give the species $(M + H)^+$ and $(M - H)^-$ respectively. Ionisation can also occur by addition or loss of metal ions if any are present. The ions produced are of much lower energy than those made by EIMS or CIMS, which means that very little fragmentation occurs.

Glycerol can interfere with the spectra produced in that it forms ionised oligomers of the type $(Gly_n + H)^+$, with itself and $(M + Gly_n + H)^+$, with the sample. The abundance of these oligomeric species decreases with increasing n, and are barely detectable at higher mass.

AII.2 EXPERIMENTAL

FABMS analysis of all species was carried out by mixing an aqueous solution of the free acids (15 μl, 0.3 M) with 2 M sodium or potassium hydroxide (10 μl). This mixture was suspended in glycerol (35 μl), giving an overall conventration of 0.075 M pyrophosphate analogue in 60% glycerol containing 4 equivalents of counterion. Alternatively, FABMS spectra were obtained from samples of the requisite salt or free acid dissolved in 60% glycerol.

Mass spectra were obtained on an MS80 mass spectrometer fitted with a standard FAB gun (Kratos Analytical Instruments Ltd., Manchester, U.K.) and data analysed using a DS55 data system. The primary argon beam energy was 4 k eV with a gun current of 300 μA. Spectra were recorded with the sample probe at room temperature using a resolution of 1000. Spectra of the free acids were recorded on a Finnigan model 3300 mass spectrometer fitted with an Ion Tech B11 NF saddle field gun as described (Caprioli, *et al.*, 1983) and using xenon as the bombarding gas at 8 k eV and 40 μA gun current.

AII.3 RESULTS AND DISCUSSION

AII.3.1 Spectra of Free Acids of Pyrophosphate Analogues

FABMS spectra of the free acids of these species were recorded in the positive mode, the general species ovserved being $(M + H + Gly_n)^+$ (where n = 0, 1, 2, 3)

Table AII. 3 Pyrophosphate Analogues Studied

	Name	Formula	Elemental Composition	Mol. Wt.
(1)	Pyrophosphoric Acid	$O(PO_3H_2)_2$	$H_4O_7P_2$	178
(2)	Phosphonoacetic Acid	$HOOCCH_2PO_3H_2$	$C_2H_5O_5P$	140
(3)	3-Phosphono-propionic Acid	$HOOC(CH_2)_2PO_3H_2$	$C_3H_7O_5P$	154
(4)	1-Hydroxy-ethane-1,1-bisphosphonic Acid	$CH_3(OH)C(PO_3H_2)_2$	$C_2H_8O_7P_2$	206
(5)	Dimethylamino-methylenebis-phosphonic Acid	$(CH_3)_2NCH(PO_3H_2)_2$	$C_3H_{11}NO_6P_2$	219
(6)	Carbonyl bis-phosphonic Acid	$OC(PO_3H_2)_2$	$CH_4O_7P_2$	190
(7)	Dichloromethyl-enebisphos-phonic Acid	$Cl_2C(PO_3H_2)_2$	$CH_4Cl_2O_6P_2$	244, 246, 248
(8)	Dibromomethyl-enebisphos-phonic Acid	$Br_2C(PO_3H_2)_2$	$CH_4Br_2O_6P_2$	332, 334, 336
(9)	Chloromethyl-enebisphos-phonic Acid	$H(Cl)C(PO_3H)_2)_2$	$CH_5ClO_6P_2$	210,212
(10)	Bromomethylene-bisphosphonic Acid	$H(Br)C(PO_3H_2)_2$	$CH_5BrO_6P_2$	254, 256
(11)	Methylchloro-methylene-bisphosphonic Acid	$CH_3(Cl)C(PO_3H_2)_2$	$C_2H_7ClO_6P_2$	224, 226
(12)	Benzylchloro-methylene-bisphosphonic Acid	$C_6H_5CH_2(Cl)C(PO_3H_2)_2$	$C_8H_{11}ClO_6P_2$	300, 302

(Table AII.3.1) which decrease in intensity with increasing n. The majority of pyrophosphate analogues would appear in the region in which glycerol oligomers occur as intense peaks (i.e. 93,185,277 a.m.u.) (e.g., Fig. AII.3.1). Thus, the spectra could be improved by use of metal counterions to shift the region of interest to higher mass, away from the glycerol dominated area.

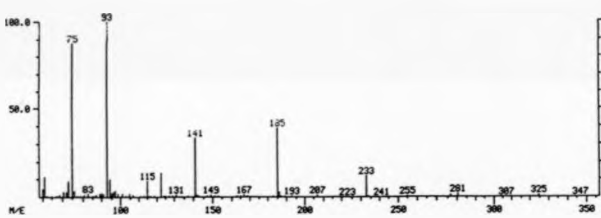

Fig. AII.3.1 FAB spectrum of PAA

Table AII.3.1 Principal ions and Relative Abundance (%) in FABMS Spectra of Free Acids of Pyrophosphate Analogues

Compound	Spectrum
(2)	93 (100), 141 (34), 185 (38), 233 (12)
(3)	93 (100), 155 (38), 185 (51), 247 (12), 277 (3), 329 (4)
(4)	93 (100), 185 (11), 207 (10), 277 (0.5), 299 (2), 391 (0.5)

AII.3.2 Spectra of Alkali Metal Salts of Pyrophosphate Analogues

In all cases, positive FABMS of pyrophosphate analogues (TablesAII.3.2(i) and AII.3.2(ii)) results in species which contain as many as five alkali metal counterions so that spectra are elongated over a much greater mass range.

Fig. AII.3.2 shows two spectra of dimethylamino-methylene bisphosphonic acid as its sodium and potassium salts, showing that the heavier potassium ions make for the clearest FAB mass spectra, in that the peaks of interest are well removed from interfering peaks. Little noise was discernible in any of the spectra recorded.

Negative FABMS was less useful since smaller total ion currents were obtained and sample peaks were barely discernible from baseline noise. The types of species observed were $(M - H)^-$, $(M - H_2 + Na)^-$, $(M - H_3 + Na_2)^-$ etc.

No advantage was gained by using triethanolamine, trigol or thioglycerol as matrix or by interchanging the bombarding gases.

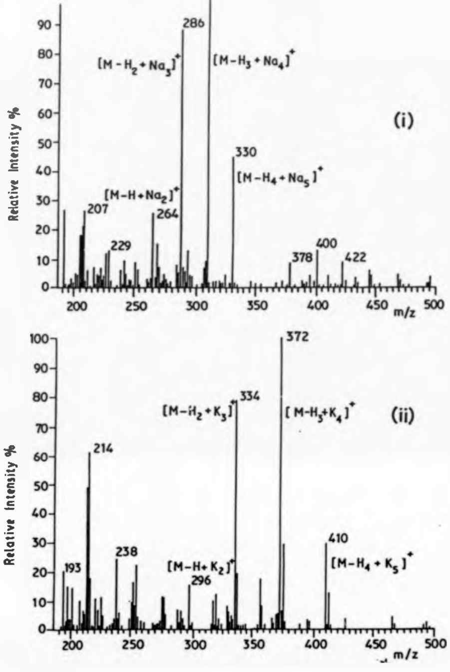

Fig. AII.3.2 Comparison of FABMS spectra of (i) sodium and (ii) potassium salts of dimethylamino-methylene bisphosphonic acid.

Table AII.3.2(i) Sample Ions and Relative Abundance (%) in the FABMS Spectra of Sodium Salts of Pyrophosphate Analogues

Compound	Spectrum
(1)	233 (13), 245 (61), 267 (100), 289 (55)
(2)	141 (12), 163 (58), 207 (27), 229 (100)
(3)	155 (16), 177 (36), 199 (100), 221 (67), 243 (19)
(4)	207 (100), 229 (56), 251 (43), 273 (20), 295 (38), 317 (34)
(5)	264 (26), 286 (88), 308 (100), 330 (45)
(6)	213 (18), 235 (98), 257 (100), 279 (20), 301 (10)
(7)	267 (39), 269 (20), 271 (4), 289 (100), 291 (65), 293 (13), 311 (85), 313 (52), 315 (9), 333 (17), 335 (12), 337 (3)
(8)	355 (24), 357 (49), 359 (28), 377 (50), 379 (100), 381 (48), 399 (32), 401 (76), 403 (37)
(9)	211 (45), 213 (15), 233 (12), 235 (7), 255 (19), 257 (8), 277 (76), 279 (27), 299 (100), 301 (30), 321 (34), 323 (12)
(10)	255 (20), 257 (23), 277 (60), 279 (62), 299 (100), 301 (99), 321 (80), 323 (78)
(11)	225 (12), 227 (5), 247 (62), 249 (20), 269 (84), 271 (29), 291 (100), 293 (34), 313 (72), 315 (26), 335 (20), 337 (8)
(12)	301 (100), 303 (33), 323 (20), 325 (8), 345 (45), 347 (15), 367 (75), 369 (25), 289 (80), 391 (27), 411 (12), 413 (5)

Table AII.3.2(ii) Sample Ions and Relative Abundances (%) in the FABMS Spectra of Potassium Salts of Pyrophosphate Analogues

Compound	Spectrum
(1)	255 (34), 293 (64), 331 (100), 369 (22)
(2)	155 (23), 193 (12), 231 (99), 269 (100), 307 (36)
(3)	179 (8), 217 (42), 255 (100), 293 (74)
(4)	207 (28), 283 (5), 321 (53), 359 (100), 397 (46)
(5)	258 (4), 296 (16), 334 (80), 372 (100), 410 (29)
(6)	191 (14), 229 (18), 267 (95), 305 (100), 343 (24)
(7)	283 (26), 285 (19), 287 (4), 321 (100), 323 (71), 325 (39), 359 (76), 361 (66), 363 (24), 397 (6), 399 (4), 401 (3)
(8)	371 (52), 373 (100), 375 (56), 409 (48), 411 (94), 413 (54), 447 (34), 449 (68), 451 (40)
(9)	211 (20), 213 (8), 249 (48), 251 (15), 287 (98), 289 (32), 325 (100), 327 (32), 363 (75), 365 (28)
(10)	293 (60), 295 (58), 331 (100), 333 (95), 369 (76), 371 (77), 407 (30), 409 (30)
(11)	263 (70), 265 (25), 301 (100), 303 (33), 339 (70), 341 (22), 377 (40), 379 (14), 415 (15), 417 (5)
(12)	301 (10), 303 (4), 339 (32), 341 (10), 377 (100), 379 (34), 415 (42), 417 (14), 453 (36), 455 (12), 491 (25), 493 (10)

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SYNTHESIS OF ALKYLATED METHYLENE BISPHOSPHONATES VIA ORGANOTHALLIUM INTERMEDIATES

DAVID W. HUTCHINSON and GRAEME SEMPLE

Department of Chemistry, University of Warwick, Coventry, West Midlands, CV4 7AL (Great Britain)

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Summary

Thallium(I) derivatives of esterified methylene bisphosphonates can be readily obtained by treating the latter with thallium(I) ethoxide under anhydrous conditions. Alkylation of the thallium(I) derivatives by a range of primary alkyl iodides takes place smoothly, and significantly higher yields are obtained than for the corresponding reactions with lithio or sodio derivatives.

Introduction

Halogenated methylene bisphosphonic acids, e.g., **1** where (R^1 = Cl or Br, R^2 = H) or (R^1 = R^2 = Cl or Br) are inhibitors of the RNA transcriptase of influenza virus A and this inhibitory activity has been correlated with the effectiveness of these compounds as chelators of zinc ions [1]. Highly polar compounds such as **1** are not taken up readily by cells and hence we have turned our attention to the synthesis of *C*-alkylated monohalogenomethylene bisphosphonic acids **1** where R^1 = alkyl, R^2 = Cl or Br as these compounds should cross lipid membranes more readily than the unalkylated analogues.

$$(HO)_2P(O)CR^1R^2P(O)(OH)_2 \quad (\mathbf{1})$$

There are two methods available for the synthesis of **1** (R^1 = alkyl, R^2 = Cl or Br). The first involves the alkylation of the tetraisopropyl ester of methylene bisphosphonic acid **1a** followed by halogenation and removal of the esterifying groups. This route has been reported previously, the sodio derivative of tetraisopropyl **1a** was alkylated and the product then halogenated with a sodium hypohalite. However, yields were poor as $\leqslant 25\%$ alkylation [2] and 45% halogenation [3] occurred. Our studies have shown that it is more efficient to invert the two steps as the yields are much higher and the products are more readily purified (Scheme 1). Monohalogenation of tetraisopropyl **1a** cannot be accomplished in a single step in good yield [2]

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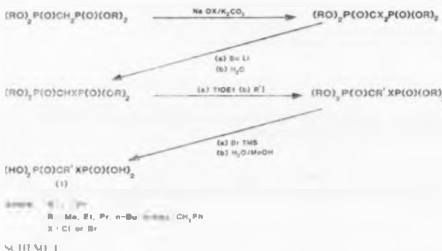

SCHEME 1

but the monodehalogenation of **1** ($R^1 = R^2 =$ Cl or Br) can readily be achieved using either fluoride ion [4] or *n*-butyllithium [5]. We find that alkylation of the tetraisopropyl esters of monochloromethylene **1e** and monobromomethylene **1m** bisphosphonic acids occurs in high yield when thallium(I) ethoxide is used as base to generate the mono-anion followed by treatment of the latter with a primary alkyl iodide. Alkylation of tetraisopropyl **1a** can be carried out in a similar manner when an excess of thallium(I) ethoxide is used as base. Hydrolysis of the tetraisopropyl esters of the *C*-alkylated products is most efficiently carried out on a small scale via the formation of the tetra(trimethylsilyl) esters with bromotrimethylsilane. The *C*-alkylated monochloromethylene bisphosphonic acids are inhibitors of the RNA transcriptase of influenza virus A, a full report of their biological activities will be published elsewhere.

Results and discussion

The alkylation of organophosphorus compounds by means of their thallium derivatives has not previously been reported although quantitative *C*-alkylation of β-diketones has been achieved via stable organothallium intermediates [6]. We have obtained isolated yields of more than 80% of the closely analogous tetra esters of *C*-alkylated monochloromethylene bisphosphonates by means of organothallium intermediates (Table 1). Such high yields could not be obtained when butyllithium, sodium hydride or sodium metal were used to form the reactive intermediates. For example, when tetraisopropyl **1e** was treated with either sodium metal or sodium hydride followed by iodomethane, ^{31}P NMR analysis of the reaction showed the presence of tetraisopropyl *C*-methyl monochloromethylene (**1f**) and tetraisopropyl *C*-dimethyl methylene **1** ($R^1 = R^2 =$ Me) bisphosphonates in the ratio 9/1. When iodoethane or iodobutane were reacted with the sodio derivative of tetraisopropyl **1e** complex mixtures of products were obtained. ^{31}P NMR analysis showed the presence of $< 45\%$ tetraisopropyl (**1**: $R^1 =$ Cl, $R^2 =$ Et or Bu) and ca. 30% of *C*-dial-

TABLE 1

CHARACTERISATION OF ALKYLATED METHYLENE BISPHOSPHONATES

Compound			Tetraisopropyl esters			Tetra sodium salts		
						Analyses (Found (calcd.)(%))		
No	R^1	R^2	Alkylation by ^{31}P NMR (%)	Yield isolated (%)	Parent ion(s) (M + H) in CIMS	C	H	P
1a	H	H		–	345	4.73 (4.55)	0.81 (0.76)	23.5 (23.5)
1b	H	Me	72	47	359	8.67 (8.63)	1.27 (1.44)	20.8 (22.3)
1c	H	Et	68	45	373	13.96 (12.33)	3.74 (2.05)	22.15 (21.23)
1d	H	n-Bu	74	49	401	18.64 (18.75)	2.70 (2.50)	19.52 (19.38)
1e	Cl	H		–	379, 381	3.84 (4.02)	1.76 (0.34)	19.30 (20.77)
1f	Cl	Me	98	80	393, 395	7.75 (7.70)	2.22 (0.96)	20.40 (19.84)
1g	Cl	Et	97	81	407, 409	11.5 (11.03)	2.19 (1.53)	18.07 (18.99)
1h	Cl	n-Pr	97	78	421, 423	14.37 (14.10)	2.66 (2.06)	19.61 (18.21)
1i	Cl	n-Bu	96	78	435, 437	14.81 (16.93)	2.90 (2.54)	16.70 (17.5)
1j	Cl	n-Hex	96	79	463, 465	21.40 (21.96)	3.89 (3.40)	16.37 (16.21)
1k	Cl	CH_2Ph	99	82	469, 471	23.11 (24.70)	2.39 (1.80)	16.75 (15.96)
1l	Cl	i-Pr	15	–	421, 423	–	–	–
1m	Br	H	–		423, 425	3.80 (3.50)	1.95 (0.30)	17.60 (18.10)
1n	Br	Me	93	70	437, 438	6.09 (6.70)	1.72 (0.84)	– (–)
1o	Br	Et	87	63	451, 453	9.70 (9.70)	1.51 (1.35)	18.01 (16.70)

kylated products (Table 2). These results confirm the observation of Quimby et al. [3] who found that monoalkylation of tetraisopropyl **1a** did not occur in high yield by this route. We obtained a good yield of tetraisopropyl **1f** when butyllithium was used to generate the mono-anion of tetraisopropyl **1e** before alkylation of the latter with iodomethane. However, poor yields of *C*-monoalkylated products were obtained with other iodoalkanes. Thus, the thallium(I) intermediate is unique in giving high yields of *C*-monoalkylated products with a range of iodoalkanes.

Treatment of the thallium salt of tetraisopropyl **1e** with bromohexane gave a 36% yield (by ^{31}P NMR) of tetraisopropyl *C*-n-hexyl monobromomethylene bisphosphonate **1j** compared with a 96% yield with iodohexane. This is in accordance with observations by Taylor et al. on the *C*-alkylation of β-diketones [6]. Although these authors reported that the alkylation of thallium(I) salts of β-diketones took place in high yield with 2-iodopropane, we have found that the equivalent reaction with esterified methylene bisphosphonates is very sluggish and we could only obtain 15% of tetraisopropyl *C*-isopropyl monochloromethylene bisphosphonate **1l** at elevated temperatures with prolonged reaction times. Presumably steric hindrance by

TABLE 2

ALKYLATIONS OF TETRAISOPROPYL SODIOCHLOROMETHYLENE BISPHOSPHONATE

	Alkylating Agent					
	MeI		EtI		BuI	
	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)	Yield by ^{31}P NMR (%)	^{31}P Chemical shift (δ)
Starting material	2	11.4	15	11.4	19	11.4
Monoalkylated product	89	14.8	44	14.7	39	14.7
Dialkylated product	9	25.6	30	25.3	26	25.3
Others	—	–	11	two peaks	16	three peaks

the bulky groups around the central carbon atom in our compounds is an important factor in this reaction with secondary iodoalkanes.

Alkylation of the monobromo derivative **1m** proceeded less cleanly than for the monochloro analogue. Fair yields of *C*-monoalkylated compounds could be isolated when the formation of the thallium salt was performed at low temperature ($-20°C$). When the salt was prepared at room temperature, up to 25% (by ^{31}P NMR) of side products were formed presumably due to positive halogen abstraction as has been observed in our earlier experiments [4] and by others [7]. The increased tendency of the monobromo derivative to undergo positive halogen abstraction can be correlated with the ionisation potentials of the two halogens [8] and presumably a monoiodo derivative would undergo dehalogenation even more rapidly. Best yields for monoalkylation of tetraisopropyl **1a** occurred at 30°C with an excess (1.4 equiv.) of thallous ethoxide. Presumably the formation of the monoanion is comparatively difficult in the absence of a chlorine atom on the central carbon atom.

All tetra esters were analysed by ammonia chemical ionisation mass spectrometry (CIMS) with ammonia as reagent gas [9]. In every case an intense ion corresponding to $(M+H)^+$ was observed, together with peaks corresponding to the successive loss of propene residues (Table 1). In the case of tetraisopropyl esters of longer chain *C*-alkylated monohalogenated methylene bisphosphonates **1h**–**j** and **1p** a second decomposition pathway of the parent $(M+H)^+$ ions was possible involving elimination of hydrogen halide from the bridge carbon atom.

The ^{1}H and ^{31}P NMR spectra of the acids are listed in Table 3. The tetraisopropyl esters had very similar ^{1}H NMR spectra with the addition of peaks in the regions δ 4.8–5.0 (m, methine protons, 4H) and 1.35–1.5 ppm (d, methyl protons, 24H, J 7 Hz). In the ^{1}H NMR spectrum of tetraisopropyl *C*-benzyl monochloromethylene bisphosphonate **1k**, four separate doublets were observed in the region δ 1.17–1.35 ppm. The extremely bulky substituents around the bridge carbon atom have restricted rotation and the separation of the doublets due to the methyl groups are presumably due to diastereotopic effects as there are several prochiral centres in the molecule.

The hydrolysis of the tetraisopropyl esters in concentrated hydrochloric acid [10] does not give high yields of the corresponding free acids. Treatment of the esters with an excess of bromotrimethylsilane followed by aqueous methanol gave quanti-

TABLE 3

NMR DATA OF ALKYLATED METHYLENE BISPHOSPHONANTES

Compound	^{31}P Chemical shift (δ(ppm))		^{1}H chemical shifts (δ(ppm)) of Na_4-salts in D_2O spin–spin coupling constants (J) in Hz
	$(i\text{-}Pr)_4$-ester ($CDCl_3$)	Na_4-salt (D_2O)	
Ia	17.2	18.0	2.40 (t, 2H, J 21)
Ib	22.0	23.2	1.44 (t, 3H (d), J 18, 8 (d), 2.25 (t, 1H (q), J 23, 7.5 (q)
Ic	21.9	22.7	1.10 (t, 3H, J 8), 1.7–2.0 (m, 3H)
Id	22.0	22.7	0.90 (t, 3H, J 7), 1.35 (2H sext, J 7), 1.55 (2H, quint, J 7), 1.75–2.0 (m, 2H), 2.25 (t, 1H (t), J_1 23, J_2 7.5)
Ie	11.4	13.2	3.90 (t, 1H, J 20)
If	14.8	17.8	1.85 (d, 3H, J 15)
Ig	14.75	17.2	1.17 (t, 3H, J 7.5), 2.25 (m, 2H)
Ih	14.8	17.3	0.87 (t, 3H, J 7.5), 1.65 (2H, sext., J 7.5), 2.10 (m, 2H)
Ii	14.7	17.3	0.86 (t, 3H, J 7.5), 1.32 (2H, sext., J 7), 1.62 (2H, quint., J 7.5), 2.15 (m, 2H)
Ij	14.75	17.3	0.86 (t, 3H, J 7.5), 1.30 (m, 6H), 1.75 (2H, quint., J 7.5), 2.15 (m, 2H)
Ik	13.7	16.9	3.40 (t, 2H, J 13), 7.25 (m, 3H), 7.40 (m, 2H)
Il	14.8	17.2	–
Im	11.4	12.9	3.75 (t, 1H, J 20)
In	14.9	17.8	2.02 (t, 3H, J 15)
Io	14.7	17.5	1.14 (t, 3H, J 8), 2.18 (m, 2H, J 8)

tative yields of the free acids [11,12]. This reaction is much cleaner than when iodotrimethylsilane [13] is used in place of the bromo derivative. Thus, the combination of the use of thallium(I) ethoxide to form the monoanions of esterified methylene bisphosphonates with the use of bromotrimethylsilane to remove the esterifying groups from the alkylated products results in significantly increased yields of C-alkylated methylene bisphosphonic acids compared with previously reported procedures.

Experimental

Instrumentation

^{1}H NMR spectra of sodium salts of the free acids were recorded at 220 MHz (Table 3) on a Perkin–Elmer R34 spectrometer using deuterium oxide as solvent and 3-(trimethylsilyl)-1-propane sulphonic acid as internal standard. ^{31}P NMR spectra were recorded to an accuracy of ± 0.5 ppm (downfield shifts positive) at 36.44 MHz on a Bruker WH90 spectrometer using H_3PO_4 as external standard. Chemical ionisation mass spectra with ammonia as reagent gas were obtained with an MS80 mass spectrometer with a DS55 data system (Kratos Analytical Instruments). Analyses were carried out by Elemental Micro-Analysis Ltd. (Beaworthy, Devon, U.K.).

Preparation of methylene bisphosphonates

(i) Tetraisopropyl dichloromethylene bisphosphonate Tetraisopropyl methylene bisphosphonate (30 g, 0.09 mol; Lancaster Synthesis Ltd., Morecambe, Lancs.,

U.K.) was treated with excess sodium hypochlorite as previously described [3]. Flash column chromatography of the product on silica with 40–60 petroleum ether/acetone (2/1, v/v) as eluant gave a white solid (29.7 g, 82%). ^{31}P NMR analysis indicated that the only phosphorus-containing species present was the title compound (δ 6.86 ppm in $CDCl_3$). The structure was confirmed by CIMS when the principal ions were $(M+H)^+$ 413, 415, 417 in the ratio 9/6/1 due to the compounds with ^{35}Cl and ^{37}Cl isotopes.

(ii) Tetraisopropyl dibromomethylene bisphosphonate. Tetraisopropyl methylene bisphosphonate (10 g, 0.029 mol) was treated with an excess of potassium hypobromite prepared in situ as described [4]. Flash column chromatography of the product on silica using 40–60 petroleum ether/acetone (8/1, v/v) as eluant gave a pale brown oil (11.4 g, 78%). ^{31}P NMR analysis indicated that the only phosphorus-containing species present was the title compound (δ 6.74 ppm in $CDCl_3$). The structure was confirmed by CIMS when the principal ions were $(M+H)^+$ 500, 502, 504, in the ratio 1/2/1.

(iii) Tetraisopropyl esters of monochloromethylene (1e) and monobromomethylene (1m) bisphosphonic acids. Tetraisopropyl dichloromethylene bisphosphonate (5 g, 0.012 mol) was treated with n-butyllithium (1 equiv.) as described [6] to give tetraisopropyl monochloromethylene bisphosphonate acid (**1e**) (3.25 g, 71%) after chromatography on silica with elution with 40–60 petroleum ether/acetone (7/1, v/v). In a similar reaction, tetraisopropyl dibromomethylene bisphosphonate (5 g, 0.01 mol) gave the tetraisopropyl ester of monobromomethylene bisphosphonic acid **1m** (2.7 g, 64%).

(iv) Tetraisopropyl C-alkyl monochloromethylene bisphosphonates. A standard procedure was used to prepare the tetraisopropyl C-alkyl monochloromethylene bisphosphonates listed in Table 1 and the preparation of tetraisopropyl ester of C-ethyl monochloromethylene bisphosphonic acid **1g** given below is a typical example. Analytical data (^{31}P NMR spectra and ammonia CIMS) are also given in Table 1.

Tetraisopropyl **1e** (0.5 g, 0.0013 mol) was dissolved in dry, freshly distilled tetrahydrofuran (10 ml) and the solution stirred for 40 min under nitrogen at room temperature in a 100 ml 3-necked flask fitted with a drying tube and a rubber septum. Thallium(I) ethoxide (95 μl, 0.0013 mol) was added and the stirring continued for 40 min during which time the solution became cloudy. Iodoethane (5 ml, 0.032 mol) was added by syringe via the septum and the mixture heated under reflux for 2 h. The solution was then cooled and the orange thallium(I) iodide removed by passing the reaction mixture through a short column of Florisil and eluting with 40–60 petroleum ether/acetone (1/1, v/v) (120 ml). The eluant was concentrated in vacuo to an oil which ^{31}P NMR analysis showed contained ≥ 97% tetraisopropyl **1g**. The latter was purified by flash chromatography on silica with elution with 40–60 petroleum ether/acetone (6/1, v/v) to give the pure tetraisopropyl **1g** (0.44 g, 81%). During the preparations of tetraisopropyl **1h** and **1i** the reaction vessel was kept dark as the alkyl iodides are light sensitive. In the preparation of tetraisopropyl **1k**, the thallium intermediate was treated with a mixture of benzyl iodide and bromide prepared, in an efficient fume hood, as follows. Benzyl bromide (5 g, 0.029 mol) was added to a solution of sodium iodide (4.4 g, 0.03 mol) in acetone (25 ml). The mixture was stirred at room temperature fo 10 min, filtered and then concentrated in vacuo. This mixture (which was shown by ^{1}H NMR to be a 4/1

mixture of benzyl iodide and benzyl bromide) was used immediately in the alkylation reaction.

(v) Tetraisopropyl C-alkyl monobromomethylene bisphosphonates. The above procedure was used for the *C*-alkylation of tetraisopropyl monobromomethylene bisphosphonate. In the case of the *C*-ethyl derivative, ^{31}P NMR analysis of the reaction showed that the tetraisopropyl esters of **1o** (87%, δ 14.7 ppm), dibromomethylene (8%, δ 17.2 ppm) and *C*-diethyl methylene (4%, δ 25.3 ppm) bisphosphonic acids were present. Column chromatography of the mixture on silica eluting with 40–60 petroleum ether/acetone (5/2, v/v) gave pure tetraisopropyl **1o** in 61% as a colourless oil.

(vi) Alkylation of tetraisopropyl sodio-chloromethylene bisphosphonate was carried out as described [3]. ^{31}P NMR Analyses of the product mixtures are shown in Table 2.

(vii) Alkylation of tetraisopropyl lithio-chloromethylene bisphosphonate. The lithium salt of tetraisopropyl monochloromethylene bisphosphonate was prepared as described above, an excess of either iodomethane or bromomethane was added and after the mixture had been heated under reflux for a short time, excess saturated sodium bicarbonate was added. The alkylated products were obtained from this reaction in the usual manner as oils. ^{31}P NMR analysis revealed the presence of the following products: (a) methylation reaction: tetraisopropyl esters of **1f** 65%, **1e** 20%, and dichloromethylene (15%) ethylation reaction: tetraisopropyl esters of **1g** 36%, **1e** 12%, and dichloromethylene 25% bisphosphonic acids together with trace amounts of four unidentified compounds.

(viii) Hydrolysis of tetraisopropyl esters of methylene bisphosphonic acids. Bromotrimethylsilane (2.5 fold excess) was added to each of the esters under dry nitrogen and the mixture stirred at room temperature for $\geqslant$ 20 h, the mixture was then lyophilised and excess aqueous methanol added. The aqueous methanol was removed in vacuo, further aqueous methanol was added and the solution evaporated again. This procedure was repeated 4 times. The residues were dissolved in a little water and isolated as the tetrasodium salt by ion exchange chromatography on Dowex 50 (2 × 15 cm). In all cases isolated yields were in excess of 90%.

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Preliminary communication

RELATIVE REACTIVITIES OF TETRAALKYL ESTERS OF METHYLENE BISPHOSPHONIC ACID

DAVID W. HUTCHINSON* and GRAEME SEMPLE
Department of Chemistry, University of Warwick, Coventry CV4 7AL, West Midlands (Great Britain)
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Summary

The synthesis in high yield of tetraisopropyl esters of *C*-alkylated methylene bisphosphonic acids can be achieved by the alkylation of the thallium salts of tetraisopropyl esters of the parent methylene bisphosphonic acids. Yields of *C*-alkylated products are markedly reduced when the tetraethyl or tetramethyl esters are used in this reaction. From a study of the ^{13}C and ^{31}P NMR spectra of the methylene bisphosphonates and their anions, this difference in reactivity is ascribed to steric factors.

Almost quantitative yields of *C*-alkylated esters (**2**) are formed from thallio derivatives of (**1**, R = i-Pr) or its bromo analogue and primary alkyl iodides (Scheme 1) [1]. However, under the same conditions, alkylation of the lithio, sodio or thallio derivatives of **1**, (R = Me or Et) gives very low (< 20%) yields of *C*-alkylated products. Furthermore, the rate of alkylation of the lithio derivative of **1** (R = i-Pr) is faster than the rate of alkylation of the lithio derivative of **1** (R = Et) and more side products are formed during the alkylation of the tetraethyl ester (Fig. 1). We

$$(RO)_2P(O)CHClP(O)(OR)_2 \xrightarrow{\text{BuLi or TlOEt}} (RO)_2P(O)C^{(-)}ClP(O)(OR)_2$$

(**1**)

$$\xrightarrow{\text{R'CH}_2\text{I}} (RO)_2P(O)CCl(CH_2R')P(O)(OR)_2$$

(**2**)

SCHEME 1. The alkylation of anions of tetra-esters of monochloromethylene bisphosphonate.

* To whom correspondence should be addressed.

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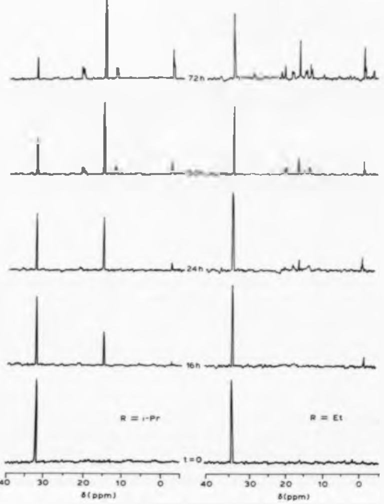

Fig. 1. The time course of alkylation of $[(RO)_2P(O)]_2CCl^- Li^+$ by n-butyl iodide in C_6D_6 at room temperature as studied by ^{31}P NMR spectroscopy. Spectra were recorded on a Bruker WH90 spectrometer at 36.4 MHz and were proton decoupled. The chemical shifts were recorded to an accuracy of ±0.5 ppm (downfield shifts positive) relative to H_3PO_4 as external standard.

have also observed by ^{31}P NMR spectroscopy that a number of products are formed when **1** (R = Me) is treated with n-butyllithium [2]. Cleavage of the C–P bond occurs and this can be avoided if t-butyllithium is used in place of n-butyllithium. While the rapid conversion of **1** (R = i-Pr) to its anion can be achieved at −78°C with one equivalent of methyllithium, the esters **1** (R = Me or Et) undergo extensive side reactions with methyllithium under these conditions.

$(RO)_2P(O)CH_2P(O)(OR)_2$ **(3)** $(EtO)_2P(O)CH_2COOEt$ **(4)** RCOCHR'COR **(5)**

TABLE 1

^{13}C AND ^{31}P NMR DATA FOR THE CENTRAL CARBON ATOM IN METHYLENE BISPHOSPHONATES (1 AND 3), THEIR ANIONS, TRIETHYL PHOSPHONOACETATE (4) AND PENTANE-2,4-DIONE (5)

(^{13}C NMR spectra were recorded in C_6D_6 at 22.63 MHz on a Bruker WH90 spectrometer and were [illegible] with benzene as internal standard. Sodium salts were prepared by the addition of 1 equivalent of sodium hydride (50% dispersion in oil) to a solution in deuterobenzene of the protonated starting materials.)

Compound	^{13}C δ (ppm)	$J(^{13}C-^{31}P)$ (Hz)	^{31}P δ (ppm)
$[(MeO)_2P(O)]_2CHCl$ [a]	42.1	141.9	36.8
$[(MeO)_2P(O)]_2CCl^- Na^+$ [a]	30.2	236.9	15.5 [c]
$[(EtO)_2P(O)]_2CHCl$	43.9	141.9	34.6
$[(EtO)_2P(O)]_2CCl^- Na^+$	31.1	236.4	13.2 [c]
$[(i\text{-}PrO)_2P(O)]_2CHCl$	45.5	143.4	32.5
$[(i\text{-}PrO)_2P(O)]_2CCl^- Na^+$	33.8	234.3	11.5
$[(MeO)_2P(O)]_2CH_2$	24.0	135.3	23.0
$[(MeO)_2P(O)]_2CH^- Na^+$	7.3	217.5	45.5 [d]
$[(EtO)_2P(O)]_2CH_2$	26.1	135.3	19.3
$[(EtO)_2P(O)]_2CH^- Na^+$	8.6	217.7	41.5 [d]
$[(i\text{-}PrO)_2P(O)]_2CH_2$	28.5	136.8	40.5
$[(i\text{-}PrO)_2P(O)]_2CH^- Na^+$	11.1	214.7	22.8
$(EtO)_2P(O)CH_2COOEt$	34.7	133.8	–
$(EtO)_2P(O)CH^-COOEtNa^+$	40.3	219.1	–
$MeCOCH_2COMe$ [b]	52.0	–	–
$MeCOCH^-COMeNa^+$ [b]	96.9	–	–

[a] Recorded on a Bruker WH400 spectrometer at 100.62 MHz. [b] Taken from reference 6. [c] Li^+ salt. [d] Taken from reference 8.

The lithio and sodio derivatives of tetra-esters of methylene bisphosphonic acid (e.g. **1** or **3**) were studied by ^{13}C and ^{31}P NMR spectroscopy as these derivatives are considerably more soluble in organic solvents than their thallium analogues. The esters were dissolved in deuterobenzene at room temperature, the anions were formed by the addition of base and then NMR spectra were also recorded (Table 1). During the alkylation experiments (Fig. 1), an excess of butyl iodide was added in the NMR tube to the preformed anions and the reactions were monitored by ^{31}P NMR. The tetra-esters of methylene bisphosphonic acid were commercially available (Lancaster Synthesis). The tetra-esters of dichloromethylene bisphosphonic acid were prepared as previously described [1,3]. Treatment of these esters with methyl, n-butyl- or t-butyl-lithium (1.6 *M* in hexane, 1 equivalent) gave the corresponding esters of **1** [2]. The yield of **1** (R = i-Pr) was 75%, ^1H NMR ($CDCl_3$): δ 1.35 (24H, d, *J* 8 Hz), 3.9 (1H, t, *J* 20 Hz), 4.9 (4H, m) ppm. Analysis: Found: C, 40.92; H, 7.35; Cl, 9.74. $C_{13}H_{33}ClO_6P_2$ calcd.: C, 41.2; H, 7.72; Cl, 9.36%. The yield of **1** (R = Et) was 57%, ^1H NMR ($CDCl_3$): δ 1.4 (12H, t, *J* 7.4 Hz), 4.05 (1H, t, *J* 18.5 Hz), 4.35 (8H, m,) ppm. Analysis: Found: C, 33.4; H, 6.65; Cl, 11.5. $C_9H_{21}ClO_6P_2$ calcd.: C, 33.5; H, 6.56; Cl, 11.0%. The yield of **1** (R = Me) was 47%, ^1H NMR ($CDCl_3$): δ 3.7 (12H, m,), 4.0 (1H, t, *J* 18 Hz) ppm. Analysis: Found: C, 22.36; H, 5.29. $C_5H_{13}ClO_6P_2$ calcd.: C, 22.52; H, 4.91%.

The one-bond $^{13}C-^{31}P$ coupling constants between the phosphorus atoms and the central carbon atom in the anions of **1** and **3** are much larger than those found

for the corresponding protonated compounds **1** and **3** (Table 1) suggesting that there is a change in hybridisation of the central atom from sp^3 to sp^2 when the anions are formed [4]. The ^{13}C chemical shifts indicate that the anions derived from **1** and **3** are not appreciably delocalised as the signals due to the central carbon atom shift upfield approximately 12–17 ppm on formation of the anions. These values may be compared with those obtained from the anion of triethyl phosphonoacetate (**4**) where the negative charge might be expected to be delocalised to some extent over the carbethoxy group. In this case, there is a downfield shift of 5.6 ppm of the signal due to the central carbon atom on formation of the anion. It has been reported for pentane-2,4-dione (**5**, R = Me; R′ = H) in solution in DMSO-d_6 that there is a large (44 ppm) downfield shift of the signal due to the central carbon atom on formation of the anion [5]. Presumably in this case there is considerable delocalisation of the negative charge, and it has been suggested from infrared evidence [6] that this anion is appreciably more delocalised than the anions of triethyl phosphonacetate or **3**.

One explanation for the difference in reactivities between the isopropyl esters of **1** or **3** and their ethyl and methyl analogues is that the bulky isopropyl groups distort the anion and prevent the two phosphoryl groups and the central carbon atom from achieving coplanarity. ^{13}C NMR evidence indicates that there is minimal delocalisation of charge in the anions of **1** or **3** and this distortion may result in the exposure of the negative charge on the central carbon atom to attack by electrophiles. Arguments invoking differences in coplanarity of anions of β-diketones have been used to explain differences in reactivities of these compounds [7].

Thus, in the synthesis of *C*-alkylated methylene bisphosphonate esters, we find that while excellent yields can be obtained when the thallio derivatives of tetraisopropyl esters are treated with primary alkyl iodides. Steric factors influence this reaction and considerably lower yields are obtained when either secondary alkyl iodides are used in this reaction or when the ethyl or methyl esters are employed.

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Stress proteins are induced by hyperosmolarity in chick embryo fibroblasts

PIER GIORGIO PETRONINI, MARIAROSARIA TRAMACERE, GIUSEPPE PIEDIMONTE and ANGELO F. BORGHETTI
Istituto di Patologia Generale, Università di Parma, 43100 Parma, Italy

A wide range of environmental stimuli including heat, oxygen deprivation, teratogens, heavy metals, ethyl alcohol, amino acid analogues etc., is known to induce the synthesis of a family of stress proteins in prokaryotic and eukaryotic cells (Ashburner & Bonner, 1979). The functions of these inducible stress proteins are poorly understood. Schlesinger *et al.* (1982) have hypothesized that the induction of these proteins could defend the cell exposed to an environmental insult from further damage by modifying the nuclear and the cytoplasmic cytoskeleton so to protect the metabolic apparatus from the stimulus persistance and to adapt consequently. In addition, the process of cell adaptation to adverse environmental factors is known to involve the cell's ability to modify the chemical complexion of the membrane and to alter the activity of membrane-associated enzymes including transport processes (Quinn, 1983).

We have recently shown that the exposure of chick embryo fibroblasts to a hyperosmolar medium results in an increase of amino acid-transport activity. This change appears to be restricted to the A system of mediation for neutral amino acids, to be dependent on both active transcription and translation, and to require a continuous exposure of the cells to the hyperosmolar stimulus (Tramacere *et al.*, 1984*a*,*b*).

In pursuing a correlation between the probable increase in the number of transport molecules and the alteration of gene expression in cells exposed to a hyperosmolar environment, we studied the pattern of polypeptides synthesized during the adaptation process to a hyperosmolar stress.

Secondary cultures of chick embryo fibroblasts were exposed to 0.5 osM in complete culture medium. Labelling was performed between 0.5 and 3 or 18.5 and 21 h of the hyperosmolar treatment. Cells were then solubilized in SDS/sample buffer and the labelled proteins were separated on 10% (w/v) SDS/polyacrylamide slab gel (Laemmli, 1970) and detected by fluorography as described by Bonner & Laskey (1974).

The effect of cell exposure for 3 or 21 h to a 0.5 osM medium on the pattern of protein synthesis was examined. When the pattern of polypeptides synthesized during the treatment was compared with those synthesized by untreated cells, the synthesis of certain proteins appeared reduced whereas for others there was a clear enhancement. As shown in Fig. 1, after 3 h of hyperosmolar treatment four proteins with apparent M_r of 76000, 59000, 51000 and 38000 were synthesized in smaller amounts than in control cells, meanwhile others (M_r 63000 and 36000) were synthesized in greater amounts. When cells were labelled between 18.5 and 21 h of hyperosmolar treatment, the synthesis of those polypeptides that at 3 h of hyperosmolar exposure appeared reduced or increased, returned to control values. Moreover, the synthesis of M_r 57000 polypeptide was markedly increased. These preliminary data suggest that the expression of some specific proteins changes at specific times during the cell adaptation to hyperosmolarity. At early times there is a change in the

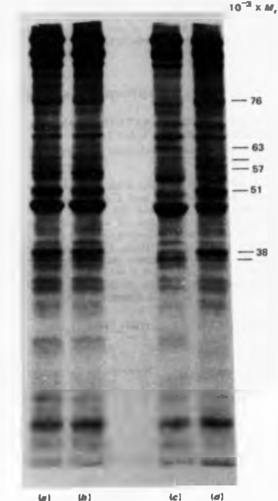

Fig. 1. *Pattern of proteins synthesized in hyperosmolarity-treated chick embryo fibroblasts*

Cells were exposed to 0.5 osM in complete culture medium. The labelling procedure was performed with 10 μCi of [^{14}C]glycine/ml between 0.5 and 3 or 18.5 and 21 h of the hyperosmolar treatment. Shown is a fluorograph of the gel. Lanes (*a*) and (*b*): controls at 3 or 21 h of incubation, respectively. Lanes (*c*) and (*d*): cells exposed to 0.5 osM for 3 or 21 h, respectively.

Abbreviation used: SDS, sodium dodecyl sulphate.

synthesis of several polypeptides, whereas at later times the protein pattern appears to be similar to control.

Our unpublished observations on the behaviour of such parameters as polyribosome profile, cation content and amino acid transport have shown a strong perturbation of these parameters in the first few hours of hyperosmolar treatment followed by a slow but persistent return to the values of the control. These results are an indication of the capacity of the cells to cope with an osmotically altered environment and are compatible with the above described modulation of protein synthesis. Of course, the possibility that the change in the expression of specific proteins might be referred to the alteration of some functions in hyperosmolarity-treated cells deserves further study.

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Pyrophosphate analogues as inhibitors of viral polymerases

DAVID W. HUTCHINSON, MATTHEW NAYLOR, GRAEME SEMPLE and PAUL A. CLOAD
Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K.

Analogues of pyrophosphoric acid, e.g. phosphonoacetic (I), phosphonoformic (II) and *C*-substituted methylene bisphosphonic acids (III) and the related tetrazole (IV) can act as product inhibitors of viral polymerases. For example, the replication of herpes viruses can be inhibited by (I) (Boezi, 1979) or (II) (Öberg, 1983), both compounds inhibiting the DNA polymerases induced by these viruses. In addition, (I), (II), (III, X = Y = Cl) and (IV) can inhibit the RNA transcriptase activity of influenza virus A (Cload & Hutchinson, 1983; Stridh & Datema, 1984).

$(HO)_2P(O)CH_2CO_2H$ **(I)** $(HO)_2P(O)CO_2H$ **(II)**

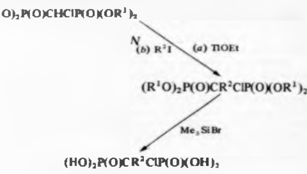
$(HO)_2P(O)CXYP(O)(OH)_2$ **(III)** tetrazolyl (N-H ring)–$CH_2P(O)(OH)_2$ **(IV)**

$(HO)_2P(S)OP(O)(OH)_2$ **(V)** $(HO)_2P(S)OP(S)(OH)_2$ **(VI)**

The inhibition of the RNA transcriptase activity of influenza virus by these compounds appears to be related to their abilities to form complexes with an essential metal ion (probably zinc) in the transcriptase (Cload & Hutchinson, 1983; Hutchinson 1985). Thus (III, X = Y = H) forms only weak complexes with zinc ions at physiological pH while (III, X = Y = Cl) forms considerably stronger complexes. This behaviour is paralleled in their inhibitory activities and (III, X = Y = Cl) is a good inhibitor of the RNA transcriptase activity while (III, X = Y = H) is a poor inhibitor.

The metal chelating properties of inorganic pyrophosphate are affected if oxygen atoms are replaced by sulphur. Oxygen is a 'hard' centre of the Pearson Hard and Soft Acid and Base Scale while sulphur is a 'soft' centre (Pearson, 1968). Thus, thiopyrophosphates might be expected to bind to Zn^{2+} ions in a manner different to that observed with pyrophosphate. We have shown by ^{31}P n.m.r. that bisthiopyrophosphate (VI) appears to bind to Zn^{2+} through sulphur while it binds to Mg^{2+} through oxygen (Hutchinson *et al.*, 1985). Mono- (V) and bis-thiopyrophosphate (VI) form strong complexes with Zn^{2+} ions under physiological conditions. They are as good inhibitors of the RNA transcriptase activity of influenza virus A as compounds (I)–(IV) and are better inhibitors of the transcriptase than inorganic pyrophosphate. Mono-and bis-thiopyrophosphate inhibit the replication of influenza virus A in MDCK cells and do not appear to be cytotoxic to these cells after 3 days at concentrations which cause marked inhibition of virus replication.

Lipid-soluble pyrophosphate analogues should be taken up by cells more readily than highly polar compounds, e.g. (I)–(III). The presence of an electron-withdrawing group on the bridge carbon atom of methylene bisphosphonates is necessary if these bisphosphonates are to be good inhibitors of the RNA transcriptase of influenza virus A (Cload & Hutchinson, 1983). We have developed a method for the synthesis of *C*-alkylated monochlorobisphosphonates (VII) in high yield:



The key steps in our synthetic route are the use of thallium(I) ethoxide as base to form the anion from a tetra-alkyl ester of monochloromethylene bisphosphonate followed by reaction with an alkyl iodide and de-esterification with trimethylsilyl bromide (Hutchinson & Semple, 1985). In this way (VII) can be prepared in over 80% yield overall. If the sodio- or lithio-salts of the monoanion of the tetra ester of monochloromethylene bisphosphonate are used in the alkylation step or if de-esterification is carried out by hydrolysis with concentrated acid, marked reductions in the yield of (VII) occur. In preliminary experiments, we have shown that (VII, R = Me, n-Pr, n-Bu, n-Hex) are effective inhibitors of

the RNA transcriptase activity of influenza virus A as this activity is inhibited by more than 50% when the analogues are present at a concentration of 250 μM

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The major P^1,P^4-bis-(5′-adenosyl)-tetraphosphate-binding protein in *Artemia* is a protein kinase

MARK PRESCOTT and ALEXANDER G. McLENNAN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Several acid-soluble nucleotides act as specific signals in the regulation of metabolic reactions (Tomkins, 1975). The past decade has seen a considerable growth in interest in the novel purine nucleotide P^1,P^4-bis-(5′-adenosyl)-tetraphosphate (Ap_4A). This apparently ubiquitous component of living cells has been shown to alter its intracellular concentration up to 1000-fold during the G_1 to S progression of a synchronized cell cycle (Weinmann-Dorsch *et al.*, 1984). It may be involved in a number of metabolic processes in the cell including the initiation of DNA replication (Weinmann-Dorsch *et al.*, 1984) and stimulation of processing of ADP-ribosylated proteins (Surowy & Berger, 1983). It is synthesized by the aminoacyl-tRNA synthetases in the presence of Zn^{2+} ions (Goerlich *et al.*, 1982). Evidence to date suggests a role as a messenger molecule in cellular proliferation.

As part of our investigations into the function of Ap_4A we have previously examined the levels of this nucleotide during development of the brine shrimp *Artemia*. When ametabolic encysted gastrulae of *Artemia* resume development they do so in the total absence of DNA replication and cell division until the point of hatching (16 h) although protein and RNA synthesis proceed unhindered. Upon re-initiation of development, a 125-fold rise in the intracellular concentration of Ap_4A was observed, the maximum level coinciding with hatching and the onset of DNA synthesis (McLennan & Prescott, 1984).

In order to clarify the relationship between Ap_4A, DNA synthesis and cellular growth and division we have sought to identify the intracellular target proteins of this nucleotide. We report here that the major protein isolated from newly hatched larvae which is capable of binding Ap_4A is a protein kinase.

When extracts of larvae are analysed by sucrose density gradient sedimentation analysis and the binding of $[^3H]Ap_4A$ determined in each fraction by a nitrocellulose filter binding assay, a major binding protein of M_r 93 000 (4.8S) is observed which co-sediments with an enzymic activity capable of incorporating $[^{32}P]$orthophosphate into histone H2B using $[\gamma\text{-}^{32}P]$ATP as substrate.

By contrast, no evidence has been obtained to suggest that this binding protein may be associated with the DNA polymerase-α-holoenzyme complex in this organism, as has been reported previously for Ap_4A-binding proteins from calf thymus (Grummt, 1978) and HeLa cells (Rapaport *et al.*, 1981).

The *Artemia* binding protein has been partially purified from newly hatched larvae by ammonium sulphate precipitation, Matrex Blue and DEAE-cellulose chromatography and AcA 34 gel filtration. This scheme was designed to remove phosphodiesterases and all other enzyme activities capable of degrading the ligand, thus allowing a more detailed study of the protein. The purified Ap_4A-binding protein still possesses protein kinase activity.

Using histone H2B (the preferred substrate) and $[\gamma\text{-}^{32}P]$ATP of medium specific activity (1.4 Ci/mmol), stimulation of the kinase activity was observed in the presence of 1 μM-cyclic AMP and 1 mM-cyclic GMP (2.1 and 2.0-fold respectively). However, at similar concentrations, Ap_4A had no detectable effect on kinase activity. Calmodulin and Ca^{2+} were also without effect.

The preparation was found to possess endogenous acceptor polypeptides when $[\gamma\text{-}^{32}P]$ATP of high specific activity was used (>5000 Ci/mmol). After separation on a 10% (w/v) polyacrylamide gel, four major acceptor species of M_r 35 000, 40 000, 42 000 and 72 000 were observed. These polypeptides all appear to be part of a single protein complex, as shown by their co-sedimentation on a 5–20% sucrose gradient. Since most protein kinases undergo autophosphorylation *in vitro* when incubated with $[\gamma\text{-}^{32}P]$ATP of high specific activity (de Jonge & Rosen, 1977), such a mechanism may be responsible for the incorporation observed here. The precise subunit composition of the native kinase is at present unclear, but our observations may be the result of partial proteolysis of a larger precursor. Such an observation has been made by several investigators (Kuo & Shoji, 1982).

The polypeptides of M_r 35 000 and 40 000 or 42 000 may represent the proteolytic products of the M_r 72 000 polypeptide which, when associated with the third polypeptide of low molecular weight, yields the native kinase of M_r 93 000.

This interpretation is supported by the fact that autophosphorylation of crude extracts in the presence of sodium fluoride, an ATPase and protein phosphatase inhibitor, yields only the polypeptide of M_r 72 000 on sodium dodecyl sulphate/polyacrylamide gel. This polypeptide co-sediments with the native Ap_4A-binding protein of M_r 93 000 on sucrose gradients.

Cyclic AMP (1 μM) or cyclic GMP (1 μM) completely suppress the phosphorylation of the bands of M_r 42 000 and 40 000 while cyclic GMP also reduces phosphorylation of the polypeptides of M_r 72 000 and 35 000. The possible role of this kinase in growth regulation and development in *Artemia* and the significance of Ap_4A binding are currently under investigation.

Abbreviation used: Ap_4A, P^1,P^4-bis-(5′-adenosyl)-tetraphosphate

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The role of an upstream sequence in the transcription of a human transfer RNA gene

JOHN P. GODDARD,* AILEEN McLAREN,* TOM CARR,* MARIANN BIENZ† and JOHN D. SMITH†
**Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and †M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.*

A 2.4 kilobase *Hind*III fragment of human genome DNA which hybridized tRNA was isolated from a larger fragment cloned in λ Charon 4A and subcloned into the plasmid pAT153. This recombinant was found to be of remarkably high transcriptional activity when microinjected into *Xenopus* oocyte nuclei, producing more tRNA than a comparable cloned cluster of seven active *Xenopus* tRNA genes (Clarkson *et al.*, 1978). Southern blot analysis (Southern, 1975) of restriction fragments and determination of the sequence (Sanger *et al.*, 1980) of 1250 residues within the 2.4 kilobase fragment revealed the presence of a single $tRNA^{Glu}$ gene (residues 356–427 inclusive of the sequenced fragment) in the middle of the 2.4 kilobase fragment. Immediately preceding this gene there is a sequence which can be folded into a tRNA-like structure (Goddard *et al.*, 1983).

The separation of the intragenic promoter elements of eukaryotic tRNA genes is variable. This variability would be eliminated, to allow common recognition and binding of RNA polymerase III and/or transcription factors, if the gene itself adopted a higher-order tRNA-like structure (Hall *et al.*, 1982). These observations, together with the finding that the 5'-flanking, or upstream, sequence of some tRNA genes inhibits transcription (see, e.g., Dingermann *et al.*, 1982; Hipskind & Clarkson, 1983), led us to ask whether the potential for a tRNA-like structure within the upstream sequence of this very active human $tRNA^{Glu}$ gene serves to enhance transcription of the gene. We therefore constructed a series of recombinants in which parts of the upstream sequence were deleted and present below our initial results and tentative conclusions on the transcriptional activity of some of these.

The $tRNA^{Glu}$ gene, cloned as a 937 base-pair fragment in M13mp9 for sequence analyses, was subcloned further as a 489 base-pair *Eco*RI–*Nae*I fragment in the same vector. There were no suitably positioned restriction sites to allow simple deletion of only the tRNA-like upstream sequence (residues 285–355). However, a unique *Sst*I site (297–302) in the recombinant allowed access to this region for sequential removal of nucleotides by partial digestion with *Bal*31 exonuclease. Subsequent cleavage by *Stu*I at another unique site after nucleotide 151 upstream from the *Sst*I site, resulted in deletions which contained a common sequence (residues 1–151) of human genomic DNA before the deletion. With no hydrolysis by *Stu*I, deletions were confined largely to the tRNA-like flanking sequence.

The extent of deletion in each of the generated mutants was estimated from the size of the fragments released on digestion of the DNA with *Sma*I (≤ 259 base-pairs or ≤ 404 base-pairs in the absence of digestion by *Stu*I). A suitable range of deletions were selected and characterized by sequence analysis. Supercoiled replicative form RNA, free of contaminating host DNA, was prepared from these recombinants and quantified spectrophotometrically.

DNA (10 ng per oocyte) was co-injected with [α-^{32}P]-GTP (410 Ci/mmol; 0.5 μCi per oocyte) into *Xenopus* oocyte nuclei (10 oocytes per recombinant) and incubated overnight (Bienz & Gurdon, 1982). Total RNA was extracted from the ten pooled oocytes and separated on 10% polyacrylamide gels containing 4 M-urea (Kressman *et al.*, 1978). The newly synthesized RNA was detected by autoradiography of the gel. The amounts of RNA transcribed from three different recombinants was quantified by measurements of the radioactivity of the tRNA and pre-tRNA in the gel bands. These recombinants were Glu6, containing the intact sequence, D5 from which residues 152–305 inclusive had been deleted to remove the first arm of the potential tRNA-like upstream sequence, and D7 where the potential for the first two arms of that structure had been destroyed by removal of residues 152–311 inclusive. The decrease in RNA produced from 100% (Glu6) to 42% (D5) to 19% (D7) indicates that deletion of increasingly large portions of the tRNA-like structure in the upstream sequence of this human $tRNA^{Glu}$ gene gradually decreases the transcriptional activity of that gene. Since these preliminary results support the hypothesis that transcription of the gene is enhanced by this unique upstream sequence, we are pursuing further studies on the transcriptional activity of a wider range of the characterized deletion mutants.

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Fast Atom Bombardment Mass Spectrometry of Salts of Substituted Methylene Bisphosphonic Acids and Other Analogues of Pyrophosphoric Acid

David W. Hutchinson† and Graeme Semple
Department of Chemistry and Molecular Sciences, University of Warwick, Coventry CV4 7AL, UK

Fast atom bombardment mass spectra have been obtained of salts of analogues of inorganic pyrophosphoric acid, e.g. substituted methylene bisphosphonates, and positive ion fast atom bombardment mass spectrometry (FABMS) has proved to be more successful than negative ion FABMS for the analysis of these salts. Owing to the low molecular weight of these analogues, FABMS of the free acids and the sodium salts does not always give results which are easy to interpret as interference by peaks from the matrix can occur. However, the potassium salts in a 60% glycerol matrix, when bombarded by argon atoms, give good FAB spectra which are relatively free of interfering peaks.

INTRODUCTION

Analogues of pyrophosphoric acid are of chemotherapeutic interest as they can inhibit the replication of viruses.[1] While the esters of these analogues can be identified and analysed by ammonia chemical ionization mass spectrometry,[2] the free acids, which are the active antiviral agents, are not sufficiently volatile for analysis by this technique. Few other analytical techniques, e.g. ^{1}H or ^{31}P NMR yield much information on these compounds, hence we have developed conditions for the analysis by fast atom bombardment mass spectrometry (FABMS) of salts of these acids.

Both positive and negative FABMS have been used to analyse simple inorganic salts[3] and these techniques have recently been shown to give good spectra with simple nucleotides.[4] The latter, however, have the advantage that they possess molecular weights in excess of 350 and hence the spectra are comparatively easy to interpret as there is little interference caused by peaks arising from the solvent matrix. Inorganic pyrophosphate has a molecular weight of 178 and hence care must be taken in the choice of counterion for this acid and its analogues in order to obtain positive FAB spectra which contain ions of sufficiently high molecular weight to avoid interference by intense peaks due to the matrix. We now report that the potassium salts of the analogues of pyrophosphoric acid give very satisfactory positive FAB spectra.

EXPERIMENTAL

All compounds are commercially available as salts unless otherwise stated. Ion exchange chromatography on Dowex 50 resin was used to interchange the salts with the corresponding acids. Tetraethyldimethylaminomethylene bisphosphonate was obtained from Lancaster Synthesis Ltd and was hydrolysed with concentrated hydrochloric acid to give the free acid (**5**) which was recrystallized from methanol before analysis. The tetraisopropyl esters of dichloro-(**3**) and dibromo-methylene (**4**) bisphosphonic acids were prepared by the method of Quimby[5] and were converted into their free acids by hydrolysis with concentrated hydrochloric acid. Carbonyl bisphosphonic acid (**6**) was prepared as described[6] and the free acid obtained by ion exchange using Dowex 50 resin (H^{+} form).

FAB mass spectrometric analysis of compounds **1–8** was carried out by mixing an aqueous solution of the free acids (15 μl, 0.3 M) with 2 M sodium or potassium hydroxide solution (10 μl). This mixture was suspended in glycerol (35 μl), giving an overall concentration of 0.075 M pyrophosphate analogue in 60% glycerol which contained four equivalents of counterion. Alternatively FAB mass spectra were obtained from solutions in 60% glycerol/water which were approximately 0.1 M in acid after the addition of potassium hydroxide solution to give the required pH.

The mass spectra of the salts were obtained on an MS 80 mass spectrometer fitted with a standard, commercially available, FAB gun (Kratos Analytical Instruments) and data were analysed using a DS55 data system. The primary argon beam energy was 4 keV with a gun current of 300 μA and all spectra were taken with the sample probe at room temperature using a resolution of 1000. The mass spectra of the free acids were recorded on a Finnigan Model 3300 mass spectrometer fitted with an Ion Tech BIINF saddle field gun,[7] and using xenon as the bombarding gas at 8 keV and 40 μA gun current.

RESULTS AND DISCUSSION

FAB mass spectra of the free acids of compounds **2**, **7** and **8** were successfully obtained, the general species observed being $[M+H+n\ \text{glycerol}]^{+}$ (where $n = 0, 1,$

† Author to whom correspondence should be addressed

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Table 1. Analogues of inorganic pyrophosphoric acid

Name	Formula	Elemental composition	Mol wt
(1) Pyrophosphoric acid	$O(PO_3H_2)_2$	$H_4O_7P_2$	178
(2) 1-Hydroxyethane 1,1-bisphosphonic acid	$CH_3(OH)C(PO_3H_2)_2$	$C_2H_8O_7P_2$	206
(3) Dichloromethylene bisphosphonic acid	$Cl_2C(PO_3H_2)_2$	$CH_4Cl_2O_6P_2$	244, 246, 248
(4) Dibromomethylene bisphosphonic acid	$Br_2C(PO_3H_2)_2$	$CH_4Br_2O_6P_2$	332, 334, 336
(5) Dimethylaminomethylene bisphosphonic acid	$(CH_3)_2NCH(PO_3H_2)_2$	$C_3H_{11}NO_6P_2$	219
(6) Carbonyl bisphosphonic acid	$OC(PO_3H_2)_2$	$CH_4O_7P_2$	190
(7) Phosphonoacetic acid	$HOOCCH_2PO_3H_2$	$C_2H_5O_5P$	140
(8) 3-Phosphonopropionic acid	$HOOC(CH_2)_2PO_3H_2$	$C_3H_7O_5P$	154

2, 3). Due to the low molecular weights of these acids (Table 1) these peaks appear in the region of the mass spectrum which is dominated by abundant ions due to the glycerol matrix. The intensities of the peaks due to the oligomers $[(\text{glycerol})_x H]^+$ become less with increasing x which suggested the use of metal ion salts to produce peaks above m/z 300. In general we have found that while sodium salts of the pyrophosphoric acid analogues do not produce ions with a sufficiently high mass (Table 2), the potassium salts give good FAB mass spectra of the analogues (Fig. 1, Table 3). In the tables the intensities of the most abundant sample ion has been taken to be 100%. In all cases the peaks due to the metal salts of the pyrophosphoric acid analogues accounted for 0.5–3% of the total ion current (TIC). As can be seen from Tables 2 and 3 a series of ions separated by $(Y-1)$ u is observed in each spectrum (where Y = atomic mass of the counterion). The series corresponds to the successive replacement of a proton in the protonated free acids by a counterion. The intensities of the peaks due to the salts do not necessarily correspond to the composition of the salt in the solid phase as free exchange between the acids and the counterions occurs on the probe. In all cases glycerol adducts of ions due to the pyrophosphoric acid analogues were observed, e.g. $[M+H+Na_n+\text{glycerol}]^+$, but these were much less abundant than the series listed in Tables 2 and 3. In the case of carbonyl bisphosphonic acid (**6**) a second series of peaks can be observed in the FAB mass spectrum 18 u higher than those due to **6** (Fig. 2). This is due to hydration of the carbonyl group in **6** which has been postulated by Quimby.[6] In our experiments, the relative concentration of the two species are approximately equal below pH 6. Under more alkaline conditions the carbonyl compound (**6**) predominates. We have replaced glycerol by triethanolamine or trigol in some experiments but there was no effect on the intensities of the peaks due to the analogues. As both these compounds are of higher molecular weight than glycerol, their oligomers cause greater inference with the peaks due to the salts of the analogues of pyrophosphoric acid and hence we do not consider that they offer any advantage over glycerol. We have also observed negative FAB spectra for the sodium salt of pyrophosphoric acid (**1**), where ions were observed at 265, 243 and 221 u corresponding to the ions $[M-H_n+Na_{n-1}]^-$ (n = 3, 4, 5). However, these

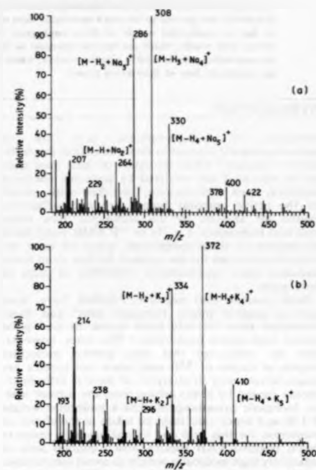

Figure 1. Comparison of FAB mass spectra of sodium (a) and potassium (b) salts of dimethylaminomethylene bisphosphonic acid.[5] Experimental details as given in the text.

ions only carried 0.01% of the TIC and were difficult to reproduce. Thus, we advocate the use of positive FAB mass spectrometry for the analysis of analogues of pyrophosphoric acid.

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Table 2. Masses and per cent relative abundance (normalized to highest sample peak) of peaks observed in the FAB mass spectra of sodium salts of analogues of pyrophosphoric acid

Analogue	Ions observed $[M+H]^+$	$[M+Na]^+$	$[M-H+Na_2]^+$	$[M-H_2+Na_3]^+$	$[M-H_3+Na_4]^+$	$[M-H_4+Na_5]^+$
1	—	—	223(13)	245(61)	267(100)	289(55)
2	207(100)*	229(56)	251(43)	273(20)	295(38)	317(34)
3	—	267(38)	289(100)	311(85)	333(17)	—
	—	269(20)	291(65)	313(52)	335(12)	—
	—	271(4)	293(13)	315(9)	337(3)	—
4	—	355(24)	377(50)	399(32)	—	—
	—	357(48)	379(100)	401(78)	—	—
	—	359(28)	381(48)	403(37)	—	—
5	—	—	264(26)	286(88)	308(100)	330(45)
6	—	213(18)	235(98)	257(100)	279(20)	301(10)
7	141(12)	163(58)	—	207(27)*	229(100)	—
8	155(16)	177(38)	199(100)	221(67)	243(19)	—

* The matrix gives rise to a significant peak at m/z 207 due to $[glycerol_2Na]^+$ which affects the calculation of the % ion current carried by peaks due to compounds 2 and 7.

Table 3. Masses and per cent relative abundance (normalized to highest sample peak) of peaks observed in the FAB mass spectra of potassium salts of analogues of pyrophosphoric acid

Analogue	Ions observed $[M+H]^+$	$[M+K]^+$	$[M-H+K_2]^+$	$[M-H_2+K_3]^+$	$[M-H_3+K_4]^+$	$[M-H_4+K_5]^+$
1	—	—	255(34)	293(64)	331(100)	369(22)
2	207(28)	—	283(5)	321(53)	359(100)	397(46)
3	—	283(26)	321(100)	359(76)	397(6)	—
		285(19)	323(71)	361(66)	399(4)	—
		287(4)	325(39)	363(24)	401(3)	—
4	—	371(52)	409(48)	447(34)	—	—
	—	373(100)	411(94)	449(68)	—	—
	—	375(56)	413(54)	451(40)	—	—
5	—	258(4)	296(16)	334(80)	372(100)	410(29)
6	191(14)	229(18)	267(95)	305(100)	343(24)	—
7	155(23)	193(12)	231(99)	269(100)	307(36)	—
8	—	179(8)	217(42)	255(100)	293(74)	

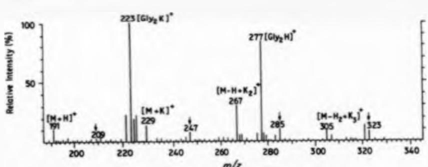

Figure 2. The FAB mass spectrum (mass range 190–340 u) of carbonyl bisphosphonic acid (**6**) in glycerol/water (80/20), pH 3.4. Arrowed peaks arise from the hydrated species, dihydroxymethylene bisphosphonic acid.

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Inhibition of Viral Nucleic Acid Synthesis by Analogues of Inorganic Pyrophosphate

D. W. Hutchinson, M. Naylor and G. Semple

Department of Chemistry, University of Warwick, Coventry, CV4 7AL, U.K.

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Abstract

Analogues of inorganic pyrophosphate can inhibit the DNA polymerase of herpes simplex virus 1, or the RNA transcriptase activity of influenza virus A. Structural features of the pyrophosphate analogues which are essential for their inhibitory activity are discussed and these are related to the ability of the analogues to bind zinc ion.

Introduction

A number of nucleoside analogues such as acyclovir [1] and (E)-5-bromovinyl-2′-deoxyuridine (BVDU) [2] have recently been shown to possess antiviral activity and much effort has been spent recently on the synthesis of nucleoside analogues which might have better therapeutic properties than these two compounds. Both acyclovir and BVDU inhibit the synthesis of DNA in herpes-infected cells. Initially these two compounds are phosphorylated by virus-induced enzymes to give their 5′-monophosphates; these are then converted into the 5′-triphosphates which inhibit DNA synthesis in the virus-infected cells. Thus, the analogues must be metabolized before they can exhibit their antiviral properties and they are only active against a narrow range of viruses of the herpes family.

Compounds which inhibit nucleic acid synthesis in virus-infected cells without the necessity of a metabolic step might be expected to be active against a wider range of viruses than acyclovir or BVDU and might have clinical value so long as toxicity problems can be overcome. One such class of inhibitors are analogues of inorganic pyrophosphate which can inhibit the reaction

$$\mathrm{pppX} \rightleftharpoons (\mathrm{pX})_n + \mathrm{pp_i}$$

Phosphonoacetic acid (**1**) [3] was the first member of this family to be shown to possess antiviral activity and the related compound phosphonoformic acid (**2**) [4] is also an active antiviral agent. Both (**1**) and (**2**) inhibit DNA synthesis in cells which have been infected by herpes viruses and these compounds inhibit the DNA polymerases induced by these viruses.

Fig. 1. Phosphonoacetic acid (**1**).

$(HO)_2P(O)COOH$ (**2**) $(HO)_2P(O)CH_2COOMe$ (**3**) $MeO(HO)P(O)CH_2COOH$ (**4**)

$(HO)_2P(O)CH_2CH_2COOH$ (**5**) $(HO)_2P(O)CR^1R^2P(O)(OH)_2$ (**6**)

The antiviral properties of a number of analogues of (**1**) and (**2**) have been examined and it has been observed that esterification of either the phosphoryl or the carboxyl groups to give, for example, (**3**) or (**4**) leads to a marked reduction in the antiviral activity [5]. Moreover, insertion of extra methylene groups between the phosphoryl and carboxyl groups as in (**5**) also reduces the antiviral activity. Enzyme kinetic [6] and other data [7] indicate that these compounds inhibit the herpes-induced DNA polymerases by acting directly on the enzymes without prior conversion into an active compound and that (**1**) and (**2**) probably act by chelating with an essential metal ion in the DNA polymerases induced by herpes viruses. Pyrophosphate analogues can also be effective inhibitors of the RNA transcriptase activity of influenza virus A. Initiation of mRNA synthesis does take place in the presence of phosphonoformate (**2**) but elongation of the RNA chain does not occur [8].

We have studied a variety of pyrophosphate analogues and believe that their ability to inhibit nucleic acid synthesis in cells which have been infected with either herpes or influenza viruses is related to their ability to form stable chelates with a metal ion (probably zinc) at the active centres of the enzyme systems [9]. We have shown that there is a good relationship between the ability of the analogue to bind zinc ion at pH 8 and its effectiveness as an inhibitor of influenza mRNA synthesis [7]. We determine the binding constant of the analogue to zinc ion by a simple gel-filtration method at the pH optimum of the transcriptase reaction and from this derive the term pK_d, which we define as $-\log_{10}$ (dissociation constant of zinc ion – pyrophosphate analogue complex). The ionic species of the analogue which binds to the zinc ion is not defined in our term pK_d as this merely refers to the ability of all ionic species present to bind zinc ion under given conditions, i.e. the conditions of the transcriptase assay. We find that if a pyrophosphate analogue has a $pK_d \geq 6$ under these conditions then the compound is a good inhibitor of the transcriptase, on the other hand compounds with a $pK_d < 5$ are, in general, poor inhibitors of the transcriptase (Table 1).

Table I. *Inhibitory effect of phosphonoacetic acid analogues and methylene bisphosphonates on the RNA transcriptase of influenza virus A and calf-thymus DNA polymerase α* [7]

Compound[a]	pK_d (Zn^{2+})	Concn (μM) producing 50% inhibition: Influenza RNA transcriptase	DNA polymerase α
RCH_2COOH	5.5	275	45
RCOOH	5.6	35	35
RCH_2CH_2COOH	< 4	> 500	> 500
RCHMeCOOH	~ 5	500	> 500
$RCONH_2$	< 4	> 500	> 500
$(EtO)_2P(O)CH_2COOH$	< 4	> 500	> 500
ROR	5.7	125	> 500
RNHR	5.7	50	> 500
RCH_2R	5.3	> 500	> 500
RCHClR	> 6	85	> 500
$RCCl_2R$	> 6	75	> 500
$RCBr_2R$	> 6	10	350
RCOR	5.4	20	100

[a] Where R = $(HO)_2P(O)$

The exact nature of the metal ion at the active centre of the transcriptase system has not been determined unequivocally. Addition of excess magnesium or manganese ion to the transcriptase assay system fails to affect RNA synthesis and hence it is unlikely that these are the essential metal ions. It has been proposed [10] that most polymerase enzymes require zinc for activity and it has been shown that the influenza viruses do contain zinc [11]. Addition of zinc ions to the transcriptase assay, which has been partially inhibited by a pyrophosphate analogue, restores the enzymic activity to a limited extent. However, zinc ion is toxic to the enzyme at high concentrations [12] and when excess zinc ion is added to the system full enzymic activity is not restored.

The results of our studies on pyrophosphate analogues can best be summarized by considering phosphonoacetic acid as the archetype pyrophosphate analogue and considering in turn the changes in biological activity which are brought about by varying the carboxyl, methylene and phosphoryl groups.

(*a*) *Variation of the carboxyl group*

As mentioned earlier, esterification of the carboxyl group in (**1**) leads to a loss of antiviral activity in cell-free systems and a similar loss in activity is observed when the carboxyl group is replaced by an amide group. In animals, however, some carboxylic esters do have anti-herpes activity, presumably because these esters are hydrolysed *in vivo* to liberate the free acid [5]. Replacement of the carboxyl group by a phosphoryl group gives rise to methylene bisphosphonic acid (**6**, $R^1 = R^2 = H$). While the parent compound is devoid of antiviral activity, as will be discussed later, replacement of hydrogen by an electron withdrawing group leads to compounds (e.g. **6**, $R^1 = R^2 = Cl$), which are good inhibitors of the RNA transcriptase of influenza virus but which have little or no anti-herpes activity.

Analogues of amino acids in which the carboxylic acid residue has been replaced by a 1H-tetrazole residue have pK_a values which are close to those of the parent amino acids and the tetrazole moiety has been used as an isostere for the carboxylic acid group in many pharmaceutically active compounds [13]. The recent report that 5-phosphonomethyl tetrazole (**7**) has anti-herpes activity [14] prompted us to synthesize a range of heterocyclic analogues of phosphonoacetic acid [15]. Treatment of 5-(chloromethyl)-1H-tetrazole with triethyl phosphite gave the diethyl ester of *N*-ethyl-5-(phosphonomethyl)-tetrazole, presumably due to intramolecular attack by the tetrazole ring on the intermediate formed in the Arbusov reaction. The esterifying groups of the phosphonic acid residue could be removed by acid but *N*-ethyl-5-(phosphonomethyl)-tetrazole (**10**) was a very poor chelator of zinc ion and had no effect on the transcriptase of influenza virus. The ethyl group could not readily be removed from the tetrazole ring in this synthetic route, so we protected this ring with the benzyloxymethyl group before the Arbusov reaction. The diethyl groups and the benzyloxymethyl residue were readily removed from the Arbusov product with acid and 5-(phosphonomethyl)-1H-tetrazole (**7**) was obtained in high yield. The 5-(phosphonoethyl) analogue (**9**) was obtained in a similar fashion. It is interesting that the Arbusov reaction between 3-(chloromethyl)-1H-1,2,4-triazole proceeded smoothly without any intramolecular alkylation of the triazole ring. The bistetrazole (**12**) and the carboxylic acid (**11**) were prepared by standard methods.

Fig. 2. Heterocyclic analogues of phosphonoacetic acid

We find that compound (**7**) and its triazole analogue (**8**) are weak inhibitors of the DNA polymerase induced by HSV-1. The 5-phosphonoethyl tetrazole (**9**) and the *N*-alkylated analogue (**10**) show no inhibitory activity against this enzyme. Of the four compounds, only (**7**) has significant inhibitory activity against the RNA transcriptase of influenza. The carboxylic acid (**11**) and the bistetrazole (**12**) do not inhibit either the DNA polymerase of HSV-1 or influenza RNA transcriptase. Only compound (**7**) binds zinc to an appreciable extent ($pK_d = 5.6$) and hence this parameter again is a pointer to the antiviral activity of these compounds (Table II).

(*b*) *Variation of the methylene group*

The homologue of phosphonoacetic acid without a methylene group is phosphonoformic acid (**2**), a compound which is in general a more active antiviral agent than (**1**). The homologue of (**1**) with two methylene groups is 3-phosphonopropionic acid (**3**) a compound with little antiviral activity. The effectiveness of these compounds as inhibitors of influenza transcriptase is again reflected by their ability to bind zinc ion (Table I). One reason for the gradation in pK_d values is that compound (**2**) forms a chelate with a five-membered ring, (**1**) forms a chelate with a six-membered ring and (**3**) forms a chelate with

Table II. *Inhibitory effect of pyrophosphate analogues and heterocyclic methylene phosphonates on the RNA transcriptase of influenza virus A and the DNA polymerase of HSV-1*

Compound	pK_d (Zn^{2+})	Concn (μM) producing 50% inhibition	
		Influenza A RNA transcriptase	HSV-1 DNA polymerase
Heterocyclic analogues			
5-(Phosphonomethyl)-tetrazole (7)	5.6	275	220
3-(Phosphonomethyl)-1,2,4-triazole (8)	4.7	580	385
5-(Phosphonoethyl)-tetrazole (9)	4.6	550	> 1000
N-ethyl-5-(phosphonomethyl)-tetrazole (10)	< 4	> 1000	870
Tetrazole-5-ethanoic acid (11)	< 4	> 1000	> 1000
5,5'-Methylene bistetrazole (12)	< 4	> 1000	> 1000
Others			
Monothiopyrophosphate (14)	5.4	60	> 500
Bisthiopyrophosphate (15)	> 6	33	> 500
Hypophosphate (16)	5.1	> 500	150
Peroxydiphosphonate (17)	4.8	~ 500	n.d.
Arsonoacetate (18)	4.7	> 500	n.d.
Methylenebisarsenate (19)	~ 5	> 500	n.d.

n.d., Not determined

Fig. 3. Metal chelates of (*a*) phosphonoformic, (*b*) phosphonoacetic and (*c*) 3-phosphonopropionic acids

a seven-membered ring. It is well known that the order of stability of cyclic metal chelates is 5 > 6 > 7 [16]. Substitution of the methylene group with an alkyl or aryl group can lead to a loss of activity as a transcriptase inhibitor in cell-free systems. However, an increase in activity can be observed in tissue culture, probably because these compounds are more lipophilic than the parent and are taken up more readily by cells [17]. Replacement of the two hydrogen atoms of the methylene group in **(1)** by two chlorine atoms has a marked effect on the properties of the carboxyl group. The ability of dichlorophosphonoacetic acid to bind zinc ion is low (pK_d < 4) and the compound does not inhibit the RNA transcriptase of influenza virus.

As mentioned above, methylene bisphosphonate (**6**, $R^1 = R^2 = H$) has little or no antiviral activity. Replacement of the bridge hydrogen atoms in **(6)** by electron withdrawing groups such as halogen increases the ability of these compounds to bind zinc ion and markedly increases their antiviral activity [7]. Replacement of the bridging methylene group by a carbonyl group **(13)** also leads to a good chelator of metal ions with both anti-herpes [18] and anti-influenza activity [7]. The presence of at least one electron withdrawing group on the bridging carbon atom in **(6)** and the observation that lipophilic pyrophosphate analogues are often more effective as inhibitors of viral replication in tissue culture than the polar parent compounds has led us to prepare [19] a number of C-alkyl monochloromethylene bisphosphonates. As may be seen in Table III, these compounds are inhibitors of the RNA transcriptase of influenza virus A in cell-free systems are inhibitors of viral replication in MDCK cells.

Table III. *Inhibitory effect of C-alkyl monochloromethylene bisphosphonates $RCCl(PO_3H_2)_2$ on the RNA transcriptase of influenza virus A and on the replication of influenza virus A in MDCK cells*

R	pK_d (Zn^{2+})	I_{250} (%)	PR_{500} (%)
H	> 6	97	22
Me	n.d.	54	n.d.
Et	n.d.	61	n.d.
n-Pr	n.d.	55	34
n-Bu	> 6	64	37
n-Hex	> 6	59	83
CH_2Ph	> 6	58	60
PAA(1)	5.5	35	[illegible]

n.d., Not determined.

I_{250} = % inhibition of transcriptase at phosphonate concentration of 250 μM.

PR_{500} = % reduction of PFU (virus control = 7.7×10^6) caused by phosphate at a concentration of 500 μM.

While the ability of these compounds to inhibit the transcriptase appears to be independent of chain length, the hexyl- and benzyl-substituted bisphosphonates are the most effective inhibitors of viral replication in cell culture. All are more effective than phosphonoacetic acid in both inhibitory systems.

(c) *Variation in the phosphoryl group*

Inorganic pyrophosphate at a sufficiently high concentration will inhibit RNA synthesis by the influenza transcriptase system (Table I) [7]. Replacement of one or both phosphoryl groups by a thiophosphoryl group leads to mono- **(14)** and bis- **(15)** thiopyrophosphate. Both these compounds are considerably more effective than the parent pyrophosphate in inhibiting the replication of influenza virus A in MDCK cells and the synthesis of RNA by the transcriptase system [20]. Sulphur is a 'soft' centre and oxygen is a 'hard' centre on the

Table IV. *Effects of pyrophosphate analogues on either the haemagglutinin titre or the virus yield reduction assay of influenza virus A grown on MDCK cells (mean of two assays)* [20]

Compound	HAU ($\log_{10}$ U/ml)	pfu/ml
Cell control	< 2.0	≤ 10
Virus control	3.65	4.77×10^7
14	2.64	1.05×10^6
15	2.10	4.2×10^6

Pearson 'hard and soft' acid and base scale [9]. Both (**14**) and (**15**) are good chelators of zinc ion and it is possible that the increased antiviral activity shown by the thiopyrophosphates is related to the formation of stronger metal chelates in the transcriptase system of influenza virus than with pyrophosphate.

Removal of the oxygen between the phosphoryl groups of pyrophosphate gives rise to hypophosphate (**16**). While this has been shown to be an inhibitor of the DNA polymerase of herpes-infected cells [18], we have shown it to have little or no effect on the transcriptase of influenza virus. Similarly, peroxydiphosphonate (**17**) is inactive against the transcriptase and the inactivity of (**16**) and (**17**) can be correlated with the abilities of these compounds to bind zinc ions (Table II). In these latter two compounds, the change in distance between the two chelating phosphoryl groups may also be important.

Replacement of the phosphoryl group in (**1**) by the arsonate group to give arsonacetate (**18**) leads to a compound with anti-herpes activity [21] and with some anti-influenza activity. Methylene bisarsonate (**19**) also shows little antiviral activity. Furthermore, toxicity problems associated with these arsenicals and with hypophosphate make them unattractive for clinical use.

$(HO)_2P(O)C(O)P(O)(OH)_2$ (**13**) $(HO)_2P(O)OP(S)(OH)_2$ (**14**) $(HO)_2P(S)OP(S)(OH)_2$ (**15**)

$(HO)_2P(O)P(O)(OH)_2$ (**16**) $(HO)_2P(O)OOP(O)(OH)_2$ (**17**) $H_2O_3AsCH_2COOH$ (**18**)

$CH_2(AsO_3H_2)_2$ (**19**)

Discussion

We believe that the evidence presented above strongly supports the suggestion that nucleic acid synthesis in cells which have been infected by either herpes or influenza viruses can be inhibited by pyrophosphate analogues which act by forming very stable chelates with an essential metal ion in the polymerase/transcriptase enzyme systems induced by the viruses. Before pyrophosphate analogues are adopted for clinical use, however, there are obvious toxicity problems to be overcome as many processes in the host cells involve the liberation of pyrophosphate and these may be affected by the presence of pyrophosphate analogues. One such problem is the accumulation of certain pyrophosphate analogues in bones and teeth [22]. This problem may be obviated by using these compounds topically to treat, for example, herpes infections. Another way in which this accumulation in bone might be overcome is to incorporate bulky groups into the analogues as this should make them less compatible with the hydroxy apatite lattice of bones [23] and hence make them less likely to be incorporated into this lattice.

It is encouraging that many of the pyrophosphate analogues which inhibit virus-induced processes do not seem to be highly toxic to cells. For example, the thiopyrophosphates (**14**) and (**15**) inhibit the replication of influenza A virus in MDCK cells at concentrations which do not appear to affect the viability of the cells after 48 h [20]. Furthermore, substituted methylene bisphosphonates inhibit the transcriptase of influenza virus A at concentrations at which they have little or no effect on the DNA polymerases of HSV-1 or -2, the DNA polymerase α [17] of mammalian cells (Table I) and the DNA and RNA polymerases of *Escherichia coli* [7].

Recently, the effect has been studied of a large number of derivatives of phosphonoacetic acid on the DNA polymerase induced by HSV-2 and on the replication of HSV-2 in tissue culture [24]. The results of these investigations broadly agree with those outlined above except that it was observed that carboxylic esters of low-molecular-weight alcohols inhibited the DNA polymerase. This result is in contrast with observations with HSV-1 [25] and our observations with the RNA transcriptase of influenza. The reason for this difference is not clear and deserves further investigation.

The hypothesis that pyrophosphate analogues inhibit virus replication by virtue of their chelating with an essential metal ion should lead to the design of compounds with significant biological activity but which incorporate features which reduce their toxicity or which lead to an increase in the uptake of the analogue into a particular region of the host.

Experimental

Materials

Arsonoacetate and methylene bisarsonate were gifts from Dr H. B. F. Dixon, Cambridge University, UK, hypophosphate was a gift from Dr B. V. L. Potter, Leicester University, UK, and peroxydiphosphonate was a gift from Professor N. J. Leonard, Univerity of Illinois at Urbana-Champaign, USA. *C*-Alkyl monochloromethylene bisphosphonates [19] and heterocyclic methylene monophosphonates [15] were prepared as described.

Biological assays

The influenza virus strain used in these experiments was A/X49, a cross between A/England/864/75 and A/PR/8/34 with the H3N2 surface antigens of the A/England strain. RNA transcriptase assays and the determination of the antiviral activity of compounds in Madin Darby Canine Kidney (MDCK) cells were carried out as previously described [20]. The herpes-simplex-1 virus strain used was KOS. The inhibition by pyrophosphate analogues of the DNA polymerase induced by this virus was carried by the method of Powell and Purifroy [26] and the DNA polymerase α assays were carried out by the method of Sabourin *et al.* [27]. The determination of pK_a values related to the strength of the binding of zinc ion to the pyrophosphate analogues was determined by a gel-filtration method [7].

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SYNTHESIS AND BIOCHEMICAL PROPERTIES OF SOME PYROPHOSPHATE ANALOGUES

D. W. HUTCHINSON, G. SEMPLE AND D. M. THORNTON

Department of Chemistry, University of Warwick, Coventry, CV4 7AL, U.K.

SUMMARY

Analogues of inorganic pyrophosphate have been prepared by the modification of tetraesters of methylene bisphosphonic acid or by the Michaelis-Becker reaction. The yields of products obtained by alkylation of the monoanions of tetraesters of methylene bisphosphonic acid depends both on the size of the esterifying groups and on the counter ion used. The antiviral properties of the pyrophosphate analogues appears to depend on their metal chelating properties and this can be altered by the introduction of a thiophosphoryl group into the analogues. The thiophosphoryl derivatives are more effective inhibitors of viral nucleic acid synthesis than their oxygenated parents.

INTRODUCTION

Analogues of inorganic pyrophosphate have interesting properties as pyrophosphate is involved in many biochemical processes. Thus, pyrophosphate analogues can (a) inhibit the replication of viruses (ref. 1), (b) have antiamoebic activity (ref. 2), (c) have herbicidal activity (ref. 3) and (d) be bone-seeking compounds (ref. 4). We have been interested in the antiviral activity of pyrophosphate analogues as it should be possible to inhibit nucleic acid synthesis in cells which have been infected by viruses when they might inhibit the following process:

$$ppX \rightleftharpoons (pX)_n + PP_i$$

Toxicity problems must obviously be overcome as the host cells are also synthesising nucleic acids. However, the mechanism of nucleic acid synthesis by virally-induced enzymes can differ from those of the host (ref. 5) and in these cases the pyrophosphate analogues might have some therapeutic value or might be used as probes to unravel some of the stages in viral nucleic acid synthesis. Phosphonoacetic acid PAA [1] was the first pyrophosphate analogue to be shown to possess antiviral properties (ref. 6), and both PAA and the related phosphonoformic acid PFA [2] (ref. 7) inhibit the replication of several viruses by

inhibiting nucleic acid synthesis (ref. 8). It is encouraging that neither PAA nor PFA appear to be particularly toxic to host cells. The antiviral activity of a number of analogues of compounds [1] and [2] have been examined and some structure-

$(HO)_2P(O)CH_2COOH$ $(HO)_2P(O)COOH$

PAA [1] PFA [2]

function relationships emerge (ref. 1). While there is some controversy over the effect of esterifying the carboxylic acid residues on the antiviral activity of these compounds, it apears that the antiviral activity is greatly reduced when the phosphoryl hydroxyl groups are esterified (ref. 9). There has been one report that simple carboxyl esters of PAA do have some inhibitory activity against the DNA polymerase of HSV-2 (ref. 10). It may be that in this case some hydrolysis of the esters occurs to release the free acid.

We have been studying the effects of pyrophosphate analogues both on the RNA transcriptase activity of influenza virus A and on the replication of this virus in MDCK cells. When we have found inhibitory activity, we have also studied their effects of the DNA polymerase of HSV-1 and on DNA polymerase-α from mammalian cells. We chose to study the RNA transcriptase of influenza virus A on account of the rather unusual mechanism of RNA synthesis shown by this system. Three distinct steps are thought to occur, (a) a virus-coded endonuclease cleaves cellular mRNA at a site 10-13 nucleotides from the caped 5'-end, (b) a GMP residue is incorporated at the 3'-end of the oligonucleotide fragment, (c) this now binds to the viral RNA and elongation takes place (ref. 11). Since our work began, it has been shown that PFA interferes with this final elongation step after the incorporation of an additional 10 or 12 nucleotides (ref. 12).

We have studied a variety of pyrophosphate analogues and believe that their ability to inhibit nucleic acid synthesis in cells which have been infected with either herpes or influenza viruses is related to their ability to form stable chelates with a metal ion (probably zinc) at the active centres of the enzyme systems. We have shown that there is a good relationship between the ability of the analogue to bind zinc ion at pH 8 and its effectiveness as an inhibitor of influenza mRNA synthesis (ref. 13). We determine the binding constant of the analogue to zinc ion by a simple gel-filtration method at the pH optimum of the transcriptase reaction and from this derive the term pK_d', which we define as $-\log_{10}$ (dissociation constant of zinc ion-pyrophosphate analogue complex). The ionic species of the analogue which binds to the zinc ion is not defined in our term pK_d' as this merely refers to the ability of all ionic species present to bind zinc ion under given conditions, i.e. the conditions of the transcriptase assay. We find that if a pyrophosphate analogue has a $pK_d' \geq 6$ under these conditions then the

compound is a good inhibitor of the transcriptase, on the other hand compounds with a pK_d. < 5 are, in general, poor inhibitors of the transcriptase (Table 1).

The exact nature of the metal ion at the active centre of the transcriptase system has not been determined unequivocally. Addition of excess magnesium or manganese(II) ion to the transcriptase assay system fails to affect RNA synthesis and hence it is unlikely that these are the essential metal ions. It has been proposed (ref. 14) that most polymerase enzymes require zinc for activity and it has been shown that the influenza viruses do contain zinc (ref. 15). Addition of zinc ions to the transcriptase assay, which has been partially inhibited by a pyrophosphate analogue, restores the enzymic activity to a limited extent. However, zinc ion is toxic to the enzyme at high concentrations (ref. 16) and when excess zinc ion is added to the system full enzymic activity is not restored.

We have prepared and studied a large number of pyrophosphate analogues as inhibitors of viral replication and some of our recent work is outlined below.

DISCUSSION

C-Alkyl halogenomethyl bisphosphonic acids [3]. We have been interested in these compounds for a number of reasons, (a) could active antiviral compounds be prepared which do not bind to bone?, (b) could the range of viruses inhibited by pyrophosphate analogues be extended?, (c) could an affinity probe discover which of the proteins of the influenza virus binds the analogue?

$(HO)_2P(O)CXRP(O)(OH)_2$

[3]

In Table 1, it may be seen that monochloromethylene bisphosphonic acid (pK_d. > 6) is an active inhibitor of the RNA transcriptase of influenza virus A while the parent methylene bisphosphonic acid (pK_d. 5.3) is not. Thus, the presence of at least one electron-withdrawing group on the central carbon atom appears to be required before the compounds show inhibitory activity against this enzyme system. In an attempt to prepare compounds which would still inhibit the transcriptase but which might not bind to bone, we have prepared C-alkylated monochloromethylene bisphosphonic acids as we wished to investigate whether a bulky substituent on the central carbon atom might hinder the binding of the analogue to the hydroxyapatite lattice of bone.

Tetraesters of monobromo- and monochloro- methylene bisphosphonic acid are difficult to prepare by direct chlorination of methylene bisphosphonic acid esters as mixtures of products are obtained and the monochloro compounds is only formed in low yield. We have found that the most efficient route to tetraesters of monohalogenomethylene bisphosphonic acids is to prepare the

dihalogeno-compound and then to remove one of the halogens with the aid of an alkyl-lithium (ref. 17, 18). In this way, tetraisopropyl monochloro- and monobromo-methylene bisphosphonates have been prepared in high yield. While this method is not applicable for the de-fluorination of difluoromethylene bisphosphonic acid esters as C-P bond cleavage occurs readily with these compounds in the presence of nucleophiles. When we attempted to prepare tetraesters of bromofluoromethylene bisphosphonic acid these were de-brominated in situ. This is, therefore, a simple route to monofluorobisphosphonates which avoids the use of vigorous reagents such as perchloryl fluoride.

Alkylation of tetraisopropyl monochloromethylenebisphosphates with sodium or lithium as the counterion proceeded in only moderate yields. However, when the thallio-derivative was prepared by addition of thallium(I) ethoxide to the tetraisopropyl ester, virtually quantitative yields of alkylated products could be obtained with primary alkyl iodides although yields were lower with secondary alkyl iodides. Interestingly, the size of the esterifying groups on phosphorus has a marked effect on the yields of products (ref. 18). When the tetraethyl or tetramethyl esters were used in this alkylation reaction, low yields (< 20%) were obtained with a range of primary alkyl iodides. Furthermore, ^{31}P n.m.r. studies show that the rate of alkylation of tetraisopropyl monochloromethylene bisphosphonate was faster than the rate with the tetraethyl ester. ^{13}C n.m.r. studies on the anions of tetraesters of monochloromethylene bisphosphonic acids suggest that the central atom is sp^2 hybridised and that the negative charge is not extensively delocalised over the P = 0 groups. This is in contrast to the case with the anion of triethyl phosphonoacetate, when ^{13}C n.m.r. indicates that there is extensive delocalisation of the negative charge over the carbethoxy group. One explanation of the variation in yields of alkylated product with the size of the esterifying group is that the bulky isopropyl groups prevent the orbitals of the P = 0 groups and the negative charge on the central carbon atom from overlapping so they do not achieve coplanarity. The smaller ethyl and methyl groups allow greater coplanarity to be achieved and there is some delocalisation of the negative charge over the P = 0 groups. Thus, the negative charge is more accessible for attack by electrophiles in the tetraisopropyl esters. Similar arguments involving differences in the coplanarities of anions of β-diketones have been used to explain differences in the reactivities of these compounds towards electrophiles (ref. 19).

Using the thallio-derivative of tetraisopropyl monochloromethylene bisphosphonic acid we have prepared a number of C-alkylated monochloromethylene bisphosphonic acids, including the benzyl derivative (3, $R=C_6H_5CH_2$, X=Cl) by de-esterifying the esters with bromotrimethylsilane, a process which goes in virtually quantitative yield. The resulting C-benzyl free acid had a pK_d. > 6 and inhibited the RNA transcriptase of influenza virus A by 50% at 180 µM.

However, it binds technetium-99 to bone in the presence of tin(II) chloride and hence the benzyl group is not sufficiently bulky to prevent the binding of this compound to bone (ref. 20).

The thallio-derivatives of tetraisopropyl monochloromethylene and methylene bisphosphonates have been used to prepare analogues [4] of the antiviral agent Arildone which inhibits the replication of rhinoviruses. Like Arildone these analogues have no activity against influenza virus replication but they are being investigated as inhibitors of rhinovirus replication.

Arildone

[4]

$R = CH_3O-C_6H_3(Cl)-O(CH_2)_6$

We have used this route to synthesise an affinity probe to ascertain which of the proteins in the RNA transcriptase complex of influenza virus A was the site of the binding of the pyrophosphate analogues. The synthesis is outlined in Scheme 1. Coupling of the tetrahydropyranyl iodide [5] with tetraisopropyl monochloromethylene bisphosphonate gave [6] in high yield. Removal of the tetrahydropyranyl group with acid and the isopropyl groups with bromotrimethylsilane left [7], which was oxidised to the aldehyde [8] with the Dess Martin periodinane (1,1,1-triacetoxy-2,1-benzoxiodol-3(3H)one (ref. 21)). The aldehyde [8] which was obtained in good yield was an effective inhibitor of the RNA transcriptase. The aldehyde was chosen as pyridoxal phosphate was used as an affinity label in an earlier investigation (ref. 22) for the transcriptase of fowl plague virus, a similar virus to influenza. Pyridoxal phosphate was a reversible inhibitor of the transcriptase and when the pyridoxal phosphate was incubated with the virus followed by treatment with [^{3}H]-borohydride, the protein PB_1 was preferentially labeled. We have carried out a similar series of experiments with our aldehyde [8] and influenza virus A/X49. Polyacrylamide gel electrophoresis of the viral proteins after incubation with the aldehyde [8] and reduction with [^{3}H]-borohydride shows that only the PA and PB proteins are labelled. These proteins are involved in RNA synthesis. No labelling of neuraminidase or other virus proteins occurs.

Finally, we have been interested in the effects on the metal chelating properties of pyrophosphate analogues of introducing sulphur, a "soft" atom on the Pearson HSAB scale (ref. 23), into the molecule. Initially, we examined the

Oxepane $\xrightarrow[\text{(b) } H_2O]{\text{(a) } B_2H_6 / I_2}$ $HO(CH_2)_6I$ $\xrightarrow{\text{dihydropyran} / H^+}$ THP–$O(CH_2)_6I$ [5]

[5] + $Tl^+ \; {}^-CCl[P(=O)(OPr^i)_2]_2$ $\longrightarrow$ THP–$O(CH_2)_6$–$CCl[P(=O)(OPr^i)_2]_2$ [6]

[6] $\xrightarrow{H^+}$ $\xrightarrow{HMDS/Me_3SiCl}$ $\xrightarrow{Me_3SiBr}$ $HO(CH_2)_6$–$CCl[P(=O)(OH)_2]_2$ [7]

[7] + (1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one; AcO, OAc, OAc) $\longrightarrow$ H–$C(=O)$–$(CH_2)_5$–$CCl[P(=O)(OH)_2]_2$ [8]

Scheme 1

inhibitory activity of mono- [9] and bis-thiopyrophosphate [10] on the replication of influenza virus A in MDCK cells. Both [9] and [10] are good chelators of zinc ion (pK_{d}. > 6) and are more effective inhibitors of the replication of influenza virus than

$(HO)_2P(O)OP(S)(OH)_2$ [9]

$(HO)_2P(S)OP(S)(OH)_2$ [10]

$(HO)_2P(S)COOH$ [11]

$(HO)_2P(S)CH_2COOH$ [12]

pyrophosphate itself (ref. 24). It is not known whether the sulphur is in the thiono- or thiolo-form but ^{31}P n.m.r. evidence suggests that the zinc ions bind to sulphur unlike magnesium ions. The thiopyrophosphates appear to be nontoxic to the MDCK cells after 3 days exposure, possibly because the thiopyrophosphates are readily hydrolysed to inorganic pyrophosphate. Encouraged by these observations, we have examined the thio-analogues of PFA [11] and PAA [12] (Table 2) (ref. 25). Compounds [11] and [12] were prepared by Michaelis-Becker reactions between the sodio-derivative of diethyl thiophosphite and ethyl chloroformate or chloroacetate, the P-OEt groups were removed with iodotrimethylsilane at high temperature over 48 hours. As may be seen in Table 2, the thio-analogues of PFA and PAA are more effective inhibitors of polymerases than their oxygenated counterparts, as is the thio-analogue of methylene bisphosphonic acid. The tetraethyl ester of the latter was prepared by a photo-activated Michaelis-Becker reaction between the sodio-derivative of diethyl thiophosphite and dibromoethane. We believe that this increased inhibitory activity against polymerases justifies further investigation into thio-phosphonates as potential antiviral agents.

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TABLE 1
Zinc chelating properties of some pyrophosphate analogues and their inhibitory effect on the RNA transcriptase of influenza virus A

Compound	pK_{d}'	Concn. (μM) producing 50% inhibition of RNA transcriptase	DNA-polymerase-α
$(HO)_2P(O)CBr_2P(O)(OH)_2$	>6	10	350
$(HO)_2P(O)CCl_2P(O)(OH)_2$	>6	75	>500
$(HO)_2P(O)CHClP(O)(OH)_2$	>6	85	>500
$(HO)_2P(S)OP(S)(OH)_2$	>6	33	>500
$(HO)_2P(O)OP(O)(OH)_2$	5.7	125	>500
$(HO)_2P(O)COOH$	5.6	35	35
$(HO)_2P(O)CH_2COOH$	5.5	275	45
Phosphonomethyl-tetrazole	5.6	275	220
$(HO)_2P(O)COP(O)(OH)_2$	5.4	20	100
$(HO)_2P(O)CH_2P(O)(OH)_2$	5.3	>500	>500
$(HO)_2P(O)CH_2CONH_2$	<4	>500	>500
$(HO)_2P(O)CH_2CH_2COOH$	<4	>500	>500
$(EtO)_2P(O)CH_2COOH$	<4	>500	>500
N-ethyl-phosphonomethyl-tetrazole	<4	>500	>500

TABLE 2
Comparison of the inhibitory effects of phosphonates and thiophosphonates on the RNA transcriptase activity of influenza virus A/X49, the DNA polymerase induced by HSV-1 (KOS) and calf thymus DNA polymerase-α (from ref. 25)

Compound	pK_a'	RNA transcriptase I_{50} (μM) [a]	HSV-1 DNA polymerase I_{50} (μM) [a]	Calf thymus DNA polymerase I_{50} (μM) [a]
$(HO)_2P(O)CH_2COOH$	5.5	350	10	40
$(HO)_2P(S)CH_2COOH$	>6	135	16	75
$(HO)_2P(O)COOH$	5.7	30	12	50
$(HO)_2P(S)COOH$	>6	45	9	150
$[(HO)_2P(O)]_2CH_2$	5.3	>500	>500	>500
$[(HO)_2P(S)]_2CH_2$	>6	350	>500	>500
$[(HO)_2P(O)]_2O$	5.7	125	>500	>500
$[(HO)_2P(S)]_2O$	>6	33	>500	>500

[a]Concentrations producing 50% of enzymic activity.